IMPACTS OF MICROBIAL SEED INOCULANTS ON GROWTH OF FIELD PEA (PISUM SATIVUM L.), AND IMPLICATIONS FOR PLANT-INSECT INTERACTIONS

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MASTER OF SCIENCE

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ABSTRACT

Soil microbes that associate with plant roots can benefit plants by increasing the supply or availability of nutrients to increase the plant's resilience to abiotic and biotic stress, crop germination rates, root and shoot growth, flower production, and yield. We evaluated the impact of five commercially available treatments: 1) B5, *Bacillus* bacteria, 2) GP, *Trichoderma* fungi, 3) N2, *Paenibacillus* nitrogen-fixing bacteria, 4) combination of B5+GP+N2, and 5) water control, and soil-dwelling Collembola on growth and biomass distribution of the specialty crop field pea in a greenhouse setting. We assessed plant growth (e.g., height, biomass of shoots and roots) and results showed that microbial inoculants positively impacted field pea plant growth under specific abiotic environmental stresses.

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CHAPTER 1. LITERATURE REVIEW

1.1. Agricultural Production and Ecological Intensification

By 2050, the human population is predicted to reach 10 billion people, with roughly 83 million people born annually (United Nations, 2017). This rapid population growth is causing heightened problems concerning food shortages, with an estimated 70% increase in food supply needed to feed a population of this magnitude per year (Abhilash et al., 2016). This is alarming given that crop yields are plateauing globally, with rice, wheat, and maize showing little to no yield growth in some of the world's biggest cereal-producing countries (Cassman et al., 2010; Bommarco et al., 2013).

Current methods for increasing productivity in modern industrial agriculture rely on fertilizers and pesticides (Tillman, 2001). Nitrogen and phosphorus chemical fertilizers can lead to groundwater pollution, accumulations of toxic chemicals, and decreased soil fertility (Alori et al., 2017). Furthermore, these fertilizers have been shown to reduce soil pH, thus making certain mineral nutrients less accessible to plants (Gupta et al., 2017). The addition of chemicals to crop fields focuses on short-term gains and can be viewed as a future detriment to agroecosystems (Gouda et al., 2018).

Increased crop production through conversion of natural ecosystems to agricultural fields has been successful, but a major consequence involves a decrease in natural habitat for plants and animals (Tsharntke et al., 2005). Currently, croplands and pastures cover up to 40% of earth's land surface and to meet human population food demands it is estimated that 2.7-4.9 million ha of farmland per year need to be added to generate enough food (Foley et al., 2005;

Ablilash et al., 2016). Relying solely on land conversion to address food demands is unsustainable and impractical for the amount of land required to be successful.

Ecological intensification is a concept within sustainable agriculture based on using biological regulation within agricultural and landscape environments (Kleijn et al., 2019). The Food and Agriculture Organization defines it as a practice of maximizing primary production within an area without reducing the ability to sustain its productive capacity (FAO, 2009). A more recent interpretation of the term focuses on ecological intensification as an attempt to increase agricultural production by implementing new and innovative ecosystem services that are environmentally friendly (Hubert et al., 2010; Dore et al., 2011). This does not exclude the use of fertilizers and pesticides but may reduce current agricultural dependence on repeated applications of chemicals (Cassman, 1999; Dore et al., 2011). Strategies to implement ecological intensification utilize natural ecosystems in the soil as a model to understand properties and interactions that could benefit plants within an agricultural setting (Malezieux et al., 2012).

1.2. The Rhizosphere

The rhizosphere consists of three main components: the soil, root surface, and the root itself (Barea et al., 2005). The soil contains elements that plants rely on, including water, nitrogen, phosphorus, and potassium (Gouda et al., 2018). Within the soil, there are beneficial and pathogenic microorganisms that have complex interactions with plant roots (Bais et al., 2006). The rhizosphere is one of the most diverse ecosystems on Earth and contains diverse taxa, including: fungi, bacteria, protists, nematodes, and arthropods (Jacoby et al., 2017). Collembola (springtails) are among the most abundant soil arthropods within the rhizosphere; they primarily feed on fungi and decaying organic material (Hopkin, 1997).

Within the rhizosphere, plant-microbe interactions have evolved over millions of years, creating a highly diverse and specialized ecosystem called the rhizosphere microbiome (Imam et al., 2016). Soil microbes can be separated into three unique groups based on how they interact with plants; 1) saprophytic, 2) pathogenic, and 3) beneficial (Lugtenberg et al., 2002). Saprophytic microbes are responsible for the decomposition of dead material and play a vital role in nutrient cycling and the release of nitrogen into surrounding soils for plants to utilize (Crowther et al., 2012). Pathogenic and beneficial microbes interact directly with living plants and impact their growth and overall plant health in different ways. Pathogenic microbes negatively impact plant roots, shoots, and leaves by causing plant diseases (Mendes et al., 2013). Beneficial microbes can increase plant nutrient acquisition, growth, yield, and ability to tolerate and respond to abiotic and biotic stress (Verma et al., 2017). Overall, soil microbes play a major role in plant health.

1.3. Plant-Microbe Communication and Colonization

Plants and soil microbes communicate via chemicals (Mhlongo et al., 2018). These interactions are facilitated by root exudates (Verma et al., 2017) that provide a steady flow of ions, oxygen, water, enzymes, and metabolites (Barea et al., 2005; Bais et al., 2006). The release of exudates is correlated with developmental stage of the plant and other key release factors including photosynthesis activity, plant size, and soil conditions (Mhlongo et al., 2018). In early plant growth stages, root structures are constantly releasing carbohydrates, amino acids, and other organic compounds into the soil that act as a primary food source or structural material for soil microbes (Walker et al., 2003). Soil microbes increase root exudation and plants allocate more carbon to roots (Canarini et al., 2019).

Plant chemical signaling strategies utilize flavonoids, strigolactones and terpenoids in exudates for the recruitment of microbes, and affect the surrounding rhizosphere (Dennis et al., 2010). When released into the soil through a process termed rhizodeposition, these chemicals attract beneficial and detrimental bacterial and fungal microbes (Jacoby et al., 2017). This can be a very specific process. For example, banana roots secrete fumaric acid, a chemo-attractant for certain plant growth-promoting *Bacillus* species (Yuan et al., 2015). Though exudates can target specific organisms, they generally attract a wide variety of beneficial, neutral, and detrimental soil microbes (Compant et al., 2019). Plants have evolved selective recognition mechanisms to allow beneficial microbes to colonize root structures. For example, arbuscular mycorrhizal fungi (AMF) grow within a plant's root cortex after receiving a chemical response from the host (Bais et al., 2006).

Microbes communicate with the roots and their signals can help plants determine if they are beneficial or pathogenic. Microbes release molecules in order to establish a mutualistic relationship, which are often in the form of volatile organic compounds (VOCs), and consist of a wide range of alkanes, alkenes, alcohols, ketones, terpenoids, and sulfur compounds (Mhlongo et al., 2018). VOCs are small molecules (< 300 Da) that easily diffuse through soil, making them a key component in communicating with plant roots (Tyc et al., 2015). Plant recognition of microbe VOCs is the first interaction between plants and microbes that colonize the rhizosphere (Lerdau et al., 1997). Using the model plant, *Arabidopsis thaliana,* it has been demonstrated that without physical contact VOCs can impact plant root system development, physiology, hormonal pathways, and biomass production (Bohm et al., 2017). Plants use fimbriae, fine hair-like projections with adhesions located on the tips, to connect with soil microbes through physical

contact (Lugtenberg et al., 2002). Colonization of plant tissue is a vital step for pathogenic and beneficial soil microbes (Schirawski and Perlin, 2018). Plant and microbe relationships can be categorized as endophytic (within plant roots), or rhizospheric (on or surrounding plant surfaces) (Imam et al., 2016). Rhizospheric microbes colonize the outer layer of root cells on the root surface (Compant et al., 2019). These microbes may colonize all root structures or a localized area as biofilms, which are densely packed communities of microbial cells growing on living surfaces (Ramey et al., 2004).

1.4. Beneficial Soil Microbes

Plant growth-promoting rhizobacteria (PGPR) are found within the rhizosphere and colonize plant roots. They make up a small percentage of microbes present in the soil, accounting for only 1-2%, and when grown in association with plants have been known to stimulate plant growth (Vessey 2003; Beneduzi et al., 2012). The most common genera include: *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Serratia* (Singh, 2011; Vejan et al., 2016). PGPR promotes plant growth through various mechanisms including decomposing organic matter, solubilization of mineral nutrients, recycling of essential elements, protecting against detrimental soil pathogens, improving soil structure surrounding plants, and regulating plant hormones, like jasmonic acid, to protect against insect herbivores (Gupta et al., 2017; Kundan et al., 2015; Rashid and Chung 2017). A list of select PGPR showing their various benefits for plant health and growth promotion are provided in Table 1.

Table 1. Plant growth-promoting rhizobacteria (PGPR) related to this study and their impact on various crop plants.

Microbial Inoculant	Crop	Finding	References
***BACILLUS* SPP. (PGPR bacteria)**			
B. subtilis	Wheat, *Triticum aestivum*	Increased productivity (yield)	Kumar et al., 2015
B. subtilis	Cotton, *Gossypium hirsutum*	Increased foliar and root growth	Medeiros et al., 2011
B. subtilis	*Arabidopsis*	Increased production of hormones in defense pathways: salicylic acid and jasmonic acid	Ryu et al., 2004
B. subtilis	Cotton, *Gossypium hirsutum*	Induction of 247 genes associated with nitrogen fixation	Baharlouei et al., 2011
B. subtilis	Banana, *Musa acuminata*	Biocontrol of *Fusarium*, a wilt causing soil pathogen, on root structures	Zhang et al., 2011
B. subtilis	Tulsi (basil), *Ocimum tenuiflorum*	Improved yield— plant biomass	Tiwari et al., 2010
B. megaterium	Drumstick tree, *Moringa oleifera*	Increased shoot/root length and dry weight	Zayed, 2012
B. megaterium	Clover, *Trifolium repens*	Drought resistance	Marulanda et al., 2009
B. polymyxa	Corn, *Zea mays*	High temperature and salinity stress resistance	Egamberdiyeva, 2007
B. pumilus	Canola, *Brassica napus*	Increased ACC-deaminase activity	Ansari et al., 2013

Table 1. Plant growth-promoting rhizobacteria (PGPR) related to this study and their impact on various crop plants. (Continued)

Microbial Inoculant	Crop	Finding	References
***PAENIBACILLUS* SP. (Nitrogen-fixing bacteria)**			
P. polymyxa	Watermelon, *Citrullus lanatus*	Biocontrol agent on *Fusarium*	Ling et al., 2011
P. polymyxa	Lodgepole pine, *Pinus contorta*	Increased levels of IAA, a growth regulator responsible for cell division and elongation, in roots	Sudha et al., 2012
***TRICHODERMA* SP. (Fungi)**			
T. harzianum	Wheat, *Triticum aestivum*; rice, *Oryza sativa*	Alleviation of soil salinity stress	Rawat et al. (2011)
T. harzianum	Tomato, *Lycopersicon esculentum*	Increased ISR (induced systemic resistance) defense activation	Moran-Deiz et al., 2009

1.4.1. Nitrogen-Fixing Bacteria

Nitrogen (N) is a major nutrient required for plant growth. It plays a key role in amino acid production, photosynthesis, and protein synthesis (Wagner, 2011). Nitrogen in the environment is found as atmospheric nitrogen (N_2), nitrate (NO_3^-), ammonium (NH_4^+), and ammonia (NH_3) (Booker, 2008). Roughly 80% of nitrogen is present in its atmospheric N_2 free state and is not biologically available for plant use, although it can be made available through biological nitrogen fixation (Mohommadi and Sohrabi, 2012). In soil, biologic nitrogen fixation

is conducted by endophytic or rhizospheric bacteria, and these organisms produce 180 x 10^6 metric tons of nitrogen annually via nitrogen-fixation (Wagner, 2011; Kundan et al., 2015). Nitrogen-fixing bacterial inoculants have been used on pulse crops such as field pea, chickpea, lentil, and soybean, and non-pulse crops including rice, switchgrass, pumpkin, and many others (Mahdi et al., 2010; Grady et al., 2016).

Some of the best studied examples of a microbe-plant symbiosis are between leguminous plants and nitrogen-fixing rhizobia, e.g., *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Backer et al., 2018). *Rhizobium* has been recognized for its benefits to legume plants for a very long time (Abdullahi et al., 2013). In the United States, *Rhizobium* received its first commercial patent as a nitrogen fixing product in 1896 (O'Callaghan, 2016). Field pea colonized by *Rhizobium* forms nodules that become sites for nitrogen fixation, and under favorable field conditions can supply 60-75% of the plant's accumulated nitrogen (Argaw and Mnalku, 2017; Mabrouk et al., 2018). Through this symbiotic relationship, legumes provide *Rhizobium* with carbohydrates and a safe environment to live in return for biologically available nitrogen (Backer et al., 2018).

In addition to *Rhizobium*, microbes in the genera *Azospirillum, Bacillus, and Pseudomonas* have been categorized as nitrogen-fixing organisms because they increase the bioavailability of nitrogen in the soil (Kundan et al., 2015; Igiehon and Babalola, 2017). The rod-shaped, endospore-forming *Bacillus* group, which can be separated into *Bacillus* and the closely related *Paenibacillus* bacteria, have been the most commercialized (Akrinrinlola et al., 2018). *Bacillus* have heat and desiccation tolerant endospores which increase cell viability and shelf life in storage (Gardener, 2004). As a rhizospheric nitrogen fixer, *Bacillus* forms thin bio-films on

root structures and produces nitrogenase to fix atmospheric N_2 while releasing ammonium from organic matter that can be used by plants (Ding et al., 2005; Hayat et al., 2010; Kuan et al. 2016; Hashem et al., 2019). Endophytic *Paenibacillus* colonize plant tissue and root surfaces and are recognized as a PGPR for their ability to fix nitrogen (Timmusk et al., 2005; Padda et al., 2016). *Paenibacillus* microbes use a molybdenum-dependent nitrogenase to catalyze the reduction of atmospheric N_2 to bioavailable ammonia (Grady et al., 2016).

1.4.2. Phosphorus Solubilizing Organisms

Like N, phosphorus (P) is a major growth-limiting nutrient for plant growth and production. Phosphorus plays an important role in photosynthesis, respiration, energy storage, cell division, and cell enlargement (Karpagam and Nagalakshmi, 2014). It is present within the rhizosphere in three forms: 1) organic phosphorus, commonly found in plant residues and manure, 2) soluble phosphorus, the form taken up by plants, and 3) bound phosphorus, which is dissolved phosphorus bound to a negative cation such as iron, aluminum, or calcium, and is therefore unavailable for plant uptake (Berg et al., 2018). Traditionally, people use fertilizers to increase phosphorus levels within the soil, however, much of this phosphorus is not readily available for plant usage. The phosphorus content within the rhizosphere is about 0.05%, with only 0.01% of that amount being soluble and available for plant use (Alori et al., 2017).

PGPR and certain fungal species, including arbuscular mycorrhizal fungi (AMF) and *Trichoderma* species, can solubilize phosphorus within the soil, making it readily available for plant uptake (Beneduzi et al., 2012). Phosphorus solubilization involves microbes releasing phosphatases and different types of organic acids to reduce the pH of the surrounding soil, thus releasing bound forms of phosphorus, and making them available for plants (Singh, 2011;

Radhakrishnan, 2017). Plants inoculated with phosphorus solubilizing microbes generally have improved growth and yield under greenhouse conditions (Karpagam and Nagalakshmi, 2014). Among bacteria, the three most studied genera of phosphate solubilizers are *Bacillus*, *Rhizobium*, and *Pseudomonas* (Kundan et al., 2015). A commercialized phosphate solubilizer, *Bacillus megaterium*, has been used to reduce crop dependence on phosphorus fertilizers by up to 75% (Backer et al., 2018).

Like bacteria, fungi secrete acids such as gluconic, citric, lactic, or acetic acids to solubilize inorganic phosphorus (Sharma et al., 2013). Fungi in the genus *Trichoderma* are known phosphorus solubilizers and can travel long distances within the soil to colonize plant roots (Alori et al., 2017). Once a plant has been colonized, *Trichoderma* spp. make phosphorus readily available for plant use, resulting in increased root development, proliferation of secondary roots, and crop yield (Hermosa et al., 2012). Beneficial mycorrhizal fungi have a high-affinity phosphorus uptake mechanism that enhances nutrient uptake in host plants (Kennedy and Stubbs., 2006; Adesemoye and Kloepper, 2009). To free bound phosphorus molecules AMF releases protons into the surrounding soil to mobilize phosphorus (Zhang et al., 2014; Bi et al., 2019). Phosphorus uptake is achieved by the AMF hyphae having a large surface area on which phosphorus is absorbed and delivered to the internal cortical root cells (Adesemoye and Kloeper, 2009; Bhardwaj et al., 2014).

1.4.3. Bio-Regulating Organisms

Biotic stress is a major constraint affecting the growth of agricultural plants. Many PGPR and fungal microbes protect crops from soil-borne pathogens through various mechanisms including inducing and synthesizing plant hormones, manipulating plant defense strategies,

producing antibiotics, competing directly with pathogens for nutrients and colonization space, and producing antimicrobial metabolites to inhibit pathogen growth (Hoitink and Boehm,1999; Whipps, 2001; Beneduzi et al., 2012; Köhl et al., 2019). Studies have shown that *Bacillus spp.* play a significant role in reducing plant stress through the synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which lowers plant ethylene levels (Hashem et al., 2019). Ethylene, a gaseous stress hormone, when found in high concentrations can increase plant stress responses resulting in premature senescence and reduce plant growth (Backer et al., 2018).

Trichoderma, spp. are well known for their ability to act as a biocontrol agents impacting soil pathogens through several direct mechanisms involving nutrient competition, mycoparasitism of phytopathogenic fungi, induction of plant systemic resistance, and production of extracellular hydrolytic enzymes (Akter et al., 2013; El-Katatny and Idres, 2014). Hydrolytic enzymes (chitinases, cellulases, and proteases) released by *Trichoderma* spp. degrade the cell walls of invading microbes killing them before they can infect their plant host (Gomes et al., 2017).

1.4.4. Co-Inoculation of Soil Microbes

Recently, the practice of using inoculants containing multiple microbial species has been used to increase the benefits provided to host plants (Santos et al., 2019). The success of this strategy comes from grouping beneficial microbes with different mechanisms to enhance plant growth and health (Trabelsi and Mhandi, 2013). For example, PGPR, including nitrogen-fixing and phosphate solubilizing microbes, work with *Rhizobium* to enhance nitrogen fixation by increasing plant root nodulation rates (Barea et al., 2005). Combinations of *A. brasilense* with *Bradyrhizobium* have been used in Brazil to increase the growth and grain yield of soybeans

(Hungria et al., 2015). Other studies have shown that there are soil fertility benefits when microbial bacteria and arbuscular mycorrhizal fungi (AMF) interact in the rhizosphere (Azćon-Aguilar and Barea, 2015). This is attributed to the bacterial microbes increasing the growth and germination rate of AMF by increasing root cell permeability (Artursson, 2005). Artursson (2005) found that inoculating clover with *Paenibacillus brasilensis* increased plant root colonization by *Glomus mosseae* (AMF).

1.5. Commercial Microbial Inoculants and Biofertilizers

Beneficial microbes hold immense potential to be commercialized into products that can benefit agricultural production (Kafle et al., 2019). A number of these living bacterial and fungal microbes have been named biofertilizers (Vessey, 2003; Mahanty et al., 2017). Specifically, biofertilizers are products containing living organisms that when applied to the soil or plant will promote plant growth through the acquisition of nutrients, induction of growth hormone production, and reduction in effect from pathogenic organisms (Mohammadi and Sohrabi, 2012; Herrmann and Lessuer, 2013). Biofertilizers are viewed as environmentally friendly, with microbes utilizing existing resources rather than relying on repeated applications of chemical treatments (Alori et al., 2017).

Formulations of microbial inoculants include powder or granular carriers, broth cultures, or liquid formulations (Albareda et al., 2008). Commercialization of microbial inoculants began in 1895 in the U.S.A. and U.K., with the first formulation being a powdered application (Brockwell and Bottomley, 1995). In 1953, peat cultures of rhizobia were used commercially in Australia (Davidson and Davidson, 1993; Deaker et al., 2004). Formulation type is a vital component to ensure the success of the microbes because the rhizosphere is a harsh, unforgiving

place. Although many microbial inoculants have seen success in a greenhouse setting it is not always replicated in field trials (Pereg and McMillen, 2015). There has even been decreased performance when microbe inoculated plants have been compared between greenhouse and field experiments (Bhattacharjee et al., 2008). When applying commercial microbial inoculants to seeds in a field setting there are many factors that can impact their efficacy. A key factor in the failure of commercial products is the rapid decline in microbe population after inoculation resulting in poor colonization (Khare and Arora, 2015). This can be caused by many of the abiotic (soil texture, pH, temperature, and moisture content) and biotic (antagonistic soil microbes and predators) stresses found in the soil ecosystem (Trabelsi and Mhandi, 2013). Studies have even shown that in rhizobial inoculants there can be competition with indigenous rhizobia populations, resulting in lower colonization rates and no added benefits from the inoculants (Thilakarathna et al., 2019).

Recently, the popularity of liquid microbial inoculants has increased, in part because they are easy to apply to seeds or the soil during planting (Herrmann and Lesueur, 2013). Liquid inoculants with higher microbe concentrations have provided increased benefits to legume host crops (Deaker et al., 2004; Schulz and Thelen, 2008; Herrmann and Lesueur, 2013). Liquid seed treatments have been shown to be equally successful in promoting plant health and growth as compared to powder and peat formulations (Brockwell et al., 1988; Deaker et al., 2004). Liquid treatments specifically tend to be more sensitive to environmental conditions compared to other formulations, but to protect the living organisms, manufacturers of microbial inoculants add certain elements to prevent product degradation. A carrier solution is added that acts as a medium for the organisms to survive and multiply (Madhi et al., 2010). Treated seeds should be

stored in containers at 5°C to prevent desiccation (Deaker et al., 2012). Some consider preventing the microbes from drying out to be the most vital factor in inoculation success (Herrmann and Lesuer, 2013). Powder and peat mixtures can be stored for up to three months at room temperature, while liquid inoculants need to be kept in a cool environment to remain viable for up to three months (Herrmann and Lesuer, 2013).

To increase the overall success of these treatments there are standards and regulations that are implemented. France has some of the strictest policies, including mandatory participation for applications of microbial inoculants and requiring that all organisms be grown in pure cultures and delivered with an application rate of 10^6 viable organisms per seed at planting (Bashan, 1998). Canada has similar rules requiring mandatory participation and an application rate between 10^3 to 10^5 viable cells per seed (Olsen et al., 1994). However, the U.S.A. lacks any regulation for the number of viable organisms per seed (Date, 2000). Generally, a standard application practice is to apply 10^3, 10^4, and 10^5 to small, medium, and large seeds respectively (Lupwayi et al., 2000). To establish the effectiveness of microbial inoculants, methods have been developed to analyze the quality of the liquid products from the distributors themselves. The two main methods include microscopic analysis and plate cell counts. A microscopic analysis includes using a Thoma slide that is filled with a broth solution in which the numbers of organisms are counted, and a plate cell count is restricted to a sterile carrier inoculant that is placed in an agar plate to be grown and counted (Lupwayi et al., 2000).

1.6. Field Pea (Fabaceae: *Pisum sativum* L.)

Field pea, *Pisum sativum* L. (Family Fabaceae) is a legume. Legumes are one of the largest and economically important plant families in the world, and are found on every continent

in the world, except Antarctica (Namestnik, 2014). Grain legumes account for 33% of dietary protein nitrogen demands in humans (Graham and Vance, 2003). Field pea is the second most important grain legume with its seed protein content ranging from 20-40%, and it serves as an important source of nutrition for humans and animals (Kumar and Paswan, 2014).

Field peas are categorized as annual, cool season, herbaceous plants (Oelke et al., 1990), and they exhibit indeterminate and determinate growth patterns. Indeterminate field peas are a climbing-type, and have an extended period of flowering, typically ranging from two to four weeks, which can be extended if the weather is cool and wet. In northern growing areas, indeterminate plants reach full plant maturity between 90 to 100 days (Bandillo et al., 2019). In contrast, field pea varieties with determinate growth are a bush or dwarf-type, have a shorter, fixed flower duration and plants reach maturity between 80 to 90 days (Bandillo et al., 2019). Field pea, *Pisum sativum* L., have a shallow root system with 75% of the biomass occuring in the top 20 cm of the soil (Bandillo et al., 2019). In root analysis experiments, researchers recovered field pea roots by digging down 100 cm to ensure they recovered the whole root system (Neilsen et al., 2001). Field pea roots are separated into a primary taproot that in early development rapidly produces lateral root branches to increase water and nutrient absorption (Weaver and Bruner, 1927).

Field pea establishes a symbiotic relationship with bacteria from the genera *Rhizobium* and *Bradyrhizobium* (Loh and Stacy, 2003). In agricultural practices, this symbiosis is viewed as a more efficient method for ensuring the proper supply of nitrogen for legume crops (Zahran, 1999). When applied correctly, *Rhizobium* can derive up to 80% of the required nitrogen supply for field peas through nitrogen fixation (Erman et al., 2008). Legumes require fewer additions of

fertilizers due to this relationship when compared to non-nitrogen fixing plants (Magrini et al., 2016). It is estimated that to replace the amount of nitrogen provided by rhizobia it would cost U.S. farmers roughly $7-10 billion dollars annually (Graham and Vance, 2003).

Establishing a symbiotic relationship between field pea and nitrogen-fixing bacteria begins with plant signaling through flavonoids that act as a chemoattractant (Skorupska et al., 2010). The bacteria then enter the root through infection threads while secreting nodulation genes that reduce plant defense mechanisms (Perret et al., 2000). Root cortical cells are mitotically activated by the nodulation genes resulting in the formation of root nodules (Tricot et al., 1997). The nodulation process takes roughly 3 to 4 weeks before they are fully functioning. Once *Rhizobium* are successfully inside the plant root tissue, they are enclosed in a membrane-bound symbiosome where they are differentiated into bacteroides capable of nitrogen fixation (Skorupska et al., 2010). The nitrogen fixation process consists of converting atmospheric nitrogen (N_2) into ammonium (NH_4), which is readily available for plant usage (Tricot et al., 1997).

1.7. Impact of Microbial Inoculants on Floral Resources for Insects

Roughly one-third of global agricultural crops rely on some form of pollination (Hellerstein et al., 2017), and approximately 75% of food crops are partially dependent on insect pollination with increases in crop yield, profit and stability tied to pollinator activity and diversity (Klein et al., 2007; Hoehn et al., 2008). Legumes are primarily self-pollinated, but when insect-pollinated they can have increased productivity and yield (Gepts et al., 2005; Milfont et al., 2013). Studies have shown that in soybean, native pollinators play an important role in pollination and increasing plant yield (Milfont et al., 2013). Recent pollinator decline has

been observed throughout the world and has been attributed to a loss or fragmentation of habitat, agrochemicals, pests and pathogens, resulting in a decline in pollinator services in agricultural and natural settings (Biesmeijer et al., 2006; Potts et al., 2010). Agricultural lands can supply insect pollinators with floral resources and nesting grounds (Tsharntke et al., 2005; Suso et al., 2016; Guzman et al., 2019), and increasing habitat and floral resources within agricultural fields could greatly benefit insect pollinator populations (Blaauw and Isaacs, 2014). Scriven et al. (2013) showed that insect visitation is predominantly influenced by flower density in an area, thus increasing the floral resources in agricultural fields could influence pollinator visitation. Pollinator visitation to flowers is also greatly influenced by flower size, display, color, scent, and number (Lebel et al., 2018).

Beneficial microbes, like arbuscular mycorrhizal fungi (AMF), *Trichoderma* fungi, and *Bacillus* bacteria are present within the flower tissue and on the flowers surface, which greatly influence a plant's ability to reproduce by altering nectar quality and visual cues used to attract pollinators (Robelleda-Gomez et al., 2019). These microbes have also been used to increase flower number, size, and pollen produced which can increase pollinator visitation rates (Gange and Smith, 2005; Lebel et al., 2018). Specifically, *Bacillus subtilis* has been used to increase the flower number and stigma size produced from saffron corms (Sharaf-Eldin et al., 2008). Other floral microbes act as a physical barrier and are a source of competition against pathogenic microbes (Olanrewaju et al., 2017).

1.8. Interactions between Collembola and Soil Microbes

Order Collembola (springtails, Arthropoda: Hexapoda: Entognatha) are found world-wide and most are associated with the soil environment in some way (Christiansen, 1990;

Hopkin, 1997). They are among the most abundant soil arthropods (Seastedt, 1984; Hopkin, 1997), with reported densities per m^2 of soil from 1×10^3 in crop fields (Coleman et al., 2004) to 1×10^5 in forests (Hopkin, 1997). Soil-dwelling Collembola are considered microbi-detritivores that primarily feed on fungi or decaying plant material (Christiansen, 1990; Hopkin, 1997; Rusek, 1998; Coleman et al., 2004), and their activity can impact plant growth and flowering time (Forey et al., 2015). Although some Collembola directly affect plants via root consumption (Hopkin, 1997; Endlweber et al.; 2009), most affect plants indirectly by influencing organic matter decomposition and nutrient cycling or regulating fungal dynamics (Warnock et al., 1982; Coleman et al., 2004; Culliney, 2013). Lussenhop (1993) found the presence of Collembola affected the species of N-fixing bacteria associated with soybean roots, and Collembola have also been shown to consume AMF hyphae and spores (Warnock et al., 1982; Moore et al., 1985; Thimm and Larink, 1995; Jonas et al., 2007). The presence and activity of Collembola impacts soil microbial communities and their function (Tiunov and Scheu, 2005; Caravaca and Ruess, 2014; Coulibaly et al., 2019).

CHAPTER 2. IMPACT OF SOIL MICROBIAL INOCULANTS AND COLLEMBOLA ON GROWTH AND BIOMASS ALLOCATION OF FIELD PEA UNDER GREENHOUSE CONDITIONS

2.1. Introduction

The human population is predicted to reach ten billion people by 2050 and it is estimated that a 70% increase in food production per year is needed to feed a population that large (Abhilash et al., 2016; United Nations, 2017). With an increasing human population, the demand for food required to sustain it can only be achieved by increasing agricultural productivity (Ahmad et al., 2018; Fróna et al., 2019). Increasing crop production through land conversion of natural ecosystems to agricultural fields in the past has been successful, but a major consequence involves a decrease in natural habitat for plants and animals (Tsharntke et al., 2005). There has been a 700% increase in fertilizer use globally in the last 40 years (Foley et al., 2005). However, fertilizer use is often unsustainable and can negatively impact soil health by causing groundwater pollution, lowering soil pH, and decreasing soil fertility (Gupta et al., 2017; Souza et al., 2015; Alori et al., 2017). A possible sustainable solution is ecological intensification, which focuses on maximizing crop outputs by implementing new and innovative ecosystem services to increase plant health and growth (Hubert et al., 2010; Dore et al., 2011).

One aspect of ecological intensification aims to manage soil microbes that directly or indirectly impact plant and crop production (Bommarco et al., 2013). Soil microbial inoculants contain beneficial microorganisms and are a potential alternative to chemical fertilizers and pesticides (Alori et al., 2017), with modern inoculants often containing multiple microbial species (Santos et al., 2019). Reported effects of soil microbial inoculants on plants include

increased foliar growth, longer shoots, heavier biomass, and increased yield (Tiwari et al., 2011; Medeiros et al., 2011; Zayed, 2012; Kumar et al., 2015). Microbial inoculants can promote plant growth and health directly by enhancing plant mineral nutrition and synthesizing compounds needed for growth and defense, and indirectly by improving surrounding soil structure, repelling or competing against pathogenic soil microbes, and impacting plant defense against herbivorous arthropods (Benduzi et al., 2012; Goh et al., 2013; Jacoby et al., 2017).

The rhizosphere is a dynamic and complex environment, and interactions among plants, microbes associated with plant roots, and soil arthropods are common (Jacoby et al., 2017). Collembola (springtails) are among the most abundant soil arthropods within the rhizosphere (Seastedt, 1984; Hopkin, 1997). Collembola impact the rhizosphere by directly consuming bacteria, fungi, and other organic material (Culliney, 2013). Onychiuridae, are categorized as having a generalist diet mainly consisting of numerous fungal species found within the soil (Sadaka-Laulana et al., 1998). When consuming fungi on root structures, Collembola have been known to damage or even kill young seedlings (Hopkin, 1997; Boetel et al., 2001). Successful germination and seedling establishment is a crucial time for young legume plants and damage to root structures could have lasting effects (Edwards, 1962; Bezerra de Melo et al., 2015).

We examined how the identity and density of commercial microbial inoculants and the presence of fungal-feeding Collembola impacted early growth of field pea under greenhouse conditions. Our predictions were that the addition of microbial inoculants would increase above- and belowground field pea biomass, inoculated plants would have a greater proportion of biomass in their root systems, and that the presence of Collembola would negatively affect plant

biomass by causing damage to plant roots and causing a in shift biomass distribution towards above-ground structures.

2.2. Materials and Methods

Greenhouse experiments were conducted to quantify the effects of microbial inoculant identity, dosage, and species combination on field pea growth and biomass allocation under controlled conditions and in the presence of Collembola. Greenhouse trials focused on exploring treatment effects on plants in early vegetative growth stages (*i.e.*, 2 to 4 weeks).

2.2.1. Experimental Organisms

2.2.1.1. Plants

Experiments focused on field pea, *Pisum sativum* L. (Fabaceae), variety Nette 2010, which is a yellow cotyledon type with small-medium seed size, early to medium maturity with determinate growth, and very good to excellent standability (Pulse USA). We used seed harvested in 2017 from field plots established at the North Dakota State University (NDSU) Research and Extension Center at Carrington, ND. Seeds were stored in 22 kg brown paper bags at room temperature (20 ± 1˚C, 40 ± 5%RH). Individual seeds were counted using a Syntron Magnetic Bowl Feeder (model EB00E; FMC Corporation, Homer City, PA).

2.2.1.2. Microbes

Microbial inoculants were purchased from Custom Biologicals (Deerfield Beach, FL, http://living-soils.com/custom-biologicals/) ≤ six months prior to use and products were purchased at different times of the year including February, July, April, and September. Formulated products were: 1) B5, blend of *Bacillus subtilis, B. laterosporus, B. licheniformus, B. megaterium, B. pumilus* bacteria, 2) GP, blend of *Trichoderma harzianum, T. koningii, T.*

polysporum, *T. viride* fungi, and 3) N2, *Paenibacillus polymyxa* bacteria. The B5 and GP inoculants were liquid formulations (50 mL bottles) that consisted of 79.9% water, 0.01% citric acid, and 20% of the previously stated bacteria and fungi, respectively. The N2 inoculant was a liquid formulation (50 mL bottle) that consisted of 80% water and 20% *P. polymyxa*. According to the company label, each inoculant consisted of 100,000,000 colony forming units (CFUs) of viable organisms per mL of liquid, although for multi-species formulations, the relative proportion of each organism was not indicated. Bottles were stored unopened in a refrigerator (5 ± 1˚C, 80% RH) until used for seed inoculation.

Seeds were surface-sterilized by placing them in a 5% (*v/v*) bleach solution (Clorox® Regular-Bleach, The Clorox Company, Oakland, CA) and agitating for 2 min by hand, after which seeds were rinsed with distilled water six times and spread out on sterile trays to dry overnight. Dried seeds were placed in sterile plastic, self-sealing bags and inoculated with microbes within 24h.

To add a specific amount of microbial CFUs to each seed, we manipulated the volume of inoculant added to a specific number of seeds or vice versa (Tables 2 to 4). We pipetted the inoculant onto the seeds contained within a sterile self-sealing plastic bag, then pipetted distilled water into each bag as a carrier medium to distribute microbes more uniformly among the seeds. Bags were then sealed and manipulated by hand for 1 min to mix the seeds and liquid to ensure each seed received an even coating of liquid. Seeds were stored in a cooler with ice packs (6 ± 1°C) while transported to the greenhouse for planting.

2.2.1.3. Collembola

Collembola were field collected and maintained in lab colonies for use in the greenhouse experiment. Soil was collected from a NDSU research farm at Prosper, ND (N 47.00191 W 97.10885) in the summer of 2018. Soil was placed in Berlese funnels (with a 25-watt light bulb) for 7 d. Arthropods were extracted into glass jars with a moistened plaster base to prevent desiccation. Live Collembola were separated from other arthropods by examining jars under a dissecting microscope (Nikon SMZ-2B, Nikon Inc., Melville, NY). Collembola with a whitish color, cylindrical body, that lacked a furcula were transferred using a moistened paintbrush to plastic containers (64 oz Natural Polypropylene Plastic Round Snap-Lock Containers, Berlin Packaging, Chicago, IL) with a 1.5 mL moistened plaster base. Each container was filled with 100 g of PRO-MIX LP15 (Premier Tech Horticulture, Richer, Manitoba, Canada) growing medium. The medium contained: 80-90% sphagnum peat moss, dolomitic limestone, calcitic limestone, perlite, and micro and macronutrients. Medium characteristics were: 5.2-6.2 pH, EC 1.0-1.8 mmhos/cm, 70-130 ppm (mg/L) NO^3-N, and 5-40 ppm (mg/L) PO^4-P. Twice a month, 2 g of active dry yeast solution (Fleischmann's, ACH Food Companies, Inc., Oakbrook Terrace, IL) was added as a food source. One ml of distilled water was added to each container once a week. Colonies were maintained at room temperature in a dark environment (20 ± 1˚C, 40 ± 5% RH). Representative specimens were sent to the NDSU Plant Diagnostics lab for identification and were identified as, *Onychiurus* sp. (Onychiuridae).

2.2.2. Design, Plant Establishment and Maintenance

Plants were grown and experiments conducted under greenhouse conditions (16:8 L:D, 23 ± 1˚C, 35 ± 5% RH) throughout the calendar year (Table 2). In the summer (*e.g.*, June) the

average temperature and relative humidity were 25.8˚C and 58.6%, and in the winter (*e.g.*, February) an average temperature of 23.5˚C and 34.4% relative humidity were recorded. The light source used in all experiments were 600-watt high pressure sodium lamps (P. L. Light Systems, Inc., Beamsville, Ontario, Canada). Field pea plants were grown in 2 L plastic pots (13 cm tall x 17 cm diam.) filled with 200 ± 20 g (1,800 mL) of PRO-MIX LP15 growing medium, which was lightly tamped down by hand before planting. However, due to the short duration of the experiment, plants in the two-week experiment on impacts of inoculant identity on field pea were grown in plastic cone-tainers (20 cm tall x 3.75 cm diam.; CN-SS-SCTR98, Greenhouse Megastore, Danville, IL) filled with 26 ± 2 g (175 mL) of PRO-MIX LP15 growing medium.

We wore nitrile gloves which were changed in-between handling seeds from different treatments to prevent inoculant cross contamination. One inoculated seed per pot was pushed 5 cm into the soil, loosely covered with additional soil, and lightly tamped down. The 2 L pots were randomly placed 2.54 cm apart on plastic bar grated tables, thus allowing water to run through the table and reducing the likelihood of microbes moving among pots during watering. Each 2 L pot received 300 mL of distilled water every 3 d for the duration of the experiment. Distilled water was stored in a 10-gal carboy and treated with 1 mL of API® Tap Water Conditioner (Mars Fishcare North America, Chalfont, PA), to remove any chlorine and detoxify heavy metals that may have been present. Cone-tainers were placed in a plastic holding tray (25.4 cm width x 35.6 cm length) with their location randomized once every week. Each cone-tainer received 100 mL of API conditioned distilled water every 3 d for the duration of the experiment. Due to the short duration of the experiments, none of the plants in any of the greenhouse experiments was fertilized.

Table 2. Planting and harvesting dates for the greenhouse experiments.

Experiment	Planting date	End date
Impact of inoculant density		
B5, *Bacillus*	23 Feb 2018	26 March 2018
N2, *Paenibacillus*	23 Feb 2018	26 March 2018
Impact of inoculant identity		
two-week cone-tainer	12 June 2018	26 June 2018
four-week, winter block	23 Feb 2018	23 March 2018
four-week, summer block	12 June 2018	9 July 2018
Impact of inoculant identity and Collembola		
pots sampled at two weeks	4 April 2019	2 May 2019
pots sampled at four weeks	4 April 2019	2 May 2019

2.2.3. Experimental Treatments

2.2.2.1. Impact of microbial inoculant density on field peas

We investigated how the number of CFUs per seed (*i.e.*, dose, or concentration) of two microbial inoculants, B5 (*Bacillus* spp.) and N2 (*Paenibacillus* sp.), impacted field pea growth and biomass allocation in two separate experiments. We established dosage treatments by pipetting 0.25 mL of either B5 or N2 onto a variable number of seeds (Table 3): 1) 0K: no inoculants added, distilled water only, 2) 20K: target of 20,000 CFUs per seed, 3) 40K: target of 40,000 CFUs per seed, 4) 80K: target of 80,000 CFUs per seed, and 5) 160K: target of 160,000 CFUs per seed. We used a completely randomized design with five replicates of each treatment.

Table 3. Seed inoculation details for the greenhouse microbial density experiments.

Inoculant density treatment	Target CFUs per seed	# of seeds	Distilled water (mL)	Inoculant (mL of B5 or N2)
0K	0	625	6 mL	0 mL
20K	20,000	1250	7.75 mL	0.25 mL
40K	40,000	625	5.75 mL	0.25 mL
80K	80,000	312	3.75 mL	0.25 mL
160K	160,000	156	1.75 mL	0.25 mL

2.2.2.2. Impact of microbial inoculant identity on field peas

We conducted two greenhouse experiments to investigate how microbial inoculants, both separately and in combination, affected growth and biomass allocation of field pea plants during early vegetative growth. The first experiment (two-week cone-tainer experiment) ended 14 days after planting (DAP), and the second experiment (four-week experiment) ended 27-28 DAP. We used a completely randomized design, and there were five inoculant treatments: 1) CON, control with distilled water only, 2) B5 only, 3) GP only, 4) N2 only, and 5) MIX, a mixture of all three inoculants that contained equal parts B5 + GP + N2. The target CFUs per seed for individual inoculants was 34,500 (Table 4). The first experiment had 19 replicates of each treatment. The second experiment was run in two blocks, the first in February 2017 (winter block) and the second in June 2018 (summer block), with 10 replications of each treatment in each block.

Table 4. Seed inoculation details for the greenhouse microbial identity experiments.

Microbial inoculant treatment	Target CFUs per seed*	# of seeds	Seed weight (g)	Distilled water (mL)	Inoculant (mL)
CON	0	2,900	680 ± 20	15 mL	0 mL
B5	34,500	2,900	680 ± 20	14 mL	1.0 mL B5 only
GP	34,500	2,900	680 ± 20	14 mL	1.0 mL GP only
N2	34,500	2,900	680 ± 20	14 mL	1.0 mL N2 only
MIX	103,500	2,900	680 ± 20	12 mL	1.0 mL B5 + 1.0 mL GP + 1.0 mL N2

*Rounded up from 34,483 and 103,448

2.2.2.3. Impact of microbial inoculant identity and Collembola on field peas

We investigated effects of individual microbial inoculants and the addition of Collembola (Onychiuridae, *Onychiurus*) on short-term vegetative growth of field pea under controlled environmental conditions. We used a factorial design, with two levels of Collembola: absent (*n* =

0) or present (*n* = 20 per pot) crossed with inoculant treatment: 1) CON, control with distilled water only, 2) B5 only, and 3) GP only. The target CFUs for individual inoculants was 34,500 per seed (Table 5). The experiment was a completely randomized design with 20 replicates of each treatment, with 10 replicates destructively sampled 14 DAP and the remaining 10 replicates destructively sampled 28 DAP.

Table 5. Seed inoculation and arthropod treatment details for the greenhouse microbial identity and Collembola experiment.

Collembola density	Microbial inoculant treatment	Target CFUs per seed*	# of seeds	Seed weight (g)	Distilled water (mL)	Inoculant (mL)
Coll- None	CON	0	2,900	680 ± 20	15 mL	0 mL
Coll+ 20 per pot	CON	0	2,900	680 ± 20	15 mL	0 mL
Coll- None	B5	34,500	2,900	680 ± 20	14 mL	1.0 mL B5 only
Coll+ 20 per pot	B5	34,500	2,900	680 ± 20	14 mL	1.0 mL B5 only
Coll- None	GP	34,500	2,900	680 ± 20	14 mL	1.0 mL GP only
Coll+ 20 per pot	GP	34,500	2,900	680 ± 20	14 mL	1.0 mL GP only

*Rounded up from 34,483

At the beginning of the experiment, mature large bodied Collembola were collected from the colonies with a paintbrush and 20 individuals were added to a 1.5 mL microcentrifuge tube with 0.5 mL of PRO-MIX LP15 soil for the Coll+ treatment. Control plants without Collembola (Coll-) only received 0.5 mL of PRO-MIX LP15 soil. Microcentrifuge tubes containing Collembola and growing medium were placed in a refrigerator (5 ± 1˚C, 80% RH) for 7 d prior to use in the experiment. Collembola were added 7 DAP by emptying the containers directly onto the field pea seed cotyledon. Each pot was immediately enclosed in a plastic Delnet®

Apertured Film bag (40.6 cm x 45.7 cm, SWM International, Alpharetta, GA) to prevent Collembola moving among pots. We used bamboo skewers (30 cm long) sunk into the soil to prop up bags to allow unrestricted plant growth and used rubber bands to secure bags to the base of the pots. Rubber bands and bags were removed during watering and data collection but were immediately reapplied.

2.2.4. Data Collection

Plant germination for all experiments was recorded 7 DAP by measuring whether the soil surface had been broken by the cotyledon. Plants in the impact of microbial inoculant density experiment were destructively sampled 31 DAP. Plants in the impact of microbial inoculant identity experiment were destructively sampled 14 DAP (two-week cone-tainer experiment), or 27 DAP (four-week experiment, summer block), or 28 DAP (four-week experiment, winter block). Destructive sampling involved removing the top layer of soil and cutting each stem at the base of the hypocotyl directly above the roots. Height of the above-ground plant material (*i.e.*, shoot) was recorded by using a ruler to measure from the cut above the hypocotyl to the shoot apex. Root systems were removed from pots and excess growing medium removed by hand. Roots were gently rinsed over a metal sieve (710 μm opening, U.S.A. standard testing sieve no. 25), and the main tap root separated from lateral roots with scissors. The length of the main tap root was measured from the base of the hypocotyl to the root calyptra using a ruler. Total plant length was calculated by combining the above-ground height and the length of the main tap root. Taproots and lateral roots from the two-week and four-week (summer block) were placed under a dissecting microscope (Nikon SMZ-2B) and root nodules were counted. There were no nodules present on plants from the four-week winter block experimental run. All shoot and root samples

were put in separate paper bags and placed in a drier at 70°C for 7 d to bring them to a constant dry mass. Samples were weighed (Type 1412, Sartorius Digital Balance, Brinkmann Instruments Co., Westbury, NY) immediately after being removed from the drier.

For the experiment on impact of microbial inoculant identity and Collembola on field pea, the height of plant above-ground material (*i.e.*, shoots) was assessed at 14, 21, and 31 DAP using a ruler. Plant height was quantified by measuring from the base of the hypocotyl directly above the root systems to the shoot apex. Growing medium was gently removed in this process to expose the hypocotyl of the plant and after the measurement was taken the soil was replaced. Rulers were rinsed in a 5% bleach solution (Clorox® Regular-Bleach, The Clorox Company, Oakland, CA) in-between each measurement.

In order to compare treatment effects at different time points between plants experiencing the same growing conditions, half the plants were destructively sampled 14 DAP and the remainder sampled 31 DAP. Plants were destructively sampled as previously described, but soil that was removed from the plant roots and pots was placed into separate, labeled, self-sealing plastic bags. Soil was then emptied into individual Berlese funnels (with a 25-watt lightbulb) for 7 d to extract arthropods and assess arthropod density, reproduction, and survival. Arthropods from plants destructively sampled at 14 DAP were extracted into plastic containers with 25 mL of 90% ethanol to kill and preserve all organisms. However, plastic containers reacted to the 90% ethanol and some samples were lost. Thus, for experimental plants destructively sampled at 31 DAP, arthropods were extracted into glass vials filled with 25 ml of 90% ethanol. All recovered arthropods (*i.e.*, white Collembola added to pots and contaminants) were counted and identified using a dissecting microscope (Nikon SMZ-2B, Nikon INC., Melville, NY) and

dichotomous identification keys (Dindal 1990). Recovered Collembola were separated into two categories: white Collembola that we had added (Onychiuridae, *Onychiurus* sp.) and gray Collembola (Hypogastruridae).

2.2.5. Statistics

Histograms, Bartlett's test for equality of variance, and residual plots were used to assess data normality. Data were analyzed using JMP®13 (SAS Institute 2016) at $\alpha = 0.05$. We considered p-values between 0.06 and 0.1 to be marginal. To investigate treatment effects on above versus belowground biomass allocation, root mass was converted to a mass fraction of total plant biomass (*i.e.*, root mass relative to total plant biomass; Evans 1972) and arcsine transformed prior to analysis.

2.2.5.1. Impact of microbial inoculant density on field peas

Data from the two experiments using different microbial inoculants, *i.e.*, *Bacillus* (B5) and *Paenibacillus* (N2), were analyzed separately. We did not need to transform the data and one control plant in the B5 experiment did not emerge and was excluded from all analyses. We used visual exploration of the data to determine whether least squares linear regression or polynomial (second order quadratic, third order cubic) regression was most appropriate for each data set. Dosage rate (first, second or third order) was the continuous independent variable and the following response variables were analyzed separately: total plant dry mass (above + belowground biomass), total root dry mass (taproot + lateral root biomass), taproot dry mass, lateral root dry mass, total plant length (shoot + taproot length), the mass fraction of plant biomass allocated to roots (arcsin transformed). Replicate was included as a random effect.

2.2.5.2. Impact of microbial inoculant identity on field peas (two-week cone-tainer experiment)

ANOVA followed by Tukey's Honest Significant Difference (HSD) *post hoc* tests for means separation were used to analyze data on dependent variables related to field pea growth (*e.g.* height, biomass, biomass distribution, number of root nodules), with microbial inoculant (MI) as a fixed effect (independent variable) and replicate as a random effect. Data from each dependent variable were analyzed separately. Dependent variables: Shoot weight and length, root weight and length, total plant weight, weight fraction of shoot versus root biomass allocation, and total root nodules were analyzed separately. Root nodule data were log transformed prior to analysis to meet model assumptions.. Contingency analysis and Pearson's correlation coefficient were used to determine effects of the independent variable (*i.e.*, microbial inoculant) on the likelihood of nodule presence at the end of the experiment (categorical binomial response variable of nodules present, yes or no). Seven data points were removed from all analyses due to two plants not germinating (CON: $n = 1$, MIX: $n = 1$), substantially delayed germination and growth of four other plants (CON: $n = 2$, B5: $n = 1$, N2: $n = 1$), and one plant being damaged during processing (B5: $n = 1$).

2.2.5.3. Impact of microbial inoculant identity on field peas (four-week experiment)

Factorial ANOVA followed by Tukey's HSD *post hoc* tests for means separation were used to analyze data on dependent variables related to field pea growth (*e.g.* height, biomass, biomass distribution, number of root nodules), with microbial inoculant (MI), experimental block, and their interaction as a fixed effects (independent variables) and replicate as a random effect. When the experimental block was found to be significant ($P < 0.05$), ANOVA was used

to analyze data from each individual experimental run (*i.e.*, the winter block or the summer block) separately. Because only plants in the summer block nodulated, root nodule data were log transformed with microbial inoculant as the sole independent variable and replicate as a random variable. Contingency analysis and Pearson's correlation coefficient were used to determine effects of the independent variable (*i.e.*, microbial inoculant) on the likelihood of nodule presence at the end of the experiment (categorical binomial response variable of nodules present, yes or no). Five data points were removed from the winter block data set due to two plants not germinating (CON: $n = 1$, N2: $n = 1$) and substantially delayed germination and growth of three other plants (CON: $n = 1$, B5: $n = 2$).

2.2.5.4. Impact of microbial inoculant identity and Collembola on field peas

Arthropod sampling dates at 14 DAP and 31 DAP dependent variables Onychiuridae (white) and Hypogastruridae (gray) were analyzed using a factorial ANOVA. Microbial inoculant (MI) was the independent variable, harvest date and Collembola addition (Coll+-) were covariates, and replicate was a random variable. When harvest date was found to be significant ($P < 0.05$) dependent variables were analyzed separately using a factorial ANOVA with MI as the independent variable, Collembola addition (Coll+-) as a covariate, and replicate as a random variable. Samples at 14 DAP were lost due to deterioration of the plastic containers caused by the alcohol used to preserve specimens. Data from samples that were lost were excluded from all analyses: CON (Coll-): n = 5, CON (Coll+): n = 5, B5 (Coll-): n = 6, B5 (Coll+): n = 5, GP (Coll-): n = 6, and GP (Coll+): n = 5.

Dependent plant variables at 14 DAP and 31 DAP including: aboveground weight and length, belowground weight and length, total plant weight and length, and belowground biomass

allocation percentage were analyzed separately using a factorial ANOVA with MI as the independent variable, Coll +- as a covariate, and replicate as a random variable. At 14 DAP one plant did not emerge and was excluded from all analysis: GP (Coll-): n = 1.

2.3. Results

2.3.1. Impact of Microbial Inoculant Density on Field Peas

2.3.1.1. *Bacillus* experiment

In the *Bacillus* (B5) experiment, the relationships between B5 dose and plant responses were non-linear. For total plant biomass and below-ground biomass there was a third-order, or cubic relationship between the variables (Table 6). For total plant weight and belowground weight, low and extremely high doses of B5 resulted in lower plant biomass compared to the control, whereas intermediate doses had slightly heavier weights compared to the control (Fig. 1a; $P = 0.064$; Fig. 1b; $P = 0.009$). For total plant length, there was a second-order, or quadratic relationship between the variables, with intermediate doses of B5 having longer total plant length (Fig. 1c; $P = 0.027$). There was no difference between plant biomass allocation and B5 dose (Fig. 1d; $P = 0.251$).

Table 6. Regression analysis on the impact of B5 (*Bacillus*) density on field pea growth and biomass allocation in a short-term (four-week) greenhouse experiment.

Variable	df	Adj. R^2	Term	*t*-Ratio	*P*-value
Total plant weight (above + belowground)	3, 16.0	0.135	Linear	2.26	**0.039**
			Quadratic	0.40	0.696
			Cubic	-2.00	*0.064*
Total belowground weight (taproot + lateral roots)	3, 16.3	0.293	Linear	2.59	**0.020**
			Quadratic	1.92	*0.072*
			Cubic	-2.96	**0.009**
Taproot weight	1, 17.9	0.486	Linear	2.89	**0.010**
Lateral root weight	3, 16.5	0.261	Linear	2.48	**0.024**
			Quadratic	1.88	*0.078*
			Cubic	-2.90	**0.010**
Total plant length (shoot + taproot)	2, 17.4	0.043	Linear	1.47	0.159
			Quadratic	-2.41	**0.027**
% Belowground biomass allocation[1]	2, 18.1	-0.383	Linear	-2.27	**0.035**
			Quadratic	1.19	0.251

[1] = arcsine transformed
Bold values indicate significance at $P = 0.05$
Italic values indicate marginal significance at $P > 0.05$ and < 0.10

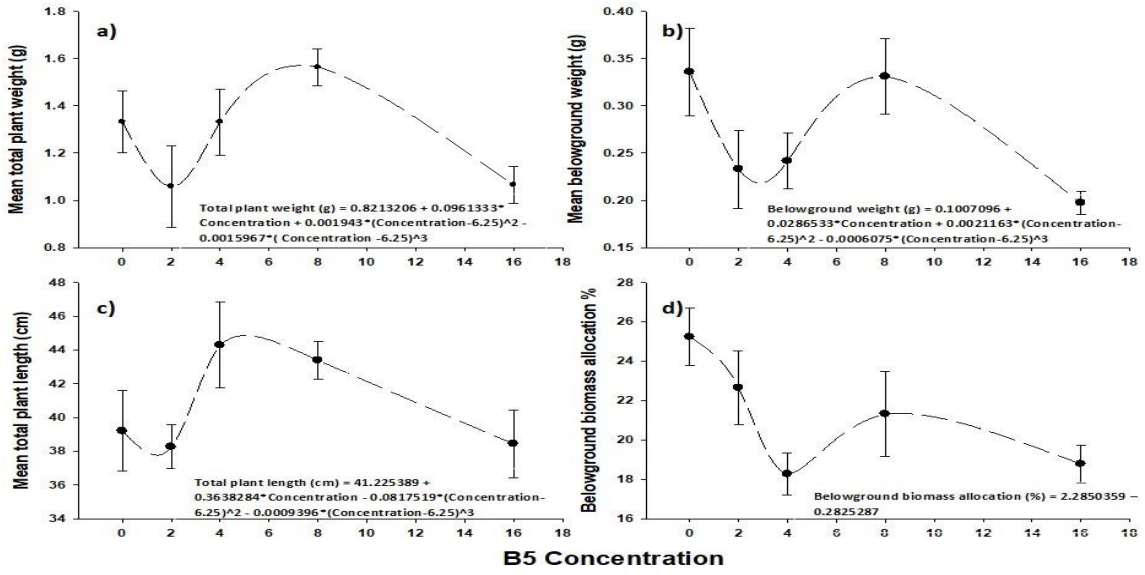

Figure 1. Impact of B5 (*Bacillus*) inoculant density in a four-week greenhouse experiment on: a) total plant weight, b) belowground weight (taproot + lateral roots), c) plant length (shoot + taproot), and d) percent of total plant biomass allocated to roots.

2.3.1.2. *Paenibacillus* experiment

In the *Paenibacillus* (N2) experiment, the relationships between N2 dose and plant responses were linear. For total plant weight and length, there was no effect of N2 dose (Table 7). There were significant differences in belowground weight with heavier root weights as N2 dose increased (Fig. 2b, $P = 0.038$). There was a marginal N2 dose effects on plant biomass allocation with more biomass in belowground structures as N2 dose increased (Fig. 3d, $P = 0.073$).

Table 7. Linear regression analysis on the impact of N2 (*Paenibacillus*) inoculant density on field pea growth and biomass allocation in a short-term (four-week) greenhouse experiment.

Variable	df	Adj. R^2	*t*-Ratio	*P*-value
Total plant weight (aboveground + belowground)	1,19	-0.20	1.15	0.264
Total belowground weight (taproot + lateral roots)	1,19	0.38	2.23	**0.038**
Taproot weight	1,19	0.24	2.09	**0.050**
Lateral root weight	1,19	0.37	2.13	**0.047**
Total plant length (shoot + taproot)	1,19	-0.17	1.28	0.215
% Belowground biomass allocation[1]	1,19	0.36	1,90	*0.073*

[1] = arcsine transformed

Italic values indicate marginal significance at $P > 0.05$ and < 0.10

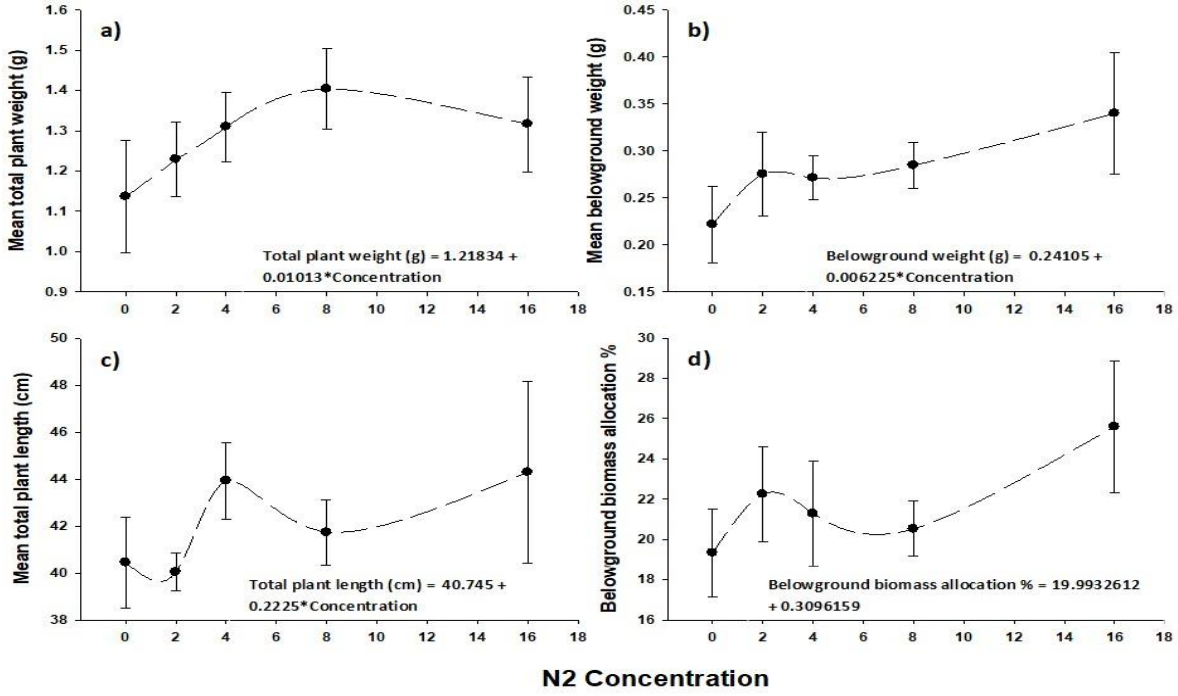

Figure 2. Impact of N2 (*Paenibacillus* sp.) inoculant density in a short-term (4 week) greenhouse experiment on: a) total plant weight, b) belowground weight, including tap and lateral root weight, c) total plant length, including length of below- and above-ground structures, and d) belowground biomass allocation percent.

2.3.2. Impact of Microbial Inoculant Identity on Field Peas

2.3.2.1. Two-week cone-tainer experiment

This experiment investigated how microbial inoculant identity, either alone or in combination, impacted field pea biomass and biomass allocation during the early stages of field pea growth. Inoculant identity impacted total plant biomass (Fig. 3a; Table 8), with control plants weighing more than plants inoculated with B5 (*Bacillus* spp.; $P = 0.049$). This was driven by heavier roots ($P = 0.0003$), as there were no differences in aboveground biomass among treatments ($P = 0.709$). Control plants had heavier tap roots compared to all other treatments (Fig. 3b; $P < 0.0001$), and their lateral roots weighed more than plants inoculated with B5 ($P =$

0.035). On average, 48.6% of plant biomass was in belowground structures. Inoculant identity affected biomass allocation (data not shown, $P = 0.007$), with control plants allocating more biomass to roots compared to B5 ($P = 0.001$), N2 (*Paenibacillus* sp.; $P = 0.006$), and MIX plants ($P = 0.002$). Total plant length was not impacted by inoculant treatments (Fig. 3c; $P = 0.659$).

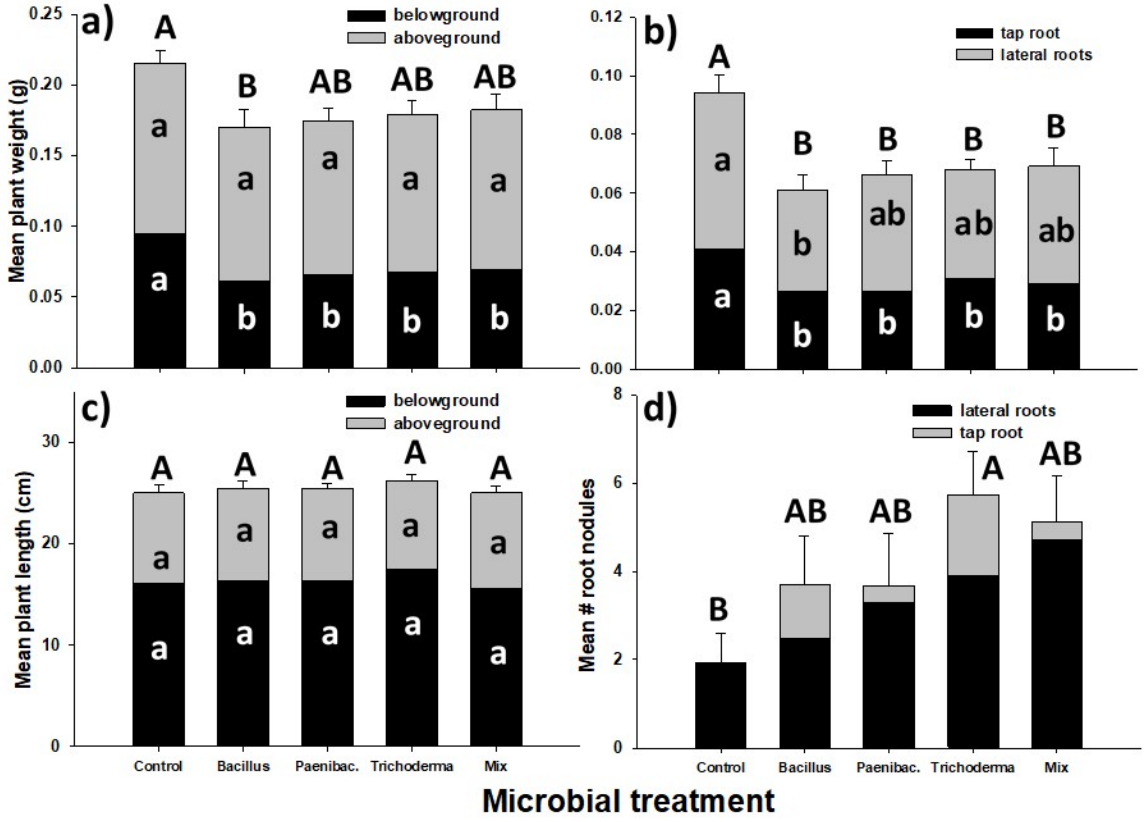

Figure 3. Impact of microbial treatments in a short-term (two-week) greenhouse experiment on a) total plant weight, including below- and aboveground biomass, b) belowground weight, including tap and lateral root biomass, c) plant length, including length of below- and above-ground structures, and d) total root nodules, including plants that didn't nodulate. Means with the same capital letter are not significantly different (Tukey's HSD, $\alpha = 0.05$) with regard to total measurements, while lower case letters represent differences among subgroups. *Bacillus* = B5, *Paenibac*. = N2, *Trichoderma* = GP, Mix = B5+N2+GP.

Table 8. ANOVA of the impact of microbial inoculant (*MI*) identity on field pea growth and biomass distribution in a two-week greenhouse experiment.

Variable	df	*F*-value	*P*-value
Total plant weight (aboveground + belowground)	4, 65.4	2.53	**0.049**
Aboveground weight (shoot)	4, 66.9	0.54	0.709
Total belowground weight (taproot + lateral roots)	4, 67.1	6.03	**0.0003**
Lateral root weight	4, 67.8	2.75	**0.035**
Taproot weight	4, 67.6	9.31	**<0.0001**
Total plant length (shoot + taproot)	4, 67.9	0.61	0.659
% Belowground biomass allocation[1]	4, 67.1	3.82	**0.007**
Total root nodules[2] (taproot + lateral)	4, 67.1	3.54	**0.011**

[1] = arcsine transformed
[2] = log (X+1) transformed
Bold values indicate significance at $\alpha = 0.05$

Microbial inoculation treatment influenced the likelihood of a plant having nodules (Pearson $\chi^2 = 11.16$, $df_{4,93}$, $P = 0.038$), with plants inoculated with GP (*Trichoderma* spp.) and the mixture more likely to be nodulated (17 of 19 = 89.5%, 15 of 18 = 83.3%, respectively) than plants in the control treatment (10 of 18 = 55.6%), or plants inoculated with B5 (10 of 19 = 52.6%) or N2 (11 of 19 = 57.9%). For nodulated plants, 81.7 ± 0.03% of nodules were located on lateral roots, and the number of nodules per plant ranged from 1 to 18, with the average across treatments being 3.9 ± 0.5. Inoculation positively impacted root nodule density (Table 9), with plants inoculated with GP having more root nodules than control plants (Fig. 3d; $df_{1, 68.1}$, $F = 10.78$, $P = 0.002$). However, when plants that did not nodulate were excluded, there was no impact of microbial inoculation on density of total and lateral root nodules (data not shown; $P >$

0.124). Although inoculant still affected nodule density on taproots (data not shown; $df_{1, 44.0}$, F = 11.58, $P < 0.0001$), with more taproot nodules on plants receiving B5 (mean ± SEM = 2.1 ± 0.9) and GP (2.1 ± 0.4) compared to plants in the other treatments ($P < 0.05$; control: 0 ± 0, N2: 0.6 ± 0.4, MIX: 0.5 ± 0.3).

Table 9. ANOVA analysis of the impact of microbial inoculant (MI) identity on field pea nodulation (including plants that didn't nodulate) in a short-term (two-week) greenhouse experiment.

Variable	df	*F*-value	*P*-value
Total nodules[1] (taproot + lateral)	4, 67.7	3.53	**0.011**
Taproot nodules[1]	4, 69.1	8.88	**<0.0001**
Lateral root nodules[1]	4, 67.5	2.72	**0.037**

[1] = log (X+1) transformed
Bold values indicate significance at $\alpha = 0.05$

2.3.2.2. Four-week experiment

This experiment was similar to the two-week cone-tainer experiment, except that plants were destructively sampled after four weeks, larger pots were used, and the experiment was run twice, first in winter 2017 again in summer 2018. Greenhouse temperature and relative humidity (%RH) data showed there was a lower average temperature and RH recorded in winter 2017 (23.5 ± 0.1˚C, 34.4 ± 0.6% RH) compared to summer 2018 (25.9 ± 0.1˚C, 58.6 ± 0.4% RH).

Microbial inoculant did not impact taproot biomass, the percent of biomass allocated to the root system, or total plant length, regardless of when the experiment was run (Fig. 4, Table 10, *MI* × *Block*, $P > 0.05$, *MI*, $P > 0.05$). Plants grown in the winter had heavier taproots (mean ± SE: winter, 32.76 ± 1.06 mg; summer; 25.18 ± 1.15 mg), more biomass allocated to the root

system (mean ± SE: winter, 32.4 ± 5.7%; summer; 16.6 ± 5.8%) and were shorter (shoot + root length) than those grown in the summer (mean ± SE: winter, 35.16 ± 1.00 cm; summer; 50.00 ±1.45 cm). The impact of microbial inoculant on total plant weight, weight of aboveground tissue, lateral root weight, and length of shoots depended on when the experiment was run (*MI* × *Block*, $P > 0.05$), with the interactive effect of microbial inoculant and block on total root biomass (*i.e.*, total belowground weight = tap + lateral roots) being marginal (*MI* × *Block*, $P = 0.082$).

Table 10. Factorial ANOVA analysis of the impact of microbial inoculant (*MI*) identity and *Block* (winter or summer) on field pea growth and biomass distribution in a four-week greenhouse experiment.

Dependent variables	Independent variables	df	*F*-value	*P*-value
Total plant weight (aboveground + belowground)	*MI*	4, 77.4	0.18	0.950
	Block	1, 77.0	179.50	**<0.0001**
	MI × *Block*	4, 77.4	3.88	**0.006**
Total aboveground weight (shoots)	*MI*	4, 77.5	0.34	0.849
	Block	1, 77.1	229.80	**<0.0001**
	MI × *Block*	4, 77.5	3.36	**0.014**
Total belowground weight (taproot + lateral roots)	*MI*	4, 77.4	0.55	*0.072*
	Block	1, 77.0	0.24	0.629
	MI × *Block*	4, 77.4	2.15	0.082
Taproot weight	*MI*	4, 76.3	0.38	0.824
	Block	1, 75.9	23.80	**<0.0001**
	MI × *Block*	4, 76.3	2.05	*0.096*
Lateral root weight	*MI*	4, 77.8	0.48	0.747
	Block	1, 77.3	0.06	0.807
	MI × *Block*	4, 77.8	3.08	**0.021**
Aboveground length (shoot)	*MI*	4, 77.0	1.18	0.327
	Block	1, 76.8	377.90	**<0.0001**
	MI × *Block*	4, 77.0	3.43	**0.012**
Total plant length (shoot + taproot)	*MI*	4, 76.6	0.96	0.437
	Block	1, 76.2	68.10	**<0.0001**
	MI × *Block*	4, 76.6	1.00	0.412
% Belowground biomass allocation[1]	*MI*	4, 77.0	0.36	0.834
	Block	1,76.6	356.20	**<0.0001**
	MI × *Block*	4,77.0	1.24	0.230

[1] = arcsine transformed
MI = Microbial inoculant
Block = winter or summer experimental block
Bold values indicate significance at $P = 0.05$
Italic values indicate marginal significance at $P > 0.05$ and < 0.10

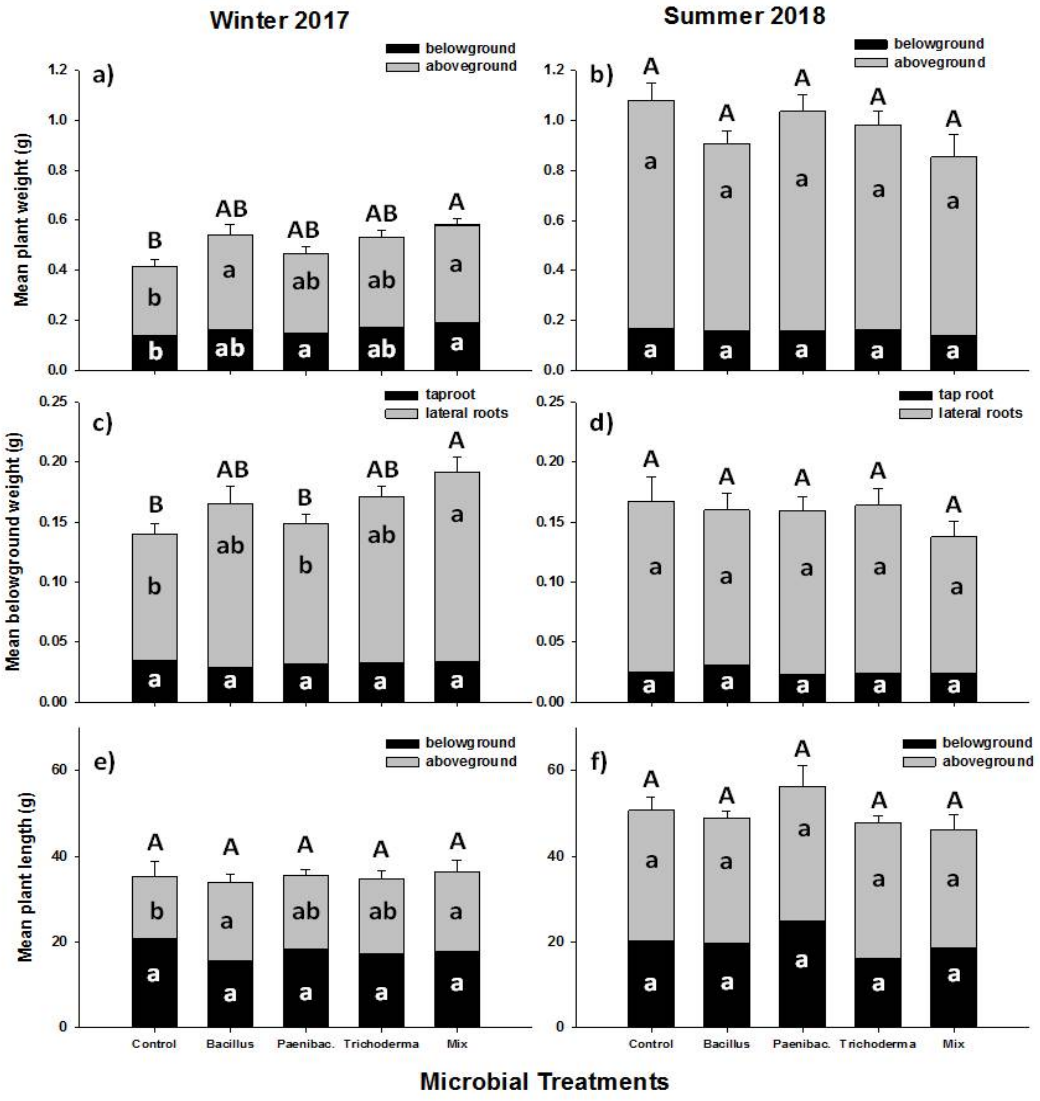

Figure 4. Impact of microbial inoculant identity in a four-week greenhouse experiment on: a-b) total plant weight (including below- and above-ground structures), c-d) root weight (including taproot and lateral roots), and e-f) total plant length (including shoot and taproot). Capital letters represent significant differences (at $P < 0.05$) among total measurements, while lower case letters represent differences among each subgroup. *Bacillus* = B5, *Paenibac*. = N2, *Trichoderma* = GP, Mix = B5+N2+GP.

Effects of microbial inoculants on field pea were only observed when plants were grown in the winter and exhibited no root nodulation. Plants receiving multiple inoculants (Mix) were heavier than control plants, while the average weight of plants receiving a single inoculant was

in-between those extremes (Fig. 4a, Table 11; total plant weight, $P = 0.004$). A similar pattern was seen for above- and belowground biomass, although plants inoculated with B5 (*Bacillus* spp.) had heavier shoots than control plants (Fig. 4a; $df_{1, 36.6}$, $F = 10.30$, $P = 0.003$), and plants inoculated with N2 (*Paenibacillus*) had lighter lateral roots than Mix plants (Fig. 4c; $df_{1, 31.9}$, $F = 9.80$, $P = 0.004$). A similar pattern was seen for shoot length (*i.e.*, aboveground length), with control plants having shorter shoots then and B5 (Fig. 4e; $df_{1, 34.3}$, $F = 10.42$, $P = 0.003$) and Mix plants ($df_{1, 32.7}$, $F = 12.85$, $P = 0.001$). Microbial inoculant did not impact taproot weight, total plant length, or biomass allocation to belowground structures (Table 11).

Table 11. ANOVA of the impact of microbial inoculant (*MI*) identity on field pea growth and biomass distribution in the four-week winter block experiment.

Variable	df	*F*-value	*P*-value
Total plant weight (g)	4, 34.7	4.63	**0.004**
Total aboveground weight (g)	4, 34.1	4.42	**0.006**
Total belowground weight (g)	4, 32.4	4.16	**0.008**
Taproot weight (g)	4, 34.1	0.84	0.512
Lateral root weight (g)	4, 32.4	5.02	**0.003**
Aboveground length (cm)	4, 32.6	3.88	**0.011**
Total plant length (cm)	4, 33.7	0.17	0.951
% Belowground biomass allocation[1]	4, 31.5	1.07	0.390

[1] = arcsine transformed
Bold values indicate significance at $P = 0.05$

In the summer block there was no impact of microbial inoculant on the weight or length of the plant, or biomass allocation to roots (Table 12). In the summer block, 41 of 50 plants (82%) formed nodules, and microbial inoculation treatment did not impact the likelihood of a plant having nodules (Pearson $\chi^2 = 5.96$, $df_{4,50}$, $P = 0.128$). Including non-nodulated plants, there was an average of 5.86 ± 0.90 root nodules per plant, with a majority (68.8%) located on the

Table 12. ANOVA analysis of the impact of microbial inoculant (*MI*) identity on field pea growth and biomass distribution in the four-week summer block experiment.

Variable	df	*F*-value	*P*-value
Total plant weight	4, 36	1.81	0.149
Total aboveground weight	4, 36	1.78	0.155
Taproot weight	4, 36	1.87	0.138
Lateral root weight	4, 36	0.69	0.606
Aboveground length	4, 36	1.76	0.159
Total plant length	4, 36	1.68	0.176
Belowground biomass allocation %[1]	4, 36	1.81	0.149

[1] = arcsine transformed

Bold values indicate significance at $P = 0.05$

lateral roots. When plants that did not form nodules were excluded, there was an average of 7.15 ± 0.99 root nodules per plant with 86.7% located on the lateral roots, and the number of nodules per plant ranged from 1 to 28.

Microbial inoculation had a marginal impact on total root nodule and lateral root nodule density (Table 13), which was driven by lower numbers of root nodules in plant receiving multiple inoculants (*i.e.*, Mix) compared to control (data not shown; $df_{1, 36}$, $F = 6.13$, $P = 0.018$) and B5 plants ($df_{1, 36}$, $F = 6.47$, $P = 0.015$). However, when plants that did not nodulate were excluded, there was no impact of microbial inoculation on root nodule density (data not shown; $P > 0.420$ for total, taproot, and lateral root nodules).

Table 13. ANOVA analysis of the impact of microbial inoculant (*MI*) identity on field pea nodulation (including plants that didn't nodulate) in a short-term (four-week) greenhouse experiment.

Variable	df	*F*-value	*P*-value
Total nodules[1]	4, 36	2.26	*0.082*
Taproot nodules[1]	4, 36	0.67	0.621
Lateral root nodules[1]	4, 36	2.32	*0.075*

[1] = log (X+1) transformed
Bold values indicate significance at $P = 0.05$
Italic values indicate marginal significance at $P > 0.05$ and < 0.10

2.3.3. Impact of Microbial Inoculant Identity and Collembola on Field Peas

2.3.3.1. Arthropod densities

This experiment investigated how microbial inoculants and the addition of *Onychiuridae* (white) Collembola impacted field pea growth and biomass allocation during the early stages of plant development. At the end of the experiment, we extracted arthropods from the soil of experimental pots to quantify arthropod densities and assess the effectiveness of our Collembola treatment. However, in addition to recovering the white Collembola we added, we also recovered several other arthropods that we loosely termed contaminants (Table 14), including a second Collembola species, which was gray in color and identified as a member of the family Hypogastruridae. Other arthropod contaminants included: mites (Acari, primarily Acaridae and Oribatida), fungus gnats (Diptera: Sciaridae), thrips (Thysanoptera), and miscellaneous (*i.e.*, Coleoptera, Phthiraptera, or unknown).

Table 14. Arthropods recovered from the soil of experimental pots using Berlese funnels at 14 and 31 DAP.

Soil arthropod taxa	Recovered at 14 DAP[1]		Recovered at 31 DAP	
	Mean	Sum	Mean	Sum
Onychiuridae (white Collembola)	3.4 ± 0.5	96	4.6 ± 0.5	274
Hypogastruridae (gray Collembola)	0.4 ± 0.3	12	2.0 ± 0.4	121
Mites	0	0	9.1 ± 5.9	182
Thrips	0	0	1.2 ± 0.2	6
Miscellaneous	0	0	1.2 ± 0.2	7
Total arthropods	3.9 ± 0.6	108	9.9 ± 2.1	595

[1] total number of samples at 14 DAP = 28
[2] total number of samples at 31 DAP = 60

Although white Collembola were only added to half the pots (*i.e.*, +Coll pots), they were recovered from 93.2% of pots (82 out of 88, across both dates), and the frequency of their recovery (presence or absence) from experimental pots was not impacted by Collembola addition treatment (Pearson $\chi^2 = 0.82$, $df_{1,88}$, $P = 0.366$). The number of white Collembola recovered from +Coll pots (mean ± SE: 4.78 ± 0.64) was marginally higher than the number recovered from -Coll pots (mean ± SE: 3.60 ± 0.43) (Table 14; *Coll*: $P = 0.092$).

The number of white Collembola recovered (Fig. 5a, c) at 14 DAP ranged from 0 to 14 (mean ± SE: 3.43 ± 0.51), and from 0 to 21 (mean ± SE: 4.57 ± 0.52) at 31 DAP, and there was a marginal *End Date* × *MI* interaction on their density (Table 15) which was driven by lower numbers of white Collembola recovered from control pots 14 DAP (mean ± SE: 2.50 ± 0.54) compared to control pots at 31 DAP (mean ± SE: 6.60 ± 1.25) (data combined across Collembola addition treatment; $df_{1, 71.9}$, $F = 7.28$, $P = 0.009$).

The number of gray Collembola contaminants recovered from pots was higher at 31 DAP compared to 14 DAP (Tables 14, 15; *End date*, $P = 0.002$), but their densities were not impacted by microbial inoculant or the Collembola addition treatment (Fig. 5b, d).

Table 15. ANOVA analysis of the impact of *End date* (14 versus 31 DAP), microbial inoculant (*MI*) identity, and Collembola addition (*Coll*) on density of Onychiuridae (experimental organism) and Hypogastruridae (contaminant) in a short-term greenhouse experiment.

Variable	Source	df	*F*-value	*P*-value
Onychiuridae (white Collembola)[1]	*End date*	1, 76.7	1.96	0.166
	MI	2, 72.7	0.32	0.726
	Coll	1, 68.8	2.93	*0.092*
	MI × *Coll*	2, 74.1	0.38	0.683
	MI × *End date*	2, 72.7	2.87	*0.063*
	End date × *Coll*	1, 68.8	0.56	0.456
	End date × *MI* × *Coll*	2, 74.1	1.57	0.214
Hypogastruridae (gray Collembola)[1]	*End date*	1, 76.7	10.8	**0.002**
	MI	2, 72.7	0.98	0.380
	Coll	1, 68.8	0.01	0.940
	MI × *Coll*	2, 74.1	0.88	0.420
	MI × *End date*	2, 72.7	0.18	0.835
	End date × *Coll*	1, 68.8	1.04	0.311
	End date × *MI* × *Coll*	2, 74.1	0.49	0.616
Aboveground length (shoot) 14 DAP[2]	*End date*	1, 98.0	0.11	0.741
	MI	2, 98.0	2.55	*0.084*
	Coll	1, 98.0	1.19	0.279
	MI × *Coll*	2, 98.0	0.92	0.402
	MI × *End date*	2, 98.0	0.71	0.499
	End date × *Coll*	1, 98.0	0.00	0.988
	End date × *MI* × *Coll*	2, 98.0	0.04	0.963

[1] = log (X+1) transformed
[2] = non-destructively assessed for all plants regardless of end date (*i.e.*, includes plants destructively sampled 14 DAP and 31 DAP)
Bold values indicate significance at $P = 0.05$
Italic values indicate marginal significance at $P > 0.05$ and < 0.10

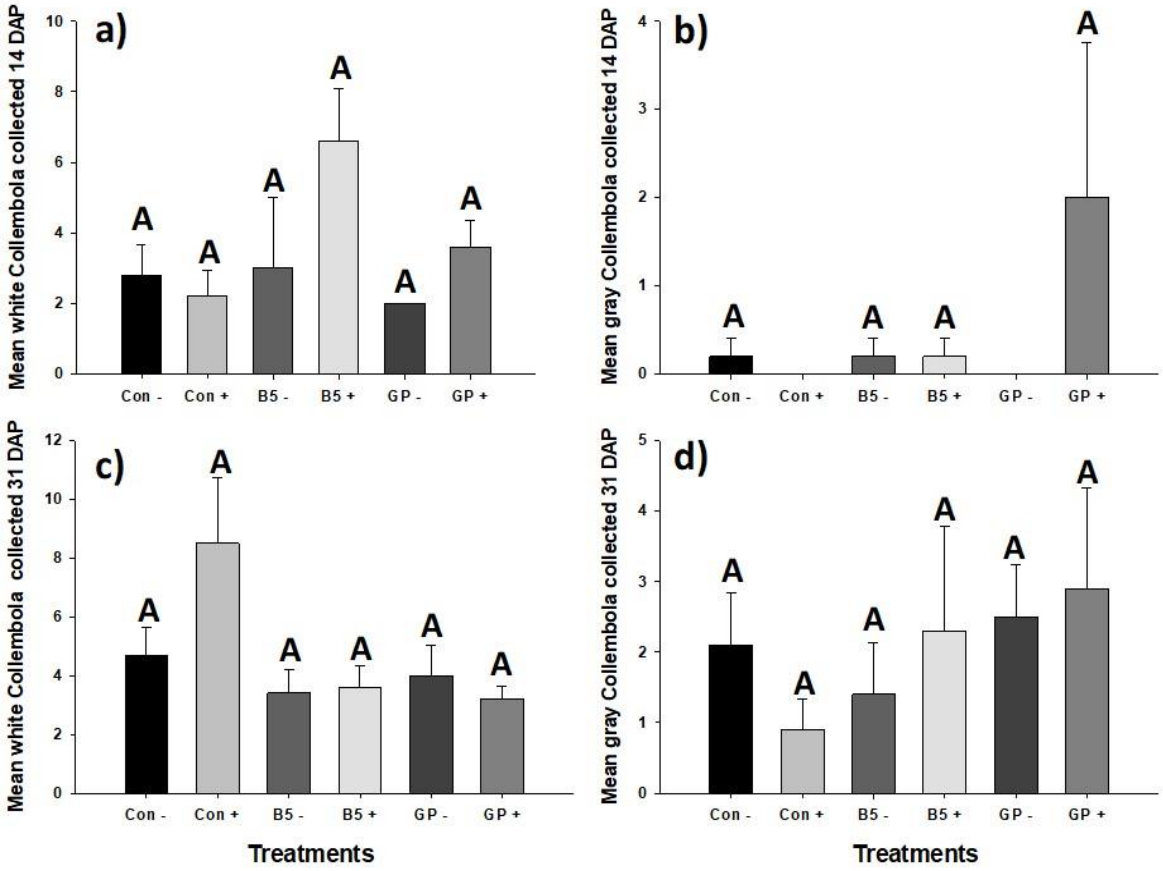

Figure 5. Impact of microbial treatments on the recovery of: a) Onychiuridae (white) Collembola collected at 14 DAP, b) Hypogastruridae (gray) Collembola collected at 14 DAP, c) white Collembola collected at 31 DAP, and d) gray Collembola collected at 31 DAP. Control = Con, *Bacillus* = B5, and *Trichoderma* = GP. Treatments with - had no Collembola added at the start of the experiment, while treatments with + had white Collembola added.

2.3.3.2. Plant growth parameters

Aboveground length was assessed non-destructively for all plants 2 weeks after planting (*i.e.*, 1 week after Collembola addition). Shoot height was similar across all treatments (Table 15) and averaged 10.27 ± 0.13 cm (mean ± SE).

Field pea total plant weight at 14 DAP was not impacted by microbial inoculant and Coll +- (Table 16; Fig. 6a; P = 0.4120). Although MI independently did have a marginal impact on total plant weight with control plants weighing more than B5 and GP plants (P = 0.0766). When analyzed separately, both aboveground (Fig. 6a: P = 0.1142) and belowground (Fig. 6b: P = 0.6175) plant weights were not affected by MI and Coll +-, but MI alone slightly impacted belowground structure resulting in B5 treatments weighing more than Con and GP plants (P = 0.0628). Overall, belowground weight accounted for 48.9±1.0% of plant biomass and Coll+- did have a marginal impact on belowground biomass allocation with control (no collembola) plants allocating more weight to belowground structures (P = 0.0755). Total plant length was not affected by any treatment effect (P=0.9167).

At 31 DAP, MI had an effect on total plant weight with Con plants weighing significantly more than GP plants (Table 17; Fig. 7b; P = 0.0172). This difference was driven by Con plant aboveground shoot being heavier than GP plants (Fig. 7b; P = 0.0445). Root systems were also impacted by MI with B5 and Con total belowground root weight weighing more than GP (Fig 7d. P = 0.0008). Lateral roots account for 92.9±0.003% of the total belowground weight and MI was significant resulting in B5 and Con lateral roots weighing more than GP. The belowground root systems made up an average of 27.2±1.0% of total plant biomass, but plant biomass allocation to root structures was not impacted by MI.

Table 16. ANOVA analysis of the impact microbial inoculant (*MI*) identity and Collembola addition (*Coll*) on field pea growth and biomass allocation for plants destructively sampled at 14 DAP.

Variable	Source	df	*F*-value	*P*-value
Total plant weight (g)	*MI*	2, 44.8	2.72	*0.077*
	Coll	1, 44.8	0.72	0.402
	MI × *Coll*	2, 44.8	0.91	0.412
Total aboveground weight (g)	*MI*	2, 44.6	2.28	0.114
	Coll	1, 44.6	2.31	0.136
	MI × *Coll*	2, 44.6	1.05	0.357
Total belowground weight (g)	*MI*	2, 44.6	2.95	*0.063*
	Coll	1, 44.6	0.00	0.990
	MI × *Coll*	2, 44.6	0.49	0.618
Total plant Length (cm)	*MI*	2, 44.7	0.21	0.810
	Coll	1, 44.7	0.01	0.978
	MI × *Coll*	2, 44.7	0.09	0.917
Belowground biomass allocation %[1]	*MI*	4, 44.3	1.20	0.311
	Coll	1, 44.3	3.27	*0.078*
	MI × *Coll*	2, 44.3	0.34	0.712

[1] = arcsine transformed

Italic values indicate marginal significance at $P > 0.05$ and < 0.10

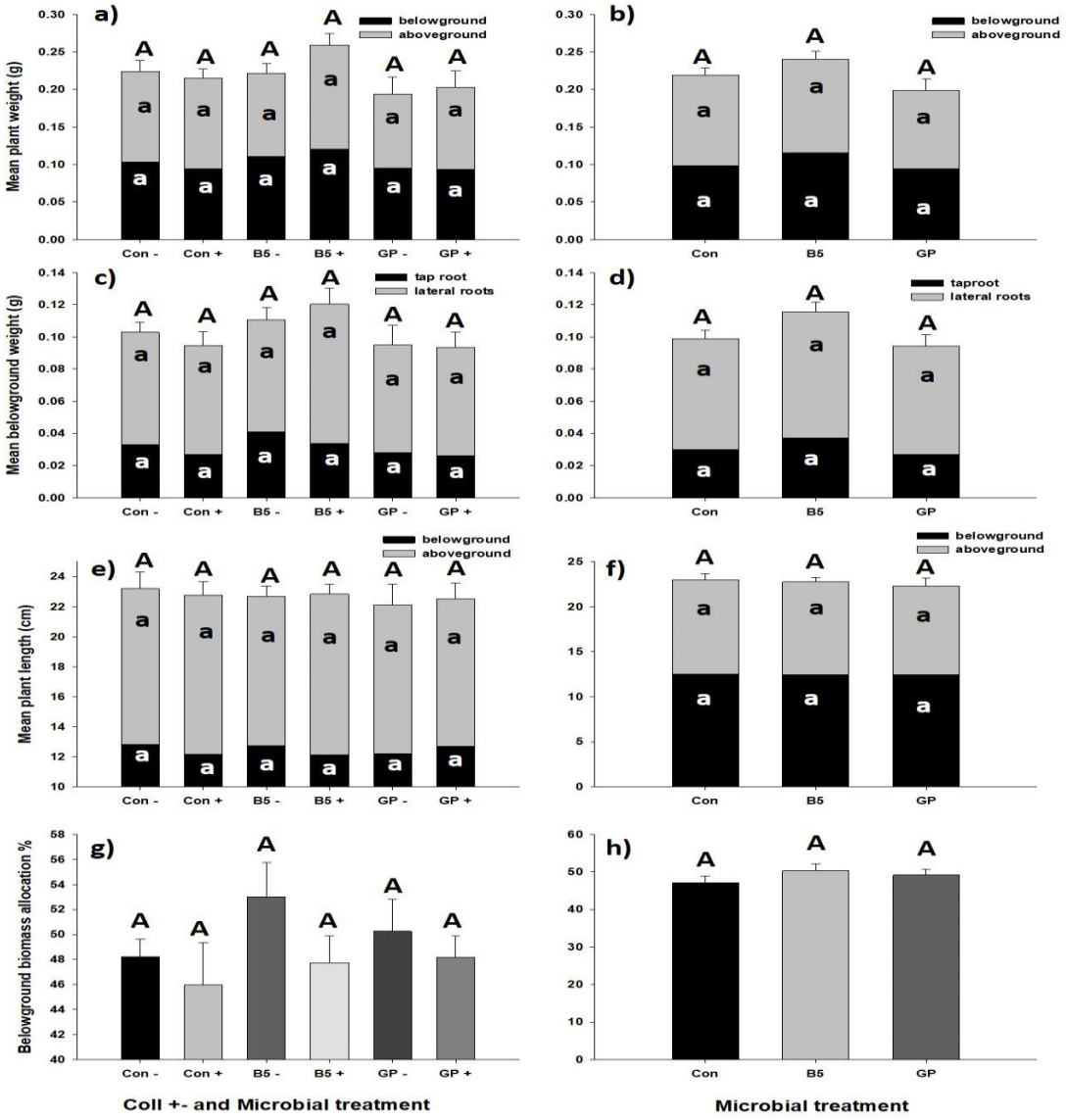

Figure 6. Impact of microbial inoculants and collembola addition (Coll+-) in a short-term (4 week): a) Coll+- distribution of total plant weight between below- and above-ground structures, b) MI distribution of total plant weight between below- and above-ground structures, c) Coll+- distribution of belowground weight between the main tap root and lateral roots, d) MI distribution of belowground weight between the main tap root and lateral roots, e) Coll+- distribution of total plant length between below- and above-ground structures, f) MI distribution of total plant length between below- and above-ground structures, g) Coll+- distribution of belowground biomass allocation percent, and h) MI distribution of belowground biomass allocation percent. Capital letters represent significant differences (at $P \leq 0.05$) among total measurements, while lower case letters represent differences among each subgroup. Control = Con, *Bacillus* = B5, and *Trichoderma* = GP. Treatments with - had no Collembola added at the start of the experiment, while treatments with + had additional Collembola added.

Table 17. ANOVA analysis of the impact microbial inoculant (*MI*) identity and Collembola addition (*Coll*) on field pea growth and biomass allocation for plants destructively sampled at 31 DAP.

Variable	Source	df	*F*-value	*P*-value
Total plant weight	*MI*	2, 45	4.45	**0.017**
	Coll	1, 45	1.79	0.188
	MI × *Coll*	2, 45	0.23	0.793
Total aboveground weight	*MI*	2, 45	3.34	**0.045**
	Coll	1, 45	2.34	0.133
	MI × *Coll*	2, 45	0.24	0.792
Total belowground weight (g)	*MI*	2, 45	8.40	**0.001**
	Coll	1, 45	0.20	0.655
	MI × *Coll*	2, 45	2.15	0.129
Lateral root weight	*MI*	2, 45	8.26	**0.001**
	Coll	1, 45	0.19	0.669
	MI × *Coll*	2, 45	2.47	0.096
Total plant length (cm)	*MI*	2, 45	1.29	0.286
	Coll	1, 45	2.48	0.122
	MI × *Coll*	2, 45	0.02	0.977
Belowground biomass allocation %[1]	*MI*	2, 45	2.04	0.141
	Coll	1, 45	1.27	0.265
	MI × *Coll*	2, 45	1.01	0.374

[1] = arcsine transformed

Bold values indicate significance at $P = 0.05$

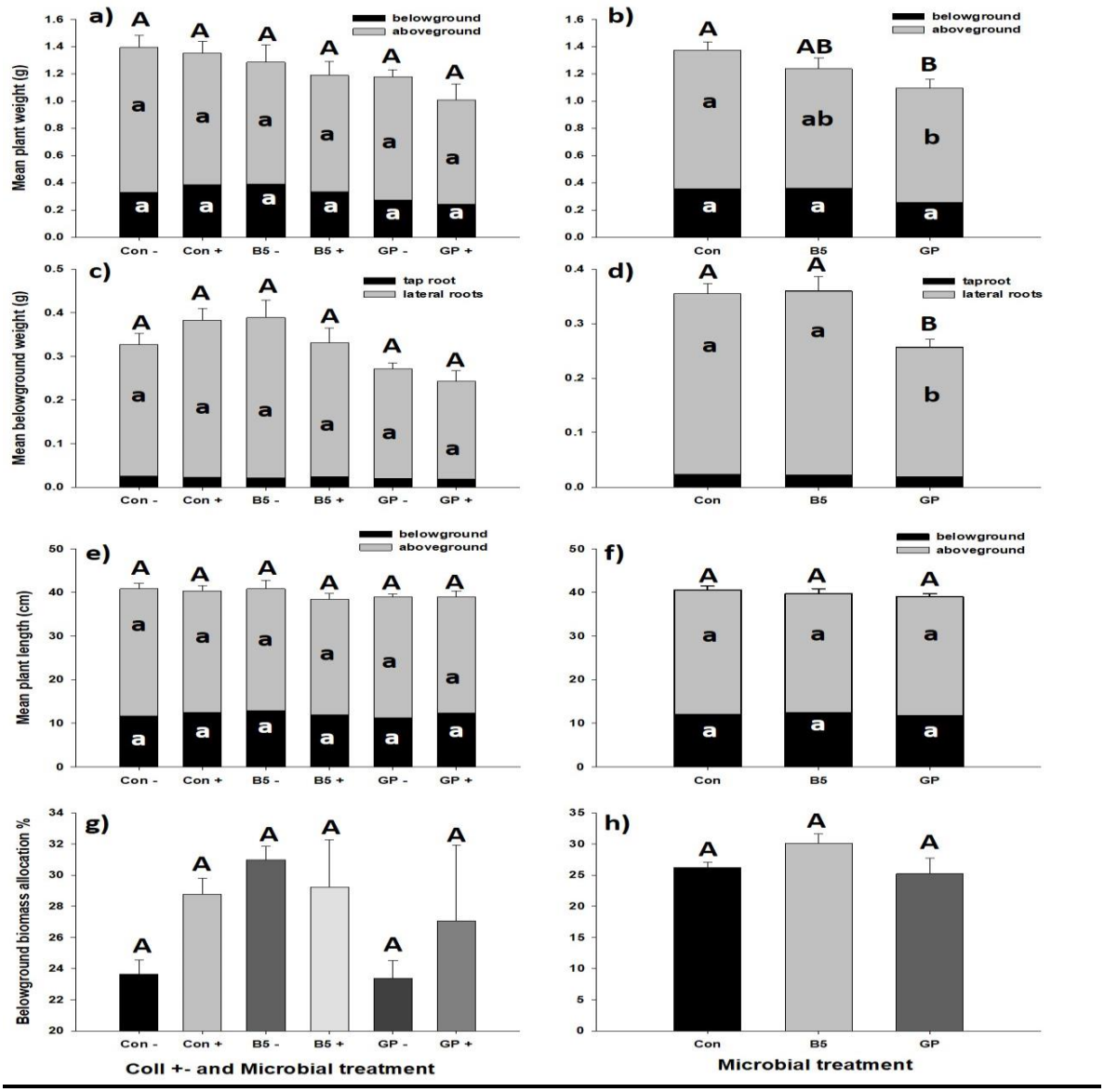

Figure 7. Impact of microbial inoculants and collembola addition (Coll+-) in a short-term (4 week): a) Coll+- distribution of total plant weight between below- and above-ground structures, b) MI distribution of total plant weight between below- and above-ground structures, c) Coll+- distribution of belowground weight between the main tap root and lateral roots, d) MI distribution of belowground weight between the main tap root and lateral roots, e) Coll+- distribution of total plant length between below- and above-ground structures, f) MI distribution of total plant length between below- and above-ground structures, g) Coll+- distribution of belowground biomass allocation percent, and h) MI distribution of belowground biomass allocation percent. Capital letters represent significant differences (at $P \leq 0.05$) among total measurements, while lower case letters represent differences among each subgroup. Control = Con, *Bacillus* = B5, and *Trichoderma* = GP. Treatments with - had no Collembola added at the start of the experiment, while treatments with + had additional Collembola added.

2.4. Discussion

A rising human population has created a higher demand for food, which can be achieved through increased crop production (Godfray and Garnett, 2014; Ahmad et al., 2018). Soil microbes are viewed as a long-term, sustainable approach to increasing food security and improving soil fertility and soil nutrient quality (Barea, 2015; Mahanty et al et al., 2017). Microbes have long been used in agriculture to promote plant health, increase yields, remove soil contaminants, compete against pathogenic microbes, acquire vital nutrients for plant growth, and produce plant growth hormones (Finkel et al., 2017; Verma et al., 2017). Beneficial microbes enhance plant growth and development, which provides an improved quality and quantity of resources for beneficial insect use (Pineda et al., 2013). Other beneficial microbes are viewed as cost-effective alternatives to pesticides through induction of plant defenses and by contributing to the production of toxic compounds that protect against detrimental organisms (Pineda et al., 2010). We used greenhouse experiments to investigate the effects of three commercial microbial inoculants, both alone and in combination, on the early growth traits and biomass allocation of the specialty crop field pea.

The relationship between root-associated microbes and their plant partners is not always straightforward but can range from symbiotic to detrimental depending on the identity and density of the microorganisms and available resources (Fukui, 2014; Friel and Friesen, 2019). We found a non-linear relationship between the dose of the B5 inoculant applied to seeds and field pea growth, with lower total plant and root biomass relative to the control at low (e.g., 20,000 CFUs per seed) and high doses (160,000 CFUs per seed), and similar or higher plant biomass at intermediate doses. Plant height responses were also non-linear, although the amount

of biomass allocated to the roots decreased as the amount of B5 CFUs applied to seeds increased, while low and intermediate rates had heavier overall plant weight. Effects of beneficial microbes can shift from mutualistic to parasitic where microbes are of little benefit to host plants and in some cases cost more to maintain then the benefit they provide (Hoeksema et al., 2010). Qiao et al., 2017 support this by showing that moderate inoculum rates of *B. subtilis* (1.4×10^7 CFUs) applied to seeds promoted plant growth in tomato plants, *Solanum lycopersicum*, while high rates (7×10^7 CFUs) led to plant death. Effects of microbial inoculants are often related to successful establishment of viable organisms on plant structures (Khare and Arora, 2015). Although we did not quantify how adding inoculants affected the density of viable organisms that colonized the plant, Massalha et al. (2017) demonstrated that *Bacillus subtilis* communities rapidly colonized root structures and then actively excluded competing bacteria. While microbes use similar strategies and principles to interact with plant roots, not all root-associated microbes function the same way (Lugtenberg et al., 2002).

Species belonging to *Paenibacillus* previously were classified under the genus *Bacillus* until rRNA sequencing and PCR probe tests separated the two (Ash et al., 1993). However, in contrast to our experiment with the B5 inoculant, we found a linear relationship between the dose of N2 inoculant applied to seeds and field pea growth, with total belowground biomass having heavier weights as N2 dose increased. This N2 dose effect was not seen when measuring plant biomass allocation or length. Hahm et al. (2012) showed that 10 mL of *P. polymyxa* was applied as a soil dredge at high application rates (10^8 CFU/mL) increased plant dry weight by up to 38% in pepper (*Capsicum annuum* L.) seedlings in a greenhouse setting. Seed applications of *P. polymyxa* have been used in greenhouse experiments at application rates of 10^8-10^9/mL on

cucumber seeds to enhance aboveground shoot weight by 60% compared to water controls (Ryu et al., 2004). Determining how inoculant dose impacted field pea growth and biomass distribution provided a context for our other experiments that used the intermediate rate of 34,500 CFUs per seed and suggests our results for microbial identity experiments may be conservative.

Other contextual limiting factors that directly affect the success of microbial inoculants include soil pH, desiccation, nutrient deficiency, and temperature (Thilakarathna and Raizada, 2017; Santos et al., 2019). The soil and root surface are hurdles for microbes due to the variety of organisms present and levels of competition between them (Timmusk et al., 2017). Soil sterilization is commonly used to eliminate microbes from experimental growing medium through heat or fumigation (Mahmood et al., 2013). Although no sterilization was used the growing medium used for all experiments was consistent for all experiments thus, the native microbial communities were consistent for all experiments. During experiments, bacterial and fungal spores could have been transported via wind or water, which could be a source of contamination (Calhim et al., 2018). Experimental pots were separated by 2.54 cm to avoid water contamination, but arial dispersal could have allowed microbes to spread to other experimental plants. Since no assessments of microbial community were conducted at the completion of experiments, we cannot conclude if there was any dispersal or contamination present.

Seeds and young developing plants are vulnerable to numerous abiotic and biotic stressors which can impact later stages of growth (Kranner et al., 2010). Expansion of the taproot and lateral roots to acquire resources from the soil are essential functions in the early growth stages of a plant's life (Lambers and Poorter, 1992). We found that our individual and combined

treatments of microbial inoculants at two weeks had smaller total belowground root structures compared to the control, which was primarily due to control plants having a larger taproot. This weight disparity was unexpected since microbial inoculants are typically viewed as beneficial organisms that provide growth and health benefits (Beneduzi et al., 2012; Gupta et al., 2017). Although, certain beneficial microbes including, *Bacillus magaterium*, when applied to *Arabodpsis thaliana* decreased primary taproot growth while increasing the number and density of fine lateral root structures (Verbon and Liberman, 2016). In our two-week experiment we used smaller pots compared to our four-week experiments, which may have limited the resources available to the plants and microbes (Poorter et al., 2012). Having a smaller pot could physically restrict root growth or result in the roots being pot bound (Bouzo and Favaro, 2015). But since the control roots were not equally hindered, it is unlikely that the size of the container was the cause of the belowground weight differences. A more likely conclusion is that there was a trade-off between growth and microbe recruitment and establishment in inoculated plants, as the latter processes are facilitated by the release of plant exudates in the form of sugars, amino acids, and organic acids, which can cost the plant anywhere from 5-30% of acquired fixed carbon (Bais et al., 2006; Jacoby et al., 2017). Plants inoculated with GP (*Trichoderma* spp.) had more root nodules present than control plants. This investment of resources could account for the disparity in belowground biomass observed at two weeks, which could impact plant growth stages later in life (Smith et al., 2018).

However, the impact of microbial inoculants changed when plants were allowed to grow two extra weeks. In the four-week summer experiment, there was no impact of microbial inoculants on field pea growth or biomass distribution. In contrast, in the four-week winter

experiment, control plants were the among the shortest and lightest, with plants inoculated with GP and N2 typically having intermediate height and weight, and plants inoculated with B5 and all three inoculants (*i.e.*, the Mix) often being tallest and heaviest. There are multiple examples of *Bacillus* spp. enhancing plant growth, which is related to production of hormones such as gibberellins, indole acetic acid (IAA) and cytokines. Arkhipova et al. (2005) showed that through increased hormone production, *B. subtilis* enhanced lettuce shoot and root weight by approximately 30% in a greenhouse setting. The ability of microbes to enhance plant growth and provide a more robust shoot system allows plants to be more efficient at gathering resources leading to a faster growth rate and higher yields (Horton, 2000; Mathan et al., 2016).

In a field setting there are numerous beneficial, detrimental, and neutral microbes interacting with plant roots in different ways, and researchers believe that combinations of beneficial microbes could be more effective for promoting plant growth than a single microbe (Baez-Rogelio et al., 2017; Compant et al., 2019). Our Mix treatment made up of three unique commercial products in the winter four-week experiment had a positive impact on plant length and overall plant weight, which was due to positive effects on both shoots and roots. Traditionally, inoculants have only contained a single microbial species or strain but combining multiple organisms in one inoculant has become more common in the past decade (Santos et al., 2019), as having multiple microbes with different mechanisms is thought to maximize beneficial effects on plant growth and yield (Pereg and McMillan, 2015; Santos et al., 2019). Korir et al. (2017) demonstrated this by combining *Rhizobium* and *B. megaterium* to enhance the dry shoot weight in common bean, *Phaseolus vulgaris*. This process of combining microbes is not always straightforward since microbes have different levels of competitiveness and colonization rates

(Oliveira et al., 2009). Co-inoculation of microbes involves using multiple unique microbes with different mechanisms to benefit the plant, but for this method to be successful antagonistic relationship studies should be conducted to determine if microbes are compatible (Molina-Romero et al., 2017).

Plants grown for four weeks in the summer (June and July) had shoots that were longer and almost twice as heavy as plants grown in the winter (February and March), although belowground biomass were similar. Experiments were set up and run in an identical manner, but environmental factors like day length and light exposure may have impacted plant growth, as they are key components of plant growth and are critical components to any greenhouse experiment (Kramer, 1936; Atkinson and Porter, 1996; Yokoya and Shimizu, 2011). Day length, light intensity, and temperature vary throughout the year in the northern hemisphere with typically shorter colder days occurring in the winter months (December – February) and longer warmer days occurring in the summer months (June – August) (Adamsson et al., 2017). Using greenhouse temperature readings from previous years there was a higher average temperature recorded in the summer months compared to winter months despite the rooms being climate controlled to have the same temperature. Abiotic pressures, such as temperature and light exposure differences could have created two unique environmental conditions between experiments. In stressed environments, beneficial microbe services were more pronounced indicating that microbes in the winter experiment were able to provide a greater benefit to stressed plants resulting in heavier and healthier plants (Al-Karaki 1998, Daei et al. 2009, Pineda et al. 2013, Aghili et al. 2014).

Root feeding arthropods like springtails can negatively impact the growth of plant roots and associated beneficial microbes through physical grazing and competition for nutrients (Kuťáková et al., 2018). We found that at two and four weeks our Collembola treatment did not have any impact on field pea growth or biomass distribution. Collembola are very abundant in soil fauna and are often under-represented in greenhouse experiments (Wurst, 2013). The number of Collembola recovered was less than the number of Collembola we added, thus, it was unlikely that added Collembola thrived, or reproduction occurred. Furthermore, at four weeks 90% of our control pots contained Onychiuridae (i.e., experimental white Collembola) and 46% of pots contained a different species of Collembola (i.e., Hypogastruridae). Having Onychiuridae and Hypogastruridae sp. present in control pots could mean that either the growing medium used was contaminated or the Delnet® Apertured Film bag failed to stop the movement of Collembola between pots. Collembola can directly impact plant growth via root consumption, although in our experiments Collembola, either due to lack of establishment or soil contamination showed no impact on plant growth, (Hopkin, 1997; Endlweber et al., 2009).

In our Collembola experiment, at two weeks there was no impact of microbial inoculants on field pea growth or biomass distribution. At four weeks, control plants had heavier total plant biomass and shoot biomass than GP, while control total root biomass was larger than both B5 and GP plant roots. Beneficial microbes influence plants directly and indirectly by protecting plants from diseases and abiotic stress and under certain circumstances with no additional pressure or stress on plants symbiotic microbes provide minimal added benefit to their host plant (Souza et al., 2015; Gupta et al., 2017). Beneficial microbe's effects in certain environments may

be lost in fertilized soils where nutrients are abundant and in some cases these symbionts can reduce plant growth (Morgan et al., 2005).

In conclusion, we found that microbial inoculants enhanced field pea plant growth under specific circumstances, in which plants inoculated with individual and a mixture of microbes had increased lateral and taproot weight, heavier aboveground shoot systems, and longer plant lengths. Our research suggests that commercial microbial inoculants played a significant role in impacting field pea growth when plants were impacted by abiotic environmental stresses. Understanding and symbiotic relationships between root-associated microbes and plant roots can lead to utilization and enhancement of agricultural crops and ultimately lead to sustainable ecological intensification.

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