CARROT ENDOPHYTES: DIVERSITY, ECOLOGY AND FUNCTION

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ABSTRACT

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Endophytes are a unique group of microorganisms that spend at least part of their life cycle within plant tissues. These microbes are increasingly being recognized for their potential to improve the health and productivity of their host plants. Recent studies indicate that endophytes could also influence human health by altering the composition of chemical compounds within plants, thereby affecting their nutritional quality and flavor. In addition, the presence of endophytes in edible plant tissues could directly affect human health by introducing microbes that can stimulate the immune system or act as opportunistic pathogens in people with compromised immune systems. However, despite their potential importance for plant and human health, these plant-associated microbes have been understudied due to a lack of visible symptoms associated with their presence and difficulty in isolating them from plant tissues. In the present studies, we hypothesized that endophytes play an important role in carrot, one of the most important vegetable crops in the world.

Carrot is well known as an important source of vitamins, antioxidants and other nutrients in the human diet, and carrot taproots are often consumed raw. Carrot crops are slow to establish and are subject to assault by a wide range of pests that negatively affect the health and productivity of this crop, as well as the storage potential of its taproots. Consequently, the aim of these studies was to examine endophyte dynamics in carrot. Studies were carried out in field, greenhouse and laboratory trials using a diverse set of carrot genotypes with broad genetic backgrounds and physiological characteristics. Endophyte communities were studied using traditional culture-based techniques, along with low and high throughput sequencing technologies.

Results of these studies demonstrate that carrot seeds and taproots are colonized by an abundant and diverse set of endophytic microbial taxa. Many of these endophytes could solubilize phosphorous, fix atmospheric nitrogen, produce siderophores and auxin and suppress infection by

a key carrot pathogen, *Alternaria dauci*, demonstrating their potential importance for maintaining carrot health and productivity. Some of the endophytes identified in these trials were vertically transmitted to progeny inside carrot seeds, indicating that they could be part of a core microbiome that evolved alongside carrot plants, and are likely to be critical in early seedling establishment. We also determined that carrot endophytes could be acquired via horizontal transmission from soil with greater soil health in an organic relative to a conventionally managed system resulting in greater populations of endophytes with antagonistic activity against *A. dauci*. Finally, endophyte communities varied among the genotypes evaluated in this study, with some being more responsive to the presence of greater populations of beneficial endophytes in their environments. This indicates that it could someday be possible to begin selecting for these beneficial plant microbial relationships in breeding programs.

Based on the results of these studies, we conclude that endophytes do indeed play an important role in carrot. Additional research aimed at determining how these microbes functionally interact with carrot plants and identifying practical approaches to manipulate these communities to enhance the productivity and quality of carrot taproots, are recommended. A new isolation technique identified in these trials will aid in these efforts.

CHAPTER 1. INTRODUCTION

Carrot (*Daucus carota L. subsp. Sativus*) is one of the top ten most consumed fresh vegetable crops worldwide (Simon, Freeman et al. 2008), and the 6th most consumed fresh vegetable in the U.S. (Rubatzky, Quiros et al. 1999). This crop is a member of the Apiaceae family, which also includes celery, cilantro, and dill. It is a biennial plant that produces a taproot during the first year and seeds during the second year following a vernalization period. The edible taproots produced by carrot plants are a good source of beta carotene, fiber, Vitamin C, and Vitamin K, as well as many other vitamins and minerals essential for human health (Rubatzky, Quiros et al. 1999).

In 2014, world production of carrot was estimated to be approximately 38.8 million tons per year. China produces 45% of the world's carrot crop, with 17.3 million tons of fresh carrot produced annually. Other significant carrot producing countries include Uzbekistan, Russia, and the United States (FAOSTAT 2017). In 2015, the total U.S. fresh market carrot acreage was estimated at 28,955.1 hectares, producing a total yield of 1.1 million tons with an average yield of 42 tons per hectare (USDA, National Ag Statistics Service, 2016).

Although carrot is a cool season crop that grows best in summer and fall in many regions of the U.S., it is available year-round in grocery stores due to winter production in southern states such as California and Texas. Over 85 % of U.S. carrot production occurs in California. The gross value of fresh carrot market production changes depending on location and mechanization, though on average, it is equal to $26,182 per hectare (Lucier and Lin 2007, NASS 2016, AgMRC 2017, Minor and Bond 2017).

1.1 Problem Statement

Like any other vegetable crop, carrot is subject to a number of biotic and abiotic stresses during its life cycle. These stresses include leaf blights caused by *Alternaria dauci*, *Cercospora carotae*, and *Xanthomonas carotae*, cavity spots caused by *Pythium sulcatum*, carrot rust fly (*Psila rosae*), nematodes (*Meloidogyne hapla*), water restriction, and heat stress (RäMerti and Ekbom 1996, Davis 2004, Kovacs, Sorvari et al. 2007, Commisso, Toffali et al. 2016). These stresses usually cause significant reduction in both carrot yield and market value, and thus are considered to be economically important diseases affecting carrot crops worldwide. For example, epidemic

infections of carrot with *Alternaria* leaf blight have been reported in many farms around the world, causing up to 100% yield loss. *Xanthomonas* leaf blight also can be destructive and cause up to 100% yield loss in regions with warm and rainy weather. Cavity spot is ranked as the third most economically significant carrot disease in the U.S, and it also has been reported in other countries around the world such as France, Australia, and Japan. This disease occurs in 50% of carrot fields in California and Washington, and 25% of Colorado carrot fields, and it usually causes complete crop loss (Davis 2004).

Many carrot growers, especially those in developing countries, lack access to chemicals that can help overcome these stresses. In addition, consumers worldwide increasingly are demanding carrots grown using organic practices. In the U.S., organic carrot production accounts for 14 % of the total U.S. carrot crop, with an estimated 4,888.6 hectares grown annually (NASS 2016). In addition, chemical pesticides and fertilizers commonly used in conventional farming systems can negatively impact human and environmental health. These factors are motivating growers to seek alternatives such as biological control agents.

Biofertilizers and biopesticides can substitute for synthetic products commonly used in conventional farming systems to manage plant stresses. These products are intensively screened to avoid applying microbes that could be considered plant or opportunistic human pathogens. In addition, they do not leave behind chemical residues, thus they are considered to be more environmental friendly. *Bacillus* and *Pseudomonas* species are two of the most commonly used microbes in formulating these kinds of products. This is because many of these species can produce metabolites with antagonistic activity against a range of phytopathogens, and they often exert a positive effect on plant health and productivity via various mechanisms. However, the beneficial effects of microbial products are often inconsistent in the field, due to their short period of effectiveness after application. This is likely related to the sensitivity of these microbes to unfavorable environmental conditions, as well as the intense competition with resident soil microbial communities they encounter after application (Rao 1982, Montesinos 2003, Rai 2006).

Endophytes, which are defined as microbes that live inside plants, for at least part of their life cycle, and are often reported to provide beneficial effects for their plant hosts, could substitute for the application of the beneficial microbial inoculants discussed above. This is because endophytes colonize internal plant tissues, which provides them with shelter from unfavorable environmental conditions and reduces competition from microbes that colonize bulk soil and plant

rhizospheres. As a result, endophytes could be isolated from plants and developed for use as inoculants to overcome the challenges discussed above. Moreover, by learning more about the factors that naturally affect endophyte community composition, such as soil health and host genetic control, it may be possible to manipulate these communities using cultural practices to manage plant stresses and enhance crop performance (Fahey, Dimock et al. 1991, Mejía, Rojas et al. 2008, Ryan, Germaine et al. 2008).

Despite the potential importance that endophytes could confer to carrot crops, only three studies have investigated endophyte communities in this important crop to date. The first, conducted by Surette et al. (2003), quantified bacterial endophyte community localization in carrot taproots, and measured the density and biodiversity of these bacteria in two carrot genotypes grown in two fields that varied given soil tillage practices. Results of this study indicated that the greatest population density of endophytes was obtained from taproot crown tissue, demonstrating that endophytes are localized within specific plant parts. The variety Red Core Chantenay had greater endophyte population density than the variety Carochoice, demonstrating that host genetic control over endophyte communities is possible in carrot. In addition, differences in bacterial endophyte population density isolated from carrot tap roots grown under the two locations were observed, providing evidence that resident soil microbial community structure also plays a role in the composition of carrot endophyte communities. Of the endophytes isolated in this study, 83% enhanced carrot seedling growth and 33% enhanced potato growth, indicating that these microbes have an important role in carrot health and could prove useful as inoculants in other crops. However, while this study provided many important insights into carrot endophyte dynamics, this study was limited to bacterial endophytes only, and the authors did not investigate the potential mechanisms these microbes use to promote carrot seedling growth. In addition, this study was conducted using only two standard carrot varieties, and therefore does not reflect the full extent to which endophyte diversity could differ among carrot germplasm. Finally, these studies were conducted using only culture-based methods, which likely only reflects a small amount of the total endophyte diversity present in carrot taproots.

The other more comprehensive carrot endophyte study conducted by Lauran et al. (2013) attempted to correlate differences in endophyte communities in carrot taproots when grown under different cropping systems with changes in proteomic profiles of carrot taproots during six months of cold storage (Louarn, Nawrocki et al. 2013). Results from this study indicated that the cropping

system only slightly affected the composition of endophytes belonging to the Ascomycota and did not affect the post-harvest storage quality of carrot taproots. However, this study was conducted using only one carrot genotype and therefore the results may not reflect how other carrot genotypes and their endophyte communities might respond to being grown in these diverse crop management systems. In addition, this study only considered the effect of endophytes on carrots during storage and did not evaluate how the presence of endophytes might affect other key carrot properties such as resistance to pathogen stress.

To our knowledge, only one study has isolated individual endophytic strains from carrot taproots and evaluated their potential to perform specific functions. This study was conducted by Rodríguez et al. (2007), who isolated bacterial endophytes identified as *Micrococcus* sp., *Pseudomonas* sp. and *Staphylococcus* sp., and a carrot yeast identified as *Pichia* sp. These species were demonstrated to mediate stereoselective reduction of ketoesters and ketones to their corresponding secondary alcohols (biotransformation) in fresh carrot fragments, indicating that these microbes could contribute to the nutritional and organoleptic properties of carrot taproots. However, they did not determine whether these endophytes could contribute to any other key carrot properties.

Clearly, there still are many unanswered questions related to the composition and potential functional role of endophyte communities in carrot that must be answered in order to realize the benefits of these plant-associated microbes in this important edible crop.

1.2 The purpose of the dissertation

The broad goals of the research described in this dissertation were to: 1) characterize endophyte communities associated with carrot, 2) identify key factors affecting the composition of these endophyte communities, and 3) begin to elucidate some of the potential functional roles of important these plant-associated microbes.

To achieve these goals, the studies described in this dissertation had four main objectives:
1) Isolate, identify and characterize the functional attributes of culturable endophytes in the seeds and seedlings of nine carrot genotypes to determine whether endophytes are vertically transmitted in carrot and could play a key role in carrot seedling establishment.

2) Determine the extent to which crop management systems (organic vs. conventional) and carrot genotype interact to alter the composition of culturable endophytes in carrot taproots and affect the potential of these microbial communities to suppress *A. dauci*, a key carrot pathogen.

3) Characterize fungal endophyte community structure in the taproots of three carrot genotypes that vary in susceptibility to soilborne pathogens when grown under organic versus conventional management using next generation sequencing (NGS), to investigate whether the composition of these microbes may be a role in pathogen suppression.

4) Quantify the potential for microfiltration, with and without a pre-enzymatic digestion step, to enhance recovery of culturable endophytes in carrot taproots.

1.3 Outline of this thesis

Chapter 1 briefly introduces the purpose of this research along with an overview of the specific goals and objectives of these studies, and a review of what is currently known about plant endophyte ecology, diversity, and the potential for endophyte communities to suppress phytopathogens. Chapter 2 focuses on three aspects: 1) the composition and plant growth promoting activity of culturable endophyte communities vertically transmitted to carrot offspring via seeds; 2) how carrot genotype affects the composition and activity of these seed-borne endophytes; and 3) how the composition and activity of these seed-borne endophytes changes following germination. Chapter 3 addresses two main questions: 1) the effect of crop management practices (organic vs. conventional) and carrot genotype on the density of culturable carrot root endophytes and their antagonistic activity against *Alternaria dauci*, a key carrot pathogen; and 2) the effect of seed treatment with specific isolates of carrot root endophytes on carrot growth and suppression of disease caused by *A. dauci* infection under greenhouse conditions. Chapter 4 describes the application of next-generation sequencing to quantify the structure of endophyte communities in the taproots of three carrot genotypes that vary in susceptibility to soilborne pathogens when grown under organic vs. conventional crop management. The 5th chapter describes the potential for using an automated hollow fiber microfiltration approach, alone or in combination with a pre-enzymatic digestion treatment, to increase the isolation and recovery of endophytes from carrot roots. Finally, the 6th chapter provides a summary of the significant results from each chapter, and also discusses the study's limitations along with suggestions for future research directions.

1.4 Review

Just like humans, plants are associated with a microbiome (Turner, James et al. 2013). Plant microbiomes consist of epiphytic and endophytic microbes. While recently much has been learned about epiphytic microbes that live on the surface of plant organs (Fig. 1.1), comparatively little is known about endophytic microbes that live within internal plant tissues (Berg, Grube et al. 2014). This review discusses what is currently known about the ecology, diversity, and interactions of endophyte communities with plants and pathogenic microbes. It also discusses new molecular techniques that can be used to address important questions related to plant endophytes. Finally, it summarizes key questions needed to advance our understanding of endophytes and how they interact with plants to promote plant health and productivity.

1.4.1 Definition of endophytes

The word "Endophyte" is derived from the Greek words ‘endon,’ meaning within, and ‘phyte,’ meaning plant (Siegel, Latch et al. 1987). Over the years, scientists have suggested several definitions for plant endophytic microbes (Hardoim, Van Overbeek et al. 2015). For a long time, the term "endophyte" was used to identify only fungi that live inside plant parts and inhabit internal plant tissues, including the spaces between living plant cells, for all or part of their lives without causing any visible disease symptoms in the plant. Later, recognizing that plants could be colonized by bacteria as well, scientists added bacteria to the endophyte definition (Chanway 1996). In 1998 (Brown, Hyde et al. 1998), researchers added latent pathogens to the above definition, since latent pathogens can colonize internal plant tissues without causing any damage until plants are under stress, or later in maturity (Agrios, Trejos et al. 2004). Included in many of these early definitions, was language indicating that endophytes were microbes that can be isolated from the surface of disinfected plant tissues or extracts. However, this definition proved problematic, because many microbes cannot be cultured in the lab. Moreover, surface disinfection does not guarantee adequate elimination of epiphytic nucleic acids, and thus researchers must take additional steps to remove surface plant tissues when using molecular techniques to characterize endophytes (Garbeva, Van Overbeek et al. 2001, Hardoim, Van Overbeek et al. 2015). The latest definition of endophytes takes into account these limitations and defines endophytes as all the culturable and unculturable microbes that live inside plants without causing any harm. In addition, some authors further distinguish endophytes from other beneficial symbionts that create external

structures such as extraradial hyphae produced by mycorrhiza and nodules created by rhizobia (Hardoim, Van Overbeek et al. 2015), though other authors include these microbes within the broad definition of endophytes. The extent function of endophytes, however, as well as the conditions under which they benefit the plant, remains to be determined.

1.4.2 Endophyte plant hosts, location within plants, and density

Endophytes colonize many different plant species as well as plant organs (Bacon and White 2000). In fact, endophytes have been found colonizing every plant species studied to date (Santoyo, Moreno-Hagelsieb et al. 2016), including field crops, horticulture crops, perennial trees, and medicinal plants (Table 1.1). They have been isolated from plant roots, leaves, stems, fruits, and even seeds (Bacon and White 2000). However, the population density of endophytes differs among plant parts. For example, plant roots often have the greatest density of bacterial endophytes, followed by stems and leaves (Schulz, Boyle et al. 2006). Other plant parts such as fruits, flowers, and seeds, host densities of bacterial endophytes that are even lower than found in stems. In other plant parts, the population density of endophytes might even be so low that they are difficult to detect (Schulz, Boyle et al. 2006). While some have suggested that high population densities of endophytic bacteria are correlated with the presence of pathogenic bacteria (Grimault and Prior 1994), up to 10^{10} colony forming units (CFU) of bacterial endophytes per gram of plant tissue have been recovered from healthy plants (McInroy and Kloepper 1995).

Other factors affecting endophyte density include, but are not limited to, plant species, variation in host growth stage, and isolation technique used (Porras-Alfaro and Bayman 2011). For instance, even the surface sterilization technique commonly used to isolate endophytic microbes can affect their population density and recovery from host plant tissues, since some of the agents used to surface sterilize plants can penetrate plant tissues, causing damage to endophyte communities (Schulz, Boyle et al. 2006).

Studies investigating endophyte communities initially were conducted using culturable endophyte communities under *in vitro* conditions, while unculturable endophytes were ignored (Kirk, Beaudette et al. 2004). Such studies were problematic, because endophyte activity under *in vitro* conditions does not necessarily exhibit the same functions as in natural environments (Sun, Guo et al. 2011). In addition, limitations associated with culturing slow groing taxa as well as obligate microbial species, which are difficult to isolate due to their special culture requirements,

obscured the potential for researchers to adequately study plant endophyte dynamics (Sun, Guo et al. 2011). Culturable endophytes are expected to account for only for 1 % of total endophytic microbes, and therefore cannot be used to represent the activities of whole plant endophyte communities (Hugenholtz 2002). Thus, new molecular and biological techniques developed to better study and understand the role of unculturable microbes and now are being successfully applied to study endophyte community diversity and function in plants (Fig. 1.2) (Handelsman 2005). For example, using the highly discriminate 16S and 18S rRNA sequences along with new "omics techniques" has made it much easier to quantify the phylogenetic structure and function of both culturable and nonculturable endophyte communities in a short amount of time and at a low cost (Handelsman 2004). By choosing from different techniques, even the resolution of microbial taxonomy can be adjusted to each researchers needs (Fig. 1.3).

1.4.3 Factors affecting endophyte composition

Plant endophyte communities are structurally and functionally heterogeneous due to a range of factors. In the next few sections, some of the factors that can affect endophyte communities will be discussed, along with the new molecular techniques that have been used to study them.

1.4.3.1 Plant host

Plant genus is a well-known factor affecting the composition of endophytic microbial communities (Hardoim, van Overbeek et al. 2008). Moreover, recent studies indicate that genetic differences among species of the same plant genus also can affect endophyte bacterial community composition (Adams and Kloepper 2002). For example, the endophyte bacterial community of two varieties of sweet pepper (Milder Spiral and Ziegenhorn Bello) differed when quantified using T-RFLP fingerprinting and 16S rRNA sequencing (Rasche, Trondl et al. 2006). Similarly, in a study investigating fungal endophytic diversity using the internal transcribed spacer region (ITS) of nuclear ribosomal DNA, Chen et al. (2011) observed differences in fungal diversity among ten Dendrobium species (Chen, Hu et al. 2011). In this study, *D. candidum* had the highest level of fungal endophyte diversity, while *D. loddigesii* and *D. christyanum* had the lowest levels. Finally, different coffee (Coffea arabica) genotypes from Colombia, Hawaii, Mexico, and Puerto Rico had different fungal endophyte diversity patterns when grown under the same conditions (Vega, Simpkins et al. 2010).

The occurrence of endophytic microbial taxa in one plant host but not another demonstrates that these microbes have specific host preferences. The mechanisms by which a plant host can control entophyte community diversity is not fully understood and additional research is needed to uncover these mechanisms (Hardoim, Van Overbeek et al. 2015)

1.4.3.2 Plant growth stage

Plant growth stage is another factor that is tightly correlated with endophyte communities. For example, using Illumina sequencing of the 16s rRNA gene, bacterial endophyte communities in sugar beet (*Beta vulgaris L.*) changed given different seedling growth stages. This change took place when plants began developing tuber and rosette formations, and during accumulation of sucrose in taproots (Shi, Yang et al. 2014). More specifically, the abundance of α- Proteobacteria and γ- Proteobacteria changed from 73 % and 25 % respectively during seedling growth to 95 % and 3 % during tuber growth, 95% and 4 % during rosette formation, and 97% and 2 % during sucrose accumulation. Thus, differences in the abundance of these key microbial groups likely were caused by the phenological stage of the sugarcane plants.

In winter wheat (*Triticum aestivum*) plants, endophytic microbial species characterized using morphological and physiological characteristics, differed between young and mature plants (Schulz, Boyle et al. 2006). The authors of this study theorized that these differences were related to smaller and weaker plant vegetative tissues that provided fewer opportunities for endophyte colonization during the winter, and greater opportunity for cold temperature fungi and other less competitive microbes to colonize plant tissues. In contrast, during summer and spring when plant biomass was actively growing, endophytic growth rates within plant tissues increased and the competitive dominance of a few endophyte species observed during winter was reduced (Schulz, Boyle et al. 2006).

1.4.3.3 Plant organs

Different plant organs within the same plant can harbor different endophyte communities. For example, using RAPD and 16S rRNA sequence analysis to characterize maize endophyte communities, researchers (Pereira and Castro 2014) observed that α- and γ-Proteobacteria diversity were higher in roots than in shoots These bacteria also were found in the plant rhizosphere,

indicating that they may have originated from soil. This finding could help explain why they were higher in roots compared to shoots (Piotrowska-Seget, Besciak et al. 2012).

In another study with *Dendrobium* plants, that characterized endophytes based on sequence-based identification using blast searches of the ITS sequence in the NCBI GenBank database and phylogenetic analysis, indicated that the abundance and composition of fungal endophytes differed among plant tissues (Chen, Hu et al. 2011). For example, in roots, *Fusarium* sp abundance represented 68.2% of the total root fungal endophyte community, while *Fusarium* sp abundance represented 20.6% of total fungi in stems and 11.2% in leaves. In addition, some fungal isolates such as *Collectotrichum* sp did not colonize roots tissues but were abundant in stem and leaf tissues. In general, most of the endophytic fungi of *Dendrobium* plants colonized roots more than stem and leaves.

The difference in endophyte community composition between roots, shoots, and stems usually reflects differences in the environment and other biological variations among plant tissues. For example, in another study, orchid leaves and roots that were equally subjected to light and air, did not differ in endophytic fungal community composition (Bayman, Lebron et al. 1997)

1.4.3.4 Climate

Environmental factors independent of plant type or plant organ also can affect endophyte communities. For example, differences in climate from one geographic region to another, or even within the same region, have been shown to affect endophyte communities within plants. In one study, endophyte communities in mature teak tree (*Tectona grandis*) leaves varied with rainfall patterns, with some taxa only being observed during rainy seasons (Nair and Padmavathy 2014).

In another study using 16s rRNA gene libraries, differences in endophyte composition were observed between chilled and non-chilled sweet pepper (*Capsicum anuum L*) plants (Rasche, Velvis et al. 2006). However, in general, weather conditions have a weaker effect on root endophyte diversity than on aerial plant tissues (Merryweather and Fitter 1998). Root endophyte assemblages only appear to respond to climate changes that last for long periods of time and are affected by the amount of precipitation as well as enhanced CO_2 concentrations (Sieber, Waisel et al. 2002). For example, using agar plating and morphological identification of endophyte communities, 45 microbial taxa were isolated from tree roots submerged in a river, while only 31 species were isolated from non-submerged roots of the same tree (Fisher, Petrini et al. 1991).

Moreover, aquatic hyphomycetes were more abundant in the submerged roots, which reflect the effect of soil moisture on root endophyte communities. However, shifts in the quality and quantity of photosynthates and other metabolites within roots, as well as root turnover rate and shifts in soil microbial communities, have a greater effect on root endophyte diversity than external climatic conditions (Sieber, Waisel et al. 2002).

1.4.3.5 Soil characteristics and management practices

The composition of resident soil microbial communities, which are influenced by both soil type as well as various soil management practices, is widely regarded as the most significant factor affecting the composition of plant microbiomes (Schulz, Boyle et al. 2006). For example, soil physical and chemical soil characteristics such as pH, texture, moisture, temperature, and the presence of macro and micronutrients have been demonstrated to affect root endophyte community composition (Schulz, Boyle et al. 2006). This has been well documented in the case of key microbial taxa such as arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE), which are well known for their potential to solubilize phosphorus, increasing its availability for uptake by host plants. After staining roots and quantifying abundance of AMF and DSE using the magnified intersect method (McGonigle, Miller et al. 1990), the abundance of these microbial groups were demonstrated to increase in plant hosts that were grown in low phosphorus soils (Ruotsalainen, Vare et al. 2002).

A wide variety of management practices also can affect endophyte diversity directly or indirectly via changes in bulk soil microbial communities. For example, when isolated using culture dependent techniques and identified based on morphological characteristics, Schulz et al. (2006) observed that cutting and clearing forest trees within rows altered the diversity of root endophytic microbiota. Another study, which used Automated Ribosomal Intergenic Spacer Analysis along with cutluter dependent techniques to characterize endophytes in grape (*Vitis vinifera*), concluded that the composition of fungal taxa differed in grapevines managed using organic practices relative to those grown on farms employing an integrated pest management approaches (Pancher, Ceol et al. 2012). Differences in endophyte diversity associated with such alternative management practices could be due to changes in soil physical and chemical characteristics that alter bulk soil microbial communities, as well as direct effects caused by the

presence of difference organic substrates that are needed to support soil microbes and application of pesticides.

1.4.3.6 Presence of plant pathogens

Infections by general as well as specific plant pathogens are known to affect plant endophyte communities. For example, in a study using 16S rRNA-based denaturing gradient gel electrophoresis (DGGE) to compare the diversity of endophytic *Pseudomonas* sp. in potato (*Solanum tuberosum*) tubers infected or not with *Erwinia Carotovora* sp. *Atroseptica*, Schulz et al. (2006) observed distinct changes in *Pseudomonas* population diversity following pathogen attack. Interestingly, plants that were resistant to Erwinia infection always had a higher diversity of *Pseudomonas* sp. present. This might be related to differences in the overall antagonistic behavior of *Pseudomonas* sp. in the resistant plants. It also could be related to chemical and physical changes in the endophytes' habitats, following upregulation of plant defense pathways upon pathogen attack.

1.4.4 Managing endophytes to control plant diseases

With the increase in environmental pollution caused by excessive use of certain pesticides, as well as development of resistance to pesticides by some plant pathogens, interest among growers in using biological methods to control plant diseases is increasing (Whipps 2001). One such biological control method is the application of beneficial microorganisms. Endophytes are one type of microorganism that has been used in biocontrol against plant diseases (Ting, Mah et al. 2012).

Numerous studies have documented the success of using endophytes in controlling plant diseases. For instance, one study (Tyvaert, França et al. 2014) used *Verticillium* Vt305, an endophytic fungus, to protect cauliflower plants from *Verticillium* wilt caused by *Verticillium dahlia*. Another study (Khastini, Ogawara et al. 2014) used a fungal endophyte characterized as *Cadophora* sp. to control Fusarium wilt caused by *Fusarium oxysporum* in watermelon. In addition, researchers (Niones and Takemoto 2014) isolated 14 fungal endophyte isolates of *Epichloe festucae* from different species and cultivars of a temperate grass that had the ability to inhibit the growth of a number of common grass pathogens when tested under *in vitro* conditions.

Another study found that bacterial endophytes related to *Acinetobacter*, *Bacillus*, and *Klebsiella* sp isolated from wheat (*Triticeae aestivum*) could inhibit the growth of the soil fungal pathogen *Gaeumannomyces graminis* under *in vitro* tests (Duran, Acuna et al. 2014). In addition, seed treatment with the endophytic fungus *Beauveria bassiana,* has been found to protect tomato and cotton seedlings against damping off caused by *Rhizoctonia solani* and *Pythium myriotylum* fungi (Ownley, Griffin et al. 2008). Six endophytic taxa characterized as *Actinomycetes* that were isolated from the roots of native plants in the Algerian Sahara significantly reduced the severity of damping-off of tomato seedlings caused by *Rhizoctonia solani* (Goudjal, Toumatia et al. 2014). *Pseudomonas aeruginosa* endophytic bacteria isolated from red chili (*Capsicum annuum L*) fruit had antagonistic effects against chili anthracnose disease caused by *Colletotrichum gloeosporioides* (Allu, Kumar et al. 2014). The endophytic fungi *Aureobasidium pullulans* isolated from sweet cherries (*Aureobasidium pullulans*) was used successfully to control post-harvest rots of sweet cherries and table grapes by *Monilinia laxa* and *Botrytis cinerea* (Schena, Nigro et al. 2003). Finally, a study (Mejia, Rojas et al. 2008) successfully controlled fungal infection caused by *Moniliophthora roreri*, *Moniliophthora perniciosa* and *Phytophthora palmivora* in *Theobroma cacao* by treating cacao plants with *Colletotrichum gloeosporioides*, *Clonostachys rosea* and *Botryosphaeria ribis* endophytic fungi.

1.4.5 Endophyte mechanisms for controlling plant diseases

While hundreds of studies have demonstrated that endophytes can be successfully used to control plant diseases, the mechanisms underlying this protection are still not totally clear, which prevents the practical application of these plant-associated microbes for controlling diseases in agriculture. In the next section, hypotheses related to the potential mechanisms responsible for disease control by endophytes will be discussed, along with new molecular techniques for testing these hypotheses.

1.4.5.1 Antagonism

One of the main mechanisms that endophytes are expected to use to inhibit pathogens, is through production of antimicrobial compounds (Ezra, Castillo et al. 2004, Niones and Takemoto 2014; Chernin, Chet et al. 2002). These antagonistic compounds weaken hypha produced by fungal pathogens, resulting in abnormalities in pathogen growth and/or prevention of further colonization

in the plant host's cells. For example, one study demonstrated that the endophytic bacteria *Bacillus subtilis* ALB629 could produce an antifungal compound that inhibited the growth of *Moniliophthora perniciosa*, *Colletotrichum* sp. and *C. gossypii*, which are pathogenic fungi of cacao, when tested under *in vitro* conditions using PDA supplemented with 80% v/v of a *B. subtilis* filtered culture (Falcäo, Silva-Werneck et al. 2014). In another study, Garyali, Kumar et al. (2014) found that certain endophytes from *Taxus baccata L. ssp. wallichiana* had the ability to produce antimitotic compounds when screened for the presence of Taxol genes involved in the biosynthetic pathway of antimitotic compounds. Selected isolates obtained in this study were identified using ITS sequencing, and their antimitotic compounds were extracted and subjected to HPLC-MS for antitumorgenic identification and activity assay (Coker, Radecke et al. 2003).

The antagonistic ability of endophytes also could play a role in inhibiting pathogens for resources, since, like any other microorganism, endophytes must be able to compete with other microbes for food, space, and even air. For instance, (Tyvaert, França et al. 2014) showed that one endophytic fungus, *Verticillium* Vt305, had a greater ability to compete for space than the plant pathogen *V. longisporum*, and as a result, could reduce wilt disease incidence in cauliflower plants. This was documented using qPCR, which demonstrated that cauliflower plants that were pre-treated with *Verticillium* Vt305 before being infested with *V. longisporum* had no or very low concentrations of *V. longisporum*, while high concentrations of Vt305 were detected.

1.4.5.2 Improving plant growth

Improving plant growth and health is a potential indirect mechanism that endophytes use to control plant diseases. This type of interaction has been reported in many studies. For example, dipping carrot seedlings in an 10^8 CFU mL−1 suspension of bacterial endophytes (*Bacillus megaterium, Bacillus subtilis, Pseudomonas fluorescens* or *Pseudomonas putida*) isolated from carrots for 30 seconds resulted in significant increases in carrot shoot and root growth, compared to untreated control seedlings four weeks after treatment (Surette, Sturz et al. 2003).

Such improvements in plant health could be related to several mechanisms, including the production of phytohormones that directly stimulate plant growth, or contributing to a plants nutrient needs by fixing atmospheric nitrogen fixation or solubilizing minerals such as phosphorus increasing their availability for plant uptake. One study found that 34% of the endophytic bacteria isolated from soybean (*Glycine max*) plants produced indoleacetic acid (IAA) (a well-known plant

growth hormone), 49% were able to solubilize mineral phosphate, and 60% of the isolates were able to produce IAA and fix atmosphere nitrogen when tested under *in vitro* conditions (Kuklinsky-Sobral, Araujo et al. 2004). Results of these studies indicate that endophytes isolated from the soybean plants could help improve their growth and health. In this experiment, a nitrogen free medium was used to grow the bacterial endophytes, and then the nitrogen fixation potential of endophytes isolates was verified by subjecting them to PCR using primers targeting the nif H gene, since this gene encodes for the nitrogenase protein Component II. Indole acetic acid production and potential to solubilize mineral phosphate was conducted by subjecting endophyte isolates to modified methods developed in Bric, Bostock et al. (1991) and Verma, Ladha et al. (2001).

In another study, endophytes isolated from wheat (*Triticum aestivum*) plants that were characterized as *Bacillus, Paenibacillus, Klebsiella,* and *Acinetobacter* sp had similar so called plant growth-promoting capabilities. This included auxin and siderophore production, phytate mineralization, and tricalcium phosphate solubilization when screened using *in vitro* techniques (Durán, Acuña et al. 2014).

1.4.5.3 Induced host resistance

Induced host resistance, also commonly referred to as induced systemic resistance (ISR), is another factor suspected to be related to the ability of endophytes to control plant diseases. Many studies have documented that some endophytes control plant disease using this mechanism. For example, *Pseudomonas fluorescens* PICF7 endophytes in olive (*Olea europaea L.*) roots upregulated genes coding for lipoxygenase2, catalase, 1-aminocyclopropane-1-carboxylateoxidase, and phenylananine ammonia-lyase (Gómez-Lama Cabanás, Schilirò et al. 2014). These genes are known to be involved in olive plant defense responses against pathogens. This was demonstrated using subtractive hybridization cDNA libraries that can help researchers identify genes that are upregulated during the interaction between plants and microbes. Expression of such genes is then subsequently verified using qRT-PCR.

In another study, endophytic *Actinobacteria* isolated from wheat plants induced systemic resistance in Arabidopsis plants against *Erwinia carotovora subsp. Carotovora* and *Fusarium oxysporum* infection via activation of defense genes involved in the jasmonic acid, ethylene, and salicylic acid defense pathways (Conn, Walker et al. 2008). Since the *Actinobacteria* primed both

the salicylic acid and jasmonic acid/ethylene pathways, the endophyte's mechanism of action in this case likely involved a combination of ISR and systemic acquired resistance (SAR) defense reactions.

1.4.6 Research needs

While lots of progress has been made in understanding how individual strains of endophytes can protect some plants against certain diseases, the mechanisms that these microbial taxa use to control diseases has yet to be fully determined. Moreover, recent studies indicate that several microbial taxa may be working synergistically to control plant diseases, indicating that additional research is needed to determine how the entire plant microbiome interacts with plants to affect their health and disease resistance. It is now well established that the diversity of endophyte communities can change depending on host specificity, as well as other factors such as resident soil microbial community structure, but does this hold for all crops? Can we breed disease-resistant crops by selecting genotypes that preferentially recruit and host beneficial endophytes that contribute to disease suppression? Can we enhance or maximize the plant growth promoting and disease suppressive effects of these beneficial endophyte communities via management practices that improve soil health?

Newly developed "omics" tools have potential to help researchers answer these questions. For example, meta-proteomics, meta-genomics, and meta-transcriptomics and associated bioinformatic analyses have widely been cited for their potential to help researchers begin to confidently link the potential functions of entire plant microbiomes to their phylogenetic identifications (Rastogi and Sani 2011). For example, in several reports published in the last few years, researchers used these techniques to begin to unravel the complex relationships underlying beneficial plant microbial interactions (Adie, Pérez-Pérez et al. 2007, Barret, Morrissey et al. 2011, Knief 2014). But will they be helpful in elucidating these relationships in an important edible root crop like carrot, or will new techniques be needed?

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Table 1.1 Summary of plant endophyte abundance, types, host plant, and plant parts that they recovered from, and techniques used in their isolation and identification. Data were collected from published articles.

Plant species	**Endophyte type and abundance of unique taxa**	**Location of recovered endophytes**	**Isolate techniques Used**	**Reference**
Corn (*Zea mays*)	373 bacterial isolates	Shoots and roots	Isolation: Culture- dependent. Identification: PCR, 16s rRNA gene sequencing, Bio Edit program and EzTaxon server.	(Pereira & Castro, 2014)
Tomato (*Solanum lycopersicum*)	174 bacterial isolates	Shoot, root and stem.	Isolation: Culture- dependent. Identification: 96 well Biology plates, PCR, 16S rRNA gene sequencing, BLAST and database from National Center for Biotechnology Information Blast.	(Rashid, Charles, & Glick, 2012)
Wheat (*Triticum aestivum)*	28 bacterial isolates	Leaves and stem	Isolation: Culture- dependent Identification: PCR, 16s rRNA gene sequencing.	(Zinniel et al., 2002)

Table 1.1 continued

Soybean (*Glycine max*)	17 bacterial isolates	Leaves and stem	Isolation: Culture- dependent Identification: PCR, 16s rRNA gene sequencing.	(Zinniel et al., 2002)
Sorghum (*Sorghum bicolor*)	151 bacterial isolates	Leaves and stem	Isolation: Culture- dependent Identification: PCR, 16s rRNA gene sequencing.	(Zinniel et al., 2002)
Cotton (*Gossypium hirsutum*)	535 bacteria isolates	Roots and stems	Isolation: Culture- dependent Identification: Fatty acid methyl Ester, gas chromatography and Microbial Identification System software.	(McInroy & Kloepper, 1995)
Sweet Corn (Zea mays L. var. rugosa)	543 bacterial isolates	Roots and stems	Isolation: Culture- dependent Identification: Fatty acid methyl Ester, gas chromatography and Microbial Identification System software.	(McInroy & Kloepper, 1995)

Table 1.1 continued

Tomato (*Solanum lycopersicum)*	15 fugal strains	leaves, stems and roots	Isolation: Culture- dependent Identification: morphological characteristics and nuclear ribosomal ITS1-5.8SITS2 Sequence analysis.	(Kim et al., 2007)
Red pepper (*Capsicum* annuum)	91 fugal strains	leaves, stems and roots	Isolation: Culture- dependent Identification: morphological characteristics and nuclear ribosomal ITS1 & TS2 Sequence analysis.	(Kim et al., 2007)
Pumpkin (*Cucurbita pepo* L.)	11 fugal strains	leaves, stems and roots	Isolation: Culture- dependent Identification: morphological characteristics and nuclear ribosomal ITS1-5.8SITS2 Sequence analysis.	(Kim et al., 2007)

Table 1.1 continued

Chinese cabbage *(Brassica campestris var. pekinensis Makino)*	17 fugal strains	leaves, stems and roots	Isolation: Culture- dependent Identification: morphological characteristics and nuclear ribosomal ITS1-5.8SITS2 Sequence analysis.	(Kim et al., 2007)
Cucumber (*Cucumis sativus* L.)	19 fugal strains	leaves, stems and roots	Isolation: Culture- dependent Identification: morphological characteristics and nuclear ribosomal ITS1-5.8SITS2 Sequence analysis.	(Kim et al., 2007)
Potatoes (*Solanum tuberosum*)	1122 fugal strains	root segments	Isolation: Culture- independent DNA isolation. Identification: DGGE of their 18S rRNA gene fragment, BOX-PCR and GenBank.	(Gotz et al., 2006)

Table 1.1 continued

Wheat (*Triticeae aestivum)*	58 actinobacterial isolates	Root tissues	Isolation: Culture- dependent Identification: PCR, 16S rDNA sequencing.	(Coombs & Franco, 2003)
Strawberry (*Fragaria* × *ananassa*)	20 bacterial isolates	Meristematic tissues	Isolation: Culture- dependent Identification: Fatty acid methyl Ester, gas chromatography and Microbial Identification System software.	(Dias et al., 2009)
Orchids (*Dendrobium*)	127 fungi isolates	Roots and seed	Isolation: Culture- dependent Identification: morphological characteristics, PCR, DNA sequencing and GenBank database (sequencing the nuclear ribosomal internal transcribed spacer)	(Chen, Wang, & Guo, 2012)
Rice *(Oryza sativa)*	28 Bacterial strains	stems, seeds, and leaf	Isolation: Culture- dependent Identification: PCR, 16S rDNA sequencing.	(Elbeltagy et al., 2000).

Table 1.1 continued

Coffee (*Coffee arabica*)	340 fungi isolates	Leaves	Isolation: Culture- dependent. Identification: PCR, DNA sequencing and GenBank database (sequencing the nuclear ribosomal) internal transcribed spacer)	(Santamaria & Bayman, 2005)
Sugarcane (*Saccharum officinarum*)	32 Bacterial isolates	leaves and stems	Isolation: Culture- dependent. Identification: PCR, 16S rRNA gene**,** BLAST and GenBank.	(Magnani et al., 2010)
Physic nut (*Jatropha curcas*)	9 fungi isolates	Leaf	Isolation: Culture- dependent Identification: rDNA sequencing of their ITS region, BLAST and NCBI database.	(Kumar & Kaushik, 2013)
Mandarin orange and tangor (Citrus)	407 fungal isolates	Leaves	Isolation: Culture- dependent Identification: RAPD variability	(Glienke-Blanco, Aguilar-Vildoso, Vieira, Barroso, & Azevedo, 2002)
Palms (*Licuala* spp.)	2237 fungal isolates	Leaves and Petiole	Isolation: Culture- dependent Identification: Morphological characteristics.	(Frohlich, Hyde, & Petrini, 2000)

Table 1.1 continued

Eucalyptus spp	76 bacterial isolates	Stem	Isolation: Culture- dependent. Identification: PCR, 16S rDNA sequencing, BLAST and ARB package software.	(Procopio, Araujo, Maccheroni, & Azevedo, 2009)
Bush & Japanese clovers *(Lespedeza* spp.*)*	39 bacterial strains	Root nodules	Isolation: Culture- dependent. Identification: PCR, 16S rDNA sequencing, BLAST and GenBank database.	(Palaniappan, Chauhan, Saravanan, Anandham, & Sa, 2010)
Soybean (*Glycine max*)	373 bacterial strains	leaves, stems and roots	Isolation: Culture- dependent Identification: PCR, 16S rDNA sequence analysis, BLAST and GenBank.	(Kuklinsky-Sobral et al., 2004)
Black pepper *(Piper nigrum*)	74 bacterial strains.	Roots and stem	Isolation: Culture- dependent. Identification: PCR,16S rDNA Sequencing, BLAST analysis and nucleotide sequence similarities were determined with the NCBI.	(Aravind, Kumar, Eapen, & Ramana, 2009)
Carrot (*Daucus carota)*	360 bacterial isolates	Roots.	Isolation: Culture- dependent. Identification: Fatty acid methyl Ester, gas chromatography and Microbial Identification System software.	(Surette, Sturz, Lada, & Nowak, 2003)

Table 1.1 continued

Milkweed (*Asclepias syriaca*)	20 bacterial isolates	Leaves and stem	Isolation: Culture- dependent. Identification: PCR, 16S rRNA gene amplification and sequencing, BLAST and GenBank.	(Zinniel et al., 2002)
23 evergreen plants	262 fungal strains	Leaves	Isolation: Culture- dependent Identification: Morphological characters.	(Liu, Liu, Yuan, & Gu, 2010)
Eggplant (*Solanum melongena*)	172 bacterial isolates	Xylem	Isolation: Culture- dependent. Identification: PCR, 16S rRNA sequencing, BLAST and database from National Center for Biotechnology Information.	(Ramesh et al., 2014)

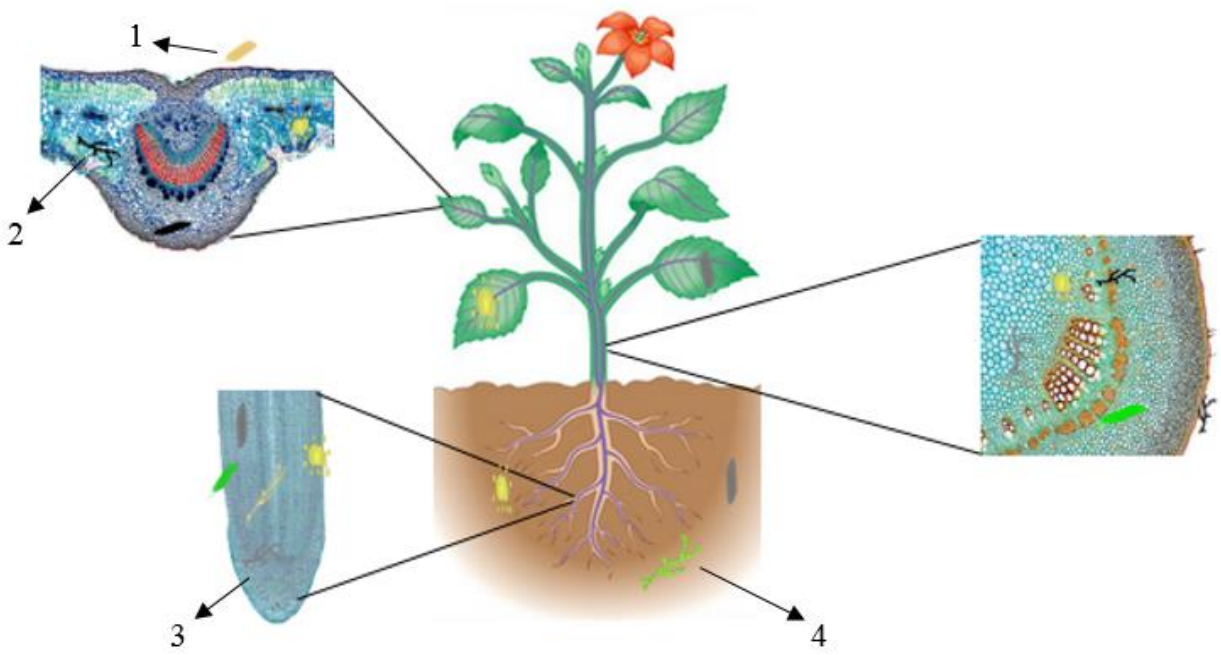

Figure 1.1 Plant microbes 1) epiphytic microbes on leaves, 2) endophytic microbes In leaves, 3) endophytic microbes in roots and 4) microbes in the plant rhizosphere.

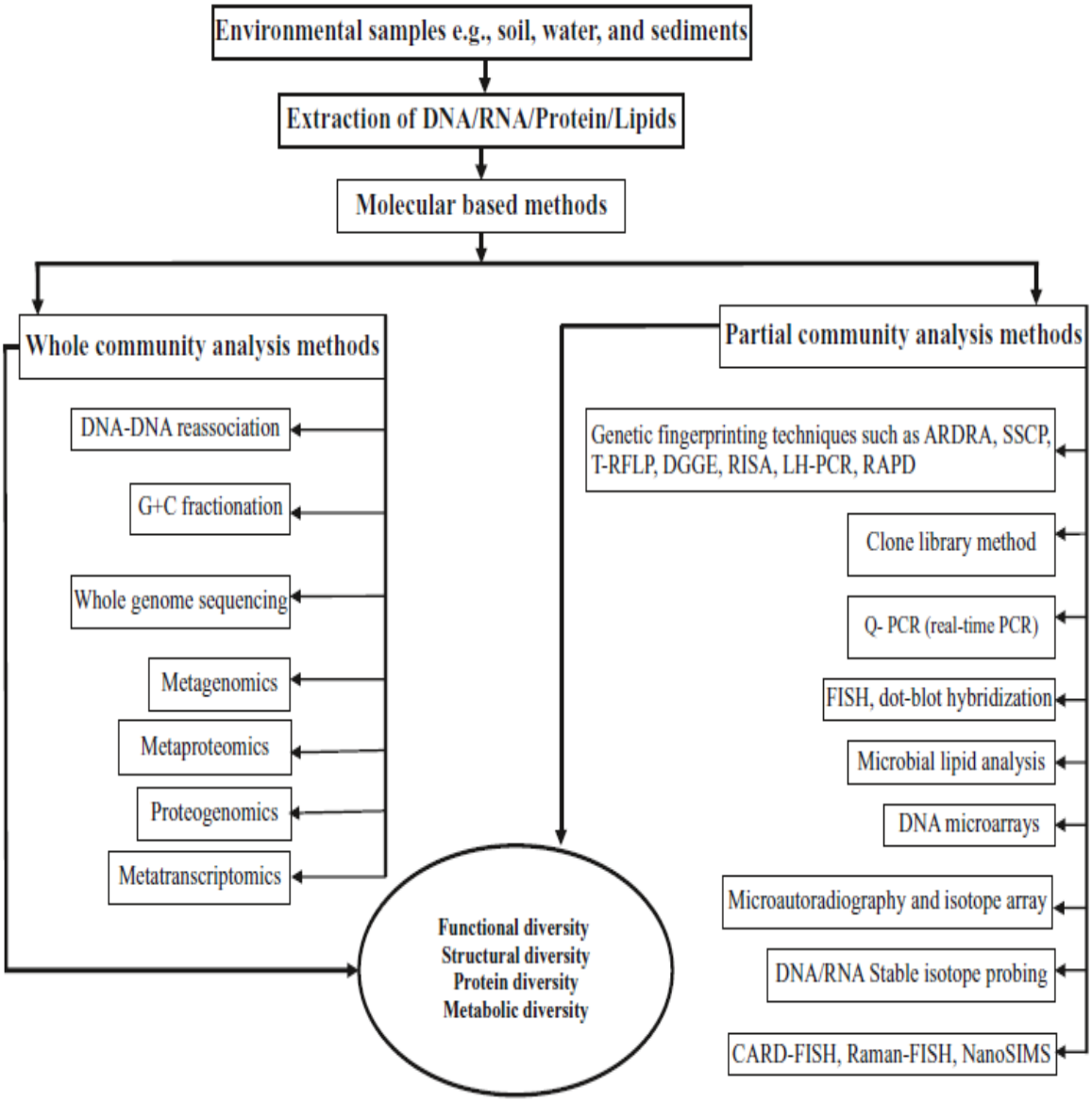

Figure 1.2 Culture-independent molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment. (Rastogi and Sani, 2011)

Approach	Sample Required	Taxonomic resolution				
		Family	Genus	Species	Sub-sp.	Strain
Culturable	Genomic DNA			<----------RAPD---------->		
	" "			<----------AFLP/RFLP---------->		
	" "			<----------Rep- and BOX-PCR---------->		
	Proteins			<----------Isozyme analysis---------->		
	Whole cell Proteins			<----Transcriptome / protein profiling---->		
	Genomic DNA		<----------DNA-DNA Hybridization---------->			
	" "		<----------ARDRA---------->			
	" "		<---16S rRNA/ tRNA PCR- Sequencing---->			
	" "			<---16S-23S rRNA-ITS/ tRNA-ITS PCR--->		
	" "		<----------*cyt C1, ctp syn, glu-tRNA syn, PKS-I, PKS-II, NPRS* genes sequencing (specific to actinobacteria)- ---------->			
	Whole cell lipids		<------FAME/ other chemical analysis ---->			
	Whole genome	<-Whole genome sequencing and Multi-Locus Sequences Analysis ->				
Unculturable	Microbial community DNA		<----------16S rRNA PCR- DGGE---------->			
	" "		<-16S rRNA/Functional genes PCR- TRFLP->			
	" "		<-16S rRNA clone libraries sequencing-->			
			<-------Direct shot-gun/ Pyro-sequencing (16S rRNA/ Functional genes)---------->			

Figure 1.3 Relative applicability of different molecular biological techniques used in the taxonomic identification and diversity analysis of endophytic *Actinobacteria* (Verma and Gange, 2014)

CHAPTER 2. VERTICAL TRANSMISSION OF CULTURABLE CARROT ENDOPHYTES: COMPOSITION, PLANT GROWTH PROMOTING ABILITIES AND EFFECT OF PLANT GENOTYPE AND DEVELOPMENTAL STAGE

2.1 Introduction

Carrot is one of the most important vegetable crops in the world due to the high nutritional value of its taproot, and potential for long-term storage (Bender 2009, Abdel-Aal, Akhtar et al. 2013). However, carrots are very slow to germinate and establish, which them make them poor competitors for nutrients in the soil and highly susceptible to competition from weeds. In addition, as carrot seeds germinate, they face and must overcome a broad range of potential pathogens that can cause damping off, negatively affect the quality of taproots, and damage above-ground biomass making taproot harvest difficult (Davis 2004, Colquhoun, Rittmeyer et al. 2017). Like all plants, carrots are naturally engaged in mutualistic relationships with a broad group of soil microorganisms (Surette, Sturz et al. 2003), which can help them overcome these challenges. Some of these are endophytes, which are defined as microbes that live part or all of their lives inside plants without causing disease (McInroy and Kloepper 1995). Endophytes form a mutualistic relationship with plants helping them tolerate biotic and abiotic stress, and in exchange, the plants provide endophytes with protection and suitable habitat to colonize and reproduce (Truyens, Weyens et al. 2015). It has been theorized that plants and their microbial symbionts evolved alongside each other for mutual benefit, and they should be considered as one 'superorganism' or 'holobiont' (Zilber-Rosenberg and Rosenberg 2008). Because of their close relationship with plants, endophytes are likely to be an important component of these holobionts.

Endophytic microbes have been isolated from roots, leaves, stems, flowers and seeds (Brader, Compant et al. 2017), though seed-borne endophytes are particularly unique because they are vertically transmitted from one generation to the next (Ringelberg, Foley et al. 2012). Seeds are vital to the life cycle of all gymnosperms and angiosperms, allowing plants to exist in a type of dormant state until environmental conditions are suitable for growth (Shahzad, Khan et al. 2018). Similarly, some microbes can produce specialized features such as flagella that allow them to migrate into seeds before they harden, and form endospores which allow them to tolerate desiccation and stay alive inside seeds for years until environmental conditions improve and seeds

germinate (Johnston-Monje and Raizada 2011, Truyens, Weyens et al. 2015). Seed-borne endophytes have an advantage over microbes that are horizontally transmitted via soil, because they are already adapted to live within plant tissues and are able to rapidly colonize new seedling tissues once seeds germinate (Neal, Larson et al. 1973, Hardoim, Hardoim et al. 2012). Microbes that are vertically transmitted in seeds can be beneficial or pathogenic, though endophytes that are exclusively transmitted via vertical transmission are not likely to be pathogens, because these symbionts depend entirely on their host for reproduction (Rodriguez, White Jr et al. 2009). Thus, it is in the best interest of vertically transmitted endophytes to promote rather than suppress plant growth, which requires specialized interactions with host plants in which a balance between plant defense and endophyte virulence is needed. Otherwise, breaking that balance will cause either endophytes to attack their host plant, or the host plant to induce defense responses that kill their endophytes (Schulz and Boyle 2005).

While seed-borne endophytes have been underappreciated because like most endophytes, they do not produce visible symptoms (Mostert, Crous et al. 2000, Hyde and Soytong 2008), recent studies indicate that they can aid in seed germination and establishment (Hardoim, Hardoim et al. 2012). For example, endophytic microbes could promote seed germination and seedling growth by secreting beneficial metabolites such as indole acetic acid (IAA) and siderophores, solubilizing phosphorus and fixing atmospheric nitrogen (Johnston-Monje and Raizada 2011, Hardoim, Hardoim et al. 2012). They could also help seedlings deal with water and other forms of plant stress by secreting bioactive secondary metabolites such as 1-aminocyclopropae-1-carboxylate (ACC) deaminase, that can cleave the precursor of stress ethylene (Qin, Miao et al. 2015, Latif Khan, Ahmed Halo et al. 2016). Endophytes have also been demonstrated to suppress pathogen infection indirectly by competing with pathogens for space and nutrients, and directly by secreting antagonistic compounds (Kaga, Mano et al. 2009). Finally, seed-borne microbial endophytes could secrete exudates that attract beneficial microorganisms from the soil to the spermosphere, further enhancing seedling establishment and helping seedlings avoid infection by pathogens (Baker and Cook 1974, Truyens, Weyens et al. 2015).

Understanding how to manipulate seed microbiomes has the potential to enhance crop performance, though unanswered questions about the ecology of seed-borne endophytes currently limit their practical application in agricultural systems. Consequently, learning more about the identify and potential functional role of seed borne endophytes, as well as characteristics and plant

factors that allow them to survive and proliferate in germinating seedlings are needed. For example, recent studies investigating specific characteristics of seed borne bacterial endophytes have indicated that the ability to produce amylases and phytases are important for mobilizing starch and phosphorous, respectively, in germinating seeds (Johnston-Monje and Raizada 2011, Truyens, Weyens et al. 2015)

Plant factors are likely to influence the composition and activity of seed borne endophytes including the developmental stage of the plant and its genotype (Adams and Kloepper 2002, Rasche, Trondl et al. 2006, Marques, da Silva et al. 2015, Rodríguez-Blanco, Sicardi et al. 2015). Once the radicle starts to grow and emerge from the seed to form a root, endophyte populations proliferate rapidly (Marques, da Silva et al. 2015), which is likely to result in a shift in endophyte community composition due to competition over limited resources (Hallmann, Quadt-Hallmann et al. 1997). Many studies have provided evidence that plant genotype is one of the most important factors influencing endophyte community composition (Hartmann, Schmid et al. 2009). Differences in endophyte communities in response to plant genotype could be related to differences in root exudates that attract or enrich specific groups that are able microbes to colonize plants (Hardoim, van Overbeek et al. 2008). It could also be due to differences in morphological and physiological characteristics of individual plant genotypes, which generate different selection pressures on endophyte communities and allow some groups of microbes to proliferate within their tissues (Nelson 2004, Johnston-Monje and Raizada 2011, Rodríguez-Blanco, Sicardi et al. 2015). The effect of plant genotype and development stage on endophyte communities could also extend to their function (Hartmann, Schmid et al. 2009). For instance, Walitang, Kim et al. (2017) showed that different rice cultivars select different bacterial seed endophytes to host in their seeds due to their potential plant growth promoting abilities. More specifically different rice cultivars were found to differ in the ability of their endophyte communities to detoxify ROS, produce polymer-degrading enzymes and tolerate osmotic stress. Govindasamy, Raina et al. (2017) found that changing the developmental stage in sorghum from seedling, to pre-flowering and flowering stages affected auxin production, phosphorus solubilization, nitrogen fixation, siderophore production and ACC deaminase activity by endophytes.

The broad goal of this study was to learn more about ecological factors affecting the structure and function of vertically transmitted endophytes in carrot. Specific objectives were to: 1) characterize the composition and PGPR activity of culturable endophytes living within carrot

seeds, 2) determine how carrot genotype affects the composition and activity of seed-borne endophytes, and 3) determine how the composition and activity of seed-borne endophytes changes following germination. Our underlying hypotheses for these objectives are that, while many microbial taxa may have characteristics that allow them to enter developing seeds and survive desiccation, they might not be able to outcompete other microbial taxa and interact effectively with plants after germination of the seeds. In addition, differences in the chemical composition and immune systems in carrots with different genetic backgrounds that were subject to selection in diverse environments are likely to select for differences in endophyte composition and functional potential. To test these hypotheses, we isolated endophytes from the seeds and seedlings of nine diverse carrot genotypes using five selective media, and quantified their potential to produce IAA and siderophores, solubilize phosphorus, and fix atmospheric nitrogen.

2.2 Material and Methods

2.2.1 Carrot germplasm

Seeds of nine diverse carrot genotypes representing a range of genetic backgrounds and phenotypic characteristics were obtained from the USDA-ARS Carrot Breeding and Genetics program in Madison, WI (Table 1). This included four commercial varieties and five experimental lines originally selected in Asia, Syria, Turkey, Europe and Brazil. Seed from three of the entries (PY191, P6306, Y8519) were propagated in cages in Madison, WI using roots grown in El Centro, CA. The remaining entries were collected from six different seed companies worldwide. All seeds were stored at 15-18C before testing.

2.2.2 Isolation and identification of carrot seed and seedling endophytes

Seed and seedlings grown from seeds under axenic conditions described below were rinsed several times with tap water before surface disinfection by soaking in 5.25% bleach for 3 minutes, followed by 3% peroxide solution for 3 minutes, and washing with sterilized water supplied with 1ml of tween (Surette et al., 2003). To confirm surface disinfection, 200 µl samples from the last washing solution was plated onto various selective media representing broad microbial groups including LGI (diazotrophic bacteria), Tryptic Soy Agar (heterotrophic bacteria), R2A (oligotrophic bacteria), 1/5th PDA (general fungal media) and Dextrose-peptone Agar supplemented with rose Bengal and antibiotics (selective for yeast and molds) (Corry et al., 2011;

Reasoner and Geldreich, 1985). All plates were incubated at 27 °C for 48 h and checked for any microbial growth.

To identify culturable endophytes inside carrot seeds, 20 g of surface sterilized seed from each genotype were homogenized using an Omni tissue master homogenizer (OMNI International, G.A, U.S) in 10 ml sterile water to prepare stock and serial dilution solutions. To identify endophytes in carrot seedlings, surface sterilized carrots seeds of each genotype were placed onto plates containing MS media (0.8% agar) supplemented with 3% sucrose and adjusted to a final pH of 5.8. The plates were maintained at 25 °C ± 2°C and exposed to 16 hours light per day for two weeks (Pant and Manandhar, 2007). After two weeks, the Omni tissue master homogenizer was used to homogenize eight seedlings from each genotype in 10 ml sterile water to prepare stock and serial dilution solutions. After homogenization, 200 µl from seed and seedling serial dilution solutions were plated onto LGI, Tryptic Soy Agar, R2A, 1/5th PDA and Dextrose-peptone plates, each with five replicates for each carrot genotype. Plates were incubated at 27°C for 48 h for bacteria and 25°C for 72 h for fungi and were checked daily to monitor bacterial or fungal growth.

Bacterial and fungal colonies that were morphologically distinct in each genotype were selected as representatives for identification using PCR followed by sequencing. Individual microbial isolates were purified following hyphae tip (Narayanasamy, 2001) and streak plate (Black, 2008) techniques for fungal and bacterial cultures respectively on agar slants. DNA was extracted from purified isolates using Microbial DNA extraction Kits from (MO BIO Laboratories, C.A., U.S.A) following the manufacturers protocol.

Fungal isolates were PCR amplified using the ITS5 forward primer 5 ′ GGAAGTAAAAGTCGTAACAAGG- 3′ and the ITS4 reverse primer 5′-TCC TCC GCT TAT TGA TAT GC- 3′ to amplify the whole ITS region (Larena et al., 1999). Bacterial isolates were PCR amplified using the universal 8F bacterial forward primer. 5 ′ - AGAGTTTGATCCTGGCTCAG-3 ′ and the 1492R reverse primer 5′-GGTTACCTTGTTACGACTT- 3′ (Turner et al., 1999) to amplify the full length 16S SSU rRNA gene. Each 25-µl PCR reaction contained 1µl of DNA template, 0.5 µl of each primer (100 mM), 12.5 µl GoTaq® colorless Master Mix from Promega and 10.5 µl free water. PCR was performed

in a Bio-Rad thermal cycler (BioRad, C.A, U.S.A). Cycle conditions for fungal endophytes included an initial denaturing step (1 cycle at 95ºC for 2 minutes), followed by 40 cycles of denaturation (95ºC for 30 seconds), annealing (55ºC for 30 seconds), and extension (72ºC for 1 minute), and a final extension step (72ºC for 10 minutes). Cycle conditions for bacterial endophytes included an initial denaturing step (1 cycle at 94ºC for 3 minutes), followed by 35 cycles of denaturation (94ºC for 45 seconds), annealing (50ºC for 60 seconds), and extension (72ºC for 90 seconds), and a final extension step (72ºC for 10 minutes).

Detection of PCR-amplified products was performed by electrophoresis on a 0.7% (wt./vol.) agarose gel stained with Bullseye DNA Safe Stain (MIDSCI., MO. U.S.A) A 100bp ladder (New England bio lab, MA. U.S.A) was run in parallel to approximate PCR product band size. Agarose gels were stained using DNA Safe Stain (MIDSCI., MO., U.S.A), and PCR products were visualized after exposure of the gel to ultraviolet (UV) light. PCR products were cleaned using UltraClean ® PCR Clean-Up Kits (MO BIO Laboratories, C.A., U.S.A) and sent to the Purdue Genomics Facility for sequencing using an ABI 3137XL capillary machine (Applied Biosystems, CA., U.S.A). Fragment analysis of the ITS region for fungi and 16S rRNA gene of bacteria were analyzed using the BLAST nucleotide sequences alignment program of the GenBank database (National Institutes of Health), and fungal identification was confirmed using the Saccharomyces Genome Database (Fungal BLAST) with a confidence level of 95% for bacteria and 97% for fungi at the genus level.

2.2.3 Enumeration of broad bacterial and fungal groups among seed and seedling endophytes:

The same seed and seedling stock and serial dilutions described above were used to enumerate endophyte abundance using the drop plate method (Herigstad et al., 2001). Briefly, 45 µl of each serial dilution solution was dispensed onto the same selective media for broad bacterial and fungal groups described above (except for Dextrose-peptone Agar) using a multichannel pipette in eight evenly spaced 5 µl drops, with five replicate plates for each genotype. After the drops dried, plates were inverted and incubated at 27°C for 48 h and 25°C for 72 h for bacterial and fungal endophytes respectively. The number of colonies within each of the eight drops was quantified, and the mean of the colony counts was used to calculate the total colony forming units for each replicate plate using the following formula:

$$\text{\# of endophytic bacteria/fungi per Carrot seed/seedling} = \frac{\text{Number of Colonies}}{\text{Volume plated (ml)} \times \text{total dilution used}}$$

2.2.4 Indole acetic acid production

To determine whether the bacterial endophytes isolated from carrot seed and seedling tissues could produce IAA under in vitro conditions, a modified Salkowski colorimetric technique employing 96 well plates was used (Sarwar and Kremer, 1995). A single bacterial colony representing each endophytic isolate was inoculated into LB broth (Corry et al., 2011) supplemented with tryptophan (1 mg/ml) and cultured under shaking at 120 rpm for 2 days at 30° C. LB broth that was not inoculated was used as a negative control, and all samples were run in triplicate for each isolate. After incubation, bacterial OD 500 nm was adjusted to 0.2 and cells were centrifuged at 3000 rpm for 10 min at 4° C. 100 µl of each bacterial supernatant was dispensed into a 96 well plate well and mixed with 200 µl of reagent, incubated for 30 minutes at room temperature, and then sample absorbance was measured at OD 530 nm in a 96-well plate (Epoch microplate spectrophotometer equipped with gene 5 software. from, Bioek, VT., U.S.A). To quantify the amount of IAA produced by the bacterial isolates, standard curves of pure IAA with known concentration were run in parallel with the samples, and final values of IAA produced by the endophytic bacteria were expressed in µg ml-1.

2.2.5 Siderophore production

Bacterial endophyte siderophore production was determined *in vitro* using a modified protocol developed by Schwyn and Neilands (1987), using chrome azole S (CAS) blue indicator dye in 96 well plates (Alexander and Zuberer, 1991). In brief, endophyte bacterial cultures were grown in LB broth medium (Corry et al., 2011) with a pH of 6.8 for 48 h at 30 °C, in triplicate for each isolate. After incubation, the OD of the bacterial cultures were adjusted to OD 500 nm = 0.1, and cultures were centrifuged at 10,000 rpm for 10 min. 100 µl of each bacterial culture was dispensed into separate wells of 96 well plates followed by the addition of 100µl of CAS reagent and incubated for a period of 20 minutes. Uninoculated LB broth was used as a reference. The absorbance of each sample was measured at 630 nm on a 96-well plate (Epoch microplate

spectrophotometer equipped with gene 5 software. from, Bioek, VT., U.S.A), and siderophore production per unit (psu) was calculated using the following formula: $\frac{(Ar-As) \times 100}{Ar.}$

Where Ar = absorbance of uninoculated LB broth with added CAS reagent (serves as a reference solution), and As = absorbance of the cell free sample supernatant with added CAS reagent.

2.2.6 Phosphate solubilization

To obtain a qualitative assessment of phosphate solubilization ability of the bacterial endophytes, isolates were inoculated onto plates containing National Botanical Research Institute's phosphate (NBRIP) solid medium (Nautiyal, 1999) supplemented with $Ca_3 (PO4)_2$ as an insoluble phosphate form. Cultures were incubated at 30º C for 13 days. Uninoculated NBRIP medium was used as a control, and all samples were run in triplicate. After incubation, bacterial colonies were visibly screened for the formation of halo zones, which indicate phosphate solubilization ability. For a quantitative assessment of phosphate solubilization ability, a modified protocol developed by Nautiyal (1999) was used. Bacterial isolates were inoculated into NBRIP broth and incubated at 30º C on a rotary shaker (180 rpm) for 7 days. Uninoculated NBRIP broth was used as a control, and all samples were run in triplicate. After incubation, the OD of the cultures were adjusted to OD 500 nm = 0.1 and centrifuged at 10,000 rpm for 20 min. 50 µl of each bacterial culture was dispensed into separate wells of a 96 well plate followed by the addition of 8 µl of molybdenum-blue reagent (Murphy and Riley, 1962), and incubated for 10 minutes. Sample absorbance was measured at OD 800 nm in a 96-well plate (Epoch microplate spectrophotometer equipped with gene 5 software. from, Bioek, VT., U.S.A), and soluble phosphate in each sample was quantified using a standard curve generated with known concentrations of KH_2PO_4.

2.2.7 Nitrogen fixation ability

Ammonia production by the bacterial endophytes, measured using Nessler's reagent (James and Natalie, 2014), was used as an indication of their ability to fix atmospheric nitrogen. Bacterial endophytes were inoculated into peptone water and incubated for 48 h at 28 ºC. Uninoculated peptone water was used as a negative control, and samples were run in triplicate. After incubation,

10 ml of each bacterial culture were mixed with 0.5 ml of the reagent, and development of brown to yellow color was used as a positive indicator for ammonia production (Ahmad et al., 2008).

2.2.8 Statistical analyses

The density, abundance and plant growth promoting activity of endophytes isolated from carrot seed and seedling tissues were analyzed using SAS JMP version 13 Software (Sall et al., 2012). For quantitative data, including Indole acetic acid, siderophore production, phosphate solubilization, endophyte density and abundance, data were subjected to analysis of variance (ANOVA) using a general liner model procedure followed by a Tukey post hoc analysis for pairwise comparison of treatment means when main effects were detected at $P \leq 0.05$. Log transformation was applied to endophyte density and abundance data prior to running the ANOVA test. To evaluate qualitative data including phosphate solubilization and nitrogen fixation ability of endophytes, a Chi-square test was used followed by Fisher's Exact Test for mean comparisons.

2.3 Results and Discussion

2.3.1 Identification of seed and seedling endophytes

Seeds are key vectors for the transmission of a wide variety of microorganisms. This includes plant pathogens as well as beneficial species that can promote plant growth. Vertical transmission of beneficial microbes inside seeds is thought to be an important component of their mutualistic relationship with plants, as some of these microbes can aid in seed preservation and promote germination in new environments (Rudgers et al., 2009). However, some beneficial plant microbes can also act as opportunistic pathogens, negatively affecting those with compromised immune systems (Berg et al., 2005). Consequently, learning more about the microbes packaged in seed is critical to plant as well as human health.

Results of the experiments described in this chapter provide evidence that many bacterial and fungal taxa are vertically transmitted to carrot progeny via seed as endophytes. There are three main pathways through which microbes can be transmitted from maternal plants to seeds. This includes: 1) via internal vascular tissue associated with developing seeds, 2) via floral pathways, especially the parental stigma, and 3) via external contact with microbes inside fruit or in the environment after seed harvesting (exogenous pathway for seed borne microbes that are not

endophytes) (Puente et al., 2009; Shahzad et al., 2018; Truyens et al., 2015). Vertical transmission of fungal and bacterial endophyte communities has been recorded in many crop plants (Gagne-Bourgue et al., 2013; Hodgson et al., 2014; Liu et al., 2017a). However, while other studies have identified seed and seedling endophytes communities and estimated their potential functional role in plants (Bailey et al., 2006; Clay, 1987; Herrera et al., 2016; López-López et al., 2010), to our knowledge, our study is the first to characterize endophyte communities in carrot seeds and seedlings, and demonstrate that they are vertically transmitted.

No bacterial or fungal isolates were cultured from the final wash solution of carrot seeds during the surface sterilization process used in our experiments, demonstrating the high efficacy of this technique and supporting our assertion that all microbial isolates obtained in this study were endophytic. This technique has been used successfully before for isolating endophytic communities from the tissues of multiple crops including red clover, potatoes and carrot (Sturz et al., 1998; Surette et al., 2003).

In total, 123 endophytic microbes exhibiting different morphological characteristics within each category, i.e. seed/seedling/genotype, were isolated in this study using five selective media. By sequencing the partial 16S rRNA and ITS region of these isolates, we were able to identify a total of 95 distinct microbial taxa (Table 2.2 & 2.3) within the bacterial phyla of Proteobacteria, Firmicutes and Actinomyces and the fungal phyla of Ascomycota and Basidiomycota. Observing these microbial communities in seed and seedlings provides evidence that at least part of the endophytic communities found residing in carrot roots and shoots is vertically transmitted via seeds. Moreover, the fact that we found individual isolates of some taxa such as *Pantoea* sp. in 8 out of 9 genotypes in seed produced in multiple locations worldwide, indicates that some of these microbes could be part of a core microbiome in carrot. Core plant microbiomes represent microbes that are systematically associated with a given host plant. These core microbiomes may or may not promote plant growth directly, but they are expected to play pivotal roles in organizing the assembly of other microbiomes in and around host plants (Toju et al., 2018).

The most common bacterial endophytes isolated from seed and seedling of the nine genotypes evaluated in this study were related to *Pantoea* sp., *Xanthomonas* sp., *Curtobacterium* sp and *Bacillus* sp., while the most common isolated fungal endophytes were related to *Alternaria* sp., *Cladosporium* sp., *Geomyces* sp. and *Stemphylium* sp. Even though some of these fungal and bacterial species and genera are well known as a plant and human pathogens such as *Pantoea*

agglomerans, *Xanthomonas campestris pv. Campestris*, *Alternaria dauci*, and *Cladosporium fulvum* (Bensch et al., 2012; Coutinho and Venter, 2009; Dutkiewicz et al., 2016; Thomma, 2003), this is not always the case. Many of these microbial species and genera have been isolated from healthy plant tissues indicating that might not be acting as pathogens. In addition, these species and genera have been noted to be able to confer plant growth promoting activities (Chutulo and Chalannavar, 2018; Cottyn et al., 2009; Li et al., 2017; Walitang et al., 2017; Walterson and Stavrinides, 2015). For example, *Bacillus pumilus*, *Pantoea agglomerans*, *Pantoea ananatis*, *Curtobacterium* sp. and *Xanthomonas* sp, endophytes were isolated from healthy rice seeds in a tropical environment, and they exhibited antifungal activity against *Sarocladium oryzae*, *Rhizoctonia solani*, *Fusarium moniliforme* and *Pyricularia grisea*, which are all rice pathogens (Cottyn et al., 2001). Moreover, *Cladosporium cladosporioides* and *Alternaria alternata*, have been isolated from healthy neem trees (*Azadirachta indica*), and have been demonstrated to produce a number of bioactive and antimicrobial compounds that fight pathogens and benefit plants (Silva et al., 2012; Verma et al., 2011b). In our study, all the recovered endophytes were isolated from healthy carrot seeds and seedling that were produced from healthy plants and seeds that did not show any symptoms of abiotic or biotic stress. Thus, we conclude that the microbes isolated from seeds and seedlings in our study are endophytes, and not pathogens. In the future, running simple pathogenicity tests would provide more support to our conclusion.

Determining how plant genetic and developmental processes affect endophyte communities that are vertically transmitted via seed is an important first step in deciphering their potential role in plants. In this study, a total of 62 individual microbial taxa were isolated from carrot seed, while only 33 taxa were obtained from their associated seedlings, and a greater total number of isolates were recovered from seeds compared to seedlings (Tables 2.2), Microbial taxa isolated from carrot seeds included *Stemphylium* sp., *Curtobacterium herbarum*, Uncultured endophytic, *Bacillus* sp., *Pseudomonas* sp., *Uncultured bacterium, Alternaria alternate, Lewia* sp., *Pantoea ananatis*, *Alternaria infectoria*, *Enterococcus casseliflavus*, *Curtobacterium* sp. *Alternaria tenuissima, Cladosporium cladosporioides, Pleospora herbarum* and *Pseudomonas* sp., while *Alternaria chenopodiicola*, *Geomyces* sp. *Xanthomonas campestris*, *Rhodotorula* sp., *Enterobacter* sp., *Xanthomonas* sp., *Uncultured Pantoea* sp. and *Xanthomonas arboricola pv. Corylina* endophytic were isolated from seedlings. Some of these microbial taxa such as *Alternaria* sp. *Alternaria burnsii*, *Cladosporium* sp., *Pantoea agglomerans*, *Pantoea vagans* and *Pantoea* sp.

were present in both seed and seedlings, indicating that these taxa have unique properties that allow them to maintain colonization and proliferate in developing plant tissues.

The number of recovered endophytes also differed among genotypes (Tables 2.2). Carrot genotypes Exp Nb3999 and Exp P6306 had the greatest number of seed and seedling endophytes isolated respectively, with a total of 15 microbial taxa isolated from genotype Exp Nb3999 and 6 from genotype Exp P6306. Carrot genotype Exp B0252 had the lowest number of recovered microbial taxa, with only one isolate obtained from seed and 2 from its seedlings. None of the endophytes were shared among all the carrot genotypes, within their seed or seedlings.

Within seedlings, a *Pantoea* sp. was the most commonly isolated taxa among the nine carrot genotypes, since it was found in Exp Nb3999, Brasilia, ExpP6306, ExpY8519 and Red Core Chantenay. *Alternaria* sp. and *Geomyces* sp. were the most commonly isolated fungal endophytes among the nine carrot genotypes. *Alternaria* sp were the most commonly recovered from genotypes Red Core Chantenay and ExpP6306, and *Geomyces* sp was the most recovered taxa from genotypes Karotan and NSFF. Within seeds, *Pantoea* sp. was the most commonly isolated bacteria taxa among the nine carrot genotypes, since it was found in Exp PY191, Brasilia and Exp Y8519. An *Alternaria* sp ws the most commonly isolated fungal taxa among the nine carrot genotypes, since, it was found in genotypes Exp Nb3999, Karotan, ExpP6306, Red Core Chantenay and ExpY8519. In contrast, most of the recovered endophytes from seed and seedlings were genotype specific such as *Rhodotorula* sp. found in only ExpY8519 seedlings, only *Pleospora herbarum* found in only NSFF seed.

Plant genotype and developmental stage have previously been reported to be important factor affecting endophyte community structure (da Silva et al., 2014; Hallmann et al., 1997; Hartmann et al., 2009). Differences in endophyte composition in response to changing plant developmental stage could be caused by differences in plant morphological and physiological characteristics associated with individual developmental stages (Hardoim et al., 2011; Marques et al., 2015). When seed start to germinate radicles and plumules first emerge, cotyledons then start to expand, and true leaves start to grow causing drastic morphological changes. These morphological changes provide seedborne endophytes with more space to colonize and propagate in comparison with the limited space they had to colonize internal seed tissues (Bewley, 1997). As a result, under normal conditions, plant seedlings are always expected to harbor larger endophyte communities in comparison with seeds.

Seed germination is also associated with changes in physiological characteristics (Black, 1970). For example, Barret et al. (2014) reported that bacterial and fungal endophyte community diversity in cotton was expected to change during seed emergence, since the abundant amount of plant exudates generated during this stage can cause extreme changes in the biochemical properties inside germinating seeds (Bewley, 1997). As a result, cotton seedlings were expected to select for copiotrophic endophytic microbial taxa, since seedlings are considered a rich environment for solubable carbon compunds for endophytes compared to seed.

In addition, genetic differences among plant genotypes that alter plant morphological and physiological characteristics could also affect endophyte communities within the same developmental stage. In support of this hypothesis, Adams and Kloepper (2002) found that different cotton cultivars harbored different endophyte communities within their seed and seedlings, which were correlated with differences in morphological and anatomical characteristics such as cotton seed size, number of ovule cells within seeds, and seed coat structural differences among the cotton cultivars. They also added that differences in physiological characteristics among different cotton cultivars such as differences in radical length and the quality and quantity of root exudates was likely to have affected the composition of endophyte communities with cotton seed and seedlings, since these compounds are considered an important source of food for endophytic microbes.

The nine carrot genotypes selected for our study differ greatly in terms of their genetic background and morphological and physiological characteristics including root color and shape, resistance to parasitic nematodes and early top growth (Table 2.1). We suspect that selection under different environmental conditions for these traits could have altered the types of endophytic microbes that are recruited and vertically transmitted by these genotypes. However, future studies using seed from these genotypes that were all grown in the same location will be needed to confirm this hypothesis.

Some microbial taxa were only observed in seedlings, despite the fact that they were grown under sterile conditions. Robinson et al. (2016) observed similar results when trying to isolate endophytes from surface sterilized wheat seeds and germinated seedlings grown under sterile conditions. In fact, no endophytes were detected in the seeds in this study. The authors theorized that seeds are subjected to a number of biotic and abiotic stresses during and after development, which results in the production of growth inhibitors and antimicrobial compounds in seed tissues,

reducing the recovery of culturable endophytes from seed macerates. Other studies have noted the presence of phenolic and antimicrobial compounds in wheat seed tissues, providing support for this hypothesis (Barbosa Pelegrini et al., 2011; Capparelli et al., 2005; Dykes and Rooney, 2007). Future studies using culture-independent tools to characterize the composition of endophyte communities within seeds are needed to confirm this hypothesis.

2.3.2 Enumeration of broad bacterial and fungal groups among seed and seedlings

To determine if carrot genotype and developmental stage would affect the density of culturable endophytes, the number of endophyte colonies of broad microbial groups including: heterotrophic, diazotrophic and oligotrophic bacteria, and total fungal in the seed and seedling of nine diverse carrot genotypes were recorded then converted to relative abundance. Oligotrophic endophytes are slow-growing microorganisms that can live under extremely low nutrient conditions within plants in a symbiotic relationship without causing any harm to their hosted plants. They have very low metabolic rates and usually present at a low density, which helps to sustain their survival in low-nutrient environments (Kuznetsov, Dubinina et al. 1979). Heterotrophic microbes are fast-growing bacteria that require the presence of organic carbon sources to survive, because they cannot produce these compounds on their own. Thus, they are commonly found living in a symbiotic relationship with plants (Bamisile et al., 2018). Diazotrophic bacteria are microorganisms that can live without external sources of nitrogen (N) because they are able to fix atmospheric nitrogen into useable forms such as ammonia. Unlike rhizobia, they do not require specialized plant organs such as nodules to fix N, but they do require anoxic conditions to protect the nitrogenase enzyme (Sona Janarthine et al., 2011). Finally, relationships between endophytic fungi and plants are usually framed as either mutualistic or commensalistic, but they can also sometimes be described as potential latent pathogens (Schulz, Römmert et al. 1999). However, the majority of fungal endophytes have been demonstrated to provide effects for their host plants such as increasing their total yield, and tolerance to stress via production of beneficial bioactive compounds.

The total number of diazotrophic bacterial endophytes ranged from log 2 to 9, heterotrophic bacterial endophytes ranged from log 3 to 9, oligotrophic bacterial endophytes ranged from log 3 to 8, and total fungal endophytes ranged from log 2 to 4 colony forming units (C.F.U) per seed or seedling (Fig. 2.2 & 2.3). Similar to our results the above endophytic groups have been recovered

before from the seeds of many crops including maize (*Zea mays*), cucmber (*Cucurbitaceae*), wheat (*Triticum sp.*) and rice (*Oryza sativa*) and their abundance were always assiosated with host high productivity and rsistance to Abiotic and biotic stress (Reinhold-Hurek and Hurek, 1998; Riggs et al., 2001)

A greater density of endophytes among all groups were observed on carrot seedlings compared to seeds (Fig. 2.1), and there were differences in the relative abundance of endophytic fungi in response to the developmental stage (Figure 2.2). Similar results have been observed among endophyte communities in cotton as well as many other crops (Adams and Kloepper, 2002; Robinson et al., 2016). The increase in endophyte density in seedlings relative to seeds could be related to the presence of a radical and root exudates produced by the germinated seedling. As mentioned above, root exudates are well known to provide an adequate source of carbon and other nutrients to signal and support root associated microbes, which likely differs significantly from lower carbon and nutrient concentrations that would be expected in seed (Baker and Snyder, 1965).

In addition, differences in the total abundance of some endophytic communities between seed and seedlings could be related to differences in plant mass, which provides endophytes with more space to propagate (Neal et al., 1973). However, competition among microbial taxa for differences in space and nutrients over time is likely to cause a shift in endophyte community structure (Weller, 1988), like we observed in this study with fungal endophytes relative abundance in this study. Since fungi are well known for their weaker competion and slower growth compared to bacteria (Agrios, 2005).

The density and relative abundance of broad bacterial groups also differed among some carrot genotypes (Figs. 2.3 & 2.4). Genotype ExpNb3999 had the greatest number of seedborne endophytes among all selective media, and the greatest number of heterotrophic endophytes among seedlings (Fig. 2.3). Within seeds, genotype ExpPY191 had the greatest amount of total fungi, while genotype Red Core Chantenay had the greatest number of diazotrophic bacteria and genotype ExpB0252 had the greatest number of oligotrophic bacteria. The lowest number of heterotrophic and diazotrophic bacteria on seed and seedlings were observed in genotype Karotan, and genotype Brasilia had the lowest oligotrophic and fungal abundance in seeds. Finally, the lowest number of fungal and oligotrophic endophytes in seedlings was recorded in genotypes ExpB0252 and ExpY8519, respectively. In addition, the relative abundance of broad bacterial and fungal groups of endophytes differed in some genotypes. In seed, the highest fungal relative

abundance was observed for genotype ExpPY191 while the lowest fungi relative abundance was related to genotype ExpNb3999. For diazotrophic bacteria in seed, the highest relative abundance was related to ExpY8519, while the lowest was associated with NSFF. Within seed, there were no differences in oligotrophic bacteria differences among the carrot genotypes. Finally, for heterotrophic bacteria within seed, the greatest relative abundance was observed in Brasilia, and the lowest in ExpB0252.

Within seedlings, the greatest fungal abundance was observed in ExpY8519, while the lowest was observed in ExpB0252. For diazotrophic bacteria within seedlings, ExpNb3999 had the greatest relative abundance and NSFF had the lowest. Among oligotrophic bacteria among seedlings, the greatest relative abundance was observed in ExpB0252, and lowest in ExpY8519. Finally, for heterotrophic bacteria in seedlings, the greatest relative abundance was associated with ExpY8519 and the lowest was associated with ExpB0252 (Fig 2.4).

The effect of plant genotype on endophyte groups density and abundance observed in our study has previously been reported before in a number of crops (Adams and Kloepper, 2002; Marques et al., 2015; Rodríguez-Blanco et al., 2015). The reason behind these differences could be related to morphological, physiological and functional differences among the crop genotypes as well as the adaptation, preferences and competitive ability of the endophytes themselves (Adams and Kloepper, 2002). For example, (Roesch et al., 2006) theorized that differences among *Zea mays* genotypes in the number of diazotrophic bacteria present in the plants roots was related to different selective pressures on endophyte community composition. Moreover, genetic and physiological differences among different *Z. mays* genotypes resulted in the elimination of diazotrophic microbes within some genotypes. With the recent landmark study published by Van Deynze et al. (2018) indicating that a specific *Z. mays* variety from the Oaxaca region of Mexico can host diazotrophic bacteria that contribute between 29-82% of its total nitrogen nutritional needs, it is exciting to think about the potential to select genotypes for their ability to host this important group of bacteria. While we did observe differences in diazotrophic density among our nine carrot genotypes, future studies that specifically quantify nitrogen fixation and the total nitrogen transferred to plants from this important group of microbial taxa are needed to determine if these differences are biologically relevant.

In another example comparing endophytes in the seed of ten *Z. mays* cultivars (Johnston-Monje and Raizada, 2011), the authors observed that most of the cultivars appeared to share a core

endophyte microbiome, though there were some differences in endophyte abundance among the different cultivars. The authors theorized that changes in abundance were related to differences in the unique ecological niche provided by individual cultivars. As mentioned above, in our study some of the carrot genotypes have distinct morphological and physiological characteristics including differences in resistance to soilborne pathogens, which might be the reason why we observed differences in the density and abundance fo their endophytes community. The fact that we did observe such differences indicates that it could potentially by possible to select for the ability to host beneficial microbial endophytes. However, further investigation will be needed to quantify the exact benefit of each endophytic taxa isolated, and determine the limited factors affecting each groups density within different carrot genotypes.

2.3.3 Indole acetic acid production

Indole acetic acid (IAA) is well known for its potential to act as a key plant hormone stimulating root cell expansion, root hair development and production of reactive oxygen species (ROS) in seedlings (Ivanchenko et al., 2013). This compound is also well known for its potential to be an important signaling molecule among microbes, and it is expected to be a key component in plant-microbial relationships as over 80% of plant-associated microbes have been found to produce IAA (Patten and Glick, 1996). Initially, production of IAA by plant-associated microbes was thought to play a key role in pathenogenesis, but it is now known that a wide variety of microbes, including those considered as PGPR, produce IAA (Spaepen et al., 2007). The exact reason that microbes produce IAA, as well as which environmental conditions stimulate its production is still unclear, though it is often assumed to be an important component of PGPR activity. For example, IAA can promote plant growth by controlling cell elongation, division, tissue differentiation and response to gravity and light (Teale et al., 2006), by altering levels ROS that can help plants tolerate biotic stress, by directly acting as a pathogen barrier (Hückelhoven and Kogel, 2003), and/or acting indirectly by regulating plant defense genes (Levine et al., 1994). The beneficial effects of IAA could also be purely coincidental, as microbes produce these compounds to increase nutrient availability for themselves by loosening plant cell walls to release saccharides from plants (Lindow and Brandl, 2003; Vorholt, 2012).

Most of the bacterial endophytes isolated in this study (55 out of 58) were able to produce indole acetic acid (IAA) under *in vitro* conditions. Levels of IAA produced by the endophytic

bacteria ranged from 0.09 to 15.3 µg ml-1. As discussed above, production of IAA by plant endophytes is very common and has been documented in many previous studies (Khan and Doty, 2009; Tian et al., 2015; Verma et al., 2017). However, it also might indicate that the seeds are vertically transmitting pathogens instead of endophytes since IAA production by microbes has previously been noted as a potential mechanism of pathogenicity (Link and Eggers, 1941). In this study, all the seeds were obtained from healthy plants that did not show any signs of infection or stress at the time of harvesting, and they were saved and handled carefully to avoid any microbial contamination. In addition, all seedlings evaluated in this study were grown *in vitro* under axenic condition, thus we do not believe that the endophytes were acting as pathogens. However, pathogenicity tests on the endophytic isolates would be required to support this observation.

Carrot developmental stage had a significant effect on IAA production, with higher levels produced by endophytes isolated from seedlings compared to seed (Fig.2 5a). Increasing the IAA produced by microbes could be related to many factors including subjecting microbes to environmental stress such as acidic pH, osmotic stress and carbon limitation (Ona et al., 2005), or even the presence of plant extracts. For example, IAA production by *Xanthomonas axonopodis* was reported to increase after subjecting bacteria to citrus leaf extracts, however the exact compound within the extract that was responsible for this increase is still unclear (Costacurta et al., 1998). In addition, genetic factors such as location of the IAA gene in the genome, and transcription factors that regulate expression can also affect the amount of IAA produced by microbes (Patten and Glick, 1996). In our study, the increase in IAA production by seedling endophytes compared to those isolated from seed, might be related to all of the above since developing from seeds into seedlings is likely associated with a change in pH, osmotic stress, the release of viable carbon for endophytes through plant exudates, and changes in the morphology of the surrounding endophytes environment. In addition, plant needs for IAA at this key developmental stage might motivate vertically transmitted endophytes to produce this compound as part of their mutualistic relationship with plant hosts (White Jr. et al., 2014). Finally, endophytes producing IAA themselves might prefer to live in seedling tissues rather than seeds, since they are more adapted to the physiological and anatomical characterstics of this environment. Following up with experiments that test if the same effect will occur when plants are subject to real life conditions that they would experience in soil and identifying the specify environmental triggers behind such an increase would support these assertions.

Carrot genotype also had a significant effect on the ability of bacterial endophytes isolated from seed and seedlings to produce IAA. Within seeds, endophytes recovered from Brasilia produced the highest level of IAA with a value of 2.76 µg ml-1, while thoese recovered from ExpP6306 had the lowest level of production with a value of 0.90 µg ml-1 (Table 2.4a). Among endophytes isolated from seedlings, IAA production by microbial isolates recovered from genotype ExpNb3999 produced the highest level of IAA with a value of 7.033 µg ml-1, while taxa isolated from genotype ExpB0252 had the lowest level of IAA production with a value of 0.37 µg ml-1 (Table 2.4b). Other studies have also observed an effect of plant genotype on IAA production by endophytes. For example, Marques et al. (2015) observed differences in IAA production among three sweet potato genotypes. Most of the IAA producing bacterial strains were isolated from one of the three sweet potato genotypes, indicating that differences in IAA production in response to plant genotype are likely due to functional diversity among the different genotypes.

Functional diversity among different plant genotypes is represented by differences in the morphological and developmental needs of individual genotypes (Hartmann et al., 2009). As discussed above, the nine carrot genotypes evaluated in our study differ significantly based on early top growth and resistance to pathogens, as well as morphological characteristics such as root color and shape (Table 2.1), therefore differences in IAA production by microbes isolated in these genotypes could theoretically play a role in these characteristics.

Differences in the internal environment among different genotypes such as differences in pH, osmotic stress, and availability of labile carbon sources in plant exudates could also be the reason behind differences in endophytes producing with the potential tor produce IAA as explained above. Further *in planta* investigation is needed to support the potential differences in IAA production among different genotypes and developmental stages to determine if they will exist under real life conditions and investigate the mechanisms that might be facilitating it.

2.3.4 Siderophore production

Siderophores are low molecular weight, high affinity iron chelating compounds that are produced by microbes under iron limiting conditions, and function in solubilization, and transporting and storing iron for future needs. Microbial siderophores are related to five main groups. However, the most common groups produced by microbes are catecholates, carboxylates and hydroxamates (Cornelis, 2010; Matzanke, 2017). Like IAA, siderophore production by

microbes has previously been reported as a potential mechanism of pathogenicity (Leong and Neilands, 1982). However, endophytic bacteria that can produce siderophores have also been reported to have high rates of antagonistic activity against phytopathogens (Compant et al., 2005; Kloepper et al., 1980; Miethke and Marahiel, 2007). Microbes that produce siderophores can also promote plant growth directly by transferring iron when their host plants need it, and/or sequestering it in their cells under high iron levels, which prevents host toxicity (Loaces et al., 2011). In addition, many microbial taxa that can produce siderophores have been found to be able to induce systemic resistance in plants against biotic and abiotic stress, and therefore promote plant growth indirectly (Loaces et al., 2011).

Most of the bacterial endophytes isolated in this study (49 out of 58) had the ability to produce siderophores under *in vitro* conditions, though production by individual isolates ranged from 0.81 to 50.58 % of S.U (siderophore unit). Production of siderophores by endophytes has been reported before in a number of studies (Rosconi et al., 2013; Verma et al., 2011a). As described above, producing siderophores by microbes might indicate that the seed is vertically transmitting pathogens rather than endophytes. However, we do not expect that this is the case as the seeds were collected from healthy plants, though again, we would need to run pathogenicity tests to confirm this assertion.

Carrot developmental stage did not affect the potential for endophytic isolates to produce siderophores (Fig 2.5b), but carrot genotype did (Table 2.4 a & b). In seed, the highest level of siderophore production was by endophytes isolated from genotype ExpB0252 with a value of 35.98 % of S.U., while the lowest level was obtained by bacteria isolated from Karotan, with a value of 9.24 % of S.U. Among seedlings, endophytes isolated from ExpPY191had the highest siderophore production with a value of 30.86 % of S.U, while. endophytes retrieved from ExpB0252 had the lowest production with a value of 4.38 % of S.U.

Differences in the production of siderophores by bacterial endophytes in response to plant factors has been reported in other studies (Lacava et al., 2008; Mukherjee et al., 2017; Tianxing et al., 2013). For example, Govindasamy et al. (2017) observed differences in siderophore production by endophytes isolated from sorghum roots at various stages of plant development (seedling, panicle initiation, pre-flowering and flowering), which differed from the results of our study in carrot. However, like our study, they also observed differences in siderophore production among endophytic isolates obtained from different sorghum genotypes. Differences in sorghum traits

associated with different genotypes were thought to have contributed to the functional diversity among their endophytic isolates. However, the exact differences in sorghum genotypes, which caused this functional diversity among endophytes were not identified.

Differences in iron concentration in microbial environments is one of the main factors that affect their siderophore production since siderophores are produced by microbes only under low iron concentration. Differences in metal ions that are present in a microbe surrounding environment, as well as the nature of nitrogen and carbon sources which are available for microbial uptake, are also factors that could affect microbial production of siderophores (Kloepper et al., 1980). For this reason, we expect that differences in iron availability or quality and quantity of carbon and nitrogen sources among the nine carrot genotypes evaluated in this study might have affected the differences in siderophore production observed. This also could have been related to differences bacterial preference for living inside some genotypes more than others, since they are adapted to specific physiological and anatomical characteristics provided by those genotypes (Loaces et al., 2011). Further physiological investigations are needed to confirm our observation under real life conditions and elucidate specific factors that might be responsible.

2.3.5 Phosphate solubilization

Phosphorus is an essential nutrient for plant growth and development, though it is often limiting to plants because it is rapidly converted to insoluble forms in soil. Consequently, the presence of phosphorus solubilizing microbes can enhance plant performance. These microbes can convert insoluble phosphorus to soluble forms through acidification and chelation processes such as solubilization of mineral phosphate, or through mineralization and solubilization of organic phosphorus (Chung et al., 2005; Rodríguez and Fraga, 1999). Many studies have documented the isolation of phosphorous solubilizing endophytes from different plants (Kuklinsky-Sobral et al., 2004; Verma et al., 2001), highlighting their critical role in plant growth promotion and productivity (Kuklinsky-Sobral et al., 2004; Rodríguez and Fraga, 1999).

All endophytic microbial taxa isolated in this study had the ability to solubilize phosphate on both solid and liquid media. Their potential to solubilize phosphate ranged from 2.8 to 12.18 mg phosphate ml $^{-1}$. Differences in the ability of individual isolates to solubilize phosphate could be detected, though no significant effect of developmental stage or genotype was observed (Fig 2.5c) & (Table 2.4 a & b). Within endophytes isolated from the seed or seedling, ExpP6306

harbored bacteria with the highest potential rate of phosphate solubilization with a value of 9.71 mg phosphate ml^{-1}, while the lowest phosphate solubilization activity was observed in endophytes isolated from Exp Nb3999 with a value of 8.07 mg phosphate ml-1. Among endophytes isolated from seedlings, isolates with the highest phosphate solubilization potential were retrieved from Exp Nb3999 with a value of 10.07 mg phosphate ml-1, and the lowest was in bacteria isolated from genotype 4 with a value of 7.18 mg phosphate ml-1.

As with other plant growth promoting activities, isolation of endophytes with the potential to solubilize phosphors has been reported to be affected by plant developmental stage and genotype. For example, in soybean (*Glycine max*), higher phosphorus solubilization activity was observed in endophytes isolated from the vegetative stage of production in comparison to initial developmental stages. Differences among soybean cultivars in the retrieval of endophytes capable of solubilizing phosphorus has also been reported (Kuklinsky-Sobral et al., 2004). However, the authors did not attribute this effect to specific differences among different genotypes or developmental stages.

In general, Factors that could affect phosphorus solubilization by microbes include environmental factors such as pH, nutrient availability and temperature, (Musarrat and Khan, 2014). The fact that we did not observe differences in this study could indicate that the carot genotypes and developmental stages did not differ in any of the factors controlling phosphorus solubilizing endophytes colonization or efficacy. However, further investigation is needed in the future to support this observation.

2.3.6 Nitrogen fixation ability

Nitrogen (N) is generally the most limiting nutrient for plant growth. Consequently, associating with microbes that are able to fix atmospheric N into forms that are available for plant uptake would be very useful for plants, especially when plant available N sources are low. There are three types of nitrogen fixing microbes that can associate with plants: those that are considered “free living”, those that live in specialized structures in a so-called endosymbiosis, and those that would be considered true endophytes (Saikia and Jain, 2007). An example of an endosymbiotic nitrogen fixing relationship is rhizobia, which are a unique group of microbes that live inside specialized nodules produced by legume plants. In this endosymbiotic relationship, rhizobia are well known for their potential to fix atmospheric N and increase their host plant growth (Saikia

and Jain, 2007). So-called free-living bacteria such as soil diazotrophs represent another group of nitrogen fixing microbes that are also able to fix atmospheric nitrogen using nitrogenase enzymes and transfer it to their hosts as ammonia (Gupta et al., 2012). As discused above, diazotrophic bacteria can colonize plants without causing any disease symptoms and in this case, and thus they are considered endophytes. Nitrogen fixing endophytes have been isolated from a wide variety of plant species, and they are widely available commercially as biofertilizers. If they can truly survive as endophytes, they have an advantage over other free-living nitrogen fixing microbes as they are sheltered inside plants and thus face less competition with other microbes. In addition, partial oxygen pressure inside plants is more suitable for efficient nitrogen fixation by endophytes than if microbes try to carry out this activity in soil, such as on the surface of a mineral particle embedded in a biofilm (Elbeltagy et al., 2001; Gupta et al., 2012; James, 2000; Muangthong et al., 2015). However, additional in-depth research is needed to understand factors that affect the survival of nitrogen fixing endophytes to improve their efficacy as biofertilizers (Gupta et al., 2012).

Only three out of 58 endophytes isolated in this study were not able to produce ammonia, which serves as a positive indication of nitrogen fixation ability. Chi-square analysis followed by Fisher's Exact Test indicates that the distribution of nitrogen fixing endophytes was significantly affected by developmental stage, and endophytes isolated from seedlings had a higher ability than seedborne endophytes to produce ammonia and as a result to fix nitrogen (Fig 2.6). Carrot genotype did not affect the distribution of nitrogen fixing endophytes in this study (Fig 2.7 a &b). Similarly, Johnston-Monje and Raizada (2011) did not observe differences in N fixation among endophytes isolated from different maize cultivars. They theorized that the lack of differences reflects the common needs of different cultivars for N acquisition. This could also explain why we did not observe differences in N fixation ability among endophytes isolated from different carrot genotypes in this study.

Once seedlings start to grow, they are likely to require more N than what is needed during seed germination, which likely reflects differences in N-fixation ability between endophytes isolated from seed vs. seedlings (Nelson, 2004). Another explanation for differences in N fixation ability among endophytes isolated from seed vs seedlings, could be due the preferences of these microbes in colonizing seedlings rather than seed, as they are more adapted to the internal environment that seedlings provide. Colonization preferences by endophytes for specific plant developmental stage have been documented before by (Shi et al., 2014). It could be also related to

extreme differences in the internal environment between seed and seedlings that controls nitrogen fixation activity such as pH, temperature, moisture and salinity. Additional experiments should be conducted to follow up on our initial studies to determine if the same dynamics will occur under field conditions and elucidate the specific factors that contribute to difference in N-fixation activitiy.

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Table 2.1 Carrot genotypes evaluated in this study

CIOA entry #	Genotype description	Origin	Taproot color (external/internal)	Tap root shape	Flavor
Experimental breeding lines with novel root colors and tall tops for weed competitiveness					
7	Exp P6306	Turkey	Purple/Yellow	Imperator	Below Average
9	Exp Y8519	Turkey	Yellow/Yellow	Imperator	Very Good
2	Exp PY191	Asia	Purple/Purple	Imperator	Average
3	Exp B0252	Syria	Purple/Orange	Imperator	Below Average
Nematode resistant breeding lines with high beta-carotene					
11	Exp Nb3999	Brazil/Europe	Orange/Orange	Imperator	Average
Open-pollinated populations with nematode resistance and tall tops for weed competitiveness					
23	Brasilia	Brazil	Orange/orange	Nantes	Average
22	Scalet fancy fruit (NSFF)	Europe	Orange/orange	Nantes	Very Good
Standard open-pollinated populations with tall tops for weed competitiveness					
4	Karotan	Europe	Orange/Orange	Flak	Average
24	Red Core Chantenay (RCC)	Europe	Orange/Orange	Chantenay	Very Good

Table 2.2 BLAST n alignment results for 16s r RNA & ITS partial sequences of endophytes isolated from the seed and seedlings of nine carrot genotypes. 98% and 97% confident level cut off were used for bacterial and fungal sequences identification at species level respectively.

Carrot genotype	Developmental stage	Isolate	Code	Closest strain on NCBI data base	E value	Identity	Accession # of closest species
Exp PY191	Seedling						
		1	2N1	*Xanthomonas campestris*	0	100%	MG597200.1
		2	2N4	*Uncultured Pantoea* spp.	0	99%	JF357619.1
		3	2N2	*Pantoea* spp.	0	98%	MF458865.1
				Pantoea vagans	0	98%	MH211327.1
				Pantoea agglomerans	0	98%	MH190052.1
				Gammaproteobacteria bacterium	0	98%	KY549067.1
				Uncultured Pantoea spp.	0	98%	KX603458.1
				Enterobacteriaceae bacterium	0	98%	LC007907.1
				Enterobacter agglomerans	0	98%	AF130948.2
				Enterobacteriaceae bacterium	0	98%	JN392831.1
				Uncultured bacterium clone	0	98%	HM450015.1

Table 2.2 continued

Exp B0252	Seedling						
		1	3N4	*Xanthomonas campestris*	0	99%	MG597200.1
				Xanthomonas arboricola pv. pruni	0	99%	LC331099.1
				Xanthomonas campestris pv. hederae	0	99%	KT862774.1
				Xanthomonas campestris pv. campestris	0	99%	KP182149.1
				Xanthomonas spp.	0	99%	KM252981.1
				Xanthomonas arboricola	0	99%	KP340804.1
		2	3N3Z	*Xanthomonas arboricola pv. corylina*	1.00E-99	98%	JQ861275.1
Karotan (Rijk Zwaan)	Seedling						
		1	4N2	*Xanthomonas campestris*	0	100%	MG597200.1
		2	4N1	*Geomyces* spp.	0	97%	MG845137.1
				Pseudogymnoascus spp.	0	97%	KY270893.1
				Uncultured Geomyces	0	97%	JN392107.1
				Fungal endophyte	0	97%	HQ335300.2
				Uncultured fungus	0	97%	HM069447.1

Table 2.2 continued

		3	4N1	*Geomyces* spp.	0	97%	JX270341.1
Exp P6306	Seedling						
		1	7N5	*Pantoea agglomerans*	0	98%	KX891543.1
				Pantoea spp.	0	98%	KX397513.1
				Pantoea vagans	0	98%	KT347523.1
		2	7N2	*Enterobacter* spp.	0	96%	AF130887.1
		3	7N2	*Pantoea* spp.	0	91%	MG544106.1
		4	7N21	*Alternaria tenuissima*	0	97%	KX073998.1
				Alternaria spp.	0	97%	KM396417.1
				Fungal endophyte	0	97%	KC178645.1
		5	7N4	*Alternaria burnsii*	0	98%	KR604838.1
		6	7N2	*Alternaria* spp.	0	99%	KC178641.1
Exp Y8519	Seedling						
		1	9N51	*Pantoea agglomerans*	0	98%	KX179633.1
				Pantoea spp.	0	98%	KM587003.1
				Enterobacter agglomerans	0	98%	AF130947.2
		2	9N22	*Rhodotorula* spp.	2.00E-156	88%	EU450883.1
		3	9N5	*Pantoea agglomerans*	0	98%	KT803093.1
		4	9N7	*Pantoea* spp.	0	96%	KR054975.1
Exp Nb3999	Seedling						
		1	11N22	*Pantoea* spp.	0	97%	KT803093.1
		2	11N1	*Pantoea agglomerans*	0	98%	KY127366.1
		3	11N12	*Pantoea vagans*	0	98%	KY127421.1

Table 2.2 continued

				Pantoea agglomerans	0	98%	AF130918.1
NSFF	Seedling						
		1	22N1	*Xanthomonas campestris*	0	100%	MG597200.1
				Xanthomonas arboricola pv. pruni	0	100%	LC331099.1
				Xanthomonas campestris pv. hederae	0	100%	KU518243.1
				Xanthomonas arboricola	0	100%	KP340804.1
				Xanthomonas campestris pv. campestris	0	100%	KP182149.1
				Xanthomonas spp.	0	100%	KM252981.1
		2	22N4	*Xanthomonas campestris*	0	100%	MF285891.1
				Xanthomonas arboricola pv. pruni	0	100%	MF351923.1
				Xanthomonas campestris pv. vitians	0	100%	MH165478.1
		3	22N6	*Xanthomonas* spp.	0	97%	KR708907.1
		4	22N2	*Geomyces* spp	0	97%	JN630629.1
		5	22N3	*Cladosporium* spp.	0	96%	GQ221853.1
Brasilia	Seedling						
		1	23N3	*Xanthomonas campestris*	0	99%	MG597200.1
		2	23N4	*Pantoea agglomerans*	0	98%	MH165381.1
		3	23N1	*Pantoea* spp.	0	96%	KT803093.1

Table 2.2 continued

		4	23N2	*Xanthomonas campestris*	0	99%	MG597200.1
				Xanthomonas arboricola pv. pruni	0	99%	LC331099.1
				Xanthomonas campestris pv. hederae	0	99%	KU518243.1
				Xanthomonas campestris pv. campestris	0	99%	KP182149.1
				Xanthomonas spp.	0	99%	KM252981.1
Red Core Chantenay	Seedling						
		1	24N1Z	*Pantoea* spp.	2.00E-57	83%	KF202778.1
		2	24N2	*Alternaria spp.*	8.00E-28	88%	MG065793.1
		3	24N2	*Alternaria chenopodiicola*	0	98%	MF077222.1
Exp PY191	Seed						
		1	2D4	*Pantoea* spp.	0	99%	KU725941.1
		2	2D21	*Pantoea agglomerans*	0	98%	MH141453.1
				Pantoea vagans	0	98%	KU605690.1
				Pantoea spp.	0	98%	KY385284.1
		3	2D31	*Pseudomonas* spp.	0	97%	KY792615.1
		4	2D5	*Penicillium corylophilum*	0	99%	MF475926.1
				Penicillium spp.	0	99%	MF475914.1

Table 2.2 continued

				Penicillium consobrinum	0	99%	MG490874.1
				Penicillium rubefaciens	0	99%	LT558907.1
				Penicillium chrysogenum	0	99%	KT898795.1
				Uncultured Penicillium	0	99%	KT334799.1
				Penicillium chloroleucon	0	99%	KP016813.1
				Fungal endophyte	0	99%	KF435253.1
				Uncultured fungus	0	99%	KM104015.1
				Penicillium rubefaciens	0	99%	KJ527453.
		5	2D3	*Alternaria* spp.	3.00E-57	80%	KJ526175.1
Exp B0252	Seed						
		1	3D13	*Pantoea* spp.	0	98%	MF458864.1
				Pantoea agglomerans	0	98%	KY127366.1
Karotan (Rijk Zwaan)	Seed						
		1	4D4	*Methylobacterium* spp.	0	99%	KT720396.1
				Methylobacterium adhaesivum	0	99%	GU992357.1
		2	4D2	*Methylobacterium marchantiae*	0	99%	KR811206.1
				Methylobacterium spp.	0	99%	JF274801.1

Table 2.2 continued

				Uncultured bacterium	0	99%	FN421878.1
		3	4D1	*Xanthomonas arboricola pv. pruni*	0	100%	MF351923.1
				Xanthomonas campestris strain	0	100%	KR708907.1
				Xanthomonas spp.	0	100%	KR922188.1
		4	4D1	*Bacillus aryabhattai*	0	100%	KU323599.1
				Bacillus megaterium	0	100%	KU362284.1
		5	4DA	*Alternaria* spp.	0	97%	MG065793.1
		6	4DA2	*Alternaria* spp.	0	99%	KR909163.1
				Embellisia spp.	0	99%	JN578612.1
Exp P6306	Seed						
		1	7D1	*Pantoea* spp.	0	98%	MF458864.1
				Pantoea agglomerans	0	98%	KY127366.1
		2	7DE	*Alternaria tenuissima*	0	99%	MG583736.1
				Alternaria alternata	0	99%	MG198619.1
				Alternaria burnsii	0	99%	KY949589.1
				Alternaria spp.	0	99%	KX611633.1
				Fungal endophyte	0	99%	KT291006.1
				Uncultured endophytic fungus	0	99%	EF505242.1
				Glomerella spp.	0	99%	JX559855.1
				Alternaria brassicae	0	99%	JX290150.1
				Ustilago tritici	0	99%	JN114420.1
				Alternaria porri	0	99%	HQ821482.1
		3	7DF	*Alternaria* spp	0	100%	KY077497.1

Table 2.2 continued

				Uncultured fungus	0	100%	JX135780.1
				Uncultured endophytic fungus	0	100%	EF505188.1
		3	7DA	*Alternaria* spp.	2.00E-80	92%	KC178630.1
Exp Y8519	Seed						
		1	9D51	*Pantoea* spp.	0	96%	JX077098.1
		2	9D2	*Pantoea agglomerans*	0	98%	JX077098.1
		3	9D35	*Pantoea* spp.	0	98%	MF458865.1
				Pantoea vagans	0	98%	MH211327.1
				Gammaproteobacteria bacterium	0	98%	KY549067.1
				Pantoea agglomerans	0	98%	MH190052.1
				Uncultured Pantoea spp.	0	98%	KX603458.1
				Enterobacteriaceae bacterium	0	98%	LC007907.1
				Enterobacter agglomerans	0	98%	AF130947.2
		4	9D5	*Pantoea vagans*	0	99%	MH211305.1
		5	905DB	*Methylobacterium extorquens*	0	100%	KY622701.1
				Methylobacterium spp.	0	100%	KX608935.1
		6	9D1	*Alternaria* spp.	0	99%	HQ025969.1
		7	9D3	*Fungal endophyte*	0	99%	KT290973.1
				Alternaria burnsii	0	99%	KR604843.1

Table 2.2 continued

Exp Nb3999	Seed						
		1	11D7	*Curtobacterium* spp.	0	100%	LT797548.1
				Curtobacterium flaccumfaciens pv. flaccumfaciens	0	100%	KP898898.1
				Uncultured bacterium	0	100%	KP843050.1
		2	11D4	*Pantoea agglomerans*	0	99%	CP016889.1
		3	11D3	*Bacillus* spp.	0	100%	LN878354.1
		4	11D11	*Paenibacillus tundrae*	0	100%	MF101179.1
				Paenibacillus spp.	0	100%	HF954523.1
		5	11D10	*Bacillus gibsonii*	0	100%	MH298508.1
				Bacillus spp.	0	100%	KX066846.1
		6	11D6	*Pantoea ananatis*	1.00E-164	99%	KJ016245.1
				Uncultured bacterium	1.00E-164	99%	HM556598.1
		7	11B2B	*Cellulomonas* spp.	0	99%	LC133615.2
				Cellulomonas hominis	0	99%	JQ660102.1
		8	11DA5	*Alternaria* spp.	0	99%	JF742667.1
		9	11DA3	*Alternaria alternata*	0	98%	KX622102.1
		10	11DA3	*Uncultured endophytic fungus*	0	98%	EF504948.1
		11	11DB2	*Alternaria burnsii*	0	99%	KY949588.1
				Uncultured fungus	0	99%	KX928737.1
		12	11D3	*Fusarium equiseti*	0	99%	MH266073.1

Table 2.2 continued

				Fusarium spp.	0	99%	MG066485.1
				Fusarium verticillioides	0	99%	KX553874.1
				Neurospora spp.	0	99%	KX058050.1
				Fusarium verticillioides	0	99%	KU204755.1
				Gibberella spp.	0	99%	KT268931.1
				Uncultured endophytic fungus	0	99%	EF505484.1
				Uncultured soil fungus	0	99%	EU675977.1
				Pteris vittata	0	99%	AM920400.1
				Uncultured Fusarium	0	99%	HG936943.1
		13	11DC	*Alternaria burnsii*	0	99%	KY949587.1
		14	11DA6	*Alternaria tenuissima*	0	99%	KX073998.1
		15	11DA	*Alternaria radicina*	0	99%	FJ958190.1
				Alternaria spp.	0	99%	FJ266485.1
NSFF	Seed						
		1	22D2	*Pantoea agglomerans*	0	99%	MH158730.1
				Uncultured Pantoea spp.	0	99%	JX852670.1
				Uncultured bacterium	0	99%	JQ047358.1
				Pantoea spp.	0	99%	FJ426593.1
		2	22D4	*Bacillus* spp.	0	99%	LN878354.1
		3	22D1	*Bacillus gibsonii*	0	100%	MG651558.1
				Bacillus spp.	0	100%	KF891396.1
				Uncultured bacterium.	0	100%	HE589833.1

Table 2.2 continued

		4	22D3	*Cladosporium cladosporioides*	0	99%	GQ221853.1
		5	22D	*Stemphylium vesicarium*	0	99%	MH383053.1
				Stemphylium majusculum	0	99%	MH037562.1
				Pleospora sp. isolate	0	99%	MG020759.1
				Uncultured fungus	0	99%	MF976723.1
				Stemphylium spp	0	99%	MF435142.1
				Pleospora herbarum	0	99%	KU752185.1
				Fungal endophyte	0	99%	KT203000.1
				Uncultured Stemphylium	0	99%	HF947051.1
				Uncultured Pleospora	0	99%	HQ650086.1
				Pleospora tarda	0	99%	HQ161160.1
				Stemphylium solani	0	99%	AB693928.1
		6	22DA	*Lewia* spp.	4.00E-101	97%	EF432279.1
		7	22D	*Pleospora herbarum*	0	97%	KP334720.1
Brasilia	Seed						
		1	23D5	*Enterococcus casseliflavus*	0	100%	MH376403.1
		2	23D4	*Pantoea* spp.	0	97%	KX129755.1
		3	23D3	*Curtobacterium* spp.	0	99%	KR906478.1
		4	23D2	*Pseudomonas* spp.	0	99%	KC822776.1

Table 2.2 continued

Red Core Chantenay	Seed						
		1	24D7	*Curtobacterium flaccumfaciens*	0	100%	MH298447.1
				Curtobacterium spp.	0	100%	MF458877.1
				Alcaligenes faecalis	0	100%	KY271067.1
				Uncultured bacterium	0	100%	KP843050.1
				Endophytic bacterium.	0	100%	GU354333.1
				Unidentified bacterium	0	100%	EF154095.1
		2	24D4	*Curtobacterium herbarum strain*	0	99%	JF706509.1
		3	24D3	*Uncultured bacterium*	0	100%	KP843007.1
		4	24D2	*Pantoea ananatis*	0	100%	KJ004603.1
		5	24D1	*Curtobacterium herbarum strain*	0	99%	JF706509.1
		6	24D1	*Pantoea agglomerans*	1.00E-77	100%	MG544108.1
				Pantoea vagans strain	1.00E-77	100%	KC139414.1
		7	24DA2	*Alternaria infectoria*	0	97%	MH399531.1
				Alternaria alternata	0	97%	MF141014.1
				Alternaria spp. *isolate*	0	97%	KX611017.1
				Alternaria quercicola	0	97%	KX228297.1
				Uncultured fungus	0	97%	KC766061.1
				Uncultured endophytic fungus	0	97%	EF505574.1
				Alternaria californica	0	97%	NR_136021.1
				Alternaria conjuncta	0	97%	FJ266475.1
				Lewia spp.	0	97%	EF432297.1

Table 2.2 continued

				Alternaria conjuncta	0	97%	NR_135929.1
		8	24DB	*Stemphylium globuliferum isolate*	0	97%	MH399295.1
				Stemphylium spp.	0	97%	GU062207.1
		9	24DA4	*Stemphylium* spp.	0	96%	KX301013.1
		10	24DA3	*Alternaria* spp.	0	96%	KY949589.1
		11	24DC	*Alternaria infectoria*	4.00E-151	97%	MG583735.1
		12	24DA	*Cladosporium* spp.	1.00E-150	94%	JN689952.1

Table 2.3 Main endophytes bacterial and fungal genera isolated from the seed and seedlings of nine carrot genotypes

			Carrot Genotypes																	
			ExpPY191		**Exp B0252**		**Exp P6306**		**Exp Y8519**		**Karotan**		**NSFF**		**Red core chaty**		**Exp Nb3999**		**Brasilia**	
Kingdom	Microbial Phylum	Microbial Genera	seed	seedling	seed	seedling	seed	seedling	seed	seedling	seed	seedling	seed	seedling	seed	seedling	seed	seedling	seed	seedling
Bacteria	Firmicutes	*Bacillus*									X		X				X			
	Actinobacteria	*Cellulomonas*															X			
		Curtobacterium													X		X		X	
	Proteobacteria	*Enterobacter*						X												
	Firmicutes	*Enterococcus*																	X	
	Proteobacteria	*Methylobacterium*							X		X									
		Pantoea	X	X	X		X	X	X	X			X		X		X	X	X	X
	Firmicutes	*Paenibacillus*															X			
	Proteobacteria	*Pseudomonas*	X																X	
		Xanthomonas		X		X					X	X		X						X
Fungi	Ascomycota	*Alternaria*	X				X	X	X		X				X		X			
		Cladosporium											X	X	X					
		Fusarium															X			

Table 2.3 continued

		Geomyces										X		X						
		Lewia											X							
		Penicillium	X																	
		Pleospora											X							
	Basidiomycota	*Rhodotorula*								X										
	Ascomycota	*Stemphylium*											X		X					

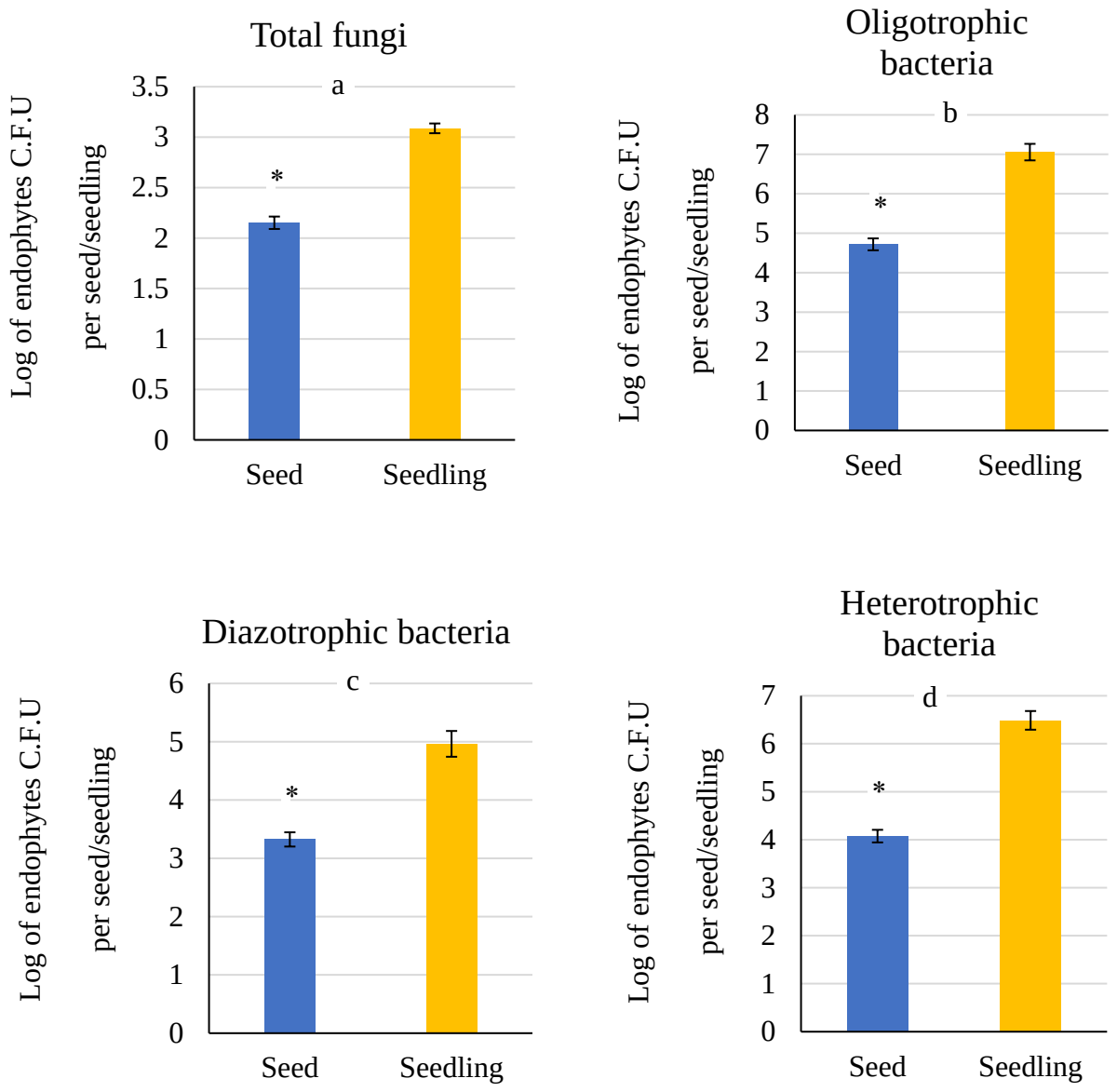

Figure 2.1 Log of seed and seedling endophyte counts averaged across nine carrot genotypes. Counts were observed on selective media for Total fungi (a), Oligotrophic bacteria (b), Diazotrophic bacteria (c) & Heterotrophic bacteria (d) with (n=5). Bars represent stander error mean & ∗ represent significant value of < 0.0001.

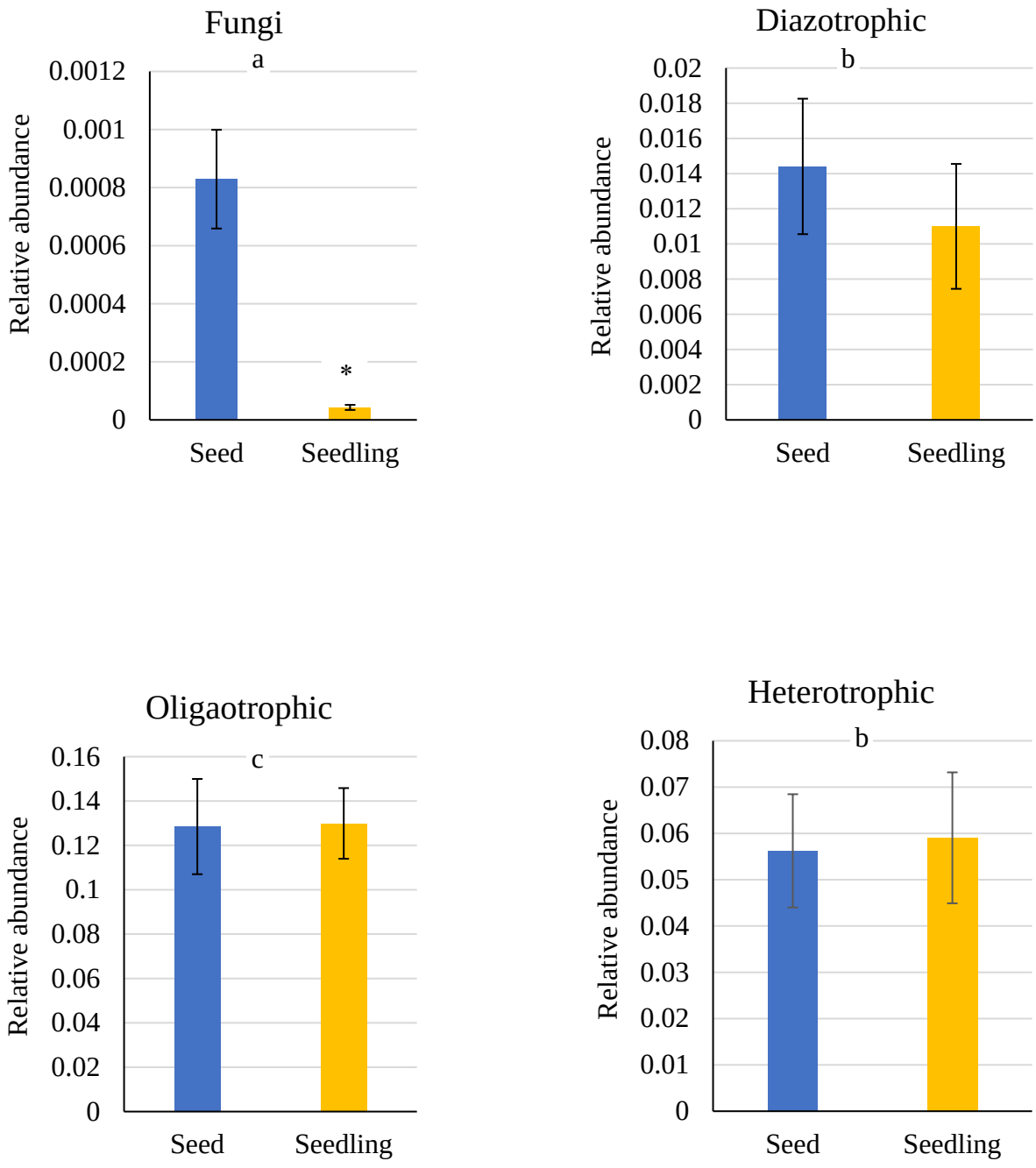

Figure 2.2 Relative abundance of a) Fungi, b) Diazotrophic, c) Oligotrophic & d) Heterotrophic endophytes isolated from seed and seedlings of nine carrot genotypes, ∗ significant P value of < 0.0001

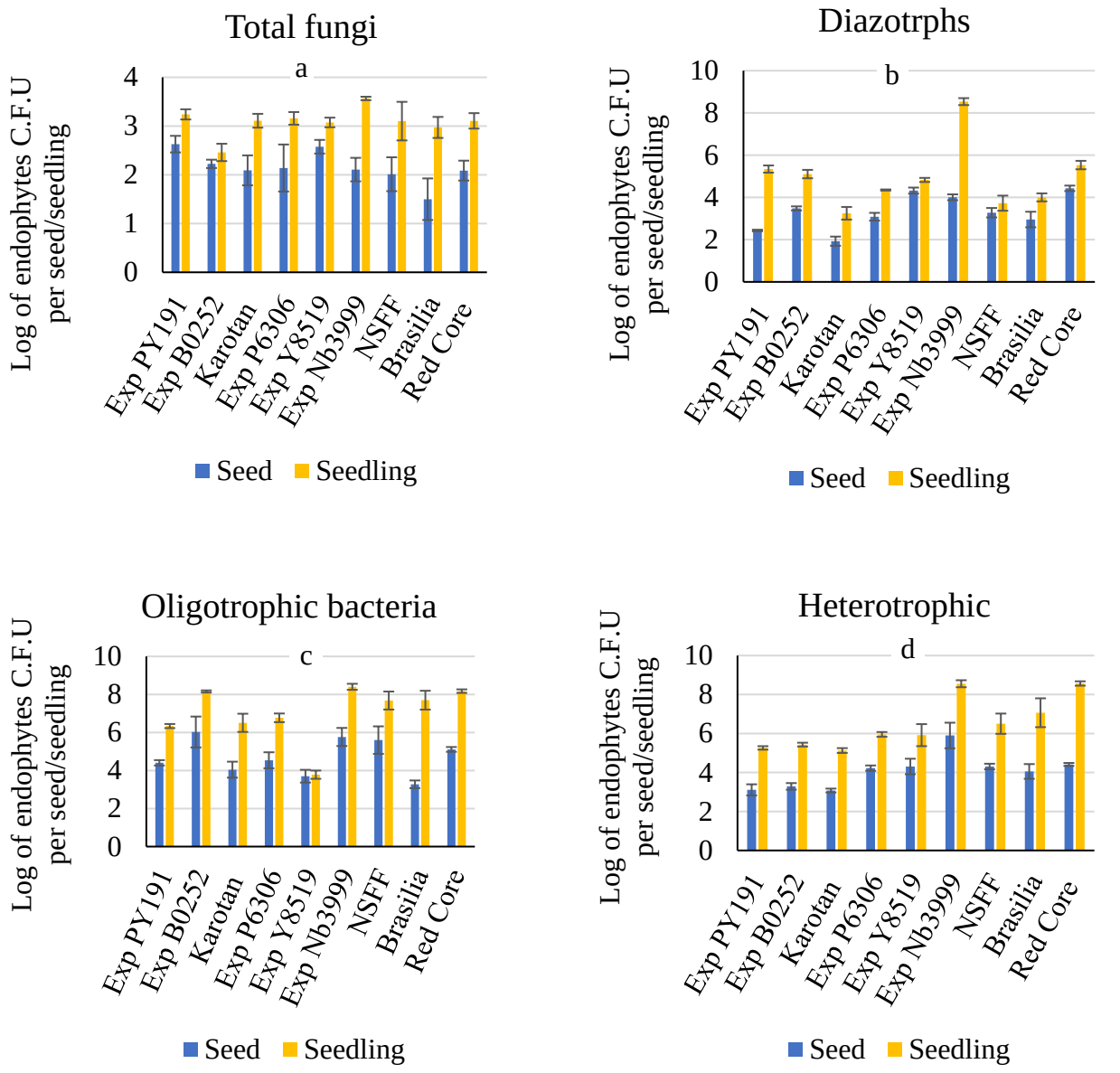

Figure 2.3 Log of seed and seedling endophytes isolated from nine carrot genotypes on four selective media for broad bacterial and fungal groups. (a) represents endophyte counts for Total fungi, (b) Diazotrophic bacteria, (c) Oligotrophic bacteria, and (d) Heterotrophic bacteria with (n=5). Bars represent stander deviation.

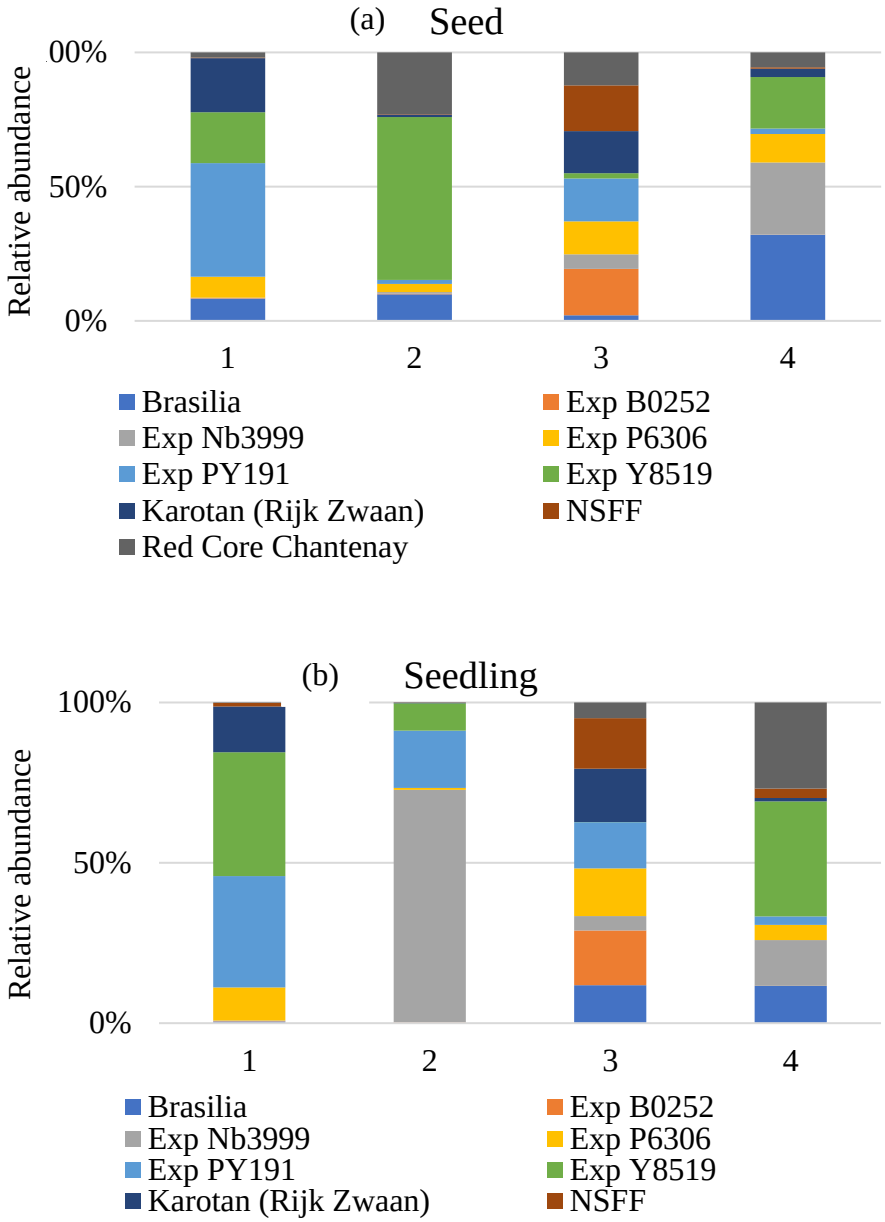

Figure 2.4 Relative abundance of 1) Fungi, 2) Diazotrophic, 3) Oligotrophic & 4) Heterotrophic endophytes isolated from seed (a) and (b) seedlings of nine carrot genotypes

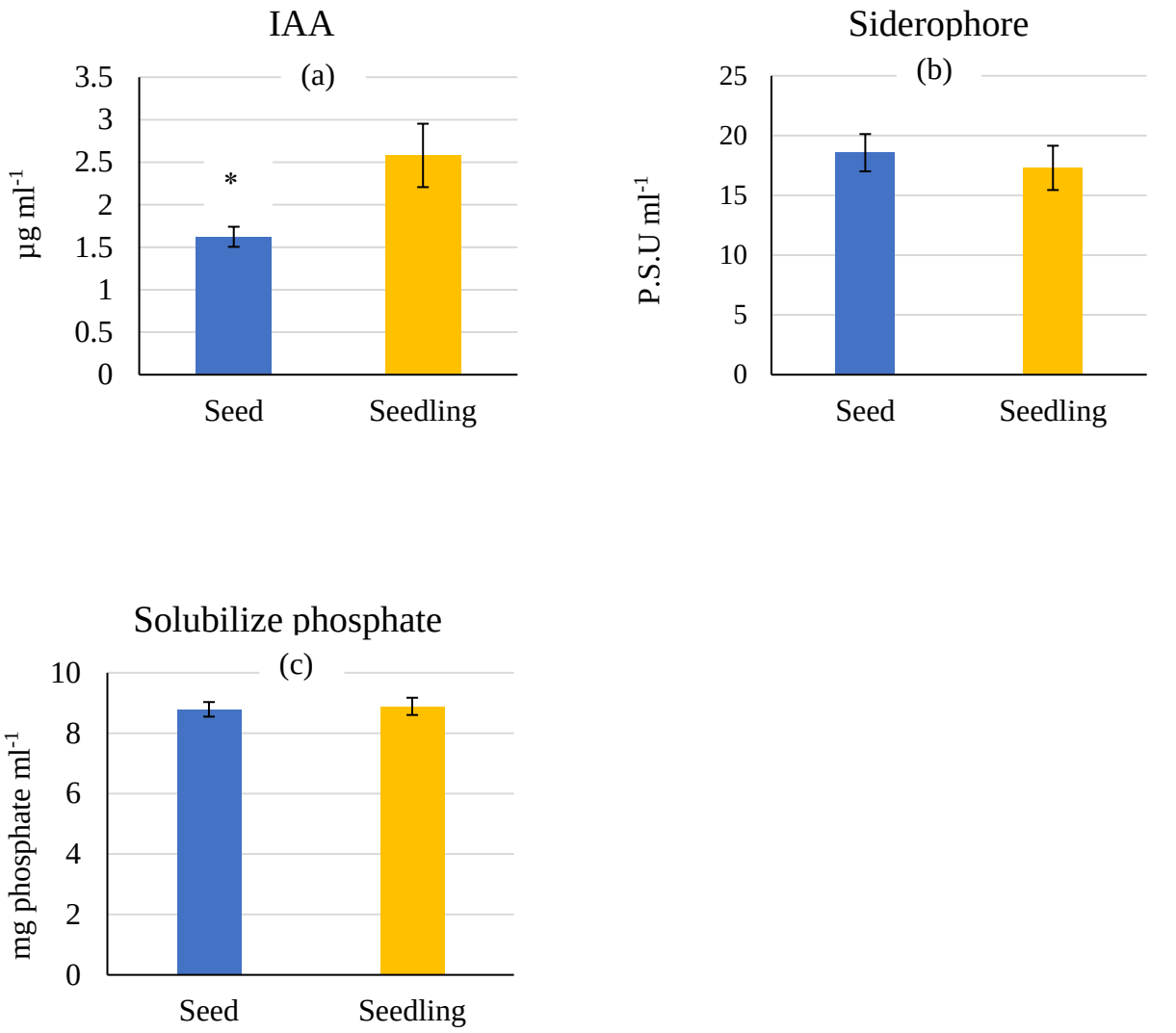

Figure 2.5 Comparison of plant growth promoting activity of endophytes isolated from the seed and seedlings averaged over nine carrot genotypes. a) Indole acetic acid production, ∗ significant P value of < 0.0060, b) Siderophore production. c) Phosphate solubilization ability. Bars represent stander error mean for n=5

Table 2.4 Comparison of plant growth promoting activity of bacterial endophytes isolated from nine carrot genotypes ± stander error mean. IAA data represented as µg ml^{-1}, Siderophores as P.S.U ml^{-1} and Phosphate solubilization as mg phosphate ml^{-1} for (n=5). a) Seed endophytes and b) seedling endophytes

a)

Carrot genotype	Mean of IAA	Mean of Siderophores	Mean of solubilized phosphate
Exp PY191	1.39 ± 0.37	24.36 ± 6.95	8.78 ± 0.65
Exp B0252	2.67 ± 0.29	35.98 ± 6.65	9.67 ± 0.28
Karotan (Rijk Zwaan)	1.38 ± 0.27	9.24 ± 3.04	9.35 ± 0.35
Exp P6306	0.90 ± 0.45	12.91 ± 1.36	9.71 ± 0.30
Exp Y8519	1.53 ± 0.20	23.30 ± 2.88	9.59971 ± 0.42
Exp Nb3999	1.45 ± 0.24	26.39 ± 4.02	8.07 ± 0.79
NSFF	1.41 ± 0.49	16.19 ± 1.72	8.15 ± 1.28
Brasilia	2.76 ± 0.46	16.21 ± 6.16	9.39 ± 0.11
Red Core Chantenay	1.48 ± 0.26	9.61 ± 3.21	8.20 ± 0.53

b)

Carrot genotype	Mean of IAA	Mean of Siderophores	Mean of solubilized phosphate
Exp PY191	3.00 ± 0.11	30.86 ± 5	9.43 ± 0.70
Exp B0252	0.37± 0.14	4.38 ± 0.53	10.23 ± 0.35
Karotan (Rijk Zwaan)	0.61 ± 0.10	0 ± 0	7.18 ± 2.04
Exp P6306	3.17 ± 0 .89	17.08 ± 6.19	8.85 ± 0.51
Exp Y8519	2.06 ± 0.58	17.20 ± 1.6	9.45 ± 0.34
Exp Nb3999	7.033 ± 2.12	23.33 ± 3.35	10.07 ± 0.19
NSFF	1.90 ± 0.36	12.68 ± 1.63	7.71 ± 1.20
Brasilia	0.90 ± 0.31	21.43 ± 5.55	7.68 ± 1.04
Red Core Chantenay	3.47 ± 0.37	0 ± 0	9.08 ± 0.30

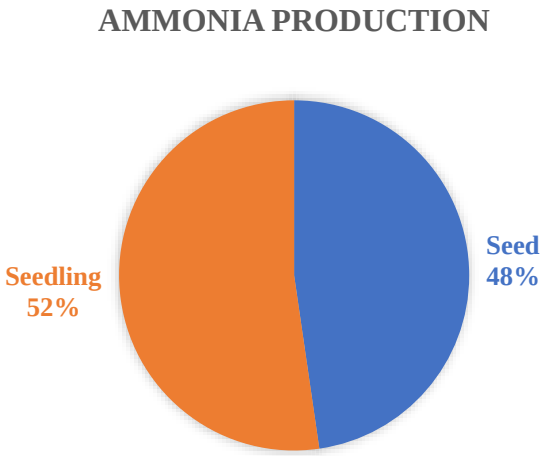

Figure 2.6 Pie graph comparing relative frequency of in vitro ammonia production by bacterial endophytes isolated from seed and seedlings averaged across nine carrot genotypes.

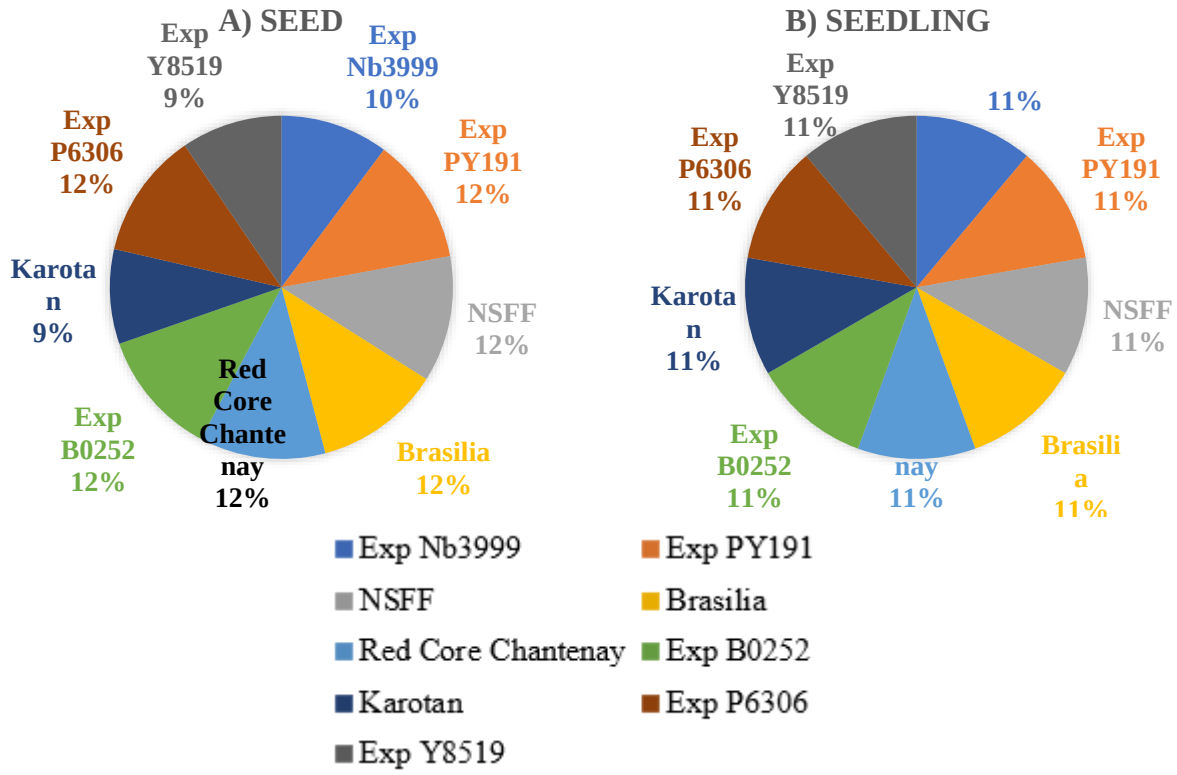

Figure 2.7 Pie graph comparing relative frequency of in vitro ammonia production by bacterial endophytes isolated from nine tested genotypes within a) seed and b) seedling.

CHAPTER 3. CARROT ENDOPHYTES: ROLE OF CROP MANAGEMENT SYSTEM AND CARROT GENOTYPE ON COMMUNITY COMPOSITION AND *A. DAUCI* DYNAMICS

3.1 Introduction

Carrot is a nutritious and high-value crop, but it is subject to several biotic and abiotic stresses that can negatively impact crop productivity and end-use quality characteristics of carrot taproots. For example, carrot is very slow to establish following seeding, which makes this crop highly susceptible to competition by weeds. Carrot is also susceptible to many pathogens including *Alternaria dauci*. This pathogen is best known for its potential to cause leaf blight (Agrios 2005), though it can also cause taproot decay and seedling damping-off like symptoms (Agrios 2005). Several herbicides and pesticides can be applied to help carrot growers overcome these challenges. However, many growers, particularly in developing countries, lack access to these inputs. In addition, many growers may choose not to use these products due to demand for carrots grown using organic management practices. In the U.S. alone, carrots hold the greatest market share of all crops in the organic sector, with approximately 14% of the entire crop now grown using organic practices (NASS 2016).

Endophytes offer an alternative approach to pesticides to help overcome biotic and abiotic challenges in crops like carrot. Endophytes are classically defined as a group of microorganisms that live within plant tissues for all or part of their life cycle without causing any visible disease symptoms (Bacon and White 2000). Recently, many positive correlations between endophytic microbial communities and crop productivity have been reported in a number of plants, including many economically important crops like tomatoes, potatoes, rice and carrots (Surette, Sturz et al. 2003, Hardoim, Van Overbeek et al. 2015). These correlations are often attributed to the potential for endophytes to promote their host plant growth, which has now been documented in many studies (Kuklinsky-Sobral, Araujo et al. 2004). Endophytes can promote host plant growth and thereby help plants overcome various stresses directly by: 1) producing phytohormones such as auxin and ethylene, which are well known for their role in stimulating plant growth (Patten and Glick 2002), and/or 2) increasing acquisition of limiting nutrients from the surrounding environment, such as by solubilizing phosphorus and fixing atmospheric nitrogen (Kuklinsky-Sobral, Araujo et al. 2004). Indirect mechanisms of plant-growth promotion that can also increase

tolerance to biotic stresses by endophytes include: 1) stimulating a plant's innate immune system, resulting in induced systemic resistance (ISR) against pathogens (Cabanas, Schiliro et al. 2014), 2) starving or outcompeting pathogens by limiting nutrient availability (Tyvaert, França et al. 2014), and/or 3) producing antimicrobial or other secondary metabolic compounds that negatively affect pathogens (Ezra, Castillo et al. 2004, Niones and Takemoto 2014). Individual endophytic isolates could contribute to one or more of these mechanisms, depending on their life cycle and environmental conditions (Chen, Bauske et al. 1995, Sturz and Matheson 1996, Sturz and Nowak 2000, Liu, Zou et al. 2001).

One of the most critical factors in establishing a beneficial plant endophyte community is likely to be soil health, since resident soil microbial community structure is generally the most significant factor affecting the composition of plant root microbiomes (Compant, Clément et al. 2010). Crop management practices are critical to this process, because they can have a major effect on the composition and functional potential of soil microbial communities (Schulz, Boyle et al. 2006). For example, Liu et al. (2007) demonstrated that soil on farms managed using organic practices had greater physical, chemical and biological soil properties than conventionally managed systems, and had greater potential to suppress disease caused by the pathogen *Sclerotium rolfsii* in greenhouse bioassays. Differences in crop management practices have also been demonstrated to effect plant endophyte communities. For example, the composition of endophytes in grapevines differed when crops were managed using an organic compared to an integrated pest management approach (Pancher, Ceol et al. 2012). In another more recent study, Estendorfer et al., (2017) observed clear differences in soil, rhizosphere and endophytic microbial communities in *Dactylis glomerata L*. in response to a gradient of land use intensity. However, endophyte communities differed less than those in the soil and rhizosphere, indicating that plants also have a significant degree of control over their endophyte communities.

Plant species is well known for its potential to be another major affecting the composition of plant microbiomes (Adams and Kloepper 2002, Roesch, Olivares et al. 2006). Moreover, recent studies have provided evidence that differences among genotypes within an individual plant species can also play a smaller, yet significant role in shaping plant microbiomes (Peiffer, Spor et al. 2013), indicating that plant breeders may someday be able to select for beneficial plant-microbial relationships. Such differences are likely to be driven by the fact that plants can selectively recruit individual microbial taxa by secreting specific compounds. For example, many

plants secrete strigolactones to recruit arbuscular mycorrhizal fungi (AMF), which are well known for their potential to help plants acquire nutrients (Parniske 2008). Once individual microbial taxa arrive at the root-soil interface, plants possess specific mechanisms such as plasma-membrane localized pattern recognition receptors (PRRs) to detect the presence of molecular signatures produced by pathogenic microbes, and up-regulate their defense processes accordingly to keep these microbes from entering plant roots in a processes known as microbe-associated molecular pattern (MAMP)–triggered immunity (Trdá, Boutrot et al. 2015). In contrast, plants likely possess similar mechanisms that allow them to identify and permit internal colonization by beneficial microbial taxa (Adams and Kloepper 2002), as well as terminate unsuccessful microbial partnerships as in the case of rhizobia (Broghammer, Krusell et al. 2012, Liang, Cao et al. 2013). At the same time, endophytes may also prefer some plant species and genotypes over others, as the environment inside these hosts is more conducive to the environmental conditions these taxa require to survive (Fisher and Petrini 1987).

Despite the potential importance of endophytes in plant health and productivity, to our knowledge, only two studies to date have investigated endophyte communities in carrot. One of these was conducted by Surette, Sturz et al. (2003), who characterized bacterial endophyte communities in the taproots of two carrot genotypes (Carochoice and Red Core Chantenay) when grown in two carrot fields with different tillage practices (Canning and Great Village, in Nova Scotia, Canada). The authors in this study determined that endophyte communities varied by carrot variety and location where the plants were grown, as well location within carrot taproots, but they did not evaluate the potential implications of these differences. The other study was conducted by Louarn, Nawrocki et al. (2013), which quantified changes in the composition of fungal endophyte communities in the taproots of one carrot variety when grown under organic vs. conventional management, along with the proteomic profiles of the carrot taproots during six months of cold storage. While there were some small differences in the composition of the fungal endophyte communities in the carrots grown under these two cropping systems, this did not appear to affect the post-harvest quality of the taproots during storage. To our knowledge, studies exploring the potential for differences in carrot endophytes to affect agronomic properties have not yet been explored.

The broad goal of this study was to determine how differences in soil chemical and biological properties in side-by-side fields managed using organic vs. conventional management,

would affect the composition and potential functional role of culturable endophyte communities in carrot taproots. In particular, we sought to determine how differences in the composition of endophytic microbial taxa between these two management systems might affect the presence of microbial taxa with antagonistic activity towards *A. dauci*. Nine carrot genotypes with broad genetic backgrounds and different morphological and agronomic characteristics were included in this study (Table 2.1) to determine the extent to which carrot genotype could also affect these beneficial plant-soil-microbial relationships. To accomplish these goals, endophytes were isolated from carrot taproots at harvest and individual isolates were subject to an *in vitro* assay to quantify potential antagonistic activity towards *A. dauci*. The potential of these isolates to help carrot plants withstand *A. dauci* stress was determined using a greenhouse bioassay. This greenhouse trial was conducted using two standard carrot varieties to provide further evidence to support the hypotheses that carrot genotypes vary in their potential to host and benefit from endophytic microbes.

3.2 Material and methods

3.2.1 Field trial

Carrot taproots were grown in a long-term crop systems trial comparing organic and conventional management at Purdue′s Meigs Horticultural Research Farm (lat. 40˚17′21″ N. long. 86˚53′02″), located approximately 10 miles south of Lafayette, IN. Soil at this site is classified in the Drummer soil series, which typically contain approximately 3.2% organic matter and a neutral pH. The mean annual precipitation at this site is 1008 mm, and summer temperatures range from 21.1 to 26.7 °C. The crop systems trial was established in 2011 on adjacent tracts of land with uniform topography that had previously been managed using either organic or conventional farming practices since 2001. The crop systems trial was arranged in a split-block design with three replicates for each system given constraints at the site. Within each crop system, four cash crops, carrot (*Daucus carota*), tomato (*Solanum lycopersicum*), popcorn (*Zea mays everta*) and soybean (*Glycine max*), were grown annually and managed using standard practices for each system. This included application of inorganic fertilizers and synthetic pesticides in the conventional system, and inclusion of a winter cover crop and organic fertilizers in the organic system. The winter cover crop planted in the organic system consisted of a custom fall green

manure mix containing winter rye (*Secale cereale L.*), hairy vetch (*Vicia villosa*), winter pea (*Pisum sativum*), annual rye (*Lolium perenne*), and timothy grass (*Phleum pratense*) (Cloverland Seed, Millersburg, OH). Cash crops were rotated in both crop systems annually in the following order: tomato -> carrot -> popcorn -> soybean.

In the carrot plots, fertilizers were applied to both systems to achieve a target rate of 134.5, 180 and 224 kg ha^{-1} of N, P and K respectively. In the organic plots, this consisted of Re-vita Pro Compost (Ohio Earth Foods, Hartville, OH), applied at a rate of 5,380 kg ha^{-1} to meet fertility needs, assuming 50% of the nutrients would be available for plant uptake in the year of application. In the conventional plots, diammonium phosphate (18-46-0) and potash (0-0-60) was applied to meet fertility needs. Sub-plots containing 36 carrot genotypes, which represented advanced breeding lines as well as commercial check varieties, were randomized within each larger carrot plot. Untreated carrot seeds provided by Dr. Phillip Simon, USDA-ARS Vegetable Crop Research Unit, Madison, WI, were planted in mid-May of each year. Seeds were planted on raised beds that were 1.8 m apart, in 1 m rows to provide approximately 60 plants m^{-1} per sub-plot, given previously determined germination rates. Seeds were sown to a depth of 10 mm. In the conventionally managed system, a pre-emergent herbicide (Prowl H2O, BASF Corporation) was applied immediately after planting. In the organically managed system, plots were hand weeded as needed. No additional pesticides were applied in either crop management system. Approximately 110 days after seeding, the percentage of infection by foliar pathogens in each plot was estimated, carrots were manually harvested, and the total number and weight of all taproots and aboveground foliage in each plot were recorded.

3.2.2 Field soil chemical and biological assays

Ten soil cores were randomly collected to a depth of 10 cm in each field rep just prior to carrot seeding in spring. The ten cores within each field rep were pooled and transferred to the laboratory on ice. After thoroughly mixing the cores from each replicate, a subsample of soil was air-dried before shipping to Midwest Labs (Omaha, NE) for a standard soil test according to common methods used in this region (Brown, 1998). Briefly, total organic matter was determined using loss of weight on ignition; available P was extracted as Weak Bray (readily available P) and Strong Bray (potentially available P) and analyzed calorimetrically; exchangeable potassium (K), calcium (Ca), and magnesium (Mg) were extracted with neutral ammonium acetate (1 N) and

quantified by inductively coupled argon plasma–mass spectrometry detection; and base saturation and cation exchange capacity [mmol (+)·kg–1] were estimated from the results of exchangeable minerals (Brown, 1998). Another subsample was placed in the cooler at 4 °C until being air-dried overnight to conduct assays to estimate microbial activity and active soil carbon. Microbial activity was estimated using the hydrolysis of fluorescein diacetate (FDA) in soil slurries using a method optimized for soil (Green et al., 2006). Active C was quantified using the permanganate oxidizable carbon (POXC) technique (Weil et al., 2003). Finally, a subsampled was lyophilized and stored at -20, before being shipped overnight on dry ice to WARD lab (Grand Island, NE) for phospholipid fatty acid analysis (PLFA) using methods described in (Buyer and Sasser, 2012).

3.2.3 Isolation and enumeration of culturable endophytes in carrot taproots

At harvest, nine of the 36 carrot genotypes planted in the larger trial were selected for use in this study, based on their country of origin, differences in top size and tap root color/shape, and resistance to pathogenic nematodes (Table 2.1). Two randomly selected carrot taproots representing each genotype were collected from each of three field replicates, placed in a cooler on ice and transferred to the lab where they were stored at 4° C until processing within 48 hours. Taproots were collected from healthy plants with no signs of disease or any other plant stress. Isolation of endophytes in the carrot taproots was conducted using methods previously described by Sturz et al. (1998). Briefly, carrot taproots were rinsed very well with tap water before being surface disinfected by soaking taproots in 5.25% bleach for 3 minutes, followed by soaking in 3% peroxide solution for 3 minutes, and finally washing with sterilized water supplied with 1ml of tween (Surette et al., 2003). To confirm surface disinfection of the carrot taproots, 200 µl samples from the last washing solution were plated onto various semi-selective media for broad microbial groups. The plates were incubated at 27 °C, and growth of any microbial communities was recorded. Five cores were collected from each surface sterilized carrot taproot using a sterile 15 mm cylinder core borer. Then 5 grams of cores were ground in 25 ml sterile water using an Omni tissue master homogenizer (OMNI International, GA., United States) to create a stock solution. The stock solutions were serially diluted ten times, and 100 μl of each dilution was spread onto plates containing the following selective media for diazotrophic bacteria (LGI), oligotrophic bacteria (R2A), heterotrophic bacteria (Tryptic Soy Agar) and total fungi (1/5th PDA media) (Corry et al., 2011; Reasoner and Geldreich, 1985), each with two replicates. The petri plates were

incubated at 27 °C or 25 °C and counted after 48 or 72 hours, for bacterial and fungal enumeration respectively. To calculate the number of bacterial and fungal colony forming units (CFU) per gram carrot taproot sample, the following equation was used:

$$\#\text{ of bacteria/fungi CFU per gram carrot taproot sample} = \frac{\text{Number of colonies}}{\text{Volume plated x total dilution used}}$$

Plates with serial dilutions of 10^3 or 10^4 and 10^4 or 10^5 were used to isolate individual fungal and bacterial colonies respectively, with unique morphologies. Each individual microbial isolate was inoculated onto a clean petri plate and incubated at 27 °C or 25 °C for fungi and bacteria respectively, to facilitate growth. The hyphael tip technique was used to further purify fungal cultures (Narayanasamy, 2001), while the streak plate technique was used to further purify bacterial cultures using agar slants (Black, 2008). Individual bacterial and fungal cultures that were morphologically distinct were selected and stored in glycerol stocks at -80C for future DNA extraction.

3.2.4 Identification of endophytes

Individual cultures of endophytic microbes stored at -80C were revived by culturing on fresh PDA or Luria-Bertani Agar media in petri plates before subjecting cultures to DNA extraction. DNA extraction was conducted using Microbial DNA extraction kits (Mo Bio, Laboratories, C.A., U.S.A) following the manufacturers recommendations. The final concentration and quality of DNA from each isolate was quantified using a nanodrop (Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers, U.S.A), before being diluted to l ng using Promega nuclease free water in preparation for DNA amplification. For amplification of fungi the universal ITS5 forward (5′ GGAAGTAAAAGTCGTAACAAGG- 3′) and ITS4 reverse (5′-TCC TCC GCT TAT TGA TAT GC- 3′) primers were used to amplify the whole ITS region (Larena et al., 1999). The 25-µl PCR reaction mixture contained 2µl of DNA template, 0.5 µl of each primer (100 mM), 12.5 µl GoTaq® colorless Master Mix from Promega and 9.5 µl Promega nuclease free water. PCR reactions were performed in a Bio-Rad T100 thermal cycler (BioRad, C.A, U.S.A) using the following cycle conditions: initial denaturing step of 1 cycle at 95ºC for 2 minutes, 40 cycles of (denaturing step: 95ºC for 30 seconds, annealing step: 49ºC for 30 seconds, extension step: 72ºC

for 1 minute), and a final extension step of 72°C for 10 minutes. Amplification of DNA from bacterial cultures were conducted using the 8F universal forward (5′-AGAGTTTGATCCTGGCTCAG- 3′) and 1492R universal reverse (5′-GGTTACCTTGTTACGACTT- 3′) primers (Turner et al., 1999) to amplify the V1-V9 (full length) hypervariable region of the 16S SSU rRNA gene of bacteria. The 25-µl PCR reaction mixture contained 1µl of DNA template, 0.5 µl of each primer (100 mM), 12.5 µl GoTaq® colorless Master Mix from (Promega, WI. U.S.A) and 10.5 µl Promega nuclease free water. PCR reactions were performed in a Bio-Rad T100 thermal cycler using the following cycle conditions: initial denaturing step of 1 cycle at 94ºC for 3 minutes, 35 cycles of (denaturing step: 94ºC for 45 seconds, annealing step: 50ºC for 60 seconds, extension step: 72ºC for 90 seconds) and a final extension step of 72ºC for 10 minutes. Verification of PCR-amplification was performed by electrophoresis on a 0.7% (wt./vol.) agarose gel stained with Bullseye DNA Safe Stain (MIDSCI., MO. U.S.A). A 100bp ladder (New England bio lab, MA. U.S.A) was run in parallel with the PCR products on each gel to approximate product band size. Presence of DNA bands stained with DNA Safe Stain were visualized after exposure of the gel to ultraviolet (UV) light. Amplified PCR products were cleaned using Ultra Clean ® PCR Clean-Up Kits (MO BIO Laboratories, Laboratories, C.A., U.S.A), before being sent to the Purdue Genomics Facility for sequencing using an ABI 3137XL low-throughput capillary machine (ABI company, CA., U.S.A) using forward primers.

Nucleotide sequences obtained through sequencing were analyzed using the Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden, 2004) of the National Institutes of Health GenBank database (Bilofsky and Christian, 1988) for precise identification of bacterial endophytes, and the identification of fungal nucleotide sequences were further confirmed using the UNITE Genome Database (Abarenkov et al., 2010). A 98% confidence level cut off was used for identification of bacteria, and a 97% confidence level cut off was used for identification of fungi at the species level. All sequences will be submitted to the NIH GenBank database to obtain accession numbers.

3.2.5 *In vitro* screening of endophyte isolates for antagonistic activity against *Alternaria dauci*

Unique endophytic isolates representing 22 bacteria and 6 fungi were screened using an *in vitro* assay to quantify their antagonistic activity against *A. dauci*. The *A. dauci* isolate used in this experiment was previously isolated from a carrot field, identified using DNA extraction, PCR amplification and sequencing as described above, and evaluated for pathogenicity using Koch's postulates under greenhouse conditions. To obtain working bacterial cultures, isolates stored in -80C glycerol stocks were streaked onto fresh LB plates (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g/L agar in 950 mL deionized water) (Corry et al., 2011) and incubated at 27°C for 2 days before single colonies were selected for use in the antagonistic screening assay. To obtain fungal endophyte and *A. dauci* working cultures, 5mm diameter mycelial plugs from each fungal stock were transferred onto fresh PDA plates and incubated at 25° C for 7 days before use in the antagonistic screening assay. All antagonistic tests were conducted on petri plates filled with PDA media. To screen fungal endophytes, 5mm diameter mycelial plugs from both *A. dauci* and the fungal isolate were placed 4.5 cm away from each other on the same petri plate. To screen bacterial endophytes, a 5 mm diameter disk of *A. dauci* was placed at the center of the PDA plate, and then the individual bacterial isolates where streaked 2.25 cm away from the *A. dauci* disk on both sides of the disk. Plates containing only *A. dauci* pathogen disks served as a control. There were three replicate plates for each endophytic isolate as well as the control, and the entire experiment was repeated to confirm initial results. Plates were incubated at 25°C until the *A. dauci* culture in the control covered the entire plate, and then the diameter of the *A. dauci* colony in the control plates and plates containing the endophytic isolates were recorded.

3.2.6 Greenhouse trial to quantify potential of selected endophytic isolates to increase carrot growth and enhance tolerance to *A. dauci*

Five bacterial isolates were selected based on their antagonistic activity towards *A. dauci* during the *in vitro* assay described above (Table 3.7). Two *Pseudomonas fluorescence* isolates that have previously been found to possess some of the most common and potent bacterial toxins responsible for plant protection against fungal pathogens in agriculture soils (F1&F2) (Garbeva et al., 2004) were also included as positive controls. This included *P. fluorescence* isolate Q2-87 (obtained from L. Thomashow, USDA-ARS Pullman, WA), which is known to produce 2,4-diacetylphloroglucinol (DAPG), and *P. fluorescence* CHA0 (obtained from the Culture Collection

of Switzerland), which is known to produce pyrrolnitrin (PRN). To obtain working cultures, the bacterial isolates were streaked onto plates containing LBA media (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g/L agar in 950 mL deionized water) (Corry et al., 2011), and incubated for 24 hours at 26° C. A single bacterial colony from each isolate was used to inculcate sterile LB broth, and incubated overnight at 26° C on a rotary shaker set at 200 rpm to obtain cultures in the log phase of growth. All cultures were then diluted to equal concentrations, by adjusting their OD_{600} to 0.6.

Untreated seed of two popular carrot varieties (Red Core Chantenay and Napoli) were obtained from High Mowing Seed (VT., U.S.A) for use in these trials. Seeds were surface sterilized following methods described in Surette et al. (2003), before being soaked in bacterial suspensions for 24 hours on a rotary shaker set at 200 rpm. Untreated carrot seeds were included as a control. All seeds were sown in sterile 5 x 25 cm deepots (Green house megastore, IL., USA) filled with Fafard Potting Mix #1 (Sungro Horticulture, MA., U.S.A) that had previously been pasteurized by subjecting the potting mix to a temperature of 60° C for 72 hours. One carrot seed was sown in each deepot, and the pots then were kept in a mist chamber to facilitate germination. There were 20 replicates for all treatments. Germination was recorded 5 days post-planting, and percentage of seed germinated was calculated and used to compare treated and untreated carrot seed. Once all carrot seeds had germinated, seedlings were moved to a greenhouse set at 22 ° C during the day and 18° C during the night ± 1° C, with 50-70% relative humidity and given 16 hours of daylight. The experiment was organized in a complete randomized block design to accommodate environmental variation within the greenhouse. In total, six replicates each of the variety Red Core Chantenay and eight replicates each of the variety Napoli were designated for inoculation with *A. dauci* or a sterile water control treatment.

A. dauci inoculum preparation and carrot seedling infection was conducted using the same isolate described above. The inoculum was produced by placing disks of the virulent *A. dauci* isolate onto petri plates containing carrot leaf agar (Strandberg, 1987), and incubating plates in the dark for 10 days at 20°± 2 C, followed by a period of alternate exposure to 12 hours of dark and 12 hours of ultraviolet light for 10-15 days (Strandberg, 1987). Petri plates containing *A. dauci* cultures were filled with sterile water and scraped with a sterile scalpel to dislodge *A. dauci* spores from mycelium and obtain a spore suspension. The spore and mycelium suspension was filtered through two layers of cheesecloth to separate the conidial spores from the mycelium for use in the

infection process. Finally, the spore suspension was adjusted to an approximate concentration of 1 x 10^4 spores per ml using a hemocytometer (Pawelec et al., 2006). Leaves of carrot seedlings (~30-day old seedlings) were sprayed with equal amounts of the *A. dauci* spore suspension using an atomizer to deliver an equal amount of inoculum to each plant. Carrot seedlings in the control treatment were sprayed with sterile water supplemented with 0.05% of tween 20 (Sigma). Sixty days after planting, carrot shoot and root length and shoot and root dry weight were recorded.

3.3 Statistical analysis

All soil chemical properties, soil microbial biomass and activity, number of colony forming units of endophytes obtained on each selective media, antagonistic activity of endophytic microbial isolates, percent germination of carrot seed, and carrot root and taproot length and dry weight were statistically analyzed using the general linear model procedure for ANOVA, and differences among treatment pairs were determined using the student's t test at a p-value of 0.05, using SAS JMP software package (Institute, 2000). All data were checked for normality, homogeneity of variance and linearity prior to analysis, and were transformed when necessary.

3.4 Results and Discussion

3.4.1 Impact of crop management system on soil chemical and biological properties and carrot yield in the field trial

Many chemical and biological soil properties, including soil pH, total organic matter, active soil organic matter, microbial activity, microbial biomass and several macro and micronutrient concentrations differed between the organic and conventionally managed systems (Tables 3.1 & 3.2 & Fig.3.1). Results of these assays confirm results of other studies, which have demonstrated that many of the soil-building practices commonly used in organic crop management systems can improve soil health relative to conventional management systems (Garbeva, Van Veen et al. 2004, Reeve, Hoagland et al. 2016).

Above- and below-ground biomass among the nine carrot genotypes evaluated in this study did not differ between the two crop management systems (Table 3.3). Disease infection percentage in the above-ground foliage was significantly greater in two of the nine carrot genotypes evaluated (Fig. 3.2). These data confirm that organic management systems can be just as productive as

conventional ones (Lotter, Seidel et al. 2003), and that like other studies (Dalal, Henderson et al. 1991, Liu, Tu et al. 2007) the organic system may be more disease suppressive. This disease suppressive activity could be related to the presence of beneficial microbes that can directly and/or indirectly suppress pathogens via the mechanisms described in the introduction. Moreover, this could indicate that the two varieties with lower disease pressure in the organic system could be more responsive to the presence of these beneficial microbes.

3.4.2 Influence of crop management system and carrot genotype on the density of culturable endophytes in carrot taproots

An abundant and diverse assortment of bacterial and fungal endophytes was isolated from the taproots of nine diverse carrot genotypes grown under organic and conventional management (Fig. 3.3 & Table 3.4). No microbial growth was recovered from the last washing solution used in surface sterilizing carrot taproots, which confirms that the taxa isolated in this study were indeed endophytes. This sterilization technique has previously been used to isolate endophytes from potato, carrot and red clover plants (Sturz, Christie et al. 1998, Surette, Sturz et al. 2003).

In this study, we focused on measuring the density of broad microbial groups including: heterotrophic and oligotrophic bacteria, and total fungal in the roots of nine diverse carrot genotypes. Oligotrophic endophytes are slow-growing microorganisms that can live under extremely low nutrient conditions within plants in a symbiotic relationship without causing any harm to their hosted plants. They have very low metabolic rates and usually present at a low density, which helps to sustain their survival in low-nutrient environments (Kuznetsov, Dubinina et al. 1979). Heterotrophic microbes are fast-growing bacteria that require the presence of organic carbon sources to survive, because they cannot produce these compounds on their own. Thus, they commonly found living in a symbiotic relationship with plants. Finally, relationships between endophytic fungi and plants are usually framed as either mutualistic or commensalistic, but they can also sometimes be described as potential latent pathogens (Schulz, Römmert et al. 1999). However, the majority of fungal endophytes have been demonstrated to provide effects for their host plants such as increasing their total yield, and tolerance to stress via production of beneficial bioactive compounds.

The density of total heterotrophic and oligotrophic bacteria was greater in carrot taproots grown under the organic relative to the conventional management system (Fig.3.3a & b), but there was no difference in total fungi between the two management systems (Fig. 3.3 c). Difference in

endophytic bacterial communities between these two systems is not surprising given the fact endophytes are often a subset of the rhizosphere microbial community (Quadt-Hallmann, Hallmann et al. 1997, Quadt-Hallmann, Kloepper et al. 1997, Surette, Sturz et al. 2003), which are known for their potential to be affected by soil resident microbial community structure. Many studies comparing organic and conventional management have previously demonstrated that management systems can play a significant role in the composition of rhizosphere microbial communities (Bowen and Rovira 1999, Peters, Sturz et al. 2003, Chaparro, Sheflin et al. 2012, Bender, Wagg et al. 2016). Such differences could be due to a greater reliance on chemical fertilizer, pesticides and herbicides in conventionally managed systems, which can directly influence soil and plant microbial community structures (Dalal, Henderson et al. 1991). For example, Bulluck III, Brosius et al. (2002) reported that using synthetic fertilizer in a conventional system significantly reduced the microbial biomass and beneficial *Trichoderma* and thermophilic bacterial species compared to organic systems that relied on organic fertilizer. Moreover, pathogenic *Phytophthora* and *Pythium* species densities were lower when using organic fertilizer compared to synthetic. In addition, other practices that can differ between these two management systems, such as inclusion of cover crops and frequency of soil tillage, could also alter soil and plant microbiomes by altering various physical and chemical soil properties (Bowen and Rovira 1999, Peters, Sturz et al. 2003, Chaparro, Sheflin et al. 2012, Bender, Wagg et al. 2016). For example, in the study comparing endophytes in the taproots of carrots grown in two fields that differed primarily in their use of minimum tillage (Great Village) vs. moldboard plowing (Canning Field), Surette, Sturz et al. (2003) theorized that the greater density of endophytic bacterial communities in the taproots grown at the Great Village site was directly related to greater amounts of organic soil matter that would support greater populations of bacterial soil communities.

In our trial, we anticipate that greater populations of bacterial endophytes in carrot taproots grown in the organic system were also likely related to greater microbial biomass and activity generated by a higher concentration of total and active organic matter fractions observed in the organic relative to the conventional system (Table 3.1).

Despite the fact that was a greater abundance of in total fungal biomass in soils from the organic system (Table 3.2), there were not differences in fungal endophyte communities in the taproos of carrot taproots grown under the two management systems evaluated in this trial (Fig. 3.3). This indicates that fungal endophyte communities in carrot taproots are likely to more

affected by other factors such as difference root morphology, exudates and pigments of the carrot genotypes than resident microbial community structure.

While not as dramatic as crop management, carrot genotype also affected the density of endophytes within the taproots of carrot, and in some cases, there was an interaction between carrot genotype and crop management system indicating that some genotypes are more plastic in their response to differences in microbial community structure (Tables 3.4). Among carrot taproots grown under conventional management, Exp Red Core had the lowest abundance of oligotrophic bacteria with a log of 3.18 CFU per g of carrot taproot tissue, while ExpP6306 genotype had the highest abundance with a log of 6.79 CFU. ExpP6306 also had the lowest abundance of heterotrophic bacteria with a log of 3.87 CFU when grown under conventional management, while ExpB0252 had the highest with a log of 8.09 CFU. Finally, ExpB0252 had the lowest abundance of fungal endophytes with a log of 1.78 CFU in the conventional system, while ExpY8519 had the greatest with a log of 4.35 CFU. In the organic system, genotype ExpNb3999 had the lowest abundance of oligotrophic bacteria with a log of 8.02 CFU, while Exp Y191 had the greatest with a log of 10.42 CFU. Among heterotrophic bacteria, Exp NSF & Nb3999 had the lowest abundance with a log of 9.7 and 9.8 respectively CFU when grown in the organic system, while genotype Exp B0252 had the highest abundance with a log of 11.23 CFU. Finally, genotype Barasilia and Exp Y8519 had the lowest abundance of fungal endophytes when grown in the organic system with logs of 1.93 CFU, while genotype ExpB0252 had the greatest abundance with a log of 4.47 CF U.

As mentioned above, while endophyte communities often represent a subset of soil and rhizosphere communities, plant genotype can also play a significant role in shaping the structure of endophyte communities (Adams and Kloepper 2002, Hardoim, van Overbeek et al. 2008). Results of our study support these assertions. Similar results were obtained by Hardoim, Andreote et al. (2011) when comparing bacterial communities in the roots of ten rice cultivars. Individual microbial taxa might be attracted to one genotype over another depending on the quality and quantity of a particular genotype's root exudates, its morphological and physiological characteristics, or its internal metabolic profile (Hardoim, van Overbeek et al. 2008). At the same time, plant genotypes can differ in the ability to relax their defense processes to let specific microbial taxa with maximum functional benefits colonize their internal tissues (Hardoim, van Overbeek et al. 2008). The nine carrot genotypes evaluated in this study differ substantially with respect to various morphological, metabolic and physiological characteristics including root size,

shape and color, and resistance to pathogenic nematodes, which might help explain differences in the composition of their endophyte communities. In addition, these genotypes were developed in very diverse environments; consequently, they could have evolved features that allow them to recruit some microbes over others when presented with ecological conditions that are more similar to the ones in which they were originally selected (Table 2.1).

Observing differences in endophyte communities in the taproots of carrots grown under the two systems, or among carrot genotypes is very promising for improving plant health and productivity, because it indicates that it may be possible to manipulate these communities to benefit plants. For example, several studies have reported the important role of some oligotrophic microbes in their potential to produce siderophores as well as key enzymes such 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, fix atmospheric nitrogen fixation and solubilize phosphate (Keshtacher-Liebso, Hadar et al. 1995, Khalaf and Raizada 2016). Consequence, plants with greater densities of these types of endophytes are likely to have higher performance and productivity. In addition, endophyte species belonging to heterotrophic bacteria, which are mainly related to Proteobacteria and Firmicutes bacterial phyla, often include taxa with activities of agricultural importance, such as the ability to fix atmospheric nitrogen, produce siderophores and promote plant growth through various mechanisms (Loaces, Ferrando et al. 2011, Malfanova 2013, Bamisile, Dash et al. 2018). Thus, plants with higher densities of these type of microbes may indicate that they are more tolerant to biotic and abiotic stress.

3.4.3 Identification of endophytic microbial taxa recovered from carrot taproots and significance of crop management system and carrot genotype

A total of 36 microbial isolates with different morphological characteristics were isolated from surface sterilized carrot taproots grown in two crop management systems representing nine diverse carrot genotypes. Following amplification and sequencing of 16s rRNA and ITS, variable regions, 28 distinct microbial species were identified; twenty-two of these microbes were bacterial isolates while the rest were fungi (Table 3.5).

Bacteria identified as *Xanthomonas*, *Pseudomonas*, *Rhizobium*, *Bacillus* and *Stenotrophomonas* spp were the most common genera isolated from carrot taproots. Endophytic microbes belonging to *Rhizobium*, *Bacillus*, *Stenotrophomonas* and *Pseudomonas* genera have been isolated before from a number of economically important crops including carrot, soybean, citrus and rice (Yanni, Rizk et al. 1997, Araújo, Maccheroni Jr et al. 2001, Araújo, Marcon et al.

2002, Surette, Sturz et al. 2003, Rosenblueth and Martínez-Romero 2006). In addition, some of the endophytic microbes isolated in these studies were reported to possess plant-growth promoting properties, as well as antagonistic activity against a number of plant and human pathogens including *Xylella fastidiosa* and *Salmonella*, respectively (Yanni, Rizk et al. 1997, Araújo, Maccheroni Jr et al. 2001, Araújo, Marcon et al. 2002, Surette, Sturz et al. 2003, Rosenblueth and Martínez-Romero 2006). Even though certain members of *Xanthomonas* and some *Pseudomonas* species are often implicated for their potential to act as plant and animal pathogens (Bergan 1981, Swings, Van den Mooter et al. 1990, Hildebrand, Palleroni et al. 1994), this is not always the case. For example, in our study endophytes were isolated from healthy carrot roots that did not show any symptoms of abiotic or biotic stress, indicating that they are not pathogens and could instead provide benefits to plants. In addition, Walitang, Kim et al. (2017) isolated *Xanthomonas* sp. from healthy rice seeds that had the ability to improve rice germination under salt stress. Similarly, endophytic microbes related to *Pseudomonas* genera were isolated from healthy grass plants grown under nutrient-poor sand dunes, which had the potential to fix atmospheric nitrogen (Dalton, Kramer et al. 2004).

Endophytes identified as *Cladosporium, Plectosphaerella, Colletotrichum* and *Epicoccum* spp. were the most common fungi isolated from carrot taproots in this study. As mentioned above Some members of *Cladosporium, Colletotrichum* and *Plectosphaerella* have previously been implicated as potential plant pathogens too (Masel, Braithwaite et al. 1990, Thomma, Van Esse et al. 2005, Carlucci, Raimondo et al. 2012); however at the same time, other members of these genera such as *Colletotrichum tofieldiae*, *Cladosporium oxysporum*, *C. sphaerospermum* and *Plectosphaerella cucumerina*, have been isolated from healthy pine (*Pinus* sp.) and soapberry (*Sapindus saponaria*) trees, as well as Arabidopsis (*Arabidopsis thaliana*) and sugar cane (*Saccharum officinarum*) plants (Wang, Jiao et al. 2007, Li, Zhang et al. 2012, Sadrati, Daoud et al. 2013, Pan, Su et al. 2017). Many of the endophytes isolated in these studies were able to produce bioactive products such as antibiotics and other anti-fungal compounds that were antagonistic to a number of plant and human pathogens, demonstrating that they could benefit their host plants. Moreover, such endophytes could also play a role in the nutritional quality of plants. For example, Gribanovski-Sassu and Foppen (1967) reported that in addition to the potential for the fungus *Epicoccum nigrum* to act as a biological control agent against the plant pathogens (Larena, Torres et al. 2005), this fungus could also produce four types of carotenoid pigments (Gribanovski-Sassu

and Foppen 1967). Carrot taproots are well known for their potential to be an important source of carotenoids in the human diet, and the presence of this particular microbe in this study indicates that it could possibly contribute to the composition of these compounds in carrot taproots.

Out of the twenty-two unique bacterial endophyte isolates recovered from surface sterilized carrot taproots in this study, fourteen were isolated from carrots grown under the organic management system, while eight were isolated from carrots grown under the conventional management system (Table 3.5). Among fungal endophyte isolates, six unique taxa were recovered from carrot taproots grown under organic management, while only two were recovered from carrot taproots grown under conventional management (Table 3.5). These results are consistent with other studies, which have observed greater diversity in organic relative to conventionally managed systems. For example, Xia, DeBolt et al. (2015) recovered 239 unique endophyte isolates from tomato, corn, melon and potato crops grown under organic management practices, while only 97 were recovered from the same vegetable crops grown under conventional management. Greater diversity in the endophytic microbial taxa isolated from organic relative to conventional management systems could be due to the different practices used under these two systems. For instance, following the application of organic fertility amendments such as compost and green manure cover crops, soil organic matter is often greater in organic relative to conventional systems, which is often correlated with greater abundance and diversity of microbial biomass in the rhizosphere as well as endophytes of plants (Dalal, Henderson et al. 1991, Fließbach and MaÈder 2000, van Diepeningen, de Vos et al. 2006). Bulluck III, Brosius et al. (2002) reported that adding organic amendment to organic tomato fields increases the nutrient concentration and soil health in comparison to synthetic fertilizer used in conventional fields. In response, the total microbial biomass and beneficial microbes were greater in organic relative to conventional fields. Chemical herbicides and pesticides applied in the conventional system could also reduce microbial biomass and diversity directly via non-target effects on potentially beneficial microbial taxa (Hartmann, Frey et al. 2015). In this study, the concentration of organic matter, Mg, Ca, FDA, POCX, gram positive and negative bacteria, actinomycetes, mycorrhiza, saprophytic fungi and protozoa biomass were all higher in organic soil, which could be responsible for the observed shifts in endophytes recovered from carrot taproots grown under these two systems (Tables 3.1 &3.2 & Fig 3.1).

3.4.4 Impact of crop management system on the antagonistic activity of endophytic isolates against *A. dauci*

All of the 22 bacterial and 6 fungal endophytes isolated from carrot taproots in this study were able to reduce the growth of *A. dauci* when compared to the control during *in vitro* assays (Table 3.6). This is consistent with results of similar studies, which have demonstrated that most endophytes have at least some level of antagonistic activity against pathogens (Sessitsch, Reiter et al. 2004, Berg, Krechel et al. 2005). The antagonistic activity of microbes against pathogens generally relies on one or more of the following mechanisms: 1) antibiosis, which results from the production of various toxins, enzymes or other antibiotic compounds, 2) parasitism, in which a microbe directly feeds on a pathogen, and 3) competition over space or other limiting resources such as oxygen or nutrients (Berg and Hallmann 2006).

In general, bacterial and fungal endophytes isolated from carrot taproots grown under organic management had greater antagonistic activity than those grown under conventional management (Fig. 3.4). In addition, differences in antagonistic activity among individual endophytes recovered from carrot taproots were also observed (Table 3.6). For example, among bacterial endophytes isolated from carrot taproots grown under conventional management, isolate CE.10 caused the greatest reduction in *A. dauci* growth, with a colony diameter = 4.6 cm, while isolate CE 11 caused the least reduction in *A. dauci* growth, with a colony diameter = 7.56 cm (Table 3.6 a). Among bacterial endophytes isolated from carrot taproots grown under organic management, isolate CE 14 had the greatest antagonistic activity against *A. dauci* with a colony diameter of 1.69, while isolate CE 8 caused the least reduction in *A. dauci* growth with a colony diameter of 7.4 (Table 3.6 a). Among fungal endophytes recovered from carrot taproots grown under organic management, isolate 3 had the greatest antagonistic activity against *A. dauci*, with a colony diameter of 2.89 cm, while isolate CE 5 had the least antagonistic activity against *A. dauci* with a colony diameter of 4.45 cm. Finally, when comparing fungal endophytes recovered from carrot taproots grown under conventional management, isolate CE 3 had the greatest antagonistic activity against *A. dauci* with a colony diameter of 2.89 cm, while isolate CE 4 had the lowest antagonistic activity with a colony diameter of 5.33 cm (Table 3.6 b).

As we mentioned before, plants grown in organic management systems often have greater microbial diversity and biomass in their rhizosphere than plants grown in conventional management systems Dalal, Henderson et al. (1991). According to Hillebrand, Bennett et al.

(2008) this is because of the higher soil microbial biomass and diversity that is often found in organic relative to conventionally managed systems, which leads to greater competition for resources and consequently, greater pathogen suppression activity among resident soil microbial communities. Since endophytes are generally a subset of rhizosphere microbes, it is not surprising that the composition of endophytes also differs between different crop management systems (Quadt-Hallmann, Hallmann et al. 1997, Quadt-Hallmann, Kloepper et al. 1997, Surette, Sturz et al. 2003). Dalal and Kulkarni (2015) also observed differences in endophytic as well as epiphytic microbes in vineyards managed using organic vs. conventional practices, with greater antagonistic activity by microbes isolated in the organic system. In this study, our PLFA data indicated that gram positive and negative bacteria, actinomycetes, mycorrhiza, saprophytic fungi and protozoa biomass were higher in organic than conventional soil. Even the ratio between predators to prey were higher in the organic system (Table 3.2), which supports the observation of greater antagonistic activity among endophytes isolated from organic carrot taoroots. Results of these studies not only provide evidence that management practices employed in organic systems have the potential to increase populations of antagonistic activity and thereby reduce the need for pesticides, but they also could be a good place to isolate individual taxa that could someday be used as inoculants for pathogen biocontrol.

3.4.5 Potential for select endophytic isolates to improve plant growth and reduce susceptibility to *A. dauci* in two popular carrot varieties

To determine whether the endophytic taxa isolated in this study can indeed promote plant growth and reduce susceptibility to *A. dauci*, greenhouse trials were conducted using bacterial isolates with the greatest *in vitro* antagonistic activity. These trials were conducted using two popular carrot varieties (Napoli and Red Core Chantenay) to determine whether carrot genotype would affect responsiveness to beneficial microbes.

Interestingly, there were no differences in germination between endophyte-treated and untreated carrot seed of the Red Core Chantenay variety, whereas germination of the Napoli variety was significantly reduced by the endophyte treatment (Table 3.8). The lowest germination rate was observed when treating Napoli seed with isolate CE 3, in which only 30% of the seeds germinated. In contrast, 90% of Napoli seeds germinated when they were treated with isolate F2. This indicates that some of these microbes could be acting as potential pathogens in Napoli or may even be outcompeting beneficial endophytic microbes that were present within the seed of this

variety. The decrease in seed germination in Napoli and lack of a response in Red Core Chantenay could also potentially be related to the methods we used to inoculate seeds. For example, it has been reported that the concentration of the endophyte inoculum, duration of the endophyte inoculation treatment and surface sterilization method used prior to endophyte treatment can affect the endophyte colonization rate (Akello, Dubois et al. 2009, Tefera and Vidal 2009). In other studies that have observed increases in seed germination in response to seed treatment with endophytes with potential antagonistic activity (Dalal and Kulkarni 2015), a different seed treatment method than the one employed in this study was used. Consequently, we expect that endophyte density might have been too low to cause differences in seed germination in Red Core Chantenay, or too high in Napoli.

Differences in plant growth characteristics in response to seed treatment with the endophyte isolates evaluated in this study also differed between the two carrot genotypes evaluated (Fig 3.5, 3.6, 3.7 & 3.8). In the absence of *A. dauci*, there were no differences in shoot or root dry weight in cv. Napoli in response to the endophyte seed treatments (Fig. 3.5 &3.6). However, when Napoli plants were inoculated with *A. dauci*, seeds treated with F1, F2, CE 2 and the all isolates combined treatment, shoot dry weight was greater than the control. The greatest shoot dry weight, with a value 2.9 g, was achieved by treating Napoli seeds with isolate CE 2. In contrast, shoot dry weight of cv. Red Core Chantenay plants were greater than the control in plants grown from seeds that were treated with endophyte CE 9 and the all isolates combined treatment, while seed inoculation with F1 resulted in lower shoot dry weight in comparison to the control. When Red Core Chantenay plants were infected with *A. dauci*, plants grown from seeds treated with isolate CE 2 and CE 3 had greater shoot dry weight in comparison to the control. The greatest increase in shoot dry weight in Red Core Chantenay plants was achieved by treating carrot seeds with isolate 3, which resulted in a dry shoot weight of 4.5 g.

Napoli root dry weight grown from carrot seed treated with isolate CE 1, CE 2, F1 and F2 was greater in comparison to the control. The greatest root dry weight of 4.6 g, was achieved by treating carrot seed with isolate CE 1. Upon infection with *A. dauci*, root dry weight in Napoli plants treated with all endophyte isolates (with the exception of isolate 3), had greater root dry weight in comparison to the control. The greatest root dry weight, of 4.8 g, was achieved by treating Napoli seed with isolate CE 1 (Fig 3.6). Root dry weight of Red Core Chantenay plants were not affected by any of the endophyte treatments, with the exception of the all isolates

combined treatment, which had lower root dry weight. In contrast, upon *A. dauci* infection, Red Core Chantenay plants grown from seeds treated with isolate CE 3 and the all endophyte isolates combined treatment, had greater root dry weight in comparison with the control; the greatest root dry weight of 2.6 g, was achieved by treating seed with isolate CE 3 (Fig. 3.6).

None of the Napoli plants treated with the various endophytic isolates had greater root length in comparison with the control, though treating seed with isolate 5 resulted in lower root length. Upon *A. dauci* infection, Napoli plants grown from seed treated with isolate CE 1, CE 9, F1 and F2 had greater root length in comparison with the control; the greatest root length was achieved by treating Napoli seed with isolate F2, resulting in a length of 22.6 cm (Fig. 3.7). The root length of Red Core Chantenay plants grown from seed treated with isolate CE 3 and CE 5 were greater than the control; the greatest root length was achieved by treating plants with isolate CE 3, resulting in a length of 19.23 cm. Upon *A. dauci* infection, root length in Red Core Chantenay plants was greater than the control in response to treatment with isolates CE 3, CE 5, F2 and the all endophyte isolates treatment; the greatest root length was achieved in response to inoculation with isolate CE 3, which resulted in a root length of 17.28 cm. The lowest root length in Red Core Chantenay plants subject to *A. dauci* infection was observed in seed treated with isolate CE 1, resulting in a length of 9.05 cm (Fig.3.7).

Seed treatment with none of the endophytic isolated evaluated in this studying resulted in greater shoot length in Napoli plants; rather, treatment with isolate 1 CE, CE 3, CE 5, F1 and F2 resulted in lower shoot length in comparison to the control (Fig. 3.8). Upon *A. dauci* infection, seed treatment of Napoli plants with isolate CE 9, the all endophytes combined treatment, F1 and F2, resulted in greater shoot length in comparison to the control, with the greatest shoot length of 42.3 cm achieved by treating with isolate CE 9, and the lowest, at 31.9 cm, in response to treatment with isolate CE 5 (Fig. 3.8) None of the endophyte treatments increased root length in Red Core Chantenay plants. However, when Red Core Chantenay plants were subject to infection with *A. dauci*, seed treatment with isolate CE 3 and the all endophyte isolates combined treatment, had greater shoot length compared to the control; the greatest length of 48.8 cm, was achieved by treating seed with isolate CE 3.

Consistent with the results of this study, others have also observed increases in plant biomass following inoculation with endophytes in the presence and absence of pathogens indicating that these microbes can promote plant growth and reduce susceptibility to diseases. For

example, Dalal and Kulkarni (2015) reported greater soybean (*Glycine max*) shoot and root dry weight and length as well as tolerance to *Rhizoctonia solani* in response to seed treatment with a number of endophytic isolates. Shoot and root dry weight and barley (*Hordeum vulgare*) grain yield were greater after treatment with the endophytic fungus *Piriformospora indica* (Khan, Hussain et al. 2015). Finally, cucumber (*Cucumis sativus*) fruit yield was significantly greater while incidence of anthracnose and leaf spot were significantly lower following treatment with the endophyte *Bacillus pumilus* under field conditions (Zehnder, Murphy et al. 2001).

As discussed above, increases in plant biomass and tolerance to pathogens in response to the presence of some endophytic microbial taxa could be due to a combination of direct and indirect effects on plant host physiology and defense responses, and the presence of pathogens. Possible direct effects of endophytes on plant growth promotion include production of phytohormones such as indole acetic acid (Ali, Charles et al. 2017). For example, in a study evaluating how some endophytic isolates belonging to *Penicillium* spp. could increase plant growth in soybean (*Glycine max*), Khan et al. (2011) found evidence that the effects were directly related to the ability of these isolates to produce gibberellin. Increasing nutrient availability to their hosts is another direct mechanism that endophytes can use to promote plant growth. For example, *Colletotrichum tofieldiae* endophytes were found to promote Arabidopsis (*Arabidopsis thaliana*) growth by increasing phosphorus solubility under conditions of low phosphorus availability (Hiruma, Gerlach et al. 2016). Indirect mechanisms of plant growth promotion include pathogen suppression. For instance, many endophytic isolates belonging to *Streptomyces, Microbispora, Micromonospora* and *Nocardioides* spp. have been demonstrated to have antifungal activity under *in vitro* conditions against a destructive wheat pathogen belonging to *Pythium, Rhizoctonia* and *Gaeumannomyces* spp. (Coombs, Michelsen et al. 2004). Another indirect mechanism of endophytes that could contribute to plant growth promotion is by inducing systemic resistance against pathogens. For example, in a recent study Lanna-Filho et al. (2017) provided evidence that two endophytic isolates belonging to *Bacillus* spp. were able to stimulate a tomato plant's immune system and thereby reduce disease caused by the foliar pathogen that causes bacterial speck (Lanna-Filho, Souza et al. 2017). Finally, endophytes could be directly competing with pathogens over limited resources, as was observed when a non-pathogenic endophytic isolate of *Fusarium oxysporum* that was co-inoculated with a pathogenic *F, oxysporum* isolate; the non-pathogen isolate was more successful at colonizing tomato root tissue, which resulted in lower root rot

(Bolwerk, Lagopodi et al. 2005). The fact that some of endophytic isolates evaluated in this study increased carrot growth indicates that it may be possible to develop them for use as commercial inoculants.

Differences in responsiveness to potentially beneficial endophytic microbial taxa have also been observed in other studies. For example, Andreote, Da Rocha et al. (2010) observed differences in the potential for three potato cultivars (Eersteling, Robijn and Karnico) to respond in the form of enhanced plant growth when inoculated with *Paenibacillus* spp and *Methylobacterium mesophilicum* bacterial endophytes. Similarly, modern and wild rice cultivars responded different to inoculation with diazotrophic bacteria, which appeared to be related to differences in rooting behavior and composition of root exudates between the cultivars (Hallmann and Berg 2006). Differences in responsiveness among plant genotypes are most likely related to differences in the endophytes lifestyle and physiology as well as the plant genotype itself. This is because some endophytes may prefer to colonize certain plant genotypes over others, because they may provide better protection or other resources that these microbial taxa need to survive. At the same time, some plant genotypes may preferentially select specific endophytes because they can fulfill some specific function that the plant needs (Adams and Kloepper 2002). The two carrot varieties evaluated in our trial, Napoli and Red Core Chantenay, vary significantly in their morphological and physiological characteristics, which likely affected their interaction with the endophytic isolates.

Differences in some plant parts but not others, such as roots vs. shoots, in response to endophyte inoculant has also been documented in other studies (Dalal and Kulkarni 2015). This phenomenon is mostly related to the fact that some endophytes prefer to colonize specific plant tissues since they are particularly adapted to conditions present in that given plant (Guo, Huang et al. 2008). As a result, those tissues that are preferentially colonized by endophytes might show a different plant growth response in comparison to other plant parts that are not colonized by endophytes. For example, it has been reported before that most of the entophytic fungi of the Dendrobium plant (*Orchidaceae*) colonize the root more than the stem and leaves (Chen, Hu et al. 2011). The differences in endophyte community composition between roots, shoots and stems in Dendrobium plants was thought to reflect differences in the environment such as biological differences among plant tissue (Bayman, Lebron et al. 1997).

Interestingly, in some cases we did not observe a benefit from endophyte inoculation until the plants were subject to stress by the presence of *A. dauci*. This is not surprising given that endophytes are classically defined as microbes that do not cause any visible symptoms on a plant, though it has been theorized that they are available to help when environmental conditions change. For example, Hiruma et al. (2016) observed that *Colletotrichum tofieldiae* endophytes only seemed to benefit Arabidopsis plants when they were subject to abiotic stress resulting from low phosphorous availability. Under these conditions, the endophyte improved Arabidopsis growth by solubilizing and transporting phosphorus to its host. Similar relationships are likely to occur when pathogens are subject to pathogen stress (Schulz, Boyle et al. 2006), since pathogens are often implicated as a major factor affecting endophyte communities structure and composition (Sessitsch, Reiter et al. 2004).

Finally, in some cases we observed a negative effect on plant growth in response to inoculation with the bacterial endophytes. As discussed above, this could have been due to the concentration of endophytes, where in some cases they might have become too abundant and began acting like a parasite. Alternatively, the negative plant growth response could have been related to environmental conditions. For example, if the plant was not under significant stress, then the endophyte ends up being a cost to the plant, whereas when the plant is under environmental conditions that are more like what it experiences in nature, the presence of the endophyte is worth the cost. Schulz and Boyle (2005) reported that symbiotic relationships among endophytes and their host is balanced between plant defense and endophyte virulence, though if this interaction becomes unbalanced, endophyte virulence takes over and causes disease in the host plant. At this point, the plant will succumb to the endophyte, which is now acting like a pathogen, or up-regulate its defense responses to kill the endophytes. This delicate balance between a plant and its endophyte could change upon different environmental conditions, such as nutrient status and developmental stage of the plant. Thus, further research to evaluate the activities of these endophytes under field conditions would be valuable.

3.5 Conclusions and potential implications of this research

Results of this study verify the assumption that soil-building practices commonly used in organic farming systems such as integrating cover crops, applying organic fertilizers and avoiding application of pesticides, can improve soil quality. However, the benefits of such soil quality

improvements may not be realized until plants are subject to some specific stress. In this study, we demonstrated that endophytic communities in the taproots of carrot are more abundant and diverse in an organic relative to a conventionally managed system. Moreover, microbial isolates recovered from carrots grown in the organic system had greater antagonistic activity to *A. dauci*, a key carrot pest. The greenhouse trial confirmed that some of these isolates can promote plant growth and help carrot plants withstand assault by this devastating pathogen. These results suggest that beneficial relationships between plant and soil health could evolve in systems managed using organic practices and help compensate for the need to use pesticides to mitigate disease outbreaks.

The carrot genotypes evaluated in this study differed in endophyte composition as well as responsiveness to individual taxa that promoted plant growth and enhanced tolerance to *A. dauci*. This indicates that plant breeders could begin to select for varieties that are better able to recruit and host microbes that can help them fight pathogens. This could have far reaching effects for managing carrot diseases and reducing the need for pesticides in organic as well as conventionally managed systems.

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Table 3.1 Organic and conventional field trials soil chemical properties

T	OM%	P - weak bray	P - strong bray	K	Mg	Ca	Soil pH	CEC	%k	%Mg	%Ca	%H
Conventional	2.6	139.7	151.3	488.3	314.3	1840.0	5.6	17.1	7.3	15.3	53.7	23.6
Organic	3.1	38.3	85.7	298.3	349.7	2190.0	6.4	15.7	4.8	18.5	69.6	7.1

Table 3.2 Organic and conventional field trials soil microbial community estimated by Phospholipid Fatty Acid. (a) Microbial biomass & (b) ratios of microbial biomass groups.

a)

	Microbial Biomass (PLFA)										
System	Total Microbe	Total Bacteria	Actinomycetes	Gram (+) Bacteria	Gram (-) Bacteria	Rhizobia	Total Fungi	Arbuscular Mycorrhizal Fungi	Saprophytes	Protozoa	Undifferentiated
Conventional	1266.1	704.2	140.4	474.1	230.1	6.4	87.6	13.3	74.3	0.0	807.6
Organic	2223.7	1219.9	259.9	795.0	424.9	0.0	203.4	68.7	134.7	8.6	791.8

b)

	Ratios of microbial biomass groups			
System	Fungi:Bacteria	Predator:Prey	Gram (+): Gram (-)	Sat:Unsat
Conventional	0.133	all prey	2.348	2.684
Organic	0.167	0.007	1.931	1.816

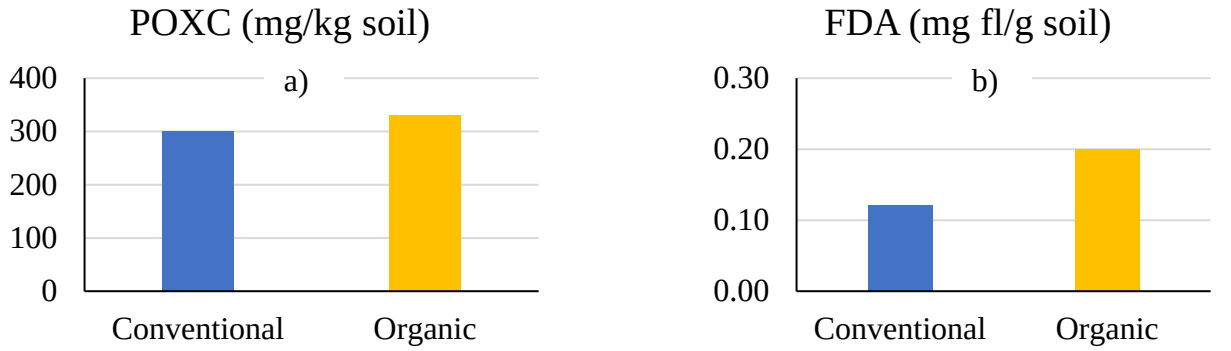
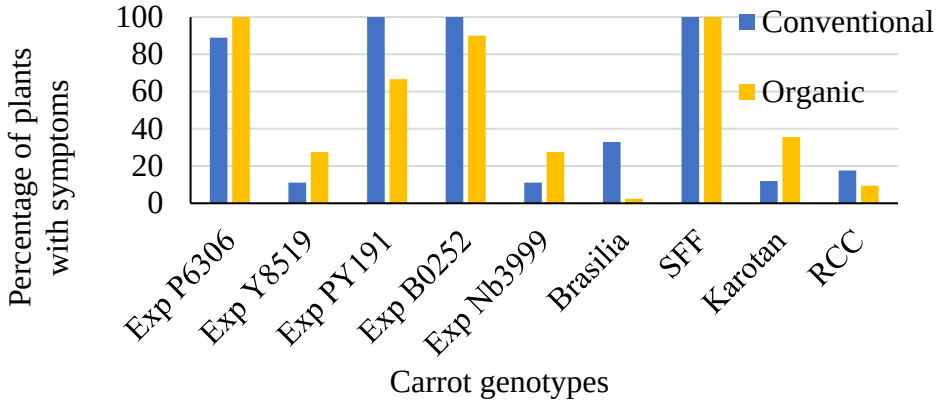


Figure 3.1 Organic and conventional field trials soil analysis. (a) Bars represent mean of active carbon as measured by Permanganate Oxidizable and (b) Bars represent mean of soil microbial activity as estimated by Fluorescein Diacetate.

Table 3.3 Above- and below-ground biomasses of the nine tested carrot genotypes grown under organic or conventional system field trials.

	# of plants		**Top wt. (g, all)**		**Root wt. (g, all)**	
Entry	**Conventional**	**Organic**	**Conventional**	**Organic**	**Conventional**	**Organic**
Exp P6306	16.00	9.00	1.19	0.50	2.32	1.03
Exp Y8519	19.67	20.00	1.28	1.08	2.74	1.55
Exp PY191	10.00	13.00	0.34	0.33	1.14	1.20
Exp B0252	12.00	11.00	0.31	0.52	0.83	0.67
Exp Nb3999	14.00	12.67	0.56	0.38	2.06	1.61
Brasilia	9.00	15.67	0.67	0.79	1.38	1.56
SFF	9.67	15.67	0.66	0.44	1.21	1.15
Karotan	9.00	15.67	0.67	0.79	1.38	1.56
RCC	13.67	21.33	0.96	0.53	2.02	1.34

Figure 3.2 Field trials data for foliar disease infection percentage of the nine tested carrot genotypes grown under organic or conventional system.



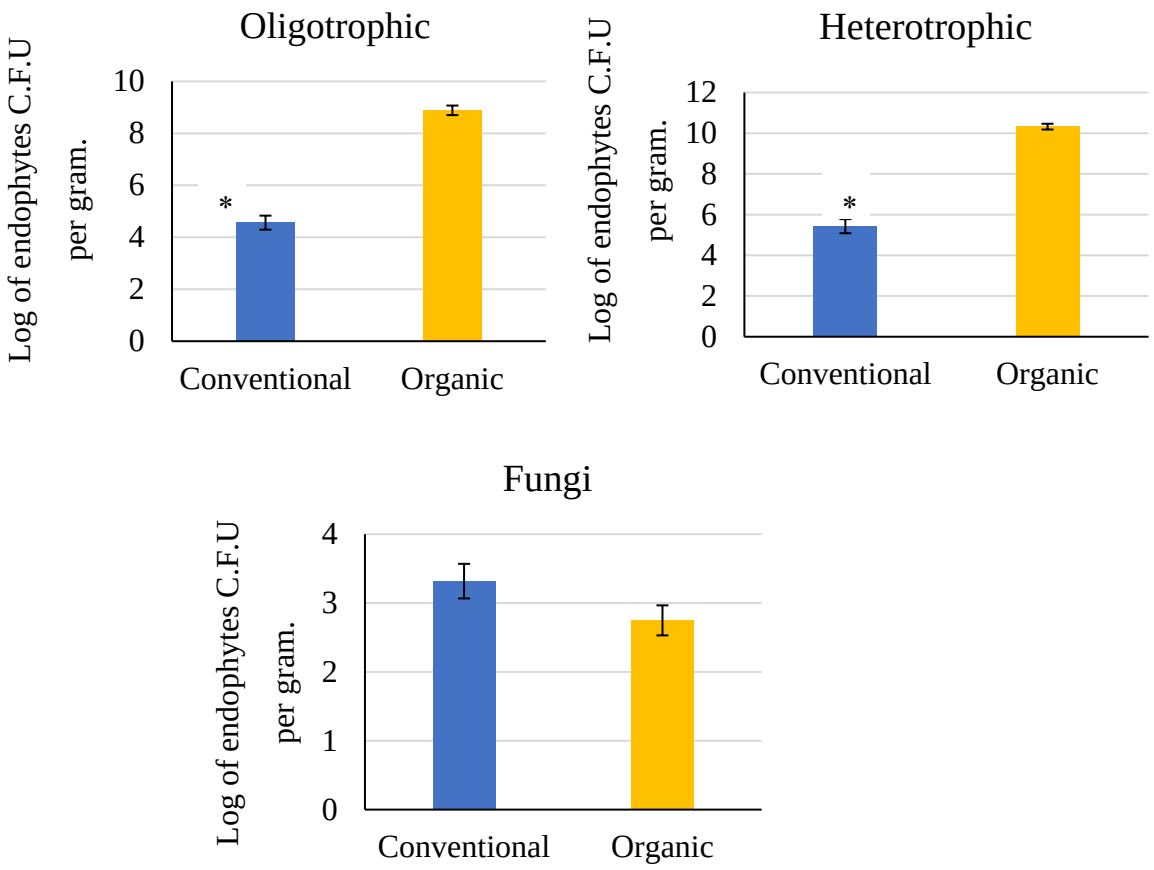


Figure 3.3 Log of carrot root endophytes colony forming unite (C.F.U) counts obtained from the nine tested genotypes grown under organic or conventional system. Counts were observed on three different selective media for Oligotrophic (a), Heterotrophic (b) & Fungi (c). With (n=2). Bars represent stander error mean & ∗ represent significant value of < 0.0001

Table 3.4 Log of carrot root endophytes colony forming unite counts (C.F.U) obtained from the nine tested genotypes grown under organic or conventional system ± stander error mean. Counts were observed on three different selective media for Oligotrophic, Heterotrophic & Fungi with (n=2). P value was < 0.001.Different letters represent significantly different between treatments when treatments means were compared using each pair student's test comparison

	Oligotrophic bacteria		Heterotrophic bacteria		Fungi	
	Conventional	Organic	Conventional	Organic	Conventional	Organic
Exp PY191	6.23 ± 0.15 b	10.42 ±0.05 a	7.77 ± 0.21 b	10.87 ± 0.13 ab	1.93 ± 0.15 c	3.65 ± 0.03 b
Exp B0252	4.08 ± 0 d	9.26 ± 0 bc	8.09 ± 0.06 a	11.23 ± 0.01 a	1.78 ± 0.0 c	4.47 ± 0.01 a
Karotan	3.7 ± 0.08 e	8.53 ± 0.45 cde	4.13 ± 0.05 de	9.84 ± 0.17 de	3.63 ± 0.06 b	2.08 ± 0.30 d
Exp P6306	6.79 ± 0.11 a	9.62 ± 0 b	3.87 ± 0.02 e	10.61 ± 0.20 bc	2.13 ± 0.35 c	2.92 ± 0.30 c
Exp Y8519	4.4 ± 0.01 c	8.38 ± 0 de	5.30 ± 0.05 c	9.84 ± 0.17 de	4.35 ± 0.01 a	1.93 ± 0.15 d
Exp Nb3999	3.99 ± 0.02 d	8.02 ± 0.24 e	5.13 ± 0.17 c	9.61 ± 0.20 e	4.2 ± 0.01 a	2.23 ± 0.15 d
Scalet fancy fruit (NSFF	4.36 ± 0.01 c	8.69 ± 0.21 cde	5.23 ± 0.01 c	9.75 ± 0.25 e	4.23 ± 0.0 a	2.02 ± 0.24 d
Brasilia	4.35 ± 0.01 c	8.20 ± 0.42 e	5.15 ± 0.01 c	10.88 ± 0.17 ab	4.31 ± 0.03 a	1.93 ± 0.15 d
Red Core Chantenay	3.18 ± 0.12 f	8.86 ± 0.18 cd	4.23 ± 0.02 d	10.3 ± 0.07 cd	3.3 ± 0.12 b	3.51 ± 0.04 bc

Table 3.5 Table (2) BLAST n alignment results for (a) 16s r RNA & (b) ITS partial sequences of carrot root endophytes retrieved from different treatment. 98% and 97% confident level cut off were used for bacterial and fungal sequences identification at species level respectively.

a)

Treatment	Sample code	*Closest strain on NCBI data base*	E value	Identity	Accession # of closest species
Organic					
	O10	*Xanthomonas arboricola pv. pruni*	0	100%	LC388644.1
		Xanthomonas campestris	0	100%	MG597200.1
		Xanthomonas campestris pv. hederae	0	100%	KU518243.1
		Xanthomonas arboricola	0	100%	KP340804.1
		Xanthomonas campestris pv. campestris	0	100%	KP182149.1

Table 3.5a continued

		Xanthomonas spp.	0	100%	KM252981.1
	O5	*Uncultured gamma proteobacterium*	0	100%	MH478520.1
		Stenotrophomonas spp.	0	100%	MH465193.1
		Stenotrophomonas maltophilia	0	100%	MH432203.1
		Gammaproteobacteria bacterium	0	100%	MH362776.1
		[Pseudomonas] geniculata	0	100%	KJ452162.2
		Xanthomonas spp. *strain*	0	100%	MF574216.1
		Pseudomonas spp.	0	100%	KP282735.1
		Staphylococcus succinus	0	100%	KJ534522.1
		Staphylococcus spp.	0	100%	KJ605128.1
		Xanthomonas oryzae	0	100%	KF822671.1
		Uncultured bacterium	0	100%	JX001217.1
		Uncultured Steroidobacter spp.	0	100%	HQ674839.1
		Uncultured Stenotrophomonas spp.	0	100%	GU569133.1
		Xanthomonas retroflexus	0	100%	AY841369.1
	O14	*Paenibacillus tundrae*	0	100%	MF101179.1
		Paenibacillus spp.	0	100%	HF954523.1
		Paenibacillus amylolyticus	0	100%	HQ284944.1
	O4	*Uncultured gamma proteobacterium*	0	100%	MH478520.1
		Stenotrophomonas spp.	0	100%	MH465193.1
		Stenotrophomonas maltophilia	0	100%	MH432203.1
		Gammaproteobacteria bacterium	0	100%	MH362776.1
		[Pseudomonas] geniculata	0	100%	KJ452162.2
		Xanthomonas spp.	0	100%	MF574216.1
		Pseudomonas spp.	0	100%	KP282735.1
		Staphylococcus succinus	0	100%	KJ534522.1

Table 3.5a continued

		Staphylococcus spp.	0	100%	KJ605128.1
		Uncultured bacterium	0	100%	JX001217.1
		Xanthomonas retroflexus	0	100%	JQ890537.1
		Uncultured Steroidobacter spp.	0	100%	HQ674839.1
		Uncultured Stenotrophomonas spp.	0	100%	HQ674838.1
	O12	*Pseudomonas fluorescens*	0	100%	AM900685.1
	O1	*Pseudomonas* spp.	0	100%	KX953866.1
		Pseudomonas brassicacearum	0	100%	MG461459.1
	O9	*Pseudomonas oleovorans*	0	99%	GQ365203.1
	O15	*Methylobacterium bullatum*	0	99%	KY012255.1
		Methylobacterium spp.	0	99%	FN868937.1
		Uncultured bacterium	0	99%	HM335898.1
	O6	*Pseudomonas* spp	0	97%	CP015225.1
	O8	*Rhizobium giardinii*	0	99%	MF101027.1
		Rhizobium spp.	0	99%	KP751382.1
		Bradyrhizobium spp.	0	99%	DQ310792.1
	O13	*Pseudomonas brassicacearum*	0	100%	KU350592.1
		Pseudomonas fluorescens	0	100%	AM900685.1
		Pseudomonas spp.	0	100%	FJ225306.1
	O7	*Xanthomonas campestris pv. campestris*	0	100%	MG149779.1
		Xanthomonas arboricola pv. pruni	0	100%	LC388645.1
		Uncultured Xanthomonas spp.	0	100%	KX456334.1
		Xanthomonas campestris	0	100%	MF285891.1
		Xanthomonas campestris pv. vitians	0	100%	MH165478.1
		Xanthomonas hortorum	0	100%	CP016878.1

Table 3.5a continued

		Xanthomonas hortorum pv. vitians	0	100%	MG262373.1
		Xanthomonas spp.	0	100%	KY446031.1
	O3	*Uncultured Pseudomonadaceae*	0	99%	MF374349.1
		Pseudomonas fluorescens	0	99%	CP025542.1
		Pseudomonas spp. *strain*	0	99%	KX758046.1
		Pseudomonas brassicacearum	0	99%	KP762561.1
		Pseudomonas brassicacearum subsp. neoaurantiaca	0	99%	KP762555.1
		Endophytic bacterium	0	99%	JF901350.1
	O2B	*Uncultured bacterium*	0	99%	JX001211.1
Conventional					
	C6	*Xanthomonas campestris pv. campestris*	0	100%	MG149778.1
		Xanthomonas arboricola pv. pruni	0	100%	LC388645.1
		Uncultured Xanthomonas spp.	0	100%	KX456334.1
		Xanthomonas campestris	0	100%	MF285891.1
		Xanthomonas hortorum pv. vitians	0	100%	MG262373.1
		Xanthomonas spp.	0	100%	KY446031.1
		Xanthomonas gardneri	0	100%	CP018731.1
		Xanthomonas vesicatoria	0	100%	CP018725.1
		Xanthomonas campestris pv. hederae	0	100%	KU518243.1
		Xanthomonas arboricola pv. juglandis	0	100%	CP012251.1
		Xanthomonas campestris pv. zinniae	0	100%	KP731996.1
		Xanthomonadaceae bacterium	0	100%	KM187230.1
		Xanthomonas oryzae pv. oryzicola	0	100%	CP011962.1
		Uncultured bacterium	0	100%	KF094329.1
	C1	*Rhizobium fabae*	0	100%	KY038069.1

Table 3.5a continued

		Rhizobium spp.	0	100%	KU947328.1
		Rhizobium lentis	0	100%	NR_137243.1
		Rhizobium phaseoli	0	100%	KU862348.1
		Rhizobium leguminosarum	0	100%	FJ715815.1
		Rhizobium etli	0	100%	EU637928.1
	C3	*Uncultured gamma proteobacterium*	0	99%	MH478520.1
		Stenotrophomonas spp.	0	99%	MH465193.1
		Gammaproteobacteria bacterium	0	99%	MH362776.1
		[Pseudomonas] geniculata	0	99%	KJ452162.2
		Xanthomonas spp.	0	99%	MF574216.1
		Stenotrophomonas maltophilia	0	99%	KU726258.1
		Staphylococcus succinus	0	99%	KJ534522.1
		Xanthomonas oryzae	0	99%	KF822671.1
		Pseudomonas geniculata	0	99%	KC247683.1
		Uncultured bacterium	0	99%	JX001217.1
		Uncultured Steroidobacter spp.	0	99%	HQ674839.1
		Pseudomonas spp.	0	99%	FJ233849.1
		Uncultured gamma proteobacterium	0	99%	EU979078.1
		Xanthomonas retroflexus	0	99%	AY841369.1
	C8	*Xanthomonas campestris pv. campestris*	0	99%	MG149779.1
		Xanthomonas arboricola pv. pruni	0	99%	LC388645.1
		Xanthomonas spp. *strain*	0	99%	MH470420.1
		Uncultured Xanthomonas spp. *clone*	0	99%	KX456334.1
		Xanthomonas campestris	0	99%	MF285891.1
		Xanthomonas campestris pv. vitians	0	99%	MH165478.1
		Xanthomonas hortorum	0	99%	CP016878.1

Table 3.5a continued

		Xanthomonas axonopodis	0	99%	MF525813.1
		Xanthomonas gardneri	0	99%	CP018731.1
		Xanthomonas vesicatoria	0	99%	CP018725.1
		Xanthomonas arboricola pv. corylina	0	99%	KP402165.1
		Xanthomonas campestris pv. hederae	0	99%	KT862774.1
		Xanthomonas arboricola pv. juglandis	0	99%	CP012251.1
		Xanthomonas campestris pv. zinniae	0	99%	KP731996.1
		Xanthomonas oryzae pv. oryzicola	0	99%	CP011962.1
	C9	*Bacillus* spp. *strain*	0	100%	KX570915.1
	C10	*Xanthomonas campestris*	0	99%	JQ698512.1
	C4	*Bacterium strain*	0	100%	KY427680.1
		Bacillus aryabhattai	0	100%	MG593997.1
		Bacillus megaterium	0	100%	MG593993.1
		Bacillus spp.	0	100%	MG593988.1
	C7	*Xanthomonas arboricola pv. pruni*	0	100%	LC388644.1
		Xanthomonas campestris strain	0	100%	MG597200.1
		Xanthomonas spp.	0	100%	KM252981.1
		Xanthomonas campestris pv. campestris	0	100%	KP182149.1
		Xanthomonas campestris pv. hederae	0	100%	KU518243.1

b)

Treatment	Sample code	*Closest strain on NCBI data base*	E value	Identity	Accession # of closest sppecies
Organic	1	*Epicoccum nigrum*	0	100%	MF993434.1
		Paecilomyces tenuis	0	100%	MH027202.1
		Fungal spp. *strain*	0	100%	KU837871.1
		Uncultured fungus	0	100%	MF510615.1
		Epicoccum spp.	0	100%	MF972508.1

Table 3.5 b) continued

		Ascochyta spp.	0	100%	MF136540.1
		Dothideomycetes spp.	0	100%	KX909007.1
		Uncultured Ascomycota	0	100%	EU490049.1
		Uncultured soil fungus	0	100%	DQ420956.1
		Uncultured Epicoccum	0	100%	KC785551.1
Organic	6	*Colletotrichum coccodes*	0	99%	JX294026.1
Organic	7	*Uncultured Engyodontium*	0	100%	KF768338.1
		Parengyodontium album	0	100%	LC092887.1
Organic	5	*Plectosphaerella cucumerina*	0	100%	MH250007.1
		Verticillium dahliae	0	100%	KY704087.1
		Trichurus spiralis	0	100%	KY365579.1
		Uncultured Phyllachoraceae	0	100%	EU754975.1
		Uncultured endophytic	0	100%	EF505612.1
		Uncultured endophytic	0	100%	EF505612.1
		Fungal spp.	0	100%	KF212305.1
		Colletotrichum pisi	0	100%	GU934514.1
		Colletotrichum pisi	0	100%	EU400150.1
Conventional	4	*Cladosporium* spp. *isolate*	0	100%	MF154612.1
		Cladosporium cladosporioides	0	100%	MF422160.1
		Cladosporium angustisporum	0	100%	MF422159.1
		Fungal spp.	0	100%	MG761093.1
		Cladosporium westerdijkieae	0	100%	MF473314.1
		Cladosporium vicinum	0	100%	MF473312.1
		Cladosporium uwebrauniana	0	100%	MF473307.1
		Cladosporium subuliforme	0	100%	MF473277.1
		Cladosporium pseudocladosporioides	0	100%	MF473221.1

Table 3.5 b) continued

		Cladosporium lycoperdinum	0	100%	MF473140.1
		Cladosporium inversicolor	0	100%	MF473125.1
		Cladosporium halotolerans	0	100%	MF473012.1
		Cladosporium funiculosum	0	100%	MF472984.1
		Cladosporium europaeum	0	100%	MF472971.1
		Cladosporium delicatulum	0	100%	MF472954.1
		Cladosporium anthropophilum	0	100%	MF472923.1
		Uncultured fungus	0	100%	KX516507.1
Organic/Conventional	3	*Dothideomycetes* spp.	0	99%	KX641948.1
		Cladosporium spp.	0	99%	KX641947.1
		Cladosporium cladosporioides	0	99%	KX067796.1

Table 3.6 In vitro screening of endophytes isolates antagonistic ability against *Alternaria dauci*. Colonies diameter of *Alternaria dauci* in different treatments recorded on P.D.A ± Error stander error mean (n=4 for bacteria & n=3 for fungi). Experiment was repeated twice. a1, a2) bacterial endophytes & b) fungal endophytes. Different letters indicate significantly different treatments when treatments means were compared using each pair student's test comparison with P value < 0.05.

a1)

Treatment	Colony Diameter (cm)
Control	8±0 a
CE3 Conventional	7.2±0.029 cd
CE4 Conventional	7.2±0.089 cd
CE6 Conventional	7.39±0.05 bc
CE7 Conventional	7.15±0.12 d
CE8 Conventional	7.14±0.08 d
CE9 Conventional	7.05±0.02 d
CE10 Conventional	4.6±0.04 e
CE11 Conventional	7.56±0.13 b

a2)

Treatment	Colony Diameter (cm)
Control	8±0 a
CE2 Organic	2.4±0.02 i
CE3 Organic	7.05±0.05 cd
CE4 Organic	2.74±0.09 h
CE5 Organic	6.89±0.06 d
CE6 Organic	7.013±0.09 d
CE7 Organic	2.79±0.06 h
CE8 Organic	7.4±0.07 b
CE9 Organic	3.85±0.03 f
CE10 Organic	4.25±0.05 e
CE11 Organic	7.01±0.14 d
CE12 Organic	3.08±0.03 g
CE13 Organic	2.39±0.10 i
CE14 Organic	1.69±0.05 j
CE15 Organic	7.28±0.21 bc

b)

Treatment	Colony Diameter (cm)
Control	8 ± 0 a
CE1 Organic	3.65 ± 0.03 e
CE5 Organic	4.45± 0 d
CE6 Organic	5.05±0.03 c
CE7 Organic	4.93± 0.03 c
CE3 Both	2.89 ± 0.04 f
CE4 Conventional	5.33 ± 0 .09 b

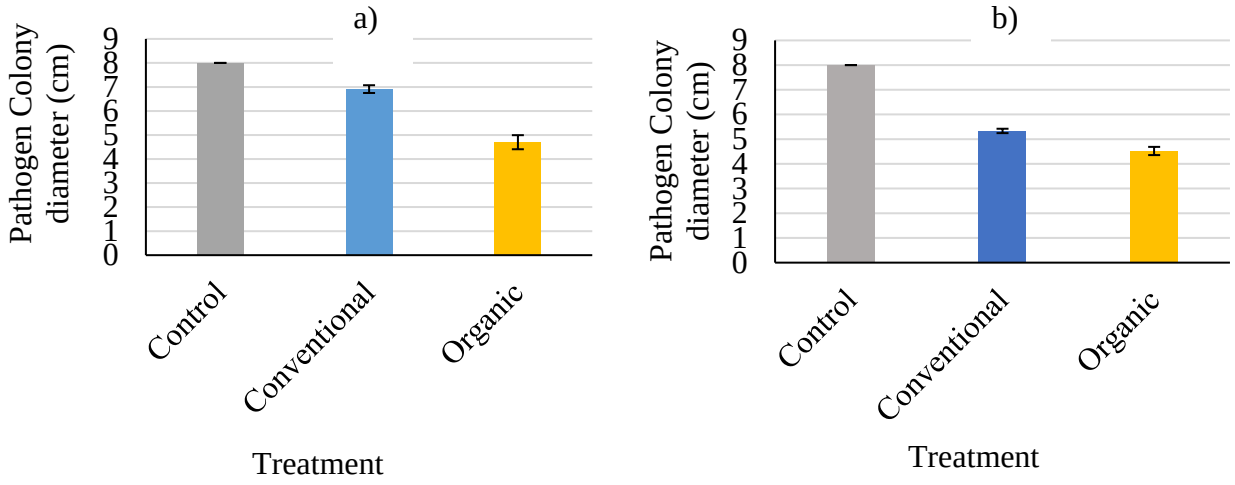


Figure 3.4 In vitro screening of endophytes isolates antagonistic ability against Alternaria dauci. Colonies diameter of *Alternaria dauci* of different treatments were recorded on P.D.A media when culture growth of *Alternaria dauci* in control treatment cover the whole plate. Error bars indicates stander error mean (n=4 for bacteria & n=3 for fungi). Experiment was repeated twice. (a) Bacterial endophytes & (b) fungal endophytes isolated from carrot grown under organic vs. conventional system. Different letters represent significantly different treatments when treatments means were compared using each pair student's test comparison with P value < 0.05

Table 3.7 Identification of selected endophytes isolates used for plant growth promoting screening under greenhouse condition

Sample code	*Closest strain on NCBI data base*	E value	Identity	Accession # of closest species
CE1	*Pseudomonas* spp.	0	100%	KX953866.1
	Pseudomonas brassicacearum	0	100%	MG461459.1
CE2	*Uncultured Pseudomonadaceae*	0	99%	MF374349.1
	Pseudomonas fluorescens	0	99%	CP025542.1
	Pseudomonas spp. *strain*	0	99%	KX758046.1
	Pseudomonas brassicacearum	0	99%	KP762561.1
	Pseudomonas brassicacearum subsp. neoaurantiaca	0	99%	KP762555.1
	Endophytic bacterium	0	99%	JF901350.1
CE3	*Pseudomonas* spp	0	97%	CP015225.1
CE5	*Xanthomonas arboricola pv. pruni*	0	100%	LC388644.1
	Xanthomonas campestris	0	100%	MG597200.1
	Xanthomonas campestris pv. hederae	0	100%	KU518243.1

Table 3.7 continued

	Xanthomonas arboricola	0	100%	KP340804.1
	Xanthomonas campestris pv. campestris	0	100%	KP182149.1
	Xanthomonas spp.	0	100%	KM252981.1
CE9	*Paenibacillus tundrae*	0	100%	MF101179.1
	Paenibacillus spp.	0	100%	HF954523.1
	Paenibacillus amylolyticus	0	100%	HQ284944.1
F1	*P. fluorescence* isolate Q2-87			
F2	*P. fluorescence* CHA0			
CE All	Combination of isolate 1, 2, 5, 9, O9, O12 and O13			

Table 3.8 The effect of carrot seed treatment with bacterial endophytes on the percentage of carrot germination. Data represents the mean of treated seed germination percentage recorded 5 days post planting using two carrot genotypes (Napoli & Red core) ± stander error mean with (n=20). Different letters indicate significantly different treatments when treatments means were compared using each pair student's test comparison with P value < 0.05

	Carrot genotype	
Endophyte treatment	Napoli	Read core
CE 1	60 ± 11.24 bcd	35 ± 10.94 a
CE 2	65 ± 10.94 abc	25 ± 9.93 a
CE 3	30 ± 10.51 e	45 ± 11.41 a
CE 5	35 ± 10.94 de	31.58 ± 10.96 a
CE 9	55 ± 11.41 cde	30 ± 10.51a
CE All	65 ± 10.94 abc	50 ± 11.47 a
(-) Control	85 ± 8.19 ab	40 ± 11.24 a
(+) Control F1	65 ± 10.94 abc	30 ± 10.51 a
(+) Control F2	90 ± 6.88 a	45 ± 11.41 a

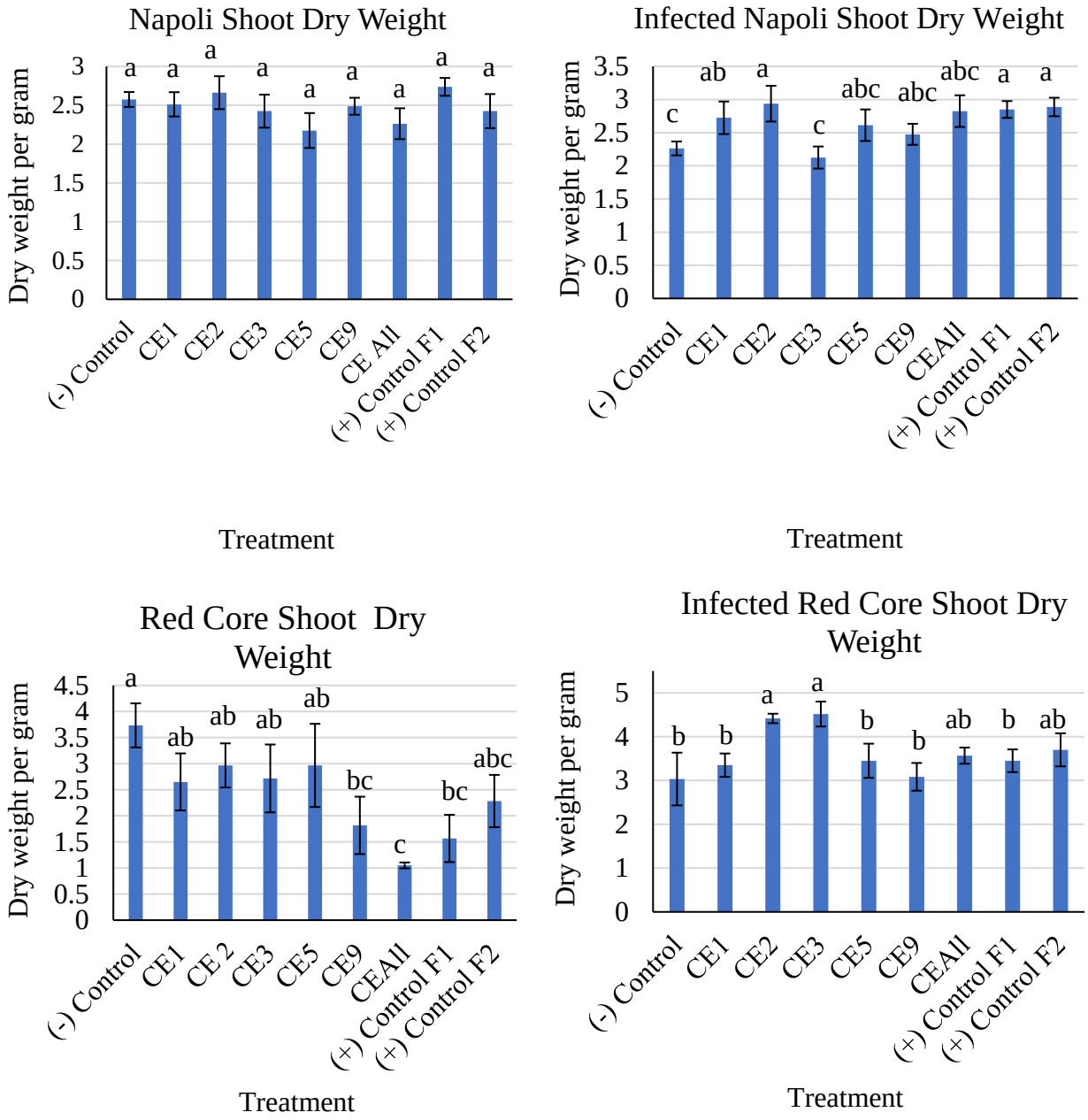


Figure 3.5 The effect of carrot seed treatment with bacterial endophytes on shoot dry weight of infected and none infected Red Core and Napoli plants 60 days after planting. Data represents shoot dry weight mean of each treatment ± stander error mean with (n=6 and n=8 for Red Core and Napoli respectively). Different letters indicate significantly different treatments when treatments means were compared using each pair student's test comparison with P value < 0.05

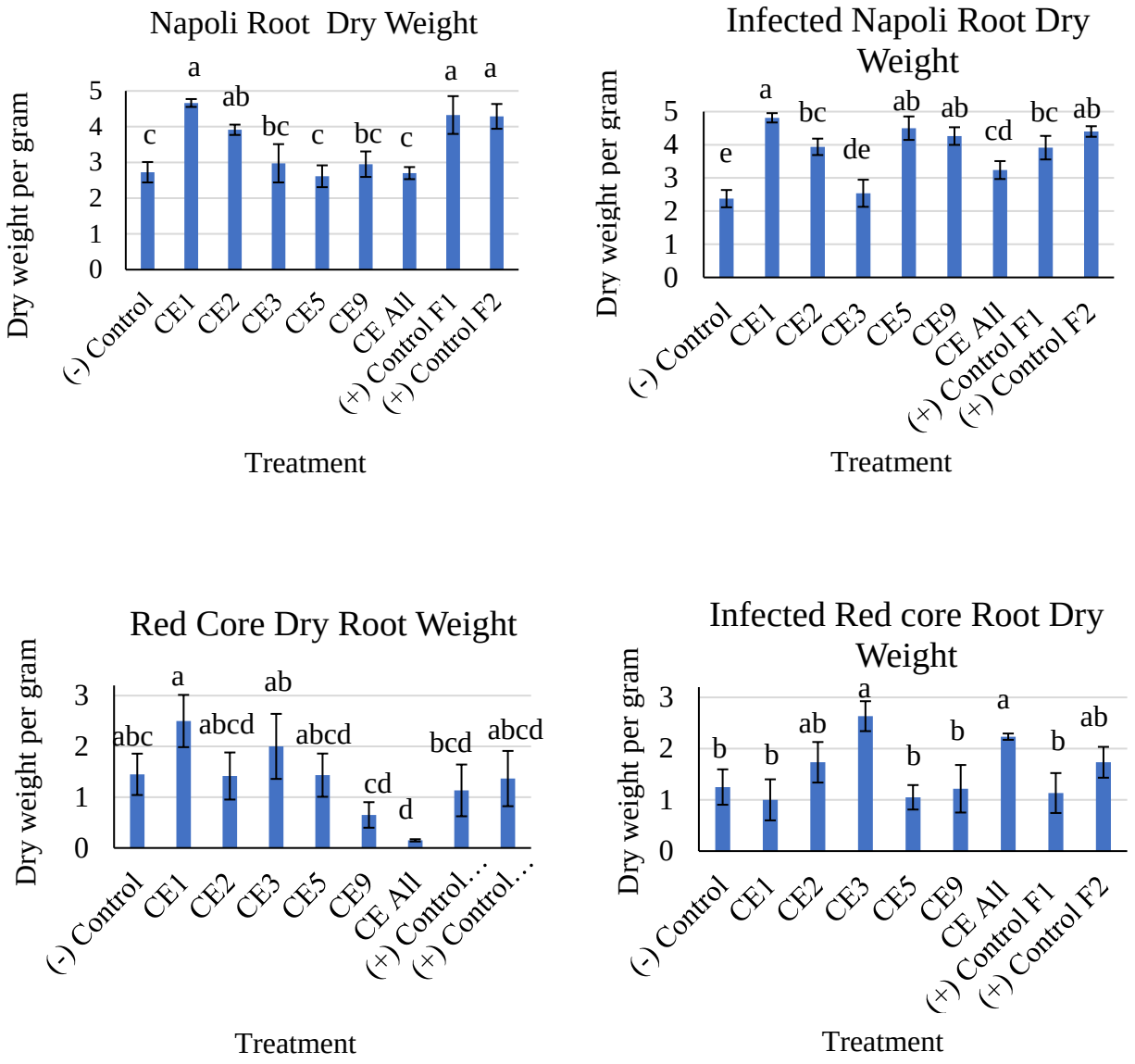


Figure 3.6 The effect of carrot seed treatment with bacterial endophytes on root dry weight of infected and none infected Red Core and Napoli plants 60 days after planting. Data represents root dry weight mean of each treatment ± stander error mean with (n=6 and n=8 for Red Core and Napoli respectively). Different letters indicate significantly different treatments when treatments means were compared using each pair student's test comparison with P value < 0.05.

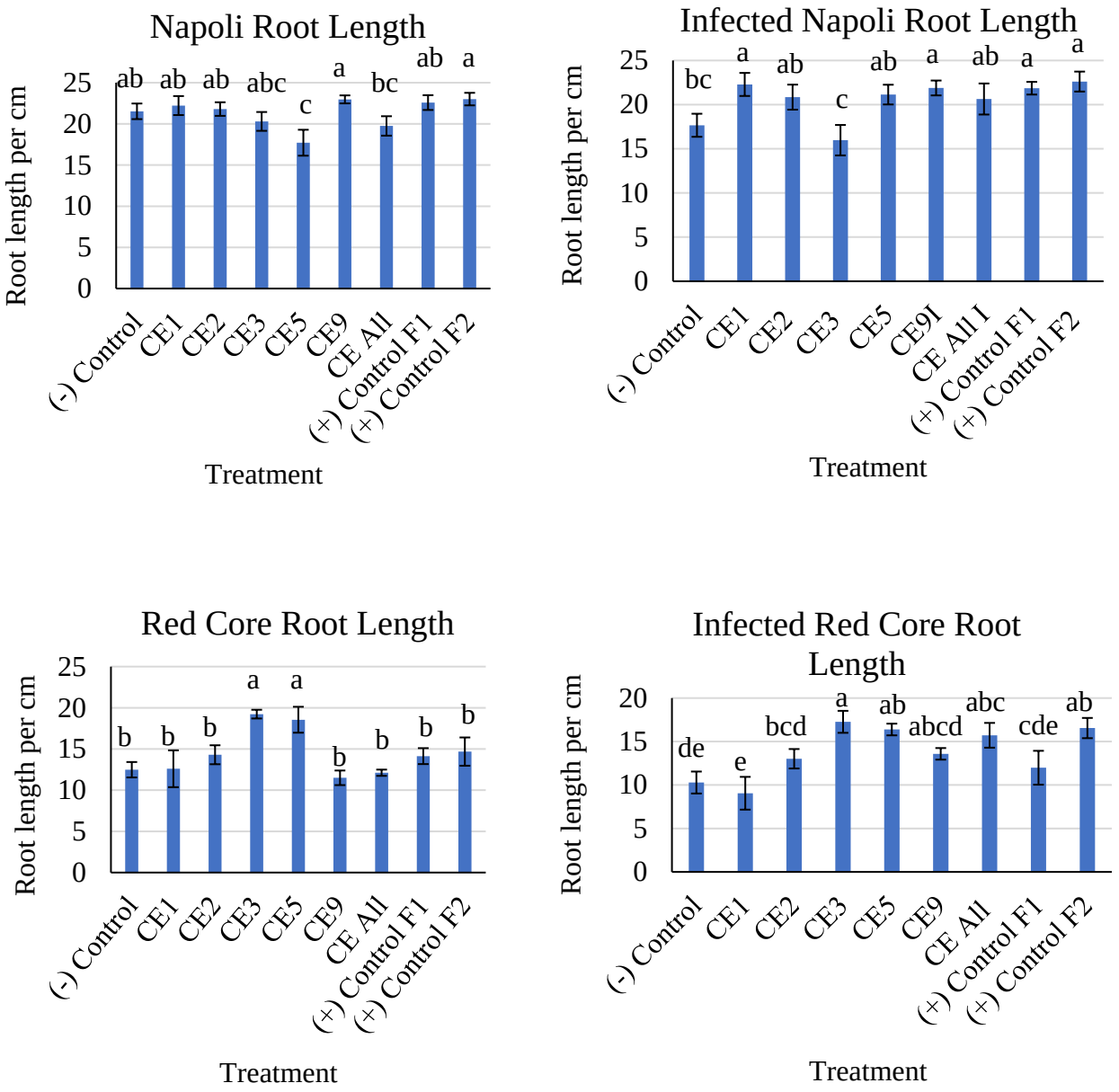


Figure 3.7 The effect of carrot seed treatment with bacterial endophytes on root length of infected and none infected Red Core and Napoli plants 60 days after planting. Data represents root dry weight mean of each treatment ± stander error mean with (n=6 and n=8 for Red Core and Napoli respectively). Different letters indicate significantly different treatments when treatments means were compared using each pair student's test comparison with P value < 0.05.

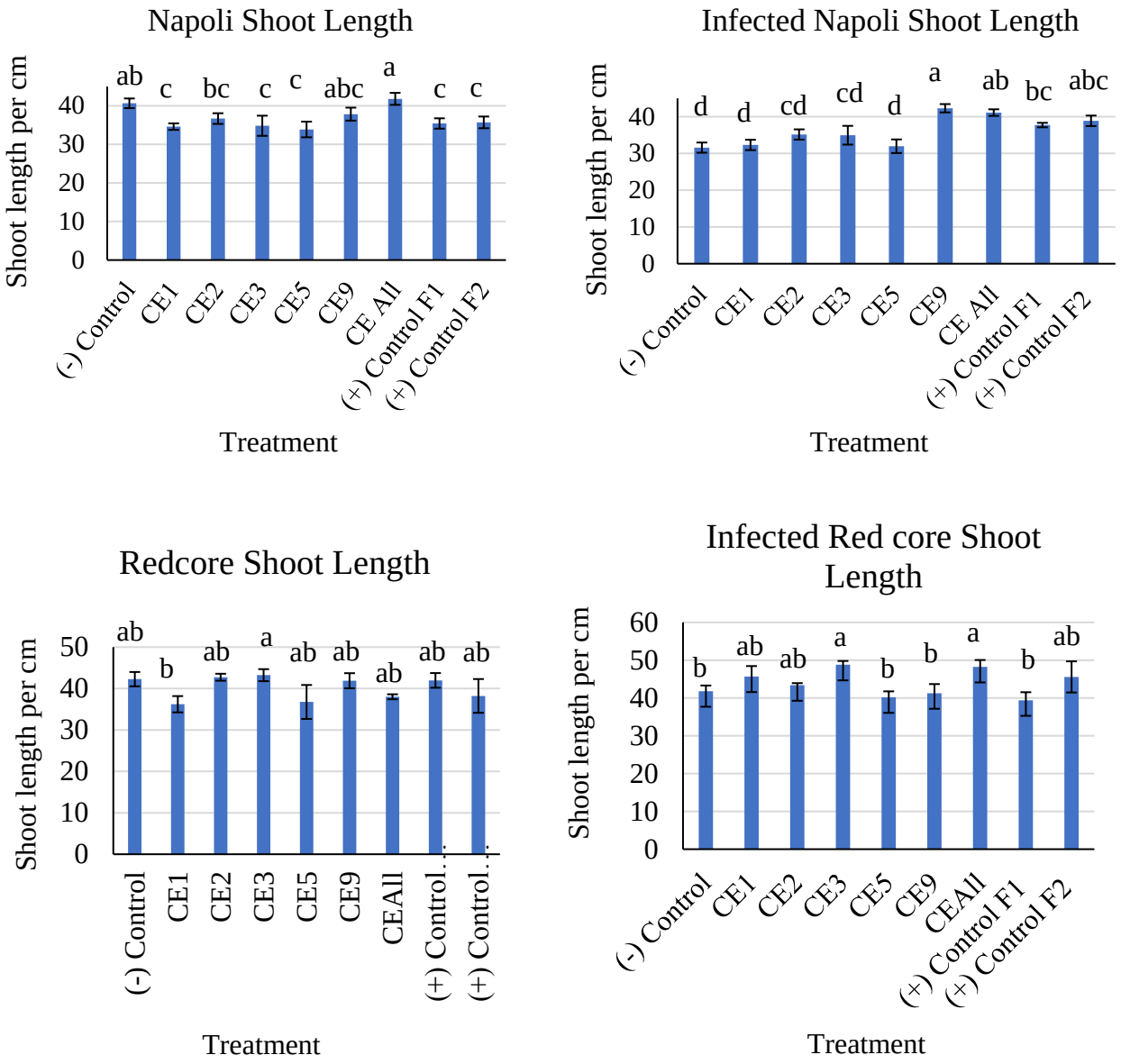


Figure 3.8 The effect of carrot seed treatment with bacterial endophytes on shoot length of infected and none infected Red Core and Napoli plants 60 days after planting. Data represents root dry weight mean of each treatment ± stander error mean with (n=6 and n=8 for Red Core and Napoli respectively). Different letters indicate significantly different treatments when treatments means were compared using each pair student's test comparison with P value < 0.05

CHAPTER 4. WHAT'S HIDING IN YOUR CARROTS? A NEW LOOK AT FUNGAL ENDOPHYTE COMMUNITIES IN TAPROOTS USING NEXT-GENERATION SEQUENCING

4.1 Introduction

It has recently become clear that plants are colonized by an abundant and diverse assortment of microorganisms. However, the exact function of these microbes, as well as factors affecting their composition and assembly, remain key unawnswered questions in plant microbial ecology. Because plants are sessile organisms, researchers have theorized that they specifically recruit and support microbes to help buffer themselves from environmental challenges (Bever, Dickie et al. 2010, Lareen, Burton et al. 2016). In particular, endophytes, which are microbes that spend at least part of their life cycle living inside plants, are suspected to play a crucial role in these processes. This is because endophytes colonize a 'restricted area' (Stone, Bacon et al. 2000), implying that plants tolerate their presence for a specific purpose. In the past, studying these plant-microbial relationships was challenging because of limitations associated with isolating and culturing endophytic microbes. However, the development of new high-throughput sequencing technologies has made it possible to overcome these limitations and begin to elucidate the identity and potential functional role of endophytic microbes in plants. Recent studies using these techniques indicate that the composition of endophytic microbial communities differ markedly from the composition of bulk and rhizosphere soils (Knief, Delmotte et al. 2012, HUANG 2018). This suggests that only a subset of microbial taxa is able to inhabit this unique ecological niche, and endophytes likely have specific roles in plant health.

Endophytes have been isolated from every plant studied on earth to date (Strobel 2018). These microorganisms inhabit a wide variety of different plant parts but do not cause any visible disease symptoms or obvious benefits. Consequently, in the past it was assumed that these microbes were simply 'latent saprotrophs' or 'secondary pathogens' waiting for plants to senesce or succumb to more virulent pathogens (Busby, Ridout et al. 2016). But now it is clear that endophytes can play important roles in plant fitness, such as helping plants acquire nutrients and withstand abiotic stress (Bacon 1993). Moreover, many fungal endophytes have potential to help defend plants against pests (Conn, Walker et al. 2008, Gómez-Lama Cabanás, Schilirò et al. 2014), so there is much interest in learning how to manage these organisms for use in biocontrol. In a

recent review of 85 studies investigating relationships between fungal endophytes and plant pathogens, Busby et al. (2016) concluded that antagonism is generally the most commonly reported ecological interaction, though this relationship could be context dependent. For example, antagonistic activities may occur only in environments where endophytic fungi confer a fitness advantage, such as those characterized by elevated disease pressure. Thus, before these microbes can be deployed in biocontrol strategies, additional studies conducted in real-world environments are needed to identify factors governing the assembly and activity of endophytes within plants.

Plant species is one factor that is well known to have a potential effect on endophyte community composition (Adams and Kloepper 2002). However, most of the recent studies using next generation sequencing to characterize fungal endophyte communities have been conducted using model crops such as Arabidopsis and Medicago (Bulgarelli, Rott et al. 2012, Lundberg, Lebeis et al. 2012, Pini, Frascella et al. 2012, Bodenhausen, Horton et al. 2013), or major agronomic crops such as rice, wheat, and maize (Siciliano, Theoret et al. 1998, Seghers, Wittebolle et al. 2004, Prakamhang, Minamisawa et al. 2009). In contrast, other important crops, including those in which endophytes could influence plant as well as human health, have been overlooked. For example, *Daucus carota L.* subsp. *Sativus* (Hoffm.) Arcang., or carrot, is one of the top ten most consumed vegetable crops worldwide (Simon, Freeman et al. 2008). Carrots are a good source of beta-carotene, fiber, Vitamin A and many other vitamins and minerals (Rubatzky, Quiros et al. 1999). They also could be considered a good model for studying root endophyte communities because they produce long-lived fleshy storage roots that are commonly subject to abiotic stress and predation by a variety of soil-borne and post-harvest pathogens. Moreover, endophytes can produce or stimulate production of secondary metabolites in plants (Kusari, Hertweck et al. 2012). Consequently, endophyte composition could play a role in the nutritional quality and organoleptic properties of this crop (Rodríguez, Barton et al. 2007). Finally, since humans commonly consume carrot taproots raw, the presence of endophytes could positively or negatively affect human health, depending on their composition.

In most studies investigating endophyte communities, soil has been identified as the most significant factor shaping endophyte composition within plants (Lundberg, Lebeis et al. 2012). This is particularly true for fungi, possibly because of their greater dispersal limitations in comparison to bacteria (Coleman-Derr, Desgarennes et al. 2016). Agricultural management practices are known to dramatically alter soil properties, and this alteration is likely to affect the

composition and activity of fungal endophytes. For example, organically managed cropping systems often have greater microbial diversity and activity than their conventionally managed counterparts (Reeve, Hoagland et al. 2016). Greater soil quality in organic systems has been correlated with increased potential to withstand biotic stress (Lotter, Seidel et al. 2003), reduced pathogen incidence and severity (Liu, Tu et al. 2007, van Bruggen, Sharma et al. 2015), and greater nutritional quality of vegetables (Wszelaki, Delwiche et al. 2005, Rodriguez, White Jr et al. 2009), among other plant traits. Fungal endophytes have previously been demonstrated to affect such traits (Rodriguez, White Jr et al. 2009), though direct relationships between soil management practices, endophyte community structure, and plant characteristics remain to be determined.

While not as significant as soil, another factor that can cause differences in the structure of root microbiomes is plant genotype (Wagner, Lundberg et al. 2016). Such differences could be related to variations in the abundance and composition of plant root exudates, which are known to play a key role in signaling and supporting colonization by root-associated microbes (Compant, Clément et al. 2010). They also could be due to differences in the chemical composition of roots. For example, the presence of anthocyanins has been shown to produce antifungal activity in plants (Zhang, Li et al. 2013, Tellez, Rojas et al. 2016), indicating that the presence of these compounds could also affect endophyte community composition. Composition of root endophyte communities also has been shown to differ between genotypes that are resistant and susceptible to plant pathogens, with cultured isolates from the resistant varieties capable of promoting plant growth and suppressing disease activities (Martin, Gazis et al. 2015, Upreti and Thomas 2015). This suggests that endophytes could play a role in pathogen suppression and that plant breeders have been inadvertently selecting for these communities while developing resistant genotypes. If this proves to be correct, then it may be possible for plant breeders to begin to actively select for such beneficial root-microbial relationships (Wagner, Lundberg et al. 2016), though additional research is needed to determine how the environment plays a role in these interactions.

The objective of this study was to determine how management system (conventional/organic), carrot genotype, and the interaction of these two factors influence endophyte fungal communities in carrot taproots. We predicted the following: 1) endophyte communities would be more abundant and diverse in the organic system due to greater availability of labile organic matter pools supporting greater abundance, diversity, and activity of soil microbes, and the absence of broad spectrum pesticides that could further alter soil microbial communities

and cause plants to allocate more resources toward supporting biocontrol activity in the absence of chemical controls; 2) carrot genotype would host distinctive fungal endophyte communities due to differences in their genetic background, the composition of chemical compounds (ie. anthocyanins) in taproots, and resistance to soil-borne pathogens; and 3) the resistant genotype would be most responsive to the management system due to differences in the abundance of antagonists present in soil and the need to recruit them to aide in pathogen suppression. To test these hypotheses, the composition of fungal endophyte communities in carrot taproots collected from field trials were identified following amplification, high throughput sequencing, analysis of ITS fragments using Illumina Miseq (Shi, Yang et al. 2014), and analysis using the Quantitative Insights into the Microbial Ecology (QIIME) (Caporaso, Kuczynski et al. 2010) bioinformatics pipeline. In addition, an indicator species analysis (Hill, Bunce et al. 1975) was used to identify fungal taxa associated with different management regimes and carrot genotypes. To our knowledge, this is the first study to employ high-throughput amplicon sequencing to quantify endophyte fungal community structure in carrot taproots.

4.2 Material and methods

4.2.1 Field treatment and plant sample collection

This experiment was performed during summer 2015 at Purdue's Meigs Horticulture Research Farm near Lafayette, IN, using three experimental carrot genotypes (E0191, E0252 and E3999). These genotypes were selected based on their broad genetic background, differences in taproot morphological and physiological characteristics, and susceptibility (E0191), moderate resistance (E0252) and resistance (E3999) to pathogenic nematodes. Carrot plants were grown in plots that had been managed using organic or conventional practices for the past ten years, in a spilt plot design with three replications for each management system. Both systems were planted with tomato (*Solanum lycopersicum*) in the previous year. A winter cover crop mixture containing winter rye (*Secale cereale L.*), hairy vetch (*Vicia villosa*), winter pea (*Pisum sativum*), annual rye (*Lolium perenne*), and timothy grass (*Phleum pratense*) (Cloverland Seed, Millersburg, OH) was sown in the organic plots during autumn 2014, mown and soil incorporated one month prior to carrot planting in spring 2015. The target fertilizer rate for both systems was 134.5, 180 and 224 kg ha^{-1} of N, P and K respectively. In the organic system, Re-vita Pro Compost (Ohio Earth Foods,

Hartville, OH) was applied at a rate of 5,380 kg ha^{-1} to meet fertility needs, assuming 50% of the nutrients would be available for plant uptake in the year of application. In the conventional system, diammonium phosphate (18-46-0) and potash (0-0-60) was applied to meet fertility needs. Carrot seeds (provided by Dr. Simon, USDA-ARS) were planted in mid-May, on raised beds spaced 1.8 m apart, in 1.0 m plots at a rate targeting 60 plants m^{-1} given previously determined germination rates, to a depth of 10 mm. A pre-emergent herbicide (Prowl H2O, BASF Corporation) was applied after planting in the conventional plots, and all organic plots were hand weeded as needed. No additional pesticides were applied in either crop management system.

At harvest (110 days after seeding), two randomly selected healthy carrot taproots were collected from each genotype in each field replicate plot and transferred to the lab in a cooler on ice for processing. Roots were rinsed thoroughly with tap water, then surface disinfected by soaking in 5.25% bleach for 3 minutes, followed by soaking in 3% peroxide solution for 3 minutes, and finally washing with sterilized water supplemented with 1% of tween (Surette et al., 2003). To confirm surface disinfection of the carrot taproots, 200µl from the last wash solution was cultured on dextrose-peptone and 1/5th PDA fungal media (Corry et al., 2011; Reasoner and Geldreich, 1985), and incubated at 27 °C to detect microbial growth. Two (15 mm) carrot cylinders from each taproot sample were obtained using a sterilized core borer, and the four cores from each field replicate were pooled for analysis. Carrot core samples were lyophilized using a lyophilizer (LABCONCO, Kansas City, U.S.A) and stored at -80C for fungal endophyte community DNA extraction.

4.2.2 Fungal endophyte DNA extraction, ITS amplification and sequencing

Endophyte community DNA was extracted in duplicate from each lyophilized carrot root sample using Qiagen DNeasy Plant Mini Kits (Qiagen, U.S.A) following the manufacturer's protocol and diluted using 100 µl of elution buffer. The two lab replicates were pooled, and DNA was quantified using a Qubit Fluorometer 2.0 and dsDNA HS Assay Kit (ThermoFisher Scientific, U.S.A.) and normalized to 1 ng/µl prior to ITS amplification. Fungal endophyte community ITS library construction was carried out in two steps. First, the ITS1 region was amplified using the universal primers ITS1F forward primer 5 ′ CTTGGTCATTTAGAGGAAGTAA- 3′ (Gardes and Bruns, 1993) and ITS2 reverse primer 5′-GCTGCGTTCTTCATCGATGC- 3′ (White et al., 1990)

modified to contain an adapter region for sequencing on the Illumina MiSeq platform, in triplicate reactions for each sample. Each 25-µl PCR reaction mixture contained 3µl of DNA template, 0.5 µl (100 mM) of each primer, 12.5 µl GoTaq® colorless Master Mix (Promega, Wisconsin, U.S.A) and 8.5 µl of nuclease free water (Promega, Wisconsin, U.S.A.). Each PCR reaction was performed using a Bio-Rad T100™ Thermal Cycler (BioRad, California, U.S.A) with the following conditions: initial denaturing using 1 cycle at 95ºC for 2 minutes, 40 cycles of the following (denaturing step 95ºC for 30 seconds, annealing step 55ºC for 30 seconds, and extension step 72ºC for 1 minute), and a final extension step of 72ºC for 10 minutes. Detection of PCR-amplified products was performed with electrophoresis on a 0.7% (wt. /vol.) agarose gel stained with Bullseye DNA Safe Stain (MIDSCI, U.S.A.). A 100bp ladder (New England bio lab, U.S.A) was also run in parallel to approximate PCR product band sizing. Presence of DNA bands stained with DNA Safe Stain (MIDSCI, U.S.A.) were visualized after exposure of the gel to ultraviolet (UV) light. PCR replicate products of the same samples were pooled and cleaned using Ultraclean ® PCR Clean-Up Kits (MO BIO, U.S.A) following the manufacturers protocol. Cleaned PCR products were subjected to a second PCR reaction, with specific tag encoded primers for each sample. The same thermocycling conditions described above were used, with the exception of 5 amplification cycles instead of 35. Again, all PCR products were confirmed by electrophoresis as described above. Final PCR product concentration was quantified and adjusted using the Qubit Fluorometer 2.0 as described above. Samples were submitted in equimolar concentrations (20 ng) to the Purdue genomic facility for sequencing of ITS libraries. A TruSeq DNA LT Sample Prep Kit (Illumina, San Diego, CA) was used to construct paired-end (2 X 250 bp) sequencing libraries. MiSeq Reagent Kit v2 (Illumina, San Diego, CA) was used to perform amplicon sequencing on a MiSeq Desktop Sequencer (San Diego, CA).

4.2.3 Processing of Illumina derived datasets

Raw fastq Illumina files were demultiplexed, quality-filtered, and converted to fasta format for use as an input into QIIME (Version 1.9.1) (Caporaso et al., 2010). Phred quality reads with score <30 were discarded. Operational taxonomic unit (OTU) picking, taxonomic assignment, and construction of phylogenetic trees were carried out using QIIME's open-reference OTU picking module using the UCLUST method (Edgar, 2010). Reads were clustered against a reference fungal database (UNITE 97, 12_11 version) sequence collection, and reads that failed to hit the reference

were subsequently clustered de novo into operational taxonomic units (OTUs) at 97 %. All the samples were taken into account without any subsampling. The suppress_align_and_tree was passed as a parameter because the trees generated from ITS sequences are generally not phylogenetically informative. Only OTUs of fungal origin were considered for further analysis.

The QIIME module identify_chimeric_seqs.py was used to screen for chimeric sequences. To report the number of sequences per sample, the QIIME module biom summarize-table was used. To estimate within-sample species richness and evenness the alpha diversity script based on Faith's phylogenetic diversity index (Faith, 1992) was used. The alpha rarefraction script was used to generate alpha rarefaction plots for each sample and estimate species richness for a given number of sequences by the number of observed phylotypes and the Chao1 richness estimate (Chao, 1984). QIIME's filter scripts were used to retain OTU's where 25% of the samples in groups being compared have OTU's. Beta diversity estimates were calculated within QIIME using Bray-Curtis distances matrices and results were used to produce principle coordinate analysis (PCoA) plots (Vázquez-Baeza et al., 2013). Community differences within all samples of a group as well as between different groups were further assessed using QIIME's make distance boxplots script and t-tests for all pairs of boxplots, while community differences between groups were assessed using QIIME's compare_categories.py script and ADONIS (Anderson, 2001) and ANOSIM methods.

In order to evaluate differential abundance for specific OTUs between groups among the different comparisons, the phyloseq software package, implemented in Bioconductor, was used to provide a platform for statistical analysis and figure generation in R (Team, 2013). With the use of DESeq2, phyloseq assigned adjusted p-values for each OTU. Tables were generated in R for each comparison that included statistical information and taxonomic assignments for each OTU as well as raw OTU counts for the samples in the comparisons. To visualize how DESeq2 fit the OTU abundance data to its model, MA plots were generated (using alpha threshold values (or FDR) of 0.2). Additionally, 2D PCoA plots based on Euclidean (non-phylogeny based) distances were generated to illustrate community differences based solely on OTU abundances. Finally, for each comparison, phyloseq was used to generate ggplot2 summary plots of the significantly differentially abundant OTUs (using alpha threshold values (or FDR) of 0.2).

4.2.4 Field soil chemical and biological assays, yield and foliar pathogen infection data

Ten soil cores were randomly collected to a depth of 10 cm in each field rep just prior to carrot seeding in spring. The ten cores within each field rep were pooled and transferred to the laboratory on ice. After thoroughly mixing the cores from each replicate, a subsample of soil was air-dried before shipping to Midwest Labs (Omaha, NE) for a standard soil test according to common methods used in this region (Brown, 1998). Briefly, total organic matter was determined using loss of weight on ignition; available P was extracted as Weak Bray (readily available P) and Strong Bray (potentially available P) and analyzed calorimetrically; exchangeable potassium (K), calcium (Ca), and magnesium (Mg) were extracted with neutral ammonium acetate (1 N) and quantified by inductively coupled argon plasma–mass spectrometry detection; and base saturation and cation exchange capacity [mmol (+)·kg–1] were estimated from the results of exchangeable minerals (Brown, 1998). Another subsample was placed in the cooler at 4 °C until being air-dried overnight to conduct assays to estimate microbial activity and active soil carbon. Microbial activity was estimated using the hydrolysis of fluorescein diacetate (FDA) in soil slurries using a method optimized for soil (Green et al., 2006). Active C was quantified using the permanganate oxidizable carbon (POXC) technique (Weil et al., 2003). Finally, a subsampled was lyophilized and stored at -20, before being shipped overnight on dry ice to WARD lab (Grand Island, NE) for phospholipid fatty acid analysis (PLFA) using methods described in (Buyer and Sasser, 2012). At 60 and 190 days after seeding, the percentage of infection by foliar pathogens in each plot was estimated. Carrots were manually harvested 190 days post planting and the total number and weight of all taproots and aboveground foliage in each plot were recorded.

4.3 Results and Discussion

Aided by the development of new genomic tools, several studies have been conducted in recent years to characterize factors that affect the composition of plant root microbiomes. Some of these studies have included the endophytic component of plant roots because of the intimate relationships these microbes have with their plant hosts. However, most of these studies have either focused on bacterial endophytes only or been conducted using only a single plant genotype or management system (Andreote et al., 2010; Campisano et al., 2014; Van Overbeek and Van Elsas, 2008). Moreover, the few studies that did investigate the combined effects of plant genotype and

management system on root endophytes were conducted using model crops, grasses or other crops that are not directly consumed by humans (Wilberforce et al., 2003; Lundberg et al., 2012; Pancher et al., 2012). This study is the first to apply ITS Illumina sequencing to characterize the combined effects of management system and plant genotype on fungal endophyte communities in carrot, an important edible root crop. In total, 36 samples of carrot taproots representing three diverse genotypes grown under organic and conventional management regimes were characterized in this study. The three carrot genotypes evaluated in this study were chosen based on their broad genetic backgrounds, differences in phenotypic characteristics such as root color, and susceptibility to pathogenic nematodes (Table 2.1).

4.3.1 Abundance and quality of fungal endophyte sequences isolated from carrot taproots

Fungal endophyte community structure in the taproots of the carrot genotypes grown under the two management regimes were determined using Illumina sequencing of the ITS gene. After quality filtering, adapter trimming, and merging of Illumina reads, approximately 3,793,627 high-quality sequences were obtained and used as input into QIIME for analysis and comparison of fungal endophyte communities. Sequences clustered into 1,480 different fungal operational taxonomic units (OTUs) when grouped at the 97% genetic similarly level. Rarefaction curves (Fig. 4.1) indicated that only 38.5 % of fungal endophyte diversity present in carrot taproots was recovered by this surveying effort, implying that a greater sequencing depth would have yielded additional information. In addition, the presence of many unidentified and unassigned sequences in our data set indicates that the availability of a more widely referenced taxonomic annotation database (or even a combination of annotation databases), would be helpful in obtaining additional information about the taxa characterized in this study (Motooka, Fujimoto et al. 2017). Since the presence of unassigned and unidentified sequences is due to the absence of these species in existing fungal databases, as well as the presence of sequences that are uninformative such as sequences for uncultured microbes for which there is no other information (Lindahl, Nilsson et al. 2013). To overcome this challenge, additional research is needed.

4.3.2 Identification of fungal taxa present within carrot taproots

Consistent with other studies exploring root fungal endophyte communities (Gazis and Chaverri 2010, Rivera-Orduña, Suarez-Sanchez et al. 2011, Vieira, Hughes et al. 2011, Singh,

Sharma et al. 2017), carrot taproots in this study were dominated by microbes in the Ascomycota phyla (73.9%) (Fig. 4.2), further demonstrating that they are uniquely adapted to thrive in plant roots as endophytes. Other abundant phyla in carrot taproots belonged to the Basidiomycota (24.8%) and Chytridiomycota (<1%) families (Fig. 4.2). In contrast, no endophytic fungi representing the Zygomycota phylum were identified, which is consistent with results of Zhang and Yao (2015), who also did not observe any fungal taxa within this phylum in the roots of several *Alpine* spp. The absence of fungal sequences related to the Zygomycota phylum indicates that carrot taproots are not a sufficient habitat for these microbes. Endophytic microbes are known to have specific preferences for individual plant hosts and even specific host plant tissues, which makes them best adapted to unique ecological niches (Schulz, Boyle et al. 2007, Chen, Hu et al. 2011). Alternatively, the lack of taxa belonging to the Zygomycota phylum also could be related to the narrow spectrum amplified by the ITS primers used in our study and/or the low efficacy of our PCR amplification method (De Beeck, Lievens et al. 2014). The choice of ITS primers has previously been reported to influence the taxonomic diversity of samples analyzed using next generation sequencing (Nilsson, Ryberg et al. 2009). Future studies to help overcome such potential biases could include the use of a PCR-free amplification method, such as shotgun metagenomics (Aird, Ross et al. 2011).

At the level of genera, *Rhizoctonia* and *Fusarium* were predominant in carrot taproots, representing 19% and 13% of all endophyte communities identified (Fig. 4.3). The predominance of these two fungal genera is consistent with previous studies. For example, Gond, Verma et al. (2007) observed that *Fusarium* spp. had the highest colonization frequency in the bark, leaves, and roots of *Aegle marmelos*, and *Rhizoctonia* spp. were among the other most dominant taxa isolated from this plant. Similarly, one study (Gamboa and Bayman 2001) found that *Rhizoctonia* spp. were among the most dominant taxa in leaves of *Guarea guidonia* (*Meliaceae*) trees. Other taxa that were observed across all samples included *Ophiosphaerella* (5.4%), *Ceratobasidium* (3.6%), *Colletotrichum* and *Gibberella* (each at 0.4%), *Cladosporium* (0.3%), *Aspergillus* (0.2%), and *Cyphellophora*, *Thanatephorus*, *Alternaria* and *Plectosphaerella* (all at 0.1%). Finally, *Cercospora, Rhizopycnis* and *Phoma* were among twenty other genera observed with less than 0.1% relative abundance.

Many of the fungal taxa identified in carrot taproots have previously been observed in the roots of a variety of plant species, demonstrating that they are well adapted to survive within

internal plant tissues (Maciá-Vicente, Jansson et al. 2008, Tao, Liu et al. 2008, Naik, Shashikala et al. 2009, Márquez, Bills et al. 2010, Sun, Guo et al. 2011, Kleczewski, Bauer et al. 2012, Liu, Li et al. 2017, Pan, Su et al. 2017). The potential functional role of many of these fungal taxa is still unclear, though some studies have begun to unravel these complex plant-microbial relationships (Brader et al., 2017). For example, while certain members of the *Rhizoctonia*, *Ceratobasidium* and *Cyphellophora* genera commonly have been characterized as plant and animal pathogens (Baker 1970, Burpee, Sanders et al. 1980, Feng, Lu et al. 2014), this is not always the case. Like the results of this study, fungal endophytes assigned to these genera have been collected from healthy plants roots, and in some cases these genera have even been observed to promote plant growth. For example, several researchers have isolated *Ceratobasidium* and *Thanatephorus* (*Rhizoctonia*) genera from healthy orchid trees and noted their role as mycorrhizal fungi that can promote seed germination and growth of orchid (Orchidaceae) plants (Otero, Flanagan et al. 2007, Porras-Alfaro and Bayman 2007). Similarly, Liu, Li et al. (2017) isolated *Cyphellophora* from healthy non-mycorrhizal plant roots, supporting previous studies suggesting that members of this genera can promote plant growth and help plants resist biotic and biotic stress (Yuan, Zhang et al. 2010, Newsham 2011). Members of the *Cladosporium*, *Aspergillus* and *Thanatephorus* (*Rhizoctonia teleomorph*) genera are well known for their production of bioactive products including antibiotics and antifungal compounds against many phyto and human pathogens (Wang, Jiao et al. 2007, Li, Zhang et al. 2012, Sadrati, Daoud et al. 2013, Pan, Su et al. 2017). Finally, endophytic members of the *Ophiosphaerella* genera have been shown to solubilize calcium, aluminum and iron phosphate in both liquid and solid media (Spagnoletti, Tobar et al. 2017), which could have important implications in plant nutrition.

Future studies conducted under more controlled conditions that quantify both the composition and activity of endophytes within plants when they are subject to specific stresses are needed to help clarify the specific role of the endophytes identified in this study. In addition, to fully understand their potential role in carrots and other plant species, it will be important to isolate and culture these fungal communities to test for potential pathogenicity against carrots and other potential plant growth promoting properties.

4.3.3 Effect of crop management system on fungal endophytes in carrot taproots

To test our hypotheses that management system could influence fungal endophyte community structure, we calculated species richness (number of observed OTUs), estimated alpha diversity within samples (represented by the Shannon index H') (Table 4.1), and calculated and compared beta diversity (ADONIS) (Table 4.2) between the conventional and organic management systems. Results indicated that fungal endophyte richness was significantly affected by management system but that alpha diversity within these samples was not (Table 4.1). Management system significantly influenced endophyte beta diversity (Table 4.2). However, only a few individual fungal genera differed between management systems, with a greater abundance of one unidentified genera in the organic system and greater abundance of one unassigned fungal genera in the conventional system (Fig 4.3). This indicates that a few individual fungal taxa are highly impacted by these two management systems, which can affect the overall relative abundance of fungal taxa in these systems and possibly their functional roles.

To identify the specific fungal taxa that were responsible for differences among the management systems, we performed an indicator species analysis. Of the 1,480 fungal groups included in this analysis, 98.3% were not significantly different in relative abundance or frequency with respect to management system (Table 4.4), indicating that they are likely part of a core microbiome in carrot. In contrast, individual endophytic taxa belonging to the *Ascomycota*, *Basidiomycota* and *Chytridiomycota* phlya, which comprised 1.6% of all fungal taxa observed in the study, were specifically associated with one management practice (Table 4.4). Of these, 87.5% were significantly associated with organic management, while only one unidentified, unassigned and uncultured fungal genus was significantly associated with conventional management. At the level of genera, *Alternaria*, *Plectosphaerella*, *Rhizoctonia* and *Fusarium* were correlated with organic management.

The effect of management practices on endophyte community composition and abundance has been reported in many previous studies (Surette, Sturz et al. 2003, Pancher, Ceol et al. 2012). Such differences are likely related, at least in part, to the impacts of individual management practices on soil health. This is because endophyte communities often are a subset of rhizosphere microbial communities (Quadt-Hallmann, Hallmann et al. 1997, Quadt-Hallmann, Kloepper et al. 1997, Surette, Sturz et al. 2003), and any factors that would affect rhizosphere communities also would affect endophyte communities. Consistent with the results of soil analyses conducted in our

trial (Table 4.6 &4.7 & Fig. 4.4), soil health is often greater in organic relative to conventional farming systems (Reeves et al., 2016), and differences in soil health are consistently correlated with rhizosphere community composition (Bowen and Rovira 1999, Peters, Sturz et al. 2003, Chaparro, Sheflin et al. 2012, Bender, Wagg et al. 2016). The inclusion of soil-building practices such as planting cover crops or amending soil with composts is likely to be a major factor in improving soil health in organic farming systems. Both of these practices have been demonstrated to increase soil organic matter (Hoagland, Carpenter-Boggs et al. 2008, Rudisill, Bordelon et al. 2015, Reeve, Hoagland et al. 2016), which serves as the primary food and energy source for soil microbes. Moreover, cover crops provide a consistent source of labile carbon compounds to sustain microbes that thrive in plant roots when cash crops are not present (Rudisill, Bordelon et al. 2015). Differences in tillage practices also can affect soil and plant microbiomes by altering soil organic matter levels. For example, when comparing bacterial endophytes in carrot taproots grown in two fields that used the same crop rotation but differed in their tillage practices, Surette, Sturz et al. (2003) found greater populations of bacterial endophytes in taproots growing in the field employing minimum tillage.

Another factor that could be responsible for differences in the fungal endophyte communities observed in this trial in relation to the two management systems is the lower use of pesticides that often occurs in organic relative to conventional farming systems (Dalal, Henderson et al. 1991). For example, in a recent study investigating fungal endophyte community composition between two vineyards managed using an organic or integrated pest management approach, Pancher, Ceol et al. (2012) theorized that differences in endophyte communities were due to long-term use of synthetic fungicides in the integrated pest management system, which likely reduced the abundance and diversity of fungal taxa. Similarly, the authors of another study concluded that differences in agrochemical use between an organic and conventional management altered the soil as well as the endophyte communities within the roots of *Zea mays* (Seghers, Wittebolle et al. 2004).

The potential implications of the differences in endophytic fungal taxa observed between the two management systems are unclear at this time. However, as discussed above, it is possible that the endophytic fungi associated with the organic regime could play a role in promoting plant growth, for example, by reducing pathogen colonization via production of antimicrobial and

antifungal compounds with activity against a number of plant pathogens (Porras-Alfaro and Bayman 2007, Wang, Jiao et al. 2007, Rosa, Tabanca et al. 2012, Aimé, Alabouvette et al. 2013).

In addition, as discussed above, to determine whether this is the case, future studies will need to specifically track the activity of these endophytes when plants are under stress. Moreover, researchers should consider conducting studies that could tease apart specific factors, such as the inclusion of cover crops and the use of fungicides, to determine which practices are most influential in structuring fungal endophyte communities in carrot taproots.

4.3.4 Effect of the carrot genotype on fungal endophytes in taproots

In our study, carrot genotype did not affect overall fungal richness, alpha (Table 4.1), or beta diversity (Table 4.2) when genotypes were compared across management systems, though several individual fungal genera did differ among genotypes. In particular, unidentified and non-assigned genera, *Cladosporium, Thanatephorus, Rhizoctonia, Ceratobasidium, Aspergillus, Cyphellophora, Ophiosphaerella* differed among carrot genotypes. Specifically, there was a greater abundance of *Cladosporium, Thanatephorus, Rhizoctonia, Ceratobasidium and Aspergillus* in genotype E0191 when compared with genotype E3999, whereas the opposite occurred with *Cyphellophora* and *Ophiosphaerella* genera. Genotype E0191 also had a greater abundance of *Aspergillus* and *Ceratobasidium* than genotype E0252. In contrast, only a few unidentified and non-assigned genera were more abundant in genotype E0252 than in E3999 (Table 4.3).

Similar to the minimal effect of genotype on fungal endophyte community structure, the indicator species analysis indicated that only three out of the 1,480 fungal taxa observed in this study were correlated with an individual carrot genotype (Table 4.5). This included one uncultured fungus and one unidentified fungus that were uniquely associated with genotype E0252, and one fungal taxon related to the Corticiaceae family that was correlated with genotypes E0252 and E3999.

Differences in the relative abundance of endophytic species among plant genotypes are likely related to differences in the genetic composition of plants that influence key physiological processes. They also are likely related to preferences of individual endophytic microbial taxa for specific plant genotypes due to the presence of unique ecological niches within these genotypes (Adams and Kloepper 2002, Chen, Hu et al. 2011). More specifically, as discussed in the

introduction, differences in endophytic communities among genotypes could be due to factors such as the chemical composition of compounds like anthocyanins present in roots, as well as differences in resistance against soil borne pathogens (Adams and Kloepper 2002, Schulz and Boyle 2005, Schulz, Boyle et al. 2007). Differences in the composition of endophytes among plant genotypes could provide a direct fitness advantage, such as helping plants resist colonization by soil borne pathogens or acquiring nutrients under limited conditions.

The potential implications of the differences observed in this study in endophyte communities between carrot genotypes are unclear at this time, however, there are some interesting possibilities. For example, the greatest difference in fungal endophyte community composition was observed between E0252 and E3999, which are susceptible and resistant, respectively, to pathogenic nematodes that reside in soil (Table 2.1). This could indicate that by selecting for resistance to these nematodes, plant breeders may have inadvertently selected against the potential for carrot taproots to be colonized by some fungal endophytes. These two genotypes also differ in carrot taproot color, which is due to the presence of anthocyanins in E0252. Consequently, the greater abundance of several fungal taxa in E0252 relative to E3999 could be the result of the unique habitat provided by the presence of these chemical compounds. In addition, the endophytic microbes themselves could be contributing to the abundance of these compounds in carrot taproots. Finally, E0252 produced a significantly greater weight of taproots than E3999 in this study (Table 4.8), indicating that the presence of unique endophytes in E0252 could have contributed to the greater productivity observed in this genotype. Of course, studies will be needed to confirm these hypotheses, but they offer intriguing possibilities.

Although many studies now provide support for the potential role of plant genotype in shaping the endophyte communities associated with plants (Elamo, Helander et al. 1999, Adams and Kloepper 2002, Cordier, Robin et al. 2012, Bálint, Tiffin et al. 2013). Most of theses studies recorded their observation on endophytes communities of arial plant part not root system. Results of our study confirm that the effect of genotype is generally not as strong as soil factors. Which consistant with perious work done by Wagner, Lundberg et al. (2016) who observed that genetic control by of *Boechera stricta* (*Brassicaceae*) and Lundberg, Lebeis et al. (2012) found that host genetic control of Arabidopsis root microbiomes was weak compared to variation contributed by differences in soil type. The authors in this paper argued that the ability of plant host-genotype to control root microbiomes in previous studies could have been related to the inclusion of small

sample sizes and low-resolution phylotyping techniques, which can be associated with chimeric and other off-target sequences. However, in the present study we used high throughput Illumina sequencing of the ITS region, followed by QIIME to provide a high phylotyping resolution of fungal endophyte communities. In addition, no chimeric amplicons were detected during our analysis. Thus, we conclude that genotypes indeed play a potentially weaker role in shaping fungal endophyte communities in carrot taproots comparing to magment system.

4.3.5 Interactions between carrot genotype and management system

When comparing interactions between management system and carrot genotype, only genotype E3999 had marginal differences in overall taxonomic richness of fungal endophytes (Table 4.1) when grown in the two production systems. In addition, there were differences in the abundance among individual taxa in E0191 and E3999 (*Fig. 2 and supplements*). More specifically, within genotype E0191, levels of *Aspergillus, Ophiosphaerella, Rhizoctonia, Thanatephorus* and *Fusarium* were greater in carrots grown in organic compared to conventional management systems, whereas the opposite result was found with *Colletotrichum* and *Ceratobasidium*. Within genotype E3999, seven fungi including an uncultured Ascomycota, an uncultured fungus, and some non-assigned taxa were greater when grown under organic compared to conventional management, while the opposite was found for only one uncultured fungi and one non-assigned fungi. In contrast, no differences in individual fungal genera were detected in genotype E0252 when grown in the organic compared to conventional management system (Table 4.3).

When comparing differences among genotypes within each individual management system, there were differences in the abundance of some individual genera (Table 4.3). Specifically, under conventional management, a greater abundance of *Ophiosphaerella* and *Cladosporium* genera was present in genotype E0252 than in E0191, whereas the opposite was found for *Ceratobasidium*. *Ophiosphaerella* was more abundant in genotype E3999 than in either E0191 or E0252, along with a few other unidentified and non-assigned genera (Table 4.3). Under organic management, *Aspergillus* and *Rhizoctonia* were more abundant in genotype E0191 than in E3999, one unidentified genera had greater abundance in genotype E0191 than in E0252, and one non-assigned genus had greater abundance in genotype E0252 than in E3999 (Table 4.3).

These results provide further support for the potential of management regime and carrot genotype to alter fungal endophyte communities in carrot taproots. It also suggests that some genotypes are more plastic than others in their responses to the presence of different microbial taxa in bulk soil.

Interactions between plant genotype and management system have been reported in other studies, which observed differences in bacterial endophyte communities among three agronomically important grass species (*Dactylis glomerata L., Festuca rubra L., and Lolium perenne L.*) (Wemheuer, Wemheuer et al. 2016, Wemheuer, Kaiser et al. 2017). The authors suggested that differences in endophyte communities observed in these studies could be related to differences in the nutritional needs and physiological characteristics of these species, as well as differences in their tolerance to individual management practices such as pesticides or fertilizer amendments. As suggested above, this could indicate that some genotypes might be more plastic in their ability to recruit and support beneficial microbes when they are present in a system and needed for some particular function. For example, in our study we did observed change in abundance of *Fusarium* while recent studies have demonstrated that endophytic members of the *Fusarium* genus can reduce colonization of plant pathogens like *Fusarium oxysporium* in tomato and *Ustilago maydis* in maize plants. Endophytes accomplish this by priming plant defense responses in tomato and interfering in early disease development in maize plants (Lee, Pan et al. 2009, Aimé, Alabouvette et al. 2013). In addition, *Colletotrichum* sp. also change in abundance between different treatment due to the interaction between genotype and mangment pratices while certain endophytic members of the *Colletotrichum* genus have been demonstrated before to produce bioactive metabolites, including plant hormones and antimicrobial compounds, that work against a number of crop pathogens (Redman, Freeman et al. 1999, Lu, Zou et al. 2000). One study found that endophytic fungi related to the *Colletotrichum* genus could improve plant fitness under phosphorous-deficient conditions by transferring phosphorus to plant shoots, thereby improving plant growth (Hiruma, Gerlach et al. 2016). But additional studies are needed to determine whether individual carrot genotypes can in fact recruit specific individual endophytic taxa and contribute to some specific functional role.

Interestingly, the one carrot genotype that did differ in productivity between the two management systems (E0252) (Table 4.8.) was also the one genotype that showed no difference in fungal endophyte communities between the two management systems. This indicates that other

factors, such as greater availability of soil phosphorous or lower pH between the two systems (Table 4.6), might have been responsible for the greater productivity of this genotype in the conventional system relative to the organic system observed in this trial (Table 4.8). In addition, these results indicate that this particular carrot genotype is more conservative in comparison to the other genotypes in permitting colonization of different endophyte communities present in field soil, and therefore this genotype might not be able to recruit and support beneficial symbionts when they are needed. It is reasonable to suppose that this is why this genotype is only moderately resistant to pathogenic nematodes (Table 2.1).

4.3.6 Conclusions

Results of this study provide evidence that the majority of fungal endophytes observed in the trial could constitute a core fungal endophyte microbiome that is conserved across carrot genotypes and management regimes. This core endophytic microbiome may reflect common biological needs among all carrot roots. For example, Johnston-Monje and Raizada (2011) observed a largely conserved core bacterial endophyte community among *Z. mays* seeds that is shared across different maize evolution, migration and domestication. The authors theorized that this core microbiome is essential for healthy seed germination. They also suspected that this core microbiome was the result of both the phylogeny of the plant as well as ancient selection conditions that shaped the bacterial endophyte community composition and abundance of individual taxa, respectively. Consequently, by integrating crop ancestors into breeding programs, it may be possible to select for the abundance of beneficial endophyte communities in modern crop plants.

Results of this study also provide further support for the fact that organic farming systems often have better soil health than their conventional counterparts, and this can lead to differences in the abundance and diversity of fungal endophytes in important edible root crops such as carrot. Out of the 24 fungal taxa that were specifically associated with management practices, 21 were specifically associated with the organic regime. These results are in line with many other studies that have observed greater abundance and diversity of microbial taxa in organic management systems (Reeve and Cheon 2016). For example, Surette, Sturz et al. (2003) theorized that the reason certain management practices lead to the enrichment of specific types (harmful or beneficial) of endophytic microbial populations is that soil generally is the main source of endophytes (Lamb, Tonkyn et al. 1996). They also suggested that establishing beneficial

rhizosphere microbial communities in the presence of superior soil health would guarantee the establishment of beneficial endophyte communities. Consequently, we suggest that growers consider including soil-building management practices such as planting cover crops and amending soil with compost to encourage greater abundance and diversity of microbes that could help crops withstand biotic and abiotic stress.

Finally, we confirm the results of previous studies that showed that plant host specific selection of endophyte microbes can occur and could have important implications for plant health and productivity (Adams and Kloepper 2002, Rasche, Trondl et al. 2006, Chen, Hu et al. 2011). As discussed above, some plants may be able to specifically recruit and support individual endophytic taxa to meet their individual nutritional needs and/or fight off pests when they are present. This indicates that it could one day be possible to begin selecting for beneficial plant-microbial relationships in breeding programs, though additional studies will be needed to confirm such assertions. Such research could have important implications for reducing agrochemical use and improving crop productivity.

4.4 Refrences

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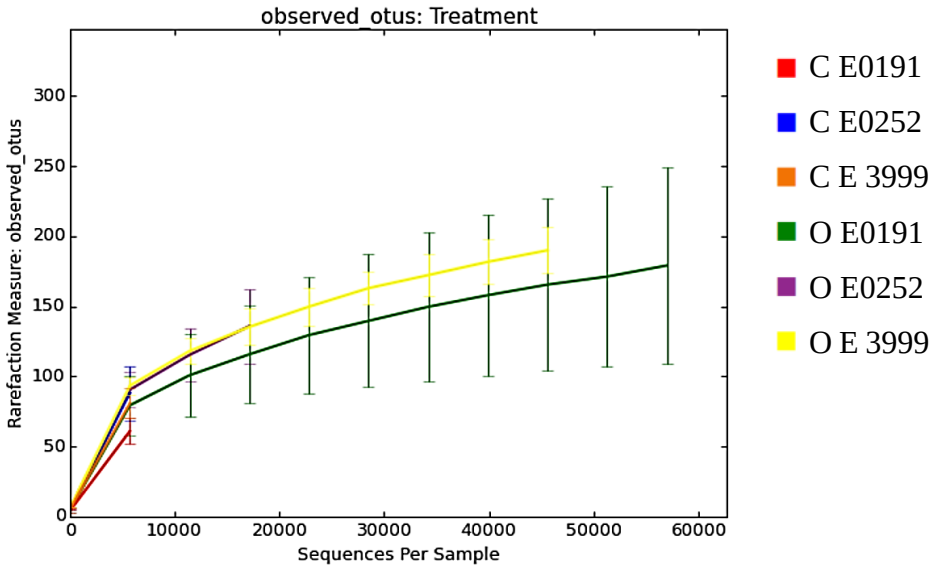

Figure 4.1 Rarefaction curves of observed operational taxonomic units of fungal endophytes species diversity (OTUS grouped at 97% genetic similarly level) of the three tested carrot genotypes E0191, E0252 & E 3999 grown under C (conventional) or O (organic system.).

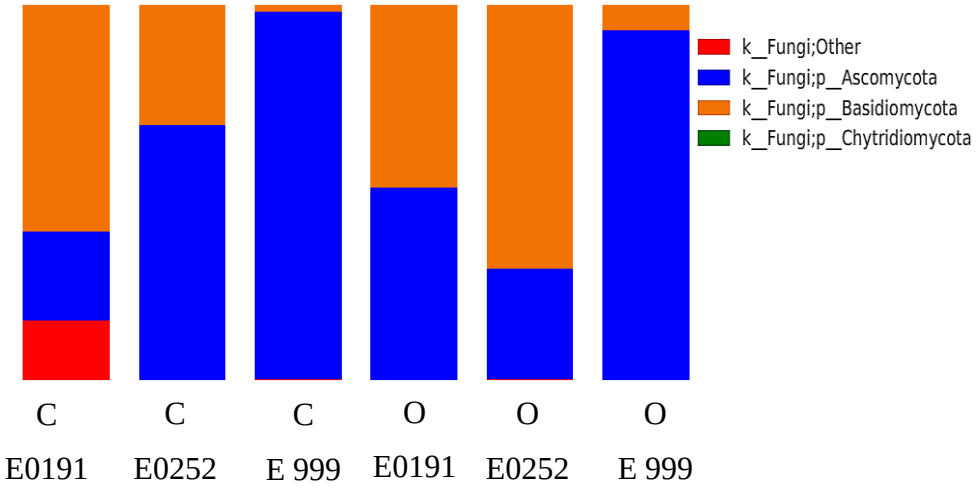

Figure 4.2 Relative abundance of fungal endophytes phyla of the three tested carrot genotypes E0191, E0252 & E 3999 grown under C (conventional) or O (organic system.).

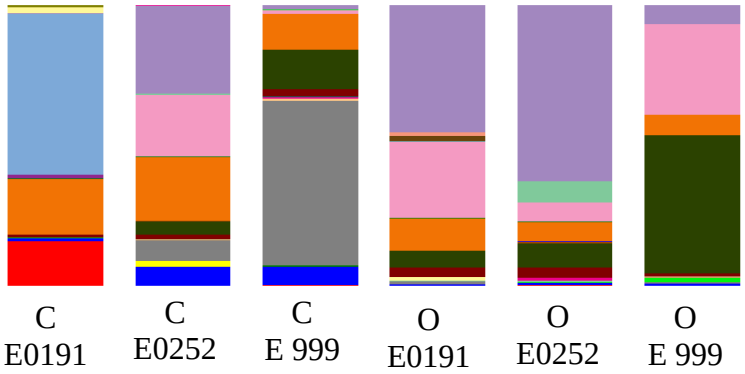

Figure 4.3 Relative abundance of fungal endophytes genera of the three tested carrot genotypes E0191, E0252 & E 3999 grown under C (conventional) or O (organic system.).

k__Fungi;Other;Other;Other;Other;Other
k__Fungi;p__Ascomycota;Other;Other;Other;Other
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Botryosphaeriales;f__Botryosphaeriaceae;Other
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Botryosphaeriales;f__Botryosphaeriaceae;g__Macrophomina
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Mycosphaerellaceae;g__Cercospora
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Mycosphaerellaceae;g__Cladosporium
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Incertae_sedis;f__Incertae_sedis;g__Rhizopycnis
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;Other;Other
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Arthopyreniaceae;g__unidentified
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Incertae_sedis;g__Phoma
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Phaeosphaeriaceae;g__Ophiosphaerella
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;Other
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Alternaria
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__unidentified;g__unidentified
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Chaetothyriaceae;g__Cyphellophora
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Eurotiales;f__Trichocomaceae;g__Aspergillus
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Eurotiales;f__Trichocomaceae;g__Penicillium
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__unidentified;g__unidentified
k__Fungi;p__Ascomycota;c__Saccharomycetes;o__Saccharomycetales;f__Dipodascaceae;g__Galactomyces
k__Fungi;p__Ascomycota;c__Sordariomycetes;Other;Other;Other
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Diaporthales;f__Valsaceae;g__Phomopsis
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;Other;Other
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Clavicipitaceae;g__Claviceps
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Clavicipitaceae;g__Nomuraea
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Incertae_sedis;g__Myrothecium
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Incertae_sedis;g__Stachybotrys
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Nectriaceae;Other
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Nectriaceae;g__Fusarium
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Nectriaceae;g__Gibberella
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__unidentified;g__unidentified
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Incertae_sedis;f__Glomerellaceae;g__Glomerella
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Incertae_sedis;f__Plectosphaerellaceae;Other
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Incertae_sedis;f__Plectosphaerellaceae;g__Plectosphaerella
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Phyllachorales;f__Phyllachoraceae;g__Colletotrichum
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Sordariales;f__Chaetomiaceae;g__Chaetomium
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Xylariales;f__Hyponectriaceae;g__Microdochium
k__Fungi;p__Ascomycota;c__unidentified;o__unidentified;f__unidentified;g__unidentified
k__Fungi;p__Basidiomycota;Other;Other;Other;Other
k__Fungi;p__Basidiomycota;c__Agaricomycetes;Other;Other;Other
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__Lyophyllaceae;g__Ossicaulis
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__Schizophyllaceae;g__Schizophyllum
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Cantharellales;f__Ceratobasidiaceae;Other
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Cantharellales;f__Ceratobasidiaceae;g__Ceratobasidium
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Cantharellales;f__Ceratobasidiaceae;g__Thanatephorus
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Corticiales;f__Corticiaceae;Other
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Corticiales;f__Corticiaceae;g__Rhizoctonia
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Polyporales;Other;Other
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Polyporales;f__Sistotremataceae;g__Sistotrema
k__Fungi;p__Basidiomycota;c__Microbotryomycetes;o__Sporidiobolales;f__Incertae_sedis;g__Rhodotorula
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Filobasidiales;f__Filobasidiaceae;g__Cryptococcus
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Tremellales;f__Tremellaceae;g__Bullera
k__Fungi;p__Basidiomycota;c__unidentified;o__unidentified;f__unidentified;g__unidentified

Table 4.1 . Impact of management system, carrot genotype and their interaction on richness and alpha diversity of fungal endophytes within samples. Significant ($P \leq 0.05$) parameters written in bold and underlined.

Sample	Diversity	Richness
Genotype	0.205	0.778
Management	0.354	**0.019**
Genotype + Management	0.524	0.284
Genotype E0191 + Management	0.513	0.275
Genotype E0252 + Management	0.827	0.513
Genotype E 3999 + Management	0.275	**0.050**

Table 4.2 ADONIS analysis showing the impact of management system, carrot genotype and their interaction on endophyte fungal community structure in carrot taproots. Significant ($P \leq 0.05$) parameters are written in bold and underlined.

	ADONIS
Comparison	**p-value**
Conventional vs. Organic	**0.03**
E0191 vs. E0252	0.74
E0191 vs. E3999	0.72
E0252 vs. E3999	0.67
Conv. E0191 vs. Conv. E0252	0.80
Conv. E0191 vs. Conv. E3999	0.80
Conv. E0252 vs. Conv. E3999	0.90
Org. E0191 vs. Org. E0252	0.80
Org. E0191 vs. Org. E3999	0.80
Org. E0252 vs. Org. E3999	0.90
Conv. E0191 vs. Org. E0191	0.20
Conv. E0252 vs Org. E0252	0.90
Conv. E3999 vs. Org. E3999	0.10

Table 4.3 Significantly different abundant endophytes species among different treatments (Adjusted p Value = 0. 2).C represent conventional treatment & O represent organic treatment.

Comparison	Significantly different abundant endophytes species Adjusted p Value = 0. 2			
Carrot Genotype		Management		
		Conventional	Organic	Conventional vs Organic
Genotype E0191 vs. E0252	*g _Aspergillus* (Specific to E0191) *g – Ceratobasidium* (E0191> E0252) *g _unidentified* NA	*g__Ophiosphaerella* (E0252> E0191) *g__Cladosporium* (E0252> E0191) *g__unidentified* *g__Ceratobasidium* (Specific to E0191) NA	*g__unidentified* (E0191> E0252)	

Table 4.3 continued

Genotype E0191 vs. E3999	*g__Cyphellophora* (Specific to E3999) *g _Ophiosphaerella* (E3999> E0191) *g__Cladosporium* (E0191> E3999) *g__Thanatephorus* (Specific to E0191) *g__Rhizoctonia* (E0191> E3999) *g__Ceratobasidium* (E0191> E3999) *g__Aspergillus* (E0191> E3999) *g__unidentified* NA	*g__Ophiosphaerella* (E3999> E0191) *g__unidentified* NA	*g__Aspergillus* (Specific to E0191) *g__Rhizoctonia* (E0191> E3999) *g__unidentified* NA	
Genotype 0252 vs. E3999	*g__unidentified* (E0252> E3999) NA (E0252> E3999)	*g__Ophiosphaerella* (E3999> E0252) *g__unidentified* (E3999> E0252) NA (E3999> E0252)	*g__unidentified* NA (E0252> E3999)	
Conventional vs. Organic				*g__unidentified* (O>C) NA (C>O)

Table 4.3 continued

Genotype E0191 vs. E0191				*g__Aspergillus* Organic specific *g__Ophiosphaerella* (O>C) *g__Rhizoctonia* (O>C) *g__Thanatephorus* Organic specific *g_Fusarium* (O>C) *g__Ceratobasidium* (C>O) *g__Colletotrichum* (C>O) *g__unidentified* NA
Genotype E0252 vs. E0252				NONE.
Genotype E3999 vs. E3999				*g__unidentified NA*

Table 4.4 List of significant indicator species analysis results for the two tested management regimes endophytes and their associated p. value

Conventional	Organic	p.value	Taxonomy
0	1	0.001	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Pleosporales; f__Pleosporaceae; g__Alternaria; s__*Alternaria_zinniae*
1	0	0.001	Unassigned
0	1	0.005	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Incertae_sedis; f__Plectosphaerellaceae; g__*Plectosphaerella*
0	1	0.006	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Corticiales; f__Corticiaceae; g__*Rhizoctonia*
0	1	0.008	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Nectriaceae; g__*Fusarium*
0	1	0.009	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Pleosporales; f__Pleosporaceae
1	0	0.016	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
0	1	0.019	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedAscomycota
0	1	0.022	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified

Table 4.4 continued

0	1	0.023	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
0	1	0.023	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Pleosporales; f__Pleosporaceae; g__*Alternaria*
0	1	0.023	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedAscomycota
0	1	0.029	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Corticiales; f__Corticiaceae; g__Rhizoctonia; s__Rhizoctonia_sp_FA59209
0	1	0.031	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Cantharellales; f__Ceratobasidiaceae; g__Thanatephorus; s__*Thanatephorus_cucumeris*
0	1	0.032	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Nectriaceae
0	1	0.035	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedAscomycota
0	1	0.042	Unassigned
0	1	0.046	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
1	0	0.046	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus

Table 4.4 continued

0	1	0.047	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
0	1	0.047	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
0	1	0.048	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified
0	1	0.048	Unassigned
0	1	0.049	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus

Table 4.5 List of significant indicator species analysis results for the three tested genotypes endophytes & associated p value

Genotype E0191	**Genotype** E0252	**Genotype** E3999	**p.value**	**Taxonomy**
0	1	0	0.011	__Fungi.p__unidentified.c__unidentified.o__unidentified..f__unidentified..g__unidentified
0	1	0	0.031	__Fungi...p__unidentified...c__unidentified.o__unidentified..f__unidentified..g__unidentified..s__unculturedsoil_fungus
0	1	1	0.031	__Fungi.p__Basidiomycota.c__Agaricomycetes..o__Corticiales..f__*Corticiaceae*

Table 4.6 Organic and conventional field trials soil chemical properties

System	OM %	P - weak bray	P - strong bray	K	Mg	Ca	Soil pH	CEC	% K	%Mg	%Ca	%H
Conventional	2.2 b	70.7 a	81.0	256.3	335.7 b	1990.7 b	6.0 b	15.9 b	4.1	17.5	15.7	62.6
Organic	3.1 a	34.3 b	67.7	229.7	426.3 a	2790.0 a	6.6 a	19.2 a	3.1	18.3	5.8	72.8

Table 4.7 Organic and conventional field trials soil microbial community estimated by Phospholipid Fatty Acid. (a) Microbial biomass & (b) ratios of microbial biomass groups

a)	Microbial Biomass (PLFA)										
System	Total Microbe	Total Bacteria	Actinomycetes	Gram (+) Bacteria	Gram (-) Bacteria	Rhizobia	Total Fungi	Arbuscular Mycorrhizal Fungi	Saprophytes	Protozoa	Undifferentiated
Conventional	1589 b	1.289	668.8 b	493.8 b	175.0 b	0 b	68.9 b	23.9 b	45.0 b	0 b	851.0
Organic	2433 a	1.311	1292.9 a	874.2 a	418.7 a	7.0 a	142.7 a	37.4 a	105.4 a	9.6 a	987.7

b)	Ratios of microbial biomass groups			
System	Fungi:Bacteria	Predator:Prey	Gram(+):Gram(-)	Sat:Unsat
Conventional	0.1	All prey	2.9	3.2
Organic	0.1	0	2.1	2.3

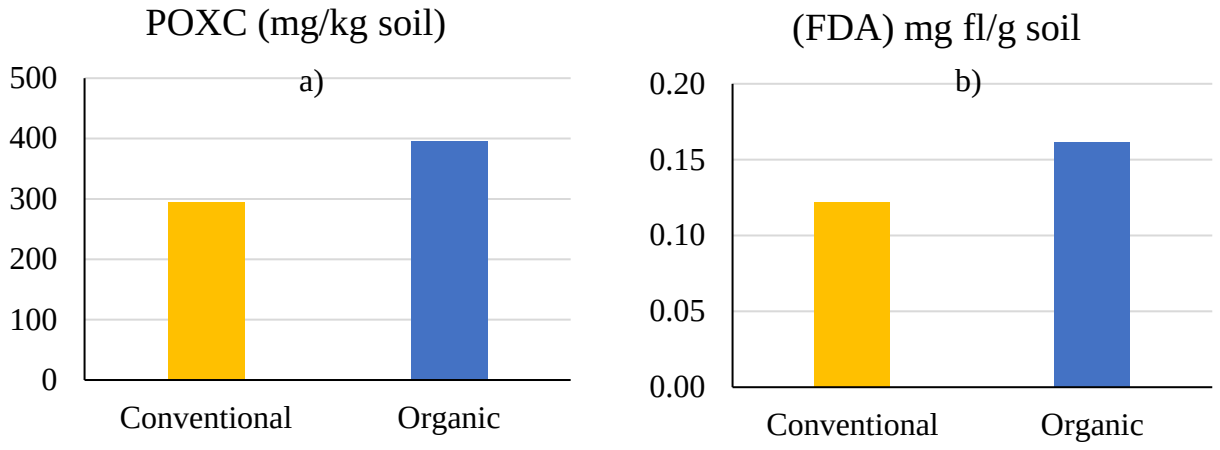

Figure 4.4 Organic and conventional field trials soil analysis. (a) Bars represent mean of active carbon as measured by Permanganate Oxidizable and (b)Bars represent mean of soil microbial activity as estimated by Fluorescein Diacetate

Table 4.8 Above- and below-ground biomasses and disease infection percentage (60 &119 Dayes psot planting) of the three tested carrot genotypes grown under organic or conventional system field trials

	Disease (60)		**Disease (119)**		**# of carrots**		**Top wt. (g, all)**		**Root wt. (g, all)**	
Entry	Conventional	Organic	Conventional	Organic	Conventional	Organic	Conventional	Organic	Conventional	Organic
E0191	3.33	0	65	71.7	5.33	8.33	0.23	0.19	0.58	0.57
E0252	0	11.67	70	90.0	9.67	3.67	0.22	0.05	0.64	0.14
E3999	16.67	23.33	83.3	91.7	13.67	5.67	0.11	0.02	0.53	0.11

CHAPTER 5. MICROFILTRATION AND ENZYMATIC DIGESTION AS METHODS OF IMPROVING ENDOPHYTE RECOVERY IN CARROT TAPROOTS

5.1 Introduction

Plant endophytes are well known for their potential to benefit their plant hosts in many ways (Chi, Shen et al. 2005, Rodriguez, White Jr et al. 2009). These benefits include promoting host plant growth, increasing tolerance to biotic and abiotic stress (Sessitsch, Reiter et al. 2004, Hardoim, van Overbeek et al. 2008), and enhancing phytoremediation of soil pollutants (Khan and Doty 2011, Ma, Prasad et al. 2011). Although it has received far less attention, another critical benefit potentially provided by endophytes is their ability to positively or negatively influence human health. For example, endophytes are commonly present within plant tissues in concentrations ranging from 10^4 to 10^9 g^{-1} (Hallmann, Quadt-Hallmann et al. 1997). Many studies have demonstrated that some of these microbes can be enteric pathogens causing foodborne pathogen outbreaks and others can be opportunistic pathogens causing disease in people with compromised immune systems (Eduardo, Seockmo et al. 2018). In contrast, recent studies have provided evidence suggesting that some of these plant-associated endophytes could directly improve human health by stimulating the immune system (Berg, Eberl et al. 2005, Flandroy, Poutahidis et al. 2018). Moreover, the presence of endophytes also could indirectly affect human health by altering the composition of chemical compounds within plants. For example, endophytes have been shown to directly produce or simulate production of important antimicrobial compounds such as taxol in *Taxus baccata* (Garyali, Kumar et al. 2013) and apingenin in *Chamomille matricaria* (Schmidt, Köberl et al. 2014). At the same time, however, endophytes can produce mycotoxins, which are among the world's most toxic and carcinogenic compounds (Wu, He et al. 2014). Consequently, there are many reasons to further study endophytes, particularly in an important edible crop such as carrot, which is well known to be an important source of vitamins and antioxidants (Simon, Freeman et al. 2008), and commonly is consumed raw.

For endophytic microbes to be used and studied, they must be cultivated under laboratory conditions. Isolation is the first step in cultivating any microorganism. However, isolating endophytic microbes is particularly difficult since unlike microbes that reside on external plant tissues in the rhizosphere or phylloplane, endophytic microbes are embedded within internal plant

tissues (Bafana 2013, Eevers, Gielen et al. 2015, Greenfield, Pareja et al. 2015). Moreover, endophytic microbes often are embedded within biofilms, which are defined as communities of microbes that are attached to inert living surfaces by self-produced matrices of extracellular polymeric substances primarily derived from polysaccharide materials (Flemming and Wingender 2010). Biofilms are produced by microorganisms to facilitate coordinated processes such as pathogenesis, and to protect microbes from various stresses such as those that might result from induction of plant defense responses (van Overbeek and Saikkonen 2016). Because of these factors, it is likely that only a fraction of the endophytic communities that live in important edible crops like carrot have been isolated.

Microfiltration is a physical separation process that has the potential to enhance the recovery of endophytes. During microfiltration, materials are passed through a special pore-sized membrane (which typically measures from about 0.1 to 10 µm) to separate microorganisms and suspended particles from a liquid. This technique is commonly used in the food industry to detect food-borne pathogens (Smith and Hill 2009, Dwivedi and Jaykus 2011). However, the concentration of microbes recovered using traditional microfiltration techniques are usually low and thus require a large initial food sample to detect a particular microbe at some level. This low recovery rate often is related to the high amount of solid contents, the viscosity of the food samples, and the frequent plugging of filter membranes (Dwivedi and Jaykus 2011, Li, Ximenes et al. 2013). To overcome this challenge, scientists at Purdue University recently developed an automated hollow fiber microfiltration unit designed to rapidly concentrate and detect food-borne pathogens in contaminated foods (Ximenes et al., 2017). This automated unit is capable of filtering and concentrating up to two liters of food samples into a 5 ml volume. The unit contains two filters. One is a 2.7 µm glass microfiber filter designed to pre-filter samples and thereby remove colloidal plant particles that can clog the final microfilter membrane. The others is an 0.2 µm polyether sulfone hollow fiber membrane used to concentrate recovery of the microbial sample. This new technique has been successfully used to enhance the recovery of food-borne pathogens present in a few cells from a range of food types, including fresh vegetables (Ximenes, Hoagland et al. 2017).

Coupling this new automated hollow fiber microfiltration technique with a short enzymatic digestion step prior to filtration has the potential to further increase detection of microbes that are embedded within plant tissues (Ku, Ximenes et al. 2016, Ku, Kreke et al. 2017). During this step, hydrolytic enzymes such as protease, cellulase, and pectinase are added to homogenized food

samples to release microbes by breaking down plant cell walls. The enzymes also can break apart the polysaccharide bonds that are present between microbes and plant surfaces or between individual microbes within biofilm structures (Furukawa, Akiyoshi et al. 2010, Vibbert, Ku et al. 2015). This combined approach has been demonstrated to significantly enhance recovery of very low populations of *Salmonella enterica* from spinach samples (Ku et al., 2017; Ximenes et al., 2017). However, to our knowledge this technology has not yet been tested for its potential to enhance recovery of a broad set of endophytic microbes from plant tissues. Therefore, the goal of this study was to determine whether this technology could be used to enhance the recovery of a diverse set of bacterial and fungal endophytes from the taproots of carrot, one of the most important vegetable crops in the world.

5.2 Material and methods

5.2.1 Field conditions and sample collection

This experiment was conducted during summer 2017 using carrots grown at Purdue's Student Farm located in West Lafayette, IN. Soils at this site are characterized as a silty clay loam belonging to the Mahalasville Treaty Complex. These soils are generally poorly drained, though tile drainage was installed in 2016 to help promote drainage at this site. A soil test conducted at this site during spring 2017 indicated that soil pH and organic matter, and concentrations of key plant nutrients were within satisfactory range for these soils (Table 5.1). A fall cover crop consisting of winter rye (*Secale cereale*), field peas (*Pisum sativum subsp. arvense*), ryegrass (*Lolium perenne*), crimson clover (*Trifolium incarnatum*) and hairy vetch (*Vicia sp.*) (Johnny's Selected Seeds, Winslow, ME) that was planted in the previous autumn was mown and disked into soil approximately one month before planning carrot seed. Re-Vita Pro Compost (Ohio Earth Foods, Hartville, OH) was applied at a rate of 5,380 kg ha^{-1} to meet fertility needs prior to further disking and creation of raised beds. Untreated carrot seed of an experimental breeding line, Exp3999 (provided by Dr. Phil Simon, USDA-ARS Vegetable Crops Research Unit, Madison, WI) was sown in mid-May. Carrot seeds were sown on raised beds that were 1.8 m apart, in 1 m rows to provide a density of approximately 60 plants m^{-1} given previously determined germination rates. Seeds were sown to a depth of 10 cm. Approximately 110 days after planting, five randomly collected healthy carrot taproots that showed no obvious signs of damage from pathogens or

insects were harvested using a spade from each of three replicate field plots. The carrot taproots were placed in a cooler on ice for transfer to the lab where they were stored at 4°C until processing on the following day.

5.2.2 Surface sterilization and processing of the carrot taproot samples

Carrot taproots were rinsed well with tap water and surface sterilized by soaking in 5.25% bleach for 3 minutes, followed by soaking in 3% peroxide solution for 3 minutes before a final rinsing step in sterile water supplemented with 1 ml of tween (Surette, Sturz et al. 2003). To confirm surface disinfection of the carrot taproots, 200 µl of the last wash solution was cultured on the following selective media: LGI (diazotrophic bacteria), R2A (oligotrophic bacteria), Tryptic soy agar (heterotrophic bacteria), and 1/5 PDA media (general fungal media) (Reasoner and Geldreich 1985, Corry, Curtis et al. 2011). Petri plates containing the inoculated media were incubated at 27 °C and 28 °C for fungal or bacteria respectively, and the growth of any colony forming units was recorded.

After surface sterilizing the carrot taproots, a sterile core borer was used to obtain 15 mm cylinders from the carrot taproots. Eighteen carrot cylinders from each field replicate were mixed carefully, then divided equally for use in the following processing treatments with three replicates for each treatment. To create stock solutions for the endophyte isolation treatments, five grams of carrot taproot cylinders were homogenized using an Omni Tissue Master Homogenizer (OMNI International, GA, U. S. A) in 25 ml of sodium phosphate buffer (pH 7). The first treatment (A) received no additional processing to separate endophytes from plant tissue. Stock solutions for the second and third endophyte isolation treatments were filtered through 90 mm filter paper prior to further processing. Carrot taproot extracts in the second treatment (B) were subject to the automated microfiltration unit to further separate endophytes from plant tissues. For this treatment 50 ml of the stock solution was injected directly into the automated hollow fiber microfiltration unit using methods described in Ximenes, Hoagland et al. (2017) (Figure 5.1). In the third treatment (C), carrot taproot extracts were subjected to a short enzymatic digestion step prior to microfiltration. For the enzymatic digestion step, a 0.5% concentration of hydrolytic enzymes (protease, cellulase and pectinase) was added to the stock solution, before incubating at 37°C for 2 hours while shaking at 200 rpm. After the enzymatic digestion step was complete, 50 ml of enriched stocks was injected into the hollow fiber microfiltration unit. All samples were run in

triplicate for each treatment, and the microfiltration unit was cleaned after each replicate using 200Mm NaOH and 70 % ethanol.

The stock solutions from each of the three treatments were used to prepare serial dilutions, and 100 µl of each serial dilution was plated onto petri dishes containing the four-selective media for broad microbial groups described above. Five replicates were used for each media type. Plates containing inoculated media were incubated at 27°C for 48 h, and 25°C for 72 h, for bacteria and fungi respectively. All of the colony forming units (CFU) that grew on each plate were enumerated and put into the following formula (Goldman and Green 2015) to estimate the number of CFU per ml stock solution.

$$\#\text{ of endophytic C.F.U per gram of carrot taproot tissue} = \frac{\text{Number of Colonies}}{\text{Volume plated (ml) x total dilution used}}$$

Each plate was stored at 4 °C for further purification and DNA extraction as described below

5.2.3 DNA extraction and identification of endophytes isolated from carrot taproots

Morphologically distinct colonies from each treatment were isolated and purified in preparation for DNA extraction. Fungal colonies were purified using the hyphael tip technique (Narayanasamy 2001) while bacterial colonies were purified using the streak plate technique (Black 2008). DNA was extracted from endophytic isolates using the DNeasy Ultraclean Microbial Kit (Mo Bio, Laboratories, C.A., U.S.A) following the manufacturer's protocol, before amplifying and sequencing the partial 16S r RNA & ITS regions for bacteria and fungi respectively. The forward ITS5 (5 ′ GGAAGTAAAAGTCGTAACAAGG- 3′) and reverse ITS4 (5′-TCC TCC GCT TAT TGA TAT GC- 3′) primers were used to amplify the whole ITS region in fungal isolates (Larena, Salazar et al. 1999). Each 25-µl PCR reaction mixture contained 1µl of DNA template, 0.5 µl of each primer (100 mM), 12.5 µl GoTaq® colorless Master Mix (Promega, WI, U.S.A) and 10.5 µl sterile ultrapure water. The PCR reactions were performed in a Bio-Rad T100 thermal cycler (BioRad, C.A, U.S.A) using the following cycling conditions: initial denaturing step: 1 cycle at 95ºC for 2 minutes, 39 cycles of the following: 95ºC for 30 seconds, 49ºC for 30 seconds, and72ºC for 1 minute, followed by a final extension step: 72ºC for 10 minutes. For amplification of bacterial isolates, the universal forward 8f (5′-AGAGTTTGATCCTGGCTCAG- 3′) and reverse

1492 (5′- GGTTACCTTGTTACGACTT- 3′) (Turner, Pryer et al. 1999) primers were used. Each 25 µl PCR reaction contained 1µl of DNA template, 1 µl of each primer (100 mM), 12.5 µl GoTaq® colorless Promega Master Mix and 9.5 µl sterile ultrapure water. Thermocycler conditions for amplifying bacterial isolates were: initial denaturing step: 1 cycle at 94ºC for 3 minutes, 35 cycles for the following steps; 94ºC for 45 seconds, 50ºC for 60 and 72ºC for 90 seconds, followed by a final extension step: 72ºC for 10 minutes. Following PCR, all products were subject to electrophoresis using a 0.7% (wt. /vol.) agarose gel stained with Bullseye DNA Safe Stain (MIDSCI, MO. U.S.A) to confirm amplification. A 100bp ladder (New England bio lab, MA. U.S.A) was run in parallel to approximate PCR product band sizing. Bands were visualized using a Bio-Rad Gel Doc EZ (Bio-Rad, C.A, U.S.A) after gel exposure to UV light. PCR products were then cleaned using PCR Clean-Up Kits (MO BIO Laboratories, Laboratories, C.A., U.S.A), before being sent to the Purdue Genomics Facility for sequencing on an ABI 3137XL low-throughput capillary machine (ABI company, CA., U.S.A), using the forward primer. Sequences were analyzed using the BLAST nucleotide sequences alignment software (Basic Local Alignment Search Tool), which is part of the NIH GenBank database (National Institutes of Health), for identification (McGinnis and Madden 2004). A confidence level of 98 and 97 % were used for identification of bacterial and fungal isolates at species level, respectively. Fungal identification was confirmed using the UNITE Database and Fungal BLAST (Abarenkov, Henrik Nilsson et al. 2010). All obtained sequences will be submitted to the NIH GenBank database to obtain accession numbers.

5.2.4 Statistical analysis

Data representing the number of CFU ml^{-1} of carrot taproot tissue collected from each treatment on the four types of selective media for broad bacterial and fungal groups were subjected to analysis of variance (ANOVA) using SAS JMP version 13 Software (Sall, Lehman et al. 2012), followed by post hoc analysis (Tukey) to conduct a pairwise comparison of treatment means when main effects were detected.

5.3 Results & Discussion

5.3.1 Enumeration of carrot taproot endophytes following alternative isolation treatments

Total fungal counts obtained from carrot taproots in this study ranged from log 4 to 5.6, diazotrophic bacterial counts ranged from log 4 to 6, oligotrophic bacterial counts ranged from log 4 to 5.6, and heterotrophic bacterial counts ranged from log 4 to 5.6 colony forming units per ml. No microbial colonies were observed growing on the selective media after plating the final wash solution was used to surface sterilize carrot taproots, confirming the efficacy of this technique and proving that all of the bacterial and fungal isolates obtained in this study were indeed endophytes.

While variations in the total number of CFU's representing each microbial group between the three treatments evaluated in this trial were not significantly different, there were trends suggesting that employing microfiltration and enzymatic digestion can enhance recovery of endophytes from carrot taproots (Figure 5.2). For example, within all four-selective media evaluated in this trial, the greatest number of CFU's were recovered from carrot taproot cores that had been subjected to enzymatic digestion followed by the automated hollow fiber microfiltration treatment (treatment C). In contrast, the lowest number of CFU's was recovered from treatment A, where extracts were directly plated onto selective media following tissue homogenization. Microfiltration treatment (treatment B) alone was intermediate between the other treatments. These results are not surprising. As pointed out in this study's introduction, the pre-enzymatic digestion step can degrade plant cell walls and bacterial biofilms, thereby enhancing retrieval of microbes embedded within these tissues (Furukawa, Akiyoshi et al. 2010, Wang, Wang et al. 2016). Using enzymatic digestion to degrade plant cell walls and bacterial biofilms has previously been shown to enhance recovery of foodborne pathogens in spinach (*Spinacia oleracea*) (Ku, Ximenes et al. 2016, Ku, Kreke et al. 2017) and increase the recovery of a broad set of endophytes within leaf tissues of *Oxalis acetosella L*. (wood sorrel) by 3X (Prior, Görges et al. 2014). Combining enzymatic digestion with this new automated hollow fiber microfiltration unit can further enhance recovery of microbes in plant samples by concentrating microbes between the two microfilters (Ku, Ximenes et al. 2016, Ku, Kreke et al. 2017)

5.3.2 Identification of endophytes recovered from carrot taproots

In total, 46 distinct microbial isolates were recovered from carrot taproots using the three processing treatments evaluated in this study (Table 5.2). These isolates were selected based on

their distinct morphological characteristics and potential to be genetically distinct based on sequence results. Only 6 distinct microbial taxa were recovered from carrot taproot extracts that were planted immediately following tissue homogenization (treatment A), and all of these were bacteria. These results demonstrate that the technique tested in treatment A has limited application in recovering a diverse set of endophytic microbial taxa from carrot taproots. The greatest number of distinct microbial isolates, 22 in total, were recovered from carrot taproot extracts that were subjected to the automated hollow fiber microfiltration treatment alone, without the pre-enzymatic digestion step (treatment B). Using this treatment, 16 different bacterial and 6 fungal isolates were recovered from carrot taproots, clearly demonstrating that microfiltration can enhance the recovery of unique endophytic microbial taxa in carrot taproots. In contrast, when the carrot taproot extracts were subjected to enzymatic digestion prior to microfiltration (treatment C), only 18 different microbial isolates were recovered (16 bacteria and 2 fungi), indicating that the enzymatic digestion treatment may have introduced a bias towards some taxa, particularly fungi. These results are not surprising, given that the enzymatic digestion step has the potential to favor the enrichment of individual microbial taxa, which then could outcompete other microbial taxa during culture on selective media. For example, bias has previously been observed in the recovery of different foodborne pathogen strains in studies that included an enrichment step to enhance the recovery of specific taxa (Bruhn, Vogel et al. 2005, Gorski, Flaherty et al. 2006). Gorski (2012) reported that there was even bias within individual *Salmonella* enterica strains in response to using different versions of the Rappaport-Vassiliadis peptone broth for enrichment. This was likely due to the fact that individual *Salmonella* strains are uniquely adapted to the presence of different growth substrates and environmental conditions (Singer, Mayer et al. 2009). In our study, we expect that by adding hydrolytic enzymes to our carrot taproot extracts, we may have released labile carbon compounds that were present in plant cell walls. We also expect that biofilms were released and then used by copiotroph bacteria that respond quickly to the presence of available nutrient sources. Interestingly, some endophytic microbial isolates were recovered from only one of the isolation treatments, which further supports our hypothesis that the availability of different substrates in these treatments might affect the recovery of unique taxa. For example, a bacterium belonging to *Comamonadaceae* was recovered only from carrot taproot extracts that were plated immediately following tissue homogenization. This could indicate that this microbe is an obligate biotroph, as this type of microbe can be cultured only when its host plant tissue is present. Treatment A likely

had the most carrot taproot tissue available on the plates while the endophytes were growing. This is why researchers often try to culture endophytes directly from plant tissue such as leaf slices (Prior, Görges et al. 2014). Because of this, researchers also have tried to establish the growth of biotrophic species by specifically adding plant extracts or even sterilized plant particles in the media (Arnold and Herre 2003, Arnold, Mejía et al. 2003). However, this approach could reduce the recovery of a greater diversity of endophytic taxa, as slower growing species are outcompeted by fast growing species.

Microbial taxa belonging to *Bjerkandera adusta*, *Torula caligans*, *Lysinibacillus fusiformis*, *Bacillus thuringiensis* and *an uncultured bacterium* (HM127153.1), were recovered only when carrot taproot extracts were subject to the automated hollow fiber microfiltration treatment alone (treatment B), whereas *Paenibacillus lautus* was recovered only when microfiltration was preceded by the enzymatic digestion step (treatment C). This provides further evidence that the microfiltration procedure evaluated here can enhance the recovery of unique endophytic microbial taxa by concentrating microbes from carrot taproots. This likely helps facilitate better dispersal of the microbes across the petri dish, which prevents slower growing species from being taken over by faster growing species and reduces the potential for competition and antagonistic behavior (Prior, Görges et al. 2014). As discussed above, by adding the enzymatic digestion step, we could have introduced bias that prevented us from isolating these unique taxa. Alternatively, it is possible that the taxa that were no longer present in treatment C, because they were damaged by the enzymatic digestion treatment, even though this approach has previously been shown not to damage bacteria (Ximenes, Hoagland et al. 2017) or fungi (Prior, Görges et al. 2014). Because we were able to recover a unique *Paenibacillus* species by including the enzymatic digestion step, it is possible that by release growth substrates, this approach still could hold promise as a way of enriching unique taxa that otherwise are difficult to isolate.

5.3.3 Potential significance of endophytic microbial taxa isolated in this study

Results obtained in this study confirm findings from our previous experiments (chapters 2, 3 and 4), demonstrating that carrot taproots are colonized by an abundant and diverse assortment of bacterial and fungal endophytes (Fig. 5.2 & table 5.2). The most commonly isolated bacterial endophytes in this study were related to *Pantoea* spp., *Xanthomonas* spp. and *Bacillus* spp., while the most commonly isolated fungal endophytes were related to *Cladosporium*. Some of these

fungal and bacterial species and genera are well known as plant and human pathogens such as *Pantoea agglomerans*, X*xanthomonas campestris pv. Campestris*, and *Cladosporium fulvum* (Thomma 2003, Coutinho and Venter 2009, Bensch, Braun et al. 2012, Dutkiewicz, Mackiewicz et al. 2016). However, these isolated were recovered from healthy plants, and these taxa have previously been noted to have plant growth promoting activity (Cottyn, Debode et al. 2009, Walterson and Stavrinides 2015, Li, Song et al. 2017, Walitang, Kim et al. 2017, Chutulo and Chalannavar 2018). For example, *Bacillus pumilus*, *Pantoea agglomerans*, *Pantoea ananatis*, *Curtobacterium* sp. and *Xanthomonas* sp, endophytes were isolated from healthy rice plants in a tropical environment and had antifungal activity against *Sarocladium oryzae, Rhizoctonia solani, Fusarium moniliforme*, and *Pyricularia grisea* fungal rice pathogen (Cottyn, Regalado et al. 2001). Moreover, *Cladosporium cladosporioides* has been isolated from neem trees (Azadirachta indica) and has been demonstrated to be able to produce a number of bioactive and antimicrobial compounds. (Verma, Gond et al. 2011, Silva, Barbosa et al. 2012). In this study, we think that many of recovered endophytes also might have the potential to help carrot withstand biotic and abiotic stress and affect the end-use quality of carrot taproots by altering their nutritional profile, flavor and storage potential (Cottyn, Debode et al. 2009, Walterson and Stavrinides 2015, Li, Song et al. 2017, Walitang, Kim et al. 2017, Chutulo and Chalannavar 2018). This includes the unique microbial taxa recovered in this trial using the more aggressive isolation techniques. For example, the presence of *Methylobacteria* could affect carrot flavor by altering its biochemical profile, since this microbe previously has been demonstrated to enhance the biosynthesis of furanone compounds and thereby improve strawberry flavor (Verginer, Leitner et al. 2010). *Bacillus thuringienses* is well known for its potential to protect plants against insects as well as pathogens (James, Miller et al. 1993, Morikawa 2006), and this microbe therefore could be protecting carrot taproots from a wide range of potential pests. Finally, *Bjerkandera* sp. and *Pseudomonas* sp. might also play an important role in carrot stress tolerance since they have been shown to reduce biotic and abiotic stress in a number of plants, such as maize and lettuce (Grover, Ali et al. 2011, Naveed, Mitter et al. 2014). However, future studies are needed to identify the exact function of the endophytes recovered from carrot taproots in this study.

5.4 Conclusions

Endophytes are an important group of plant-associated microbes that have been understudied due to difficulties associated with isolating them. Here we show that by subjecting carrot taproot extracts to enzymatic digestion and microfiltration, we can enhance recovery of endophytes in this important vegetable crop. Additional studies need to be conducted to fully understand the potential significance of endophyte community structure in important edible crops such as carrot and identify practical approaches for manipulating these communities to enhance the productivity and quality of our crops. This work provides a foundation for these future studies by identifying a new approach to enhance recovery of these important plant associated microbes. Future studies evaluating different enrichment conditions, such as the addition of different types of hydrolytic enzymes as well as various incubation temperatures and time periods, also are recommended to further enhance endophyte recovery in carrot taproots. In addition, researchers might want to consider including additional centrifugation steps, as this technique has also been demonstrated to further enhance recovery of microbes embedded in plant tissues (Ximenes, Hoagland et al. 2017).

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Table 5.1 Field trials soil chemical characteristics

			ppm							Percentage base saturation		
Crop System	**pH**	**% total OM**	**Nitrate**	**Bray -1 P**	**Bray -2 P**	**K**	**Mg**	**Ca**	**CEC** (meq/100g)	**%K**	**%Mg**	**%Ca**
ORG	6.8	3.9	183	26	55	239	567	2179	16.2	3.8	29.1	67.1

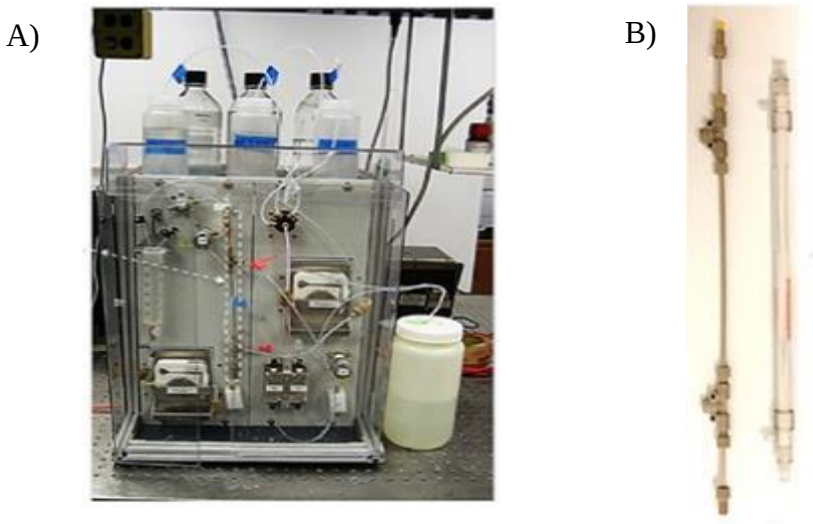

Figure 5.1 A) Automated Hollow fiber microfiltration unit used (developed by Dr. Ximenes lab Purdue University) equipped with B) (left) glass microfiber filter for pre-samples filtration and (right) polyether sulfone hollow fiber membranes for micro-filtration and concertation of the samples. Pictures taken from (Ximenes et al., 2017)

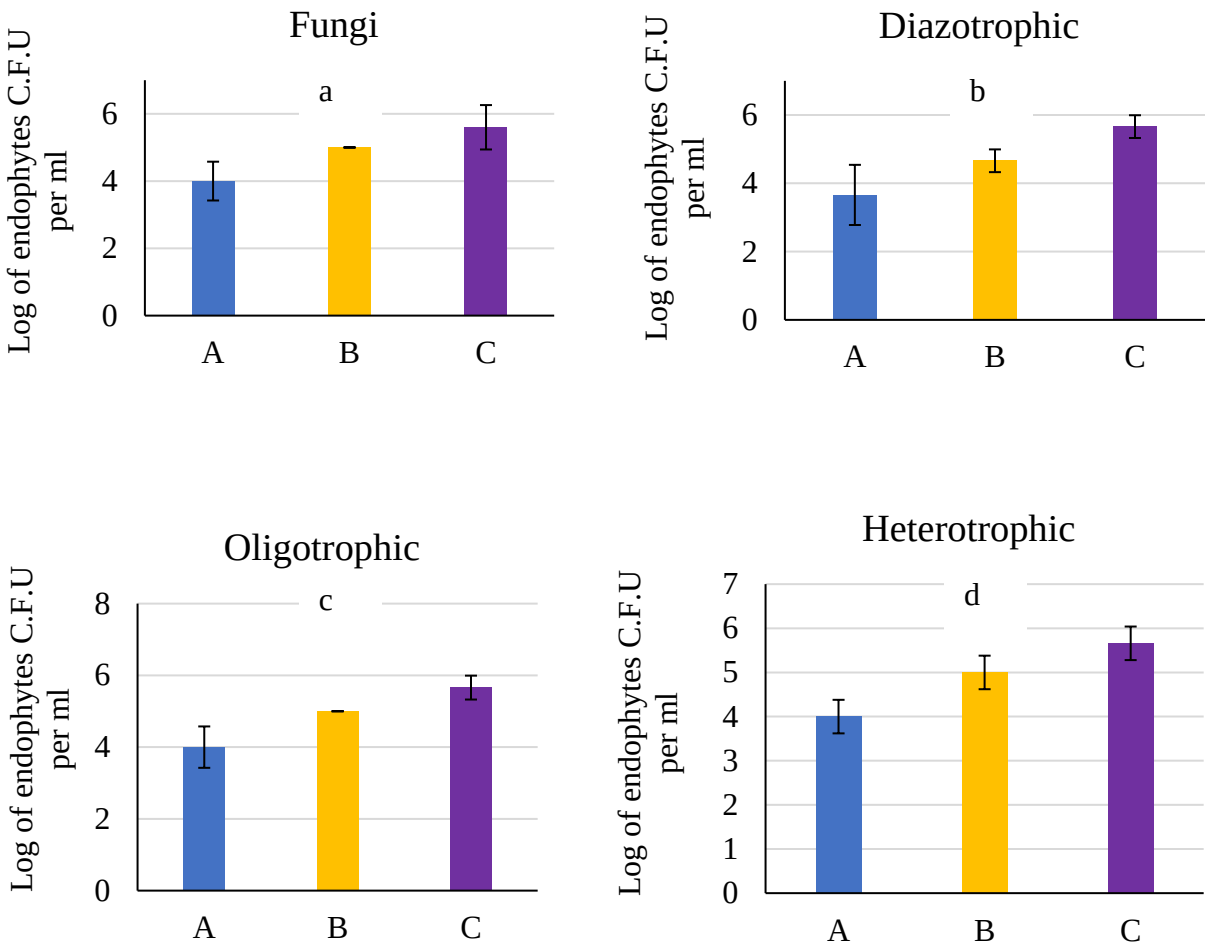

Figure 5.2 Log of carrot root endophytes counts obtained from different treatment. A) Endophytes recovered from untreated carrot root extracts, B) Endophytes recovered from hollow fiber microfiltration treatment & C) Endophytes recovered from none selective enrichment and hollow fiber microfiltration treatment. Endophytes counts were recorded on four different selective media for a) Fungi, b) Diazotrophs, c) Oligotrophic & d) Heterotrophic with three replicates for each media. Bars represent stander error mean (n=3)

Table 5.2 BLAST n alignment results for 16s r RNA & ITS partial sequences of carrot root endophytes retrieved from different treatment. A) Endophytes recovered from untreated carrot root extracts, B) Endophytes recovered from hollow fiber microfiltration treatment & C) Endophytes recovered from none selective enrichment and hollow fiber microfiltration treatment. 98% and 97% confident level cut off were used for bacterial and fungal sequences identification at species level respectively

Treatment	Morphologically distinct isolate	Most closely related strains on NCBI data base in order of closest potential match	E value	Identity	Accession # of closest species
A	A7	***Bacillus aryabhattai***	0	99%	MF662245.1
		Bacillus megaterium	0	99%	MG430231.1
		Bacillus spp. *strain*	0	99%	MG266304.1
		Bacterium R11	0	99%	KU561913.1
		Uncultured prokaryote	0	99%	KP410241.1
		Falsibacillus spp.	0	99%	HF912256.1
A	A3	***Comamonadaceae bacterium***	0	100%	KT591346.1
A	A6	***Pantoea eucalypti***	0	99%	KC139447.1
A	A1	***Xanthomonas arboricola pv. pruni***	0	100%	LC388644.1
		Xanthomonas campestris	0	100%	MG597200.1
		Xanthomonas campestris pv. hederae	0	100%	KU518243.1
		Xanthomonas arboricola	0	100%	KP340804.1
		Xanthomonas campestris pv. campestris	0	100%	KP182149.1
		Xanthomonas spp.	0	100%	KM252981.1
A	A2	***Xanthomonas arboricola pv. pruni***	0	100%	LC388644.1
		Xanthomonas campestris	0	100%	MG597200.1
A	A5	***Xanthomonas arboricola pv. pruni***	0	100%	LC388644.1
		Xanthomonas campestris strain	0	100%	MG597200.1
		Xanthomonas spp.	0	100%	KM252981.1

Table 5.2 continued

B (*maybe C*)	B23	***Uncultured bacterium***	5.00E-86	98%	HM127153.1
B	B28	***Agrobacterium tumefaciens***	0	99%	MH503856.1
		Rhizobium spp.	0	99%	MH236588.1
		Agrobacterium spp.	0	99%	MF443189.1
		Bacterium WQS21	0	99%	KU240584.1
B	B21	***Aureobasidium*** spp.	0	98%	MF154607.1
		Aureobasidium melanogenum	0	98%	MH424604.1
		Aureobasidium pullulans	0	98%	MH102060.1
		Uncultured fungus	0	98%	KX515518.1
		Fungal endophyte	0	98%	KR016954.1
		Uncultured Aureobasidium voucher	0	98%	KR012917.1
		Dothioraceae spp.	0	98%	KF573612.1
		Uncultured eukaryote	0	98%	GU942324.1
		Fungal endophyte spp.	0	98%	EU054400.1
		Uncultured ascomycete	0	98%	AM901847.1
		Aureobasidium spp.	0	98%	AM901687.1
B	B22	***Bacillus cereus***	0	99%	KY312781.1
		Bacillus thuringiensis	0	99%	KT986127.1
		Bacillus spp.	0	99%	KP992110.1
		Uncultured prokaryote	0	99%	KP410032.1
B (*maybe C*)	B30	***Bacillus thuringiensis***	0	99%	HF584925.1
B	B1	***Bjerkandera adusta***	0	99%	KC176339.1
B	B31	***Cladosporium tenuissimum***	0	98%	MF473296.1
		Uncultured fungus	0	98%	GQ851690.1
B	B21	***Dothioraceae* spp.**	0	97%	EF060818.1
		Aureobasidium pullulans	0	97%	KF938449.1

Table 5.2 continued

B	B2	***Fungal* spp.**	1E-156	97%	JQ747685.1
		Pseudozyma spp.	6E-155	97%	AJ876488.1
B (*maybe C*)	B29	***Lysinibacillus fusiformis***	0	99%	FJ174591.1
	B19	***Lysinibacillus fusiformis***	0	99%	KP192008.1
		Lysinibacillus spp.	0	99%	JQ844161.1
	B20	***Pantoea* spp.**	0	98%	KX129755.1
		Pantoea vagans	0	98%	JX113240.1
	B21	***Pantoea eucalypti***	0	97%	KC139438.1
B	B20	***Pantoea eucalypti***	0	98%	KC510026.1
		Uncultured bacterium	0	98%	JQ357574.1
		Pantoea spp.	0	98%	HM591487.1
		Pantoea ananatis	0	98%	HM591484.1
		Pantoea vagans	0	98%	CP020820.1
		Enterobacteriaceae bacterium	0	98%	LC007859.1
		Uncultured bacterium	0	98%	JF181231.1
		Uncultured microorganism	0	98%	EU181033.1
B	B24	***Providencia spp.***	0	99%	KY964269.1
		Uncultured bacterium	0	99%	HM126931.1
B	RB16	***Pseudomonas spp.***	0	100%	MH517510.1
		Methylobacterium spp.	0	100%	MG807354.1
		Pseudomonas plecoglossicida	0	100%	MG674376.1
		Pseudomonas putida	0	100%	MG011581.1
		Pseudomonas taiwanensis	0	100%	KY514158.1
		Pseudomonas monteilii	0	100%	KX785170.1
		Uncultured bacterium	0	100%	KX242396.1
		Pseudomonas parafulva	0	100%	KP190118.1

Table 5.2 continued

B	B17	***Pseudomonas spp.***	0	99%	KM253122.1
		Pseudomonas salomonii	0	99%	KC139424.1
B	B16	***Uncultured Pseudomonas* spp.**	0	99%	LT576258.1
		Pseudomonas monteilii	0	99%	KT735168.1
		Pseudomonas putida	0	99%	KF767898.1
B	B10	***Rhizobium* spp.**	0	100%	MH569334.1
		Bacillus spp.	0	100%	MH558375.1
		Agrobacterium tumefaciens	0	100%	LC388673.1
		Bacterium strain	0	100%	MF664534.1
		Agrobacterium spp.	0	100%	MH465150.1
		Rhizobium leguminosarum	0	100%	MH401128.1
		Uncultured bacterium	0	100%	KY609421.1
		Beijerinckia fluminensis	0	100%	MG547695.1
		Uncultured bacterium	0	100%	MF581286.1
		Agrobacterium rhizogenes	0	100%	CP019702.1
B	B225	***Torula caligans***	0	98%	FJ478093.1
B	B30	***Xanthomonas arboricola pv. pruni***	0	99%	LC388645.1
		Xanthomonas spp.	0	99%	MH470420.1
		Xanthomonas oryzae pv. oryzae	0	99%	MH464904.1
		Uncultured Xanthomonas spp.	0	99%	KX456334.1
		Xanthomonas campestris	0	99%	MF285891.1
B	B27	***Xanthomonas arboricola pv. pruni***	0	100%	LC388644.1
		Xanthomonas campestris	0	100%	MG597200.1
C	C101	***Uncultured fungus***	1E-163	90%	HQ611305.1
		Claussenomyces atrovirens	5E-157	91%	KY633591.1
C	C14	***Bacillus anthracis***	0	100%	KP813786.1

Table 5.2 continued

		Bacillus spp.	0	100%	LN829559.1
		Bacillus cereus	0	100%	KJ812209.1
		Bacillus thuringiensis	0	100%	KC911634.1
C	C14	***Bacillus cereus***	0	98%	KX350001.1
		Uncultured bacterium	0	98%	JN236259.1
C	C1	***Cladospporium* spp.**	0	98%	KT826656.1
		Cladospporium cladospporioides	0	98%	MG655161.1
C	C11	***Enterobacter* spp.**	0	100%	JN975210.1
		Enterobacter asburiae	0	100%	KC568143.1
C	C4	***Enterobacter asburiae***	0	100%	KC568143.1
		Enterobacter spp.	0	100%	JN975210.1
C	C10	***Lysinibacillus* spp.**	0	100%	KT204468.1
		Lysinibacillus fusiformis	0	100%	MF083065.1
		Bacillus spp.	0	100%	FR849924.1
		Bacillus fusiformis	0	100%	AM292996.1
C	C20	***Lysinibacillus fusiformis***	0	100%	MF521962.1
		Lysinibacillus spp.	0	100%	MF429547.1
		Uncultured bacterium	0	100%	HG798878.1
C	C12	***Pantoea* spp.**	0	99%	KR610525.1
		Enterobacteriaceae bacterium	0	99%	MF155193.1
		Uncultured Enterobacter spp.	0	99%	LC318238.1
		Kluyvera spp.	0	99%	MG593857.1
		Pantoea rodasii	0	99%	MG571649.1
C	C6	***Pantoea agglomerans***	0	99%	MF407401.1
		Pantoea eucalypti	0	99%	KC139447.1
		Uncultured bacterium	0	99%	JF181231.1

Table 5.2 continued

C	C17	***Paenibacillus lautus***	0	99%	KT719472.1
C	C8	***Paenibacillus lautus***	0	98%	MH298461.1
		Paenibacillus spp.	0	98%	KU904494.1
C	C13	***Pseudomonas* spp.**	0	100%	KM253151.1
		Pseudomonas salomonii strain	0	99%	KC139424.1
C	C18	***Xanthomonas arboricola pv. Pruni***	0	100%	LC388644.1
		Xanthomonas campestris	0	100%	MG597200.1
C	C18	***Xanthomonas arboricola pv. pruni***	2E-166	99%	LC388645.1
		Xanthomonas spp.	2E-166	99%	MH470420.1
		Uncultured Xanthomonas spp.	2E-166	99%	KX456334.1
		Xanthomonas campestris	2E-166	99%	MH211280.1
C	C15	***Xanthomonas arboricola pv. pruni***	0	100%	LC388644.1
		Xanthomonas campestris	0	100%	MG597200.1
		Xanthomonas spp.	0	100%	KM252981.1
C	C9	***Xanthomonas arboricola pv. pruni***	0	99%	LC388642.1
		Xanthomonas campestris	0	99%	MG597200.1
C	C1	***Xanthomonas* spp.**	0	97%	KP419705.1
		Xanthomonas oryzae pv. oryzae	0	97%	JQ269244.1

CHAPTER 6. SUMMARY AND FUTURE DIRECTIONS

6.1 Introduction

Endophytes are an important group of plant-associated microorganisms with the potential to improve plant productivity and help plants tolerate a wide range of biotic and abiotic stresses. The broad goal of the research described in this dissertation was to begin to characterize endophytes associated with carrots and elucidate some of their potential functional roles. Sppecifically, this project: 1) identified culturable endophytes in the seed and seedling of nine carrot genotypes and estimated some of their potential plant-growth promoting properties, 2) isolated and identified culturable endophytes in the taproots of the same nine carrot genotypes grown under organic versus conventional management, and quantified their potential antagonistic activity against *A. dauci*, a key carrot pathogen, 3) identified the fungal endophyte community in the taproots of three of the carrot genotypes grown under organic versus conventional management using NGS sequencing, and 4) evaluated the potential for microfiltration, with and without a pre-enzymatic digestion step, to enhance recovery of culturable endophytes in carrot taproots. This final chapter provides a broad summary of the results of each of these studies, including their significant contributions to the literature as well as the industry, their limitations, and suggestions for future research to further elucidate the role of endophytes in carrots.

6.2 Vertical transmition of culturable carrot endophytes: composition, plant growth promoting abilities and effect of plant genotype and developmental stage

Plants evolved alongside their beneficial microbial symbionts and likely developed several mechanisms to ensure the long-term survival of these important relationships as part of their core microbiome. One of these potential mechanisms is the vertical transmission of endophytes via seeds. Learning more about factors that affect the transmission of seed-borne endophytes, along with their potential functional role in plants, could help researchers better understand how plant microbiomes are assembled and maintained, and could someday be used to improve crop productivity. While others have quantified the effect of plant genotypes on endophyte community structures in seeds along with their potential functional role in several crops (Mastretta, Taghavi et al. 2009, López-López, Rogel et al. 2010, Hardoim, Hardoim et al. 2012, Truyens, Weyens et

al. 2015), this is the first study to conduct these assays in carrots, one of the most important vegetable crops in the world. Carrots are well known for challenges associated with crop establishment (Davis 2004, Simon, Freeman et al. 2008), therefore the presence of beneficial symbionts in seeds has important implications.

Results of this research provide evidence that carrot seeds are inhabited by a diverse assortment of bacterial and fungal endophytes. Some of the endophytes isolated in this study have potential plant-growth promoting properties, including the ability to produce IAA and siderophores, solubilize phospphorous, and fix atmosppheric nitrogen. The relative abundance and density of some of these endophyte communities varied among genotypes, as well as the presence in seeds versus germinated seedlings. For example, there were greater numbers of total fungi, and total oligotrophic, diazotrophic and heterotrophic bacteria in seedlings compared to seeds, as well as greater IAA production and nitrogen fixation among isolates collected from seedlings. These results indicate that many microbial taxa possess features that allow them to colonize the reproductive tissues of carrot plants and tolerate desiccation to survive in seeds and proliferate in newly developing seedlings. Whether these endophytes sppecifically benefit carrot plants via potential plant-growth promoting properties or are simply acting as parasites and using carrot seeds and carrot seedlings for their own survival, is yet to be determined. The fact that the density and relative abundance of some of seed-borne endophytes varied among some genotypes indicates that individual genotypes could possess mechanisms that allow them to sppecifically recruit and harbor microbial taxa with sppecific characteristics. However, because not all of the genotypes evaluated in this study were grown in the same location, it is unclear whether these differences were due to genotype alone or to the presence of the resident microbial community where the seeds were produced. Future studies, including those suggested below, are needed to answer these questions.

One of the main limitations of this study was the failure to achieve precise identification of many of the endophytic isolates at the sppecies level following amplification of 16 rRNA or ITS genes using Sanger sequencing. Moreover, there were many isolates where it was impossible to recover any sequence data at all using the techniques employed in this study (Janda and Abbott 2007). This prevented an accurate assessment of the endophyte community in carrots seeds and seedlings, along with the ability to place them in a phylogenetic tree or accurately evaluate differences in community structure between genotypes. To achieve better taxonomic resolution in future studies, researchers should consider sequencing the entire genome of these isolates using

Illumina HiSeq. Alternatively, researchers could consider conducting various biochemical, morphological and physiological assays alongside partial gene sequences to aid in identification. They can also think about combining the partial gene sequences with microarray technology or other housekeeping genes to achieve precise identification of endophytes at the sppecies level (Maiden, Bygraves et al. 1998, Janda and Abbott 2007)

Another limitation of this study was that evaluation of the potential plant-growth promoting activities of the isolated endophytes were conducted under *in vitro* conditions in the laboratory. This is a limitation because the functional activities of microbes might be very different when they are living inside their plant host, as well as when the host is subject to various environmental constraints. To overcome this limitation, researchers could consider conducting these assays *in planta* in the future, by quantifying production of compounds such as IAA by endophytes within germinating seeds and young seedlings using techniques such as gas/liquid chromatography, stable isotopic probing, and/or in situ hybridization (Simonet, Normand et al. 1990, Wang, Li et al. 2000, Rasche, Lueders et al. 2009). The challenges of these studies, however, would be to generate a sterile control. For example, heat-treating seeds to eliminate microbial endophytes can damage seeds; therefore, researchers may want to consider using systemic pesticides. Other alternative techniques to characterize the functional role of endophytes during *in planta* studies would be to use transcriptomic or proteomic analyses to quantify activities at the gene or protein level, resppectively (Gómez-Vidal, Salinas et al. 2009, Lery, Hemerly et al. 2011, Zuccaro, Lahrmann et al. 2011, Shidore, Dinse et al. 2012, Kaul, Sharma et al. 2016). To ensure that such studies are realistic, researchers should consider conducting these assays when the plant is subject to sppecific biotic and abiotic stresses which plants are likely to experience in the field. This would allow researchers to better understand mechanisms affecting endophyte community assembly, identify factors that regulate why endophytes might shift between different lifestyles (e.g. commensal, mutualistic, parasitic), and uncover mechanisms linking microbial activities with sppecific plant benefits.

Finally, to determine if the microbes inhabiting seeds are truly part of a core microbiome, researchers should consider conducting experiments that track the composition of seed-borne endophytes over multiple generations. In addition, as mentioned above, it would be interesting to conduct these studies in plants that are subject to realistic environmental constraints. For example, how does being subject to pathogen stress alter the composition and functional roles of seed-borne

endophytes, and does this relate to enhanced pathogen suppression in subsequent generations? Results of such studies could have important implications for plant breeders as well as the seed industry.

6.3 Carrot endophytes: role of crop management system and carrot genotype on community composition an *A. dauci* dynamics

Carrots produce a long-lived taproot that is subject to a wide variety of stresses over the course of its lifetime. In particular, pathogens such as *Alternaria dauci*, which causes foliar blight in carrots as well as taproot decay and seedling damping-off like symptoms are problematic in carrot (Davis 2004). Endophytes present in seedlings and carrot taproots could help reduce the damage caused by such pathogens directly by outcompeting pathogens for sppace and resources (Tyvaert, França et al. 2014) and/or producing antagonistic substances (Ezra, Castillo et al. 2004, Niones and Takemoto 2014). They could also suppress pathogens indirectly by stimulating a plants immune system (Gómez-Lama Cabanás, Schilirò et al. 2014), reducing disease severity in roots as well as foliar tissues. Thus, characterizing the presence of endophytic microbes with antagonistic activity in carrot taproots and identifying factors that can affect their abundance and activity could have important implications for disease control. Moreover, carrot taproots are often eaten raw, so characterizing the composition of endophytes within carrot taproots could have important implications for human health (Van Overbeek, van Doorn et al. 2014, Hardoim, Van Overbeek et al. 2015). While the effect of management practices and crop genotype on the identification of culturable endophytes communities and their antagonistic ability towards pathogens have been investigated before in several crops (Sessitsch, Reiter et al. 2004, Berg, Krechel et al. 2005, Hardoim, van Overbeek et al. 2008), this is the first study to conduct this research in carrots, an important root crop that is one of the most widely consumed vegetables worldwide. In addition, this study is unique because it was conducted using genotypes that are still undergoing the breeding process and have not yet been released to the market. Consequently, results of these studies could have immediate implications for carrot breeding programs.

Results of this research demonstrate that like seeds and seedlings, carrot taproots are colonized by a diverse assortment of microbial taxa. Moreover, agricultural management practices that can affect soil and plant health can alter the density and antagonistic ability. Sppecifically, growing carrots under organic management increased the total number of heterotrophic and

oligotrophic bacteria in carrot taproots compared to those grown under conventional management. Carrots grown in the organic management system also had greater abundance of endophytic isolates with antagonistic ability against *A. dauci* under *in vitro* conditions. Whether differences in the endophyte communities observed in this study were due to the sppecific inclusion or absence of sppecific practices, such as the presence of winter cover crops, the application of organic fertilizers, or the lack of pesticides used in the organic relative to the conventional management system cannot be determined at this time. In addition, whether these results were due to differences in the resident soil microbial community structure altered by these two management systems, and/or if they were due to differences in plant physiological processes caused by differences in biotic and/or abiotic stresses caused by these management systems, also cannot be determined at this time. In the future, more targeted studies designed to identify the exact factors respponsible for these differences are recommended. In the meantime, inclusion of soil-building practices such as planting cover crops and applying organic soil amendments to improve soil health is highly recommended in any cropping system to encourage colonization by beneficial endophytic communities in plant roots.

Another important result from this study was the observation of differences in endophyte density among carrot genotypes evaluated in this study. For example, within the conventional system, Red Core Chantenay had the lowest oligotrophic bacterial numbers and ExpP6306 had the highest. In contrast, within the organic system, ExpNb3999 had the lowest count for oligotrophic bacterial endophytes and ExpPY191 had the highest. Moreover, the two carrot genotypes evaluated in the greenhouse trials differed in their growth and susceptibility to *A. dauci* when inoculated with endophytes isolated from the field trial. These results provide further evidence that different genotypes might possess sppecific mechanisms that allow them to recruit, host and/or resppond to the presence of beneficial microbes that promote growth and can help plants withstand assault by pathogens. If this proves to be true, then it could someday be possible to select for crop genotypes that can better interact with beneficial microbes to improve productivity in crop breeding programs. Identifying the sppecific mechanisms respponsible for these beneficial plant-microbial relationships would aid in this effort. In the meantime, agronomists and breeders could screen existing varieties and advanced breeding lines under organic management systems where soil microbial communities are generally more abundant and diverse, to identify varieties best adapted for beneficial plant-microbial relationships.

Finally, another important result of the studies described in this chapter is that because some of the endophytic microbes isolated in this study improved carrot growth and helped carrots withstand *A. dauci* stress when inoculated onto carrot seed, they could someday be commercialized for use as seed inoculants. This would require future studies to determine whether these microbes can effectively colonize carrot roots, persist in the presence of the wide range of other microbial taxa carrot roots would encounter in the field, promote plant growth, and reduce disease incidence under actual field conditions. In addition, such studies could be designed in ways that would help researchers identify factors that promote or reduce the long-term survival of microbial inoculants in the field, along with other ecological implications of applying microbial seed treatments.

As discussed in the seed-borne endophyte section, researchers could consider using other technologies such as transcriptomic or proteomic approaches in future studies designed to further study endophyte communities in carrot taproots. These types of assays could help them better quantify the potential activities of endophyte communities living within plants in these studies, as well as the potential effects of endophytes on the plants themselves, which would allow them to begin to start teasing out the mechanisms regulating these plant-microbial relationships. If possible, researchers should consider increasing the number of field replicates, as well as the number of replicates under more controlled greenhouse or growth chamber studies, to increase the power of the statistical analyses (VanVoorhis and Morgan 2007).

Like the seed-borne endophyte section, a major limitation of this study was the ability to accurately identify the endophytic isolates, esppecially at the sppecies level, using the techniques employed in this study. In addition, another limitation of both of these sections is the fact that these studies relied on culture-based techniques. It has been estimated that only 1-10% of microbial taxa can be cultured (Hugenholtz 2002), which seriously limits the ability of researchers to quantify the presence and potential functional role of plant endophytes. One way to overcome this challenge is to use new molecular tools such as next generation sequencing to better characterize the composition of plant endophytes.

6.4 What's hiding in your carrots? A new look at fungal endophyte communities in taproots using NGS sequencing

As discussed above, plant microbial endophytes have the potential to improve plant growth, help plants tolerate stress and potentially influence human health (Van Overbeek, van Doorn et al.

2014, Hardoim, Van Overbeek et al. 2015). Therefore, learning more about factors that affect the composition of plant endophytes has important implications. However, studies involving plant endophytes have been constrained by the fact that only a small set of endophytes have been cultured in the laboratory to date (Hugenholtz 2002). New molecular tools such as next-generation sequencing that selectively amplify a sppecific diagnostic gene sequence, such as the 16S rRNA hypervariable region in bacteria and archaea and the ITS region in fungi, and amplify these gene sequences using massively parallel gene sequencing technology, have potential to help overcome this challenge (Handelsman 2004). Other researchers have successfully used these tools to characterize the effect of crop management practices and/or plant genotypes on endophyte community structure in several angiospperm and gymnospperm plants (Wilberforce, Boddy et al. 2003, Van Overbeek and Van Elsas 2008, Andreote, Da Rocha et al. 2010, Pancher, Ceol et al. 2012, Campisano, Antonielli et al. 2014). To our knowledge, this is the first study to use these tools to study endophytes in carrot.

Results of the research described in this section confirm results of the previous one, demonstrating that carrot taproots are colonized by an abundant and diverse assortment of fungal microbial taxa, and that crop management practices can significantly affect fungal endophyte community richness, abundance and structure in carrot taproots. They also confirm that carrot genotype can affect the abundance of individual fungal genera. Moreover, richness and abundance of fungal endophytes in one of three carrot genotypes differed when grown in an organic vs. a conventional management system. This indicates that carrot genotypes could differ in their ability to resppond to the presence of different soil microbial communities in these alternative systems, and/or alter the composition of endophytic fungal communities in respponse to differences in the physiological parameters expressed when plants are grown under these two divergent management systems. As discussed above, these results could have important implications in the identification of sppecific management practices that could improve crop productivity, and aid in the selection of beneficial plant-microbial relationships in carrot breeding programs. Again, further research to tease apart sppecific factors that might be affecting these relationships, as well as more targeted studies that can begin to elucidate some of the mechanisms regulating these interactions are needed.

Some of the limitations associated with the research described in this section include the fact that this study was conducted using carrot taproots grown in the field during only one year. This is due to the fact that we were unable to successfully amplify fungal communities in carrot

taproots grown in the previous year due to the quality of the DNA isolated and/or stored. Differences in climatic conditions between years can dramatically alter the effects of agronomic studies conducted in the field, which is why most field studies are repeated for at least two years. In addition, another limitation of this study as well as the study discussed in the previous section is that the assays of endophyte community structure were only conducted at one location. Management practices used with farms that fit within the definition of "organic" and "conventional" can vary dramatically. Moreover, site-sppecific soil and environmental factors in different locations can dramatically alter soil and crop health. Consequently, including additional field sites with different soil types and environmental conditions are recommend in future trials to better understand the variability of carrot taproot endophyte communities and how they are influenced by site and crop management practices.

One of the biggest limitations of this study was our inability to identify bacterial endophyte communities using a standard V3-V4 16S rRNA primer set commonly used in studies or bulk soil and rhizospphere microbial communities. A set of blocking primers, which were sppecifically developed to avoid the challenge associated with the homology of the 16S rRNA gene with plant mitochondria and plastids that has previously been demonstrated to limit the potential of researchers to amplify bacteria in plant endophyte studies (Hanshew, Mason et al. 2013, Arenz, Schlatter et al. 2015, Shen and Fulthorpe 2015), did not work in carrot taproots. Employing so-called mismatch primer sets that target the V5-V7 16S rRNA region can help overcome this challenge (Beckers, Op De Beeck et al. 2016), however, the libraries represented by these primers sets are much less robust than those associated with the V3-V4 region, which can bias the results of these studies (Thijs, Op De Beeck et al. 2017). In addition, though we were successful in amplifying many fungal genera in this study, many of the OTU's could not be assigned to the sppecies level, and many could not be assigned at all. Other technologies that can sequence longer gene fragments (third generation sequencing), such as Single molecule real time sequencing (SMRT), nanopore technology sequencing platforms, Hi-C Technology and Illumina Tru-seq synthetic long read technology, might be able to help overcome this challenge (Schadt, Turner et al. 2010, Rhoads and Au 2015)

6.5 Microfiltration and enzymatic digestion as a method of improving endophyte recovery in carrot taproots

As mentioned above, studies of plant-microbial relationships have long been constrained by the fact that only 1-10% of the microbial community can be cultured. Consequently, knowledge of plant-microbial relationships is currently highly biased towards a small number of phylogenetic groups. New culture-independent techniques such as NGS sequencing can aid in studying plant microbiomes, but to accurately identify individual taxa and elucidate their functions, researchers need to isolate individual taxa and find ways to culture them if possible. One of the challenges associated with isolating and culturing some microbial taxa is that they are very low in abundance and therefore escape detection. Hollow fiber microfiltration has the potential to help overcome this challenge by filtering out plant debris and concentrating microbial cells that pass through the filter. Another challenge associated with culturing plant-associated microbes, is the fact that many microbes are embedded in biofilms, which are an aggregation of microorganisms to each other and/or plant surfaces in a self-produced matrix of extracelluarly polysachharide materials (López, Vlamakis et al. 2010). One potential way to help overcome this challenge is to subject plant materials to enzymatic digestion with cellulases, hemicellulases and pectinases, which can disrupt biofilms, as well as break down plant materials where microbial cells could also be tightly bound, as in the case of endophytes. Coupling enzymatic digestion with microfiltration has previously been shown to significantly enhance the recovery of food-borne pathogens associated with vegetable tissues in samples where these pathogens were undetectable without these treatments (Dwivedi and Jaykus 2011, Vibbert, Ku et al. 2015, Ximenes, Hoagland et al. 2017). To our knowledge, this is the first study to evaluate the potential for using these two techniques, alone and in combination, to enhance recovery of broad groups of culturable endophytes from carrot taproots.

Results from this experiment demonstrate that combining these two approaches has the potential to enhance the recovery of culturable endophytes in carrot taproots. In particular, the combination of enzymatic digestion and hollow fiber microfiltration resulted in the highest overall density of total endophytic microbial taxa. The hollow fiber microfiltration treatment alone, was intermediate to the combined treatment and untreated control. Although there were no significant differences in total endophyte counts among broad microbial groups between the treatments evaluated in this study, there were differences in the number of unique taxa isolated. The hollow

fiber microfiltration had the greatest number of recovered bacterial and fungal taxa. This indicates that carrot taproot endophyte communities are even more diverse than initially thought and coupling microfiltration and enzymatic digestion has the potential to aid in the study of plant endophytes in many crops. Efforts to determine if using these techniques will aid in the amplification and NGS-sequencing of bacterial communities using various bacterial primer sets are underway.

In addition to the challenges associated with identifying microbial isolates to the sppecies level that was discussed above, another limitation of this study and other culture-based studies in this dissertation, is that many microbial taxa could be phenotypically indistinct, yet are actually physiologically and genetically distinct. Consequently, unique microbial taxa may have been overlooked when colonies were picked based on morphological differences. To overcome this challenge, researchers would need to isolate and sequence all potential colony-forming units on a plate, which would be extremely time consuming. One way to overcome this challenge, as well as address the fact that some microbial taxa are slow growing and therefore outcompeted on a petri plate or have very unique growth requirements that are not captured using standard culture-based techniques, is to use single cell sorting to isolate individual microbial cells. For example, this technique was previously used to isolate rare endophytic microbial taxa in *Populus* roots and conduct whole genome sequencing on a few of these unique isolates (Utturkar, Cude et al. 2016). Other innovative technologies with potential to help overcome the challenge associated with culturing unculturable microbes and evaluating their potential function include co-cultivation studies such as those that employ an "i-chip" (Berdy, Sppoering et al. 2017) or membrane systems (Ferrari, Winsley et al. 2008), which may require co-factors produced by other microbes or environmental signals to grow, as well as CHIP-SIP (Mayali, Weber et al. 2012), and Raman excitation with optical tweezers (Huang, Ward et al. 2009).

6.6 Relevance of Dissertation Results

The research described in this dissertation will significantly contribute to scientific literature by providing further evidence that: 1) carrot seeds and taproots are colonized by a diverse assortment of endophytic microbial taxa; 2) seed-borne endophytes are key components of carrot microbiomes and these microbes may have the potential to aid in germination and early seedling vigor; 3) crop management practices that alter soil microbial community structures are critical to

the composition of carrot root endophytes, particularly those with the potential to help plants withstand stress imposed by *A. dauci*; 4) endophytic microbiomes in carrot taproots are influenced by carrot genotypes, which could have important implications for varietal selection and breeding; 5) coupling enzymatic digestion with microfiltration has the potential to enhance recovery of culturable endophytes in carrot taproots; and 6) NGS-sequencing tools have the potential to help researchers better characterize carrot root microbiomes, though additional work is needed to identify the best approach for amplifying bacterial endophytes and improving taxonomic resolution of endophytes using these tools. Results of these studies can provide immediate benefit to carrot growers, by demonstrating that management practices that improve soil health have the potential to reduce disease incidence, thereby resulting in greater productivity and reducing the need for pesticides that could have negative environmental effects. Soil microbial ecologists can use the results of these studies to design new experiments that will uncover factors affecting plant microbial community assembly and the sppecific mechanisms by which beneficial endophytes use to aid in carrot establishment and resistance against pathogens. Knowing that carrot genotypes differ in their composition and respponse to beneficial microbial endophytes, carrot breeders can begin to identify ways to integrate selection for these beneficial relationships in breeding programs. Finally, further research related to factors affecting the composition of seed microbiomes could have important implications for the seed industry and growers by aiding in carrot germination and establishment.

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