PHENOLIC ANTIOXIDANT-LINKED BIOACTIVE ENRICHMENT IN BLACK BEANS (*PHASEOLUS VULGARIS* L.) TO SCREEN FOR HEALTH BENEFITS AND ENHANCEMENT OF SALINITY RESILIENCE

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ABSTRACT

Dietary consumption of black bean has diverse human health benefits which can be targeted as part of dietary strategies for chronic disease management. A gap in knowledge currently exists regarding how to advance the efficient production of black bean under abiotic stress conditions with the potential to simultaneously enrich human health-relevant bioactives in harvested beans. Therefore, the objective was to evaluate the effects of seed and foliar elicitation treatments on metabolic regulation and health-relevant bioactive markers in dark-germinated sprouts and field-grown black beans using *in vitro* assays. Further, a study was designed to evaluate the combined effects of salinity stress, seed elicitation, and genotypes on regulation of salinity stress response. Significant improvement in total soluble phenolic content, and total antioxidant activity following seed and foliar elicitation treatments were found. Further seed elicitation resulted in improvement in antioxidant enzyme responses and associated higher salinity stress tolerance.

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DEDICATION

To Dipayan Sarkar, whose genuine kindness, compassion, and friendship made it possible for me to face each day with positivity and focus.

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To my parents Peter and Dawn Orwat, who guided me to become who I am today, and who gave me the support to pursue my dreams all the way to North Dakota.

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LIST OF ABBREVIATIONS

T2D.…...……..Type 2 Diabetes Mellitus

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(3-ethylbenzothiazoline-6-sulphonic acid

CHAPTER 1. INTRODUCTION

Global Paradigm Shifts: in Food Crop Production, Climate Change, and Human Health

Since the end of World War 2 the focus of global economies shifted to address major challenges, especially to modernize the world's agriculture and healthcare systems underpinned by the strategy to provide food security and safety for rising global populations (Ericksen, 2008). The resulting decades have shown substantial successes of this global food transition that have yielded unprecedented improvements to global agricultural and food infrastructures that allowed global populations to be sustained with macronutrients and energy needs (Godfray et al., 2010). This global food economy only became possible by structural rearrangements with our relationship towards food and its production by transferring the prevailing economic model of goods commodification to the agricultural sector. Coupled with technological advancements and a globally connected distribution chain, this transition has made possible the mass production of food crops to be exchanged as a commodity in globally connected markets (Friedmann, 1993).

Such rapid transformations to agriculture and food paradigms has caused unintended externalized costs and consequences to both the environment in which food is grown, as well as on the overall health of consumers (Battisti and Naylor, 2009). Industrial agriculture can cost-effectively convert food commodities (such as corn, rice, wheat, and soybean) into hyper-processed foods that are high in refined and soluble carbohydrates as well as dense in calories, while also typically are deficient in micronutrients and human health relevant bioactive compounds (Welch and Graham, 1999). These rapid changes and depletion in food quality while increasing food quantity have led to the rise in incidence and prevalence of diet-linked non-communicable chronic diseases (NCDs) such as obesity, type 2 diabetes (T2D), and cardiovascular disease – all of which were considered rare in populations of pre-commoditized

agricultural systems. For example, a recently published article projected T2D incidence will increase from affecting 285 million adults in 2010 to 439 million by 2030 (Shaw, 2010). Additionally, agricultural production in the last few years has begun to plateau, primarily through a reduction of major advancements in innovative technologies as well as increasing susceptibility to climate change being cited as major causal factors (Rosegrant, 2003). Novel innovative strategies are required to facilitate crop defense against environmental stresses in a rapidly shifting climate extremes that can simultaneously improve the nutritional properties of food crops in order to meet the micronutrient and bioactive benefits required by consumers across different communities and countries.

Climate Change and Global Food Security

According to the Food and Agriculture Organization (FAO), food security is defined as "when all people, at all times, have physical and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (FAO, 2008). Ensuring such year around supply of nutritionally balanced foods to the increasing global population (which will reach 10-11 billion maximum by 2100) is an enormous challenge for both growers and agricultural scientists, particularly in developing countries where projected global population rise will grow more rapidly in numbers and overall density per unit area (Godfray et al., 2010). Meeting global food security needs will only get more challenging as climate change threatens to further hinder food crops production which will greatly affect the sustainability of our current agricultural system (Batisti and Naylor, 2009).

Climate change is characterized by fluctuations in natural phenomenon contributed to by human intervention, with extreme changes within and above earth surface potentially leading to land degradation, changes in hydrology, depletion of soil resources, and changes in climate

pattern, especially due to increasing emission of greenhouse gases with serious consequences on global food security and agricultural sustainability (Cakmak, 2005; Ericksen, 2008). The present upward trend of atmospheric carbon dioxide and subsequent deviations in global mean temperature and rainfall will affect the aggregate global productivity of grains, legumes, and other important food crops and presents a major challenge to current food production outputs (Lobell and Gourdji, 2012). Saline soils have historically been one of the most critical abiotic stresses in agriculture, with estimates showing most of global arable land will face some degree of salinization by 2050 largely focused in developing countries and in areas most impacted from climate change (Wang et al., 2004). Most major agricultural commodities will be impacted by increasing salinization, as estimations indicate that more than 50% of food crops subjected to increased levels of salt have hyperionic and hyperosmotic effects that can cause membrane disintegration, cellular toxicity and accumulation of reactive oxygen species (ROS) in cells (Hasegawa and Bressan, 2000). The rapid progression of climate change-linked abiotic stress such as salinity will exponentially increase losses to global agricultural commodity yields, and this must be addressed quickly and effectively in order to mitigate detrimental impacts to the global food economy. Furthermore, such rapid climate changes will not only just affect productivity of important food crops but will also impose novel challenges in the maintenance and preservation of food quality during both the pre- and post-harvest crop stages.

Rise of Lifestyle and Diet-Linked Chronic Diseases

The urgent challenge of addressing food security in the midst of rapid climate change becomes even more critical due to the upsurge in the incidence and prevalence of diet-linked chronic diseases (T2D, obesity, cardiovascular diseases, and cancer) in recent decades, largely associated with macronutrient-dense foods, rapid urbanization, and sedentary lifestyles

(Rosegrant, 2003). Type 2 Diabetes is among the most common of such NCDs globally with 415 million people diagnosed in 2015 and an estimated five million T2D-associated deaths in 2015; with many more undiagnosed (IDF, 2015). This global health burden also disproportionately impacts developing countries such as China and India, with 80% of T2D-related mortality occurring in low- and middle- income populations; a common trend within the new global food system (IDF, 2015). The trend of total mortality associated with T2D continues to steadily rise, with the World Health Organization projecting that diabetes will be the 7th leading cause of death in 2030 (WHO, 2016).

Type 2 Diabetes is a multifactorial chronic disease resulting from a systematic breakdown of glucose balance and metabolic regulation, and is known to progress and develop along with several other chronic diseases which are collectively referred to as metabolic syndrome, or "syndrome X" (Shaw et al., 2010; Wilson et al., 2005). Mounting evidence suggests that while T2D and other NCDs are symptomatically diverse, a shared pathogenesis of development and incidence exists: chronic inflammation induced from oxidative stresses (Ceriello and Motz, 2004; Lin and Beal, 2006). Over-nutrition from hyper-processed macronutrients in combination with decreased physical activity leads to excessive loads 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 ROS that cannot be quenched through normal cellular defense mechanisms (Ceriello and Motz, 2004; Singh et al., 2009). To counter such chronic oxidative stress, plant-based foods rich in natural antioxidants are excellent dietary targets that can be utilized as safe, cost-effective, and complimenting dietary strategy to manage NCDs, such as type 2 diabetes and associated macro- and micro-vascular complications (Sarkar and Shetty, 2014a).

Role of Phenolic Bioactives in Plant-Based Foods to Counter Abiotic Stresses and NCDs

The antioxidant defense systems of plants are composed of antioxidant enzymes and non-enzymes, such as hydrophilic and lipophilic metabolites and play a critical role in countering cellular oxidative damages induced by abiotic and biotic stresses (Gutteridge and Halliwell, 1990; Polle, 1997). Plants' secondary metabolites such as phenolics (and subsequent derivatives) are potent antioxidants and also provide additional protection against oxidative damages (Bartwal et al., 2013). These phenolic metabolites are a large group of bioactive compounds (8000 phenolic and polyphenolic metabolites currently known with typical phenolic structural features) distributed widely in fruits, vegetables, cereals, legumes, oilseeds, and other edible plants and can be targeted in the development of dietary strategies to counter oxidative stress-linked NCDs as well improved health outcomes (Bravo, 1998; Levin, 2016; Tsao, 2010). Thus, phenolic phytochemicals have a crucial role in the development of functional foods and ingredient technologies from staple food crops (Buse et al., 2009; McCue and Shetty, 2002; Sarkar and Shetty, 2014a). Higher consumption of phenolic rich plant-based foods could potentially provide cost-effective prevention, and a compliment to align strategies with pharmaceutical therapy for the management of early stages of major chronic diseases, such as type 2 diabetes, cardiovascular disease, and potentially cancer (Labriola and Livingston, 1999; Shetty and McCue, 2003). When consumed through the diet one of the primary mechanism of phenolic metabolites is its biological activity as antioxidants, either by directly quenching undesirable excess ROS or indirectly stimulating antioxidant enzyme responses that in turn support environment for insulin sensitivity, signaling, and function, which is key for maintaining glucose homeostasis (Pandey and Rizvi, 2009; Randhir et al., 2008; Tsao, 2010).

Dual Strategy Approach: Simultaneous Improvement of Food Functionality and Abiotic Stress Tolerance

Based on the above insights of the overall defense mechanisms of plants, which in part is due to the combination of phenolic biosynthesis and its correlation with stimulation of protective antioxidant enzyme responses to counter mitochondrial oxygen malfunction, novel food innovations that are safe and cost-effective can be designed (Randhir and Shetty, 2005; Shetty and Wahlqvist, 2004). Within plant systems, exogenous elicitors (such as phenolics, microbial, and other chemical compounds) can be used to stimulate the biosynthesis of phenolics and antioxidant enzymes to counter oxidative stress in plants (Sarkar and Shetty, 2014a). This redox pathway-linked model provides a scientific foundation for the development of dynamic metabolic stimulation strategies to harness the benefits of phenolic bioactives and antioxidants in food crops. Furthermore, this stimulation of phenolic bioactives also provides protection to plant for countering abiotic and biotic stresses (Schützendübel and Polle, 2002; Shetty and McCue, 2003). Such pathways are accessible in a diverse range of crop plants across diverse ecology dealing with range of abiotic and biotic stresses and reproductive functions. Using this understanding of the dual function nature of stress-induced phenolic metabolites, strategies can be developed to protect food crops in the field against abiotic stresses while simultaneously producing a product of higher nutritional value for the consumer, especially to counter oxidative stress-linked NCDs.

Selection of Black Bean (*Phaseolus vulgaris* L.*)* as a Model Crop

Edible dry beans are major staples in Central America and Africa and are an integral part to many traditional dietary systems due to their balanced nutritional compositions (protein, dietary fiber, and carbohydrates) and provide good complementation to cereal crops (Leterme,

2002; Messina, 2014; Vandemark et al., 2014). Edible dry beans are gaining worldwide attention due to the diverse human health benefits associated to their rich content of phenolic metabolites and other bioactive compounds, as well as recognition of their role in a plant-based, whole-food diet rich in fiber and micronutrients (Messina, 2014; Mitchell et al., 2009; Silva-Cristobal et al., 2010). Such characteristics have focused the use of dry beans to the world stage as target crops for designing safe and cost-effective dietary strategies to manage NCDs such as T2D by providing a high quality, inexpensive plant-based protein to fight undernutrition while simultaneously providing bio protection against the chronic diseases of over nutrition (Limón et al., 2014; Shetty and McCue, 2003). Although the use of new improved genotypes has led to modest and yet steady yield increases over time, edible dry bean growers will face increasing challenges to productivity under the extreme variations and fluctuations in growing conditions and disease associated withclimate change (Vandemark et al., 2014). New innovations are needed to improve the sustainable production of edible dry bean. In particular black beans are of special interest due to their excellent phenolic content from the high anthocyanin content in their seed coats while they are susceptible to abiotic stresses (such as saline soils) that affect productivity. Black bean production is increasing in the Northern Plains region of the United States and this region is a major global exporter. In recent years, soil salinity and waterlogging have become major challenges for the growers of the Northern Plains, and they require safe and cost-effective strategies to improve salinity tolerance in black bean without drastically changing the current agronomic practices (Franzen, 2007). Such improvement of salinity resilience in black bean and its concurrent improvement of human health relevant bioactive profiles will ensure growers higher productivity and economic security which in turn will in part address global climate change-linked food security and diet-linked chronic disease challenges.

CHAPTER 2. LITERATURE REVIEW

Oxidative Stress and Reactive Oxygen Species (ROS)

Environmental conditions that induce or favor photo-oxidative stress are common events in the growth and development of plants (Paliyath, 1992). Within various critical biochemical pathways, particularly for respiratory energy production, stable O_2 is an important part of the electron transfer resulting in stable water molecule at the mitochondrial level. However, this mechanism is a mixed blessing because while it permits versatility and productivity in the biosphere (including crop diversity), it simultaneously acts as an oxidizer that leads to the formation of variety of ROS in plant cells (Apel and Hirt, 2004; Polle, 1997). Reactive Oxygen species are highly reactive molecules primarily derived from the sequential reduction of molecular oxygen and split into two broad categories: free radicals (Superoxide, singlet 0_2-, and Hydroxyl) and non-radical molecular forms (H_2O_2 and $^1O_2$ singlet oxygen) (Apel and Hirt, 2004; Polle, 1997). ROS are produced continuously within most biochemical systems located in several organelles with high metabolic activity, primarily in the mitochondria, chloroplasts, and peroxisomes. ROS production is important for different cellular functions, such as in signal transduction, genetic stress responses, destruction of invading pathogens, and cellular homeostasis. ROS are formed primarily in the mitochondria as oxygen is reduced along the electron transport chain (ETC) in cellular respiration for overall energy generation in Eukaryotic systems. O_2•- is the primary ROS produced in this process and will lead to the formation of other ROS, hydroxyl, and hydrogen peroxide. Despite their essential activities for cellular function, ROS can clearly be toxic to cellular organelles and cell membranes. Higher ROS concentration as a result of breakdown in cellular redox balance leads to damage of macromolecules such as carbohydrates, proteins, lipids, and nucleic acids (Apel and Hirt, 2004; Gutteridge and Halliwell,

1990; Polle, 1997). Thereby those unstable oxygen intermediates are able to react with the polyunsaturated fatty acids and cholesterol present in cell membrane and generate oxidized lipids which finally lead to apoptosis or cell death (Halliwell, 2006). ROS may also cause damage to DNA or can induce rapid depletion of cellular NADP/NADPH pools for needs of anabolism, leading to depletion of cellular energy (ATP) reserves from inefficient NADH production (Apel and Hirt, 2004). As ROS are toxic and also participate in signaling processes, plant cells require at least two different mechanisms to regulate their intracellular ROS concentration by directly scavenging them. One that will enable the fine modulation of low levels of ROS for signaling purposes, and one that will ensure the detoxification of excess cellular ROS (Foreman et al., 2003).

Abiotic Stress-Induced Oxidative Damages in Plants

Plants subjected to abiotic stresses go through protective physiological and molecular adjustment which, if unsuccessful, leads to the complete breakdown of metabolic regulation associated with overall plant integrity. It is well known that the generation of ROS due to oxidative stress and breakdown of cellular homeostasis are common mechanisms through which abiotic stress factors exerts harmful effects on the cells and tissues of susceptible plants (Akula and Ravishankar, 2011; Apel and Hirt, 2004). Abiotic and biotic stresses (such as UV radiation, drought, temperature extremes, salinity, pathogens, and insects) can directly or indirectly disrupt the critical balance between ROS and the antioxidant defense systems in plant cells, which can in turn induce a chronic state of redox imbalances that significantly affect the overall fitness (Gill and Tuteja, 2010; Zhu, 2001). Being highly reactive, ROS will interact with all major macromolecules of the cell and cause widespread damage and alterations to normal metabolic functioning, a cellular state referred to as oxidative stress (Apel and Hirt, 2004; Gill and Tuteja,

2010). Reactive oxygen intermediates (ROI) can also play critical roles in plant endogenous defense responses, especially against biotic stresses and they are at the interface between abiotic and biotic stress responses of plants. Other than directly quenching and scavenging ROS, plants also have mechanism to avoid the ROS and ROI production, which dictates overall fitness against abiotic stresses. Reducing the risk of ROI production helps plants to mitigate oxidative damages induced by abiotic stresses (Mittler, 2002). Thus all terrestrial plants have evolved complex and organized defense mechanism to counter chronic oxidative stresses and subsequent maintenance of cellular homeostasis.

Oxidative Stress-Linked Chronic Diseases in Humans

As an aerobic Eukaryotic organism, human cells can also be impacted by redox imbalances from metabolic and environmental fluctuations, where ROS interact with major macromolecules which inevitably lead to cellular damages and dysfunction, similarly to plants (Aruoma, 1998; Wilson et al., 2005). Demand of excessive cellular energy (ATP) usually results in an incomplete reduction of oxygen in the mitochondria and leading to the subsequent generation of excess ROS. While oxidizing environment favors cell death, reducing environments favor cell proliferation. Environmental stress, pathogenic attack, and oxidative stress-linked diseases can easily breakdown redox homeostasis in the cell and can induce dysfunction of oxidative phosphorylation at the mitochondria (Aruoma, 1998; Lin and Beal, 2006). Perturbation of pro-oxidant/antioxidant balance can lead to the alteration of cellular function and oxidative damage of macromolecules (lipids, proteins or DNA). When cellular stress defenses become overwhelmed, oxidative stress-linked damages will accumulate and ultimately lead to dysfunction or apoptosis of cells that results in chronic disease and aging (Lin and Beal, 2006). As the damage inflicted is widespread and diverse, every major organ system

can be impacted with increasing severity over time by degrading molecules and causing chronic inflammation (Singh et al., 2009). Mounting evidence suggests that while most NCDs are symptomatically diverse, a shared pathogenesis for development and incidence exists: chronic inflammation induced from oxidative stresses (Ceriello and Motz, 2004; Lin and Beal, 2006). Pathogenesis of vascular degeneration involves oxidative stress either by triggering or exacerbating the biochemical processes accompanying metabolic syndrome (Hanhineva et al., 2010; Wilson et al., 2005). Chronic antioxidant deficiency may favor the propagation of oxidative alterations from intra- to extra-cellular spaces that induces a systemic state of oxidative stress. ROS not only damage cellular structures, but also contribute to cellular aging, mutagenesis, carcinogenesis, coronary heart disease and apoptosis (Kaneto, 2007). Thus, improved protection against chronic oxidative stress is an integral part for the prevention and management of chronic diseases in humans.

Antioxidant Defense Systems in Plants

Plants have evolved an elaborate antioxidant defense system that consists of both enzymatic and non-enzymatic components within the cell to counter ROS in order to maintain cellular homeostasis (Polle, 1997). These include enzymatic scavengers such as superoxide dismutase (SOD), which hasten the dismutation of $O_2\bullet^-$ to H_2O_2, and catalase and glutathione peroxidase, which convert H_2O_2 to water; hydrophilic radical scavengers such as ascorbate, urate and glutathione; lipophilic radical scavengers such as tocopherols (α-tocopherol, γ-tocopherol), flavonoids (quercetin, epigallocatechin, gallate), carotenoids (β-carotene, lycopene) and ubiquinone; enzymes involved in the regeneration of oxidized forms of small molecular antioxidants (GSH reductase, dehydroascorbate reductase) or responsible for maintenance of protein thioles (thiredoxin reductase); the cellular machinery that maintains a reducing

environment (glucose-6-phosphate dehydrogenase, which regenerates NADPH); and secondary metabolites such as inducible phenolics (Apel and Hirt, 2004; Beckman, 1997). Antioxidant protective systems are present in all cellular and subcellular compartments of plants, including the apoplastic region. Balancing ROS with antioxidant responses is a key function of eukaryotic cells to counter oxidative challenges and maintaining cellular homeostasis (Mittler, 2002). The ratio of ROS and antioxidants are an interconnected system and closely associated with primary and secondary metabolism of plants. Provided external sources of radical formation are minimal, the antioxidant defense system can adequately maintain balance of the potentially deleterious nature of ROS (Halliwell, 2006). Thus, diverse protective roles of antioxidants and their subcellular localization determine the fitness of plants under abiotic stress-induced oxidative pressure.

Phenolics: Biological Role as Antioxidant

The most important biological function of phenolic compounds with structural significance is to antioxidant activity. The antioxidant activity of phenolics is actually governed by their redox properties, which play crucial role in absorbing and neutralizing free radicals, quenching single and triplet oxygen or decomposing peroxides (Rice-Evans et al., 1997). Such free radical scavenging property of phenolics is mainly due to their ability to donate hydrogen from hydroxyl groups positioned along the aromatic ring to terminate free radical oxidation of lipids and other biomolecules and by forming aryloxyl radicals (Bors and Michel, 2002; L Bravo, 1998; Tsao, 2010). Among phenolics, monophenols are less potent as hydrogen-donating radical scavengers than polyphenols (Figueroa-Espinoza and Villeneuve, 2005). Phenolics either directly quench free radicals or combat free radicals through the stimulation of host antioxidant enzyme responses to counter oxidative pressure (Tsao, 2010). The most widely distributed

phenolic compounds in plant tissues are hydroxicinamic acids, p-coumaric, caffeic and ferulic acids, which are synthesized via shikimate pathway (Tsao, 2010). Different mechanisms are involved in determining antioxidant activity potential of these phenolic compounds, which ultimately determine their role as antioxidants to counter abiotic stresses (Bartwal et al., 2013).

Role of Plant Phenolics to Counter Abiotic Stresses

In response to biotic and abiotic stresses, biosynthesis of phenolic antioxidants takes place through stimulation of plant secondary metabolite pathways. Many phenylpropanoid compounds such as flavonoids, isoflavonoids, anthocyanins, and phenolic acids are induced in response to wounding, nutritional stress, cold stress, salinity, UV-B stress, drought, and flooding (Akula and Ravishankar, 2011; Mittler, 2002; Zhu, 2001). It is now generally accepted that the development of phenolic polymer biosynthesis is partly induced by UV-B and has played a major role in the evolution of land plants (Rozema et al., 2002). Study with *Arabidopsis* mutants further strengthens the hypothesis that flavonoids are the main UV absorbents in plant tissues (Bieza et al., 2016). Phenolics present in plant cells can also stimulate synthesis and activity of other antioxidant enzymes, which in turn also provide protection against abiotic stresses (Akula and Ravishankar, 2011).

Under abiotic stresses, phenolics not only just act as antioxidants but also play critical role in metabolic and structural adjustments, which dictate overall fitness of plants against abiotic stresses (Akula and Ravishankar, 2011; Gill and Tuteja, 2010). The role of phenolic antioxidants in plants abiotic stress tolerance is gaining attention for improving rapid adjustment, robustness and productivity of major crops under limited resources and extreme environments. Further due to its antioxidant potential, these inducible phenolics from plant-based foods may

also help to counter oxidative stress-linked chronic diseases in humans when consumed as a part of diet (Pandey and Rizvi, 2009).

Role of Plant Phenolics in Dietary Management of Chronic Disease

Although human cells cannot produce phenolic antioxidants themselves, dietary consumption of such plant-based secondary metabolites has shown to prevent and manage many NCDs by neutralizing the destructive reactivity of undesirable excess ROS (Pandey and Rizvi, 2009; Rice-Evans et al., 1997). While phenolic antioxidants can directly offset damages caused by ROS, plant phenolics have also shown strong evidence of other important protective bioactive benefits. Numerous protective effects are associated with phenolic antioxidants, including cardio-protection, anti-cancer, anti-aging, neuro-protection, and anti-diabetes (Fernandez-Panchon et al., 2008; Pandey and Rizvi, 2009; Shahidi and Ambigaipalan, 2015). Cells typically respond to phenolic phytochemicals mainly through direct interactions with receptors or enzymes involved in metabolic processes of digestion, signal transduction, or through modifying gene expressions which may results in modification of the redox status of the cell that may trigger a series of redox-dependent reactions (Shalaby and Horwitz, 2014; Tsao, 2010). Dietary phenolic phytochemicals and their metabolites may also the influence digestion, absorption and metabolism of dietary carbohydrates (such as starch and sucrose) that may improve type 2 diabetes management (McCue and Shetty, 2003). Dietary phenolics from different plant-based sources influence glucose metabolism by several mechanisms, such as inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from liver, activation of 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 obvious targets for better glycemic control after high carbohydrate meals, with α-amylase and α-glucosidase being key enzymes responsible for the digestion of dietary carbohydrates to glucose. Inhibition of these digestive enzymes could reduce the rate of glucose release and absorption in the small intestine and consequently suppress postprandial hyperglycemia (Hanhineva et al., 2010). Many *in vitro* studies have been reported that phenolic metabolites including flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanindins and ellagitanins) inhibit α-amylase and α-glucosidase activities (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 showed 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). Thus, improving biosynthesis of phenolics in food crops has dual advantages; to enhance climate resilience for countering abiotic stresses, and to improve nutritional quality to counter oxidative stress-linked NCDs, such as type 2 diabetes and associated complications.

Proline-Associated Pentose Phosphate Pathway and Phenolic Biosynthesis in Plants

The proline-associated pentose phosphate pathway (PAPPP) is a proposed plant metabolic response model that suggests phenolic metabolites in plants can efficiently be induced through an alternative mode of metabolism, linking proline synthesis with the pentose phosphate pathway (PPP) regulation (Sarkar and Shetty, 2014b; Shetty and Wahlqvist, 2004). In effect, proline synthesis in the cytosol is coupled with the PPP through up-regulating NADP+/NADPH ratio in favor of NADPH, and activation of NADPH-dependent PPP stimulates secondary metabolite biosynthetic pathways such as phenylpropanoid and shikamate pathways (Shetty and

Wahlqyist, 2004). At the same time, proline serves as a reducing equivalent, instead of NADH for oxidative phosphorylation (ATP synthesis) in the mitochondria (Hare and Cress, 1997). PPP also generates erythrose-4-phosphate which along with phosphoenolpyruvate from glycolysis, which is channeled to the shikimate pathway to produce phenylalanine, and then directed through the phenylpropanoid pathway to produce phenolic compounds (Shetty and McCue, 2003) (Figure 2.1.).

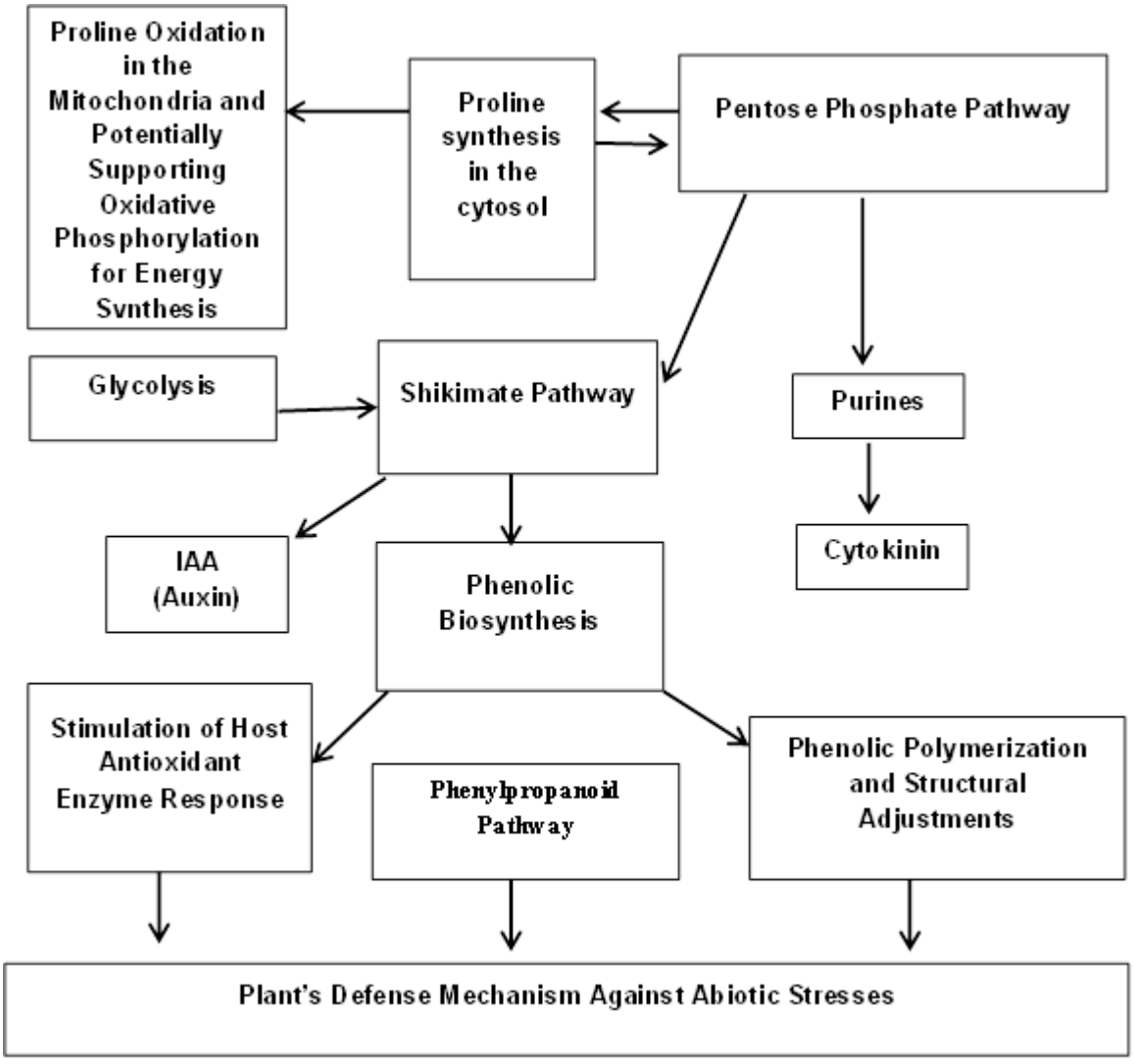

Figure 2.1. The role of redox-linked proline-associated pentose phosphate pathway for biosynthesis of secondary metabolites (phenolics), plant growth regulators (auxin & cytokinin), and stimulation of antioxidant enzyme responses to counter abiotic stresses (Adapted from Shetty, 1997).

Up-regulation of PAPPP provides NADPH, which is an anabolic co-factor for the biosynthesis of endogenous antioxidant enzymes such as SOD, CAT, and GPX. Increased biosynthesis of inducible phenolics coupled with up regulation of PAPPP also stimulates host endogenous antioxidant enzyme responses (Duval and Shetty, 2001; Randhir and Shetty, 2005; Shetty and McCue, 2003). Abiotic stresses favor the stimulation of both the shikimate and phenylpropoanoid pathways in plants, and therefore, the up-regulation of PAPPP could lead to the optimum stimulation of inducible phenolic biosynthesis under abiotic stresses. The first committed step of PPP is carried out by glucose-6-phosphate-dehydrogenase (G6PDH). 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 power for nitrite reduction. It is evident that plant nitrate assimilation is coupled with activation of G6PDH and thus have important role in crop production especially for leguminous plants like black bean (Hare and Cress, 1997; Singh et al., 2009). Up-regulation of PAPPP may not only just improve secondary metabolite biosynthesis (such as phenolics), but may additionally support the growth-regulating anabolic needs (such as hormones, polymeric phenolics and purines) that are critically important during seed germination and early establishment (Shetty and Wahlqvist, 2004). Thus, the advantages of improved biosynthesis of inducible phenolics through PAPPP are not only with its direct role, but also have a significant role in plant redox and metabolic regulation resulting into higher fitness and resilience against abiotic stresses. Similarly, such

enhancement of phenolic biosynthesis through up-regulation of PAPPP may potentially improve phenolic bioactive-linked functionalities in food crops, which is relevant for dietary management of NCDs, such as type 2 diabetes, cardiovascular diseases, and cancer.

Stimulation of Phenolic Biosynthesis through Elicitation

Elicitation techniques using naturally-derived elicitors and other bio-processed compounds provide a metabolic strategy to stimulate the biosynthesis of phenolic bioactives in plants to improve resilience against abiotic stresses, while concurrently improving the bioactive profile of the harvested food crops targeting dietary management of NCDs (Fig 2.2). Several compounds have already shown empirical evidence of eliciting phenolic biosynthesis *in vitro*, primarily proline, proline precursors, and proline analogs (Limón et al., 2014; Shetty and McCue, 2003; Yang and Shetty, 1998). Earlier research under controlled environments has shown improved seed vigor, enhancement of phenolic content and overall antioxidant-enzyme stimulation in plants through application of exogenous elicitors (Horii et al., 2007; Gavhane, 2013; Randhir and Shetty, 2007; Sarkar et al., 2010a). External elicitors and abiotic stress treatments also showed up-regulation of PPP and stimulation of phenolic biosynthesis in different dicot and monocot plants. UV-B treated fava bean seeds showed stimulation of PPP regulation with increase in nutraceutical–relevant phenolic metabolites, and guiaicol peroxidase (GPX) activity (potential polymerization of phenolics) in dark germinated fava beans (Randhir et al., 2002). Similarly, up-regulation of PPP and enhancement of levo dihydroxy phenylalanine (L-DOPA) concentration was observed in lactoferrin and fish protein hydrolysate treated fava bean (Randhir and Shetty, 2003). Microwave treated fava bean seeds also showed increase in G6PDH and concurrent increase in phenolic concentration and GPX activity (Randhir and Shetty, 2004).

Stimulation of PPP and enhancement of phenolic and antioxidant activity in sprouted corn was observed after external elicitor treatments (Randhir and Shetty, 2005).

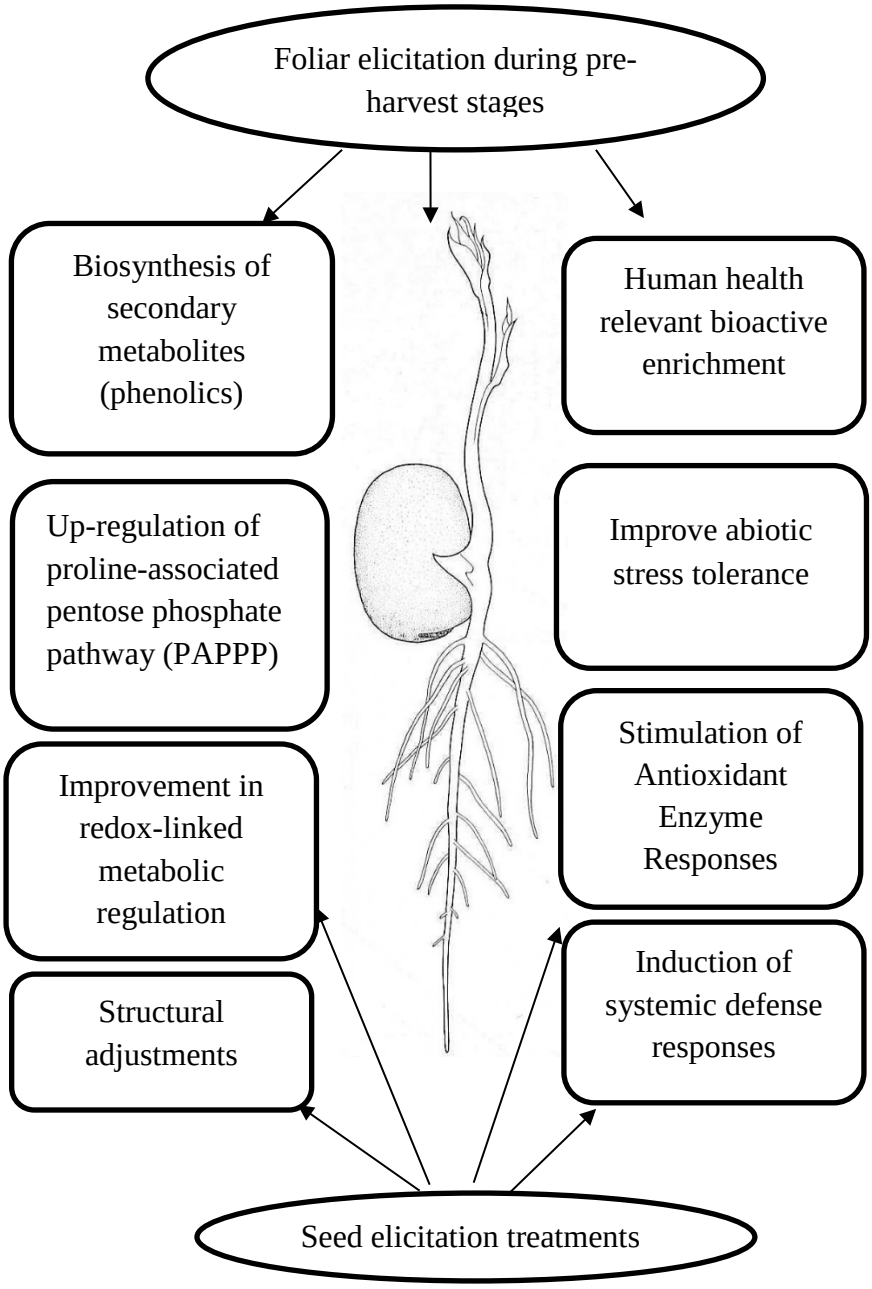

Figure 2.2. Model for improving abiotic stress tolerance and human health-relevant bioactive enrichment in food crops using seed and foliar elicitation treatments.

Similarly, improvement of phenolic content, antioxidant activity, and antimicrobial properties through up-regulation of PPP was observed in mung bean sprouts with natural elicitor

treatments (Randhir et al., 2004a). PPP mediated and phenolic-linked antioxidant elicitation was also observed in dark germinated fenugreek sprouts with natural elicitors (Randhir et al., 2004a). Stimulation of phenolic content, antioxidant activity, in relation to improved seed vigor was found in dark germinated pea seedling, treated with genetically transformed and high cytokinin root extracts of anise (Duval and Shetty, 2001). Acid-induced cell growth and elongation of pea seedling in association with PPP-linked stimulation of phenolic biosynthesis was observed in post germinated pea seedlings (Zheng and Shetty, 2000). Exogenous application of natural antioxidants, such as vitamin C and folic acid, showed enhanced seed vigor and improved seedling performance through PPP regulation in pea (Burguieres et al., 2007). Similar improvement in germination and in seed vigor was found in soybean, tomato, and corn seedlings with fish protein hydrolysates treatments (Horii et al., 2007). Improvements in the phenolic bioactive profiles in both seeds and sprouts of dietary plants through elicitation have also been demonstrated. Dark germinated bean sprouts have been shown to have improvement in antioxidant and antimicrobial properties as well as improved health-relevant functionality using natural elicitors of the PAPPP (Limón et al., 2014; Randhir et al., 2004a)

The role of PAPPP in modulating phenolic and antioxidant enzyme responses under abiotic stresses has been demonstrated in other studies with mature plants. Long-life span oregano clonal line showed higher phenolic biosynthesis and antioxidant enzyme activity through stimulation of PAPPP regulation with UV treatment (Kwon et al., 2006). Study with cool season turfgrasses, such as creeping bentgrass, Kentucky bluegrass, and perennial ryegrass also showed similar role of PAPPP for stimulating phenolic biosynthesis and antioxidant enzyme responses under UV-B stress (Sarkar et al., 2011). Enhanced cold tolerance with improved photochemical efficiency, and up-regulation of PPP was observed in single-seed origin creeping

bentgrass clonal lines (Sarkar et al., 2009). Role of PPP to improve cold tolerance through stimulation of phenolic biosynthesis and antioxidant enzyme responses was observed in cold acclimated cool-season grasses (Sarkar and Bhowmik, 2009). Similar response with PPP mediated stimulation of phenolics and antioxidants enzymes was found in cold acclimated perennial ryegrass lines (Sarkar et al., 2013). Further, foliar treatments with natural elicitors, such as COS) and Gro-Pro enhanced PAPPP regulation coupled with inducible phenolic biosynthesis to induce cold stress resilience in cool-season grasses (Sarkar et al., 2011). These above studies suggest that external elicitor treatments or abiotic stress induction can potentially up-regulate PPP function with concurrent stimulation of defense related pathways linked to inducible phenolics and antioxidant enzyme responses. Such protective mechanism through wider system-based metabolic function of PAPPP regulation improves seed vigor, and emergence, while allowing plants to maintain cellular redox balance by countering oxidative stresses. Further such stimulation also improves human health relevant phenolic bioactive profiles in food crop and can enhance nutritional values relevant for dietary management of NCDs.

Role of Soluble Chitosan Oligosaccharide as Elicitor

Chitosan and its derivatives have shown various functional properties and can be used as a bioactive material in many applications including, food, biomedicine, agriculture, environmental protection, and wastewater management (Kim and Rajapakse, 2005). COS is a highly soluble chitosan oligomer produced with enzymatic hydrolysis of chitosan. COS can be used for improving agricultural production system, especially overall crop quality, fitness, and resilience (Gavhane, 2013). Recently, the antioxidant activity of chitosan and its derivatives attracted a greater attention and studiesrevealed that hydroxyl radical scavenging potency of

COS is partly due to chelating ability of transition Fe2+, molecular charge properties and proton donation via hydroxyl and amino groups (Huang et al., 2005). Being a compound made from fungal cell walls, COS 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). Soluble COS derivatives can be found in different forms and can utilized according to their chemical structure and biological functions. COS-C has ascorbic acid as a side chain, which is alone an important bioactive antioxidant compound. Bioprocessed soluble COS as seed or foliar treatments are effective against different fungal pathogens, microbes and stimulate secondary metabolite synthesis in plants for improving biotic and abiotic stress tolerances (Agrawal et al., 2002; Gavhane, 2013; Khan et al., 2003; Prapagdee et al., 2007; Sarkar et al., 2010b). There is still limited understanding about the mechanism and the method of application of chitosan and chitosan derivatives such as COS in field crops for alleviating abiotic stresses and improving resilience.

Black Bean as a Model Crop for Enhancing Climate Resilience and Improving Human Health Relevant Phenolic Bioactives

Black beans are a rich source of dietary fiber, resistant starches, amino acids, vitamins, and plant phytochemicals (Chimmad et al., 2005; Guajardo-Flores et al., 2013; Lai and Varriano-Marston, 1979; Lin et al., 2008). Protective function of black beans in preventing genetic damage induced by chemical mutagens was observed in mice and other *in vivo* models (Azevedo et al., 2003; Luna Vital et al., 2014; Thompson et al., 2009; Zhu et al., 2012). Similarly, water soluble condensed tannins isolated from black beans showed inhibition of prostatic cancer cell growth (Bawadi et al., 2005). Black beans also have potential to improve maternal and child health due to their rich composition of bioactives along with minerals and vitamins (Mitchell et al., 2009).

2Another potential function of black beans bioactives is due to its antiproliferative and antioxidative activities (Guajardo-Flores et al., 2013; Oomah et al., 2010). Most black bean genotypes have a low glycemic index and phenolic bioactives in black beans have potential in improving carbohydrate metabolism in humans when consumed in diet (McCue and Shetty, 2003; Silva-Cristobal et al., 2010). The American Diabetes Association, the American Heart Association, and the American Cancer Society have all recommended legumes (including black beans) as important dietary components for preventing and managing NCDs and T2D (Leterme, 2002).

Black bean cultivation is gaining popularity among growers in the Northern Plains of the United States due to its soil enrichment potential (nitrogen fixation), superior health benefits, and better value added export opportunities (Miller et al., 2002). North Dakota is the largest producer of dry beans including black beans in the United States but growers are facing increasing challenges to maintain better fitness and higher productivity under extreme variations in climate and soil conditions (Franzen, 2007). Edible dry beans including black beans are very susceptible to salinity stress, especially during germination and early emergence stages in spring (Abdul Qados, 2011). Increasing salinization in different regions of North Dakota and in other parts of the Northern Plains significantly affecting food crop production including black beans, and growers are seeking safe and sustainable solution to improve resilience against salinity stress (Franzen, 2007). Improving salinity resilience through elicitor –induced stimulation of endogenous defense responses in black beans offer excellent strategy and provide new tool to the growers for maintaining higher crop productivity under marginal lands.

While the synergies between plant stress defense responses and bioactive improvement are clear, currently no study in black beans has examined both mechanisms concurrently. The

aim of this proposed research was to determine the potential evidence that stimulation of the phenolic biosynthesis through up-regulation of PAPPP, by using elicitors as seed and foliar treatments, can simultaneously improve abiotic stress resilience as well as health relevant bioactive profiles in black beans.

CHAPTER 3. OBJECTIVES

Based on the above scientific rationale the broad objective of this study was to evaluate the dual role of phenolic antioxidants for enhancement of salinity resilience and human health relevant bioactive profiles in black beans using seed and foliar elicitation treatments. Therefore, the specific objectives are

1. To improve phenolic-linked antioxidant functionalities and their metabolic responses in dark-germinated sprouts of black beans using seed elicitation treatments and screening bioactives for dietary management of early stages T2D,
2. To evaluate the effects of seed elicitors, salinity stress, and black bean genotypes on phenolic biosynthesis, antioxidant enzyme responses, and salinity resilience using metabolic markers of PAPPP regulation.
3. To study the effects of chitosan oligosaccharide applied as a foliar elicitor treatment during pod-filling stages to enhance phenolic antioxidants in black bean genotypes and evaluate their health benefits for T2D.

CHAPTER 4. ELICITATION OF PHENOLIC ANTIOXIDANT-LINKED FUNCTIONALITY IN DARK-GERMINATED BLACK BEAN SPROUTS AND SCREENING FOR MANAGEMENT OF EARLY-STAGE TYPE 2 DIABETES USING *IN VITRO* MODELS

Abstract

Sprouting and elicitation provides an easy and cost-effective solution for improving the phenolic antioxidant-linked functionalities in legumes that can be screened and targeted for dietary management of chronic diseases. Prior to its successful application in food and health industry, it is essential to optimize appropriate elicitation technique along with selection of ideal legume species and genotypes with high human health relevant bioactive profiles. Therefore, the major aim of this study was to determine dual roles of phenolic antioxidants in black bean sprouts for improving plant fitness associated with critical metabolic regulation of pentose phosphate pathway (PPP) and its subsequent enhancement of human health relevant bioactive profile after seed elicitation. Eclipse black bean seeds were subjected to two different seed priming treatments (soluble chitosan-COS-C and marine hydrolysate-Gro-Pro) for four hours and enzyme analysis associated with PPP regulation along with bioactive functionality studies screening for early stages type 2 diabetes were carried out in dark germinated black bean sprouts using *in vitro* assay models. In this study, improvement of phenolic-linked antioxidant enzyme responses through up-regulation of PPP (G6PDH activity) in black bean sprouts with seed elicitation treatments was observed. But such improvement in antioxidant activity with seed elicitation did not show any further improvement in inhibition of key enzyme associated with glucose metabolism, such as α-amylase and α-glucosidase using model *in vitro* assays. The

results showed that both elicitors have significant promise to improve phenolic antioxidants in black bean sprouts, but further evaluation is required to prove its relevance for enhancing wider human health benefits.

Introduction

Agrobiodiversity, sustainable agriculture, and accessibility to balanced nutrition are essential to ensure global food security and to mitigate the epidemic of diet-linked chronic diseases (Thrupp, 2002). Legumes can play critical roles to address such interconnected challenges of agroecology, food, and human health through improving soil health (with nitrogen fixation), by adding agronomic values as ideal rotational and intercrop with cereals, and by compli8menting cereals in diet as a source of nutritionally-balanced amino acids and minerals (Akibode, 2011). Legumes are also important dietary sources to address protein malnutrition in developing countries (Messina, 2014). Further due to its rich and diverse human health relevant bioactive profiles and soluble fiber, legumes in diet have potential to counter many chronic diseases, such as type 2 diabetes and cardiovascular diseases (Leterme, 2002; Nakamura et al., 2001; Zhu et al., 2012). Even with such high agronomic and nutritional values, global food legume acreage and production is significantly less than the major cereal crops. But with the rise of diet-linked non-communicable chronic diseases (NCDs), the demand for higher supply of food legumes is increasing rapidly, especially in many developing countries and in food insecure regions of the world (Bazzano et al., 2001). South-East Asian countries have demand of around 4 million tons of assorted legumes annually with India being the largest importer, as legume production in this region have reduced significantly in post-Green Revolution era (Russell et al., 1989; Vandemark et al., 2014; Welch and Graham, 1999). Further, legumes act as a main source of dietary protein to traditional vegetarian population of India and to other Asian and non-Asian

countries. As dietary strategies offer most cost effective and safe alternatives and complimentary solutions to pharmaceutical drugs for prevention and management of NCDs, plant-based foods with diverse human health benefits such as legumes are excellent dietary targets to address such public health challenges (Shaw et al., 2010).

The current major challenge is to provide better access and availability of legumes with affordable prices, especially to impoverished population of urban and rural food deserts. To improve the consumption and year around supply of legumes, it is important to have alternative production system as global arable land is shrinking significantly due to rapid urbanization as well as due to the deterioration of soil and water quality (Godfray et al., 2010). Since ancient times, legumes have been consumed as sprouts in traditional diet and thus offer excellent scope to be incorporated as regular food or as a functional food in modern dietary system (Gaut, 2014; Tang et al., 2014). Such integration of sprouts in regular diet will also help in part to prevent and manage diet and lifestyle-linked NCDs, such as type 2 diabetes and associated cardiovascular risks (Nakamura et al., 2001). Seed sprouting is considered as natural processing methods to improve nutritive values and health qualities of foods (Plaza and Cano, 2003). The spouting technique is also easy and inexpensive processing method and can be adopted in urban-indoor food production system where accessibility of fresh and healthy food is a major constraint. Similarly, in the temperate region during winter, grains and legume sprouts are excellent source of fresh and nutritionally balanced foods.

During germination legume seed goes through different physiological changes and as a result nutritive and digestive values in sprouts such as increase in antioxidant and phenolic acid contents can be improved (Limón et al., 2014). Currently sprouting has become a popular technique for food enrichment in the global food market due to a rise in interest of health-

focused, minimally processed, additive free food processing over industrial approaches (Plaza and Cano, 2003). The germination process used in sprouting has been well documented with evidence that many nutrients are increased or have higher bioavailability for assimilation, particularly in the formation of free amino acids from the breakdown of storage proteins in the endosperm (Hofsten, 1979; Kim et al., 2012; López et al., 2013). Due to such rapid biochemical, and physiological changes during germination, sprouts are excellent model for improving human health relevant bioactives and associated functionalities through modulation of critical metabolic regulation.

Previous research indicated that elicitation with safe and edible products during seed priming followed by dark germination in different legumes and in non-legumes sprouts resulted in improved phenolics and antioxidant enzyme profile through up-regulation of pentose phosphate pathway (PPP) (Randhir and Shetty, 2004; Randhir et al., 2009; Vattem et al., 2005). Thus the use of external elicitors as seed treatment can improve oxidation-linked metabolic adjustment through stimulation of system-based critical pathways such as PPP which can be targeted to improve phenolic antioxidant-linked functionalities in bean sprouts for dietary management of early stages type 2 diabetes. Selection of the proper legume species and genotypes, combined with a suitable germination process could provide good sources of bioactive compounds from legumes and their germinated products for nutraceutical and functional food applications (Donkor et al., 2012). While knowledge exists regarding the characterization and biosynthesis of phenolic bioactives throughout varying stages of sprouting, little understanding exists examining how elicitation can improve phenolic-linked antioxidants and the subsequent health-targeted bioactive enrichments in black bean sprouts. Therefore, the aim of this research was to evaluate the effect of seed elicitation in black bean (*Phaseolus*

vulgaris L.) sprouts to improve phenolic antioxidant –linked functionalities screening and targeting dietary management of early stages type 2 diabetes using *in vitro* assay models. Additionally, the critical metabolic role of pentose phosphate pathway (PPP) to stimulate phenolic biosynthesis and endogenous antioxidant enzyme responses during sprouting after seed elicitations was also evaluated.

Materials and Methods

Plant Material: Eclipse, an early maturing and high yielding black bean genotype, was selected for this study based on being the highest yielding variety and 90% of total black bean acreage in North Dakota, the largest dry bean producer in the U.S. (Osorno et al., 2009). Eclipse seeds were acquired from the 2014 fall harvest.

Analytical Material: Porcine pancreatic a-amylase (EC 3.2.1.1), baker's yeast glucosidase (EC 3.2.1.20), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and 3,5-Dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO) and of analytical grade.

Elicitors: Two elicitors were used as seed treatment for this study: soluble chitosan oligosaccharide- vitamin C (COS-C), derived from marine chitin obtained from Kong Poong Bio, Jeju Korea and marine peptide hydrolysate (Gro-Pro), derived from seaweed and marine fish extracts and obtained from Icelandic Bioenhancer, Iceland. The elicitors and subsequent concentrations were selected based on previous studies that indicated significant elicitation of PPP at the selected concentrations in multiple crops, as well as the dose being practical for application in conventional agriculture (Sarkar and Shetty, 2014b). COS was investigated using a 1% solution (1 g COS-C /1 L distilled water) and Gro-Pro in a 1% solution (1 mL Gro-Pro /1 L

distilled water). All elicitor treatments were prepared 12 hours prior to seed incubation and stored in refrigerator at 0-4℃.

***Seed Treatments*:** A seed treatment incubation strategy was used, with a four hour incubation period at 25°C prior to dark germination of the seeds. Black bean seeds were treated with COS (1 g/L) and Gro-Pro (1 mL/L) solution. The control was treated with distilled water. Seed (50 g) were added to 450 mL of solution for each experimental group. Treated and untreated solution was drained after 4 hours and seeds were kept in a dark incubator set to 25°C for the duration of the study in 500 mL beakers, with each treatment being rinsed and drained periodically to maintain germination.

Sampling: Black bean sprout sample were taken for both pentose phosphate pathway and for bioactive functionalities assays after five, seven, and nine days of seed treatment. Time points were selected based on germination stages: day five (½ inch cotyledon emergence), day seven (rootlet formation), and day nine (first leaf formation) of black bean sprouts. The days required to reach these stages the specific germination conditions used (80% humidity, 25°C, lightly soiled tray flats). For bioactive analysis seed coats were included in the extraction.

Statistical Analysis: A completely randomized design (CRD) was used with elicitors as experimental groups. The experiment was repeated twice, with six replications for all *in vitro* bioactive assays and three replications for pathway regulation assays. All experimental groups were subjected to identical conditions in order to control extraneous variables. Univariate analysis was conducted using statistical analytical software (SAS) 9.3 version. Differences among treatments were determined using Tukey test at 0.05 probability level.

The experiment was divided in two main sections i) to evaluate the pentose phosphate pathway mediated phenolic biosynthesis and antioxidant enzyme responses in black bean

sprouts, and ii) to evaluate phenolic antioxidant-linked functionalities in black bean sprouts screening and targeting early stages type 2 diabetes management through *in vitro* assay models.

Part I: Pentose Phosphate Pathway Mediated Phenolic Biosynthesis and Antioxidant Enzyme Responses

Enzyme Extraction: Black bean sprouts (400 mg) were collected and thoroughly macerated by using a cold pestle and mortar with 4 mL cold enzyme extraction buffer [0.5% polyvinylpyrrolidone (PVP), 3 mmol L^{-1} EDTA, and 0.1 mol L^{-1} potassium phosphate buffer of pH 7.5]. The extracted sample was centrifuged at 10,188 g_n for 10 min at 2-5 °C and stored in ice. Supernatant was used for further protein and enzyme analysis.

Extraction Procedure to Determine Total Soluble Phenolic Content and Total Antioxidant Activity: A quantity of 100 mg (fresh weight-FW) black bean sprouts was immersed in 5 mL of 95% ethanol and kept in the freezer for 48-h. After 48-h, the sample was homogenized and centrifuged at 12,225 g_n for 10 min using tissue tearer (VWR Model 200, Randor, PA).

Total soluble phenolic assay: The total phenolics were determined by the Folin–Ciocalteu method which was based on a method modified by Shetty et al. (1995). Briefly, 0.5 mL of the sample extract and 0.5mL of distilled water were transferred into a test tube and mixed with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50% (vol/vol) Folin–Ciocalteu reagent was added and vortexed. After 5 min, 1 mL of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The standard curve was established using various concentrations of gallic acid in 95% ethanol, and results were expressed as mg per gram fresh weight (FW).

***ABTS Inhibition Assay*:** The total antioxidant activity of black bean sprout extract was measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$ radical cation (Re et al., 1999). ABTS (Sigma Chemical Co, St. Louis, MO) was dissolved in water to a 7 mmol L^{-1} concentration. $ABTS^+$ radical cation was prepared by reacting 5 mL of 7 mmol L^{-1} ABTS stock solution with 88 µL of 140 mmol L^{-1} potassium persulphate, and mixture was allowed to stand in the dark at room temperature for 12-16 h before use. Prior to assay $ABTS^+$ stock solution was diluted with 95% ethanol (ratio 1:88) to give an absorbance at 734 nm of 0.70 ± 0.02, and was equilibrated to 30 °C . One milliliter ABTS was added to glass test tubes containing 50 µL of each tissue extract, and mixed by vortex mixer for 30 s. After 2.5 min incubation, mixtures were read at 734 nm. The readings were compared with controls, which contained 50 µL of 95% ethanol instead of the extract. The Trolox reference standard for relative antioxidant activities was prepared with 5 mmol L^{-1} stock solution of Trolox in ethanol for introduction into the assay system at concentrations within the activity range of the assay (0-20 µmol L^{-1} final concentration) for preparing a standard curve to which all data were referenced. The percent inhibition was calculated by:

$$\% \text{ inhibition} = \frac{([A_{734}^{control} - A_{734}^{extract}])}{[A_{734}^{control}]} \times 100$$

Total Protein Assay: Protein content was determined by the method of Bradford assay (Bradford, 1976). One parts of dye reagent (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted with 4 parts of distilled water. A volume of 5 mL of diluted dye reagent was added to 50 µL of the black bean sprout extract. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a blank (5 mL reagent and 50 µL buffer solution) by using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

Glucose-6-phosphate dehydrogenase (G6PDH) Activity: A modified method originally described by Deutsch (Deutsch, 1983) was used. The enzyme reaction mixture containing 5.88 µmol L^{-1} ß-NADP, 88.5 µmol L^{-1} magnesium chloride ($MgCl_2$), 53.7 µmol L^{-1} glucose-6-phosphate, and 0.77 mmol L^{-1} maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 mL of this mixture, 100 µL of the extracted enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme activity in the mixture with the help of the extinction co-efficient of NADPH (6.22 mmol L^{-1} cm^{-1}) and expressed as nanomoles per milligram protein.

Succinate Dehydrogenase (SDH) Activity: To assay the activity of SDH a modified method of Bregman (Bregman, 1987) was used. The assay mixture containing 1.0 mL of 0.4 mol L^{-1} potassium phosphate buffer (pH 7.2), 40 µL of 0.15 mol L^{-1} sodium succinate (pH 7.0), 40 µL of 0.2 mol L^{-1} 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 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 minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP (19.1 mmol L^{-1} cm^{-1}).

Proline Dehydrogenase (PDH) Activity: A modified method described by Costilow and Cooper (Costilow and Cooper, 1978) was carried out to assay the activity of PDH. The enzyme reaction mixture containing 100 mmol L^{-1} sodium carbonate buffer (pH 10.3), 20 mmol L^{-1} L-proline solution and 10 mmol L^{-1} mM nicotinamide adenine dinucleotide (NAD) was used. To 1 mL of this reaction mixture, 200 µL of extracted enzyme sample was added. The increase in absorbance was measured at 340 nm for 3 min, at 32 °C. 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).

Catalase (CAT) Activity: The CAT activity was assayed according to the methods of Beers and Sizer (1952). To 1.9 mL of distilled water 1 mL of 0.059 mol L^{-1} hydrogen peroxide (Merck's Superoxol or equivalent grade, Merck Co. & Inc., Whitehouse Station, NJ) in 0.05 mol L^{-1} potassium phosphate, pH 7.0 was added. This mixture was incubated in a spectrophotometer for 4-5 min to achieve temperature equilibration and to establish blank rate. To this mixture 0.1 mL of diluted enzyme sample was added and the disappearance of peroxide was followed spectrophotometrically by recording the decrease in absorbance at 240 nm for 2-3 min. The change in absorbance ΔA_{240}/min from the initial (45 s) linear portion of the curve was calculated. One unit of CAT activity was defined as amount that decomposes 1 micromole of H_2O_2.

Guaiacol Peroxidase (GPX) Activity: The activity of GPX was assayed by a modified method (Laloue, H Weber-Lotfi et al., 1997)The enzyme reaction mixture containing 0.1 mol L^{-1} potassium phosphate buffer (pH 6.8), 56 mmol L^{-1} guaiacol solution, and 50 mmol L^{-1} hydrogen peroxide (H_2O_2) was used. To 990 µL of this reaction mixture, 10 µL of enzyme sample was added. The absorbance was recorded at zero time and then after 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture by using the extinction coefficient of the oxidized product tetraguaiacol (26.6 mmol L^{-1} cm^1).

Superoxide Dismutase (SOD) Activity: A competitive inhibition assay was performed that used xanthine-xanthine oxidase generated superoxide to reduce nitroblue tetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (Oberley, Larry W., 1984). The reaction mixture contained 13.8 mL of 50 mmol L^{-1} potassium phosphate buffer (pH 7.8) containing 1.33 mmol L^{-1}

diethylenetetraaminepentaacetic acid (DETAPAC); 0.5 mL of 2.45 mmol L^{-1} NBT; 1.7 mL of 1.8 mmol L^{-1} xanthine and 40 IU/mL catalase. To 0.8 mL of reagent mixture 100 µL of phosphate buffer and 100 µL of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 s for 2 min and the concentration of xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme sample and the change in absorbance was monitored every 20 s for 2 min. One unit of SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum.

Part II: Phenolic Antioxidant-Linked Functionalities Targeting Early Stages Type 2 Diabetes Using In Vitro Assay Models

Bioactive Extraction: Ten grams of black bean sprout sample were taken and added to 50 mL distilled water in 50 mL tube. Samples were kept in ice bucket in between steps to avoid excess foaming. Un-sprouted beans were removed from sampling. The mixture was blended for 3 minutes on using 100 mL volume in a Waring commercial blender. After blending sample was again homogenized using a Tissue-Tearer for 2 minutes on "30" speed setting. Then blended sample was centrifuged at 8500 RPM twice for 20 min and 10 minutes. Final supernatant was used for bioactive assays.

Total Soluble Phenolic Content: Total soluble phenolic content of the above extract was determined from to correlate with other *in vitro* functionality assays from same extracts. Same Folin-Ciocalteu method described earlier was used.

Antioxidant Activity by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) Inhibition Assay: The DPPH scavenging activity was determined by an assay method modified by Kwon et al. (2006). 1.25 mL of 60 µM DPPH in 95% ethanol was added to 250 µL of each sample

extract, with the decrease in the absorbance monitored after 5 min at 517 nm (A517 extract). The absorbance of a control (distilled water instead of sample extract) was also recorded after 5 min at the same wavelength (A517 control). The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A517(control)} - \text{A517(extract)}}{\text{A517(control)}}\right) \text{x } 100$$

α-Amylase Inhibition Assay: The α-amylase inhibitory activity was determined using an assay method modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a). A total of 500 µL of each sample extract and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α -amylase solution (0.5 mg/mL) were incubated at 25 ℃ for 10 min. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube and then incubated at 25 ℃ for 10 min. Next, 1.0 mL of dinitrosalicylic acid color reagent was added. The test tubes were then incubated in a boiling water bath for 10 min and cooled to room temperature to stop the reaction. The reaction mixture was then diluted after adding 10 mL of distilled water, and absorbance was measured at 540 nm. The absorbance of sample blank (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were recorded as well. The final extract absorbance (A540 extract) was obtained by subtracting its corresponding sample blank reading. The *a*-amylase inhibitory activity was calculated according to the equation below:

$$\% \text{ Inhibition} = \left(\frac{\text{A540(Control)} - [\text{A540(Extract)} - \text{A540(Sample blank)}]}{\text{A540(Control)}}\right) \text{x } 100$$

α-Glucosidase Inhibition Assay: The assay method was modified from the Worthington Enzyme Manual for *a* -glucosidase inhibition (Worthington Biochemical Corp., 1993b; McCue et al., 2005). A volume of 50 µL of sample extract diluted with 50 µL of 0.1 M potassium

phosphate buffer (pH 6.9) and 100 µL of 0.1 M potassium phosphate buffer (pH 6.9) containing glucosidase solution (1.0 U/mL) was incubated in 96-well plates at 25 ℃ for 10 min. After preincubation, 50 µL of 5mM p-nitrophenyl- a-D-glucopyranoside solution in 0.1 M potassium phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 ℃ for 5 min. Before and after incubation, absorbance readings (A405 extract) were recorded at 405 nm by a microplate reader (Molecular Devices Co., Sunnyvale, CA) and compared to a control which had 50 µL of buffer solution in place of the extract (A405 control). The *a*-glucosidase inhibitory activity was expressed as a percentage of inhibition and calculated as follows:

$$\%\ \text{Inhibition} = \left(\frac{\triangle\ \text{A405(control)} - \triangle\ \text{A405(extract)}}{\triangle\ \text{A405(control)}}\right) \text{x } 100$$

High performance liquid chromatography (HPLC): The sample extracts (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample was injected using an Agilent ALS 1100 autosampler into an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 - 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 306 nm and 333 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of chlorogenic acid, gallic acid, ellagic acid and quercetin in 100% methanol were used to calibrate the standard curves and retention time.

Results and Discussion

Part I: Pentose Phosphate Pathway Mediated Phenolic Biosynthesis and Antioxidant Enzyme Responses

Total Soluble Phenolic Content and Total Antioxidant Activity (ABTS) of Black Bean Sprouts: Shifts in primary metabolism, along with mobilization of nutrient reserves from seed and cotyledon to shoot, are common phenomenon during germination. Bound phenolics and antioxidant compounds are mobilized in the seed to aid in the germination process (McCue et al., 2000). When consumed as sprouts, these compounds have potential to reduce oxidative stress-linked cellular damages, as well as improving post-prandial glucose metabolism (Shetty and Walquist, 2004). Total soluble phenolic content and radical scavenging potential (ABTS inhibition) in de-coated black bean sprouts were measured to determine the potential link between such metabolic shift and phenolic-linked and enzyme-linked antioxidant mobilization in sprouts after seed elicitation. Overall, the TSP content of black bean sprouts indicated a decreasing trend throughout germination, reducing slightly from day 5 to 7 and then slightly improving again on day 9. (Figure 4.1.). The average TSP content across treatments showed 250 µg/g F.W. on day 5, 200 µg/g F.W. on day 7, and 270 µg/g F.W on day 9. Sprouts of black bean from day 9 following seed elicitation with soluble COS showed the highest TSP content (300 µg/g F.W.), followed by sprouts from Gro-Pro primed seeds (280 µg/g F.W.). This trend indicated that free phenolic compounds might be highly utilized in the mobilization processes of germination. With seed elicitation treatments (both COS and Gro-Pro treated), a higher TSP content trend was observed compared to control, although only COS at day 9 was found to be statistically significant (Figure 4.1).

These results were not similar to a previous study, where improvement in TSP content was found in 2 days post-germinated pea seedlings after seeds were primed with salicylic acid (McCue et al., 2000). Enhancement in TSP content and total antioxidant activity was observed in dark germinated mung bean sprouts after fish protein hydrolysate and lactoferrin elicitation treatment (Randhir et al., 2004b). Kidney bean sprouts treated with glutamic acid also showed higher TSP content and higher antioxidant potentials in treated sprouts, similar to the findings presented in this experiment (Limón et al., 2014).

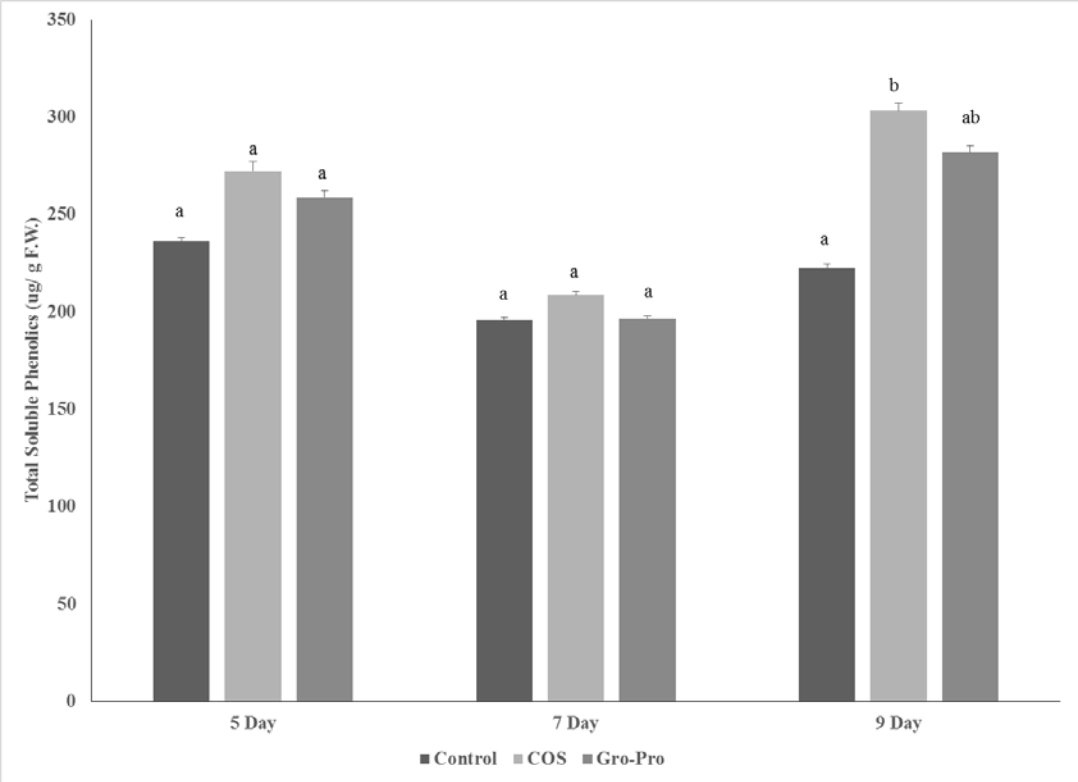

Figure 4.1. Total soluble phenolic content (µg/ g F.W.) of black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

In general, the total antioxidant activity of black bean sprouts was found to decrease from day 5 to day 9 in all post-seed priming treatments (Figure 4.2).This general trend suggests that the seed utilized soluble phenolics throughout germination, in which some may have been

associated with antioxidant function. Among seed elicitation treatments, no significant difference was found in total antioxidant activity at day 5 (emergence of cotyledon). The only exception to this trend was at day 9 (first leaf emergence) in Gro-Pro treated sprouts, which had a statistically higher total antioxidant activity (5% higher activity than control) (Figure 4.2.). Similar improvement in antioxidant activity was observed in fava bean sprouts with fish protein hydrolysate elicitor treatments, which has a similar elicitation formulation using Gro-Pro. Enhanced total antioxidant activity was also observed in pea seedlings after seeds were treated with fish protein hydrolysate in combination with acetyl salicylic acid (Andarwulan and Shetty, 1999). Black bean sprouts from COS primed seeds showed higher TSP content, while higher total antioxidant activity was observed with Gro-Pro seed treatments. The result suggests mobilization and utilization of phenolic and antioxidants may occur as germination progresses, as the total phenolic and antioxidant contents decreased during later stages of germination. Further, while elicitor treatments did show some statistically significant improvements at certain early stages of germination, a consistent pattern could not be established. This may be due to the removal of the seed coat step before enzyme extraction, as improvements in TSP and antioxidants may have occurred in the seed coat.

G6PDH and SDH Activity of Black Bean Sprouts: To understand the effect of seed priming treatments for inducing shifts in critical metabolic regulation that is associated with primary and secondary metabolism, the activities of key regulatory enzymes (G6PDH and SDH) were evaluated. Higher G6PDH activity indicates stimulation of the pentose phosphate pathway via the synthesis of sugar phosphates, which is critical for all anabolic pathways (Shetty, 2004; Shetty and McCue, 2003).

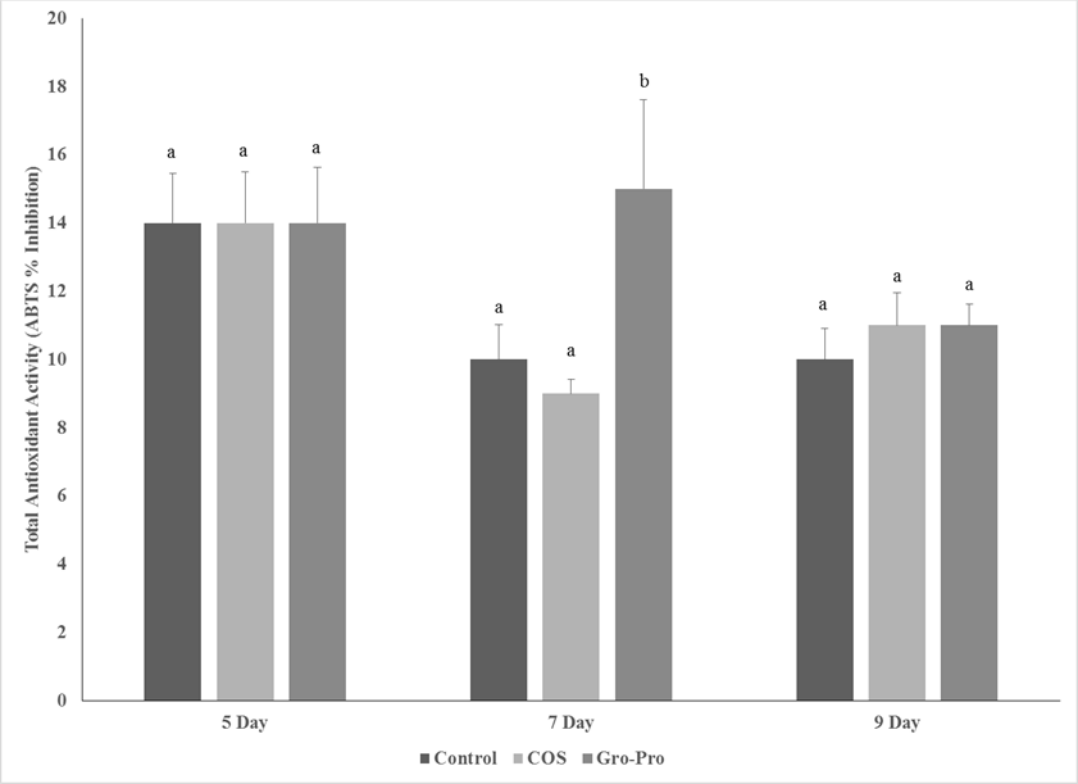

Figure 4.2. Total antioxidant activity (ABTS % inhibition) of black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

Overall the G6PDH activity trend increased at the earlier stages of sprouting (60 nmol/mg protein to 100 nmol/mg protein) until first leaf emergence, where activity did not change significantly (Figure 4.3.). The trend at days 5 (COS) and 9 with Gro-Pro treatments indicates a higher G6PDH activity, although no statistical difference could be established (Figure 4.3.). This finding of the stimulation of G6PDH activity in black bean sprouts suggests that PPP-mediated biosynthesis increases throughout germination, although this biosynthesis may not be solely linked to phenolic metabolites. Further, while elicitor treatments did indicate a modest increasing trend in G6PDH activity, this trend could not be validated as statistically significant. A similar relationship between up-regulation of PPP and subsequent stimulation in phenolic

biosynthesis was observed in other legumes (McCue et al., 2000; Randhir and Shetty, 2003; Shetty et al., 2003.)

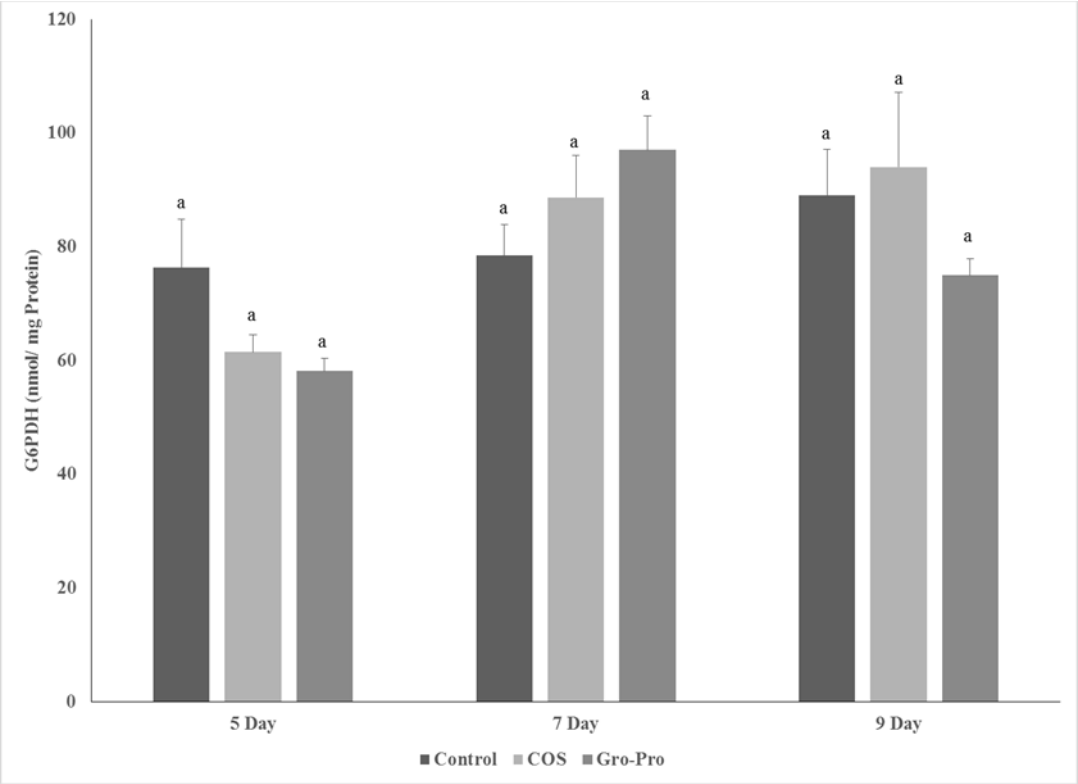

Figure 4.3. Glucose-6-phosphate dehydrogenase (G6PDH) activity (nmol.mg^{-1} protein) in black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

To understand the regulation of tricarboxylic acid/Kreb's cycle in black bean sprouts after seed priming treatments, the activity of an important enzyme, succinate dehydrogenase (SDH) was measured. Increasing SDH activity implies that more of the carbon flux is being shifted towards primary metabolism (catabolism) and away from secondary metabolism (anabolism). Contrary to the trend of G6PDH activity, the overall SDH activity decreased until day 7 (0.60 nmol/mg protein to 0.38 nmol/mg protein) while remaining constant though days 7 to 9 (Figure 4.4.). No significant differences among seed treatments were found (Figure 4.3.)

These finding indicates a slowing down of respiration rates in black bean sprouts as photosynthetic activity begins to increase in seedlings (from day 5 to 9). Interestingly, this trend correlates inversely with G6PDH activity, implying a shift towards secondary metabolic pathways while decreasing respiration rates as sprouting progresses.

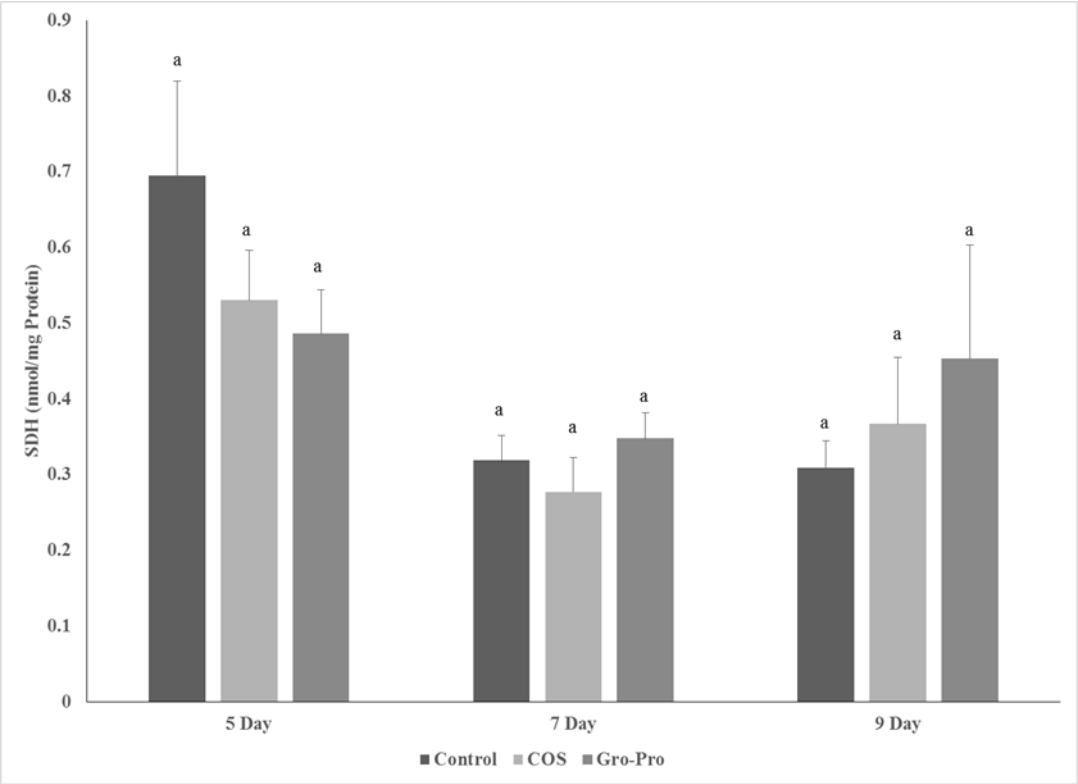

Figure 4.4. Succinate dehydrogenase (SDH) activity (nmol.mg^{-1} protein) in black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

PDH Activity of Black Bean Sprouts: Proline dehydrogenase (PDH) activity of black bean sprouts was measured to determine the potential oxidation of proline in the mitochondria and its association with up-regulation of PPP. In general, PDH activity decreased at day 7 from day 5 and then slightly increased at day 9 (Figure 4.5.). The trend was similar with SDH activity and it might be due to the link between proline synthesis and Kreb's cycle through α-

ketoglutarate. Although COS seed priming treatment showed higher PDH activity in black bean sprouts at 5 and 9 days but no clear correlation with G6PDH activity was observed (Figure 4.5.). This results indicate that stimulation of PPP in black bean sprouts from seeds primed with COS may not be directly associated with proline synthesis in the cytosol and its oxidation in the mitochondria. The germination and development of these sprouts took place without any external stress and thus black bean sprouts may not need to switch towards alternative energy synthesis pathway with proline being critical metabolic regulator (Hare and Cress, 1997; Shetty, 2004).

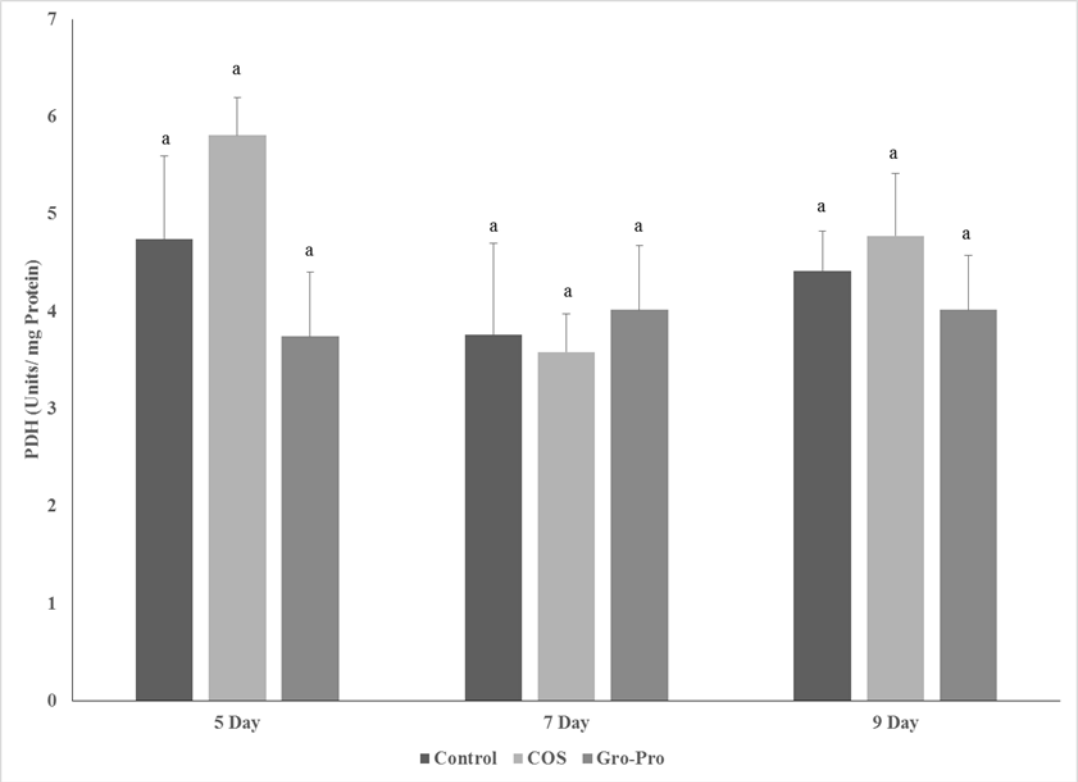

Figure 4.5. Proline dehydrogenase (PDH) activity (Units.mg^{-1} protein) in black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

CAT, GPX, and SOD Activity of Black Bean Sprouts: Activities of three important antioxidant pathway enzymes (catalase, guaiacol peroxidase, and superoxide dismutase) were

measured to determine the potential stimulation of antioxidant enzyme responses in black bean sprouts after post-seed priming treatments with elicitors. Catalase activity of black bean sprouts increased from day 5 to day 9 (Figure 4.6.). Black bean sprouts treated with COS and Gro-Pro showed a higher CAT activity at days 7 and 9, although only Gro-Pro treated ay day 7 showed statistically relevant improvements (Figure 4.6.). Higher CAT activity found in this study may potentially indicate peroxides are produced throughout sprouting, which the plant then in turn uses it through increase in the expression of CAT to manage them. Stimulation of CAT activity in black bean sprouts from COS and Gro-Pro treated seeds suggest that these seed priming treatments with elicitors could have enhanced antioxidant enzyme responses in these sprouts to counter cellular peroxidation, although this trend cannot be statistically validated from the results of this study.

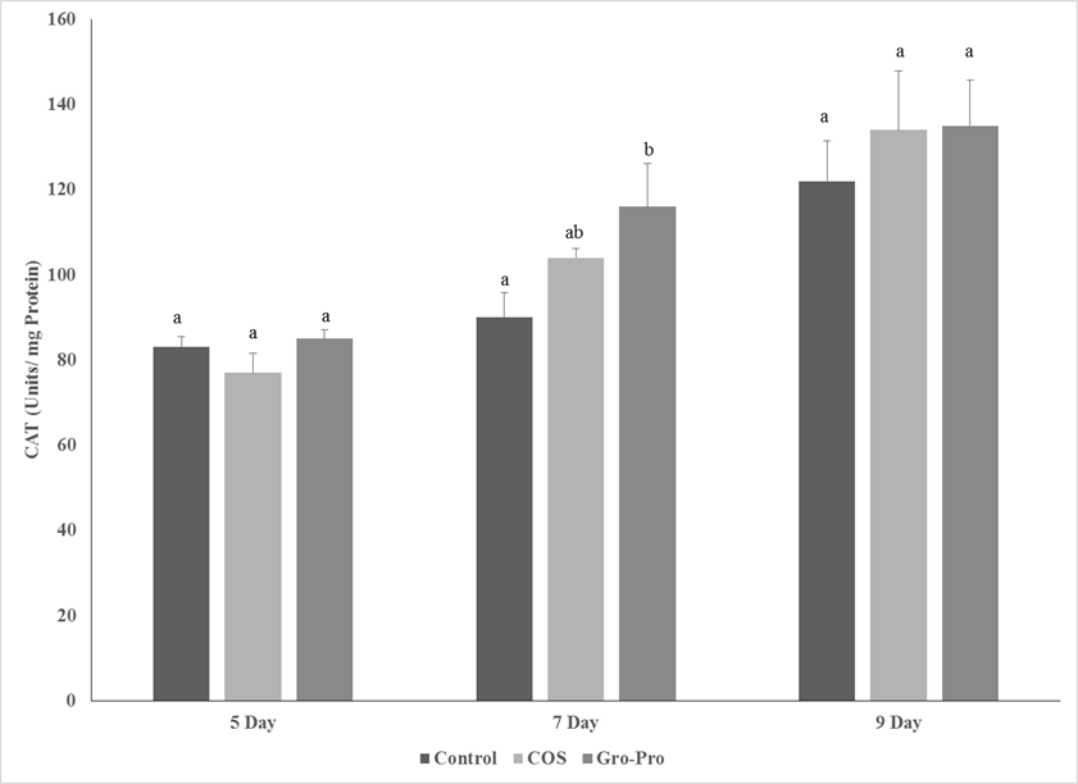

Figure 4.6. Catalase (CAT) activity (Units.mg^{-1} protein) in black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine

hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

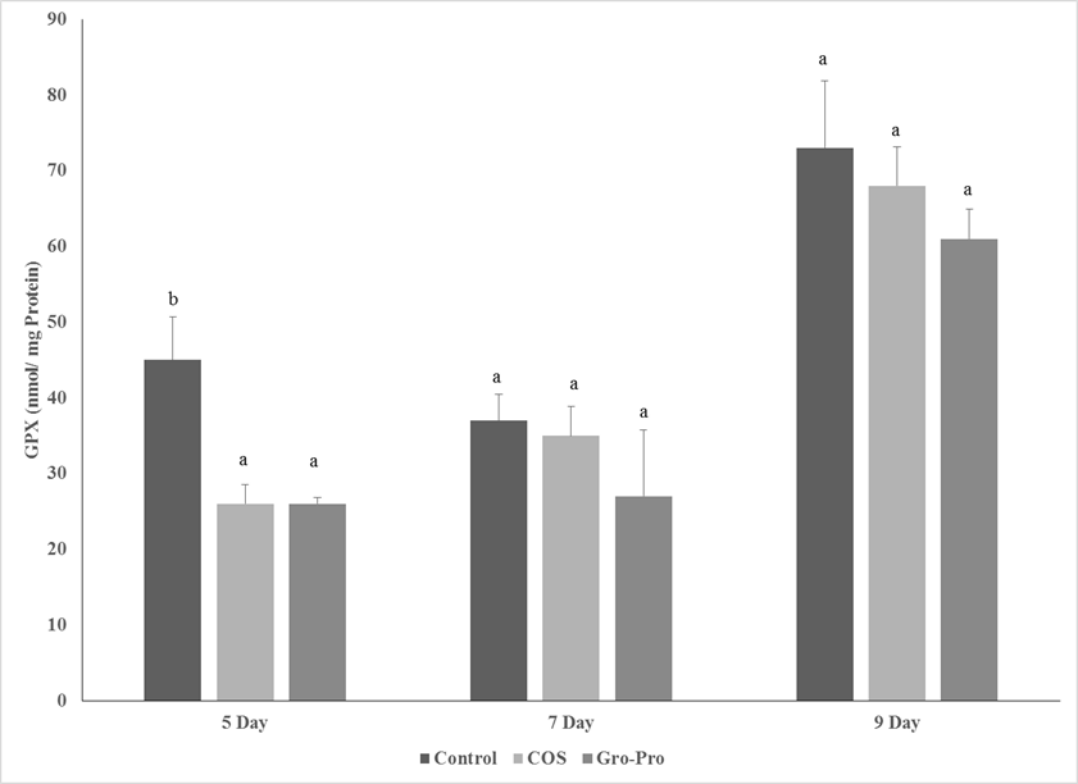

Figure 4.7. Guaiacol peroxidase (GPX) activity (nmol.mg^{-1} protein) in black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

The activity of another important enzyme, GPX, responsible for phenolic polymerization was measured to determine if elicitor treatments can improve phenolic polymerization that is connected to cellular osmotic adjustments. Similar to CAT activity, GPX activity also increased throughout sprouting progression (30 nmol/mg protein to 40 nmol/mg protein to 70 nmol/mg protein) (Figure 4.7.). The increased activity of GPX in the later stages of sprout development suggests that GPX may have a critical role in cellular structural adjustments before the plant begins photosynthesis. Surprisingly, seeds primed with elicitors exhibited an overall decreasing trend in GPX activity compared to control treatments, being statistically significant only at day 5

(Figure 4.7.). Although black bean sprouts from elicitor treated seeds may stimulate PPP and phenolic biosynthesis, the GPX data suggests that this response is not linked to the upregulation of phenolic polymerization (which is typically associated with cellular structural adjustments to stress).

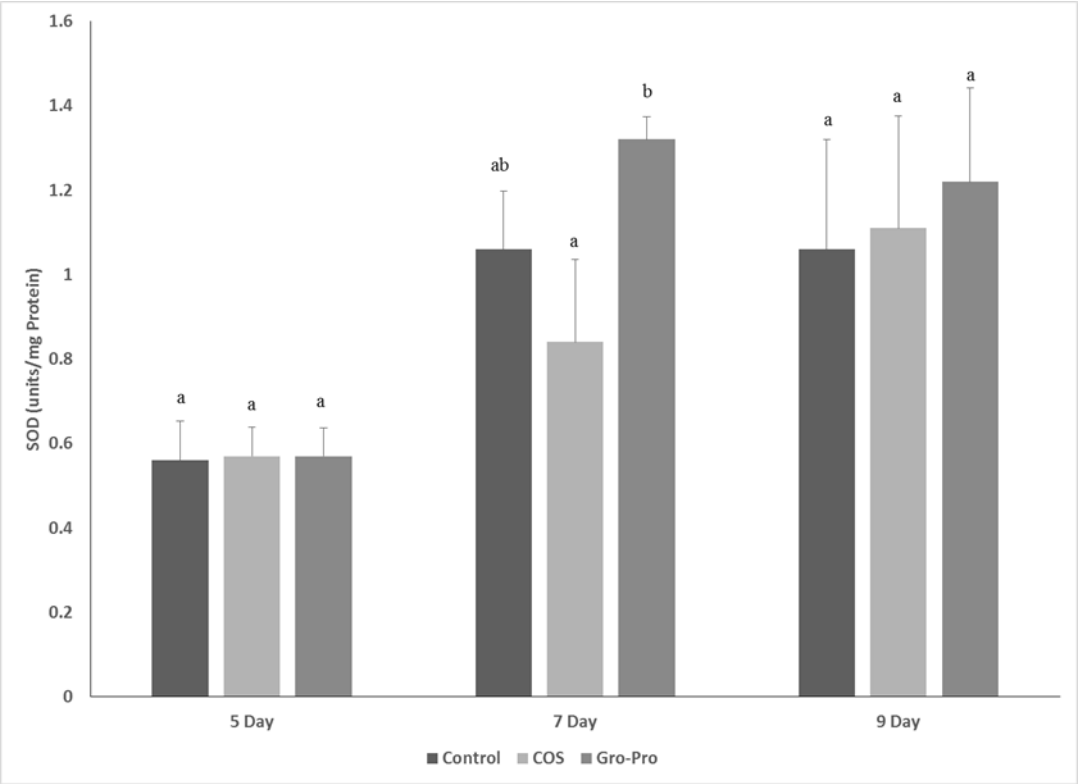

Figure 4.8. Superoxide dismutase (SOD) activity (Units.mg^{-1} protein) in black bean sprouts at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

Superoxide Dismutase (SOD) activity was determined in order to examine if overall antioxidant activity changes occurred throughout black bean sprouting and also in response to seed elicitors.. Similar to CAT and GPX activity, SOD activity also increased in black bean sprouts over days 5 to 9, showing an average increase from 0.55 units/mg protein (day 5) to 1.15 units/mg protein (day 9) (Figure 4.8.). No major difference in SOD activity was found among

elicitation treatments (Figure 4.8.). Black bean sprouts from Gro-Pro treated seeds indicated a minor increase in SOD activity at 7 and 9 days, but was not found to be statistically different than COS and control (Figure 4.8.). The general stimulation of both CAT and SOD activity in black bean sprouts after seed priming may reflect on the role of antioxidant enzyme defense responses in these sprouts to counter peroxidation and other oxidative stresses that occur during germination and sprouting.

Part II: Phenolic Antioxidant-Linked Functionalities Targeting Early Stages Type 2 Diabetes through In Vitro Assay Models

Current interest in legume sprouts is mainly due its nutritive value and its potential role as a part of daily diet as a health relevant functional food to compliment improved food balance for better nutrition and to potentially counter diet-linked chronic diseases. Thus in this study phenolic antioxidant-linked functionalities targeting dietary management of early stages type 2 diabetes was evaluated in black bean sprouts after seed priming treatments with elicitors. While the previous section (part one) of results showed that seed priming may modestly modify PPP-linked phenolic biosynthesis and antioxidant enzyme activities (CAT) in black bean sprouts, Part two of the results section examined if elicitation treatments have any effect on biosynthesis of the total phenolic content and antioxidant (using DPPH% inhibition), which then reflect in e bioactive potential of the consumed sprouts to mitigate oxidative damages within the digestive system. Secondly, the inhibitory effects on α–amylase and α-glucosidase using *in vitro* enzyme models was determined to evaluate if elicitor treatments had any significant effects on modulation of bioactives that have relevance for post-prandial glucose metabolism.

Total Soluble Phenolic Content and Total Antioxidant Activity (DPPH Inhibition) of Black Bean Sprouts: Although TSP content and total antioxidant activity (ABTS) was measured

previously, the extraction method for bioactive functionality used in part two was done in a higher concentration (10 g/ 25 mL distilled water) as well as containing the sprout seed coats that were removed by the initial extraction method. In addition, the DPPH antioxidant assay was selected to evaluate the higher total activities compared to the previous extraction. Measurement of TSP in new extraction is also essential to determine the correlation between TSP content and functionalities associated with targeting the inhibition of key enzymes in glucose digestion and absorption (such as α-amylase and α-glucosidase) using *in vitro* models which subsequently could reflect use in dietary consumption after animal and clinical studies.

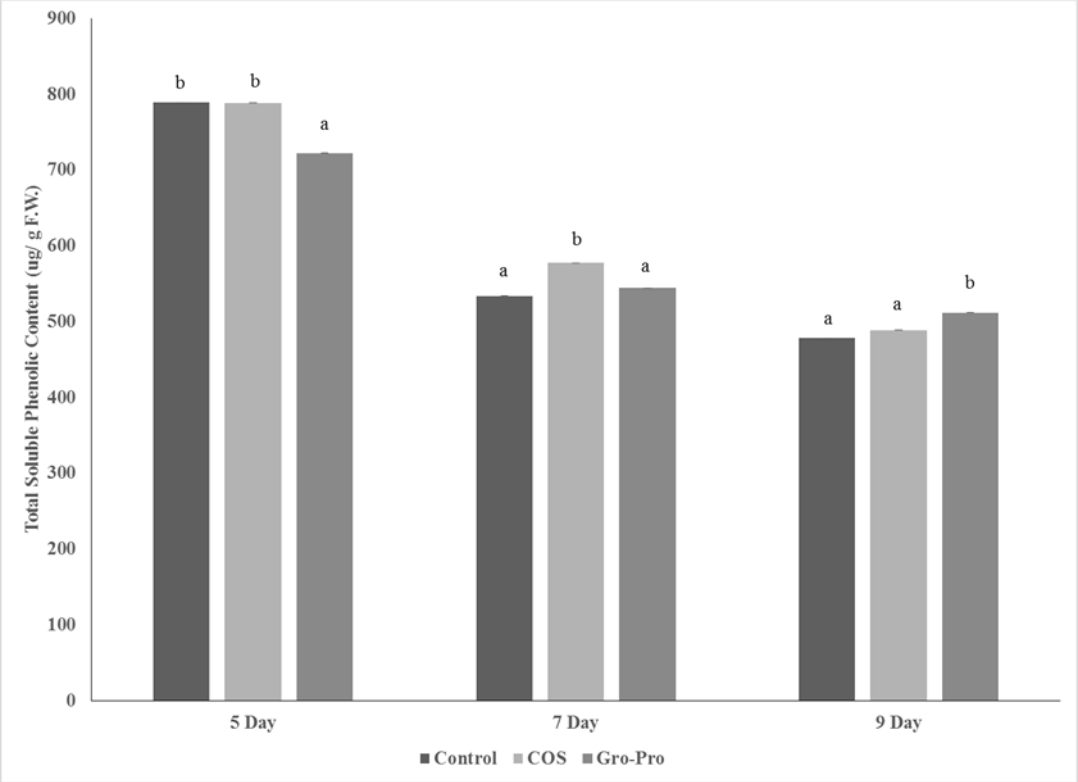

Figure 4.9. Total soluble phenolic content (µg/ g F.W.) of black bean sprouts (bioactive extraction) at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

In black bean sprout extraction for bioactive analysis, TSP content gradually decreased across all treatments with progression in days of sprouting, 700 mg/g, 525 mg/g, and 475 mg/g fresh weight, respectively (Figure 4.9.). Use of different extraction protocol and different concentration of sample might have contributed to such differences in findings compared to part 1. Significantly higher TSP content was found in black bean sprouts from seeds treated with COS at day 7 and Gro-Pro at day 9 (Figure 4.9.), suggesting that even though the overall TSP content may be decreasing throughout the sprouting process, elicitation treatments may be able to improve the overall content compared to control . This trend was similar to part 1 of the study where TSP content, although part 2 showed a stronger general trend and correlation as well as significant differences among elicitor treatments.

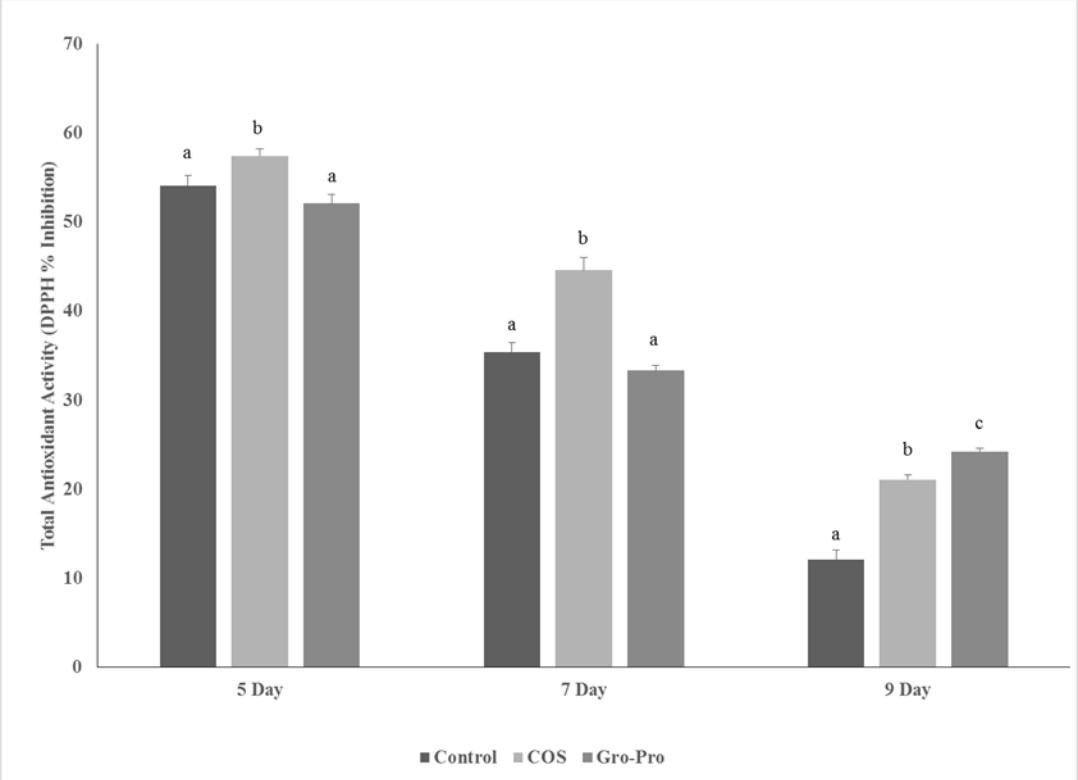

Figure 4.10. Total antioxidant activity (DPPH % inhibition.) of black bean sprouts (bioactive extraction) at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide

(COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

Total antioxidant activity of black bean sprouts was determined again using another radical scavenging (DPPH) assays. An improved DPPH % inhibition activity among treatments would suggest that either phenolic or enzyme linked biosynthesis has been upregulated through stress response to elicitation. As previously observed, the total antioxidant activity (as DPPH % inhibition) decreased over days 5 and 9 of sprouting from 55%, to 40% and 20% (Figure 4.10.). Interestingly, COS treated sprouts exhibited significantly higher antioxidant activity at all recorded stages of sprouting, with 10% higher total inhibitory activity at days 7 and 9 (Figure 4.10.). Gro-Pro did not result in any significant improvements to antioxidant activity until day 9, with a 15% improvement of total DPPH inhibitory activity compared to control (Figure 4.10.). Due to varying sensitivity of these radical scavenging assays depending on the concentration of the sample and the extraction method used in the study an additional ABTS assay method was used. Interestingly both ABTS and DPPH radical scavenging assays showed similar trends, even from two different extraction methods and concentrations (Figure 4.2. and 4.9.). These results have important relevance in functional food applications, as it suggests that both of the evaluated elicitors (COS and Gro-Pro) as seed treatments may potentially improve antioxidant activity in throughout black bean germination and sprouting stages, and this may be coupled to the enhanced mobilization of antioxidant enzymes observed previously. Similar improvement in antioxidant activity was observed in kidney beans after seed elicitation (Limon et al., 2014). Improvement in antioxidant activity in legume sprouts with fish protein hydrolysate treatment was also observed in previous research (Randhir and Shetty, 2003).

Alpha-amylase Inhibitory Activity of Black Bean Sprouts: To evaluate the relevance of phenolic antioxidants of black bean sprouts for potentially improving glucose metabolism, α-

amylase inhibitory activity was targeted using *in vitro* assay model. Pancreatic α-amylase is key enzyme responsible for digestion and breakdown of polysaccharides into mono-and di-saccharides and thus inhibition of this enzyme after pancreatic release helps in maintaining post-prandial blood glucose level (Hanhineva et al., 2010; McCue and Shetty, 2003). Many plant-based foods and food ingredients have been previously shown to have α –amylase inhibitory potential and are safe targets for use in the dietary management of early-stage type 2 diabetes (Kwon et al., 2007; Sarkar and Shetty, 2014).

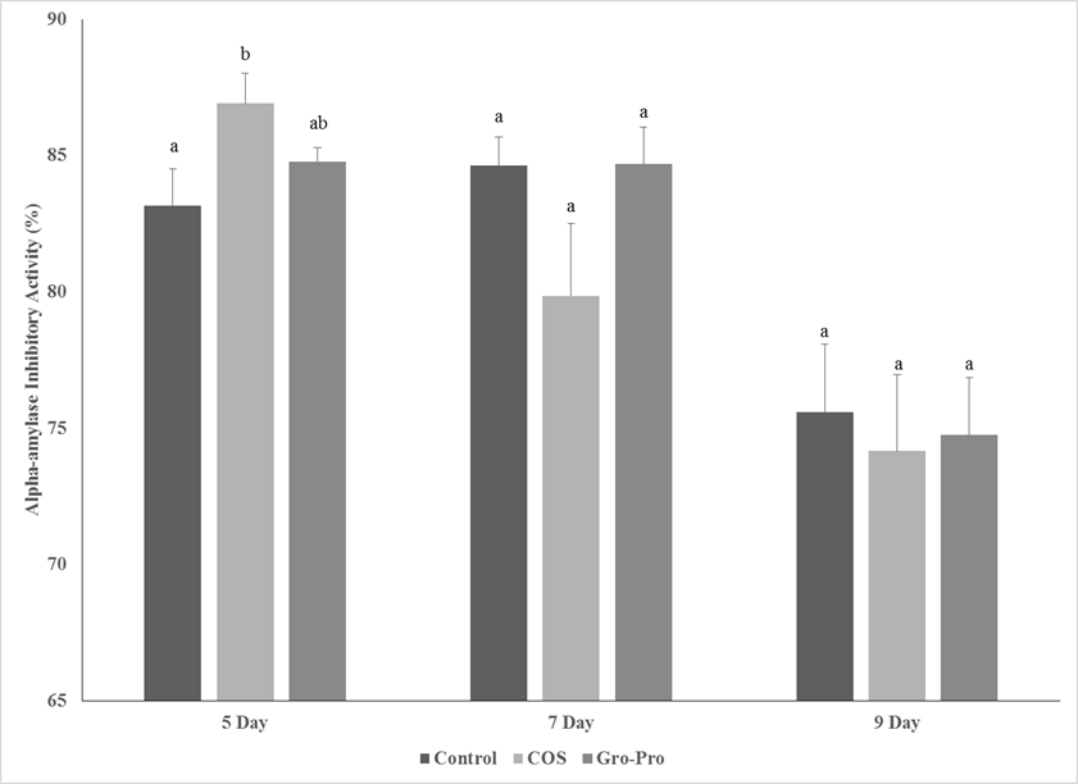

Figure 4.11. Alpha-amylase inhibitory activity (%) of undiluted black bean sprouts (bioactive extraction) at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

In this study, high α –amylase inhibitory activity (74-86% inhibition) was found in all black bean sprout extracts, decreasing slightly by day 7 (80% inhibition) and modestly by day 9

(73% inhibition) (Figure 4.11.). The α –amylase inhibitory activity of black bean sprouts exhibited a decreasing trend with sprouting stages when compared with TSP content and total antioxidant activity. Interestingly, seed priming elicitors treatments (COS and Gro-Pro) did not show any significant enhancement of α –amylase inhibitory activity besides in day 5 COS treated sprouts (Figure 4.11.). Therefore, seed elicitation treatments do not appear to have a significant enhancement of this functionality relevant to glucose management. Other genotypes of black bean and other edible dry beans need to be evaluated to confirm the wider trends and responses of this study.

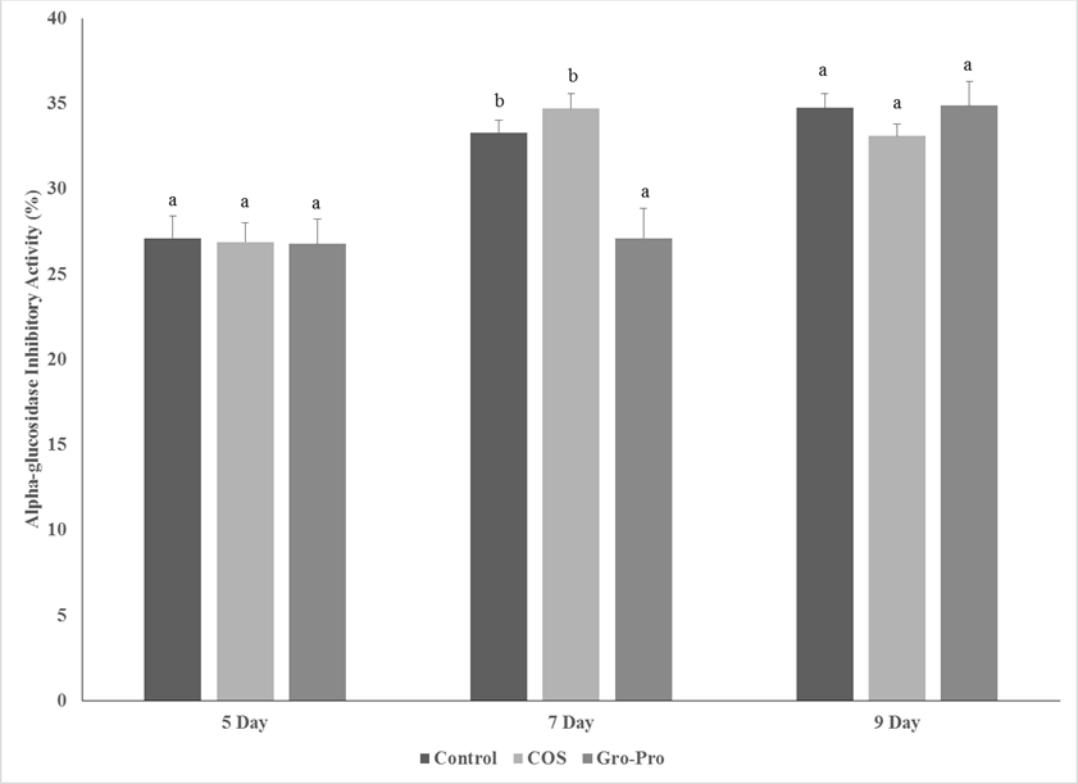

Figure 4.12. Alpha-glucosidase inhibitory activity (%) of undiluted black bean sprouts (bioactive extraction) at 5, 7, and 9 days post seed priming treatments with soluble chitosan oligosaccharide (COS) and marine hydrolysate (Gro-Pro). Different letters indicate significant differences between seed elicitation treatments within each time point at the $p<0.05$ level.

Alpha-glucosidase Inhibitory Activity of Black Bean Sprouts: Another important enzyme responsible for breakdown of polysaccharides and disaccharide and subsequent absorption of glucose in small intestine is α –glucosidase (McDougall et al., 2008). In this study black bean sprouts exhibited low overall α –glucosidase inhibitory activities while increasing modestly over sprouting stages (26% on day 5 up to 34% on day 9) (Figure 4.12.). This trend is contrary to the TSP content, total antioxidant activity, and α –amylase inhibitory activity. This may suggest that α –glucosidase inhibitory activity in black bean sprouts are likely not dependent on TSP content and biosynthesis and other factors (such as amino acid profiles or protein) may be responsible for the inhibitory activities. Similar to α–amylase, black bean seeds primed with seed elicitors also did not show any significant improvement in α–glucosidase inhibitory activity over time, and in fact it decreased at day 7 in Gro-Pro treated when compared to control (Figure 4.12.).

Conclusions

The above results suggest that overall seed priming with COS and Gro-Pro elicitors has relevance in enhancing total antioxidant activity and TSP content in Eclipse black bean sprouts. But, such improvements in antioxidant activity and TSP content did not correlate to improvements in the inhibitory activities of key enzymes of glucose metabolism linked to management of type 2 diabetes using *in vitro* models. Therefore, seed elicitation techniques developed in this study do have relevance for improving black bean bioactive profile relevant to chronic inflammation and cellular oxidative stress. Further other functionalities relevant to glucose and lipid metabolism can be targeted in future studies. Overall, the results of part 1 indicate that seed priming treatments stimulated phenolic biosynthesis through up-regulation of PPP, while Gro-Pro seed priming treatment improved total antioxidant activity and individual

antioxidant enzyme activity such as SOD in Eclipse black bean sprouts. The PPP mediated metabolic regulation in these sprouts may be dependent on the energy demands associated with respiration and through maintenance of critical balance between catabolic and anabolic needs. This study provides new insights on the critical metabolic regulation of black bean sprouts during germination and role of seed elicitation treatments for improving PPP mediated phenolic biosynthesis and associated antioxidant enzyme responses. Further studies with other genotypes of black bean, different legume species, and different doses of elicitor treatments are required to further validate this concept for wider application and use. This safe and easy to use technique of legume sprouting has significant merit to contribute to low cost processing technologies. This can be part of the solution to address global food security and public health challenges through improvement of accessibility and availability of vegetable protein, minerals, fiber, and other bioactive to the wider global population in food deserts where dried legume and other nutritionally enriched seeds can be transported and locally sprouted in any season to enhance diet and health relevance.

CHAPTER 5. IMPROVEMENT OF SALINITY RESILIENCE IN BLACK BEANS THROUGH STIMULATION OF ANTIOXIDANT-LINKED ENDOGENOUS DEFENSE RESPONSES WITH SEED ELICITATION TREATMENTS

Abstract

Plants adaptive response to abiotic stress, including salinity stress, in part involves stimulation of the biosynthesis of secondary metabolites and induction of endogenous antioxidant enzyme responses. This is regulated through the up-regulation of critical defense related anabolic pathway, such as pentose phosphate pathway (PPP). We have targeted seed elicitation strategy to enhance such phenolic and antioxidant-linked defense responses in black bean to improve salinity stress resilience. Therefore, the primary aim of this study was to evaluate the effects of two seed elicitor treatments for the potential improvement of salinity resilience in two black bean genotypes through up-regulation of PPP. In this study, Eclipse and Zenith black bean genotypes were selected and subjected to seed elicitor treatments (chitosan oligosaccharide-COS and marine hydrolysate-Gro-Pro) and then grown under three levels of soil salinity (no salt stress, 2-3 ds/M, and 5-6 ds/M) in greenhouse condition. Black bean leaf samples were collected biweekly to conduct biochemical and enzyme assays. No black bean plants survived high salt stress (5-6 ds/M) more than 55 days post-germination. Both black bean genotypes demonstrated contrasting adaptive responses under salinity stress. While Eclipse showed evidence of the stimulation of phenolic biosynthesis through up-regulation of PPP, Zenith exhibited higher reliance on stimulating endogenous antioxidant enzyme responses (catalase, guaiacol peroxidase, and superoxide dismutase) to counter salinity stress. Among seed

elicitors, Gro-Pro showed modest improvements in total soluble phenolic content (60 day) as well as high glucose-6-phosphate dehydrogenase activity (45 day) in Eclipse, while in Zenith, Gro-Pro stimulated antioxidant enzyme activity, particularly during later growth stages (45 and 60 days).

Introduction

Soil salinization is one of the most serious threats to the current global agricultural paradigm, and is currently affecting significant areas of cultivated and arable land resources worldwide, typically combined with drought conditions (Ladeiro, 2012). Salinization is most widely impacting arid and semi-arid regions, particularly from excessive irrigation- induced secondary salinization from human-related activities (Qadir et al., 2000; Yeo, 1998). Around 50% of global arable land will face some degree of salinization by 2050, and with each year there is a deterioration of an additional two million Hectares of global-arable land due to soil salinization (Parida and Das, 2005; Wang et al., 2004; Yadav et al., 2011). During the same period the human population is expected to rise and will consequently increase global demand for food production, while arable land is rapidly declining due to climate change pressure coupled with such breakdowns as salinity (Lobell and Gourdji, 2012; Yeo, 1998). Only less than 10% of global arable lands are now free from any kind of environmental stresses, and will likely decline further if the projections of increasing irrigation demand and climate change-linked environmental transformations occur (Vadez et al., 2012). The present trend of carbon-dioxide increase in atmosphere and subsequent changes in mean temperature and rainfall is going to affect the aggregate productivity of grains, legumes, and other important food crops globally (Lobell and Gourdji, 2012; Vadez et al., 2012). Due to such an erratic and unpredictable climate,

in conjunction with limited soil and water resources, farmers are facing increasing challenges to grow crops and to maintain high productivity (Lobell and Gourdji, 2012; Parry et al., 2004).

Soil salinity is defined as the presence of excessive concentrations of salts within the soil that surpasses a plants threshold to counter its higher concentration in the root zones (Rengasamy, 2010). The major cations common in saline soils are sodium, calcium, and magnesium, while the major anions are typically chloride, sulfate and carbonate. The concentration and combination of various types of such salts can greatly diversify the impacts of overall soil salinity to plants (Qadir et al., 2000). Excessive salt accumulation in top soils is a major causal factor attributed to declining agricultural productivity by seriously impeding growth, development, and productivity of food crops (Ahuja et al., 2010; De Azevedo Neto et al., 2006; Parida and Das, 2005).

Salt stress affects all the major physiological and metabolic processes in plants such as growth, photosynthesis, protein synthesis, energy and lipid metabolism (Parida and Das, 2005). When subjected to increased levels of salt, plants indicate hyperionic and hyperosmotic effects that can cause membrane disintegration, cellular toxicity and accumulation of reactive oxygen species (ROS) in cells (Mittler, 2002). To counter the multifactorial nature of salinity stress, plants have evolved intricate biochemical and molecular defense responses including: a) selective accumulation of ions, b) regulation of ion uptake by roots c) synthesis of compatible osmolytes such as proline, glycine betaine, sugars and polyols, d) change in photosynthetic pathway, e) alteration of membrane structure and f) stimulation of antioxidant enzymes (Parida and Das, 2005). Increased accumulation of reactive oxygen species is one of the consequences of osmotic imbalance and mitochondrial dysfunction in plants due to salinity stress (Apel and Hirt, 2004; Mittler, 2002). Many of the osmolytes and stress proteins with unknown functions likely

detoxify plants by scavenging ROS and prevent oxidative stress from damaging cellular structures (Zhu, 2001). The plants defend against these reactive oxygen species by induction of activities of certain antioxidante enzymes such as catalase, peroxidase, glutathione reductase, and superoxide dismutase, which scavenge ROS (Parida and Das, 2004). While this growth suppression due to abiotic stresses occurs in most plants, the tolerance levels and reduction of growth varies greatly among food crops and among their genotypes (Hasegawa et al., 2000).

Under abiotic stresses, including salinity stress plants undergo significant metabolic adjustments and biochemical changes including accumulation of amino acids (especially proline), synthesis of secondary metabolites (such as phenolics), and up-regulation of defense related pathways (PPP, and shikimate pathway) (Shetty and Wahlqvist, 2004). Synthesis of proline in the cytosol is potentially associated with PPP through NADP/NADPH regulation which drives this pathway to provide chemical and structural protection to plants under abiotic stresses (Shetty and Wahlqvist, 2004). In coupling of the PPP, proline plays a key active role in this metabolic regulation as under oxidative stresses and it potentially supports mitochondrial oxidative phosphorylation to generate energy, while allowing anabolic biosynthesis to continue through PPP (Shetty and Wahlqvist, 2004). This proline-associated pentose phosphate pathway (PAPPP) regulation improves protective defense responses in plants including secondary metabolite synthesis, stimulating antioxidant enzyme responses, supporting plants anabolic needs, and providing energy (ATP) synthesis by diverting carbon flux from energy intensive pathways (Hare and Cress, 1997; Shetty and Wahlqvist, 2004). Therefore in effect PAPPP potentially serves as the critical system based metabolic control to improve plant fitness against abiotic stresses including salinity.

While a substantial body of evidence exists illustrating plant responses to salinity, little practical and applicable knowledge demonstrates the impact of climate change on food crop production. With climate change already beginning to alter temperate zones and the salinization of arable soils, new strategies designed to target salinity tolerance and resilience in nutritive food crops are essential for the continuation of a sustainable and high-quality food production systems (Lobell and Gourdji, 2012; Sarkar and Shetty, 2014b). One such strategy is the induction of systemic defense responses by elicitors to counter abiotic stresses, which potentially involves energy efficient metabolic regulation such as up regulation of PPP (Shetty and Wahlqvist, 2004). Thus use of external elicitors that can improve oxidation-linked metabolic adjustment through system-based critical pathways can be targeted to improve overall salinity resilience by directly mobilizing the protective bioactive metabolites during early growth stages, when plants are more susceptible to abiotic stresses. This strategy concurrently could also induce salinity stress tolerance in targeted food crops by stimulating endogenous antioxidant defense responses. Previous research in our laboratory in controlled environments has shown improved seed vigor, enhancement of phenolic content and overall stimulation of antioxidant enzyme response in seedlings due to exogenous application of elicitors such as chitosan oligosaccharide and fish protein hydrolysate (Randhir and Shetty, 2006, Sarkar et al. 2010). Therefore, the major aim of this study was to enhance PPP mediated phenolic biosynthesis and antioxidant enzyme responses in salt susceptible black bean genotypes through seed elicitation treatments and subsequently improving their salinity stress resilience.

Materials and Methods

Plant Material: Eclipse, an early maturing and high yielding black bean genotype released in 2004, and Zenith, an upright and full-season black bean genotype released in 2014

were selected for this study. Eclipse was selected for being one of the best adapted black bean genotypes in the Northern Plains and currently accounts for 90% of total black bean acreage (Osorno et. al., 2009). Zenith was selected for its improved resistance to certain biotic stress compared to Eclipse (Kelly et.al. 2014). Eclipse and Zenith black bean samples were collected from the 2015 fall harvest from the NDSU bean trial.

Analytical Material: All chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Waltham, MA), all being of analytical grade.

Elicitors: Two elicitors were used as seed treatment for this study: soluble chitosan oligosaccharide- vitamin C (COS-C), derived from marine chitin obtained from Kong Poong Bio, Jeju Korea and marine peptide hydrolysate (Gro-Pro), derived from seaweed and marine fish extracts and obtained from Icelandic Bioenhancer, Iceland. The elicitors and subsequent concentrations were selected based on previous studies that indicated significant elicitation of PPP at the selected concentrations in multiple crops, as well as the dose being practical for application in conventional agriculture (Sarkar et al., 2010). COS was investigated using a 1% solution (1 g COS-C /1 L distilled water) and Gro-Pro as well in a 1% solution (1 mL Gro-Pro /1 L distilled water) in order to replicate conditions from chapter 4. Solution was prepared ahead of time prior to seed incubation. Elicitor solutions were stored for up to two weeks in the refrigerator at 0-4℃. All solutions were brought to room temperature prior to seed incubation.

Seed Treatments: A similar seed treatment incubation strategy as stated in chapter 4 was used for this study. The ratio of 100 seeds (30 g) to 200 mL elicitor stock solutions (mL) was used for both genotypes. Seeds (30 g) were weighed and surface sterilized by being soaked in a 0.5% hypochlorite solution for two minutes. Soaked beans were then drained and rinsed using a colander several times to remove any remaining 0.5% hypochlorite solution. Washed seeds were

then added to previously prepared treatment solutions in separate 250 mL beakers, with Para film used to lightly seal each beaker. Each beaker was placed into a ThermoShaker for eight hours using the setting of 180 RPM at 23℃.

Preparation of Saline Soil Treatments: Four salts were selected for this study in order to simulate salts common in the Northern Plains region: sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), sodium sulfate ($Na2SO_4$), and calcium sulfate ($CaSO_4$) (Franzen, 2007). Electrical Conductivity (Ec) was used to determine salt concentrations within the soil through use of an EcProbe. Using the aforementioned salts, three levels of soil salinity was investigated: Control with no salt added (Ec: 0.5-1.0 ds/M), mild salinity stress (Ec: 2.0-3.0 ds/M), and high salinity stress (Ec: 5.0-6.0 ds/M). Each salt was measured (g) according to salt stock calculations to produce 12 liters of 0.035M (mild salinity stress) and 0.1 0M (moderate salinity stress) stock solutions, with each salt used in equal molar concentrations. Using a separate Sterlite plastic bin for each saline treatment, professional grade potting mix was combined with the previously prepared 12 liters of stock salt solution, mixed thoroughly, sealed, and allowed to equilibrate for 12 hours. Initial Ec baseline measurement was then taken to insure it was within specifications. Soil was then transferred to individual, pre-labeled, six inch-wide standard greenhouse pots. The fertilizer 'Osmocote 15-9-12 (3-4 Month)' was added at a ratio of 1.5 teaspoons per individual pot and mixed thoroughly.

Germination and Transplanting: Two starter trays (1.5in x 2in x 1in dimensions, each holding 24 individual beans) were prepared for each elicitor treatment prior to sowing. All starter trays were filled with professional-grade potting mix and soaked with distilled water until field capacity was achieved, and then allowed to equilibrate 6-8 hours prior to sowing of treated beans. After 8 hour incubation, treated beans were drained and transferred to starter trays in the

greenhouse using commonly accepted dry bean planting practices. Beans were allowed to germinate and grow until they reached the two-leaf stage of plant development. Individual bean seedlings were then directly transferred to saline treated pots, with a total of 5 separate replications (pots) for each elicitor/saline soil treatment combination. After transplanting, 100 mL of saline solution was added near the bean roots of each individual plant to bring Ec into specifications. As stated in chapter 4, all experimental groups were subjected to identical conditions in order to control extraneous variables. A randomized complete block design (RCBD) was selected using a split-split plot arrangement. Genotypes was the main plot, soil salinity level is the sub plot, and elicitor treatment as the sub-sub plot.

Greenhouse Growing Conditions: Greenhouse temperature was kept constant between 25-30℃. A photoperiod split of 14 hours light and 10 hours dark was selected for optimal bean growth and development. Beneficial nematodes were used to minimize various forms of common biotic greenhouse pests and were applied every week. Insecticide applications were additionally applied twice a week, with insecticide selection and application discretion given to the greenhouse management staff. Due to overly saline soils, a water filtration system was attached to the greenhouse water supply to minimize the effect of unaccounted dissolved salts that are common in North Dakotan public water systems.

Leaf Tissue Sampling: Bean sprouts were sampled seven days post-transplanting to saline treatment pots, corresponding to plant development at the two-leaf growth stage. Additional sampling occurred in 15-days intervals continually for a total of 3 observations taken throughout lifecycle, prior to the pod filling stage. Two leaves were selected from an individual plant (experimental unit) and replicated in triplicates for each treatment. Each sample was sealed

with tin foil and immediately submerged into liquid nitrogen for enzyme preservation during the move to the laboratory from greenhouse to conduct biochemical assays.

Enzyme Extraction: Black bean leaf tissue (200 mg) were collected and thoroughly macerated by using a cold pestle and mortar with 2 mL cold enzyme extraction buffer [0.5% polyvinylpyrrolidone (PVP), 3 mmol L^{-1} EDTA, and 0.1 mol L^{-1} potassium phosphate buffer of pH 7.5]. The extracted sample was centrifuged at 15,300 RPM for 10 min at 2-5 °C and stored in ice. Supernatant was used for total protein and enzyme analysis.

***Extraction Procedure to Determine Total Soluble Phenolic Content and Total Antioxidant Activity*:** A quantity of 100 mg (fresh weight-FW) black bean leaf tissue was immersed in 5 mL of 95% ethanol and kept in the freezer for 72-h. After 72-h, the sample was homogenized using a Tissue Tearer (VWR Model 200, Randor, PA) and centrifuged at 13,500 RPM for 10 min in a 2ml Eppendorf centrifugation tube. Samples were kept in ice concurrently with analytical analysis to avoid degradation.

Total Soluble Phenolic Assay: The total soluble phenolic content was determined by the Folin–Ciocalteu method which was based on a method modified by Shetty et al. (1995). Briefly, 0.5 mL of the sample extract and 0.5mL of distilled water were transferred into a test tube and mixed with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50% (vol/vol) Folin–Ciocalteu reagent was added and vortexed. After 5 min, 1 mL of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The standard curve was established using various concentrations of gallic acid in 95% ethanol, and results were expressed as mg of gallic acid per µg of sample in fresh weight (FW).

***ABTS Inhibition Assay*:** The total antioxidant activity of black bean leaf tissue extract was measured by the $ABTS^+$ radical cation-decolorization assay involving preformed $ABTS^+$

radical cation (Re et al., 1999). ABTS (Sigma Chemical Co, St. Louis, MO) was dissolved in water to a 7 mmol L^{-1} concentration. $ABTS^{+}$ radical cation was prepared by reacting 5 mL of 7 mmol L^{-1} ABTS stock solution with 88 µL of 140 mmol L^{-1} potassium persulphate, and mixture was allowed to stand in the dark at room temperature for 12-16 h prior to use. Prior to assay $ABTS^{+}$ stock solution was diluted with 95% ethanol (ratio 1:88) to give an absorbance at 734 nm of 0.70 ± 0.02, and was equilibrated to 30 °C . One milliliter ABTS was added to glass test tubes containing 50 µL of each tissue extract, and mixed by vortex mixer for 30 s. After 2.5 min incubation, mixtures were read at 734 nm. The readings were compared with controls, which contained 50 µL of 95% ethanol instead of the extract. The Trolox reference standard for relative antioxidant activities was prepared with 5 mmol L^{-1} stock solution of Trolox in ethanol for introduction into the assay system at concentrations within the activity range of the assay (0-20 µmol L^{-1} final concentration) for preparing a standard curve to which all data were referenced. The percent inhibition was calculated by:

$$\% \text{ inhibition} = \left(\frac{[A_{734}^{\text{control}} - A_{734}^{\text{extract}}]}{[A_{734}^{\text{control}}]}\right) \times 100$$

Total Protein Assay: Protein content was determined by the method of Bradford assay (Bradford, 1976). One parts of dye reagent (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted with 4 parts of distilled water. A volume of 5 mL of diluted dye reagent was added to 50 µL of the black bean leaf extract. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a blank (5 mL reagent and 50 µL buffer solution) by using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

Glucose-6-phosphate dehydrogenase (G6PDH) Activity: A modified method originally described by Deutsch (1983) was used. The enzyme reaction mixture containing 5.88 µmol L^{-1} ß-

NADP, 88.5 µmol L^{-1} magnesium chloride ($MgCl_2$), 53.7 µmol L^{-1} glucose-6-phosphate, and 0.77 mmol L^{-1} maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 mL of this mixture, 100 µL of the extracted enzyme sample was added. The rate of change in absorbance per minute was used to quantify the enzyme activity in the mixture with the help of the extinction co-efficient of NADPH (6.22 mmol L^{-1} cm^{-1}) and expressed as nanomoles per milligram protein.

Succinate Dehydrogenase (SDH) Activity: To assay the activity of SDH a modified method of Bregman (1987) was used. The assay mixture containing 1.0 mL of 0.4 mol L^{-1} potassium phosphate buffer (pH 7.2), 40 µL of 0.15 mol L^{-1} sodium succinate (pH 7.0), 40 µL of 0.2 mol L^{-1} 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 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 minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP (19.1 mmol L^{-1} cm^{-1}).

Proline Dehydrogenase (PDH) Activity: A modified method described by Costilow and Cooper (1978) was carried out to assay the activity of PDH. The enzyme reaction mixture containing 100 mmol L^{-1} sodium carbonate buffer (pH 10.3), 20 mmol L^{-1} L-proline solution and 10 mmol L^{-1} mM nicotinamide adenine dinucleotide (NAD) was used. To 1 mL of this reaction mixture, 200 µL of extracted enzyme sample was added. The increase in absorbance was measured at 340 nm for 3 min, at 32 °C. 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).

Catalase (CAT) Activity: The CAT activity was assayed according to the methods of Beers and Sizer (1952). To 1.9 mL of distilled water 1 mL of 0.059 mol L^{-1} hydrogen peroxide (Merck's Superoxol or equivalent grade, Merck Co. & Inc., Whitehouse Station, NJ) in 0.05 mol L^{-1} potassium phosphate, pH 7.0 was added. This mixture was incubated in a spectrophotometer for 4-5 min to achieve temperature equilibration and to establish blank rate. To this mixture 0.1 mL of diluted enzyme sample was added and the disappearance of peroxide was followed spectrophotometrically by recording the decrease in absorbance at 240 nm for 2-3 min. The change in absorbance ΔA_{240}/min from the initial (45 s) linear portion of the curve was calculated. One unit of CAT activity was defined as amount that decomposes 1 micromole of H_2O_2.

Guaiacol Peroxidase (GPX) Activity: The activity of GPX was assayed by a modified method (Laloue et al., 1997). The enzyme reaction mixture containing 0.1 mol L^{-1} potassium phosphate buffer (pH 6.8), 56 mmol L^{-1} guaiacol solution, and 50 mmol L^{-1} hydrogen peroxide (H_2O_2) was used. To 990 µL of this reaction mixture, 10 µL of enzyme sample was added. The absorbance was recorded at zero time and then after 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture by using the extinction coefficient of the oxidized product tetraguaiacol (26.6 mmol L^{-1} cm^1).

Superoxide Dismutase (SOD) Activity: A competitive inhibition assay was performed that used xanthine-xanthine oxidase generated superoxide to reduce nitroblue tetrazolium (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 mmol L^{-1} potassium phosphate buffer (pH 7.8) containing 1.33 mmol L^{-1} diethylenetetraaminepentaacetic acid (DETAPAC); 0.5 mL of 2.45 mmol L^{-1} NBT; 1.7 mL of 1.8 mmol L^{-1} xanthine and 40 IU/mL catalase. To 0.8 mL of reagent mixture 100 µL of phosphate

buffer and 100 µL of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 s for 2 min and the concentration of xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme sample and the change in absorbance was monitored every 20 s for 2 min. One unit of SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum. Extracted samples were diluted (20 µL sample + 980 µL 50 mmol L^{-1} potassium phosphate buffer (pH 7.8)) prior to the assay to allow for measurements within readable spectrum.

High Performance Liquid Chromatography (HPLC): The sample extracts (1 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample was injected using an Agilent ALS 1100 autosampler into an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 - 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 306 nm and 333 nm and the chromatogram was integrated using Agilent Chem station enhanced integrator. Pure standards of chlorogenic acid, gallic acid, ellagic acid and quercetin in 100% methanol were used to calibrate the standard curves and retention time.

Statistical Analysis: The experiment was carried out according to the split-split design with genotype as main plot, salinity level as sub plot, and elicitation treatment as sub-sub plot. Data analysis was run in triplicates per treatment group and per data point. Sample from three

replications per treatment were collected and one individual run of extraction was conducted for each black bean genotype. Each individual pot was used as an experimental unit. The mixed procedure analysis was conducted using statistical analytical software (SAS) 9.3 version and considering main plot, sub plot, and sub-sub plot as fixed effect and replication as random. Differences among all main factors and their respective interactions were determined using Tukey least square means at $p<0.05$ and at $p<0.001$ levels.

Results and Discussion

Visual Observation on Growth and Survival of Black Bean under Salt Stress: Overall, both black bean genotypes showed high susceptibility to salt stress. Stunted growth, drooping, and foliar desiccation were observed under high salt stress (5-6 ds/M) in both genotypes (Figure 5.1.).

Figure 5.1. Zenith under three salinity levels (no salt stress-control, mild salt stress-2-3 ds/M, and high salt stress 5-6 ds/M) one month after germination.

Figure 5.2. Comparison of three seed elicitation treatments in black bean (Eclipse & Zenith) under high salt stress (5-6 ds/M EC) three weeks after germination.

Both black bean genotypes did not survive under high salt stress (5-6 ds/M) after 60 days from germination. Even under mild salt stress (2-3 ds/M) a significant reduction of growth was observed, although leaf and shoot tissues of black bean plant did not show any other significant

damages. Among elicitation treatments across genotypes, better growth and improved leaf coloration was found with COS, followed by Gro-Pro seed treatments (Figure 5.2.). While most of the untreated and Gro-Pro treated black bean plants did not survive after 50 days under high salt stress, plants treated with COS survived extra 10 days (60 days). Overall, Eclipse showed higher vigor and growth (number of leaves, height, and branches) when compared to Zenith across all salinity levels.

Seed Weight: The average seed weight of black bean plants was measured to determine the effect of seed elicitation and salt stress treatment combinations on the yield after harvest. A reduction in the average seed weight was observed with salt stress across genotypes and elicitation treatments, although this effect was more pronounced in Zenith. Under mild salt stress, Eclipse had a significantly higher seed weight when compared to Zenith (Table 5.1.). COS exhibited a modestly higher average seed weight per plant among all seed elicitation treatments, with the only exception in Zenith under mild salt stress where Gro-Pro exhibited higher seed weight.

Table 5.1. Average seed weight (g) per plant of Eclipse and Zenith (post-harvest) with three seed elicitation treatments (control, COS, and Gro-Pro) under two salinity levels (no salt stress, and 2-3 ds/M).

Elicitation Treatments	Salinity Levels			
	No Salt Stress		Mild Salt Stress (2-3 ds/M)	
	Eclipse	Zenith	Eclipse	Zenith
Control	27.35	26.55	20.55	6.376
COS	29.9	27.01	21.43	8.578
Gro-Pro	28.95	22.82	19.15	12.94

*No black bean plant survived under high salt stress, and thus seed weight data could not be included.

Total Soluble Phenolic Content and Total Antioxidant Activity (ABTS): Plants have evolved dynamic endogenous defense system to counter deleterious effects induced by environmental stresses. One such adaptive response of plant against abiotic stresses is stimulation of biosynthesis of protective secondary metabolites such as phenolics and associated antioxidant enzyme responses to maintain cellular redox balance (Bartwal et al., 2013). Total soluble phenolic content was measured using Folin-Ciocalteu method to determine salinity stress induced secondary metabolite biosynthesis in black bean after seed elicitation. Overall, total soluble phenolic content increased with the maturity of black bean plants from day 30 to day 60 (Figure 5.3.).

Table 5.2. Analysis of variance (ANOVA) table indicating significant differences between sample/genotype (main plot), salinity levels (sub plot), elicitation treatments (sub-sub plot) and their respective interactions for all biochemical parameters associated with plants defense response involving pentose phosphate pathway (PPP) mediated secondary metabolite biosynthesis and antioxidant enzyme responses in black beans at 30 (A), 45 (B), and 60 (C) days.

(A)

	df	Total Soluble Phenolics	Total Antioxidant Activity	G6PDH	SDH	PDH	CAT	GPX	SOD
Sample/genotype	1	**	**	ns	*	*	ns	*	*
Salinity Levels	2	*	*	ns	ns	*	**	**	*
Sample × Salinity	2	ns	ns	*	**	ns	**	*	ns
Treatments	2	ns	ns	ns	ns	ns	ns	ns	*
Sample × Treatment	2	ns	ns	ns	ns	ns	ns	*	ns
Salinity × Treatment	4	ns	ns	ns	ns	ns	ns	ns	ns
Sample × Salinity × Treatment	4	ns	ns	ns	ns	ns	ns	ns	ns

Table 5.2. Analysis of variance (ANOVA) table indicating significant differences between sample/genotype (main plot), salinity levels (sub plot), elicitation treatments (sub-sub plot) and their respective interactions for all biochemical parameters associated with plants defense response involving pentose phosphate pathway (PPP) mediated secondary metabolite biosynthesis and antioxidant enzyme responses in black beans at 30 (A), 45 (B), and 60 (C) days (continued).

(B)

	df	Total Soluble Phenolics	Total Antioxidant Activity	G6PDH	SDH	PDH	CAT	GPX	SOD
Sample/genotype	1	**	**	**	ns	**	*	*	ns
Salinity Levels	2	**	**	ns	*	**	**	**	*
Sample × Salinity	2	**	**	*	*	**	**	**	ns
Treatments	2	ns	ns	ns	ns	ns	*	ns	ns
Sample × Treatment	2	ns	ns	*	ns	ns	*	ns	ns
Salinity × Treatment	4	ns	ns	ns	ns	ns	*	ns	ns
Sample × Salinity × Treatment	4	ns	ns	ns	ns	ns	**	ns	ns

(C)

	df	Total Soluble Phenolics	Total Antioxidant Activity	G6PDH	SDH	PDH	CAT	GPX	SOD
Sample/genotype	1	**	**	**	**	**	**	**	*
Salinity Levels	2	ns	ns	**	ns	ns	*	*	ns
Sample × Salinity	2	ns	*	*	ns	ns	*	*	ns
Treatments	2	*	ns	ns	ns	ns	ns	ns	ns
Sample × Treatment	2	ns	ns	ns	ns	ns	*	ns	ns
Salinity × Treatment	4	ns	ns	ns	**	*	ns	ns	ns
Sample × Salinity × Treatment	4	ns	ns	ns	ns	ns	ns	ns	ns

*Significance at $p<0.05$

**Significance at $p<0.001$

ns Non significant

Significant differences in total soluble phenolic content among genotypes ($p<0.001$) and salinity levels ($p<0.05$) were observed at day 30 (Table 5.1. A). While at day 45 total soluble

phenolic content between sample/genotype ($p<0.001$), salinity levels ($p<0.001$), and sample × salinity interactions ($p<0.001$) showed significant differences (Table 5.1. B). Similarly, at day 60 significant differences on total soluble phenolic content was observed between sample/genotype ($p<0.001$), and genotype/sample × salinity interactions ($p<0.05$) (Table 5.1. C).

Black bean plants under mild and higher salt stress (2-3ds/M & 5-6 ds/M) also showed significantly higher soluble phenolic content compared to the no salt stressed plants at day 30 ($p<0.05$) and day 45 ($p<0.001$). But at day 60 higher level of salinity did not result in any further increment of total soluble phenolic content when compared to control. Reduction of overall photosynthetic activity and concurrent reduction in respiration rate under high salinity stress may have resulted in lower levels of carbon flux being directed towards both energy synthesis (ATP) in the mitochondria as well as the biosynthesis of secondary metabolites like phenolics (through pyruvate from glycolysis and sugars from PPP) in the cytosol. Such strategy may have helped black bean plants to conserve more energy under higher salinity stress by slowing down the catabolic process and subsequent energy expense. The baseline soluble phenolic content of Eclipse (4-7.8 mg/g FW) was significantly higher when compared with Zenith (1.8 -5 mg/g FW) at all developmental phases (30, 45, 60 days) ($p<0.001$). Stimulation of phenolic biosynthesis with increasing levels of salt stress (mild and high) was observed in Eclipse at day 45 ($p<0.001$), but was not found in Zenith. Therefore the biosynthesis of phenolics in black bean under salt stress may be dependent on the genetic make-up of the genotypes and their specific responses towards salinity stress.

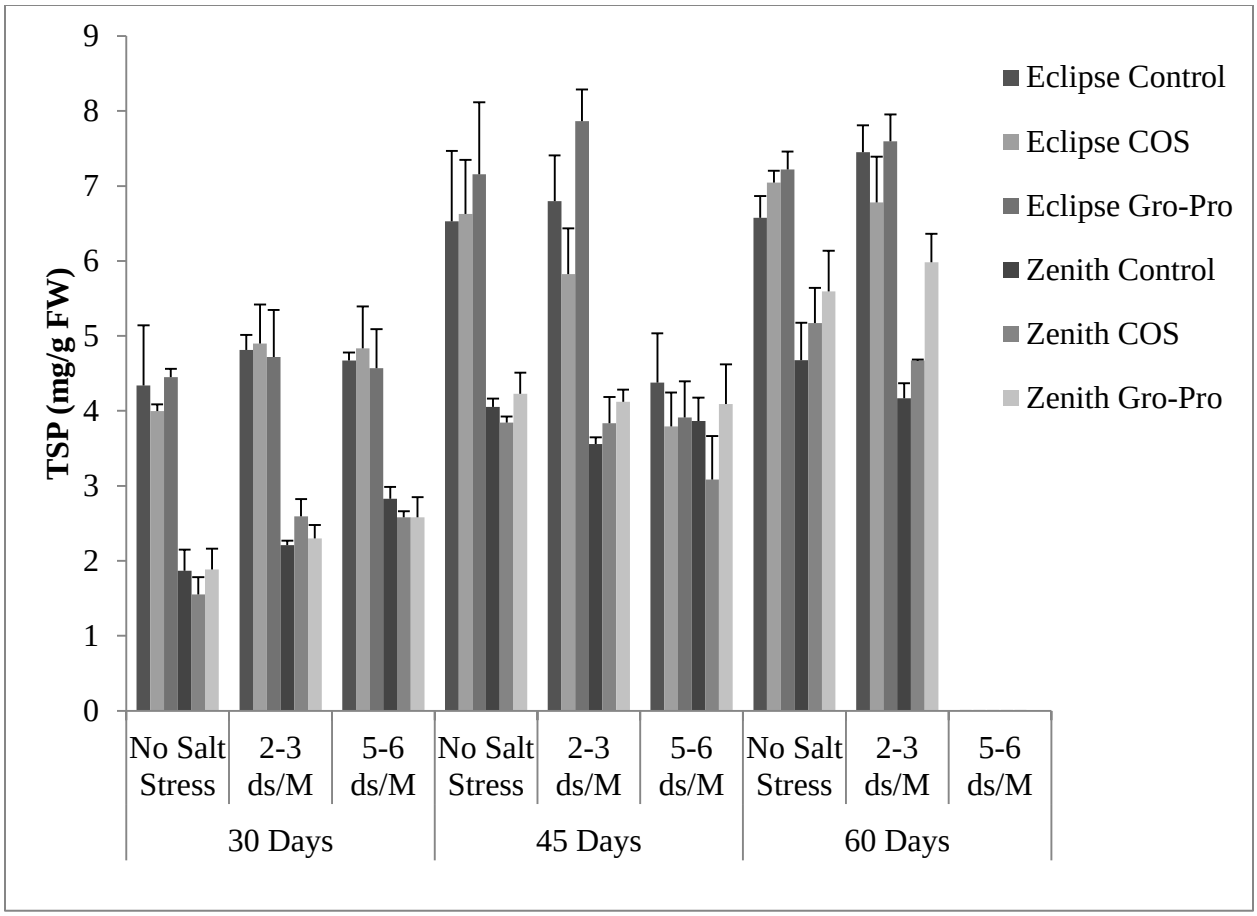

Figure 5.3. Total soluble phenolic content (mg. g^{-1} FW) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

Among seed elicitation treatments, Gro-Pro significantly improved total soluble phenolic content in both black bean genotypes at day 60 under mild salt stress ($p<0.05$). Similar stimulation of phenolic biosynthesis was observed in sprouted corn and in sprouted fenugreek bean with external elicitor treatments (Randhir and Shetty, 2005; Randhir et al., 2004a)**.** Dark germinated pea seedling also showed higher phenolic content after treated with high cytokinin root extracts of anise (Duval and Shetty, 2001). Enhancement in phenolic biosynthesis and concurrent improvement in cold tolerance was found in cold acclimated cool-season grasses (Sarkar and Bhowmik, 2009).

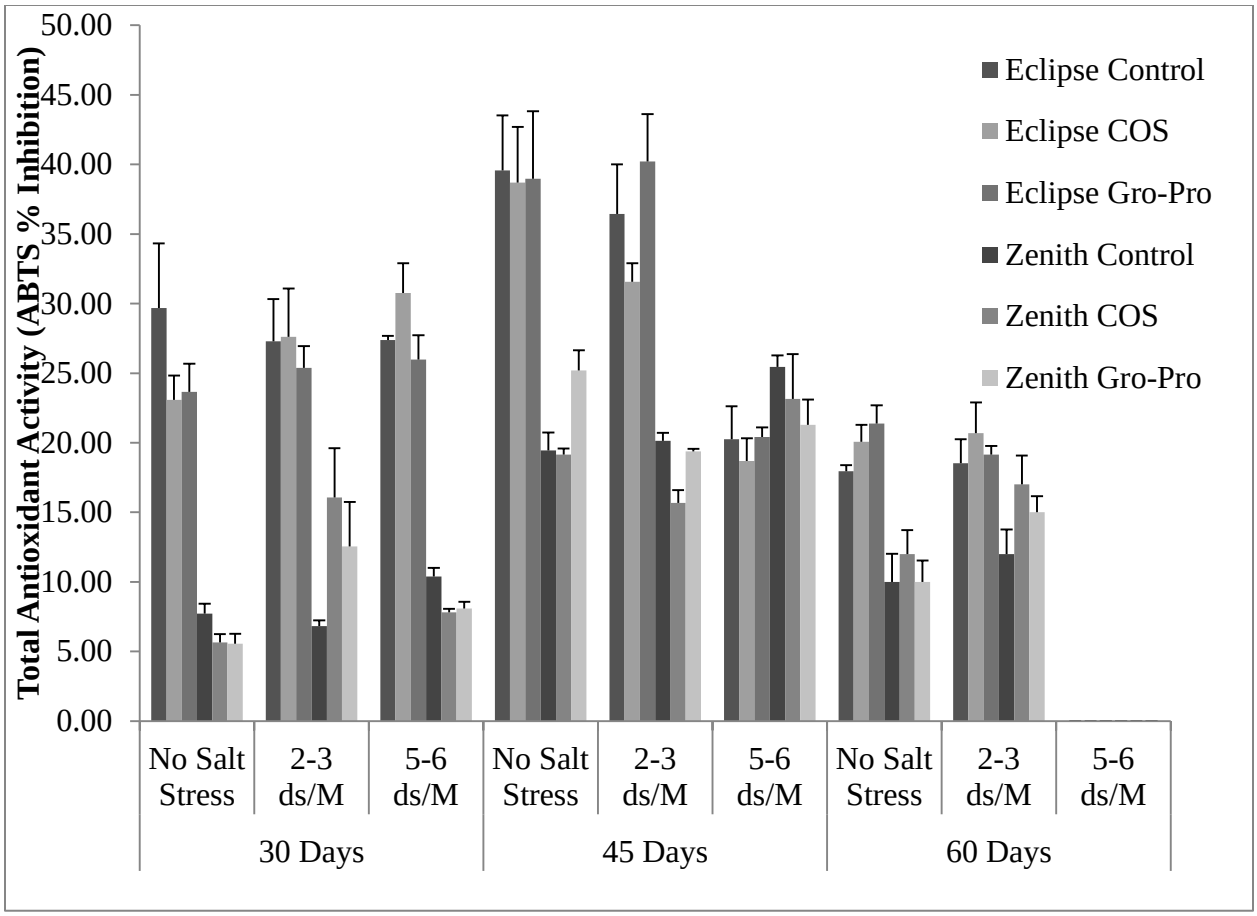

Figure 5.4. Total antioxidant activity (ABTS % Inhibition) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

Overall, total antioxidant activity of black bean plants increased from day 30 to day 45, but then decreased at day 60 (Figure 5.4.). Similar to total soluble phenolic content, Eclipse showed significantly higher baseline antioxidant activity (18-40% ABTS radical scavenging activity) when compared with Zenith (5-20%) at all developmental stages ($p<0.001$) (Table 5.1. A; B; C). Significant increase in total antioxidant activity under mild and high salt stress was observed in Zenith at 30 ($p<0.05$) and 45 ($p<0.001$) day. No statistical significance among elicitation treatments was observed in this study, but COS showed slightly higher trend in total antioxidant activity at day 60 in both black bean genotypes.

Higher antioxidant activity in COS treated black bean genotype with and without salt stress at 60 days has significant relevance for ensuring higher resilience as well as better fitness to black bean, especially during reproductive stage. Such improvement in antioxidant activity with COS seed elicitation may provide better protection to black bean plants against oxidative stresses for the entire life cycle and valuable to ensure higher fitness during pre-harvest stages. Higher antioxidant activity and cold stress tolerance improvement was observed in creeping bentgrass with COS foliar treatments (Sarkar et al., 2010b).

G6PDH and SDH Activity: To determine the critical metabolic role of pentose phosphate pathway (PPP) in black bean under salt stress, G6PDH enzyme activity (key enzyme responsible for first rate limiting step of PPP) was measured. In this study, salt stress alone did not result in increased G6PDH activity at day 30 and day 45, but at day 60 mild salt stress (2-3ds/M) showed higher G6PDH activity in both black bean genotypes ($p<0.001$) (Figure 5.5.) (Table 5.1. C). Gro-Pro seed elicitation significantly improved activity of this key enzyme in Eclipse under mild salt stress at day 45 (Figure 5.5.) when compared to control and COS ($p<0.001$). The activity of G6PDH had positive correlation with the soluble phenolic content of Eclipse with Gro-Pro seed elicitation treatment under mild and high salt stress. Thus this result potentially suggests that Gro-Pro seed elicitation improved phenolic biosynthesis in Eclipse black bean through up-regulation of PPP, especially under mild salt stress.

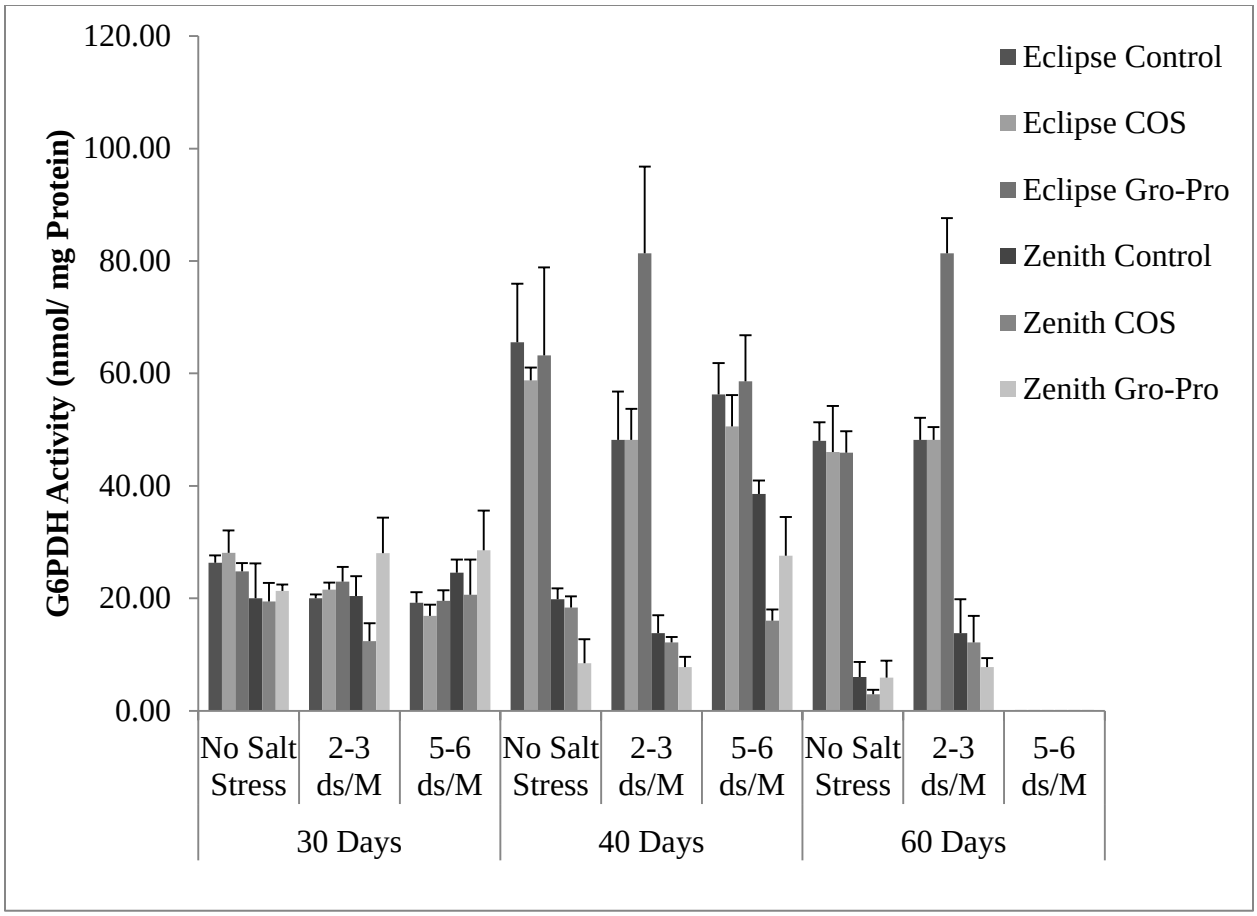

Figure 5.5. Glucose-6-phosphate dehydrogenase (G6PDH) activity (nmol.mg^{-1} Protein) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

Up-regulation of PPP in Eclipse may also contribute to improved anabolic support (through NADPH), biosynthesis of plant growth regulator hormones (IAA and cytokinin), and phenolic secondary metabolites. Such an adaptive response involving PPP helps plant to undertake different metabolic, biochemical, and structural adjustments to counter abiotic stress induced breakdown of cellular integrity. Initially, Gro-Pro seed elicitation also improved G6PDH activity slightly in Zenith, but such improvement was not observed during later growth stages (at 45 and 60 days) and no significant differences among treatments were observed. Similar to total soluble phenolic content and total antioxidant activity, the baseline G6PDH activity in Eclipse

was significantly higher when compared with Zenith at 45 and 60 day ($p<0.001$) (Table 5.1. B; C). Seed elicitation with COS did not show any significant up-regulation of PPP in either black bean genotypes under salt stress.

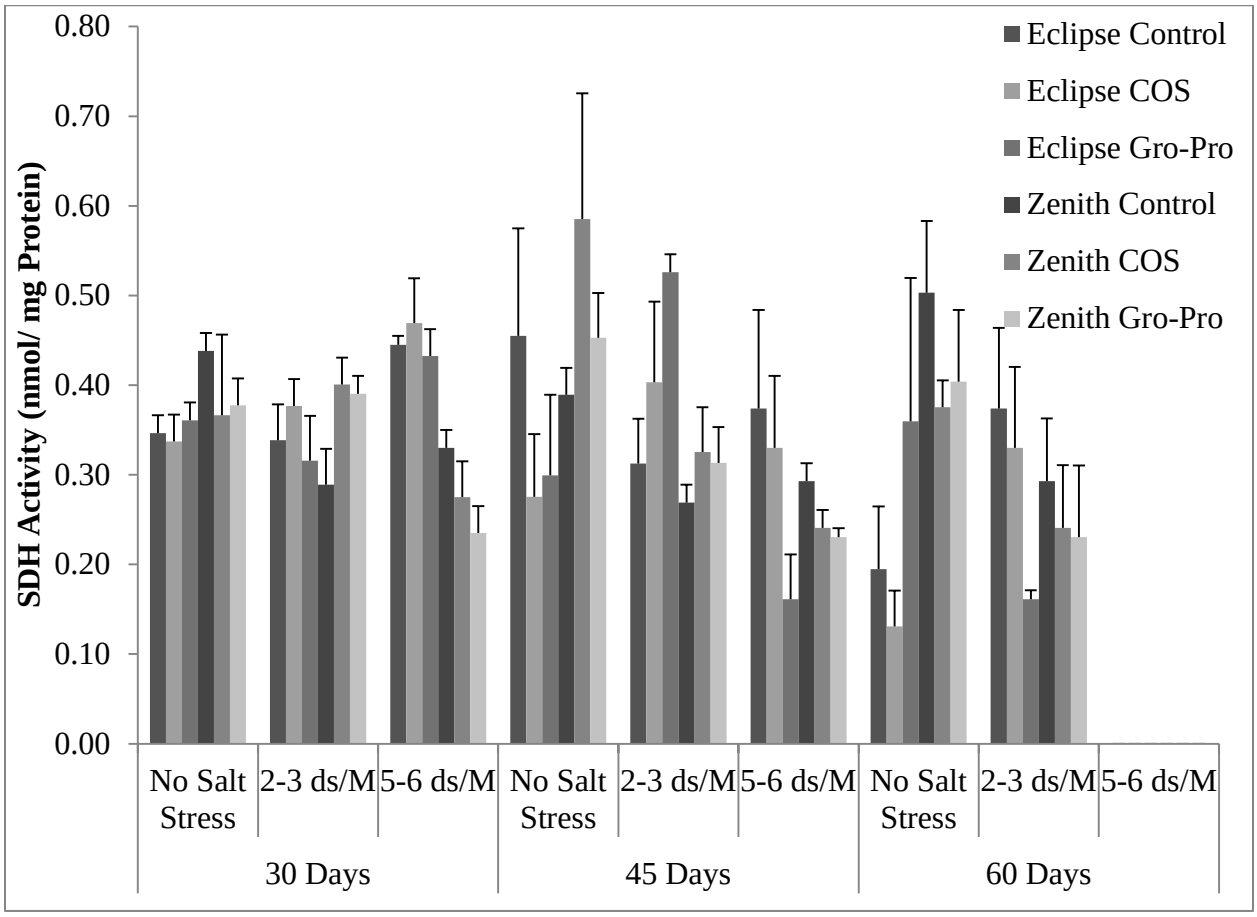

Figure 5.6. Succinate dehydrogenase (SDH) activity (nmol.mg^{-1} Protein) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

Succinate dehydrogenase (SDH) activity was measured to determine the relevance of TCA/Kreb's cycle functions and related respiration rate in black bean under salt stress. Overall, SDH activity decreased with seed elicitation treatments (COS and Gro-Pro) under mild salt stress at day 60 (Figure 5.6.). This result suggests that Gro-Pro seed elicitation may up-regulate PPP

(high G6PDH) by diverting carbon flux away from respiration processes to balance energy expenditure under salt stress. Only under mild salt stress (2-3 ds/M), both seed elicitation treatments showed higher trend of SDH activity in both genotypes at day 30 and day 45 but no statistical significance was observed. Interestingly, the baseline SDH activity in Zenith was at par with Eclipse at day 45, while G6PDH, total phenolic content, and antioxidant activity in Zenith was significantly lower when compared with Eclipse at same developmental stage. Under high salt stress at 30 day and under mild salt stress at 60 day Eclipse exhibited higher SDH activity. This finding indicates that Eclipse and Zenith have potentially adapted alternative strategies to mitigate salt stress. At day 45, high salt stress reduced the SDH activity in both genotypes. Such responses may help black bean plants to reduce their energy expenditure under higher oxidative pressure induced by salt stress. Similar balance between PPP regulation and respiration related energy expenditure was observed in cool season grasses under cold stress (Sarkar et al., 2011).

PDH Activity: To understand the role of proline oxidation in black bean under salt stress after seed elicitation treatments, PDH activity was evaluated. Overall, PDH activity decreased significantly as black bean plants matured from day 30 to day 60 (Figure 5.7.). Such a trend indicates during early growth stages black bean plants require higher proline oxidation, while the need is reduced during later reproductive stages of the growth. There was no clear and consistent trend observed in PDH activity with seed elicitation treatments. Interestingly at day 60, Zenith had significantly higher PDH activity when compared with Eclipse ($p<0.001$), while at day 45 Eclipse had higher PDH activity compared to Zenith ($p<0.001$) irrespective of the elicitation treatments (Table 5.1. B; C). At day 30 and day 45 significant decreases in PDH activity were observed under high salt stress (mild and high) (Table 5.1. A). Both black bean genotypes

exhibited the above trend decrease in PDH activity at day 45. This is may be due to higher demand of proline accumulation in the cytosol under high salt stress.

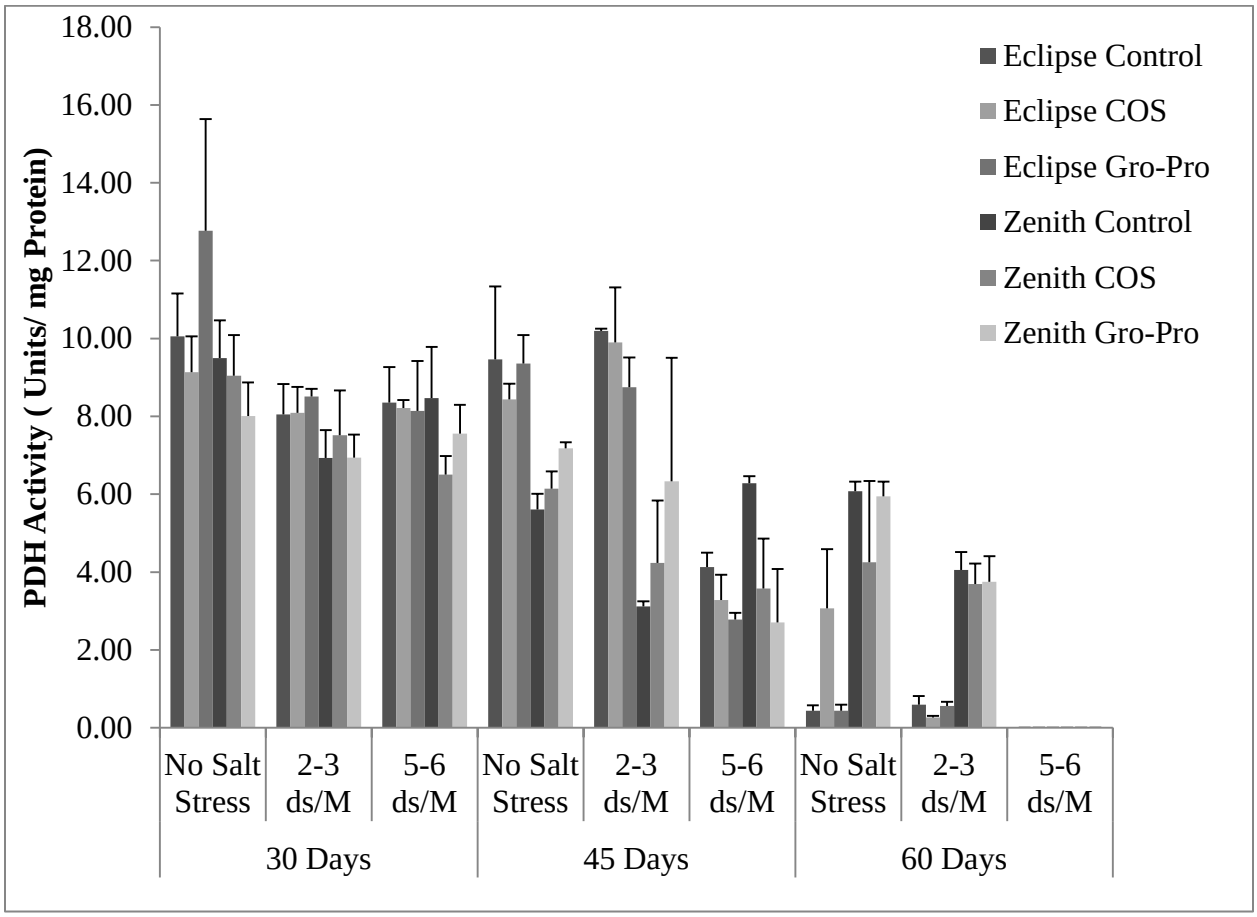

Figure 5.7. Proline dehydrogenase (PDH) activity (Units.mg^{-1} Protein) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

CAT, GPX, and SOD Enzyme Activity: The antioxidant enzyme activity of CAT, GPX, and SOD was evaluated to understand the role of antioxidant enzyme responses in black bean under salt stress with seed elicitation treatments. Except CAT, higher activities of other two antioxidant enzymes (GPX and SOD) were observed at day 60 in both genotypes irrespective of the seed elicitation treatments (Fig 5.8.; 5.9.; 5.10.).

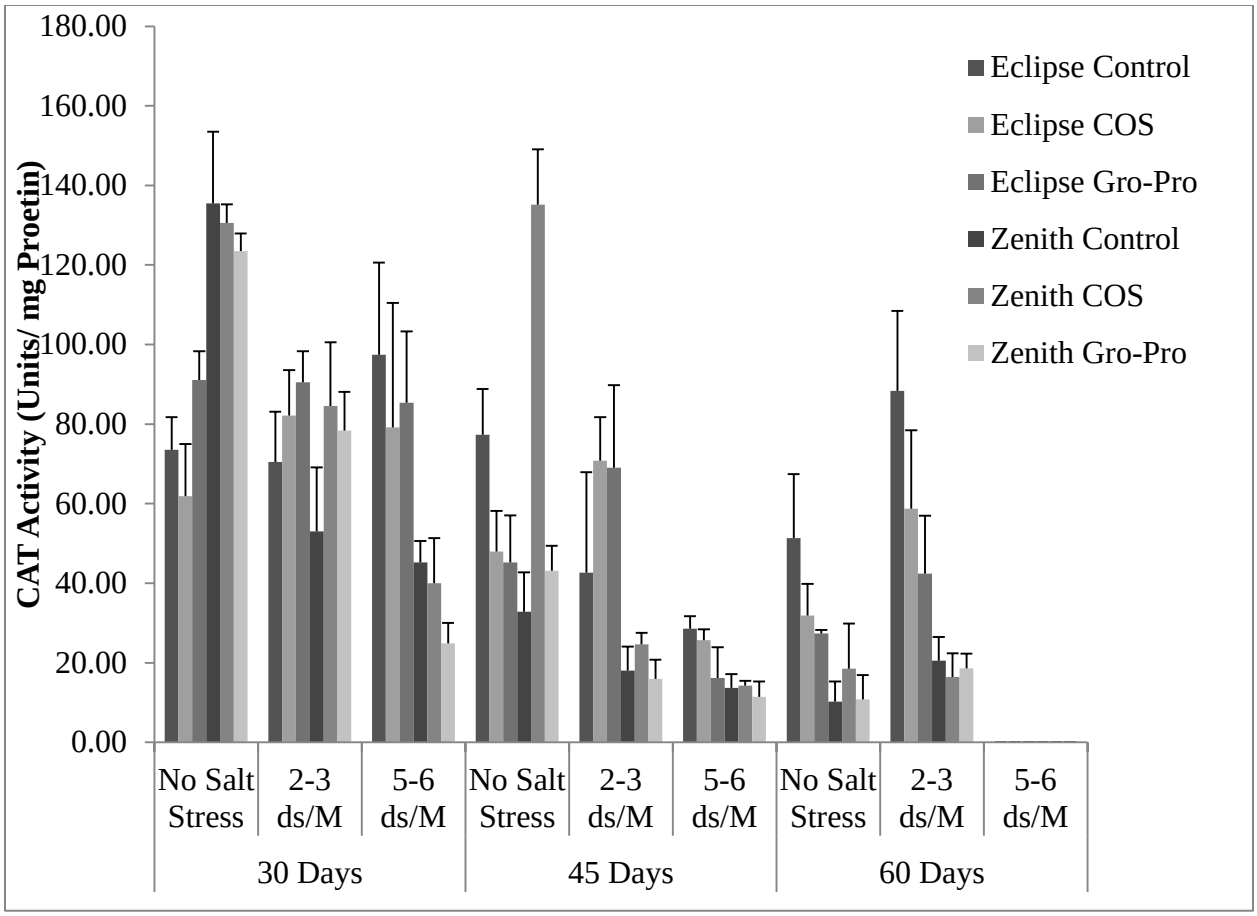

Figure 5.8. Catalase (CAT) activity (Units.mg^{-1} Protein) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

Catalase activity of Zenith was significantly lower under high salt stress (5-6 ds/M) at 30 and 45 days across all elicitor treatments ($p<0.001$) (Figure 5.8). The black bean genotypes evaluated in this study did not show CAT dependent antioxidant enzyme response under high salt stress and may be susceptible to higher peroxidation damages induced by salinity. Black bean in general are highly susceptible to salt stress and this result further provides evidence that corroborates this observation. At day 45, under high salt stress control and COS seed elicitation treatments in Eclipse resulted in significantly higher CAT activity when compared to the Gro-Pro treated black bean plants ($p<0.05$). Overall, Eclipse had higher CAT activity than Zenith at day 45 and day 60 under mild and high salt stress. This finding suggests that Eclipse had

significantly higher need for CAT during these developmental stages to counter cellular peroxidation irrespective of salt stress.

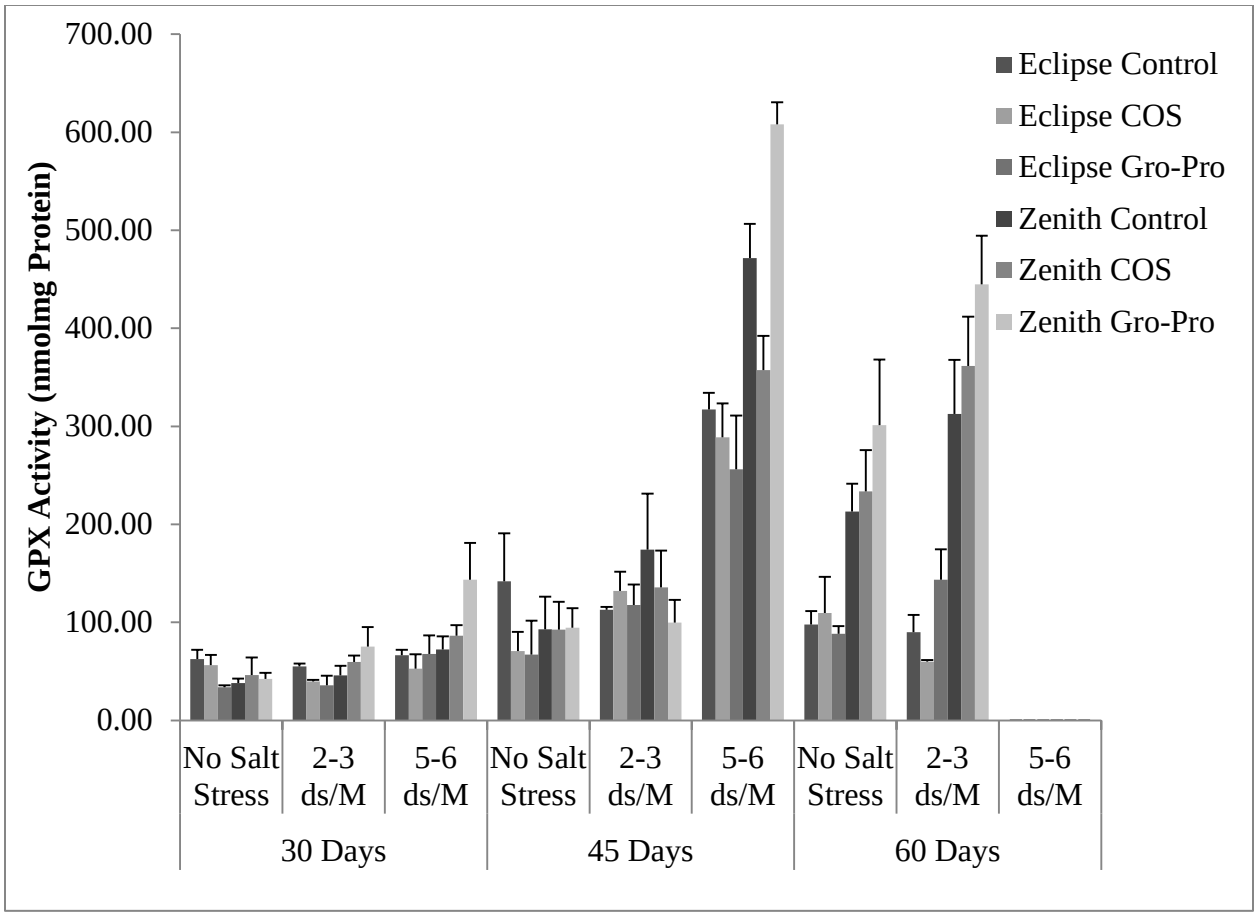

Figure 5.9. Guaiacol peroxidase (GPX) activity (nmol.mg^{-1} Protein) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

Contrary to the CAT activity the GPX activity in black bean plants increased with increasing levels of salt stress irrespective of their seed elicitation treatments (Figure 5.9.). Zenith showed significantly higher GPX activity when compared with Eclipse, especially under high salt stress at all developmental stages (Table 5.1. A; B; C). Gro-Pro seed elicitation significantly enhanced GPX activity in Zenith at day 30 under high salt stress (5-6 ds/M). Similar trend was observed at day 45, but no statistically significant difference among treatments was

observed. This result suggests a more important role of GPX in black bean under high salt stress which may be coupled to the specific function of this antioxidant enzyme in phenolic polymerization for structural adjustments especially during early developmental stages.

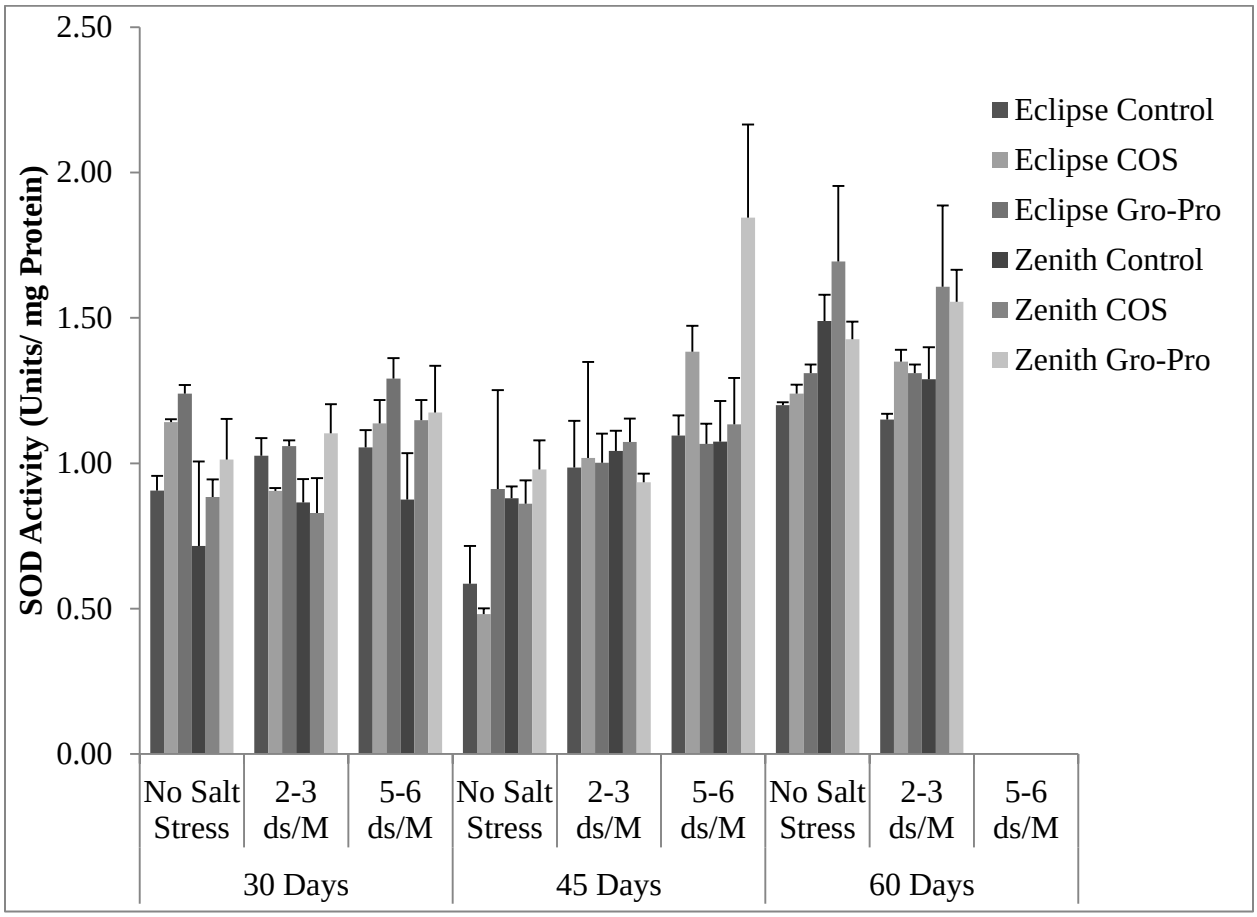

Figure 5.10. Superoxide dismutase (SOD) activity (Units.mg^{-1} Protein) of two black bean genotypes (Eclipse and Zenith) at 30, 45, and 60 days post-germination after treatment with seed elicitors (COS and Gro-Pro) and grown under three salinity levels (no salt stress, 2-3 ds/M, 5-6 ds/M EC).

Higher phenolic content coupled with high GPX activity was also observed in oregano clonal lines with application of seed elicitors such as acetyl salicylic acid and fish protein hydrolysate (Andarwulan and Shetty, 1999). Similarly, UV-B treated fava bean seeds showed stimulation of PPP regulation and increase in GPX activity (potential polymerization of phenolics) in dark germinated fava beans (Shetty et al. 2002). Previously higher GPX activity

along with high total soluble phenolic content was also observed in cool-season turfgrasses after their exposure to UV-B treatment illustrating the interconnected roles of GPX activity and phenolics in plants for determining fitness and resilience under abiotic stress. Therefore, the observed trend of higher GPX activity in black bean under high salt stress in this study indicates a similar function of GPX to counter salt stress-induced oxidative pressure and subsequent loss in structural integrity of cells.

Similar to the activities of the other two antioxidant enzymes (CAT and GPX), overall SOD activity increased overtime as black bean plants matured from day 30 to day 60 (Figure 5.10.). Gro-Pro seed elicitor treatments increased SOD activity in both black bean genotypes at day 30 ($p<0.05$) (Table 5.1. A). While at day 30 Eclipse had significantly higher baseline SOD activity when compared to Zenith, the complete opposite trend was observed at day 60, where Zenith showed high SOD activity. High salt stress also increased SOD activity in both genotypes when compared to no salt stress at day 30 and day 45. Like other two antioxidant enzyme activities, Zenith treated with Gro-Pro also showed higher trend in SOD activity under high salt stress (5-6 ds/M) at day 45. This result indicates that at later growth stages, Zenith had significantly higher demands of antioxidant enzymes such as GPX, and SOD to counter salt stress induced oxidative pressure and to maintain cellular redox balance. In general, Zenith is more tolerant to biotic stresses (anthracnose) compared to Eclipse and these higher antioxidant enzyme responses in Zenith under high salt stress may have relevance for the improvement of both biotic and abiotic stress tolerance (Kelly et al., 2015). Alternatively, Eclipse showed higher dependence on PPP mediated phenolic biosynthesis to counter salt stress induced metabolic breakdown and for determining overall fitness.

Conclusions

Both black bean genotypes evaluated in this study demonstrated high susceptibility to salinity stress, but their biochemical responses against salt stress varied. While higher phenolic content, up-regulation of PPP, and high total antioxidant activity was observed in Eclipse, Zenith demonstrated stimulation of individual antioxidant enzyme responses (GPX, and SOD) under high salt stress during later developmental stages. Gro-Pro seed elicitation treatment showed higher stimulation of these adaptive responses in both black bean genotypes, particularly under high salt stress. These results provide basic scientific insights about the potential of antioxidant-linked adaptive responses of black beans under salt stress. Further evaluation comparing with other black bean genotypes and other edible dry bean species is needed in both greenhouse and field conditions to determine the agronomic impact of these seed elicitors for improving salinity resilience and to provide this tool to bean growers of the Northern Plains and globally. While this study showed some positive evidences for the benefits of the seed elicitation model to improve salinity resilience in black beans, experiments with varying concentrations of seed elicitors, along with different application methods are required to validate the outcome of this study and to prove overall concept across other legumes and food crops.

CHAPTER 6. THE STIMULATION OF PHENOLIC ANTIOXIDANT-LINKED BIOACTIVE FUNCTIONALITIES IN FIFTEEN BLACK BEAN GENOTYPES USING COS AS A FOLIAR ELICITOR TREATMENT DURING PRE-HARVEST STAGES

Abstract

Increasing empirical evidence suggests that edible dry beans play an important role in the dietary management of non-communicable chronic diseases (NCDs) such as type 2 diabetes (T2D). One of the major elucidated mechanisms of such health benefits is associated with antioxidant-linked functionalities from phenolic bioactive compounds abundantly present in plant-based foods. Therefore dietary management strategies for T2D can be developed by targeting and subsequently improving the phenolic-linked bioactive compounds in plant foods through use of safe elicitation strategy. The goal of this project was to broadly enrich the phenolic-linked antioxidant bioactive properties present in fifteen commercial black bean genotypes through use of foliar elicitation with soluble chitosan oligosaccharide (COS) at pre – harvest stages. Total soluble phenolic content, antioxidant activity, and associated inhibitory potential of these bioactives of key enzymes of starch digestion such as α-amylase and α-glucosidase were evaluated using *in vitro* assay models. Total soluble phenolic content and total antioxidant activity of most black bean genotypes were improved with COS foliar treatment. While improvement in α-amylase inhibitory activity with COS foliar treatment was found in few black bean genotypes, but no significant improvement was observed in α-glucosidase inhibitory

activity. The results suggest that pre-harvest foliar COS application has important role for improving phenolics and antioxidant potential in black beans, but its role to improve glucose metabolism need further evaluation.

Introduction

Human relationship with food has progressively transformed in the aftermath of the World War 2 by adopting prevailing economic models of goods commodification to build a novel, global food system through advances in plant breeding, agricultural technologies, food science (food safety, quality, access), and worldwide distribution networks. While the global transition towards a globalized food system has yielded vital and unprecedented successes in constructing a global food infrastructure that has alleviated many of the pressing food security concerns of the post-war era (Godfray et al., 2010), this shift has caused a subsequent paradox: while one billion of the world population are currently food insecure/malnourished from a lack of quality food and/or quantity, more than another one billion people are considered to be overweight/obese linked to chronic overnutrition from hyper-processed, macronutrient and calorie-dense foods as well as the increasingly sedentary nature of modern urban lifestyles (Ericksen, 2008 FAO 2016). In order to counteract the deleterious effects that affordable, calorie-dense foods have caused to global health quality and outcomes, previously-known cultural knowledge must coordinate with emerging scientific consensus to demonstrate that whole, unprocessed plant-based foods (rich in micronutrients, fiber, and numerous health-promoting bioactives) can play a pivotal role in the maintenance of human nutrition in diverse societies, and that the re-addition of such foods into the modern diet is essential to counter the rising non-communicable chronic diseases (Liu, 2007; Slavin, 2004).

Of the numerous crops common in traditional and pre-industrial diets, edible dry beans (*Phaseolus vulgaris* L.) have been recognized for their critical role in numerous food cultures as foundational food, linked to their diverse growth adaptations, well-established rich nutritional compositions (protein, carbohydrates, dietary fiber, and vitamins), as well their association with a diverse range of human health benefits (Luthria and Pastor-Corrales, 2006; Silva-Cristobal, 2010). Further, edible dry beans are gaining worldwide attention and popularity in recent years due to consumers' desire for delicious and convenient food that also promotes overall health and dietary management of NCDs. Edible dry bean cultivation is also gaining popularity among growers globally, particularly in the Northern Plains of United States, due to its soil enrichment potential (nitrogen fixation), superior health benefits, and better value added export opportunities. North Dakota is the largest producer of edible dry beans, including black beans, in the United States and currently is the largest supplier of black bean for export (Knodel et al., 2015).

Many of the aforementioned health benefits of edible dry beans are due to their content of several secondary plant metabolites, particularly phenolic compounds (Leterme, 2002). While once considered as just to be by-products of primary metabolism and/or metabolic and toxic waste substances stored in vacuoles, plant secondary metabolites are now recognized widely as valuable molecules with diverse critical biological functions in plants as well as their role in human nutrition as health relevant bioactives when consumed regularly in the diet (Hanhineva et al., 2010). One of the most important biological functions of phenolic compounds with structural significance is antioxidant activity (Rice-Evans et al., 1997). Higher consumption of phenolics rich plant-based food could potentially provide cost-effective prevention, and a complimentary strategy to pharmaceutical drugs for the management of early stages of major chronic diseases,

such as cardiovascular disease, type 2 diabetes and potentially cancer (Labriola and Livingston, 1999).

Significant evidence exists that demonstrates the beneficial effect of dry bean bioactive compounds, and their associated role in reducing chronic inflammation, anti-oxidation activities, and management of NCDs (Leterme, 2002; Luthria and Pastor-Corrales, 2006; Silva-Cristobal, 2010). In 2015, black beans became the leader of dry bean production market classes in the United States for the first time in U.S. history, surpassing both navy and pinto beans. Production is primarily concentrated in Michigan and North Dakota in the U.S. (USDA-NASS, 2014; Vandemark et al., 2014). The national rise of black bean consumption stems from growing awareness of the well-established health benefits of consuming beans (Mitchell et al., 2009). Black bean is a rich source of dietary fiber, resistant starch, amino acids, vitamins, and plant phytochemicals (Luthria and Pastor-Corrales, 2006; Silva-Cristobal, 2010). Protective function of black bean in preventing genetic damage induced by chemical mutagens was observed in mice (Azevedo et al., 2003). Similarly, water soluble condensed tannins isolated from black beans showed inhibition of prostatic cancer cell growth (Bawadi et al., 2005). Another potential function of black bean bioactives is due to its antiproliferative and antioxidative activities (Dong et al., 2007). Most black bean genotypes have low glycemic index and phenolic bioactives of black bean has potential in improving *carbohydrate metabolism* in humans when consumed as diet (Silva-Cristobal, 2010). American Diabetes Association, American Heart Association, and American Cancer Society have recommended legumes including black bean as important dietary component for preventing and managing NCDs such as type 2 diabetes-linked cardiovascular complications (Leterme, 2002).

Improvement of human health relevant phenolic bioactive profile in food crops using novel strategies are gaining attention worldwide. One such approach is through stimulation of endogenous defense responses in plants and subsequent enhancement of phenolic metabolites in food crops. Different plant-based and marine-based sources have previously shown potential for improving phenolic bioactive profile when applied during pre- and post-harvest stages (Sarkar and Shetty, 2014). Chitosan oligosaccharide (COS) is one such compound which is highly soluble chitosan oligomer produced with enzymatic hydrolysis of chitosan. It has shown various functional properties and can be used as a bioactive material in many applications including, food, biomedicine, agriculture, environmental protection, and wastewater management (Kim and Rajapakse, 2005). Bioprocessed soluble COS as seed or foliar treatments has shown to be effective against different fungal pathogens and abiotic stresses during crop growth while simultaneously stimulating secondary metabolite synthesis in plants for improving the bioactive properties of the subsequently harvested food crops (Agrawal et al., 2002; Khan et al., 2003; Prapagdee et al., 2007; Sarkar et al., 2010).

These above studies suggest that external elicitor treatments and abiotic stress induction can potentially stimulate defense related pathways linked to inducible phenolics and antioxidant enzyme responses in food crops. Thus, the overall objective of this study was to demonstrate that the phenolic linked antioxidant responses and bioactive profile of black bean can be improved using a foliar-application of COS during pod-filling stage. Further the role of such bioactive enrichment in black bean to potentially improve glucose metabolism when consumed as diet was evaluated using *in vitro* assays.

Materials and Methods

Plant Material: The following fifteen black bean genotypes were selected in coordination with Dr. Osorno research group (Plant Sciences department NDSU, ND) under the 2015 Black Bean Variety Trial conducted at Prosper, ND. The genotypes evaluated were: ND071206, ND71256, NDF09304, ECLIPSE, T-39, ZORRO, LORETO, GTS-1103, MS001, BLACK CAT, 12576, 13489, 13496, 13503, and 13505.

Analytical Material: Porcine pancreatic a-amylase (EC 3.2.1.1), baker's yeast glucosidase (EC 3.2.1.20), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 3,5-Dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO) and of analytical grade.

Field Design: Sowing and maintenance of black bean genotypes was conducted in collaboration with the Dr. Osorno dry bean research group through the Black Bean variety trial, conducted in the 2015 growing season in Prosper, ND. RCBD with four replications was employed for this project, with each aforementioned replication containing fifteen randomized plots. Each plot contained four rows; two rows treated with COS, and two rows as control.

Foliar COS Treatment: Soluble chitosan oligosaccharide- vitamin C (COS-C), derived from marine chitin obtained from Kong Poong Bio, Jeju Korea was selected as the elicitor treatment. A concentration of 5g/L distilled water was selected based on positive results from previous studies involving foliar applied elicitors (Sarkar et al., 2010). This concentration was further selected on the basis of practicality and economic feasibility for conventional agriculture. Using a conventional backpack sprayer, a 5% COS solution was sprayed at 50 mL m^{-2} (within plots and replication) using two passes during the pod filling stage of growth (August 2015).

Black Bean Milling: Black bean seeds were harvested and cleaned of all physical contaminants. Next, a randomly selected sample of 200 mL was measured from the total harvest population of each treatment. The measured black bean sample was then milled using a WonderMill Grain Mill set to the "bread" setting until a fine dry powder was produced. The produced black bean powder was collected for use in bioactive extraction.

Bioactive Extraction: Ten grams of black bean powdered sample were taken and added to 50 mL distilled water in 50 mL tube. Samples were kept in ice bath in between each extraction step to avoid excess foaming. The mixture was blended for 5 minutes at "LOW" setting using a Waring commercial blender with a 100 mL volume blender attachment. Then blended sample was the transferred into a 50 mL tube and centrifuged at 8500 RPM twice for 20 min and 15 minutes, respectively. Final supernatant collected and stored in ice bath for use in bioactive assays.

Sampling & Statistical Analysis: Three separate replications were collected and analyzed separately. Three of the four collected replications were separately extracted and analyzed using *in vitro* assays. Each separate extraction was analyzed in triplicate for all assays. This triplicate was then combined for all three replications, for a total nine observations for each genotype and elicitor combination. The experiment was carried out using randomized complete block design (RCBD). Univariate analysis (ANOVA) was conducted using the 9.3 version of statistical analytical software (SAS). Differences among treatments were determined using Tukey's test through critical differences at the $p<0.05$ level.

Total Soluble Phenolic Content Assay: The total phenolics were determined by the Folin–Ciocalteu method, which was based on a method modified by Shetty et al. (1995). Briefly, 0.5 mL of the sample extract and 0.5 mL of distilled water were transferred into a test tube and

mixed with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50% (vol/vol) Folin–Ciocalteu reagent was added and vortexed. After 5 min, 1 mL of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The standard curve was established using various concentrations of gallic acid in 95% ethanol, and results were expressed as mg of gallic acid per gram of sample in fresh weight (FW).

Antioxidant Activity by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) Inhibition Assay: The DPPH scavenging activity was determined by an assay method modified by Kwon et al. (2006). Before conducting the experiment, a ½ dilution (125 µL sample + 125 µL distilled water) was used to dilute the sample due to the high antioxidant activity of the extractions. A 1.25 mL volume of 60 µM DPPH in 95% ethanol was added to 250 µL of each sample extract solution, with the decrease in the absorbance monitored after 5 min at 517 nm (A517 extract). The absorbance of a control (distilled water instead of sample extract) was also recorded after 5 min at the same wavelength (A517 control). The percentage of inhibition was then calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A517(control)} - \text{A517(extract)}}{\text{A517(control)}}\right) \text{x } 100$$

α-Amylase Inhibition Assay: The α-amylase inhibitory activity was determined using an assay method modified from the Worthington Enzyme Manual (Worthington Biochemical Corp., 1993a). A total of 500 µL of each sample extract (Sample extract first diluted to 1:2 ratio [250 µL +250 µL of distilled water]) and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α -amylase solution (0.5 mg/mL) were incubated at 25 ℃ for 10 min. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube and then incubated at 25 ℃ for 10 min. Next, 1.0

mL of dinitrosalicylic acid color reagent was added. The test tubes were then sealed with tin foil and incubated in a boiling water bath for 10 min, and immediately cooled using an ice water bath to bring samples to room temperature to stop the reaction. The reaction mixture was then diluted after adding 8-10 mL of distilled water, and absorbance was measured at 540 nm. The absorbance of sample blank (buffer instead of enzyme solution) and a control (buffer in place of sample extract) were also recorded. The final extract absorbance (A540 extract) was obtained by subtracting its corresponding sample blank reading. Additionally, a 1:5 dilution of the sample extract (100 µL +400 µL of distilled water) was performed to investigate potential dose dependence. The *a*-amylase inhibitory activity was calculated according to the equation below:

$$\% \text{ Inhibition} = \left(\frac{\text{A540(Control)} - [\text{A540(Extract)} - \text{A540(Sample blank)}]}{\text{A540(Control)}} \right) \text{x } 100$$

α-Glucosidase Inhibition Assay: The assay method was modified from the Worthington Enzyme Manual for *a* -glucosidase inhibition (Worthington Biochemical Corp., 1993b; McCue et al., 2005). A volume of 50 µL of sample extract diluted with 50 µL of 0.1 M potassium phosphate buffer (pH 6.9) and 100 µL of 0.1 M potassium phosphate buffer (pH 6.9) containing glucosidase solution (1.0 U/mL) was incubated in 96-well plates at 25 ℃ for 10 min. After preincubation, 50 µL of 5mM p-nitrophenyl- a-D-glucopyranoside solution in 0.1 M potassium phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 ℃ for 5 min. Before and after incubation, absorbance readings (A405 extract) were recorded at 405 nm by a microplate reader (Molecular Devices Co., Sunnyvale, CA) and compared to a control which had 50 µL of buffer solution in place of the extract (A405 control). The *a*-glucosidase inhibitory activity was expressed as a percentage of inhibition and calculated as follows:

$$\% \text{ Inhibition} = \left(\frac{\triangle \text{A405(control)} - \triangle \text{A405(extract)}}{\triangle \text{A405(control)}} \right) \text{x } 100$$

High Performance Liquid Chromatography (HPLC): The sample extracts (2 mL) were filtered through a 0.2 µm filter. A volume of 5 µL of sample was injected using an Agilent ALS 1100 autosampler into an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250 - 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 306 nm and 333 nm and the chromatogram was integrated using Agilent Chemstation enhanced integrator. Pure standards of chlorogenic acid, gallic acid, ellagic acid and quercetin in 100% methanol were used to calibrate the standard curves and retention time.

Results and Discussion

Total Soluble Phenolic Content and Total Antioxidant Activity (DPPH Inhibition) of Black Bean Genotypes: The TSP content of all 15 black bean genotypes was determined using the Folin–Ciocalteu method to evaluate the effect of Foliar COS application on improving phenolic bioactives in black beans prior to harvest. The overall general trend observed between treatments was that 6 black bean genotypes treated with COS (ND071206, NDF09304, ECLIPSE, 12576, 13489, and 13503) had a significant improvement in TSP content compared to control, while 9 genotypes (ND712256, T-39, ZORRO, LORETO, GTS-1103, MS001, BLACK CAT, 13496, and 13505) had no significant difference between treatments, and no genotypes exhibited a decrease in TSP content in COS treated plants (Figure 6.1.). While most genotypes

had similar baseline contents of total soluble phenolics, 4 genotypes in particular had higher baseline total phenolic contents in addition to having significant differences with elicitor treatment: NDF09304 (3.9 to 4.15 mg/g FW), GTS-1103 (4.06 to 4.15 mg/g FW), 13503 (3.86 to 3.97 mg/g FW) and 13505 (4.28 to 4.36 mg/g FW).

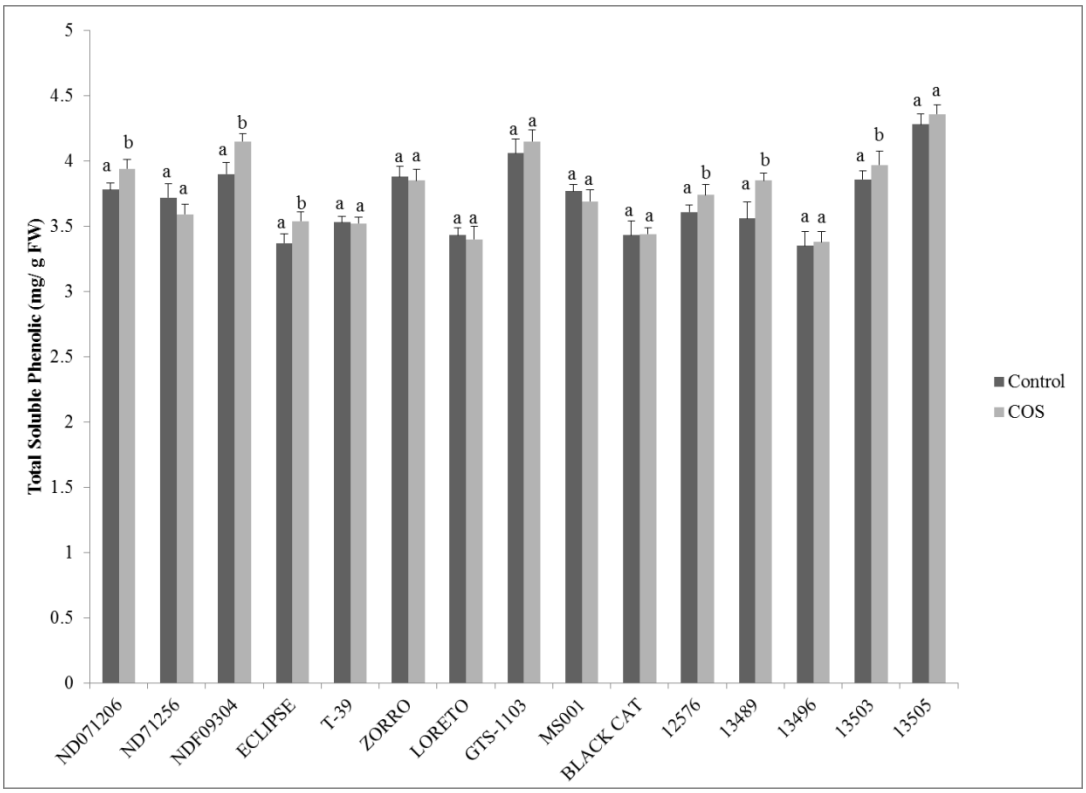

Figure 6.1. Total Soluble Phenolic Content (mg/g FW) of 15 black bean genotypes after treated with chitosan oligosaccharide-COS foliar treatment at pod-filling stage along with untreated control. Different letters indicate significant differences between foliar elicitation treatments within each genotype at the $p<0.05$ level.

Total antioxidant activity of the 15 black bean genotypes was determined using a radical scavenging (DPPH) assay to evaluate the effect of Foliar COS on the total antioxidant activity in black beans compared to a control. The trend of total antioxidant activity in the evaluated 15 black bean genotypes was comparable to that of total soluble phenolics, with COS-treated black beans largely having statistically significant increases (Figure 6.2.). Of the 15 genotypes

evaluated; ND071206, NDF09304, ECLIPSE, T-39, LORETO, MS001, 12576, 13489, 13496, 13503, 13505, had significant increase in total antioxidant activity in COS-treated plants compared to the control, with only the Zorro variety with the reverse trend. Baseline DPPH activities had higher overall variance when compared to total phenolics, with 13505, 13503, and MS001 with overall higher antioxidant activities and also exhibited significant improvements with COS treatments.

In all 45% of the evaluated black bean genotypes had significant improvements in both total phenolic content and total antioxidant activity (ND071206, NDF09304, ECLIPSE, 12576, 13489, 13503, 13505) while 22% only had significant improvements in antioxidant activity (T-39, LORETO, MS001, and 13496) and only 1 genotype had significant improvements in total phenolic content (GTS-1103). In general 15% had none or negative significant differences between COS treated and control black bean plants (ND71256, ZORRO, and BLACK CAT). Black bean genotypes such as 13505 and 13503 outperformed all other genotypes on the basis of baseline levels in total antioxidant activity and COS-treated plants exhibited increase in both total phenolic content and antioxidant activity. Interestingly, while COS-treated plants appeared to stimulate a stronger response in increasing total antioxidant activity, only a modest improvement was observed in total phenolic content.

Similar improvement in total antioxidant activity in cool season grasses were observed with COS foliar treatments (Sarkar et al., 2010). This may indicate that the COS foliar application has larger role in stimulating antioxidant enzyme responses and lesser impact on improving overall phenolic biosynthesis in black bean plants. Overall, total soluble phenolic content as well as total antioxidant activity results suggest that COS has potential to improve phenolic-linked antioxidant activity in black beans and can be targeted to develop bioactive

enriched black beans. Chitosan oligosaccharide is cost effective, safe, and easy to use and may provide a useful tool to the bean growers for improving human health relevant bioactive profile in black bean and in other legume and non-legume food crops.

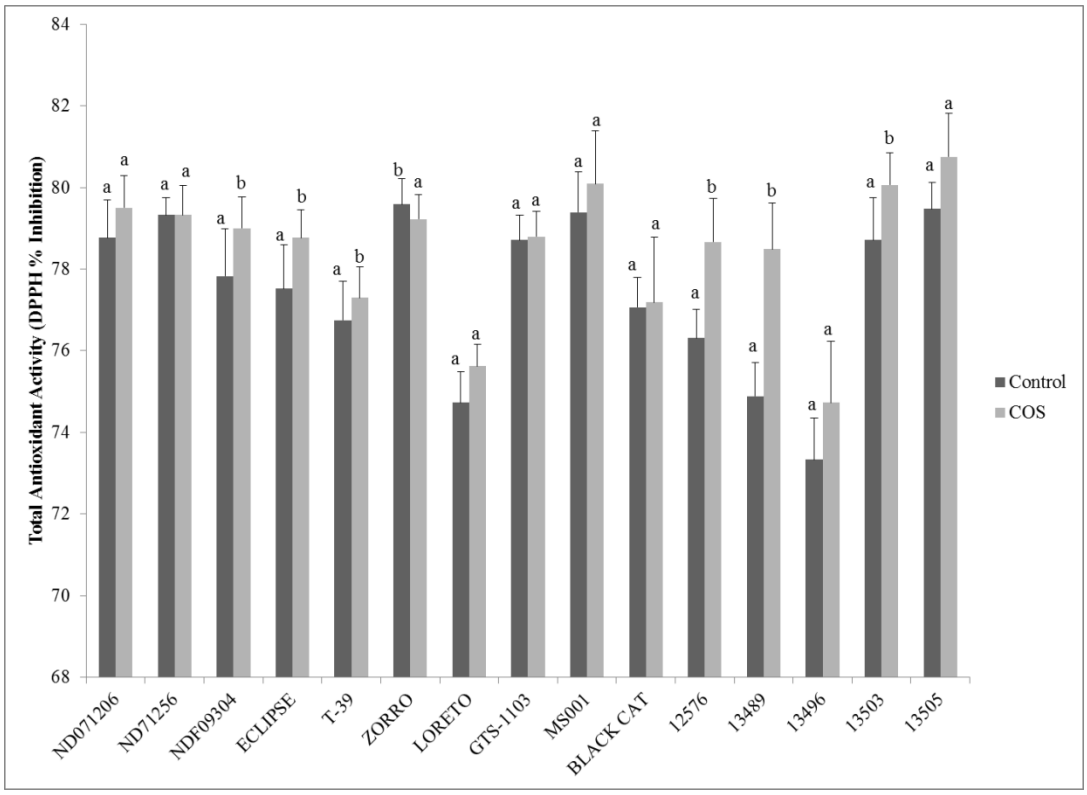

Figure 6.2. Total antioxidant activity (DPPH % Inhibition) of 15 black bean genotypes after treated with chitosan oligosaccharide-COS foliar treatment at pod-filling stage along with untreated control. Different letters indicate significant differences between foliar elicitation treatments within each genotype at the $p<0.05$ level.

***Alpha-glucosidase Inhibitory Activity of Black Bean Genotypes*:** The α-glucosidase inhibition activity was determined using *in vitro* assays to evaluate if COS foliar treatments at the pod-filling growth stages could provide potential indications of improved carbohydrate digestion. All fifteen black bean genotypes exhibited low baseline inhibitory activities (22% average inhibition). In addition, the α-glucosidase inhibitory activity also did not show any further improvement in COS treated plants across all black bean genotypes (Figure 6.3.). Furthermore, only the 4 black bean genotypes NDF09304, GTS-1103, 13496, and 13503

exhibited minor improvements in α-glucosidase inhibitory activity in COS-treated plants without showing any significant differences, while 8 genotypes ECLIPSE, T-39, ZORRO, MS001, BLACK CAT, 12576, 13489, and 13505, showed decrease in α-glucosidase inhibitory activity after COS treatment. Such evidence indicates that either COS may not elicit bioactive compounds linked to α-glucosidase inhibition, or that COS elicitation may actually divert the biosynthesis and polymerization of various phenolic compounds by utilizing metabolites involved in α-glucosidase inhibitory activity. Further, α –glucosidase inhibitory activity of black beans in this study was likely not dependent on their phenolic contents, and was more likely influenced by other factors such as: amino acid profiles, total protein content or soluble oligosaccharides.

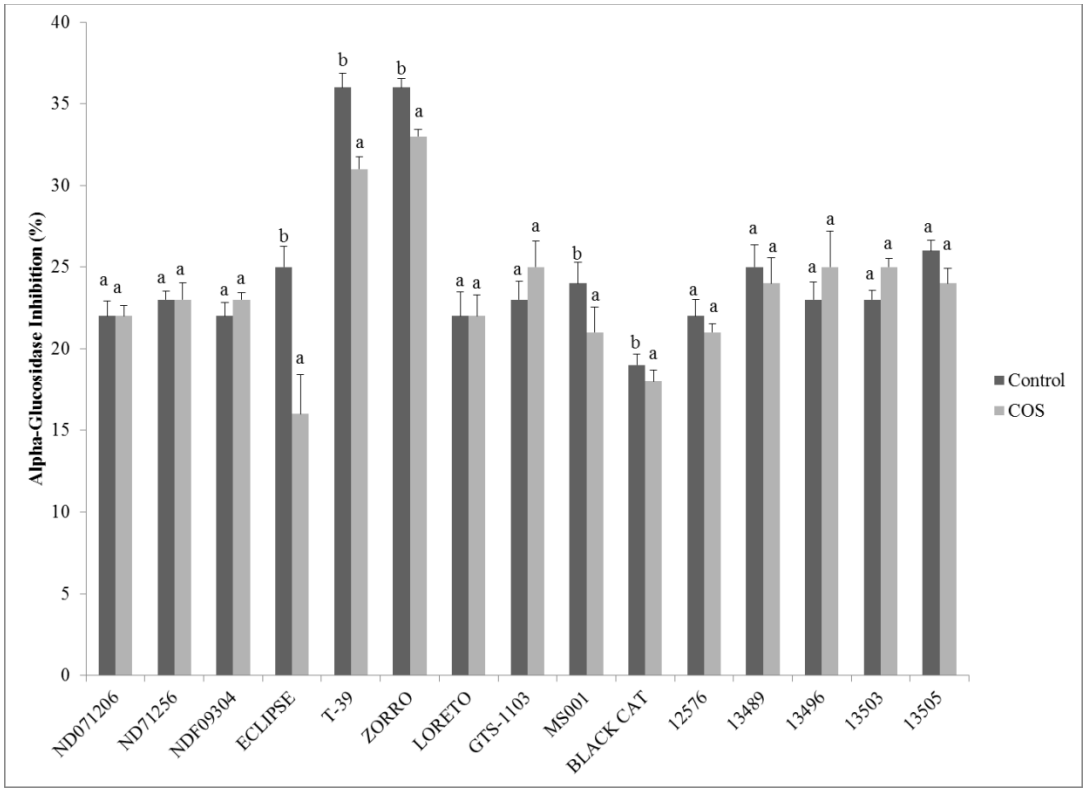

Figure 6.3. Alpha-glucosidase inhibitory activity (%) of 15 black bean genotypes after treated with chitosan oligosaccharide-COS foliar treatment at pod-filling stage along with untreated

control. Different letters indicate significant differences between foliar elicitation treatments within each genotype at the $p<0.05$ level.

***Alpha-amylase Inhibitory Activity of Black Bean Genotypes*:** Pancreatic α-amylase is key enzyme responsible for digestion and breakdown of starch into mono-and di-saccharides and thus inhibition of this enzyme in intestine helps in maintaining post-prandial blood glucose level (Hanhineva et al., 2010) Thus, the α-amylase inhibitory activity for all 15 black bean genotypes was determined using *in vitro* assays in order to evaluate if their phenolic compounds and phenolic- linked antioxidants are: 1.) linked to α-amylase inhibition 2.) and can improve α-amylase inhibition through COS foliar treatments at the pod-filling stage. In order to determine if a dose-dependent nature exists, all black bean genotype extracts were evaluated using one-half and one-fifth dilutions as undiluted sample showed very high α-amylase inhibitory activity.

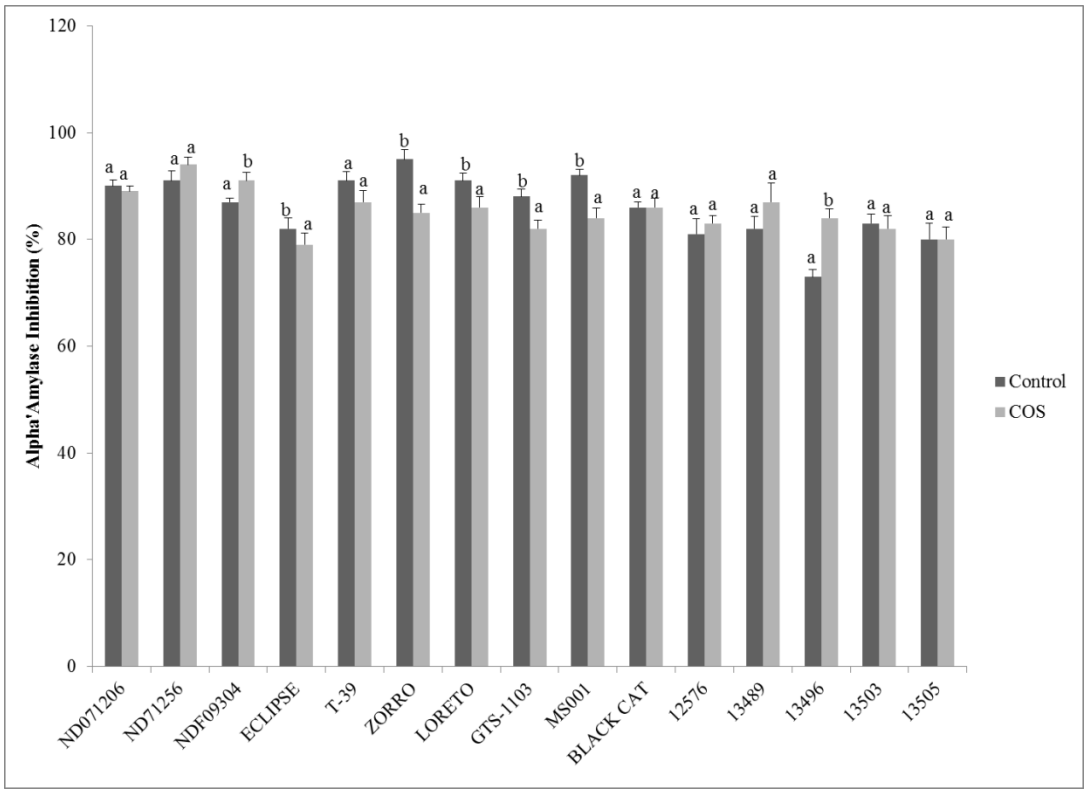

Figure 6.4. Alpha-amylase inhibitory activity (%) (Half dilution) of 15 black bean genotypes after treated with chitosan oligosaccharide-COS foliar treatment at pod-filling stage along with

untreated control. Different letters indicate significant differences between foliar elicitation treatments within each genotype at the $p<0.05$ level.

The overall baseline activity appeared to vary greatly by genotypes, with a range of 80-95% inhibition in one-half dilution extracts, while one-fifth extracts activity ranged widely between 30-60% (Figure 6.4. & 6.5.). This finding suggests that more critical factors than dilution are involved in determining α-amylase inhibitory activity, and that the evaluated black bean extracts did not appear to exhibit any dose dependence. At one-half dilution, 4 genotypes (ND71256, NDF09304, 12576, 13489, and 13496) had improved α-amylase inhibitory activities, while 6 genotypes (ECLIPSE, T-39, ZORRO, LORETO, GTS-1103, and MS001) had reduced activities (Figure 6.5.). At one-fifth dilution 5 genotypes (ND071206, ND71256, ZORRO, LORETO, and BLACK CAT,) showed improvements in their α-amylase inhibitory activities, while 3 genotypes (12576, 13489, and 13503) exhibited reduced activities.

Disregarding ND71256, none of the evaluated black bean genotypes had a consistent pattern from one-half to one-fifth dilutions, indicating that a dose-dependent response may not be present. For example, ZORRO and LORETO showed a decrease in inhibition at one-half dilution, but showed a completely opposite trend in the one-fifth dilution. Interestingly, no notable pattern within genotypes or treatments influencing α-amylase inhibition was evident, indicating that black bean phenolics may not play a significant role in α-amylase inhibitory activity, and said aforementioned activity was not improved with foliar COS treatment during the pod filling stage of growth.

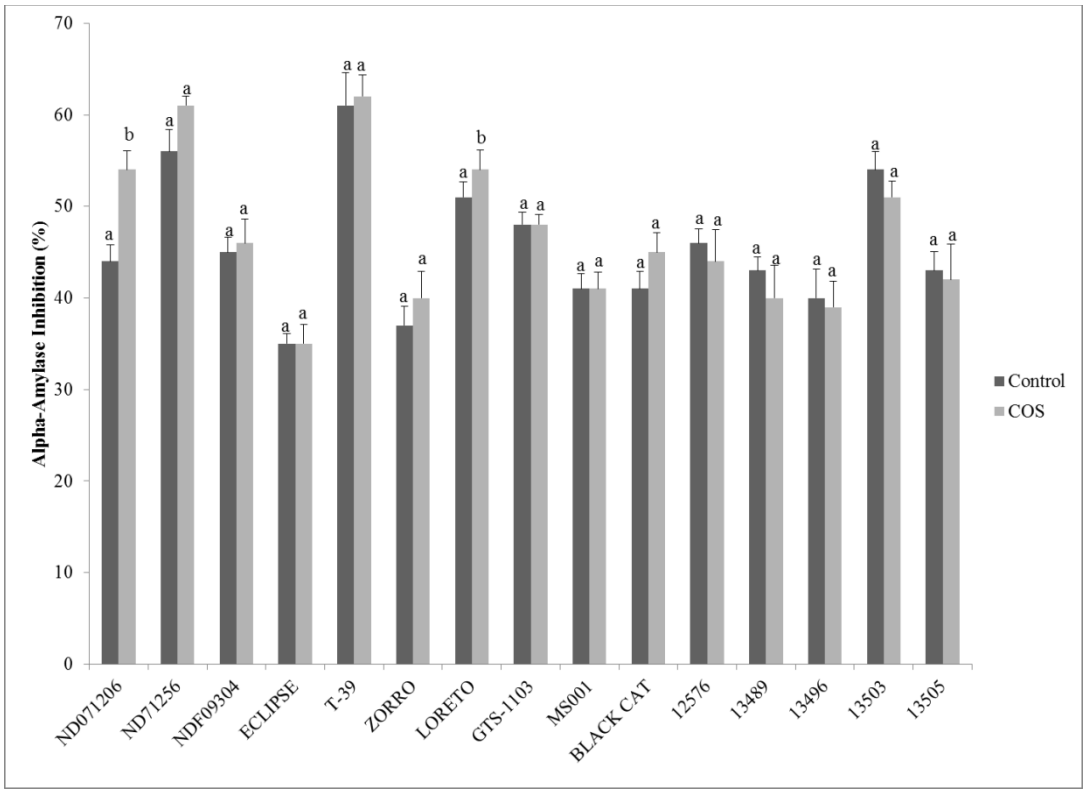

Figure 6.5. Alpha-amylase inhibitory activity (%) (One-fifth dilution) of 15 black bean genotypes after treated with chitosan oligosaccharide-COS foliar treatment at pod-filling stage along with untreated control. Different letters indicate significant differences between foliar elicitation treatments within each genotype at the $p<0.05$ level.

Conclusions

The overall trend of several black genotypes provided evidence in improvements to total phenolic content and total antioxidant activities, but no strong link between such improvements in phenolic antioxidants with functionalities relevant to carbohydrate digestion was observed in *in vitro* assays. These results suggest that while elicitation using COS did improve the phenolic content and antioxidant activities in black bean, these elicited compounds may not be linked to subsequent improvements in the type 2 diabetes relevant bioactive markers, such as inhibition of key enzymes of starch digestion. An important area of subsequent studies will be to evaluate if the phenolic antioxidant improvements can be linked to other markers of black bean bioactivity responsible for T2D management, particularly model assays characterizing anti-lipase activity,

inflammatory control and inhibition of AGE formation. The improvement in antioxidant activity with COS foliar treatment in black bean has overall human health relevance, especially to provide cellular protection against oxidative stress linked to chronic diseases associated with type 2 diabetes and wound healing linked complications linked to oxidative stress. Since this study provided positive evidence of elicited phenolic and antioxidant activity, further studies must be conducted to optimize the concentration and volume of COS applied as well as which plant developmental stage best responds to foliar COS treatments. We now have evidence for the use of COS as an elicitor for stimulating phenolic linked-antioxidant activity in one time field trial but need further evaluation in multiple trials over many seasons for practical applications. This model must be expanded to determine if this strategy can also improve range of human health relevant bioactives in other edible dry beans and in other food crops.

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