DUAL ROLE OF PHENOLIC BIOACTIVES IN IMPROVING FUNCTIONAL HEALTH BENEFITS AND ABIOTIC STRESS RESILIENCE IN BARLEY

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Sciences

By

Ramnarain Ramakrishna

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Cereal Science

February 2021

Fargo, North Dakota

North Dakota State University
Graduate School

Title

DUAL ROLE OF PHENOLIC BIOACTIVES IN IMPROVING FUNCTIONAL HEALTH BENEFITS AND ABIOTIC STRESS RESILIENCE IN BARLEY

By

Ramnarain Ramakrishna

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Kalidas Shetty
Chair

Dr Richard Horsley

Dr Paul Schwarz

Dr Eric Berg

Approved:

04-13-2021
Date

Dr. Richard Horsley
Department Chair

ABSTRACT

Food insecurity, climate change, and public health challenges are interconnected and pressing issues facing humanity in the 21st century. Therefore, it is imperative to address key obstacles underlying food and nutritional insecurity by developing strategies to concurrently improve the nutritional benefits and environmental stress resilience of food crops. The metabolic and physiological roles of plant-derived phenolic compounds are particularly relevant in addressing these challenges. Phenolic compounds are an integral part of plant adaptive defense responses against biotic and abiotic stresses. When consumed as part of diet, certain phenolic compounds from plant-based foods also provide wider protection against metabolic breakdowns and related health risks associated with non-communicable chronic diseases (NCDs). Therefore, the central theme of this dissertation is to delineate the dual protective roles of phenolic compounds in improving crop stress resilience and their human health protective functions, specifically antioxidant and anti-hyperglycemic benefits supporting management of early stages of type 2 diabetes. Barley was used as a model crop; initially several malting barley samples were screened based on their phenolic-linked antioxidant and anti-hyperglycemic functions using *in vitro* assay models. Following metabolically driven screening, novel bioprocessed elicitor treatments such as marine protein hydrolysates and chitosan oligosaccharides were used as seed and foliar treatments to improve type 2 diabetes supporting dietary functions through upregulation of proline-associated pentose phosphate pathway (PAPPP), which is linked to the biosynthesis of phenolic metabolites. Improvement of phenolic biosynthesis and type 2 diabetes related benefits were observed in grains and sprouts with targeted elicitor treatments. Further, elicitor treatments were also found to positively improve PAPPP-linked metabolic responses under abiotic stresses, such as high soil salinity and water logging stress. From a human health

perspective, food barley tea and coffee were found to have significant phenolic-linked antioxidant, and anti-hyperglycemic benefits. Additionally, integrated bioprocessing strategy by combining sprouting with mixed Kefir culture-mediated fermentation was found to improve phenolic-linked antioxidant, anti-hyperglycemic, and human gut health benefits relevant functionalities in pigmented and unpigmented food barley substrates. Therefore, these metabolically driven strategies can be targeted to screen both abiotic stress resilience and human health protective functions in malting and food barley varieties.

ACKNOWLEDGEMENTS

I owe an immense debt of gratitude to Dr. Kalidas Shetty for his constant support and for providing me with the opportunity to learn under him. I am equally indebted to Dr. Dipayan Sarkar for being a mentor and philosopher and helping me through every step I have taken while pursuing this doctoral degree. I thank the members of my research committee – Dr. Richard Horsley, Dr. Paul Schwarz and Dr. Eric Berg – for their technical inputs and support during my research. I thank the NDSU Malting Barley Breeding Program for providing barley grains for my research. I thank Julie Hochhalter and her team at the NDSU AES greenhouse facility for their assistance with the greenhouse studies. I am also thankful to my professors, fellow students, technicians and all other members of the Cereal Science program and the Department of Plant Sciences at NDSU for all their guidance, assistance, and inspiration.

DEDICATION

For Appa, Amma, Anu – my family, my everything.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

T2D……Type 2 diabetes

PAPPP…….……...Proline-Associated Pentose Phosphate Pathway

NCD…….……..Non-Communicable Chronic Diseases

ROS…..……..Reactive Oxygen Species

ROI……..……...Reactive Oxidative Intermediates

PPP..……...Pentose Phosphate Pathway

ATP……..……..Adenosine Triphosphate

G6PDH…..…….Glucose-6-phophate dehydrogenase

SDH.……..…….……Succinate dehydrogenase

PDH….……..…….Proline dehydrogenase

GPX……..……..Guaiacol peroxidase

SOD..……..Superoxide dismutase

CAT……...…….Catalase

DPPH..……2,2-Diphenyl-1-picrylhydrazyl

ABTS…….……2,2-Azino-bis 5(3-ethylbenzothiazoline-6-sulphonic acid)

CHAPTER 1. INTRODUCTION

1.1. Global Food and Nutritional Insecurity Challenges – An Overview

Food and nutritional insecurity and associated public health challenges represent one of the most pressing global issues of the 21st century. As the global population is projected to rise to about 9.5-10 billion by the year 2050, a commensurate and sustainable rise in overall food production is required to ensure overall accessibility and availability of nutritionally balanced foods to support good health and well-being (United Nations Department of Economic and Social Affairs, 2019). Additionally, the utilization of nutritionally balanced foods from diverse sources is also essential to addressing issues of malnourishment (under- and over nutrition)-linked public health challenges globally. The current state of global hunger and related malnutrition, which affects around 1 billion individuals worldwide, further confounds and complicates the challenges of food and nutritional insecurity. The United Nations has identified the management of global malnourishment as a vital component of its sustainable developmental goals (SDGs), as both undernutrition and over nutrition-linked health challenges coexist within the populations of several developing and developed countries.

In the last century, scientific advancements in food crop breeding strategies and agricultural production practices have contributed to significant improvements in global food production and profoundly helped to address global hunger and calorie- based malnutrition challenges. Such success in global food production improvements were achieved in part due to the adoption of resource intensive agricultural practices and widespread adoption of high yielding food crop cultivars (especially cereals) that were relevant to support the global calorie demands. However, many challenges have arisen with the application of such practices, such as the: i) increase in global greenhouse gas emissions (GHGEs) from intensive agricultural

production systems, ii) over and injudicious use of chemical fertilizers and pesticides contributing to a plateauing crop yields and increased risk of contamination of soil and groundwater, iii) reduction in diversity of food crops and varietal choices due to extensive monocropping and iv) increased risk of soil erosion and rapid depletion of fresh ground water due to over use of arable land and water resources. These factors have collectively contributed to the overall increase in global agricultural carbon footprint with significant adverse effects on the environment. Therefore, many of these current agricultural practices, which are not ecologically and environmentally sustainable, add serious burdens to global food and nutritional insecurity challenges and even become more complex in the midst of rapid climate change.

In addition to the challenges associated with intensive agricultural production, the rapid changes in industrial food processing and engineering, and emergence of multiple commercial food chains in post-World War 2 era has led to the increase in consumption of highly processed, refined, and high soluble carbohydrate rich calorie dense foods worldwide. Higher consumption of such hyper-processed calorie dense foods has partly contributed to nutritional imbalance and led to the rapid rise in diet and lifestyle-linked non-communicable chronic diseases (NCDs) in recent decades. In such industrial food production systems, improving crop yields with high-yielding species and varieties of soluble carbohydrate rich food crops were favored over the health relevant nutritional qualities and nutritional diversities of the food. The resulting shift in the overall focus of global food production is evidenced by the fact that currently, 95% of the world's calories come from 30 species, with more than half of the global calorie demand is met by only three food crops, i.e., corn (maize), rice, and wheat. This current food systems lack nutritional diversity and nutritional balance (micronutrients, fiber, phytochemicals), and have

partly contributed to rapid emergence and prevalence of food and nutritional insecurity-linked public health challenges globally.

Therefore, global agri-food systems will need to find sustainable solutions to feed the growing populace, while minimizing their impact on human health and the environment (Searchinger, 2013). Consequently, novel, and holistic integrated approaches must be adopted to design sustainable food systems, which can also address the health targeted nutritional requirements of the growing population. It is necessary to rethink current global food production systems with their structural components and feedback loops to improve linkages between agri-food production, public health, chronic disease management, and environmental impact, to ensure overall food and nutritional security with sustainable solution strategies. Therefore, to substantially improve productivity of health relevant and nutritionally balanced food crops, varieties and cultivars with desirable traits such as improved human health relevant nutritional qualities and tolerance to increasing climate change-linked abiotic and biotic stresses need to be identified. In order to address these food and nutritional insecurity issues the underlying obstacles must be systematically targeted, especially by improving resilience to climate change, overall fitness, productivity, and nutritional qualities of food crops.

1.2. Crop Production is Undermined by Climate Change and Related Abiotic Stress Pressures

The phenomenon of climate change is the single largest, overarching threat to food production and associated food insecurity worldwide. The pace of climate change has accelerated substantially since the 20th century, due to increase in greenhouse gas emissions (GHGEs) arising from anthropogenic factors such as industrialization, rapid increase in burning of fossil fuels, and drastic reduction of forest cover for agricultural and industrial uses. Such

rapid climate change has been strongly associated with marked shifts in precipitation patterns, which are expected to result in alternating periods of increased and/or erratic precipitation (rainfall) and more intense, intermittent dry spells. Additionally, climate change has also been linked to higher global mean temperatures leading to more frequent cold-season thaws and higher temperature-induced heat stress during the primary agricultural production season of summer both in Northern and Southern hemisphere (Schmidhuber and Tubiello, 2007).

As a direct consequence of such shifts in large-scale weather patterns, impact of climate change-linked abiotic stresses on food plants - which at present are responsible for 60-70% of yield losses in agricultural crops – is also expected to be exacerbated (Federoff *et al*., 2010; Rouphael *et al*., 2017; Yakhin *et al*., 2017). While several forms of abiotic stresses are known to limit crop productivity, salt stress and waterlogging are two common abiotic stress factors with serious adverse effects on food crop production and are widespread worldwide having impact on; a) large tracts of arable lands; b) adversely impacting the ecological balance and soil fertility, especially hindering nutrient uptake which prevents maximum yield potential; and c) altering the availability and quality of fresh water, which is the most important agricultural resource for food crop production. To counter such abiotic stress-induced agricultural production challenges, understanding and recruiting the abiotic stress-associated metabolic regulation of plants to improve salt and waterlogging stress resilience of food crops has significant merit and relevance to overall economic security.

1.2.1. Salt Stress

Among several abiotic stresses, salt stress is one of the most serious environmental issues that affects growth, fitness, productivity, and end-use quality of food crops. Currently, 20% of the world's irrigated lands are being affected by varying degrees of salinity, and some scholars

suggest that up to 50% of total arable land in the world will be affected by 2050 (Jamil *et al*., 201l; Qadir *et al*., 2014). Several anthropogenic and non-anthropogenic factors, such as overuse of arable land, over irrigation, continuous drilling of ground water for agricultural and non-agricultural purposes, use of salt or sodic products for agriculture, irrigation, construction, oil fracking have significantly contributed to increased salinization of arable land worldwide. Overall, salt stress affects all the major physiological and biochemical processes such as germination, growth, photosynthetic pigments and photosynthesis, water retention, nutrient imbalance, oxidative stress, and productivity especially through impeding availability of water and nutrients to the plant. These detrimental effects occur due to the presence of excessive soluble mineral ions in soil, which compete with the uptake of essential mineral nutrients, and induce ion toxicity in plant tissues (Parihar *et al*., 2014).

Most commonly, salt stress occurs in soils that are naturally rich in mineral ions (weathering of parent rock materials rich in soluble salts or deposition of oceanic salts carried in wind and rain). As described above, soil salinity may result from anthropogenic factors that alter the hydrologic balance of the soil, such as large-scale agriculture involving the replacement of perennial vegetation with annual crops, irrigation with salt-rich water, and overuse of ground water that pulls out and allows salt to deposit in topsoil. Additionally, industrial extraction of fossil fuels (fracking-oil sand processing), which is common practice in different pockets of the United States and Canada also increase the risk of soil salinization (Franzen, 2003; Renault *et al*., 2003). Further, climate change is expected to exacerbate this phenomenon due the impact of rising sea levels on coastal areas, while rising temperatures can lead to increased evaporation and further salinization (Shahid *et al*., 2018). As most food crops are extremely susceptible to salt

stress, it is important to address salinity resilience issue to improve overall agricultural productivity and profitability.

1.2.2. Waterlogging

Similar to soil salinization, waterlogging and flooding is another environmental factor that imposes high agricultural production risks with potential crop failure, significant loss in yield, and reduction in quality. Waterlogging and flooding are said to occur when soils are completely saturated with water for prolonged periods, either due to poor soil drainage, or in regions with low water tables and / or high rates of precipitation, which may be transient (seasonal) or permanent. In such waterlogged soil, gaseous exchange in the root zone is inhibited, leading to a state of anaerobiosis that can lead to hypoxic, and eventually anoxic tissues in the affected plant (Vartapetian and Jackson, 1997). As a result of impaired root function in affected plants, nutrient uptake is severely restricted, and carbohydrate catabolism is encouraged. Additionally, photosynthates are diverted from sinks such as grains and pods to vegetative tissues to meet the cellular energy demands, causing a reduction in overall biomass and end product quality. The geographical extent of soils impacted by flooding and waterlogging is projected to increase due to changes in global weather events, including erratic precipitation patterns. A majority of crops that currently constitute global food systems are mesophytes, therefore, prolonged exposure to waterlogged soils could diminish their fitness and productivity, and therefore represents a key challenge to global agricultural output.

The increased risk of climate change-linked abiotic stresses is expected to impact sustainable food production goals and further complicate the global food and nutritional insecurity challenges. In addition to undermining global food production, abiotic stresses may also diminish the nutritional qualities of food crops, which could potentially impact public health

goals, especially in the context of addressing emerging nutritional insecurity-linked non-communicable chronic disease challenges (NCDs) worldwide. Therefore, improving both climate change resilience and nutritional qualities of food crops using sound metabolic rationale are equally important and can help to address global food and nutritional insecurities issues and related public health challenges, such as global epidemic of non-communicable chronic diseases (NCDs).

1.3. Increasing Prevalence of Non-Communicable Chronic Diseases (NCDs)

The sustainable developmental goals to achieve global food and nutritional security is further confounded by disparities in equitable access to and availability of nutritious food. The issues of hunger and malnourishment from undernutrition are persistent problems that are yet to be eradicated, with an estimated 821 million individuals being affected as of 2017 (WHO, 2018). However, in recent decades, "over nutrition" that is also part of malnutrition issues has led to a rapid increase in incidence and prevalence of several diet-linked non-communicable chronic diseases (NCDs), which further complicate the food and nutritional insecurity challenges and their solutions.

This rise in the prevalence and progression of such NCDs results from a complex interaction between genetic and environmental factors, of which diet, especially imbalanced nutrition is a critical component. Rising incomes and urbanization are driving a global dietary transition, whereby modern diets consisting of energy-dense and hyper-processed foods, comprising refined sugars, fats, oil, and high animal protein are being increasingly adopted. Such diets lack diversity, containing inadequate amounts of whole grains, protein rich pulses, fresh vegetables, and fruits; thus, deficient in beneficial micronutrients and phytochemicals (Arndt *et al*., 2009). The widespread adoption of this "Western/Modern" diet even in non-western

developing countries has led to an unprecedented NCD-linked public health challenges, whereby over nutrition coexists with undernutrition contributing to severe malnourishment.

The dietary transition from diverse and balanced diets, to a more calorie-dense hyper-processed dietary patterns coincides with the epidemiological transition, as chronic NCDs have overtaken infectious diseases as the leading causes of mortality and morbidity globally. In the context of diet and lifestyle linked NCD epidemic, type 2 diabetes (T2D), obesity, and cardiovascular disease (CVD) are key contributors to the global public health burden.

Among major NCDs, type 2 diabetes is a chronic and complex syndrome of interlinked metabolic conditions caused by a systemic breakdown of glucose homeostasis (Shaw *et al*., 2010; Wilson *et al*., 2005). The International Diabetes Federation (2017) estimates the global prevalence of T2D to be 425 million individuals, with another estimated 212 million undiagnosed individuals. Low- and middle-income countries are disproportionately affected by this disease as they account for 79% of the adults with T2D, with evidence linking between nutrition patterns and epidemiological transition (International Diabetes Federation, 2017). Additionally, another 352 million individuals have been identified as being prediabetic or having an elevated risk of developing T2D (International Diabetes Federation, 2017).

1.3.1. Etiology of Type 2 Diabetes

In general, diabetes mellitus or T2D is a chronic disease of metabolic dysregulation, characterized by the existence of impaired glucose metabolism. Most often, it is accompanied by various complications including cardiovascular disease, nephropathy, neuropathy, and retinopathy (Nolan *et al*., 2011). While the risk of developing T2D is governed to an extent by genetic predisposition, environmental factors such as the quality of lifestyle and diet play a very important role too.

The impairment of glucose homeostasis in individuals affected by T2D is due to insufficient insulin secretion by the pancreatic β-cells, and/or the resistance to the action of insulin at the target cells. This leads to the establishment of hyperglycemia, which is a key risk factor in the progression of T2D and associated complications. Brief periods of hyperglycemic exposure can lead to acute changes in cellular metabolism, which on recurrence, can cause cumulative changes in cellular function and structure, even after restoring glycemic balance. Chronic hyperglycemia, on the other hand, is the causal link between T2D and long-term degenerative complications associated with it.

Glucose toxicity, a condition closely linked with chronic hyperglycemia, results in deleterious effects induced from elevated oxidative stress due to impaired mitochondrial function, where generation of ROS occurs at an elevated rate and the activity of antioxidant defensive systems declines. Under hyperglycemic conditions, the products of increased glucose flux passing through the mitochondria from Krebs cycle leading to an increase in the concentration of reducing factors (NADH and FADH2), which then lead to increased proton-linked metabolic flux through the electron transport chain (ETC). This increases mitochondrial membrane potential, which then partially inhibits flux through complex III of the ETC. Coenzyme Q (CoQ) becomes reduced, causing the diversion of electrons from CoQ to oxygen, thus forming superoxide anions (Nishikawa *et al*., 2000; Brownlee, 2001; Rolo and Palmeira, 2006).

Epidemiological studies have identified four major metabolic pathways that contribute to hyperglycemia-induced cell damage: a) increased polyol pathway flux, b) increased production of advanced glycated end (AGE) products, c) activation of protein kinase C (PKC) isoforms, and d) increased hexosamine pathway flux. Hyperglycemia induced overproduction of superoxide

anions in the mitochondria has been identified as the trigger that drives each of these pathways (Du *et al*., 2000; Nishikawa *et al*., 2000; Brownlee *et al*., 2001).

Alteration of molecular and cellular components by oxidative stress affects cellular mechanisms such as autophagy or inflammation. These processes are altered or disrupted, which contributes to a further increase in oxidative stress and insulin resistance. Vascular endothelial cells are particularly vulnerable to damage caused by inflammatory response triggered by the action of AGE and ROS, and these are key steps in the pathogenesis of endothelial dysfunction, which leads to micro-and macrovascular complications (Burgos-Moron *et al*., 2019; Wellen and Hotamisligil, 2005). Also, insulin resistance can cause an increase in serum levels of free fatty acids (FFA). The normal function of vascular tissues deteriorates with high concentrations of FFA, causing macrovascular complications such as coronary artery disease, peripheral arterial disease, and stroke, which often lead to premature death of the diabetic patient (Burgos-Moron *et al*., 2019). Given the implication of oxidative stress in the onset and progression of T2D, the use of exogenous antioxidants through dietary sources represents a feasible and effective component in therapeutic strategies to prevent or manage chronic hyperglycemia in humans. Therefore, understanding the role of chronic oxidative stress on T2D pathogenesis and prevention of oxidative breakdown is important for addressing global T2D epidemic challenge. Diet, especially nutritional composition of daily dietary intake plays critical role in maintaining cellular redox (reduction and oxidation) balance and helps to counter chronic oxidative stress of intracellular systems.

Consuming hyper-processed macronutrient-rich foods in combination with decreased physical activity leads to excessive levels of soluble carbohydrates and free-fatty acids within cells, which over time can lead to an overload of mitochondrial oxidative reactions that then

produces an excess of reactive oxygen species (ROS) that cannot be quenched through normal cellular defense mechanisms (Ceriello and Motz, 2004; Singh *et al*., 2009). On the contrary, plant-based foods that are rich in natural antioxidants are excellent dietary targets to manage chronic cellular oxidation and potentially support prevention and management of NCDs, such as T2D, CVD, and obesity (Sarkar and Shetty, 2014).

The abundance of natural antioxidants in plants is mainly to provide protection against abiotic and biotic stress-linked oxidative breakdowns, which are extremely common in oxygen-driven cellular energy production system under aerobic environment. When consumed as part of diet, these antioxidants present in plant-based foods can also help to counter NCD-linked oxidative breakdown and can be targeted as dietary antidote against T2D, CVD, and other NCDs, especially to manage chronic inflammation state.

1.4. Chronic Oxidative Stress: A Common Physiological Challenge of Aerobic Environment that Affects Both Plant and Animal Health

Eukaryotic organisms living in aerobic environments derive energy to drive normal physiological functions from mitochondrial respiration. It is evolutionarily conserved process common to both plants and animals, involving a tightly regulated series of redox reactions that converts energy dense compounds to adenosine triphosphate (ATP). However, when eukaryotes are subjected to some form of stress, the cellular redox homeostasis is vulnerable to disruption. If unchecked, this can lead to the excessive generation of ROS, that can harm various cellular processes and functions (Addabbo *et al*., 2015).

1.4.1. Oxidative Stress and Plant Health

In plants, adverse environmental conditions such as abiotic stresses undermine the normal physiological and metabolic functions and can disrupt regular redox regulation. These abiotic

stress-induced cellular redox breakdowns favor the uncontrolled and excessive production of deleterious free radicals, including reactive oxygen species (ROS), thus resulting in chronic oxidative stress. Like other abiotic stresses, salt and waterlogging stress also results in excessive formation of ROS in plant cells and subsequent damages of cellular organelles.

Specifically, salt stress leads to stomatal closure, leading to a reduction in the amount of CO2 available for carbon fixation, while exposing chloroplasts to excessive excitation energy which in turn increase the generation of ROS such as superoxide (O_2 •–), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and singlet oxygen. Also, excessive levels of mineral ions exert significant osmotic effects, leading to a condition of water deficit which impacts several key metabolic activities and also leads to the formation of ROS (Greenway and Munns 1980; Cheeseman 1988).

On the other hand, in the case of waterlogging, oxygen deprivation disrupts the mitochondrial electron transport chain and ATP synthesis via diminished tricarboxylic acid cycle (TCA), causing affected cells to shift to less efficient process of fermentation, which is unsustainable. Furthermore, diminished photosynthetic efficiency reduces the ability of chloroplasts to effectively utilize incident photons under stress, leading to a breakdown in cellular redox balance and exertion of oxidative stress due to excessive formation of ROS. The risk of cellular damage may increase several folds even upon the removal of flooding stress, due to the abrupt exposure of affected tissue to elevated levels of oxygen. Excessive accumulation of ROS which can cause peroxidation of lipids, oxidation of protein, inactivation of enzymes, DNA damage, and/or interaction with other vital constituents of plant cells, leading to the disruption of vital cellular processes. Therefore, the maintenance of bioenergetic and redox homeostasis at the

cellular level represents a vital target for improving overall crop fitness in regions prone to salt and waterlogging stress.

1.4.2. Oxidative Stress and Human Health

As with plants, humans are also eukaryotic organisms, and therefore vulnerable to ROS mediated oxidative stress, when exposed to extraneous stresses that disrupt the normal physiological functions. Data emerging from mechanistic studies indicate that oxidative stress, and consequent inflammatory responses plays a crucial factor in the development and progression of various chronic NCDs such as obesity, diabetes, cancer, cardiovascular disease, chronic respiratory and neurological diseases (Camps and Garcia-Heredia, 2014).

Chronic oxidative stress is a key component in the etiology of T2D. In this disease state, hyperglycemic conditions lead to the mitochondrial apparatus being overloaded by excessive glucose flux and can directly lead to elevated levels of ROS production, beginning with the superoxide anion. In this state, oxidative stress can be further exerted due the disruption of inherent cellular antioxidant systems, such as regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG), and the activity of enzymes like catalase (King and Loeken, 2004).

The resultant oxidative stress can contribute to the destruction of pancreatic β-cells, leading to impaired insulin production. Additionally, oxidative stress can damage vascular tissue, by causing modifications in the proteins, lipids, and nucleic acids in the endothelial and smooth muscle cells, leading to diabetic complications (Schaffer *et al*., 2012). Therefore, just as in the case of plant health, maintenance of cellular redox balance is critical to maintaining physiological balance and managing the risk of chronic NCDs such as T2D.

Inclusion of beneficial and natural antioxidants in the diet has been recognized as a viable strategy to supplement the innate redox homeostatic mechanisms in humans and reduce the risk

of chronic degenerative diseases (Hung *et al*., 2004). Specifically, plant-based foods that are rich in natural antioxidants can be targeted for this purpose, as they are inexpensive, safe, and easily accessible. Further, they may be suitable for supplementing pharmacological approaches to T2D management, thereby providing a holistic means of disease management (Sarkar and Shetty, 2014). Among diverse natural antioxidants, secondary metabolites of plants are gaining increasing attention, especially for integrating dietary intervention and therapeutic strategies to counter chronic oxidative breakdowns commonly associated with NCDs.

1.5. Phenolic Metabolites – Bioactive Compounds of Plants with Dual-Relevance in Improving Plant and Human Health

As discussed in the previous section, the maintenance of redox homeostasis is vital for ideal physiological and metabolic regulation in eukaryotes, including in plants and humans. In this context, phenolic compounds, most abundant secondary metabolites of plants, may offer a unique opportunity to address the goals of improving plant resilience to environmental stresses and to improve human health relevant bioactive functionalities in foods derived from such stress resilient plants.

Overall, phenolic compounds constitute a diverse, heterogeneous class of plant secondary metabolites, that can perform a wide range of physiological functions. The primary function of plant phenolics is to provide protection against a broad spectrum of pathogens and insects and also to counter abiotic stress-induced damages. They also participate in various signal transduction pathways and have been shown to modulate plant-microbe interactions, including symbiotic processes such as nitrogen fixation.

One of the most significant properties of phenolic metabolites is their potent antioxidant capacity, due to which they can function as efficient free radical scavengers at the cellular level.

Additionally, they are also capable of stimulating a range of other antioxidant defense responses, which include the mobilization of protective antioxidant enzymes in various cellular compartments (Shetty and Wahlqvist, 2004).

Interestingly, the increased dietary intake of phenolic metabolites from plant-based food sources has demonstrated great potential in improving human health as well. Phenolic compounds from plant-based foods have exhibited several beneficial biological functions in humans such as antioxidant, antimutagenic, anticarcinogenic and hypoglycemic activities. Of specific interest is the potential of phenolic compounds to help prevent and manage chronic NCDs, such as T2D and associated vascular complications. In addition to their antioxidant potential, phenolic compounds of plant-based foods are also having other health protective functions such as anti-hyperglycemic properties, which can help to modulate the metabolism of soluble carbohydrates, and therefore aid in maintain glycemic control. Further, their antioxidant capacity may help to maintain cellular redox homeostasis and protecting cells against oxidative stress induced impairment and related pathophysiological pathways commonly associated with chronic hyperglycemia (Sarkar and Shetty, 2014).

Therefore, phenolic metabolites derived from plant sources and their diverse biological functions can be targeted for dual benefits; i) to improve abiotic and biotic stress resilience in food crops, as well as ii) to improve human health through dietary intake of phenolic enriched plant-based foods. In this context, enhancing the biosynthesis of phenolic compounds potentially provide a means to simultaneously improve abiotic stress resilience, and NCD benefits relevant nutritional qualities of food crops (Shetty and Wahlqvist, 2004). However, the phenolic content and composition of phenolics vary widely among different plant species, and even within different varieties of same species. Additionally, environmental factors and growing conditions

also play large role in distribution of phenolics in different plant systems. Therefore, it is important to understand and optimize the phenolic biosynthesis relevant metabolic regulation of a target food and beverage crop such as barley, which is targeted for scientific investigations in this dissertation, prior to advancing the dual benefits of phenolic metabolites for improving abiotic stress resilience and human health relevant nutritional qualities.

1.6. Selection of Barley as a Candidate Crop for Climate Change-Linked Food and Nutritional Insecurity Solutions

Barley (*Hordeum vulgare* L.) is a widely cultivated food crop (cereal) that has been adapted to both high-input, industrial-scale cropping systems as well as low-input, subsistence agriculture systems. It is an important source of feed and forage for livestock, and food for humans. Barley has move with human migration widely across the world since its initial domestication about 10 millennia ago and is currently grown in a wide range of agro-ecological conditions, evidencing its strong potential for adaptation to various environmental niches.

Barley has been identified as a very good plant model for understanding plant responses to climate change. The primary reason is that barley is resilient to certain abiotic stresses, which are detrimental to most other cereal crops, such as soil salinity and water deficit (Ogle and John, 2010). However, barley is relatively susceptible to abiotic stresses such as waterlogging and high temperature stress (Setters and Waters, 2003). In either case, limited information is available with regard to the phenolic biosynthesis and associated mobilization of endogenous antioxidant enzyme responses and potential metabolic role of critical amino acid proline to dictate such redox regulation in barley under salinity and waterlogging stresses.

Furthermore, a growing body of epidemiological evidence suggests that barley is a significant source of beneficial phytochemicals, including phenolic compounds, capable of

positively influencing human health, especially in relation to glycemic control. Further to this, the baseline phenolic profile of barley grains may be improved upon to enhance the content, availability and activity of these compounds using metabolic driven strategies. Therefore, barley was chosen as a candidate crop to study the metabolic regulation and stimulation of phenolic biosynthesis, and to understand the dual benefits of barley phenolics for improving abiotic stress resilience, as well as for NCD-linked human health benefits (Figure 1.1).

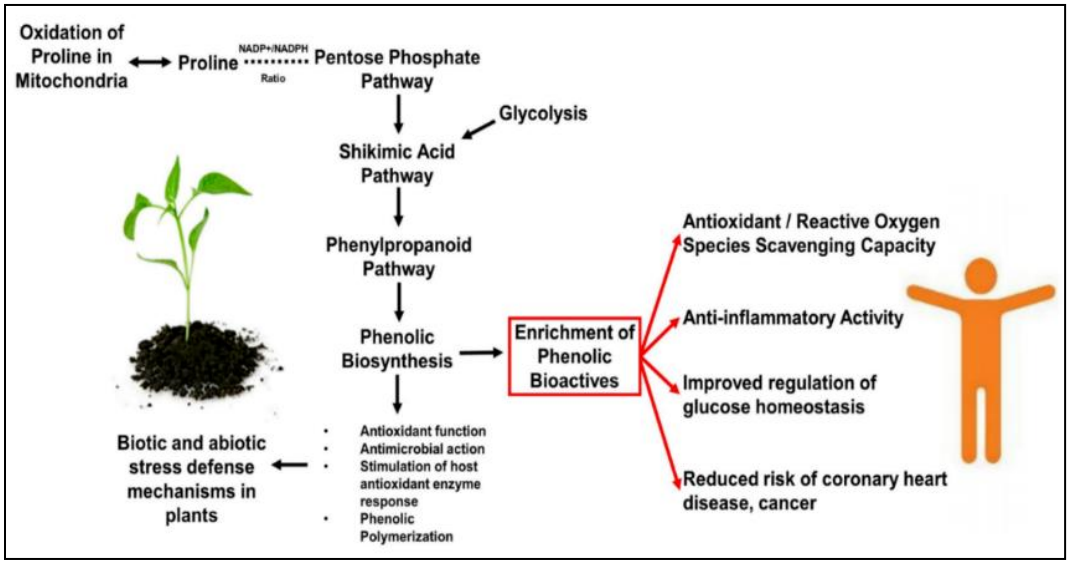

Figure 1.1. Mechanisms for metabolically driven strategies to potentially improve barley phenolics for dual benefits of abiotic stress resilience improvement and NCD-linked health benefits.

CHAPTER 2. REVIEW OF LITERATURE

2.1. Barley: An Overview of the Production, Usage and Nutritional Profile

Barley is among the most widely grown cereal grains in the world and has been utilized as a source of animal feed, food, and beverages since ancient times. Globally, over 48 million hectares were under barley cultivation with a combined output of 144 million metric tons (Shewry and Ullrich 2014). It is one of the major cereal crops grown for malt production (for beer) and animal feed purposes in the United States, and the Northern Great Plains alone accounts for 51% of the total barley currently produced in the country (USDA Climate Hubs, 2019) Among cereal crops, it ranks as the fourth most widely cultivated in terms of acreage, and ranking fifth among major agricultural commodities, in terms of total dry matter produced, after corn, rice and soybean (Tricase *et al*., 2018; Zhou, 2009).

Most of the barley cultivated globally is utilized primarily for animal feed (65%), followed by malt production (33%), with only a very small fraction (2%) being consumed as or processed into food products (Ullrich, 2011; Sullivan *et al*., 2013). Barley has been relatively underutilized as food source since it has been traditionally recognized and consumed as a food in fewer traditional cultures historically, compared to certain other cereals such as wheat and rice. Furthermore, barley is a cool season crop best suited for temperate climatic zones. Certain agronomic traits also restrict the cultivation, and availability of barley such as its susceptibility to winter kill (compared to wheat, *Triticum aestivum* L.), intolerance to waterlogging (as opposed to rice, *Oryza sativa* L.), susceptibility to diseases in regions that experience hot and humid climates (Jacobs and Whitten, 2016) and requirement of well-drained soil for almost all growth and developmental stages.

Recent years have witnessed a resurgent interest in incorporating barley into food products, driven by a growing body of epidemiological evidence linking the regular consumption of barley with reduced risk of NCDs such as hypertension, chronic heart disease and hyperglycemia (Baik and Ullrich, 2008; Shewry and Ullrich, 2014). Barley grains have also been reported to have immuno-stimulatory properties and to be involved in improving immune response and potentially preventing certain types of cancer (Kanauchi *et al*., 1999; Tada *et al*., 2009).

The health benefits of barley have been attributed to the presence of the functional constituents of barley grains, that include insoluble and soluble dietary fiber (β-glucans) as well as various phytochemicals. The role of β-glucans in imparting health benefits in humans has been extensively studied and firmly established, particularly in the context of regulating blood cholesterol levels and exerting glycemic control (Behall *et al*., 2004; Braaten *et al*., 1991; Cavallero *et al*., 2002; Fadel *et al*., 1987; Newman *et al*., 1989; Pins and Kaur, 2006; Wood *et al*., 1990). Consequently, foods containing substantial levels of barley β-glucans are technically considered to be functional foods, and are permitted by the Food and Drug Administration (FDA) to carry health claims specifically for their blood cholesterol lowering capacity (Baik and Ullrich, 2008).

While the dietary fiber component of barley grains contributes to the improvement of human health significantly, several studies also suggest that barley is a significant source of several other phytochemical constituents, such as phenolic compounds that can potentially play a role in mitigating risk factors and therefore, the development of chronic diseases (Idehen *et al*., 2017; Okarter and Liu, 2010; Ward *et al*., 2008). Barley is on par with other major cereals, including wheat, oat (*Avena sativa* L.), rye (*Secale cereale* L.) and rice in terms of the diversity

and content of such bioactive compounds. Major classes of barley phytochemicals associated with human health benefits include phenolic compounds, tocols, sterols, folates, γ-aminobutyric acid and bioactive lipids (Idehen *et al*., 2017). These compounds have been reported to individually possess health beneficial effects on physiological and metabolic risk factors for NCDs in various *in vitro* and *in vivo* studies, and thus may collectively contribute to the therapeutic potential of barley grains (Idehen *et al*., 2017). These same bioactive compounds of barley also potentially play critical roles in determining the abiotic and biotic stress tolerance of different barley varieties/cultivars.

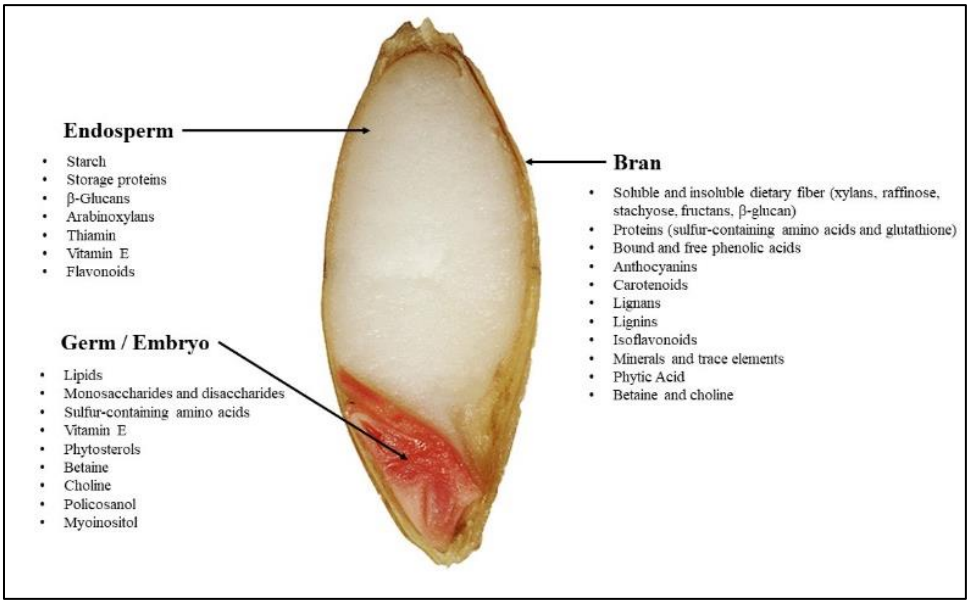

Figure 2.1. The distribution of major biologically active phytochemicals within barley grain (Adapted from The Maltster's Association of Great Britain; http://www.ukmalt.com).

2.2. Biologically Active Phenolic Compounds in Barley

Phenolic compounds are the most complex and diverse class of phytochemicals in cereal grains. They are characterized by the presence of a benzene ring, containing one or more hydroxyl functional groups, as the basic structural unit. They can broadly be categorized as derivatives of either benzoic acid (C6-C2) or cinnamic acid (C6-C3). In plants, phenolic

metabolites are derived from aromatic amino acids via the shikimic acid and phenylpropanoid pathways.

Barley has been recognized as a good source of phenolic compounds in comparison with other conventional cereal grains. In barley kernels, phenolic compounds are mostly concentrated in the pericarp and the aleurone layer. The phenolic acid profile of barley grains may be influenced by the factors like genotype (G), the growing environment (macro and microenvironment) (E) and their interactions. However, processing steps such as malting and kilning have been found Furthermore, post-harvest storage conditions and different processing strategies also influence the content and profile of phenolic bioactives and associated nutritional qualities of foods and beverages that are produced from barley (Cai *et al*., 2015; Zhou *et al*., 2018; Wannenmacher *et al*., 2018).

2.2.1. Simple Phenolic Acids

Phenolic acids are by far the most abundant class of phenolic compounds found in barley kernels. They can broadly be categorized as derivatives of either hydroxybenzoic acid or hydroxycinnamic acid (Figure 2.2) (Shahidi and Naczk, 2003). Based on the ease of extraction, they can be further classified as (i) free phenolic acids, (ii) conjugated acids (soluble forms that are esterified with moieties of low molecular weight such as sugars) and (iii) bound phenolics (insoluble forms that are esterified with non-starch polysaccharides in the cell wall such as arabinoxylans, cellulose, hemicelluloses, and lignin) (Shahidi and Naczk, 2003; Wannenmacher *et al*., 2018). In terms of relative concentration, the free phenolic and conjugated phenolic acid fractions account for up to 3% and 25% respectively, making the bound insoluble fraction the most dominant form in barley kernels (Baik and Ullrich, 2008).

A great degree of variation in the phenolic acid profile of barley grains has been reported by various investigators, primarily due to the differences in the extraction and analytical methodology employed, thus making comparisons between studies challenging. The free phenolic fraction was found to range between 8.1 – 17.5 µg/g in nine barley cultivars (Goupy *et al*., 1999) while the bound phenolic fraction in sixteen lines ranged between 604 – 1347 µg/g (Holtekjølen *et al*., 2006) and the total phenolic acid content in 11 lines ranged between 357 – 604 µg/g (Quinde-Axtell and Baik, 2006).

Andersson *et al*., (2008) evaluated the content of phenolic acids and their concentrations in the free, bound, and conjugated fractions in 10 barley cultivars grown at the same location. The study found that the free phenolic fraction was rich in ferulic, vanillic, syringic and 2,4-dihydroxybenzoic acids; the conjugated fraction in ferulic, 4-hydrobenzoic, vanillic, sinapic and 2, 4-dihydroxybenzoic acids and the bound fractions in ferulic and p-coumaric acids.

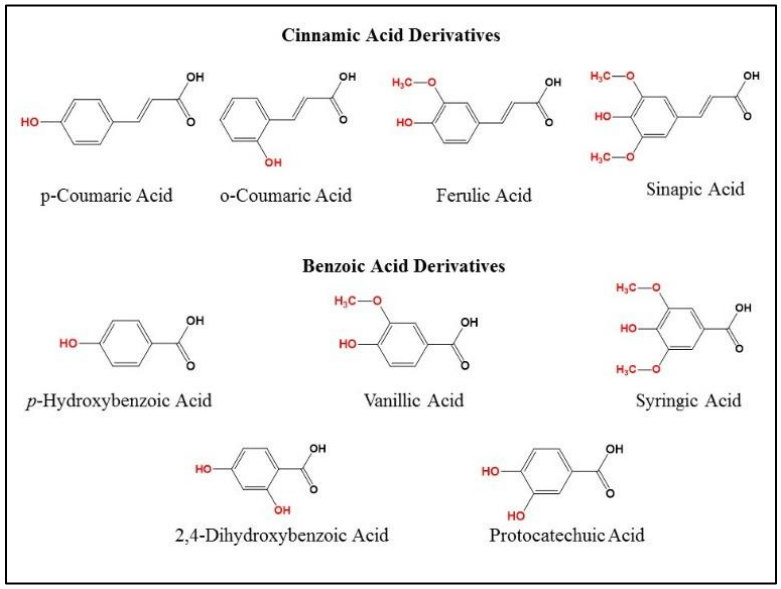

Figure 2.2. Structures of key phenolic acids found in barley.

Various studies have identified ferulic acid as the major phenolic acid in barley, accounting for approximately 68% of the total phenolic acid content. The total ferulic acid

content in barley grains varies between 149 – 413 µg/g (Andersson *et al*., 2008). Additionally, several dimeric forms of ferulic acid were also found in barley, such as (Z)-β-{4-[(E)-2-carboxyvinyl]- 2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamic acid; (E, E)-4,40 -dihydroxy-5,50 -dimethoxy-30 -bicinnamic acid; trans-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3- carboxylic acid and (E, E)-4,4-dihydroxy-3,50 -dimethoxy- β,30 -bicinnamic acid (Hernanz *et al*., 2001).

2.2.2. Flavonoids

In general, flavonoids constitute a vast class of diverse polyphenolic compounds; whose members share a common 'C6-C3-C6' skeleton. The dominant classes of flavonoids in barley grains include flavan-3-ols and polymeric forms such as proanthocyanidins and anthocyanins. Flavonoids impart pigmentation in the hulls and aleurone of cereal grains, and consequently influence the overall hue and color of the grain. In general, flavonoids are found in greater proportion in barley cultivars with pigmented grains, such as blue- and purple-colored grains containing the highest total flavonoid content. Most of these compounds exist as soluble glycosides and are localized in the pericarp and the aleurone layers of the grain (Idehen *et al*., 2017).

Among different flavonoids, flavanols, anthocyanins, and proanthocyanidins are the most dominant types in barley kernels. Flavanols and anthocyanins are located in the pericarp of barley grains where they exist mostly as glycoside derivatives, including cyanidin-3-glucoside, penidin-3-glucoside, and delphinidin-3-glucoside (El-Sayed *et al*., 2006; Gamel and El-Sayed, 2012). Flavonoid content in barley grains shares a strong positive correlation with the degree of color depth (Liu *et al*., 2013).

The flavonoid content of 127 lines of hulled and unhulled colored barley was found to range between 62.0 and 300.8 μg/g (Kim *et al*., 2007). Further, the monomeric, dimeric, and trimeric flavanol components were found to account for 58–68% of the total phenolic content of barley with trimeric flavanols being the most abundant flavanols in barley (Goupy *et al*., 1999). The proanthocyanidin content in barley studied by Kim *et al*., (2007) varied between 15.8 μg/g and 131.8 μg/g. The proanthocyanidin content of the unhulled barley group (75.9 μg/g) was higher than that of the hulled group (56.29 μg/g), whereas the proanthocyanidin content of the blue and purple group (83.0 μg/g) was significantly higher than that of the black group (55.3 μg/g) (Kim *et al*., 2007).

Dvorakova *et al*., (2008) found that the major proanthocyanidins in barley kernels included two proanthocyanidin dimers (prodelphinidin B3 and procyanidin B3) and four proanthocyanidin trimers (procyanidin C2, prodelphinidin C2, and 2 other prodelphinidin isomers), across 10 barley cultivars (Figure 2.3). Prodelphinidin B3 (90–197 μg/g) accounted for majority of proanthocyanidin present in barley, whereas procyanidin C2 (5–19 μg/g) was reported to be present only in minor quantities.

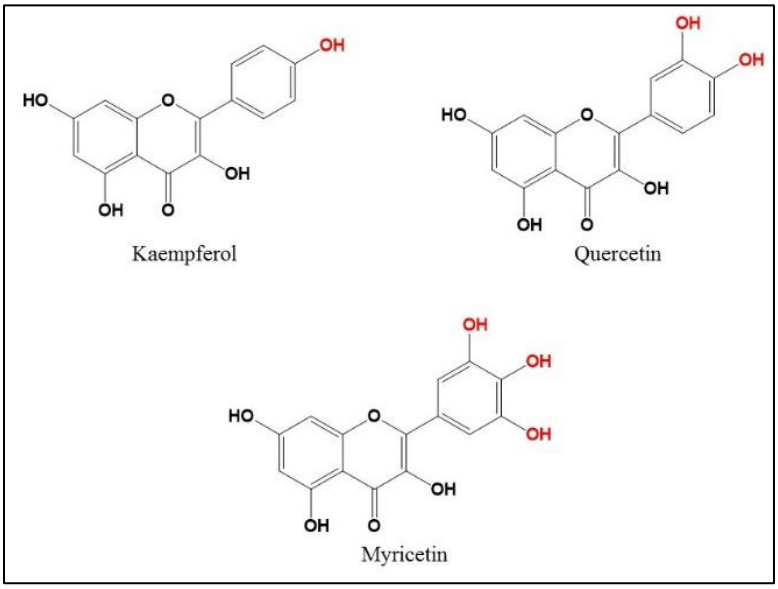

Figure 2.3. Structures of key flavonoids found in barley.

2.2.3. Anthocyanins and Proanthocyanidins

Anthocyanins are the most predominant group of flavonoids in colored barley cultivars and found in the pericarp and aleurone layers of the bran, causing the kernels to appear blue or purple. The most commonly detected anthocyanins in barley include cyanidin, cyanidin 3-glucoside, delphinidin, pelargonidin, pelargonidin glycosides, and petunidin 3-glucoside (Figure 2.4, 2.5) (El-Sayed *et al*., 2006; Mazza and Gao, 2005). Common anthocyanins in purple barley include cyanidin 3-glucosode, peonidin 3-glucoside and pelargonidin 3-glucoside, which account for up to 70% of the total anthocyanin content. In blue and black barley cultivars, delphinidin-3-glucoside is the most abundant anthocyanin. Jende-Strid (1993) reported the presence of delphinidin and cyanidin in yellow, blue, and black barley varieties, whereas pelargonidin was present exclusively in purple barley.

Yang *et al*., (2013) evaluated unhulled purple, normal, and hulled purple barley cultivars. This study determined that the flavonoids were located mainly in the bran fraction, whereas the hull had negligible flavonoid content. The mean flavonoid content of hulled purple barley

(124.8 μg/g) was significantly higher than that of unhulled purple barley (69.40 μg/g) and normal barley (48.50 μg/g), while total catechin content of up to 21.85 μg/g was reported. The mean catechin content in whole grain normal barley was significantly higher than that in hulled purple barley and unhulled purple barley, while myricetin content in hulled purple barley was significantly higher than that in unhulled purple barley and normal barley. The mean quercetin content of hulled purple barley (60.98 μg/g) was significantly higher than that in the unhulled purple barley (24.35 μg/g) and normal barley. The total average kaempferol content in unhulled purple barley (36.00 μg/g) was considerably higher than that in hulled purple barley (32.56 μg/g) and normal barley (26.65 μg/g).

All these bioactive compounds of barley have specific role in the defense responses against various biotic and abiotic stresses including their role in physiological and metabolic regulation and to provide structural supports under stresses. These same bioactive compounds also have diverse human health protective functions and when consumed as part of a diet may provide benefits to prevention and management of diet-linked chronic diseases, such as T2D, CVD, and other NCDs. Based on such potential dual beneficial roles of barley secondary metabolites, their specific role in salinity and waterlogging stress resilience as well as their T2D relevant anti-hyperglycemic function were investigated as part of the objectives of this dissertation.

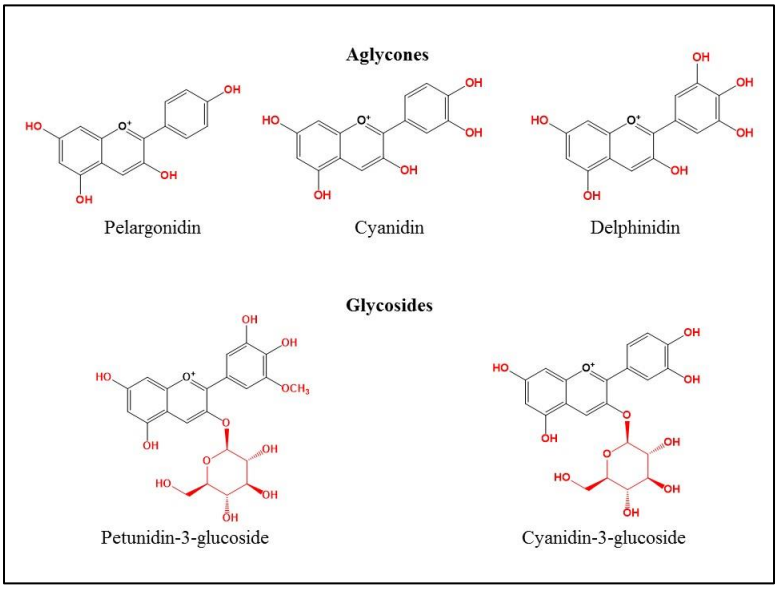

Figure 2.4. Structures of key anthocyanins found in barley.

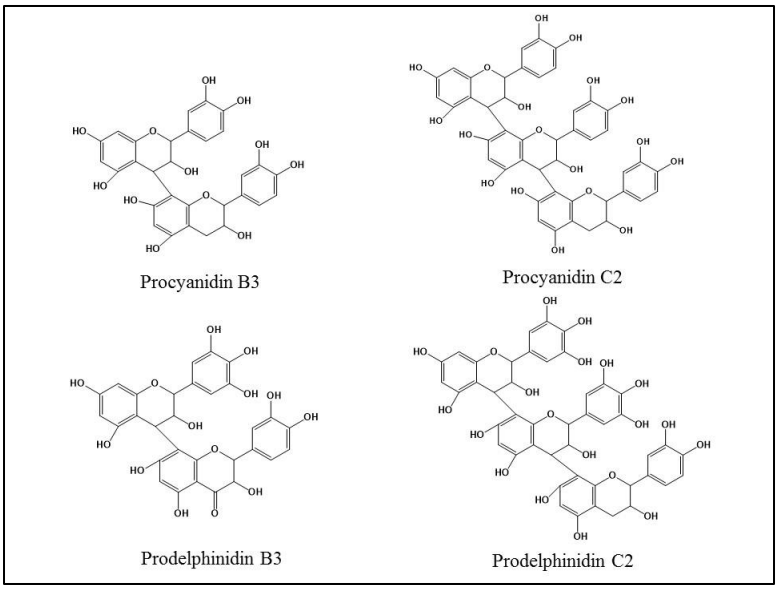

Figure 2.5. Structures of key proanthocyanidins found in barley.

2.3. Role of Dietary Phenolics in Improving Glycemic Control

A significant body of research has indicated a positive correlation between the consumption of plant-based foods that are rich in phenolic compounds and improved glycemic control in various cell and animal-based study models. The beneficial properties of phenolic compounds from grains, herbs, vegetables, and fruits have been extensively reported and

reviewed. However, in recent times, whole grains such as barley have been increasingly gaining attention as viable dietary sources of phenolic compounds, which in combination with other functional components such as soluble fiber (beta-glucans) can help manage and regulate post-prandial glucose levels (Belobrajdic and Bird, 2013).

Dietary phenolic phytochemicals can influence the digestion, absorption, and metabolism of dietary carbohydrates, which may aid in managing T2D (Hahnineva *et al*., 2010; Kardum and Glibetic, 2018; McCue and Shetty, 2003). Such carbohydrate metabolism relevant benefits can be achieved by various mechanisms, such as inhibiting carbohydrate digestion and glucose absorption in the brush border cells of the small intestine; stimulating insulin secretion from β-cells of the pancreas, modulating the release of glucose from hepatic cells, activating insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Hanhineva *et al*., 2010; McCue and Shetty, 2003). Carbohydrate digestion and glucose absorption are key control points for post-prandial glycemic response, with α- amylase and α-glucosidase being the key enzymes responsible for the digestion of dietary carbohydrates to glucose. It is posited that, like pharmacological agents currently prescribed for glycemic control, phenolic compounds can inhibit the activity of these key glycosidic hydrolases prior to absorption into the bloodstream (Hanhineva *et al*., 2010). Several studies indicate that phenolic metabolites including flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanidins and ellagitannins) inhibit α-amylase and α-glucosidase activities *in vitro* (Hanhineva *et al*., 2010). Phenolics of plant-based foods including berries (strawberries, raspberries, blueberries, and black currants), vegetables (pumpkin, beans, and eggplants), green and black tea and red wine, and whole grains (cite some

paper) shown significant α-amylase and α-glucosidase inhibitory activities in *in vitro* studies (Cheplick *et al*., 2010; da Silva Pinto *et al*., 2008; Kwon *et al*., 2008; McCue *et al*., 2005).

As an additional layer of protection, phenolic compounds also exert significant antioxidant activity (Adom and Liu, 2002; Gamel and El-Sayed, 2012). Phenolic acids in cereals have demonstrated similar or higher antioxidant activities than catechins and have been linked to prevention of chronic disease due to the presence of unsaturated carboxylic group (Sidhu *et al*., 2007). Zielinski and Kozlowska (2000) found a very strong positive correlation between total phenolic compounds and total antioxidant activity of methanolic extracts of barley whole grains. Goupy *et al*. (1999) studied the antioxidant properties of barley phytochemicals. The antioxidant properties of barley flavonoids, phenolic acids (especially FA), and tocopherols (α, β, γ) determined by their quenching of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were approximately 4.76, 0.34, and 0.89 antiradical power, respectively. Furthermore, the antioxidant activity determined by the inhibition of co-oxidation of β-carotene in linoleate model system for various barley flavonoids ranged from 16.4% - 65.5%, whereas those for phenolic acids ranged from 12% -31.2%, while those of tocopherol ranged from 49.1 - 55.2%. Antioxidant activities of barley components were also evaluated based on their ability to inhibit lipoxygenase. The antioxidant activities recorded were 24.8 - 65.4% for flavonoids, 2.9 - 10.8% for phenolic acids, and 49.1 - 63.9% for tocopherols. Zieliński and Kozłowska (2000) ranked the AOA of various whole grains in descending order as barley > oats > wheat > rye.

The above evidence suggests that barley phenolics and other bioactives have potential beneficial roles for glycemic control and for countering oxidative breakdown-linked damages in humans. As part of their evolutionary function in plant cells, these same secondary metabolites of barley also provide protection against biotic and abiotic stresses, especially to ameliorate

stress-induced oxidative breakdowns and to counter other metabolic dysregulation and to support physiological and structural adjustments. However, the role of phenolic compounds in endogenous defense responses of barley vary among cultivars/varieties, different growth stages, and based on different stress factors (cold/heat/salinity/drought/waterlogging). Therefore, it is important to study the specific function and regulation of phenolics and phenolic biosynthesis based on metabolic rationale prior to targeting them for improving abiotic stress resilience of barley.

2.4. Barley under Salt Stress

Barley is a relatively salt tolerant crop, in comparison to wheat, rice or corn (*Zea mays* L.), but there exists a great degree of genetic variation across cultivars for this phenotypic attribute. In some cases, barley plants have reportedly withstood electrical conductivity (Ec) levels of up to 8 dS m-1 in the rootzone without significant loss of yield (Ogle and John, 2010).

It is notable that barley can continue to grow while accumulating high concentrations of Na+ in the leaf tissue (Rengasamy *et al*., 2003). Such high tissue tolerance of barley most likely involves the ability to sequester Na+ into intracellular vacuoles and the synthesis of compatible solutes in the cytoplasm to counterbalance the osmotic potential of the vacuolar Na+ (Widodo *et al*., 2009). In a previous study, Tilbrook *et al*. (2017), evaluated 24 Australian barley lines for salt tolerance and found that the most resilient lines had considerable shoot ion-independent tolerance. Other studies have attributed the salt tolerance of barley plants to different mechanisms such as selective exclusion of Na+ and Cl- ions in the root zone, maintenance of high cellular K+:Na+ ratios, accumulation of osmoprotectants, elevated levels of protective antioxidant enzyme activity and the increased synthesis of organic acids which can serve as

readily available carbon sources under salinity stress (Ahmed *et al*., 2015; Chu *et al*., 1976; Colmer *et al*., 2005; Munns *et al*., 1995; Widodo *et al*., 2009).

The ability of barley to sustain growth even under salt stress could make it a suitable alternative annual crop in salt-affected areas and a cover or forage crop in non-cultivated areas affected by moderate salinity (Tober *et al*., 2007). Additionally, the application of barley cover as a means of sequestering excess Na+ and Cl- ions from saline effluents resulting from the processing of tar sands to crude oil, thereby aiding the reclamation of affected soils, has also shown potential (Renault *et al*., 2003). More importantly, it can serve as an important cereal model for metabolomic studies, especially those aiming to transfer salt tolerance traits to other grain crops with lower tolerance, such as wheat or rice (Widodo *et al*., 2009). Therefore, understanding metabolic regulation and its role in determining susceptibility and tolerance of barley cultivars under salinity stress has significant merit.

2.5. Barley under Waterlogging Stress

Being a dryland crop, barley has very low waterlogging tolerance and performs poorly in soils with insufficient drainage (USDA, 2016). It has the least resilience to flooding stress in comparison to all other major cereal crops, and the yield of barley may be reduced by up to 50% in regions that receive high rainfall and have poor drainage (Setters and Waters, 2003). Waterlogged soils diminish vegetative growth and development of barley plants, while increasing their vulnerability to other abiotic and biotic stresses such as fungal attacks. Current efforts to overcome waterlogging stress in barley are aimed at breeding and screening barley germplasm for tolerant genotypes. However, such strategies are complicated by the fact that waterlogging resilience is a complex trait in barley, which has relatively low heritability (Mendiondo *et al*., 2016).

Setter *et al*. (1999) demonstrated a genetic diversity in waterlogging tolerance of barley exposed to intermittent waterlogging over 4 weeks. Yordanova and Popova (2001) found that exposure of barley plants to flooding for 72-120 hours led to a decrease in the rate of CO2 assimilation, transpiration, chlorophyll content and dry biomass. They also observed a reduction in stomatal conductance and the activities of ribulose 1,5-bisphosphate carboxylase and phosphoglycolate phosphatase and glycollate oxidase. However, phosphoenolpyruvate carboxylase activity, proline content and leaf acidity increased in response to flooding (Yordanova and Popova, 2001). In a separate study, Yordanova *et al*. (2005) found catalase, ascorbate peroxidase and peroxidase activity to be significantly increased in response to flooding. Regardless of the increased activity of hydrogen peroxide scavenging enzymes, flooding treatment caused a substantial rise in total endogenous peroxide concentration. The results suggested that root oxygen deficiency caused photooxidative damage to barley leaves via an increased generation of active oxygen species.

Thus, it is of interest to better understand the underlying metabolic and physiological responses of susceptible plants such as barley under waterlogging stress and target critical control points in metabolically relevant defense response pathways to improve crop fitness and productivity. Moreover, while most studies dealing with waterlogging stress on barley focus on improvement of agronomic traits, there is ample scope to delineate possible means by which cellular redox balance can be improved in stressed plants. In this context, understanding the redox-linked metabolic regulation, such as role of pentose phosphate pathway (PPP) to drive carbon flux towards secondary metabolite synthesis and to support diverse endogenous defense related anabolic responses under abiotic stresses is extremely relevant.

2.6. Phenolic Biosynthesis in Plants and the Role of Proline Associated Pentose Phosphate Pathway (PAPPP)

Phenolic compounds in plants are synthesized through the sequential activities of the pentose phosphate (PPP), shikimate, and phenylpropanoid pathways (Shetty 1997, Shetty 2004). The first rate-limiting step of the PPP is the conversion of glucose-6-phosphate (G6P), mediated by glucose-6-phosphate dehydrogenase (G6PDH), which converts G6P into ribulose-5-phosphate, while simultaneously producing reducing equivalents - nicotinamide adenosine di-phosphate hydrogen (NADPH) - for cellular anabolic reactions (Phang, 1985). The PPP also generates erythrose-4-phosphate from glycolysis, which is channeled together with phosphoenol pyruvate (PEP) into the shikimate pathway to produce phenylalanine, which is further directed towards the phenylpropanoid pathway to produce phenolic metabolites (Shetty and Wahlqvist, 2004).

Being a component of the inducible defensive mechanism of plants, phenolic metabolites can be synthesized in response to several biotic and abiotic stress induced stimuli. However, given their broad spectrum of beneficial biological actions, the delineation of a mechanism to overexpress consistently and efficiently holds great merit.

Shetty (1997) proposed a proline-associated pentose phosphate pathway (PAPPP) model in plant systems, whereby phenolic metabolites in plants can efficiently be induced by bridging cellular proline metabolism with the regulation of the pentose phosphate pathway (PPP) (Shetty, 1997; Shetty and Wahlqvist, 2004). The model suggests that cytosolic proline synthesis may be coupled with the PPP by favoring the reduction of NADP+: NADPH ratio (i.e., favoring NADPH production) thereby activating the NADPH-dependent PPP. This in turn can drive carbon flux from primary metabolism towards biosynthetic pathways associated with secondary

metabolism, such as the phenylpropanoid and shikimic acid pathways (Shetty and Wahlqvist, 2004).

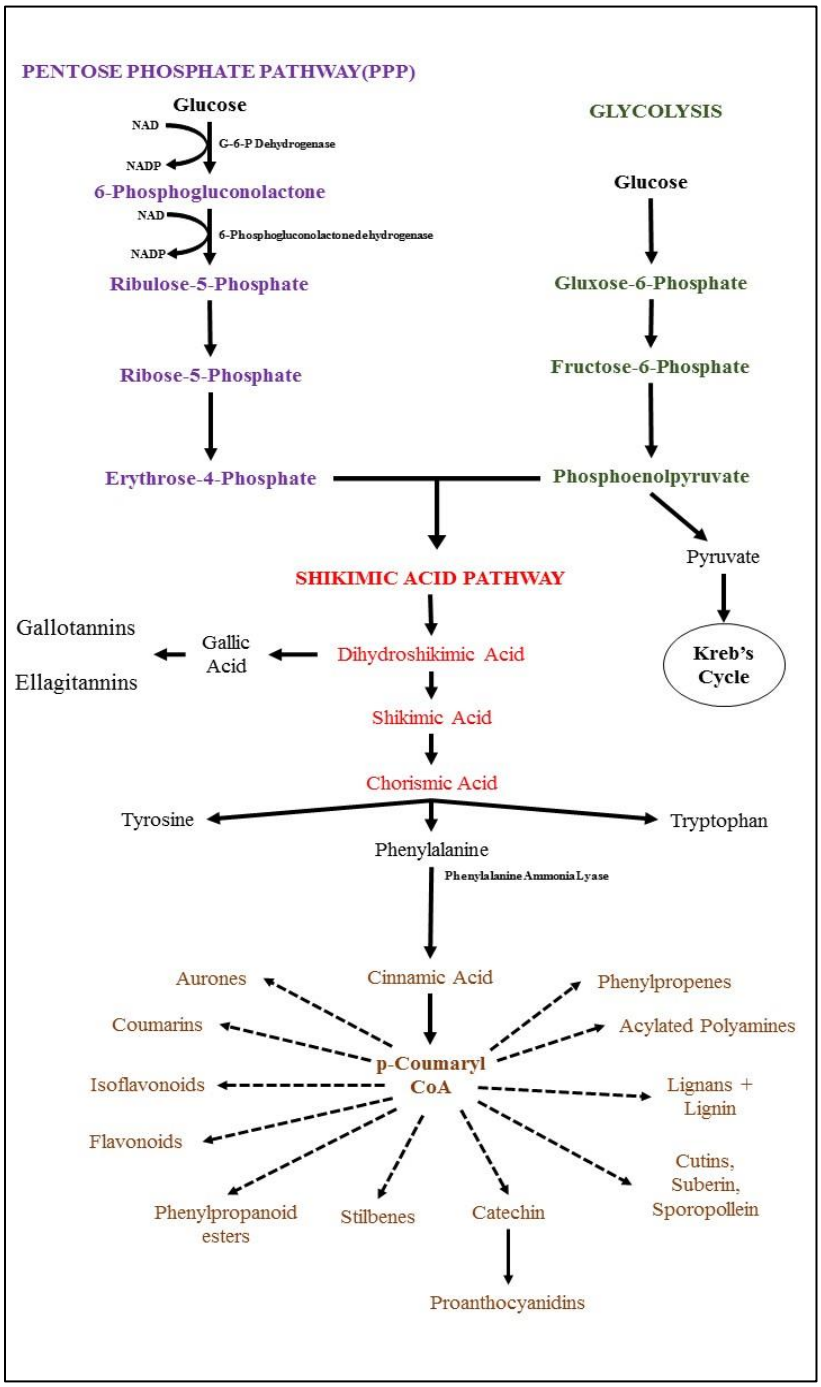

Figure 2.6. Overview of the biosynthesis of phenolic compounds in plants.

Furthermore, the model posits that proline may act as a reducing equivalent instead of NADH during ATP synthesis and support mitochondrial oxidative phosphorylation (Hare and Cress, 1997), which is more energy efficient under abiotic stresses. The PPP also serves as the source of carbon skeletons by supplying erythrose-4-phosphate, which along with phosphoenolpyruvate from glycolysis, are channeled into the shikimic acid pathway to produce the aromatic amino acid phenylalanine, and which moves subsequently to the phenylpropanoid pathway to produce phenolic compounds (Shetty and McCue, 2003).

Additionally, the upregulation of PAPPP provides NADPH, an anabolic co-factor essential for the biosynthesis and activation of endogenous antioxidant enzymes such as SOD, CAT, and GPX. Therefore, the induction of elevated levels of phenolic biosynthesis coupled with the regulation of PAPPP also stimulates endogenous antioxidant enzyme responses in the host plant (Duval and Shetty, 2001; Randhir and Shetty, 2005; Shetty and McCue, 2003). As abiotic stresses favor the stimulation of shikimate and phenylpropoanoid pathways simultaneously in plants, the up regulation of PAPPP could lead to the optimum stimulation of inducible phenolic biosynthesis under such stress condition.

During abiotic stresses, a coupled enzymatic and non-enzymatic defense system could involve low molecular weight antioxidants, such as ascorbate, glutathione, α-tocopherol, carotenoids and phenylpropanoids, in conjunction with several enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases, glutathione reductase, ascorbate peroxidase, and non-enzymes like inducible phenolics (Gill and Tuteja, 2010; Mittler, 2002; Rao *et al*., 1996). Generation of NADPH in PPP can also potentially help nitrate reduction in the cytosol, as it provides reducing factors for nitrite reduction. Beyond the induction of phenolic biosynthesis, the upregulation of PAPPP may potentially support the synthesis and mobilization of key growth

regulators such as phytohormones and purines too (Shetty and Wahlqvist, 2004). As a result, the redox-linked PAPPP regulation provides a possible means of stimulating the production of beneficial secondary metabolites like phenolic compounds, and other bioactive molecules that can aid in establishing and maintaining redox and metabolic regulation, leading to improved overall fitness and resilience to abiotic stresses. Several metabolically driven pre-harvest and post-harvest strategies can be advanced to improve phenolic biosynthesis and subsequent improvement of abiotic stress tolerance through stimulation of redox-linked PAPPP regulation in food crops such as barley.

2.7. Pre-harvest Seed Priming to Stimulate Phenolic Biosynthesis for Improving Abiotic Stress Resilience and Health Relevant Nutritional Qualities

Seed priming refers to the induction of a desired physiological state in plants, by treating the seeds with natural and/or synthetic compounds, prior to germination. It has been established as an effective method of physiologically activating seeds prior to emergence, to improve their germination efficiency, seedling emergence, growth, abiotic stress tolerance, and overall fitness without adversely affecting the end use quality of harvested grains or pods (Jisha *et al*., 2012).

In general, seed priming improves seed vigor, which then helps reduce emergence time, accomplish uniform emergence, and establish better crop stand (Ashraf and Foolad, 2005). Enhanced, rapid and uniform emergence, with high vigor and better yields were observed with seed priming in vegetables and floriculture (Bruggink *et al*., 1999) and in some field crops (Basra *et al*., 2005; Kaur *et al*., 2005). The primed seeds acquire the potential to rapidly imbibe water and revive the seed metabolism thus enhancing the germination rate (McDonald, 2000). Such revitalization of seeds also helps to mobilize nutrients, which is important for improving seedling emergence and seedling vigor during early growth stages. Additionally, seed priming

treatments can also stimulate endogenous defense responses in plant through mimicking biotic and abiotic stress induction, and therefore are relevant for improving abiotic stress tolerance of food crops. In this seed priming context, several bio-based and bioprocessed elicitors are available to improve seed and seedling vigor, however it is important to optimize the efficacy of these natural compounds prior to integrating them in sustainable food production strategies.

2.7.1. Bioprocessed Elicitors as Seed and Foliar Treatments

A plant elicitor or biostimulant is either a natural or synthetic chemical compound or microbial culture, applied exogenously on plants, to enhance vigor, fitness, nutrient uptake efficiency, stress tolerance and/or crop quality traits (du Jardin, 2015). This comprises a very broad range of formulations containing carbohydrates, proteins and amino acids, lipids, glycoproteins, and volatile compounds, which may be of plant, microbial or animal origin (Angelova *et al*., 2014).

One of the most widely studied applications of elicitor compounds is based on their ability to stimulate inducible defensible responses in plants. Specific elicitor compounds may be used to upregulate plant-secondary metabolite biosynthetic pathways, consequently stimulating an overexpression or beneficial metabolites which are crucial for improving crop fitness, and have commercial relevance (Angelova *et al*., 2015).

With regard to the targeted induction of PAPPP, and stimulation of associated secondary metabolite biosynthesis and defensive antioxidant responses, which is the metabolic rationale of this dissertation, the efficacy of various types of plant and animal based bioprocessed elicitor compounds have been studied in several food and non-food plant models (Sarkar and Shetty, 2014). Among these natural elicitors, two in particular – namely, marine protein hydrolysates (MPH) and chitosan oligosaccharides (COS) - were found to stimulate biologically significant

levels of PAPPP-mediated phenolic biosynthesis and antioxidant enzyme responses in different plant models (Andarwulan and Shetty, 1999; Horii *et al*., 2007; Orwat *et al*., 2017; Randhir and Shetty, 2003; Randhir and Shetty, 2005; Randhir *et al*., 2004; Randhir *et al*., 2009; Sarkar *et al*., 2010)

Such bioprocessed elicitor treatments may function by serving as sources of free proline or proline analog and/or mimicking external biotic stresses such as fungal attacks, in order to drive the oxidation-linked metabolic stimulation of anabolic PPP to synthesize defensive secondary metabolites, while simultaneously activating antioxidant enzyme complexes. As a result, seed priming with such elicitors may potentially improve the seedling emergence and seedling establishment in the early stages of vegetative growth, while enhancing endogenous defense relevant metabolic and physiological adjustments with which food crops can respond better to abiotic stresses from germination to pre-harvest stages.

2.7.1.1. Chitosan Oligosaccharide (COS)

Chitosan oligosaccharides (COS) are produced by the deacetylation and subsequent hydrolysis of marine chitin. These water-soluble, polycationic compounds can vary widely in their degree of polymerization and deacetylation and are often processed to incorporate functional moieties, such as ascorbic acid, that can influence their biological function (du Jardin, 2015).

Chitosan oligosaccharides are widely used in various agronomic applications, especially in the improvement of crop quality, fitness, and resilience (Gavhane, 2013). COS-mediated biostimulation has been applied in improving crop resilience towards biotic stresses such as fungal pathogens as well as abiotic stresses like drought, salinity, and cold-stress (du Jardin, 2015). The physiological effects of COS derivatives are attributed to their ability to bind to

cellular components and receptors which are involved in the activation of defensive genes (du Jardin, 2015). Further, they resemble fungal cell wall fragments, and therefore can trigger critical host adaptive responses in plants at very low doses without causing harmful effects (El Hadrami *et al*., 2010; Kim and Rajapakse, 2005). The possible mechanism involved in such a response is the induction of pathogen and damage associated molecular pattern (DAMP / PAMP) signal cascades, which include elevated synthesis and accumulation of phenolic compounds and phytoalexins (Sarkar and Shetty, 2014; Trouvelot *et al*., 2014). Bioprocessed COS, when applied as seed or foliar treatments have proven to be effective against in stimulating secondary metabolite synthesis in plants for improving biotic and abiotic stress resilience (Agrawal *et al*., 2002; Gavhane, 2013; Khan *et al*., 2003; Orwat *et al*., 2016; Prapagdee *et al*., 2007; Sarkar *et al*., 2010).

2.7.1.2. Marine Protein Hydrolysates

Like chitosan oligosaccharides, protein hydrolysates (PHs) are a category of elicitors or biostimulants comprising a mixture of polypeptides, oligopeptides, and amino acids, derived from protein sources using partial chemical or enzymatic hydrolysis (Schaafsma, 2009). They are largely produced from agro-industrial by-products of plant or animal origin (du Jardin, 2015). Marine protein hydrolysates (MPHs) are a subset of PHs that are produced from proteolytic digestion of the by-products of fish processing and seaweeds.

Protein hydrolysates have demonstrated multi-functional properties that help improve crop performance, physiological status, and tolerance to environmental stresses. Protein hydrolysates are able to impart these benefits directly by modulating the signaling pathway of nitrogen acquisition in roots, thus improving nitrogen assimilation. They also regulate enzymes in the tricarboxylic acid cycle (TCA), thereby contributing to the physiological cross talk

between nitrogen and carbon metabolisms (du Jardin, 2015). Further, certain PHs and nitrogenous compounds contribute to soil remediation and protection against environmental stresses by chelating heavy metal ions and scavenging deleterious free radicals. Further, they significantly improve aeration, water retention, fertility, and microbial biomass in soil, thus also being able to influence crop health indirectly. Additionally, the chelating and complexing properties of PHs containing certain peptides and amino acids also aid in enhanced availability and absorption of essential micronutrients in the rootzone (du Jardin, 2015).

Several PHs have been found to induce secondary metabolic pathways and defensive response in plants, and tolerance to stresses including salinity, drought, oxidative conditions, and extreme temperatures (Kauffman *et al*., 2007; Apone *et al*., 2010; Ertani *et al*., 2013). Ertani *et al*. (2013). Previous study reported a concurrent increase in the synthesis of protective flavonoids and activity of the enzyme phenylalanine ammonia lyase (PAL) involved in the phenylpropanoid pathway, in response to alfalfa PH treatment, in salt-stressed maize plants. In this case, enhanced phenolic biosynthesis has been attributed to the PAPPP hypothesis, whereby the increased biosynthesis and availability of free proline in response to PH treatment encourages carbon flux towards the oxidative pentose phosphate pathway and subsequently towards the phenylpropanoid pathway (Shetty *et al*., 2003; Shetty and Wahlqvist, 2004). Several previous studies have reported the improvement of seed vigor, seedling emergence and mobilization of phenolics through protein hydrolysate seed and foliar treatments (Andarwulan and Shetty, 1999; Horii *et al*., 2007; Randhir and Shetty, 2003; Randhir and Shetty, 2005; Randhir *et al*., 2002; Randhir *et al*., 2004a, 2004b; Randhir *et al*., 2009). Like these elicitor-based pre-harvest strategies, there are several bio-based innovations that can be advanced at post-harvest stage to improve human health relevant phenolic metabolites and their associated functionalities in plant-based foods.

2.8. Post-harvest Grain Bioprocessing Strategy to Improve Human Health Relevant Functionalities Targeting Type 2 Diabetes Relevant Benefits

Barley grains contain a broad range of bioactive phytochemical constituents with the potential to impart various health benefits and help mitigate the risk factors of systemic metabolic disorders commonly associated with NCDs. However, the accessibility, availability, and thus the bioactivity of many of these compounds are limited in their native, unprocessed form. A key consideration to make is that certain compounds such as bound phenolics are insoluble and would require some form of intense physical or chemical processing or extraction step or biocatalysis to release them and improve their availability. Further, other compounds such as gamma amino butyric acid (GABA) that may be present in relatively low quantities in intact grains can be enhanced via bioprocessing steps such as germination. Therefore, the following section outlines various processing methods that can be employed to improve the availability and the overall content and bioactivity of barley-based food matrices to improve their functional properties and to provide health benefits to consumers.

2.8.1. Sprouting

Grain sprouting is an inexpensive primary processing method that finds usage even in traditional food customs around the world. It refers to the biological activation of grains upon the imbibition of enough moisture, followed by physiological and biochemical modification of the grain components to meet the needs of the germinating embryo. From a food processing perspective, the net result of sprouting is the enzymatic hydrolysis of complex nutrient reserves to simple sugars and amino acids, and the mitigation of anti-nutrients such as phytic acid. However, during this process, the carbon and nitrogen flux from the solubilization of macronutrients may also be partitioned towards the synthesis and the mobilization of free and

bound phytochemicals. Several studies have reported a substantial increase in the content and resultant bioactivity of various phytochemicals, such as GABA, ferulic acid, tocols and sterols, in response to sprouting (Singh and Sharma, 2017).

Germination impacts the overall content, availability, and bioactivity of phenolic compounds than most other phytochemicals in cereal grains such as barley. The total phenolic content (TPC) of barley grains has largely been found to increase steadily during germination and sprouting (Ha *et al*., 2016; Kim *et al*., 2013; Lee *et al*., 2017). The mobilization of various classes of phenolic compounds including simple phenolic acids, flavonoids, and other polyphenols contribute to the overall increase in total phenolic content (Kim *et al*., 2013). This can be attributed to the hydrolysis of insoluble phenolic compounds bound to non-starch polysaccharides by esterases, and the conversion of phenolic glycosides to aglycones by glycolytic enzymes (Singh and Sharma, 2017). Upon biological activation during germination, the anabolic pentose phosphate, shikimic acid and phenylpropanoid pathways are also activated to synthesize a broad spectrum of compounds, including phenolic compounds for growth, mechanical support and biological defense (Sarkar and Shetty, 2014; Ti *et al*., 2014). In certain cases however, a decrease in the total phenolic content of barley grains has been observed during the initial stages of germination, which may be attributed to the leaching of soluble phenolic compounds from the hull during steeping (Sharma and Gujral, 2010).

The concentrations of various phenolic compounds are differentially affected during germination. Post germination, the content of certain metabolites such as epigallocatechin (10.94 → 36.59 µg/mL), epicatechin (1.51 → 2.71 µg/mL) and ferulic acid (12.53 → 14.03 µg/mL) increased, whereas that of gallic acid (4.7 → 2.9 µg/mL), catechin (20.81 → 18.94 µg/mL), epigallocatechin gallate (4.67 → 1.73 µg/mL), p-coumaric acid (2.43 → 0.68 µg/mL) and

luteolin (159 → 12.29 µg/mL) decreased (Kim *et al*. 2013). A strong positive correlation exist between the phenolic content of plant-based matrices, including barley grains, and total antioxidant capacity and free radical scavenging capacities (Adom and Liu, 2002; Rice-Evans, *et al*., 1996). Further to this, germination also appears to have a positive effect on the antioxidant potential of barley grains (Donkor *et al*., 2012; Ha *et al*., 2016; Kim *et al*., 2013; Sharma and Gujral 2010).

Germination was also found to positively influence the human-health relevant functionality of barley sprouts. Donkor *et al*. (2012) observed that methanolic extracts of 5-day old barley sprout demonstrated moderate and very high inhibitory activity against α-glucosidase and α-amylase enzymes *in vitro*, thereby indicating the positive influence of germination on the anti-hyperglycemic potential of barley. Similarly, Ha *et al*. (2016) noted that the *in vitro* α-glucosidase and maltase inhibitory activity of germinated sprouts increased after 48 h of steeping (IC50 18.88 mg/mL and 79.33 mg/mL respectively). The content of GABA, a potent neurotransmitter and antihypertensive agent, was also significantly increased in sprouted barley extracts, compared to the non-germinated grain (Donkor *et al*.,, 2012; Lee *et al*., 2017). Additionally, Lee *et al*. (2017) also detected an increase in the content of the alkaloid hordenine (reported to inhibit melanogenesis and the activity of monoamine oxidase, in addition to demonstrating antibacterial and antibiotic properties), and γ-oryzanol, a composite fraction containing ferulic acid esters of triterpene alcohols and plant sterols (reported to have strong antioxidant activity and the ability to lower plasma and serum cholesterol levels and decrease platelet aggregation), in germinated barley. Kim *et al*. (2013) further reported that the anti-proliferative activity of barley extracts at high concentrations (3 mg/mL) towards human liver (HepG2) and colon (HCT-116) cells were improved post germination. However, in the same

study, germinated barley extracts were shown to have lower anti-proliferative effects compared to other cereal grains such as oats. Therefore, sprouting, and subsequent bioprocessing can be an effective strategy to improve human health relevant nutritional qualities in barley and can be targeted for designing functional foods and functional ingredients relevant for NCD-linked health benefits.

2.8.2. Fermentation

Like sprouting, biotransformative process of fermentation has been widely recognized as a potent means of enhancing the nutritional profile of plant and dairy-based foods. It is a highly adaptable and versatile method of beneficially influencing a comprehensive array of attributes in a given substrate such as shelf-life and food safety attributes, organoleptic profile, nutrient to anti-nutrient ratio, and in certain cases, enhanced processing and functional qualities (Singh *et al*., 2015).

During fermentation, biochemical changes induced by microbial metabolism can alter the physical matrix of the grain substrate, and thereby its phytochemical profile. These changes are predominantly mediated by the action of microbial enzymes such as glucosidases, amylases, cellulases, chitinase, inulinase, phytase, xylanase, tannase, esterase, invertase and lipase (Hur *et al*., 2014). Further, changes in pH brought about by microbial action may favor the activity of endogenous grain enzymes such as amylases, proteases, hemicellulases and phytases (Singh *et al*., 2015). The combined effect of these enzymes leads to the release of beneficial phytochemicals from the grain matrix, thereby increasing their potential availability and bioactivity.

The effect of fermentation on the bioavailability of phenolic compounds in cereals such as barley has been widely studied. Đorđević, Šiler-Marinković and Dimitrijević-Branković

(2010) evaluated the impact of fermentation on the phenolic-linked AOA of various cereals and pseudocereals by lactic acid bacteria (LAB; *Lactobacillus rhamnosus*) and yeast (*Saccharomyces cerevisiae*). In this previous study, *L. rhamnosus* was found to significantly improve the total phenolic content of barley (20.1 mg GAE/g D.W.), compared to *S. cerevesiae* and control (18.5 and 16.4 mg GAE/g D.W. respectively). Concurrently, the L. rhamnosus-fermented barley substrate also demonstrated a higher antioxidant capacity as measured by the ferric reducing antioxidant power and lipid peroxidation inhibition methods, than the control and S. cerevisiae-fermented barley samples.

Similarly, Hole *et al*. (2012) reported that fermentation of whole grain barley flour by specific strains of LAB with the ability to synthesize exogenous ferulic acid esterase (FAE) aided in the mobilization of free and bound phenolics in whole grain barley. Of the various strains evaluated, *Lactobacillus johnsonii* LA1 (66.91 µg/g D.M), *Lactobacillus reuteri* SD2112 (55.8 µg/g D.M) and *Lactobacillus acidophilus* LA-5 (48.02 µg/g D.M) were found to improve the total free phenolic acid content from a baseline of 2.55 µg/g D.M. in the unfermented barley flour. These LAB strains were found to favor the increase of ferulic acid content over other phenolic acids in the substrate. On the other hand, all evaluated LAB strains (including those without FAE activity), along with unfermented water extracts, effectively increased the total bound phenolics in the fermented flour by 23± 3 %. The increase in total bound phenolic content was attributed primarily to the mobilization of previously insoluble p-coumaric, ferulic, and diferulic acids from the non-starch polysaccharide matrix of barley flour.

The increase of phenolic compound content and their associated AOA of barley-based substrates may be influenced directly and indirectly by fermentation. Apart from mobilizing phenolic compounds from the grain matrix, fermentative microbes may specifically hydrolyze

phenolic glycosides to their more biologically active aglycones, by synthesizing ligninolytic and carbohydrate-metabolizing enzymes. Additionally, the acidification of the fermented medium also aids in the formation and stabilization of the phenoxide cations, which possess greater antioxidant and free radical scavenging capacity (Singh *et al*., 2015).

Bacterial fermentation may also be used to influence the content non-phenolic bioactive metabolites as well. Rizzello *et al*. (2012) reported exogenous proteolytic activity associated in LAB-mediated sourdough fermentation, resulting in the improvement of bioactive lunasin content. This study also reported that fermentation with specific LAB strains such as *L. curvatus* SAL33, *L. rossiae* CD76, *L. brevis* AM7 and *L. pentosus* 12H6 resulted in a 2-3-fold increase in the lunasin content compared to the unfermented control, after 30 hours of fermentation.

Various studies have also identified fungal/yeast fermentation to be a viable means of manipulating the bioactive content and corresponding functionality of barley based substrates. Gibreel *et al*. (2009) found S. cerevisiae mediated fermentation to significantly enhance the total phenolic and phytosterol content of barley distillers' dry grains with solubles (DDGS), pretreated with Stargen (a mixture of α-amylase and glucoamylase). Notably, improvements in bioactive accumulation occurred to a greater extent in hulled cultivars (Bold and Xena), with the final total phenolic content being comparable to fruits such as figs. These results also reinforce the viability of targeting barley hulls as a potential source of beneficial bioactive compounds. Sandhu and Punia (2017) reported that solid-state fermentation (SSF) of barley grains with *Aspergillus awamorinakazawa* improved the phenolic profile in six evaluated cultivars. The increase in total phenolic content (2890-3922 to 4082-5403 µg GAE/g) and TFC (1968-2198 to 3059-3686 µg GAE/g) was directly proportional to the duration of fermentation over the first 5 days, after which a sharp decrease was observed in both attributes. The AOA of the fermented substrates

measured by metal chelating (31-51% to 82-99 %), ABTS+ scavenging (132-17.8 µmol/g to 119-172 µmol/g) and DPPH scavenging activities (18.3-25.8% to 40-66%) also followed a similar pattern and demonstrated a positive correlation with the phenolic content. Apart from mediating the mobilization and biotransformation of endogenous bioactive phytochemicals, fermentative microbes may also synthesize exogenous bioactive compounds that are of potential benefit to human health. Probiotic microbes, such as LAB, can synthesize and promote the release a wide spectrum of enzymatic and non-enzymatic antioxidant compounds such as catalase, glutathione, exopolysaccharides and antioxidant peptides (Coda *et al*., 2012; Hur *et al*., 2014). Certain strains of LAB may also produce highly potent compounds such as angiotensin-I-converting enzyme (ACE) inhibitor peptides and GABA (Torino *et al*., 2013). All these previous studies suggest that fermentation is an effective biotransformation strategy to improve human health relevant nutritional qualities of food grains such as barley.

However, the application of barley in functional food development is relatively under investigated compared to other cereal grains such as oat. Further, the efficacy of sprouting and fermentation strategies to improve T2D-linked health benefits of barley were also not studied widely. Therefore, the overall goal of this dissertation was to holistically improve and mobilize phenolic metabolites with dual function benefits of improving abiotic stress resilience and T2D-linked health benefits in barley using above mentioned pre-harvest and post-harvest strategies.

CHAPTER 3. OBJECTIVES

The overarching theme of this dissertation was to study the dual function benefits of phenolic metabolites and its' elicitation through metabolically-driven strategies for improving abiotic stress resilience, and T2D-associated health benefits in barley and barley–based foods. With such overarching goals, the objectives of potential improvement of abiotic stress tolerance and human health relevant benefits of barley (outlined in Figure 3.1) were investigated using *in vitro* assay models.

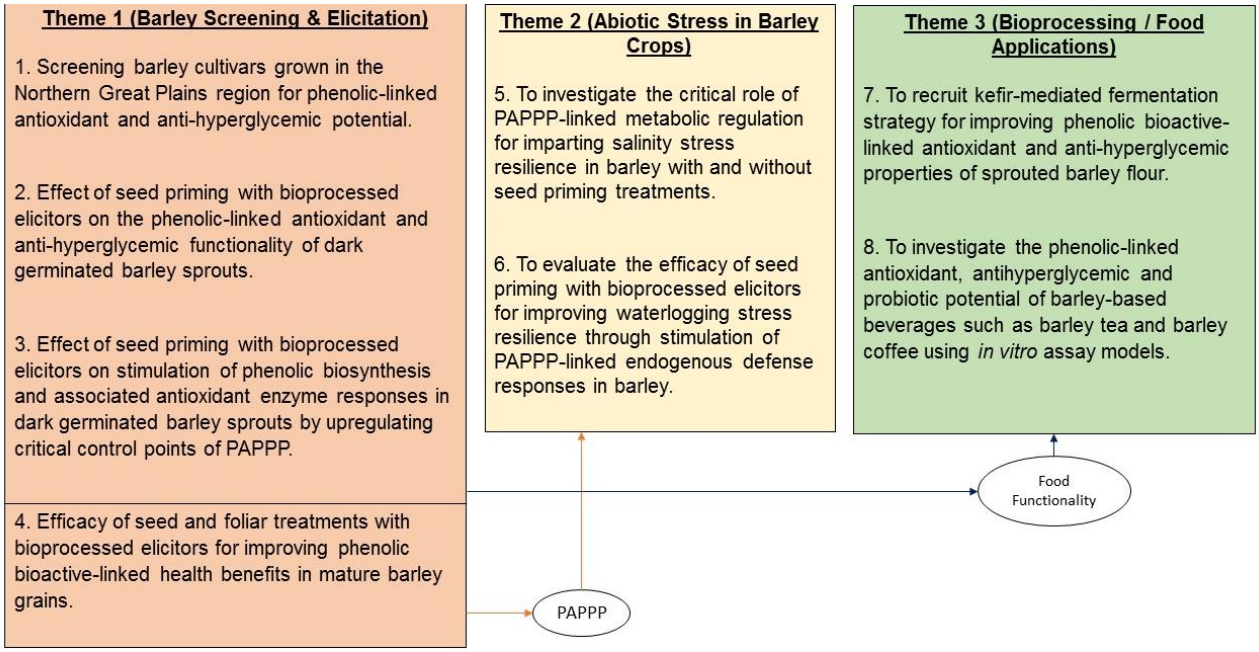

Figure 3.1. Scheme of research objectives.

CHAPTER 4. *IN VITRO* SCREENING OF PHENOLIC LINKED ANTI-HYPERGLYCEMIC BIOACTIVES OF BARLEY CULTIVARS AS NUTRACEUTICALS FOR TARGETING TYPE 2 DIABETES

4.1. Abstract

Wholegrain cereals rich in phenolic bioactives and antioxidant activity are excellent targets for developing non-food nutraceuticals and functional food ingredients to counter non-communicable chronic diseases (NCDs) such as Type 2 Diabetes (T2D) and associated complications. Barley (*Hordeum vulgare* L.) is an important commercial and industrial crop with several applications. It is known to have moderate to high content of phenolic bioactives with potential human health benefits. In this study, 13 barley types from a single crop year were screened for phenolic-linked functionalities targeting antioxidant and anti-hyperglycemic properties using *in vitro* assay models. Total soluble phenolics (TSP) content, individual phenolic acid profile, and antioxidant potential of barley were measured. The antioxidant potential was determined based on ABTS (2, 2'-azino-bis 5(3-ethylbenzothiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals scavenging activities. The α-amylase and α-glucosidase inhibitory activities of barley extracts were also evaluated using *in vitro* assays. The TSP content of barley varied between 0.44 and 0.63 mg/g GAE on a dry weight basis across barley samples and extraction types, with hot water extracts having highest TSP content. Across barley samples, cv. Pinnacle had the highest antioxidant activity, while among extraction types, ethanol extracts had highest antioxidant activity. Cold water and ethanol extracts of most barley samples had significantly high α-amylase inhibitory activity; however, significant differences were not observed among different barley types. The black barley had the highest α-glucosidase inhibitory activity (34%) across all extraction types, and a dose-dependent

pattern was observed in all barley samples. Therefore, barley types with potential to modulate the activity of key enzymes that regulate carbohydrate metabolism were screened and identified in this *in vitro* study. Consequently, grains of such barley screening may be utilized for further clinical and animal studies to develop phenolic bioactive-rich non-food type nutraceuticals and bioactive functional ingredients.

Keywords: α-Amylase; α-Glucosidase; Antioxidant; Bioactive; Nutraceuticals; Oxidative Stress

4.2. Introduction

Barley can be targeted as an industrial crop source to design non-food nutraceuticals and functional food ingredients which can be part of innovative strategies for managing early stages of Type 2 diabetes (T2D). Type 2 diabetes is currently among the most commonly occurring diet and lifestyle-linked non-communicable chronic diseases (NCDs) worldwide. Around 415 million individuals worldwide are suffering from diabetes with more than 90% of the estimated total have T2D (World Health Organization, 2016; International Diabetes Federation, 2016). The global prevalence of T2D and its associated morbidity and mortality is projected to continually increase by around 50% by 2040 AD. In particular, the prevalence and occurrence of T2D among children and young adults is on the rise, making it a serious global public health challenge (International Diabetes Federation, 2016; World Health Organization, 2016). Type 2 diabetes develops in multiple different phases and thus it is important to understand the pathogenesis of this disease to halt and prevent the progression in the early stages (Pratley, 2013).

Chronic hyperglycemia, a condition characterized by persistent and abnormally high postprandial blood glucose levels, has been established as a key contributing factor to the onset of T2D (American Diabetes Association, 2006). It is also linked with the development of micro-

and macrovascular complications associated with T2D, which include cardiovascular disease (CVD), diabetic retinopathy, nephropathy, and neuropathy (Fowler, 2008). Diets comprising of hyper-processed, calorie dense foods, in the form of refined sugars and fats, are a major risk factor for hyperglycemia. Prolonged consumption of calorie-dense and rapidly soluble and digestible carbohydrate rich foods leads to a state of energy imbalance and induce state of chronic oxidative stress. This in turn can trigger a series of metabolic consequences such as protein glycosylation, oxidative damage to proteins and DNA, chronically high plasma insulin and lipid levels, leading to partial or complete metabolic breakdown (Cabalerro, 2007; Popkin *et al.*, 2012). Therefore, the management of chronic hyperglycemia is a critical in the treatment of T2D, especially in its early stages.

A key target in the treatment of chronic hyperglycemia is the modulation of the postprandial absorption of glucose in the human gut (Hanhineva *et al.*, 2010; Inzucchi *et al*., 2015). This is accomplished by using inhibitors to reduce the activities of α-amylase and α-glucosidase, the major digestive enzymes responsible for carbohydrate catabolism *in vivo*. By retarding the activities of these enzymes, the release of glucose from starch is delayed, thereby reducing the rate of absorption and assimilation of glucose into the bloodstream (Hanhineva *et al.*, 2010; Inzucchi *et al*., 2015). Current clinical treatment regimens utilize synthetic inhibitors (i.e., Acarbose, Voglibose, Metformin etc.) that are effective modulators of blood glucose levels. However, drug such as Acarbose can cause excessive inhibition of digestive enzymes, leading to adverse effects on the gastrointestinal and hepatic systems (Murai *et al*., 2002; Kwon *et al*., 2006; Kwon *et al*., 2007; Asgar, 2013). However, natural enzyme inhibitors derived from plant and agricultural crop sources, especially phenolic bioactives have moderate to high inhibitory activity against α-amylase and α-glucosidase without the negative side-effects associated with

synthetic inhibitors (Tundis *et al*., 2010; Kwon *et al*., 2006). These phenolic bioactives also possess strong free radical scavenging potentials reflecting in their high antioxidant activity and could potentially aid in mitigating the effects of hyperglycemia-induced chronic oxidative stress (Rice-Evans *et al*., 1996; Kahkonen *et al*., 1999; Sarkar and Shetty, 2014). Therefore, common agricultural crops rich in phenolic bioactives such as barley grown in industrial scale hold significant potential for the development of safe non-food nutraceutical ingredients, which have the potential to be used to complement or replace synthetic enzyme inhibitors in the management of chronic hyperglycemia in early stages of T2D (Sarkar and Shetty, 2014).

Barley grains are a rich source of phenolic bioactives among the major cereal grains (Ullrich, 1999; Dykes and Rooney, 2007, Wannenmacher *et al*., 2018). Currently, barley is utilized primarily in animal feed and brewing applications. However, barley kernels contain a wide range of phenolic bioactives, and consequently have potential to be used to develop non-food nutraceuticals and bioactive functional ingredients to aid in managing and mitigating hyperglycemia associated with early stages of T2D and its complications. While previous studies have identified and characterized the various classes of phenolic metabolites in barley and determined their antioxidant activity (Carvalho *et al*., 2015; Ferreres *et al*., 2009; Holtekjølen *et al*., 2006; Kim *et al*., 2007; Zhao *et al*., 2006), a gap exists in understanding the effect of phenolic compounds as non-food nutraceuticals in specific areas of human health such as T2D prevention and management. Such human health benefits of major industrial agricultural crops vary widely among genotypes and phenotypes, growing environments, and interactions between genotype × environment (Yu *et al*., 2013). More specifically in this study the potential inhibitory activity of phenolic bioactives from samples of different types of barley against key carbohydrate metabolizing enzymes associated with early stages of T2D was investigated using *in vitro* assay

models. Therefore, the aim of the current study was to screen various types of barley for their phenolic antioxidant-linked functionalities relevant for the management of early stage T2D linked to carbohydrate modulation using *in vitro* assay models, which can then be targeted as a source of non-food nutraceuticals or bioactive functional ingredients.

4.3. Materials and Methods

All chemical reagents including enzymes, and substrates for *in vitro* and bioactive analysis were purchased from Sigma Aldrich Chemical Co. (St Louis, MO, USA).

4.3.1. Sample Preparation

4.3.1.1. Barley Milling

Seeds of various barley cultivars from a single growing season and location were obtained from the Malting Barley Breeding Program, NDSU (2014 barley variety trial at North Dakota State University Experiment Stations- 2-rowed barley cultivars - Black Barley, Conlon, Hockett, ND Genesis, Pinnacle, Rawson; 6-rowed barley cultivars- Celebration, Innovation, Quest, Robust, Tradition, and Lacey). Additionally, a commercial food-grade hull-less barley (Bob's Red Mill brand, Milwaukie, OR) was obtained from Walmart, Fargo, ND. Sixty grams of barley grains were randomly sampled, and ground for 10 min using a disk mill (WonderMill, Pocatello, ID) to obtain fine flour. This study aimed to screen different malting and food barley types for their potential phenolic-associated antioxidant and anti-hyperglycemic functionalities. However, future studies with barley cultivars from multiple growing seasons and multiple locations are required for better understanding of cultivar differences and its effective integration in functional food design with additional *in vivo* models.

4.3.1.2. Preparation of Extracts

Hot water extracts were prepared by mixing 5g of barley flour with 50 mL of distilled water (preheated to 100ºC) in a glass beaker, and constantly stirred on a hot plate at 750 rpm for 15 min. The temperature of the hot plate was maintained at 100°C during this period. The mixture was cooled to room temperature, transferred to polypropylene tubes, and centrifuged at 8500 rpm for 30 min. The supernatant was transferred to another polypropylene tube, and centrifuged again at 8500 rpm 30 min. The supernatant from the second centrifugation step was decanted, stored at 4°C and used for subsequent biochemical and *in vitro* assays.

Cold water and 12% ethanol extracts were prepared by blending 10g of barley flour with 50 mL of solvent (precooled in ice bath to 4°C) for 5 min using a commercial-grade food blender (Waring, Torrington, CT, USA). The resultant mixture was transferred to polypropylene tubes, and centrifuged at 8500 rpm for 20 min. The supernatant was transferred to another polypropylene tube, and centrifuged again at 8500 rpm for 15 min. The supernatant from the second centrifugation step was decanted, stored at 4°C and used for subsequent assays. The ethanol content of 12% was used for extraction reflecting high end barley alcoholic beverage made from malt.

4.3.2. Total Soluble Phenolic (TSP) Content

The total soluble phenolics (TSP) content of the barley extracts were measured using the Folin-Ciocalteu (FC) based assay (Shetty *et al*., 1995). The barley extract (1 mL) was combined with 1 mL of 95% ethanol and 5 mL of distilled water in a test tube. To this mixture, 0.5 mL of FC reagent (50% v/ v), followed by 1 mL Na_2CO_3 (5% v/v) were added, vortexed thoroughly and incubated in the dark for 60 min. Absorbance values were measured at 725nm using a spectrophotometer (Genesys UV-visible, Milton Roy Inc., Rochester, NY). Standard curves were

prepared using various concentrations of gallic acid (10 - 300µg/mL) in 95% ethanol. Absorbance values were converted to TSP and expressed as milligram equivalents of gallic acid per gram dry weight of barley kernels. The FC based assay method to determine TSP content is also relevant for measuring the reducing equivalent and in this study specifically aligned with *in vitro* antioxidant assays for better understanding of overall antioxidant potential of targeted barley samples.

4.3.3. DPPH Free Radical Scavenging Activity

Antioxidant activity was measured using a modified DPPH (1, 1- diphenyl-2-picrylhydrazyl, Sigma Chemical Co.) free radical scavenging assay (Cervato *et al*., 2000). A volume of 0.25 mL of barley extract was added to 1.25 mL of 60 µM DPPH stock solution (adjusted to absorbance 2.0 at 517 nm) in 95% ethanol. The samples were vortexed and after 5 min the absorbance was measured at 517 nm. Control samples contained 0.25 mL of 95% ethanol instead of sample extracts. The antioxidant activity of the extracts was expressed as % inhibition of DPPH free radical formation and was calculated per the following formula:

$$Inhibition\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\ \text{x100}$$

4.3.4. ABTS Free Radical Scavenging Activity

Antioxidant activity of barley extracts was also measured using the ABTS [2, 2–azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay (Pellegrini *et al*., 2002). The ABTS radical cation was prepared by mixing 5 mL of 7 mM ABTS solution with 88 mL of 140 mM $K_2S_2O_4$ solution. This stock solution was stored in the dark at 4°C for 12–16 h before use. Prior to the assay, this mixture was diluted with ethanol at an approximate ratio of 1:88 (ABTS: ethanol) and adjusted to yield an absorbance of 0.70 ± 0.02 units at 734 nm. One mL of ABTS was added to 50 µL of extract and the mixture was vortexed thoroughly. The

sample was incubated at room temperature (22- 24°C) for 2.5 min and absorbance was measured at 734 nm. Determining total antioxidant activity by using both DPPH and ABTS based assays is relevant to measure hydrophilic and lipophilic antioxidants present in the aqueous food matrix and was particularly used with the context of TSP content and phenolic profile. The antioxidant activity of the barley extracts was expressed as percentage (%) inhibition of ABTS free radical formation and was calculated per the following formula:

$$Inhibition\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \text{ x } 100$$

4.3.5. α-Amylase Inhibitory Activity

The α-amylase inhibitory activity was determined by an assay modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a). A volume of 500 µL of barley sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine α-amylase (0.5 mg/mL) were incubated at 25°C for 10 min. Following this, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixtures were further incubated at 25 °C for 10 min. The reaction was stopped with 1 mL of dinitrosalicylic (DNS) acid and incubated in a boiling water bath for 10 min and then cooled to room temperature. The reaction mixture was then adjusted using distilled water such that absorbance could be measured at 540 nm. The absorbance of sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were also recorded. The final absorbance of barley extracts was obtained by subtracting its corresponding sample blank reading. The α-amylase inhibitory activity was calculated as percentage (%) inhibition per the following equation:

$$Inhibition\ (\%) = \frac{Abs_{control} - (Abs_{extract} - Abs_{sample\ blank})}{Abs_{\ control}} \times 100$$

4.3.6. α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity was determined using an assay modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a; McCue *et al*., 2005). A volume of 50 µL of sample solution and 100 µL of 0.1M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 unit/mL) were incubated in 96-well microplates at 25°C for 10 min. Following this, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) solution in 0.1M phosphate buffer (pH 6.9) was added to each well at timed intervals and reaction mixtures were incubated at 25°C for 5 min. Absorbance values at 405 nm were recorded before and after incubation using a microplate reader (Thermomax, Molecular Device Co., Sunnyvale, CA) and compared to a control containing 50 µL of buffer solution instead of sample extract. The α-glucosidase inhibitory activity was expressed as percentage (%) inhibition and was calculated per the following equation:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

4.3.7. Determination of Major Phenolic Compounds using HPLC

Sample extracts were micro-centrifuged for 10 min at 13000 rpm, from which a small volume (5 µL) of the sample was subjected to chromatographic analysis using reverse phase HPLC (Agilent 1260 Infinity Series equipped with DAD 1100 diode array detector; Agilent Technologies, Palo Alto, CA). A gradient elution method, involving 10 mM phosphoric acid (pH 2.5; Solvent A) and 100% methanol (Solvent B), was used. Sample extracts were eluted on a C-18 analytical column (Agilent Supelco SB-C18 250 x 4.6 mm internal diameter) with a packing material particle size of 5 µm, at a flow rate of 0.7 mL/min at ambient temperature, with a total run time of 25 min. Pure standards of gallic acid, protocatechuic acid, catechin, chlorogenic acid,

caffeic acid, quercetin, and p-coumaric acid in 100% methanol were used to calibrate retention times on the standard curve. The chromatograms so obtained were analyzed using the Agilent Chemstation integration software.

4.3.8. Statistical Analysis

A completely randomized design (CRD) was used for all *in vitro* assays in this study. For each barley type, 2 separate lots of grain from the same location and single crop year were analyzed with 6 samples from each lot. The means of these 12 samples for each cultivar, with standard error are reported in this study. Analysis of variance (ANOVA) using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC) was performed and mean separations between barley types and extraction types were determined using the Tukey's least mean square test at a confidence level of 95% ($p < 0.05$).

4.4. Results

4.4.1. Total Soluble Phenolic Content and Total Antioxidant Activity

Total soluble phenolic (TSP) content was determined using the modified Folin-Ciocalteu method described by Shetty *et al*. (1995). The TSP content of the barley samples analyzed in this study ranged between 0.41 and 0.63 mg/g D.W. across all extraction types (Figure 4.1). Significant differences in TSP content between all three extraction types were observed ($p < 0.05$). The TSP content of hot water extracts was significantly higher (0.42-0.63 mg/g D.W.), followed by ethanol (0.47 – 0.62 mg/g D.W.) and cold-water extracts (0.45-0.54 mg/g D.W.). However, in the commercial food grade hull-less barley samples, TSP content of cold-water extracts was higher than ethanol extracts of the same barley type (Figure 4.1). Among the barley samples, Pinnacle had the highest TSP content in hot water extracts (0.64 mg/g D.W), while in cold water and ethanol (12%) extracts hull-less barley had higher TSP (0.54 and 0.62 mg/g

respectively). The TSP of hot water extracts of Pinnacle was found to be significantly higher than Hockett, Conlon, Black Barley and Robust ($p < 0.05$), while being at par with the other barley samples (Figure 4.1).

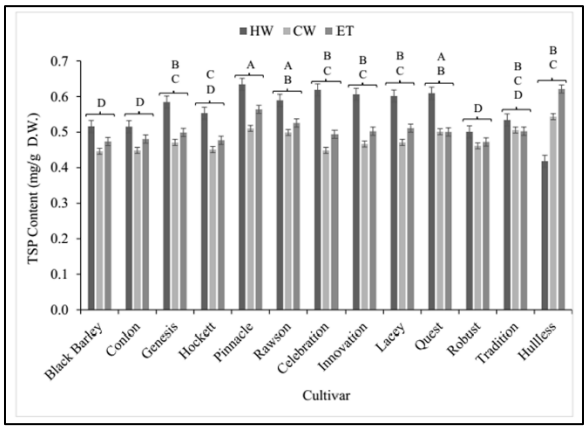

Figure 4.1. Total soluble phenolics content (mg/g dry weight) (n = 12) of hot water (HW), cold water (CW), and 12% ethanol (ET) extracts from samples of 13 different types of barley. Different capital letters represent significant differences between barley samples at $p < 0.05$.

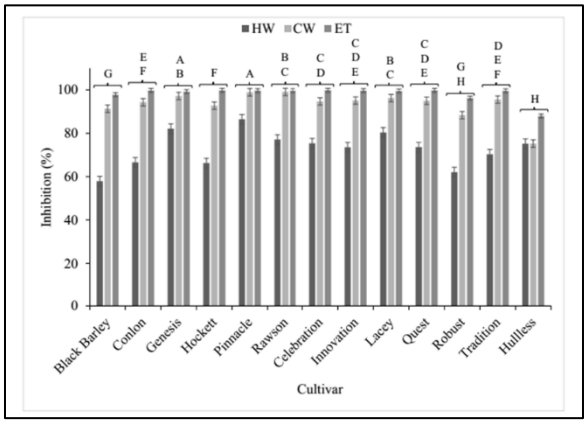

Figure 4.2. ABTS free radical scavenging capacity (% inhibition) (n = 12) of hot water (HW), cold water (CW), and 12% ethanol (ET) extracts from samples of 13 different types of barley. Different capital letters represent significant differences between barley samples at $p < 0.05$.

The antioxidant activity of the various barley extracts was determined using the DPPH (Cervato *et al*., 2000) and ABTS (Pellegrini *et al*., 2002) free radical scavenging assays. Both antioxidant assays were used in the context of soluble phenolics present in the aqueous extracts of the barley samples. The ABTS-based antioxidant activity in samples of various types of barley

ranged between 57.91% and 100% (Figure 4.2). Antioxidant activity (ABTS-based) of Pinnacle was found to be significantly higher than all cultivars, except Genesis (Figure 4.2).

The ABTS-based antioxidant activity also positively correlated with the TSP content in the barley sample. The DPPH–based antioxidant activity of various barley samples ranged between 19.25% and 57.83% (Figure 4.3). The antioxidant activity (DPPH) of Pinnacle was found to be significantly higher than all other barley samples (Figure 4.3). Significant differences ($p < 0.05$) in antioxidant activity between the three extraction types were also observed in both DPPH and ABTS-based free radical scavenging assays. In both cases, ethanol (12%) extracts of barley kernels were found to have the highest antioxidant activity, followed by cold and hot water extracts (Figures 4.2, 4.3).

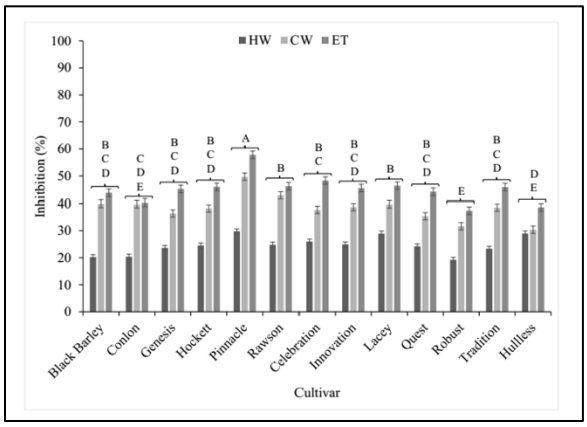

Figure 4.3. DPPH free radical scavenging capacity (%) (n = 12) of hot water (HW), cold water (CW), and 12% ethanol (ET) extracts from samples of 13 different types of barley Different capital letters represent significant differences between barley samples at $p < 0.05$.

4.4.2. α-Amylase Inhibitory Activity

In this study, the α-amylase inhibitory activity of the barley extracts was measured using *in vitro* assay (Worthington Enzyme Manual, 1993a) to assess their relevance in delaying the enzymatic breakdown of starch to mono and disaccharides. No significant differences in α-amylase inhibitory activity were observed among extraction types ($p < 0.05$). Cold water

(59.08% - 84.82%) and 12% ethanol (56.85% - 97.78%) extracts were found to have moderate to high α-amylase inhibitory activity (Figure 4.4). But significant differences in α-amylase inhibitory activity among various types of barley were observed, with higher inhibitory activity in Hockett when compared with Conlon and Genesis barley samples. Further, in the case of hot water extracts except for Black Barley and hull-less food barley, none of the samples had any inhibitory activity. Therefore, the results for hot water extracts were not presented (Figure 4.4).

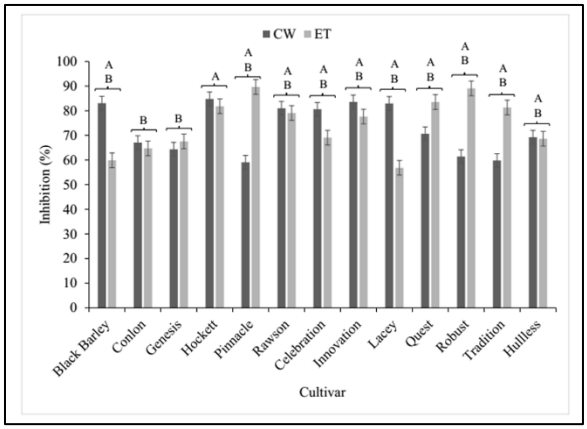

Figure 4.4. α-Amylase inhibitory activity (%) (n = 12) of hot water (HW), cold water (CW), and 12% ethanol (ET) extracts from samples of 13 different types of barley. Different capital letters represent significant differences between barley samples at $p < 0.05$.

4.4.3. α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity of the barley extracts was measured using *in vitro* assays as described by McCue *et al*. (2005). When the sample were not diluted, cold water and ethanol (12%) extracts of various types of barley had moderate α-glucosidase inhibitory activity (Figure 4.5A). The ability of hot water extracts of barley samples to inhibit α-glucosidase activity was found to be significantly lower than both cold water and ethanol (12%) extracts ($p < 0.05$) even without dilution. Black Barley and Genesis had significantly higher α-glucosidase inhibitory activity, when compared with other types of barley (Figure 4.5.A). Even at half dilution, Black Barley had significantly higher α-glucosidase inhibitory activity when compared

to other barley samples but was at par with Genesis. However, at one-fifth dilution, Pinnacle and Black barley were found to have significantly higher α-glucosidase inhibitory activity than other barley samples. The α-glucosidase enzyme inhibitory activity of all barley extracts was also found to have a dose-dependent response (Figures 4.5 A, B, C).

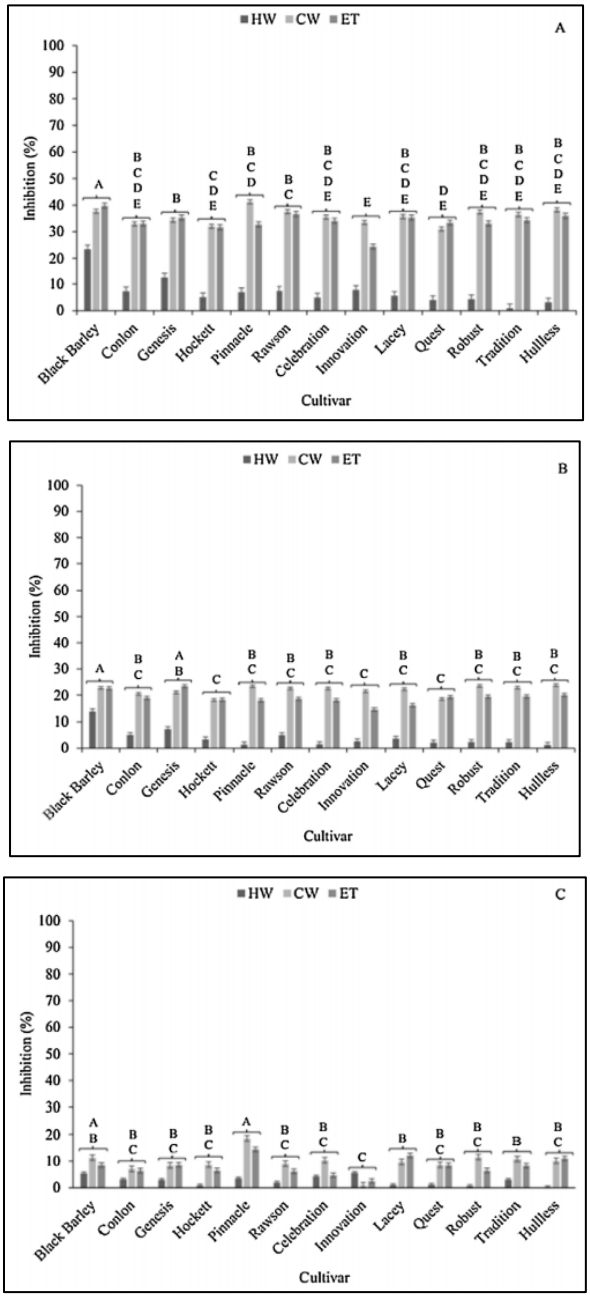

Figure 4.5. α-Glucosidase inhibitory activity (%) (n = 12) of hot water (HW), cold water (CW), and 12% ethanol (ET) extracts from samples of 13 different types of barley at no dilution (A), half-dilution (B) and one-fifth dilution (C). Different capital letters represent significant differences between barley samples at $p < 0.05$.

4.4.4. Characterization of Phenolic Compounds using HPLC

Major soluble phenolic acids of barley extracts were determined using HPLC method.

Gallic acid was the predominant phenolic compound among all barley samples and across all

extraction types. Among various types of barley, Pinnacle had the highest concentration of gallic acid, as well as across extraction types (Table 7.1), which positively correlated with total soluble phenolic content result. Protocatechuic acid was detected in hot water extracts of certain types of barley such as Black Barley, Celebration, Conlon, Genesis and Robust (Table 4.1).

Catechin was found in ethanol (12%) and hot water extracts of almost all barley samples, whereas, in the case of cold-water extracts, it was detected only in certain samples, such as Celebration, Genesis, Conlon, Hockett, and Innovation (Table 4.1). Similarly, caffeic acid was detected only in cold water extracts of very small number of barley samples such as Black Barley, Rawson, Robust, and Tradition (Table 4.1).

Table 4.1. Major phenolic compounds detected in barley grain extracts (expressed in µg/g D.W.).

Cultivar	Gallic acid			Protocatechuic acid			Catechin			Caffeic acid		
	CW	ET	HW	CW	ET	HW	CW	ET	HW	CW	ET	HW
Black Barley	0.014jklm	0.010rst	0.033fghi	n.d.	n.d.	0.017b	n.d.	0.003	0.005	0.004bc	n.d.	n.d.
Celebration	0.013lmno	0.010qrst	0.034efgh	n.d.	n.d.	0.015bc	0.006	0.003	0.005	n.d.	n.d.	n.d.
Conlon	0.004u	0.011opqr	0.041bc	n.d.	n.d.	0.013cd	0.005	n.d.	0.006	n.d.	n.d.	n.d.
Genesis	0.005u	0.012mnop	0.032ghij	n.d.	n.d.	0.010d	0.003	0.006	0.006	n.d.	n.d.	n.d.
Hockett	0.005u	0.009st	0.018st	n.d.	n.d.	n.d.	0.004	0.006	0.006	n.d.	n.d.	n.d.
Hull-less	0.008t	0.011pqrs	n.d	n.d.	n.d.	n.d.	n.d.	0.001	n.d.	n.d.	n.d.	n.d.
Innovation	0.016hij	0.013lmnop	0.037cdef	n.d.	n.d.	n.d.	0.004	0.008	0.019	n.d.	n.d.	n.d.
Lacey	0.016hijk	0.014hijk	0.037cde	n.d.	n.d.	n.d.	n.d.	0.005	0.007	n.d.	n.d.	n.d.
Pinnacle	0.018defg	0.014klmn	0.046a	n.d.	n.d.	n.d.	n.d.	0.004	0.007	n.d.	n.d.	n.d.
Quest	0.016hijk	0.013lmno	0.036def	n.d.	n.d.	n.d.	n.d.	0.006	0.004	n.d.	n.d.	n.d.
Rawson	0.006u	0.012nopq	0.044ab	0.011 a	n.d.	n.d.	n.d.	0.005	0.008	0.002c	n.d.	n.d.
Robust	0.014jkl	0.013lmno	0.031hijk	n.d.	n.d.	0.004e	n.d.	0.005	0.007	0.008ab	n.d.	n.d.
Tradition	0.015jkli	0.129a	0.040cd	n.d.	n.d.	n.d.	n.d.	0.002	0.009	0.012a	n.d.	n.d.

n.d. – Not detected, CW- cold water, HW- hot water, ET- ethanol (12%)

4.5. Discussion

Phenolic phytochemicals derived from plant sources have been shown to have a wide range of bioactive functionalities that are relevant in the prevention and management of various NCDs, including T2D and its complications. (Slaving *et al,* 1997; Balasundaram *et al*., 2006; Dykes and Rooney, 2007, Carter *et al*., 2010, Sarkar and Shetty, 2014). Most of the existing literature on the health benefits of phenolic compounds focusses on fruits, vegetables and beverages like wine and tea (Dykes and Rooney, 2007). However, cereal grains such as barley have also been reported to have significant levels of phenolic compounds but their bioactive functionalities and therapeutic potential for managing T2D and associated chronic diseases have not been sufficiently explored. Further, such cereal grains are relatively inexpensive, non-perishable, and amenable to being produced on a large industrial scale and can be processed into non-food-based nutraceutical ingredients targeted to manage early stages of T2D and other chronic diseases (Bach-Knudsen *et al*., 2016; Belobrajdic and Bird, 2013; Dykes and Rooney, 2007; Liu, 2007).

The TSP content of barley grains varies widely and is largely influenced by differences in cultivars and growing environments (Yu *et al*., 2013). In general, pigmented cultivars have a higher content of free soluble phenolics, compared to non-pigmented cultivars (Bonoli *et al*., 2004; Dykes and Rooney, 2007; Tokusoglu and Hall, 2010; Suriano *et al*., 2018). The overall TSP content of barley extracts measured in this study ranged between 0.42 and 0.6 mg GAE/g D.W., which is comparable to the range of free phenolics reported by Dykes and Rooney (2007) and Madhujith and Shahidi (2009), but higher than the values reported by Dvořáková *et al*. (2012). The extraction methods used in this current study did not involve acidic or alkaline hydrolysis, which may have led to the release of bound phenolics from the cell walls in previous

studies (Pandey and Rizvi, 2009; Kandil *et al*., 2012). Therefore, it is logical to conclude that free phenolics were the major contributors to the TSP content of the barley extracts in this study. Whole milled flour was found to have lower TSP content compared to bran fractions due to the dilution effect caused by inclusion of the endosperm (Madhujith and Shahidi, 2009). The hull-less barley was found to have higher TSP content compared to other malting barley samples. Lower phenolic content can be expected in malting barley samples as high phenolic content is not desirable to the challenges, they pose to beer quality and stability and therefore are likely to contain lower levels of free phenolics (Ullrich, 1999).

All barley extracts had significantly high free radical (DPPH and ABTS-based) scavenging potentials, indicating high antioxidant activities, and positively correlated with the soluble phenolic content. Though the antioxidant activity of the barley extracts as determined by the two free radical scavenging assays showed comparable trends a difference in magnitude of antioxidant activity was observed. This can be attributed to the DPPH free radical being more stable than $ABTS^{\bullet+}$ cation and the resulting delay in attaining a steady state due to the quenching of $DPPH^{\bullet}$ radical by phenolic antioxidants (Halaby *et al*., 2013). In this *in vitro* assay model-based screening study, all barley samples had higher antioxidant activity and thus have the potential to counter chronic oxidative stress associated with T2D. However, future studies using *in vivo* assays or animal models are required to further confirm the antioxidant potential of targeted barley samples. Chronic hyperglycemia associated with T2D leads to an imbalance in the redox homeostasis at the cellular level due to the uncontrolled production of harmful reactive oxygen species (ROS) (Rolo and Palmeira, 2006; Sarkar and Shetty, 2014). The ROS react with and damage cellular biomolecules such as proteins and DNA. Further, they are also implicated in the development of various micro- and macro-vascular complications which can lead to

impairment of vision, renal function, the nervous system, and ultimately death (Brownlee, 2005; Evans *et al*., 2002; Rahimi *et al*., 2005; Sarkar and Shetty, 2014). Thus, the phenolic linked-antioxidant activity indicated by barley samples in this *in vitro* study is of significance, as they may potentially aid in mitigating hyperglycemia-mediated oxidative stress.

Type 2 diabetes is a disease characterized by impaired glucose homeostasis. Its onset is preceded by a chronic hyperglycemia, caused by insufficient insulin production and sensitivity (Leahy *et al*., 1992). Therefore, one of the critical points for the prevention and management of chronic hyperglycemia is the regulation of postprandial blood glucose levels, by inhibiting the activities of α-amylase and α-glucosidase enzymes. In this study, the anti-glycemic potential of various types of barley was determined using *in vitro* assay models with the aim of screening them on the basis of their bioactive functionalities relevant to T2D management.

Cold water and ethanol (12%) extracts of almost all barley samples had moderate to high α-amylase inhibitory activity. However, a distinct pattern in inhibitory activity was not observed among extraction types, and these trends had no positive correlation with the total soluble phenolic content of the barley extracts. These observations are indicative of components other than phenolics potentially contributing to the α-amylase inhibitory activity of the barley extracts. Mature cereal grains have been reported to contain proteins capable of inhibiting the activities of digestive enzymes such as α-amylase and trypsin (Marshall, 1977; Piasecka-Kwiatkowska, 2012). Further, the heating of the extracts during the preparation of hot water extracts may have caused the denaturation of these proteins and resulted in lack of α-amylase inhibitory activity in hot water extracts. In this study, moderate levels of inhibition of α-glucosidase were observed among the ethanol (12%) and cold-water extracts, with significant dose dependence response. The results are indicative of the impact of extraction procedure on the enzyme inhibitory

potential, in addition to cultivar differences. The general trend observed in this study agrees with previous findings, where barley kernel extracts were found to exhibit stronger inhibition towards α–amylase enzyme, while showing low to moderate inhibition of α-glucosidase enzyme (Donkor *et al*., 2012; Kim *et al*., 2014).

Barley grains are among the richest sources of phenolic compounds among the major cereal grains. In this current study the barley extracts were analyzed using HPLC to determine the main water-soluble phenolic compounds with potential bioactive functionalities relevant for managing chronic hyperglycemia. Gallic acid was detected in most barley samples and across all three extraction types. Among extraction types, hot water extracts of barley had highest gallic acid content. Other identified phenolic acids include protocatechuic acid, catechin, and caffeic acid. Earlier studies indicated that the main phenolic acids found in non-pigmented barley kernels were ferulic, p-, m-, o-coumaric, syringic, vanillic, protocatechuic, salicylic and p-hydroxybenzoic acids (Ullrich, 1999; Tokusoglu and Hall, 2010). Also, ferulic acid was found to be the dominant phenolic acid in studies which utilized acid hydrolysis in the sample preparation stages (Ullrich, 1999; Tokusoglu and Hall, 2010). Variations between observations in the current study and previous findings may be attributed to differences in sample extraction and processing methods, type of solvent used in extraction and HPLC analysis protocols (use of different mobile phases and reference scales).

4.6. Conclusions

In the current study 13 barley types from single crop year were screened to determine the phenolic bioactive-linked functionalities for developing non-food nutraceuticals targeting the management of early stage T2D associated chronic hyperglycemia. Samples of the Pinnacle and high antioxidant activity. Further, the Black Barley sample used in this study was found to have

moderate levels of α-glucosidase inhibitory activity, which was significantly higher than other types of barley. However, further delineation of the impact of different barley types on phenolic content and bioactive functionality warrants investigation of these parameters on barley cultivars from multiple growing years and locations. Cold water and ethanol (12%) extracts of all barley samples had moderate to high *in vitro* α-amylase inhibitory activity. In conclusion, preliminary data from this study suggests that barley types such as Pinnacle and Black Barley may be studied further using *in vivo* models for designing non-food nutraceutical and bioactive functional ingredients for improving glycemic control. The antioxidant potential of these barley samples may also aid in mitigating chronic oxidative stress associated with chronic hyperglycemia, thereby potentially preventing the onset and development of micro- and macro-vascular complications associated with T2D. Additionally, this study also outlined the practical utility of *in vitro* screening models to rapidly select suitable types of barley for further development into non-food nutraceuticals and bioactive functional ingredients targeting of the potential benefits against early stage of type 2 diabetes and its complications and needs *in vivo* models for validation.

CHAPTER 5. IMPROVING PHENOLIC BIOACTIVE-LINKED ANTI-HYPERGLYCEMIC FUNCTIONS OF DARK GERMINATED BARLEY SPROUTS (*HORDEUM VULGARE* L.) USING SEED ELICITATION STRATEGY

5.1. Abstract

Sprouts of cereal grains, such as barley (*Hordeum vulgare* L.), are a good source of beneficial phenolic bioactives. These bioactive compounds can play a role in managing chronic oxidative stress and hyperglycemia due to their antioxidant capacity and ability to regulate the activity of enzymes involved in carbohydrate digestion. Improving such phenolic bioactives by stimulating plant endogenous defense responses during sprouting has significant merit. Based on this metabolic rationale, this study aimed to enhance phenolic bioactive content and associated anti-hyperglycemic functions in dark germinated barley sprouts using exogenous elicitor treatments. Dark-germinated sprout samples of two malting barley types (Pinnacle and Celebration), treated with chitosan oligosaccharide (COS) and marine protein hydrolysate (GP), were studied. Total soluble phenolic content (TSP), phenolic acid profile, total antioxidant activity and *in vitro* enzyme inhibitory activities of hyperglycemia relevant α-amylase and α-glucosidase of the sprouts were evaluated at day 2, 4, and 6 post seed treatments. Overall, TSP content, total antioxidant activity, and α-amylase inhibitory activity of dark germinated barley sprouts decreased, while α-glucosidase inhibitory activity and gallic acid increased from day 2 to day 6. Among barley samples, high phenolic antioxidant-linked anti-hyperglycemic functions were observed in cv. Celebration, compared to cv. Pinnacle. Furthermore, GP and COS seed treatments in selective doses improved T2D relevant phenolic-linked anti-hyperglycemic functions of barley spouts at day 6. These results suggest that seed-priming based elicitation strategies under controlled conditions may further be explored as a bioprocessing strategy to

potentially improve the phenolic-linked health functionalities of barley sprouts targeting T2D relevant dietary benefits using further *in vivo* validation.

Keywords: Antioxidants; Anti-hyperglycemia; Elicitors, Phenolic Bioactives; Type 2 Diabetes

5.2. Introduction

Type 2 diabetes (T2D), a non-communicable chronic disease characterized by impaired glucose and fat metabolism, is among the most significant global public health challenges currently (Stovall *et al*., 2005). Its pathogenesis is directly linked to chronic hyperglycemia, a metabolic condition wherein fasting blood glucose levels remain abnormally high over a prolonged period of time (Kahn, 2003). The regulation of postprandial absorption of glucose in the small intestine, by modulating the activities of carbohydrate solubilizing enzymes such as α-amylase and α-glucosidase is a key strategy for controlling chronic hyperglycemia (Nair *et al*., 2013). Current pharmaceutical strategies involve the use of synthetic inhibitors to regulate the activity of these enzymes, to delay starch digestion and reduce the rate of glucose absorption at the brush border cells of the gut (Hanhineva *et al*., 2010; Inzucchi *et al*., 2015). However, such drugs have been associated with adverse effects such as severe gastrointestinal distress and hepatic damage, due to excessive inhibition of α-amylase and α-glucosidase, thereby warranting a wider search for safer oral hypoglycemic agents from natural and commonly used food sources, with minimal side effects (Murai *et al*., 2002; Kwon *et al*., 2006; Kwon *et al*., 2007; Ali Asgar, 2013).

Phenolic metabolites from plant food sources have shown significant inhibitory activity against α-amylase and α-glucosidase, with minimal or less adverse side-effects (Stumvoll *et al*., 2005; Kwon *et al*., 2006; Fowler, 2008; Tundis *et al*., 2010). Being potent antioxidants, these compounds may offer an additional level of protection against the detrimental effects of

oxidative stress arising from chronic hyperglycemia, such as formation of harmful advanced glycated end products (AGEs) and oxidative degradation of nucleic acids and cell membranes. Therefore, the development of plant-based food matrices, enriched in phenolic metabolites, that can potentially aid in mitigating and managing early stage T2D-associated chronic hyperglycemia, is a strategy that holds significant merit (Rice-Evans *et al*., 1997; Marja P. Kähkönen *et al.*, 1999).

The upregulation of phenolic biosynthesis can be triggered as a broad-spectrum defensive mechanism in plants, in response to biotic and abiotic stresses (Dixon and Paiva, 1995). Several studies have demonstrated that a broad range of elicitor treatments, in conjunction with sprouting, may be employed to stimulate endogenous defense responses, as a means of improving the content of beneficial phenolic metabolites (Andarwulan and Shetty, 1999; Randhir *et al*., 2004; Randhir *et al*., 2009). From among the various elicitors evaluated in such studies, chitosan oligosaccharide and marine protein hydrolysate were selected, due to their relatively low cost and ease of availability. Chitosan oligosaccharides, manufactured via the deacetylation of marine chitin, are capable of inducing pathogen and damage associated molecular pattern (DAMP / PAMP) signal cascades, which include elevated synthesis accumulation of phenolic compounds and phytoalexins (Sarkar and Shetty, 2014; Trouvelot *et al*., 2014). Marine protein hydrolysates are derived from proteolysis of fish and seaweed processing byproducts. It has been shown to influence the activity of enzymes of the TCA cycle, and interactions between carbon and nitrogen metabolic pathways (du Jardin, 2015). Furthermore, it may also upregulate the proline associated pentose phosphate pathway (PAPPP), by supplying free proline, resulting in over expression of phenolic metabolites (Sarkar and Shetty, 2014).

Sprouting – a form of controlled germination – is a simple and cost-effective strategy capable of enhancing the overall nutritional profile and food quality of cereal and legumes by breaking down anti-nutrients, improving digestibility, and availability of amino acids, soluble carbohydrates, and dietary fiber (Hubner and Arendt, 2013). Moreover, the total soluble phenolic content of sprouts may be improved due to solubilization of bound phenolics, and synthesis of phenolics as a protective mechanism to cope with elevated oxidative stress, and for structural adjustments which is important during early stages of emergence. Seed priming with bioprocessed elicitors has been used in conjunction with controlled germination in earlier studies to enhance the phenolics-linked bioactive functionality of various cereal grains and legumes (Andarwulan and Shetty, 1999; Randhir *et al*., 2004; Randhir *et al*., 2009; Orwat, 2016).

While most of the barley under current production is utilized for malting and brewing, and feed purposes, interest in barley as a food grain and as a source of phytochemical components with health relevant functionalities is steadily growing (Baik and Ullrich, 2008). The abundance and diversity of phenolic compounds in barley also makes it unique source of beneficial phenolic bioactives among the major cereal grains. Additionally, barley grains have a high, uniform rate of germination and are physiologically well adapted for controlled germination and malting (Baik and Ullrich, 2008; Hübner and Arendt, 2013). Thus, barley grains were chosen to evaluate a seed elicitor-mediated approach for improvement of phenolic bioactive content through sprouting, to develop anti-hyperglycemic nutraceuticals and functional food ingredients.

The objective of this study was to evaluate the efficacy of marine protein hydrolysates (GroPro®; GP) and chitosan oligosaccharide (COS) as seed priming treatments to stimulate phenolic biosynthesis in dark germinated barley sprouts. Additionally, the sprouts were also

evaluated at various growth stages (i.e., 2, 4- and 6-days post-priming) for their phenolic antioxidant-linked anti-hyperglycemic functions and potential relevance in targeting early stages of T2D benefits, using *in vitro* enzyme inhibition (i.e., α-amylase and α-glucosidase) assay models.

5.3. Materials and Methods

5.3.1. Materials

All reagents and enzymes used in this study were purchased from Sigma Aldrich Chemical Co. (St Louis, MO), unless otherwise mentioned.

5.3.1.1. Preparation of Elicitor Treatment Solutions

Two types of bioprocessed elicitors were evaluated in the current study – marine protein hydrolysate (GroPro / GP; derived from seaweed and marine fish extracts) obtained from Icelandic Bio-Enhancers (Westchester, NY) and soluble chitosan oligosaccharide (derived from shells of marine crustaceans) cross linked with ascorbic acid, obtained from Kong Poong Bio (Jeju, South Korea). Both elicitors were dissolved in distilled water separately to obtain solutions of the following concentrations – GP1 and COS1 (1000 ppm), GP2 and COS2 (2000 ppm), GP5 and COS5 (5000 ppm) and GP10 and COS10 (10,000 ppm). Thus, a total of 8 different elicitor treatments were evaluated, and distilled water was used as a control treatment.

5.3.1.2. Seed Priming and Dark Germination

Seeds of two malting barley types (Pinnacle - 2 rowed; Celebration- 6 rowed) from a single growing season and location, were procured from the Malting Barley Breeding Program at North Dakota State University (Fargo, ND). The seeds were disinfected in 0.5% sodium hypochlorite solution for 5 mins, rinsed thoroughly with distilled water and the excess water was blotted out using paper towels. The seeds were transferred to conical flasks containing 150 mL of

elicitor treatment solution and then incubated on a rotary shaker for 8 hours at 20 °C and 150 rpm.

Post incubation, the seeds were rinsed with distilled water and blotted with paper towels. Treated seeds were distributed into multiple sterile perforated plastic trays lined with moist paper towels and covered with aluminum foil to simulate dark conditions and sprouted at room temperature (20 - 24 °C). Germination was monitored daily, and sprouts were moistened regularly to prevent desiccation. Germination rate (%) was recorded prior to harvesting samples. Dark germinated sprouts were assayed on day 2, 4 and 6 post seed elicitation treatments using *in vitro* assay models.

5.3.1.3. Sample Extraction

Dark germinated barley sprouts were excised to exclude rootlets and homogenized in pre-chilled distilled water (3g in 30 mL) using a laboratory blender (Waring, Torrington, CT, USA). The homogenate was centrifuged first at 8500 rpm for 20 min, followed by a second round of centrifugation at 8500 rpm for 15 min. The resulting supernatant was stored at 4 °C for *in vitro* assays.

5.3.2. Total Soluble Phenolic (TSP) Content

Total soluble phenolic (TSP) content of the dark germinated sprout extracts was determined using a modified Folin-Ciocalteu (FC) method (Shetty *et al*., 1995). Sample extracts (1 mL) were combined with 95% ethanol (1 mL), distilled water (5 mL), 50% v/v FC reagent (500 µL), and 5% w/v Na_2CO_3 (1 mL) in a test tube, vortexed, and incubated in the dark for 60 min. The absorbance of the resultant reaction mixtures was measured at 725nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). From this data, TSP content of the samples was calculated using a gallic acid standard curve (10 – 300 µg/mL;

dissolved in 95% ethanol) and expressed as milligram equivalents of gallic acid (GAE) per gram fresh weight (FW) of the barley sprout samples.

5.3.3. Determination of Major Phenolic Compounds using HPLC

Sample extracts were centrifuged for 10 min at 13,000 rpm and subjected to chromatographic analysis using an Agilent 1260 Infinity Series reverse phase HPLC, equipped with DAD 1100 diode array detector (Agilent Technologies, Palo Alto, CA). A gradient elution method, involving 10 mM phosphoric acid (pH 2.5; Solvent A) and 100% methanol (Solvent B), was used. Sample extracts were eluted on a C-18 analytical column (Agilent Supelco SB-C18 250 x 4.6mm internal diameter) with a packing material particle size of 5 μm, at a flow rate of 0.7 mL/min at ambient temperature, with a total run time of 25 min. Pure standards of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, quercetin, resveratrol and p-coumaric acid in 100% methanol were used to calibrate retention times on the standard curve. Each sample was run in duplicate, and chromatograms obtained were analyzed using Agilent Chemstation integration software. Phenolic acid concentrations were expressed in micrograms per gram F.W. of barley sprouts.

5.3.4. DPPH Free Radical Scavenging Assay

The total antioxidant activity of the barley samples was measured using a modified DPPH (1, 1- diphenyl-2-picrylhydrazyl; Sigma Chemical Co.) free radical scavenging assay (Cervato *et al*., 2000). A working solution of DPPH was prepared by diluting a 10mM stock solution with 95% ethanol such that its absorbance was adjusted to a range of 1.8 – 2.0 units at 517 nm. Barley sprout extracts (250 μL) were combined with DPPH working solution (1.25 mL), mixed thoroughly using a vortex mixer, incubated in a dark cabinet for 5 mins. Following this, the absorbance of the reaction mixture was measured at 517 nm. For each sample, a

corresponding control was prepared by replacing the extract with 95% ethanol (250 µL) instead of barley sprout extract. Standard solutions of ascorbic acid dissolved in distilled water (50, 75, 125, 250, 500, and 1000 µg/mL) were used as positive controls. The DPPH free radical scavenging capacity of barley sprout extracts was calculated per the equation:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

5.3.5. ABTS Free Radical Scavenging Assay

The antioxidant activity of barley sprout extract was also measured by the $ABTS^{+}$ free radical cation-decolorization assay (Re *et al.,* 1999). A stock solution of ABTS (2, 2 –azinobis (3-ethylbenzothiazoline-6-sulfonic acid) was prepared by combining 7 mM ABTS (5 mL) with 140 mM potassium persulphate (88 µL). The mixture was incubated for at least 16 hours prior to the assay. The stock solution was then diluted with 95% ethanol to prepare a working solution with an absorbance of 0.70 ± 0.02 at 734 nm. Barley sprout extract (50 µL) was added to the working solution (1 mL), mixed on a vortex mixer for 30 sec and incubated for 2.5 min at room temperature in a dark cabinet. Absorbance values of the reaction mixtures were measured at 734 nm. For each sample, a corresponding control was prepared by replacing the extract with 95% ethanol (50 µL). As with the DPPH radical scavenging assay, standard solutions of ascorbic in distilled water (50, 75, 125, 250, 500, and 1000 µg/mL) were used as positive controls for comparison. The $ABTS^{+}$ free radical scavenging capacity of barley sprout extracts was calculated per the equation:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

5.3.6. Oxygen Radical Absorbance Capacity (ORAC) Assay

Oxygen radical absorbance capacity (ORAC) of dark germinated barley sprouts was determined using an assay modified from Huang *et al*. (2002). Zen Bio (Zen Bio Inc. Research Triangle Park, NC, USA) antioxidant assay kit was used to determine the ORAC values of barley spouts. A stock solution of fluorescein (4.19×10^{-3} mM) was prepared in 75 mM potassium phosphate buffer (PBS; pH 7.4) and stored at 4°C until further use. Prior to the performing the assay, the stock solution was diluted with PBS to produce a working solution (8.16×10^{-5} mM). A solution of 2, 2´-azobis (2- amidinopropane) dihydrochloride (AAPH; 153 mM) was prepared by dissolving the salt in PBS and placed in an ice bath during the assay.

A 96-well black polypropylene microtiter plate was used for the assay, whose outer wells were filled with distilled water (300 μL) to serve as a thermal buffer. The inner wells were loaded with fluorescein working solution (150 μL), followed by sprout extracts (25 μL). For blanks, sprout extracts were replaced with PBS (25 μL). A separate row was filled with Trolox standard solutions (prepared in PBS) of varying concentrations (100, 50, 25, 12.5, 6.25 μM) in place of sprout extracts. The microtiter plates were placed in the pre-heated (37 ºC) holding chamber of a BioTek Synergy H-1 Hybrid Multi –Mode Fluorescence reader (Bio Tek Instruments Inc. Winooski, VT, USA) and incubated for 10 min. Following this, AAPH solution (25 μL) was added to each inner well and the plate was immediately returned to the reader for fluorescence measurements. The fluorescence was measured every minute over 30 minutes at 485 nm excitation and 538 nm emission. Further to this, area under curve (AUC) value for each sample was obtained, from which their net AUC was calculated by subtracting blank AUC value and these values were plotted against Trolox standard curve (net AUC). The ORAC values of barley sprouts were expressed as μM Trolox equivalent.

5.3.7. α-Amylase Inhibitory Activity

The α-amylase inhibitory activity was determined by an assay utilized by Kwon *et al*. (2006). Sprout extracts (500 µL) were mixed with 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl; 500 µL) containing porcine pancreatic α-amylase (0.5 mg/mL) and incubated at 25 °C for 10 min. Starch solution (1% w/v, prepared in 0.02 M sodium phosphate buffer; 500 µL) was added to the mixture and further incubated at 25°C for 10 min. Finally, 3, 5-dinitrosalicylic acid (DNS; 1 mL) solution was added and the reaction was stopped by incubating the mixtures in a boiling water bath (90 - 100 °C) for 10 min. Reaction mixtures were cooled to room temperature and diluted with distilled water to bring control absorbance readings within a range of 0.8 to 1.0 units at 540 nm. Sample blank (containing sodium phosphate buffer instead of enzyme solution) absorbance and a control (containing sodium phosphate buffer in place of sprout extract) were also recorded. The α-amylase inhibitory activity was calculated as percentage (%) inhibition per the equation:

$$Inhibition\ (\%) = \frac{Abs_{control} - (Abs_{extract} - Abs_{sample\ blank})}{Abs_{\ control}} \times 100$$

Sample results were compared with acarbose standard solutions (0.05, 0.07, 0.12, 0.25, 0.50, and 1.0 mg/mL).

5.3.8. α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory potential was determined using an assay modified from the Worthington Enzyme Manual (Kwon *et al*., 2006). Sprout extracts (10 µL, 25 µL, 50 µL) were loaded in 96-well microtiter plates, and the volume in each well was made up to 100 µL with 0.1M phosphate buffer (pH 6.9). To each well, α-glucosidase solution (1 unit/mL, prepared in 0.1M phosphate buffer; 100 µL), was added, and the plate was incubated at 25°C for 10 min. Then, 5mM p-nitrophenyl-α-D-glucopyranoside (pNPG; 50 µL) solution prepared in 0.1M

phosphate buffer was added to all wells, and the plate was incubated at 25°C for 5 min. Absorbance values were recorded before and after incubation at 405 nm using a microplate reader (Thermomax, Molecular Device Co., Sunnyvale, CA) and compared to a corresponding control containing buffer solution (50 µL) instead of sample extract. The α-glucosidase inhibitory activity was expressed as percentage (%) inhibition and was calculated per the equation:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

Sample results were compared with acarbose standard solutions (0.05, 0.07, 0.12, 0.25, 0.50, and 1.0 mg/mL).

5.3.9. Statistical Analysis

A completely randomized design (CRD) was used for this study. Each plastic tray in which sprouts were grown was considered an experimental unit, and at each time point sprout samples were collected from 6 different trays (each constituting a biological replicate) for each biochemical analysis and the whole experiment was repeated twice. Calculation of means and standard deviations of 12 data points (6 biological replicates with 2 repetitions) along with Pearson's correlation coefficient (PCC, *r*) was performed using Microsoft Excel 2016. Analysis of variance (ANOVA) for the data was performed using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC). Statistically significant mean separations in bioactive functionality parameters, due to differences in elicitor treatments, barley samples and the interaction between barley samples × treatments were determined using the Tukey's least mean square test at a confidence level of 95% ($p < 0.05$).

5.4. Results and Discussion

5.4.1. Rate of Germination

The rate of germination was recorded at each assay time point prior to harvesting of sprout samples for assays and the observations are presented in Table 5.1.

Table 5.1. Mean germination rate (%) of dark germinated barley sprouts post priming with elicitor treatments.

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	96	92	98	96	100	100
GP1	97	95	100	100	100	100
GP2	97	95	100	100	100	100
GP5	98	96	100	100	100	100
GP10	97	96	100	100	100	100
COS1	96	97	100	100	100	100
COS2	97	96	100	100	100	100
COS5	96	97	100	100	100	100
COS10	95	97	100	100	100	100

5.4.2. Total Soluble Phenolic Content

The total soluble phenolic (TSP) content of dark germinated barley sprouts after seed elicitation treatments was measured using the Folin-Ciocalteu method, and the effects of the types of barley, elicitor treatment, and sprouting stage on the phenolic content were compared. Overall, the TSP content of the dark germinated barley sprouts across both barley types and elicitor treatments ranged from 0.33 to 0.76 mg/GAE. g^{-1} FW and the TSP content decreased with the progression of the germination process from day 2 to day 6 of sprouting (Figure 5.1. A, B, C). Ha *et al*. (2016) found similar trends in barley sprouts treated with distilled water and observed that TSP content initially increased at 48 hours post soaking and then sharply decreased afterward. The decrease in phenolic content with progression of sprouting might be due to the potential utilization of phenolic compounds in the mobilization processes of germination

(Randhir, Lin and Shetty 2004). In this study, significant differences in TSP content of dark germinated barley sprouts were observed between samples of the two barley types, treatments, and barley types × treatment interactions at day 2 and day 6 of sprouting. However, no significant difference in TSP content between the samples of the two barley types was found at day 4 of sprouting. At day 2 and day 6, dark germinated barley sprouts of Celebration had significantly higher TSP content when compared with the Pinnacle ($p < 0.05$).

The highest TSP content was found in dark germinated sprouts of Celebration with elicitor treatments GP5 (0.76 and 0.44 mg/GAE. g^{-1} FW), GP10 (0.73 and 0.45 mg/GAE. g^{-1} FW), COS 1 (0.73 mg/GAE. g^{-1} FW only at day 2), COS 5 (0.45 mg/GAE. g^{-1} FW only at day 4) at day 2 and day 4 of sprouting. However, it was statistically at par with the control ($p < 0.05$) (Figure 5.1. A, B). At day 6, GroPro (GP5 and GP10) and COS (COS2 and COS5) elicitor treatments resulted in significantly higher TSP content in dark germinated sprouts of Celebration when compared to the control ($p < 0.05$) (Figure 5.1.C). Based on the TSP content results of this study, Gro-Pro (5000 and 10000 ppm concentration) and COS (2000 ppm) can be targeted to improve phenolic bioactive content of barley and other grain or legume sprouts in future food and ingredient design studies. Previously, Randhir *et al*. (2009) reported that fish protein hydrolysates (2 mL/L), with a composition similar to Gro-Pro improved TSP content in dark germinated mung bean (*Vigna radiata*) sprouts during the initial stages of germination, specifically at day 1 post seed priming treatment. Therefore, this seed elicitation strategy with bioprocessed elicitor such as Gro-Pro and COS has significant merit to improve human health relevant phenolic bioactives and such strategy can be used for developing phenolic-enriched functional food ingredients.

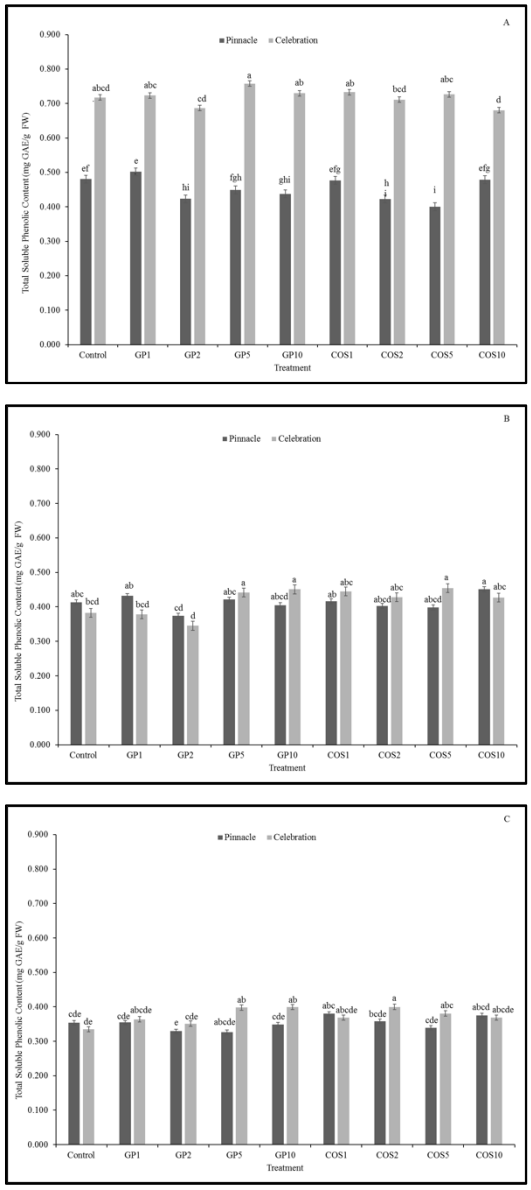

Figure 5.1. Total soluble phenolic content (μg GAE/g F.W.) of dark germinated barley sprouts, measured on day 2 (A), day 4 (B) and day 6 (C) after priming with elicitor treatments. Different alphabets represent significant differences in TSP content due to barley types × treatment interactions at 95% confidence level ($p < 0.05$).

5.4.3. Phenolic Acids Profile

The major phenolic acids in the primed, dark germinated barley sprout extracts were determined using reverse phase HPLC method. The major phenolic acids detected in this study were catechin, gallic acid, protocatechuic acid, and dihydroxybenzoic acid (Table 5.1). Among

the phenolic acids, the concentration of gallic acid was found to be the highest in both types of barley and across all treatment types. Although TSP content of barley sprouts decreased with the progression of the sprouting, the gallic acid content increased during sprouting and this was more evident in Pinnacle. At day 2 and day 6 of sprouting, GP1 (1000ppm) elicitor treatment resulted in higher gallic acid content in both types of barley when compared to the control and other elicitor treatments. Gallic acid content of food has relevance for managing oxidative stress and other metabolic disorders commonly associated with chronic hyperglycemia (Lee *et al.*, 2015; Moneim *et al.*, 2016). Inhibition of pro-inflammatory cytokine production in human monocytes in the presence of gallic acid was observed previously (Lee *et al.*, 2015). Hypoglycemic and hepato-renal protective effects of gallic acid was also reported in diabetic rats (Moneim *et al*., 2016) Addition (50%) of gallic acid in proportion with Acarbose (50%) also resulted in higher α-glucosidase inhibitory activity (Oboh *et al*., 2016). In this study while TSP content decreased during sprouting, both gallic acid concentration (Table 5.1) and α-glucosidase inhibitory activity (Table 5.4) increased from day 2 to day 6 which indicated a potential role of gallic acid for inducing α-glucosidase inhibitory activity in these dark germinated barley sprouts. Similarly, significant improvement in catechin content was also observed with Gro-Pro and COS elicitor treatments (GP5, GP10, COS5) in Celebration at day 2, 4, and 6 of sprouting. Catechin is a known antioxidant with potential anti-hypertensive, anti-inflammatory, and anti-proliferative functions (Anandh Babu and Liu, 2008; Iacopini *et al*., 2008). Therefore, improving catechin content (Table 5.1) through elicitor treatment in dark germinated barley sprouts may have significant relevance against oxidative stress-induced macro-vascular complications which is commonly associated with early stages T2D. Overall higher concentration of dihydroxybenzoic acid was found in Pinnacle, while higher protocatechuic acid was observed in Celebration.

Similar to gallic acid, protocatechuic acid is also potent antioxidant with diverse human health relevant and pharmacological functions (Kakkar and Bais 2014). Higher *in vitro* antioxidant activity of protocatechuic acid compared to Trolox was reported previously (Li *et al*., 2011). Higher antioxidant activity of Celebration might be associated with presence of higher concentration of protocatechuic acid (Table 5.1) compared to the Pinnacle (no protocatechuic acid was detected in Pinnacle at day 2 and day 4). Therefore, the phenolic acid profile and the response of barley to seed elicitation treatments may be dependent on the barley type × environment interactions.

In general, phenolic acids have been found to be the most abundant class of phenolic metabolites in cereal grains such as barley, and the findings of this study agree with the previous study (Carvalho *et al*., 2015). However, while ferulic acid has been reported as the most dominant phenolic acid in previous studies involving barley grains, it was not detected in the dark germinated sprout extracts of this study (Dykes and Rooney, 2007). Various factors may have contributed for not finding ferulic acid in this study, such as the use of dietary relevant aqueous extracts, the choice of mobile phase and HPLC protocols used in this study. However, improvement of gallic acid, catechin, and protocatechuic acid (in Celebration; Table 5.1) content in dark germinated barley sprouts with seed elicitation treatments has significant relevance and such strategy can be used to enhance targeted human health relevant phenolic bioactives in barley sprouts.

Table 5.2. Dominant phenolic acids in dark germinated barley sprouts 2 days (A), 4 days (B) and 6 days (C) post priming with elicitor treatments (expressed in µg/g D.W.)

(A)

Treatment	Catechin		Gallic Acid		Protocatechuic Acid		Dihydroxybenzoic Acid	
	Pin[#]	Cel[$]	Pin	Cel	Pin	Cel	Pin	Cel
Control	1.13a*	0.40a	0.75f	7.36cde	n.d.	n.d.	n.d.	0.41cd
GP1	0.42a	0.64a	6.72cde	10.0bcd	n.d.	0.62d	9.43a	4.52abcd
GP2	0.36a	0.62a	5.52def	9.21bcde	n.d.	0.30e	9.28a	0.50cd
GP5	0.37a	0.59a	5.15def	7.36cde	n.d.	0.53d	1.19cd	0.41cd
GP10	0.30a	0.70a	4.98def	9.11bcde	n.d.	1.07b	8.19cd	0.48cd
COS1	0.38a	0.71a	4.96def	8.26bcde	n.d.	0.18ef	7.38a	1.06cd
COS2	0.35a	0.55a	5.49def	15.4a	n.d.	1.61a	5.97abc	1.53bcd
COS5	0.29a	0.57a	4.56ef	12.7ab	n.d.	0.84c	0.67cd	0.34cd
COS10	0.38a	0.59a	6.32cde	11.0abc	n.d.	1.44a	7.09ab	1.45bcd

(B)

Treatment	Catechin		Gallic Acid		Protocatechuic Acid		Dihydroxybenzoic Acid	
	Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Control	0.34j	0.48e	14.7a	12.5cde	n.d.	1.48a	5.66a	0.69a
GP1	0.43g	0.48ef	14.1ab	12.0cdef	n.d.	1.15a	4.22a	0.45a
GP2	0.31	0.58ab	11.5def	13.2bc	n.d.	0.66a	0.86a	0.48a
GP5	0.54cd	0.57abc	13.0bc	10.9wf	n.d.	1.05a	1.05a	0.45a
GP10	0.45fg	0.59a	13.0bc	12.5cd	0.20a	1.66a	1.25a	0.48a
COS1	0.39i	0.43gh	11.1ef	11.4def	0.18a	0.97a	0.99a	0.56a
COS2	0.40j	0.60a	12.4cde	12.1cdef	1.35a	0.98a	0.60a	0.41a
COS5	0.55bcd	0.54d	11.8cdef	9.3g	1.31a	0.55a	1.16a	0.42a
COS10	0.46efg	0.53d	12.3cdef	12.2cdef	0.16a	0.89a	0.76a	0.40a

(C)

Treatment	Catechin		Gallic Acid		Protocatechuic Acid		Dihydroxybenzoic Acid	
	Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Control	0.34def	0.25gh	12.4a	12.5a	0.15fgh	n.d.	0.78a	0.46d
GP1	0.50b	0.22h	16.2a	17.7a	0.04h	1.22a	0.70abc	0.40d
GP2	0.30efg	0.34def	14.6a	13.3a	0.34def	0.34def	0.73ab	0.41d
GP5	0.34def	0.35cdef	14.2a	8.81a	0.07gh	0.87h	0.54bcd	0.43d
GP10	0.08i	0.50b	12.4a	17.2a	0.12fgh	0.59cd	0.48cd	0.45d
COS1	0.35def	0.29fg	14.5a	15.2a	0.22fgh	0.31efg	0.54bcd	0.43d
COS2	0.59a	0.41c	14.1a	1.62a	0.33def	0.51cde	0.87bcd	0.41d
COS5	0.20h	0.40cd	11.5a	14.1a	0.05h	0.61c	0.34d	0.39d
COS10	0.37cde	0.19h	9.00a	14.6a	0.15fgh	0.99ab	0.32d	0.35d

n.d. – Not detected; # - Pinnacle; $ - Celebration

*Different lowercase letters represent statistically significant differences between barley types x elicitor treatment interactions at 95% confidence level ($p < 0.05$)

5.4.4. Antioxidant Activity

The antioxidant activity of the dark germinated barely sprout extracts was evaluated using DPPH and ABTS free radical scavenging-based assays. Furthermore, ORAC values of dark germinated barley sprout extracts were also measured to corroborate with antioxidant activity based on DPPH and ABTS free radical scavenging assay. Additionally, results of the antioxidant activity were presented with respect to the soluble phenolic content of the barley sprout extracts. Overall, significant differences in total antioxidant activity (based on both DPPH and ABTS assay) of dark germinated barley sprouts were observed between barley types, treatments, and barley type × treatment interactions ($p < 0.05$) at all stages of the sprouting (Figure 5.2 A, B, C and 5.3 A, B, C). However, significant differences in antioxidant capacity (ORAC) were only observed between two barley types at day 2 and day 4 (Table 5.2). Dark germinated sprout of Celebration had significantly higher antioxidant capacity (67-114 µM of Trolox Equivalent) when compared with Pinnacle (61-71 µM of Trolox Equivalent/ g of sample) at day 2. Like the TSP content and antioxidant capacity (based on ORAC), dark germinated barley sprouts of Celebration had significantly ($p < 0.05$) higher antioxidant activity (DPPH and ABTS) than Pinnacle barley sprouts during all three sprouting stages (Figure 5.2 A, B, C and 5.3 A, B, C). When compared with ascorbic acid which was used as a positive control, DPPH inhibition of Celebration (average TSP of 70.0 µg/mL) at day 2 was equivalent to 350 µg/mL ascorbic acid, while for Pinnacle (average TSP of 45.0 µg/mL) it was 200 µg/mL ascorbic acid. However, at day 6 the DPPH % inhibition of both types of barley (average TSP of 37.0 and 35.0 µg/mL for Celebration and Pinnacle) was equivalent to 100-120 µg/mL ascorbic acid. Similarly, antioxidant activity based on ABTS % inhibition for Celebration (70.0 µg/mL TSP) and Pinnacle (45.0 µg/mL TSP) at day 2 was equivalent to 50 and 35 µg/mL ascorbic acid. The antioxidant

activity results indicated better response of barley extracts and ascorbic acid towards ABTS radical scavenging assay when compared with DPPH radical scavenging assay as reported previously (Re *et al*., 1999). While Celebration barley sprouts treated with GP5 were found to have the highest antioxidant activity based on the DPPH free radical scavenging assay at day 2 (22.45 %) and day 4 (13.65%), however statistically significant difference ($p < 0.05$) with control was only observed at day 2 (Figure 5.2, B, C). Further, at day 6, GP2, COS1, and COS2 seed elicitation treatment resulted in significantly higher antioxidant activity (based on DPPH) in Celebration and this result corroborated with TSP content result of the same barley type. Overall, antioxidant activity (based on DPPH and ABTS) and antioxidant capacity (based on ORAC) was found to steadily decline over the course of sprouting and this trend is consistent with that of decreasing TSP content during the same stages, across barley types and elicitor treatments. A strong positive correlation was found between TSP content and DPPH free radical scavenging capacity at day 2 ($r = 0.96$) reflecting the relevance of determining antioxidant activity by aligning with TSP content.

A similar trend was observed in dark germinated mung bean (Randhir *et al*., 2009) and in barley sprouts (Ha *et al*., 2016), where the antioxidant activity peaked during the first 24 -48 hours of germination. However, this can be attributed to an increased demand for oxygen for respiration by sprouts in the initial stages of emergence (Randhir *et al*., 2009). Further, total antioxidant activity of the barley sprouts decreased commensurately with declining TSP content, which indicates the significant antioxidant function of soluble phenolic compounds in the barley sprout extracts. Overall, higher total antioxidant activity of dark germinated barley sprouts was observed based on ABTS free radical scavenging assay, when compared to the DPPH free radical scavenging assay results. The differences between DPPH and ABTS based assays are

potentially linked to the water-soluble phenolic fractions responsible for radical scavenging activity, and which are generally more responsive to the ABTS assay (Re *et al*., 1999).

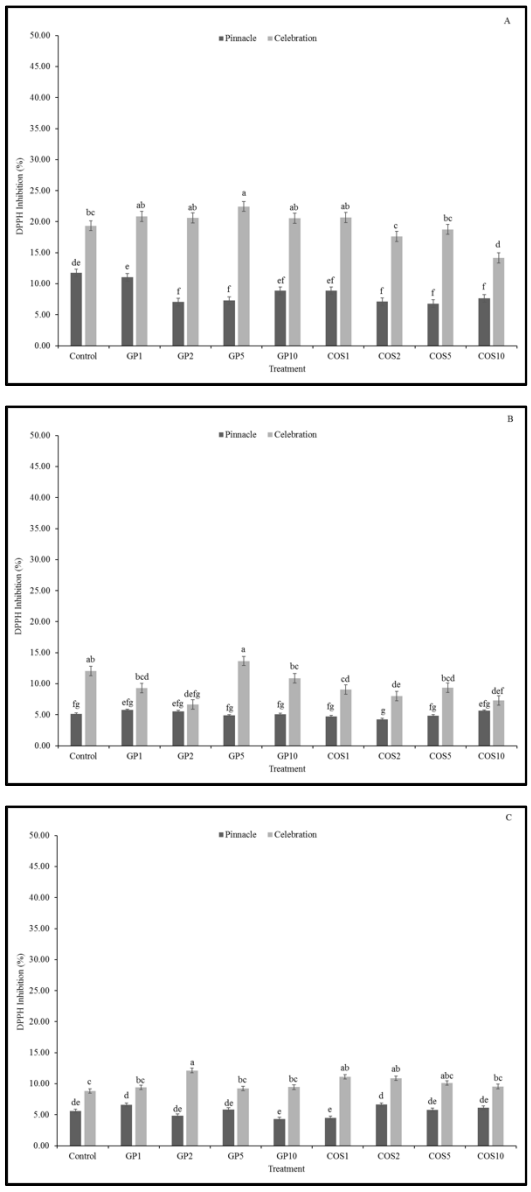

Figure 5.2. DPPH free radical inhibitory activity (%) of dark germinated barley sprouts, measured on day 2 (A), day 4 (B) and day 6 (C) after priming with elicitor treatments. Different alphabets represent significant differences in DPPH free radical scavenging capacity due to barley type × treatment interactions at 95% confidence level ($p < 0.05$).

Dark germinated sprouts of Celebration with COS2 treatment had highest antioxidant activity based on ABTS free radical scavenging assay (41.05%) at day 2, however this value was

not statistically different than control and other elicitor treatments (Figure 5.3.A). At day 4, Pinnacle treated with GP1 (26.59%) had significantly higher ($p < 0.05$) total antioxidant activity (based on ABTS) when compared with the control (23.29%) (Figure 5.3.B). Similarly, at day 6 dark germinated barley sprouts of Celebration treated with GP5 had significantly ($p < 0.05$) higher total antioxidant activity (based on ABTS) when compared with the control (Figure 5.3.C).

Like the DPPH based antioxidant results, strong and positive correlations between TSP content and ABTS based antioxidant activity ($r = 0.82$) was observed at day 2 of the sprouting. The correlation between two antioxidant assays (DPPH and ABTS) was also found to be higher and positive at day 2 ($r = 0.77$) and day 4 ($r = 0.48$) of sprouting. These results suggest that total antioxidant activity of barley sprouts was closely linked with the TSP content. Therefore, enhancement of TSP content might result in the improvement of antioxidant functions of barley sprouts which has potential relevance for targeting multi-functional dietary therapy of chronic hyperglycemia-linked oxidative stresses commonly associated with early stages of T2D.

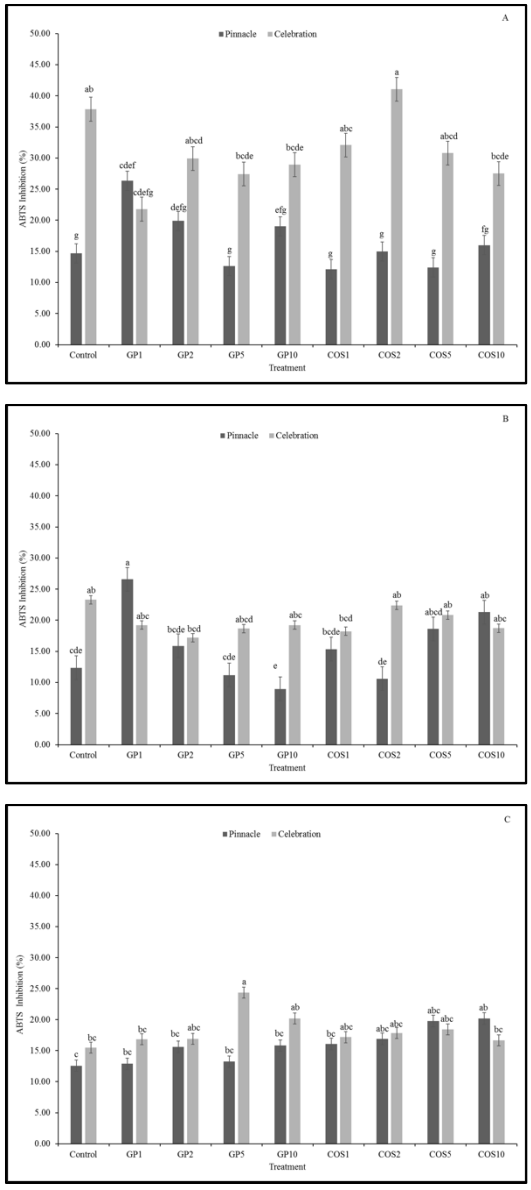

Figure 5.3. ABTS free radical inhibitory activity (%) of dark germinated barley sprouts, measured on day 2 (A), day 4 (B) and day 6 (C) after priming with elicitor treatments. Different alphabets represent significant differences in ABTS free radical scavenging capacity due to barley type × treatment interactions at 95% confidence level ($p < 0.05$).

5.4.5. α-Amylase and α-Glucosidase Inhibitory Activities

To evaluate anti-hyperglycemic functions of dark germinated barley sprouts, relevant α-amylase and α-glucosidase enzyme inhibitory activities were targeted and determined using rapid

in vitro assay models. Overall, like the TSP content and total antioxidant activity, α-amylase inhibitory activity of dark germinated barley sprouts decreased with the progression of the sprouting from day 2 to day 6 (Table 5.2. A, B, C). Randhir *et al*. (2009) in comparison found higher α-amylase inhibitory activity of mung bean sprout at day 1 than day 4 following seed elicitation treatments. However, completely opposite trend was observed for α-glucosidase, as inhibitory activity against this key targeted enzyme increased steadily from day 2 to day 6 (Table 5.3. A, B, C). Further for both enzyme inhibitory activities, significant dose dependent response was also observed in dark germinated sprout samples of two types of barley. Overall, significant differences in α-amylase and α-glucosidase enzyme inhibitory activities of barley sprouts due to differences in barley types, treatments, and barley type × treatment interactions were also observed in all three stages of the sprouting ($p < 0.05$). For all dilutions (undiluted, half, and one-fifth) and across all treatments significantly ($p < 0.05$) higher α-amylase inhibitory activity was observed in Celebration at day 2, however Pinnacle had significantly higher inhibitory activity against this enzyme at day 4 and day 6 (Table 5.2. A, B, C). When compared with the positive control, the α-amylase inhibitory activity of Celebration (100 mg/mL) at day 2 was equivalent to 1 to 1.2 mg/mL of Acarbose, however for Pinnacle (100 mg/mL) it was equivalent to 0.25 mg/mL of Acarbose. Like the TSP content and antioxidant activity of Celebration, α-amylase inhibitory activity decreased significantly with the progression of the sprouting and this result suggests that the α-amylase inhibitory activity might be linked to the TSP content of the barley sprouts. Positive and moderately higher correlations between TSP content and α-amylase inhibitory activity was observed at all three stages of the sprouting ($r = 0.66$; 0.59; 0.39 at day 2, 4, and 6 respectively).

In this study, seed elicitation treatments also resulted in higher α-amylase inhibitory activity in dark germinated barley sprouts. In undiluted sample, COS1 treated barley sprouts of Pinnacle had significantly higher α-amylase inhibitory activity when compared to the control at day 2 of sprouting ($p < 0.05$) (Table 5.2.A). However, at day 4, COS2 and at day 6, GP5 seed elicitor treatments resulted in significantly higher α-amylase inhibitory activity in undiluted sample of dark germinated barley sprouts of Pinnacle. Therefore, these seed elicitor treatments can be targeted selectively to improve α-amylase inhibitory activity of barley sprouts in the future food and ingredient design studies.

Overall, high α-glucosidase inhibitory activity was observed in undiluted sample of both types of barley at day 4 and day 6 of sprouting (Table 5.3.A). Like the TSP content and total antioxidant activity, significantly higher α-glucosidase inhibitory activity was observed in dark germinated barley sprouts of Celebration when compared to the Pinnacle barley sprouts at all sprouting stages ($p < 0.05$). α-Glucosidase inhibitory activity of undiluted sample of Celebration barley sprouts (100 mg/mL) at day 2 and day 6 was equivalent to 0.15 and 1 mg/ mL Acarbose (positive control) respectively. Similarly, for undiluted sample of Pinnacle barley sprouts (100 mg/ mL), α-glucosidase inhibitory activity at day 2 and day 6 was equivalent to 0.07 and 0.6 mg/mL of Acarbose. Among seed elicitor treatments, statistically significant ($p < 0.05$) improvement in α-glucosidase inhibitory activity of dark germinated barley sprouts of Pinnacle was observed with COS2 and COS5 seed treatments at day 4 and day 6 of sprouting. However, for dark germinated barley sprouts of Celebration, GP2 seed elicitor treatment resulted in significantly higher α-glucosidase inhibitory activity, especially at half and one-fifth diluted samples (Table 5.3. B, C). Improvement of α-glucosidase inhibitory activity with COS seed elicitor treatment was also observed in black bean (*Phaseolus vulgaris*) sprouts previously

(Orwat 2016). The α-glucosidase inhibitory activity of germinated barley sprouts correlated positively with the TSP content in previous study (Ha *et al*., 2016).

Moreover, the enzyme inhibition response was found to be dose dependent, which is essential in the design of functional food ingredients for glycemic control (Ha *et al*., 2016). In this study, high and positive correlations between α-glucosidase inhibitory activity and DPPH based antioxidant activity was observed in dark germinated barley sprouts (r = 0.82, 0.75, 0.63 at day 2, 4 and 6).

Results of this *in vitro* study indicated that anti-hyperglycemic and antioxidant functions of dark germinated barley sprouts are associated with the TSP content and phenolic acids profile of specific type of barley. The use of seed elicitation strategy with safe and food grade edible elicitors has significant merit to improve phenolic antioxidant-linked anti-hyperglycemic functions in barley and can be targeted for designing functional food ingredients to targeting chronic diseases, such as early stages of T2D.

Table 5.3. α-Amylase inhibitory activity (%) of undiluted (A), 1-in-2 part (B) and 1-in-5 part diluted (C) dark germinated barley sprout extracts. Different alphabets represent significant differences in inhibition due to barley type × treatment interactions at 95% confidence level ($p < 0.05$).

(A)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	68.94c	91.09ab	75.67cd	51.10e	37.97defg	27.99fgh
GP1	86.67ab	91.83ab	73.80cd	63.01e	45.10abcd	12.06i
GP2	77.07bc	94.06a	47.05fgh	33.98h	54.66ab	13.17hi
GP5	66.44c	94.82a	63.72de	44.00gh	58.41a	31.55efg
GP10	28.76e	89.37ab	55.69ef	89.04 ab	44.21abcde	14.16hi
COS1	87.50ab	92.04a	75.53dc	82.29abc	44.06abcdefg	37.49cdefg
COS2	46.33e	86.78ab	91.04ab	94.81a	29.63efg	29.52gh
COS5	87.79ab	87.36ab	45.10fgh	75.00bc	52.30abc	41.31abcdef
COS10	63.80c	91.38a	86.78abc	45.30fgh	43.92bcdefg	43.49abcdef

(B)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	58.89cdef	82.60a	56.97a	13.42fg	22.13bcd	0.34e
GP1	53.67efg	74.14abc	42.76c	49.05abc	36.71a	2.24e
GP2	55.86defg	78.15ab	40.31cd	10.47g	37.08ab	0.27e
GP5	41.35h	76.52b	53.25ab	12.03fg	32.02ab	11.78de
GP10	15.33i	79.40ab	37.75d	57.01a	33.52ab	0.95e
COS1	54.91defg	65.72bcde	43.99bc	39.06cd	26.87abc	31.55ab
COS2	36.33h	36.93h	42.96bc	56.88a	24.18bc	14.57cd
COS5	42.57fgh	71.89abc	23.49ef	55.16a	22.42bcd	17.28cd
COS10	47.11fgh	71.40abcd	48.54abc	29.83de	13.24e	26.83abc

(C)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	29.46fghi	75.01a	34.26a	3.21efg	8.07bc	0.00c
GP1	19.03hij	59.49bc	20.94bcd	15.83cde	24.67a	0.00c
GP2	33.05efgh	63.79ab	11.51defg	0.76fg	13.60ab	0.00c
GP5	34.98efg	65.22ab	14.70cdef	0.00g	21.67a	0.00c
GP10	10.79j	47.69cd	19.95bcd	31.77ab	13.36ab	0.24c
COS1	36.95ef	51.20cd	10.55cdefg	18.55cd	4.65bc	11.94ab
COS2	18.13ij	16.02j	13.80cde	24.37abc	0.30c	6.27bc
COS5	14.45j	42.74de	13.30cde	24.65	7.08bc	6.19bc
COS10	22.65ghij	34.80ef	15.98cde	15.94bcd	4.13bc	0.00bc

Table 5.4. α-Glucosidase inhibitory activity (%) of undiluted (A), half (B) and one-fifth diluted (C) dark germinated barley sprout extracts. Different alphabets represent significant differences in inhibition due to barley type × treatment interactions at 95% confidence level ($p < 0.05$).

(A)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	47.80abcd	46.53bcd	58.66de	88.68a	72.73de	86.75ab
GP1	38.02de	49.36abcd	57.80de	88.74a	74.53cd	70.57de
GP2	42.68cde	55.97ab	61.11cd	90.97a	67.65e	92.07a
GP5	25.34f	49.91abcd	59.65cde	89.12a	79.82c	87.30ab
GP10	31.27ef	44.88bcd	56.98de	91.22a	68.60de	85.92c
COS1	22.04f	55.17ab	65.87bc	92.04a	79.22c	86.92ab
COS2	22.75f	58.85a	68.57b	90.47a	91.07ab	87.38bc
COS5	31.07ef	54.56abc	69.19b	92.18a	87.74ab	86.65bc
COS10	24.88f	50.30abc	53.27e	92.92a	70.97de	86.62bc

(B)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	32.05bcd	31.06cde	44.53de	80.15b	57.52ef	76.53c
GP1	23.34efg	30.33cde	42.28e	80.60ab	63.01de	56.62ef
GP2	27.70def	36.77abc	46.39de	86.20ab	52.56f	86.35ab
GP5	18.41gh	32.09bcd	44.26de	80.26b	67.53d	77.91c
GP10	21.43fgh	27.34def	43.42e	86.85ab	55.66ef	78.08c
COS1	17.28gh	37.91abc	51.45cd	85.70ab	66.83d	77.33c
COS2	15.32gf	42.15a	54.33c	84.28ab	87.83a	79.54bc
COS5	18.42	39.32ab	55.32c	86.08ab	78.74bc	76.97c
COS10	13.27h	35.07abcd	39.44e	87.89a	56.77ef	78.94bc

(C)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	17.26bcde	13.06ef	26.03g	64.95bcd	39.41fgh	58.70dc
GP1	11.71efg	12.49efg	24.76g	63.88cd	48.01ef	38.96fgh
GP2	14.58cdef	20.69abc	26.79g	68.72abcd	36.13h	70.78a
GP5	13.37def	17.25bcde	25.65g	62.13d	52.20de	60.72bcd
GP10	13.97def	13.47def	29.30fg	69.97abcd	35.76h	58.12cd
COS1	11.55efg	20.49abcd	31.88efg	71.62abc	47.80efg	61.60bc
COS2	9.28fg	26.41a	37.40ef	69.53abcd	69.39ab	61.87abc
COS5	5.52g	23.20ab	37.99e	73.26ab	64.59abc	57.76cd
COS10	5.56g	20.38abcd	25.01g	75.62a	39.57fgh	58.24cd

5.5. Conclusions

Dark germination and sprouting in combination with seed elicitation may provide a simple and cost-effective strategy for improving human health relevant phenolic bioactives and associated anti-hyperglycemic functions of barley grains. Such a bioprocessing strategy based on sound metabolic rationale can potentially be targeted for developing functional food ingredients and nutraceuticals. However, finding suitable elicitor and optimizing effective doses is essential prior to utilizing this metabolic strategy for broader food and health relevant applications. Therefore, two different bioprocessed elicitor treatments in several doses and two different types of barley were strategically targeted and evaluated in this study. Overall, TSP content, antioxidant activity, α-amylase inhibitory activity and concentrations of catechin and dihydroxybenzoic acid decreased from day 2 to day 6, while α-glucosidase inhibitory activity and gallic acid concentration increased during these sprouting stages. Seed elicitation treatments with marine protein hydrolysate and COS in selected doses improved phenolic bioactive-linked anti-hyperglycemic functions in dark germinated barley sprouts. The data suggests that both elicitors may be targeted for improving phenolic bioactives and anti-hyperglycemic functions associated with early stages T2D in future food and ingredient design studies involving barley sprout models. However, a difference in response to elicitor treatments was observed between samples of the two types of barley utilized in the current experimental setup. Therefore, further research to evaluate other barley cultivars grown across multiple locations and growing seasons and different doses of elicitor treatments is vital to validate this concept for wider application and use.

CHAPTER 6. METABOLIC STIMULATION OF PHENOLIC BIOSYNTHESIS AND ANTIOXIDANT ENZYME RESPONSE IN DARK GERMINATED BARLEY SPROUTS USING BIOPROCESSED ELICITORS

6.1. Abstract

Sprouting coupled with seed elicitor treatments stimulate the biosynthesis of health relevant phenolic bioactives in plants by upregulating proline-associated pentose phosphate pathway (PAPPP). This study aimed to understand the upregulation of PAPPP-linked and antioxidant enzyme associated metabolic responses in elicitor-treated barley sprouts previously established with stimulation of health relevant phenolic bioactives. Barley seeds were treated with bioprocessed elicitors; marine protein hydrolysates (GroPro®, GP) and soluble chitosan oligosaccharide (COS) and germinated under dark. Upregulation of PAPPP and subsequent stimulation of phenolic biosynthesis and antioxidant enzyme responses were monitored at day 2, 4, and 6 of sprouting. High PAPPP-linked antioxidant enzyme responses were observed at early stages of germination with selected doses of GP treatments, especially in cv. Pinnacle. Total soluble phenolic content remained at higher level, while guaiacol peroxidase (GPX) activity increased over the course of sprouting indicating increased phenolic polymerization to support structural needs of sprouts.

Keywords: Antioxidant enzymes; Elicitors; Pentose Phosphate Pathway; Phenolics; Proline.

6.2. Introduction

Phenolic compounds are an important class of plant secondary metabolites with diverse protective functions towards plant and human health (Naczk and Shahidi, 2004). Due to their significant antioxidant property the role of phenolic compounds in human health relevant therapeutic applications has been widely investigated in recent decades (Briskin, 2000; Crozier *et*

al., 2006). Phenolic biosynthesis in plants is associated with the regulation of the protective pathways such as pentose phosphate (PPP), shikimate, and phenylpropanoid pathways (Shetty and Wahlqvist, 2004). The first-rate limiting step of PPP involves the glucose-6-phosphate dehydrogenase (G6PDH) catalyzed conversion of glucose-6-phosphate (G6P) to ribulose-5-phosphate, while generating reducing equivalents for other anabolic cellular processes (Shetty and Wahlqvist, 2004). Based on this rationale a model was proposed for biosynthesis of phenolic metabolites in plants through up-regulation of protective PPP coupled with active metabolic role of proline under stress (Shetty, 1997). In proline-associated pentose phosphate pathway (PAPPP) model, the demand for $NADPH_2$ during proline synthesis from glutamate in the cytosol can potentially increase the $NADP^+$ / $NADPH_2$ ratio, which favors the activation of G6PDH (Shetty, 2004; Shetty and Wahlqvist, 2004). Simultaneously, during its catabolism in the mitochondria proline can potentially act as a reducing equivalent instead of NADH to facilitate ATP synthesis via oxidative phosphorylation (Hare and Cress, 1997). Therefore, stimulation of the PAPPP can drive carbon flux in the form of erythrose-4-phosphate towards the shikimate and phenylpropanoid pathways, potentially elevating the rate of phenolic biosynthesis (Shetty and Wahlqvist, 2004). Further, this mechanism may also stimulate various antioxidant enzyme responses to counter stress-induced oxidative damage of cellular structures and their functions (Shetty, 1997; Shetty and McCue, 2003).

Various novel strategies have been previously investigated in plant models to stimulate protective phenolic–linked antioxidant enzyme responses through up-regulation of PAPPP-linked metabolic responses (Randhir *et al*., 2004; Randhir *et al*., 2002; Shetty and Wahlqvist, 2004; Shetty, 2004). Among these strategies, bioprocessed elicitors from natural sources, such as marine protein hydrolysates and soluble chitosan oligosaccharide (COS) have been found to be

particularly effective (Orwat, 2016; Sarkar *et al*., 2011). Further, PAPPP-linked metabolic responses were especially improved with dark germination, as respiration-linked biosynthetic processes could potentially be favored over photosynthesis, under such conditions (McCue and Shetty, 2002; Orwat, 2016; Randhir *et al*., 2009). In the absence of light, a greater amount of carbon flux derived from the amylolytic degradation of starch, may be channeled towards the PPP (McCue and Shetty, 2002). Thus, elicitor-primed dark germination of seeds represents a novel approach that effectively partitions the carbon flux between primary and secondary metabolism and could potentially improve human health relevant phenolic bioactive profile in targeted food crops (Sarkar and Shetty, 2014; Shetty, 2004). Based on this rationale, dark germination of primed seeds can be employed as effective viable strategy to produce grain sprouts with enhanced phenolic bioactive profiles with human health benefits.

Among cereal grains, barley is widely consumed, containing a diverse profile of phenolic metabolites that are of relevance to human health (Baik and Ullrich, 2008). Therefore, barley is an ideal choice for studying the overexpression of phenolic bioactives through PAPPP mediated metabolic regulation during germination. In a previous study, improvement of phenolic-linked antioxidant and anti-hyperglycemic properties were observed in dark germinated barley sprouts with seed elicitor treatments [(marine protein hydrolysate (GroPro ®) and COS] (Ramakrishna *et al*., 2017). Therefore, the aim of this study was to investigate the efficacy of these bioprocessed elicitors (GroPro and COS) in stimulation of human health relevant phenolic biosynthesis and associated antioxidant enzyme responses in dark germinated barley sprouts through upregulation of critical control points of the anabolic PAPPP.

6.3. Materials and Methods

6.3.1. Chemical Reagents

All reagents and enzymes used in this study were purchased from Sigma Aldrich Chemical Co. (St Louis, MO, USA), unless otherwise mentioned.

6.3.2. Bioprocessed Elicitors

Two different types of bioprocessed elicitors were targeted in the current study – marine protein hydrolysate derived from seaweed and marine fish extracts (GroPro® / GP; Icelandic Bio-Enhancers, Westchester, NY, USA) and soluble chitosan oligosaccharide derived from shells of crustaceans, with vitamin-C side chain (Kong Poong Bio, Jeju, South Korea). Both elicitors were dissolved in distilled water to obtain solutions of the following concentrations – 1 mL/L or 1 g/L (GP1 / COS1), 2 mL/L or 2 g/L (GP2 / COS2), 5 mL/L or 5 g/L (GP5 / COS5) and 10 mL/L or 10 g/L (GP10 / COS10). In all two different elicitors with four different doses were used along with control (water seed treatment).

6.3.3. Seed Treatment and Germination

Seeds of two malting barley types (Pinnacle - 2 rowed; Celebration - 6 rowed) from a single crop year were obtained from the Malting Barley Breeding Program at North Dakota State University (Fargo, ND, USA). The seeds were disinfected in 0.5% sodium hypochlorite solution for 5 minutes (min), rinsed 5 times with distilled water and extra water was blotted out using dry paper towels. The disinfected seeds were then transferred to conical flasks containing seed treatment solution (100 mL) and incubated on a rotary shaker at 150 rpm and at room temperature for 8 hours. The seed treatment solutions were drained, the seeds were rinsed with distilled water and excess treatment solution was then blotted out with paper towels. Treated seeds were placed in sterile Petri dishes lined with moist paper towels (10 seeds per plate) and

transferred to an incubator for germination. Internal temperature and relative humidity were maintained throughout the experiment at 20°C and > 95% respectively. Germination was monitored daily, and the sprouts were moistened as required. Prior to harvesting the sprout samples, their rate of germination was recorded at each assay time point. The developing dark germinated sprouts were used for biochemical analysis at 2, 4 and 6-days post elicitor treatment.

6.3.4. Sample Extraction for Total Soluble Phenolic Content and Antioxidant Activity Assays

Barley sprouts (100 mg fresh weight F.W.) were transferred to a glass vial containing 95% ethanol (5 mL) and stored at -10°C in the freezer. After 48 hours, samples were homogenized using tissue tearor and centrifuged at 13,000 rpm for 5 min. The supernatant was analyzed to determine the total soluble phenolic content and antioxidant activity of the sprout samples *in vitro*.

6.3.5. Total Soluble Phenolic (TSP) Content

Total soluble phenolics content of the barley sprout extracts was measured using the Folin-Ciocalteu (FC) method, modified from Shetty *et al*., (1995). Sprout extracts (1 mL) were combined with 95% ethanol (1 mL), distilled water (5 mL), FC reagent (50% v/ v; 0.5 mL), and sodium carbonate solution (5% v/v; 1 mL) in a test tube, mixed thoroughly and incubated in the dark for 60 min. Absorbance of the reaction mixtures were measured at 725nm using a spectrophotometer (Genesys 10S UV-Vis, ThermoFisher Scientific; Waltham, MA, USA). Standard curves were prepared using various concentrations of gallic acid (10 – 300 μg/mL) in 95% ethanol. Absorbance values were converted to total soluble phenolic content and expressed as milligram equivalents of gallic acid per gram fresh weight of tissue sample.

6.3.6. ABTS Free Radical Scavenging Assay

Antioxidant activity was measured using the ABTS [2, 2 –azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay (Re *et al*., 1999). A stock solution of 7 mM ABTS containing 140 mM of potassium persulphate was prepared and stored in the dark at 4°C for 12–16 h before use. Prior to performing the assay, a working solution of ABTS was prepared by diluting the stock solution with 95% ethanol, to reduce its absorbance to 0.70 ± 0.02 units at 734 nm. To determine the antioxidant activity, 1 mL of ABTS working solution was mixed with 50 µL of sprout extract, incubated at room temperature for 2.5 min and absorbance was measured at 734 nm. The antioxidant activity of the sprout extracts was expressed as percentage (%) inhibition of ABTS radical formation and was calculated as per the following formula:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

6.3.7. Enzyme Extraction

Barley sprouts (200 mg F.W.) were macerated thoroughly using a cold pestle and mortar, placed in an ice bath, with 2 mL of cold enzyme extraction buffer (0.5% polyvinylpyrrolidone (PVP), 3 mM EDTA, and 0.1 M potassium phosphate buffer; pH 7.5). The sample extract was then centrifuged at 13,500 rpm for 10 min and immediately stored on ice. The supernatant was used for further biochemical analysis.

6.3.8. Total Protein Assay

Protein content was measured using the method described by Bradford (1976). One part of dye reagent (Bio-Rad protein assay kit II, Bio-Rad Laboratory; Hercules, CA, USA) was diluted with four parts of distilled water. A volume of 5 mL of diluted dye reagent was added to 50 µL of the barley extract; the mixture was then vortexed and incubated in the dark for 5 min at

room temperature. The absorbance of the reaction mixture was measured at 595 nm against a blank (5 mL reagent and 50 µL buffer solution) using a Genesys 10S UV-Vis Spectrophotometer (Thermo-Fisher Scientific; Waltham, MA, USA). A calibration curve was prepared with standard solutions of bovine serum albumin dissolved in distilled water (1, 2.5, 5, 7.5, 10 µg/mL), and used to calculate the total protein content of the extracts.

6.3.9. Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

A modified method described by Deutsch (1983) was used. The enzyme reaction mixture containing 5.88 µmol ß-NADP, 88.5 µmol $MgCl_2$, 53.7 µmol glucose-6-phosphate, and 0.77 mmol maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer (Evolution 200 UV-Vis, Thermo Fisher Scientific; Waltham, MA, USA) reading at 340 nm wavelength. To 1 mL of this mixture, 100 µL of the extracted sample was added. The rate of change in absorbance per min was used to quantify the enzyme in the mixture with the help of the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}). The activity of G6PDH was expressed in nmol/g protein.

6.3.10. Guaiacol Peroxidase (GPX) Assay

A modified method described by Laloue *et al*., (1997) was used to measure the activity of guaiacol peroxidase. The enzyme reaction mixture containing 0.1 M potassium phosphate buffer (pH 6.8), 56 mM guaiacol solution, and 50 mM hydrogen peroxide was used. To 990 µL of this reaction mixture, 10 µL of enzyme sample was added. The absorbance was recorded immediately after the addition of reaction mixture and after 3 min. The rate of change in absorbance per min was measured using an Evolution 200 UV-Vis spectrophotometer (ThermoFisher Scientific; Waltham, MA) and used to quantify GPX activity in the sample based

on the extinction coefficient of the oxidized product, i.e., tetraguaiacol (26.6 mM^{-1} cm^{-1}). The activity of GPX was expressed in nmol/g protein.

6.3.11. Superoxide Dismutase (SOD) Assay

A competitive inhibition assay was performed that used xanthine oxidase generated superoxide to reduce nitrobluetetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (Oberley and Spitz, 1984). The reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33 mM diethylenetetraaminepentaacetic acid (DETEPAC); 0.5 mL of 2.45 mM NBT; 1.7 mL of 1.8 mm xanthine and 40 IU/mL catalase. Then 100 µL of phosphate buffer and 100 µL of xanthine oxidase were added to 0.8 mL of reagent mixture. The change in absorbance at 560 nm was measured immediately after adding the reagents and after 1 min using an Evolution 200 UV-Vis spectrophotometer (ThermoFisher Scientific; Waltham, MA, USA). The concentration of xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.024 to 0.026 absorbance per min. The phosphate buffer was replaced by the sprout sample and the change in absorbance was monitored immediately after the addition of reaction mixture and after 1 min. One unit of SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum. The activity of SOD was expressed in units/mg protein.

6.3.12. Catalase (CAT) Assay

A method originally described by Beers and Sizer (1952) was used to measure the activity of catalase. To 1.9 mL of distilled water, 1 mL of 0.059 M hydrogen peroxide (Fisher Scientific; Waltham, MA) in 0.05 M potassium phosphate (pH 7.0) was added. This mixture was incubated in a spectrophotometer (Evolution 200 UV-Vis, ThermoFisher Scientific; Waltham, MA) for 4–5 min to achieve temperature equilibration and to establish blank rate. Then 0.1 mL

of diluted enzyme was added, and the disappearance of peroxide was followed spectrophotometrically by recording the decrease in absorbance at 240 nm for 1 min. The change in absorbance (ΔA_{240}/min) from the initial linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2. The activity of CAT was expressed in units/mg protein.

6.3.13. Proline Dehydrogenase (PDH) Assay

A method described by Costilow and Cooper (1978) was used to measure the activity of proline dehydrogenase. The enzyme reaction mixture containing 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline solution and 10 mM NAD was used. To 1 mL of this reaction mixture, 200 µL of enzyme extract was added. The increase in absorbance was measured at 340 nm for 3 min, at 32°C using an Evolution 200 UV-Vis spectrophotometer (ThermoFisher Scientific; Waltham, MA). The absorbance was recorded at zero time and then after 3 min. In this spectrophotometric assay, one unit of enzyme activity is equal to the amount causing an increase in absorbance of 0.01 per min at 340 nm (1.0 cm light path). The activity of PDH was expressed in units/mg protein.

6.3.14. Succinate Dehydrogenase (SDH) Assay

To measure the activity of succinate dehydrogenase a modified method described by Bregman (1987) was used. The reaction mixture containing 1.0 mL of 0.4 M potassium phosphate buffer (pH 7.2), 40 µL of 0.15 M sodium succinate (pH 7.0), 40 µL of 0.2 M sodium azide, and 10 µL of 6.0 mg/mL 2, 6-dichlorophenolindophenol (DCPIP) was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer (Evolution 200 UV-Vis, ThermoFisher Scientific; Waltham, MA) reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 µL of the enzyme sample was added. The rate of change of absorbance per min was

used to quantify the enzyme in the mixture based on the extinction coefficient of DCPIP (19.1 mM^{-1} cm^{-1}). The activity of SDH was expressed in nmol/g protein.

6.3.15. Determination of Free Proline Content using HPLC

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1260 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector (DAD 1100). A C-18 analytical column (Agilent Zorbax Eclipse Plus; 4.5 mm × 250 mm) with a packing material particle size of 5 μm was used as the stationary phase, and a 20 mM ortho-phosphoric acid solution was used as the mobile phase. The column temperature was maintained at 25°C. Each sample extract was eluted isocratically for 10 mins, at a flow rate of 1.0 mL/min and free proline was detected at 210 nm. Standard solutions of L-proline dissolved in 20 mM ortho-phosphoric acid were used to prepare the standard curve. Four samples were analyzed per sample, and the mean free proline content was expressed in mg/g fresh weight.

6.3.16. Statistical Analysis

A completely randomized design (CRD) was used for this study. Each Petri-dish in which sprouts were grown was considered an experimental unit, and sprout samples corresponding to each treatment combination were collected from three different dishes (each constituting a replicate) for biochemical analysis. The entire experiment was repeated thrice. Calculation of means and standard deviations of a total of 9 data points was performed using Microsoft Excel 2016. Analysis of variance (ANOVA) for the data was performed using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC). Statistically significant differences for mean separations` between elicitor treatments, barley types, and barley

type × elicitor treatment interactions were determined using the Tukey's least mean square test at a confidence level of 95%.

6.4. Results and Discussion

6.4.1. Rate of Germination

The rate of germination was recorded at each assay time point prior to harvesting sprout samples for assays and the observations are presented in Table 6.1.

Table 6.1. Mean germination rate (%) of dark germinated barley sprouts post priming with elicitor treatments.

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	95	96	99	100	100	100
GP1	97	96	100	100	100	100
GP2	97	98	100	100	100	100
GP5	98	97	100	100	100	100
GP10	97	97	100	100	100	100
COS1	96	98	100	100	100	100
COS2	97	96	100	100	100	100
COS5	96	98	100	100	100	100
COS10	95	97	100	100	100	100

6.4.2. Total Soluble Phenolic Content and Antioxidant Activity

Phenolic compounds are mostly found in the hull and aleurone layers of cereal grains bound to cell wall polysaccharides such as arabinoxylans providing structural support (Baik and Ullrich, 2008). Previous studies have reported that seed treatment with bioprocessed elicitors prior to sprouting further enhanced the total soluble phenolic (TSP) content of various cereal grains and legumes (Randhir *et al*., 2004; Randhir *et al*., 2002), which may have relevance in improving the health-oriented functionality of grain sprouts. In our previous study with barley sprouts to study hyperglycemia related health benefits, improvement of TSP and individual phenolic acids (catechin, gallic acid, protocatechuic acid, and dihydroxybenzoic acid) content

were observed with seed elicitor treatments (Ramakrishna *et al*., 2017). Therefore, in this study the metabolic control associated with TSP content of dark-germinated barley sprouts was investigated. In the current study, the TSP content of sprouts of both Celebration and Pinnacle slightly reduced from day 2 to day 4, followed by a subsequent increase at day 6 post seed elicitor treatments (Table 6.1.A). The TSP content of sprouts of cultivar Celebration was found to be statistically higher than that of Pinnacle at day 4 ($p < 0.05$). However, at day 2 and 6, TSP content was found to be statistically at par between the two barley types. Previously, Ramakrishna *et al*., (2017) found high TSP content at day 2 in aqueous extracts of Pinnacle and Celebration sprouts following GP and COS seed elicitor treatments. Similarly, fish protein hydrolysates, lactoferrin, and oregano extracts also elicited TSP content in 1-day old mung bean sprouts, followed by a gradual decline in TSP content as sprouting progressed (Randhir *et al*., 2004). This trend may be attributed to an increase in TSP content immediately after the sprouting induced activation of the seed, due to cleaving and solubilization of cell-wall bound phenolics, as well as the biosynthesis of new phenolic compounds to meet the anabolic needs of the developing plants (Singh and Sharma, 2017). The decline of TSP content over further seedling emergence may be linked to the conversion of simple soluble phenolics to more complex insoluble compounds of greater molecular weight, associated with developmental processes such as lignification (Randhir *et al*., 2004). However, in the current study, such a pattern of decline in TSP content was not observed over the course of sprouting and TSP content remained comparatively high. The high baseline value of TSP content in the current study over the period of germination and sprouting might have relevance for targeting barley sprout from different stages for designing phenolic bioactive enriched functional foods.

No significant differences in TSP content were observed due to barley type × elicitor treatment interaction at day 2 and 4. However at day 6, sprouts of cv. Pinnacle treated with GP1 (GroPro 1mL/L) had significantly higher TSP content when compared to the control and other seed elicitor treatments ($p < 0.05$). For Celebration barley sprouts COS1 seed treatment had significantly higher TSP content, however it was at par with respective control. Previously, seed treatment with fish protein hydrolysates (5 mL/L) elicited the phenolic biosynthetic during the later stages of sprouting in primed sprouts of corn (Randhir and Shetty, 2005), while in mung bean sprouts 1 mL/L treatment corresponded with maximum phenolic stimulation on day 1 post priming (Randhir *et al*., 2004). Such variability could potentially arise from differences in response between monocots and dicots and anabolic demands of the developing plants during sprouting.

The ability of phenolic compounds to function as effective antioxidants and free radical scavengers is an important aspect of their biological activity, and it is vital for countering any deleterious deviations from optimal cellular redox homeostasis in plant tissues (Rice-Evans *et al*., 1997). Previous studies observed that elicitor-mediated stimulation of TSP content has corresponded with a simultaneous increase in the free radical scavenging capacities of sprout extracts of grains and legumes such as corn (*Zea mays*), pea (*Pisum sativum*), and mung beans (*Vigna radiata*) (Andarwulan and Shetty, 1999; Randhir *et al*., 2004; Randhir and Shetty, 2005). In the current study antioxidant activity of the dark germinated sprouts was found to be highest at day 2, and steadily decreased over the course of sprouting (Table 6.1.B). A similar trend was observed in corn sprouts (Randhir and Shetty, 2005), where antioxidant activity (based on DPPH assay) was found to be highest at early stages of germination. Respiration is the dominant physiological process in the seed tissues immediately after water imbibition, which can lead to

elevated levels of free radical generation and therefore necessitating a greater demand for antioxidants during this time (Randhir and Shetty, 2005). In earlier studies, declining antioxidant activity of barley and mung bean sprouts has corresponded with a decrease in TSP content (Ramakrishna *et al*., 2017; Randhir *et al*., 2004). The antioxidant activities of samples of the two types of barley used in this study were significantly different at all three timepoints ($p < 0.05$). Pinnacle had significantly higher antioxidant potential at days 2 and 6, whereas Celebration was higher at day 4. At all time points, significant differences were observed in antioxidant activity due to barley type × treatment interactions. At day 2 and 4, higher antioxidant activity was observed in control. However, at day 6, higher total antioxidant activity in Pinnacle sprouts was observed with GP1 seed elicitor treatment, while in cv. Celebration, GP10 had significantly higher antioxidant activity when compared to the respective control. For cv. Pinnacle, same elicitor treatment (GP1) resulted in higher TSP content at day 6 which has significant relevance for improving phenolic-linked antioxidant activity in dark germinated barley sprouts. Therefore, GroPro in lower doses (1mL/L) can be targeted further to induce phenolic-linked antioxidant activity in barley sprout, especially at later sprouting stages.

Table 6.2. Total soluble phenolics (TSP) (A) (mg/g GAE F.W.) and ABTS free radical inhibition capacity (B) (%) of dark germinated barley sprouts following seed elicitor treatments. Different lowercase letters indicate significant differences in TSP content and antioxidant activities due to barley type × treatment interactions, during the corresponding sampling time point at 95% confidence level ($p < 0.05$).

(A)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	0.886	0.797	0.882	0.891	0.754cdef	0.820abcd
GP1	0.784	0.618	0.839	0.739	0.927a	0.784bcde
GP2	0.742	0.699	0.659	0.813	0.748cdef	0.571g
GP5	0.722	0.767	0.647	0.72	0.701defg	0.860abc
GP10	0.734	0.864	0.669	0.72	0.782bcde	0.708defg
COS1	0.76	0.706	0.667	0.829	0.759cdef	0.909ab
COS2	0.794	0.781	0.777	0.746	0.733cdef	0.658efg
COS5	0.706	0.759	0.58	0.707	0.739cdef	0.721cdef
COS10	0.759	0.768	0.687	0.748	0.638fg	0.833abcd

(B)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	24.88a	16.25bcd	21.19a	13.33cdef	12.33abc	6.74gh
GP1	20.55ab	10.91defg	10.50defgh	18.17ab	14.34a	7.30efgh
GP2	8.77fg	12.75cdefg	6.08ij	13.86cde	9.59bcdefg	7.71defgh
GP5	8.36g	10.50efg	8.33ghij	16.07bc	12.75ab	8.93cdefg
GP10	9.65efg	9.98efg	7.13hij	16.28bc	10.40bc	11.01bcde
COS1	10.41efg	9.40efg	10.28efgh	12.65cdef	10.68bcde	4.41h
COS2	17.15bc	13.03cdefg	12.65cdef	9.72fghi	8.32defg	9.90bcdefg
COS5	13.96cdefg	15.03bcde	11.06defg	5.30j	10.24bcdef	8.30defg
COS10	14.14cdef	10.99defg	13.95cd	7.55ghij	9.47bcdefg	6.89fgh

6.4.3. Glucose-6-Phosphate Dehydrogenase (G6PDH) and Succinate Dehydrogenase (SDH) Activities

The pentose phosphate pathway (PPP) is a critical protective pathway through which carbon flux are channeled into various anabolic pathways such as shikimic acid and phenylpropanoid pathways and leads to phenolic biosynthesis. The first-rate limiting step of the PPP is the oxidation of glucose-6-phosphate to 6-phosopho-glucose-δ-lactone, catalyzed by G6PDH. Consequently, G6PDH activity in a specific tissue can be indicative of the amount of carbon flux from primary metabolism that is being potentially directed towards secondary metabolic pathways, including phenolic biosynthesis (Shetty and Wahlqvist, 2004).

In barley sprouts of cv. Celebration and Pinnacle, G6PDH activity was at the highest levels at day 2 post seed elicitor treatments following which it steadily declined over the course of sprouting (Figure 6.1.A, B). Overall, Pinnacle sprouts had higher G6PDH enzyme activity than Celebration sprouts at all time points (at day 2, 4, and 6). At day 2, GP 10 treated Pinnacle sprouts had significantly higher G6PDH activity compared to its corresponding control as well as other elicitor treatments ($p < 0.05$). At day 4, G6PDH activity of most GP treated Pinnacle sprouts were significantly higher than that of Celebration sprouts with an exception for the GP2 seed elicitor treatments where it was statistically at par. No significant differences in G6PDH activities between Celebration and Pinnacle sprouts were observed at day 6.

Previously, Randhir and Shetty (2005) found that corn seeds primed with fish protein hydrolysate (5 mL/L) had similar results whereby G6PDH activity was highest at the early stages of germination (day 3 post treatment), before declining in the intermediate stages. This may be attributed to elevated levels of carbohydrate solubilization and mobilization to meet the anabolic and energy demands of the developing seedling (Singh and Sharma, 2017). Furthermore, Randhir *et al*., (2004) also reported a positive correlation between G6PDH activity and TSP content in the intermediate stages of sprouting in elicitor treated mung bean sprouts, indicating the potential role of PPP upregulation in promoting phenolic biosynthesis. However, in the current study, TSP content maintained at higher level from day 2 to day 6, while G6PDH activity declined during the same course of sprouting.

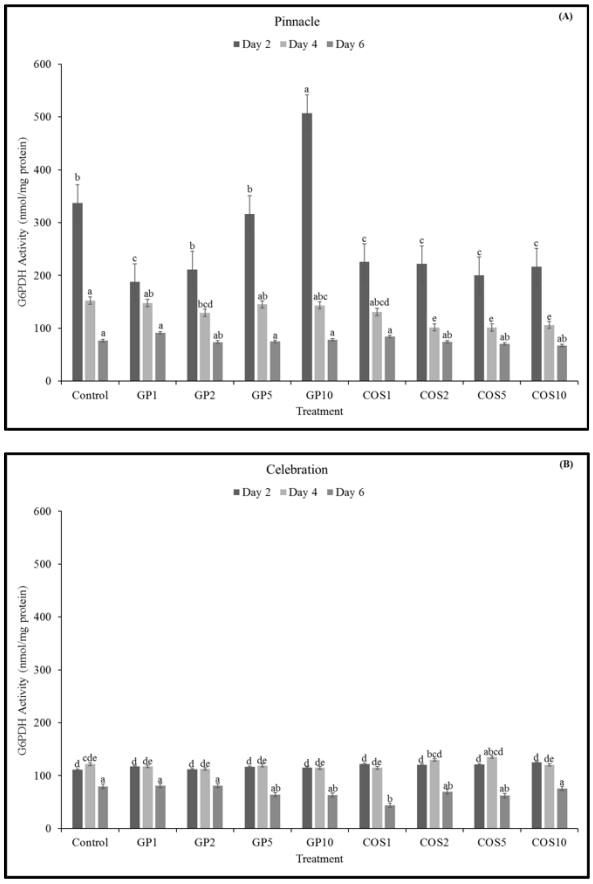

Figure 6.1. Glucose-6-phosphate dehydrogenase (G6PDH) activity (nmol/mg protein) of dark germinated barley sprouts of Pinnacle (A) and Celebration (B) at day 2, day 4, and day 6 following seed elicitor treatments (GroPro and COS). Different lowercase letters indicate significant differences in G6PDH activity between barley type × treatment interactions, during the corresponding sampling time point ($p < 0.05$).

Succinate dehydrogenase (SDH) is a critical redox enzyme that couples the oxidation of succinic acid to fumaric acid in Krebs's cycle with the reduction of ubiquinone to ubiquinol in the mitochondrial electron transport chain (ETC), accompanied by the formation of reducing equivalents ($FADH_2$) and ATP (Huang and Millar, 2013). The activity of SDH activity was measured to evaluate whether the COS and GP elicitor treatments could upregulate the activity of Krebs's cycle and overall respiration rate (Sarkar *et al*., 2011). For samples of both barley types, SDH activity was found to increase from day 2 to the highest level at day 4, and subsequently decreasing to the lowest level at day 6 (Figure 6.2.A, B). Among the samples of

barley, sprouts of Pinnacle had higher SDH activity than those of Celebration at day 2 and 4, but statistically significant differences in SDH activity were observed only at day 2. However, at day 6 Celebration sprouts had higher SDH activity than those of Pinnacle for most seed elicitor treatments.

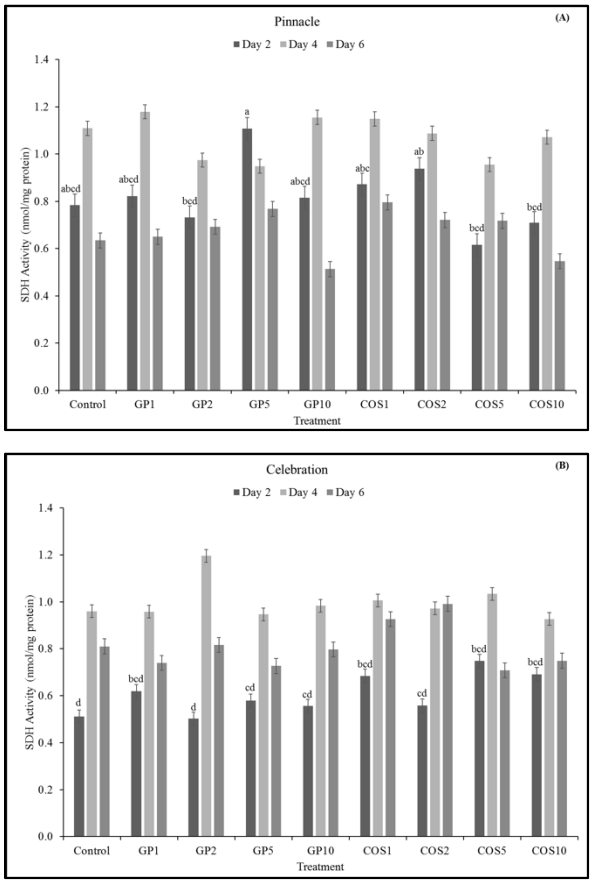

Figure 6.2. Succinate dehydrogenase (SDH) activity (nmol/mg protein) of dark germinated barley sprouts of Pinnacle (A) and Celebration (B) at days 2, 4, and 6 after seed elicitor treatments. Different lowercase letters indicate significant differences in SDH activity between cultivar × treatment interactions, during the corresponding sampling time point, at 95% confidence level. Bars without lowercase letters do not have statistically significant differences between barley type × treatment interactions ($p < 0.05$).

Statistically significant difference in SDH activity due to barley type × treatment interaction was observed only at day 2. At day 2, GP5 treated Pinnacle sprouts had the highest SDH activity (1.11 nmol/mg protein). However, this value was statistically at par with the SDH activities of other Pinnacle sprouts treated with other doses of GP, as well as those treated with

COS1 and COS2. The improvement of both G6PDH and SDH activity at day 2 in Pinnacle with GroPro seed elicitor treatments (GP10 and GP5) might have relevance for mobilization of carbon flux through PPP and Krebs's cycle to meet higher energy demands during germination and seedling emergence of barley. Therefore, GroPro can be targeted in selective doses to upregulate PPP to support anabolic needs during germination and seedling emergence of barley and other grain sprouts.

6.4.4. Proline Content and Proline Dehydrogenase (PDH) Activity

The free proline content of dark germinated barley extracts was measured to evaluate the modulation of proline metabolism and its coupling with PPP, in response to seed elicitor treatments. The free proline content of dark germinated sprouts of Celebration and Pinnacle was found to decline over the course of germination, with the highest levels being observed at day 2 (Figure 6.3. A, B). Extracts of Celebration sprouts treated with all doses of COS, as well as GP1 and GP10 treatments were found to have higher proline content than Pinnacle sprouts with same doses of elicitor treatments. In this study, Celebration sprouts treated with GP1 and GP10 had the highest free proline content at day 2 and 4. Overall, significantly higher proline content was observed in Pinnacle at day 2, while it was higher in Celebration at day 6. However, significant differences in proline content due to barley type × treatment interactions were not observed at any stage of the study.

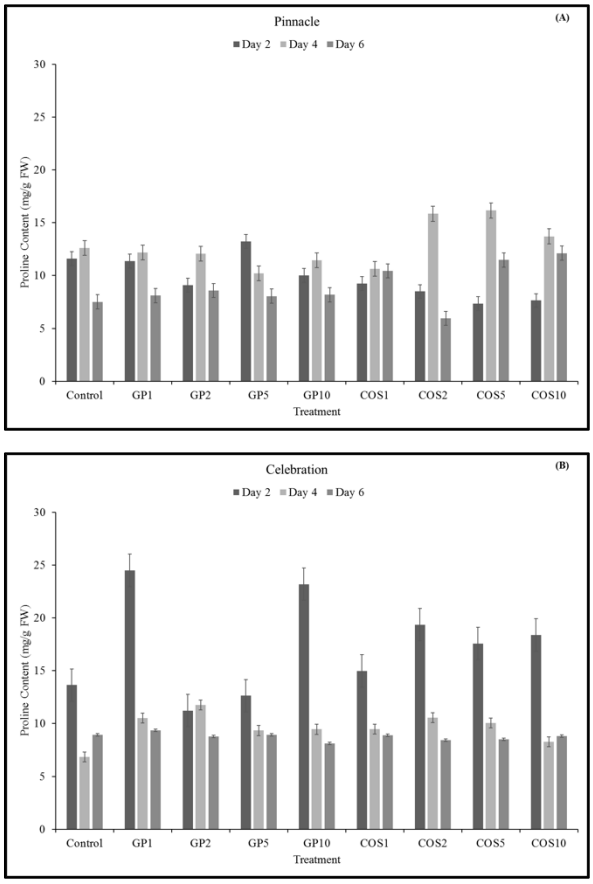

Figure 6.3. Total proline content (mg/g F.W.) of dark germinated barley sprouts of Pinnacle (A) and Celebration (B) at days 2, 4, and 6 after seed elicitor treatments (GroPro and COS).

The enzyme PDH catalyzes the oxidation of L-proline to pyrroline-5-carboxylate in the mitochondria. Activity of the PDH in the barley sprouts was measured to assess the degree of cellular proline oxidation, and thereby to evaluate the role of elicitor treatments in upregulating proline mediated mitochondrial ATP synthesis. The highest PDH activity of dark germinated barley sprouts were observed at day 2. Activity of PDH decreased rapidly from day 2 to day 4, before reaching an intermediate level at day 6 (Figure 6.4. A, B). At day 2, Celebration sprouts treated with COS1, COS2, and GP10 elicitor treatments were found to have significantly higher PDH activities than that of the corresponding control and other treatments. However, differences in PDH activity due to barley type × treatment interactions were found to be statistically

significant only in the early stages of sprouting (day 2), and not in the intermediate or later stages.

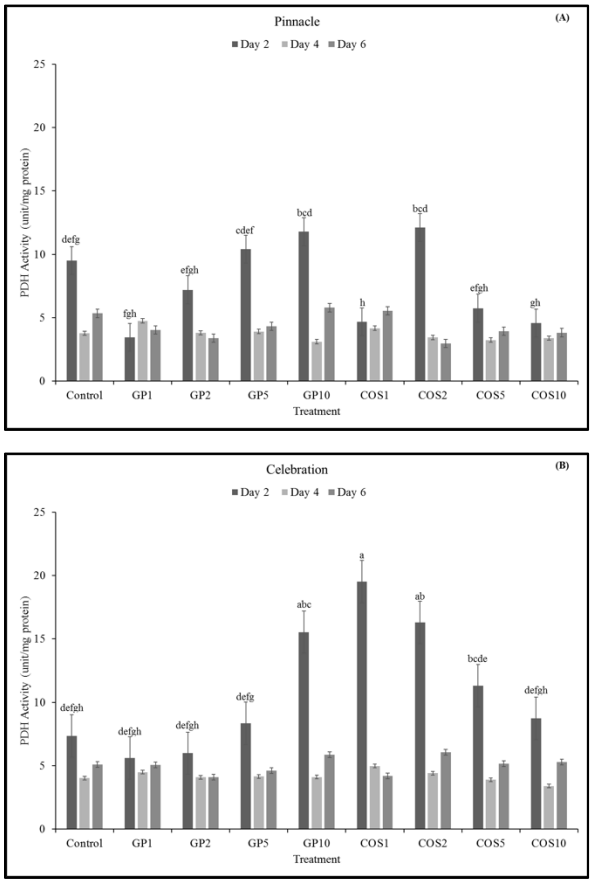

Figure 6.4. Proline dehydrogenase (PDH) activity (unit/mg protein) of dark germinated barley sprouts of Pinnacle (A) and Celebration (B) at day 2, day 4, and day 6 following seed elicitor treatments (GroPro and COS). Different lowercase letters indicate significant differences in PDH activity between barley type × treatment interactions, during the corresponding sampling time point, at 95% confidence level. Bars without lowercase letters do not have statistically significant differences between barley type × treatment interactions at 95% confidence level.

Overall, the trend observed for PDH activity during sprouting is very similar to that of free proline content. Therefore, the observed trends indicate that the seed treatment with elicitors such as COS and GP may play a potential role in stimulating proline oxidation by PDH which has relevance for improving abiotic stress modulation of food crops. Such active metabolic role of proline may support proline-linked ATP synthesis in the early stages of sprouting to meet the energy demand in the developing barley seedlings.

6.4.5. Guaiacol Peroxidase (GPX), Catalase (CAT) and Superoxide Dismutase (SOD) Activities

The effect of COS and GP seed elicitor treatments on stimulating the protective antioxidant enzyme responses in barley sprouts at various growth stages was investigated by measuring the activities of GPX, SOD, and CAT enzyme activities. Peroxidases such as GPX are responsible for the polymerization of phenolic compounds by catalyzing the formation of oxidation-mediated cross linkages between phenolic precursors, during the biosynthesis of lignin in plant cell walls (Morales and Barceló, 1997).

The activity of GPX increased steadily over the course of sprouting, with the highest levels being observed at day 6 post seed elicitor treatments (Table 6.2.A.). The concurrent increase in GPX activity with the development of the barley seedlings is consistent with the trends observed in various other elicitor treated sprouts of corn, fava bean (*Vicia faba*), black bean (*Phaseolus vulgaris*), velvet bean (*Mucuna pruriens*), and mung bean (Orwat, 2016; Randhir *et al*., 2009; Randhir and Shetty, 2004; Randhir and Shetty, 2003). This can be attributed to the increasing necessity for lignification whereby free phenolics are utilized for the formation of robust polymeric complexes to lend structural and mechanical support and defense against environmental stresses to the growing plant. The early stages of germination are associated with the mobilization and production of phenolic precursors for the anabolic needs of the emerging seedling, because of which GPX levels are expected to have relatively low levels of activity (Randhir and Shetty, 2003). Dark germinated Pinnacle sprouts had higher GPX activity than those of Celebration. GroPro treated sprouts showed higher GPX activity at day 2 and 4 post seed treatment, while COS treated sprouts exhibited higher GPX activity in the later stages.

Differences in GPX activity due to barley type × treatment interactions were statistically significant ($p < 0.05$) at day 4 and 6.

Catalase is another key antioxidant enzyme which catalyzes the decomposition of hydrogen peroxide to water and oxygen, thereby aiding in protecting sub-cellular structures from the toxic effects of excessive hydrogen peroxide accumulation (Sarkar *et al*., 2009). In this study, the observed levels of CAT activity varied widely among barley types across all time points (Table 6.2.B). Significant differences in CAT activity due to barley type × treatment interactions were observed at day 2 and 4 ($p < 0.05$). However, at day 4, CAT activity of Celebration sprouts was significantly higher than those of Pinnacle. Whereas the CAT activity of Celebration sprouts treated with COS2 and COS5 treatments were found to be statistically at par with the corresponding control treatment. However, it was significantly higher than that most GP treatments.

Superoxide dismutase (SOD) regulates the elimination of highly deleterious superoxide free radicals by catalyzing its conversion to molecular oxygen and hydrogen peroxide and is critical to maintaining cellular redox homeostasis. It is a key component of the enzymatic antioxidant response mechanism that aids plants in overcoming ROS mediated cellular damage induced by biotic and abiotic stresses (Sarkar *et al*., 2009). During sprouting, the highest level of SOD activity was observed at day 6 for all combinations of barley type × elicitor treatments (Table 6.2.C). Among the sprout samples of the two barley types under consideration, Pinnacle demonstrated significantly higher SOD activity when compared to Celebration ($p < 0.05$). High SOD activity of Pinnacle also positively correlated with G6PDH activity, TSP content, and total antioxidant activity. From the trends observed in this study, it was evident that while seed

treatment with elicitors did influence the activity of key antioxidant enzymes to varying extents, their response was specific to barley type (genetic make-up).

Table 6.3. Guaiacol peroxidase (GPX) (A) (nmol/mg protein), catalase (CAT) (B) (unit/mg protein), and superoxide dismutase (SOD) (C) (unit/mg protein) enzyme activity of dark germinated barley sprouts following seed elicitor treatments. Different lowercase letters indicate significant differences in enzyme activities due to barley type × treatment interactions, during the corresponding sampling time point at 95% confidence level ($p < 0.05$).

(A)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	523.8	249.2	863.1ab	637.2c	1459.1abcd	1117.6ef
GP1	525.9	251.2	795.5abc	699.5bc	1414.3bcde	1172.1def
GP2	560.9	275.3	723.7bc	742.4bc	1341.5bcdef	1115.9ef
GP5	582.1	281.7	772.2abc	730.7bc	1307.5cdef	1061.1f
GP10	525.3	295.7	933.9a	824.2ab	1430.1abcd	1165.3def
COS1	547.7	277	789.5abc	766.0abc	1337.0bcdef	1247.0cdef
COS2	441.6	272.2	851.1ab	819.5ab	1616.5ab	1231.3cdef
COS5	445.7	251.1	836.0ab	870.7ab	1489.9abc	1179.2def
COS10	554.7	282.7	751.8abc	783.0abc	1720.5abcd	1226.0def

(B)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	210.7a	219.3ab	47.4d	47.4d	42.7	24.3
GP1	230.2ab	227.2ab	50.9d	50.9d	43.9	25.5
GP2	238.8a	204.8ab	51.8d	51.8d	44.8	26.2
GP5	255.7a	237.4ab	51.0d	51.0d	41.4	27.1
GP10	230.9ab	217.3ab	58.3d	58.3d	33.7	23
COS1	242.5a	229.3ab	64.5d	64.5d	47.5	27.3
COS2	208.6ab	223.1ab	55.3d	55.3d	40.9	26.6
COS5	184.3b	256.8a	56.1d	56.1d	39.6	24.2
COS10	220.0ab	235.4ab	51.7d	51.7d	40.5	27.5

(C)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
Control	0.01	0.01	0.02	0.01	0.07	0.01
GP1	0.03	0.01	0.02	0.01	0.05	0.01
GP2	0.02	0.01	0.02	0.02	0.02	0.01
GP5	0.02	0.01	0.02	0.01	0.03	0.01
GP10	0.03	0.01	n.d.	0.01	0.05	0.03
COS1	0.02	0.01	0.02	0.02	0.02	0.01
COS2	0.02	0.02	0.03	0.01	0.03	0.02
COS5	0.03	0.01	0.03	0.02	0.04	0.01
COS10	0.01	0.01	0.02	0.02	0.04	0.02

n.d.- Not detected

Overall, the results of the current study indicate that GPX antioxidant enzyme has more relevance during germination and sprouting stages of barley and seed elicitor treatments in selective doses can be targeted to improve the antioxidant enzyme responses of some barley cultivars.

6.5. Conclusions

Sprouting is a viable bioprocessing strategy for functional food enrichments as many nutrients are mobilized or have higher bioavailability for assimilation during germination and seedling emergence of cereal grains and legumes. When used in conjunction with seed elicitation treatments, sprouting could offer a simple strategy for improving phenolic biosynthesis and antioxidant enzyme responses through up-regulation of critical protective pathways such as PAPPP-linked metabolic regulation. This study investigated the critical control points of PAPPP-linked metabolic responses for stimulating phenolic biosynthesis and antioxidant enzyme responses in dark germinated barley sprouts following seed elicitor treatments. Overall, PAPPP-linked improvement of phenolic biosynthesis and antioxidant enzyme response was observed in dark germinated barley sprouts with selected doses of GP as seed elicitor treatment. Thus, the current data suggests that bioprocessed elicitors such as GP in selected doses may improve phenolic biosynthesis and antioxidant enzyme responses in barley sprouts. Moreover, the response was more prominent in sprout extracts of cv. Pinnacle compared to those of cv. Celebration suggesting that response to seed elicitation may be specific to the type of barley used. Further studies are required with other barley cultivars, replicated across growing seasons and different location is also required to ascertain the extent of variability in response.

CHAPTER 7. BIOELICITATION STRATEGY TO IMPROVE PHENOLIC-LINKED ANTIOXIDANT AND ANTIHYPERGLYCEMIC FUNCTIONALITIES IN BARLEY GRAINS

7.1. Abstract

Improving stress inducible and human health protective phenolic bioactives in barley using metabolically driven bio-elicitation strategy has potential dietary and therapeutic relevance. Specifically, such bioactive enriched barley grain and processed ingredients can be integrated in dietary and therapeutic support strategies against diet-linked chronic diseases, such as early stages of type 2 diabetes (T2D). Based on this rationale, the current greenhouse-based study focused on improving post-harvest phenolic bioactive profile and associated antioxidant and anti-hyperglycemic functionalities in grain samples of two barley types (one malting hulled-Tradition and one hulless food barley- BG012) using exogenous and pre-harvest bio-elicitor treatments. Bio-elicitors targeted in this study were bioprocessed marine protein hydrolysate (GP-2 and 5 g/L) and soluble chitosan oligosaccharides (COS-2 and 5 g/L), applied individually either as pre-sowing seed treatment or in combination with foliar spray during grain filling stage. After harvest, total soluble phenolic (TSP) content, phenolic acid profile, antioxidant activity, and anti-hyperglycemic relevant α-amylase and α-glucosidase enzyme inhibitory activities of milled flour from bio-elicited barley grain were determined using *in vitro* assay models. Overall, significantly high phenolic bioactive-linked antioxidant and anti-hyperglycemic relevant α-glucosidase enzyme inhibitory activity was observed in the hull-less food barley (cv. BG012) when compared to the hulled malting barley (cv. Tradition). Improvement of TSP content with foliar application of COS, and enhanced antioxidant activity with COS seed elicitor treatment were also observed in barley flour extracts. Additionally, combination of seed and foliar

treatments with GP resulted in significantly high antioxidant activity. Therefore, the results of this study provide critical insights on efficacy of targeted metabolically driven seed and foliar elicitation strategy for enrichment of therapeutically relevant inducible phenolics and to improve antioxidant activity in barley.

Keywords: Antioxidant; Bioactive Enrichment; Bio-elicitation; Chitosan Oligosaccharide; Marine Protein Hydrolysate; Phenolics; Type 2 Diabetes

7.2. Introduction

Higher consumption of whole cereal grains as part of regular diet positively contribute to the reduced risk of developing diet-linked non-communicable chronic diseases (NCDs) such as type 2 diabetes (T2D), obesity, and cardiovascular disease (Okarter and Liu, 2010; Borneo and León, 2012). The ability of such plant-based whole foods to provide health benefits and protect against chronic diseases has partly been attributed to the presence of various phytochemicals, minerals, and fibers in whole cereal grains (Liu 2007; Okarter and Liu, 2010). Phenolic compounds are a dominant class of grain phytochemicals that include simple phenolic acids, flavonoids, and condensed tannins, which may occur as free acids, soluble conjugates in bran and aleurone layers, or insoluble forms bound to cell wall polysaccharides (Dykes and Rooney, 2007). As potent antioxidants, they may potentially play a protective function against degenerative diseases involving chronic oxidative stress when integrated into diet (Dykes and Rooney, 2007). Certain phenolic compounds are also reported to possess anti-inflammatory, anti-carcinogenic, gastroprotective, glucose metabolism relevant functionalities (Del Rio *et al*., 2013; Okarter and Liu, 2010). Therefore, due to diverse protective functionalities of the phenolic compounds of whole cereal grains, they may promote optimal health and reduce the risk of chronic disease in humans, beyond just providing basic nutrition.

In recent years, phenolic compounds from natural dietary components have garnered considerable attention for their potential therapeutic role in the management of chronic hyperglycemia, a major risk factor for the onset of T2D (Kahn, 2003; Lin *et al*., 2016). Phenolic compounds can modulate the activity of key digestive enzymes such as α-amylase and α-glucosidase by non-competitive inhibition, thereby regulating the rate of carbohydrate digestion and assimilation (Nair *et al*., 2013). Phenolic compounds can also function as free radical scavengers and counter the detrimental effects of hyperglycemia-induced oxidative stress (Kähkönen *et al*. 1999). Moreover, phenolic compounds from natural food sources are relatively safer as they do not manifest adverse side-effects such as bloating, gastrointestinal discomfort and impairment of hepatic function, that commonly accompany with the administration of synthetic antihyperglycemic drugs (Kwon *et al*., 2006; Tundis, 2010). Therefore, the screening and development of food matrices with enhanced phenolic content and their bioavailability to potentially aid in mitigating chronic hyperglycemia and oxidative stress linked to T2D holds significant merit.

Fundamentally, inducible phenolic compounds are produced in plants as a defense mechanism against abiotic and biotic stresses (Shetty and Wahlqvist, 2004; Shalaby and Horwitz, 2014). Additionally, plants can modulate the rate of phenolic biosynthesis and accumulation in accordance with their external environment, especially under diverse abiotic stress pressures. Consequently, such mild stress -induced endogenous defense responses of food plants may be utilized to develop food matrices and ingredients with an improved profile of inducible phenolics and associated therapeutic and medicinal properties (Shetty and Wahlqvist, 2004; Sarkar and Shetty, 2014).

Such rationally targeted modulation of plant's endogenous defense responses through exogenous application of bioprocessed elicitors can induce metabolically linked pathway regulation, resulting in increased biosynthesis and accumulation of inducible phenolics (Shetty and Wahlqvist, 2004; Sarkar and Shetty, 2014). This strategy is based on the NADPH dependent redox regulation of the anabolic pentose phosphate pathway (PPP), which provides sugar phosphate precursors for phenolic biosynthesis via shikimic and phenylpropanoid pathways (Shetty and McCue, 2003; Shetty and Wahlqvist, 2004). Previously, stimulation of phenolic biosynthesis and subsequent improvement of abiotic stress tolerance and human health protective functional properties were observed in several food and non-food plants with chitosan oligosaccharide (COS) and marine protein hydrolysates (GP) seed and foliar elicitor treatments (Randhir and Shetty, 2005; Randhir *et al*., 2009; Sarkar *et al*., 2010; Orwat 2016; Cheplick *et al*., 2017; Ramakrishna *et al*., 2017a). Chitosan oligosaccharide is a biopolymer consisting of (1-4)-β linked *N*-acetyl-D-glucosamine units, derived primarily from marine crustacean shells (Kim, 2011). It is most commonly applied as a foliar spray or seed priming treatment. Upon exposure to COS, signal cascades associated with tissue wounding or fungal pathogen attack are activated in plants, resulting in the overexpression of defensive responses, including the biosynthesis of stress protective secondary metabolites such as phenolics. During such stress induced response, plants tend to upregulate redox-linked PPP regulation and drive carbon flux towards shikimate and phenylpropanoid pathways, which eventually lead to enhanced biosynthesis of protective phenolic compounds (Shetty and Wahlqvist, 2004; Sarkar and Shetty, 2014; El Hadrami *et al*., 2010). The second bioprocessed elicitor targeted in this study was marine protein hydrolysates (GP), produced from proteolysis of fish and seaweed processing byproducts. It is rich in soluble peptides and free amino acids, especially glutamic acid, and aspartic acid. Application of such

bioprocessed natural extracts has been shown to enhance the function of Krebs cycle and drive oxidative PPP by favoring $NADP^+$ formation via increased proline synthesis (Sarkar and Shetty, 2014; Colla *et al*., 2015, du Jardin, 2015). Additionally, both of these bioprocessed compounds are non-toxic, biodegradable and water soluble, rendering them safe and compatible with agricultural and human health relevant applications.

In the current study, barley was selected as a model crop as it is an excellent dietary source of beneficial phytochemicals, especially phenolic compounds and can be targeted for medicinal and therapeutic applications against non-communicable chronic diseases (NCDs), such as early stages of T2D (Idehen, 2017). Among diverse phenolic compounds, ferulic acid is the most abundant low molecular weight phenolic compound in barley grains, followed by p-coumaric acid. Varying levels of flavonols, anthocyanins and proanthocyanidins may also be present depending on the genotype and growing environment (Idehen, 2017; Wannenmacher *et al*., 2018). In previous *in vitro* screening study with 13 barley cultivars, high antihyperglycemic property relevant α-amylase and moderate α-glucosidase inhibitory activities in conjunction with high soluble phenolic content and antioxidant activity were observed (Ramakrishna *et al*., 2017b). Additionally, COS and GP seed elicitor treatments in selective concentrations were able to stimulate phenolic biosynthesis, and the phenolic-linked antihyperglycemic activity in dark-germinated barley sprouts (Ramakrishna *et al*., 2017a).

However, the efficacy of these bio-elicitors as seed and pre-harvest foliar treatments to improve phenolic-linked health benefits of barley were not studied previously. Therefore, the aim of this greenhouse-based controlled environment study was to evaluate the efficacy of COS and GP seed priming and pre-harvest foliar spraying treatments (at critical maturity stage) for improving the phenolic content and associated antioxidant activity of the mature barley kernels

after harvest. Additionally, the phenolic-linked α-amylase and α-glucosidase enzyme inhibitory activities of elicitor-treated barley kernels were also investigated using *in vitro* assay models to assess the potential improvements in their anti-hyperglycemic functionality.

7.3. Materials and Methods

7.3.1. Chemical Reagents

All reagents and enzymes used in this study were purchased from Sigma Aldrich Chemical Co. (St Louis, MO), unless otherwise mentioned.

7.3.2. Barley Cultivars and Elicitor Treatments

Two barley cultivars – Tradition (malting barley cultivar) and BG012 (food barley cultivar) were evaluated in this study. Seeds of cv. Tradition (hulled) were procured from the Malting Barley Breeding Program at North Dakota State University (Fargo, ND), while seeds of BG012 (Bob's Red Mill hull-less food barley) were purchased from the local supermarket (Walmart, Fargo, ND).

The efficacy of two types of bioprocessed elicitors in potentially improving the post-harvest phenolic bioactive profile of barley grains were investigated. Marine protein hydrolysate (GroPro® / GP) was procured from Icelandic Bio-Enhancers (Westchester, NY) and soluble chitosan oligosaccharide cross linked with ascorbic acid (COS) was obtained from Kong Poong Bio (Jeju, South Korea). Both elicitors were dissolved in distilled water separately to obtain solutions with 2 levels of concentration (2 and 5 g/ L) and used for subsequent seed and foliar treatments.

7.3.3. Seed Priming and Planting

Barley seeds were disinfected by soaking in 0.5% sodium hypochlorite solution for 10 min, rinsed thoroughly with distilled water and the excess water was blotted out using paper

towels. The seeds were then transferred to conical flasks containing 150 mL of elicitor treatment solution, as outlined in Table 7.1. For treatment combinations A and F, elicitor solution was replaced with distilled water. The seeds were then incubated on a rotary shaker for 8 h at 20 °C and 150 rpm. Post incubation, treatment solutions were drained, and the seeds were transferred to labelled plastic pots containing potting soil (5 seeds per pot) and grown in the greenhouse. The temperature in the greenhouse was maintained between 20 - 25℃, and a photo regime of 14 h: 10 h (light: dark) was maintained.

7.3.4. Foliar Application

Table 7.1. Scheme for application of elicitor treatment solutions.

Treatments	Concentration (g/L)	Seed treatment	Foliar application	Treatment code
A-Control	-	-	-	Control
B-GP	2	+	-	GP2-S
C-GP	5	+	-	GP5-S
D-COS	2	+	-	COS2-S
E-COS	5	+	-	COS5-S
F-COS	2	-	+	COS2-F
G-GP	2	+	+	GP2-SF
H-COS	2	+	+	COS2-SF

Foliar application of elicitor treatments was performed during the grain filling stage, as outlined in Table 7.1. The barley plants were sprayed with the elicitor treatments in a spray booth. Approximately 100 mL of elicitor treatment solution was sprayed to each individual barley plant at a pressure of 290 kPa, while sprayer speed was maintained at 1 mph. Per Table 7.1, plants corresponding to only seed treatments were sprayed with distilled water instead of elicitor solutions.

7.3.5. Barley Harvest and Extract Preparation

Mature barley heads were left to dry on the plant and then harvested. Following drying, the barley kernels were manually threshed and separated from the chaff. The grains were milled

using a benchtop disk mill (WonderMill®, Pocatello, ID, USA). A mixture of the milled barley flour and cold water (1:5; w: v) was homogenized for 5 min using a benchtop blender. The homogenate was centrifuged at 8500 rpm for 20 min, and the supernatant was centrifuged again at 8500 rpm for 15 min. The supernatant from the second centrifugation step was decanted, stored at 4 °C and used for subsequent biochemical assays.

Total soluble phenolic (TSP) content of barley flour extracts was determined using a modified Folin-Ciocalteu (FC) method. Barley sample extract (1 mL) was combined with 95% ethanol (1 mL), distilled water (5 mL), FC reagent (500 µL; 50% v/ v), and Na_2CO_3 (1 mL; 5% v/v) in a test tube, vortexed, and incubated in the dark for 60 min. The absorbance of the resultant reaction mixtures was measured at 725nm using a UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). From this data, TSP content of the samples was calculated using a gallic acid standard curve (10 - 300µg/mL; dissolved in 95% ethanol) and expressed as milligram equivalents of gallic acid (GAE) per gram dry weight (DW) of the barley flour samples.

Barley flour extracts were micro-centrifuged for 10 min at 13000 rpm and subjected to chromatographic analysis using reverse phase HPLC (Agilent 1260 Infinity Series equipped with DAD 1100 diode array detector; Agilent Technologies, Palo Alto, CA). A gradient elution method, involving 10 mM phosphoric acid (pH 2.5; Solvent A) and 100% methanol (Solvent B), was used. Sample extracts were eluted on a C-18 analytical column (Agilent Supelco SB-C18 250 x 4.6mm internal diameter) with a packing material particle size of 5 µm, at a flow rate of 0.7 mL/min at ambient temperature, with a total run time of 25 min. Pure standards of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, quercetin, resveratrol and p-coumaric acid in 100% methanol were used to calibrate retention times on the standard curve.

Each sample was run in duplicate, and the chromatograms obtained were analyzed using the Agilent Chemstation integration software. Phenolic acid concentrations were expressed in µg/g D.W. of barley flours.

7.3.6. DPPH Free Radical Scavenging Assay

The total antioxidant activity of barley samples was measured using a modified DPPH (1, 1- diphenyl-2-picrylhydrazyl; Sigma Chemical Co.) free radical scavenging assay. A working solution of DPPH was prepared by diluting a 10mM stock solution with 95% ethanol such that its absorbance was adjusted to a range of 1.8 – 2.0 units at 517 nm. Barley flour extracts (250 µL) were combined with DPPH working solution (1.25 mL), mixed thoroughly using a vortex mixer, incubated in a dark cabinet for 5 mins. Following this, the absorbance of the reaction mixture was measured at 517 nm. For each sample, a corresponding control was prepared by replacing the extract with 95% ethanol (250 µL) instead of barley flour extract. Ascorbic acid was dissolved in distilled water and in different doses (50, 75, 125, 250, 500, & 1000 µg/mL) was used as a positive control for comparison. The antioxidant activity of barley flour extracts was then calculated from the change in absorbance of the reaction mixture during the incubation period, per the following formula:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

7.3.7. ABTS Free Radical Scavenging Assay

The antioxidant activity of barley flour extract was also measured by the $ABTS^{+}$ (2, 2 – azinobis (3-ethylbenzothiazoline-6-sulfonic acid) free radical cation-decolorization assay. A stock solution was prepared by combining 7 mM ABTS (5 mL) with 140 mM potassium persulphate (88 µL). The mixture was incubated for at least 16 h prior to the assay. The stock solution was then diluted with 95% ethanol to prepare a working solution with an absorbance of

0.70 ± 0.02 at 734 nm. Barley flour extract (50 µL) was added to the working solution (1 mL), mixed on a vortex mixer for 30 sec and incubated for 2.5 min at room temperature in a dark cabinet. Absorbance values of the reaction mixtures were then measured at 734 nm. For each sample, a corresponding control was prepared by replacing the flour extract with 95% ethanol (50 µL). As with the DPPH radical scavenging assay, solutions of ascorbic in distilled water of varying concentrations were used as positive controls (50, 75, 125, 250, 500, & 1000 µg/mL) for comparison. The percent inhibition was calculated by the following equation:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

7.3.8. α-Amylase Inhibitory Activity

The α-amylase enzyme inhibitory activity was determined by an assay modified from the Worthington Enzyme Manual. Flour extracts (500 µL) were mixed with 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl; 500 µL) containing porcine pancreatic α-amylase enzyme (0.5 mg/mL) and incubated at 25 °C for 10 min. Following this, starch solution (1% w/v, prepared in 0.02 M sodium phosphate buffer; 500 µL) was added to the mixture and further incubated at 25°C for 10 min. Finally, 3, 5-dinitrosalicylic acid (DNS; 1 mL) solution was added and the reaction was stopped by incubating the mixtures in a boiling water bath (90 - 100 °C) for 10 min and cooled to room temperature. The reaction mixtures were then diluted with distilled water to reduce control absorbance readings within a range of 0.8 to 1.0 units at 540 nm. The absorbance of sample blanks (which contained sodium phosphate buffer instead of enzyme solution) and a control (which contained sodium phosphate buffer in place of flour extract) were also recorded. The α-amylase inhibitory activity was calculated as percentage (%) inhibition per the following equation:

$$Inhibition\ (\%) = \frac{Abs_{control} - (Abs_{extract} - Abs_{sample\ blank})}{Abs_{\ control}} \times 100$$

Sample results were compared based on equivalent concentration of undiluted sample with Acarbose solutions of various concentrations (0.05, 0.07, 0.12, 0.25, 0.50, & 1.0 mg/mL).

7.3.9. α-Glucosidase Inhibitory Activity

The α-glucosidase enzyme inhibitory potential was determined using an assay modified from the Worthington Enzyme Manual. Flour extracts (10 µL, 25 µL, 50 µL) were loaded in 96-well microtiter plates, and the volume in each well was made up to 100 µL 0.1M phosphate buffer (pH 6.9).

To each well, α-glucosidase solution (1 unit/mL, prepared in 0.1M phosphate buffer; 100 µL), was added, and the plate was incubated at 25°C for 10 min. Following this, of 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG; 50 µL) solution prepared in 0.1M phosphate buffer was added to all wells at the plate was further incubated at 25°C for 5 min. Absorbance values were recorded before and after incubation at 405 nm using a microplate reader (Thermomax, Molecular Device Co., Sunnyvale, CA) and compared to a corresponding control containing buffer solution (50 µL) instead of sample extract. The α-glucosidase inhibitory activity was expressed as percentage (%) inhibition and was calculated per the following equation:

$$Inhibition\ (\%) = \frac{\Delta Abs_{control} - \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

Sample results (undiluted) were compared (equivalent concentration) with standard curve developed from various concentrations of Acarbose solution (0.05, 0.07, 0.12, 0.25, 0.50, & 1.0 mg/mL).

7.3.10. Determination of Major Phenolic Compounds using HPLC

Milled grain extracts were collected and stored in micro-centrifuge tubes in the freezer prior to the analysis. The frozen samples were thawed and micro-centrifuged for 10 min at 13000 rpm, from which a small volume (5 μL) of the sample was subjected to chromatographic analysis using reverse phase HPLC (Agilent 1260 Infinity Series equipped with DAD 1100 diode array detector; Agilent Technologies, Palo Alto, CA). A gradient elution method, involving 10 mM phosphoric acid (pH 2.5; Solvent A) and 100% methanol (Solvent B), was used. Sample extracts were eluted on a C-18 analytical column (Agilent Supelco SB-C18 250 x 4.6mm internal diameter) with a packing material particle size of 5 μm, at a flow rate of 0.7 mL/min at ambient temperature, with a total run time of 25 min. All phenolic compounds of barley flour and fermented samples were quantified at 230 nm. Each sample was run in duplicate, and the chromatograms obtained were analyzed using the Agilent Chemstation integration software.

7.3.11. Statistical Analysis

A randomized complete block design, with split-plot arrangement was utilized in this study. The type of barley (hulled and hull-less) and elicitor treatment combinations were treated as the main plot and sub-plot, respectively. The process of growing and treating the barley plants in the greenhouse was repeated twice. During each run, 2 sets of extracts were prepared, and 6 samples (using as biological replicate) were used for all *in vitro* assays, per set. Therefore, the results presented in this study represent the combined analysis of the means from 24 data points per treatment combination for both types of barley, averaged across time. Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC) was used to perform analysis of variance (ANOVA) and the significant difference of each parameter between interactions of barley type ×

elicitor treatment combinations were determined using the Tukey's least mean square test at a confidence level of 95% ($p < 0.05$).

7.4. Results and Discussion

7.4.1. Total Soluble Phenolic Content

Whole cereal grains, such as barley, is a rich source of dietary phenolic metabolites with high antioxidant potentials and can be targeted and integrated in dietary and therapeutic interventions to maintain optimal physiological redox balance and mitigation of chronic oxidative stress associated NCDs, such as early stages of T2D (Okarter and Liu, 2010; Sarkar and Shetty, 2014; Shetty and Wahlqvist, 2004). Therefore, improvement in the free phenolic content and associated antioxidant activity of barley grains could potentially lead to its application for various therapeutic solutions, such as countering chronic oxidative stress and inflammation commonly associated with T2D. To improve such health protective phenolic profile and associated antioxidant functionality of harvested barley grains, metabolically driven bio-elicitation using bioprocessed elicitors as seed and foliar treatments were targeted in this study.

Overall, significant differences ($p < 0.05$) in TSP content between barley type, among bio-elicitor treatments, and barley type × elicitor treatment combinations were observed. Between the samples of the two barley types, flour extracts of hull-less food barley (cv. BG-012) had significantly ($p < 0.05$) higher mean TSP content when compared to the flour extracts of hulled malting barley type (Tradition) (Figure 7.1). Previously, Šimić *et al*. (2017) also reported significantly high TSP content in hull-less food barley cultivars when compared to several malting barley cultivars. Additionally, in the current study, foliar COS treatment (2 g/L) resulted in significantly higher TSP content in barley grains when compared to other seed and foliar

elicitor treatments. The highest improvement in TSP content was observed in the samples of hulled malting barley type (Tradition), with a 19% increase over the corresponding control (no seed elicitor treatment). The higher improvement of TSP content in flour extracts of hulled malting barley type (Tradition) with COS foliar elicitor treatment might be due to having the lower mean (baseline) phenolic content of this type of barley.

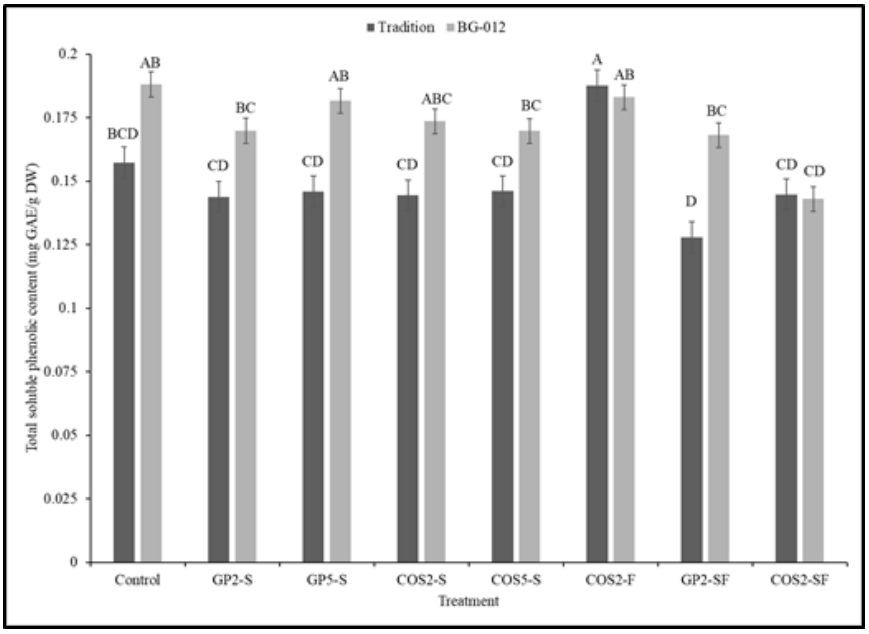

Figure 7.1. Total soluble phenolic content (mg GAE/ g D.W.) of flour of two barley types (hulled Tradition & hull-less BG012). Different capital letters represent significant differences between barley type × treatment interactions at $p < 0.05$.

The foliar elicitation with COS would likely drive carbon flux towards biosynthesis of secondary metabolites such as phenolics through stimulation of endogenous PPP response and unlocked the genetic potential and its interaction with growing environment (different due to several potential factors as malting vs food barley, hulled vs hull-less, and due to different growing locations and respective environment) of this barley sample to reach optimum phenolic content in barley grain. In our previous study, significant improvement of TSP content was observed in 6-day old dark germinated spouts of hulled barley Tradition with COS and GP seed elicitor treatments (Ramakrishna *et al*. 2017a).

Similarly, improvement of TSP content and stimulation of PPP regulation was also found in 6-day old barley sprouts of malting barley type Pinnacle with GP seed elicitor treatment (Ramakrishna *et al*. 2019). In the current study, the high TSP content of hull-less barley BG-012 was observed with foliar COS elicitor (2 g/L) and GP (5 g/L) seed elicitor treatments. However, it was statistically at par with no elicitor treatment (control). Therefore, the results of this study indicated that COS foliar treatment at pre-harvest grain filling stage may be an effective strategy to improve phenolic content of barley with low baseline phenolic, such as Tradition. Additionally, with validation from future field-based, multiple-year, and multiple locations investigations, such metabolically driven bio-elicitation strategy can be rationally targeted to improve human health protective and inducible phenolics in barley prior to integrating them in dietary and therapeutic support strategies against common NCDs, such as T2D.

7.4.2. Qualitative and Quantitative Analyses of Individual Phenolic Compounds

In addition to the TSP content, phenolic acid profile of aqueous extracts of milled barley grain was also determined using gradient-elution based high performance liquid chromatography (HPLC) method. The major phenolic acids detected in this study were gallic acid, cinnamic acid, and protocatechuic acid (Table 7.2). However, protocatechuic acid was only found in samples of hull-less BG012. Among three major phenolic acids, higher concentration of cinnamic acid was observed samples of hulled barley type Tradition. Previously, Idehen *et al*. (2017) reported benzoic acid and cinnamic acid and their derivatives as two major classes of phenolic acids in barley grain. Additionally, Zhu *et al*. (2015) reported high concentration of protocatechuic acid in dehulled highland barley cultivars. Though, major forms of free phenolic acids of barley grains are ferulic acid, vanillic acid, syringic acid, and p-coumaric acid (Gamel and Abdel-Aal, 2012), this study did not detect any of these phenolics in aqueous extracts of barley flour. The

food grade extraction (cold water extracts) of barley flour and the HPLC protocol used in this study might not have resulted in detection of all these free phenolic fractions of food and malting barley. Among different elicitor treatments, only improvement of cinnamic acid content was observed in hull-less barley (BG012) with COS seed elicitor treatment (5 g/L). However, in samples of BG012, higher cinnamic acid content was observed with no elicitor treatment (control). Similarly, high protocatechuic acid was also found in hull-less food barley with no elicitor treatment. All three phenolic acids of the barley flour detected in this study contributed to high antioxidant potentials, indicating relevance for health applications. Therefore, targeting food barley type with high phenolic content and high antioxidant potentials as part of dietary and therapeutic support strategy for countering chronic oxidative stress associated complications of T2D has significant merit.

Table 7.2. Dominant phenolic acids in barley grains from barley plants primed with elicitor treatments prior to sowing (expressed in µg/g D.W.).

Treatment	Cinnamic Acid		Gallic Acid		Protocatechuic Acid	
	Tradition	BG 012	Tradition	BG 012	Tradition	BG 012
Control	38.72	3.93	1.75	1.34	n.d.	1.62
GP2-S	21.76	4.6	1.61	0.63	n.d.	0.31
GP5-S	24.79	2.12	0.5	0.07	n.d.	0.15
COS2-S	28.77	1.11	1.46	0.05	n.d.	0.17
COS5-S	15.11	6.1	0.94	1.47	n.d.	0.52
COS2-F	25.5	2.1	1.77	1.12	n.d.	0.18
GP2-SF	35.15	n.d.	1.71	0.23	n.d.	0.51
COS-SF	23.1	2.37	1.1	0.23	n.d.	0.47

n.d.- Not detected

7.4.3. Total Antioxidant Activity

Whole barley grains contain a diverse array of bioactive compounds that function as potent antioxidants, among which phenolic compounds constitute an important group (Idehen *et al*., 2017; Ramakrishna *et al*., 2017a, b). Previously, various studies indicated a strong positive

correlation between the overall phenolic content and antioxidant property of whole grains, including barley (Gamel and Abdel-Aal, 2012; Idehen *et al*., 2017). The phenolic-linked antioxidant activity is a crucial component of the overall human health relevant functionality of barley-based foods, especially in mitigating chronic oxidative stress-induced complications (Ha *et al*., 2016; Sharma *et al*., 2020). Therefore, to determine the impact of bio-elicitation on phenolic-linked antioxidant property of barley, the total antioxidant activity of milled barley flour from elicitor treated barley plants were assessed based on their ability to scavenge DPPH and ABTS free radicals using *in vitro* assay methods.

Similar to the result of TSP content, significantly ($p < 0.05$) high total antioxidant activity (both based on ABTS and DPPH free radical scavenging assays) was found in flour of hull-less food barley (cv. BG-012) when compared to malting barley (cv. Tradition) (Figure 7.2 & 7.3). Overall, antioxidant activity (ABTS based) of hull-less food barley flour varied between 78-80 % (inhibition), while for samples of hulled Tradition, it ranged between 68-80% (inhibition) (Figure 7.3). When measured using DPPH based free radical scavenging assay, total antioxidant activity of flours of BG012 and Tradition samples ranged between 71-80 % (inhibition) and 53-65 % (inhibition) respectively (Figure 7.2). Previously, Šimić *et al*. (2017) also found high antioxidant activity in hull-less barley cultivars. In the current study, significant effect of elicitor treatments and barley type × elicitor treatment combinations on total antioxidant activity of barley flour were observed with DPPH-based free radical scavenging assay.

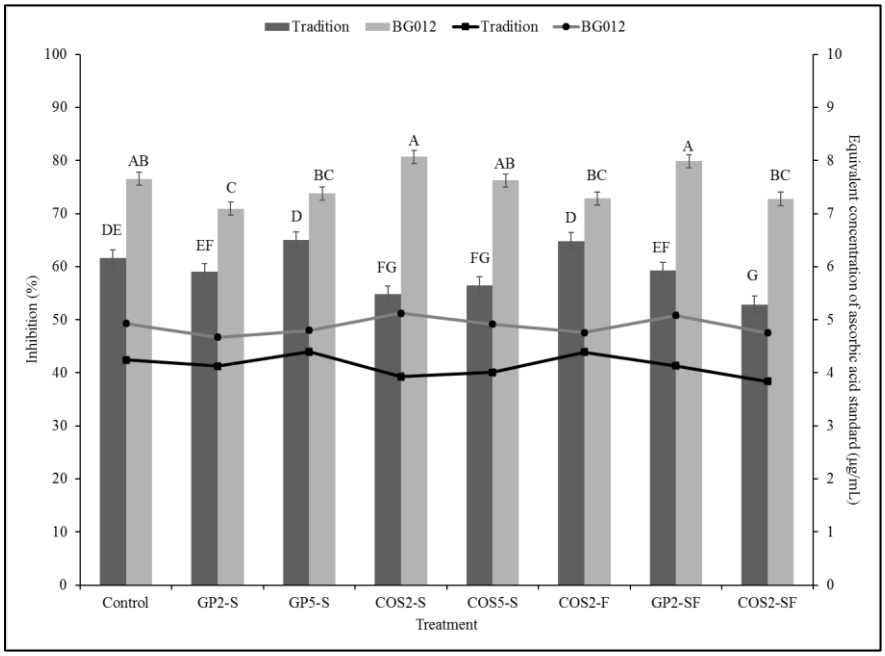

Figure 7.2. DPPH free radical scavenging activity of flour of two barley types (A - Tradition; B - BG012) along with equivalent concentrations of ascorbic acid standard. Different capital letters represent significant differences between barley type × treatment interactions at $p < 0.05$.

Overall, COS seed elicitor treatment (2 g/L) and combination of GP seed and foliar elicitor treatments (2 g/L) resulted in higher total antioxidant activity (both DPPH and ABTS) in samples of hull-less BG-012. The same elicitation treatments also improved total antioxidant activity of hulled barley Tradition samples when measured using ABTS based free radical scavenging assay. Seed elicitation with COS (2 g/L) resulted in significantly ($p < 0.05$) higher total antioxidant activity (ABTS based) in Tradition, when compared to no elicitor treatment (control) of the same hulled barley. Previously, improvement of antioxidant activity in dark germinated barley sprouts were observed with select seed elicitor treatments (COS and GP) (Ramakrishna *et al.*, 2017a). The results of previous and current study indicated that elicitation with bioprocessed elicitor, such as COS seed priming treatment in select dose (2 g/L) had positive impact on antioxidant activity of barley grains.

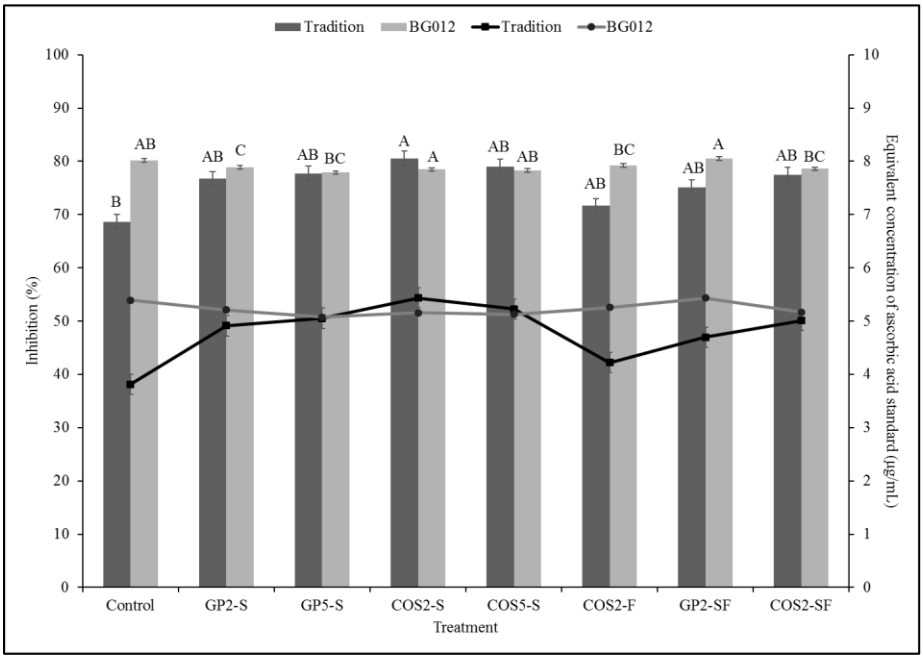

Figure 7.3. ABTS free radical scavenging activity of two barley types (A - Tradition; B - BG012). Different capital letters represent significant differences between barley type × treatment interactions at $p < 0.05$.

Therefore, such bio-elicitation tool can be targeted to improve antioxidant properties of select barley grain, especially for their high-value integration in dietary and therapeutic interventions for managing chronic oxidative stress, which is a common risk factor associated with T2D. However, it is also important to understand the overall impact of such bio-elicitation strategy on lowering other T2D associated risk factors, such as anti-hyperglycemic functionalities of whole and processed barley grains.

7.4.4. α-Amylase Inhibitory Activity

The enzyme α-amylase is involved in the digestion and solubilization of dietary starch and is responsible for converting starch to simple carbohydrate fraction for further digestion by other digestive enzymes (Sales *et al*., 2012). Regulating the degree of activity of α-amylase, especially inhibition of this enzyme represents a crucial component of dietary and pharmaceutical regimens to slow down carbohydrate digestion, thereby managing post-prandial

blood glucose levels (Barrett *et al*., 2018). Dietary phenolics, including those from barley are known to bind to enzymes such as α-amylase, and decrease their activity by non-competitive inhibition (Piasecka-Kwiatkowska *et al*., 2012; Ramakrishna *et al*., 2017a).

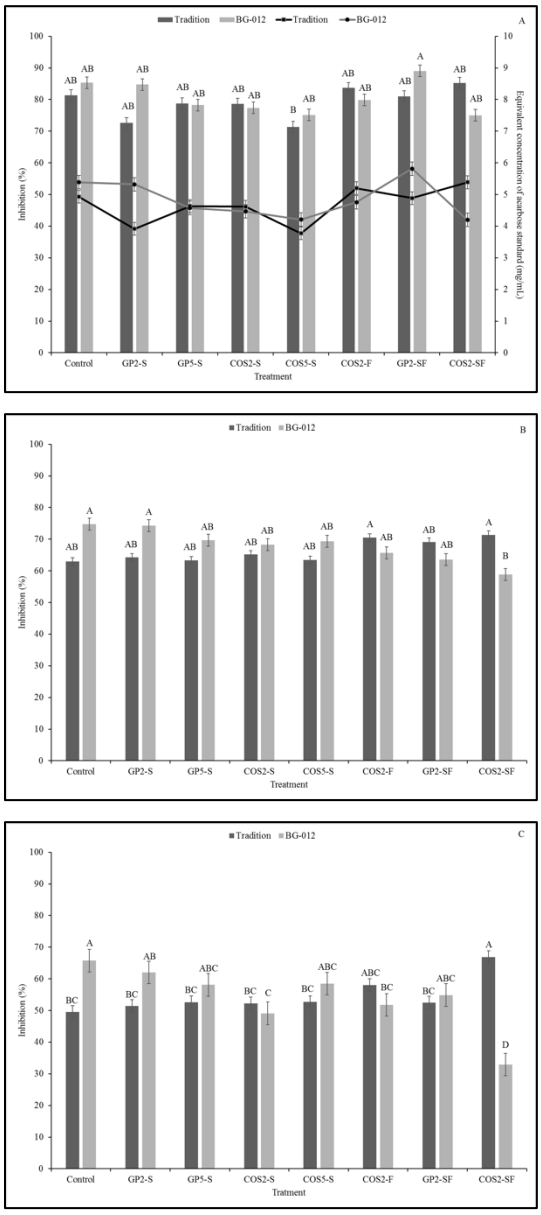

Figure 7.4. *In vitro* α-amylase enzyme inhibitory activity of flour extracts (A - undiluted, B - half diluted, C - one-fifth diluted) of two barley types (Tradition & BG012) along with equivalent concentrations of Acarbose standard.

Therefore, the *in vitro* α-amylase inhibitory activity of milled (flour) barley grains from select elicitor treated barley plants were assessed to investigate potential improvements in this anti-hyperglycemic functionality. Additionally, equivalent concentration based on synthetic anti-diabetic drug Acarbose as positive control was also determined.

In the current study, very high *in vitro* α-amylase enzyme inhibitory activity and significant dose dependent response was observed in sample extracts of both barley types (Figure 7.4 A, B, C). Overall, undiluted sample of barley flour extracts had 71-89% α-amylase enzyme inhibitory activity. The equivalent concentration of Acarbose in respect to α-amylase enzyme inhibitory activity of undiluted barley flour extracts (200 mg/mL) ranged between 4-5.8 mg/mL. In our previous studies, a similar trend in α-amylase enzyme inhibitory activity of dark germinated barley sprout extracts was observed (Ramakrishna *et al*., 2017a; b). However, in this study, no significant difference in α-amylase enzyme inhibitory activity was observed between two different types of barley types. Similarly, elicitation treatments also did not result in any statistically significant effect on α-amylase enzyme inhibitory activity of milled barley grains. This α-amylase enzyme inhibitory activity results of this study indicated that barley grain and processed flour is a good dietary and therapeutic target for potentially controlling chronic hyperglycemia, especially to regulate post-prandial blood glucose level and to manage early stages of T2D. However, further studies with other barley cultivars, from multiple years, different locations and with different processing and extraction method is needed to understand any potential impact and efficacy of targeted bioprocessed elicitors on α-amylase enzyme inhibitory activity of barley grains.

7.4.5. α-Glucosidase Inhibitory Activity

The digested products of α-amylase catalytic activity on starch, are further digested by α-glucosidase in the lumen of the large intestine, resulting in the formation and assimilation of simple sugars, specifically glucose into the bloodstream (Nair *et al*., 2013).

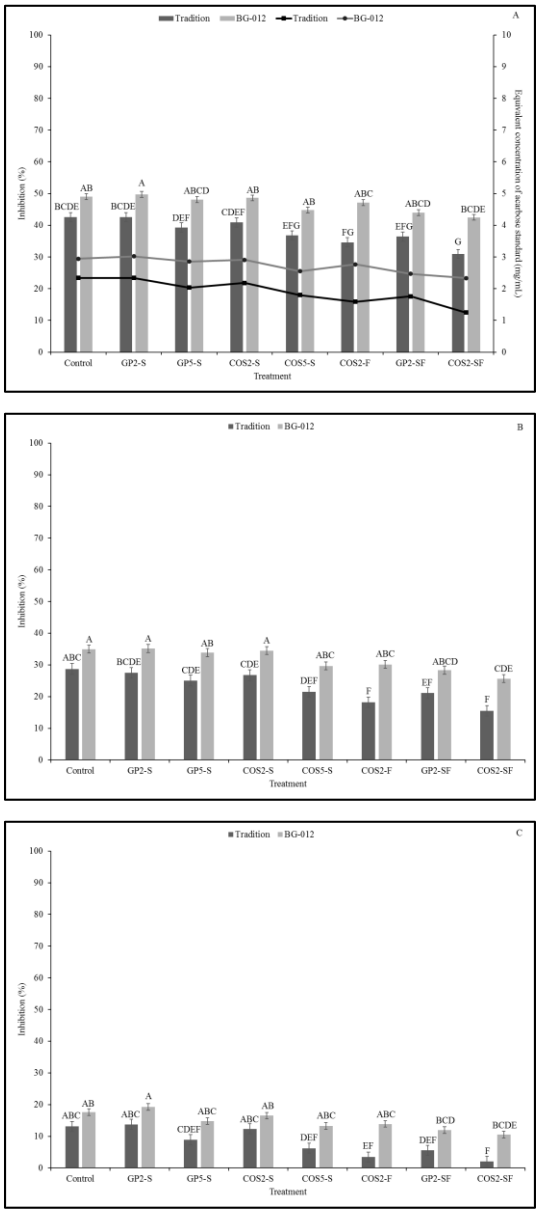

Figure 7.5. *In vitro* α-glucosidase enzyme inhibitory activity of flour extracts (A - undiluted, B - half diluted, C - one-fifth diluted) of two barley types (Tradition & BG012) along with equivalent concentrations of Acarbose standard.

Therefore, modulating α-glucosidase enzyme activity is a key control point in dietary and therapeutic strategies targeting post prandial glycemic regulation, which is of relevance to manage chronic hyperglycemia in early stages of T2D (Sarkar and Shetty, 2014). In the current study, the α-glucosidase enzyme inhibitory activity of milled flour of barley grains from elicitor-treated barley plants were evaluated to assess potential improvements in their anti-hyperglycemic function.

Overall, moderate α-glucosidase enzyme inhibitory activity was observed in undiluted extracts of all barley flours investigated in this study (Figure 7.5 A). Among the samples of the two barley types, significantly ($p < 0.05$) high α-glucosidase enzyme inhibitory activity was observed in flour extracts of hull-less BG-012 when compared to flour extracts of hulled barley Tradition, and it was consistently high in all three dilutions (undiluted, half, and one-fifth diluted) (Figure 7.5 A, B, & C). The mean α-glucosidase enzyme inhibitory activity of undiluted sample of hull-less food barley extract was 47% (inhibition), while it was 38% (inhibition) for undiluted sample extracts of hulled barley Tradition. Previously, similar range of α-glucosidase enzyme inhibitory activity was observed in undiluted extracts of 2-day old dark germinated barley sprouts (Ramakrishna *et al*., 2017a). However, in the same study (Ramakrishna *et al*., 2017a), high α-glucosidase enzyme inhibitory activity (up to 86 % inhibition) was observed in extracts of 6-day old barley sprouts. Similar trend in α-glucosidase enzyme inhibitory activity of germinated barley grain was also reported by Ha *et al*. (2016).

Therefore, high α-amylase and moderate α-glucosidase enzyme inhibitory activities of barley grain has significant therapeutic relevance and can be targeted in functional foods or ingredients to slow down digestion of carbohydrate and subsequent absorption of glucose in the bloodstream, which can contribute to maintaining post-prandial blood sugar and lowering the

risk of T2D. In the current study, GP seed elicitor treatment (2 g/L) resulted in highest α-glucosidase enzyme inhibitory activity (50% inhibition) in flour extracts of hull-less food barley (BG-012). However, it was statistically at par with no elicitor treatment (control) and COS seed and foliar elicitor treatments. Therefore, the improvement of phenolic content and associated antioxidant activity with elicitor treatments did not result in improved anti-hyperglycemic functions in samples of the two targeted barley types. Future multiple-year field-based study with combined elicitor treatments and with different doses and different barley cultivars will be needed to better understand the potential efficacy of these elicitor treatments for improving anti-hyperglycemic function in barley grains.

7.5. Conclusions

The stimulation of endogenous, protective metabolic pathways to enhance biosynthesis of inducible phenolics, in response to elicitor treatments has been previously studied in various cereal and legume models, specifically during the early stages of emergence and sprouting. However, little data exists regarding the effect of applying such elicitor treatment in later stages of physiological development, particularly in barley, as a means of improving the post-harvest phenolic profile and associated health benefits of the mature grains. Therefore, the current study assessed the effect of applying marine protein hydrolysate emulsion and soluble chitosan oligosaccharides, using varying combinations of seed and foliar treatments on phenolic-linked antioxidant and antihyperglycemic functionalities of matured barley grain. In the study, the improvement of total soluble phenolic content was observed in hulled barley Tradition following COS foliar treatment (2 g/L). Contrasting results were obtained for the antioxidant activity, whereby Tradition plants treated with COS-seed treatment (2 g/L) produced the greatest improvement in terms of DPPH free radical scavenging capacity. On the other hand, the highest

ABTS free radical scavenging capacity was observed in flour extracts from grain of BG012 plants treated with a seed and foliar application of GP (2 g/L). While all combinations of barley type × elicitor treatment combinations demonstrated moderate to high α-amylase enzyme inhibitory activity, the application of elicitor treatments did not significantly improve the level of this antihyperglycemic function. Similarly, all barley type × elicitor treatment combinations demonstrated moderate dose dependent α-glucosidase enzyme inhibitory activity, but none of the elicitor treatment had any significant impact. While the current study provides preliminary information regarding the post-harvest modulation of phenolic profile using elicitor treatments, extensive field-based studies are further needed to optimize the methods of applying such elicitor treatments. Further, more extensive work on optimizing treatment dose and the ideal physiological stage for application is required. Optimized elicitor application protocols may then serve as a potential strategy to improve the post-harvest soluble phenolic content, and targeted health benefits of barley, which is critical for integration of such bioactive enriched barley in dietary and therapeutic support strategies against early stages of T2D and other NCDs with further *in vivo* validation.

CHAPTER 8. BIOELICITATION STRATEGY TO IMPROVE SALINITY STRESS TOLERANCE IN BARLEY THROUGH STIMULATION OF PROTECTIVE PROLINE-ASSOCIATED PENTOSE PHOSPHATE PATHWAY

8.1. Abstract

Increasing salinization of arable land and related loss in agricultural productivity is a growing global challenge amid rapid climate change. Among different crops, barley (*Hordeum vulgare* L.) is one of the most salt tolerant plant species and can be targeted for commercial cultivation in salt affected regions of the world. However, high salt stress can affect germination, seedling growth, head formation, and grain filling with significant economic impacts on barley production. Therefore, improving salinity stress tolerance of barley using metabolically driven bio-elicitation strategy has significant scientific merit and economic relevance. The aim of this study was to recruit bioprocessed elicitors, water soluble chitosan oligosaccharide (COS) and marine protein hydrolysate (GP) as seed priming treatments to improve salinity stress tolerance in samples of two types of malting barley (Pinnacle – 2-rowed; Celebration – 6-rowed). Following seed treatments and germination, barley seedlings (1 week old) were subjected under three soil salinity levels (<1-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}) in the greenhouse and photochemical efficiency, plant height, grain weight, protective endogenous defense response relevant proline-associated pentose phosphate pathway (PAPPP) regulation and associated phenolic biosynthesis and antioxidant enzyme responses of barley leaf tissues were determined (at 35, 50, and 65 day from seed priming treatment). Overall, reduced plant height, low photochemical efficiency and significant variations in critical PAPPP-linked defense regulation was observed in barley under high salt stress. Samples of cultivar Pinnacle showed relatively higher fitness and resiliency with greater plant height, high photochemical efficiency, and

improved soluble phenolic (TSP) content under high salt stress. Additionally, seed elicitor treatments, specifically GP, improved grain weight, photochemical efficiency, TSP content, CAT antioxidant enzyme activity in barley plants under high salt stress. Therefore, such bio-elicitation strategy using seed elicitor treatments can be targeted as an effective tool to improve salinity stress resilience in barley.

Keywords: Antioxidant Enzymes; Chitosan Oligosaccharide, Marine Protein Hydrolysate; Phenolic Metabolites; Proline; Salinity Tolerance; Seed Priming

8.2. Introduction

Soil salinity is among the dominant abiotic stresses that affect crop productivity globally, with over 20% of the world's irrigated land currently being affected to varying degrees of salinity (Qadir *et al.*, 2014). In the United States, soil salinization is a significant challenge to crop production, especially in the Northern Great Plains region, which accounts for approximately 25% of the total area under cultivation and represents over $27 billion of the total revenue from agriculture in the country (Wienhold *et al.*, 2018). Several tracts of this vast region are naturally prone to salinity due to hydrogeological (weathering of sodium-rich parent rock material; presence of discharge soils and saline seeps; capillary rise of saline ground water due to excessive surface evaporation) and climatic (excessive precipitation) factors (Daniels, 1987; Franzen, 2007; Kharel, 2016). Anthropogenic factors such as intensive large-scale agriculture (excessive irrigation; use of saline water for irrigation) and extraction of fossil fuels (fracking-oil sand processing) have further increased the risk of soil salinization (Renault, MacKinnon and Qualizza, 2003; Franzen, 2007). Additionally, dramatic changes in regional weather patterns are also projected to contribute to increases in salt-affected areas, thereby detrimentally impacting the overall ecology and agricultural productivity of this region (Wienhold *et al.*, 2018).

Overall, elevated levels of salt concentration and electrical conductivity (E_c) restrict the normal growth and development of several crops currently grown in the Northern Great Plains, resulting in reduced biomass production, yield, and end-use quality. Screening and selection of crops that possess resilience towards salinity is a key strategy in offsetting the ecological and economic costs of soil salinization. Salinity stress resilience is a quantitative trait involving genotype × environment interplay and complex interactions between multiple endogenous defense response related metabolic pathways (Munns and Tester, 2008). Plants may exhibit significant variability in stress tolerance, even within a given species, depending on factors such as growth stage and cultivars. Thus, an understanding of the underlying molecular and metabolic mechanisms involved in stress tolerance is critical for screening food and industrial crops that can potentially survive in saline soils (Parihar *et al.*, 2015).

Barley, which is grown primarily for utilization as malt, food, and feed – is one of the major cereal crops grown in the United States, and the Northern Great Plains alone accounted for 60% of the total barley produced in the U.S. (Colmer, Munns and Flowers, 2006). Barley is relatively tolerant to salinity and can reportedly withstand root zone E_c levels of up to 8 dS m^{-1} without its yield being greatly affected (Ogle and St John, 2010). As a result, it may serve as a suitable alternative annual crop in salt-affected areas and a cover or forage crop in non-cultivated areas affected by moderate salinity (Tober *et al*. 2007). The application of barley as a cover crop and as a means of sequestering excess Na^+ and Cl^- ions from saline effluents resulting from the processing of tar sands to crude oil, thereby aiding the reclamation of affected soils, has also shown potential (Renault, MacKinnon and Qualizza, 2003). Previous studies have attributed the salt tolerance of barley plants to various mechanisms including selective exclusion of Na^+ and Cl^- ions in the root zone, maintenance of high cellular K^+/ Na^+ ratios, accumulation of

osmoprotectants, and improved antioxidant enzyme activity (Chu, Aspinall and Paleg, 1976; Munns, Schachtman and Condon, 1995; Colmer, Munns and Flowers, 2006; Patterson *et al.*, 2009; Ahmed *et al.*, 2015).

Therefore, critical redox-linked metabolic regulation involving endogenous antioxidant enzymes, amino acids such as proline, and other defense related pathways are critical to induce salinity stress tolerance in plants. In this context, proline metabolism and its association with other redox-linked defense related pathways such as pentose phosphate pathway (PPP) may play a critical role to induce abiotic stress resilience in crops such as barley (Hare and Cress, 1997; Shetty and Wahlqvist, 2004). Proline is one of the dominant cellular osmoprotectants and a reserve source of carbon and nitrogen under stress (Hare and Cress, 1997; Verbruggen and Hermans, 2008). Phang (1985) first reported the coupling of the reduction of glutamate to proline, with redox regulation and hydride-ion mediated stimulation of the PPP in an animal model. Further to this, Shetty (1997) proposed that a proline-associated pentose phosphate pathway (PAPPP) can help plants meet the demands of reductant-intensive anabolic requirements under abiotic stress, while simultaneously stimulating the shikimate and phenylpropanoid pathways leading to the synthesis of protective secondary metabolites such as phenolics. Proline may also potentially replace NADH as a reducing equivalent in mitochondrial oxidative phosphorylation, therefore enabling greater efficiency in ATP synthesis by bypassing the energy intensive Krebs cycle and NADH-dependent respiration under abiotic stress conditions (Hare and Cress, 1997; Shetty and Wahlqvist, 2004). Furthermore, PAPPP may also aid in scavenging reactive oxygen species (ROS) by supporting anabolic needs, in addition to serving as a component of stress signal cascades and stimulating various enzyme-mediated antioxidant mechanisms (Shetty and Wahlqvist, 2004; Verbruggen and Hermans, 2008).

Therefore, the regulation of PAPPP may potentially provide a critical metabolic control point, at the intersection between primary and secondary metabolic pathways, to improve the resilience of food crops to counter the adverse effects of high soil salinity and associated oxidative stress.

There is an emerging interest in developing novel strategies within the current paradigm of agricultural practices and systems to improve crop fitness under salt stress and mitigate losses to crop productivity. In this context, the application of seed priming with metabolically relevant and stress modulation associated elicitor treatments could be an effective tool, as previous studies indicated the ability to enhance endogenous defense mechanisms by the stimulation of PAPPP and associated antioxidant enzyme responses (Shetty and Wahlqvist, 2004; Randhir and Shetty, 2005; Sarkar, Bhowmik and Shetty, 2010; Orwat *et al.*, 2017). Seed priming may enhance oxidation-linked metabolic regulation though a critical anabolic pathway (PPP), by mobilizing protective secondary metabolites and antioxidant enzymes at vegetative stages where crops are most vulnerable to external stress, such as seedling emergence and seedling establishment, thereby providing an avenue to improve the overall resilience to soil salinity.

Therefore, the objective of the current study is to gain insight into the metabolic responses of seed primed barley under high salt stress from seedling establishment until full maturity stages by examining temporal changes in redox-linked critical control point of anabolic PAPPP regulation, thereby assessing the potential of such strategies in improving salinity stress resilience in barley.

8.3. Materials and Methods

8.3.1. Chemical Reagents

The reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO), unless mentioned otherwise.

8.3.2. Barley Cultivars and Seed Elicitor Treatments

Seeds of 2 malting barley types (Pinnacle – 2-rowed; Celebration – 6-rowed) from a single growing season and location were obtained from the Malting Barley Breeding Program at North Dakota State University (Fargo, ND). Two types of bioprocessed elicitors– marine protein hydrolysate (GroPro® / GP; Icelandic Bio-Enhancers, Westchester, NY) and soluble chitosan oligosaccharide with ascorbic acid sidechain (COS; Kong Poong Bio, Jeju, South Korea) - were evaluated as seed priming treatments. Elicitor treatment solutions were prepared by dissolving these compounds in distilled water to a concentration of 0.1%, while distilled water was used as negative control.

8.3.3. Sample Preparation

8.3.3.1. Preparation of Saline Soil Treatments

Soil salinity was measured as a function of its electrical conductivity (EC). Barley plants were subjected to moderate (EC: 5 dSm^{-1}) and high salinity stress (EC: 9 dSm^{-1}), while non-saline soil (EC: <1 dS/m) was used as a control. A combination of 4 salts - sodium chloride, magnesium sulfate, sodium sulfate, and calcium sulfate, which are most commonly associated with salt-damaged soil in the Northern Great Plains region were used to simulate soil salinity in this study (Franzen, 2007).

Solutions containing equimolar mixtures of the above salts were combined with potting mix in a soil mixer and allowed to equilibrate for 24 h. Salinity levels were regularly monitored using a FieldScout Direct Soil EC meter (Spectrum Technology Inc, Aurora, IL), and adjusted by adding saline solution as required. Saline potting mix was distributed to plastic pots (165 cubic inch) and Osmocote 15-9-12 fertilizer pellets (The Scotts Company, Marysville, OH) were added at a rate of 2 teaspoons per pot.

8.3.3.2. Sowing and Transplanting

Approximately 50 seeds per treatment were randomly sampled and disinfected in 0.5% sodium hypochlorite solution for 10 min. Disinfected seeds were rinsed thoroughly with distilled water, patted dry with paper towel, and transferred to conical flasks containing 100 mL of each elicitor treatment solution. The seeds were incubated on a rotary shaker for 8 h at 20°C and 150 rpm. Following incubation, the treatment solutions were drained, and primed seeds were sown in seed starter trays containing potting mix and germinated. One week after seedling emergence, the barley seedlings were transplanted to the prepared pots with saline soil at the rate of 3 seedlings per pot. Soil salinity was measured on a weekly basis and adjusted to maintain EC levels within the required range. The temperature in the greenhouse was maintained between 20 – 25ºC and a photoperiod of 14 h: 10 h (light: dark) was used. Leaf tissue samples were collected from barley plants and were subjected to biochemical analysis at three time points – 35 days**,** 50 days, and 65 days after seed priming treatment.

8.3.4. Photochemical Efficiency and Plant Height

Chlorophyll fluorescence of the barley leaves were measured using an OS-1 Fluorometer (Opti-Sciences, Inc., Tyngsboro, MA) 24 h prior to harvesting samples, to evaluate their photochemical efficiency. The test was carried out in the dark-adapted mode and the photochemical efficiency, i.e., the ratio of variable fluorescence to maximal fluorescence (F_v/F_m) was calculated. Additionally, prior to harvesting leaf tissue, plant height was measured at each time point.

8.3.5. Sample Extraction for Enzyme Assays

Barley leaf samples (200 mg fresh weight; FW) were excised, immediately wrapped in aluminum foil, and stored in liquid nitrogen until being assayed. Harvested tissue was

thoroughly macerated in 2 mL of cold enzyme extraction buffer (0.5% polyvinylpyrrolidone / PVP; 3 mM EDTA; 0.1 M potassium phosphate buffer; pH 7.5) using a mortar and pestle placed in an ice-bath. The homogenate was centrifuged at 13,500 rpm for 10 min and stored in an ice-bath until biochemical assays were performed.

8.3.6. Protein Content and *In Vitro* Enzyme Assays

8.3.6.1. Total Protein Assay

Total protein content was measured using the Coomassie brilliant blue dye binding assay (Bradford, 1976). A working solution was prepared by combining one part of dye reagent (Bio-Rad pro8.tein assay kit II, Bio-Rad Laboratory, Hercules, CA) with four parts of distilled water. Enzyme extracts (50 µL) were thoroughly mixed with working solution (5 mL) using a vortex mixer and incubated in a dark cabinet for 5 min. A corresponding blank solution was prepared by replacing the sample extract with distilled water (50 µL) and used to set the baseline of the spectrophotometer. The absorbance of the incubated solutions was measured at 595 nm, and these values were used to calculate the activity of enzymes in the subsequent enzyme assays.

8.3.6.2. Glucose-6-Phosphate Dehydrogenase (G6PDH) Activity

A modified method originally described by Deutsch (1983) was used. An enzyme reaction mixture containing 5.88 µmol β-nicotinamide adenine dinucleotide phosphate (ß-NADP), 88.5 µmol $MgCl_2$, 53.7 µmol glucose-6-phosphate, and 0.77 mmol maleimide was prepared. This solution was used to set the baseline (zero) of the spectrophotometer at 340 nm. Barley leaf homogenate (100 µL) was added to the reaction mixture (1 mL) and the shift in absorbance over 5 min was used to quantify G6PDH activity in the samples based on the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}).

8.3.6.3. Succinate Dehydrogenase (SDH) Activity

Activity of SDH was measured by a modified method described by Bregman (1987). A reaction mixture containing 0.4 M potassium phosphate buffer (1.0 mL; pH 7.2), 0.15 M sodium succinate (40 µL; pH 7.0), 0.2 M sodium azide (40 µL), and 2,6-dichlorophenolindophenol (DCPIP; 10 µL; 6.0 mg/mL) was prepared. The enzyme extraction buffer was used to establish the baseline of the spectrophotometer at 600 nm. Enzyme extract (0.2 mL) was added to the reaction mixture (1 mL) and incubated at room temperature for 5 mins. The rate of change of absorbance per min was measured to quantify SDH activity in the sample, based on the extinction coefficient of DCPIP (19.1 mM^{-1} cm^{-1}).

8.6.3.4. Guaiacol Peroxidase (GPX) Activity

A modified method described by Laloue *et al.* (1997) was used to measure the activity of guaiacol peroxidase. An enzyme reaction mixture, containing 0.1 M potassium phosphate buffer (pH 6.8), 56 mM guaiacol solution, and 50 mM hydrogen peroxide was prepared (Sarkar *et al.*, 2009). Enzyme extracts (10 µL) were combined with the reaction mixture (990 µL) and absorbance shift over 3 min was measured. The rate of change in absorbance per min was used to quantify the activity of GPX in the extracts, based on the extinction coefficient of tetraguaiacol (26.6 mM^{-1} cm^{-1}).

8.3.6.5. Superoxide Dismutase (SOD) Activity

The activity of SOD was measured by monitoring the reduction of nitroblue tetrazolium (NBT) by barley leaf homogenate at 560 nm (Oberley and Spitz, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8; 13.8 mL), 1.33 mM diethylenetetraaminepentaacetic acid (DETEPAC), 2.45 mM NBT (0.5 mL), 1.8 mm xanthine (1.7 mL) and catalase (40 IU/mL). Phosphate buffer (100 µL) and xanthine oxidase (100 µL)

were added to the reagent mixture (800 µL) and the change in absorbance at 560 nm was measured over 1 min. The concentration of xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.024 - 0.026 units of absorbance per min. Phosphate buffer was replaced with enzyme extracts and the shift in absorbance over 1 min was calculated. One unit of SOD activity was defined as the amount of protein that inhibited NBT reduction to 50% of the maximum value.

8.3.6.6. Catalase (CAT) Activity

A method originally described by Beers and Sizer (1952) was used to measure catalase activity. A reaction mixture was prepared by combining 0.257 mL of 50 mM H_2O_2 (Fisher Scientific, Waltham, MA, USA) with 50 mL of 0.05 M potassium phosphate buffer solution (pH 7.0) and used to establish the baseline of the spectrophotometer at 240 nm. Enzyme extracts (50 µL) were added to the reaction mixture (1.45 mL) and the shift in absorbance arising from the catalase-mediated decomposition of peroxide was monitored by measuring the change in absorbance at 240 nm over 1 min. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2.

8.3.6.7. Proline Dehydrogenase (PDH) Activity

A modified method described by Costilow and Cooper (1978) was carried out to measure PDH activity. The enzyme reaction mixture comprising 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline and 10 mM nicotinamide adenine dinucleotide (NAD) was used. Enzyme extract (200 µL) was added to the reaction mixture (1 mL) and incubated in a warm water bath at 32ºC for 3 mins. The reaction mixture's absorbance was measured at 340 nm, before and after incubation, and the change in absorbance was calculated. One unit of PDH

activity was defined as amount required to cause a shift of 0.01 absorbance units per min at 340 nm (1.0 cm light path).

8.3.6.8. Determination of Proline Content using HPLC

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector (DAD 1100). The analytical column was a reverse phase Nucleosil C18, 250 nm 4.6 mm with a packing material of 5 µm particle size. The samples were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 mL/min and detected at 210 nm. Standard solutions of L-proline prepared in 20 mM potassium phosphate solution were used to calibrate the standard curve. Proline concentration was reported as millimoles (mM) of proline per gram (FW) of barley leaf tissue.

8.3.7. Sample Extraction for Total Soluble Phenolic Content and Antioxidant Assays

Harvested leaf tissue (100 mg FW) was transferred to a glass vial containing 95% ethanol (5 mL) and stored at -20°C for 48 h. Following this, the tissue was homogenized and centrifuged at 13,000 rpm for 5 min. The resulting supernatant was used to determine the total soluble phenolic content and antioxidant capacity of the leaf samples.

8.3.7.1. Total Soluble Phenolic (TSP) Content

The total soluble phenolic content in the ethanol extracts of barley leaves was measured using the Folin-Ciocalteu (FC) method, modified by Shetty *et al.* (1995). The extract (1 mL) was combined with 95% ethanol (1 mL) and distilled water (5 mL) in a test tube. To this mixture, FC reagent (0.5 mL; 50% v/ v) and Na_2CO_3 (1 mL; 5% v/v) were added, mixed thoroughly with a vortex shaker, and incubated in the dark for 60 mins. The absorbance of the resulting solution

was measured spectrophotometrically at 725nm (Genesys 10S UV-Vis spectrophotometer, Milton Roy Inc., Rochester, NY). A standard curve was prepared using various concentrations of gallic acid (10 – 300 μg/mL), dissolved in 95% ethanol. Absorbance values were converted to total soluble phenolic concentration and expressed as milligrams of gallic acid equivalent per gram (FW) of barley leaf tissue.

8.3.7.2. ABTS Free Radical Scavenging Capacity

Antioxidant activity was measured using the ABTS [2, 2 –azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay (Re *et al.*, 1999). A stock solution containing $ABTS^{+}$ radical cations was prepared by mixing 7 mM ABTS solution (5 mL) with 140 mM K_2S_2O4 solution (88 mL). This mixture was stored in foil wrapped container, in the dark at $4^{\circ}C$ for 12–16 h before use. Prior to the performing the assay, the stock solution was diluted with 95% ethanol, to prepare a working ABTS solution with an absorbance of 0.70 ± 0.02 units at 734 nm. The ABTS working solution (1 mL) was added to the ethanol extracts (50 μL), mixed using a vortex shaker and incubated at room temperature for 2.5 mins in a dark cabinet. The absorbance of the mixture was measured at 734 nm. For each sample set, a corresponding control was prepared by replacing the extract with 95% ethanol (50 μL). The antioxidant activity of the extracts was expressed as percentage (%) inhibition of ABTS radical formation and was calculated per the following formula:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

8.3.8. Grain Weight Measurement

Barley heads were harvested upon reaching maturity and were threshed and cleaned by hand. The 1000 grain weight was measured based on the average weight of three 100-grain samples for each treatment × cultivar combination across all levels of soil salinity.

8.3.9. Experimental Design and Statistical Analysis

The experiment was carried out according to the randomized complete block design (RCBD) with 3-way factorial arrangement and 4 biological replicates. Each pot was considered as an experimental unit. Four samples from each unit were assayed per elicitor treatment × salinity level combination, for both barley types, and the entire experiment was repeated thrice. Analysis of variance (ANOVA) was performed using SAS software (version 9.4; SAS Institute, Cary, NC). Statistically significant mean differences of three main effects (soil salinity, cultivars, and seed priming treatment) and their corresponding interactions (three 2 pair interactions and one 3-way interaction) were determined by first performing mean separation by Tukey's least mean square test, followed by an F-test at a confidence level of 95%.

8.4. Results and Discussion

8.4.1. Visual Observations, Plant Height and Photochemical Efficiency

Most food crops are susceptible to high soil salinity as it impairs critical physiological and metabolic processes, thereby adversely impacting their phenotypic characteristics (Eisa *et al.*, 2012; Tsegay and Gebreslassie, 2014). In the current study, the efficacy of bioprocessed elicitors as seed priming treatment was investigated for minimizing the impact of soil salinity on key phenotypic and biological parameters such as plant height and photochemical efficiency of barley.

Overall, plants of both barley types were found to survive until the reproductive stage (grain filling and maturity) even under high saline condition (~ 9 dSm^{-1}), supporting previously published evidence on barley being relatively tolerant to high salt stress (Gürel *et al.*, 2016). However, from seedling stage to maturity, plant height was found to decrease significantly ($p \leq 0.05$) with a proportional increase in soil salinity levels, for both barley types and across all seed

elicitor treatments. Additionally, under high saline condition, the growth was stunted between 50 and 65 days. Furthermore, as the barley plants approached the grain filling stage, those subjected to high salt stress (~ 9 dSm^{-1}) were found to be susceptible to the onset of early foliar desiccation compared to plants with no salt stress (control <1 dSm^{-1}) and moderate salt stress (~ 5 dSm^{-1}). Previous study also reported significant reduction (25%) of barley plant height under high salt stress (irrigation with ~16 dSm^{-1} saline water) (Al-Busaidi *et al.*, 2008). Similarly, Hussain *et al.* (1997) observed significant reduction in plant height and lower number of tillers in barley under high salinity level (irrigation with ~9 and 16 dSm^{-1} saline water). Most of these previous studies with barley were focused on salt level of irrigation water rather than maintaining constant soil salinity level which was targeted in our experiment.

In this current study, plants of barley cv. Pinnacle were consistently found to be of significantly higher ($p \leq 0.05$) plant height when compared to barley plants of cv. Celebration at all time points (35, 50, and 65 days after seed priming treatment) (Figure 8.1 & Table 8.1). Significant improvement ($p \leq 0.05$) of plant height with seed priming treatments (both GP and COS) was also observed at early growth stage (35 days). Among seed treatments, GP and COS treated plants of both barley types had higher plant height when compared to the no seed treatment under non-saline and highly saline (~ 9 dSm^{-1}) soil conditions, especially at early growth stage (day 35). However, under moderate saline condition (~5 dSm^{-1}), only COS treated plants were observed to have slight improvements in plant height when compared to the control and GP treated plants of both barley types. These results indicated that seed elicitor treatments (GP and COS) targeted in our study had positive impact on improving growth of barley plants under salinity stress, especially during early growth stages of seedling establishment to advanced tiller formation, when plants are generally more susceptible to several abiotic and biotic stresses.

Previously, improvement of shoot dry weight of barley plant with application of seaweed extract (similar to our marine protein hydrolysate-GP) were reported under high salt stress (El-Sharkawy *et al.*, 2017). Therefore, application of such bioprocessed elicitors either as seed priming (in our study) or as soil amendment treatments can be an effective bio-elicitation strategy to counter the deleterious effect of salt stress on barley plant, especially during critical growth stages of 2-leaf stage to advanced tiller formation.

The adverse impact of high salt stress and other abiotic stresses on barley is not only just restricted to plant height and tiller formation, but also affect the most critical biological process such as photosynthesis (Karami and Sepehri, 2018; Mahlooji *et al.*, 2018). The deleterious effects of soil salinity on photosynthesis are multifaceted such as through reduction of leaf area, decrease in chlorophyll content and stomatal conductance, loss of tissue turgidity, and to a lesser extent through a decrease in photosystem II efficiency (Sudhir and Murthy, 2004; Liu *et al.*, 2017). Therefore, understanding the impact of salt stress on photochemical efficiency of barley plants and the improvement of such key biological process through seed elicitation treatments have significant relevance.

In this experiment, significant reduction of photochemical efficiency (F_v/F_m ratio-maximum potential quantum efficiency of photosystem II) was observed in barley plants with increased soil salinity levels at all time point (Figure 8.2). Overall, soil salinity levels, differences between barley types, and seed priming treatments had statistically significant effect ($p \leq 0.05$) on photochemical efficiency of barley plants at all time points (Table 8.1).

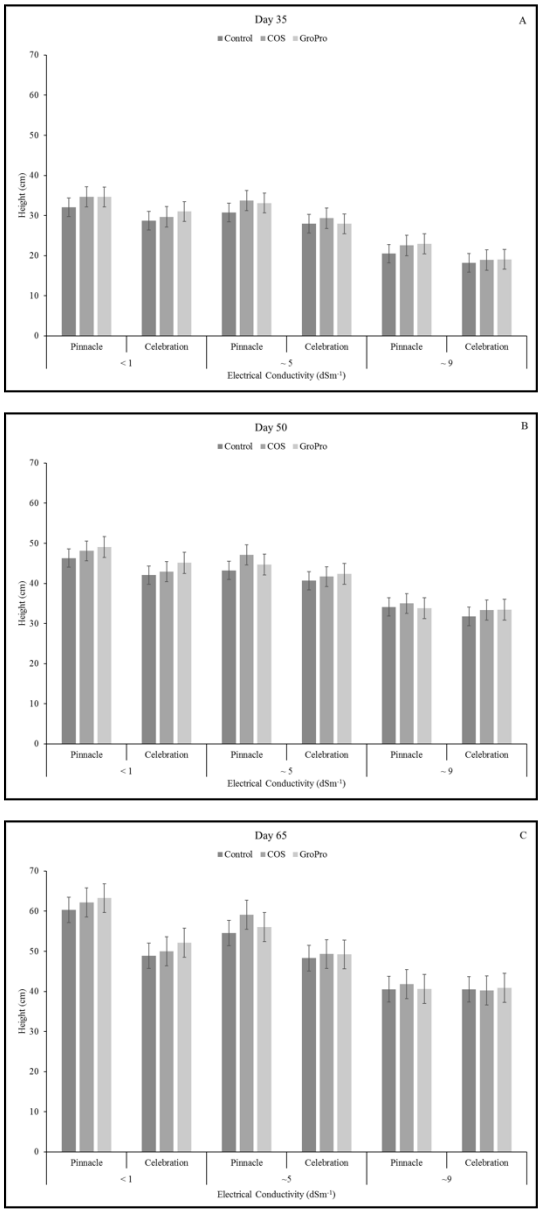

Figure 8.1. Height (cm) of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

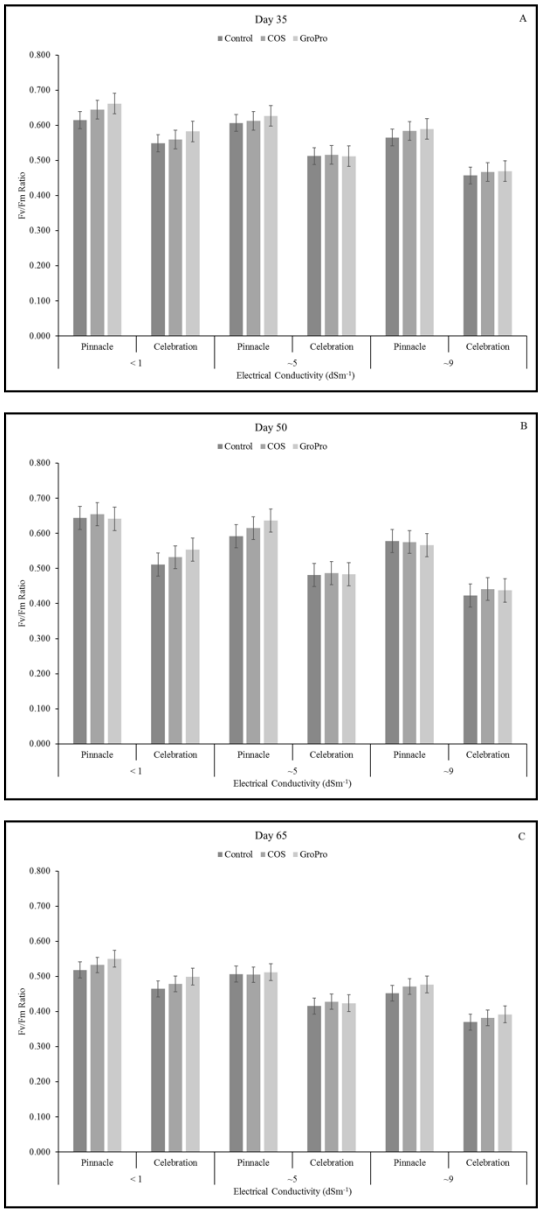

Figure 8.2. Photochemical efficiency (F_v/F_m) of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

Previously, reduction of net photosynthetic rate, stomatal conductance, and transpiration rate was reported in barley under high salt stress (Mahlooji *et al.*, 2018). Similarly, Allel, Ben-Amar and Abdelly (2018) observed that salt tolerant barley genotypes had higher photosynthetic efficiency, especially fluorescence attributes when compared to susceptible genotypes of barley

under high soil salinity. In our experiment, like the results of plant height, barley plants of cv. Pinnacle had significantly ($p \leq 0.05$) higher photochemical efficiency when compared to cv. Celebration at all growth stages and across all seed elicitation treatments. Furthermore, significant improvement of photochemical efficiency was found in barley plants of cv. Pinnacle with GP seed elicitor treatments at day 35 irrespective of soil salinity levels. Additionally, slight improvement of photochemical efficiency was also observed in barley plants of cv. Celebration with GP seed elicitor treatments under high soil salinity level (~ 9 dSm^{-1}) at all growth stages. Previously, improvement of photochemical efficiency was observed with GP foliar treatment in creeping bentgrass clonal lines under cold stress (Sarkar, Bhowmik and Shetty, 2010). Such improvement of photochemical efficiency with seed or foliar elicitor treatments has significant relevance to target this bio-elicitation strategy for sustaining optimum photosynthetic activity which in turn support better recovery of barley plants under high salt stress. Furthermore, improved photosynthetic activity and high carbon assimilation potentially support other metabolic processes including protective defense related pathway regulations (such as PPP), which is critical to counter salinity stress induced cellular dysfunction and subsequent tissue damages in barley and other food crops.

Table 8.1. Analysis of variance table indicating mean square (MS) and significant differences between barley types (C), soil salinity levels (S) and seed elicitation treatments (T), with their respective 2 and 3-way interactions for physical and biochemical parameters associated with the pentose phosphate pathway (PPP) mediated secondary metabolite biosynthesis and antioxidant enzyme responses in two barley types at days 35 (A), 50 (B) and 65 (C) after seed priming treatment.

(A)

Source	df	Plant height	PSE	G6PDH ‡	SDH	Proline	PDH	CAT	GPX	SOD	TSP	ABTS
C	1	493.7**	0.4099**	1654.9 ns†	1.08**	1654.8ns	56.2ns	71242.0**	30.6ns	71242.0**	35.21**	934.7*
S	2	1957.5**	0.0826**	3863.0 ns	0.39**	3863.0ns	452.1**	15317.0**	28250.0**	15317.0**	0.04ns	22.8ns
C×S	2	11.3ns	0.0031**	18492.0**	0.43**	18492.0**	33.1ns	1705.0ns	5438.8**	1705.0ns	0.19ns	211.0ns
T	2	62.4**	0.0049**	9222.4*	0.04 ns	9222.4*	160.1**	2127.5ns	1819.9*	2127.5ns	0.54**	192.8ns
C×T	2	8.67ns	0.0001 ns	18897.0**	0.06 ns	18897.0**	39.7ns	2753.4ns	104.9ns	2753.4ns	0.06ns	65.8ns
S×T	4	1.71ns	0.0010**	19408.0**	0.08 ns	19408.0**	41.2*	10976.0**	1550.9*	10976.0**	0.03ns	237.0*
C×S×T	4	2.27ns	0.0006*	19226.0**	0.15*	19226.0**	40.4*	1211.9ns	885.5ns	1211.9ns	0.51**	201.8ns

(B)

Source	df	Plant height	PSE	G6PDH	SDH	Proline	PDH	CAT	GPX	SOD	TSP	ABTS
C	1	313.6**	0.0098**	210.4ns	0.34ns	38546.0*	14.0ns	2700.8ns	1163.1*	2700.8ns	3.91**	57.8ns
S	2	2311.5**	0.0819**	4946.2**	2.10ns	28411.0**	351.1**	2227.2ns	1483.4**	2227.2ns	4.59**	747.2**
C×S	2	50.0*	0.0045**	469.5*	1.04ns	15937.0*	19.9*	3165.1ns	112.1ns	3165.1ns	1.23**	113.4ns
T	2	57.5*	0.0076**	196.7ns	1.38ns	10021.0ns	1.66ns	7855.6*	1003.1*	7855.6*	0.96*	192.0*
C×T	2	16.5ns	0	81.4ns	1.74ns	2341.5ns	7.42ns	66.2ns	885.4*	66.2ns	0.12ns	269.0*
S×T	4	13.6ns	0.0013**	66.0ns	1.46ns	2381.2ns	1.60ns	2181.6ns	353.0ns	2181.6ns	0.21ns	223.8**
C×S×T	4	5.15ns	0.0001ns	64.8ns	1.41ns	5852.5ns	6.80ns	1076.2ns	65.08ns	1076.2ns	0.22ns	60.2ns

(C)

Source	df	Plant height	PSE	G6PDH	SDH	Proline	PDH	CAT	GPX	SOD	TSP	ABTS
C	1	2397.2**	0.4632**	5618.0**	0.06ns	651.8ns	294.6**	10613.0**	33597.0**	10613.0**	0.10ns	1180.2**
S	2	307.6**	0.0326**	1519.2**	1.38**	8.9E+07	1075.7**	13434.0**	6132.5**	13434.0**	2.12**	41.4ns
C×S	2	108.2*	0.0038*	378.9*	0.77*	2474.5*	60.7**	11332.**	4881.4**	11332.0**	1.62**	145.1*
T	2	27.9ns	0.0052*	41.07ns	1.16**	5416.2*	11.3*	916.3ns	513.9*	916.3ns	0.08ns	59.3ns
C×T	2	32.3ns	0.0003ns	1088.7**	0.55*	357.4ns	5.73ns	2769.4*	454.2ns	2769.4*	0.04ns	64.3ns
S×T	4	36.1ns	0.0014ns	123.3ns	0.32ns	2173.2*	15.2**	12623.0**	437.1*	12623.0**	0.43**	61.8ns
C×S×T	4	16.0ns	0.0006ns	346.2**	0.38ns	2311.4*	32.1**	4849.7**	169.8ns	4849.7**	0.60**	127.2*

*Denotes significance at $p < 0.05$; **Denotes significance at $p < 0.001$; † ns, non-significant; ‡ G6PDH, glucose-6-dehydrogenase; SDH, succinate dehydrogenase; PDH, proline dehydrogenase; CAT, catalase; GPX, guaiacol peroxidase; SOD, superoxide dismutase

8.4.2. Glucose-6-Phosphate Dehydrogenase (G6PDH) and Succinate Dehydrogenase (SDH) Activities

The G6PDH is generally localized in the cytosol (often in plastids) of plant cells and it catalyzes the first-rate limiting step of the pentose phosphate pathway (PPP) (Shetty, 1997; Shetty and Wahlqvist, 2004; Gao *et al.*, 2016). The activity of G6PDH of barley leaves was determined to assess the potential metabolic role of the PPP for driving carbon flux towards downstream of protective defense (shikimate and phenylpropanoid) related pathways and supporting anabolic responses (through generation of NADPH), which are critical endogenous defense responses to counter salt stress induced physiological and metabolic breakdowns of plants.

Statistically significant differences in G6PDH activity between mean separation for 3 ways interaction of barley type × salinity level × elicitor treatment was observed at days 35 and 65 after seed priming treatment. At day 50, only 2-way interaction between barley type × salinity level had statistically significant effect on G6PDH activity of barley leaf samples. Overall, G6PDH activity of barley gradually decreased from day 35 to day 65 across barley types and irrespective of seed elicitation treatments.

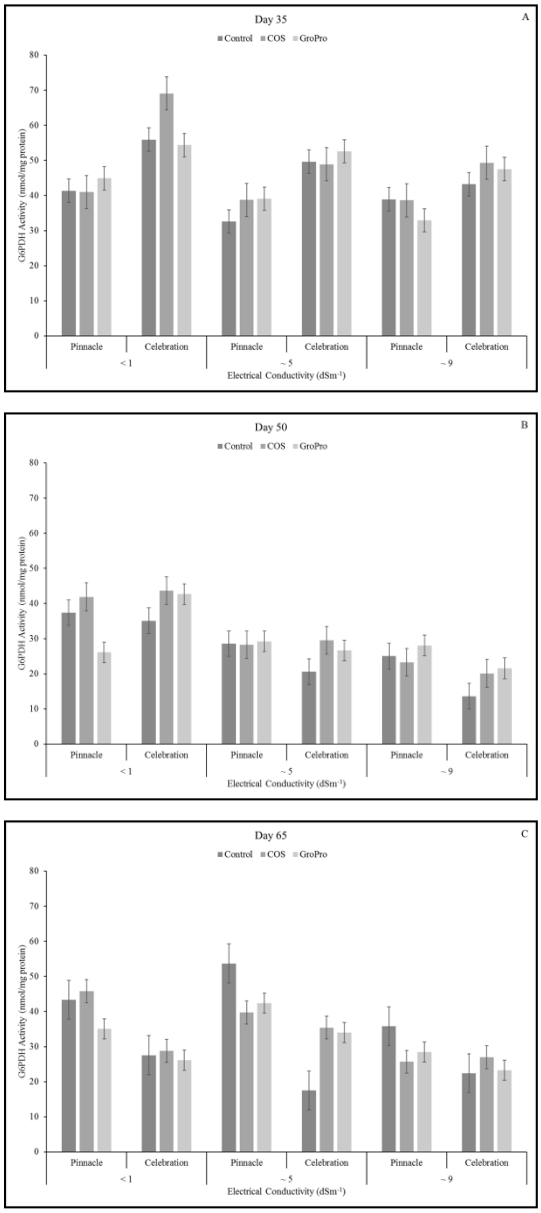

Figure 8.3. Glucose-6-phosphate dehydrogenase (G6PDH) activity (nmol/mg protein) of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

Seed priming treatment with COS resulted in significantly ($p \leq 0.05$) higher G6PDH activity in cv. Celebration both under high salt stress (~ 9 dSm^{-1}) and without salt stress (control) and such response was greater at early growth stage (35 days after seed priming treatment).

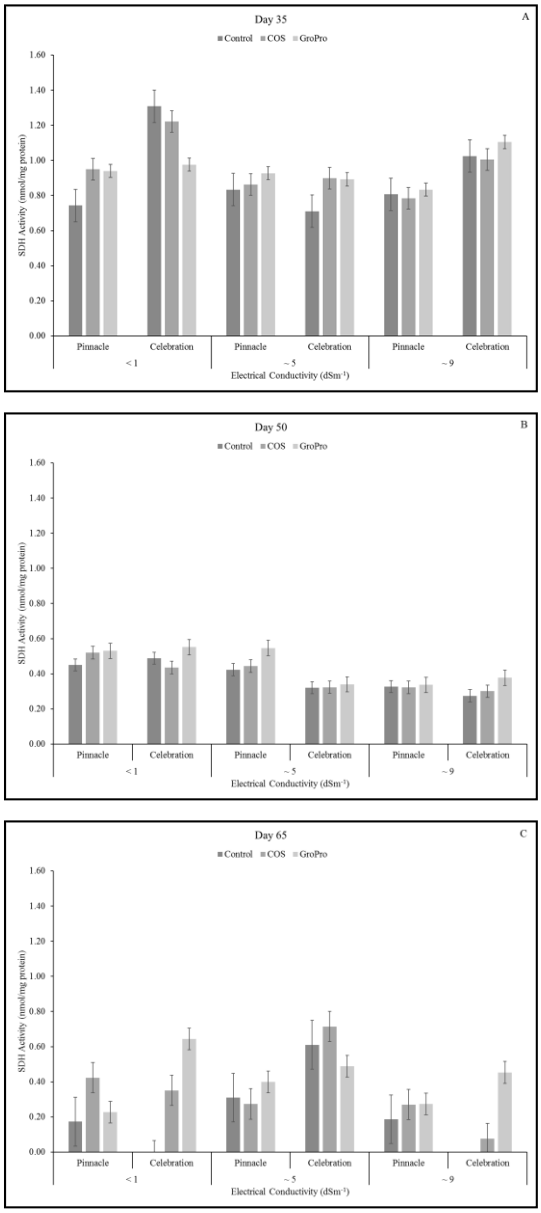

Figure 8.4. Succinate dehydrogenase (SDH) activity (nmol/mg protein) of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

Gao *et al.* (2016) previously reported that inhibiting G6PDH (glucosamine-GLUCm reagent) reduced cyclic electron flow around PSI and decreased NADPH content in spreading earth moss (*Physcomitrella patens*) under salt stress. In another study, enhanced G6PDH activity was observed in black bean with GP seed elicitor treatment under moderate salt stress (Orwat *et*

al., 2017). Similarly, improvement of salinity stress resilience through upregulation of PPP (high G6PDH activity) was also observed in *Swertia chirayita* clonal lines with inoculation of *Lactiplantibacillus plantarum* (Phoboo *et al.*, 2016). Therefore, all these results indicated that enhanced G6PDH activity and upregulation of PPP is critical for diverse protective defense responses (both catabolic and anabolic) of plant cells under high soil salinity and other abiotic stresses. The activity of another key enzyme, succinate dehydrogenase (SDH) was also measured to determine the possible modulation of the Krebs's/citric acid cycle, which indicates the potential distribution of carbon flux between PPP (energy conserving) and more energy expensive respiration pathways, and related bioenergetic functions (Huang and Millar, 2013) in the cells of barley plants under high salt stress. Like the results of G6PDH, the activity of SDH was also highest at day 35 (Figure 8.4). Statistically significant mean separation differences in SDH activity due to barley type × salinity level interaction was observed at day 35 and day 65 after seed priming treatment. Irrespective of soil salinity levels, the SDH activity drastically decreased between day 35 and 50, which indicated reduction in respiration rate during boot stage to head emergence of barley plants. Depletion of acids and intermediates related to Krebs cycle was previously observed in barley with high salt stress (Jarošová *et al.*, 2016). In the current study, the significant effects ($p \leq 0.05$) of soil salinity levels and seed priming treatments on SDH enzyme activity of barley leaves were observed at late growth stage (65 days after seed priming treatment) (Table 8.1). Statistically higher mean SDH activity was found in moderate soil salinity level when compared to no salt stress and high salt stress at day 65. Additionally, barley plants which were treated (seed elicitation treatment) with COS and GP showed significantly ($p \leq 0.05$) higher SDH activity when compared to barley plants with no seed elicitation treatment (control) at same time point of day 65. This result indicated that seed priming treatments (both

COS and GP) sustained critical and essential metabolic regulation of barley plants much longer (until critical reproductive stage) when compared to no seed elicitation treatment under moderate and high salt stress. Previously, enhanced SDH activity was observed in *Swertia chirayita* clonal lines with inoculation of *Lactiplantibacillus plantarum* under high salt stress (Phoboo *et al.*, 2016). Saha, Kunda and Biswas (2012) found reduced SDH activity in roots, while increased in shoots of mungbean (*Vigna radiata*) seedling under high salt stress. Therefore, sustaining critical metabolic regulation such as mitochondrial respiration for energy production (ATP) and PPP-related anabolic and protective defense responses are essential for plants to cope with and counter stress induced cellular breakdowns and for their recovery under high salt stress. In this context, seed priming with biological elicitors such as COS and GP can be an effective strategy to enhance metabolically linked defense responses and to improve overall fitness of crops such as barley under high salt stress.

8.4.3. Total Proline Content and Proline Dehydrogenase (PDH) Activity

Proline biosynthesis and accumulation in the cytosol is a common defense response of plant cells under salinity and other abiotic stresses (Per *et al.*, 2017). Proline, a free amino acid is largely known as an effective osmoprotectant and plays a critical role in cellular redox regulation to counter abiotic stress-induced cellular breakdowns (Szabados and Savouré, 2010). However, the active metabolic role of proline as a reducing equivalent instead of NADH to support mitochondrial oxidative phosphorylation (for ATP synthesis) and the association of proline synthesis from glutamate in the cytosol with PPP regulation ($NADP^+$/NADPH) under abiotic stresses such as salinity stress (Shetty and Wahlqvist, 2004) were not previously investigated in barley.

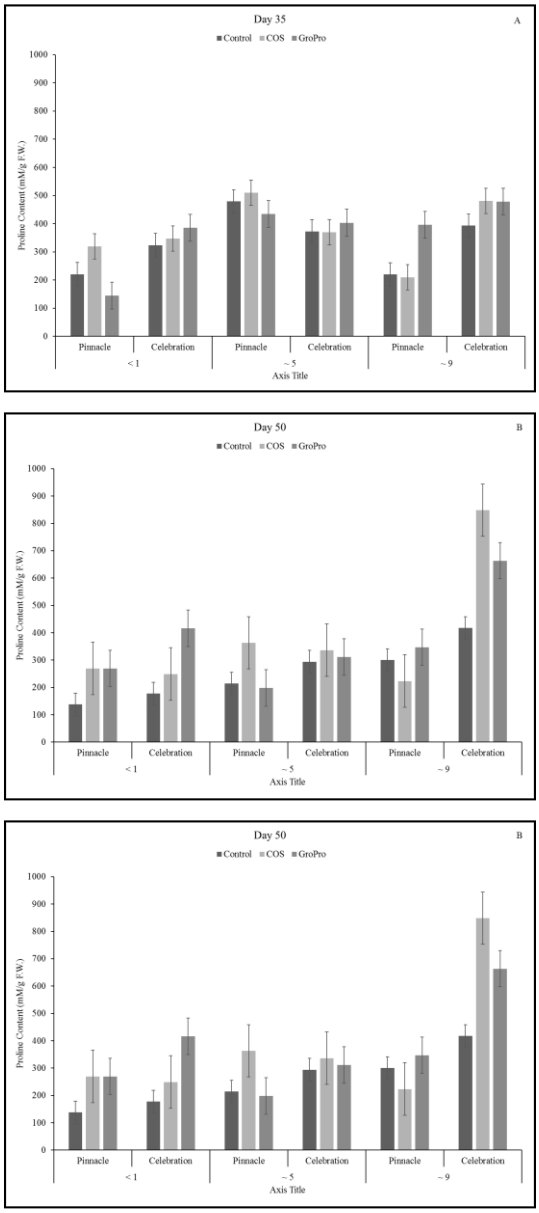

Figure 8.5. Total proline content (mM/g of leaf fresh weight) of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

Therefore, one of the aims of this study was to investigate the efficacy of seed elicitation strategy to stimulate proline-associated pentose phosphate pathway (PAPPP) and potential metabolic role of proline to improve resilience of barley under high salt stress. Overall, the proline content of barley leaves decreased significantly from day 50 to 65 and the rate of

reduction was higher under high salt stress (Figure 8.5). This trend of reduction of proline content in barley leaves positively correlated with reduced G6PDH enzyme activity during same time interval, which indicated an association of proline biosynthesis in the cytosol with PPP regulation. Statistically significant effect of 3 ways interaction of barley type × salinity level × elicitor treatment on proline content was observed at day 35 and day 65, while at day 50 only 2 ways interaction between barley type × salinity level had statistically significant effect. The mean proline content in barley plants of cv. Celebration increased under high salt stress at day 35 and 50, while at day 65 proline content of same barley type reduced under high salt stress. Additionally, barley plants of cv. Celebration treated with seed priming treatments (COS and GP) had significantly higher total proline content when compared to plants not treated with seed elicitors at days 35 and 50. At the same growth stages (day 35 and 50) high proline content was also observed in cv. Pinnacle with GP seed elicitor treatment under high salt stress. Previously, Shelden *et al*. (2016) found higher accumulation of proline and organic acids related to shikimate pathway in salt tolerant barley genotype (Clipper) when compared to salt susceptible barley landrace (Sahara). Similarly, Ma *et al.* (2019) observed higher accumulation of proline and increased activity of phenylalanine ammonia lyase (PAL) with exogenous application of γ-aminobutyric acid (GABA) in germinated hulless barley under salt stress. However, in the current study, no significant effect of seed priming treatments on total proline content of both barley types was observed at late growth stage (day 65). As barley plants showed relatively higher salt stress resilience, the maximum salinity level (~ 9 dSm^{-1}) targeted in this study may not have had significant stress induced metabolic responses such as proline synthesis and accumulation.

Proline dehydrogenase (PDH) is a key enzyme that catalyzes the oxidation of L-proline to Δ1-pyrolline-5-carboxylate and released electron from this oxidation process can be transferred to electron transport chain to support energy synthesis (ATP) in the mitochondria (Shetty and Wahlqvist, 2004; Phang, Liu and Zabirnyk, 2010; Servet *et al.*, 2012). Therefore, the activity of PDH was measured to assess the extent of proline oxidation and potential metabolic role of proline in barley leaf tissues under salt stress. Similar to the result of proline, 3 ways interaction of barley type × salinity level × elicitor treatment had statistically significant effect on PDH activity of barley leaves at day 35 and day 65 after seed priming treatment. In the current study, PDH activity was found to decrease in response to increasing levels of soil salinity at all growth stages. At day 50, under no saline (<1 dSm^{-1}) and high saline conditions (~ 9 dSm^{-1}), barley plants of cv. Celebration treated with GP showed significantly ($p \leq 0.05$) higher PDH activity when compared to no seed elicitor treatment (Figure 8.6). In the subsequent time point (day 65) same seed elicitor treatment (GP) resulted in significantly ($p \leq 0.05$) higher PDH activity in cv. Celebration under moderate (~ 5 dSm^{-1}) and high salt stress (~ 9 dSm^{-1}). Higher PDH activity of cv. Celebration with GP seed elicitor treatment under high salt stress at late growth stage indicated a potential increase in proline oxidation and active metabolic role of proline to support mitochondrial energy synthesis under high salt stress in this barley type, which is a critical defense response of plants under abiotic stresses.

Huang *et al.* (2013) observed reduced PDH activity and higher accumulation of proline in roots of relatively salt tolerant Jerusalem artichoke, while it did not change in the shoots of the same plant under high salt stress. In the current study both barley types exhibited different level of tolerance against soil salinity, their metabolic regulation such as proline metabolism and

associated redox regulation also varied significantly under high salt stress irrespective of the seed elicitor treatments.

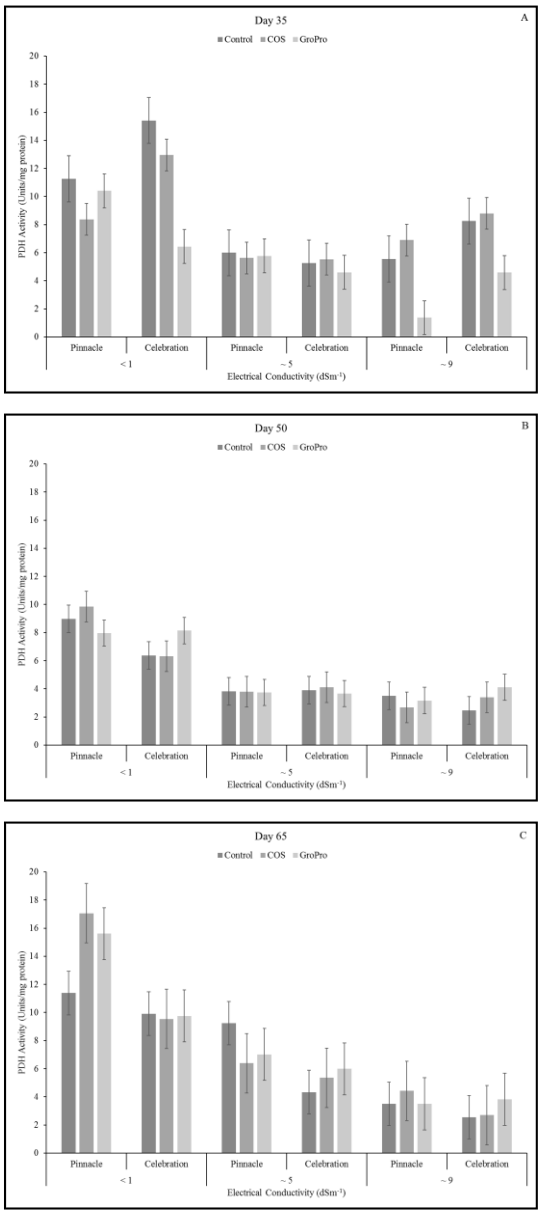

Figure 8.6. Proline dehydrogenase (PDH) (units/mg protein) activity of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

8.4.4. Activities of Antioxidant Enzymes – Catalase (CAT), Superoxide Dismutase (SOD) and Guaiacol Peroxidase (GPX)

Similar to the stress protective role of proline, endogenous antioxidant enzyme responses are also an integral part of plants intricate defense mechanism, specifically for maintaining cellular redox balance under abiotic stresses such as salinity stress (Foyer and Noctor, 2013; Hossain and Dietz, 2016). In the current study, the antioxidant enzyme activities of CAT, SOD, and GPX of barley leaf tissues were determined to understand the critical role of antioxidant enzyme responses in barley under salt stress after bio-elicitation with seed elicitor treatments.

In this study statistically significant effect of 3 ways interaction of barley type × salinity level × elicitor treatment on CAT activity of barley was observed on day 65, while at day 35 and day 65, 2 ways interaction between salinity level × elicitor treatment also had statistically significant impact. Among all three antioxidant enzymes, significant ($p \leq 0.05$) increase of CAT activity was observed in barley plants with high salt stress (~ 9 dSm^{-1}) at early growth stage (day 35) (Table 8.1 & 8.2). Salt stress induced increase of CAT activity of barley leaves (Giza 124) was previously reported by Agami (2014). Similarly, Unal, Aktas and Guven (2014) observed 7-fold increase of CAT activity in 2 weeks old barley seedlings under high salt stress and concluded that CAT might be the key antioxidant enzyme in barley under salt stress, especially during early growth stages. In the current study, no significant differences in CAT activity of barley plants among different soil salinity levels was noted at day 50, while only barley plants of cv. Pinnacle had significantly high CAT activity under moderate salt stress (~ 5 dSm^{-1}) at late growth stage (day 65). Overall, at early (day 35) and late (day 65) growth stages, barley plants of cv. Pinnacle had significantly ($p \leq 0.05$) high CAT activity when compared to cv. Celebration. Additionally, under high salt stress (~ 9 dSm^{-1}), barley plants with GP seed elicitor treatment had

significantly high CAT activity at all growth stages, with only exception of cv. Pinnacle at day 65. Previously, Erdal *et al.* (2011) reported enhanced CAT activity in wheat with salicylic acid foliar treatment under high salt stress. Overall, decomposition of hydrogen peroxide (H_2O_2) to water and hydrogen, which is catalyzed by CAT, is a critical defense response to counter oxidative stress induced damages and maintaining cellular homeostasis under high salt stress (Gondim *et al.*, 2012). Therefore, high salt stress (~ 9 dSm^{-1}) induced increase of CAT activity and stimulation of this antioxidant enzyme activity with GP seed elicitor treatment during early and late growth stage has significant relevance for targeting such bio-elicitation tool to counter salt stress-induced oxidative breakdowns in barley.

Table 8.2. Catalase (CAT) (units/mg protein) antioxidant enzyme activity of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}).

Electrical Conductivity ($dS\ m^{-1}$)	Treatment	Day 35		Day 50		Day 65	
		Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
<1	Control	242.6	203.9	178.6	183.8	214.0	197.8
	COS	256.7	217.0	201.5	176.4	229.3	217.6
	GP	254.4	183.9	180.9	190.0	156.4	193.7
5	Control	237.4	198.9	173.5	149.5	200.8	126.4
	COS	233.2	201.7	169.8	156.2	208.9	158.8
	GP	238.3	166.4	169.7	180.0	267.4	189.7
9	Control	234.9	225.4	165.7	166.9	189.6	180.3
	COS	246.6	198.9	160.6	168.5	170.4	139.6
	GP	300.2	270.5	189.5	191.6	165.0	198.7

Table 8.3. Guaiacol peroxidase (GPX) (nmol/mg protein) antioxidant enzyme activity of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}).

Electrical Conductivity ($dS\ m^{-1}$)	Treatment	Day 35		Day 50		Day 65	
		Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
<1	Control	112.3	135.7	66.1	72.8	106.3	68.8
	COS	128.8	168.6	76.7	78.4	115.4	70.5
	GP	116.5	119.3	70.0	87.6	87.5	67.2
5	Control	101.7	82.1	62	67.1	108.1	72.3
	COS	100.6	56.1	64.9	64.3	117.9	76.4
	GP	93.0	86.0	71.8	70.7	108	75.8
9	Control	92.2	83.4	68.7	67.2	63.5	66.8
	COS	98.4	83.0	70.7	65.0	72.4	74.5
	GP	94.2	88.9	74.0	90.3	75.5	73.6

Table 8.4. Superoxide dismutase (SOD) (units/mg protein) antioxidant enzyme activity of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}).

Electrical Conductivity ($dS\ m^{-1}$)	Treatment	Day 35		Day 50		Day 65	
		Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
<1	Control	n.d.	n.d.	n.d.	0.0143	n.d.	0.0137
	COS	0.0073	0.0043	0.0033	0.001	n.d.	0.087
	GP	-0.0003	n.d.	n.d.	0.0033	n.d.	0.0613
5	Control	n.d.	n.d.	0.0133	n.d.	n.d.	0.0317
	COS	0.0093	0.0067	0.01	0.0053	n.d.	0.0003
	GP	n.d.	n.d.	n.d.	0.0017	0.0033	0.0017
9	Control	0.0077	0.0003	0.0233	0.0057	0.0033	0.006
	COS	0.013	0.0107	n.d.	0.004	n.d.	0.001
	GP	n.d.	n.d.	n.d.	0.0013	n.d.	0.003

n.d. – Not detected

However, different trend was observed for GPX activity of barley leaves under all three levels of soil salinity (Table 8.3). At early growth stage (day 35), significantly ($p \leq 0.05$) high GPX activity of both barley types was observed under no salt stress when compared to plants grown under moderate and high salt stress. Similarly, at late growth stage (day 65) high GPX antioxidant activity was observed in barley under no (control <1 dSm^{-1}) and moderate (~ 5 dSm^{-1}) salt stress when compared to plants under high (~ 9 dSm^{-1}) salt stress. Irrespective of soil

salinity levels, high GPX activity was observed in barley plants of cv. Pinnacle with COS seed elicitor treatment, especially at early (day 35) and late (day 65) growth stage. Same seed elicitor treatment also enhanced GPX activity of cv. Celebration under no salt stress at day 35, while at day 50, GP seed elicitor treatment resulted in high GPX activity in cv. Celebration under high salt stress (~ 9 dSm^{-1}). Therefore, GP seed elicitor treatment can be targeted to stimulate antioxidant enzyme responses (CAT and GPX) for improving salinity stress resilience in barley. Previously, improved GPX, CAT, and SOD antioxidant enzyme activity was observed in salt tolerant wild barley (Tibetan) when compared to salt susceptible cultivated barley cultivar (Ahmed *et al.*, 2013). In the current experiment, very low and inconsistent SOD antioxidant enzyme activity was observed in barley leaves under all salinity levels and at all growth stages (Table 8.4). Overall, at early growth stage (day 35) significantly high SOD activity was observed in barley plants with COS seed elicitor treatment. However, at late growth stage (day 50 and 65) and under moderate and high salt stress, no improvement of SOD activity was observed in barley leaves with seed elicitor treatments. These results indicated that for barley cultivars targeted in this study, CAT and GPX played more critical protective roles in leaf tissues than SOD under salt stress. Previously, Wang *et al.* (2011) observed high SOD activity in alfalfa roots and shoots when compared to leaves of same plant under high salt stress. Therefore, further studies by investigating SOD and other antioxidant enzyme activity of different plant parts of barley are needed to understand the more comprehensive role of this antioxidant enzyme to counter oxidative stress under high soil salinity.

8.4.5. Total Soluble Phenolic Content and Antioxidant Activity

Enhanced biosynthesis of protective phenylpropanoid metabolites, such as soluble phenolic compounds is a critical defense response of plants under the exposure to abiotic and

biotic stresses. These stress protective secondary metabolites are often mobilized together with protective antioxidant enzyme responses to maintain cellular redox balance (Bartwal *et al.*, 2013). Based on this rationale, TSP content and total antioxidant activity of the barley leaf tissues were measured to determine bio-elicitation induced biosynthesis of phenolics and its protective antioxidant function under salinity stress.

Overall, statistically significant ($p \leq 0.05$) effect of 3-way interaction between barley type × salinity level × elicitor treatment on TSP content of barley leaves was observed at day 35 and day 65 (Table 8.1 & Figure 8.7). At later growth stages of day 50 and day 65, high mean TSP content of both barley types was observed under high salt stress (~ 9 dSm^{-1}) irrespective of seed elicitation treatments. Corroborating the results of plant height and photochemical efficiency, significantly high TSP content was also observed in barley leaves of cv. Pinnacle when compared to cv. Celebration across all salinity levels, especially at early growth stage of day 35. Additionally, under high salt stress (~ 9 dSm^{-1}), cv. Pinnacle had significantly ($p \leq 0.05$) high TSP content at later growth stages (day 50 and 65). High TSP content of cv. Pinnacle may indicate higher fitness of this barley type under salinity stress. Previously, Ahmed *et al.* (2015) reported over expression of genes related to secondary metabolism such as expression of Phenylalanine ammonia-lyase (PAL), a key enzyme of phenylpropanoid pathway, in salt tolerant Tibetan wild barley under salinity stress. Similarly, higher accumulation of phenolics in leaves of salt tolerant barley cultivar (Acsad 1230) was also observed under high salt stress (Lilia *et al.*, 2005). In the current study, significant ($p \leq 0.05$) effect of seed elicitor treatments on TSP content of barley leaves was also observed. At day 35 and 50, barley plants of cv. Pinnacle with COS seed elicitor treatment had significantly higher TSP content under moderate and high salt stress.

The same seed elicitor treatment also resulted in higher TSP content in cv. Celebration under high salt stress at day 35, and under moderate and high salt stress at day 50. The positive effect of GP seed elicitor treatment on TSP content of barley leaves was observed under moderate and high salt stress at day 50 and under high salt stress at day 65. Therefore, the result of TSP content of barley leaves at different growth stages indicated that biosynthesis of phenolic metabolites is a critical protective defense response of barley under high salt stress, and seed elicitation with COS and GP can be targeted to further enhance biosynthesis of such secondary metabolites for improving overall salinity stress resilience in barley. As one of the major biological function of phenolics under abiotic stresses is to provide protection against oxidative stress induced cellular breakdowns, total antioxidant activity (based on ABTS radical scavenging activity) of barley was determined.

In the current study, statistically significant effect of 3 ways interaction of barley type × salinity level × elicitor treatment on ABTS-based antioxidant activity was observed at day 65, while 2 ways interaction of salinity level × elicitor treatment had statistically significant impact on total antioxidant activity of barley at day 35 and day 50 after seed priming treatment. Additionally, high salt stress-induced (~ 9 dSm^{-1}) enhancement of total antioxidant activity of both barley types was only observed at day 50 (Figure 8.8). Among two barley types, high antioxidant activity was found in cv. Pinnacle when compared to cv. Celebration at day 35 and 65. The antioxidant activity positively correlated with TSP content of the barley types under salt stress.

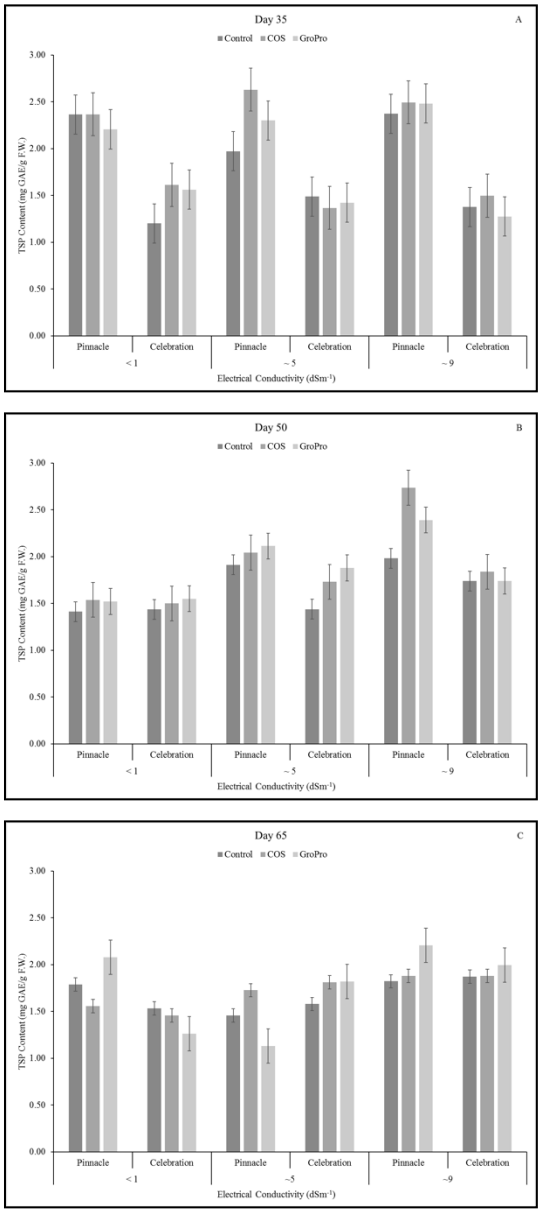

Figure 8.7. Total soluble phenolic (TSP) (mg/g fresh weight) content of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

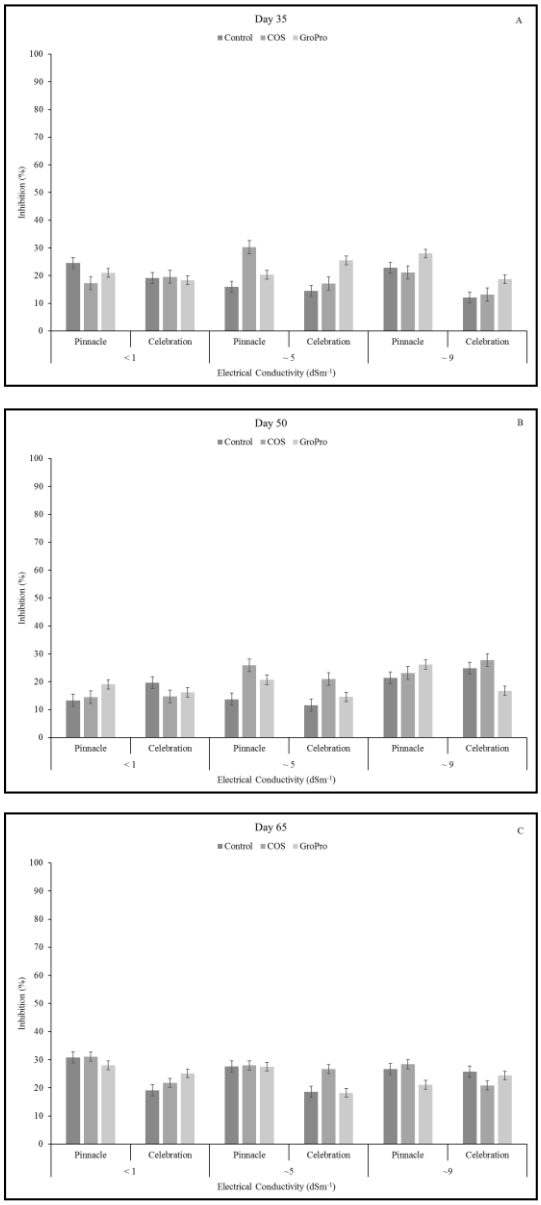

Figure 8.8. Total antioxidant activity (ABTS free radical scavenging based % inhibition) of two barley types (cv. Pinnacle and cv. Celebration) at 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm-1, and ~9 dSm-1). Vertical bars represent standard error.

Additionally, improvement of antioxidant activity was observed in cv. Pinnacle with COS seed elicitor treatment under moderate salt stress (~ 5 dSm^{-1}) at day 35 and 50. In cv. Celebration, same seed elicitor treatment (COS) improved antioxidant activity at day 50 and 65 under moderate salt stress. At early growth stage (day 35), GP seed elicitor treatment also

enhanced total antioxidant activity of both barley types under high (~ 9 dSm^{-1}) and moderate (~ 5 dSm^{-1}) salt stress. Previously, Genisel, Erdal and Kizilkaya (2015) observed mitigation of oxidative stress and low level of cellular ROS production with exogenous application of cysteine in barley. However, this study concluded that antioxidant property of cysteine was responsible to counter salt-induced ROS production, rather than its modulating effect on endogenous enzymatic and non-enzymatic antioxidants. Though, in the current study, response of seed elicitor treatments on total antioxidant activity varied between barley types and with different growth stages, such metabolically driven bio-elicitation strategy can be targeted for improving phenolic and other antioxidant enzyme-linked cellular redox balance under high salt stress. However, for effective and economically viable application of such elicitation strategy, it is also important to determine its impact on grain yield and overall productivity of barley under high salt stress.

8.4.6. Grain Weight

Grain (seed) weight is an effective indicator to understand the extent of adverse impact of abiotic stresses on food crops. To determine the combined effect of seed elicitation treatments and salt stress on grain weight of two barley types, the heads of the mature barley plants were harvested, cleaned, and weighed. In this experiment, an increase in soil salinity levels did not lead to a significant reduction in the grain weight of both barley types (Figure 8.9). On the contrary, the grain weight was found to increase slightly under moderate salt stress (~ 5 dSm^{-1}). Additionally, under high salt stress (~ 9 dSm^{-1}), both seed elicitation treatments were found to help maintain a greater grain weight when compared to control (without seed elicitor treatment). Between two barley types, higher mean grain weight was observed in cv. Pinnacle when compared to cv. Celebration across all salinity levels. Previously, Pakar *et al.* (2016) reported significant reduction of grain yield, grain dry weight, and harvest index in barley with high salt

stress (≥ 10 dSm^{-1}). However, they observed improvement of these yield components of barley with foliar application of salicylic acid. The grain weight result of the current study suggested that barley type cv. Pinnacle were relatively tolerant to salt stress of 9-10 dSm^{-1}, and seed elicitors such as GP and COS can be advanced as an effective agronomic tool to improve growth, photosynthetic activity, and productivity of barley under moderate to high salt stress.

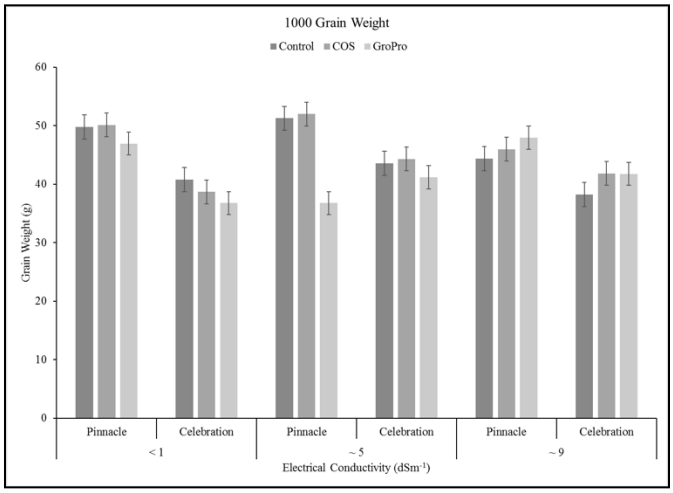

Figure 8.9. Grain weight (g) of two barley types (cv. Pinnacle and cv. Celebration) with seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three soil salinity levels (<1 dSm^{-1}-no salt stress, ~5 dSm^{-1}, and ~9 dSm^{-1}). Vertical bars represent standard error.

8.5. Conclusions

To understand critical endogenous defense response related metabolic regulations of barley for improving their overall fitness and resiliency under high salt stress, two malting barley types were screened under moderate and high salt stress and critical metabolic role of proline-associated pentose phosphate pathway (PAPPP) regulation and related modulation of phenolic biosynthesis and antioxidant enzyme responses for determining salt stress resilience of barley were also investigated. Furthermore, bio-based, and metabolically driven seed elicitation strategy (with bioprocessed elicitors such as COS and GP) was optimized and recruited to improve overall salt stress resiliency of barley. In the current study, adverse effect of high salt stress (~9

dSm^{-1}) on plant height, growth, and photochemical efficiency of barley was observed, while it did not affect the grain weight of both barley cultivars. Enhanced TSP content, total antioxidant activity, CAT antioxidant enzyme activity, and proline accumulation were also observed in barley plants at day 50 under high salt stress. Additionally, elicitation with GP seed elicitor resulted in higher grain weight, TSP content, CAT antioxidant enzyme activity (day 50 and 65), proline accumulation (day 50), and higher SDH enzyme activity (day 65) in barley leaves under high salt stress. Therefore, the results of the current study indicated that seed elicitation with GP can be an effective strategy to improve salinity stress resilience in barley. However, field-based, multiple year, and multiple location studies with more malting and food barley types are required for large-scale and commercial application of such seed elicitors to improve production and profitability of barley under high saline soil.

CHAPTER 9. ENHANCING WATERLOGGING STRESS TOLERANCE OF BARLEY DURING SEEDLING ESTABLISHMENT WITH BIOELICITATION STRATEGY

9.1. Abstract

Barley is highly susceptible to waterlogging stress as it affects germination, seedling emergence, seedling establishment, grain filling, and can lead to complete crop failure. Therefore, recruiting metabolically driven seed bio-elicitation strategy to improve waterlogging stress tolerance of barley during critical seedling emergence and seedling establishment stage through stimulation of redox-linked anabolic pentose phosphate pathway (PPP) regulation and associated stress protective endogenous responses has multiple agronomic and economic advantages. To explore this rationale, seeds of two malting barley types (Pinnacle & Celebration) were treated with bioprocessed elicitors, marine protein hydrolysate (GP 1mL/L) and water-soluble chitosan oligosaccharide (1 g/L), and emerging barley seedlings (at 3 leaf stage) were subjected under waterlogging stress (W0-no stress control; W1-hypoxic condition with constant 3 inches water level; W2-anoxic condition with constant 9 inches water level) for two weeks. Following waterlogging stress, barley plants were recovered and grown under normal soil moisture condition in the greenhouse. Physical (plant height, shoot and root biomass), biological (photochemical efficiency), and biochemical (PPP associated enzyme regulation, biosynthesis of stress protective phenolic metabolites, and associated antioxidant enzyme responses) parameters of bio-elicited barley plants were monitored during (20 days after seed treatment) and after withdrawal from waterlogging stress (35, 50, and 65 days after seed treatment). Additionally, several yield parameters such as 1000 grain weight, spikes/plant, grains/spike, and grain yield/plant were also determined after harvest. Significant reduction of plant height, root weight, shoot weight, and photochemical efficiency of barley plants were observed under waterlogging

stress (anoxic condition). Overall, barley plants treated with GP seed elicitor treatment had better recovery with improved shoot and root growth, photosynthetic activity and through upregulation of critical redox-linked PPP regulations. Results of this study indicated that barley is highly susceptible to waterlogging stress. However, bio-elicitation with GP seed treatment is an effective bio-elicitation strategy to improve resiliency and fitness of barley seedlings under waterlogging stress.

Keywords: Antioxidant Enzymes; Bio-elicitors; Pentose Phosphate Pathway; Phenolics; Seed Treatment; Waterlogging Stress

9.2. Introduction

Flooding and waterlogging are among the most significant and widespread abiotic stress challenge affecting agricultural production and profitability worldwide. It occurs predominantly in soils with poor drainage, in regions with low water tables, and / or high rates of precipitation (Pacetti *et al*. 2017). However, the geographical extent and number of incidences of soils impacted by flooding are projected to increase in major agricultural belts worldwide due to rapid changes in global weather events, specifically with constantly varying precipitation patterns (Alam *et al*., 2011). In the United States, flooding becomes a serious agricultural production challenge for vast regions of the Northern Plains and Mid-West regions due to increasing mean spring and fall precipitations (Wuebbles *et al*. 2017). All current climate forecast models suggest even further increase of spring precipitation in the Northern Plains in coming decades. Therefore, growers of this region are facing enormous challenge from constantly varying spring precipitation and subsequent waterlogging of crop fields, especially during the planting season (Wuebbles *et al*. 2017). Since majority of food crops that currently constitute global food systems and grown widely in the Northern Plains are mesophytes, exposure to prolonged

flooding/waterlogging could diminish their fitness and productivity, and therefore represents a key challenge to agricultural output and economic profitability.

Overall, prolonged waterlogged condition severely affects normal physiological and metabolic functions of plants at multiple levels. Primarily, waterlogged soils impede gaseous exchange resulting in a state of anaerobiosis at the root zone. This leads to the formation of hypoxic, and eventually anoxic tissues in the affected plant (Vartapetian and Jackson, 1997; Herzog *et al*., 2016; Shiono *et al*., 2019). The impact of oxygen deprivation on cellular respiration is particularly severe as the mitochondrial electron transport chain is obstructed and ATP synthesis from oxidative phosphorylation via tricarboxylic acid cycle (TCA) is diminished and eventually replaced by the less efficient process of fermentation (Irfan *et al*., 2010). The rate of carbohydrate catabolism also increases and photosynthates are diverted from sinks such as grains to vegetative tissues to meet the cellular energy demands, leading to a reduction in overall biomass and end product quality (Bailey-Serres *et al*., 2012). Furthermore, a reduction in photosynthetic efficiency and ability of chloroplasts to effectively utilize incident photons under stress can lead to a breakdown in cellular redox balance and exertion of oxidative stress due to excessive formation of reactive oxygen species (ROS), which may further affect the integrity of cellular structures and processes (Paradiso *et al*., 2016). The risk of ROS-mediated cellular damage may increase manifold even upon the removal of flooding/waterlogging stress, due to the abrupt exposure of affected tissue to elevated levels of oxygen (Sairam *et al*., 2011; Li *et al*., 2012). Therefore, the maintenance of bioenergetic and redox homeostasis at the cellular level represents a vital target for improving overall crop fitness under flooding/ waterlogging stress.

The cellular metabolism of amino acids, specifically proline, can play an important role in improving abiotic stress resilience by supporting critical physiological and metabolic

functions (Hare and Cress, 1997; Verbruggen and Hermans, 2008). Additionally, proline can also play a key role in defense response to abiotic stresses via its association with critical protective and anabolic processes (Verbruggen and Hermans, 2008). The synthesis of proline from glutamate in the cytosol was reported to be coupled with hydride-ion mediated stimulation of the pentose phosphate pathway (PPP) and redox regulation in animal cells (Phang, 1985). Such critical metabolic role of proline in plant model was proposed by Shetty (1997), whereby a proline-associated pentose phosphate pathway (PAPPP) regulation can increase the carbon-flux towards shikimate and phenylpropanoid pathways, resulting in the synthesis of secondary metabolites, including phenolic compounds which can serve as antioxidants and building blocks for structural tissue adjustments under abiotic stresses. Additionally, after entering the mitochondria, proline may substitute NADH as a reducing equivalent for ATP synthesis by bypassing the energy intensive NADH-dependent oxidative phosphorylation, especially under abiotic stress pressure (Hare and Cress, 1997; Shetty and Wahlqvist, 2004). Furthermore, the stimulation of PAPPP has been found to correspond with the activation of stress signal cascades resulting in the mobilization of enzymatic antioxidant responses, as additional protection against ROS-induced oxidative stress (Shetty and Wahlqvist 2004; Verbruggen and Hermans 2008). Therefore, the regulation of PAPPP can potentially function as a critical metabolic control point which modulates the interplay between primary and secondary metabolic pathways and may aid in enhancing the ability of plants to counter the adverse effects of abiotic stresses such as flooding/waterlogging -linked anaerobiosis and breakdown of redox homeostasis. In this context, metabolically driven strategies can be advanced to improve waterlogging stress resilience through up-regulation of endogenous defense response, such as anabolic PAPPP-linked protective functions in plant models.

Seed priming with bioprocessed elicitors is one of such metabolically driven strategy, which can be targeted for physiologically activating seeds prior to emergence, to improve their germination efficiency and abiotic stress tolerance (Jisha *et al*., 2012). In recent years, various bioprocessed compounds known as elicitors (or biostimulants) have been investigated as seed priming treatments, specifically for inducing redox-linked PAPPP and associated antioxidant enzyme responses (Shetty and Wahlqvist, 2004; Sarkar and Shetty, 2014). Among the evaluated elicitors, compounds derived as byproducts of marine wastes, namely fish protein hydrolysates (FPH) and soluble chitosan oligosaccharides (COS), have found to stimulate PAPPP-mediated phenolic biosynthesis and antioxidant enzyme responses in several food and non-food plants (Orwat *et al*., 2017; Randhir and Shetty, 2005; Sarkar *et al*., 2010; Shetty and Wahlqvist, 2004). Such elicitor treatments may function by serving as sources of free proline and/or mimicking the stimuli similar to external challenges such as fungal attacks, in order to drive the oxidation-linked metabolic stimulation of anabolic PPP for enhancing biosynthesis of stress protective secondary metabolites, while simultaneously activating antioxidant enzyme complexes. As a result, seed priming with such elicitors may potentially improve germination, seedling emergence and seedling establishment in the early stages of vegetative growth, while also improving overall fitness to counter abiotic stress-induced metabolic breakdowns and tissue damages. Based on the above metabolic rationale, bioprocessed elicitors such as fish protein hydrolysates (GP) and soluble chitosan oligosaccharides (COS) were targeted to improve seedling emergence, seedling establishment, and abiotic stress resilience of barley under waterlogging stress.

Barley is a major dryland crop that is grown for food, feed, and malt applications. It has the least resilience to flooding stress in comparison to all other major cereal crops, and the yield

of barley may be reduced by up to 50% in regions that receive high rainfall and have poor drainage (Setters and Waters, 2003). Waterlogged soils diminish vegetative growth and development of barley plants, while increasing their vulnerability to other abiotic and biotic stresses. Current efforts to overcome waterlogging stress in barley are aimed at breeding and screening barley germplasm for tolerant genotypes. However, such strategies are complicated by the fact that waterlogging resilience is a complex trait in barley, which has relatively low heritability (Mendiondo *et al*., 2016). Therefore, it is of interest to better understand the underlying metabolic and physiological responses of barley under waterlogging stress and target critical control points of these protective defense pathways such as PAPPP to improve fitness and productivity.

The current study was aimed to understand the possible role of elicitor-based seed priming towards improving resilience to waterlogging stress in barley plants. The efficacy of two elicitor treatments – GP and COS – in improving the PAPPP linked metabolic response under waterlogging stress were compared in two malting barley types (Pinnacle and Celebration), which are popular in the Northern Great Plains of the USA. The barley plants were grown under two levels of waterlogging stress (fully saturated- constant 3 inches of water above soil for mimicking hypoxic condition and constant 9 inches of water above soil level for anoxic condition), while plants grown in well-drained soil at field capacity was used as a control. Various critical control points of redox-linked PAPPP, phenolic content and the activity of key antioxidant enzymes (catalase, superoxide dismutase, and guaiacol peroxidase) of shoots were monitored while barley plants were subjected to waterlogging conditions and during the recovery period upon removal of stress.

9.3. Materials and Methods

9.3.1. Barley Cultivars

Two malting barley types – Pinnacle (2-rowed) and Celebration (6-rowed) were procured from the Malting Barley Breeding Program of North Dakota State University, Fargo, ND.

9.3.2. Elicitor Treatments

Two bioprocessed elicitors were used in the current study – marine fish and seaweed-based protein hydrolysate (GroPro® / GP; Icelandic Bio-Enhancers, Westchester, NY), and soluble chitosan oligosaccharide from crustacean shells, containing ascorbic acid side chains (COS; Kong Poong Bio, Jeju, South Korea). The elicitors were dissolved in distilled water at a ratio of 1:1000 (1 mL GP and 1 g COS in 1 L water). Distilled water was used as the negative control.

9.3.3. Chemical Reagents

All reagents and enzymes used in this study were purchased from Sigma Aldrich Chemical Co. (St Louis, MO), unless otherwise mentioned.

9.3.4. Bioprocessed Elicitors Seed Treatments

Barley seeds were disinfected in 0.5% sodium hypochlorite solution for 10 min, and rinsed thoroughly with distilled water, drained, and placed on Whatman filter paper (#14) to remove excess water. The seeds were transferred to conical flasks containing elicitor solution and incubated on a rotary shaker for 8 h at 20°C with 180 rpm.

9.3.5. Sowing and Induction of Waterlogging Stress

After incubation with elicitors, treatment solutions were drained, and the seeds were patted dry with dry paper towels to remove excess solution. The treated seeds were sown in plastic pots (8 × 5 × 5 inch) containing potting mix, at a rate of one seed per pot. One week later,

pots containing barley seedling with complete and uniform emergence were identified and used for the study.

When the plants were at the 3-leaf stage (2 weeks), they were subjected to waterlogging stress. The pots were transferred to plastic tubs (32.5 × 20 × 19 inch) in such a way that there were 9 pots per tub and 5 inches between each pot. Two levels of waterlogging stress were imposed to mimic hypoxic (W1) and anoxic (W2) conditions at the root zone. This was achieved by filling the tubs with water up to 3 inches and 9 inches for W1 and W2, respectively. In the case of tubs representing W2, stone pebbles were placed in the pots above the soil layer to prevent the pots from toppling.

Unstressed plants were not placed in tubs and were watered to field capacity (W0). Waterlogging stress was maintained for 2 weeks, following which the stressed plants were removed from the tub. During stress recovery all plants were watered to field capacity. Measurements and leaf sample collection for biochemical analysis were performed beginning from one week of waterlogging stress, and every 2 weeks thereafter until maturity.

9.3.6. Plant Height and Shoot Biomass

Prior to collecting samples at each assay time point, the total plant height was measured. Then, the sample plants were gently removed from the pots and washed thoroughly to remove adhering soil and debris, patted dry with paper towels and the fresh weights of the root, and shoot portions were recorded separately.

9.3.7. Photochemical Efficiency

The photochemical efficiency of barley plants studied in this experiment was measured using an OS-1 Fluorometer (Opti-Sciences Inc., Tyngsboro, MA) 24 hours prior to harvesting

samples. Measurements were made in the dark-adapted mode and the photochemical efficiency, i.e., the ratio of variable fluorescence to maximal fluorescence (F_v/F_m) was calculated.

9.3.8. Sample Extraction for the Determination of Total Soluble Phenolic Content and Antioxidant Activity

Frozen barley leaf tissue (100 mg FW) was transferred to glass vials containing 95% ethanol (5 mL) and stored at -10°C for 48 h. After this, samples were homogenized using tissue tearor (BioSpec Products Inc., Bartlesville, OK) and the homogenate was centrifuged (13,000 rpm: 5 min). The supernatant was used to determine the total soluble phenolic content and antioxidant activity of the leaf samples *in vitro*.

9.3.9. Total Soluble Phenolic (TSP) Content

The total soluble phenolic content in the ethanol extracts of barley leaf tissue was measured using the Folin-Ciocalteu (FC) method, modified by Shetty *et al*. (1995). The extract (1 mL) was combined with 95% ethanol (1 mL) and distilled water (5 mL) in a test tube. To this mixture, FC reagent (0.5 mL; 50% v/ v) and Na_2CO_3 (1 mL; 5% v/v) were added, mixed thoroughly with a vortex shaker, and incubated in the dark for 60 min. Absorbance of the resultant solutions were measured spectrophotometrically at 725nm (Genesys UV-visible, Milton Roy Inc., Rochester, NY). A standard curve was prepared using various concentrations of gallic acid (10 – 300 μg/mL) in 95% ethanol. Absorbance values were converted to total soluble phenolic concentration and expressed as milligram equivalents of gallic acid equivalent per gram FW of barley leaf sample.

9.3.10. ABTS Free Radical Scavenging Capacity Assay

Antioxidant activity was measured using the ABTS [2,2′ –azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay (Pellegrini *et al*.,

2002). A stock solution containing ABTS radical cations was prepared by mixing 7 mM ABTS solution (5 mL) with 140 mM K_2S_2O4 solution (88 mL). This mixture was stored in foil wrapped container, in the dark at 4°C for 12–16 h before use. Prior to the performing the assay, the stock solution was diluted with 95% ethanol, to prepare a working ABTS solution with an absorbance of 0.70 ± 0.02 units at 734 nm. The ABTS working solution (1 mL) was added to the ethanol extract of barley leaf tissue (50 µL) in a plastic vial and mixed thoroughly. The mixture was incubated at room temperature for 2.5 min and absorbance was measured at 734 nm. The antioxidant activity of the extracts was expressed as percentage (%) inhibition of ABTS radical formation and was calculated per the following formula:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

9.3.11. Sample Extraction for the Determination of Enzyme Activities *In Vitro*

At each time point, 200 mg (fresh weight; FW) of barley leaf tissue was excised, flash frozen in liquid nitrogen, and stored at -80˚C for biochemical analysis. Prior to conducting the biochemical assays, leaf samples were macerated using a chilled pestle and mortar in an ice bath, with 2 mL of pre-chilled enzyme extraction buffer (0.5% polyvinylpyrrolidone (PVP), 3 mM EDTA, and 0.1 M potassium phosphate buffer; pH 7.5). Tissue homogenates was centrifuged at 13,500 rpm for 10 min and immediately transferred to an ice bath. The supernatant was used for further biochemical analysis.

9.3.12. Total Protein Assay

The Bradford protein binding assay was used to measure the amount of total protein present in the sample extracts (Bradford, 1976). To 50 µL of extract, 5 mL of dye reagent (diluted with water in the ration 1:4) was added. The mixture was thoroughly mixed on a vortex

shaker, incubated in a dark cabinet for 5 min and its absorbance was measured at 595 nm, against a blank (50 µL distilled water in 5 mL dye reagent).

A standard curve was prepared using standard solutions of bovine serum albumin, dissolved in distilled water, based on which the protein content of the sample extracts was calculated. These values were used in subsequent calculations to determine the activities of various enzymes involved in ROS mitigation mechanisms and the PAPPP regulation.

9.3.13. Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

A modified method originally described by Deutsch (1983) was used. The enzyme reaction mixture containing 5.88 µmol ß-NADP, 88.5 µmol MgCl2, 53.7 µmol glucose-6-phosphate, and 0.77 mmol maleimide was prepared. This solution was used to set the baseline (zero) of the spectrophotometer at 340 nm. Barley leaf extract (100 µL) was added to the reaction mixture (1 mL) and the shift in absorbance over 5 min was used to quantify G6PDH activity in the samples based on the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}).

9.3.14. Succinate Dehydrogenase (SDH) Assay

To assay the activity of succinate dehydrogenase a modified method described by Bregman (1987) was used. A reaction mixture containing 0.4 M potassium phosphate buffer (1.0 mL; pH 7.2), 0.15 M sodium succinate (40 µL; pH 7.0), 0.2 M sodium azide (40 µL), and 2,6-dichlorophenolindophenol (DCPIP; 10 µL; 6.0 mg/mL) was prepared. The baseline of the spectrophotometer at 600 nm was established using the enzyme extraction buffer solution. Barley leaf extract (0.2 mL) was added to the reaction mixture (1 mL) and the rate of change of absorbance per min was measured to quantify SDH activity in the sample, based on the extinction coefficient of DCPIP (19.1 mM^{-1} cm^{-1}).

9.3.15. Guaiacol Peroxidase (GPX) assay

A modified method described by Laloue *et al*. (1997) was used to assay the activity of guaiacol peroxidase. The enzyme reaction mixture containing 0.1 M potassium phosphate buffer (pH 6.8), 56 mM guaiacol solution, and 50 mM hydrogen peroxide was used. Barley leaf extract (10 µL) was combined with the reaction mixture (990 µL) and the shift in absorbance over 3 min was measured. The rate of change in absorbance per min was used to quantify the enzyme in the mixture, based on the extinction coefficient of the oxidized product tetraguaiacol (26.6 mM^{-1} cm^{-1}).

9.3.16. Superoxide Dismutase (SOD) Assay

The activity of SOD was measured by monitoring the reduction of nitroblue tetrazolium (NBT) by barley leaf extracts at 560 nm (Oberley and Spitz, 1984). The reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33 mM diethylenetetraaminepentaacetic acid (DETEPAC); 2.45 mM NBT (0.5 mL); 1.8 mm xanthine (1.7 mL) and catalase (40 IU/mL). Phosphate buffer (100 µL) and xanthine oxidase (100 µL) were added to 0.8 mL of reagent mixture. The change in absorbance at 560 nm was measured over 1 min and the concentration of xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.024 - 0.026 units of absorbance per min. Further to this, phosphate buffer was replaced with barley leaf extracts and the shift in absorbance over 1 min was calculated. One unit of SOD activity was defined as the amount of protein that inhibited NBT reduction to 50% of the maximum.

9.3.17. Catalase (CAT) Assay

A method originally described by Beers and Sizer (1952) was used to assay the activity of catalase. A reaction mixture was prepared by combining distilled water and 0.059 M hydrogen

peroxide (Merck's Superoxol or equivalent grade, Merck Co. & Inc., Whitehouse Station, NJ) in 0.05 M potassium phosphate buffer solution (pH 7.0) was added. This mixture was incubated in a spectrophotometer for 4–5 min to achieve temperature equilibration and to establish a baseline. Further to this, barley leaf extract (0.1 mL) was added to the reaction mixture and the disappearance of peroxide due to catalase activity was monitored by measuring the shift in absorbance at 240 nm in 1 min. The change in absorbance from the initial linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2.

9.3.18. Proline Dehydrogenase (PDH) Assay

A modified method described by Costilow and Cooper (1978) was carried out to assay the activity of proline dehydrogenase. The enzyme reaction mixture containing 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline solution and 10 mM NAD was used. Barley leaf extract (200 µL) was added the reaction mixture (1 mL) and the increase in absorbance was measured at 340 nm over an interval of 3 min at 32°C was measured. One unit of PDH activity was defined as amount required to cause a shift of 0.01 absorbance units per min at 340 nm (1.0 cm light path).

9.3.19. Determination of Proline Content using HPLC

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1100 liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector (DAD 1100). The analytical column was a reverse phase Nucleosil C18, 250 nm 4.6 mm with a packing material of 5 µm particle size. The samples were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 mL/min and detected at 210 nm. A known quantity of L-

Proline dissolved in 20 mM potassium phosphate solution was used to calibrate the standard curve. The amount of proline in the sample was reported as milligram of proline per gram FW.

9.3.20. Determination of Yield Parameters

Mature barley spikes were harvested, threshed, and cleaned by hand. Yield parameters such as the total number of spikes per plant and number of grains per spike were determined. Additionally, the 1000 grain weight was calculated based on the mean weight of three 100-grain samples. The grain yield per plant for each treatment × cultivar combination across all levels of waterlogging was calculated per the formula:

$$Grain\ yield = \frac{100\ grain\ weight \times Total\ number\ of\ spikes\ per\ plant \times Number\ of\ grains\ per\ spike}{100}$$

9.3.21. Experimental Design and Statistical Analysis

The experiment was carried out according to 3-way factorial arrangement with a randomized complete block design (RCBD) , with barley types, waterlogging stress treatments, and elicitation treatment considered as fixed effects. Each pot was considered as an experimental unit, and three samples (biological replicate) were assayed per barley type × waterlogging level × elicitor treatment combination, and the entire experiment was repeated thrice and average for each replicate were taken for statistical analysis. Analysis of variance (ANOVA) was performed using SAS software (version 9.4; SAS Institute, Cary, NC).

Statistical differences among all mean separation for the main factors and their corresponding 2-way interactions, and one 3-way interaction were determined using Tukey's least mean square test at a confidence level of 95%.

9.4. Results and Discussion

9.4.1. Visual Observations, Physical Parameters and Photochemical Efficiency

Barley is best suited to grow in cool and dry areas with well-drained soils. It performs poorly when excessive moisture is present and barley seedlings are particularly vulnerable to physiological stress induced by waterlogged soil conditions (SARE, 2007). In the current study, plants of both barley types (Celebration and Pinnacle) grown under anoxic condition for 2 weeks exhibited stunted growth and reduced number of tillers. However, the effect of waterlogging stress on physical growth parameters was more severe on barley plants of cv. Celebration when compared to cv. Pinnacle (Table 9.1 A, B, C, & D). Additionally, barley plants under same waterlogging stress (anoxic) condition had chlorosis in the leaf tissues beginning around 30 days after seed treatment and remained chlorotic until maturity. Overall, photochemical efficiency (F_v/F_m) of barley seedlings reduced significantly under waterlogging stress (anoxic and hypoxic), which affected the recovery of barley plants even after removal of the stress (Table 9.1 A).

Previously, Xiao *et al*. (2005) reported reduced net photosynthetic rate and stomatal conductance in barley plants under waterlogging stress. Similarly, Luan *et al*. (2018) observed greater reduction in photosynthetic performance and total biomass in waterlogging sensitive barley genotype (TF57) under hypoxic condition. In the current study, the impact of barley type, waterlogging stress treatments, and seed elicitation treatments on photochemical efficiency of barley plants were statistically significant ($p \leq 0.05$) at all growth stages, while the effect of 3-way interactions between barley type × waterlogging level × elicitor treatment combination on photochemical efficiency of barley was statistically significant at day 50 and 65 (Table 9.2). The reduced photochemical efficiency under anoxic condition affected the amount of available photosynthates, which were essential for regular metabolic functions and subsequent recovery of

barley plants during vegetative and reproductive growth stages. However, significant ($p \leq 0.05$) improvement of photochemical efficiency of barley plants was observed with GP seed elicitor treatment irrespective of soil water level. Such improvement of photochemical efficiency of barley seedlings with GP seed elicitor treatment under anoxic soil condition remained at higher level even during late reproductive stages (50 & 65 days after seed elicitation treatment). Furthermore, barley plants with COS seed elicitor treatment also had higher photochemical efficiency at day 65 under anoxic soil condition. Therefore, this result indicated that seed priming treatments (GP & COS) not only just improved photosynthetic activity of barley seedlings, but also supported the recovery, reproductive growth, and overall productivity of barley plants under waterlogging stress (hypoxic and anoxic).

Like, photochemical efficiency, shoot and root biomass (shoot weight and root weight) of barley plants also reduced significantly ($p \leq 0.05$) under anoxic soil condition (Table 9.1 B & C: Table 9.2). Even after removal of waterlogging stress, it remained significantly lower when compared to the barley plants grown under field capacity. Interestingly, plants of both barley types exposed to hypoxic root condition had no significant impact on shoot and root biomass and were statistically at par with barley plants grown under normal moisture condition (field capacity).

Table 9.1. Photochemical efficiency (F_v/F_m) (A), shoot fresh weight (g) (B), root fresh weight (g) (C) and plant height (cm) (D) of two barley types (Pin, cv. Pinnacle, Cel, cv. Celebration) at 20, 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate), and grown under three waterlogging levels.

(A)

Waterlogging level	Treatment	Day 20		Day 35		Day 50		Day 65	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	0.709	0.668	0.676	0.638	0.673	0.592	0.600	0.556
	COS	0.759	0.719	0.706	0.642	0.640	0.623	0.611	0.562
	GP	0.772	0.731	0.726	0.677	0.712	0.644	0.637	0.579
Hypoxic	Control	0.704	0.694	0.635	0.602	0.631	0.577	0.589	0.474
	COS	0.707	0.671	0.666	0.594	0.674	0.571	0.584	0.534
	GP	0.715	0.680	0.704	0.595	0.672	0.566	0.601	0.504
Anoxic	Control	0.627	0.643	0.517	0.495	0.462	0.474	0.444	0.432
	COS	0.671	0.644	0.512	0.493	0.517	0.488	0.456	0.462
	GP	0.691	0.639	0.540	0.508	0.529	0.509	0.464	0.480

(B)

Waterlogging level	Treatment	Day 20		Day 35		Day 50		Day 65	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	2.84	2.48	4.81	3.48	6.89	5.84	8.48	8.04
	COS	3.13	2.50	5.21	3.58	7.06	6.42	9.36	7.87
	GP	3.20	3.27	5.01	3.47	8.82	6.46	9.55	8.56
Hypoxic	Control	2.55	2.29	5.20	3.52	8.03	5.62	8.45	7.02
	COS	3.14	2.46	5.71	3.60	7.34	5.64	8.65	7.58
	GP	3.35	3.07	5.79	4.31	8.87	7.18	9.35	8.10
Anoxic	Control	2.91	1.62	3.37	2.07	4.08	2.41	5.11	2.88
	COS	3.35	2.37	3.15	2.21	4.36	2.98	5.40	4.37
	GP	2.78	1.93	3.57	2.27	4.55	2.84	4.92	3.63

Table 9.1. Photochemical efficiency (F_v/F_m) (A) shoot fresh weight (g) (B), root fresh weight (g) (C) and plant height (cm) (D) of two barley types (Pin, cv. Pinnacle, Cel, cv. Celebration) at 20, 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate), and grown under three waterlogging levels (continued).

(C)

Waterlogging level	Treatment	Day 20		Day 35		Day 50		Day 65	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	2.95	2.94	4.94	3.61	7.02	5.97	8.61	8.17
	COS	3.25	2.97	5.33	3.70	7.18	6.55	9.49	8.00
	GP	3.32	3.39	5.14	2.60	8.94	6.59	9.68	8.69
Hypoxic	Control	3.03	2.76	5.61	3.97	8.48	6.07	8.88	7.48
	COS	3.60	2.91	6.14	4.04	7.79	6.03	9.13	8.02
	GP	3.78	3.51	6.29	4.76	9.29	7.63	9.80	8.52
Anoxic	Control	3.00	3.03	3.44	2.15	4.15	2.49	5.20	2.97
	COS	3.43	3.11	3.22	2.29	4.45	3.05	5.47	4.44
	GP	2.86	3.00	3.65	2.35	4.63	2.92	5.00	3.70

(D)

Waterlogging level	Treatment	Day 20		Day 35		Day 50		Day 65	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	19.3	18.0	30.7	22.2	46.1	38.8	59.9	50.7
	COS	20.9	18.6	32.9	23.1	50.0	40.8	61.6	52.4
	GP	22.8	19.5	34.0	20.1	52.8	42.6	64.4	55.2
Hypoxic	Control	18.2	16.5	32.3	23.2	48.1	38.9	55.4	45.6
	COS	19.8	18.4	35.5	24.7	51.5	42.3	60.1	49.9
	GP	20.3	19.3	36.2	26.5	53.3	43.3	59.4	48.0
Anoxic	Control	19.3	18.7	22.3	12.7	26.6	16.3	31.4	22.5
	COS	21.6	17.9	22.5	13.9	27.9	20.2	35.7	26.5
	GP	20.0	19.7	23.9	14.7	29.0	20.4	33.6	24.4

Table 9.2. Analysis of variance table indicating mean square (MS) and significant differences between barley type (C), waterlogging levels (W) and seed elicitation treatments (T), with their respective interactions for physical and biochemical parameters associated with the pentose phosphate pathway (PPP) mediated secondary metabolite biosynthesis and antioxidant enzyme responses in two barley cultivars at days 20 (A), 35 (B), 50 (C) and 65 (D) after sowing.

(A)

Source	df	Shoot Fresh Weight	Root Fresh Weight	Plant height	†PSE	G6PDH	SDH	PDH	CAT	GPX	SOD	TSP	ABTS
C	1	10.4*	13.16**	64.6*	0.0228**	149.3ns‡	0.57*	11.8ns	340.9ns	3497.6*	0.00403*	8.42*	6110.5*
W	2	3.79*	2.50ns	43.9*	0.0922*	3561.0*	0.73*	537.7*	4153.4**	3221.3*	0.00375*	0.04**	57.2*
C×W	2	3.28*	1.31ns	1.83ns	0.0001ns	553.3*	0.30*	13.6ns	958.5ns	439.2**	0.00117*	0.28*	237.5*
T	2	4.29*	4.37ns	67.7*	0.0163*	276.8**	0.45*	50.2*	3849.9**	2863.5*	0.01076*	1.87*	327.8*
C×T	2	0.28ns	0.28ns	0.75ns	0.0037ns	317.7**	0.07**	184.8*	1298.1ns	90.2ns	0.00041ns	0.24*	94.1*
W×T	4	1.04*	1.53ns	8.6*	0.0093**	172.3**	0.22*	8.8ns	1873.7**	1166.4*	0.00383*	0.15*	172.2*
C×W×T	4	0.24ns	1.32ns	23.3*	0.0036ns	157.4**	0.08**	77.2*	2363.1**	138.6ns	0.00017ns	0.47*	5.04ns

(B)

Source	df	Shoot Fresh Weight	Root Fresh Weight	Plant height	PSE	G6PDH	SDH	PDH	CAT	GPX	SOD	TSP	ABTS
C	1	70.5*	82.3*	3070.7*	0.0627*	5516.6*	0.00ns	1.21ns	61661.0*	1940.5**	0.00004ns	21.7*	2971.1*
W	2	60.3*	74.5*	2187.5*	0.4591*	2142.1*	0.31*	470.0*	9562.7*	26013.0*	0.00006ns	0.07**	36.2**
C×W	2	2.03*	1.21ns	26.9*	0.0104**	50.80ns	0.06**	0.71ns	565.5ns	2215.6**	0.00032	0.88*	73.8*
T	2	2.52*	2.26**	152.8*	0.0142**	695.7*	0.11**	31.3*	6332.0*	39.6ns	0.00168**	1.26*	72.1*
C×T	2	0.56ns	1.75ns	80.3*	0.0061ns	1.68ns	0.01ns	3.80ns	5631.2*	1741.7**	0.00069ns	0.21*	29.1**
W×T	4	2.50*	4.7*	95.6*	0.0049ns	285.4*	0.01ns	4.50ns	18441.0*	1167.3**	0.00040ns	0.35*	80.4*
C×W×T	4	1.22**	2.56**	90.6*	0.0034ns	148.2**	0.04**	1.90ns	1729.0**	907.3**	0.00075ns	0.49*	22.1**

*Denotes significance at $p < 0.05$; **Denotes significance at $p < 0.001$; † PSE, photosynthetic efficiency; G6PDH, glucose-6-dehydrogenase; SDH, succinate dehydrogenase; PDH, proline dehydrogenase; CAT, catalase; GPX, guaiacol peroxidase; SOD, superoxide dismutase; ‡ns, non-significant

Table 9.2. Analysis of variance table indicating mean square (MS) and significant differences between barley type (C), waterlogging levels (W) and seed elicitation treatments (T), with their respective interactions for physical and biochemical parameters associated with the pentose phosphate pathway (PPP) mediated secondary metabolite biosynthesis and antioxidant enzyme responses in two barley cultivars at days 20 (A), 35 (B), 50 (C) and 65 (D) after sowing (continued).

(C)

Source	df	Shoot Fresh Weight	Root Fresh Weight	Plant height	PSE	G6PDH	SDH	PDH	CAT	GPX	SOD	TSP	ABTS
C	1	74.6*	74.8*	2447.7*	0.0712*	4177.4*	0.09**	126.6*	18076.0*	19184.0*	0.00225**	2.26*	308.0*
W	2	243.6*	271.4*	9800.8*	0.3942*	1773.5*	0.50*	1186.3*	11425.0*	7451.7*	0.00156**	3.62*	378.8*
C×W	2	7.02*	7.13**	83.3*	0.0219*	370.5**	0.11*	14.9**	566.2ns	6607.1*	0.00002ns	1.26*	122.5*
T	2	12.9*	12.8*	407.6*	0.0220*	1218.7*	0.06**	137.6*	2877.0*	400.5ns	0.00205**	0.86*	113.0*
C×T	2	8.64*	8.28**	95.5*	0.0066ns	793.6*	0.00ns	20.9*	58.8ns	505.1**	0.00095ns	0.01ns	3.95ns
W×T	4	6.25*	6.31**	62.8*	0.0019ns	496.9*	0.00ns	54.0*	6046.9*	1677.9*	0.00003ns	0.06**	3.12ns
C×W×T	4	6.11*	6.30**	62.1*	0.0160*	119.7ns	0.01ns	5.25ns	839.1ns	627.0**	0.00130**	0.09*	22.7*

(D)

Source	df	Shoot Fresh Weight	Root Fresh Weight	Plant height	PSE	G6PDH	SDH	PDH	CAT	GPX	SOD	TSP	ABTS
C	1	45.5*	46.2*	2765.2*	0.0712*	50.1ns	0.21**	5.75ns	40784.0*	8333.3*	0.00281*	0.92*	117.6*
W	2	320.7*	348.2*	13883.0*	0.3942*	1882.3*8	0.32*	424.6*	6338.4**	11669.0*	0.00063ns	4.38*	484.2*
C×W	2	4.45*	4.54ns	102.0*	0.0219*	351.8**	0.04ns	44.3*	12051.0*	1470.6*	0.00005ns	0.47*	30.0**
T	2	10.4*	10.4**	403.9*	0.0220*	394.7**	0.05ns	0.35ns	7280.9*	170.0ns	0.00096**	0.96*	94.2*
C×T	2	1.96**	1.86ns	77.7*	0.0066ns	802.8*	0.58*	7.15ns	8471.6*	214.0ns	0.00138**	0.07*	5.33ns
W×T	4	2.21**	2.13ns	18.2*	0.0019ns	321.5*	0.06**	8.95ns	13756.0*	789.9*	0.00008ns	0.59ns	64.5*
C×W×T	4	0.79ns	0.81ns	71.1*	0.0160*	140.0ns	0.06ns	7.16ns	4793.32*	420.09**	0.00054**	0.63*	74.34*

*Denotes significance at $p < 0.05$; **Denotes significance at $p < 0.001$; † PSE, photosynthetic efficiency; G6PDH, glucose-6-dehydrogenase; SDH, succinate dehydrogenase; PDH, proline dehydrogenase; CAT, catalase; GPX, guaiacol peroxidase; SOD, superoxide dismutase; ‡ns, nonsignificant

Therefore, mild waterlogging stress (hypoxic) did not have deleterious effects on biomass and height of barley plants (Table 9.2 B, C, & D). In a previous study, Pang *et al*. (2004) reported adverse effect of waterlogging stress on shoot and root weight of 6 barley genotypes. They also suggested that susceptibility of barley plants to waterlogging stress might depend on the pattern of aerenchyma tissue formation in barley roots, which influence the gaseous exchange between shoots and roots. In the context of root development, Luan *et al*. (2018) reported higher number of adventitious roots in waterlogging stress tolerant genotypes of barley. In the current study, we also observed decaying of adventitious roots of barley plants, particularly for cv. Celebration at day 35 under waterlogging stress. However, significant improvement ($p \leq 0.05$) of shoot and root biomass and plant height of barley plants were observed with GP and COS seed elicitation treatments, especially under high waterlogging stress (anoxic). Therefore, the results of this study suggested that bioelicitation strategy with GP and COS seed treatments had positive impacts on shoot and root development of barley plants under high waterlogging stress. Such enhanced growth of barley seedling with seed elicitation also supported recovery and improved reproductive growth of barley plants after removal of waterlogging stress. However, wider field-based experiments with multiple crop year and locations are required to prove the concept and for effective integration of seed elicitation strategy to improve fitness waterlogging stress resiliency and productivity of barley.

9.4.2. Total Soluble Phenolic Content and Total Antioxidant Activity

Stimulation of endogenous defense responses, such as enhanced biosynthesis of secondary metabolites (phenolics) is a natural protective response of plants to counter abiotic stress-induced physiological and metabolic breakdowns (Sharma *et al*. 2019; Shetty and Wahlqvist 2004). The stress protective role of plant phenolics is not only just restricted to their

antioxidant activity and maintaining cellular redox homeostasis, but also involves structural and physiological adjustments of shoot and root tissues that are essential for improving abiotic stress resilience (Akula and Ravishankar, 2011). In the current study, statistically significant ($p \leq 0.05$) effects of 3-way interactions between barley type × waterlogging level × elicitor treatment combination on phenolic content of barley shoot sample were observed (Figure 9.1 & Table 9.2).

Overall, phenolic content of cv. Pinnacle was significantly higher under anoxic soil condition when compared to plants of same barley type grown under hypoxic and field capacity (Figure 9.1). This result indicated that higher phenolic content of cv. Pinnacle, specifically during stress recovery and reproductive stages might have relevance for their higher waterlogging stress resilience and overall fitness. Previously, seed treatment with exogenous application of phenolic compounds (dihydroquercetin) showed improvement of root growth of barley seedling under soil flooding (Balakhnina *et al*., 2008). Similarly, exogenous application of protocatechuic acid and vanillic acid enhanced root growth, chlorophyll content, endogenous phenolic content, expression of genes encoding antioxidant enzymes and improved survival of rice seedling under water submergence (Xuan and Khang, 2018).

In the current study, the enhanced phenolic content was positively correlated with improved photosynthetic activity, root and shoot growth of barley plants under anoxic soil condition. Due to the dual benefits of phenolic for improving abiotic stress resilience and human health relevant end use qualities, selecting barley type cv. Pinnacle, and using mild abiotic stress induction might be an effective strategy to address climate change-linked food and nutritional insecurity challenges. Additionally, GP seed elicitor treatment enhanced phenolic content of barley leaves under waterlogging stress, and it was statistically significant ($p \leq 0.05$) when compared to control (water) and COS seed elicitation treatment. Therefore, the improvement of

waterlogging stress resiliency with GP seed elicitor treatment might be due to the enhanced level of phenolics and associated protective functions, such as enhanced antioxidative activity, structural tissue support from phenolic-linked lignification, and stimulation of endogenous antioxidant enzyme responses through phenylpropanoid pathway regulation.

The antioxidant defense response of plants is critical to counter abiotic stress induced oxidative breakdowns and associated cellular damages. In this study, antioxidant activity of barley shoot samples was analyzed during and after removal of waterlogging stress using ABTS-based free radical scavenging assay. Like phenolic content, 3-way interactions between barley type × waterlogging level × elicitor treatment combinations had statistically significant effect on total antioxidant activity of barley at day 35, 50 and 65. (Figure 9.2). After removal of waterlogging stress (at day 50 & day 65), higher antioxidant activity was observed in barley plants which were grown under anoxic soil condition for 2 weeks. Therefore, this result indicated that high antioxidant activity of barley leaves might be part of the overall protective defense response, especially to counter waterlogging stress-induced oxidative breakdowns of cells. The improvement of antioxidant activity with GP seed elicitor treatment was also observed in barley plants grown under hypoxic and anoxic soil conditions, with only exception of cv. Pinnacle under hypoxic condition at day 65.

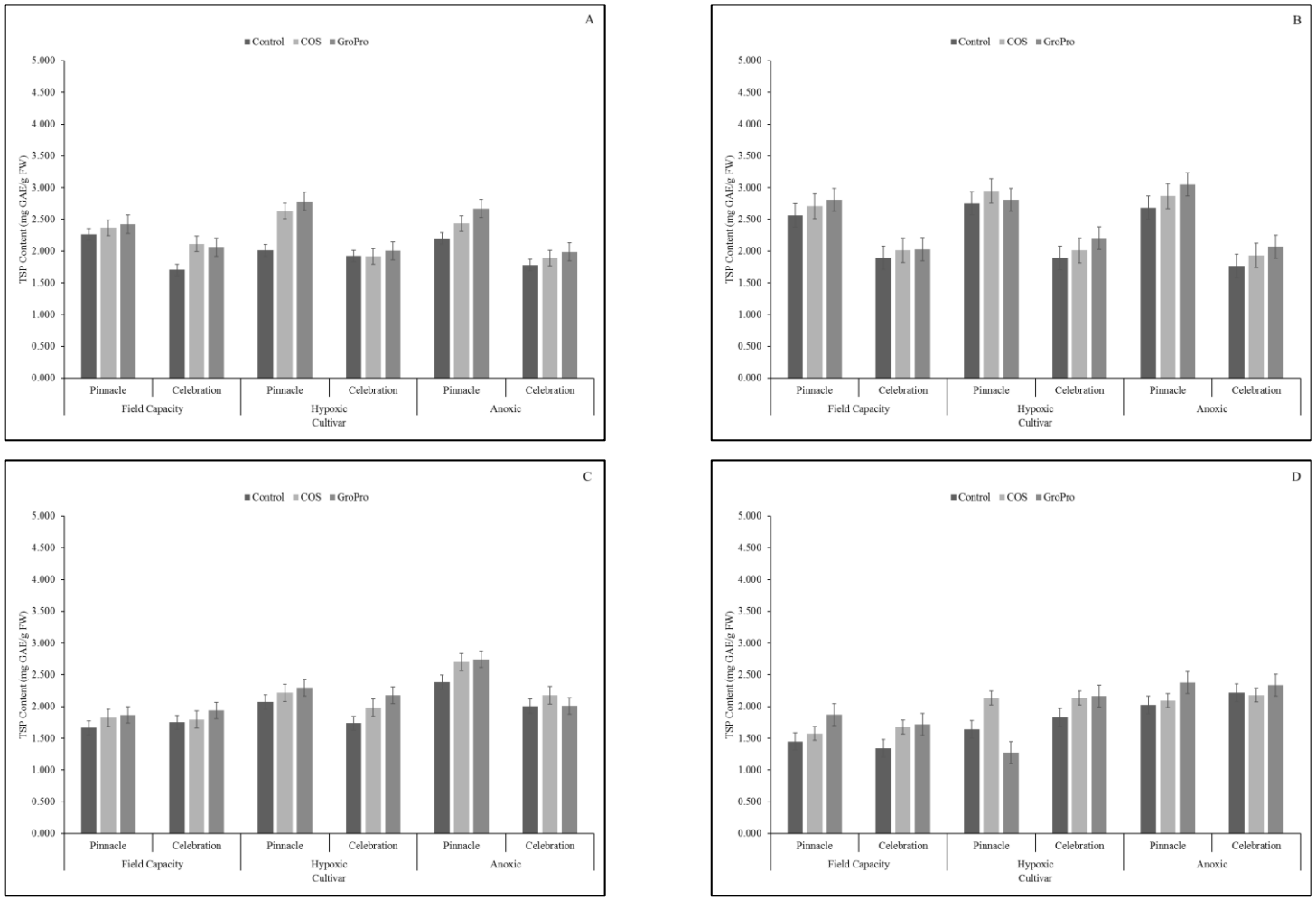

Figure 9.1. Total soluble phenolic content (mg GAE/g F.W.) of barley leaves (Pinnacle and Celebration) with two bioprocessed seed elicitor treatments (COS and GroPro) along with no seed treatment (control) and under three levels of soil moisture conditions (field capacity, hypoxic, and anoxic) at 20 (A), 35 (B), 50 (C), and 65 (D) days after emergence.

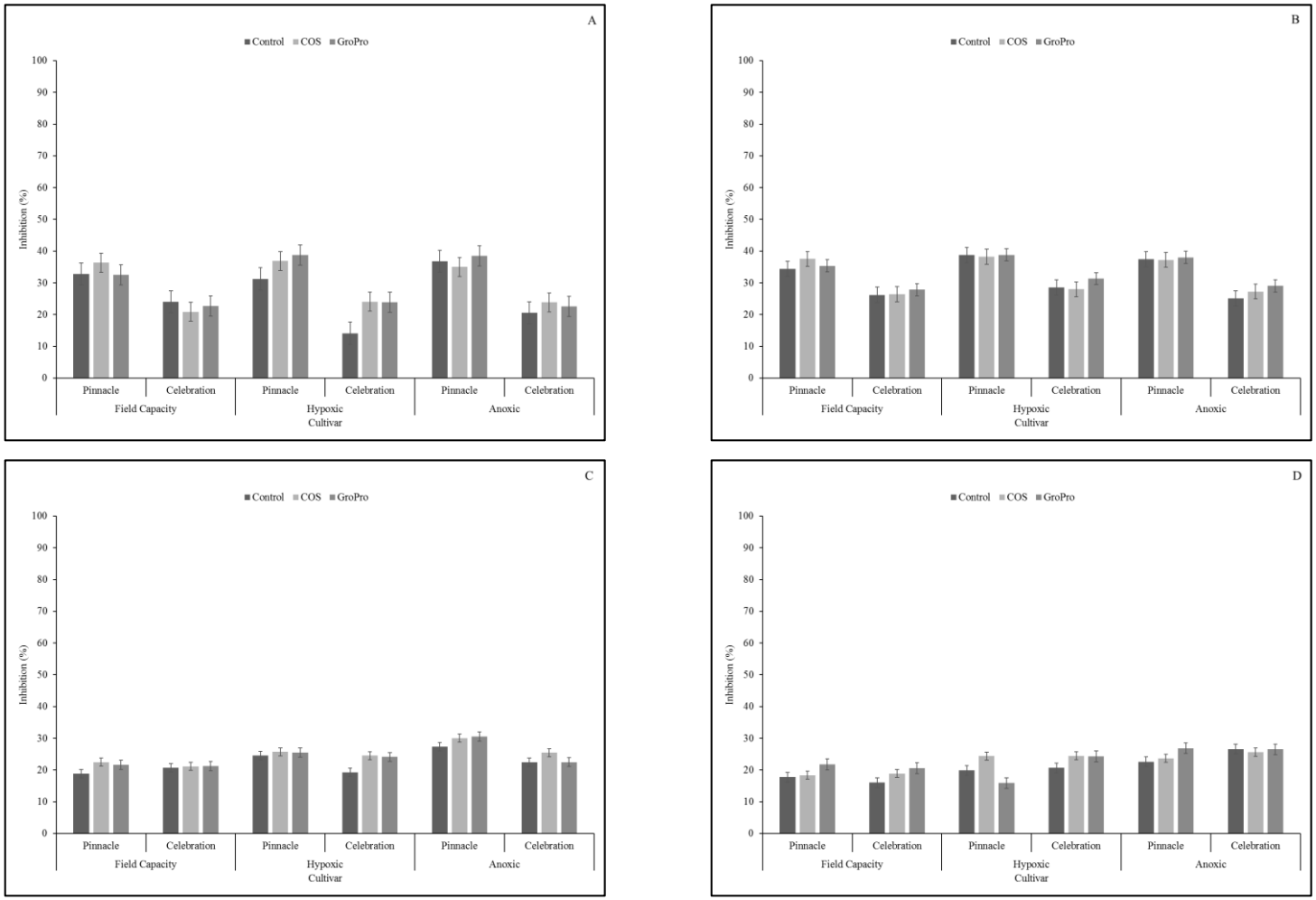

Figure 9.2. Total antioxidant activity (ABTS-based % Inhibition) of barley leaves (Pinnacle and Celebration) with two bioprocessed seed elicitor treatments (COS and GroPro) along with no seed treatment (control) and under three levels of soil moisture conditions (field capacity, hypoxic, and anoxic) at 20 (A), 35 (B), 50 (C), and 65 (D) days after emergence.

Therefore, targeting GP seed elicitation treatment to improve antioxidant defense response and waterlogging stress resiliency has significant merit. Previously, Xiao *et al*. (2005) reported high antioxidant activity with enhanced malondialdehyde (MDA) content in barley plants under waterlogging stress. In another study, Gill *et al* (2019) found excessive accumulation of reactive oxygen species (ROS) in barley plants under waterlogged soils. The oxygen deficiency under partial anaerobic condition of waterlogged soil can lead to oxygen-linked metabolic malfunction and accumulation of superoxide anion and hydrogen peroxide, which cause excessive oxidative stress. Therefore, improved antioxidant activity is essential to counter waterlogging stress-induced generation of ROS and associated oxidative breakdowns of cells and cellular organelles. In this context, bioelicitaton with natural elicitor such as GP seed treatment is and inexpensive and effective tool to stimulate phenolic biosynthesis and associated antioxidant activity in barley to mitigate waterlogging stress-induced damages and for improving overall abiotic stress resiliency. However, it is also important to understand the role of redox-linked metabolic pathway regulation of plants associated with phenolic biosynthesis and antioxidant enzyme response under abiotic stress condition such as waterlogging stress.

9.4.3. Glucose-6-Phosphate Dehydrogenase (G6PDH), Succinate Dehydrogenase (SDH) and Proline Dehydrogenase (PDH) Activities

As part of physiological and biochemical adaptation to abiotic stress-induced oxidative stress, shift in primary carbon metabolism such as driving carbon flux towards redox-linked pentose phosphate pathway (PPP) is a protective cellular function, which potentially dictates susceptibility and tolerance of genotypes/cultivars/varieties to diverse abiotic stresses (Shetty, 2004; Kruger *et al*., 2011; Lu *et al*. 2016). Therefore, up-regulation of PPP and associated anabolic responses, including stimulation of secondary metabolite biosynthesis and potential

structural adjustments in roots and shoots by diverting primary carbon metabolic flow for stress protection is an effective strategy to improve abiotic resilience of food plants. Based on this rationale, activity of G6PDH, a key enzyme responsible for the first rate-limiting step of PPP of barley leaves was determined following seed elicitor treatments. Overall, statistically significant effect of 3 way interactions between barley type × waterlogging level × elicitor treatment combinations on G6PDH activity of barley leaves was observed at day 20 and 35, while 2 way interactions between barley type × waterlogging level and waterlogging level × elicitor treatment had statistically significant effect at day 20, 50 and 65. Additionally, G6PDH enzyme activity increased significantly from day 20 to day 35 irrespective of the soil water level and seed elicitor treatments (Figure 9.3). After removal of the waterlogging stress (at day 35 & 50), higher G6PDH activity, which was statistically significant ($p \leq 0.05$) was observed in leaves of cv. Pinnacle when compared to cv. Celebration. The results of high G6PDH activity of cv. Pinnacle also positively correlated with phenolic content, photosynthetic activity and high root and shoot weight in respective growth stages of barley plants. Therefore, these results indicated the critical role of PPP for diverse anabolic responses supporting metabolic and tissue structural adjustments of barley plants under waterlogging stress. In a previous study, enhanced PPP flux, along with high photosynthetic activity, and effective anaerobic respiration were observed in a mangrove species (*Kandelia candel* L.) under flooding stress (Pan *et al*., 2018).

Additionally, increased G6PDH activity ($p \leq 0.05$) was also observed in barley plants following GP seed elicitor treatment (Figure 9.3). The higher G6PDH activity of barley leaves were more evident under hypoxic and anoxic soil conditions. This result indicated a positive impact of GP seed elicitor treatment for up-regulation of redox-linked PPP and stimulation of associated anabolic responses in barley under waterlogging stress. Such bioelicitation-induced

upregulation of PPP, especially in cv. Pinnacle have scientific relevance and such seed elicitation-based strategy can be targeted to improve biochemical and physiological adaptation of barley to waterlogging stress.

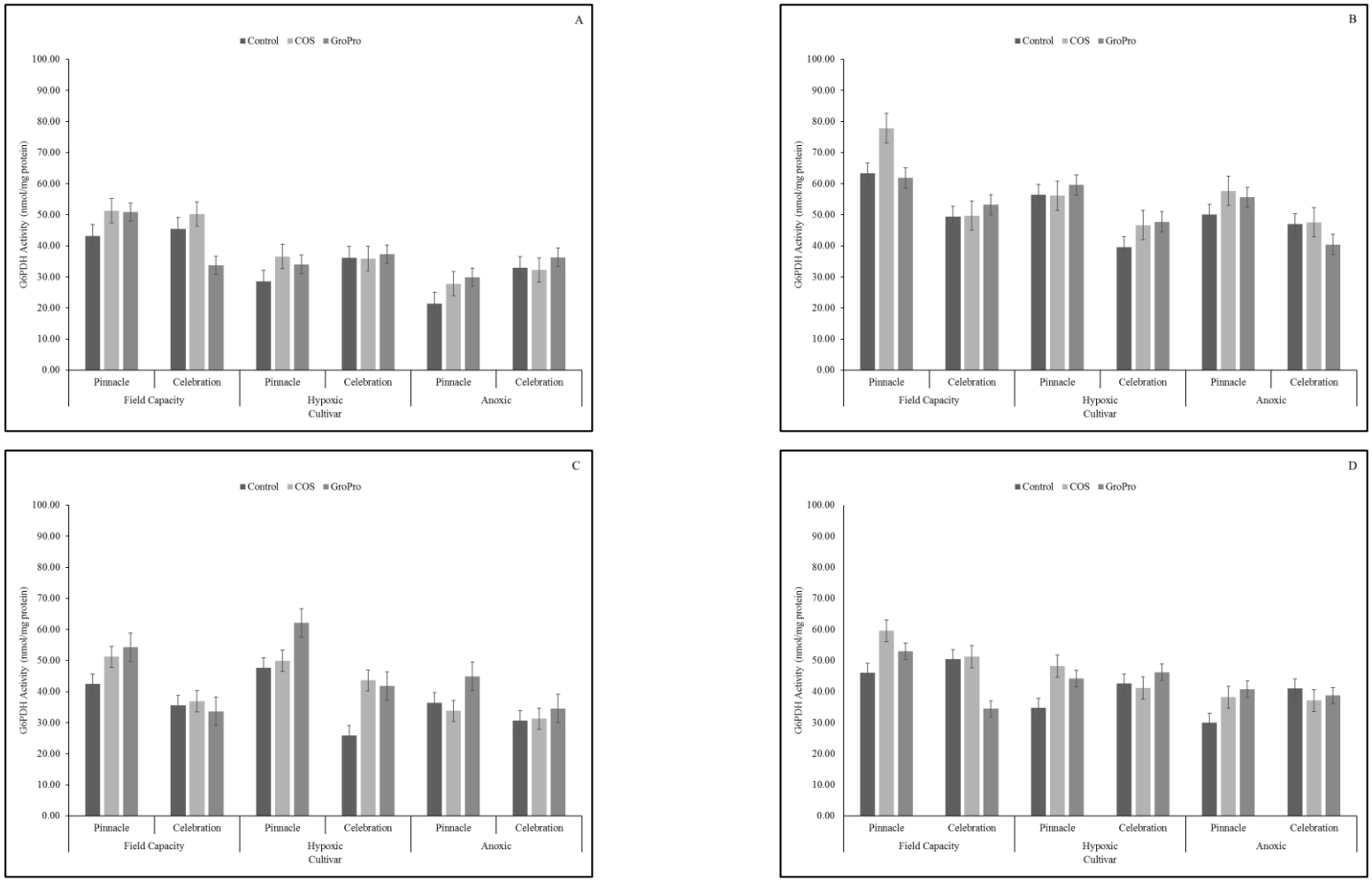

Figure 9.3. Glucose-6-phosphate dehydrogenase (G6PDH) activity (nmol/mg of protein) of barley leaves (Pinnacle and Celebration) with two bioprocessed seed elicitor treatments (COS and GroPro) along with no seed treatment (control) and under three levels of soil moisture conditions (field capacity, hypoxic, and anoxic) at 20 (A), 35 (B), 50 (C), and 65 (D) days after emergence.

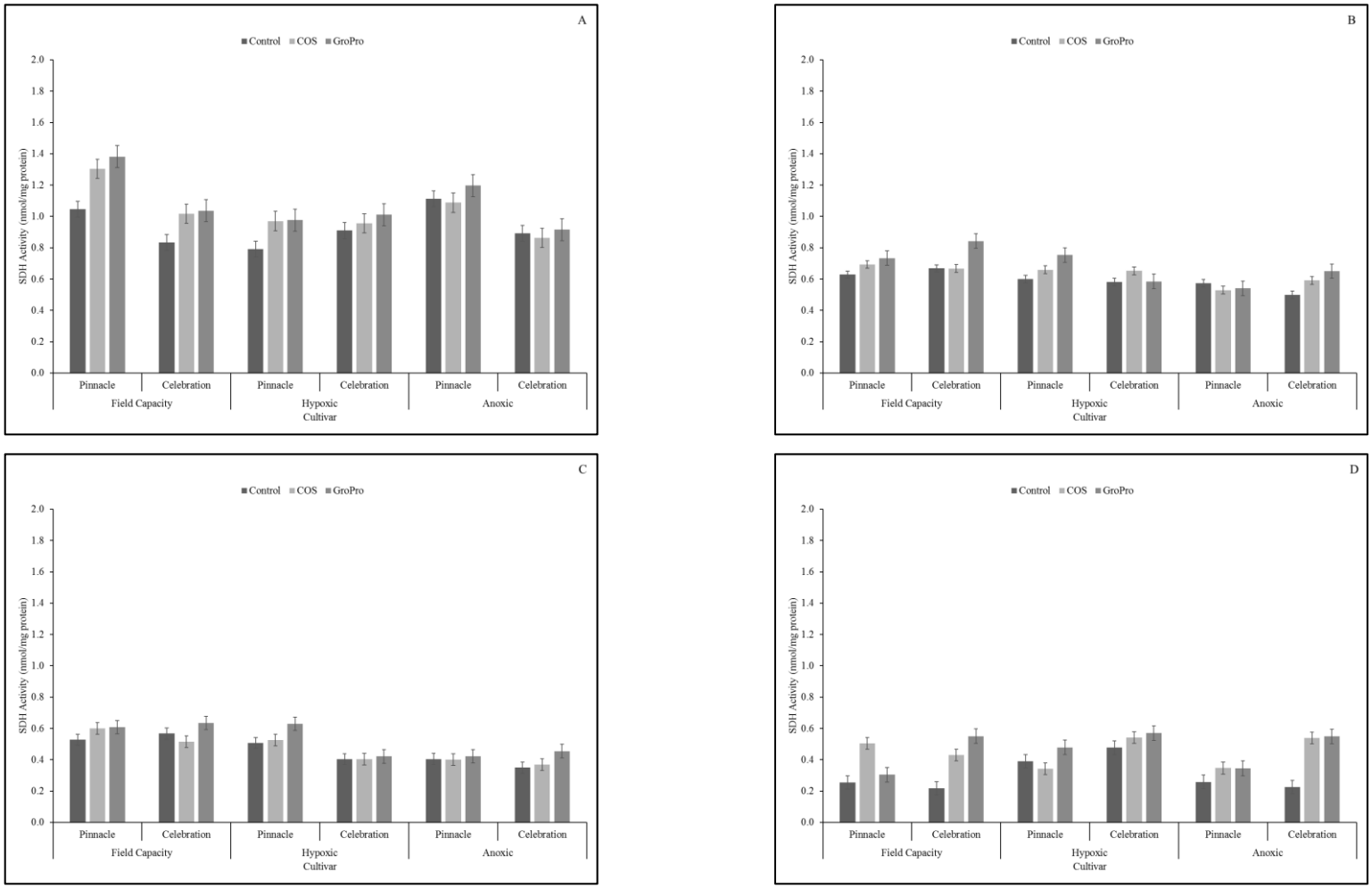

Figure 9.4. Succinate dehydrogenase (SDH) activity (nmol/mg of protein) of barley leaves (Pinnacle and Celebration) with two bioprocessed seed elicitor treatments (COS and GroPro) along with no seed treatment (control) and under three levels of soil moisture conditions (field capacity, hypoxic, and anoxic) at 20 (A), 35 (B), 50 (C), and 65 (D) days after emergence.

Activity of succinate dehydrogenase (SDH), which oxidizes succinate to fumarate as part of tricarboxylate cycle (TCA)/ Krebs's cycle was also determined in barley leaves samples following seed elicitation and waterlogging stress treatment. The SDH activity of barley leaves reduced significantly with increased level of soil water level (hypoxic and anoxic) at day 35 and day 50, during the recovery of barley plants from waterlogging stress (Figure 9.4). Like, results of G6PDH activity, 3-way interactions of barley type × waterlogging level × elicitor treatment combinations had statistically significant effect on SDH activity of barley at day 20, and 35. At day 50, 2-way interaction of barley type × waterlogging level, and at day 65, 2-way interactions of barley type × elicitor treatment and waterlogging level × elicitor treatment had statistically significant effect on SDH activity of barley. Although, higher SDH activity was observed in cv. Pinnacle at day 20 and day 50, an opposite trend with high SDH activity was found in cv. Celebration at day 65. Higher flow of carbon through energy expensive TCA cycle in cv. Celebration during reproductive stage might have relevance for the reduced fitness and resiliency of this barley type under waterlogging stress. Interestingly, statistically significant ($p \leq 0.05$) effect of GP seed elicitor treatment on SDH activity was also observed in both barley types. The improved photosynthetic activity along with high G6PDH and SDH activity in barley leaves with GP seed elicitor treatment indicated a higher level of available photosynthate and subsequent distribution of carbon flow towards stress-adaptive and redox-linked metabolic regulation, which was critical to determine waterlogging stress resilience of barley.

The activity of another key enzyme, proline dehydrogenase (PDH) was also measured to understand the potential metabolic role of proline and its association with PPP regulation under waterlogging stress. Overall, 3-way interactions of barley type × waterlogging level × elicitor treatment combinations only had statistically significant effect ($p \leq 0.05$) on PDH activity of

barley leaves at day 20. However, at day 50, all 2-way interactions had significant effect on PDH activity, while only effect of barley type × waterlogging level interactions was statistically significant at day 65 (Figure 9.5). The PDH activity decreased significantly with increased soil water level. However, GP seed elicitor treatment resulted in high PDH activity in barley leaves of cv. Pinnacle under anoxic soil condition.

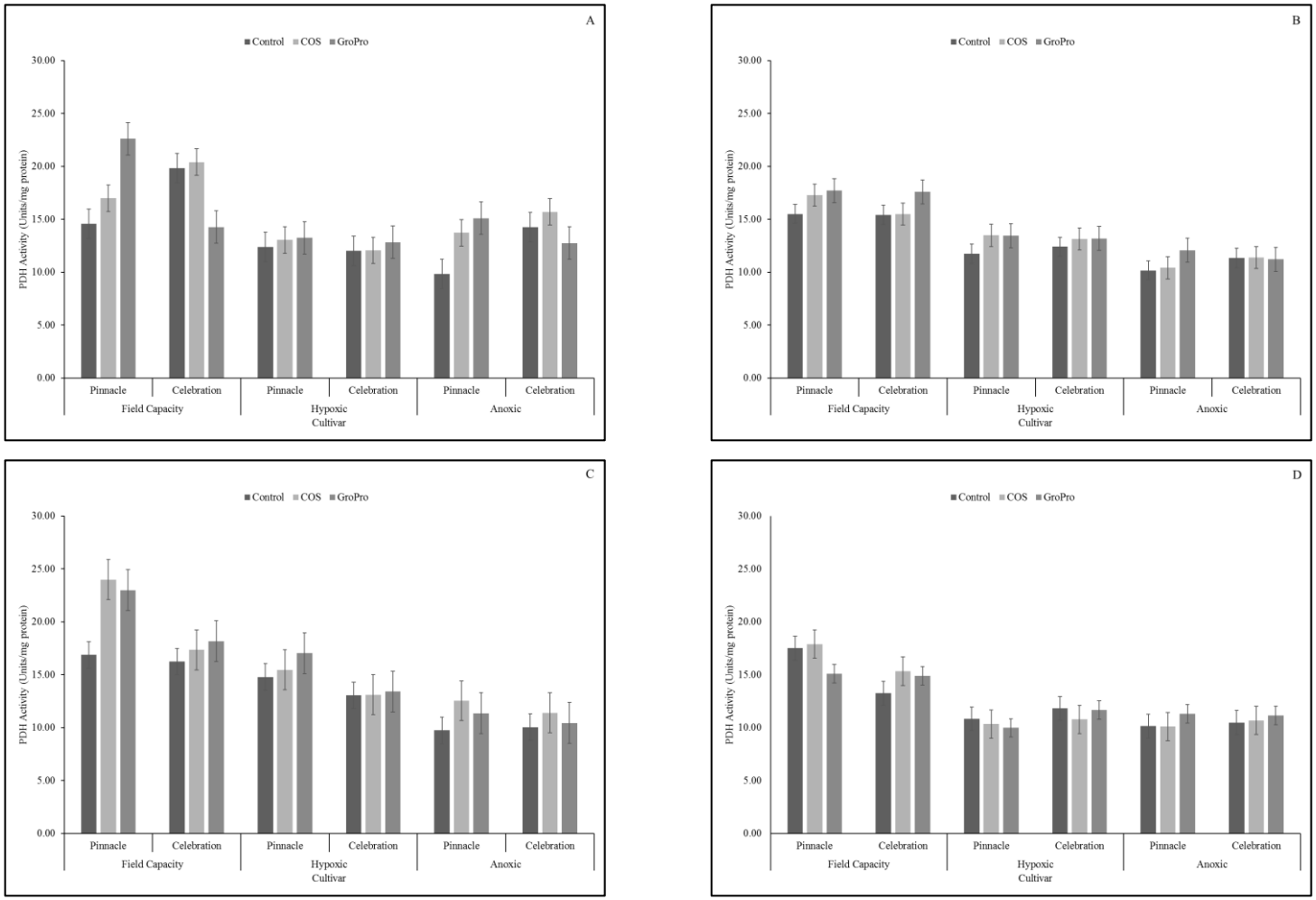

Figure 9.5. Proline dehydrogenase (PDH) activity (units/mg of protein) of barley leaves (Pinnacle and Celebration) with two bioprocessed seed elicitor treatments (COS and GroPro) along with no seed treatment (control) and under three levels of soil moisture conditions (field capacity, hypoxic, and anoxic) at 20 (A), 35 (B), 50 (C), and 65 (D) days after emergence.

Therefore, higher waterlogging stress resiliency of cv. Pinnacle might be related to its' stress adaptive responses such as active metabolic role of proline under waterlogging stress. Previous study reported increase in proline content of barley plants under waterlogging stress (Yodanova and Popova, 2001). However, the metabolic role of proline to determine waterlogging stress resiliency of barley was not studied extensively. Therefore, our study provided a metabolic rationale and potential role of proline-associated PPP regulation for improving waterlogging stress resiliency of barley.

9.4.4. Antioxidant Enzyme (CAT, GPX and SOD) Activities

Like proline metabolism, antioxidant enzyme response is also critical for plants to counter abiotic stress-induced damages, especially to mitigate excessive oxidative stress. In this study, 3-way interactions of barley type × waterlogging level × elicitor treatment combinations had statistically significant effect on CAT activity at day 20, 35, and 65 (Table 9.3A). Overall, CAT activity decreased with increased level of soil water. Under anoxic soil condition, GP seed elicitor treated barley plants showed high CAT activity, which was also statistically significant ($p \leq 0.05$) when compared to control and COS seed treatment. Previously, Yodanova *et al* (2004) reported increase in CAT activity in barley leaves after 120-h flooding. However, Zhang *et al*. (2015) did not find any positive correlation between antioxidant enzyme activities and waterlogging stress tolerance of barley. Scavenging hydrogen peroxide (H_2O_2) is extremely important to prevent cellular damages and for maintaining redox homeostasis under abiotic stresses such as waterlogging stress. Therefore, results of this study, indicated improved CAT activity after GP seed elicitation treatment, which provided scientific rationale to target this novel bioelicitation strategy to improve antioxidant enzyme response in barley under waterlogging stress.

Activity of another key antioxidant enzyme, GPX also varied significantly between barley types and with different soil water levels (Table 9.3B). Statistically significant differences in GPX activity due to 3-way interactions between barley type × waterlogging level × elicitor treatment combinations were observed at day 35, 50, and 65. Reduction of GPX activity was observed with increased soil water level (hypoxic and anoxic). At day 20, significant improvement of GPX activity was found in barley plants with GP seed elicitor treatments. However, the effect of GP seed elicitor treatment on GPX activity was not statistically significant after removal of waterlogging stress (at day 35, 50 & 65). The high GPX activity during early seedling stage might have influence on lignification and structural adjustments of barley plants under hypoxic and anoxic soil conditions. Similarly, high SOD activity was observed in barley plants under hypoxic and anoxic soil conditions at day 20 (Table 9.3C). Statistically significant effect of 3-way interactions between barley type × waterlogging level × elicitor treatment combinations on SOD activity was observed at day 50, and 65. Overall, GP seed elicitor treatment did not have any significant effect of SOD activity of barley plants. In a previous study, Yordanova *et al*. (2004) observed decrease in SOD activity of barley leaves after 120-h flooding. They reported that reduction of SOD activity was due to progressive reduction in the activity of Fe-containing SOD, which is commonly located in the chloroplast (Yordanova *et al*., 2004). Zhang *et al*. (2007) observed higher SOD activity in waterlogging susceptible barley genotype. In another study, Luan *et al*. (2018) found increased activity of SOD and CAT in barley root and shoot after waterlogging stress treatment. Therefore, distribution and stimulation of antioxidant enzymes (and different isomers of these enzymes) in plant cells are largely dependent on specific stress response mechanism and biochemical adjustment of plants under abiotic stresses.

Table 9.3. Activity of antioxidant enzymes catalase (CAT; units/mg protein) (A), guaiacol peroxidase (GPX; nmol/mg protein) (B) and superoxide dismutase (SOD; units/mg protein) (C) of two barley types (Pin, cv. Pinnacle; Cel, cv. Celebration) at 20, 35, 50, and 65 days after seed elicitation treatments (control-no seed treatment, COS-soluble chitosan oligosaccharide, GP-marine protein hydrolysate) and grown under three waterlogging levels.

(A)

Waterlogging level	Treatment	Day 21		Day 35		Day 50		Day 65	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	206.5	220.8	271.8	235.0	254.8	234.5	252.8	221
	COS	226.0	208.3	301.1	250.4	260.4	220.9	263.3	256.6
	GP	209.4	223.7	292.0	216.5	248.2	221.3	193.4	220.2
Hypoxic	Control	208.2	174	275.1	229.6	236.8	203.1	231.3	158.9
	COS	204.1	193.5	266.7	248.6	222.6	200.1	236.4	188.4
	GP	192.7	219.5	276.9	206.5	236.3	208.0	301.3	228.5
Anoxic	Control	198.0	210.2	260.2	245.7	227.5	218.1	215.1	208.9
	COS	185.2	203.6	273.1	231.9	246.3	211.4	205.4	161.8
	GP	230.3	231.9	344.8	304.7	272.4	270.5	204.1	236.6

(B)

Waterlogging level	Treatment	Day 21		Day 35		Day 50		Day 65	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	67.6	60.0	143.8	177.1	140.8	100.3	142.3	138.7
	COS	73.1	67.9	157.8	208.1	145	96.8	153.9	152.5
	GP	79.7	72.2	154.5	148.1	124	95.1	139.2	121.6
Hypoxic	Control	61.2	50.6	125.7	113.3	145.9	96.8	135.8	105.1
	COS	58.6	48.1	122.8	93.4	138.1	95.6	130.5	94.5
	GP	67.5	54.1	119.1	117.6	131.3	111.6	125.2	114.6
Anoxic	Control	65.0	49.1	119.6	107.4	99.7	102.8	109.7	105.1
	COS	59.8	44.6	142.9	117.6	90.6	104.2	116.8	110.9
	GP	84.9	75.4	122.5	117.5	106.9	101.1	114.7	109.3

(C)

Waterlogging level	Treatment	Day 21		Day 35		Day 50		Day 65	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	n.d	n.d	0.003	0.021	n.d	0.017	n.d	0.02
	COS	0.011	0.01	0.006	0.007	0.004	0.019	0.007	0.01
	GP	0.003	n.d	0.002	0.008	n.d	0.006	n.d	0.005
Hypoxic	Control	0.006	0.002	0.016	0.002	0.005	0.026	0.004	0.021
	COS	0.014	0.012	0.014	0.01	n.d	0.005	0.008	0.011
	GP	n.d	n.d	n.d	0.006	0.008	0.006	n.d	0.005
Anoxic	Control	0.015	0.008	0.027	0.01	0.01	0.014	0.009	0.015
	COS	0.019	0.018	0.006	0.009	n.d.	0.007	0.004	0.008
	GP	0	n.d.	n.d.	0.006	0.006	0.007	n.d.	0.007

n.d.-Not Detected

Table 9.4. Yield parameters of two barley types (cv. Pinnacle and cv. Celebration) treated with seed elicitors and grown under three waterlogging levels.

Waterlogging level	Treatment	1000 grain weight (g)		Spikes per plant		Grains per spike		Grain yield per plant (g)	
		Pin	Cel	Pin	Cel	Pin	Cel	Pin	Cel
Field capacity	Control	32.6	30.1	1.67	2.52	64.6	27	3.51	2.04
	COS	34.3	31.6	2.67	2.81	67.6	27.9	6.19	2.48
	GP	35.5	32.6	2.67	3.04	71.7	30.1	6.78	2.99
Hypoxic	Control	33.1	29.2	1.44	1.89	56.9	23.6	2.72	1.3
	COS	32.6	30.5	1.78	2.33	57.6	25.9	3.34	1.84
	GP	34.4	30.7	2.56	2.67	61.2	27.9	5.38	2.28
Anoxic	Control	24.6	19.4	1.33	1.19	35.6	16.3	1.17	0.38
	COS	25.3	22.7	1.44	1.00	37.6	17.4	1.37	0.4
	GP	27.0	23.2	1.56	1.78	41.0	19.7	1.72	0.81

Table 9.5. Analysis of variance table indicating mean square (MS) and significant differences between types (C), waterlogging levels (W) and seed elicitation treatments (T) and their respective 2-way and 3-way interactions.

Source	df	Spikes per plant	Grains per spike	1000 grain weight	Grain yield per plant
C	1	0.0093ns‡	11001.0*	63.9**	154.9*
W	2	12.1*	5163.5*	834.1*	125.6*
C×W	2	0.23ns	415.5**	1.03ns	14.2*
T	2	0.68ns	1220.2*	331.2*	29.2*
C×T	2	0.95ns	125.7ns	10.6ns	7.04*
W×T	4	1.03**	828.4*	135.3*	4.68*
C ×W×T	4	2.23**	593.1**	15.7**	2.81**

*Denotes significance at $p < 0.05$; **Denotes significance at $p < 0.001$; ‡ns, not-significance

9.4.5. Yield Parameters

It is important that improvement of physiological and biochemical parameters should translate into increase in yield attributes, which helps to determine economic viability and efficacy of any abiotic stress resilience strategy. In this study, anoxic soil condition had adverse effect on yield attributes (1000 grain weight, spikes/plant, grains/spike, and grain yield/plant) of barley plants (Table 9.4 & 9.5) confirming the earlier findings that barley is highly susceptible to waterlogging stress (Pang *et al*., 2004; Zeng *et al*., 2013; Romina *et al*., 2014). Additionally, 3-way interactions of barley type × waterlogging level × elicitor treatment combinations had statistically significant effect on all yield parameters investigated in this study. Like results of physiological and biochemical parameters, the effect of waterlogging stress (hypoxic and anoxic)

on yield attributes was more severe on cv. Celebration when compared to cv. Pinnacle. Overall, positive effect of seed priming treatments on grains/spike, 1000 grain weight, and grain yield/plant of barley was observed. GP seed elicitor treatment improved all these yield attributes. Therefore, these results suggested that benefits of such bioelicitation strategy was not only just restricted to enhancing stress related adaptive responses and vegetative growth of seedlings, but also contributed to improving reproductive growth and yield attributes of barley plants. However, future field-based studies are needed to prove this concept and for successful integration of GP-based bioelicitation strategy for improving waterlogging stress resiliency of barley.

9.5. Conclusions

Prolonged flooding or waterlogging of agricultural field adds serious abiotic stress pressure that affect seedling emergence, seedling establishment, survival, fitness, and growth of major food plants. Barley is best suited to well-drained sandy loams with cool dry climate, and excessive moisture and waterlogged soil affect overall growth, productivity, and profitability of barley. Therefore, finding effective and inexpensive tool to improve waterlogging stress resiliency of commercial malting barley cultivars have significant economic relevance. In this study, metabolically driven bioelicitation strategy using natural seed elicitor treatments (COS & GP) was targeted to improve waterlogging stress resiliency of barley during critical seedling establishment stages. Overall, anoxic soil condition reduced photosynthetic activity, root and shoot growth, and plant height of barley seedlings, which also resulted in poor yield of barley plant during harvest. Statistically significant effects of 3-way interactions of barley type × waterlogging level × elicitor treatment combinations on physical, physiological, and yield parameters of barley were observed in select growth stages. Additionally, upregulation of stress-

protective and redox-linked PPP and associated anabolic responses such as enhanced phenolic content, antioxidant activity, and improved tissue structural adjustments were observed, which suggests the relatively higher waterlogging stress resiliency of cv. Pinnacle compared to cv. Celebration, within the current experimental setup. Furthermore, GP seed elicitor treatment improved photosynthetic activity, vegetative growth, different yield attributes and stimulated PPP-associated biosynthesis of phenolic and antioxidant enzyme responses of barley plants. Therefore, GP seed elicitor treatment could potentially be targeted for multiple-year and multi-locations field-based experiments to prove and validate the above findings and for wider application of such strategy to improve waterlogging stress resiliency of barley.

CHAPTER 10. PHENOLIC BIOACTIVE-LINKED ANTIOXIDANT AND ANTIHYPERGLYCEMIC BENEFITS OF ROASTED BARLEY TEA AND COFFEE

10.1. Abstract

Barley tea and coffee are traditional beverages prepared from roasted, unmalted barley grains. These barley-based beverages are rich sources of phenolic compounds, which are known to possess potent antioxidant and anti-hyperglycemic functionalities. Therefore, novel barley-based beverages with high health protective phenolic compounds can be integrated into dietary support strategies to address chronic oxidative stress and chronic hyperglycemia commonly associated with early stages of type 2 diabetes (T2D) with additional *in vivo* validation based on this study. In the current study, two different types of barley tea and coffee with different steeping times were compared and investigated for phenolic linked functionalities targeting antioxidant and anti-hyperglycemic benefits. Total soluble phenolic (TSP) content, phenolic acid profile, total antioxidant activity based on ABTS (2, 2'-azino-bis 5(3-ethylbenzothiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals scavenging activities, and anti-hyperglycemic property relevant α-amylase and α-glucosidase enzyme inhibitory activities of barley tea and coffee samples with different steeping times were determined using *in vitro* assay models. Additionally, the potential function of the barley tea and coffee to support growth of beneficial lactic acid bacteria (LAB) (*Bifidobacterium longum* and *Lactobacillus helveticus*), as well as anti-bacterial activity against ulcer causing pathogenic bacteria *Helicobacter pylori* were also evaluated. Overall, higher phenolic content and associated antioxidant and anti-hyperglycemic functionalities were observed in targeted barley coffee when compared to barley tea samples investigated in this study. In barley tea sample, higher content of catechin was observed while cinnamic acid was the major phenolic compound in barley coffee

samples. Furthermore, with increased in steeping time, soluble phenolic content and associated antioxidant activity increased proportionately in both barley tea and coffee. However, in most samples anti-hyperglycemic property relevant α-amylase enzyme inhibitory activity and individual phenolic acid content decreased with increased in steeping time. Interestingly, improvement in growth of beneficial LAB such as *Bifidobacterium longum* after 24 h incubation with barley tea sample was observed, which indicated potential gut health benefits of barley tea. The results of this study suggested that barley tea and coffee sample from two sources but from different growing locations and processing strategies can be advanced as novel functional beverage as part of dietary solution strategies to improve early stages of T2D benefits with further validation from future *in vivo* studies. These results when combined with the relative growth promotion of beneficial LAB with barley tea provides metabolic and microbiological rationale to further investigate and validate the human gut health benefits for overall health-focused food applications to address T2D and associated health risks.

Keywords: Anti-Hyperglycemic; Antioxidant; Antibacterial, Human gut health; Phenolic Compounds; Functional Beverage

10.2. Introduction

Consumer interest in functional foods and ingredients, which potentially provide several health benefits beyond basic nutrition has emerged as a key trend in the global food market over the past decade (Corbo *et al*., 2014). The steady growth of commercially available functional foods globally is driven by factors such as increased awareness of consumers about the health protective role of nutritionally balanced diet; increasing prevalence of unhealthy diet and lifestyle-linked non-communicable chronic diseases (NCDs); and growing demand for nutritious and value-added convenience foods (Granato *et al*., 2010). Within the functional food category,

beverages are arguably the fastest growing segment, with an estimated global value of $93.68 billion in 2019 (Grand View Research Report, 2019). This trend is driven by the fact that beverages provide the ease of; (i) adapting to consumer demands for convenient packaging; (ii) easy storage and distribution of refrigerated and shelf-stable products; and (iii) simple soluble matrices to incorporate desirable and health-targeted nutrients and bioactive compounds (Sanguansri and Augustin 2015; Wootton-Beard and Ryan 2011; Nasir *et al*., 2019).

Therefore, with the targeted efforts to develop new commercial functional beverages, attention has increasingly shifted towards products derived from whole grain cereals for many key reasons. Whole grain cereals contain a wide range of bioactive components that have potential of supporting beneficial physiological activities, are amenable to various processing and technological considerations, and may be suitable for individuals with specific dietary needs and restrictions (for example, lactose-free, caffeine-free, vegan, vegetarian products) (Corbo *et al*., 2014; Kreisz *et al*., 2008; Nyanzi and Jooste, 2012; Sonawane and Arya, 2018). More specifically, traditional cereal grain-based beverages are of interest to the food industry as they tend to have an established history of consumption and acceptance across many communities worldwide, thereby offering a suitable platform to innovate and develop value-added products with improved health-relevant functionalities (Charalampopoulos, Wang and Pandiella, 2002; Corbo *et al*., 2014).

Therefore, functional foods and beverages with health protective benefits such as antioxidant, anti-hyperglycemic, and anti-hypertensive properties are valuable dietary target to address increasing prevalence of diet linked NCDs, such as T2D, cardiovascular disease, chronic lung disease, and some specific types of cancer. This is significant, considering that the prevalence of T2D has reached epidemic proportions globally resulting in premature mortality

and co-morbidity in the wider populations of developed and low/middle income countries alike (Alkhatib *et al*., 2017; Mattei *et al*., 2015; Wareham and Herman; 2016). As the importance of adopting a preventive dietary strategy towards T2D benefits have become increasingly apparent, several studies have focused on identifying and formulating plant-based foods and beverages from various sources including whole grains that contain functional compounds capable of exerting positive anti-hyperglycemic functions (Adefegha, 2017). Such foods may help complement existing pharmacological strategies of mitigating the symptoms of T2D and reduce the risk of adverse side-effects of conventional medication, especially in high-risk individuals (Ernst and Pittler, 2001). Therefore, innovation and development of cereal-based functional beverages with the ability to improve glycemic control, especially benefitting prediabetic and diabetic individuals holds significant merit.

Barley is a rich source of diverse beneficial phytochemicals such phenolic compounds, phytosterols, biologically active peptides, and soluble dietary fiber (β-glucans) (Ramakrishna *et al*., 2019). These phytochemicals exhibit strong antioxidant, antihyperglycemic, antiproliferative, and cholesterol lowering abilities, which are potentially useful in lowering the risk of certain diet-linked diseases such as T2D and other NCDs (Idehen *et al*., 2017; Ramakrishna *et al*., 2019). As a result, barley-based foods and beverages may be viable options for the dietary interventions to address chronic conditions such as chronic hyperglycemia and chronic oxidative stress, two most common risk factors associated with early stages of T2D and related NCDs.

Barley tea and barley coffee are non-alcoholic beverages prepared from roasted, unmalted barley grains, which although have a long history of consumption, have not been widely investigated in regard to their potential benefits in managing chronic hyperglycemia, a common risk factor associated with the pathogenesis of T2D. Barley tea is traditionally

consumed in many Asian countries, including Korea (boricha), China (dàmàichá or màicha), and Japan (mugicha), Tibet and to a certain extent in parts of India (Newman and Newman, 2008; Oh *et al*., 2014). Barley coffee, also referred to as caffè d'orzo, is a similar beverage that is produced from roasted ground barley, originating in Italy (Papetti *et al*., 2007). It has long been used as a caffeine-free coffee substitute, especially for children and pregnant women (Newman and Newman, 2008).

The consumption of barley tea has been linked to various health benefits, attributed to the presence of diverse human health protective phytochemicals. Etoh *et al*. (2004) observed the presence of several phenolic compounds, including p-hydroxyacetophenone, 5,7-dihydroxychromone, naringenin, quercetin, and iso-americanol A, many of which were found to exhibit more potent antioxidant activity than butylated hydroxytoluene (BHT). Omwamba *et al*. (2013) demonstrated the antioxidant and antiaging properties of roasted barley grain using different *in vitro* and *in vivo* assays. Apart from diverse phenolic compounds, which are naturally present in the barley kernels, the roasting process also results in the synthesis of phenolic compounds as products of the Maillard reaction (MR) (Duh *et al*., 2001; Etoh *et al*, 2004). The antioxidant potency of the aqueous extract of roasted hulled barley (AERB) has been reported (Oh and others 2014). Similarly, barley coffee has been found to contain significant amounts of polyphenols, in addition to melanoidins developed during roasting (Papetti *et al*., 2007). Additionally, barley coffee extracts were found to prevent the colonization of detrimental microbes, such as *Streptococcus mutans* (Papetti *et al*, 2007; Papetti *et al*. 2014).

In the context of health benefits, few previous studies indicated that barley tea and coffee are rich sources of potent phenolic bioactives (Etoh *et al*., 2004; Duh *et al*., 2001). Barley rich in health protective phenolics, demonstrated moderate to high antihyperglycemic potential

especially regarding modulating the activity of enzymes like α-amylase and a-glucosidase, in addition to its high antioxidant activity (Idehen, 2017; Ramakrishna *et al*., 2017a; Ramakrishna *et al*, 2019). Therefore, an investigation into the potential antihyperglycemic activity of barley tea and coffee has merit. Thus, the current study focused on investigating phenolic-linked antioxidant capacity and the ability of roasted barley tea and coffee to modulate the activity of key enzymes involved in carbohydrate digestion such as, α-amylase and α-glucosidase relevant for managing postprandial blood glucose level using *in vitro* assay models. Additionally, the potential of barley tea and coffee to support the growth of gut bacteria capable of exerting beneficial probiotic activity, such as *Bifidobacterium longum* and *Lactobacillus helveticus*, was also studied. Furthermore, the potential of these barley-based beverages to inhibit the growth of *Helicobacter pylori* - a widely prevalent bacterial pathogen known to cause gastric complications in the stomach and small intestine such as ulcers and cancer, was also investigated. Overall, the broad objective of this study was to investigate phenolic-linked antioxidant, anti-hyperglycemic and human gut health benefits of roasted barley tea and coffee for their potential health-focused dietary application targeting early stages T2D benefits.

10.3. Materials and Methods

All chemical reagents including enzymes, and substrates for *in vitro* and bioactive analysis were purchased from Sigma Aldrich Chemical Co. (St Louis, MO, USA).

10.3.1. Sample Preparation

Barley tea (BT) and coffee (BC) samples were obtained from local supermarkets (Walmart, Fargo, ND). In each case, two products from different manufactures were used, and designated as BT1, BT2, BC1 and BC2 in the present study. Barley tea brands tested were "Tokuya Hiyashi Mugicha Roasted Barley Tea" (BT1; country of origin – Japan) and "Daesang

Organic Roasted Barley Tea" (BT2; country of origin –South Korea). Barley coffee brands tested were "Orzo di Siena Barley Coffee" (BC1; country of origin – Italy) and "Delta Cafes Cevada" (BC2; country of origin – Portugal).

Hot water extracts of the barley tea and coffee samples were prepared separately. The BT samples were steeped in a beaker containing boiling distilled water (5 g/ L) separately for 5, 10 and 15 min. The BC samples were steeped in boiling distilled water (50 g / L) in a French press (Bodum French Press Coffee Maker, Switzerland) for a duration of 3-, 6- and 9-min. Extracts were decanted to remove granular debris and filtered under vacuum through Whatman filter paper (Grade # 4) to remove remaining suspended particulate matter. The supernatants were collected, labelled appropriately, and stored at -20 ºC prior to *in vitro* analysis.

10.3.2. Total Soluble Phenolic (TSP) Content

The total soluble phenolic (TSP) content of the barley tea and coffee were measured via the Folin-Ciocalteu (FC) based assay (Shetty *et al*., 1995). To the sample (1 mL), 95% ethanol (1 mL), distilled water (5 mL), FC reagent (50% v/ v; 0.5 mL) and Na_2CO_3 (5% v/v; 1 mL) were successively added, thoroughly vortexed, and incubated in a dark cabinet for 60 min under room temperature. Post incubation, the absorbance of the mixture was measured at 725nm using a spectrophotometer (Genesys UV-visible, Milton Roy Inc., Rochester, NY). The steps mentioned above were repeated with various concentrations of gallic acid (10 – 300 μg/mL) in 95% ethanol to prepare a standard curve, which was then used to convert sample absorbance values to TSP content, expressed as milligram equivalents of gallic acid per gram dry weight of barley the original samples.

10.3.3. Determination of Major Phenolic Compounds using HPLC

Sample extracts were micro-centrifuged for 10 min at 13000 rpm, from which a small volume (5 μL) of the sample was subjected to chromatographic analysis using reverse phase HPLC (Agilent 1260 Infinity Series equipped with DAD 1100 diode array detector; Agilent Technologies, Palo Alto, CA). A gradient elution method (Mishra *et al*., 2020), involving 10 mM phosphoric acid (pH 2.5; Solvent A) and 100% methanol (Solvent B), was used. Sample extracts were eluted on a C-18 analytical column (Agilent Supelco SB-C18 250 x 4.6 mm internal diameter) with a packing material particle size of 5 μm, at a flow rate of 0.7 mL/min at ambient temperature, with a total run time of 25 min. Pure standards of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, quercetin, and p-coumaric acid in 100% methanol were used to calibrate retention times on the standard curve. The chromatograms so obtained were analyzed using the Agilent Chemstation integration software**.**

10.3.4. DPPH Free Radical Scavenging Activity

Antioxidant activity was measured using a modified DPPH (2, 2- diphenyl-1-picrylhydrazyl, Sigma Chemical Co.) free radical scavenging assay (Cervato *et al*., 2000; Kwon *et al*., 2007). Sample extracts (0.25 mL) were mixed with 60 μM DPPH stock solution (adjusted to absorbance 2.0 at 517 nm; 1.25 mL) prepared with 95% ethanol. The mixture was thoroughly vortexed, incubated at room temperature for 5 min, and its absorbance was measured at 517 nm. For each sample, a corresponding control containing 95% ethanol (0.25 mL) instead of sample extracts was also included. The antioxidant activity of the extracts was expressed as percentage (%) inhibition of DPPH free radical formation was calculated as per the following formula:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

10.3.5. ABTS Free Radical Scavenging Activity

Antioxidant activity of sample extracts was also measured using the ABTS [2, 2 – azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay (Pellegrini *et al*., 2002). The ABTS radical cation was prepared by mixing (5 mL) of 7 mM ABTS solution with 140 mM $K_2S_2O_4$ solution (88 mL). The stock solution allowed to mature in the dark at 4°C for 12–16 h before use. Prior to the assay, the stock solution was diluted with 95% ethanol at an approximate ratio of 1:88 (ABTS: ethanol) to prepare a working solution with an absorbance of 0.70 ± 0.02 units at 734 nm. The working solution (1 mL) was added to the sample extract (50 µL), vortexed thoroughly and incubated at room temperature (22- 24°C) for 2.5 min and absorbance was measured at 734 nm. The antioxidant activity of the sample extracts was expressed as percentage (%) inhibition of ABTS free radical formation and was calculated as per the following formula:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

10.3.6. α-Amylase Inhibitory Activity

The α-amylase enzyme inhibitory activity was determined by an assay modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a). Sample extracts (500 µL) were mixed with 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl; 500 µL) containing porcine α-amylase (0.5 mg/mL) and incubated at 25°C for 10 min. To this, 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl; 500 µL) was added and incubated again at 25°C for 10 min. 3,5-dinitrosalicylic (DNS) acid solution (1 mL) was further added and the reaction was halted by incubating this final mixture in a boiling water bath for 10 min. The reaction mixture was cooled to room temperature and diluted with distilled water such that its absorbance could be measured at 540 nm. The absorbance of respective sample

blanks (enzyme solution replaced by buffer) and a control (sample extract and enzyme solution replaced by buffer) were also recorded. The α-amylase enzyme inhibitory activity was calculated as percentage (%) inhibition based on the following equation:

$$Inhibition\ (\%) = \frac{Abs_{control} - (Abs_{extract} - Abs_{sample\ blank})}{Abs_{\ control}} \times 100$$

10.3.7. α-Glucosidase Inhibitory Activity

The α-glucosidase enzyme inhibitory activity was determined using an assay modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a; McCue *et al*., 2005). Sample extracts (50 µL) were incubated with 0.1M phosphate buffer (pH 6.9; 100 µL) containing α-glucosidase enzyme (1 unit/mL) in 96-well microplates at 25°C for 10 min. For the dose dependent response half (25 µL) and one-fifth (10 µL) of sample was added in individual well and mixed with buffer to reach final volume of 50 µL. Following this, 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG; 50 µL) solution dissolved in 0.1M phosphate buffer (pH 6.9) was added to each and reaction mixtures were incubated at 25°C for 5 min. Absorbance values at 405 nm were recorded before and after incubation using a microplate reader (Thermomax, Molecular Device Co., Sunnyvale, CA) and compared to a corresponding control in which the sample extracts were substituted with buffer solution (50 µL). The α-glucosidase enzyme inhibitory activity was expressed as percentage (%) inhibition and calculated per the following equation:

$$Inhibition\ (\%) = \frac{\Delta Abs_{control} - \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

10.3.8. Proliferation of Lactic Acid Bacteria (LAB) and Viable Cell Count

The ability of the sample extracts to support the proliferation of beneficial LAB was evaluated using an assay described by Ranilla *et al*. (2012). Two species of LAB were used in this study namely *Bifidobacterium longum* (BL) and *Lactobacillus helveticus* (LH). Upon

procurement from ATCC, the LAB cultures were preserved in De-Mann-Rogosa-Sharpe (MRS) broth (Becton Dickinson and Co. Sparks, MD) containing 20% glycerol and stored at −80 °C. Prior to conducting the assay, the frozen stock cultures (100 μL) were thawed and inoculated into MRS broth (10 mL) and incubated for 24 h at 37°C under hypoxic conditions. The revived cultures were further re-inoculated into 10 mL MRS broth for 24 h at 37°C.

Sample extracts were passed through sterile Millex GP 0.22 μm filters (Millipore Corp., Bedford, MA). Filter-sterilized sample extracts (1 mL) and 100 μL of the fully revived LAB cultures (diluted 100 times with sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37°C for 48 h. Corresponding controls containing sterile distilled water (1 mL) instead of sample extract were also included.

At 0, 6, 12, 24 and 48 h. post inoculation, 100 μL of the inoculated sample extracts were drawn, serially diluted and plated on MRS agar and incubated in anaerobic BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak EZ anaerobe container system sachets (Becton Dickinson and Co. Sparks, MD) so as to create an anaerobic atmosphere with less than 1% of oxygen, at 37 °C for 48 h to determine the viable cell count of the respective bacterial cultures in the sample (expressed in log CFU/mL).

10.3.9. *Helicobacter pylori* Inhibitory Activity

The ability of the barley tea and coffee extracts to potentially inhibit the growth of *H. pylori* was evaluated using a modified agar-diffusion disc assay method described by Ranilla *et al*. (2012). Under aseptic conditions, filter sterilized sample extracts (100 μL) were added to sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, N.H., U.S.A.) and these saturated disks were transferred onto the surface of seeded agar plates. Controls comprised disks saturated with sterilized-distilled water. Treated plates were incubated at 37°C for 48 h in BBL

GasPak jars with BD GasPak Campy container system sachets (Becton Dickinson and Co., Sparks, MD) to create a microaerophilic environment. Post incubation, the diameter of the clear zone of inhibition (no growth) surrounding each disk was measured and expressed in mm.

10.3.10. Statistical Analysis

A completely randomized design (CRD) was used for all *in vitro* assays in this study. From each type of product (experimental unit), 6 samples were analyzed during each run, and all assays were repeated twice. Calculation of means and standard deviations for a total of 12 data points were performed using Microsoft Excel 2016. Analysis of variance (ANOVA) on the data obtained, was performed using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC) separately for BT and BC samples due to differences in steeping times used in each case. Statistical differences between the means of the main effects (sample type and steeping time) and effects of their interactions were determined using the Tukey's least mean square test at a confidence level of 95% ($p < 0.05$).

10.4. Results and Discussion

10.4.1. Total Soluble Phenolic Content

Soluble phenolic content of functional foods and beverages are gaining increasing interest due to their wider health benefits, especially the health protective functionalities relevant for dietary application against common NCDs, such as early stages of T2D. In this study, the TSP content of tea and coffee derived from roasted barley grains was determined based on the Folin-Ciocalteu-based colorimetric assay (Shetty *et al*., 1995). The overall trend among BT and BC samples indicated a positive correlation between steep time and TSP content (Fig 10.1A, B). Additionally, a significant impact of interactions between sample type × steeping time combinations were statistically different from each other at $p < 0.05$.

Among the two different types of beverage samples, BC samples exhibited considerably higher TSP content when compared to BT samples. Among the barley tea samples, BT1 (3.08 – 3.99 mg GAE/g D.W.) was found to have a higher range of TSP content compared to BT2 (1.03 – 1.87 mg GAE/g D.W). In both BT samples, highest TSP content was observed after 15 min steeping, which was also statistically significant when compared to BT with 5 and 10 min of steeping.

In the case of BC sample extracts, differences in TSP content due to sample type were not statistically significant at $p < 0.05$. However, as mentioned earlier, longer steep times of 6 and 9 min did correspond to improved soluble phenolic content into the aqueous extracts compared to a steep time of 3 min. However, at 6 and 9 min of steeping, TSP content of both samples were statistically at par. The ranges of TSP content observed in both BT and BC samples in this study were considerably higher than previous studies focusing on barley-based beverage products. For instance, the highest TSP content among hot water extracts of 13 barley cultivars was found to be around 0.64 mg GAE/g D.W (Ramakrishna *et al*., 2017a). Similarly, in a comparison of different types of coffees, Kreicbergs *et al*. (2011) reported a maximum of approximately 1.5 mg GAE 100^{-1}g in two barley-based coffee substitutes. Differences in the values observed between previous and current studies may be attributed to differences in roasting and extraction conditions, especially after thermal processing (Omwamba and Hu, 2010). The positive correlation between steep time and TSP content found in this study agrees with the trend reported by Oh *et al*. (2014). Overall, the result of this current study suggested that barley tea and coffee with optimum steeping time are good sources for dietary phenolics and can be advanced for health-focused dietary solutions, especially for potential antioxidant and anti-hyperglycemic benefits. However, it is also important to understand the changes in phenolic acid profile of

barley tea and coffee with different steeping times, as phenolic acid composition potentially play critical role in determining human health relevant functionalities of food and food ingredients.

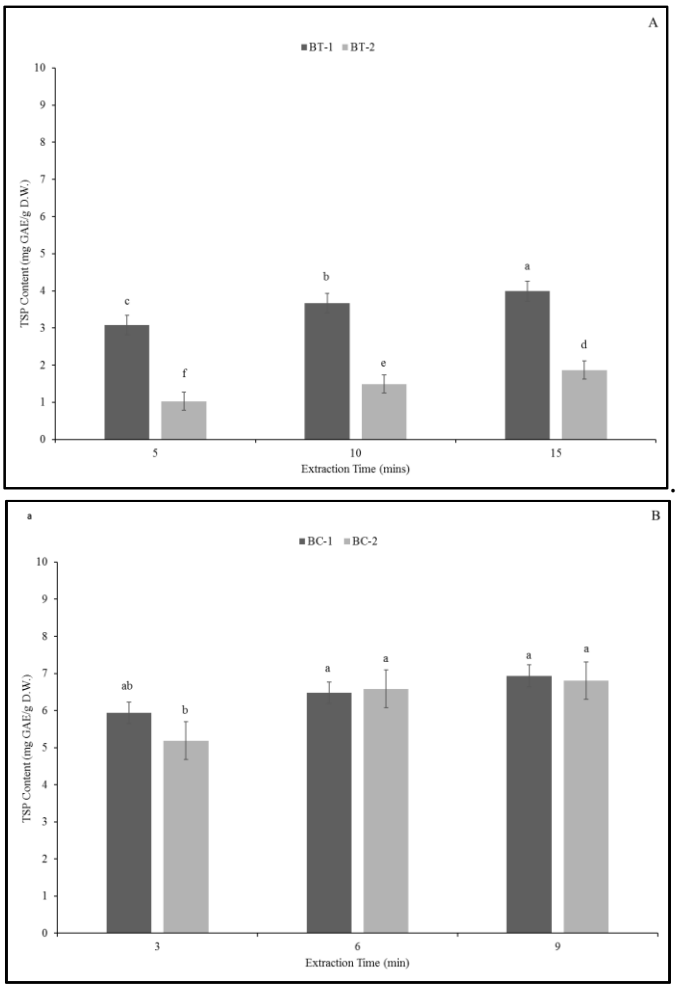

Figure 10.1. Total soluble phenolic (TSP) content (mg GAE/g dry weight) of barley tea (A) and barley coffee (B) extracts. Different alphabets represent significant differences in TSP content due to interaction between sample × steeping time at 95% confidence level ($p < 0.05$) separately for BT and BC.

10.4.2. Phenolics Profile

In this study, individual major phenolics and particularly phenolic acids of barley tea and coffee samples were detected and quantified using high performance liquid chromatography (HPLC) method as described by Mishra *et al*. (2020). The major phenolic acids found in the barley tea samples were catechin, cinnamic acid, dihydroxybenzoic acid, gallic acid and *p*-

coumaric acid (Table 10.1 A). Among these specific phenolics, higher concentration of catechin was detected in both barley tea samples (BT1 & BT2). Previously, Etoh *et al*., (2004), reported higher content of specific phenolic acids such as hydroxybenzoic acid, vanillic acid and *p*-coumaric acid in barley tea samples. In another study, gallic acid, *p*-coumaric acid, ferulic acid, epicatechin gallate, and kaempferol were found in roasted barley malts (Chen *et al*., 2019). Additionally, higher concentration of catechin, ferulic acid, and procyanidin were also observed in free phenolic fractions of barley flour (Mosele *et al*., 2018).

In the current study, *p*-coumaric acid was only detected in BT1 sample. The statistically significant ($p < 0.05$) effect of sample type × steeping time interactions was observed on major phenolic compounds of barley tea samples. However, for gallic acid content, only statistically significant effect of steeping time was observed. Overall, in BT2 sample, individual specific phenolic content decreased proportionately with increased in steeping time from 5 to 15 min.

However, for sample BT1, highest phenolics content was found with 10 min steeping time. Additionally, BT1 sample had significantly higher catechin content, when compared to other barley tea (BT2) and both barley coffee samples. In barley coffee samples (BC1 &BC2), the major specific phenolics found were cinnamic acid, catechin, protocatechuic acid, and *p*-coumaric acid (Table 10.1B).

Previously, Omwamba and Hu (2009) found *p*-hydroxybenzaldehyde, gallic acid, vanillic acid, tocopherol, syringic acid, catechin and *p*-hydroxybenzoic acid in roasted barley grain extracts. The results from previous and current study indicated that source of barley sample, roasting parameters, and type of extractions had significant effect on specific phenolics profile of roasted barley samples (food and malting barley).

Table 10.1. Major phenolics identified in barley tea (A) and barley coffee (B) samples by HPLC analysis. Different lowercase letters represent significant differences in individual phenolic acid concentration due to sample type × steeping time interactions at 95% confidence level ($p < 0.05$). Uppercase letters in the gallic acid column represent significant differences in phenolic acid content due to different steeping times at 95% confidence level ($p < 0.05$) (Interaction effects are not significant for gallic acid).

(A)

Sample	Steeping Time (min)	Average concentration (µg/g D.W.)				
		Dihydroxybenzoic Acid	Gallic Acid	Cinnamic Acid	*p*-Coumaric Acid	Catechin
BT1	5	14.0c	1.11B	23.6b	1.05b	134.1a
	10	16.0b	1.57A	25.2a	1.17a	143.9a
	15	13.6c	1.27B	23.4b	1.16a	28.9b
BT2	5	22.6a	1.19B	23.2b	n.d.*	75.9b
	10	13.0c	1.50A	20.4c	n.d.	53.0b
	15	10.0d	1.04B	20.9c	n.d.	78.7b

(B)

Sample	Steeping Time (min)	Average concentration (µg/g D.W.)			
		Protocatechuic Acid	Cinnamic Acid	*p*-Coumaric Acid	Catechin
BC1	3	0.85c	30.9a	0.55c	1.41c
	6	0.53d	21.7e	0.29c	5.37a
	9	0.38e	14.2f	0.20c	3.66ab
BC2	3	1.34a	78.3a	6.43ab	0.52c
	6	0.99b	55.0c	7.89a	2.00bc
	9	0.99b	61.4b	5.10b	1.77bc

n.d. – Not detected

Like barley tea samples, phenolic acids such as protocatechuic acid, cinnamic acid, and *p*-coumaric acid content of both barley coffee samples decreased proportionately with increased in steeping time from 3 to 9 min. However, the catechin content increased from 3 min to 6 min steeping time and then slightly decreased with 9 min steeping time. The changes in specific phenolics profiles of roasted barley tea and coffee samples with different steeping times might be due to the differences in phenolic fractions, especially free vs bound phenolic constituents. In general, thermal processing can lead to the dissociation of conjugated phenolic forms followed by some polymerization/oxidation of phenolic constituents, which can increase the free phenolic

content in processed food samples (Randhir *et al*., 2008; Ranilla *et al*., 2009). Previously, Clifford (2000) suggested that cinnamic acid derivatives such as caffeic acid, ferulic and *p*-coumaric acid are mostly found in conjugated form and they are generally released following thermal processing treatment. Therefore, the specific phenolics profile found in this study was positively corroborated with the previous findings of thermal processed grain and bean samples. The changes in specific phenolics profile of barley tea and coffee sample with different steeping times is not only just relevant for understanding the bioactive constituents but is important in human health associated functionalities such as antioxidant and anti-hyperglycemic properties of these barley-based beverages.

10.4.3. Total Antioxidant Activity

The total antioxidant activity of barley tea and coffee samples was measured based on their capacity to reduce two types of free radicals, i.e., DPPH and ABTS. Like with the TSP content of the barley beverage samples, BC1 and BC2 had higher total antioxidant activity based on both DPPH and ABTS-based scavenging activity, when compared to two BT samples. Proportionate increase in total antioxidant activity (DPPH) with increased in steeping time was also observed, however the difference between sample and steeping time interactions was not statistically significant (Fig 10.2A & 2B).

Overall, higher mean antioxidant activity was found in ABTS free radical scavenging assay (Fig. 10.3A & B), when compared to the results of DPPH based antioxidant activity. The higher antioxidant activity with ABTS based assay indicated presence of more water-soluble antioxidants in barley tea and coffee, as ABTS free radicals showed higher affinity towards hydrophilic compounds (Re *et al*., 1999). In the case of BT samples, main effects such as sample type and steeping time and their interactions had statistically significant (at $p < 0.05$) impact on

ABTS free radical scavenging capacity. Among barley tea samples, BT1 (25.25 – 29.78 %) showed a much greater ability to scavenge DPPH free radicals when compared to BT2 (6.21 – 13.96%) (Fig 10.2A).

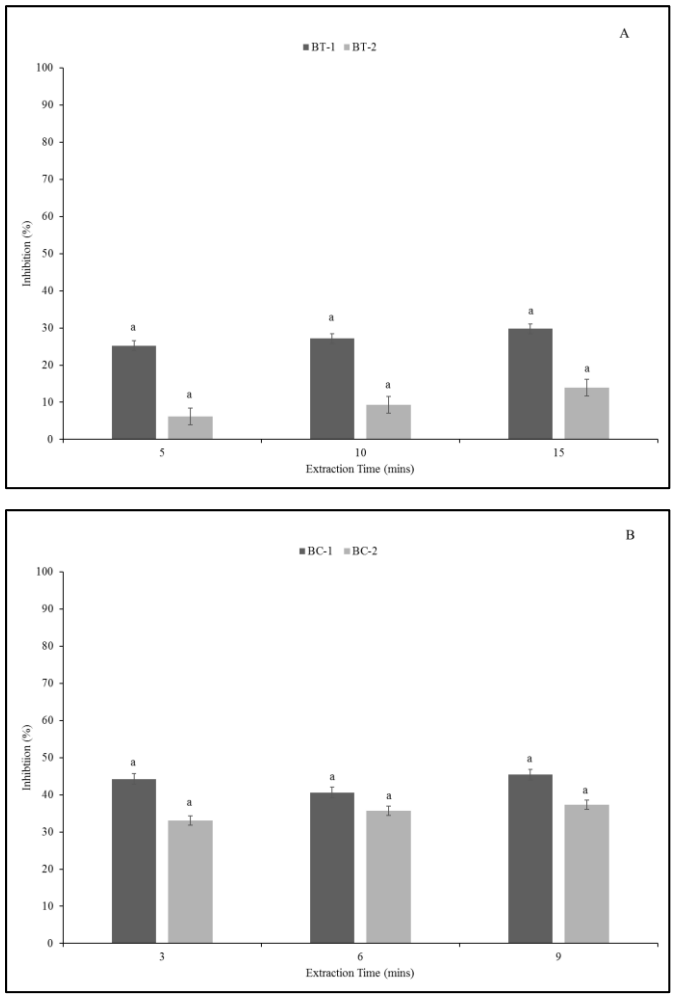

Figure 10.2. Total antioxidant activity based on DPPH free radical scavenging activity (%) of barley tea (A) and barley coffee(B) extracts. Different alphabets represent significant differences in antioxidant activity due to interaction between sample × steeping time at 95% confidence level ($p < 0.05$) separately for BT and BC.

The observations in this study corroborated with previous studies, as significantly high levels of antioxidant activity were reported in roasted barley-based substrates (Etoh *et al*., 2004; Oh *et al*., 2014). Oh at al. (2014) found that longer steep times significantly improved the ABTS and DPPH free radical scavenging capacity in aqueous extracts of roasted hulless barley. The

high antioxidant activity of barley-based beverages may be attributed to their phenolic bioactive content, especially the free phenolics that were observed in the current study (Table 10.1 A &B).

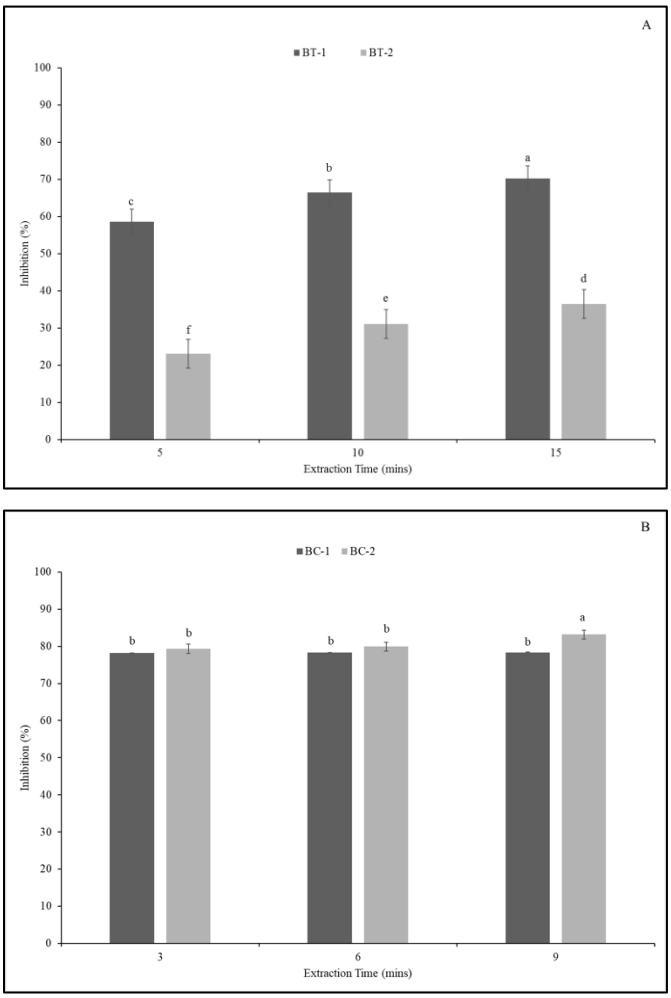

Figure 10.3. Total antioxidant activity based on ABTS free radical scavenging activity (%) of barley tea (A) and barley coffee (B) extracts. Different alphabets represent significant differences in antioxidant activity due to interaction between sample × steeping time at 95% confidence level ($p < 0.05$) separately for BT and BC.

Etoh *et al*. (2004) reported the presence of several phenolic compounds with potent antioxidant activity in barley tea, including p-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, p-hydroxybenzoic acid, vanillic acid, and *p*-coumaric acid. Among these compounds, 3,4-dihydroxybenzaldehyde, *p*-coumaric acid, quercetin, and isoamericanol A showed more potent antioxidant activities than that of BHT (butylated hydroxytoluene), a

commonly used commercial synthetic antioxidant. In the case of barley coffee, the antioxidant activity may arise from the presence of antioxidants such as gallic acid, catechin, caffeic acid, vanillin, chlorogenic acid, epicatechin and ferulic acid (Kreicbergs *et al*., 2011). In the current study, higher content of catechin was found in barley tea, while barley coffee had high cinnamic acid content. Both catechin and cinnamic acid of plant-based foods have shown strong antioxidant potentials, relevant for food quality and human health benefits (Amic *et al*., 2018; Zaiter *et al*., 2016). Furthermore, Omwamba *et al*. (2013) reported roasted barley grain extracts had very strong antioxidant and antiaging activity *in vitro* and *in vivo*, which was attributed to high concentrations of potent antioxidants such as individual phenolic acids and several polyphenolic compounds.

Additionally, the presence of Maillard reaction products (MRPs such as reductones and melanoidins) formed during the roasting process may also contribute to high antioxidant activity in barley tea and coffee (Goupy *et al*., 1999; Samaras, Gordon, and Ames, 2005). Papetti *et al*. (2006) found that the strong antioxidant activity of roasted barley coffee extract *in vitro* and *ex vivo* to a form of high molecular mass melanoidinic component, which was resistant to acid hydrolysis. Moreover, Papetti *et al*. (2006) also reported the naturally occurring components in unroasted barley grains to have relatively weak antioxidant capacity compared to the melanoidinic component. Furthermore, the roasting process may also lead to release of phenolic compounds generally bound to MRPs, which are capable of having significant antioxidant activity. Perrone, Farah and Donangelo (2012) reported an increase of melanoidin-bound phenolic compounds from 1% in common green coffee to 29% in roasted coffee. However, in the same study, it was observed that free phenolic acids, such as chlorogenic acid, contributed to the overall antioxidant activity than the combined forms by a significant margin. Similarly,

Samaras, Gordon, and Ames (2005) observed that the total antioxidant activity in kilned and roasted malt was due to the combination of glucose, proline and ferulic acid during Maillard reaction. Therefore, it can be inferred that the strong antioxidant activities observed in the samples of BT and BC in this study are due to the combined effects of diverse phenolic and non-phenolic compounds with high antioxidant potentials. Overall, the results of the current study indicated that both barley tea and coffee with high antioxidant activity are good dietary targets to counter chronic oxidative stress and associated pathophysiology of common NCDs such as early stages of T2D. However, for effective integration in dietary interventions to address early stages of T2D, it is also important to investigate potential anti-hyperglycemic properties of barley-based functional beverages, such as tea and coffee.

10.4.4. α-Amylase Inhibitory Activity

The ability of food ingredients or a food matrix to modulate the activity of α-amylase enzyme, and in turn regulating the hydrolytic digestion of starch into dextrins and reducing sugars in the upper digestive tract is an important metabolic target for determining its anti-hyperglycemic potential. Therefore, α-amylase enzyme inhibitory activity of the barley tea and coffee samples was measured using equivalent *in vitro* model assay to assess their potential efficacy in delaying the post-prandial breakdown of starch and for maintaining glucose homeostasis, which is critical for managing chronic hyperglycemia at pre- and post-diabetic stages.

In this study, low α-amylase enzyme inhibitory activity was observed in undiluted barley tea samples (Fig. 10.4 A), while undiluted barley coffee samples had moderate α-amylase enzyme inhibitory activity (Fig. 10.5A). Among barley tea samples, BT1 had significantly (at $p < 0.05$) higher α-amylase enzyme inhibitory activity when compared to BT2.

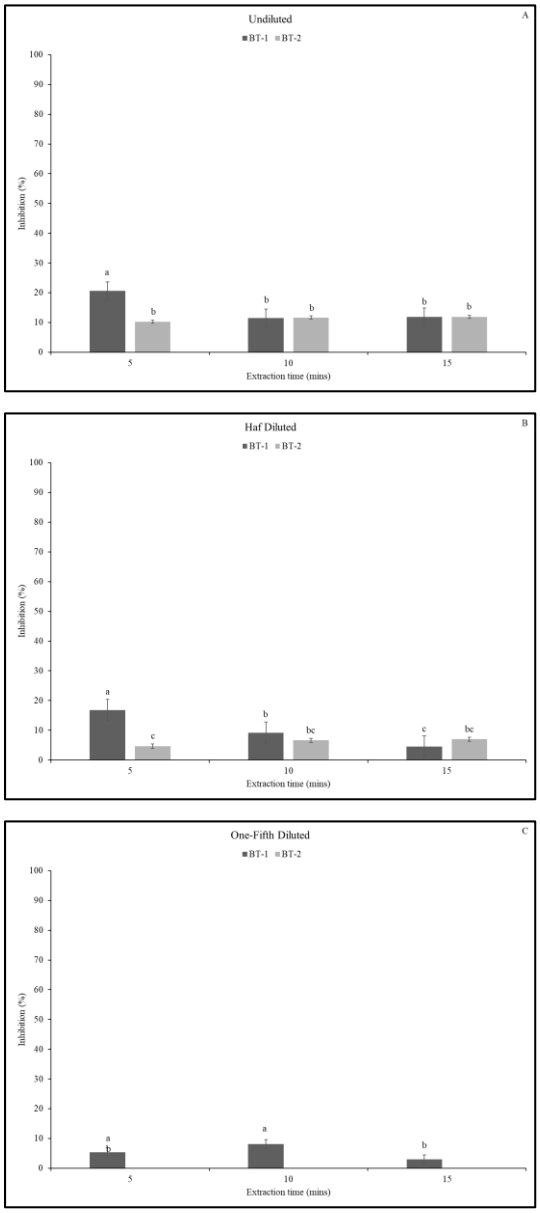

Figure 10.4. Inhibition of α-amylase enzyme activity (%) by barley tea samples. Different alphabets represent significant differences in α-amylase enzyme inhibitory activity in undiluted (A), half diluted (B) and one-fifth diluted (C) tea samples due to interactions between sample × steeping time at 95% confidence level ($p < 0.05$).

Additionally, a steep time of 5 min (lowest) was found to result in a relatively higher degree of α-amylase enzyme inhibitory activity, which was significantly different than values observed in extracts steeped for 10 and 15 min. Specifically, BT1 with 5 min steeping time (20.62%) combination had the highest and most significant level of α-amylase enzyme inhibitory

activity when compared to other sample × steeping time combinations. Therefore, the result indicated that increased steeping time had overall negative impact on α-amylase enzyme inhibition relevant anti-hyperglycemic functionalities of barley tea.

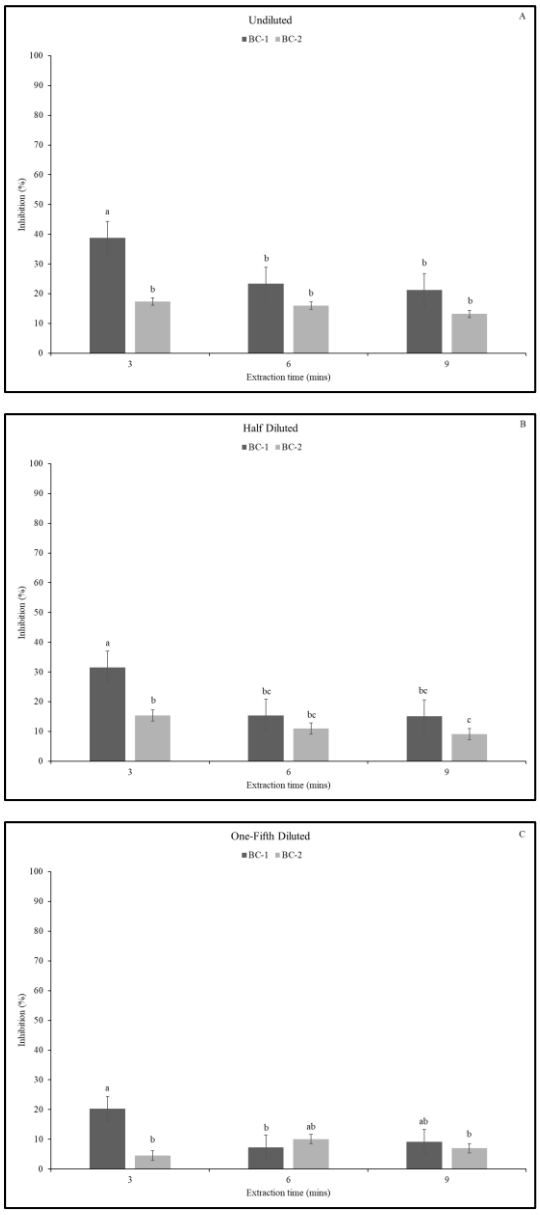

Figure 10.5. Inhibition of α-amylase enzyme activity (%) by barley coffee samples. Different alphabets represent significant differences in α-amylase enzyme inhibitory activity in undiluted (A), half diluted (B) and one-fifth diluted (C) coffee samples due to interactions between sample × steeping time at 95% confidence level ($p < 0.05$).

In the case of barley coffee samples, BC1 after 3 min of steeping exhibited a significantly higher level of α-amylase enzyme inhibitory activity (34.21%) when compared to other steep times. Therefore, in both barley tea and coffee samples, α-amylase enzyme inhibitory activity decreased with increased in steeping time. Like differences between barley tea samples, the α-amylase enzyme inhibitory activity of BC1 was significantly higher than those of BC2 across all steep times (Fig 10.5A). Notably, contrary to the trends observed in the case of TSP content and total antioxidant activity, an increase in steep time was inversely proportional to α-amylase enzyme inhibition, as seen in Fig 10.4A and 10.5A.

Overall, significant dose dependent response of α-amylase enzyme inhibitory activity was observed in both BT (Fig 10.4 A, B, & C) and BC samples (Fig 10.5 A, B, & C), which also corroborated with previous findings from barley grains and sprout studies (Ramakrishna *et al*., 2017a, 2017b Chapter 4 and 5). Furthermore, Anand *et al*. (2018) reported that roasted barley extracts aided in preventing symptoms of sucrose withdrawal in dysglycemic and dyslipidemic rats and the level of α-amylase inhibition observed (20.9±0.3%) was comparable with the range of values observed in undiluted BT and BC samples in the current study.

Ramakrishna *et al*. (2017a Chapter 4) reported that heat treatment during hot water extraction negatively impacted α-amylase inhibitory activity, and most hot water extracted samples did not show any inhibition, while cold water and 12% ethanol extracts of the same grains had moderate inhibitory activity. Interestingly, in the present study barley tea and coffee samples were subjected to heat treatment during roasting and subsequent extraction into hot aqueous solutions, which might have contributed to low α-amylase enzyme inhibitory activity in BT and BC samples, especially after longer steep time.

While some of this carbohydrate digestion relevant enzyme inhibitory activity of food and food ingredients may be attributed to the presence of free soluble phenolic compounds, as in the case of total antioxidant activity, it seems likely that other amylase inhibitors such as proteins and Maillard reaction products (MRP), or combined forms of MRPs and phenolic compounds may contribute to the overall α-amylase enzyme inhibitory activity of BT and BC samples. The degree and duration of roasting was found to be inversely proportional to the free total soluble phenolic content in cocoa, with the proportion of phenolic compounds becoming structurally incorporated into melanoidins depending on the severity of thermal treatment (Quiroz-Reyes and Fogliano; 2018). While roasting promoted formation of melanoidins and depolymerization of polyphenols, high phenolic content was also shown to promote melanoidins formation, which then may impart biological activities to the final product, like that of the original phenolic compounds (Quiroz-Reyes and Fogliano; 2018). In the current study, BT1, and BC1 exhibited high TSP content, high antioxidant, and low to moderate α-amylase enzyme inhibitory activity, which are relevant to manage chronic oxidative stress and chronic hyperglycemia and therefore can be targeted in dietary support strategies focusing on early stages of T2D benefits.

10.4.5. α-Glucosidase Inhibitory Activity

α-Glucosidase catalyzes the successive hydrolysis of terminal α-d-glucose residues from the non-reducing ends of polysaccharide chains with the release of α-d-glucose (Frandsen and Svensson, 1998). The ability of a food matrix to modulate the activity of α-glucosidase enzyme allows for the regulation of final breakdown and absorption of glucose in the brush border cells of the small intestine (Frandsen and Svensson, 1998).

Therefore, the ability of the barley tea and coffee samples to modulate the activity of α-glucosidase enzyme was determined *in vitro* to assess their efficacy reflecting potential in

delaying the post-prandial breakdown of sugars and dextrins and absorption of glucose in the lower gastrointestinal tract.

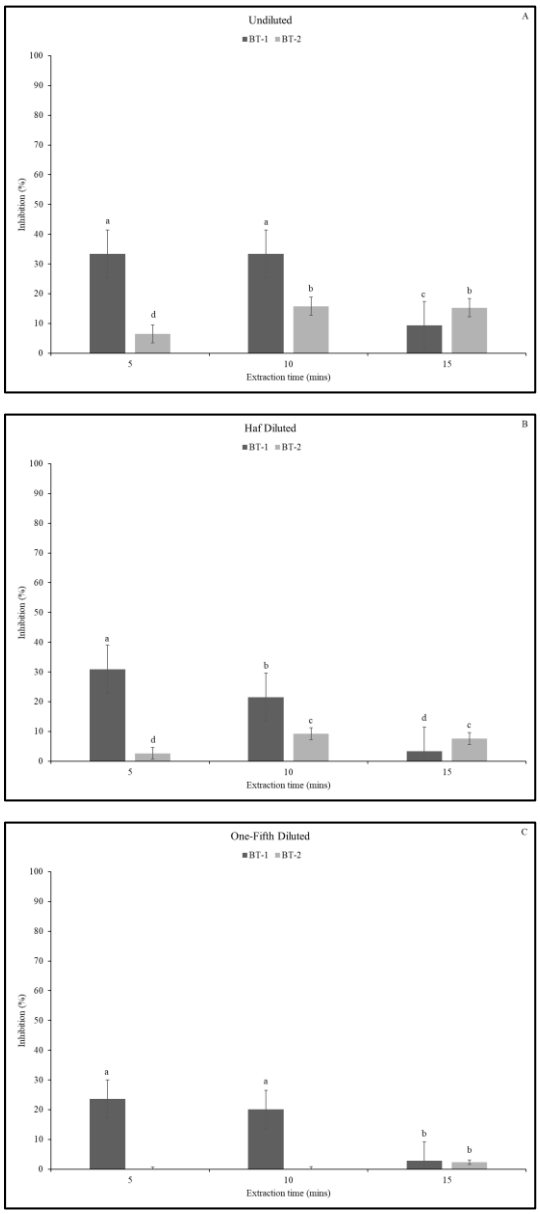

Figure 10.6. Inhibition of α-glucosidase enzyme activity (%) by barley tea samples. Different alphabets represent significant differences in α-glucosidase enzyme inhibitory activity in undiluted (A), half diluted (B) and one-fifth diluted (C) tea samples due to interaction between sample × steeping time at 95% confidence level ($p < 0.05$).

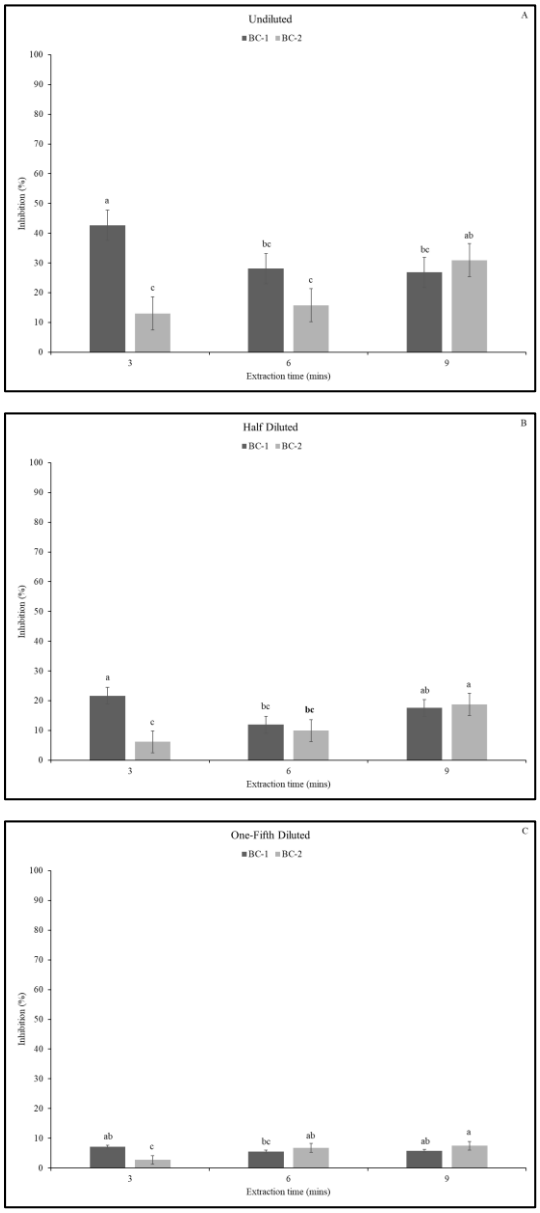

Figure 10.7. Inhibition of α-glucosidase enzyme activity (%) of barley coffee samples. Different alphabets represent significant differences in α-glucosidase enzyme inhibitory activity in undiluted (A), half diluted (B) and one-fifth diluted (C) coffee samples due to interaction between sample × steeping time at 95% confidence level ($p < 0.05$).

In the current study, low to moderate α-glucosidase enzyme inhibitory activity was observed in most BT and BC samples across all steeping times. Additionally, certain sample × steep time combinations had statistically significant effect on α-glucosidase enzyme inhibitory activity. As in the case of α-amylase inhibitory activity, a clear dose dependent response of α-

glucosidase enzyme inhibitory activity was observed among BT and BC samples with three different dilution levels (undiluted, half-diluted, and one-fifth diluted) (Fig 10.6 A, B, C and Fig 10.7 A, B, C).

For barley tea sample, differences in α-glucosidase enzyme inhibitory activity due to sample type and steeping time were statistically significant. Among two samples, BT1 had significantly higher level of α-glucosidase enzyme inhibitory activity when compared to the BT2. Interestingly, α-glucosidase enzyme inhibitory activity did not change or reduced from steeping time of 5 to 10 min, however it decreased significantly ($p < 0.05$) with further increased in steeping time to 15 min (Fig 10.6A). Therefore, while high α-amylase enzyme inhibitory activity was observed with 5 min steeping, for α-glucosidase enzyme inhibitory activity the optimum steeping time for barley tea was between 5-10 min.

Like barley tea sample, significant variations in α-glucosidase enzyme inhibitory activity were also observed between two barley coffee samples, as BC1 extracts had higher inhibitory activity when compared to BC2. More specifically, BC1 steeped for the shortest duration (3 min) had the highest α-glucosidase enzyme inhibition among barley coffee samples (42.7 %) (Fig 10.7A). For BC1, longer steeping time corresponded with a decrease in α-glucosidase inhibitory activity, while the opposite trend was observed in the case of BC2 (Fig 10.7A).

The levels of α-glucosidase enzyme inhibitory activity of BT and BC samples in the present study were higher than the results of the previous study where hot water extracts of 13 barley cultivars were evaluated for antioxidant and anti-hyperglycemic functionalities using same *in vitro* assays (Ramakrishna *et al*., 2017a). Previously, Anand *et al*. (2018) reported that roasted barley extracts helped to normalize the glycemic response in chronic sucrose-induced dysglycemia and dyslipidemia in rat model. Furthermore, the level of α-glucosidase enzyme

inhibition observed (29.0±0.6%) in the previous study by Anand *et al*. (2018) was comparable to the values found in this current study with barley tea and coffee. The results of both α-amylase and α-glucosidase enzyme inhibitory activities suggested that barley tea and coffee had low to moderate anti-hyperglycemic functionalities. However, it is important to optimize roasting (heat treatment) and steeping time to improve these anti-hyperglycemic functionalities prior to integrating them in dietary support strategies for early stages of T2D benefits.

10.4.6. LAB Proliferation and *Helicobacter pylori* Inhibition

A key aspect of value addition to cereal-based beverages and foods is to improve their health-relevant functionalities such as antioxidant, anti-hyperglycemic and human gut health benefits. In the context of human gut health improvement, supporting the growth of beneficial microorganisms in the lower gastrointestinal tract is critical for diverse human health protective metabolic functions, including glucose metabolism. The proliferation of microorganisms in the gut, which predominantly tend to be beneficial lactic acid bacteria (LAB), impart additional health benefits such as improved gut function and digestion, and release of protective bioactive compounds including transformed phenolic bioactives from the food matrices during digestive processes. Therefore, it was of interest to determine whether the barley tea and coffee samples exerted a positive or negative effect on the growth of specific beneficial LAB strains (*B. longum* and *L. helveticus*).

Based on the results of preliminary bioactive functionality assays, sample × steep time combinations with the highest TSP and high antioxidant activity were chosen for the LAB proliferation assays. From the various combinations, samples corresponding to 15 min and 9 min steep times were chosen for barley tea and coffee samples respectively (hereafter referred to as BT1-15, BT2-15, BC1-9, and BC2-9).

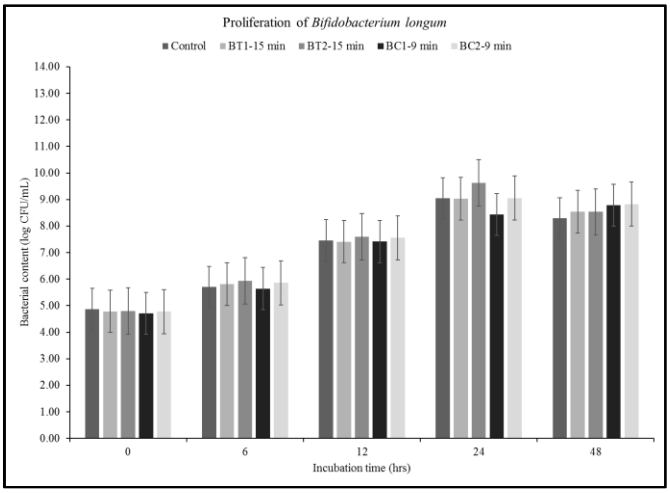

Figure 10.8. Viable cell counts of *Bifidobacterium longum* (log CFU/mL) in select combinations of barley tea and barley coffee samples over a period of 48 hours.

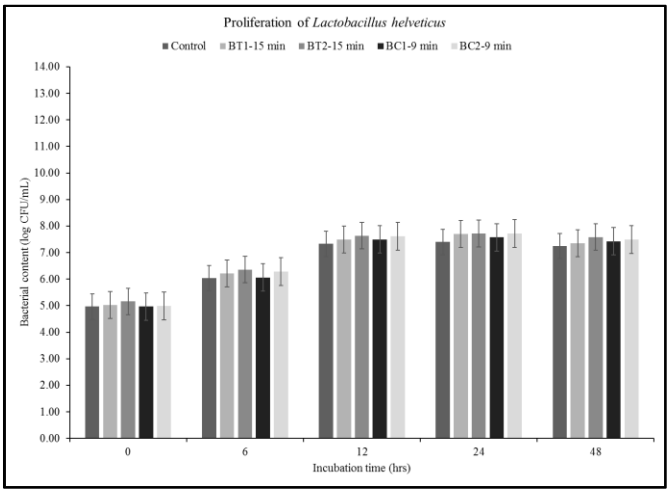

Figure 10.9. Viable cell counts of *Lactobacillus helveticus* (log CFU/mL) in select combinations of barley tea and barley coffee samples over a period of 48 hours.

Overall, for the samples inoculated with both *B. longum* and *L. helveticus*, the viable cell counts increased proportionally with the duration of incubation (Fig 10.8 and 10.9). In the case of *B. longum*, the number of viable cells in the samples and controls were comparable at 0, 6 and 12 h. However, BT2- with 15 min steeping time exhibited a relatively higher cell count compared to the corresponding control at 24 h. Furthermore, the cell count of *B. longum* was higher in all sample × steep time combinations when compared to the control at 48 h.

In the case of *L. helveticus*, the trend observed was slightly different than that of with *B. longum* growth, as a steady increase in viable cell count from 0 to 12 h was observed. Beyond this stage, cell growth seemed to have reached a plateau, and did not change significantly between 12 to 48 h incubation. Among the samples under consideration, BT2-15 exhibited higher viable cell counts compared to the controls and other sample × steep time combinations at 0 and 6 h of incubation.

The results indicated that the select BT and BC samples with high TSP content and antioxidant activities did not negatively impact the growth of beneficial LAB. On the contrary, certain samples (BT2-15) were found to promote the growth of these beneficial gut bacteria at specific time points. These observations revealed the potential for developing barley-based functional beverages such as barley tea with integrated antioxidant, antihyperglycemic and gut-health benefits. However, it is also important to understand the effect of these potential functional beverages on pathogenic gut bacteria such as *Helicobacter pylori* for their more comprehensive and wider health-focused applications.

Individuals who are prediabetic or fully diabetic are known to be at a greater risk of developing infections, including by *H. pylori* (Bener *et al*., 2007). Conversely, certain studies have indicated that persistent *H. pylori infections* may be associated with an elevated risk of developing T2D and other NCDs (Jeon *et al*., 2012). Therefore, in this study it was of interest to investigate the potential inhibitory activity of BT and BC extracts on *H. pylori.* However, the combinations of barley tea and coffee with different steeping time studied did not produce any visible inhibitory activity against *H. pylori,* and therefore data is not presented for this assay. Further studies with different concentrations and extraction protocols are needed to better understand the potential anti-bacterial activity of barley-based tea and coffee. However, the

positive impact of barley tea on beneficial LAB also has relevance, as beneficial gut bacteria help to protect integrity of intestinal barrier and can suppress the growth of pathogenic bacteria such as *H. pylori* (Wong *et al*., 2019). Therefore, beyond its potential active anti-bacterial activity, barley-based functional beverage can also provide gut health support by promoting beneficial microorganisms and subsequently potentially supporting diverse health protective metabolic functions such as anti-inflammation, anti-hyperglycemic, immune response, and protection against chronic infection.

10.5. Conclusions

In this study, barley-based beverages, tea, and coffee derived from unmalted and roasted barley and from two different commercial sources were compared and investigated for their potential phenolic-linked antioxidant, anti-hyperglycemic, and human gut health benefits using *in vitro* assay models. Overall, high phenolic-linked antioxidant activity and moderate anti-hyperglycemic functionalities were observed in both barley tea and coffee samples. High catechin content was found in barley tea samples, while cinnamic acid was the predominant phenolic compound in barley coffee samples. However, this phenolic-linked antioxidant and T2D relevant benefits of barley tea and coffee targeted for early stages of disease varied widely between types of the commercial products and also due to the variations in steeping times. The phenolic-linked antioxidant activity of barley tea and coffee samples investigated in this study increased proportionately with increase in steeping time, while individual phenolic compounds content and anti-hyperglycemic property relevant α-amylase and α-glucosidase enzyme inhibitory activities decreased with increase in steeping time. Most significantly, mild proliferation of beneficial LAB (*B. longum*) was observed in barley tea samples with high phenolic content and high antioxidant activity. Therefore, the results of the present study

indicated that targeted barley tea and coffee samples have potential to be good dietary targets as functional beverages to address complex metabolic breakdowns commonly associated with early stages of T2D and provides foundation for further studies towards *in vivo* validation. Further in *vivo* models using animal studies are required to optimize food designs with improved roasting processes and other processing and beverage preparation variables prior to the effective integration of barley-based functional beverages such as tea and coffee in dietary solutions targeting early stages of T2D.

CHAPTER 11. INTEGRATED BIOPROCESSING STRATEGY TO IMPROVE PHENOLIC-LINKED ANTIOXIDANT, ANTIHYPERGLYCEMIC AND HUMAN GUT HEALTH BENEFITS IN FOOD BARLEY

11.1. Abstract

Novel bioprocessing strategy by integrating sprouting with beneficial lactic acid bacteria (LAB)-based fermentation of cereal grains, like barley is an effective approach to improve bioavailability and stability of bioactive compounds for designing functional foods and ingredients with wider health benefits. In the thesis earlier studies indicated improvement of phenolic-linked antioxidant and anti-hyperglycemic functionalities in sprouted/germinated malting barley seeds. Based on these promising findings the primary aim of this study was to advance sprouting strategy (0-48 h) in three hull-less food barley, such as unpigmented, purple, and black barley to improve phenolic-linked antioxidant and anti-hyperglycemic properties. Additionally, Kefir culture with mixed beneficial LAB strains was recruited to ferment aqueous extracts of sprouted barley flour for up to 72 h and changes in phenolic-linked antioxidant and anti-hyperglycemic functionalities were evaluated using *in vitro* assay models. The biochemical parameters investigated were total soluble phenolic content, profile of phenolic compounds, total antioxidant activity, and anti-hyperglycemic property relevant α-amylase and α-glucosidase enzyme inhibitory activities. Furthermore, human gut health benefits relevant properties of sprouted and fermented barley flour extracts were also evaluated based on growth of dairy Kefir culture containing mixed LAB strains and through determining potential anti-bacterial property against pathogenic human ulcer causing bacteria *Helicobacter pylori*. Sprouting barley up to 48 h alone and in combination with Kefir-culture mediated fermentation significantly improved the total soluble phenolic content of aqueous extracts of food barley flours. The major phenolic

compounds found in sprouted-unfermented and fermented barley flour were protocatechuic acid, cinnamic acid, gallic acid and dihydroxybenzoic acid, while ferulic acid was only observed in pigmented (purple and black) food barley samples. Higher protocatechuic acid content was observed in barley flour extracts with combination of 48 h sprouting and 72 h Kefir culture-mediated fermentation, when compared to the un-germinated and unfermented control. Additionally, 48 h sprouted barley flour extracts had significantly high antioxidant activity (based on DPPH and ABTS free radical scavenging capacity) as well as moderate anti-hyperglycemic property relevant α-amylase and α-glucosidase enzyme inhibitory activities in unpigmented food barley. Similarly, improvement of antioxidant and anti-hyperglycemic functionalities were also observed after 72 h of Kefir-mediated fermentation of sprouted barley flour extracts. Additionally, anti-bacterial property relevant inhibition of *H. pylori* and active growth of viable LAB cells above the minimum level required for probiotic activity were also observed in combination of sprouted and fermented food barley flour extracts. The results of this study indicated that sprouting/germination in combination with mixed LAB-culture (Kefir) mediated fermentation is a novel and viable strategy for developing functional phenolic enriched food matrices and such integrated bioprocessing strategy can be rationally recruited to improve antioxidant, anti-hyperglycemic and gut health benefits in food barley for integrating in type 2 diabetes relevant dietary support strategies validated with further *in vivo* studies.
Keywords: Antioxidant; Anti-hyperglycemic; Food Barley; Fermentation; Kefir culture; Phenolics; Sprouting

11.2. Introduction

The overall awareness of healthy diets is growing rapidly among consumers, which has increased demand for new functional foods and beverages with added health benefits. Among

diverse healthy food choices, probiotic foods are fermented formulations containing sufficient numbers of selected live microorganisms that beneficially modify the intestinal microbiota of the host and concurrently provide several health benefits (Alander *et al*., 1999; Fuller, 1989; Havenaar and Huis Veld, 1992). In contemporary market, most common probiotics are dairy based. However, cereal grains can be an healthier alternative for developing novel non-diary probiotic foods to mitigate disadvantages associated with fermented dairy products like lactose intolerance, allergy, and the adverse impact on body cholesterol levels.

Due to such increasing demands for whole grain based healthy and probiotic foods, there is a resurgent interest in extending the utilization of barley beyond feed, malting, and brewing applications. Previously published studies have reported that food barley is rich in health-promoting functional compounds, including phytochemicals such as phenolic bioactives (Ramnarain *et al*., 2019; Idehen, Tang and Yang, 2017; Hung, 2015; Okarter and Liu; 2010; Dykes and Rooney, 2007; Holtekjølen, Kinitz and Knutsen, 2006; Wannenmacher *et al*., 2018). Phenolic antioxidants from barley have demonstrated significant antiproliferative, anti-carcinogenic, and anti-inflammatory properties in various *in vitro* models (Ramnarain *et al*., 2019; Idehen, Tang and Yang, 2017; Hung, 2015; Okarter and Liu, 2010). In previous studies of this thesis, germinated sprouts of malting barley cultivars have shown high antioxidant and anti-hyperglycemic functionality in *in vitro* model based assays (Ramakrishna, 2017a, b).

Due to their carbohydrate metabolism relevant enzyme inhibitory potential and other functional benefits, dietary phenolic bioactives, including those from whole grains like barley may play a critical role in augmenting current pharmacological treatment strategies for preventing type 2 diabetes (T2D) linked chronic hyperglycemia (Ramakrishna *et al*., 2019; Panhwar *et al*., 2018; Belobrajdic and Bird, 2013). Sprouting/germination potentially liberate

insoluble, cell-wall bound phenolic compounds into biologically available and active soluble forms during grain modification (Singh and Sharma, 2017; Wang, He and Chen, 2014; Donkor *et al*., 2012). Beyond its antioxidant and glucose metabolism modulating functions, the solubilized phenolic compounds also exert anti-bacterial effects, by virtue of the toxicity of the phenolic groups and by complementing the activity of short chain fatty acids produced by beneficial gut bacteria against pathogenic bacteria (Espín, González-Sarrías and Tomás-Barberán, 2017; Vitaglione, Napolitano, Fogliano; 2008). Additionally, the products derived from the grain modification during sprouting – such as hemicellulose and oligosaccharide fractions and glutamine-rich peptides, can aid the growth and proliferation of beneficial bacteria in the human gut resulting in potential probiotic and gastro-protective benefits (Slavin, 2010; Bamba *et al*., 2002; Kanauchi *et al*., 1999). Therefore, sprouted food barley potentially serves as a phenolic-rich functional food ingredient with several human health protective benefits, such as antioxidant, antihyperglycemic and gut health improvements (Ramakrishna *et al*., 2019; Idehen, Yang and Tang, 2017).

Current published evidence in this area mostly focused on the sprouting/germination-linked mobilization of phenolic compounds in barley in the context of malting and brewing applications (Baik and Ullrich, 2008; Newman and Newman, 2008). However, sprouting-induced phenolic mobilization is regarded as detrimental to malt (and beer) quality. Currently, there is limited published literature on phenolic mobilization and solubilization in food barley, and their potential impact on improving human health-related functional properties of barley-based food ingredients such as barley flour. In general, controlled germination, or sprouting, improves the phenolic profile and associated antioxidant activity due to mobilization and solubilization of bound insoluble phenolic compounds in the cell wall of the grain (Wang *et al*.,

2014; Singh and Sharma, 2017). Consequently, functional parameters of sprouted barley flour such as anti-hyperglycemic potential, probiotic LAB proliferative potential and anti-microbial such as *Helicobacter pylori* inhibitory potential would also be expected to improve (Ramakrishna *et al*., 2017b).

Like sprouting, fermentation of whole grain cereals with beneficial lactic acid bacteria (LAB) is also gaining increased interest from food industry and consumers. During fermentation, the grain constituents are modified by the action of both endogenous and bacterial enzymes, including esterases, xylanases, and phenoloxidases, thereby affecting their structure, bioactivity, and bioavailability (Nkhata *et al*., 2018, Wang, He and Chen, 2014). Cereal-based LAB fermentation has been shown to increase the levels of nutrients including folates, soluble dietary fiber, and total phenolic content in cereals (Perez-Jimenez *et al*., 2009) and to improve the protein digestibility and short chain fatty acid (SCFA) production *in vitro* (Anson *et al*., 2009; El Hag *et al*., 2002). Most cereal-based fermentation studies focused on the fermentation of rye and wheat for the baking industry, although barley has been shown to be appropriate substrates for LAB fermentation with diverse health-focused food application potential. Interestingly, barley-based fermented products have been reported to decrease total cholesterol and increase fecal concentration of probiotic bacteria like *Bifidobacterium* spp. in healthy individuals (Mårtensson *et al*., 2002). However, the effect of LAB fermentation on the content of free and bound phenolic compounds and their bioavailability and human health relevant functionalities such as antioxidant and anti-hyperglycemic properties has not been studied extensively in food barley.

Therefore, the broad objective of this study was to investigate the combined effect of controlled germination/sprouting and fermentation with beneficial LAB on the mobilization and

solubilization of phenolic compounds in food barley types that may support diets and ingredients in addressing chronic oxidative stress and chronic hyperglycemia commonly associated with early stages of type 2 diabetes. The specific aim of this study was to evaluate the changes in the total phenolic content, antioxidant activity, anti-hyperglycemic property, and beneficial gut health benefits relevant anti-bacterial activity (*H. pylori*) in sprouted barley flour extracts during 72 h of fermentation with beneficial mixed culture bacteria obtained from commercial Kefir product. The selection of commercial Kefir-based mixed culture for fermentation of sprouted barley flour extract was based on the previous promising findings of phenolic mobilization and improved antioxidant and anti-diabetic functionalities in Kefir culture mediated fermentation of soymilk (McCue and Shetty, 2005; Kwon *et al*., 2006). Therefore, in this current study, both sprouting, and Kefir culture mediated fermentation were integrated and targeted as a novel bioprocessing strategy to improve mobilization of health protective phenolics and to enhance associated antioxidant, anti-hyperglycemic, and gut health benefits relevant functionalities in food barley for type 2 diabetes benefits relevant functional food application.

11.3. Materials and Methods

11.3.1. Preparation of Sprouted Barley Flour

Three types of food barley with varying pigmentation (unpigmented/colorless – UB; black – BB; purple – PB) were obtained from a local grocery store (Walmart, Fargo, ND, USA). Barley kernels (100 g) were steeped in distilled water (400 mL) for 24 h at 20ºC, while being agitated on a rotary shaker (150 rpm). Steeped barley kernels were then wrapped in clean cheese cloth and sprouted/germinated (20ºC; > 90% relative humidity) inside controlled environment incubator. The sprouts were moistened and turned periodically to avoid the matting of rootlets, and to ensure adequate aeration.

Sprouted grains were removed from the containers at 24 h and 48 h after steeping and desiccated in an incubator (40ºC; 48 h) until the dry weight of the kernels remained constant. Prior to collecting sprout samples, the rate of germination (%) was also recorded at each assay time point. The rootlets of the desiccated barley sprouts were removed by hand and the grains were milled to a fine flour using a disc-mill (WonderMill, Pocatello, ID), sealed in polypropylene zip-lock bags and refrigerated until further analysis.

11.3.2. Sample Extract (Cold Water) Preparation

Milled barley flour samples were combined with cold water (1:5, w: v) and homogenized using a benchtop blender (Waring Products Co, CT, USA) for 5 min. The homogenate was transferred to polypropylene tubes and centrifuged at 8500 rpm for 20 min. The supernatant was decanted and centrifuged for the second time at 8500 rpm for 15 min. The decanted supernatants were collected and stored at 4°C for further biochemical analysis.

11.3.3. Kefir Culture Preparation, Inoculation and Fermentation

The method used in Kefir culture preparation was adapted from Fujita *et al*. (2017) and Kwon *et al*. (2006). Commercially available non-fat milk Kefir (100 µL; Lifeway Foods Inc., Morton Grove, IL) was aseptically inoculated into MRS broth (10 mL; Difco, Becton, Dickinson and Co., Franklin Lakes, New Jersey, USA) for 24 h at 37 °C. The same steps were repeated with an aliquot (100 µL) of this culture, and the second set of cultures were used to inoculate the fermented barley flour slurries.

Samples of food barley slurries were aliquoted (25 mL) into sterile polypropylene centrifuge tubes, sealed and pasteurized by immersion in a water bath (80ºC, 10 min) and immediately transferred into an ice bath for 10 min.

Samples were aseptically inoculated with the Kefir cultures (2.5 mL). Corresponding controls, to which 2.5 mL of sterile distilled water was added instead of inoculum, were also included for each sample × culture combination. All tubes were placed in a closed incubator (VWR) maintained at 37°C and samples at 0, 24, 48 and 72 h post-inoculation along with a corresponding control tube were removed for analysis. At each time point, half of the sample was transferred to a separate tube and the pH was adjusted to the level of the corresponding control, in order to evaluate the effect of acidification from lactic acid production on the evaluated functional biochemical parameters. After viable cell counts were measured, samples were centrifuged at 15000g for 15 min prior to carrying out *in vitro* assays as outlined in later sections.

11.3.4. Total Soluble Phenolic (TSP) Content

Total soluble phenolic (TSP) content of the sprouted barley flour extracts with and without Kefir mediated fermentation was determined using the Folin-Ciocalteu (FC) method as described by Shetty *et al*. (1995). The sample extract (1 mL) was combined with 95% ethanol (1 mL), distilled water (5 mL), FC reagent (0.5 mL; 50% v/ v), and Na_2CO_3 (1 mL; 5% v/v) in a test tube, vortexed, and incubated in the dark for 60 min. Absorbance values were measured at 725 nm using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). From this data, TSP content of the samples was calculated using a gallic acid standard curve (10 – 300 μg/mL; dissolved in 95% ethanol) and expressed as milligram equivalents of gallic acid (GAE) per gram dry weight (DW) of the samples.

11.3.5. Determination of Major Phenolic Compounds using HPLC

Sprouted barley flour extracts before and during fermentation were collected and stored in micro-centrifuge tubes in the freezer prior to the analysis. The frozen samples were thawed

and micro-centrifuged for 10 min at 13000 rpm, from which a small volume (5 µL) of the sample was subjected to chromatographic analysis using reverse phase HPLC (Agilent 1260 Infinity Series equipped with DAD 1100 diode array detector; Agilent Technologies, Palo Alto, CA). A gradient elution method, involving 10 mM phosphoric acid (pH 2.5; Solvent A) and 100% methanol (Solvent B), was used. Sample extracts were eluted on a C-18 analytical column (Agilent Supelco SB-C18 250 x 4.6mm internal diameter) with a packing material particle size of 5 µm, at a flow rate of 0.7 mL/min at ambient temperature, with a total run time of 25 min. All phenolic compounds of barley flour and fermented samples were quantified at 230 nm, except ferulic acid, which was quantified at 280 nm. Pure standards of gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, quercetin, cinnamic acid, dihydroxybenzoic acid, benzoic acid and p-coumaric acid in 100% methanol were used to calibrate retention times on the standard curve. Each sample was run in duplicate, and the chromatograms obtained were analyzed using the Agilent Chemstation integration software.

11.3.6. DPPH Free Radical Scavenging Assay

The total antioxidant activity of the sprouted and fermented barley flour extracts was measured using a modified DPPH (2, 2- diphenyl-1-picrylhydrazyl; Sigma Chemical Co.) free radical scavenging assay (Cervato *et al*., 2000; Kwon *et al*., 2007). Barley flour extracts (0.25 mL) were combined with 60 µM DPPH working stock solution (adjusted to an absorbance range of 1.8 – 2.0 at 517 nm), vortexed, and incubated in the dark for 5 min. For control, 95% ethanol (0.25 mL) was combined with the DPPH solution, instead of the sample extracts. Antioxidant activity of the flour extracts was then calculated based on the change in absorbance of the reaction mixture during the incubation period as per the following formula:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

11.3.7. ABTS Free Radical Scavenging Assay

The antioxidant activity of sprouted and fermented barley flour extracts was also measured by the ABTS+ free radical cation-decolorization assay (Re *et al*., 1999). A stock solution of ABTS (2,2–azinobis (3-ethylbenzothiazoline-6-sulfonic acid); Sigma Chemical Co., St. Louis, MO) was prepared by combining 7 mM ABTS (5 mL) with 140 mM potassium persulphate (88 µL). The mixture was incubated for at least 16 h prior to the assay. The stock solution was then diluted with 95% ethanol to prepare a working solution with an absorbance of 0.70 ± .02 at 734 nm. Flour extract (50 µL) was mixed with the working solution (1 mL), vortexed for 30 sec, incubated for 2.5 min. Absorbance values of the mixtures was then read at 734 nm. Controls were prepared for each sample extract substituting 50 µL of 95% ethanol in place of the extract. The percent inhibition was calculated by the following equation:

$$Inhibition\ (\%)\ =\ \frac{\Delta Abs_{control}\ -\ \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

11.3.8. α-Amylase Inhibitory Activity

The α-amylase enzyme inhibitory activity was determined by an assay modified from the Worthington Enzyme Manual (McCue *et al*., 2005). Sprouted barley flour extracts (0.5 mL) before and after fermentation were mixed with 0.5 mL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine α-amylase (0.5 mg/mL) and incubated at 25°C for 10 min. Further, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixtures were further incubated at 25°C for 10 min. After this, 1 mL of 3, 5-dinitrosalicylic (DNS) acid was added and the reaction was stopped by incubating the mixtures in a boiling water bath (90- 100°C) for 10 min and then cooled to room temperature. The reaction mixtures were then diluted with distilled water to obtain control absorbance reading between 0.8 and 1.0 units at 540 nm. The absorbance of

sample blanks (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were also recorded. The final extract absorbance was obtained by subtracting its corresponding sample blank reading. The α-amylase enzyme inhibitory activity was calculated as percentage (%) inhibition as per the following equation:

$$Inhibition\ (\%) = \frac{Abs_{control} - (Abs_{extract} - Abs_{sample\ blank})}{Abs_{\ control}} \times 100$$

11.3.9. α-Glucosidase Inhibitory Activity

The α-glucosidase enzyme inhibitory activity was determined using an assay modified from the Worthington Enzyme Manual and as described by McCue *et al*. (2005). A volume of 50 µL of sample solution and 100 µL of 0.1M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 unit/mL) were incubated in 96-well microplates at 250C for 10 min. Following this, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals and reaction mixtures were incubated at 25°C for 5 min. Absorbance values were recorded before and after incubation at 405 nm using a microplate reader (Thermomax, Molecular Device Co., Sunnyvale, CA) and compared to a control containing 50 µL of buffer solution instead of sample extract. The α-glucosidase inhibitory activity was expressed as percentage (%) inhibition and was calculated per the following equation:

$$Inhibition\ (\%) = \frac{\Delta Abs_{control} - \Delta Abs_{sample}}{\Delta Abs_{control}}\ x\ 100$$

11.3.10. *Helicobacter pylori* Inhibition Assay

Helicobacter pylori was cultured according to Stevenson, Lucia, and Acuff (2000). Nutrient medium for *H. pylori* was prepared with special peptone (10 g/L), granulated agar (15 g/L), sodium chloride (5 g/L), yeast extract (5 g/L) and beef extract (5 g/L) in distilled water.

Broth media was prepared special peptone (10 g/L), sodium chloride (5 g/L), yeast extract (5 g/L), and beef extract (5 g/L) in distilled water. Prepared stock of *H. pylori* (1 mL) was added to test tubes containing 10 mL of sterile broth media and incubated at 37 °C for 24 h prior to inoculation by spread plate technique. The activated culture was later spread evenly into plates containing *H. pylori* growth agar to prepare bacterial lawn for the agar-diffusion assay.

The inhibitory activity of sample extracts towards *H. pylori* was analyzed by the agar-diffusion disc-assay method (Kwon *et al*., 2007). Under aseptic conditions, 100 µL of filter sterilized sprouted barley flour extracts with and without fermentation was dispensed on to sterile 12.7-mm diameter paper discs, while sterile distilled water was used as a negative control. The saturated discs were transferred to the surface of agar plates containing *H. pylori* microbial lawn and incubated at 37 °C for 48 h under anaerobic conditions in GasPak jars (Becton, Dickinson and Co., Sparks, MD., U.S.A.) with BD GasPak Campy container system sachets (Becton, Dickinson and Co., Sparks, MD., U.S.A.). The diameter of clear zones which indicate the region of *H. pylori* inhibition surrounding each disc was measured and expressed in millimeters. To determine dose-dependence, 50 and 75 µL of the sample were used. The entire procedure was repeated twice, consisting of duplicates for each sample (three discs per sample within each Petri dish).

11.3.11. Viable Cell Count of Beneficial LAB

Samples inoculated with the mixed Kefir LAB cultures were serial diluted using autoclaved distilled water, until 10−6 dilutions. Aliquots of the diluted sample (100 µL) were inoculated onto plates containing MRS Agar using the spread plate method and incubated for 48 h at 37 °C within an anaerobic BBL GasPak jar (Becton, Dickinson and Co., Sparks, MD., U.S.A.) with BD GasPak EZ anaerobe container system sachets (Becton, Dickinson and Co.,

Sparks, MD., U.S.A.). Plates containing 20–350 colonies were then selected for counting colonies, from which viable cell count (log CFU/ mL) of mixed LAB strains was calculated.

11.3.12. Statistical Analysis

A completely randomized design (CRD) was used for all *in vitro* assays in this study. The entire fermentation experiment was repeated twice (each run constituting a replicate), and during each repetition, 6 samples were analyzed from each treatment combination. Analysis of variance (ANOVA) was performed using data obtained from the *in vitro* assays using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC). In the case of unfermented food barley samples, statistical differences in bioactive functionality parameters due to the two main effects, i.e., barley type and duration of sprouting, and their interactions were determined using Tukey's least mean squares test at a confidence level of 95% ($p < 0.05$). For Kefir-fermented food barley samples, statistically significant differences between means in bioactive functionality parameters due to three main effects, i.e., barley type, pH adjustment and duration of sprouting, and their 2-way and 3-way interactions were determined using Tukey's least mean squares test at a confidence level of 95% ($p < 0.05$) and the results were presented in a separate ANOVA table (Table 11.1).

11.4. Results and Discussion

11.4.1. Rate of Germination

The rate of germination of all sprout samples was recorded prior to being transferred to the dryer, and the observations are presented in Table 11.1.

Table 11.1. Mean germination rate (%) of food barley grains post steeping.

Food barley type	0 hrs	24 hrs	48 hrs
UB	0	93	97
BB	0	93	96
PB	0	92	96

11.4.2. Total Soluble Phenolic Content and Individual Phenolic Compound Profile

Phenolic compounds are the most dominant type of secondary metabolites with diverse human health protective functions in cereal grains, such as barley (Idehen, Tang and Yang, 2017; Okarter and Liu, 2010). While barley grains are known to contain a considerable number of various types of phenolic compounds, their composition and content in barley-based food and beverages can vary widely depending on genotype, phenotype and based on growing environment and different food processing strategies (Ramnarain *et al*., 2019; Idehen, Tang and Yang, 2017). In this study, the impact of different types of food barley varying in pigmentation and the effect of integrated bioprocessing strategy involving sprouting and mixed Kefir LAB culture-based fermentation on total soluble phenolic (TSP) content of barley flour extracts were determined using the Folin-Ciocalteu method.

In the current study, statistically significant differences in TSP content between food barley type × sprouting duration interactions was observed. Among different food barley types with different pigmentation, unpigmented (UB) food barley had the highest TSP content when compared to the purple (PB) and black (BB) barley (at $p < 0.05$) (Fig. 11.1). Additionally, TSP content increased proportionally with increased duration of sprouting as aqueous extracts of 48 h sprouted flour extracts had significantly higher TSP content when compared to 24 h and un-germinated/sprouted barley flour extracts (Fig 11.1).

Interestingly, UB was counterintuitively found to have higher TSP content when compared to pigmented varieties – PB and BB. Similar reductions in TSP content were observed

with steeping of barley grains in other studies, and this may be attributed to the leaching of free, water soluble phenolic compounds such as anthocyanins and phenolic acids from the pericarp and testa during the soaking stage prior to germination, or due to formation of insoluble complexes with proteins (Lu *et al*, 2007; Sharma and Gujral, 2010). To understand the impact of steeping and potential leaching of phenolics, TSP content of steeped water (excluding barley sample) was also determined. Significantly higher TSP content in the purple barley (PB) and black barley (BB) steeped water sample (results not presented) was observed, while steeped water of UB had no detectable amount of TSP. The result indicated that PB and BB food barley samples had high free and water soluble phenolics that leached in the steeped water, which resulted in lower TSP content in its sprouted and un-sprouted flour (Fig. 11.1).

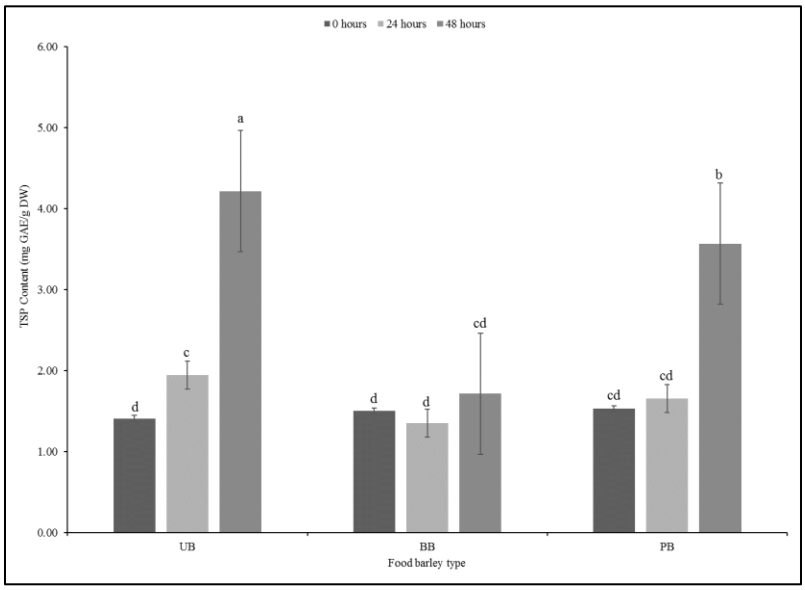

Figure 11.1. Total soluble phenolic (TSP) content of barley flour samples derived from unpigmented barley (UB), black barley (BB) and purple barley (PB) after varying durations of sprouting/germination (0, 24, 48 h). Different alphabets represent significant differences in TSP content due to interactions between barley type × sprouting duration at 95% confidence level ($p < 0.05$).

Several previous studies indicated that sprouting/germination of barley grains improved the TSP content of the sprouts (Gujral and Sharma, 2010; Ha *et al*., 2017; Ramakrishna *et al*.,

2017b; Singh and Sharma, 2017; Niroula *et al*., 2019). The increase in TSP content, especially phenolic compounds can result from – (a) biosynthesis during germination, and (b) the combined hydrolytic action of cell wall-degrading enzymes synthesized *de novo* (cellulases and endoxylanases) and endogenous cinnamoyl esterases and feruloyl esterases on cell wall bound phenolics (Singh and Sharma, 2017).

Improvement in TSP content was reported until 48 h of sprouting, following which it either plateaued or decreased due to the potential utilization of phenolic compounds in other anabolic processes such as lignification (Sharma and Gujral, 2010; Ha *et al*., 2017; Ramakrishna *et al*., 2017b). In conjunction with the current findings, 48 h of sprouting appears to be the ideal duration for sprouting-mediated improvement of TSP and its associated health functionalities. Improvements in TSP content was found to be specific for the food barley with varying pigmentations, as it increased by 198%, 133%, and 13% for UB, PB, and BB, respectively. The TSP results found for UB and PB particularly agreed with results of Ha *et al*. (2017) (217% increase from 0 to 48 h). This may be attributed to the differences in types of food barley types, as UB and PB had significantly higher increment in TSP content after 48 h sprouting. The findings of previous as well as current study suggested that sprouting is a highly viable and effective bio-processing strategy to improve TSP content, especially in food-grade barley grains (Wang, He and Chen, 2014; Ha *et al*., 2017, Ramakrishna *et al*., 2017b).

Like sprouting, Kefir- culture mediated fermentation was also observed to considerably impact the TSP content of the fermented food barley flour slurries. Statistically significant differences in TSP content due to the 2-way interaction between pH (P) × barley sprouting duration (G) as well as three main effects, i.e., pH, food barley type and duration of sprouting was observed at all fermentation time points ($p < 0.05$) (Table 11.1). Within each sample type,

48-h sprouted barley flour extracts was found to have the highest TSP content with and without fermentation (Fig. 11.2 A, B, C, & D). Overall, as fermentation progressed from 0 to 72 h, the TSP content increased proportionately across all sample combinations. While sprouting and Kefir-mediated fermentation both improved the overall TSP content of food barley extracts, the mechanism involved in improved TSP content might be different. Solubilization of low molecular weight phenolic compounds following LAB-fermentation can be attributed to the action of exogenous bacterial enzymes, such as feruloyl esterases, β-glucosidases, decarboxylases, hydrolases, reductases, and xylanases, on grain cell wall bound forms of phenolic compounds (Topakas, Vafiadi and Christakapoulous, 2007; Adebo and Medina-Meza, 2020).

Table 11.2. Analysis of variance table indicating mean square (MS) and significant differences between pH (P), food barley sample type (S) and barley sprouting times (G), with their respective interactions for phenolic content, antioxidant activity and anti-hyperglycemic activity in sprouted flours from three types of food barley samples at 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation with Kefir culture.

(A)

					α-Amylase Inhibition			α-Glucosidase Inhibition		
Source	df	TSP†	ABTS	DPPH	Undiluted	Half diluted	One-fifth diluted	Undiluted	Half diluted	One-fifth diluted
P	2	3.23*	9956.1*	1268.2*	2996.0*	9551.5*	1467.8*	4811.7*	2650.3*	951.4*
S	2	1.26*	4904.4*	48742.0*	1816.1*	1174.4*	2739.9*	542.5*	224.0*	108.9*
P×S	4	0.01 ns‡	0.81 ns	264.9*	0.92 ns	0.30 ns	2.93 ns	45.5*	36.0*	58.6*
G	2	3.92*	6770.5*	1905.9*	598.9*	11744.0*	11294.0*	5163.0*	3104.6*	2449.9*
P×G	4	0.47*	1695.0*	199.0*	4085.2*	8834.3*	2861.2*	1566.6*	785.1*	580.4*
S×G	4	0.00 ns	1.22 ns	32.2 ns	1.57 ns	0.82 ns	8.86 ns	136.8*	67.9*	16.7**
P×S×G	8	0.00 ns	3.04 ns	84.15*	3.99 ns	3.00 ns	5.84 ns	72.4*	59.3*	33.6*

(B)

					α-Amylase Inhibition			α-Glucosidase Inhibition		
Source	df	TSP	ABTS	DPPH	Undiluted	Half diluted	One-fifth diluted	Undiluted	Half diluted	One-fifth diluted
P	2	8.69*	7018.9*	1746.7*	3851.8*	1874.6*	1268.2*	1653.9*	795.7*	412.5*
S	2	1.19*	2986.5*	44174.0*	2036.71*	1207.8*	3109.4*	228.1*	76.0*	19.8**
P×S	4	0.00 ns	2720.7*	633.0*	6.71 ns	8.51 ns	0.63 ns	9.51 ns	2.56 ns	5.05 ns
G	2	10.1*	639.0**	1095.2*	2642.*	2500.6**	429.9*	3188.5*	2530.1*	917.*
P×G	4	2.25*	670.0*	245.5*	2777.7*	1724.7 ns	206.3*	3463.0 *	859.9*	289.6*
S×G	4	0.00 ns	87.6 ns	194.7*	10.4**	5.39 ns	1.57 ns	1.65 ns	0.80 ns	6.05 ns
P×S×G	8	0.00 ns	548.7 ns	12.1 ns	8.49**	3.18 ns	4.39 ns	6.66 ns	6.09 ns	2.29 ns

*Denotes significance at $p < 0.05$; **Denotes significance at $p < 0.001$; † TSP, total soluble phenolic content; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl free radical scavenging capacity; ‡ns, non-significant

Table 11.2. Analysis of variance table indicating mean square (MS) and significant differences between pH (P), food barley sample type (S) and barley sprouting times (G), with their respective interactions for phenolic content, antioxidant activity and anti-hyperglycemic activity in sprouted flours from three types of food barley samples at 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation with Kefir culture (continued).

(C)

					α-Amylase Inhibition			α-Glucosidase Inhibition		
Source	df	TSP	ABTS	DPPH	Undiluted	Half diluted	One-fifth diluted	Undiluted	Half diluted	One-fifth diluted
P	2	1.17*	8643.1*	673.8*	3804.2*	8505.2*	3395.8*	12400.0*	4075.4*	721.6*
S	2	1.32*	2016.4*	51148.0*	1936.0*	1307.1*	2827.0*	191.4*	51.4*	17.2**
P×S	4	0.00ns	724.6*	362.9*	4.34 ns	3.77 ns	3.93 ns	1.63 ns	1.72 ns	2.60 ns
G	2	12.28*	22.4 ns	427.1*	5666.6*	3249.2*	2003.0*	3279.1*	2183.5*	1090.1*
P×G	4	0.87*	793.8*	371.2*	3067.2*	2536.6*	1993.6*	710.0*	411.4*	65.0*
S×G	4	0.00 ns	1.39 ns	232.3*	2.68 ns	3.70 ns	5.24 ns	7.62 ns	1.45 ns	3.00 ns
P×S×G	8	0.00 ns	6.07 ns	68.7*	5.66 ns	4.52 ns	4.12 ns	4.21 ns	2.98 ns	4.14 ns

(D)

					α-Amylase Inhibition			α-Glucosidase Inhibition		
Source	df	TSP	ABTS	DPPH	Undiluted	Half diluted	One-fifth diluted	Undiluted	Half diluted	One-fifth diluted
P	2	0.47*	18762.0*	2822.7*	4156.5*	70172.0*	6889.8*	6749.5*	1567.6*	480.3*
S	2	1.27*	2602.7*	35911.0*	1080.5*	1798.1*	2778.4*	197.5*	35.6*	3.31 ns
P×S	4	0.00 ns	404.6*	785.2*	189.9*	8.45**	10.9**	7.16 ns	8.63 ns	11.5**
G	2	17.2*	509.3*	404.7*	668.3*	5729.4*	1277.4*	2361.5*	1435.1*	437.5*
P×G	4	2.65*	2064.8*	596.3*	343.3*	2104.3*	268.2*	565.3*	349.7*	65.63*
S×G	4	0.00 ns	24.6 ns	63.3*	28.3*	43.6*	2.56 ns	0.80 ns	2.07 ns	0.55 ns
P×S×G	8	0.00 ns	13.2 ns	52.8*	27.0*	36.4*	2.54 ns	2.09 ns	7.52 ns	4.66 ns

*Denotes significance at $p < 0.05$; **Denotes significance at $p < 0.001$; † TSP, total soluble phenolic content; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl free radical scavenging capacity; ‡ns, non-significant

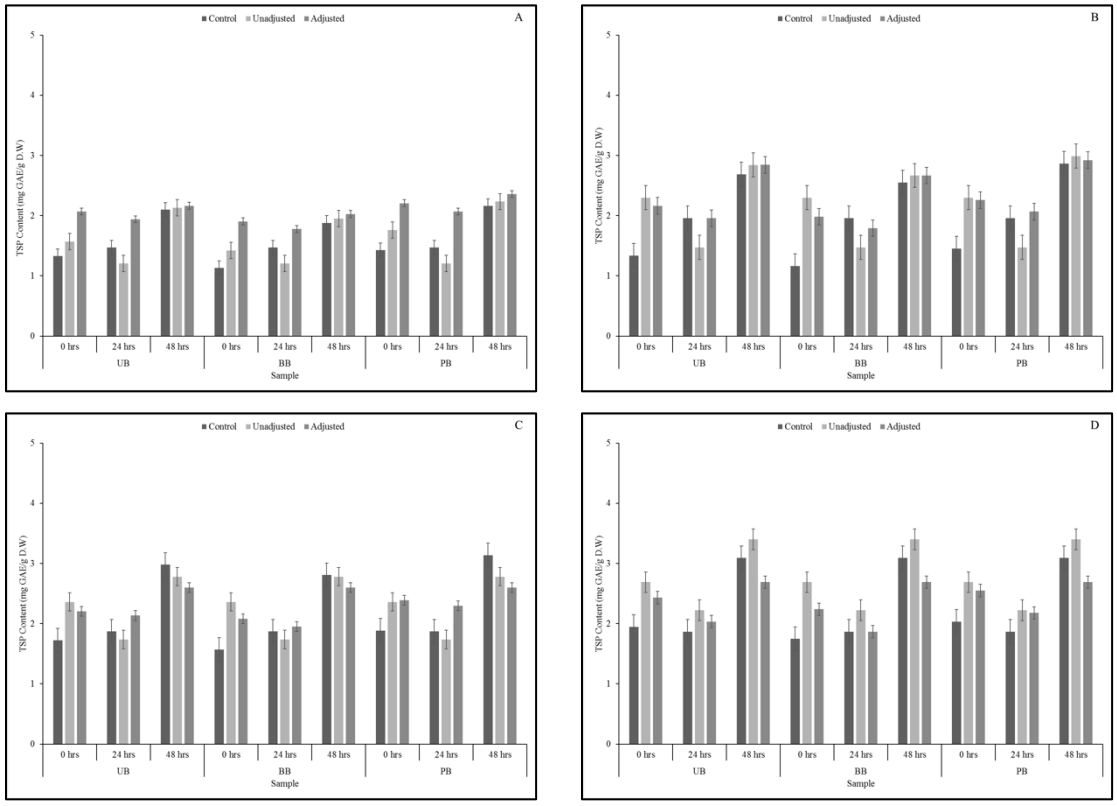

Figure 11.2. Total soluble phenolic (TSP) content of flour extracts derived from germinated (0, 24, 48 h) unpigmented barley (UB), black barley (BB) and purple barley (PB) after 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation with Kefir culture.

The mixed Kefir culture used in this study contains strains such as *L. reuteri, L. acidophilus* and *L. plantarum*, which are known to exhibit significant feruloyl esterase activity (FEA), as observed by Hole *et al*. (2012). Additionally, specific individual LAB strains screened for high FEA were found to result in as much as a 20-fold increase in free phenolic content in whole grain oat groats and barley (Hole *et al*. 2012).

As changes in matrix acidity may affect the phenolic solubilization post fermentation the TSP content of pH adjusted fermented samples (same level as that of the corresponding control) was also measured. At the beginning of fermentation (0 h), most of the pH-unadjusted samples showed lower levels of TSP content compared to the corresponding controls and pH-adjusted samples. However, as fermentation progressed, certain sample combinations indicated a pH-

dependent improvement of TSP content, including UB-0 at 24, 48 and 72 h (Fig. 11.2 B, C, D). Acidification of the food barley slurries during fermentation may hydrolyze phenolic acid esters and flavonoid glucosides from cell wall polysaccharides, resulting in the observed trend (Sarkar, Ankolekar and Shetty, 2020). Additionally, some strains of LAB are capable of metabolizing phenolic acids by decarboxylation, yielding products such as vinyl catechol, vinyl phenol, vinyl guaiacol and catechol; and by reduction, yielding dihydrocaffeic acid and dihydroferulic acid (Sanchez-Maldonaldo *et al*., 2011; Curiel *et al*., 2010; Svensson *et al*., 2010). Thus, Kefir-mediated fermentation may influence TSP content via the combined effect of pH changes and exogenous bacterial enzyme activity, with the former being more dominant, especially in the later stages of fermentation. In this study, grain bioprocessing via sprouting, separately and in combination with Kefir-mediated fermentation resulted in improved TSP content. Specifically, barley flour with significantly enhanced phenolic content was generated after 48 h of sprouting, which even further improved with Kefir-culture mediated fermentation for 72 h.

In addition to the TSP content, profile of phenolic compounds of the sprouted barley flour extracts with and without Kefir culture mediated fermentation was also determined and quantified using HPLC method. The major phenolic compounds found in three types of food barley (UB, BB, PB) were cinnamic acid, gallic acid, protocatechuic acid, catechin, and benzoic acid (Table 11.2). Additionally, ferulic acid and dihydroxybenzoic acid were also observed in pigmented (PB & BB) food barley samples. Previously, Kim *et al*. (2007) observed gallic acid, protocatechuic acid, chlorogenic acid, vanillic acid, syringic acid, ferulic acid, caffeic acid, coumaric acid, benzoic acid, catechin, rutin, quercetic, myrecetin, kaempferol in hulled and un-hulled samples of pigmented barley (black, blue, and purple) samples. Similarly, diverse phenolic compounds, such as chlorogenic acid, gallic acid, benzoic acid, hydroxybenzoic acid,

vanillic acid, coumaric acid, and catechin were found in hulless pigmented (white, yellow, black, and blue) barley samples (Ge *et al*., 2021) with LC-MS-IT-TOF analytical method. The concentration of individual phenolic compound found in the current study was similar with the previous findings of the Kim *et al*. (2007).

In the current study, barley type × sprouting duration interactions had statistically significant effect on individual phenolic profile. Higher gallic acid content was found in unpigmented (UB) barley sample, while purple barley (PB) had significantly ($p < 0.05$) high protocatechuic, ferulic, benzoic, and dihydroxybenzoic acid content (Table 11.2 A). When steeped water was analyzed, higher gallic acid and cinnamic acid were found in PB and BB steeped water (Table 11.2 B). Additionally, m-coumaric acid was also found in PB steeped water, however it was not detected in the barley flour samples. The results of this study indicated that during steeping, phenolics such as gallic acid, cinnamic acid, ferulic acid, and m-coumaric acid from pigmented (PB & BB) barley leached into the steeped water. Like the results of the TSP content, significant enhancement in cinnamic acid, protocatechuic acid, gallic acid, and ferulic acid content was observed in barley flour extracts after 48 h of sprouting. However, benzoic acid, dihydroxybenzoic acid, and catechin content decreased proportionately with increased duration of sprouting. The results indicated that changes in phenolic content and phenolic profile during sprouting of food barley is based on the composition of free and bound phenolic fractions in the grain and due to the potential variations in their chemical structure and functions.

Like the phenolic profile results of the sprouted barley flour extracts, major phenolic compounds found in Kefir -mediated fermented barley samples were protocatechuic acid, gallic

acid, cinnamic acid, catechin, and dihydroxybenzoic acid (Table 11.3 A, B, C, & D). However, benzoic acid was only found in UB and PB flour extracts at 0 h of fermentation time point.

On the contrary, in UB flour extracts, cinnamic acid was found in 24, 48, and 72 h fermented sample, while it was not observed in 0 h fermentation time point. Overall, at 0, 48, and 72 h time points 3-way interactions between pH treatment × barley sample type × sprouting duration had statistically significant effect on all individual phenolic compounds. However, at 24 h time point, effect of 2-way interaction between pH treatment × barley sample type and barley sample type × sprouting duration on phenolic compounds were statistically significant. Interestingly, significant improvement in catechin, cinnamic acid, and protocatechuic acid content was observed in fermented barley samples, especially in pigmented barley (BB & PB) flour extracts after 48 and 72 h of fermentation. Except for catechin, the content of all other phenolic compounds mostly improved after 48 and 72 h of fermentation. Previously, Hole *et al*. (2012) also observed increase in free phenolic acid (caffeic, *p*-coumaric, and ferulic acid) content in beneficial LAB-fermented flour extracts of whole grain barley and oat groat. The results of the current study indicated that both sprouting, and Kefir-culture (mixed LAB) mediated fermentation are effective food processing strategies to improve the bioavailability and bioactivity of health protective phenolic compounds in unpigmented and pigmented food barley. Therefore, this integrated bioprocessing strategy combining sprouting with LAB fermentation can be targeted for developing phenolic-rich barley-based substrates in a simple and cost-effective manner. Consequently, these bioprocesses may be further optimized to develop phenolic-rich functional foods and beverages with dietary and therapeutic functionalities to address poor quality diet and lifestyle-linked NCDs such as T2D and cardiovascular diseases. The major phenolic compounds, such as gallic acid, cinnamic acid, protocatechuic acid, and

catechin found in sprouted and fermented barley flour extracts are also potent antioxidants and can be targeted for designing antioxidant enriched functional foods and beverages (Kim *et al*. 2007; Ge *et al*. 2020). Therefore, it is also important to investigate the impact of sprouting and LAB fermentation integrated bioprocessing strategy on the health relevant functionalities such as antioxidant and anti-hyperglycemic properties of food barley.

Table 11.3. Major phenolic acids identified in sprouted and unfermented barley flour (A) and steep water (B) by HPLC analysis. Different lowercase letters represent significant differences in individual phenolic acid concentration due to (A) barley type × sprouting duration interactions and (B) barley type, at 95% confidence level ($p < 0.05$).

(A)

Food barley type	Germination time (h)	Average concentration (µg/g D.W.)						
		Cinnamic acid	Protocatechuic acid	Catechin	Gallic acid	Ferulic acid	Dihydroxybenzoic acid	Benzoic acid
UB	0	2.11f	3.18e	1.83bc	3.42f	n.d.*	n.d.	0.18d
	24	5.70d	10.3c	1.03cd	6.71c	n.d.	n.d.	0.43bcd
	48	7.89c	12.7b	0.60d	8.07a	n.d.	n.d.	0.32bcd
BB	0	2.57f	2.22ef	1.91bc	1.31i	0.98a	0.98bc	1.72a
	24	3.99e	1.89f	3.44a	2.00h	0.60b	0.68cd	0.66b
	48	14.4a	12.5b	1.52cd	4.91d	0.96a	0.59cd	0.25cd
PB	0	1.74f	1.78f	2.73ab	2.53g	0.33c	1.65a	1.47a
	24	2.61f	5.74d	1.81bc	4.28e	0.34c	1.31ab	0.62bc
	48	10.4b	15.6a	1.49cd	7.24b	1.00a	0.18d	0.15d

(B)

Food barley type	Average concentration (µg/g D.W.)					
	Protocatechuic acid	Cinnamic acid	Benzoic acid	Gallic acid	Ferulic acid	m-Coumaric acid
UB	0.01c	0.50c	0.06a	0.18b	n.d.	n.d.
BB	0.03b	0.72b	0.04a	0.65a	0.1b	n.d.
PB	0.06a	0.78a	0.05a	0.30b	0.18a	0.47

*n.d. – not detected

Table 11.4. Major phenolic acids in sprouted barley flour extracts during Kefir culture-mediated fermentation by HPLC analysis.

(A)

Food barley type	Germination time (h)	Dihydroxybenzoic Acid		Catechin		Cinnamic Acid		Protocatechuic Acid		Gallic Acid		Benzoic Acid	
		Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermnted	Control	Fermented
UB	0	0.33	0.37	0.17	0.12	n.d.	n.d.	2.11	2.63	1.28	1.42	0.04	0.4
	24	0.59	0.66	0.22	0.17	n.d.	n.d.	2.49	3.31	1.71	1.78	0.2	0.3
	48	0.97	0.95	0.29	0.27	n.d.	n.d.	4.19	4.43	2.42	2.98	0.22	0.37
BB	0	0.38	0.6	0.25	0.14	2.56	11.6	1.94	3.69	1.01	1.62	n.d.	n.d.
	24	0.87	1.05	0.23	0.25	5.81	13.9	3.92	5.99	2.32	2.79	n.d.	n.d.
	48	1.55	1.46	0.3	0.33	6.56	16.7	7.4	7.85	3.68	5.16	n.d.	n.d.
PB	0	0.2	0.28	0.3	0.16	2.67	8.23	2.1	2.75	2.14	2.23	0.04	0.31
	24	0.84	0.93	0.26	0.21	5.34	14.8	4.18	4.93	2.99	3.53	0.02	0.26
	48	1.39	1.32	0.36	0.55	7.63	23.1	6.81	7.69	4.26	4.95	0.03	0.32

(B)

Food barley type	Germination time (h)	Dihydroxybenzoic Acid		Catechin		Cinnamic Acid		Protocatechuic Acid		Gallic Acid	
		Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermented
UB	0	0.45	0.56	0.26	0.18	4.66	12.6	3.06	5.98	1.19	2.35
	24	0.9	0.87	0.3	0.29	6.47	13.5	4.86	7.4	1.95	2.54
	48	1.61	1.35	0.39	0.45	7.69	20.3	8.61	11.0	4.65	5.49
BB	0	0.74	0.9	0.19	0.41	5.88	4.73	1.32	8.69	0.97	1.22
	24	1.44	1.27	0.32	0.48	5.47	5.22	20.8	10.5	3.61	1.83
	48	2.29	1.97	0.4	0.62	7.09	6.09	13.6	18.3	6.93	5.25
PB	0	0.49	0.39	0.21	0.31	6.61	2.93	1.52	5.56	4.01	1.51
	24	1.35	1.27	0.37	0.29	7.93	5.04	0.5	11.0	4.14	3.62
	48	1.95	1.99	0.68	0.68	7.36	7.49	2.18	16.4	6.70	8.61

n.d. – Not detected;

Table 11.4. Major phenolic acids in sprouted barley flour extracts during Kefir culture-mediated fermentation by HPLC analysis (continued).

(C)

Food barley type	Germination time (h)	Dihydroxybenzoic Acid		Catechin		Cinnamic Acid		Protocatechuic Acid		Gallic Acid	
		Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermented
UB	0	0.66	0.65	0.29	0.64	4.25	4.48	4.75	8.33	1.12	1.56
	24	1.36	0.87	0.26	0.33	4.6	4.44	6.69	10	1.85	1.93
	48	2.32	1.44	0.54	0.28	5.86	5.93	13.3	14	6.51	3.1
BB	0	1.08	0.96	0.24	0.43	0.84	4.18	0.15	1.34	0.99	1.72
	24	1.75	1.15	0.36	0.19	0.77	6.57	0.09	0.68	0.43	2.54
	48	3.14	2.38	0.39	0.26	1.46	7.22	0.04	1.13	0.48	4.73
PB	0	0.79	0.57	0.26	0.14	0.44	3.35	1.82	9.26	1.86	2.3
	24	1.78	1.14	1.12	0.21	2.65	5.07	1.61	12.2	5.5	2.97
	48	2.62	2.11	0.32	0.36	5.75	7.54	3.2	18.7	3.17	9.2

(D)

Food barley type	Germination time (h)	Dihydroxybenzoic Acid		Catechin		Cinnamic Acid		Protocatechuic Acid		Gallic Acid	
		Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermented	Control	Fermented
	0	0.92	0.61	0.3	0.67	19.1	28.4	0.64	0.79	0.74	2.51
UB	24	1.25	0.85	0.21	0.25	20.9	30.5	0.7	0.27	2.46	2.75
	48	2.7	1.64	0.1	0.37	48.1	38.6	0.16	0.53	7.3	4.44
	0	1.48	1.07	0.21	0.19	1.41	1.96	0.21	0.79	1.53	3.09
BB	24	2.23	133	0.06	0.23	0.54	3.57	0.14	1.55	5.95	3.76
	48	2.97	2.76	0.09	0.15	0.6	3.6	0.22	2.21	9.72	6.9
	0	0.82	0.59	0.24	0.15	0.2	1.79	4.59	8.67	2.21	3.42
PB	24	2.27	1.18	0.44	0.29	0.45	4.24	4.53	13.4	6.73	4.38
	48	3.48	2.07	0.09	0.4	1.27	5.76	16.6	20.1	9.02	9.13

n.d. – Not detected

Table 11.5. Analysis of variance table indicating mean square (MS) and significant differences between pH treatment (T), food barley sample type (S) and barley sprouting times (G), with their respective interactions for catechin, cinnamic acid, gallic acid, protocatechuic acid and benzoic acid contents in sprouted flours from three types of food barley samples at 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation with Kefir culture.

(A)

Source	df	Catechin	Cinnamic acid	Gallic acid	Protocatechuic acid	Benzoic acid
T	1	0.01*	1110.1 ns	7.23*	22.0*	0.66*
S	2	0.18*	2365.3*	36.6*	75.9*	1.20*
T×S	2	0.01*	560.0 ns	1.67*	3.86*	0.35**
G	2	0.5 ns	417.1*	95.9*	270.8*	0.02*
T×G	2	0.11**	64.6*	2.11*	2.19*	0.04**
S×G	4	0.09 ns	297.2*	9.56*	33.3*	0.03*
T×S×G	4	0.09**	90.2*	0.68*	2.83**	0.07 ns

(B)

Source	df	Catechin	Cinnamic acid	Gallic acid	Protocatechuic acid
T	1	0.12*	115.8*	0.99 ns	22.0*
S	2	0.26*	57.0*	62.9*	75.9*
T×S	2	0.24*	687.2*	17.2*	3.86**
G	2	1.42*	179.7*	378.1*	270.8*
T×G	2	0.02 ns	45.7*	4.28**	2.19**
S×G	4	0.27*	63.8*	11.8*	33.3*
T×S×G	4	0.07**	33.8*	33.3*	2.83 ns

(C)

Source	df	Catechin	Cinnamic acid	Gallic acid	Protocatechuic acid
T	1	2.76*	517.7*	22.2*	644.4*
S	2	1.62*	115.6*	101.9*	1619.5*
T×S	2	6.85*	343.8**	52.0*	544.2*
G	2	1.05*	421.1*	162.3*	335.3*
T×G	2	9.22*	4.22**	27.8*	13.0*
S×G	4	13.2*	130.9*	33.6*	181.6*
T×S×G	4	12.0*	40.0*	131.6*	103.0*

(D)

Source	df	Catechin	Cinnamic acid	Gallic acid	Protocatechuic acid
T	1	0.31*	221.7*	9.35*	140.8*
S	2	0.49*	1995.0*	115.2*	2716.3*
T×S	2	0.21*	6.23 ns	4.28*	146.8*
G	2	0.17*	1171.6*	55.6*	325.1*
T×G	2	0.18*	180.4*	60.3*	14.3*
S×G	4	0.59*	1671.8*	17.3*	601.7*
T×S×G	4	0.44*	560.1*	26.7*	42.0*

*Denotes significance at $p < 0.05$; **Denotes significance at $p < 0.001$; ‡ns, non-significant

11.4.3. Total Antioxidant Activity

The presence of phenolic compounds in plant-food substrates has been positively correlated with high antioxidant activity and free radical scavenging abilities, as observed in several previous studies (Shahidi and Ambigaipalan, 2015; Sarkar and Shetty, 2014; Balasundaram, Sundaram and Samman, 2006; Kähkönen *et al*, 1999; Rice-Evans, Miller and Paganga, 1997).

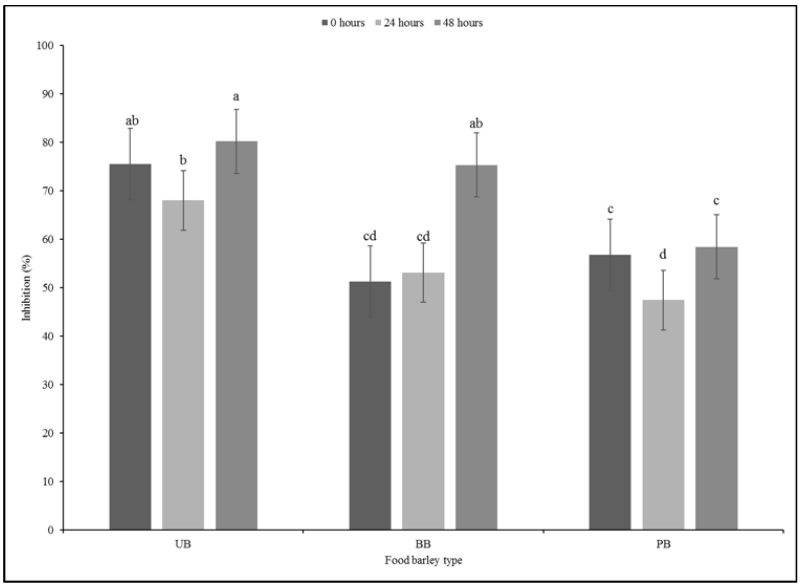

Figure 11.3. ABTS free radical scavenging capacity of flour extracts from unpigmented barley (UB), black barley (BB) and purple barley (PB) after varying durations of sprouting (0, 24, 48 h). Different alphabets represent significant differences in ABTS-based antioxidant activity due to interactions between barley type × sprouting durations at 95% confidence level ($p < 0.05$).

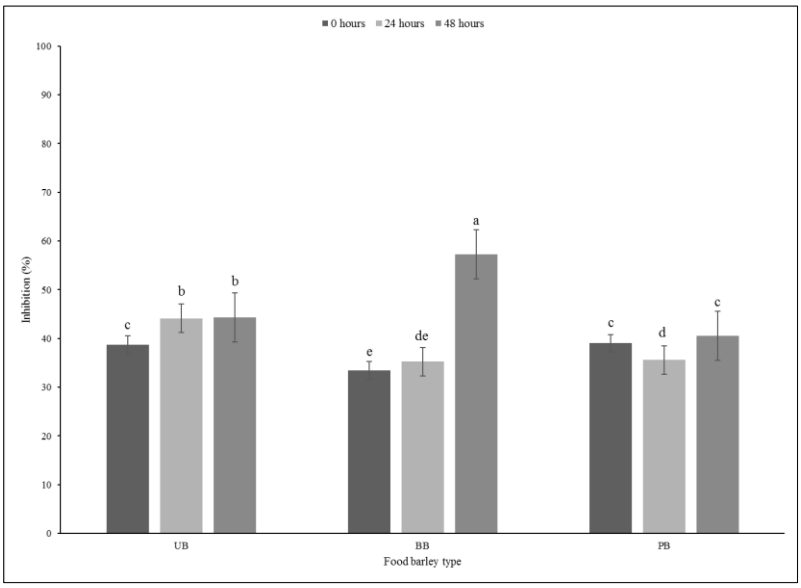

Figure 11.4. DPPH free radical scavenging capacity of flour extracts from unpigmented barley (UB), black barley (BB) and purple barley (PB) after varying durations of sprouting (0, 24, 48 h). Different alphabets represent significant differences in DPPH-based antioxidant activity due to interactions between barley type × sprouting duration interactions at 95% confidence level ($p < 0.05$).

In the current study, it was of interest to assess how changes in TSP content observed in the food barley samples after sprouting and during Kefir culture-mediated fermentation translated to total antioxidant capacity relevant for managing chronic oxidative stress commonly associated with NCDs such as T2D, and cardiovascular diseases. Therefore, total antioxidant activity of sprouted barley flour extracts before and during fermentation was determined by measuring the ability of the sample extracts to scavenge ABTS and DPPH free radicals using *in vitro* assays. In this study, statistically significant effect of barley type × sprouting duration interactions on ABTS and DPPH based antioxidant activity of sprouted barley flour was observed. Additionally, the total antioxidant activity based on ABTS free radical scavenging assay, varied significantly among different food barley types (unpigmented and pigmented) ($p < 0.05$) (Table 11.1). Like the results of the TSP content, aqueous extracts of UB flour extracts had significantly higher antioxidant activity, when compared to BB and PB flour extracts. The

duration of sprouting also positively contributed towards antioxidant activity, with flours derived from 48 h germinated barley had the highest levels of antioxidant activity, specifically in the case of UB and BB flour extracts. Surprisingly, in UB and PB flour extracts, the antioxidant activity was the lowest in 24 h sprouted flour (Fig 11.3). However, high antioxidant activity was observed in PB and BB steeped water (result not presented), which indicated leaching of bioactive compounds with antioxidant capacity from pigmented food barley during steeping.

Similarly, with the DPPH free radical assay, both aqueous extracts of UB and BB flour had significantly higher antioxidant activity when compared to PB flour extracts at $p < 0.05$. However, in DPPH free radical scavenging assay, extract of BB sprouted for 48 h had highest antioxidant activity than corresponding UB sample, which differed from the results of the ABTS assay. The differences in results of antioxidant activity between 2 different assays (ABTS and DPPH) might be due to different affinity and activity of these free radicals towards hydrophilic and lipophilic antioxidants that were potentially present in the food barley flour extracts (Celik *et al*.,2010). As seen with the ABTS assay, the flours derived from barley grains sprouted for 48 h had relatively higher antioxidant activity (Fig11. 4).

In this study, the improvement in total antioxidant activity observed in both ABTS and DPPH-based free radical scavenging assays positively correlated with the increased TSP content of corresponding barley flours. The mobilization of free and bound phenolic compounds within the grain matrix into water soluble forms during sprouting might have resulted in higher antioxidant activity (Singh and Sharma, 2017). While most previous studies have focused primarily on malting barley, the results obtained for the food barley varieties in the current study are in good agreement with prior findings (Ha *et al*., 2017, Ramakrishna *et al*., 2017b; Rico *et al*., 2020). Therefore, the results of this study further supported the viability of using flour

derived from sprouted food barley as a functional food ingredient for phenolic-linked antioxidant benefits.

Like the results of unfermented barley, the total antioxidant activity of the fermented barley flour samples was also determined by the ABTS and DPPH free radical scavenging assays. The results of the ABTS assay indicated that the overall free radical scavenging capacity of fermented food barley samples increased consistently during fermentation (Fig. 11.5). In this study, statistically significant differences between 3-way interactions of pH treatment × barley sample type × sprouting duration were observed in DPPH-based antioxidant activity at 0, 48, and 72 h fermentation time points. However, for ABTS-based antioxidant activity result, only 2 -way interaction between pH treatment × sprouting duration was statistically significant at all fermentation time points (Table 11.1). Additionally, across all fermentation assay time points, the main effects of pH, barley type, and sprouting duration had statistically significant (except germination duration at 48 h) effect on antioxidant activity. Interestingly, the ABTS free radical scavenging capacity of all fermented (unadjusted pH) barley samples was found to be significantly higher than the corresponding controls and pH-adjusted fermented sample. This is indicative of a pH-dependent improvement in the antioxidant capacity of the samples, rather than being solely due to the phenolic mobilization. However, unlike in the case of the unfermented barley samples, the trend of DPPH-based antioxidant activity differed considerably from the results of the antioxidant activity based on ABTS assay (Fig. 11.6). While UB-0 samples had the highest DPPH scavenging capacity among the food barley types, the pH-unadjusted samples had lower antioxidant activity than the corresponding controls and pH-adjusted solutions.

Overall, fermentation, and more specifically with beneficial LAB-based biotransformation, has been shown to improve the antioxidant activity in various whole grain

substrates (Adebo and Medina-Meza, 2020; Wang *et al*., 2019; Đorđević *et al*. 2010). The production of lower molecular weight phenolic compounds such as phenolic acids and aglycones during fermentation is particularly relevant, as they tend to be the end products of solubilization from the bound forms (Adebo and Medina-Maza, 2020). However, the pH-dependent improvement of antioxidant activity in fermented food barley slurries may be attributed to the production and combined activity of organic acids (Liu *et al*, 2019). In addition to lactic acid, mixed microbial cultures - such as in Kefir – may produce other organic acids such as acetic, citric, butyric, propionic, and pyruvic acids (Güzel-Seydim *et al*., 2000).

These acids may also exert significant antioxidant activity, in combination with or exceeding that of phenolic compounds (Liu *et al*, 2019). Further, differences in trends observed in the two antioxidant assays may arise from the DPPH free radical being more stable in the fermented matrix than $ABTS^{+}$ cation and the resulting delay in attaining a steady state due to the quenching of DPPH radical by phenolic antioxidants, leading to lower values overall (Shalaby and Shanab, 2013).

Improvement of the antioxidant activity is recognized as an important strategy to enhance the overall human health relevant functionalities of foods and beverages (Sarkar, Ankolekar and Shetty, 2020). This is especially true for dietary strategies aimed at addressing T2D and associated complications, because the underlying pathophysiology fundamentally involves chronic oxidative stress due to the breakdown of cellular redox balance (Burgos-Morón *et al*., 2019). The current study indicated a considerable increase in total antioxidant activity of food barley slurries, with and without sprouting in response to Kefir-mediated fermentation. This trend was sustained throughout duration of fermentation (72 h), as seen in the DPPH free radical scavenging assay. Therefore, the improvement of total antioxidant activity of food barley flour

(with and without germination) by Kefir-mediated fermentation is of relevance to designing functional foods for maintaining cellular redox homeostasis, which is a key targ*et al*ong with anti-hyperglycemic function in the dietary interventions for prevention and long-term management of T2D.

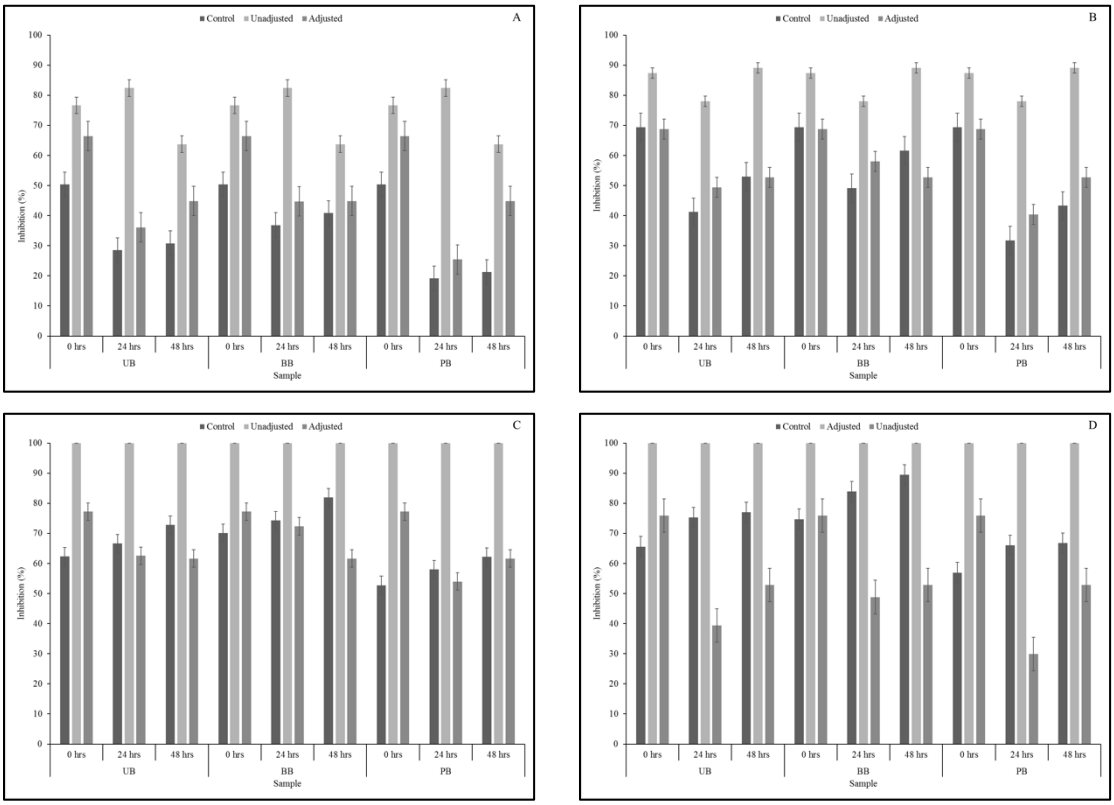

Figure 11.5. ABTS free radical scavenging capacity of aqueous extracts derived from sprouted (0, 24, 48 h) unpigmented barley (UB), black barley (BB) and purple barley (PB) after 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation with Kefir culture.

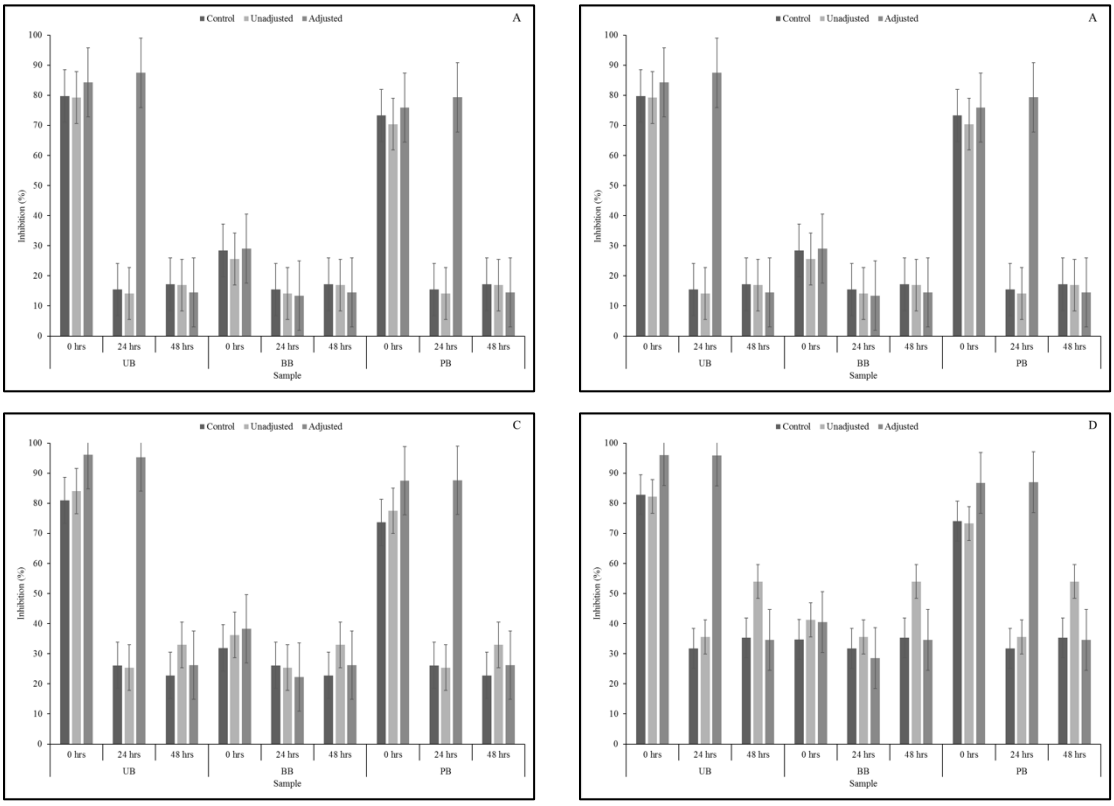

Figure 11.6. DPPH free radical scavenging capacity of extracts derived from sprouted (0, 24, 48 h) unpigmented barley (UB), black barley (BB) and purple barley (PB) after 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation with Kefir culture.

11.4.4. α-Amylase Inhibitory Activity

The ability of a food substrate to modulate the activity of α-amylase, in turn moderating the digestion of starch, is a key factor in determining its ability to effect glycemic control (Ansari *et al*. 2017; McCue *et al*., 2004). In this context, the ability of food barley flours derived from sprouted and un-sprouted grains to inhibit α-amylase was determined using *in vitro* assay model.

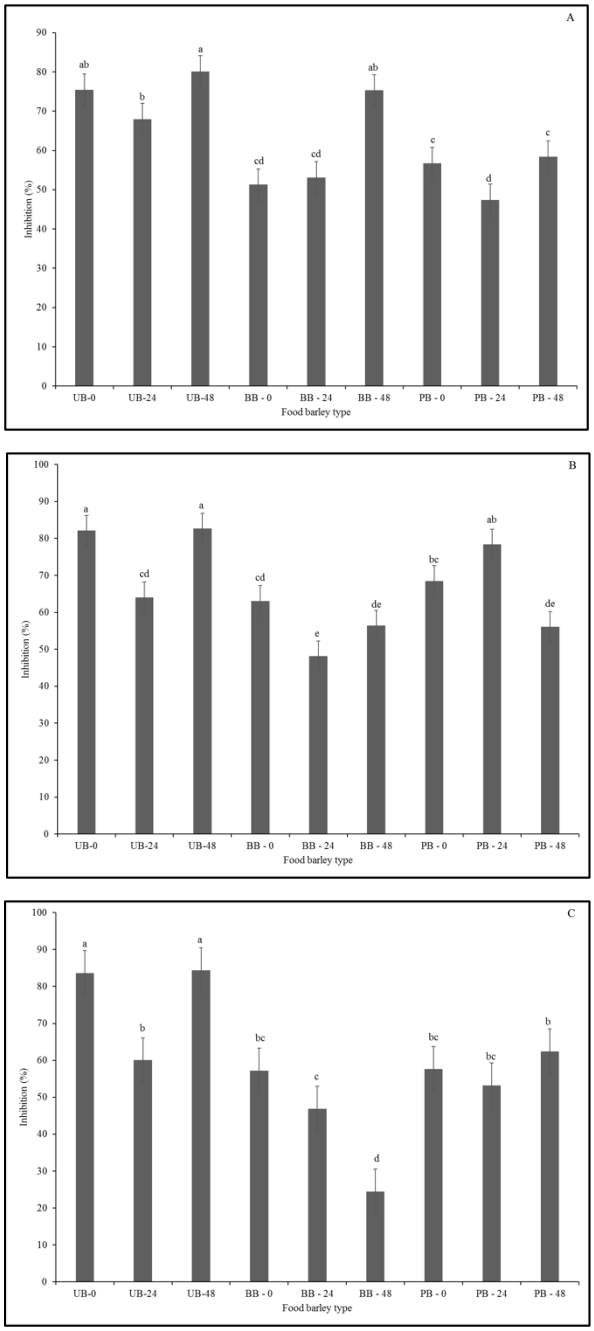

Figure 11.7. α-Amylase enzyme inhibitory activity of extracts derived from sprouted (0, 24, 48 h) unpigmented barley (UB), black barley (BB) and purple barley (PB) at zero dilution (A), half dilution (B) and one-fifth dilution (C). Different alphabets represent significant differences in α-amylase enzyme inhibitory activity due to interactions between barley type × sprouting duration at 95% confidence level ($p < 0.05$), separately within each level of dilution.

In this *in vitro* assay model-based study, the barley flour extracts had moderate to high levels of α-amylase enzyme inhibitory activity with notable dose-dependent response (Fig. 11.7

A, B, C). Statistically significant effect of barley type × sprouting duration interaction on α-amylase enzyme inhibitory activity was observed. Among the different barley sample types, aqueous extracts of UB flour had highest α-amylase enzyme inhibitory activity, regardless of sprouting durations, followed by PB and BB. However, the duration of sprouting indicated an inverse relationship with α-amylase enzyme inhibition, with un-sprouted flour extracts showing a greater level of inhibitory activity when compared to sprouted flour extracts (Fig 7 A, B, C).

In the undiluted samples, the α-amylase enzyme inhibitory activity was found to be comparable between un-sprouted and 48 h sprouted samples, while it reduced in 24 h sprouted samples. The values observed in un-sprouted barley flour were like the results reported previously by Ramakrishna *et al*. (2017a) (Chapter 5). Furthermore, in dark germinated barley sprouts, Ramakrishna *et al* (2017b) reported α-amylase inhibition to be the highest in 2-day old (48 h) sprouts, followed by a linear decrease over the next 4 days (Chapter 5). Such α-amylase enzyme inhibitory activity in 48 h sprouted barley flour may be attributed to high TSP content as evidenced by the results presented in the earlier sections (11.3.1). These findings also support the fact that the 48-h sprouting threshold is most conducive for the improvement of TSP content and associated bioactive functionalities including anti-hyperglycemic property relevant α-amylase enzyme inhibitory activity. As in the case of unfermented food barley samples, the fermented samples exhibited a clear dose dependent relationship, however, only values indicating data from the undiluted samples are presented here. All three main effects on α-amylase enzyme inhibitory were found to be statistically significant at $p < 0.05$, across all fermentation time points (Table 11.1).

The results from Kefir culture-mediated fermentation indicated that the α-amylase inhibitory activity of food barley flour extracts improved due to fermentation with probiotic

mixed LAB culture. The effect of 3-way interactions of pH treatment × barley sample type × sprouting duration on α-amylase enzyme inhibitory activity was statistically significant for undiluted sample at 24, and 72 h fermentation time points. At 0 and 48 h fermentation time points, 2-way interactions between pH treatment × sprouting duration had statistically significant effect on α-amylase enzyme inhibitory activity. The fermented samples exhibited moderate to high levels of enzyme inhibitory activity. At the beginning of fermentation, the 24 h sprouted flour extracts of all barley sample types had moderate to high levels of α-amylase enzyme inhibitory activity which was pH-independent. However, as fermentation progressed, increase in α-amylase enzyme inhibition in most sample combinations was found to be pH dependent. This can be observed in Fig 11.8B, 11.8C, and 11.8D, where enzyme inhibition of the pH-unadjusted samples was higher than that of the corresponding controls and pH-adjusted samples. Thus, improvements in α-amylase enzyme inhibitory activity may be attributed to the acidification of the medium due to LAB fermentation to a greater extent in addition to the mobilization of soluble phenolic compounds in fermented food matrix.

As fermentation progressed from 0-72 h, α-amylase enzyme inhibitory activity of the fermented barley samples was also found to increase concurrently (Fig. 11.8 A, B, C, D). Overall, the highest α-amylase enzyme inhibition was observed after 72 h of fermentation. At this fermentation time point, the inhibitory activity of specific sample combinations (UB-24, UB-48, BB-24, BB-48) reached complete saturation in terms of α-amylase enzyme inhibition (i.e., 100% inhibition), while remaining barley samples had 70-90% α-amylase enzyme inhibitory activity.

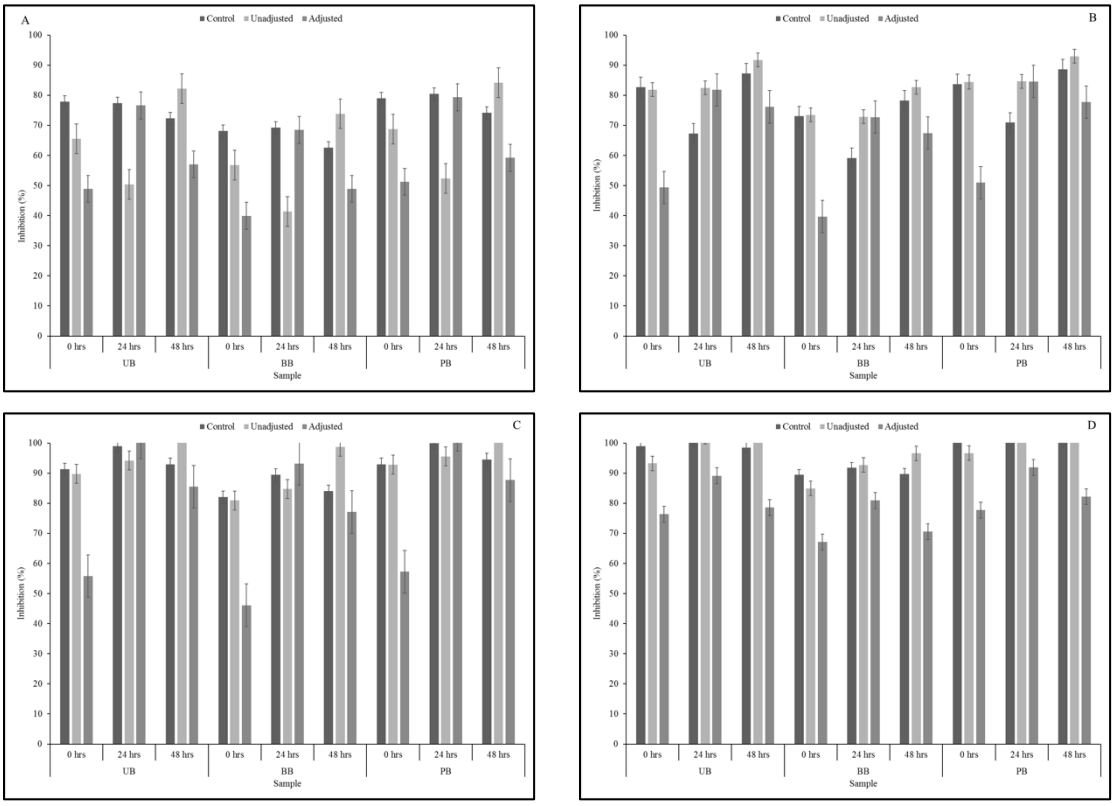

Figure 11.8. α-Amylase inhibitory activity of Kefir-fermented extracts derived from sprouted (0, 24, 48 h) unpigmented barley (UB), black barley (BB) and purple barley (PB) after 0 h (A), 24h (B), 48 h (C) and 72 h (D) of fermentation.

The linear improvement of α-amylase enzyme inhibitory activity relative to the duration of fermentation is like the previous findings in camu-camu substrate fermented with *L. plantarum* (Fujita *et al*. 2017). However, *L. acidophilus*-fermented pear juice did not show any visible improvements in α-amylase enzyme inhibition in response to fermentation (Ankolekar *et al*., 2012). Therefore, it may be inferred that LAB-fermentation based improvement of α-amylase enzyme inhibitory activity varies according to the fermentative strains used and their growth dynamics in specific plant-based food substrates.

As in the case of antioxidant activity, α-amylase enzyme inhibitory activity of sprouted food barley flour was found to be improved in response to Kefir-mediated fermentation in this study. Food barley grains sprouted for 24 and 48 h and later fermented for 72 h with Kefir

culture can be specifically targeted for this purpose. It is important to note that excessive α-amylase enzyme inhibition may cause detrimental side-effects such as abdominal distension, flatulence, and diarrhea, due to the fermentation of undigested starch in the large intestine (Rosenstock *et al*., 1998; Bischoff, 1994). However, the dose dependent response observed in this study suggested the scope for formulating food barley substrates with optimal modulation of starch breakdown. These results further support the relevance of the combined application of these two bioprocessing strategies as an integrated food processing approach to improve α-amylase enzyme inhibitory activity related anti-hyperglycemic functions in cereal grains such as barley.

11.4.5. α-Glucosidase Inhibitory Activity

The ability of a food or beverage to regulate the formation and uptake of glucose in the small intestine by the controlled modulation of α-glucosidase represents another key target for glycemic control (Hedrington and Davis, 2019). Therefore, in this study, the α-glucosidase enzyme inhibitory activity of aqueous extracts from sprouted and un-sprouted food barley flour was measured using *in vitro* assay model. Overall, flour extracts from the food barley types under consideration in the current study indicated low to moderate levels of α-glucosidase enzyme inhibitory activity with clear dose-dependent response (Fig. 11.9 A, B, C). Statistically significant effect between barley type × sprouting duration on α-glucosidase enzyme inhibitory activity was observed. Across all levels of sprouting, UB flour extracts were found to have highest α-glucosidase enzyme inhibitory activity, when compared to those of BB and PB, at $p < 0.05$ significance level. The duration of sprouting positively influenced α-glucosidase enzyme inhibitory activity, with barley grains sprouted for 48 h had highest level of enzyme inhibition when compared to un-sprouted and 24 h sprouted barley grain flours.

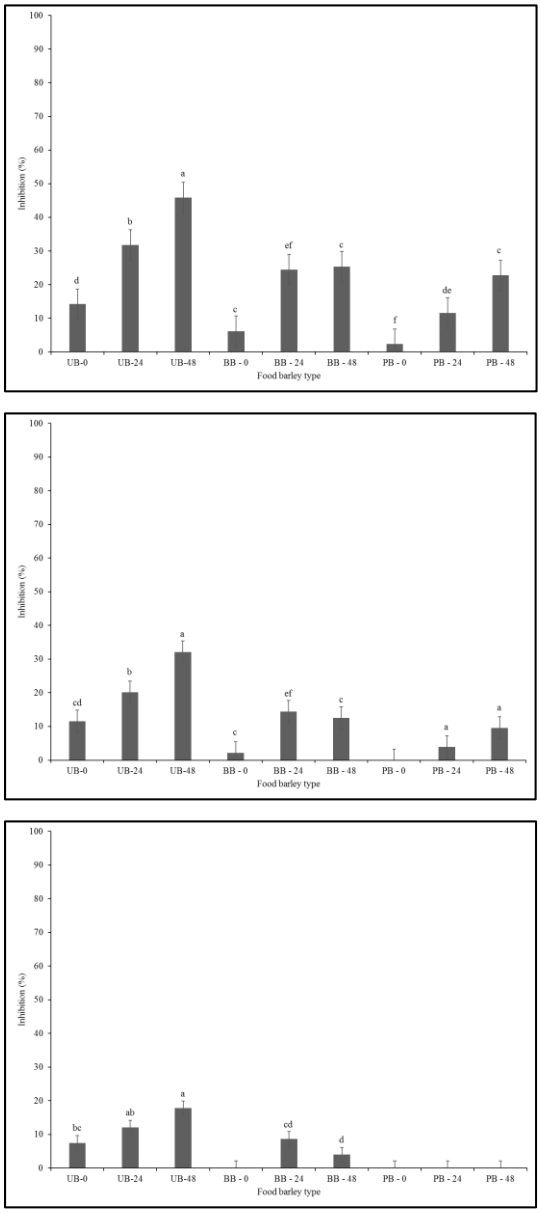

Figure 11.9. α-Glucosidase enzyme inhibitory activity of extracts derived from sprouted (0, 24, 48 h) flour extracts from unpigmented barley (UB), black barley (BB) and purple barley (PB) at zero dilution (A), half dilution (B) and one-fifth dilution (C). Different alphabets represent significant differences in α-glucosidase inhibitory activity due to interactions between barley type × sprouting duration at 95% confidence level ($p < 0.05$), separately within each level of dilution.

Like the current findings, Ramakrishna *et al*. (2017b) (Chapter 5) reported that α-glucosidase enzyme inhibitory activity increased proportionally with sprouting time, in elicitor-treated dark germinated malting barley sprouts. Additionally, Ha *et al*. (2017) reported an

increase in α-glucosidase inhibition by barley sprout extracts up to 48 h of germination, following which this anti-hyperglycemic property relevant enzyme inhibitory activity plateaued and finally decreased at 67 h of sprouting. In both these previous studies, α-glucosidase inhibitory activity was reported to have a strong positive correlation with TSP content, like that observed in the current study.

The α-glucosidase enzyme inhibitory activity of fermented food barley flour extracts was found to be low to moderate (Fig. 11.10 A, B, C, D). As in the case of the α-amylase assay, clear dose dependent response was observed in α-glucosidase enzyme inhibition, data from only the undiluted samples are presented in Fig. 11.10.

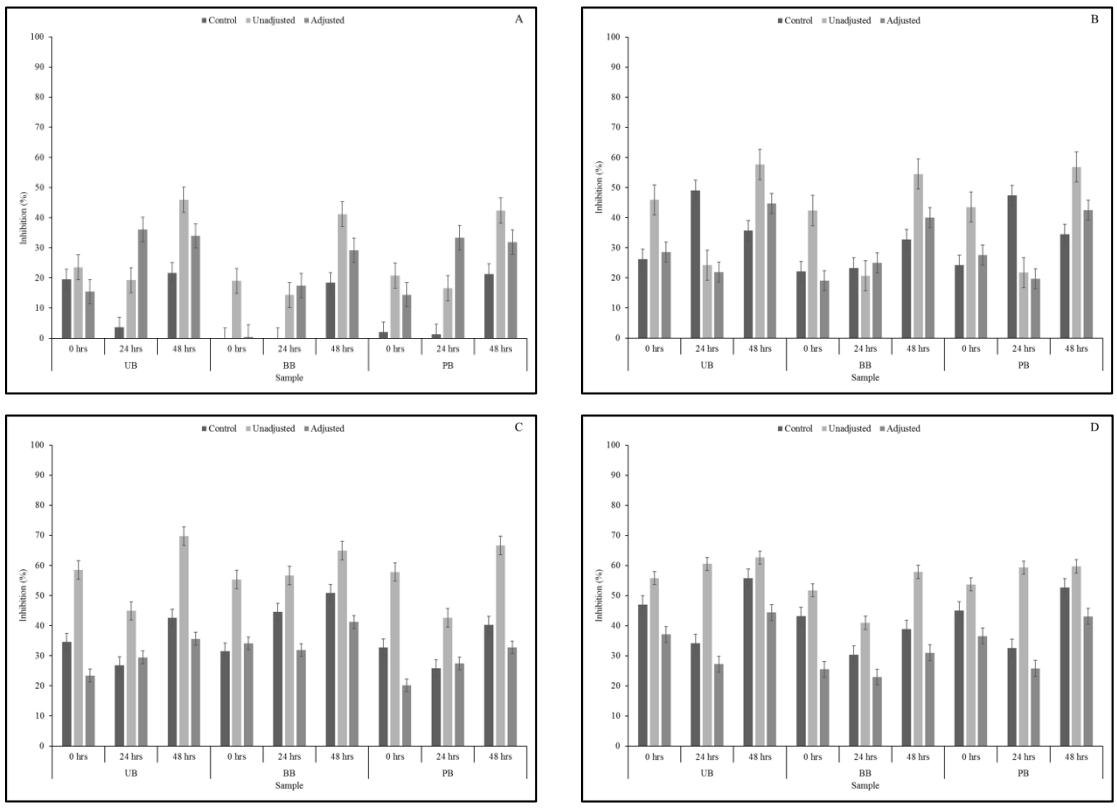

Figure 11.10. α-Glucosidase inhibitory activity of Kefir-fermented extracts derived from sprouted (0, 24, 48 h) from unpigmented barley (UB), black barley (BB) and purple barley (PB) after 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation.

Kefir culture-mediated fermentation was found to improve α-glucosidase inhibitory activity in food barley flour extracts. The 3-way interaction and all 2-way interactions had statistically significant effect on α-glucosidase enzyme inhibitory activity at 0 h time points. However, only 2-way interaction between pH treatment × sprouting duration had statistically significant effect on α-glucosidase enzyme inhibitory activity at 24, 48, and 72 h fermentation time points. Additionally, all three main effects were found to be statistically significant on α-glucosidase enzyme inhibitory activity at $p < 0.05$ (Table 11,1). In most sample combinations, α-glucosidase enzyme inhibition was found to be maintained or improved as fermentation progressed. Particularly, α-glucosidase enzyme inhibitory activity peaked at 48 h of fermentation, and subsequently decreased slightly at 72 h. In terms of the duration of sprouting, 48 h sprouted barley flours of all three food barley types were found to induce the highest level of α-glucosidase enzyme inhibitory activity across all fermentation time points, and this was consistent with other parameters investigated in this study such as TSP content, antioxidant activity, and α-amylase enzyme inhibition.

As seen in the current study, LAB-mediated fermentation was found to improve the α-glucosidase inhibitory activity in other plant-based substrates such as pear juice and camu-camu (Ankolekar *et al*., 2012; Fujita *et al*., 2017). However, it is important to note that improvements in α-glucosidase inhibition by Kefir-fermented food barley extracts was not found to be pH-independent. From Figure 11.10, it is apparent that the pH adjusted samples had lower α-glucosidase enzyme inhibitory activity than the corresponding pH unadjusted samples, which was found consistently across barley sample type and fermentation duration. This trend is comparable to the findings of Ankolekar *et al*. (2012), where the inhibitory activity of *L. acidophilus*-fermented pear juice was significantly higher at fermented acidic pH, than the

corresponding pH adjusted samples at every fermentation time point. Therefore, this anti-hyperglycemic relevant function of fermented plant substrates may be influenced more by the change in acidity of the medium induced by organic acid production than the release of phenolic compounds in fermented matrix.

Like the results of α-amylase enzyme inhibition, the ability of food barley flour – especially after sprouting - to inhibit α-glucosidase enzyme *in vitro* was also positively influenced by Kefir-mediated fermentation. However, in this case, high levels of α-glucosidase inhibition are favored in order to effectively control post-prandial blood glucose levels (Rosenstock *et al*., 1998). In this context, the study identified Kefir-mediated fermentation of sprouted food barley flour for 48 h as an effective integrated bioprocessing strategy for optimal α-glucosidase enzyme inhibitory activity potentially supporting desirable post-prandial blood glucose control. The integrated application of sprouting and Kefir- culture mediated fermentation to improve this anti-hyperglycemic property relevant functions in cereal grains such as barley has not been explored previously. Therefore, the current study delineates a novel integrated bioprocessing approach to develop functional foods and ingredients from food barley with antioxidant and anti-hyperglycemic properties, which may be further optimized to complement current therapeutic and dietary support strategies to target reduced health risks associated with T2D.

11.4.6. Viable Cell Count of LAB in Fermented Barley Flour and *Helicobacter pylori* Inhibition

To achieve higher probiotic function in fermented plant food substrates, it is important to have higher concentration of active and viable cells of beneficial LAB strains. In the current study, the viable cell count of the inoculated mixed LAB strains in the pH-unadjusted slurry of

barley flour extracts at each fermentation time point was found to increase from the initial level at 0 h. As the duration of the fermentation increased, the cell count was found to either increase in some sample combinations or remain at a relatively high level (Fig 11.11 A, B, C, D).

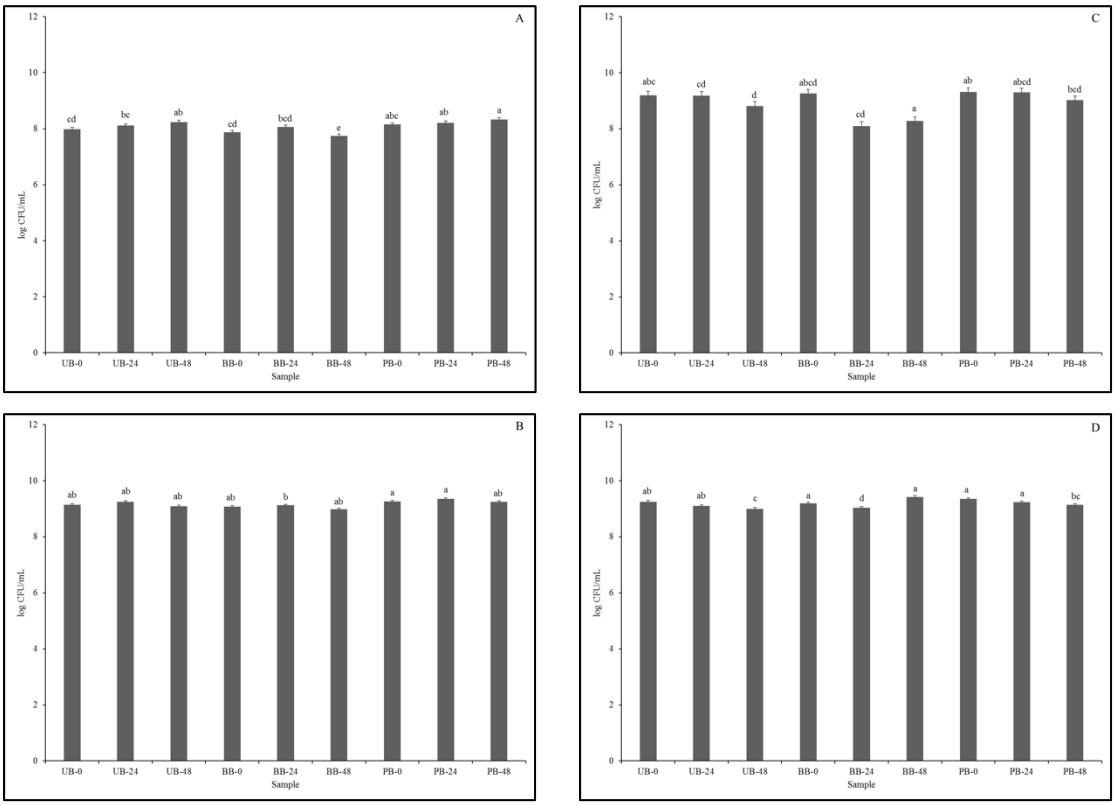

Figure 11.11. Viable cell count of lactic acid bacteria in unpigmented barley (UB), black barley (BB) and purple barley (PB) extracts at 0 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation with Kefir culture.

Notably, there was no decrease in the cell count below initial levels, even after 72 h of fermentation. Interestingly, during fermentation, the cell count levels remained above 8 log CFU/mL, which is the minimum level for a food or beverage to be considered as a probiotic (Kandyllis *et al*., 2016). Barley may serve as a highly suitable substrate for the growth of beneficial microbes, including LAB as it contains various fermentable substrates required for their growth (Enujiugha and Badejo, 2017). Charalampopoulos, Pandiella and Webb (2002) found that the growth of individual probiotic LAB strains of *L. plantarum, L. reuteri, L.*

fermentum and L, acidophilus was strongly supported in malt, wheat, and barley flours. Similar observations were made in various other studies, as the inclusion of whole and flours of barley and wheat have supported the growth of various probiotic LAB strains (Mridula and Sharma, 2015; Sharma, Mridula and Gupta, 2014; Chavan *et al*., 2018). The results obtained in this current study are in good agreement with previous studies and indicated that both whole and sprouted food barley can be a suitable carrier for beneficial LAB strains, with potential prebiotic properties.

Additionally, certain combinations of sprouted and fermented food barley samples were found to exhibit inhibition against ulcer causing bacteria *H. pylori* (Table 11.2). Interestingly, sprouting was observed to positively influence this targeted anti-bacterial parameter, and flours from barley grains sprouted for a longer duration (48 h) tended to have higher anti-bacterial activity. Among the different types of barley used in this study, PB had the greatest *H. pylori* inhibition followed by UB and BB. Further, as fermentation progressed the zone of inhibition produced by these sprouted barley flour extracts also increased (Table 11.5).

Table 11.6. Diameter (mm) of the circular zone of inhibition of *H. pylori* by unpigmented barley (UB), black barley (BB) and purple barley (PB) fermented with Kefir culture.

Sample	Duration of fermentation (h)			
	0	24	48	72
UB-0	n.d.	n.d.	n.d.	n.d.
UB-24	n.d.	12.9	12.9	13.0
UB-48	13.8	13.1	14.0	14.2
BB-0	n.d.	n.d.	n.d.	n.d.
BB-24	n.d.	12.9	13.1	13.0
BB-48	12.9	13.0	13.3	13.2
PB-0	n.d.	n.d.	n.d.	n.d.
PB-24	13.0	13.2	13.1	14.0
PB-48	14.3	15.1	15.8	16.3

n.d.: Not detected

These results of increased *H. pylori* targeted anti-bacterial activity with increased duration of fermentation might be due to the production of lactic acid by the mixed LAB culture.

Furthermore, the higher inhibition by sprouted barley samples may be due to the greater availability of fermentable sugars to support higher growth of mixed LAB strains. Previous studies indicated that lactic acid exert antimicrobial activity and may further enhance the ability of phenolic compounds present in fermented extracts to inhibit pathogenic bacteria such as *H. pylori* (Alakomi *et al*., 2000, Ankolekar *et al*., 2011, Midolo *et al*., 1995). Lactic acid can compromise the integrity of bacterial outer membrane by releasing lipopolysaccharide or other components and it can enable phenolic antioxidants to scavenge electrons from electron transport chain along the bacterial membrane (Alakomi *et al*., 2000, Vattem *et al*., 2005). Therefore, the sprouted food barley substrates may offer an additional layer of gastrointestinal benefit by imparting anti-*H. pylori* properties, apart from their potential prebiotic action and can be targeted for human gut health improvements, essential for T2D and other NCD relevant benefits.

11.5. Conclusions

Integrated bioprocessing strategy such as controlled germination (sprouting) and beneficial LAB-mediated fermentation represent viable and cost-effective means of developing grain-based functional food ingredients, with enhanced bioactive profiles and targeted health benefits. In this study, barley type × sprouting duration had statistically significant effect on the soluble phenolic content and individual phenolic compounds of food barley grains, which consequently led to enhancements in specific biological activities such as antioxidant capacity and modulation of glycemic control based on *in vitro* assay models. Interestingly, the study identified unpigmented barley as being particularly suited to sprouting, due to the comparatively lower loss of soluble phenolics during steeping, than pigmented varieties. Additionally, Kefir-mediated fermentation of sprouted barley flour, the second layer of bioprocessing targeted in this study, was found to further improve the phenolic content, phenolic profile, and associated

bioactivity such as antioxidant, anti-hyperglycemic and anti-bacterial properties. Sprouted barley flour, specifically 48 h sprouted sample was found to support beneficial LAB growth during the fermentation process at levels sufficient to impart probiotic benefits, without supplementation of additional nutrients, indicative of a possible prebiotic effect supporting probiotic bacterial growth. Therefore, this study achieved its objectives of identifying sprouted food barley with 48 h sprouting as a promising ingredient source for functional food development, which can be further enhanced by integrating sprouting with fermentation by mixed LAB culture such as Kefir culture. Future studies focusing on fermenting food barley substrates with various single and mixed cultures, the long-term survivability of these cultures and sensory analysis may further help in optimizing sprouted food barley-based functional foods and beverages supporting wider T2D health benefits validated by *in vivo* models.

REFERENCES

Abdel-Aal, E. S. M., Young, J. C. and Rabalski, I., 2006. Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry*, 54(13), pp. 4696–4704.

Adebo, O. A. and Gabriela Medina-Meza, I., 2020. Impact of fermentation on the phenolic compounds and antioxidant activity of whole cereal grains: A mini review. *Molecules*, 25(4), pp. 927.

Adefegha, S. A., 2018. Functional foods and nutraceuticals as dietary intervention in chronic diseases; novel perspectives for health promotion and disease prevention. *Journal of Dietary Supplements*, pp. 977–1009.

Adom, K. K. and Liu, R. H., 2002. Antioxidant activity of grains. *Journal of Agricultural and Food Chemistry*, 50(21), pp. 6182–6187.

Agami, R. A., 2014. Applications of ascorbic acid or proline increase resistance to salt stress in barley seedlings. *Biologia Plantarum*, 58(2), pp. 341–347.

Ahmed, I. M. *et al.*, 2013. Genotypic differences in physiological characteristics in the tolerance to drought and salinity combined stress between Tibetan wild and cultivated barley. *Plant Physiology and Biochemistry*, 63, pp. 49–60.

Ahmed, I. M. *et al.*, 2015. Secondary metabolism and antioxidants are involved in the tolerance to drought and salinity, separately and combined in Tibetan wild barley. *Environmental and Experimental Botany*, 111, pp. 1–12.

Akar, T., Avci, M. and Dusunceli, F., 2004. *Barley: Post-harvest operations.* Available at: http://www.fao.org/fileadmin/user_upload/inpho/docs/Post_Harvest_Compendium_-_BARLEY.pdf (Accessed: 16 July 2018).

Akula, R. and Ravishankar, G. A., 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, 6(11), pp. 1720–1731.

Al-Busaidi, A. *et al*., 2008. Effects of zeolite on soil nutrients and growth of barley following irrigation with saline water. *Journal of Plant Nutrition*, 31(7), pp. 1159–1173.

Alakomi, H. L. *et al.*,2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology*, 66(5), pp. 2001–2005.

Alam, M. M. *et al.*,2011. Rainfall variation and changing pattern of agricultural cycle. *American Journal of Environmental Sciences*, 7(1), pp. 82–89.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T. and von Wright, A., 1999. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus GG*, after oral consumption. *Applied and Environmental Microbiology*, *65*(1), pp.351-354.

Ali Asgar, M., 2013. Anti-diabetic potential of phenolic compounds: A review. *International Journal of Food Properties*, 16(1), pp. 91–103.

Alkhatib, A. *et al.*, 2017. Functional foods and lifestyle approaches for diabetes prevention and management. *Nutrients*, 9(12), p. 1310.

Allel, D., Ben-Amar, A. and Abdelly, C., 2018. Leaf photosynthesis, chlorophyll fluorescence and ion content of barley (*Hordeum vulgare*) in response to salinity. *Journal of Plant Nutrition*, 41(4), pp. 497–508.

American Diabetes Association (2017) *Statistics About Diabetes: American Diabetes Association*. Available at: http://www.diabetes.org/diabetes-basics/statistics/?referrer=https://www.google.com/ (Accessed: 20 March 2017).

Amić, A. *et al.*, 2018. Theoretical study of the thermodynamics of the mechanisms underlying antiradical activity of cinnamic acid derivatives. *Food Chemistry*, pp. 481–489.

Anand, A. *et al.*, 2018. Administration of roasted barley and roasted horse gram powders pacified chronic sucrose-induced dysglycemia and dyslipidemia in rats and exerted In Vitro potent antioxidative stress effect. *Pharmacognosy Magazine*, 14(59), p. 578.

Anandh Babu, P. and Liu, D., 2008. Green tea catechins and cardiovascular health: An update. *Current Medicinal Chemistry*, 15(18), pp. 1840–1850.

Andarwulan, N. and Shetty, K., 1999. Improvement of pea (*Pisum sativum*) seed vigour response by fish protein hydrolysates in combination with acetyl salicylic acid. *Process Biochemistry*, 35(1), pp. 159–165.

Andersson, A. A. M. *et al.*, 2008. Phytochemical and dietary fiber components in barley varieties in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry*, 56(21), pp. 9767–9776.

Ankolekar, C. *et al.*, 2011. Inhibitory potential of tea polyphenolics and influence of extraction time against *Helicobacter pylori* and lack of inhibition of beneficial lactic acid bacteria', *Journal of Medicinal Food*, 14(11), pp. 1321–1329.

Ankolekar, C. *et al.*, 2012. *In vitro* bioassay based screening of antihyperglycemia and antihypertensive activities of *Lactobacillus acidophilus* fermented pear juice. *Innovative Food Science and Emerging Technologies*, 13, pp. 221–230.

Ansari, P. *et al.*, 2017. Anti-hyperglycemic activity of *Aegle marmelos* (L.) corr. is partly mediated by increased insulin secretion, α-amylase inhibition, and retardation of glucose absorption. *Journal of Pediatric Endocrinology and Metabolism*, 30(1), pp. 37–47.

Anson, N. M. *et al.*, 2009. Bioprocessing of wheat bran improves *in vitro* bioaccessibility and colonic metabolism of phenolic compounds. *Journal of Agricultural and Food Chemistry*, 57(14), pp. 6148–6155.

Augustin, M. A. and Sanguansri, L., 2015. Challenges and solutions to incorporation of nutraceuticals in foods. *Annual Review of Food Science and Technology*, 6(1), pp. 463–477.

Awad, A. B. and Fink, C. S., 2000. Phytosterols as anticancer dietary components: Evidence and mechanism of action. *Journal of Nutrition*, 130(9), pp. 2127–2130.

Bach Knudsen, K. E. *et al.*, 2017. Dietary fibers and associated phytochemicals in cereals. *Molecular Nutrition & Food Research*, 61(7), p. 1600518.

Baik, B.-K. K. and Ullrich, S. E., 2008. Barley for food: characteristics, improvement, and renewed interest. *Journal of Cereal Science*, 48(2), pp. 233–242.

Bailey-Serres, J., Lee, S. C. and Brinton, E., 2012. Waterproofing crops: Effective flooding survival strategies. *Plant Physiology*, 160(4), pp. 1698–1709.

Balakhnina, T. I. *et al.*, 2009. Dihydroquercetin protects barley seeds against mold and increases seedling adaptive potential under soil flooding. *Plant Growth Regulation*, pp. 127–135.

Balasundram, N., Sundram, K. and Samman, S., 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99(1), pp. 191–203.

Bamba, T. *et al.*, 2002. A new prebiotic from germinated barley for nutraceutical treatment of ulcerative colitis. *Journal of Gastroenterology and Hepatology*, 17(8), pp. 818–824.

Bartwal, A. *et al.*, 2013.Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. *Journal of Plant Growth Regulation*, pp. 216–232.

Battacharyya, D. *et al.*, 2015. Seaweed extracts as biostimulants in horticulture. *Scientia Horticulturae*, pp. 39–48.

Becker, F., 2013. *Tocopherols in wheat and rye : a review of research within the subject*, Swedish University of Agricultural Sciences, Uppsala.

Beers, R. F. and Sizer, I. W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*,195, pp. 133–140.

Behall, K. M., Scholfield, D. J. and Hallfrisch, J., 2004. Diets containing barley significantly reduce lipids in mildly hypercholesterolemic men and women. *The American Journal of Clinical Nutrition*, 80(5), pp. 1185–1193.

Belobrajdic, D. P. and Bird, A. R., 2013. The potential role of phytochemicals in wholegrain cereals for the prevention of type-2 diabetes. *Nutrition Journal*, 12 (1), pp. 1–12.

Bener, A. *et al.*, 2007. Association between type 2 diabetes mellitus and *Helicobacter pylori* infection. *Turkish Journal of Gastroenterology*, 18(4), pp. 225–229.

Benkeblia, N. (ed.) (2014) *Polysaccharides: Natural fibers in food and nutrition.* Boca Raton, Florida: CRC Press.

Berhanu, A. T. and Berhane, G., 2014. The effect of salinity (NaCl) on germination and early seedling growth of *Lathyrus sativus* and *Pisum sativum* var. abyssinicum', *African Journal of Plant Science*, 8(5), pp. 225–231.

Bischoff, H., 1994. Pharmacology of α-glucosidase inhibition. *European Journal of Clinical Investigation*, 24(3 S), pp. 3–10.

Bonoli, M. *et al.*, 2004. Antioxidant phenols in barley (*Hordeum vulgare* L.) flour: Comparative spectrophotometric study among extraction methods of free and bound phenolic compounds. *Journal of Agricultural and Food Chemistry*, 52(16), pp. 5195–5200.

Braaten, J. T. *et al.*, 1991. Oat gum lowers glucose and insulin after an oral glucose load. *The American Journal of Clinical Nutrition*, 53(6), pp. 1425–1430.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), pp. 248–254.

Bregman and A.A., 1987. Laboratory investigations in cell biology. Second edition. John Wiley and Sons Inc.,New York, NY.

Brownlee, M., 2005. The pathobiology of diabetic complications: A unifying mechanism, in *Diabetes*. American Diabetes Association, pp. 1615–1625.

Bulgari, R. *et al.*, 2015, Biostimulants and crop responses: A review. *Biological Agriculture and Horticulture*, 31(1), pp. 1–17.

Burger, W.C., Qureshi, A.A., Elson, C.E. and Wisconsin Alumni Research Foundation, 1984. *Cholesterol lowering method of use*. U.S. Patent 4,603,142.

Burgos-Morón *et al.*, 2019. Relationship between oxidative stress, ER stress, and inflammation in type 2 diabetes: The battle continues'. *Journal of Clinical Medicine*, 8 (9), pp. 1385.

Burton, G. W. and Ingold, K. U., 1989. Vitamin E as an *in vitro* and *in vivo* antioxidant. *Annals of the New York Academy of Sciences*, 570, pp. 7–22.

Caballero, B., 2007. The global epidemic of obesity: An overview'. *Epidemiologic Reviews*, 29(1), pp. 1–5.

Carter, P. *et al.*, 2010. Fruit and vegetable intake and incidence of type 2 diabetes mellitus: systematic review and meta-analysis. *British Medical Journal (Clinical research ed.)*, 341, p. c4229.

Carvalho, D., Curto, A. and Guido, L., 2015. Determination of phenolic content in different barley varieties and corresponding malts by liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry. *Antioxidants*, 4(3), pp. 563–576.

Cavallero, A. *et al.*, 2004. Tocols in hull-less and hulled barley genotypes grown in contrasting environments. *Journal of Cereal Science*, 39(2), pp. 175–180.

Cavazos, A. and Gonzalez de Mejia, E., 2013. Identification of bioactive peptides from cereal storage proteins and their potential role in prevention of chronic diseases. *Comprehensive Reviews in Food Science and Food Safety*, 12(4), pp. 364–380.

Çelik, S. E. *et al.*, 2010. Solvent effects on the antioxidant capacity of lipophilic and hydrophilic antioxidants measured by CUPRAC, ABTS/persulphate and FRAP methods. *Talanta*, 81(4–5), pp. 1300–1309.

Cerna, J. and Kas, J.,1983. Folacin in cereals and cereal products. *Developments in Food Science*, 5, pp. 501–506.

Cervato, G. *et al.*, 2000. Antioxidant properties of oregano (*Origanum vulgare*) leaf extracts. *Journal of Food Biochemistry*, 24(6), pp. 453–465.

Charalampopoulos, D. *et al.*, 2002. Application of cereals and cereal components in functional foods: A review. *International Journal of Food Microbiology*, 79(1–2), pp. 131–141.

Chavan, M. *et al.*, 2018. Development of non-dairy fermented probiotic drink based on germinated and ungerminated cereals and legume. *LWT - Food Science and Technology*, 91, pp. 339–344.

Chen, Y. *et al.*, 2019. Comparative study on the phytochemical profiles and cellular antioxidant activity of phenolics extracted from barley malts processed under different roasting temperatures. *Food and Function*, 10(4), pp. 2176–2185.

Cheng, L. and Lai, M., 2003. Aberrant crypt foci as microscopic precursors of colorectal cancer. *World Journal of Gastroenterology*, pp. 2642–2649.

Chu, T., Aspinall, D. and Paleg, L., 1976. Stress Metabolism. VII. Salinity and Proline accumulation in barley. *Australian Journal of Plant Physiology*, 3(2), p. 219.

Chung, H.J. *et al.*, 2009. Effects of steeping and anaerobic treatment on GABA (γ-aminobutyric acid) content in germinated waxy hull-less barley. *LWT - Food Science and Technology*, 42(10), pp. 1712–1716.

Clark, A. (ed.) (2007) 'Barley', in *Managing cover crops profitably*. 3rd edn. Sustainable Agriculture Research and Education (SARE) program, pp. 77–80.

Clarke, R. *et al.*, 2012. Homocysteine and coronary heart disease: Meta-analysis of MTHFR Case-control studies, avoiding publication bias', *PLoS Medicine*, 9(2), p. e1001177.

Clifford, M.N., 2000. Chlorogenic acids and other cinnamates–nature, occurrence, dietary burden, absorption and metabolism. *Journal of the Science of Food and Agriculture*, *80*(7), pp.1033-1043.

Coda, R. *et al.*, 2012. Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours. *Applied and Environmental Microbiology*, 78(4), pp. 1087–1096.

Colla, G. *et al.*, 2016. Protein hydrolysate-based biostimulants: origin, biological activity and application methods. *Acta Horticulturae*,1148, pp. 27–34.

Corbo, M. R. *et al.*, 2014. Functional beverages: The emerging side of functional foods: commercial trends, research, and health implications. *Comprehensive Reviews in Food Science and Food Safety*, 13(6), pp. 1192–1206.

Costilow, R. N. and Cooper, D., 1978. Identity of proline dehydrogenase and delta1-pyrroline-5-carboxylic acid reductase in *Clostridium sporogenes*. *Journal of Bacteriology*, 134(1), pp. 139–46.

Crozier, A., Jaganath, I. B. and Clifford, M. N, 2007. Phenols, polyphenols and tannins: An overview, in Crozier, A., Clifford, M.N. and Ashihara, H. (eds.), *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*. Oxford, UK: Blackwell Publishing Ltd, pp. 1–24.

Curiel, J. A. *et al.*, 2010. Ability of *Lactobacillus brevis* strains to degrade food phenolic acids. *Food Chemistry*, 120(1), pp. 225–229.

Daniels, R. B. (1987) 'Saline seeps in the Northern Great Plains of the USA and the Southern Prairies of Canada', in Wolman, M. and Fournier, F. (eds) *Land transformation in agriculture* . Chichester : John Wiley and Sons, pp. 381–406. Available at: https://agris.fao.org/agris-search/search.do?recordID=US201301400225 (Accessed: 29 December 2020).

Deutsch J (1983) *Methods of enzymatic analysis*. 3rd edn. Deerfield Beach, Florida: Verlag Chemie Academic Press.

Donkor, O. N. *et al.*, 2012. Germinated grains - sources of bioactive compounds. *Food Chemistry*, 135(3), pp. 950–959.

Dordević, T. M., Šiler-Marinković, S. S. and Dimitrijević-Branković, S. I., 2010. Effect of fermentation on antioxidant properties of some cereals and pseudo cereals. *Food Chemistry*, 119(3), pp. 957–963.

Duh, P. Der *et al.*, 2001. Antioxidant effects of water extracts from barley (*Hordeum vulgare* L.) prepared under different roasting temperatures. *Journal of Agricultural and Food Chemistry*, 49(3), pp. 1455–1463.

Dvořáková, M. *et al.*, 2008. Characterization of monomeric and oligomeric flavan-3-ols from barley and malt by liquid chromatography-ultraviolet detection-electrospray ionization mass spectrometry. *Journal of Chromatography A.*, 1189(1–2), pp. 398–405.

Dvořáková, M., Guido, L.F., Dostálek, P., Skulilová, Z., Moreira, M.M. and Barros, A.A., 2008. Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. *Journal of the Institute of Brewing*, *114*(1), pp.27-33.

Dykes, L. and Rooney, L., 2007. Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World*, 52(3), pp. 105–111.

Edelmann, M. *et al.*, 2013. Folate in barley grain and fractions. *Journal of Cereal Science*. Academic Press, 58(1), pp. 37–44.

Ehrenbergerova, J. *et al.*, 2006. Effect of cultivar, year grown, and cropping system on the content of tocopherols and tocotrienols in grains of hulled and hulless barley. *Plant Foods for Human Nutrition*, 61(3), pp. 145–150.

Eisa, S. *et al.*, 2012. Effect of NaCl salinity on water relations, photosynthesis and chemical composition of Quinoa ("*Chenopodium quinoa*" Willd.) as a potential cash crop halophyte. *Australian Journal of Crop Science*, 6(2), pp. 357–368.

El-Sharkawy, M. *et al.*, 2017. Effect of plant growth stimulants on alfalfa response to salt stress. *Agricultural Sciences*, 8(04), pp. 267–291.

Enujiugha, V. N. and Badejo, A. A., 2017. Probiotic potentials of cereal-based beverages. *Critical Reviews in Food Science and Nutrition*, 57(4), pp. 790–804.

Erdal, S. *et al.*, 2011. Effects of salicylic acid on wheat salt sensitivity. *African Journal of Biotechnology*, 10(30), pp. 5713–5718.

Ernst, E. and Pittler, M. H., 2001. Assessment of therapeutic safety in systematic reviews: Literature review. *British Medical Journal*, p. 546.

Espín, J. C., González-Sarrías, A. and Tomás-Barberán, F. A., 2017. The gut microbiota: A key factor in the therapeutic effects of (poly)phenols. *Biochemical Pharmacology*, pp. 82–93.

Evans, J. L. *et al.*, 2002. Oxidative stress and stress-activated signaling pathways: A unifying hypothesis of type 2 diabetes', *Endocrine Reviews*, 23(5), pp. 599–622.

Fadel, J. G. *et al*., 1987. Hypocholesterolemic effects of beta-glucans in different barley diets fed to broiler chicks. *The Journal of Nutritional Biochemistry*, 35, pp. 1049–1058.

Fardet, A., 2010. New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre?.*Nutrition Research Reviews*, 23, pp. 65–134.

Feng, X. M., Eriksson, A. R. B. and Schnürer, J., 2005. Growth of lactic acid bacteria and *Rhizopus oligosporus* during barley tempeh fermentation. *International Journal of Food Microbiology*, 104(3), pp. 249–256.

Fernandes, C. G., Sonawane, S. K. and Arya, S. S., 2018. Cereal based functional beverages: A review. *Journal of Microbiology, Biotechnology and Food Sciences*, 8(3), pp. 914–919.

Ferreres, F. *et al.*, 2009. Free Water-soluble phenolics profiling in barley (*Hordeum vulgare* L.), *Journal of Agricultural and Food Chemistry*, 57(6), pp. 2405–2409.

Floridi, S. *et al.*, 2003. Determination of free phenolic acids in wort and beer by coulometric array detection. *Journal of Agricultural and Food Chemistry*, 51(6), pp. 1548–1554.

Fowler, M. J., 2008. Microvascular and macrovascular complications of diabetes. *Clinical Diabetes*, 26(2), pp. 77– 82.

Foyer, C. H. and Noctor, G., 2009. Redox regulation in photosynthetic organisms: Signaling, acclimation, and practical implications. *Antioxidants and Redox Signalling*, 11(4), pp. 861–905.

Fraga, C. G. *et al.*, 2010. Basic biochemical mechanisms behind the health benefits of polyphenols. *Molecular Aspects of Medicine*, pp. 435–445.

Frandsen, T. P. and Svensson, B., 1998. Plant α-glucosidases of the glycoside hydrolase family 31. Molecular properties, substrate specificity, reaction mechanism, and comparison with family members of different origin. *Plant Molecular Biology*, pp. 1–13.

Franzen, D. (2007) *Managing Saline Soils in North Dakota*. Available at: https://library.ndsu.edu/ir/bitstream/handle/10365/5441/sf1087.pdf?sequence=1 (Accessed: 4 May 2020).

Frølich, W., Åman, P. and Tetens, I., 2013. Whole grain foods and health - A Scandinavian perspective. *Food and Nutrition Research*, 57(1), pp. 18503.

Fujita, A. *et al.*, 2017. Improving anti-hyperglycemic and anti-hypertensive properties of camu-camu (*Myriciaria dubia* Mc. Vaugh) using lactic acid bacterial fermentation. *Process Biochemistry*, 59, pp. 133–140.

Fuller, R.,1989. Probiotics in man and animals. *Journal of Applied Bacteriology*, 66(5), pp. 365–378.

Havenaar, R., Ten Brink, B. and Huis, J.H., 1992. Selection of strains for probiotic use. *Probiotics*. pp. 209–224.

Grand View Research, 2018. *Functional Drinks Market Worth $93.68 Billion by 2019 | CAGR: 6.1%* (Online). Available at: https://www.grandviewresearch.com/press-release/global-functional-drinks-market (Accessed: 29 December 2020).

Gamel, T. H. and Abdel-Aal, E. S. M., 2012. Phenolic acids and antioxidant properties of barley wholegrain and pearling fractions. *Agricultural and Food Science*, 21(2), pp. 118–131.

Gao, S. *et al.*, 2016. G6PDH activity highlights the operation of the cyclic electron flow around PSI in *Physcomitrella patens* during salt stress. *Scientific Reports*, 6(1), pp. 1–9.

Ge, X. *et al.*, 2021. The phenolic compounds profile, quantitative analysis and antioxidant activity of four naked barley grains with different color. *Food Chemistry*, 335, p. 127655.

Genisel, M., Erdal, S. and Kizilkaya, M., 2014. The mitigating effect of cysteine on growth inhibition in salt-stressed barley seeds is related to its own reducing capacity rather than its effects on antioxidant system. *Plant Growth Regulation*, 75(1), pp. 187–197.

Gibreel, A. *et al.*, 2009. Fermentation of barley by using *Saccharomyces cerevisiae*: Examination of barley as a feedstock for bioethanol production and value-added products. *Applied and Environmental Microbiology*, 75(5), pp. 1363–1372.

Gibson, G. R. *et al.*, 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews*, 17(2), pp. 259–275.

Gill, M. B. *et al.*, 2017. Cell-based phenotyping reveals QTL for membrane potential maintenance associated with hypoxia and salinity stress tolerance in barley. *Frontiers in Plant Science*, 8, p. 1941.

Gondim, F. A. *et al.*, 2012. Catalase plays a key role in salt stress acclimation induced by hydrogen peroxide pretreatment in maize. *Plant Physiology and Biochemistry*, 56, pp. 62–71.

Goupy, P. *et al.*, 1999. Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture*, 79(12), pp. 1625–1634.

Granato, D. *et al.*, 2010. Functional foods and nondairy probiotic food development: Trends, concepts, and products. *Comprehensive Reviews in Food Science and Food Safety*, 9(3), pp. 292–302.

Guilloteau, P. *et al.*, 2010. From the gut to the peripheral tissues: The multiple effects of butyrate. *Nutrition Research Review*, 23(2), pp. 366–384.

Gujska, E. and Kuncewicz, A., 2005. Determination of folate in some cereals and commercial cereal-grain products consumed in Poland using trienzyme extraction and high-performance liquid chromatography methods. *European Food Research and Technology*, 221(1–2), pp. 208–213.

Gupta, M., Abu-Ghannam, N. and Gallaghar, E., 2010. Barley for brewing: characteristic changes during malting, brewing and applications of its by-products. *Comprehensive Reviews in Food Science and Food Safety*, 9(3), pp. 318–328.

Gürel, F. *et al.*, 2016. Barley genes as tools to confer abiotic stress tolerance in crops. *Frontiers in Plant Science*, 7, pp. 1137.

Güzel-Seydim, Z. B. *et al.*, 2000. Determination of organic acids and volatile flavor substances in kefir during fermentation. *Journal of Food Composition and Analysis*, 13(1), pp. 35–43.

Ha, K. S. *et al.*, 2016. Stimulation of phenolics, antioxidant and α-glucosidase inhibitory activities during barley (*Hordeum vulgare* L.) seed germination. *Plant Foods for Human Nutrition*, 71(2), pp. 211–217.

El Hadrami, A. *et al.*, 2010. Chitosan in Plant Protection. *Marine Drugs*, 8(4), pp. 968–987.

El Hag, M. E., El Tinay, A. H. and Yousif, N. E., 2002. Effect of fermentation and dehulling on starch, total polyphenols, phytic acid content and *in vitro* protein digestibility of pearl millet. *Food Chemistry*, 77(2), pp. 193–196.

Hallikainen, M. A., Sarkkinen, E. S. and Uusitupa, M. I. J., 2000. Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner. *Journal of Nutrition*, 130(4), pp. 767–776.

Han, Y. H., Yon, M. and Hyun, T. H., 2005. Folate intake estimated with an updated database and its association to blood folate and homocysteine in Korean college students. *European Journal of Clinical Nutrition*, 59(2), pp. 246–254.

Hanhineva, K. *et al.*, 2010. Impact of dietary polyphenols on carbohydrate metabolism. *International Journal of Molecular Sciences*, 11(4), pp. 1365–1402.

Hare, P. D. and Cress, W. A., 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation*, 21(2), pp. 79–102.

Hedrington, M. S. and Davis, S. N., 2019. Considerations when using alpha-glucosidase inhibitors in the treatment of type 2 diabetes. *Expert Opinion on Pharmacotherapy*, 20(18), pp. 2229–2235.

Hegedüs, M., Pedersen, B. and Eggum, B. O., 1985. The influence of milling on the nutritive value of flour from cereal grains. 7. Vitamins and tryptophan. *Qualitas Plantarum Plant Foods for Human Nutrition*, 35(2), pp. 175–180.

Hendriks, H. F. J. *et al.*, 1999. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *European Journal of Clinical Nutrition*, 53, pp. 319–327.

Hernanz, D. *et al.*, 2001. Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. *Journal of Agricultural and Food Chemistry*, 49(10), pp. 4884–4888.

Herzog, M. *et al.*, 2016. Mechanisms of waterlogging tolerance in wheat - a review of root and shoot physiology. *Plant Cell and Environment*, pp. 1068–1086.

Hole, A. S. *et al*., 2012. Improved bioavailability of dietary phenolic acids in whole grain barley and oat groat following fermentation with probiotic *Lactobacillus acidophilus*, L*actobacillus johnsonii*, and *Lactobacillus reuteri*. *Journal of Agricultural and Food Chemistry*, 60(25), pp. 6369–6375.

Holtekjølen, A. K., Kinitz, C. and Knutsen, S. H., 2006. Flavanol and bound phenolic acid contents in different barley varieties. *Journal of Agricultural and Food Chemistry*, 54(6), pp. 2253–5560.

Hossain, M. S. and Dietz, K. J., 2016. Tuning of redox regulatory mechanisms, reactive oxygen species and redox homeostasis under salinity stress. *Frontiers in Plant Science*, pp. 548.

Howitt, C. A. and Pogson, B. J., 2006. Carotenoid accumulation and function in seeds and non-green tissues. *Plant, Cell and Environment*, 29(3), pp. 435–445.

Huang, D. *et al.*, 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry*, 50(16), pp. 4437–4444.

Huang, S. and Millar, H. A., 2013. Succinate dehydrogenase: the complex roles of a simple enzyme. *Current Opinion in Plant Biology*, 16(3), pp. 344–349.

Huang, Z. *et al.*, 2013. Salt stress encourages proline accumulation by regulating proline biosynthesis and degradation in Jerusalem artichoke plantlets. *PLoS ONE*, 8(4), p. e62085.

Hübner, F. and Arendt, E. K., 2013. Germination of cereal grains as a way to improve the nutritional value: A review. *Critical Reviews in Food Science and Nutrition*, 53(8), pp. 853–861.

Hur, S. J. *et al.*, 2014. Effect of fermentation on the antioxidant activity in plant-based foods. *Food Chemistry*, 160, pp. 346–356.

Hussain, G. *et al.*, 1997. Effect of saline irrigation on germination and growth parameters of barley (*Hordeum vulgare* L.) in a pot experiment. *Agricultural Water Management*, 34(2), pp. 125–135.

Iacopini, P. *et al.*, 2008. Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, *in vitro* antioxidant activity and interactions. *Journal of Food Composition and Analysis*, 21(8), pp. 589–598.

Idehen, E., Tang, Y. and Sang, S., 2017. Bioactive phytochemicals in barley. *Journal of Food and Drug Analysis*, 25(1), pp. 148–161.

IDF Diabetes Atlas (2017). Available at: http://www.diabetesatlas.org/ (Accessed: 20 March 2017).

Iimure, T. *et al.*, 2009. A method for production of γ-amino butyric acid (GABA) using barley bran supplemented with glutamate. *Food Research International*, 42(3), pp. 319–323.

Inoue, K. *et al.*, 2003. Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. *European Journal of Clinical Nutrition*, 57(3), pp. 490–495.

Inzucchi, S. E. *et al.*, 2015. Management of hyperglycaemia in type 2 diabetes, 2015: a patient-centred approach. Update to a position statement of the american diabetes association and the European association for the study of diabetes. *Diabetologia*, 58(3), pp. 429–442.

Irfan, M. *et al*., 2010. Physiological and biochemical changes in plants under waterlogging. *Protoplasma*, 241(1–4).

Jacobs, A. A. and Whitten, J.L., 2016. *Plant guide for common barley (Hordeum vulgare L.)*. Available at: http://plants.usda.gov/ (Accessed: 6 January 2018).

Jägerstad, M. *et al.*, 2005. Increasing natural food folates through bioprocessing and biotechnology. *Trends in Food Science & Technology*, 16(6–7), pp. 298–306.

du Jardin, P., 2015. Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae*, 196, pp. 3–14.

Jarošová, M. *et al.*, 2016. Humic acid protects barley against salinity. *Acta Physiologiae Plantarum*, 38(6), pp. 1–9.

Jende-Strid, B., 1993. Genetic control of flavonoid biosynthesis in barley. *Hereditas*, 119(2), pp. 187–204.

Jeon, C. Y. *et al.*, 2012. *Helicobacter pylori* infection is associated with an increased rate of diabetes', *Diabetes Care*, 35(3), pp. 520–525.

Jeong, G.-T. and Park, D.-H., 2005. Enhancement of growth and secondary metabolite biosynthesis: Effect of elicitors derived from plants and insects. *Biotechnology and Bioprocess Engineering*, 10(1), pp. 73–77.

Jeong, H. J. *et al*., 2007. The cancer preventive peptide lunasin from wheat inhibits core histone acetylation. *Cancer Letters*, 255(1), pp. 42–48.

Jeong, H. J. *et al.*, 2010. Lunasin is prevalent in barley and is bioavailable and bioactive in *in vivo* and *in vitro* studies. *Nutrition and Cancer*, 62(8), pp. 1113–1119.

Jeong, H. J., Lam, Y. and de Lumen, B. O., 2002. Barley Lunasin suppresses ras -induced colony formation and inhibits core histone acetylation in mammalian cells. *Journal of Agricultural and Food Chemistry*, 50(21), pp. 5903–5908.

Jin, B. J. *et al.*, 2007. Cancer-preventive peptide lunasin from *Solanum nigrum* L. inhibits acetylation of core histones H3 and H4 and phosphorylation of retinoblastoma protein (Rb). *Journal of Agricultural and Food Chemistry*, 55(26), pp. 10707–10713.

Jisha, K. C., Vijayakumari, K. and Puthur, J. T., 2013. Seed priming for abiotic stress tolerance: An overview. *Acta Physiologiae Plantarum*, pp. 1381–1396.

Jones, P. J. *et al.*, 2000. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. *Journal of Lipid Research*, 41, pp. 697–705.

Kähkönen, M. P. *et al.*,1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*. 47(10), pp. 3954–3962.

Kahn, S. E., 2003. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia*, 46(1), pp. 3–19.

Kakkar, S. and Bais, S., 2014. A review on protocatechuic acid and its pharmacological potential. *ISRN Pharmacology*, 2014, p. 952943.

Kanauchi, O. *et al.*, 1999. Increased growth of *Bifidobacterium* and *Eubacterium* by germinated barley foodstuff, accompanied by enhanced butyrate production in healthy volunteers. *International Journal of Molecular Medicine*, 3 (2), pp. 175–184.

Kanauchi, O. *et al.*, 2008. Modulation of intestinal environment by prebiotic germinated barley foodstuff prevents chemo-induced colonic carcinogenesis in rats. *Oncology Reports*, 20(4), pp. 793–801.

Kandil, A. *et al.*, 2012. Phenolic acids in some cereal grains and their inhibitory effect on starch liquefaction and saccharification. *Journal of Agricultural and Food Chemistry*, 60(34), pp. 8444–8449.

Kandylis, P. *et al.*, 2016. Dairy and non-dairy probiotic beverages. *Current Opinion in Food Science*, pp. 58–63.

Karami, A. and Sepehri, A., 2018. Beneficial role of mwcnts and snp on growth, physiological and photosynthesis performance of barley under nacl stress. *Journal of Soil Science and Plant Nutrition*, 18(3), pp. 752–771.

Kawabata, K. *et al.*, 2000. Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats. *Cancer Letters*, 157(1), pp. 15–21.

Kharel, T., 2016. *Soil salinity study in Northern Great Plains sodium affected soil*, PhD Thesis, South Dakota State University. Available at: https://openprairie.sdstate.edu/etd/999 (Accessed: 28 December 2017).

Kihara, M. *et al.*, 2007. Accumulation and degradation of two functional constituents, gaba and β-glucan, and their varietal differences in germinated barley grains. *Breeding Science*, 57, pp. 85–89.

Kim, H. *et al.*, 2008. Saengshik, a formulated health food, prevents liver damage in CCl4-induced mice and increases antioxidant activity in elderly women. *Journal of Medicinal Food*, 11(2), pp. 323–30.

Kim, M. *et al.*, 2014. The ingredients in *Saengshik* , a formulated health food, inhibited the activity of α-amylase and α-glucosidase as anti-diabetic function. *Nutrition Research and Practice*, 8(5), p. 602.

Kim, M. J. *et al.*, 2007. Relationship between phenolic compounds, anthocyanins content and antioxidant activity in colored barley germplasm. *Journal of Agricultural and Food Chemistry*, 55(12), pp. 4802–4809.

Kim, S.K. (ed.), 2011. *Chitin, chitosan, oligosaccharides and their derivatives: Biological activities and applications*. Boca Raton, Florida: CRC Press.

Kim, S. *et al.*, 2016. Comparative DNA adduct formation and induction of colonic aberrant crypt foci in mice exposed to 2-amino-9H-pyrido [2, 3-b] indole, 2-amino-3, 4-dimethylimidazo [4, 5-f] quinoline, and azoxymethane. *Environmental and Molecular Mutagenesis*, 57(2), pp. 125–136.

Kreicbergs, V. *et al.*, 2011. Biologically active compounds in roasted coffee, in *Proceedings of the 6th Baltic Conference on Food Science and Technology*. Jelgava, Latvia: FOODBALT 2011, pp. 110–115.

Krüger, A. *et al.*, 2011. The pentose phosphate pathway is a metabolic redox sensor and regulates transcription during the antioxidant response. *Antioxidants and Redox Signaling*, 15(2), pp. 311–324.

Kumar, N. and Pruthi, V., 2014. Potential applications of ferulic acid from natural sources. *Biotechnology Reports*, pp. 86–93.

Kwon, Y.I., Vattem, D. A. and Shetty, K., 2006. Evaluation of clonal herbs of Lamiaceae species for management of diabetes and hypertension. *Asia Pacific Journal of Clinical Nutrition*, 15(1), pp. 107–118.

Kwon, Y.-I. *et al*., 2007. Health benefits of traditional corn, beans, and pumpkin: In vitro studies for hyperglycemia and hypertension management. *Journal of Medicinal Food*, 10(2), pp. 266–275.

Laloue, H. *et al.*, 1997. Identification of ascorbate and guaiacol peroxidase in needle chloroplasts of spruce trees', *Plant Physiology and Biochemistry*, 35(5), pp. 341–346.

Lam, Y., Galvez, A. and De Lumen, B. O., 2003. Lunasin™ suppresses E1A-mediated transformation of mammalian cells but does not inhibit growth of immortalized and established cancer cell lines. *Nutrition and Cancer*, 47(1), pp. 88–94.

Lampi, A.-M. *et al.*, 2004. Pearling barley and rye to produce phytosterol-rich fractions. *Lipids*, 39(8), pp. 783–7.

Landberg, R. *et al.*, 2008. Localization of alkylresorcinols in wheat, rye and barley kernels. *Journal of Cereal Science*, 48(2), pp. 401–406.

Leahy, J. L., Bonner-Weir, S. and Weir, G. C., 1992. β-Cell dysfunction induced by chronic hyperglycemia: Current ideas on mechanism of impaired glucose-induced insulin secretion. *Diabetes Care*, 15(3), pp. 442–455.

Lee, J. H. *et al.*, 2015. Barley sprout extract containing policosanols and polyphenols regulate AMPK, SREBP2 and ACAT2 activity and cholesterol and glucose metabolism *in vitro* and *in vivo*. *Food Research International*, 72, pp. 174–183.

Lee, N. Y. *et al.*, 2010. Biological activity of barley (*Hordeum vulgare* L.) and barley by-product extracts. *Food Science and Biotechnology*, 19(3), pp. 785–791.

Lee, W. *et al.*, 2015. Gallic acid decreases inflammatory cytokine secretion through histone acetyltransferase/histone deacetylase regulation in high glucose-induced human monocytes. *Journal of Medicinal Food*. 18(7), pp. 793–801.

Levitan, E. B. *et al.*, 2008, Dietary glycemic index, dietary glycemic load, blood lipids, and C-reactive protein. *Metabolism: Clinical and Experimental*, 57(3), pp. 437–443.

Li, H. *et al*., 2012. Generation and scavenging of reactive oxygen species in wheat flag leaves under combined shading and waterlogging stress. *Functional Plant Biology*, 39(1), p. 71.

Li, X. *et al.*, 2011. Antioxidant activity and mechanism of protocatechuic acid *in vitro*', *Functional Foods in Health and Disease*, 1(7), pp. 232–244.

Lilia, E. *et al.*, 2005. Effect of salt treatment on the expression of phenolics and peroxidase activity assessed in two barley cultivars Acsad 1230 and Arig 8. *Journal of Agronomy*, 4(3), pp. 196–202.

Lin, D. *et al.*, 2016. An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. *Molecules*, 21(10), p. 1374.

Liu, K. S. and Moreau, R. A., 2008. Concentrations of functional lipids in abraded fractions of hulless barley and effect of storage. *Journal of Food Science*, 73(7), pp.C569-C576.

Liu, R. H., 2007. Whole grain phytochemicals and health. *Journal of Cereal Science*, 46(3), pp. 207–219.

Liu, Z. *et al.*, 2013. Regulation, evolution, and functionality of flavonoids in cereal crops. *Biotechnology Letters*, pp. 1765–1780.

Liu, X., Fan, Y., Mak, M., Babla, M., Holford, P., Wang, F., Chen, G., Scott, G., Wang, G., Shabala, S. and Zhou, M., 2017. QTLs for stomatal and photosynthetic traits related to salinity tolerance in barley. *BMC genomics*, 18(1), pp.1-13.

Lu, J. *et al.*, 2007. Evolution of phenolic compounds and antioxidant activity during malting. *Journal of Agricultural and Food Chemistry*, 55(26), pp. 10994–11001.

Lu, X., Huan, L., Gao, S., He, L. and Wang, G., 2016. NADPH from the oxidative pentose phosphate pathway drives the operation of cyclic electron flow around photosystem I in high-intertidal macroalgae under severe salt stress. *Physiologia Plantarum*, *156*(4), pp.397-406.

Luan, H. *et al.*, 2018. Elucidating the hypoxic stress response in barley (*Hordeum vulgare* L.) during waterlogging: A proteomics approach. *Scientific Reports*, 8(1), pp. 1–13.

Lucas, E. A. *et al*., 2004. Flaxseed reduces plasma cholesterol and atherosclerotic lesion formation in ovariectomized Golden Syrian hamsters. *Atherosclerosis*, 173(2), pp. 223–229.

Ma, Y. *et al.*, 2019. GABA mediates phenolic compounds accumulation and the antioxidant system enhancement in germinated hulless barley under NaCl stress. *Food Chemistry*, 270, pp. 593–601.

Madhujith, T. and Shahidi, F., 2007. Antioxidative and antiproliferative properties of selected barley (*Hordeum vulgare* L.) cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. *Journal of Agricultural and Food Chemistry*, 55(13), pp. 5018–5024.

Madhujith, T. and Shahidi, F., 2009. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chemistry*, 117(4), pp. 615–620.

Mahlooji, M. *et al.*, 2018. Effect of salt stress on photosynthesis and physiological parameters of three contrasting barley genotypes. *Photosynthetica*, 56(2), pp. 549–556.

Maillard, M.N. *et al.*, 1996. Antioxidant Activity of barley and malt: Relationship with phenolic content. *LWT - Food Science and Technology*, 29(3), pp. 238–244.

Maillard, M.N. and Berset, C., 1995. *E*volution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. *Journal of Agricultural and Food Chemistry*, 43(7), pp. 1789–1793.

Marshall, J., 1977. Pancreatic alpha-amylase inhibitors in cereals. *Carbohydrate Research*, 57, pp. C27–C30.

Mårtensson, O., Öste, R. and Holst, O., 2002. The effect of yoghurt culture on the survival of probiotic bacteria in oat-based, non-dairy products. *Food Research International*, *35*(8), pp.775-784.

Mattei, J. *et al.*, 2015. Reducing the global burden of type 2 diabetes by improving the quality of staple foods: The Global Nutrition and Epidemiologic Transition Initiative. *Globalization and Health*, 11(1), pp. 1–20.

Mattila, P., Pihlava, J. M. and Hellström, J., 2005. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *Journal of Agricultural and Food Chemistry*, 53(21), pp. 8290–8295.

Mazza, G. and Gao, L., 2005. Blue and purple grains. in Abdel-Aal, E. and Wood, P. (eds) *Speciality grains for food and feed*. St. Paul, Minnesota: AACC International, pp. 313–350.

Mccue, P., Kwon, Y.-I. I. and Shetty, K., 2005. Anti-amylase, anti-glucosidase and anti-angiotensin I-converting enzyme potential of selected foods. *Journal of Food Biochemistry*, 29(3), pp. 278–294.

McCue, P. and Shetty, K., 2002. Clonal herbal extracts as elicitors of phenolic synthesis in dark-germinated mungbeans for improving nutritional value with implications for food safety. *Journal of Food Biochemistry*, 26(3), pp. 209–232.

McIntosh, G. H., Jorgensen, L. and Royle, P., 1993. The potential of an insoluble dietary fiber-rich source from barley to protect from DMH-induced intestinal tumors in rats. *Nutrition and Cancer*, 19(2), pp. 213–221.

Mendiondo, G. M. *et al.*, 2016. Enhanced waterlogging tolerance in barley by manipulation of expression of the N-end rule pathway E3 ligase PROTEOLYSIS6. *Plant Biotechnology Journal*, 14(1), pp. 40–50.

Midolo, P. D. *et al.*, 1995. *In vitro* inhibition of *Helicobacter pylori* NCTC 11637 by organic acids and lactic acid bacteria. *Journal of Applied Bacteriology*, 79(4), pp. 475–479.

Miettinen, T. A. *et al.*, 1995. Reduction of Serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *New England Journal of Medicine*, 333(20), pp. 1308–1312.

Mishra, L. K. *et al.*, 2020. Evaluation of phenolic bioactive-linked anti-hyperglycemic and *Helicobacter pylori* inhibitory activities of Asian Basil (*Ocimum* spp.) varieties. *Journal of Herbal Medicine*, 20, pp. 100310.

Miyauchi, S. *et al.*, 2004. Functional Identification of SLC5A8, a tumor suppressor down-regulated in colon cancer, as a Na+-coupled Transporter for short-chain fatty acids. *Journal of Biological Chemistry*, 279(14), pp. 13293–13296.

Molloy, A. M., 2002. Folate bioavailability and health. *International Journal for Vitamin and Nutrition Research*, 72(1), pp. 46–52.

Morales, M. and Barceló, A. R., 1997. A basic peroxidase isoenzyme from vacuoles and cell walls of *Vitis vinifera*. *Phytochemistry*, 45(2), pp. 229–232.

Moreau, R. A. and Fang, X., 2016. Analysis of alkylresorcinols in wheat germ oil and barley germ oil via HPLC and fluorescence detection: Cochromatography with tocols. *Cereal Chemistry*, 93(3), pp. 293–298.

Moreau, R. A., Flores, R. A. and Hicks, K. B., 2007. Composition of functional lipids in hulled and hulless barley in fractions obtained by scarification and in barley oil. *Cereal Chemistry*, 84(1), pp. 1–5.

Mosele, J. I., Motilva, M. J. and Ludwig, I. A., 2018. Beta-glucan and phenolic compounds: Their concentration and behavior during *in vitro* gastrointestinal digestion and colonic fermentation of different barley-based food products. *Journal of Agricultural and Food Chemistry*, 66(34), pp. 8966–8975.

Mridula, D. and Sharma, M., 2015. Development of non-dairy probiotic drink utilizing sprouted cereals, legume and soymilk. *LWT - Food Science and Technology*, 62(1), pp. 482–487.

Munns, R., Schachtman, D. and Condon, A., 1995. The significance of a two-phase growth response to salinity in wheat and barley. *Australian Journal of Plant Physiology*, 22(4), p. 561–569

Munns, R. and Tester, M., 2008. Mechanisms of salinity tolerance. *Annual Review of Plant Biology*. Annual Reviews , 59(1), pp. 651–681.

Murai, A. *et al.*, 2002. Control of postprandial hyperglycaemia by galactosyl maltobionolactone and its novel anti-amylase effect in mice. *Life Sciences*, 71(12), pp. 1405–1415.

Naczk, M. and Shahidi, F., 2004. Extraction and analysis of phenolics in food. *Journal of Chromatography A*, 1054(1–2), pp. 95–111.

Nair, S. S., Kavrekar, V. and Mishra, A., 2013. In vitro studies on alpha amylase and alpha glucosidase inhibitory activities of selected plant extracts. *European Journal of Experimental Biology*, 3(1), pp. 128–132.

Nazir, M. *et al.*, 2019. Opportunities and challenges for functional and medicinal beverages: Current and future trends. *Trends in Food Science and Technology*, pp. 513–526.

Ndolo, V. U. and Beta, T., 2013. Distribution of carotenoids in endosperm, germ, and aleurone fractions of cereal grain kernels. *Food Chemistry*, 139(1–4), pp. 663–671.

Nesaretnam, K., Yew, W. W. and Wahid, M. B., 2007. Tocotrienols and cancer: Beyond antioxidant activity. *European Journal of Lipid Science and Technology*, 109(4), pp. 445–452.

Newman, R. K. *et al.*,1990. Hypocholesterolemic effect of barley foods on healthy men. *Nutrition Reports International*, 39, pp. 749–760.

Newman, R. K. and Newman, C. W., 2008. *Barley for Food and Health: Science, Technology, and Products*. Hoboken, New Jersey: John Wiley and Sons.

Niki, E. *et al.*, 1985. Effect of phytyl side chain of vitamin E on its antioxidant activity. *The Journal of Biological Chemistry*, 260, pp. 2191–2196.

Niroula, A. *et al.*, 2019. Total phenolic contents and antioxidant activity profile of selected cereal sprouts and grasses. *International Journal of Food Properties*, 22(1), pp. 427–437.

Nkhata, S. G. *et al.*, 2018. Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. *Food Science and Nutrition*, pp. 2446–2458.

Nogata, Y. *et al.*, 2012. Determination of γ-aminobutyric acid and free amino acid contents in barley seeds and amounts produced by water soaking treatment. *Food Science and Technology Research*, 18(2), pp. 263–269.

Oberley, L. W. and Spitz, D. R., 1984. Assay of superoxide dismutase activity in tumor tissue. *Methods in Enzymology*, pp. 457–464.

Oboh, G. *et al.*, 2016. Influence of gallic acid on α-amylase and α-glucosidase inhibitory properties of acarbose. *Journal of Food and Drug Analysis*, 24(3), pp. 627–634.

Ogle, D. and John, S.T., 2010. Plants for saline to sodic soil conditions. USDA. *Natural Resources Conservation Service. Boise, Idaho. Technical Note*, (9A), p.p10150.

Oh, S.H., Moon, Y.-J. and Oh, C.-H., 2003. γ -Aminobutyric acid (GABA) content of selected uncooked foods. *Preventive Nutrition and Food Science*, 8(1), pp. 75–78.

Oh, S. *et al.*, 2014. Evaluation of *in vitro* antioxidant properties of roasted hulled barley (*Hordeum vulgare* L.). *Food Science and Biotechnology*, 23(4), pp. 1073–1079.

Okarter, N. and Liu, R. H., 2010. Health Benefits of Whole Grain Phytochemicals. *Critical Reviews in Food Science and Nutrition* , 50(3), pp. 193–208.

Omwamba, M. *et al.*, 2013. Antioxidant effect of roasted barley (*Hordeum vulgare* L.) grain extract towards oxidative stress *in vitro* and *in vivo. Food and Nutrition Sciences*, 4(8), pp. 139–146.

Omwamba, M. and Hu, Q., 2010. Antioxidant activity in barley (*Hordeum vulgare* L.) grains roasted in a microwave oven under conditions optimized using response surface methodology. *Journal of Food Science*, 75(1), pp. C66–C73.

Orwat, J., 2016. *Phenolic antioxidant-linked bioactive enrichment in black beans (Phaseolus vulgaris l.) to screen for health benefits and enhancement of salinity resilience,* Masters thesis, North Dakota State University. Available at: http://gradworks.umi.com/10/15/10150593.html (Accessed: 24 February 2017).

Orwat, J. *et al.*, 2017. Improved Salinity resilience in black bean by seed elicitation using organic compounds. *Agronomy Journal*, 109(5), p. 1991.

Pacetti, T., Caporali, E. and Rulli, M. C., 2017. Floods and food security: A method to estimate the effect of inundation on crops availability. *Advances in Water Resources*, 110, pp. 494–504.

Pakar, N. *et al.*, 2016. Barley growth, yield, antioxidant enzymes, and ion accumulation affected by PGRs under salinity stress conditions. *Journal of Plant Nutrition*, 39(10), pp. 1372–1379.

Pan, D. *et al.*, 2018. Phosphoproteomics unveils stable energy supply as key to flooding tolerance in *Kandelia candel*. *Journal of Proteomics*, 176, pp. 1–12.

Pandey, K.B. and Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), pp.270-278.

Panfili, G., Fratianni, A. and Mario, I., 2004. Improved normal-phase high-performance liquid chromatography procedure for the determination of carotenoids in cereals. *Journal of Agricultural and Food Chemistry*, 52, pp. 6373–6377.

Pang, J. *et al.*, 2004. Growth and physiological responses of six barley genotypes to waterlogging and subsequent recovery. *Australian Journal of Agricultural Research*, 55(8), pp. 895.

Panhwar, R. B. *et al.*, 2018. Phytochemical components of some minor cereals associated with diabetes prevention and management. *Journal of Biosciences and Medicines*, 6(2), pp. 9–22.

Papetti, A. *et al.*, 2006. Isolation of an *in vitro* and *ex vivo* antiradical melanoidin from roasted barley, *Journal of Agricultural and Food Chemistry*, 54(4), pp. 1209–1216.

Papetti, A. *et al.*, 2007. Effect of barley coffee on the adhesive properties of oral *streptococci*. *Journal of Agricultural and Food Chemistry*, 55(2), pp. 278–284.

Papetti, A., Mascherpa, D. and Gazzani, G., 2014. Free α-dicarbonyl compounds in coffee, barley coffee and soy sauce and effects of *in vitro* digestion. *Food Chemistry*, 164, pp. 259–265.

Paradiso, A. *et al.*, 2016. ROS production and scavenging under anoxia and re-oxygenation in *Arabidopsis* cells: A balance between redox signaling and impairment. *Frontiers in Plant Science*, 7, pp. 1803.

Parihar, P. *et al.*, 2015. Effect of salinity stress on plants and its tolerance strategies: a review. *Environmental Science and Pollution Research*, 22(6), pp. 4056–4075.

Peltonen, S., Mannonen, L. and Karjalainen, R.,1997. Elicitor-induced changes of phenylalanine ammonia-lyase activity in barley cell suspension cultures. *Plant Cell, Tissue and Organ Culture*, 50(3), pp. 185–193.

Per, T. S. *et al.*, 2018. Jasmonates in plants under abiotic stresses: Crosstalk with other phytohormones matters. *Environmental and Experimental Botany*, 145, pp. 104–120.

Peterson, D. M., 1994. Barley tocols: Effects of milling, malting and mashing. *Cereal Chemistry*, 71(1), pp. 42–44.

Phang, J. M., 1985. The regulatory functions of proline and pyrroline-5-carboxylic acid. *Current topics in cellular regulation*, 25, pp. 91–132.

Phang, J. M., Liu, W. and Zabirnyk, O., 2010. Proline Metabolism and microenvironmental stress. *Annual Review of Nutrition*, 30(1), pp. 441–463.

Phoboo, S. *et al.*, 2016. Improving salinity resilience in *Swertia chirayita* clonal line with *Lactobacillus plantarum. Canadian Journal of Plant Science*, 96(1), pp. 117–127.

Piasecka-Kwiatkowska, D. *et al.*, 2012. Digestive enzyme inhibitors from grains as potential components of nutraceuticals. *Journal of Nutritional Science And Vitaminology*, 58(3), pp. 217–20.

Piironen, V., Toivo, J. and Lampi, A. M., 2002. Plant sterols in cereals and cereal products. *Cereal Chemistry*, 79(1), pp. 148–154.

Pins, J. J. and Kaur, H., 2006. A review of the effects of barley β-glucan on cardiovascular and diabetic risk. *Cereal Foods World*, pp. 8–11.

Popkin, B. M. B. *et al.*, 2012. Global nutrition transition and the pandemic of obesity in developing countries. *Nutrition reviews*, 70(1), pp. 3–21.

Prasad, K., 2000. Oxidative stress as a mechanism of diabetes in diabetic BB prone rats: Effect of secoisolariciresinol diglucoside (SDG). *Molecular and Cellular Biochemistry*, 209(1–2), pp. 89–96.

Prasad, K. and Jadhav, A., 2016. Prevention and treatment of atherosclerosis with flaxseed - derived compound secoisolariciresinol diglucoside. *Current Pharmaceutical Design*, 22(2), pp. 214–220.

Pratley, R. E., 2013. The early treatment of type 2 diabetes. *The American Journal of Medicine*, 126(9), pp. S2–S9.

Qadir, M. *et al.*, 2014. Economics of salt-induced land degradation and restoration. *Natural Resources Forum*, 38(4), pp. 282–295.

Quinde-Axtell, Z. and Baik, B. K., 2006. Phenolic compounds of barley grain and their implication in food product discoloration. *Journal of Agricultural and Food Chemistry*, 54(26), pp. 9978–9984.

Quiroz-Reyes, C. N. and Fogliano, V., 2018. Design cocoa processing towards healthy cocoa products: The role of phenolics and melanoidins. *Journal of Functional Foods*, pp. 480–490.

Qureshi, A. A. *et al.*, 1986. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. *Journal of Biological Chemistry*, 261, pp. 10544–10550.

Rahimi, R. *et al.*, 2005. A review on the role of antioxidants in the management of diabetes and its complications. *Biomedicine & Pharmacotherapy*, 59(7), pp. 365–373.

Ramakrishna, Ramnarain *et al.*, 2017b. Improving phenolic bioactive-linked anti-hyperglycemic functions of dark germinated barley sprouts (*Hordeum vulgare* L.) using seed elicitation strategy. *Journal of Food Science and Technology*, 54(11), pp. 1–13.

Ramakrishna, R. *et al.*, 2017a. Phenolic linked anti-hyperglycemic bioactives of barley (*Hordeum vulgare* L.) cultivars as nutraceuticals targeting type 2 diabetes. *Industrial Crops and Products*, 107, pp. 509–517.

Randhir, R., Kwon, Y.I. and Shetty, K., 2009. Improved health-relevant functionality in dark germinated *Mucuna pruriens* sprouts by elicitation with peptide and phytochemical elicitors. *Bioresource Technology*, 100(19), pp. 4507–4514.

Randhir, R., Lin, Y.-T. and Shetty, K., 2004. Stimulation of phenolics, antioxidant and antimicrobial activities in dark germinated mung bean sprouts in response to peptide and phytochemical elicitors. *Process Biochemistry*, 39(5), pp. 637–646.

Randhir, R. and Shetty, K., 2003. Light-mediated fava bean (*Vicia faba*) response to phytochemical and protein elicitors and consequences on nutraceutical enhancement and seed vigour. *Process Biochemistry*, 38(6), pp. 945–952.

Randhir, R. and Shetty, K., 2005. Developmental stimulation of total phenolics and related antioxidant activity in light- and dark-germinated corn by natural elicitors. *Process Biochemistry*, 40(5), pp. 1721–1732.

Randhir, R., Shetty, P. and Shetty, K., 2002. L-DOPA and total phenolic stimulation in dark germinated fava bean in response to peptide and phytochemical elicitors. *Process Biochemistry*, 37(11), pp. 1247–1256.

Ranilla, L. G., Apostolidis, E. and Shetty, K., 2012. Antimicrobial activity of an Amazon medicinal plant (Chancapiedra) (*Phyllanthus niruri* L.) against *Helicobacter pylori* and lactic acid bacteria. *Phytotherapy Research*, 26(6), pp. 791–799.

Ranilla, L. G., Genovese, M. I. and Lajolo, F. M., 2009. Effect of different cooking conditions on phenolic compounds and antioxidant capacity of some selected Brazilian bean (*Phaseolus vulgaris* L.) cultivars. *Journal of Agricultural and Food Chemistry*, 57(13), pp. 5734–5742.

Rao, A. V. and Janezic, S. A.,1992. The role of dietary phytosterols in colon carcinogenesis. *Nutrition and Cancer*, 18(1), pp. 43–52.

Re, R. *et al.*, 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26, pp. 1231–1237.

Renault, S., MacKinnon, M. and Qualizza, C., 2003. Barley, a potential species for initial reclamation of saline composite tailings of oil sands. *Journal of Environment Quality*, 32(6), pp. 2245–2245.

Rhee, Y., 2016. Flaxseed secoisolariciresinol diglucoside and enterolactone down-regulated epigenetic modification associated gene expression in murine adipocytes. *Journal of Functional Foods*, 23, pp. 523–531.

Rice-Evans, C. A., Miller, N. J. and Paganga, G.,1996, Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, 20(7), pp. 933–956.

Rice-Evans, C., Miller, N. and Paganga, G., 1997. Antioxidant properties of phenolic compounds. *Trends in Plant Science*, 2(4), pp. 152–159.

Rico, D. *et al.*, 2020. Sprouted barley flour as a nutritious and functional ingredient', *Foods*, 9(3), p. 296.

Rizzello, C. G. *et al.*, 2012, Synthesis of the cancer preventive peptide lunasin by lactic acid bacteria during sourdough fermentation. *Nutrition and Cancer*, 64(1), pp. 111–120.

Rolo, A. P. and Palmeira, C. M., 2006. Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress. *Toxicology and Applied Pharmacology*, 212(2), pp. 167–178.

Rosenstock, J. *et al.*, 1998. Efficacy and safety of acarbose in metformin-treated patients with type 2 diabetes. *Diabetes Care*, 21(12), pp. 2050–2055.

Ross, A. B. *et al.*, 2003. Alkylresorcinols in cereals and cereal products. *Journal of Agricultural and Food Chemistry*, 51(14), pp. 4111–4118.

Ruiz-García, Y. and Gómez-Plaza, E., 2013. Elicitors: A tool for improving fruit phenolic content. *Agriculture*, 3(1), pp. 33–52.

Saha, P., Kunda, P. and Biswas, A. K., 2012. Influence of sodium chloride on the regulation of Krebs cycle intermediates and enzymes of respiratory chain in mungbean (*Vigna radiata* L. Wilczek) seedlings. *Plant Physiology and Biochemistry*, 60, pp. 214–222.

Sairam, R. K. *et al.*, 2011. NADPH oxidase as the source of ROS produced under waterlogging in roots of mung bean. *Biologia Plantarum*, 55(4), pp. 741–746.

Salas-Salvadó, J. *et al.*, 2011. The role of diet in the prevention of type 2 diabetes. *Nutrition, Metabolism and Cardiovascular Diseases*, 21(SUPPL. 2), pp. B32–B48.

Saleem, M. *et al.*, 2005. An update on bioactive plant lignans. *Natural Product Reports*, 22(6), pp. 696–716.

Samaras, T. S. *et al.*, 2005. Antioxidant properties of kilned and roasted malts. *Journal of Agricultural and Food Chemistry* , 53, pp. 8068–8074.

de San Celedonio, R. P., Abeledo, L. G. and Miralles, D. J., 2014. Identifying the critical period for waterlogging on yield and its components in wheat and barley. *Plant and Soil*, 378(1–2), pp. 265–277.

Sánchez-Maldonado, A. F., Schieber, A. and Gänzle, M. G., 2011. Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *Journal of Applied Microbiology*, 111(5), pp. 1176–1184.

Sánchez, A. and Vázquez, A., 2017. Bioactive peptides: A review. *Food Quality and Safety*, pp. 29–46.

Sandhu, K. S. and Punia, S., 2017. Enhancement of bioactive compounds in barley cultivars by solid substrate fermentation. *Journal of Food Measurement and Characterization*, 11(3), pp. 1355–1361.

Santilli, F., Davì, G. and Patrono, C., 2016. Homocysteine, methylenetetrahydrofolate reductase, folate status and atherothrombosis: A mechanistic and clinical perspective'. *Vascular Pharmacology*, 78, pp. 1–9.

Sarkar, D. *et al.*, 2009. Cold acclimation responses of three cool-season turfgrasses and the role of proline-associated pentose phosphate pathway. *Journal of the American Society for Horticultural Science*, 134(2), pp. 210–220.

Sarkar, D. *et al.*, 2011. The role of proline-associated pentose phosphate pathway in cool-season turfgrasses after UV-B exposure. *Environmental and Experimental Botany*, 70(2–3), pp. 251–258.

Sarkar, D., Ankolekar, C. and Shetty, K., 2020. Beneficial lactic acid bacteria (LAB)-based biotransformation of plant and dairy substrates to enhance type 2 diabetes-relevant health benefits, in Shetty, K. and Sarkar. D. (eds) *Functional foods and biotechnology: Biotransformation and analysis of functional foods and ingredients*. Boca Raton, Florida: CRC Press, pp. 345–360.

Sarkar, D., Bhowmik, P. C. and Shetty, K., 2010. Antioxidant enzyme response of creeping bentgrass clonal lines with marine peptide and chitosan oligosaccharide. *Agronomy Journal*, 102(3), p. 981.

Sarkar, D. and Shetty, K., 2011. Postharvest strategies to enhance bioactive ingredients for type 2 diabetes management and heart health, in Paliyath, G. amd Bakovic, M. (eds.) *Functional Foods, Nutraceuticals, and Degenerative Disease Prevention*. Oxford, UK: Wiley-Blackwell, pp. 357–379.

Sarkar, D. and Shetty, K., 2014. Metabolic stimulation of plant phenolics for food preservation and health. *Annual review of food science and technology*, 5(1), pp. 395–413.

Sen, C. K., Khanna, S. and Roy, S., 2007. Tocotrienols in health and disease: The other half of the natural vitamin E family. *Molecular Aspects of Medicine*, pp. 692–728.

Sena, L. A. and Chandel, N. S., 2012. Physiological roles of mitochondrial reactive oxygen species. *Molecular Cell*, pp. 158–167.

Servet, C., Ghelis, T., Richard, L., Zilberstein, A. and Savoure, A., 2012. Proline dehydrogenase: a key enzyme in controlling cellular homeostasis. *Front Biosci*, *17*(1), pp.607-620.

Setter, T. L. and Waters, I., 2003. Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. *Plant and Soil*, pp. 1–34.

Shahidi, F. and Ambigaipalan, P., 2015. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of functional foods*, *18*, pp.820-897.Shahidi, F. and Naczk, M., 2003. *Phenolics in food and nutraceuticals* . Boca Raton, Florida: CRC Press.

Shahidi, F. and Zhong, Y., 2008. Bioactive peptides. *Journal of AOAC International*, 91(4), pp. 914–931.

Shalaby, E. A. and Shanab, S. M. M., 2013. Comparison of DPPH and ABTS assays for determining antioxidant potential of water and methanol extracts of Spirulina platensis. *Indian Journal of Marine Sciences*, 42(5), pp. 556–564.

Sharma, A. *et al.*, 2019. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. *Molecules*, 24(13), p. 2452.

Sharma, M., Mridula, D. and Gupta, R. K., 2014. Development of sprouted wheat based probiotic beverage. *Journal of Food Science and Technology*, 51(12), pp. 3926–3933.

Sharma, P. and Gujral, H. S., 2010. Antioxidant and polyphenol oxidase activity of germinated barley and its milling fractions. *Food Chemistry*, 120(3), pp. 673–678.

Shelp, B. J., Bown, A. W. and McLean, M. D., 1999. Metabolism and functions of gamma-aminobutyric acid. *Trends in Plant Science*, 4(11), pp. 446–452.

Shen, Y. *et al.*, 2016. *In vitro* and *in vivo* antioxidant activity of polyphenols extracted from black highland barley. *Food Chemistry*, 194, pp. 1003–1012.

Shetty, K. *et al.*, 1995. Prevention of vitrification associated with *in vitro* shoot culture of Oregano. (*Origanum vulgare*) by *Pseudomonas* spp. *Journal of Plant Physiology*, 147(3–4), pp. 447–451.

Shetty, K., 1997. Biotechnology to harness the benefits of dietary phenolics; focus on Lamiaceae. *Asia Pacific Journal of Clinical Nutrition*, 6(3), pp. 162–171.

Shetty, K., 2004. Role of proline-linked pentose phosphate pathway in biosynthesis of plant phenolics for functional food and environmental applications: A review. *Process Biochemistry*, 39(7), pp. 789–804.

Shetty, K. and McCue, P., 2003. Phenolic antioxidant biosynthesis in plants for functional food application: integration of systems biology and biotechnological approaches. *Food Biotechnology*, 17(2), pp. 67–97.

Shetty, K. and Wahlqvist, M., 2004. A model for the role of the proline-linked pentose-phosphate pathway in phenolic phytochemical bio-synthesis and mechanism of action for human health and environmental applications. *Asia Pacific Journal of Clinical Nutrition*, 13(1), pp. 1–24.

Shetty, P., Atallah, M. T. and Shetty, K., 2001. Enhancement of total phenolic, l-DOPA and proline contents in germinating fava bean (*Vicia faba*) in response to bacterial elicitors. *Food Biotechnology*, 15(1), pp. 47–67.

Shewry, P. R. and Ullrich, S. E. (2014). *Barley : Chemistry and technology*. 1st edn. Wallingford, United Kingdom: American Association of Cereal Chemists International / CAB International.

Shiono, K. *et al.* (2019) 'Improved waterlogging tolerance of barley (Hordeum vulgare) by pretreatment with ethephon', *Plant Production Science*. Taylor and Francis Ltd., 22(2), pp. 285–295.

Sidhu, J. S., Kabir, Y. and Huffman, F. G., 2007. Functional foods from cereal grains. *International Journal of Food Properties*, 10(2), pp. 231–244.

Silva-Sánchez, C. *et al.*, 2008. Bioactive peptides in amaranth (*Amaranthus hypochondriacus*) seed. *Journal of Agricultural and Food Chemistry*, 56(4), pp. 1233–1240.

Šimić, G., Horvat, D., Dvojković, K., Abičić, I., Vuletić, M.V., Tucak, M. and Lalić, A., 2017. Evaluation of total phenolic content and antioxidant activity of malting and hulless barley grain and malt extracts. *Czech Journal of Food Sciences*, *35*(1), pp.73-78.

Singh, A. K. *et al.*, 2015. Enhancement of attributes of cereals by germination and fermentation: a review. *Critical Reviews in Food Science and Nutrition*, 55(11), pp. 1575–1589.

Singh, A. and Sharma, S., 2017. Bioactive components and functional properties of biologically activated cereal grains: A bibliographic review. *Critical Reviews in Food Science and Nutrition*, 57(14), pp. 3051–3071.

Slavin, J., 2010. Whole grains and digestive health. *Cereal Chemistry*, 87(4), pp. 292–296.

Slavin, J., Jacobs, D. and Marquart, L., 1997. Whole-grain consumption and chronic disease: Protective mechanisms. *Nutrition and Cancer*, 27(1), pp. 14–21.

Smeds, A. I. *et al.*, 2007. Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. *Journal of Agricultural and Food Chemistry*, 55(4), pp. 1337–1346.

Stevenson, T. H., Lucia, L. M. and Acuff, G. R., 2000. Development of a selective medium for isolation of *Helicobacter pylori* from cattle and beef samples. *Applied and Environmental Microbiology*, 66(2), pp. 723–727.

Stewart, B. W. and Wild, C. P. (2014) *World Cancer Report , International Agency for Research on Cancer*. Available at: http://publications.iarc.fr/Non-Series-Publications/World-Cancer-Reports/World-Cancer-Report-2014 (Accessed: 1 June 2018).

Stumvoll, M., Goldstein, B. J. and Van Haeften, T. W., 2005. Type 2 diabetes: Principles of pathogenesis and therapy. *Lancet*, pp. 1333–1346.

Sullivan, P., Arendt, E. and Gallagher, E., 2013. The increasing use of barley and barley by-products in the production of healthier baked goods. *Trends in Food Science & Technology*, 29(2), pp. 124–134.

Suriano, S., Iannucci, A., Codianni, P., Fares, C., Russo, M., Pecchioni, N., Marciello, U. and Savino, M., 2018. Phenolic acids profile, nutritional and phytochemical compounds, antioxidant properties in colored barley grown in southern Italy. *Food Research International*, *113*, pp.221-233.

Svensson, L., Sekwati-Monang, B., Lutz, D.L., Schieber, A. and Ganzle, M.G., 2010. Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor* (L.) Moench). *Journal of Agricultural and Food Chemistry*, *58*(16), pp.9214-9220.

Szabados, L. and Savouré, A., 2010. Proline: a multifunctional amino acid', *Trends in Plant Science*, pp. 89–97.

Tada, R. *et al.*, 2009. Barley-derived β-d-glucan induces immunostimulation via a dectin-1-mediated pathway. *Immunology Letters*, 123(2), pp. 144–148.

Tan, B., Watson, R. R. and Preedy, V. R. (eds.) (2012) *Tocotrienols: Vitamin E beyond tocopherols*. Boca Raton, Florida: CRC Press.

Tang, Y. *et al.*, 2014. Lipids, tocopherols, and carotenoids in leaves of amaranth and quinoa cultivars and a new approach to overall evaluation of nutritional quality traits. *Journal of Agricultural and Food Chemistry*, 62(52), pp. 12610–12619.

Tang, Y. *et al.*, 2015. Characterisation of fatty acid, carotenoid, tocopherol/tocotrienol compositions and antioxidant activities in seeds of three *Chenopodium quinoa* Willd. genotypes', *Food Chemistry*, 74, pp. 502–508.

Temelli, F. *et al.*, 2013. Tocol composition and supercritical carbon dioxide extraction of lipids from barley pearling flour. *Journal of Food Science*, 78(11), pp. C1643–C1650.

Ti, H. *et al.*, 2014. Dynamic changes in the free and bound phenolic compounds and antioxidant activity of brown rice at different germination stages. *Food Chemistry*, 161, pp. 337–344.

Tiwari, U. and Cummins, E., 2009. Nutritional importance and effect of processing on tocols in cereals. *Trends in Food Science and Technology*, pp. 511–520.

Tober, D., Duckwitz, W. and Sieler, S. (2007) *Plant materials for salt-affected sites in the Northern Great Plains*. Bismarck.

Tokusoglu, O. and Hall, C. (2010) *Fruit and cereal bioactives*. Boca Raton, Florida: CRC Press.

Topakas, E., Vafiadi, C. and Christakopoulos, P., 2007. Microbial production, characterization and applications of feruloyl esterases. *Process Biochemistry*, pp. 497–509.

Torino, M. I. *et al.*, 2013. Antioxidant and antihypertensive properties of liquid and solid state fermented lentils', *Food Chemistry*, 136(2), pp. 1030–1037.

Trouvelot, S. *et al.*, 2014. Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. *Frontiers in Plant Science*, 5, p. 592.

Tundis, R., Loizzo, M. R. and Menichini, F., 2010. Natural products as alpha-amylase and alpha-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: an update. *Mini Reviews In Medicinal Chemistry*, pp. 315–331.

Ullrich, S. E., 2010. Significance, adaptation, production, and trade of barley. in Ullrich, S.E. (ed.) *Barley: Production, Improvement, and Uses*. Oxford: Wiley-Blackwell, pp. 3–13.

Unal, B. T., Aktas, L. Y. and Guven, A., 2014. Effects of salinity on antioxidant enzymes and proline in leaves of barley seedlings in different growth stages. *Bulgarian Journal of Agricultural Science*, 20(4), pp. 883–887.

Vartapetian, B. B. and Jackson, M. B.,1997. Plant adaptations to anaerobic stress. *Annals of Botany*. 79(Suppl 1), pp. 3–20.

Vattem, D.A., Lin, Y.T. and Shetty, K., 2005. Enrichment of phenolic antioxidants and anti-Helicobacter pylori properties of cranberry pomace by solid-state bioprocessing. *Food Biotechnology*, *19*(1), pp.51-68.

Verbruggen, N. and Hermans, C., 2008. Proline accumulation in plants: A review. *Amino Acids*, 35(4), pp. 753–759.

Vitaglione, P., Napolitano, A. and Fogliano, V., 2008. Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. *Trends in Food Science and Technology*, pp. 451–463

Wang, F. *et al.*, 2015. Dipeptidyl peptidase IV inhibitory peptides derived from oat (*Avena sativa* L.), buckwheat (*Fagopyrum esculentum*), and highland Barley (*Hordeum vulgare* trifurcatum (L.) Trofim) proteins. *Journal of Agricultural and Food Chemistry*, 63(43), pp. 9543–9549.

Wang, L. *et al.*, 1997. Comparative cholesterol-lowering effects of barley β-glucan and barley oil in golden Syrian hamsters. *Nutrition Research*, 17(1), pp. 77–88.

Wang, M., Wichienchot, S., He, X., Fu, X., Huang, Q. and Zhang, B., 2019. In vitro colonic fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and changes in microbiota. *Trends in Food Science & Technology*, *88*, pp.1-9.

Wang, T., He, F. and Chen, G., 2014. Improving bioaccessibility and bioavailability of phenolic compounds in cereal grains through processing technologies: A concise review. *Journal of Functional Foods*, 7, pp. 101–111.

Wannenmacher, J., Gastl, M., & Becker, T. (2018). Phenolic substances in beer: Structural diversity, reactive potential and relevance for brewing process and beer quality. *Comprehensive Reviews in Food Science and Food Safety*, *17*(4), 953-988.Wang, X. *et al.*, 2011. Effects of NaCl and silicon on activities of antioxidative enzymes in roots, shoots and leaves of alfalfa. *African Journal of Biotechnology*, 10(4), pp. 545–549.

Ward, J. L. *et al.*, 2008. The HEALTHGRAIN cereal diversity screen: Concept, results, and prospects. *Journal of Agricultural and Food Chemistry*, pp. 9699–9709.

Wareham, N. J. and Herman, W. H., 2016. The clinical and public health challenges of diabetes prevention: A search for sustainable solutions. *PLOS Medicine*, 13(7), p. e1002097.

Widodo *et al.*, 2009. Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. *Journal of Experimental Botany*, 60(14), pp. 4089–103.

Wienhold, B. J. *et al.*, 2018. Vulnerability of crops and croplands in the US Northern Plains to predicted climate change. *Climatic Change*, 146(1–2), pp. 219–230.

Wood, P. J. *et al.*, 1990. Comparisons of viscous properties of oat and guar gum and the effects of these and oat bran on glycemic index. *Journal of Agricultural and Food Chemistry*, 38(3), pp. 753–757.

Wootton-Beard, P. C. and Ryan, L., 2011. Improving public health: The role of antioxidant-rich fruit and vegetable beverages. *Food Research International*, pp. 3135–3148.

World Health Organization (2016) *The global burden of chronic diseases*. Available at: http://www.who.int/nutrition/topics/2_background/en/ (Accessed: 11 January 2016).

World Health Organization (2018) *Cardiovascular Diseases (CVDs)*. Available at: http://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (Accessed: 1 September 2018).

Worthington, K. and Worthington, V. (eds) (1993) 'Alpha amylase', in *Worthington Enzyme Manual*. Freehold, New Jersey: Worthington Biochemical Corporation, pp. 36–41.

Worthington, V. and Worthington, K. (eds) (1993) 'Maltase', in *Worthington Enzyme Manual*. Freehold, New Jersey: Worthington Biochemical Corporation, pp. 261.

Wuebbles, D. *et al.* (2017) *Climate Science Special Report: Fourth National Climate Assessment (NCA4), Volume 1, Agronomy Reports*.

Xiao, Y.P., Wei, K., Chen, J.X., Zhou, M.X. and Zhang, G.P., 2005. Effects of waterlogging on photosynthesis and antioxidant enzyme activities of six barley genotypes with different waterlogging tolerance. *Agricultural Sciences in China*, *4*(4), pp.310-316.

Xuan, T. and Khang, D., 2018. Effects of exogenous application of protocatechuic acid and vanillic acid to chlorophylls, phenolics and antioxidant enzymes of rice (*Oryza sativa* L.) in submergence. *Molecules*, 23(3), p. 620.

Yang, T. *et al.*, 2013. HPLC analysis of flavonoids compounds of purple, normal barley grain. *Advanced Materials Research*, 634–638, pp. 1486–1490.

Yordanova, R. Y., Christov, K. N. and Popova, L. P., 2004. Antioxidative enzymes in barley plants subjected to soil flooding. *Environmental and Experimental Botany*, 51(2), pp. 93–101.

Young Kim, H. *et al.*, 2013. Antioxidant and antiproliferation activities of winter cereal crops before and after germination. *Food Science and Biotechnology*, 22(1), pp. 181–186.

Yu, F.L. *et al.*, 2013. Genotype, environment and their interactions of major bioactive components in 2-year licorice (*Glycyrrhiza uralensis*) population. *China Journal of Chinese Materia Medica*, 38(10), pp. 1479–83.

Zaiter, A. *et al.*, 2016. Effect of particle size on antioxidant activity and catechin content of green tea powders. *Journal of Food Science and Technology*, 53(4), pp. 2025–2032.

Zarnowski, R. *et al.*, 2002. Alkylresorcinols in barley (*Hordeum vulgare* L. distichon) grains. *Zeitschrift fur Naturforschung - Section C Journal of Biosciences*, 57(1–2), pp. 57–62.

Zarnowski, R. and Suzuki, Y., 2004. 5-n-Alkylresorcinols from grains of winter barley (*Hordeum vulgare* L.). *Zeitschrift fur Naturforschung - Section C Journal of Biosciences*, 59(5–6), pp. 315–317.

Zeng, F. *et al.*, 2013. Barley responses to combined waterlogging and salinity stress: separating effects of oxygen deprivation and elemental toxicity. *Frontiers in Plant Science*, 4(AUG), p. 313.

Zhao *et al.*, 2006. Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.). *Journal of Agricultural and Food Chemistry* , 54(19), pp. 7277–7286.Zhang, G. *et al.*, 2007. Influence of waterlogging on some anti-oxidative enzymatic activities of two barley genotypes differing in anoxia tolerance. *Acta Physiologiae Plantarum*, 29(2).

Zhang, X. *et al.*, 2015. Waterlogging tolerance in barley is associated with faster aerenchyma formation in adventitious roots. *Plant and Soil*, 394(1–2), pp. 355–372.

Zieliński, H. and Kozłowska, H., 2000. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *Journal of Agricultural and Food Chemistry*, 48(6), pp. 2008–2016.