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BACTERIAL INOCULANTS, ENDOPHYTIC BACTERIA AND THEIR INFLUENCE ON *NICOTIANA* PHYSIOLOGY, DEVELOPMENT AND MICROBIOME

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BACTERIAL INOCULANTS, ENDOPHYTIC BACTERIA AND THEIR INFLUENCE ON *NICOTIANA* PHYSIOLOGY, DEVELOPMENT AND MICROBIOME

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

By
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2018

ABSTRACT OF DISSERTATION

BACTERIAL INOCULANTS, ENDOPHYTIC BACTERIA AND THEIR INFLUENCE ON *NICOTIANA* PHYSIOLOGY, DEVELOPMENT AND MICROBIOME

Soil and root microbial communities have been studied for decades, and the incorporation of high-throughput techniques and analysis has allowed the identification of endophytic/non-culturable organisms. This has helped characterize and establish the core microbiome of many model plant species which include underground and aboveground organs. Unfortunately, the information obtained from some of these model plants is not always transferable to other agronomic species. In this project, we decided to study the microbiome of the *Nicotiana* genus because of its importance in plant physiological and plant-microbe interactions studies. The data obtained was used as baseline information that allowed us to better understand the effect of microbial inoculums on the assembly of the microbiome of the plant. We analyzed 16s rRNA amplicons to survey the microbiome in different plant organs and rhizosphere from four different species. Bacterial strains evaluated were screened for a consistent reduction or improvement in plant growth. Four bacterial strains were tested and used as seed inoculum (Lf-*Lysinobacillus fusisormis*, Ms –*Micrococcus sp*., Bs–*Bacillus sp*., Bc–*Bacillus cereus*). Bs and Bc inoculants caused plant growth promotion, and in contrast Ms caused retarded growth, while Lf acted as a neutral or non-inducing phenotype strain. Data supported that microbial inoculum used as seed treatment caused systemic changes in the host plant microbiome. Functionality of the inoculum was studied and the response in plant growth was linked to hormonal changes (evaluated in the plant and in the bacterial strains). Gene expression analysis using a genome-scale approach revealed that genes that could possibly be involved in stress response are down-regulated for Bc and Bs treatments and up-regulated for Ms. Flexibility variability of the inoculum was also evaluated to have a better understanding of the main factors involved in the promotion or suppression of growth, and possibly its effect in following generations. In summary, the findings of this project support that the plant functional microbiome responds to exogenous stimulation from abiotic and biotic factors by adapting endogenous hormone responses.

Key words: *Nicotiana benthamiana*, microbiome, core, inoculums, morphological traits, PAT-Seq, high-throughput, hormones

Andrea M. Sanchez Barrios

04/09/2018

BACTERIAL INOCULANTS, ENDOPHYTIC BACTERIA AND THEIR INFLUENCE ON *NICOTIANA* PHYSIOLOGY, DEVELOPMENT AND MICROBIOME

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04/09/2018

To my family, my greatest treasure

To my husband and best friend, Ezequiel

To my beloved grandmother, Maria Concepcion Manzanilla and my godfather Dante Barros, two of my biggest career fans and angels

To my country, always in my heart

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Abstract

The term microbiome refers to the existence of multiple microbial genomes present in an environment in an association with a host. With the development of more precise sequencing approaches, identification of genus and families that were uncultivable microbes has been made possible. The current chapter explores the importance of understanding microbial communities and their association with agricultural production systems with particular attention to endophytic microorganisms. Agri-management practices and their relationship to the selection of microbial variation of taxa by plants and soil have been discussed in detail. The article also discusses how farming practices such as cover cropping and mulching mediates microbial community dynamics. Future perspectives on advancing sustainability by microbiome optimization are discussed.

Keywords: Soil, Microbiome, Plant growth, Expansion, Endophyte, Organic

1.1 Evolving concepts of the plant microbiome

1.1.1 General

The soil is a complex environment where there is a vast mix of organic matter, minerals, nutrients, gases, among others, enclosing a myriad of organisms –micro and macro- that are capable of supporting and retarding plant life and growth. The heterogeneity that exists in these environments is controlled by a series of biological and ecological interactions combined by soil properties, allow for the proliferation and establishment of certain groups of microbial organism, changing the dynamics of the ecosystem (Gale et al., 2000).

The importance of understanding microbial communities and their association with agricultural production systems lies on the premises of a future with more sustainable approaches to challenges in agriculture.

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Although many efforts have been directed towards a better understanding on how these microbial communities work, there are still a great number of questions related to the most influential factors dictating the identity or core participants, the diversity and niche specificity, establishment and maintenance of association with plants and retrograde signaling networks that could functionalize associations.

1.1.2 Looking deeper into the plant microbiome using developing technologies

The term microbiome refers more to the existence of multiple microbial genomes present in an environment in an association with a host. For the purpose of this chapter, we are focused on the plant bacterial microbiome in and agricultural context. The soil microbial community has received an abundance of attention over past decades, but the broader plant microbiome includes organisms that dwell in the phyllosphere, inside the plant as endophytic organisms as well as those in the rhizosphere and soil. Bacterial organisms are classified as endophytic if they inhabit plant tissue during its life cycle. In contrast, some rhizospheric bacteria colonize plants as opportunistic organisms that interact at some point with the plant but don't inhabit it in an obligate manner. An interest in endophytes, particularly obligate endophytes and the benefits they are able to confer to plants, and how some of these changes may be transferred genetically have emerged recently.

Recent advances in sequencing technology have advanced our understanding of this community (Lundberg et al., 2012; Bulgarelli et al., 2012; Wagner et al., 2016). In terms of the plant microbiome and its relationship to agricultural production, studies have proved that the presence of certain groups of organisms are capable of processing and absorbing nutrients (Manzoni et al., 2008) rendering them available for plant growth (Schardl et al., 2004; Barrow et al., 2008; Xia et al., 2013), repression of disease and the capacity to mediate the impact of extreme environmental stress factors (Plett and Martin, 2011). What remains complicated is how to foment the presence of those beneficial groups and how they could be used for improvement of many important agronomical crops. Indeed, it will important to establish how soil conditions and agronomical practices affect the selection of these microbial organisms by the plant. Agri-management practices and their relationship to the selection for variation of taxa by plants and soil is the main reason for the development of this chapter. We will be looking at how managing practices could be important when trying to understand the strength or

weaknesses of these relationships, since they are able to influence the development and dominance of a bacterial community.

1.2 The microbiome and agriculture

The interaction between plants and individual microorganisms has been studied for the last several decades. Isolation and testing of strains present in soil and plants have largely aimed to understand the capacity that these microorganisms have for plant improvement or pathogenicity. Until the last 5 years, most of the isolation and identification was done via culture-dependent techniques. However, with the development of more precise sequencing approaches, identification of genus and families that were unculturable has been made possible, even to the point of looking at functional genes (Tsurumaru et al., 2015). These advances have provided more insight into the selection and structure of bacterial communities by plants under different environments (Lundberg et al., 2012; 2013; Lebeis et al., 2015; Birtel et al., 2015; Ding and Melcher, 2016). Identifying the variability as well as functionality of communities that colonize plants could be used to select for bacteria (or groups of bacterial community members) that can positively modify the plant morphology or interaction with its environment. Despite the attractiveness of being able to inject a single or collection of microorganisms into an agricultural production system to enhance crop performance, there are many reasons that this will be challenging in practice. The complexity of the microbial community and competitiveness of a single microbial factor is unlikely to be dominant enough to sustain any influence on a cropping system. Furthermore, the ability to genetically optimize or engineer microbes to enhance agricultural systems will be a regulatory and environmental containment challenge. As related to agricultural production systems, the notion that understanding the plant microbiome and how it functions and then adapting our management practices to maximize the most interesting members of the microbiome is perhaps the most rational area for future work. Furthermore, plant breeding has not taken into account any influence of a microbiome and it remains possible that the intersection between plant breeding and microbiome functionality will be a fruitful area for research (Gopal and Gupta, 2016). Finally, knowledge of the mechanisms by which a microbiome element influence the plant anatomy is still developing and should shed light on hormonal networks and functional gene networks influenced by the microbiome.

How a bacterial microbiome colonizes and establishes itself in living plant tissue will involve not just the physical entry into the plant but also how to avoid the plant immune system (friend versus foe association) (Downie et al., 1999; Iniguez et al., 2005). As the field of microbial inoculums matures it will be important to understand the complexity of this association window and whether it is under passive or active control by the host plant. It is expected that numerous non-obligate bacterial genera enter the plant during germination and seedling establishment. As the main contact point for the plant with the microbe rich soil, microbes are thought to enter into their host (plant) through the root system due to their vast adhering area with soil particles (Hansen et al., 1997; Tokala et al., 2002; Iniguez et al., 2005; Rosenblueth and Martinez-Romero 2006; Seipke et al., 2012) (Figure 1). The rhizosphere is the area that is described as the zone of the soil that is subjected to the influence of the roots. At the same time, another term that will be highly important to mention while talking about entrance of microbial organism to the plant is the spermosphere. This is related to the seed exterior layers that are in contact with the soil and over which microbes will be interacting before germination.

1.3 Insection between agricultural management practices and microbiome

It seems that through the use of culturing techniques and next generation sequencing, there have been signs that show higher amounts of organisms being identified, as well as more consistent phyla types of endophytic microbes being present when looking into microbiome elements in organic production systems when compared to conventional farming practices (Xia et al., 2015; Schlaeppi et al., 2015; Hartmann et al., 2015). The reasons behind those differences among bacterial communities still remain slightly unclear, but data supporting increased soil microbial diversity in organically managed soils have been well documented (Wang et al., 2016). More work has been put towards the elucidation of the effect that the systems may have on the selection of the taxa present in the soil. These results supported findings by Soltani et al., (2010) and Bacon et al., (2016) that many endophytic bacterial genotypes increased plant growth and induced a defense system with low cost.

As mentioned before, the differences found among isolates identified as endophytic microbial species comparing conventional and organic crops are of interest as they may be linked to the crop productivity. Since one of the main goals is to be able to replicate these environments for crop enhancements, or at least to influence selection by plants

towards some of these communities. Hall et al., (1990) suggest that certain Bacilli move through the plant using the vascular system rather than symplastic movement. Base on physiological aspects, the older the plant may be, the harder will be for certain endophytic bacteria to translocate from tissue type to tissue and therefore it is anticipated that as we develop a more sophisticated understanding of tissue type endophyte colonization, we may see different levels of abundance or community members. Some research supports that age of the plant may not be one of the limitations for the colonization of obligated bacteria when tissue type was held consistent (roots) (Lundberg et al., 2012). This could be due to the fact that some of these endophytes may be present at early stages and stay there, and that the variation of the presence or absence of other species may be related to those that are not strictly necessary to inhabit the plant. Interestingly, it was found by Lundberg et al. (2012) that genotype was a critical determinant in root microbiome community analysis suggesting that the intersection between breeding and agricultural farming practices may be critical for future work.

An interesting concept to examine is how farming practices and the types of crops that are being produced display variance in microbial community metrics. For instance, cover cropping, mulching and soil composition (Kumar et al., 2014), the use of alternative tillage systems (Carbonetto et al., 2014) and overall soil nutrient composition (Stagnari et al., 2014) have an impact in the structure and composition of the soil microbial communities. Carbonetto and co-workers (2014) suggested that soils exposed to high use of fertilizers displayed a shift in the metabolic strategies used by the microbial communities which exasperated community shifts. Metabolism seems to also become more “flexible” for those organisms that were present under tillage practices vs those in non-till areas, but the metabolic flexibility does not mean that they were better adapted, on the contrary, they showed that if conditions were considered unfavorable (example: lower nutrient content in soil) some of those microbial organism are unlikely to adapt, which differed from the non-tillage system. Similar results were found in cotton crops that were maintained under conventional tillage and no-tillage (Feng et al., 2003). It seems like the use of non-tillage, for example, and not so many applications of fertilizers, among other things can have a positive effect in microbial communities in the soil. Kennedy and Smith (1995) support that heavy tillage as a farming practice can be negative for microbial diversity and abundance by the alteration of the properties of the

soil. Overall, high population and biodiversity of microorganisms in the soil is an indicator of soil health. Healthy soil has a normal amount of aggregation and percent of air, water and nutrients; thus, the soil does not need many fertilizers or pesticides to increase plant productivity or to control stresses as the plant will be tolerant (Paul, 2007). This parlays with good farming practices, not necessarily organic versus conventional practices.

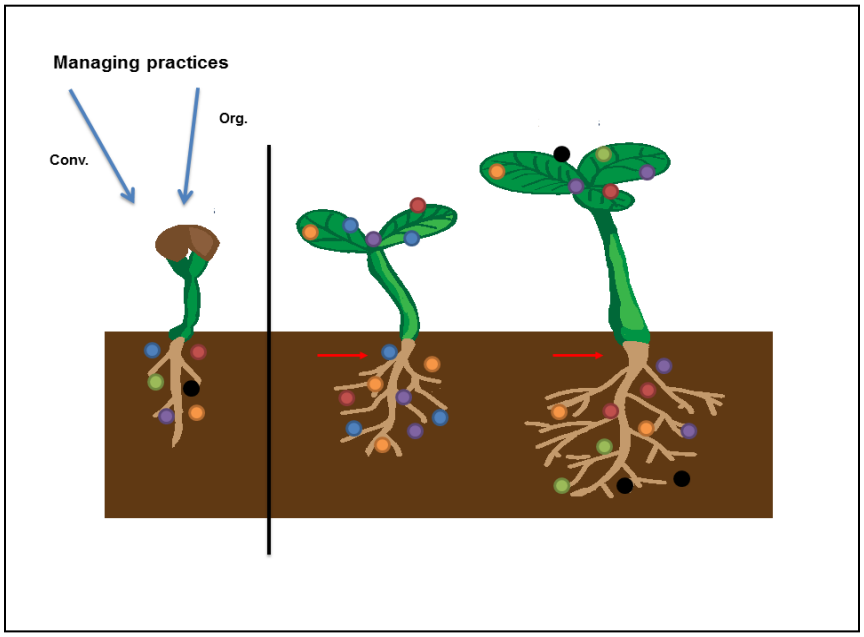


Figure 1.1 Schematic of the microbiome. Image of a broadleaf seedling planted (left) and conceptualizing the overlay of management practices. As the seedling grows, bacterial community members from the soil, which are represented as orange, blue, purple and red dots occupy various components of the root (red arrow), phyllosphere (green components of plant aerial tissue).

Both practices, organic and conventional have systems that follow the application of chemicals to treat and maintain their crops during their production process. Some of the chemicals used tend to be more long lasting within the farming system than others and could have small but progressive impacts on an indigenous microbial community present in the soil. Thus, when comparing results in this area, one must consider numerous environment and cultural factors that vary greatly and are different to compare. A question remains whether the use of pesticides affect microbial communities in the soil in a non-target manner and in turn influence the selection of the plant microbiome? Even though pesticides are made to target insects and other types of organism that have no relationship with the fungi or bacteria present in soil, it is feasible that in a more

individual scale some species in particular may be affected (Foley et al., 2005). To date, further research is needed on a case by case basis to interrogate this postulate.

Herbicides or the surfactants used in their application to a target crop may also have an impact in the microbial communities since some of these, for instance octylamines can be slightly bacteriotoxic (https://www.echa.europa.eu/sv/web/guest/registration-dossier/-/registered-dossier/1996/7/7/2) but are non-target and have been unstudied as environmental risk factors in agricultural microbiome systems. Other herbicidal or pesticidal molecules will remain in the soil (predominantly in conventional systems) for years, for example the pre-emergent herbicide used on railroad lines indaziflam (Brabham et al., 2013) has an extremely long residual time. While off target influences of commercially available pesticides and herbicides are typically non-lethal and modest, if a product can be mildly class specific bacteriotoxic, it can easily be envisioning how this could shift the balance in an agricultural crop microbiome (Wilkinson and Lucas, 1969). To date, we have an insufficient understanding of this.

It is important to take in consideration that long time exposure to a specific managing practice could alter the soil environment by a simple selection mechanism. It seems that although change is part of both systems, organic farming may be a better option to also increase richness, among others, by shifting the structure of the microbiota compared to conventional practices (Hartmann et al., 2015). Still, more parameters and variables need to be tested to fully confirm these hypotheses and address better the full impact that these practices have on the microbial communities' structure (Hartmann and Widmer, 2006).

1.4 Employing microbial elements in agricultural systems

It is known that obligated microbes have to follow usually a more elaborated process for their colonization. They can be considered pathways, which usually ramify into production of supernatants, rates of production of them, quorum sensing, hormone metabolisms, among others. Supernatants are considered to be molecules produced and released either by the plant or bacteria to the rhizosphere (Li et al., 2016 PNAS). Some of the molecules present in these supernatants are a combination of: sugars, amino acids, alkaloids, flavonoids, among others (Biedrzyckiet al., 2010; Kumar and Bais, 2012). Rates of the exudate production can also have an impact on how the plant

selects the microbes from the rhizosphere. Now, the fact that some microbes are capable to produce their own chemicals and modulate the communication with the plant through molecule signaling, it is probably one of the future uses of studying the microbiomes of different systems. Indeed, some endophytic microbiome elements have been used to identify target herbicides in plants (Xia et al., 2014). The idea will be to find ways into isolating, producing or stimulating the production of these chemicals for the manipulating of the selection power of the plant and at least inhabit it for a small time frame (or long, depending on the effect that has in the host development and health). It may be suitable to bypass the microbial soil feature and grow it in vitro to harvest the target chemical for organic farming purposes, which is already the case for *Bacillus thuringiensis*.

Promoting plant growth by manipulating microbiomes may have a modest capacity to support the positive traits in a cropping species, thus decreasing the use of synthetic chemicals or nutrients (Singh et al., 2010). Using microbes in agriculture as bio-fertilizers to and bio-pesticides has been well established, but lately it has received more attention, and scientists are currently focusing on the plant microbiome itself instead of just using microbes (Deake ret al., 2004). Using microbes is less practical than using synthetic chemicals because variation in soil and environmental conditions will almost certainly be a selection force and will therefore require regional solutions in agriculture. Modern agriculture has not accepted regionality of trait solutions from major crop biotechnology companies and therefore it is unclear whether microbial systems will be poorly accepted. Organic farmers may be more willing to work with such regional/environment specific products simply due to scale (Bacon et al., 2016).

There are select studies that show that application of bacterial isolates could support plant growth and productivity under specific conditions, possibly modulating plant microbiomes (Xia et al., 2015). However, these rarely translate from greenhouse or in vitro conditions to the field and even more rarely into a wide variety of agricultural-ecozones. The plant growth promoting fungal inoculum *Trichoderma sp*. is still the best example of a successful strategy for this (Altmore et al., 1999). It is hoped that the use of beneficial microbes in organic production system could buffer plant productivity by providing nutrients and other growth promoting compounds to the crop not only for a

short time but also for many seasons because this organic system maintains soil fertility and health.

Treatments and inoculation with bacterial organisms showed in Xia et al (2014) that plant cell walls were susceptible to the presence and production of certain chemicals (supernatants) by the bacteria. This is a good growth indicator during the interaction between plant and microbes because of the importance of plant cell wall, since it plays an essential role in being a barrier against stresses, connecting extracellular and intracellular environments and regulating plant growth. Their work also showed that the combination of techniques for identification and isolation were crucial for their selection of candidate strains and their capacity of inhabiting the plant during long periods of its life. Even though manipulating the microbiome is important to increase plant productivity, it is currently a challenge to adopt bacterial strains grown in a lab environment and implement their use in the farmers' fields. These artificially cultured "strains may lack key characteristics for widespread distribution in sustainable and productive agricultural systems" (Parnell et al. 2016). Most of the studies related to bacterial strains as an alternative to synthetic chemicals represent either lab or greenhouse experiments (Adesemoye et al., 2009). They do not represent the real environment that plants may be exposed in a farm setting (Parnell et al., 2016).

1.5 Conclusion

The overall outcome of studies into the functionality of the plant microbiome has been satisfactory to maintain research and agricultural interest. The compelling idea of establishing a more sustainable production system through increasing the abundance or functionality of members of a natural community is highly attractive and potentially cost effective. Several conclusions and future directions exist. A combined focus on plant breeding in association with detailed microbiome assessment is needed based on the genotype specificity identified in recent studies (Lundberg et al., 2012). Organic farming systems are modestly less likely to drive selection on the microbiome community due to their inherent focus on soil quality rather than external inputs. Because genotype and environmental conditions both influence the microbiome in plants, long-term studies are needed across numerous species and eco-zones to adequately assess results.

Chapter 2: Plant microbiome of *Nicotiana* species

2.1 Introduction

Soil is a complex environment where a vast diversity of organisms constantly interacts. Soil and root microbial communities have been studied for decades (Lauber et al., 2013), but a more in-depth analysis of community structure was not possible until the last five years, when the development of high-throughput culture-independent sequencing provided a more holistic view and a new approach to studies of the plant microbiome. New technologies have allowed the characterization of the core microbiome of many model plant species -including underground and aboveground organs- (Lundberg et al., 2012; Bodenhausen et al., 2013; Coleman-Derr et al., 2016).

Some of the highlights from the research done in this field are the understanding of the importance and role that indigenous microbial communities have in the host plant, altering its development, health and response to environmental changes (Mendes et al., 2011; Sugiyama et al., 2013). The microbiome has been analyzed from multiple aspects using the data available up to date, establishing connections among host-microbiome and microbial organisms with other microbes, allowing a better understanding of the effect that they have as a community and the importance of their presence (Raaijmakers et al., 2016).

Unfortunately, the information obtained from some of these model plants is not always transferable to crops of interest. To date, there are a limited number of plant species for which both above and below ground microbiota have been described, making it harder to understand how the whole plant microbiome assembles. As of today, *Nicotiana* species are increasingly becoming tools for biotechnology research, as they serve as a great model organism that allow us to evaluate laboratory and greenhouse approaches to a more field environment, with a more robust genetic information available when compared to other plant crops.

Because of the importance of the *Nicotiana* genus in plant physiology and in plant-microbe interactions studies (*Nicotiana benthamiana* to be specific) (Goodin et al., 2008; Bombarely et al., 2012), we decided to analyze and characterize a core microbiome for the genus. For *Nicotiana* species, there is no description of an established microbiome

from a single plant, but there are independent descriptions of the root and rhizosphere communities (Saleem et al., 2016) and composition of bacterial organisms present in leaves with different amounts of nitrosamines in *Nicotiana tabacum* lines (Law et al., 2016).

Characterizing a microbiome provides information that can be used to design and develop studies to target more specific questions, either for development of crop improvement or better understanding of biological processes. We hypothesized that the microbiome in the aboveground tissue might exhibit differences from the root alone when looking at different species, possibly arising from exposure to different biotic (Lugtenburg et al., 2009) and abiotic environmental pressures, genetic history and native habitat.

2.2 Methods and materials

2.2.1 Soil collection and species selected for genotype study

Topsoil from the University of Kentucky North Farm, Lexington, Kentucky (Spindletop Farm, (GPS coordinates: 38°07.555′N, 84°30.901′W) was collected, homogenized and mixed with perlite prior to use. Nutrient profiling of the soil was performed by core University of Kentucky Soil Regulatory Services. Selection of species used to establish the microbiome for *Nicotiana* genus was based on the contrast between domesticated vs non domesticated plants. Based on this, *Nicotiana benthamiana*, *Nicotiana glutinosa*, *Nicotiana rustica* and *Nicotiana tabacum* (KY14) were our final selection, and seeds were sourced from University of Kentucky Tobacco Research and Development Center (KTRDC). Seed germination of all species evaluated was performed to ensure that phenotypes observed would not be related to problems with seed viability.

2.2.2 Sample selection and processing

Plants were grown under greenhouse conditions for ~4-6 weeks (juvenile stage), with constant temperatures of ~25±3°C, and 16h of light followed by 8 h of dark. Plants were harvested at ~4 and 6 weeks following the methodology developed by Lundberg et al., (2012). All plants were harvested and processed the same day. Plants organs were aseptically removed and loose soil was manually removed from the roots by kneading, shaking and patting with sterile gloves (sprayed with 70% EtOH). Roots were place in clean and sterile 50ml falcon tubes, place in a cooler with ice and transported to the

laboratory for further processing. Samples were washed with di water to remove debris, and then placed in a clean and sterile 50ml tube containing 25ml of 95% ethanol (EtOH) for 2 min, then immersed into a solution of 30% Clorox (household grade) for 5-10min, and then rinsed with sterile di water for a total of 10 times. A total of 5 plants per species were grown and 4 were randomly selected for further DNA extraction. Segments of sterile plant organs were frozen by adding liquid nitrogen and then stored in -80°C until DNA were performed.

From the two lines of *Nicotiana tabacum*, only KY14 was selected to represent the more domesticated specie, keeping *N. benthamiana*, *N. glutinosa* and *N. rustica* as wild species to evaluate or non-domesticated (Figure 2.1)

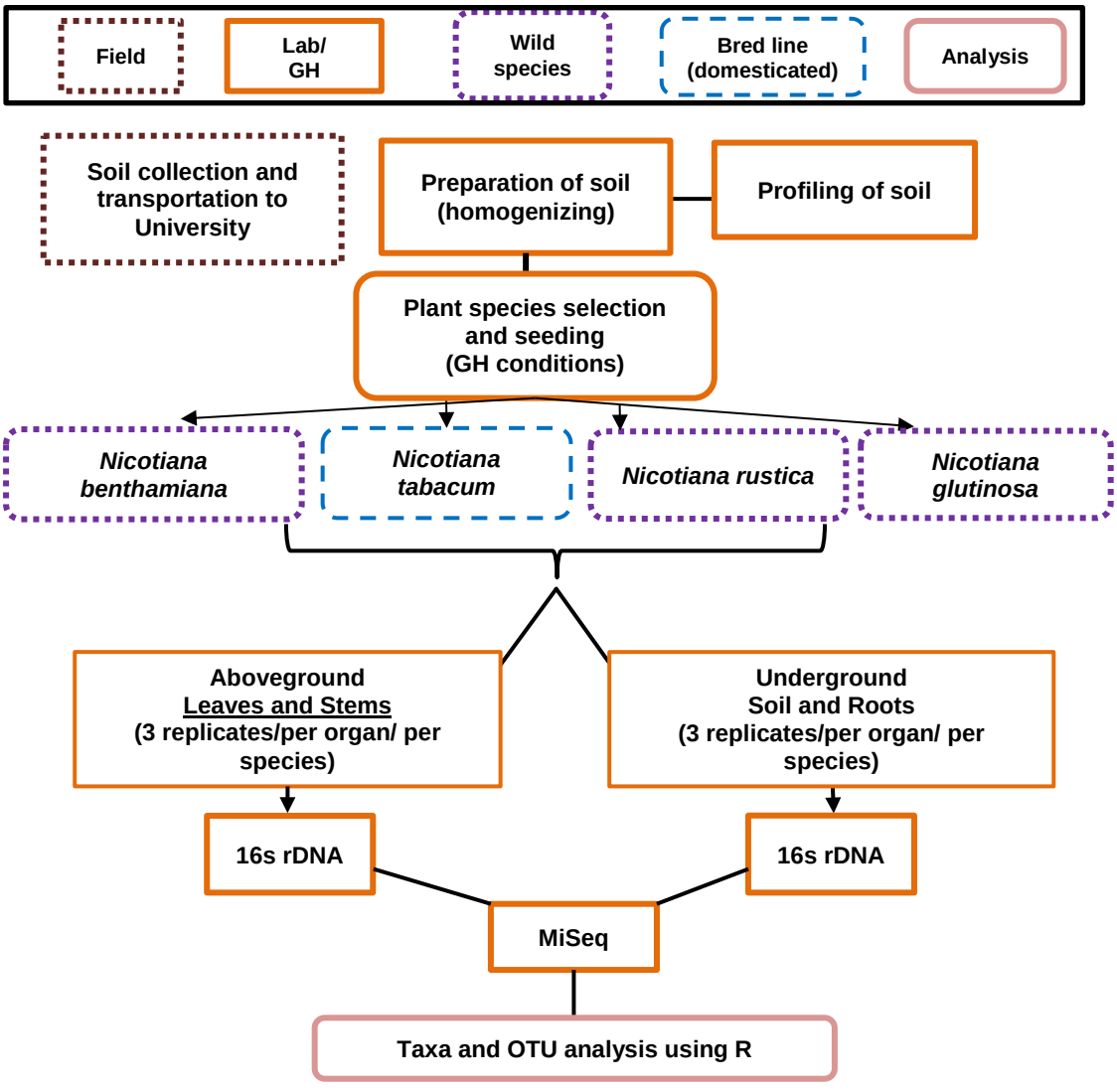


Figure 2.1 Summary of experimental design for genotype analysis.

2.2.3 DNA extractions and library preparation

DNA extraction was performed using FASTDNA™-96 Soil Microbe DNA Kit (MP Biomedical, LLC). After extraction, libraries were prepared following the protocol established by Lundberg et al., (2013), where Peptide Nucleic Acid (PNA) for mitochondrial (5'-GGCAAGTGTTCTTCGGA-3') and plastid (5'-GGCTCAACCCTGGACAG-3') 16s rRNA and plastid sequences were used as elongation arrest clamps to prevent ribosomal 16s from the plant of being amplified.

Library amplification was performed by following three different steps: Reverse molecular tagging, forward molecular tagging, and polymerase chain reaction (PCR) with barcoded primers. Primers utilized for library preparation were Ultramers™ from IDT, purified by standard desalting. Diagram of forward, reverse and barcoded are described in Table 2.1.

Template DNA was tagged with MT-FS primers in two separated reactions. Reverse tagging step was performed by using where each working stock had an equimolar mix of three primers to give a total concentration of the mixed stock was 0.5µM. High-fidelity Kapa Robust Taq (Kapa Biosystems) was used and included in a final mix for 25µL reaction that was prepared on ice in which we had: 5µL Kapa Enhancer, 5µL Kapa Buffer A, 2µL 0.5uM reverse primer mix, 0.5µL Kapa dNTPs, 0.25µL Kapa Robust Taq, DNA + water to 25µL.

Incubation of samples were done in a thermocycler using a program of denaturing at 95°C for 1min, reverse MT-FS primer annealing at 50°C for 2min, and extension at 72°C for 1min, followed by a cool down to 4°C. Following this step, the obtained template, was cleaned with Agencourt AMPure XP beads (Beckman Coulter) using the manufacturers' protocol with a modification in the bead to DNA ratio (instead of 1:1 ratio we used a 0.6: 1). DNA was eluted the DNA in 11µL water.

For the forward tagging we used the working stocks that contained a mix of three different forward primers in equimolar concentrations (same as for reverse tagging). The use of PNA was necessary to include in this step. The reaction used for this second tagging step was: 5µL Kapa Enhancer, 5µL Kapa Buffer A, 2µL 0.5uM forward-tagging

primer mix, 0.5µL Kapa dNTPs, 0.25µL Kapa Robust Taq, 2.5µL PNA working stock (mPNA and pPNA) and 10µL of the reverse-tagged DNA from the previous step.

Samples were incubated in a thermocycler using a program of denaturing at 95°C for 1min, PNA annealing at 78°C for 10s, forward tagging-primer annealing at 50°C for 2min, and extension at 72°C for 1min, followed by a cool down to 4°C. DNA tagged with both forward and reverse-tagging primers, was cleaned with Agencourt beads using a bead:DNA ratio of 0.7 : 1. Elution was done in 16µL water.

Last, PCR was performed by using a 50µL reaction mix containing a reverse primer which differed for each individually-barcoded sample. The mix contained: 25µL 2x KAPA HiFi Ready Mix (Kapa Biosystems), 2.5µL PCR_F forward primer, 2.5µL PCR_R_bc reverse primer, 5µL mixed PNA working stock, 15µL DNA from the last tagging step.

The PCR program was denaturing at 95°C for 45s followed by 34 cycles of denaturation at 95°C for 15s, PNA annealing at 78°C for 10s, primer annealing at 63°C for 30s, and extension at 72°C for 30s, ending with a cool down to 4°C. All amplicons that were obtained were cleaned with Agencourt beads using 0.7:1 ratio. DNA was eluted in 50µL of di water.

Table 2.1 List of reverse, forward and barcoded primers.

Reverse barcoded primers, 5-3

Name	Sequence
Ind1_MiSeq	CAAGCAGAAGACGGCATACGAGATTTACCGACGGTGACTGGAGTTCAGACGTGTGCTC
Ind2_MiSeq	CAAGCAGAAGACGGCATACGAGATATTGGACACGTGACTGGAGTTCAGACGTGTGCTC
Ind3_MiSeq	CAAGCAGAAGACGGCATACGAGATTCGCATGGAGTGACTGGAGTTCAGACGTGTGCTC
Ind4_MiSeq	CAAGCAGAAGACGGCATACGAGATAGCGAACCTGTGACTGGAGTTCAGACGTGTGCTC
Ind5_MiSeq	CAAGCAGAAGACGGCATACGAGATAGCTTCGACGTGACTGGAGTTCAGACGTGTGCTC
Ind6_MiSeq	CAAGCAGAAGACGGCATACGAGATGTCAGCCGTGTGACTGGAGTTCAGACGTGTGCTC
Ind7_MiSeq	CAAGCAGAAGACGGCATACGAGATTCCAGATAGGTGACTGGAGTTCAGACGTGTGCTC
Ind8_MiSeq	CAAGCAGAAGACGGCATACGAGATGAGAGTCCAGTGACTGGAGTTCAGACGTGTGCTC
Ind9_MiSeq	CAAGCAGAAGACGGCATACGAGATGCTCACAATGTGACTGGAGTTCAGACGTGTGCTC
Ind10_MiSeq	CAAGCAGAAGACGGCATACGAGATTTGACGACAGTGACTGGAGTTCAGACGTGTGCTC

Table 2.1 List of reverse, forward and barcoded primers (continued)

Ind11_MiSeq	CAAGCAGAAGACGGCATACGAGATCTTAGAACGGTGACTGGAGTTCAGACGTGTGCTC
Ind12_MiSeq	CAAGCAGAAGACGGCATACGAGATCGGTTCACAGTGACTGGAGTTCAGACGTGTGCTC
Ind13_MiSeq	CAAGCAGAAGACGGCATACGAGATCGATAGGCCGTGACTGGAGTTCAGACGTGTGCTC
Ind14_MiSeq	CAAGCAGAAGACGGCATACGAGATGCTATATCCGTGACTGGAGTTCAGACGTGTGCTC
Ind15_MiSeq	CAAGCAGAAGACGGCATACGAGATGTCTTCAGCGTGACTGGAGTTCAGACGTGTGCTC
Ind16_MiSeq	CAAGCAGAAGACGGCATACGAGATTAGACACCGGTGACTGGAGTTCAGACGTGTGCTC
Ind17_MiSeq	CAAGCAGAAGACGGCATACGAGATTCAGCTGACGTGACTGGAGTTCAGACGTGTGCTC
Ind18_MiSeq	CAAGCAGAAGACGGCATACGAGATTAAGTCGGCGTGACTGGAGTTCAGACGTGTGCTC
Ind19_MiSeq	CAAGCAGAAGACGGCATACGAGATGCTCCTTAGGTGACTGGAGTTCAGACGTGTGCTC
Ind20_MiSeq	CAAGCAGAAGACGGCATACGAGATATGGCCTGAGTGACTGGAGTTCAGACGTGTGCTC
Ind21_MiSeq	CAAGCAGAAGACGGCATACGAGATTTGCAAGTAGTGACTGGAGTTCAGACGTGTGCTC
Ind22_MiSeq	CAAGCAGAAGACGGCATACGAGATCCTAGTAAGGTGACTGGAGTTCAGACGTGTGCTC
Ind23_MiSeq	CAAGCAGAAGACGGCATACGAGATCTAGGATCAGTGACTGGAGTTCAGACGTGTGCTC
Ind24_MiSeq	CAAGCAGAAGACGGCATACGAGATTATGAACGTGTGACTGGAGTTCAGACGTGTGCTC
Ind25_MiSeq	CAAGCAGAAGACGGCATACGAGATCTTGTGCGAGTGACTGGAGTTCAGACGTGTGCTC
Ind26_MiSeq	CAAGCAGAAGACGGCATACGAGATCACGATGGTGTGACTGGAGTTCAGACGTGTGCTC
Ind27_MiSeq	CAAGCAGAAGACGGCATACGAGATACGTGCCTTGTGACTGGAGTTCAGACGTGTGCTC
Ind28_MiSeq	CAAGCAGAAGACGGCATACGAGATTGAACTAGCGTGACTGGAGTTCAGACGTGTGCTC
Ind29_MiSeq	CAAGCAGAAGACGGCATACGAGATTATTCAGCGGTGACTGGAGTTCAGACGTGTGCTC
Ind30_MiSeq	CAAGCAGAAGACGGCATACGAGATTAATCGGTGGTGACTGGAGTTCAGACGTGTGCTC
Ind31_MiSeq	CAAGCAGAAGACGGCATACGAGATGCGTCCATGGTGACTGGAGTTCAGACGTGTGCTC
Ind32_MiSeq	CAAGCAGAAGACGGCATACGAGATCGTAAGATGGTGACTGGAGTTCAGACGTGTGCTC
Ind33_MiSeq	CAAGCAGAAGACGGCATACGAGATCTGTTACAGGTGACTGGAGTTCAGACGTGTGCTC
Ind34_MiSeq	CAAGCAGAAGACGGCATACGAGATACGATCATCGTGACTGGAGTTCAGACGTGTGCTC
Ind35_MiSeq	CAAGCAGAAGACGGCATACGAGATGTAACGGCTGTGACTGGAGTTCAGACGTGTGCTC
Ind36_MiSeq	CAAGCAGAAGACGGCATACGAGATCCATGCTTAGTGACTGGAGTTCAGACGTGTGCTC
Ind37_MiSeq	CAAGCAGAAGACGGCATACGAGATGTACGCACAGTGACTGGAGTTCAGACGTGTGCTC
Ind38_MiSeq	CAAGCAGAAGACGGCATACGAGATTTAGAGCCAGTGACTGGAGTTCAGACGTGTGCTC
Ind39_MiSeq	CAAGCAGAAGACGGCATACGAGATATAAGGTCGGTGACTGGAGTTCAGACGTGTGCTC
Ind40_MiSeq	CAAGCAGAAGACGGCATACGAGATAGTGGCACTGTGACTGGAGTTCAGACGTGTGCTC
Ind41_MiSeq	CAAGCAGAAGACGGCATACGAGATCCAGAAGTGGTGACTGGAGTTCAGACGTGTGCTC
Ind42_MiSeq	CAAGCAGAAGACGGCATACGAGATCTACTAGCGGTGACTGGAGTTCAGACGTGTGCTC
Ind43_MiSeq	CAAGCAGAAGACGGCATACGAGATTAGCGTTCCGTGACTGGAGTTCAGACGTGTGCTC

Table 2.1 List of reverse, forward and barcoded primers (continued)

Ind44_MiSeq	CAAGCAGAAGACGGCATACGAGATGTGAGTCATGTGACTGGAGTTCAGACGTGTGCTC
Ind45_MiSeq	CAAGCAGAAGACGGCATACGAGATTGGTCCTACGTGACTGGAGTTCAGACGTGTGCTC
Ind46_MiSeq	CAAGCAGAAGACGGCATACGAGATTACGCGTACGTGACTGGAGTTCAGACGTGTGCTC
Ind47_MiSeq	CAAGCAGAAGACGGCATACGAGATGAGCCATCTGTGACTGGAGTTCAGACGTGTGCTC
Ind48_MiSeq	CAAGCAGAAGACGGCATACGAGATCGTCCGTATGTGACTGGAGTTCAGACGTGTGCTC
Ind49_MiSeq	CAAGCAGAAGACGGCATACGAGATGATACGTTCGTGACTGGAGTTCAGACGTGTGCTC
Ind50_MiSeq	CAAGCAGAAGACGGCATACGAGATCAGCTGGTTGTGACTGGAGTTCAGACGTGTGCTC
Ind51_MiSeq	CAAGCAGAAGACGGCATACGAGATTTAAGCGCCGTGACTGGAGTTCAGACGTGTGCTC
Ind52_MiSeq	CAAGCAGAAGACGGCATACGAGATCCTGCGAAGGTGACTGGAGTTCAGACGTGTGCTC
Ind53_MiSeq	CAAGCAGAAGACGGCATACGAGATTTGTAGCCGGTGACTGGAGTTCAGACGTGTGCTC
Ind54_MiSeq	CAAGCAGAAGACGGCATACGAGATTCTGTAGAGGTGACTGGAGTTCAGACGTGTGCTC
Ind55_MiSeq	CAAGCAGAAGACGGCATACGAGATCTATTAAGCGTGACTGGAGTTCAGACGTGTGCTC
Ind56_MiSeq	CAAGCAGAAGACGGCATACGAGATCTCTGAGGTGTGACTGGAGTTCAGACGTGTGCTC
Ind57_MiSeq	CAAGCAGAAGACGGCATACGAGATCAGGATTCGGTGACTGGAGTTCAGACGTGTGCTC
Ind58_MiSeq	CAAGCAGAAGACGGCATACGAGATTCACTGCTAGTGACTGGAGTTCAGACGTGTGCTC
Ind59_MiSeq	CAAGCAGAAGACGGCATACGAGATACATGTCACGTGACTGGAGTTCAGACGTGTGCTC
Ind60_MiSeq	CAAGCAGAAGACGGCATACGAGATATTCTGCCGGTGACTGGAGTTCAGACGTGTGCTC
Ind61_MiSeq	CAAGCAGAAGACGGCATACGAGATTACACGCTGGTGACTGGAGTTCAGACGTGTGCTC
Ind62_MiSeq	CAAGCAGAAGACGGCATACGAGATTGCATACACGTGACTGGAGTTCAGACGTGTGCTC
Ind63_MiSeq	CAAGCAGAAGACGGCATACGAGATACGCAATGTGTGACTGGAGTTCAGACGTGTGCTC
Ind64_MiSeq	CAAGCAGAAGACGGCATACGAGATGCTCGAAGAGTGACTGGAGTTCAGACGTGTGCTC
Ind65_MiSeq	CAAGCAGAAGACGGCATACGAGATAGACGTTGCGTGACTGGAGTTCAGACGTGTGCTC
Ind66_MiSeq	CAAGCAGAAGACGGCATACGAGATTAGAGCTGCGTGACTGGAGTTCAGACGTGTGCTC
Ind67_MiSeq	CAAGCAGAAGACGGCATACGAGATGGTAACCTCGTGACTGGAGTTCAGACGTGTGCTC
Ind68_MiSeq	CAAGCAGAAGACGGCATACGAGATGACTTCATGGTGACTGGAGTTCAGACGTGTGCTC
Ind69_MiSeq	CAAGCAGAAGACGGCATACGAGATCTGCATACTGTGACTGGAGTTCAGACGTGTGCTC
Ind70_MiSeq	CAAGCAGAAGACGGCATACGAGATTAAGGCATCGTGACTGGAGTTCAGACGTGTGCTC
Ind71_MiSeq	CAAGCAGAAGACGGCATACGAGATAGTATTCGCGTGACTGGAGTTCAGACGTGTGCTC
Ind72_MiSeq	CAAGCAGAAGACGGCATACGAGATTTCGCAGATGTGACTGGAGTTCAGACGTGTGCTC
Ind73_MiSeq	CAAGCAGAAGACGGCATACGAGATGCACCTGTTGTGACTGGAGTTCAGACGTGTGCTC
Ind74_MiSeq	CAAGCAGAAGACGGCATACGAGATCTCATGGTAGTGACTGGAGTTCAGACGTGTGCTC
Ind75_MiSeq	CAAGCAGAAGACGGCATACGAGATACTAGTTGGGTGACTGGAGTTCAGACGTGTGCTC
Ind76_MiSeq	CAAGCAGAAGACGGCATACGAGATGCGGACTATGTGACTGGAGTTCAGACGTGTGCTC

Table 2.1 List of reverse, forward and barcoded primers (continued)

Ind77_MiSeq	CAAGCAGAAGACGGCATACGAGATATCGCTTAAGTGACTGGAGTTCAGACGTGTGCTC
Ind78_MiSeq	CAAGCAGAAGACGGCATACGAGATTCAGGACGTGTGACTGGAGTTCAGACGTGTGCTC
Ind79_MiSeq	CAAGCAGAAGACGGCATACGAGATGCATTACTGGTGACTGGAGTTCAGACGTGTGCTC
Ind80_MiSeq	CAAGCAGAAGACGGCATACGAGATGCTATGGAAGTGACTGGAGTTCAGACGTGTGCTC
Ind81_MiSeq	CAAGCAGAAGACGGCATACGAGATGATTGTGCAGTGACTGGAGTTCAGACGTGTGCTC
Ind82_MiSeq	CAAGCAGAAGACGGCATACGAGATAGCCTCATGGTGACTGGAGTTCAGACGTGTGCTC
Ind83_MiSeq	CAAGCAGAAGACGGCATACGAGATAACTCCTGTGTGACTGGAGTTCAGACGTGTGCTC
Ind84_MiSeq	CAAGCAGAAGACGGCATACGAGATTAGAAGGCTGTGACTGGAGTTCAGACGTGTGCTC
Ind85_MiSeq	CAAGCAGAAGACGGCATACGAGATGACTAGTCAGTGACTGGAGTTCAGACGTGTGCTC
Ind86_MiSeq	CAAGCAGAAGACGGCATACGAGATGGATACTCGGTGACTGGAGTTCAGACGTGTGCTC
Ind87_MiSeq	CAAGCAGAAGACGGCATACGAGATCCGACATTGGTGACTGGAGTTCAGACGTGTGCTC
Ind88_MiSeq	CAAGCAGAAGACGGCATACGAGATTCGTGACGCGTGACTGGAGTTCAGACGTGTGCTC
Ind89_MiSeq	CAAGCAGAAGACGGCATACGAGATGGCCTATAAGTGACTGGAGTTCAGACGTGTGCTC
Ind90_MiSeq	CAAGCAGAAGACGGCATACGAGATGTAGCACTCGTGACTGGAGTTCAGACGTGTGCTC
Ind91_MiSeq	CAAGCAGAAGACGGCATACGAGATCTAAGACGTGTGACTGGAGTTCAGACGTGTGCTC
Ind92_MiSeq	CAAGCAGAAGACGGCATACGAGATCGTGCACAAGTGACTGGAGTTCAGACGTGTGCTC
Ind93_MiSeq	CAAGCAGAAGACGGCATACGAGATTGTAACGCCGTGACTGGAGTTCAGACGTGTGCTC
Ind94_MiSeq	CAAGCAGAAGACGGCATACGAGATATGCGAGACGTGACTGGAGTTCAGACGTGTGCTC
Ind95_MiSeq	CAAGCAGAAGACGGCATACGAGATCCGTCAAGAGTGACTGGAGTTCAGACGTGTGCTC
Ind96_MiSeq	CAAGCAGAAGACGGCATACGAGATTAGTAGCACGTGACTGGAGTTCAGACGTGTGCTC

forward primer, 5-3

AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGTG

forward

515_fs0_DL
GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG NNNNNNNN GA
GTGCCAGCMGCCGCGGTAA
515_fs1_DL
GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG NNNNTNNNN GA
GTGCCAGCMGCCGCGGTAA
515_fs2_DL
GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG NNNNCTNNNN GA
GTGCCAGCMGCCGCGGTAA
515_fs3_DL
GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG NNNNACTNNNN GA
GTGCCAGCMGCCGCGGTAA
515_fs4_DL
GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG NNNNGACTNNNN GA
GTGCCAGCMGCCGCGGTAA
515_fs5_DL

2.2.4 PCR quantification and sequencing

The DNA concentrations of the final reactions obtained from the PCR step were measured in 96-well plate format using PicoGreen fluorescent dye (Invitrogen) for double stranded DNA quantification in a fluorescent plate reader format (475nm to 530nm). After quantifying the amounts of DNA present, we ran a portion of the samples in an 1.5% agarose gel to ensure the presence of bands with a size of 448bp. Pooling of all samples from the prepared library was performed using equimolar ratios, and cleaned using AMPure beads at a 0.7:1 ratio, to later be eluted in 20 µl of di water to be denature and loaded in the MiSeq machine by following the Illumina protocol and the standards established by Lundberg et al., (2013).

2.2.5 Demultiplexing and heatmap generation

After sequencing, data obtained from the Illumina machine was demultiplexed by utilizing the CASAVA software from Illumina, v.1.8.2. A FASTA file was generated in which all the consensus sequences obtained per sample were stored. Software utilized was the Molecular Tag Toolbox (MT-Toolbox, Google sites, Yourstone, 2014). R scripts were made and used to create graphs that showed the abundance of the presence of the different microbial organism present in the roots of the plants that were treated and non-treated. Rarefaction values varied based on the type of heatmap that was generated (Family, phylum or OTU).

2.2.6 Phylum analysis and abundance for genotype and inoculation

Only the non-plant reads were classified to the phylum level. Reads from the same phyla were pooled, and read counts were normalized and converted to a ratio by dividing the reads from each phyla by the total number of phylum-classifiable reads in that sample. For better visualization, those phyla representing less than 5% of the total in any sample were reclassified as “Low Abundance”. Data was plotted in R using this “Hist” function of ggplot2 (Wickham).

2.3 Results and Discussion

To develop a *Nicotiana* microbiome, we selected three genotypes that included the domesticated commercial crop species *N. tabacum* (KY14), and three wild varieties *N. benthamiana, N. glutinosa* and *N. rustica*. These were grown in a single soil type

collected at the University of Kentucky, North Research Farm (Table 2.2) for six weeks under greenhouse conditions (28°C, 16:8 light:dark regime).

Root and above ground samples were surface sterilized in order to avoid sequencing microbes that did not inhabit the plant. We sequenced the 16S ribosomal RNA (rRNA) and created community assessments for the rhizosphere soil, root EC, stem EC and leaf EC (Figure 2.1).

Community sequencing generated 1,491,297 merged paired-end reads across 92 samples (from which 36 belong to genotype related-study), which after bioinformatics removal of low quality, plant-derived, and rare singleton sequences that did not cluster into OTUs of at least 2 sequences resulting in 318,860 reads (1225 reads and 243 OTUs per sample; Table 2.3).

Table 2.2 Soil profile nutrients. Nutrient composition of soil collected from the University of Kentucky Spindletop Farm.

Lab #	Sample#	1M KCL soil pH	Calculated soil-water pH	Sikora II Buffer pH	P (lbs/a)	K (lbs/a)	Ca (lbs/a)	Mg (lbs/a)	Zn (lbs/a)
22889	1926	5.43	6.28	6.83	375	485	4018	360	9.1
Boron = 1.4 lb/ac, Meh3_Cu = 2.78 lb/ac, Meh3_Mn = 368 lb/ac, Meh3_Fe = 342 lb/ac,									

OTUs were classified taxonomically using the Greengenes database, grouped into operational taxonomic units (OTUs) using a ≥97% identity criteria and read counts from the same phyla were pooled to visualize taxonomic distribution across tissues for genotypes *N. benthamiana*, *N. glutinosa*, *N. rustica* and *N. tabacum* (Table 2.4).

We observed that the rhizosphere soil community was different than that of the root/stem/leaf EC, but that it did not differ by plant genotype or place of origin (Figure 2.2; Figure 2.3). Prior studies found that a stable resident "core microbiome" exists in both rhizosphere and root EC (Gottel et al., 2011; Bulgarelli et al., 2012; Lundberg et al., 2012; Schreiter et al., 2014). In our data, rhizosphere samples from all genotypes showed similarities in abundance and phyla present (Figure 2.3). The resident communities of the stem and leaf EC (Figure 2.3) differed from the root EC in numerous ways. Some common trends across all genotypes existed, while other differences were unique to specific genotypes.

Table 2.3 Summary of all reads obtained from 16s rRNA sequenced libraries of *Nicotiana* genotypes and *N. benthamiana* treatments.

Sample type	Bar code	Total_Seq_Count	Merged_Count
Soil_Bent_1	P0_GTGTATGC	143229	6664
Stem_Bent_2	P10_CAGTAATG	88412	14142
Root_Bent_2	P11_CAGCGTGT	83260	16621
Root_447_2	P12_GTCAGCTG	255469	19620
Stem_41_2	P13_AGTGCCAC	237168	19407
Leaf_343_2	P14_ATGACTCA	92316	1126
Root_Control_2	P15_TAAGCATG	154748	8649
Soil_Bent_3	P16_AGCCGTTA	110257	7278
Leaf_Bent_3	P17_AACAGGTG	195254	11940
Stem_Bent_3	P18_AAGGCACG	141331	4157
Root_Bent_3	P19_GCTTAATA	250570	16285
Leaf_Bent_1	P1_GCAACGTC	320877	222464
Root_447_3	P20_AGCCTTCT	233910	3912
Stem_41_3	P21_TCAGGCCA	277327	12975
Leaf_343_3	P22_GTGACATG	161530	4867
Root_Control_3	P23_AACCAGCT	98491	29748
Soil_Rus_1	P24_ACGTTCAT	155533	40957
Leaf_Rus_1	P25_TGCACAAT	171406	11407
Stem_Rus_1	P26_TCTTGACG	90799	14361
Root_Rus_1	P27_TCTTCGAG	205606	60172
Stem_447_1	P28_GCTGAAGA	53550	4005
Leaf_41_1	P29_TGACTAGT	137706	8315

Table 2.3 Summary of all reads obtained from 16s rRNA sequenced libraries of *Nicotiana* genotypes and *N. benthamiana* treatments (continued).

Sample type	Bar code	Total_Seq_Count	Merged_Count
Stem_Bent_1	P2_GATGCCTT	184580	5888
Root_413_1	P30_GGCGTTAC	131041	4253
Stem_Control_1	P31_TGGACTCT	153054	7205
Soil_Rus_2	P32_GGCGCTTA	189489	1649
Leaf_Rus_2	P33_AGATGGCT	134220	11572
Root_Rus_2	P35_GAGGTTAC	97929	4722
Stem_447_2	P36_GTAGGACC	56061	1638
Leaf_41_2	P37_CGACCTTA	140915	9170
Stem_Control_2	P39_GCAGCTCT	107816	6606
Root_Bent_1	P3_CATCTTAC	245516	16478
Soil_Rus_3	P40_CACTTCTG	139897	1329
Leaf_Rus_3	P41_ATAGTCCG	68803	3710
Stem _Rus_3	P42_TCCATGCG	130589	3865
Root_Rus_3	P43_ATACGGAC	181591	20495
Stem_447_3	P44_CTTACTAG	171659	2629
Leaf_41_3	P45_TACCATGA	227375	13763
Root_413_3	P46_CGTTCTAA	159725	2807
Stem_Control_3	P47_ACATTGCG	277330	8150
Soil_Glu_1	P48_TTATAGGC	549581	24691
Root_447_1	P4_CATGAAGT	101183	2786
Stem_Glu_1	P50_ACGTCTTA	181242	13605
Root_Glu_1	P51_GTACGCGT	70698	8014
Leaf_447_1	P52_CCAACTAG	172798	8418
root_343_1	P53_CGGCTACA	90106	12270
Stem_413_1	P54_TAGCAGTG	105513	6803
Leaf_Control_1	P55_CACCGATT	104403	4027
Soil_Glu_2	P56_CTTCGCAG	190301	74726
Leaf_Glu_2	P57_GCTAGTTC	77408	3405
Stem_Glu_2	P58_GATGATCG	44486	4721
Root_Glu_2	P59_CAATGTCG	132747	6379
Stem_41_1	P5_GGAACGCT	78288	5451
Leaf_447_2	P60_TGTGCGTA	108273	3227
root_343_2	P61_CGCTGAAT	165039	3209
Stem_413_2	P62_CGGTGTCT	234616	75577

Table 2.3 Summary of all reads obtained from 16s rRNA sequenced libraries of *Nicotiana* genotypes and *N. benthamiana* treatments (continued).

Sample type	Bar code	Total_Seq_Count	Merged_Count
Leaf_control_2	P63_TCGCACAA	162455	45885
Soil_Glu_3	P64_CTGTAACA	156290	67513
Leaf_Glu_3	P65_CTCTACAG	270505	10436
Stem_Glu_3	P66_GTGTCCAA	102075	18632
Root_Glu_3	P67_AGGTTCGC	198469	5457
Leaf_447_3	P68_TTAAGCGA	158426	5484
root_343_3	P69_CTATCTGG	93311	13281
Leaf_343_1	P6_TGGCTCTA	57305	5840
Stem_413_3	P70_GTCTCGCA	383055	7528
Leaf_Control_3	P71_GCGAATAC	190571	3571
Soil_Tab_1	P72_GAGTGCTA	121928	5935
Leaf_Tab_1	P73_CGTCGGTA	260135	92769
Stem_Tab_1	P74_ACCATCGT	57327	6436
Root_Tab_1	P75_GGATATAG	183630	4307
Root_41_1	P76_AGTATGCA	174629	5634
Stem_343_1	P77_ACGGCTGA	115740	4045
Leaf_413_1	P78_CGAGTATC	176143	2739
Empty	P79_CGGCAGAA	424203	3821
Root_Control_1	P7_ACCTCAGA	144079	46279
Soil_Tab_2	P80_ATCTGCGA	126092	29525
Leaf_Tab_2	P81_TGATCCTA	83220	13992
Stem_Tab_2	P82_CGCTAGTA	61432	6806
Root_41_2	P84_CATGAGGC	159176	17489
Stem_343_2	P85_GTCGAAGC	118827	18487
Leaf_413_2	P86_TGTCGTCA	82137	18267
Empty	P87_GGCCTATC	167453	4202
Soil_Tab_3	P88_GCCGACTT	213707	29429
Leaf_Tab_3	P89_ACGTCCTG	125207	2761
Soil_Bent_2	P8_TACTTGCA	103650	9730
Stem_Tab_3	P90_GAACGTAT	261575	7970
Root_Tab_3	P91_ACAGGAGT	101966	7071

Table 2.3 Summary of all reads obtained from 16s rRNA sequenced libraries of *Nicotiana* genotypes and *N. benthamiana* treatments (continued).

Sample type	Bar code	Total_Seq_Count	Merged_Count
Root_41_3	P92_CGAATCCT	187245	4258
Stem_343_3	P93_CTAAGGAG	190150	13273
Leaf_413_3	P94_ATTGTGAG	283575	3677
Empty	P95_TGTGAACC	284334	18901
Leaf_Bent_2	P9_CATGGACG	161926	7557

Sample type	Percent_Merged	Match_Count	MT_Count	SRC_Count
Soil_Bent_1	4%	2642	1810	1222
Stem_Bent_2	15%	6003	5477	5005
Root_Bent_2	19%	7451	6891	6397
Root_447_2	7%	7876	3075	1476
Stem_41_2	8%	7104	4252	2646
Leaf_343_2	1%	314	175	106
Root_Control_2	5%	3351	2223	1530
Soil_Bent_3	6%	1987	1649	1368
Leaf_Bent_3	6%	4742	2504	1419
Stem_Bent_3	2%	1096	854	678
Root_Bent_3	6%	5970	3692	2387
Leaf_Bent_1	69%	103278	71404	49152
Root_447_3	1%	1159	525	284
Stem_41_3	4%	4757	2141	1143
Leaf_343_3	3%	1576	1394	1248
Root_Control_3	30%	9813	6861	5092
Soil_Rus_1	26%	18158	13898	10720
Leaf_Rus_1	6%	4883	2345	1174
Stem_Rus_1	15%	6286	5630	5054
Root_Rus_1	29%	27013	21358	17206
Stem_447_1	7%	1712	1622	1534
Leaf_41_1	6%	3436	2783	2265
Stem_Bent_1	3%	2232	1601	1151
Root_413_1	3%	1522	1237	1003
Stem_Control_1	4%	2696	2036	1548
Soil_Rus_2	0%	331	76	52
Leaf_Rus_2	8%	4552	3264	2338
Root_Rus_2	4%	1754	1420	1178

Table 2.3 Summary of all reads obtained from 16s rRNA sequenced libraries of *Nicotiana* genotypes and *N. benthamiana* treatments (continued).

Sample type	Percent_Merged	Match_Count	MT_Count	SRC_Count
Stem_447_2	2%	579	502	439
Leaf_41_2	6%	3505	2578	1913
Stem_Control_2	6%	1941	1737	1565
Root_Bent_1	6%	6131	3943	2651
Soil_Rus_3	0%	339	142	72
Leaf_Rus_3	5%	1492	1259	1060
Stem _Rus_3	2%	1268	1029	839
Root_Rus_3	11%	7591	4761	3113
Stem_447_3	1%	823	723	635
Leaf_41_3	6%	5063	3615	2732
Root_413_3	1%	732	408	249
Stem_Control_3	2%	2784	1624	983
Soil_Glu_1	4%	10058	2455	1177
Root_447_1	2%	919	555	340
Stem_Glu_1	7%	5185	3889	2980
Root_Glu_1	11%	3465	3144	2855
Leaf_447_1	4%	3009	2259	1754
root_343_1	13%	5283	4772	4314
Stem_413_1	6%	2509	2332	2177
Leaf_Control_1	3%	1659	1474	1311
Soil_Glu_2	39%	28614	4860	2788
Leaf_Glu_2	4%	1233	932	704
Stem_Glu_2	10%	1881	1709	1555
Root_Glu_2	4%	2366	2023	1733
Stem_41_1	6%	1798	1528	1305
Leaf_447_2	2%	1029	785	601
root_343_2	1%	918	646	465
Stem_413_2	32%	34617	24787	18087
Leaf_control_2	28%	20557	12655	7759
Soil_Glu_3	43%	30669	22180	15956
Leaf_Glu_3	3%	3740	2391	1547
Stem_Glu_3	18%	7854	6407	5288
Root_Glu_3	2%	1675	1205	901
Leaf_447_3	3%	2067	1594	1231

Table 2.3 Summary of all reads obtained from 16s rRNA sequenced libraries of *Nicotiana* genotypes and *N. benthamiana* treatments (continued)

Sample type	Percent_Merged	Match_Count	MT_Count	SRC_Count
root_343_3	14%	5728	5220	4766
Leaf_343_1	10%	2487	2324	2175
Stem_413_3	1%	2245	1370	880
Leaf_Control_3	1%	1039	737	532
Soil_Tab_1	4%	1849	1317	966
Leaf_Tab_1	35%	41161	22401	12547
Stem_Tab_1	11%	2484	2120	1811
Root_Tab_1	2%	1219	919	718
Root_41_1	3%	1901	1358	981
Stem_343_1	3%	1377	1122	917
Leaf_413_1	1%	766	660	577
Empty	0%	864	449	273
Root_Control_1	32%	20206	17664	15562
Soil_Tab_2	23%	12614	11463	10445
Leaf_Tab_2	16%	6135	5552	5023
Stem_Tab_2	11%	2878	2613	2379
Root_41_2	10%	6712	5157	4076
Stem_343_2	15%	8283	6778	5521
Leaf_413_2	22%	7742	6821	6027
Empty	2%	1416	1099	861
Soil_Tab_3	13%	11711	7947	5646
Leaf_Tab_3	2%	631	498	398
Soil_Bent_2	9%	4110	3566	3085
Stem_Tab_3	3%	2923	572	294
Root_Tab_3	6%	2556	2053	1674
Root_41_3	2%	1348	379	187
Stem_343_3	6%	4788	3506	2654
Leaf_413_3	1%	924	434	239
Empty	6%	6798	3137	1712
Leaf_Bent_2	4%	2765	2009	1472

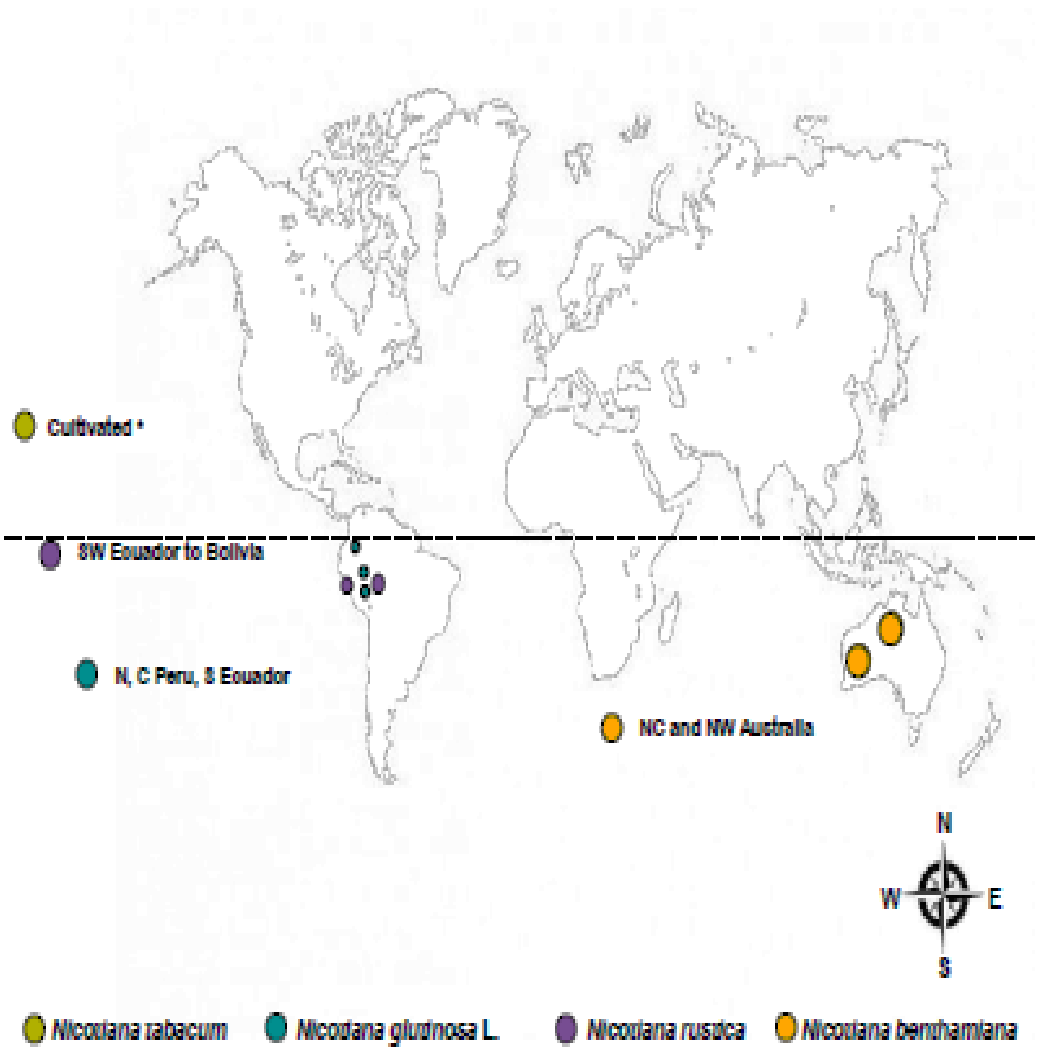


Figure 2.2 Geographical distribution of genotypes used to build a "core" microbiome of the *Nicotiana genus*. All seed used came from plants grown in the US, but the evolutionary origin of the seed spans different continents. Different colored dots represent the different species selected. *<u>*Nicotiana tabacum* provenance is established by the place where is cultivated and the original parental lines used.</u>

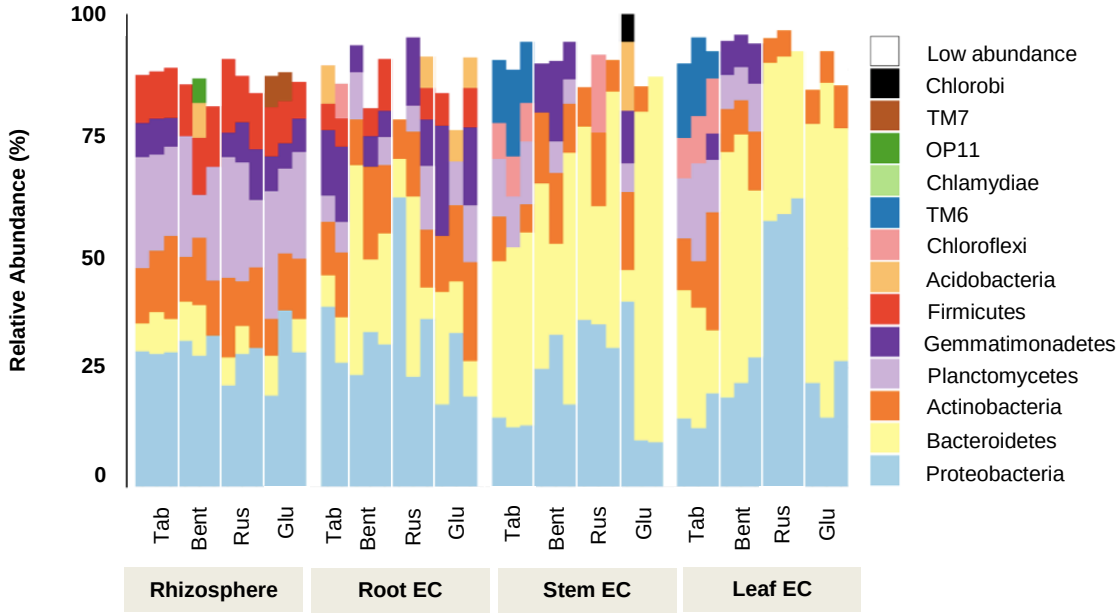


Figure 2.3 Relative abundance and establishment of the *Nicotiana* species microbiome using 16s rRNA data sequenced shows that: A) Reads grouped to phyla level had abundance differences for each phylum (represented as percentage) when comparing *Nicotiana tabacum*, *Nicotiana benthamiana*, *Nicotiana rustica* and *Nicotiana glutinosa*. Samples with reads belonging to phylum level that were less than 5% present were all classified under the "low abundance" category.

General trends included a lower diversity of phyla and an increased abundance of *Bacteroidetes* in stem and leaf EC compared with root (Figure 2.3). A decreased abundance of *Proteobacteria*, *Plantomycetes*, *Bacteroidetes* and *Actinobacteria* was also evident. These phyla are abundant in soil (Buckley et al., 2006, Youssef et al., 2008, Lundberg et al., 2012, Saleem et al., 2016), and were generally decreased in stem and leaf EC of the species evaluated (Table 2.3).

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes. After removal of OTUs classified as chloroplasts and mitochondria, remaining OTUs were grouped into their respective phyla. The abundance of each phylum in a sample was represented as a percentage, and any phylum which made up less than less than 5 percent in a given sample was classified as "Low abundance" for that sample.

	Phylum	Percentage	Sample
P17	low_abundance	13.51351351	Soil_Tab_1
P17	Firmicutes	10.13513514	Soil_Tab_1
P17	Gemmatimonadetes	7.175032175	Soil_Tab_1
P17	Planctomycetes	23.29472329	Soil_Tab_1
P17	Actinobacteria	11.67953668	Soil_Tab_1
P17	Bacteroidetes	5.823680824	Soil_Tab_1
P17	Proteobacteria	28.37837838	Soil_Tab_1
P25	low_abundance	12.87005298	Soil_Tab_2
P25	Firmicutes	9.878466812	Soil_Tab_2
P25	Gemmatimonadetes	7.510127766	Soil_Tab_2
P25	Planctomycetes	20.25553132	Soil_Tab_2
P25	Actinobacteria	12.74540355	Soil_Tab_2
P25	Bacteroidetes	8.974758492	Soil_Tab_2
P25	Proteobacteria	27.76565908	Soil_Tab_2
P48	low_abundance	12.18936773	Soil_Tab_3
P48	Firmicutes	10.41207927	Soil_Tab_3
P48	Gemmatimonadetes	6.055363322	Soil_Tab_3
P48	Planctomycetes	18.77949041	Soil_Tab_3
P48	Actinobacteria	17.36395093	Soil_Tab_3
P48	Bacteroidetes	7.03051274	Soil_Tab_3
P48	Proteobacteria	28.16923561	Soil_Tab_3
P73	low_abundance	15.63523652	Soil_Bent_1
P73	Firmicutes	10.85203057	Soil_Bent_1
P73	Planctomycetes	25.34606037	Soil_Bent_1
P73	Actinobacteria	9.355077313	Soil_Bent_1
P73	Bacteroidetes	8.189214892	Soil_Bent_1
P73	Proteobacteria	30.62238033	Soil_Bent_1
P94	low_abundance	14.28571429	Soil_Bent_2
P94	OP11	5.117270789	Soil_Bent_2
P94	Acidobacteria	7.462686567	Soil_Bent_2
P94	Firmicutes	11.94029851	Soil_Bent_2
P94	Planctomycetes	8.955223881	Soil_Bent_2
P94	Actinobacteria	14.07249467	Soil_Bent_2
P94	Bacteroidetes	10.66098081	Soil_Bent_2
P94	Proteobacteria	27.50533049	Soil_Bent_2
P12	low_abundance	20.21140878	Soil_Bent_3

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes (continued)

	Phylum	Percentage	Sample
P94	Actinobacteria	14.07249467	Soil_Bent_2
P12	Firmicutes	12.64807727	Soil_Bent_3
P12	Planctomycetes	23.85638783	Soil_Bent_3
P12	Actinobacteria	11.51813377	Soil_Bent_3
P12	Proteobacteria	31.76599235	Soil_Bent_3
P63	low_abundance	10.37006913	Soil_Rus_1
P63	Firmicutes	15.34770232	Soil_Rus_1
P63	Gemmatimonadetes	5.083367222	Soil_Rus_1
P63	Planctomycetes	25.33550224	Soil_Rus_1
P63	Actinobacteria	16.73851159	Soil_Rus_1
P63	Bacteroidetes	5.961773078	Soil_Rus_1
P63	Proteobacteria	21.16307442	Soil_Rus_1
P64	low_abundance	13.84591096	Soil_Rus_2
P64	Firmicutes	9.683191243	Soil_Rus_2
P64	Gemmatimonadetes	8.372802863	Soil_Rus_2
P64	Planctomycetes	25.07630776	Soil_Rus_2
P64	Actinobacteria	9.299021156	Soil_Rus_2
P64	Bacteroidetes	5.930954636	Soil_Rus_2
P64	Proteobacteria	27.79181139	Soil_Rus_2
P40	low_abundance	17.39130435	Soil_Rus_3
P40	Firmicutes	11.80124224	Soil_Rus_3
P40	Gemmatimonadetes	10.55900621	Soil_Rus_3
P40	Planctomycetes	14.28571429	Soil_Rus_3
P40	Actinobacteria	16.77018634	Soil_Rus_3
P40	Proteobacteria	29.19254658	Soil_Rus_3
P90	TM7	6.554216867	Soil_Glu_1
P90	Firmicutes	10.36144578	Soil_Glu_1
P90	Gemmatimonadetes	7.373493976	Soil_Glu_1
P90	Planctomycetes	26.74698795	Soil_Glu_1
P90	Actinobacteria	7.614457831	Soil_Glu_1
P90	Bacteroidetes	8.481927711	Soil_Glu_1
P90	Proteobacteria	19.08433735	Soil_Glu_1
P92	low_abundance	13.05389222	Soil_Glu_2
P92	Firmicutes	8.862275449	Soil_Glu_2
P92	Gemmatimonadetes	5.389221557	Soil_Glu_2
P92	Planctomycetes	17.7245509	Soil_Glu_2
P92	Actinobacteria	11.9760479	Soil_Glu_2
P92	Proteobacteria	37.00598802	Soil_Glu_2
P1	low_abundance	15.00634621	Soil_Glu_3
P1	Firmicutes	7.805838513	Soil_Glu_3

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes (continued)

	Phylum	Percentage	Sample
P1	Gemmatimonadetes	6.979203957	Soil_Glu_3
P1	Planctomycetes	22.41351255	Soil_Glu_3
P1	Actinobacteria	12.59966804	Soil_Glu_3
P1	Bacteroidetes	6.964558857	Soil_Glu_3
P1	Proteobacteria	28.23087187	Soil_Glu_3
P41	low_abundance	11.58730159	Root_Tab_1
P41	Acidobacteria	8.095238095	Root_Tab_1
P41	Firmicutes	5.555555556	Root_Tab_1
P41	Gemmatimonadetes	13.65079365	Root_Tab_1
P41	Planctomycetes	5.555555556	Root_Tab_1
P41	Actinobacteria	11.11111111	Root_Tab_1
P41	Bacteroidetes	6.666666667	Root_Tab_1
P41	Proteobacteria	37.77777778	Root_Tab_1
P20	low_abundance	15.35714286	Root_Tab_2
P20	Chloroflexi	7.5	Root_Tab_2
P20	Firmicutes	5.714285714	Root_Tab_2
P20	Gemmatimonadetes	15.89285714	Root_Tab_2
P20	Planctomycetes	6.428571429	Root_Tab_2
P20	Actinobacteria	13.57142857	Root_Tab_2
P20	Bacteroidetes	9.464285714	Root_Tab_2
P20	Proteobacteria	26.07142857	Root_Tab_2
P67	low_abundance	7.325383305	Root_Bent_1
P67	Gemmatimonadetes	5.792163543	Root_Bent_1
P67	Planctomycetes	9.880749574	Root_Bent_1
P67	Actinobacteria	9.540034072	Root_Bent_1
P67	Bacteroidetes	43.95229983	Root_Bent_1
P67	Proteobacteria	23.50936968	Root_Bent_1
P95	low_abundance	20.65514104	Root_Bent_2
P95	Firmicutes	5.732484076	Root_Bent_2
P95	Gemmatimonadetes	6.521079769	Root_Bent_2
P95	Actinobacteria	19.4722475	Root_Bent_2
P95	Bacteroidetes	15.19563239	Root_Bent_2
P95	Proteobacteria	32.42341523	Root_Bent_2
P21	low_abundance	10.3515625	Root_Bent_3
P21	Firmicutes	10.859375	Root_Bent_3
P21	Gemmatimonadetes	5.4296875	Root_Bent_3
P21	Planctomycetes	5.8984375	Root_Bent_3
P21	Actinobacteria	14.3359375	Root_Bent_3
P21	Bacteroidetes	23.1640625	Root_Bent_3
P21	Proteobacteria	29.9609375	Root_Bent_3

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes (continued)

	Phylum	Percentage	Sample
P57	low_abundance	22.92682927	Root_Rus_1
P57	Actinobacteria	8.292682927	Root_Rus_1
P57	Bacteroidetes	8.130081301	Root_Rus_1
P57	Proteobacteria	60.6504065	Root_Rus_1
P15	low_abundance	5.615292712	Root_Rus_2
P15	Gemmatimonadetes	14.33691756	Root_Rus_2
P15	Planctomycetes	5.615292712	Root_Rus_2
P15	Actinobacteria	13.50059737	Root_Rus_2
P15	Bacteroidetes	37.87335723	Root_Rus_2
P15	Proteobacteria	23.05854241	Root_Rus_2
P14	low_abundance	9.696969697	Root_Rus_3
P14	Acidobacteria	6.666666667	Root_Rus_3
P14	Firmicutes	6.666666667	Root_Rus_3
P14	Gemmatimonadetes	9.696969697	Root_Rus_3
P14	Planctomycetes	13.33333333	Root_Rus_3
P14	Actinobacteria	12.12121212	Root_Rus_3
P14	Bacteroidetes	6.666666667	Root_Rus_3
P14	Proteobacteria	35.15151515	Root_Rus_3
P56	low_abundance	17.46533442	Root_Glu_1
P56	Firmicutes	6.846451876	Root_Glu_1
P56	Gemmatimonadetes	23.05261011	Root_Glu_1
P56	Actinobacteria	11.7862969	Root_Glu_1
P56	Bacteroidetes	23.62357259	Root_Glu_1
P56	Proteobacteria	17.22573409	Root_Glu_1
P79	low_abundance	25.06963788	Root_Glu_2
P79	Acidobacteria	6.685236769	Root_Glu_2
P79	Planctomycetes	9.192200557	Root_Glu_2
P79	Actinobacteria	15.87743733	Root_Glu_2
P79	Bacteroidetes	10.86350975	Root_Glu_2
P79	Proteobacteria	32.31197772	Root_Glu_2
P4	low_abundance	9.848484848	Root_Glu_3
P4	Acidobacteria	6.439393939	Root_Glu_3
P4	Firmicutes	8.333333333	Root_Glu_3
P4	Gemmatimonadetes	16.28787879	Root_Glu_3
P4	Planctomycetes	11.93181818	Root_Glu_3
P4	Actinobacteria	20.83333333	Root_Glu_3
P4	Bacteroidetes	7.386363636	Root_Glu_3
P4	Proteobacteria	18.93939394	Root_Glu_3
P52	low_abundance	10.43956044	Stem_Tab_1
P52	TM6	13.32417582	Stem_Tab_1

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes (continued)

	Phylum	Percentage	Sample
P52	Chloroflexi	7.417582418	Stem_Tab_1
P52	Planctomycetes	12.22527473	Stem_Tab_1
P52	Actinobacteria	9.340659341	Stem_Tab_1
P52	Bacteroidetes	32.82967033	Stem_Tab_1
P52	Proteobacteria	14.42307692	Stem_Tab_1
P91	low_abundance	12.46153846	Stem_Tab_2
P91	TM6	18.15384615	Stem_Tab_2
P91	Chloroflexi	8.461538462	Stem_Tab_2
P91	Planctomycetes	10.61538462	Stem_Tab_2
P91	Bacteroidetes	37.84615385	Stem_Tab_2
P91	Proteobacteria	12.46153846	Stem_Tab_2
P50	low_abundance	6.703470032	Stem_Tab_3
P50	TM6	12.85488959	Stem_Tab_3
P50	Chloroflexi	8.044164038	Stem_Tab_3
P50	Planctomycetes	13.170347	Stem_Tab_3
P50	Actinobacteria	5.914826498	Stem_Tab_3
P50	Bacteroidetes	40.5362776	Stem_Tab_3
P50	Proteobacteria	12.77602524	Stem_Tab_3
P42	low_abundance	11.20448179	Stem_Bent_1
P42	Gemmatimonadetes	10.36414566	Stem_Bent_1
P42	Actinobacteria	14.84593838	Stem_Bent_1
P42	Bacteroidetes	38.93557423	Stem_Bent_1
P42	Proteobacteria	24.64985994	Stem_Bent_1
P46	low_abundance	10.62271062	Stem_Bent_2
P46	Gemmatimonadetes	16.84981685	Stem_Bent_2
P46	Planctomycetes	6.593406593	Stem_Bent_2
P46	Actinobacteria	15.01831502	Stem_Bent_2
P46	Bacteroidetes	19.04761905	Stem_Bent_2
P46	Proteobacteria	31.86813187	Stem_Bent_2
P93	low_abundance	6.563421829	Stem_Bent_3
P93	Gemmatimonadetes	7.96460177	Stem_Bent_3
P93	Planctomycetes	5.162241888	Stem_Bent_3
P93	Actinobacteria	10.25073746	Stem_Bent_3
P93	Bacteroidetes	52.80235988	Stem_Bent_3
P93	Proteobacteria	17.25663717	Stem_Bent_3
P18	low_abundance	16.25344353	Stem_Rus_1
P18	Actinobacteria	8.26446281	Stem_Rus_1
P18	Bacteroidetes	40.49586777	Stem_Rus_1
P18	Proteobacteria	34.9862259	Stem_Rus_1
P16	low_abundance	9.309967141	Stem_Rus_2

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes (continued)

	Phylum	Percentage	Sample
P16	Chloroflexi	16.42935378	Stem_Rus_2
P16	Actinobacteria	15.44359255	Stem_Rus_2
P16	Bacteroidetes	24.64403067	Stem_Rus_2
P16	Proteobacteria	34.17305586	Stem_Rus_2
P5	low_abundance	10.51004637	Stem_Rus_3
P5	Actinobacteria	6.491499227	Stem_Rus_3
P5	Bacteroidetes	53.94126739	Stem_Rus_3
P5	Proteobacteria	29.05718702	Stem_Rus_3
P32	Chlorobi	6.666666667	Stem_Glu_1
P32	Acidobacteria	14.54545455	Stem_Glu_1
P32	Gemmatimonadetes	10.90909091	Stem_Glu_1
P32	Planctomycetes	6.060606061	Stem_Glu_1
P32	Actinobacteria	16.36363636	Stem_Glu_1
P32	Bacteroidetes	6.666666667	Stem_Glu_1
P32	Proteobacteria	38.78787879	Stem_Glu_1
P22	low_abundance	15.89537223	Stem_Glu_2
P22	Actinobacteria	5.432595573	Stem_Glu_2
P22	Bacteroidetes	69.01408451	Stem_Glu_2
P22	Proteobacteria	9.657947686	Stem_Glu_2
P35	low_abundance	13.8769671	Stem_Glu_3
P35	Bacteroidetes	76.82403433	Stem_Glu_3
P35	Proteobacteria	9.298998569	Stem_Glu_3
P45	low_abundance	11.12224449	Leaf_Tab_1
P45	TM6	15.73146293	Leaf_Tab_1
P45	Chloroflexi	8.416833667	Leaf_Tab_1
P45	Planctomycetes	12.7254509	Leaf_Tab_1
P45	Actinobacteria	10.82164329	Leaf_Tab_1
P45	Bacteroidetes	26.95390782	Leaf_Tab_1
P45	Proteobacteria	14.22845691	Leaf_Tab_1
P84	low_abundance	5.794205794	Leaf_Tab_2
P84	TM6	16.48351648	Leaf_Tab_2
P84	Chloroflexi	9.89010989	Leaf_Tab_2
P84	Planctomycetes	20.47952048	Leaf_Tab_2
P84	Actinobacteria	9.79020979	Leaf_Tab_2
P84	Bacteroidetes	25.37462537	Leaf_Tab_2
P84	Proteobacteria	12.18781219	Leaf_Tab_2
P72	low_abundance	8.695652174	Leaf_Tab_3
P72	TM6	5.565217391	Leaf_Tab_3
P72	Chloroflexi	11.65217391	Leaf_Tab_3
P72	Gemmatimonadetes	5.565217391	Leaf_Tab_3

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes (continued)

	Phylum	Percentage	Sample
P72	Planctomycetes	10.95652174	Leaf_Tab_3
P72	Actinobacteria	24.69565217	Leaf_Tab_3
P72	Bacteroidetes	13.2173913	Leaf_Tab_3
P72	Proteobacteria	19.65217391	Leaf_Tab_3
P66	low_abundance	6.497987349	Leaf_Bent_1
P66	Gemmatimonadetes	7.188039103	Leaf_Bent_1
P66	Planctomycetes	7.015526164	Leaf_Bent_1
P66	Actinobacteria	8.9706728	Leaf_Bent_1
P66	Bacteroidetes	51.63887292	Leaf_Bent_1
P66	Proteobacteria	18.68890167	Leaf_Bent_1
P86	low_abundance	5.177743431	Leaf_Bent_2
P86	Gemmatimonadetes	6.800618238	Leaf_Bent_2
P86	Planctomycetes	6.877897991	Leaf_Bent_2
P86	Actinobacteria	7.187017002	Leaf_Bent_2
P86	Bacteroidetes	52.08655332	Leaf_Bent_2
P86	Proteobacteria	21.87017002	Leaf_Bent_2
P88	low_abundance	7.012296444	Leaf_Bent_3
P88	Gemmatimonadetes	8.474576271	Leaf_Bent_3
P88	Planctomycetes	10.036557	Leaf_Bent_3
P88	Actinobacteria	12.296444	Leaf_Bent_3
P88	Bacteroidetes	35.06148222	Leaf_Bent_3
P88	Proteobacteria	27.11864407	Leaf_Bent_3
P74	low_abundance	5.972222222	Leaf_Rus_1
P74	Actinobacteria	5.138888889	Leaf_Rus_1
P74	Bacteroidetes	33.19444444	Leaf_Rus_1
P74	Proteobacteria	55.69444444	Leaf_Rus_1
P58	low_abundance	4.238921002	Leaf_Rus_2
P58	Actinobacteria	5.587668593	Leaf_Rus_2
P58	Bacteroidetes	32.94797688	Leaf_Rus_2
P58	Proteobacteria	57.22543353	Leaf_Rus_2
P82	low_abundance	8.61423221	Leaf_Rus_3
P82	Bacteroidetes	30.83645443	Leaf_Rus_3
P82	Proteobacteria	60.54931336	Leaf_Rus_3
P23	low_abundance	16.79711017	Leaf_Glu_1
P23	Actinobacteria	7.104154124	Leaf_Glu_1
P23	Bacteroidetes	54.36484046	Leaf_Glu_1
P23	Proteobacteria	21.73389524	Leaf_Glu_1
P54	low_abundance	8.695652174	Leaf_Glu_2
P54	Actinobacteria	6.52173913	Leaf_Glu_2
P54	Bacteroidetes	70.28985507	Leaf_Glu_2

Table 2.4 Percentages of abundance of phylum classification in different *Nicotiana* genotypes (continued)

	Phylum	Percentage	Sample
P54	Proteobacteria	14.49275362	Leaf_Glu_2
P39	low_abundance	15.78947368	Leaf_Glu_3
P39	Actinobacteria	9.090909091	Leaf_Glu_3
P39	Bacteroidetes	48.80382775	Leaf_Glu_3
P39	Proteobacteria	26.31578947	Leaf_Glu_3

It was found that *Chlorobi*, *Acidobacteria* and *Plactomycetes* were low-abundance in the stem EC of the three wild varieties (*N. benthamiana*, *N. glutinosa* and *N. rustica*), and absent in leaf EC (Figure 2.3, Table 2.3).Of potential interest, *Gemmatimonadetes* was present only in the stem and leaf EC of *N. benthamiana,* and in a similar way*, TM6* for *N. tabacum.* It appears that the stem and leaf EC community had a more constrained diversity than the root EC.

In order to gain more information about the microbial composition beyond phyla classification, we decided to cluster our data for *N. benthamiana* and *N. tabacum* in heatmaps of OTU obtained. Heatmaps for aboveground and underground tissue comparison between the two species revealed that root EC and rhizosphere soil separated by sample type but not genotype (Figure 2.4).

Possibly, a genotype signal is marked by the high microbial diversity of the soil. As seen in the aboveground plant data, the opposite situation occurs; stems and leaves do not separate as sample type, but rather by genotype. Due to sampling and sequencing limitations, we could not investigate all four genotypes at sufficient sequencing resolution; for this reason, and because they are the two species most often used for plant biology studies, we concentrated analysis on *N. benthamiana* and *N.tabacum.*

Similarities found among species between the rhizosphere and root endophytic compartment could be explained by the linkage between and the adoption of immunity traits, evolutionary history among plant genus and microbes, among other scenarios (Maekawa et al., 2011; Lebeis et al., 2015, Schlaeppi *et al*., 2014).
Beyond finding differentially abundant phyla among the species, domestication alone did not explain the striking differences between presence or absence of specific phyla

groups (Bulgarelli *et al*., 2015; Haney *et al*., 2015). Groups like Coleman-Derr et al., (2016) showed that geographical distribution and habitats + genotype can have an influence on the composition of bacterial organisms that inhabit a plant. Here, data seems to show that the genetic history among plant and microbes does not get altered easily even when plants have been grown and exposed to different environments for decades. Thus, we conclude that although the rhizosphere and root EC seem to be more flexible/permeable areas for microbes and plant to select based on the environment, and aerial organs are subjected to greater taxonomic changes than those observed in root EC or rhizosphere soil (Figure 2.3, Table 2.3).

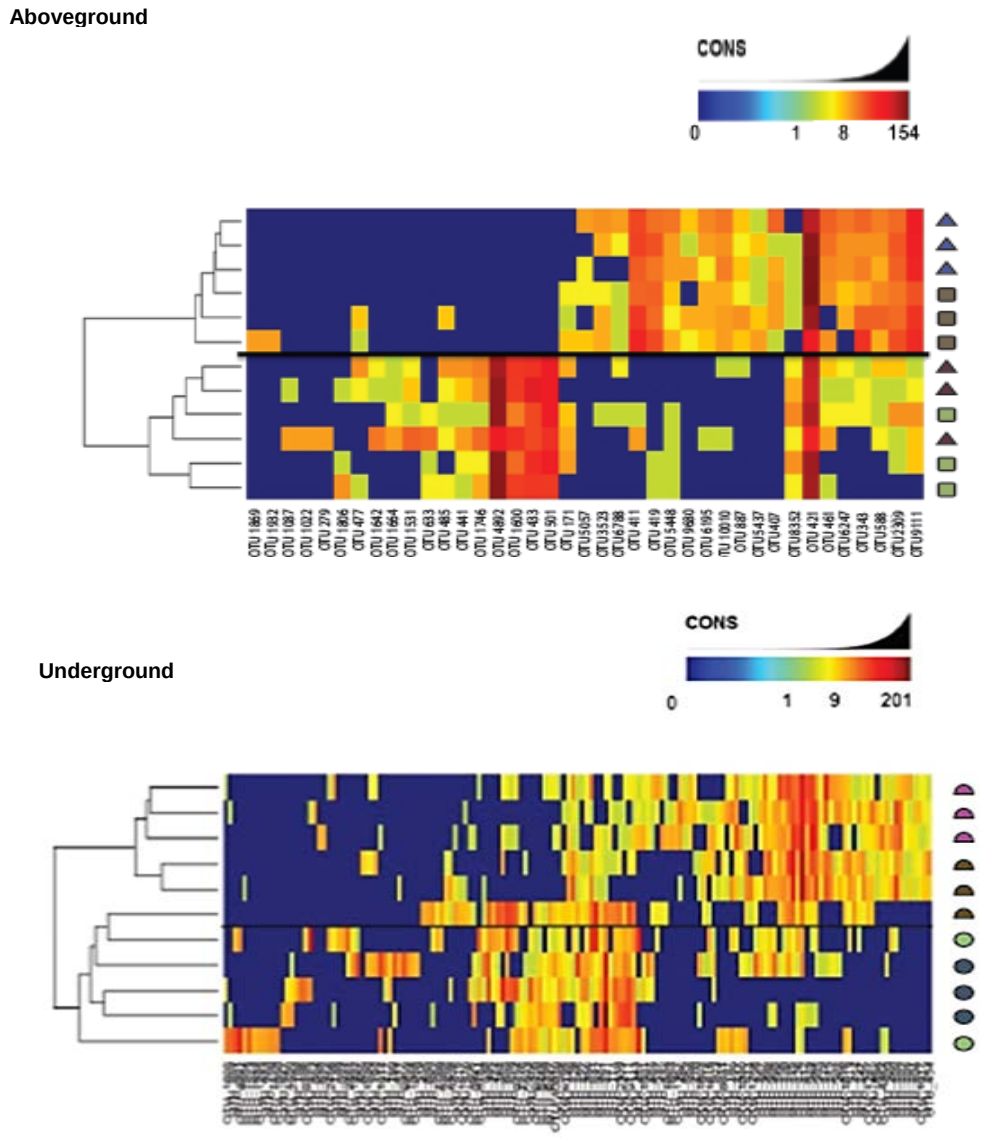


Figure 2.4 OTU heatmaps created for *Nicotiana tabacum* and *Nicotiana benthamiana* (two of the most used species for research) show that for aboveground organs –stem and leaves- samples do not separate by organ, but on the contrary they remained merged as the aerial portion of the plant, although they do cluster together by species. Presence and absence of more selective OTU's groups can be seen for the aboveground portion and that on the contrary, for underground portion –soil and roots- separation is based on type of sample, but not by specie. Triangles represent leaves and squares stem (blue and olive for *N. benthamiana*, burgundy and green for *N. tabacum*). Half circles and circles represent the soils and roots for both species.

Chapter 3: Effects of bacterial inoculums used as seed treatments in bacterial populations inhabiting *Nicotiana benthamiana* and its development.

3.1 Introduction

Bacterial organisms that inhabit or are in contact with plants have become a major topic of interest in agricultural sciences, due to the positive effects that they are attributed to have in plant development (Bashan Y., 1998; Gousterova et al., 2008; Dimkpa et al., 2009). As of today, new technologies have allowed us to gain more knowledge and understanding of the interactions and effects that arise as a consequence of the introduction of "new" or already existing bacterial organisms in higher numbers, when used as growth enhancement treatments for plants.

As we know, higher plants are organisms that predominantly exist in a soil environment (Dumbrell et al., 2010), with an extensively studied microbial dynamic at the soil root interface (also known as the rhizosphere), and more recently, in the aerial parts of the plant too (Kembel et al., 2014, Coleman-Derr et al., 2016). The major focus has been directed towards the microbial communities residing inside of the plant tissue, termed the endophytic compartment (EC) (Schulz et al., 2006), which has been studied using culture-dependent (Coombs and Franco, 2003; De Oliveira et al., 2012; Gagne-Bourgue, F., et al. 2013) and culture-independent sequencing techniques (Tian et al., 2015). Studies of the EC have provided additional information about the complexity of core microbiome of plants (Lugtenberg et al., 2009*;* Lundberg et al., 2012; Bodenhausen et al., 2013; Coleman-Derr et al., 2016) and how it interacts with plant immunity (Lebeis et al., 2015).

In sterile conditions, there have been discoveries about the functionality and effect in the plant phenotype from some of these bacterial organisms (Mantelin et al., 2004; Compant et al., 2005; Macky and Mcfall, 2006; Schuhegger et al., 2006). The problem comes when translating some of these phenomena to a more realistic environment, because of the interaction that occurs among introduced bacteria with indigenous organisms present in the microbial niche, making necessary the dissection of the questions of interest in a 1) holistic or 2) reductionist approach (Matsumura et al., 2003; de Torres-Zabala et al., 2007).

Deploying microbial inoculum as seed (spermosphere) adjuncts in agricultural settings (Emerson and Gillespie, 2008; Vamosi *et al.*, 2009) is being increasingly used for yield optimization (O'Callaghan, 2016), suppression of pests and disease (Beneduzi et al., 2012) and maintenance of fertility (Vessey, 2003; Adesemoye et al., 2008). However, it remains poorly understood whether adding a microbial inoculum to the seed spermosphere to drive functional association alters the hosts' capacity to recruit and maintain a "core" or "accessory" microbiome in the rhizosphere or EC of the host plant. This is complicated by the fact that only the roots of the plant are in contact with the soil, and therefore if functional changes in the aerial tissues are to arise, there either must be systemic responses in the EC microbiome, or more general systemic changes in the plants response to below ground events. In this study, we investigate the effect that overrepresentation of bacterial organisms have in plant growth and development and how it links to the complex microbiome of plants.

3.2 Methods and materials

3.2.1 Strain selection, seed selection and inoculation

Originally, 5 species of *Nicotiana* (*N. benthamiana, N.rustica, N. glutinosa, N.bigelou* and *N.tabacum*) had been selected to test the different bacterial strains that were previously isolated by the Debolt lab. From a total of 1000 bacterial strains, we reduced the organisms to use to a total of 24 (Table 3.1), based on bibliographical references/ information available. Germination was tested for all using a cutoff of 90-100%, as a way to confirmed that any positive or negative effect was not related to the seed viability. Surface sterilization of seeds was achieved via a 30% bleach rinse for 20 min followed by 70% ethanol for 1 min, with a final step of washing cycles of sterile water (minimum of 3 rinse cycles). Bacterial library strains were sourced from the Switchgrass (*Panicum virgatum*) and giant burpee tomato library (Xia et al., 2012). Bacterial strains selected were applied directly to seeds as an amendment (or seed treatment), with the intention of evaluating potential growth promotion, growth suppression or no influence. Strains were grown in YPD broth medium flasks overnight (11± 2 hours) at 28°C on a rotary shaker. For inoculations, strains were grown at 28°C on a rotary shaker until OD_{600} = 0.2 to add the seeds, and were kept in the media until reaching an OD_{600} = 0.6. A total of ~20 seeds were placed in each bacteria culture for spermosphere inoculation for 12h at 28°C and kept on the rotary shaker/incubator. Media with no bacterial culture was used as a mock (Control) treatment.

Table 3.1 Bacterial strains selected. Previously isolated bacterial organisms from plants grown under conventional and organic systems were sequenced for identification and archived in a bacterial and fungal library that belongs to the Debolt lab. All bacterial organisms mentioned in the list were assayed for their effect on plant development and growth.

Bacterial strains	Isolated from
Bulkholderia gladioli strain 33A	
Stenotrophomonas sp. 3c_5	
Microbacterium sp. Fek04	
Stenotrophomonas maltophilia strain H258	
***Microoccus sp.* HPABA07**	**Seed- Switchgrass**
Chryseobacterium sp. JA37A1	
Bacillus cereus strain TT15	
Bacillus sp. TZQ2	
Paracocus sp. Zy-3	
Microbacterium oleivorans strain 1P06AB	
Bacillus thuringiensis srain DW-1T	
***Bacillus sp.* AS6**	**Stem- org. tomato**
Pseudomonas sp. SaCs17	
***Lysinibacillus fusiformis* strain Ba10**	**Leaf- Indian grass**
Lysinibacillus fusiformis strain R2	
Bacillus simplex strain X9	
Brevibacillus sp. Z0-YC6800	
Sphingomonas sp. PVS17	
***Bacillus cereus* strain EI-8**	**Stem- conv. tomato**
Denococcus sp. X-121	
Flavobacterium sp. CK18	
HQ324912.1 *Pseudomona putida* strain P-1017-1	
Bacillus cereus isolate T1-9	
Paenibacillus polymyxa strain SAZ2-6	

The bacteria-treated *Nicotiana* seeds were placed into pots containing aseptically and partially steamed Pro-Mix (Premier Horticulture Inc., PA, Quakertown, USA) potting media. Potting media was tested to see microbial growth by plating fractions of it in YPDA.

From general screening of 24 bacterial organisms, final selection of bacterial strains included: *Lysinibacillus fusiformis* (Lf)/s447, *Bacillus cereus* (Bc)/s413, *Bacillus sp.* (Bs)/s343, *Micrococcus sp*. (Ms)/s41 and control. A total of 12 replicates were utilized for each treatment and control.

A volume of 500ul of bacterial culture was also added to the soil containing the treated seeds in order to create an overrepresentation of the microorganisms in the rhizosphere. All treatments were kept in a greenhouse with constant temperatures of ~25±3°C, and 16h of light followed by 8 h of dark for 60 days.

3.2.2 Sample selection and processing

Morphological analyses followed the methods of Kelemu et al., (2011) with some modification. Specifically, at ~2-3 weeks' seedlings were checked for visual differences in root system, and at after ~3-4 weeks of inoculation, measurements were taken and recorded for traits like: Height, number of leaves (NL), leaf length (LL), width (LW) and number of flowers. Plants were evaluated till week 12 (90 days). Plants from each treatment and control were harvested at ~4-5 weeks following the methodology developed by Lundberg et al., (2012), and after surface sterilization and processing, they were stored at -80°C until further use.

3.2.3 DNA extractions and library preparation

DNA extraction was performed using FASTDNA™-96 Soil Microbe DNA Kit (MP Biomedical, LLC). Samples were previously separated base on organ type (root, stem, leaves) and soil (rhizosphere) and were all placed in the freeze dryer before being pulverized. Daisy bb gun beads were used to pre-pulverize the samples allowing stem and root samples to homogenize for an optimal DNA isolation. After extraction, libraries were prepared following the protocol established by Lundberg et al., (2013), where Peptide Nucleic Acid (PNA) for mitochondrial (5'-GGCAAGTGTTCTTCGGA-3') and plastid (5'-GGCTCAACCCTGGACAG-3') rRNA and plastid sequences were used as elongation arrest clamps to prevent ribosomal 16S from the plant from being amplified.

3.2.4 PCR quantification and sequencing

The DNA concentrations of the final reactions obtained from the PCR step were measured in 96-well plate format using PicoGreen fluorescent dye (Invitrogen) for

double stranded DNA quantification in a fluorescent plate reader format (475nm to 530nm). After quantifying the amounts of DNA present, we ran a portion of the samples in an 1.5% agarose gel to ensure the presence of bands with a size of 448bp. Pooling of all samples from the prepared library was performed using equimolar ratios, and cleaned using AMPure beads at a 0.7:1 ratio, to later be eluted in 20 µl of di water to be denature and loaded in the MiSeq machine by following the Illumina protocol and the standards established by Lundberg et al., (2013).

3.2.5 Demultiplexing and heatmap generation

After sequencing, data obtained from the Illumina machine was demultiplexed by utilizing the CASAVA software from Illumina, v.1.8.2. A FASTA file was generated in which all the consensus sequences obtained per sample were store. Software utilized was the Molecular Tag Toolbox (MT-Toolbox, Google sites, Yourstone, 2014). R scripts were made and used to create graphs that showed the abundance of the presence of the different microbial organism present in the roots of the plants that were treated and non-treated. Rarefaction values varied base on the type of heatmap that was generated (Family, phylum or OTU).

3.2.6 Phylum analysis and abundance for genotype and inoculation

Only the non-plant reads were classified to the phylum level. Reads from the same phyla were pooled, and read counts were normalized and converted to a ratio by dividing the reads from each phyla by the total number of phylum-classifiable reads in that sample. For better visualization, those phyla representing less than 5% of the total in any sample were reclassified as “Low Abundance”. Data was plotted in R using this “Hist” function of ggplot2 (Wickham).

3.2.7 CAPSCALE analysis

A constrained ordination routine analysis was used to determine if samples separated based on the treatment to which they were previously exposed. These analyses used a distance matrix between the samples, showing the coordinates of each sample determined by the profile of OTU counts for that sample using Bray-Curtis distance. R packages used for the analysis were: vegan, for capscale, ordination, and pscl.

3.2.8 Poly(A) Tag library preparation and sequencing

Total RNA was isolated from *N. benthamiana* plants of ~4-5 weeks of age using RNAeasy kit (Qiagen). *Nicotiana* poly(A) tags (PATs) were generated with 1 µg of total RNA using the Method B1 as described in (Ma et al., 2014). The resulting poly(A) tags were sequenced on an Illumina high-throughput sequencing DNA platform. In all cases, three independent biological replicates were used. The sequenced PAT-seq reads were processed using the pipeline as detailed in (Bell et al., 2016). Briefly, sequences were demultiplexed and trimmed to remove the oligo-dT tracts and sequencing adapters. The processed tags were then mapped to the Nicotiana reference genome. The mapping output was saved in bam file format and used with BEDTools to determine the total count of PATs that mapped to individual annotated genes. The gene expression was determined using the empirical analysis of EDGE tool in CLC Genomics Workbench. Genes were considered significantly different using a p-value $\leq$ 0.01 and a 2-fold change and a total of 3 replicates were used per treatment.

3.2.9 CARD-FISH in roots

We utilized a modified protocol taken from Lebeis et al., (2015). For this analysis, we collected roots from plants of 10 and 21 days old, which were slightly cleaned with quick washes (10 sec) of bleach 5% and ethanol 70% and multiple changes of sterile water (in order to only obtain hybridization of endophytic bacteria and only for our 10 and 21 days old samples). Later, they were place in 15ml Falcon tubes with 4% formaldehyde in PBS at 4°C for ~4h, washed three times in PBS, and stored in 1:1 PBS: ethanol at -20°C. We proceeded to treat them with lysozyme solution (1 h at 37°C) and achromopeptidase (30 min at 37°C; Sigma) in order to make permeable the prokaryotic cell-wall. All endogenous peroxidases were inactivated with a solution of methanol amended by 0.15% H_2O_2 and incubated at room temperature for 40 min. Samples were washed one more time before storing them at -20°C again before starting hybridization.

Probes used to target target the 16S or the 23S rRNA (EUB338 (59-GCTGCCTCCCGTAGGAGT-39, 35% formamide), were provided by the Lebeis lab at University of Tennessee, Knoxville, which were selected using probeBase38 (http://www.microbial-ecology.net/default.asp), and labelled with enzyme horseradish peroxidase on the 59 end (Invitrogen).

For 10 and 21 days old samples Hybridization was performed by incubation samples at 35°C for ~2h. Unbound probes were washed away from samples in wash buffer (NaCl content adjusted according to the formamide concentration in the hybridization buffer) at 37°C for 30 min. Fluorescently labelled tyramide was used for signal amplification, and samples were washed before mounting on glass slides. Roots were mounted on glass slides using Vectashield with DAPI (Vector Laboratories, catalogue no. H-1200) for mounting solution.

3.2.10 Statistics for morphological data analyses

All data collected were analyzed with SAS using GLM to generate means for each trait. We used Tukey's test to separate means using a p-value of $p< 0.05$. Boxplots graphs were generated using BoxPlotR: a web-tool for generation of box plots, an application created by the Tyers and Rappsilber labs (http://shiny.chemgrid.org/boxplotr/). Sample size was represented by the width of each box and notches represent a 95% confidence between medians difference. Tukey was used to define the whiskers for each group sample. Number of samples was thirty-six (36) total per year/per trait/per treatment (12 samples in each season per treatment).

3.3 Results and discussion

3.3.1 Bacterial inoculation studies: Morphology

Using the information obtained from the sequenced *Nicotiana species*, we determined *a* tractable microbiome. It is known that genetic factors within the host plant are capable of altering interactions related to microbiome assembly (Lebeis et al., 2015*,* Bulgarelli *et al*., 2015, Hartman et al., 2017). Thus, we wanted to evaluate whether overrepresentation of a growth-modifying microbial inoculum applied as a seed treatment impacted the microbiota of a plant. To achieve this goal, we sought to identify whether bacterial seed inoculum could 1) induce robust changes in plant growth and development and 2) subsequently, investigate whether these changes were associated by changes to the microbiome.

From our original screen of multiple species of *Nicotiana* plants, we determine that it was complicated to evaluate strong differences for all of them, since some species responded favorably to some of the original 24 inoculums selected and extremely negatively or neutral for most of the species. Although there was potential for some of

them, we decided to reduce our inoculum list to those that strongly displayed a phenotype in more than one species and that were competitive but not dominant based on the literature information available (Kinsella et al., 2009; Tan et al., 2013; Qiao et al., 2017, Figure 3.1).

Figure 3.1 *Nicotiana* species evaluated in greenhouse conditions after seeds were treated with the original list of 24 bacterial strains that had potential to be growth promoters.

Based on this, our screen for functional microbial seed inoculum was performed by over-representing bacterial organisms on the seed of *Nicotiana benthamiana and Nicotiana tabacum,* growing the plants for twelve (12) weeks to observe growth metrics. The final bacterial strains selected from our Debolt Lab microbial library, we chose one isolated from Switchgrass (*Panicum virgatum)* and 3 from Giant Burpee tomato (*Solanum lycopersicum* L.) (Xia et al., 2013, 2014, 2015), which were consistent and reproducible for induction or suppression of growth. Our treatments were composed by: *Micrococcus sp.* (Ms), which induced growth suppression, *Lysinibacillus fusiformis* (Lf), which caused no growth influence, and two growth promoting *Bacilli, B. sp.* (Bs) and *B. cereus* (Bc) (Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5).

As mentioned, these final groups of inoculums were applied as monoculture bacterial treatments to *N. benthamiana* and *N. tabacum* to test overall effect in plant, and in order to obtain a more in detail explanation of morphological changes, we selected only *N. benthamiana* as a way to reduce the complexity inherently associated with numerous genotypes. Strains selected did not arrest germination, but they did have an influence on how fast some of them were able to complete germination, especially Ms treatment that took longer to complete germination in *Nicotiana benthamiana* when compared to untreated seeds. In *Nicotiana tabacum* lines KY14 treated seeds (all treatments) behaved similar to non-treated, but for the line TN90, Ms treated seeds took almost a month to complete germination when compared to control and the rest of the treatments (Figure 3.3B).

Morphologically, treated plants presented variable responses. Treatments Bs and Bc had a growth promoting effect on the plants that were treated, but Ms had a reduction growth effect in plants, showing a much slower development when compared with control (Figure 3.3).

Nicotiana tabacum lines (KY14 and TN90) had a favorable response to treatments Bs and Bc, but contrary to *Nicotiana benthamiana* results, KY14 plants treated with Ms had a better response in terms of growth promotion than treatments like Bs, which was much similar to control samples for this specific line (Figure 3.3). On the other hand, TN90 treated with Ms had a much stronger phenotype for slow growth than *Nicotiana benthamiana* due to a late completion of germination. Plants of all treatments had heights of 10-20cm by 8 weeks, which differed from Ms treatments that were only ~5 cm total.

As a way of reducing complexity when understanding and determining striking differences among treatments, we evaluated these monocultures as potential inoculums for seeds only in *N. benthamiana* plants. Response to the four target microorganisms was found to be reproducible over 9 generations (3 years, 12 replicates per time point – Spring, Summer and Fall/Winter- recording changes in phenotypic traits at 3, 6 and 12 weeks) (Fig. 3.3, n = 36 individuals per phenotype per year; Figure 3.4, Figure 3.5).

The consistency of the phenotype being present in multiple trials led us towards finding what factors could be playing a role in enhancing growth in the plants, beyond being the regular descriptions of soil nutrients availability (which we did not study because all treatments had a regular application of fertilizer in the same way that a tobacco plant will be treated for crop production, but under a greenhouse environment).

3.3.2 Bacterial inoculation studies: Transcriptomics and microbiome assembly effect

In addition to the phenotypic responses, we cataloged the transcriptome of plants treated with each inoculum using the draft genome information of *N. benthamiana* (Bombarely et al., 2012).

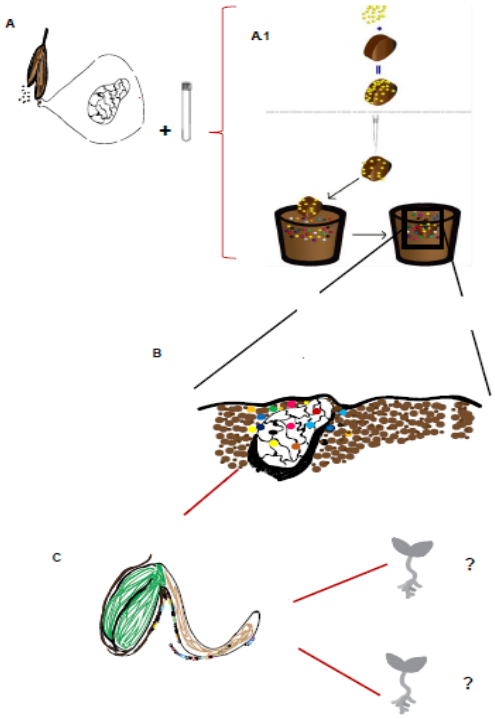


Figure 3.2 Diagram of the inoculation and screening process. A) Seeds (previously tested for germination percentage) were surface sterilized and co-cultured with the bacterial strains used as an inoculum (monoculture). A.1) inoculation of seeds: seeds were surface-sterilized and only colonized by the bacteria of interest. The overrepresented bacterial organism should have ample opportunity to colonize the plant due to sheer abundance. B) Spermosphere colonization by native bacteria from the soil (can vary based on the inoculum used) C) Seedling developed with a first microbiome (from the spermosphere bacterial community) could have a response to it by developing a phenotype that could be positive (growth promoting) or negative (growth restrictive).

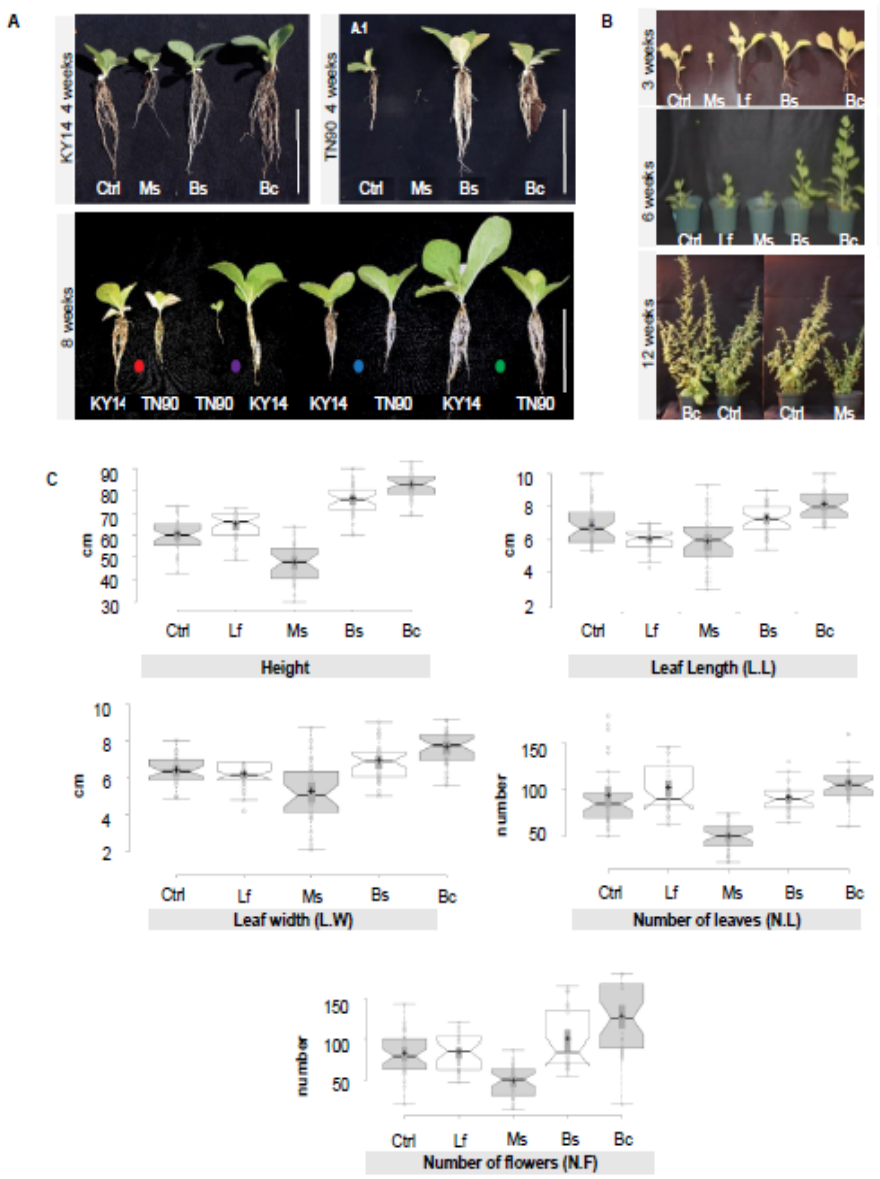


Figure 3.3 Morphological differences between *Nicotiana tabacum* and *Nicotiana benthamiana* plants treated with bacterial inoculums as seed treatments. A) *Nicotiana tabacum* line KY14 and TN90 at 4 weeks of growth. Plants at 8 weeks old (comparison side to side of all treatments per tobacco line). Dots represent different treatments: Red: Control, Purple: Ms, Blue: Bs and Green: Bc. B) Control, Ms, Lf, Bs and Bc treated plants grown in greenhouse after 3 weeks showing differences in morphology and root architecture. Plant at 6 weeks display a more accentuated phenotype, having Bc and Bs as the plants with the most growth, and Ms having the smallest phenotype. At 12 weeks, plants still show a distinctive phenotype. C) Control, Ms, Lf, Bs and Bc treated plants grown in greenhouse during a whole year period (2013-Spring, late Summer and Fall/Winter) Measurements of Height, leaf length (L.L), leaf width (L.W), number of leaves (N.L), and number of flowers (N.F). Means were separated using Tukey's test, notches represent a significant difference among treatments at a $p < 0.05$.

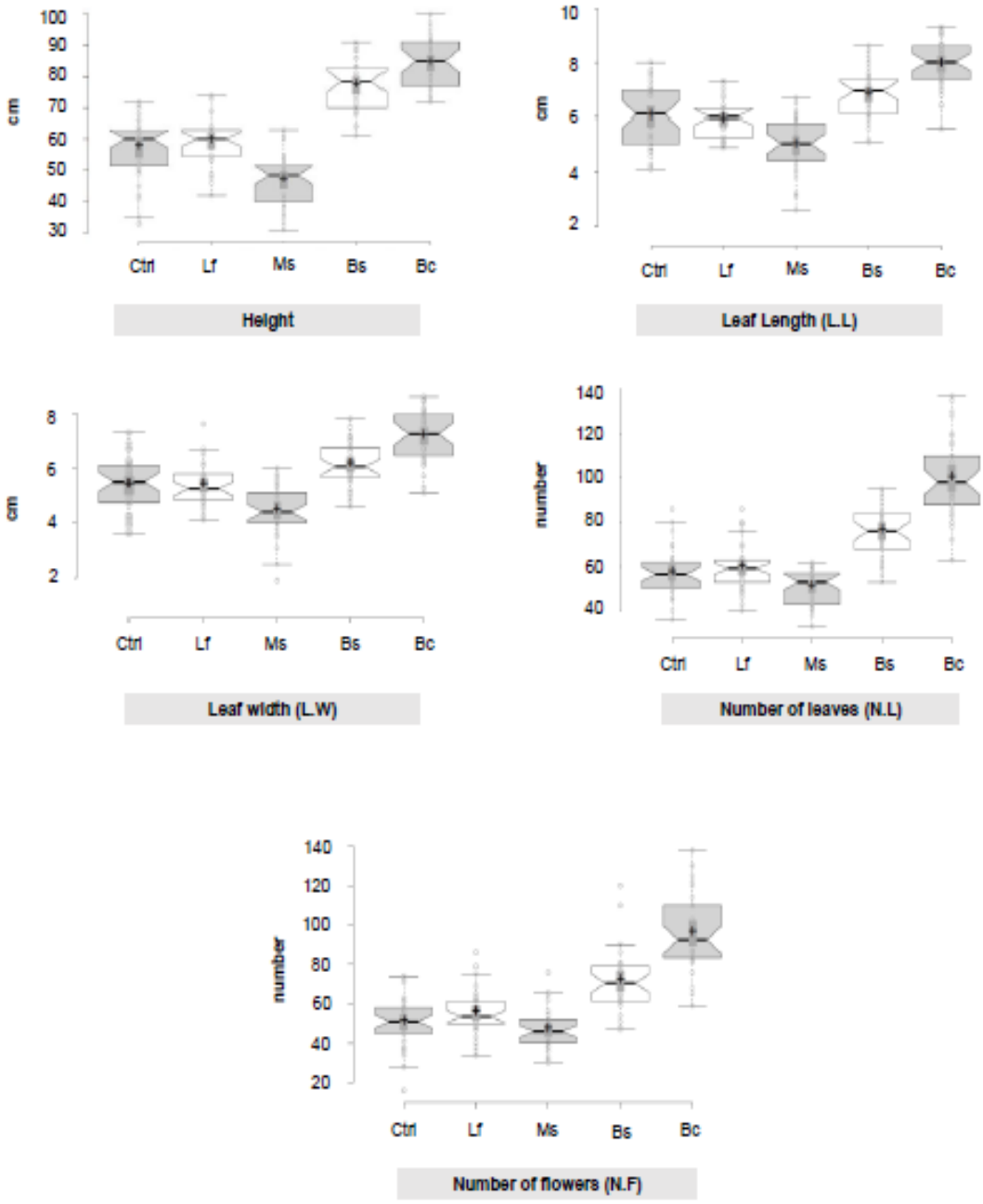


Figure 3.4 Morphological data of different traits evaluated in *Nicotiana benthamiana* plants. A) Control, Ms, Lf, Bs and Bc treated plants grown in the greenhouse during the 2014 year (composite of Spring, late Summer and Fall/Winter data) in 12 week old plants. Measurements of Height, leaf length (L.L), leaf width (L.W), number of leaves (N.L), and number of flowers (N.F). Means were separated using Tukey's test, notches represent a significant difference among treatments ($p < 0.05$).

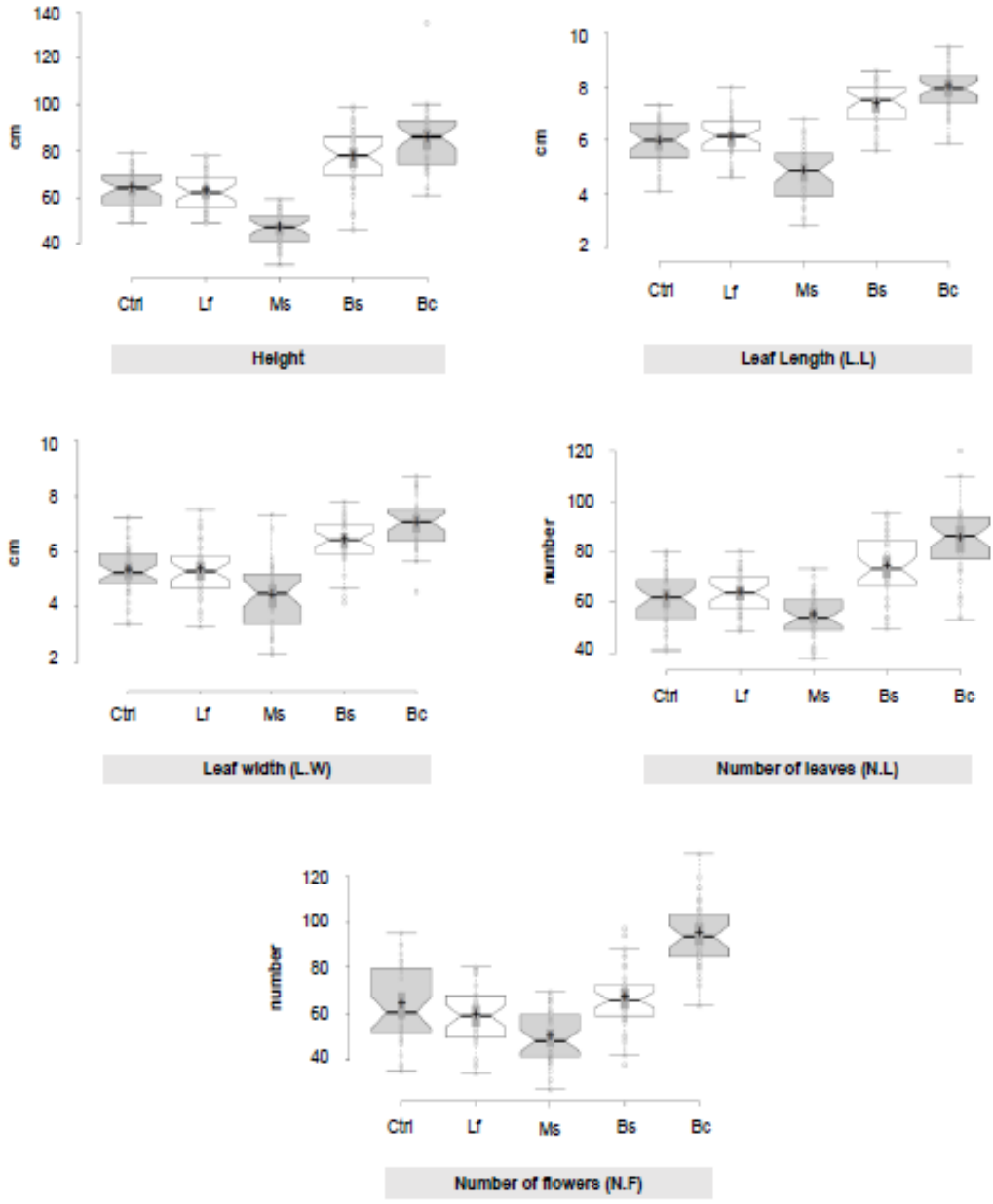


Figure 3.5 Morphological data of different traits evaluated in *Nicotiana benthamiana* plants. Control, Ms, Lf, Bs and Bc treated plants grown in greenhouse during the 2015 year (composite of Spring, late Summer and Fall/Winter data) in 12 week old plants. Measurements of Height, leaf length (L.L), leaf width (L.W), number of leaves (N.L), and number of flowers (N.F). Means were separated using Tukey's test, notches represent a significant difference among treatments ($p < 0.05$).

We identified 342, 663 and 668 differentially expressed genes (DEGs) after Ms, Bs and Bc colonization, respectively. Approximately 75% of transcripts were commonly induced in plants treated with the growth promoting Bs and Bc (fold change > 2, FDR p-value < 0.05). In contrast, only 20% of the induced transcriptome was shared between the growth promoting Bs and suppressing Ms (Figure 3.6A, 3.6B, Figure 3.7 and Table 3.2).

Data support an expected correlation between single inoculum induced phenotype and the transcriptional output of the host plant. In this group, genes associated with signaling and protein regulation were over-represented in Bs and Bc; and transport and metabolism-related genes were over-represented in Ms (Figure 3.8). Commonalities found between differentially downregulated genes also were more relevant between Bs and Bc (fold change < 2, FDR p-value < 0.05); genes associated with metabolism were over-represented in this set (Figure 3.8). Interestingly, a significant number of downregulated genes after Ms treatment were found to be upregulated in Bc and Bs, and a large number of these seem to be related to hormone genes (data is not disclosed due to regulations from the group that build the genome for *N. benthamiana*). Based on our transcriptome data, we hypothesized that *N. benthamiana* express a differential response to *Micrococcus* and *Bacillus*.

Lebeis et al., (2015) evaluated various mutants with either overexpression or repression of certain hormones involved in defense responses. Under genetic conditions lacking hormonal control (particularly of SA), the microbiome of the plant changed. Even though their findings were done using mutants that display a deficiency or an overexpression of a specific hormone related gene, we see here that treatments where overrepresentation of an organism is used can induce a similar response in the plant. It seems that soil community composition, pressures in the microenvironment, the natural ability of bacteria for colonization and plant physiological processes; have to work together in order for the microbiome to be assembled.

In order to have a better understanding of the movement and localization of bacterial organisms in plants for some of the time points (or close) to those used to evaluated morphology as well as gene expression and microbial community composition, we used target inoculum samples grown under greenhouse conditions to image the presence of

EC localized bacteria, with and without inoculation (Control and treatments), using catalyzed reporter deposition and fluorescence *in situ* hybridization (CARD–FISH).

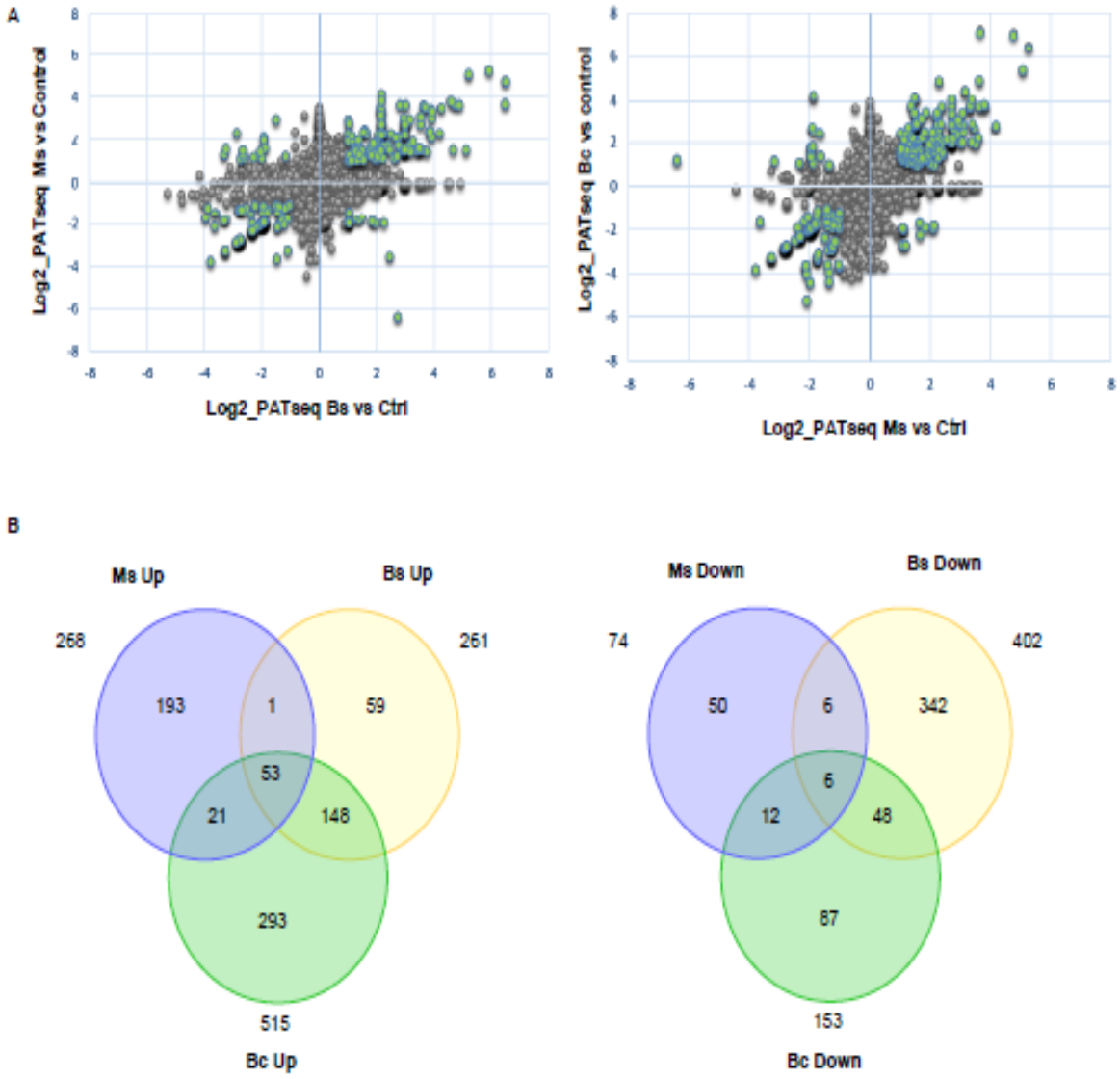
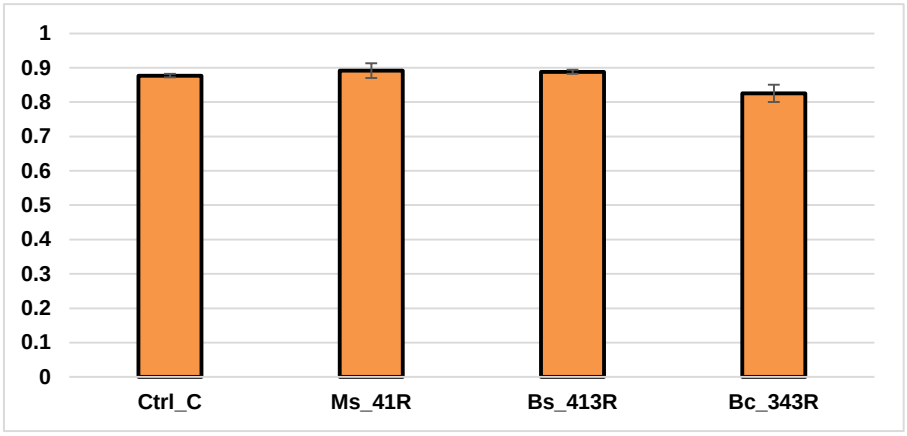


Figure 3.6 Gene expression comparison between Ms and Bc treated plants. A) Scatter plot comparing gene expression results from plants growth with Ms, Bs and Bc strains using PAT-seq. In this plot, the log2-transformed values for expression ratios for genes present in both of the two experiments were plotted as shown. B) Overlapping genes among all selected genes for each treatment.

Table 3.2 Summary of the mapping statistics for the individual poly(A) tag libraries and the pooled replicates for each condition. The library names reflect the different treatments and also include the bar code used to differentiate each of the libraries after pooling. Each read was mapped to *Nicotiana benthamiana* genome.

Samples	Name / Barcode	Number of reads	Avg.length
Control condition (Ctrl)	C1:CGGTTTT	759,915	91
	C2:ACGTTTT	8,776,310	73.4
	C3:AACTTTT	1,639,512	90.8
Micrococcus sp (Ms)	41R1:CCGTTTT	3,224,909	88.6
	41R2:AGATTTT	2,238,169	106.3
	41R3:AGCTTTT	11,105,167	75.7
Bacillus sp (Bs)	413R1:TCGTTTT	5,026,225	89.7
	413R2:CAATTTT	7,197,301	74.7
	413R3:TAGTTTT	418,872	135.7
Bacillus cereus (Bc)	343R1:CCATTTT	2,603,805	67.1
	343R2:CAGTTTT	1,405,288	67.3
	343R3:CACTTTT	3,854,541	67

Samples	Name / Barcode	Count mapped to Nicotiana benthamiana	Percentage mapped Nicotiana benthamiana
Control condition (Ctrl)	C1:CGGTTTT	655,832	87.00%
	C2:ACGTTTT	7,694,072	88.28%
	C3:AACTTTT	1,426,508	87.79%
Micrococcus sp (Ms)	41R1:CCGTTTT	2,801,594	87.75%
	41R2:AGATTTT	2,046,638	92.14%
	41R3:AGCTTTT	9,655,964	87.50%
Bacillus sp (Bs)	413R1:TCGTTTT	4,313,076	88.00%
	413R2:CAATTTT	6,379,259	89.23%
	413R3:TAGTTTT	369,971	89.24%
Bacillus cereus (Bc)	343R1:CCATTTT	2,162,072	86.04%
	343R2:CAGTTTT	1,038,270	80.13%
	343R3:CACTTTT	3,005,835	81.60%



		Average	Standard desviation
Samples	**Name**	**Percentage mapped *Nicotiana benthamiana***	**Percentage mapped *Nicotiana benthamiana***
Control condition	Ctrl_C	87.69%	0.005273203
Micrococcus sp (Ms)	Ms_41R	89.13%	0.021308371
Bacillus sp (Bs)	Bs_413R	88.82%	0.005821989
Bacillus cereus (Bc)	Bc_343R	82.59%	0.0251225

Figure 3.7 Means and standard deviations for these mapped reads. The library names reflect the different treatments and also include the bar code used to differentiate each of the libraries after pooling. Each read was mapped to *Nicotiana benthamiana* genome.

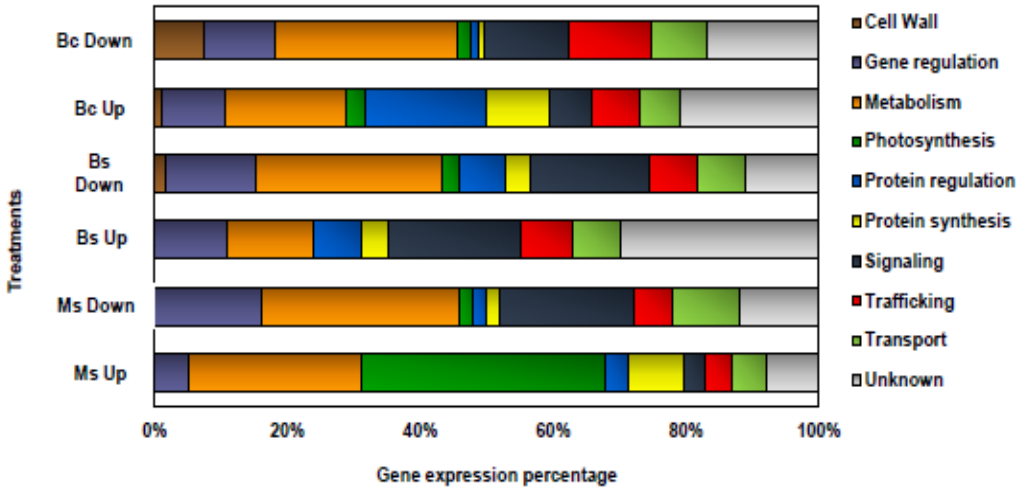


Figure 3.8 Gene expression data analysis from treated and untreated plants. CLC was used for statistical analysis and EDG was performed. Genes were considered significantly different using a p-value ≤ 0.01 and a 2-fold change. Total number of different expressed genes was 341 for Ms, 663 for Bs and 641 for Bc. Genes were assigned categories using Interpro-ID, GO and Human readable information.

Whole root segments were imaged during two different time points, which were selected based on 1) initial host cotyledon development and emergence of first true leaves, being an early stage for the seedling which represents the early colonization and points of entry and localization of the bacterial organisms selected by the plant (10 day), and 2) emergence of second set of true leaves, representing a more mature stage of the seedlings, but still prior to the plant fully transitioning to a more reproductive stage (21 days). After 10 days, the inoculated samples displayed increased fluorescence arising from bacterial organisms compared with the non-inoculated control (Figure 3.9).

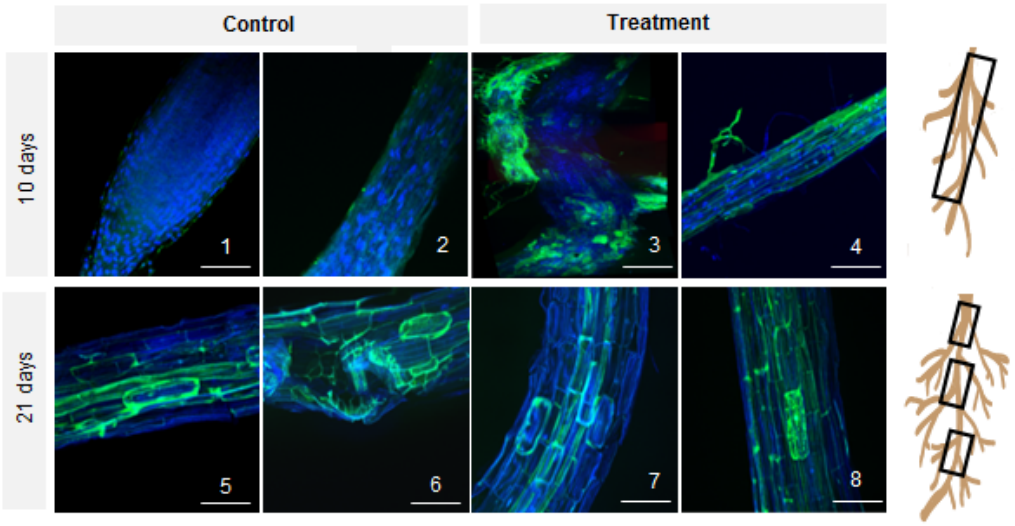


Figure 3.9 CARD-FISH imaging in treated and control plants using a eubacteria probe to determine localization of bacteria at different time points during growth and development. 1-2 & 5-6) Control at 10 days and 3 weeks, 3-4 & 7-8) Bs & Bc plants at 10 days and 3 weeks. At 10 days, most of the fluorescence is observed in the secondary roots and root hairs, at 3 weeks it can be observed mostly in the vascular system.

These were localized in the parenchymatic tissue, in lateral roots and hairs. By contrast, after 21 days, the control and inoculation treatment revealed similar levels of fluorescence, suggesting that colonization normalized between 10-21 days. Simply, these data suggested that seed inoculation created an initial short duration increase in bacteria colonizing in the plant root EC (Figure 3.9). To further evaluate the influence of seed inoculum on the plant microbiome, we sequenced the EC community composition after inoculation (Figure 3.10). We initially looked for the presence of the inoculum strain

in the community. It was noted that due to amplicons for identification it is not feasible to distinguish individual strains. At the phyla and family level the inoculum was taxonomically represented in the plant EC (Fig. 3.10).

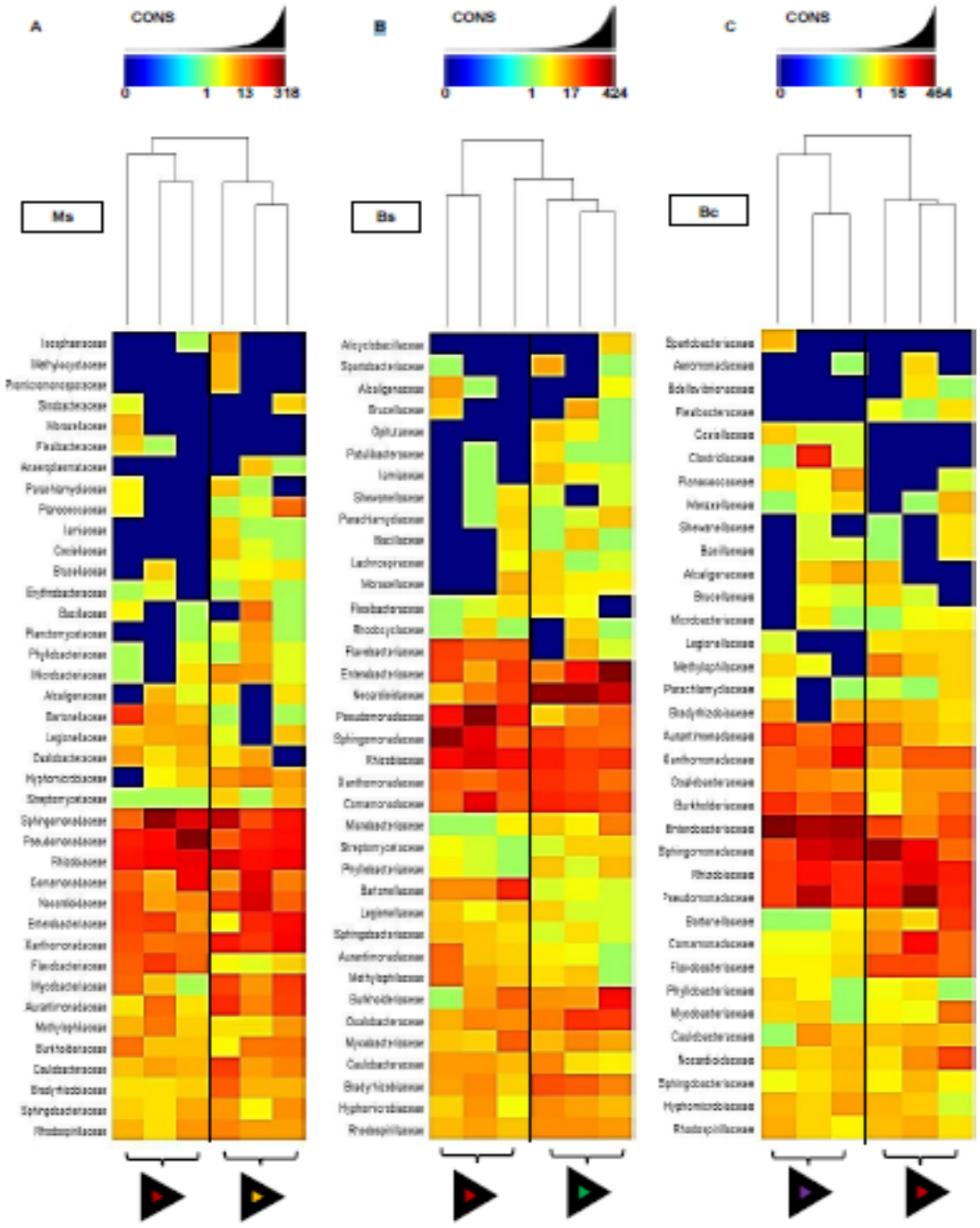


Figure 3.10 Heatmap shows the clustering of endophytic bacterial 16S rRNA amplicons of roots from treatments vs control at the A) Ms vs Control, B) Bs vs Control and C) Bc vs Control at the Family level. Red triangles represent control samples.

In terms of diversity and abundance, at least four general trends were observed from the inoculation studies. Firstly, treatments and tissue specific EC communities shared a structured “core” microbiome similar to what was observed in our genotype community description (Figure 2.3). Secondly, we observed modest variations in microbial organisms arising from treated plants, to which we decided to call, the “accessory” microbiome. For example, *Acidobacteria* was present in low quantities in control and Ms samples, but abundance of this phyla increased among Bs and Bc samples. Further, *OP11* was only found in control and Ms samples, and *Chlorobi* only in Bs samples (Figure 3.11). Thirdly, seed inoculums altered the root EC community more than was observed for the stem and leaf EC (Figure 3.11 and 3.12), which could be partially explained by the lower rarefaction used to generate our map comparisons (especially at OTU level).

This trend was similar to that seen in the previously analyzed *N. benthamiana* (Figure 2.3; Figure 3.13) samples. In stem and leaf EC, all treatments displayed a similar phyla composition, but the abundance of these classes differed. Taken together, stem and leaf ECs were more restrictive of their EC inhabitance, which was also seen in the reference microbiome (Figure 2.3; Table 2.3 and Table 3.3). A fourth observation was made when we combined all tissue EC data and performed a constrained analysis of principal coordinates to ask which treatments were most impactful on the data (Figure 3.14). PCA data suggested that Bc and Ms, which induced growth promotion or reduction, respectively *in planta*, accounted for the greatest proportion of variance in the data.

Several plausible interpretations exist to explain this result. Firstly, the microbial inoculum contributes to an EC community alteration in the host. Alternatively, the phenotype of the host plant, and ensuing architectural and metabolic structure directly influence the EC community structure. Based on prior studies into the consistent nature of the core microbiome (Lundberg et al., 2012) and the response to the plant host (Lebeis et al., 2015), we suspect that the two hypotheses are not mutually exclusive and a somewhat dynamic interplay exists between inoculum, phenotype and EC community structure.

Table 3.3 Percentages of abundance of phylum classification in different *Nicotiana benthamiana* treatments. After removal of OTUs classified as chloroplasts and mitochondria, remaining OTUs were grouped into their respective phyla. The abundance of each phylum in a sample was represented as a percentage, and any phylum which made up less than less than 5 percent in a given sample was classified as "Low abundance" for that sample.

	Phylum	Percentage	Sample
P31	low_abundance	13.04347826	Root_Control_1
P31	Firmicutes	8.074534161	Root_Control_1
P31	Gemmatimonadetes	7.453416149	Root_Control_1
P31	Planctomycetes	6.211180124	Root_Control_1
P31	Actinobacteria	11.00266193	Root_Control_1
P31	Bacteroidetes	24.75598935	Root_Control_1
P31	Proteobacteria	29.45874002	Root_Control_1
P62	low_abundance	12.47401247	Root_Control_2
P62	OP11	5.472667237	Root_Control_2
P62	Firmicutes	5.955729485	Root_Control_2
P62	Gemmatimonadetes	12.0276385	Root_Control_2
P62	Actinobacteria	11.35502018	Root_Control_2
P62	Bacteroidetes	11.24495536	Root_Control_2
P62	Proteobacteria	41.46997676	Root_Control_2
P24	low_abundance	9.089770878	Root_Control_3
P24	Acidobacteria	22.26117441	Root_Control_3
P24	Firmicutes	6.635783148	Root_Control_3
P24	Gemmatimonadetes	7.462125955	Root_Control_3
P24	Actinobacteria	12.08213347	Root_Control_3
P24	Bacteroidetes	20.7462126	Root_Control_3
P24	Proteobacteria	21.72279955	Root_Control_3
P85	low_abundance	23.00838574	Root_447_1
P85	Firmicutes	9.512578616	Root_447_1
P85	Actinobacteria	24.9475891	Root_447_1
P85	Bacteroidetes	14.1509434	Root_447_1
P85	Proteobacteria	28.38050314	Root_447_1
P87	low_abundance	6	Root_447_2
P87	Chlamydiae	18.2	Root_447_2
P87	Planctomycetes	5.8	Root_447_2
P87	Actinobacteria	10.2	Root_447_2
P87	Bacteroidetes	10.4	Root_447_2
P87	Proteobacteria	34.4	Root_447_2
P8	low_abundance	15.05646173	Root_447_3
P8	Firmicutes	12.04516939	Root_447_3
P8	Gemmatimonadetes	5.457967378	Root_447_3
P8	Actinobacteria	21.706399	Root_447_3

Table 3.3 Percentages of abundance of phylum classification in different *Nicotiana benthamiana* treatments (continued)

	Phylum	Percentage	Sample
P8	Bacteroidetes	18.13048934	Root_447_3
P8	Proteobacteria	27.60351317	Root_447_3
P68	low_abundance	12.11764706	Root_S41_1
P68	Acidobacteria	10.94117647	Root_S41_1
P68	Firmicutes	5.529411765	Root_S41_1
P68	Gemmatimonadetes	7.647058824	Root_S41_1
P68	Actinobacteria	17.76470588	Root_S41_1
P68	Bacteroidetes	9.529411765	Root_S41_1
P68	Proteobacteria	36.47058824	Root_S41_1
P29	low_abundance	21.6080402	Root_S41_2
P29	Chlamydiae	5.27638191	Root_S41_2
P29	Firmicutes	12.39530988	Root_S41_2
P29	Actinobacteria	8.793969849	Root_S41_2
P29	Bacteroidetes	13.4840871	Root_S41_2
P29	Proteobacteria	38.44221106	Root_S41_2
P30	low_abundance	19.54022989	Root_S41_3
P30	OP11	5.172413793	Root_S41_3
P30	Firmicutes	10.1532567	Root_S41_3
P30	Gemmatimonadetes	7.279693487	Root_S41_3
P30	Actinobacteria	9.578544061	Root_S41_3
P30	Bacteroidetes	13.2183908	Root_S41_3
P30	Proteobacteria	35.05747126	Root_S41_3
P9	low_abundance	11.77394035	Root_413_1
P9	Chlorobi	5.259026688	Root_413_1
P9	Acidobacteria	6.200941915	Root_413_1
P9	Gemmatimonadetes	5.416012559	Root_413_1
P9	Actinobacteria	14.12872841	Root_413_1
P9	Bacteroidetes	6.279434851	Root_413_1
P9	Proteobacteria	50.94191523	Root_413_1
P37	low_abundance	17.91553134	Root_413_2
P37	Acidobacteria	7.629427793	Root_413_2
P37	Firmicutes	6.675749319	Root_413_2
P37	Actinobacteria	26.83923706	Root_413_2
P37	Bacteroidetes	12.46594005	Root_413_2
P37	Proteobacteria	28.47411444	Root_413_2
P33	low_abundance	14.16400426	Root_413_3
P33	Acidobacteria	5.48455804	Root_413_3
P33	Firmicutes	6.07028754	Root_413_3
P33	Gemmatimonadetes	8.359957401	Root_413_3

Table 3.3 Percentages of abundance of phylum classification in different *Nicotiana benthamiana* treatments (continued)

	Phylum	Percentage	Sample
P33	Actinobacteria	29.39297125	Root_413_3
P33	Bacteroidetes	9.318423855	Root_413_3
P33	Proteobacteria	27.20979766	Root_413_3
P65	low_abundance	20.89190499	Root_343_1
P65	Acidobacteria	9.161415414	Root_343_1
P65	Actinobacteria	6.592341251	Root_343_1
P65	Bacteroidetes	7.755695589	Root_343_1
P65	Proteobacteria	55.59864275	Root_343_1
P0	low_abundance	19.05487805	Root_343_2
P0	Chlamydiae	6.402439024	Root_343_2
P0	Acidobacteria	16.15853659	Root_343_2
P0	Gemmatimonadetes	5.487804878	Root_343_2
P0	Bacteroidetes	5.868902439	Root_343_2
P0	Proteobacteria	47.02743902	Root_343_2
P2	low_abundance	18.2665424	Root_343_3
P2	Gemmatimonadetes	6.24417521	Root_343_3
P2	Actinobacteria	6.4305685	Root_343_3
P2	Bacteroidetes	7.455731594	Root_343_3
P2	Proteobacteria	61.60298229	Root_343_3
P2	Proteobacteria	61.60298229	Root_343_3
P3	low_abundance	8.149084018	Stem_Control_1
P3	Gemmatimonadetes	13.20277953	Stem_Control_1
P3	Planctomycetes	10.67593178	Stem_Control_1
P3	Actinobacteria	12.25521162	Stem_Control_1
P3	Bacteroidetes	25.26847757	Stem_Control_1
P3	Proteobacteria	30.44851548	Stem_Control_1
P13	low_abundance	11.25163541	Stem_Control_2
P13	Gemmatimonadetes	12.73440907	Stem_Control_2
P13	Planctomycetes	10.64108155	Stem_Control_2
P13	Actinobacteria	12.34191016	Stem_Control_2
P13	Bacteroidetes	24.7710423	Stem_Control_2
P13	Proteobacteria	28.2599215	Stem_Control_2
P43	low_abundance	10.05785492	Stem_Control_3
P43	Gemmatimonadetes	12.37205162	Stem_Control_3
P43	Planctomycetes	9.746328438	Stem_Control_3
P43	Actinobacteria	12.41655541	Stem_Control_3
P43	Bacteroidetes	25.45616377	Stem_Control_3
P43	Proteobacteria	29.95104584	Stem_Control_3
P61	low_abundance	19.6	Stem_447_1

Table 3.3 Percentages of abundance of phylum classification in different *Nicotiana benthamiana* treatments (continued)

	Phylum	Percentage	Sample
P61	Gemmatimonadetes	8.4	Stem_447_1
P61	Planctomycetes	8.8	Stem_447_1
P61	Actinobacteria	21.6	Stem_447_1
P61	Bacteroidetes	10.4	Stem_447_1
P61	Proteobacteria	31.2	Stem_447_1
P60	low_abundance	12.86764706	Stem_447_2
P60	Gemmatimonadetes	9.191176471	Stem_447_2
P60	Planctomycetes	6.25	Stem_447_2
P60	Actinobacteria	16.17647059	Stem_447_2
P60	Bacteroidetes	17.64705882	Stem_447_2
P60	Proteobacteria	37.86764706	Stem_447_2
P36	low_abundance	7.826086957	Stem_447_3
P36	Gemmatimonadetes	10.43478261	Stem_447_3
P36	Planctomycetes	9.565217391	Stem_447_3
P36	Actinobacteria	12.17391304	Stem_447_3
P36	Bacteroidetes	13.04347826	Stem_447_3
P36	Proteobacteria	46.95652174	Stem_447_3
P77	low_abundance	8.722741433	Stem_S41_1
P77	Gemmatimonadetes	11.52647975	Stem_S41_1
P77	Planctomycetes	12.14953271	Stem_S41_1
P77	Actinobacteria	9.345794393	Stem_S41_1
P77	Bacteroidetes	33.33333333	Stem_S41_1
P77	Proteobacteria	24.92211838	Stem_S41_1
P75	low_abundance	10.28938907	Stem_S41_2
P75	Gemmatimonadetes	11.5755627	Stem_S41_2
P75	Planctomycetes	10.61093248	Stem_S41_2
P75	Actinobacteria	14.46945338	Stem_S41_2
P75	Bacteroidetes	20.90032154	Stem_S41_2
P75	Proteobacteria	32.15434084	Stem_S41_2
P44	low_abundance	7.602339181	Stem_S41_3
P44	Gemmatimonadetes	8.187134503	Stem_S41_3
P44	Planctomycetes	11.69590643	Stem_S41_3
P44	Actinobacteria	9.356725146	Stem_S41_3
P44	Bacteroidetes	27.48538012	Stem_S41_3
P44	Proteobacteria	35.67251462	Stem_S41_3
P19	low_abundance	7.508305648	Stem_413_1
P19	Gemmatimonadetes	12.09302326	Stem_413_1
P19	Planctomycetes	8.837209302	Stem_413_1
P19	Actinobacteria	16.34551495	Stem_413_1

Table 3.3 Percentages of abundance of phylum classification in different *Nicotiana benthamiana* treatments (continued)

	Phylum	Percentage	Sample
P19	Bacteroidetes	24.11960133	Stem_413_1
P19	Proteobacteria	31.09634551	Stem_413_1
P47	low_abundance	11.87989556	Stem_413_2
P47	Gemmatimonadetes	14.49086162	Stem_413_2
P47	Planctomycetes	9.007832898	Stem_413_2
P47	Actinobacteria	14.62140992	Stem_413_2
P47	Bacteroidetes	11.09660574	Stem_413_2
P47	Proteobacteria	38.90339426	Stem_413_2
P71	low_abundance	10.49382716	Stem_413_3
P71	Firmicutes	5.24691358	Stem_413_3
P71	Gemmatimonadetes	8.950617284	Stem_413_3
P71	Planctomycetes	7.716049383	Stem_413_3
P71	Actinobacteria	22.22222222	Stem_413_3
P71	Bacteroidetes	23.14814815	Stem_413_3
P71	Proteobacteria	22.22222222	Stem_413_3
P89	low_abundance	14.94845361	Stem_343_1
P89	Gemmatimonadetes	9.278350515	Stem_343_1
P89	Planctomycetes	13.91752577	Stem_343_1
P89	Actinobacteria	17.5257732	Stem_343_1
P89	Bacteroidetes	15.46391753	Stem_343_1
P89	Proteobacteria	28.86597938	Stem_343_1
P78	low_abundance	14.5631068	Stem_343_2
P78	Gemmatimonadetes	11.16504854	Stem_343_2
P78	Planctomycetes	5.825242718	Stem_343_2
P78	Actinobacteria	17.47572816	Stem_343_2
P78	Bacteroidetes	20.38834951	Stem_343_2
P78	Proteobacteria	30.58252427	Stem_343_2
P70	low_abundance	10.59431525	Stem_343_3
P70	Gemmatimonadetes	11.11111111	Stem_343_3
P70	Planctomycetes	6.201550388	Stem_343_3
P70	Actinobacteria	11.49870801	Stem_343_3
P70	Bacteroidetes	30.10335917	Stem_343_3
P70	Proteobacteria	30.49095607	Stem_343_3
P7	low_abundance	7.496917386	Leaf_Control_1
P7	Gemmatimonadetes	7.570900123	Leaf_Control_1
P7	Planctomycetes	6.7324291	Leaf_Control_1
P7	Actinobacteria	6.239210851	Leaf_Control_1
P7	Bacteroidetes	50.45622688	Leaf_Control_1
P7	Proteobacteria	21.50431566	Leaf_Control_1

Table 3.3 Percentages of abundance of phylum classification in different *Nicotiana benthamiana* treatments (continued)

	Phylum	Percentage	Sample
P27	low_abundance	5.319843342	Leaf_Control_2
P27	Gemmatimonadetes	8.648825065	Leaf_Control_2
P27	Planctomycetes	9.611618799	Leaf_Control_2
P27	Actinobacteria	8.485639687	Leaf_Control_2
P27	Bacteroidetes	44.71279373	Leaf_Control_2
P27	Proteobacteria	23.22127937	Leaf_Control_2
P80	low_abundance	7.051282051	Leaf_Control_3
P80	Gemmatimonadetes	10.8974359	Leaf_Control_3
P80	Planctomycetes	12.40842491	Leaf_Control_3
P80	Actinobacteria	8.012820513	Leaf_Control_3
P80	Bacteroidetes	33.74542125	Leaf_Control_3
P80	Proteobacteria	27.88461538	Leaf_Control_3
P53	low_abundance	8.149779736	Leaf_447_1
P53	Gemmatimonadetes	11.56387665	Leaf_447_1
P53	Planctomycetes	10.57268722	Leaf_447_1
P53	Actinobacteria	9.691629956	Leaf_447_1
P53	Bacteroidetes	29.18502203	Leaf_447_1
P53	Proteobacteria	30.83700441	Leaf_447_1
P11	low_abundance	5.569007264	Leaf_447_2
P11	Gemmatimonadetes	10.89588378	Leaf_447_2
P11	Planctomycetes	9.523809524	Leaf_447_2
P11	Actinobacteria	8.716707022	Leaf_447_2
P11	Bacteroidetes	35.51251009	Leaf_447_2
P11	Proteobacteria	29.78208232	Leaf_447_2
P76	low_abundance	9.137055838	Leaf_447_3
P76	Gemmatimonadetes	16.24365482	Leaf_447_3
P76	Planctomycetes	9.898477157	Leaf_447_3
P6	Actinobacteria	9.598214286	Leaf_S41_2
P6	Bacteroidetes	43.30357143	Leaf_S41_2
P6	Proteobacteria	27.23214286	Leaf_S41_2
P51	low_abundance	5.431754875	Leaf_S41_3
P51	Gemmatimonadetes	7.799442897	Leaf_S41_3
P51	Planctomycetes	7.103064067	Leaf_S41_3
P51	Actinobacteria	7.242339833	Leaf_S41_3
P51	Bacteroidetes	53.34261838	Leaf_S41_3
P51	Proteobacteria	19.08077994	Leaf_S41_3
P10	low_abundance	6.020408163	Leaf_413_1
P10	Gemmatimonadetes	9.795918367	Leaf_413_1
P10	Planctomycetes	9.897959184	Leaf_413_1

Table 3.3 Percentages of abundance of phylum classification in different *Nicotiana benthamiana* treatments (continued)

	Phylum	Percentage	Sample
P10	Actinobacteria	8.163265306	Leaf_413_1
P10	Bacteroidetes	34.59183673	Leaf_413_1
P10	Proteobacteria	31.53061224	Leaf_413_1
P59	low_abundance	10.06036217	Leaf_413_2
P59	Gemmatimonadetes	11.46881288	Leaf_413_2
P59	Planctomycetes	10.86519115	Leaf_413_2
P59	Actinobacteria	11.87122736	Leaf_413_2
P59	Bacteroidetes	28.57142857	Leaf_413_2
P59	Proteobacteria	27.16297787	Leaf_413_2
P26	low_abundance	9.807208718	Leaf_413_3
P26	Gemmatimonadetes	13.66303437	Leaf_413_3
P26	Planctomycetes	12.9086337	Leaf_413_3
P26	Actinobacteria	13.16010059	Leaf_413_3
P26	Bacteroidetes	20.87175189	Leaf_413_3
P26	Proteobacteria	29.58927075	Leaf_413_3
P69	low_abundance	6.129032258	Leaf_343_1
P69	Gemmatimonadetes	8.467741935	Leaf_343_1
P69	Planctomycetes	5.64516129	Leaf_343_1
P69	Actinobacteria	9.919354839	Leaf_343_1
P69	Bacteroidetes	50.72580645	Leaf_343_1
P69	Proteobacteria	19.11290323	Leaf_343_1
P28	low_abundance	6.504065041	Leaf_343_2
P28	Gemmatimonadetes	5.691056911	Leaf_343_2
P28	Planctomycetes	5.420054201	Leaf_343_2
P28	Actinobacteria	8.401084011	Leaf_343_2
P28	Bacteroidetes	52.03252033	Leaf_343_2
P28	Proteobacteria	21.95121951	Leaf_343_2
P81	low_abundance	9.364261168	Leaf_343_3
P81	Gemmatimonadetes	7.04467354	Leaf_343_3
P81	Planctomycetes	6.701030928	Leaf_343_3
P81	Actinobacteria	7.64604811	Leaf_343_3
P81	Bacteroidetes	43.55670103	Leaf_343_3
P81	Proteobacteria	25.68728522	Leaf_343_3

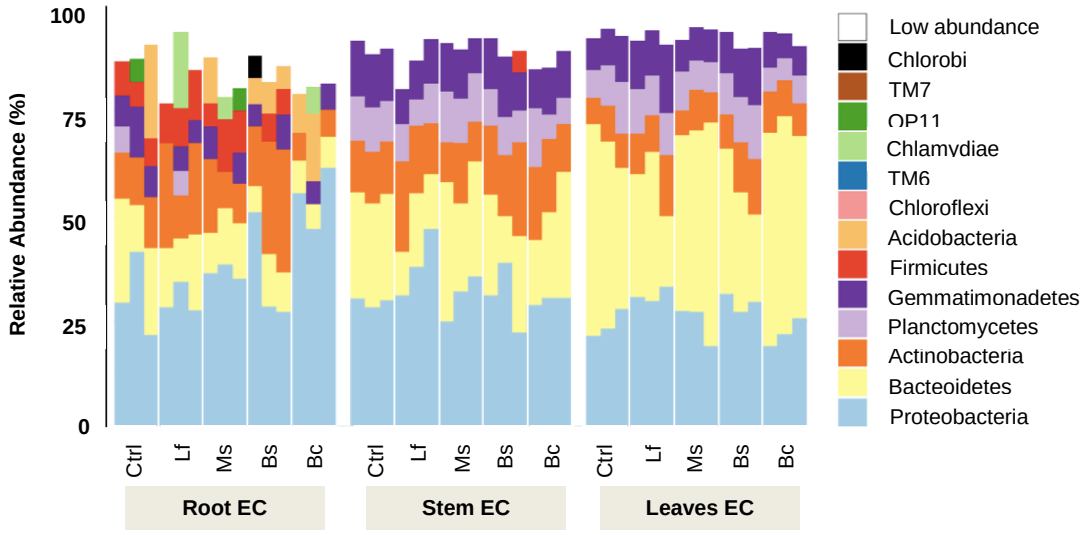


Figure 3.11 Reads grouped to phyla level had abundance differences for each phylum (represented as percentage) when comparing *Nicotiana benthamiana* plants that had been exposed to bacterial inoculums as seed treatments. Samples with reads belonging to phylum level that were less than 5% present were all classified under the "low abundance" category.

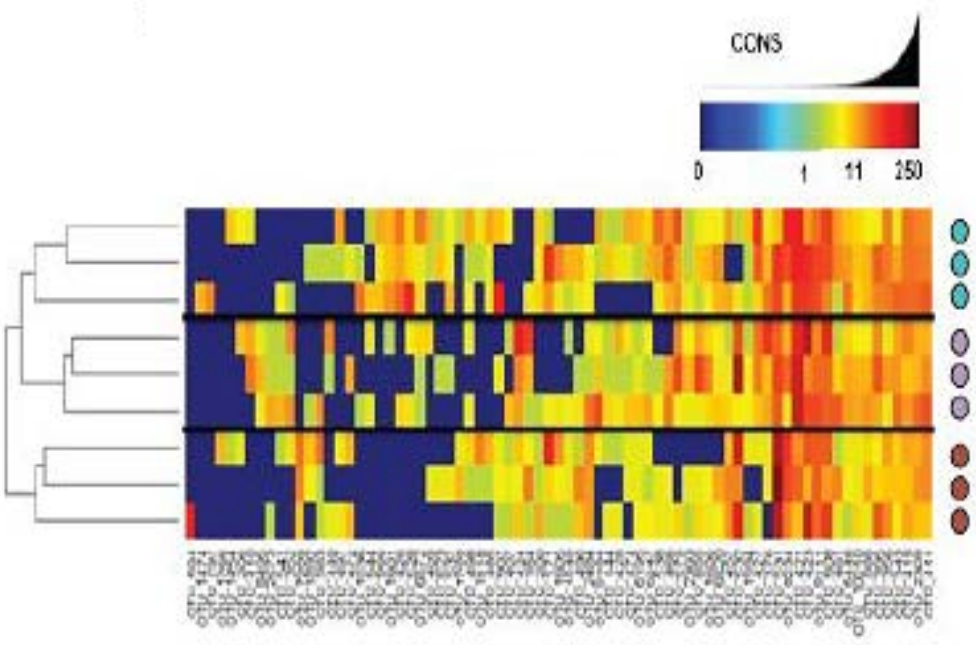


Figure 3.12 Heatmap of bacterial OTUs present in samples from roots of treated and control plants. Treatments Bc and Ms were compared to Control samples at the root level to show if at a deeper classification, samples separated in composition. Circles represent roots from different treatments. Teal: Ms roots, light purple: control roots, and red: Bc roots.

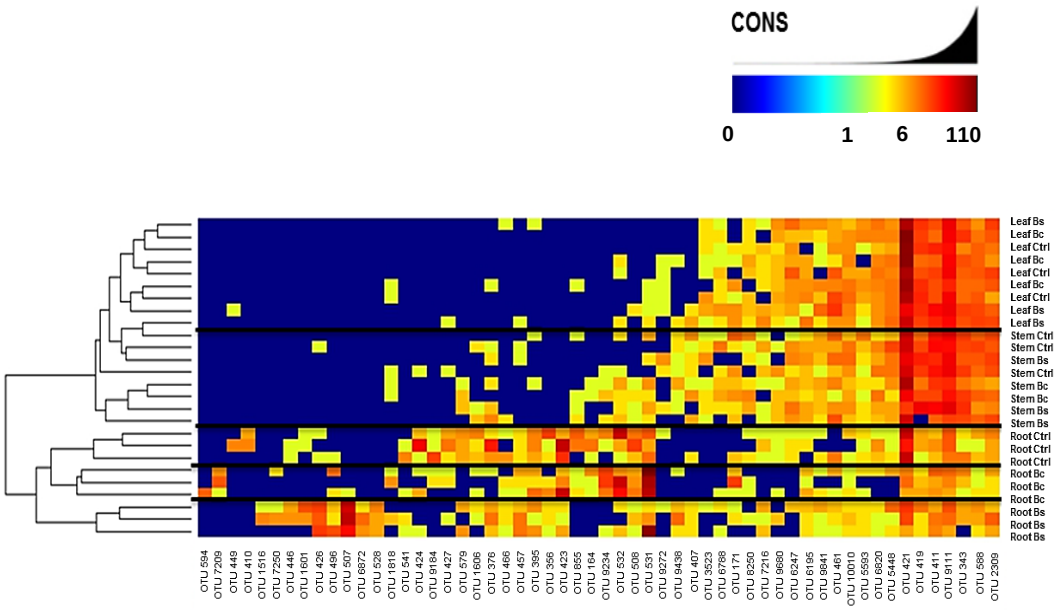


Figure 3.13 Similarities and differences among treated plant organs community evaluation. Heatmap of Bs anc Bc plant organs compared to Control samples at an OTU level. Sample organs separate when comparing root EC samples, but did not separated for aerial EC samples (like stem and leaves).

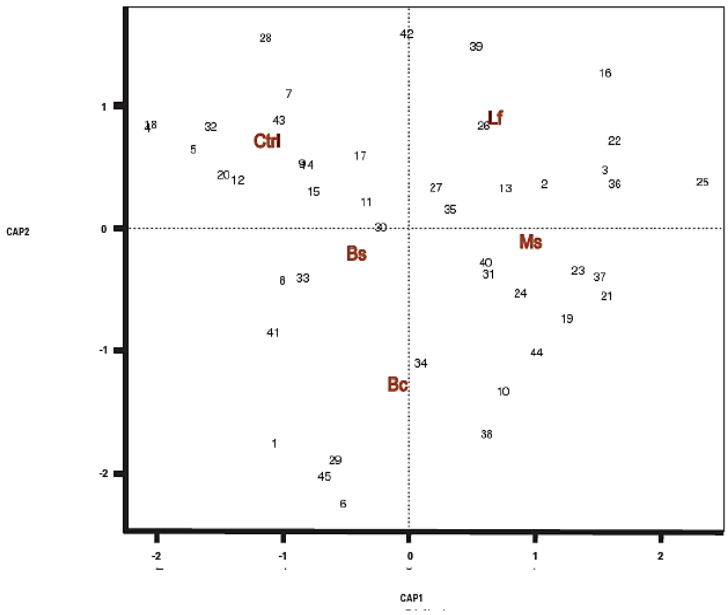


Figure 3.14 Coordinates analysis (CAPSCALE analysis) of OTUs present in each treatment. For this, all OTU's from all treatments were used to build the comparison in which we were able to differentiate those treatments that were more closely related and those that were the most different. Data showed that Lf was similar to control. In a similar manner, Bs was closely related to both the control and Bc, but different from Ms and Lf. Treatments like Bc and Ms clustered the farthest apart from control, consistent with microbial composition differences.

Taking in account the results obtained up to this point, we could say that introduction of an overrepresented bacterial strain to an environment may be setting up certain parameters at a microenvironment level, leading to a competition process among native species and introduced. Competition has been studied by many groups through time, and even though it is not the only ecological process involved in community assembly (not only at a microbial level, but for many other macro organisms too), it does play a fundamental role in it. Cavender-Bares et al., (2009) and Wiens *et al.*, (2010) explain that in cases were species are closely related, the higher are the similarities and therefore, the more competitive they will be.

At the same time, seeing changes in the gene expression patterns are also link to those changes observed phenotypically and in the community assembly. Bacterial strains like those belonging to the genus *Bacillus* are known to not be as competitive as other organisms (Tilman, 2004). We predict that ecological factors and dynamics (like competition) and the possibility of organisms to produce chemical compounds (supernatants) could be inducing the response from the plant at a molecular level.

Chapter 4: Inoculum functionality, variations and its link to morphology

4.1 Introduction

Environmental and hormonal cues interact and cause defined organization of tissue physiology and cellular growth dynamics in higher plants (Nordström et al., 2004; Aloni et al., 2006). However, the environmental signals derived from microbiome elements, such as seed spermosphere inoculum, and how it influences responses from the plant are still questions requiring more answers. Since it has been determined that some microbial organism are able to promote growth in the plant without having to be pathogenic (Chen et al., 2007; Chen et al., 2009; Kierul et al., 2015), the identification of those microbial organism and how they stimulate molecular changes in the plant could be the tool that can lead us towards a better understanding of physiology and development.

In the last decade, many companies have developed an increasing interest towards microbial inoculant potential (http://news.monsanto.com/press-release/corportate/novozymes-and-monsanto-complete-closingbioag-alliance), broadening the spectrum for more holistic approaches when studying bacterial inoculum potential.

One of the many points of interest in the plant-microbe study dynamics is the study of the changes in the microenvironments where plants are growing can lead to the production of chemical molecules (also known as supernatants) from the plant and from the microbes present (Smalla et al., 2006).

These mechanisms can have an impact on other factors related to the relationship between plant and microbes present in the niches were plants are (for example: nutrient availability) (Hacquard et al., 2015). The relationships between hormones, temperature variation effect in the soil environment were microbes, and their host is interacting, has been studied for many decades (Olsen and Baker, 1968; Paulitz and Baker, 1987; Landa et al., 2001). Previous research has shown that at the level of the rhizosphere, alterations of biotic and abiotic factors have a significant effect on the development and immunity of the plant, by influencing different dynamics in the soil community, which ultimately is selected by the plant (Landa et al., 2001).

At the same time, environmental changes not only affect the microbial community, but the viability of the seed and the tolerance of the plant too, making studies that involve environmental factors+endophytes+plant development/physiology, a necessary area to be studied in depth in order to use the potential of the inclusion of microbial organisms in agriculture (Hallmann et al., 2001; Ait Barka et al., 2006).

In the previous chapter, we showed that microbiome shift could serve as the basis for a mechanism to alter hormonal systems *in planta,* thereby driving plant physiological response. Our observations suggest that the microbiome may be tunable in plants despite the complexity of environmental and genetic factors.

4.2 Materials and Methods

4.2.1 Biochemical test for indole production from bacteria

In order to determine if microbes could degrade tryptophan and produce indole (as a possible reason for expansion/growth promotion), each bacterial strain was grown (plus a mock solution), with a total of 3 replicates in a tryptophan broth for 24-48 hours at 37°C. Ehrlich's reagent was added to all tubes including control and after shaking gently, we observed if color formation occurred (Harley, 2005). Color variation was recorded and used to determine response from each organism to the test.

4.2.2 Hormonal profile

Seeds were treated following the methods mentioned in previous chapters, and placed in soil under greenhouse conditions. After ~4 weeks, samples were harvested and clean (soil being removed from roots by patting) and snap freeze with liquid nitrogen. Seedlings from treatments Ms/s41 and Bc/s343 were selected because of their antagonist phenotype to each other, and compared to control samples. All samples were lyophilized and homogenized –root-stem-leaves-, before being send for metabolite analysis. We looked into auxins and cytokinins because of their importance in expansion, cell division and root development. A number of compounds namely DPA, ABA-GE, PA, 7'- OH-ABA, *neo*PA, *trans*-ABA and IAA-Glu were synthesized and prepared at the National Research Council of Canada, Saskatoon, SK, Canada; ABA, IAA-Leu, IAA-Ala, IAA-Asp, IAA, Z, ZR, iPR, and iP were purchased from Sigma–Aldrich; dhZ, dhZR and Z-O-Glu were purchased from OlChemim Ltd. Auxin (IAA Indole-3-acetic acid, IAA-Asp N-(Indole-3-yl-acetyl)-aspartic acid, IAA-Glu N-(Indole-3-yl-

acetyl)-glutamic acid, IAA-Ala N-(Indole-3-yl-acetyl)-alanine, IAA-Leu N-(Indole-3-yl-acetyl)-leucine, and IBA Indole-3-butyric acid) and cytokinins (*t*-ZOG (trans) Zeatin-O-glucoside, c-ZOG (cis) Zeatin-O-glucoside, t-Z (trans) Zeatin, c-Z (cis) Zeatin, dhZ Dihydrozeatin, t-ZR (trans) Zeatin riboside, c-ZR (cis) Zeatin riboside, dhZR Dihydrozeatin riboside, iP Isopentenyladenine, and iPR Isopentenyladenine riboside) were studied following Zaharia et al., (2005) and Lulsdorf et al., (2013); as a fee for service product by the National Research Council of Canada.

Calibration curves were created for all compounds of interest and quality control samples (QCs) were run along with the tissue samples.

4.2.3 Instrumentation

Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system, equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE quadrupole tandem mass spectrometer via a Z-spray interface. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and data analysis.

4.2.4 Hormone quantification by HPLC-ESI-MS/MS

The analysis and the quantification of the hormones studied was performed as a fee for service product by the National Research Council of Canada (NRC-CNRC), the quantification procedure of cytokinin and auxin in plant tissue was performed using a modified procedure described in Lulsdorf et al. (2013). Briefly, the analyses utilize the Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces are quantified off-line by the QuanLynx v4.1 software (Waters Inc) wherein each trace is integrated and the resulting ratio of signals (non-deuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard. The QC samples, internal standard blanks and solvent blanks were also prepared and analyzed along each batch of tissue samples

4.2.5 Auxin expression related to bacterial treatments

Plants expressing DR5:Green Fluorescence Protein were PCR verified using for the

presence of GFP (Mendu et al., 2011). Seeds of *N. tabacum* DR5:GFP were inoculated them with Bs, Bc, Ms, or mock control (media only), seedling treated with two concentrations of IAA (1uM and 10uM) and 2,4-D (100 nM). Seeds were plated in Murashige and Skoog medium, and after 3 days' post completing germination they were visualized using a laser scanning confocal microscope (Olympus IX83). A total of 10 replicates per treatment were analyzed. Data obtained was analyzed using a Dunnett's multiple comparison test against control with a $p<0.05$.

4.2.6 Supernatants and live cells

Strains were grown in YPD broth medium flasks overnight (11± 2 hours) at 28°C on a rotary shaker until reaching an OD_{600} = 0.6. Cultures were later transfer to 15ml Falcon tubes and taken to the centrifuge to separate bacterial cells from supernatant (supernatants). All cultures were spin down for 30-40min at 3,600rpm. Liquid fraction of each culture was placed to new tubes, adding new media to the cells remaining in the tube (4ml per tube). Supernatants were filtered using syringe filters with a pore size of 0.22um to remove possible remaining bacterial cells. A total of ~20 surface sterilized seeds were added to each fraction from the different treatments and placed back in the rotary shaker for 12 hours more. All treatments were placed in pots containing soil pot mix in the greenhouse under constant temperatures of ~25±3°C, and 16h of light followed by 8 h of dark for 30 days. Morphological evaluations were performed and data was analyzed using a triple factorial approach to determine differences among treatments.

4.2.7 Cold treatment for inoculants

Knowing that bacterial organisms can have variable responses to changes in their environment, we decided to expose seeds and bacterial strains to temperature changes as a way of having a better understanding of the effect that the inoculation has in stimulating or inhibiting growth in the plant. For this, strains were grown in YPD broth medium flasks overnight (11± 2 hours) at 28°C on a rotary shaker until reaching an OD_{600} = 0.6. As in the original inoculation process, cultures were grown until reaching OD_{600} = 0.2, surface sterilized seeds were added, and mix of seeds and inoculum were kept in shaker until reaching final OD. Tubes were removed from shaker and place in the fridge at 4°C for 12 hr. After time finished, all tubes were transferred to room temperature and shaken for an additional 2 hr prior to being place in soil. Treatments

were placed in pots containing soil pot mix in the greenhouse under constant temperatures of ~25±3°C, and 16h of light followed by 8 h of dark for ~3-5 weeks.

4.2.8 Cold treatment for bacterial cells and supernatants *in vitro*

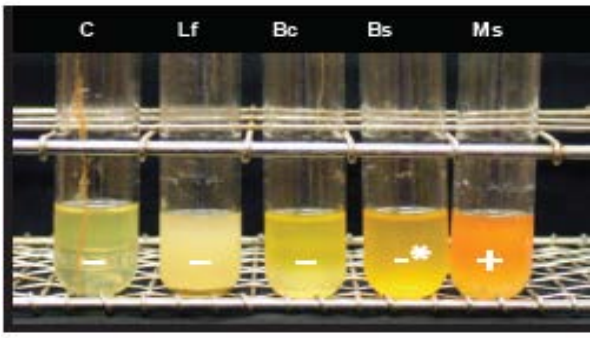
Once again, cultures for each strain were grown and place overnight (11± 2 hours) at 28°C on a rotary shaker until reaching an OD_{600} = 0.4-0.5. Fractions were separated and new media was added to the cell portion and placed back to the shaker for 1-2 hours more. Both fractions were placed in the fridge at 4°C for ~4-6 hr. For the supernatants, we decided to have our original concentration (obtained from the two phases in the culture), and have one dilution (50%). Surface sterilized seeds were added to each fraction and kept at 4°C for 12 hours. All treatments were plated and grown in vitro in ½ MS plates and kept in a grown chamber at 26±2°C.

4.3 Results and Discussion

Since it is known that certain bacterial organisms are able to produce metabolites involved in normal hormonal events in plants, such as the expansion mediator auxin (Doornbos et al., 2011, Barbez et al., 2017), we decided to test our bacterial strains for their ability to break down tryptophan and produce indole derivatives (Figure 4.1). Using a microbial biochemical assay, we evaluated which strains produced indole breakdown products when exposed to Elrich's reagent. A positive test results in visual hue shifts in the reagent mixture. Cultures corresponding to Lf, mock/control, and Bc were negative. Bs produced an intermediary positive response, which means that an alternative indole cleavage product like skatol could have been produced. The Ms bacterial cultures clearly revealed an ability to break-down tryptophan to produce indole (Figure 4.1).

Cultures corresponding to Lf (included once again as a reference for a negative control for morphology), our mock/control and Bc were primarily our fully negative responses. No color per se was produced. Although, Bs did not really have a red color, we did visualize an orange color, which under this test means that an alternative product like skatol could have been produced. Bacterial organisms have multiple pathways for producing derivatives of indole products, so these alternative products could still have an effect.

Tubes corresponding to Ms treatments were the only ones that truly had a positive response to the test, meaning that they are capable of breaking down tryptophan and producing indole. The reason why indole is so important as a test is because auxin is a type of indole product, as a hormone in plants. Crosstalk is common to occur among all hormones, and in many cases, regulation is an effect of the activation of some of them.



Treatment	Strain	Reaction	Explanation
Ctrl	Mock	-	Negative
Lf	*Lysinibacillus fusiformis*	-	Negative
Bc	*Bacillus cereus*	-	Negative
Bs	*Bacillus sp.*	-*	Negative/ *possible production of 3-methylindol
Ms	*Micrococcus sp.*	+	Positive

Figure 4.1 Indole evaluation A) Biochemical bacterial test for tryptophan breakdown (indole test) for the bacterial inoculants used as treatments. Control, Lf and Bc samples showed a negative response, and Bs had a positive response but for an alternative product for indole. Ms was the only treatment that showed a positive result for the production of indole.

Now, knowing that our microbial organisms are capable or not of producing compounds that could influence change in morphology is a step forward into understanding the relationship between microbiome changes (accessory, to be specific) with development. In our previous chapter, we were able to determine that our gene expression data had some genes possibly related to hormones, but unfortunately, we were not able to say how significant some of these genes were because *Nicotiana benthamiana* possess only

a draft genome, and a vast amount of annotations and information are incomplete. Still, we were able to see the percentage of genes involved in each category by using the overall number of genes obtained. This information serves as a base line for the next set of studies that could serve to have a better understanding between possibly microbial community effects on the phenotypes evaluated.

Based on this, we then performed HPLC-ESI-MS/MS on samples that distinctively had a phenotypic modification (Ms and Bc) plus a control, and measured changes in biologically active IAA and conjugate with aspartic acid IAA-Asp. Data showed that IAA was present in all samples, which was expected. However, it's conjugate with aspartic acid IAA-Asp was found in Ms and Bc samples and not in the control.

IAA-Asp is commonly found in bacteria and plant interactions (Gonzalez-Lamothe et al., 2012), which would explain why it is only found in the treated samples and not in the control (Fig. 4.2). Studies in *Arabidopsis thaliana* have shown that bacterial and fungal pathogens (or not) are able to "hijack" host auxin metabolism by orchestrating the accumulation of a conjugated form of the hormone, (IAA)-Asp, as a mechanism to promote disease development or colonization (Gonzalez-Lamothe et al., 2012).

Some of the other hormones evaluated were cytokinins, from which iPR was detected in treatments Bc and Ms, being more abundant in Bc treated plants than in the control samples. Data also suggest that overall cytokinin levels were the lowest in Ms samples. iPR is known to be involved in shoot elongation, which helps explain the promotion of growth related to Bc treatment compared to Bs and control (Werner et al., 2001) (Fig. 4.3).

ABA was also analyzed during the study, and the overall result of the metabolites identified in the samples showed that when compared to control, Ms was less abundant in ABA and ABA catabolites than Bc (Table 4.1). Groups like Porcel et al., (2014) showed that endogenus ABA can increase after inoculating a plant with a possible plant growth promoter (in this case, Bacillus to be specific), linking ABA levels with growth as a positive correlation since the hormone acts as a negative feedback with other hormones that can inhibit growth.

Table 4.1 ABA and ABA metabolites values obtained from plant material analyzed.

	ABA	DPA	ABAGE	PA	7'OH-ABA	neo-PA	t-ABA
Ms	520	1388	234	1062		18	64
Ctrl	326	1271	174	709		15	9
Bc	1439	1931	496	1575	7	41	41

As shown in other studies, these responses have been characterized mostly in leaf tissue, and some of the responses generated in plants have an effect on programmed cell death, cell wall thickening and phytohormones production (Torres and Dangl, 2005).

Lebeis et al., (2015) evaluated various mutants with either overexpression or repression of certain hormones involved in defense responses. What their work found was that when having uncontrolled expression of hormones, or no expression at all, the microbiome of the plant changed. Even though their findings were done in synthetic environments with a selection of organisms, we can see that in our case, any alteration presented to the environment in which the plant is growing could lead to a similar response as evaluated in mutants. It seems that soil community composition, pressures in the microenvironment, the natural ability of bacteria for colonization and plant physiological processes; have to work together in order for the microbiome to be assembled to fulfill the needs of the plant.

Based on the results obtained, our interest focused on the observation association with auxin and expansion. Using this as our foundation, we evaluated the effect that strains had on stimulation of auxin using an *in situ* reporter. DR5 promoter constructs paired with a reporter have been previously used to visualize auxin flux in plants (Ottenschlager et al., 2003).

Therefore, we generated transgenic plants expressing the translational GFP fusion DR5:GFP in *N. tabacum*. We surface sterilized NtDR5:GFP seeds and then inoculated with Bs, Bc, Ms, or mock control. Pharmacological controls were plants treated with two concentrations of IAA (1uM and 10uM) or with the auxinic herbicide 2,4-D (100nM), which were based on our own screening of multiple concentrations and their effect in seedling growth (positive, neutral and severe) (Figure 4.4).

Seedlings were grown for 5-7 days on vertical ½ MS plates in a 16:8 hr light dark regime, and then carefully visualized for DR5:GFP localization in roots with a laser scanning confocal microscope (Olympus IX83). Results showed significant differences in DR5:GFP fluorescence in Bs and Ms roots compared to control (Figure 4.5A). Quantitative assessment of DR5:GFP fluorescence showed significant increase after Ms application (P>0.05, Bonferroni test). The Bs treatment induced a modest yet significant increase from the control (P>0.05, Bonferroni test), but was significantly lower than Ms (P>0.05) (Figure 4.5B). These results suggest that these inoculums produced indole derivatives which are known expansion mediators (Rayle and Cleland, 1992; Cosgrove, 2000). The foremost observation arising from these data support that the microbial inoculum Bs and Bc appear to be influencing the hormonal levels in a secondary manner whereas Ms was capable of *in vitro* tryptophan break-down to produce indole and consistently induced IAA and IAA-asp production in the root.

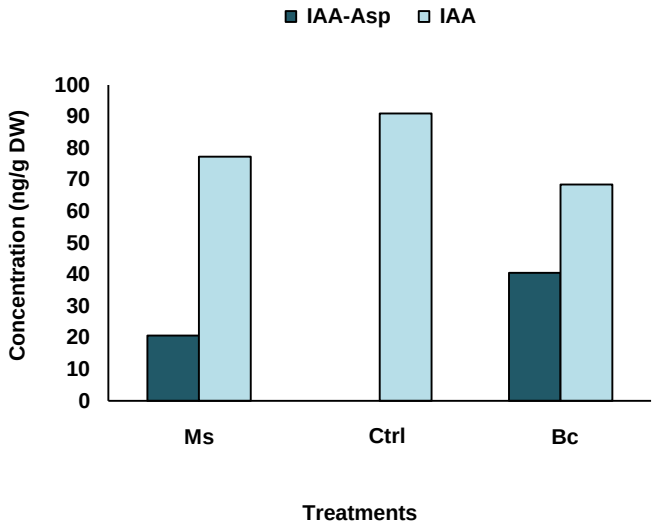


Figure 4.2 Auxin expression and production evaluation. A) Auxin metabolites in control, Bc and Ms samples. Presence of IAA-Asp form (which could be microbial or plant derived) only shows in treatments Bc and Ms which have been exposed to bacterial inoculums as seed treatments.

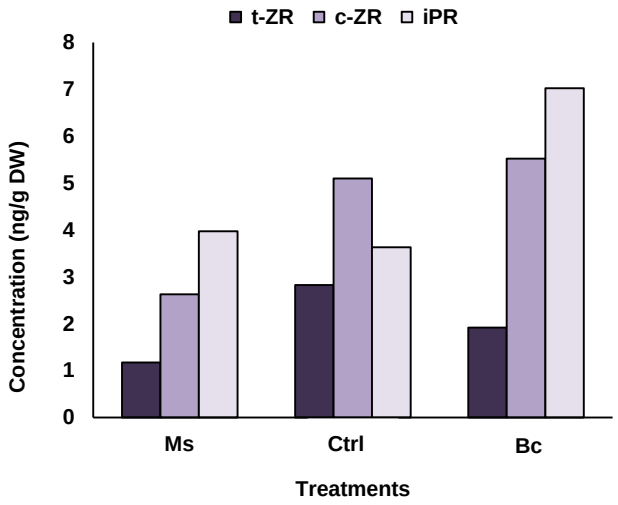


Figure 4.3 Cytokinin metabolite concentration of Control, Bc and Ms, where Bc shows a bigger presence of cytokinin.

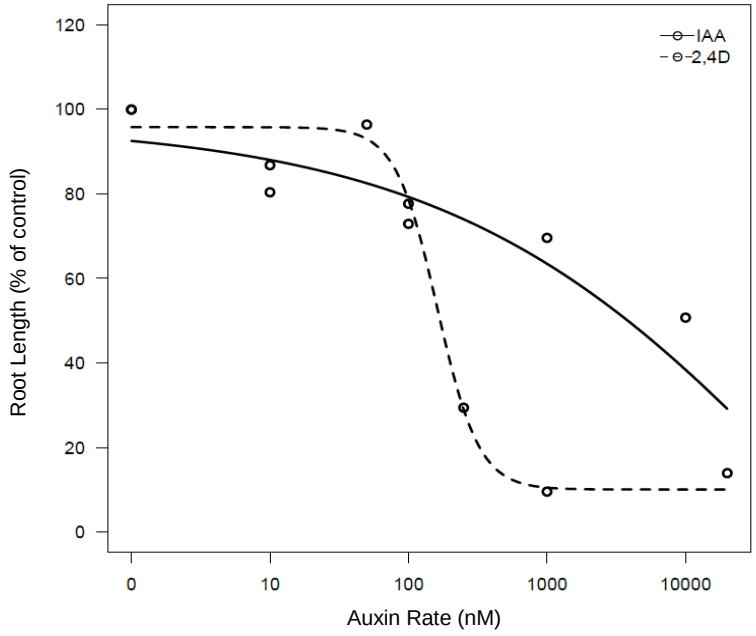


Figure 4.4 Indole evaluation IAA and 2,4D treated seedlings root length percentage difference used to determine the concentrations used to compared with the DR5:GFP seedlings treated with inoculums

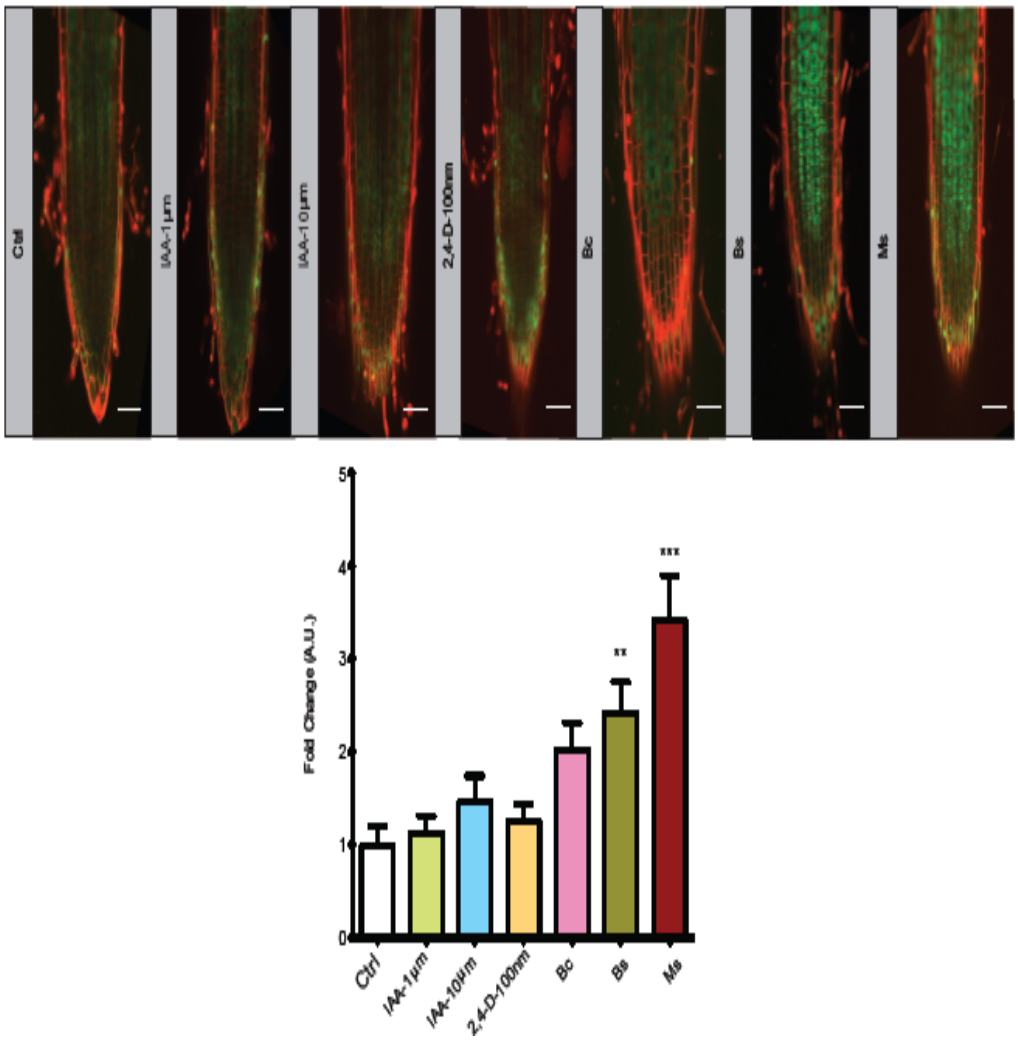


Figure 4.5 A) Differential expression of DR5:GFP in *Nicotiana tabacum* seedling roots, Control, IAA-1uM, IAA-10uM, 2,4-D-100nM, Bc, Bs and Ms stained with PI dye and evaluated under confocal microscopy. A total of 10 replicates for each treatment and control were observed under a confocal microscope using a magnification of 200X. B) Fold change difference among all treatments. Dunnett's multiple comparison test against water-control for each one suggests significance for Ms and $p<0.05$ which is represented in asterisks. Ms was significantly different for GFP expression. Scale bars represent 50µm.

When grown under no media conditions, just filter paper, we see that the roots from some of the treatments have a tendency of looking swollen and the growth promoting phenotype does not exist for those treatments that originally were beneficial (Figure 4.6). Although we do not have an explanation to why it is so drastic the phenotype under this

treatment, we think that not having a media with nutrients available for the plant to use (besides their reserves in the cotyledons), makes it harder for the plant to overcome the overrepresentation and higher concentration of the chemicals compounds produced by the organisms when they do not have to compete with other microbes.

To better understand the functional component of the inoculums, we separated each inoculant into supernatants (filtered culture media) and live cells, and applied each independently to surface-sterilized *N. benthamiana* seeds. Seeds were exposed to exudate or live cell treatments for a period of 12 hours before placing them in potting mix in the greenhouse. We observed variable phenotypic responses for Bc and Bs. Plants exposed as seeds to live cell inoculum exhibited a spindly appearance with shorter internodes in all treatments (Figure 4.7A, Table 4.2), differing from the original phenotypes obtained when inoculum fractions were unseparated. In contrast, plants that developed from seeds exposed only to the exudate treatment exhibited a phenotype indistinguishable from a normal plant (Fig. 4.7B, Table 4.2). For Ms, live cell treatment produced smaller plants with bigger leaves compared to cells plus exudate. Still, it consistently produced a smaller phenotype when compared to control plants. Plants developing from seeds treated with Ms supernatants were dramatically smaller than those treated with live cells, and unable to survive longer than 6 weeks. These results could indicate that traits like height are not necessarily a consequence of plants having more cell division; instead, molecular and physiological level alterations may influence cell expansion as a consequence of the production and expression of hormones. Furthermore, these two postulates are not mutually exclusive (Rayle and Cleland, 1992; Claussen et al., 1997; and Barbez et al., 2017).

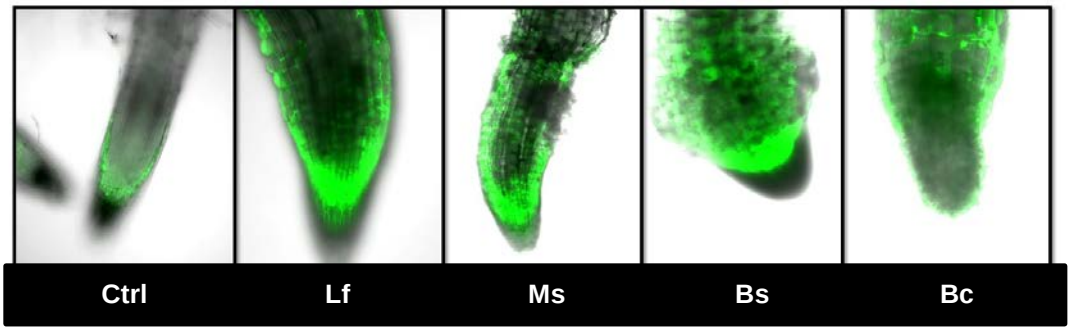


Figure 4.6 Roots from treated seedlings grown in sterile filter paper. All treated roots but Lf show signs of swelling, most predominantly for Bs and Bc.

Table 4.2 Live cell and exudate treatments in *Nicotiana benthamiana* plants. Cultures of bacterial treatments were separated in live cell and supernatants, and were used as seed treatments to evaluate their effect in growth and development of the plant. Traits like height, leaf length (L.L), leaf width (L.W), number of leaves (N.L) and number of flowers (N.F) were evaluated as a way to determine morphological changes in plants. Tables show significant differences in blue for the evaluated traits and the comparison among treatments. Asterisks mean significant difference (LC, DC, Ex) when it comes to number of flowers.

Treatments*TYPE (12h exposition)				
Variables	**Source**	**DF**	**Mean sq**	**Pr > F**
Height	Type	2	25.36	<.0001
	Treatment	4	229.36	<.0001
	Type*treatment	8	17.21	<.0001
L.L	Type	2	4.81	<.0001
	Treatment	4	34.38	<.0001
	Type*treatment	8	3.71	<.0001
L.W	Type	2	3.60	<.0001
	Treatment	4	31.78	<.0001
	Type*treatment	8	3.11	<.0001
N.L	Type	2	37.35	<.0001
	Treatment	4	277.03	<.0001
	Type*treatment	8	29.13	<.0001
N.F	Type	2	1.088	0.0015
	Treatment	4	13.25	<.0001
	Type*treatment	8	1.42	<.0001

GLM procedure
t Test (LSD)

Variables	**t Grouping**	**N**	**Mean**	**TYPE**
Height	A	15	15.70	**LC**
	A		14.16	**EX**
	B		8.16	**DC**
L.L	A	15	3.84	**LC**
	A		3.64	**EX**
	B		1.97	**DC**
L.W	A	15	3.40	**LC**
	A		3.35	**EX**
	B		1.68	**DC**
N.L	A	15	12.66	**LC**
	A		12.40	**EX**
	B		6.13	**DC**
N.F	A	15	2.46	**LC***
	B		1.80	**EX***
	C		1.06	**DC***

Natural ecological or agricultural conditions are subject to fluctuating climatic or soil conditions. Thus, we decided to evaluate whether a temperature shift imposed on each of our bacterial treatments would alter the way it interacted with the host plant.

Here, we used our spermosphere inoculum preparations (Figure 4.8A) but before placing them in the soil, all treatments (*N. benthamiana* seeds + inoculum) were exposed to a single low temperature (4°C) overnight (12hr). Treated seeds were then kept at room temperature for 2 hours before placing them in soil. Unexpectedly, plants treated with Bc and Bs no longer exhibited growth promotion and were indistinguishable from control plants (Figure 4.8B). Furthermore, Ms treated plants reverted from a growth suppressor to a growth promoter compared to the control. This trend was observed from seedling stage to week 5; inducing flowering earlier on treated Ms plants (Figure 4.8B).
In order to determine if cold treatment had an effect on decreasing the activity of the organisms and their metabolic activity (usually found in the supernatants), we decided to repeat this study adding new variables: 1) in vitro environment and 2) fractionated portions of the culture. We separated the fractions of the bacterial culture (liquid/solid phases -cells/supernatants-) and exposed our sterile seeds to them. Our preliminary results showed that seedlings (~2-3 weeks old) that were exposed to live cells (LC) under cold treatment did not display any major differences morphologically for Bc and Ms when compared to control. On the contrary, for Bs it seems like the exposure to cells+temperature seems to decrease the growth of the plant (Figure 4.9A). Germination rates varied among treatments and control, with faster completion of germination in all the treatments compared to control (3 days for treated seeds, 5-6 for control).

On the contrary, when evaluating the supernatants/cold treatment, we saw that Ms seedlings were more developed than Bc and Bs (Figure 4.9B). Although early in age, seedlings displayed visible signs of growth promotion, showing most of its differences in the aboveground portion of the plant. As a way of developing a better understanding of how the microbial inoculum exudate fraction could be involved in the variation of the plant phenotype, we diluted to half the exudate portion for each treatment and exposed our surface sterilized seeds to it. As mentioned in previously described approach, treatments were kept for 12 hours before platting them in media. What we found was that seedlings of treatments like Bs and Ms were the ones that displayed the most difference in root development and overall growth (Figure 4.9B). On the contrary, Bc

seedlings, although with a more developed root system compared to control, did not display major differences in the aboveground portion of the plant (Figure 4.9C).

As mentioned before, Ms treatments showed a reverse phenotype in the greenhouse trials when exposed to cold. We believed that separating the inoculum in fractions and evaluating in a sterile and more controlled environment should show us if the cause for the phenotype resided in the organism itself or the chemical products produced by them. Cold temperatures are known to have an effect in the activity and metabolism of many organisms (Amato and Christner, 2009; Scherrer et al., 2011), and it is this scenario, that temperature change seems to affect the rate of the compounds normally produced by the bacterial organisms evaluated, as well as the expression of genes involved with many developmental processes in the seed and in the inoculum, as has been seen before in studies of inoculation and interactions at the rhizosphere level (Landa et al., 2001).

Although we cannot give today a definite explanation to why this phenomena occurs, our previous results accompanied with the ones found here show that phenotypes like the ones found in Ms and Bc, are more tunable because the capacity of the bacteria to produce indole products could explain why a growth repressive phenotype can be reverted if the microbe is exposed to an environment that would decrease its activity.

Feasibly, these observations are linked to sensitive hormonal cues that interplay between plant development, microbiome and environment. These data reflect the challenge of deploying microbial inoculum in agricultural settings (Emerson and Gillespie, 2008; Vamosi *et al.*, 2009), where environmental fluctuation, variability in soil type and nutrient status exist across relatively short spatial distances. Thus, further information will be of great interest when trying to develop new ways of using microbes in agriculture for food production in a scenario of shifting climatic and agricultural condition

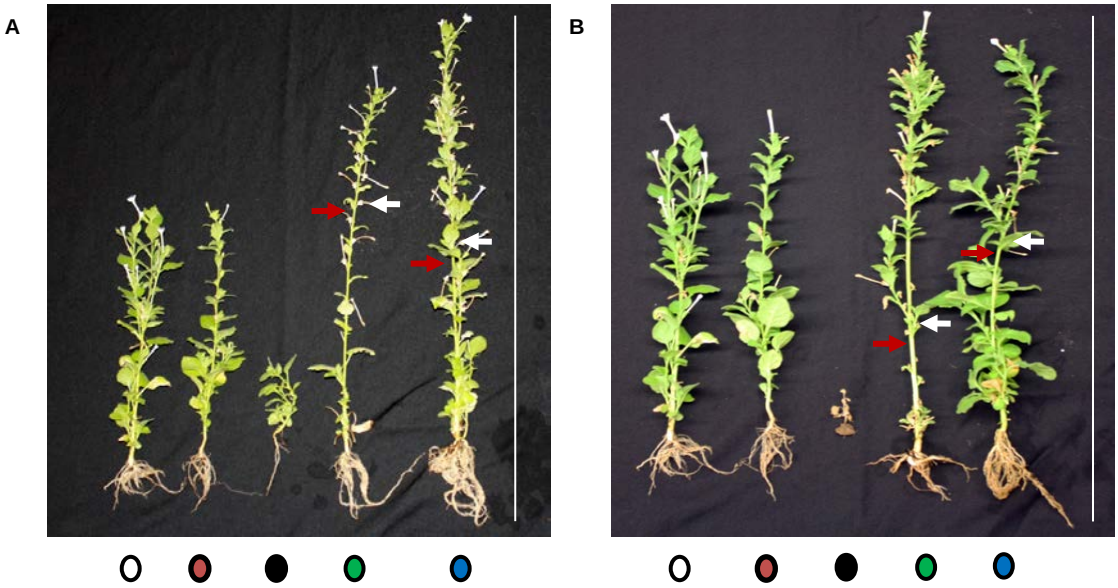


Figure 4.7 Live cell and exudate effect evaluation of bacterial strains. Panel A) shows treatments and control samples after seeds have been treated with live cell cultures after separating the supernatants produced from them. Panel B) shows the groups of plants which seeds were treated with supernatants separated from those live cells. Morphological changes can be seen among all treatments, but the variability among their architecture seems to change based on what inoculum is used. Supernatants from Ms applied as a treatment seem to not allow a proper or strong root system which leads them towards death after passing 4-5 weeks of age. Dots represent different treatments: White: Control, Orange: Lf, Black: Ms, Green: Bs and Blue: Bc. Arrows represent nodes (white) and internodes (red). Scale bars represent 30cm.

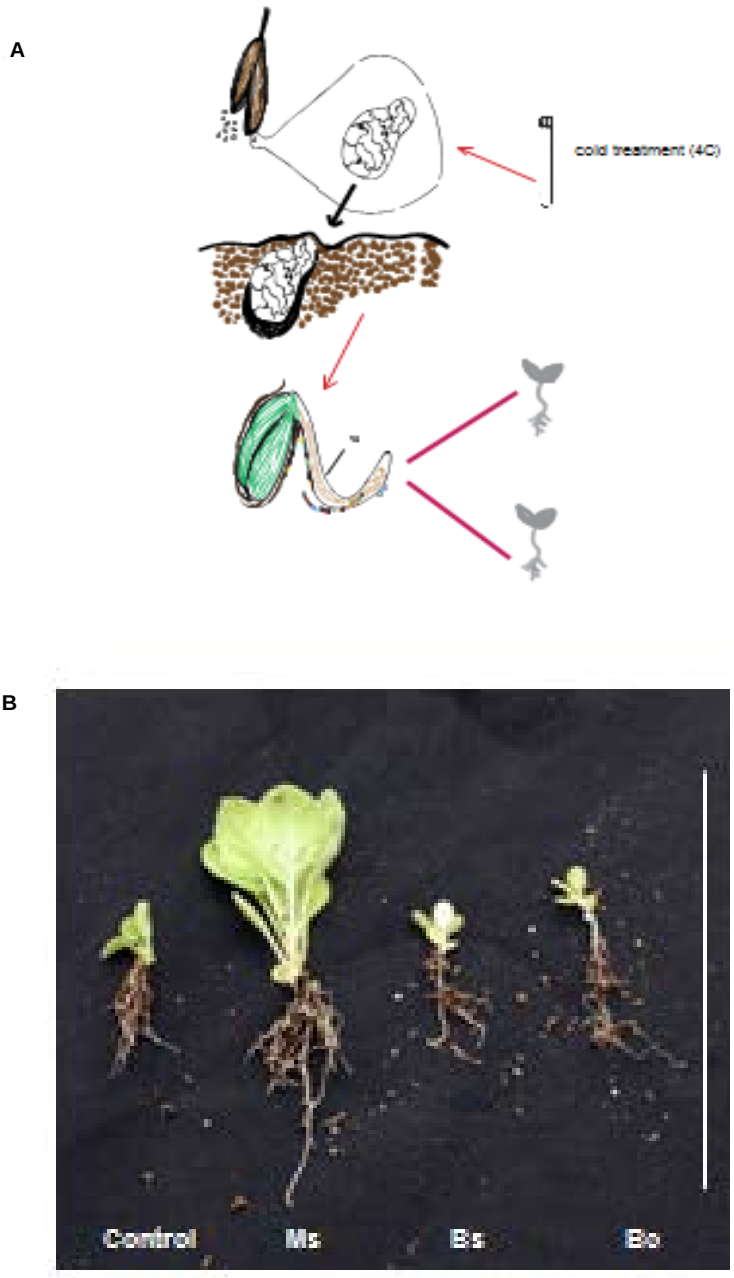


Figure 4.8 Cold treatments for seeds and inoculums. A) Seeds treated with bacterial cultures were kept at 4°C before being place in soil and evaluated for morphological differences. B) Seedlings showed differences in growth. Control, Bc and Bs were similar in above ground features, but with some minor differences in root system architecture. In terms of development, Ms treated seedlings grew faster than control. Early flowering was also visible in these plants, possibly due to temperature treatment exposure. Bar represent 5cm.

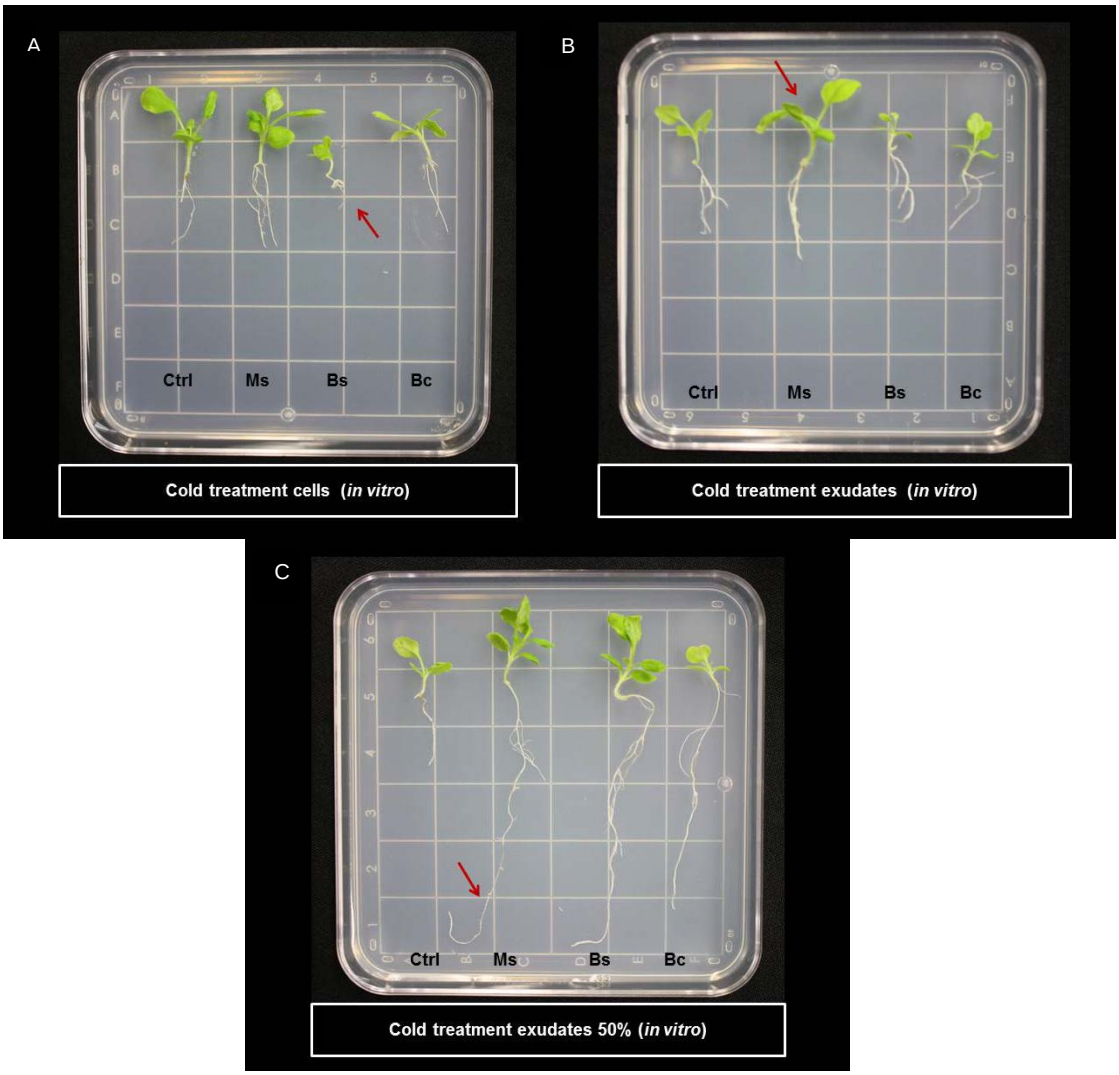


Figure 4.9 Seedlings of cold treated supernatants and live cells of Ms, Bs and Bc phenotype evaluation. Early stage seedlings (~2-3 week old seedlings) display phenotypic differences when compared to control samples. A) Cold treated live cell treated seedlings show that Bs had the less developed root system and aboveground. B) Exudate cold treated seedlings showed that Ms compared to control and the rest of the treatments had the most overall growth promoting phenotype. C) Diluted supernatants treated seedlings from Bs and Ms had displayed the most striking phenotype, with Ms having longer roots. Bc root development was bigger than that of control, but aboveground portions did not have major differences. Red arrows show main differences found among seedlings from different treatments.

Chapter 5: Bacterial inoculums effects in following generations. Preliminary results and future studies

5.1 Introduction

Endophytic organisms are known to have an effect in development, growth and physiology of the plant that they inhabit, and base on their localization, their presence and function varies. The most studied parts of the microbiome are the rhizosphere and the endophytic compartments (root, stem, leaves, flowers, etc.). Seeds are not as studied as other plant components, but it has been propose many decades ago that seed microbes may be the main drivers of selection of the microbial communities that inhabit the plant at the rhizosphere zone (Trolldenier, 1987).

Groups like Johnston-Monje et al., (2014) showed that the microbial endophytic communities in maize were responsible for the majority of the microbial organism later identify as endophytes when evaluating the rhizosphere composition. In other cases, it has been shown that the initial microbiome present in the spermosphere of the seeds of certain types of cactus is necessary for the plant to be able to stablish an interaction for colonization among the rhizosphere organisms and the plant as a way to absorb nutrients (Puente et al., 2009).

The debate between function and existence of bacterial organisms inhabiting the inside and the outside of the seed has always led most people to think that the community of organisms present in the surface of the seed coat may be responsible on for any major changes in the plant. This is due mostly to the fact that there are not many organisms identified as endophytic for many plant species. Research conducted by groups like Ait Barka et al., (2002) showed that organisms that are transmitted through the seed could be working as founders of the dynamics that occur in the rhizosphere, possibly competing with other microbes present, and restricting the colonization process for some of the organisms that are less competitive or easily adapter to environmental conditions present at the moment of the plant-microbe interaction.

It is thought that endophytic microbes in seeds should be capable of remaining in the next plant generation. The mechanism of how this can occur are still not completely understood, but based on the information available about localization and movement of

bacteria inside of plants, it is possible that after completing germination, bacteria is capable of exit towards the rhizosphere to later be re-selected by the plant (after interacting with those native in the soil), or to inhabit the plant during its whole life cycle (Adams and Kloepper, 1996, Kaga et al., 2009).

As we mentioned in previous chapters, abiotic and biotic factors can influence the interaction between plants and microbes, leading to changes in the microbial community composition, development and physiological responses from the plant. Ultimately, we predict that all of these variables can also affect the selection of the microbes that could be transfered through the seed of the progeny of the originally treated plants. In this chapter, we present some of our preliminary data from evaluated seedlings and plants grown from seeds collected from the originally treated plants.

5.2 Materials and methods

5.2.1 Germination test and morphological study

Seeds collected from mother plants (seed treated originally with inoculums) were classified as our E1 group of seeds (progeny). To assess the effect of inoculants in seeds, we evaluated germination rates under three different conditions; 1) pot mix soil under greenhouse environment, 2) Murashige and Skoog media, and filter paper. All seeds were treated for ~12 hours before being place in the different environments, and evaluated every day at the hour for 2 weeks to determine if there was a relation between environment + treatment in their ability of completing germination.

Once germination was assessed, we decided to analyze the effect of the treatments applied to the original group of plants in the following generation. Without applying any new seed treatment, we decided to conduct a morphological study to evaluate changes in certain traits of interest of the plant. We used the methodology by Kelemu et al., (2011) with some modification, in which at 2 weeks' seedlings were checked for differences in root system, and at 3-4 weeks after inoculation, measurements were taken and recorded for traits like: plant height (PH), number of leaves (NL), leaf length (LL), wide (LW) and number of flowers. Repetitions of measurements were done once a week until reaching 60 days. All data collected was analyzed with SAS using GLM to generate means for each trait. We used Dunnett's test to separate means using a p-value of $p< 0.05$.

5.2.2 Isolation of organisms in NB-E1

Base on the observations made to plants from E1, we decided to attempt to do isolation of microbial organisms from seeds. For this, we imbibed seeds for 24 hours to help loosen the seed coat. All seeds from each treatment were previously surface sterilize, to later be sectioned using a surgical scalpel (flamed in between groups, as a way of keeping it sterile) inside a flow laminar chamber, and the sectioned pieces were placed in YPDA (agar) plates and YPD liquid media

Plates and media were amended with 50ug/ml of Nystatin to inhibit the development of fungi, since we were only interested in bacteria. A total of 15 seeds were sectioned per treatment. Plates were incubated during a week at 28°C in an incubator. In the same way, we had non-surface sterilized seeds plated too to see if our colonies came from inside the seed or from the seed coat.

Re-isolation and purification of all organisms obtained from cut seeds were done, and a final pure culture of each organism isolated was kept in a glycerol stock at -80°C.

5.3 Results and Discussion

Morphological traits observed in *Nicotiana benthamiana* plants were significantly different when they were evaluated all together. Based on the consistent phenotypes, we decided to let some of the plants complete flowering and collect seeds from treated plants. When seeds were placed in soil, phenotypes comparable to originally treated plants appeared and last it about 4-6 weeks, but after flowering, no phenotypic differences were detected. Even for treatments like Ms, although a bit smaller at first, after the first months will reach the high and develop similarities to traits seem in Control plants (Figure 5.1, Figure 5.2).

Inoculants were not used as seed treatments, still plants grown from seeds of treated plants showed some sort of memory in terms of what parental plants have been through. Because of this, we let E1 plants grow past flowering, and seeds were also collected.

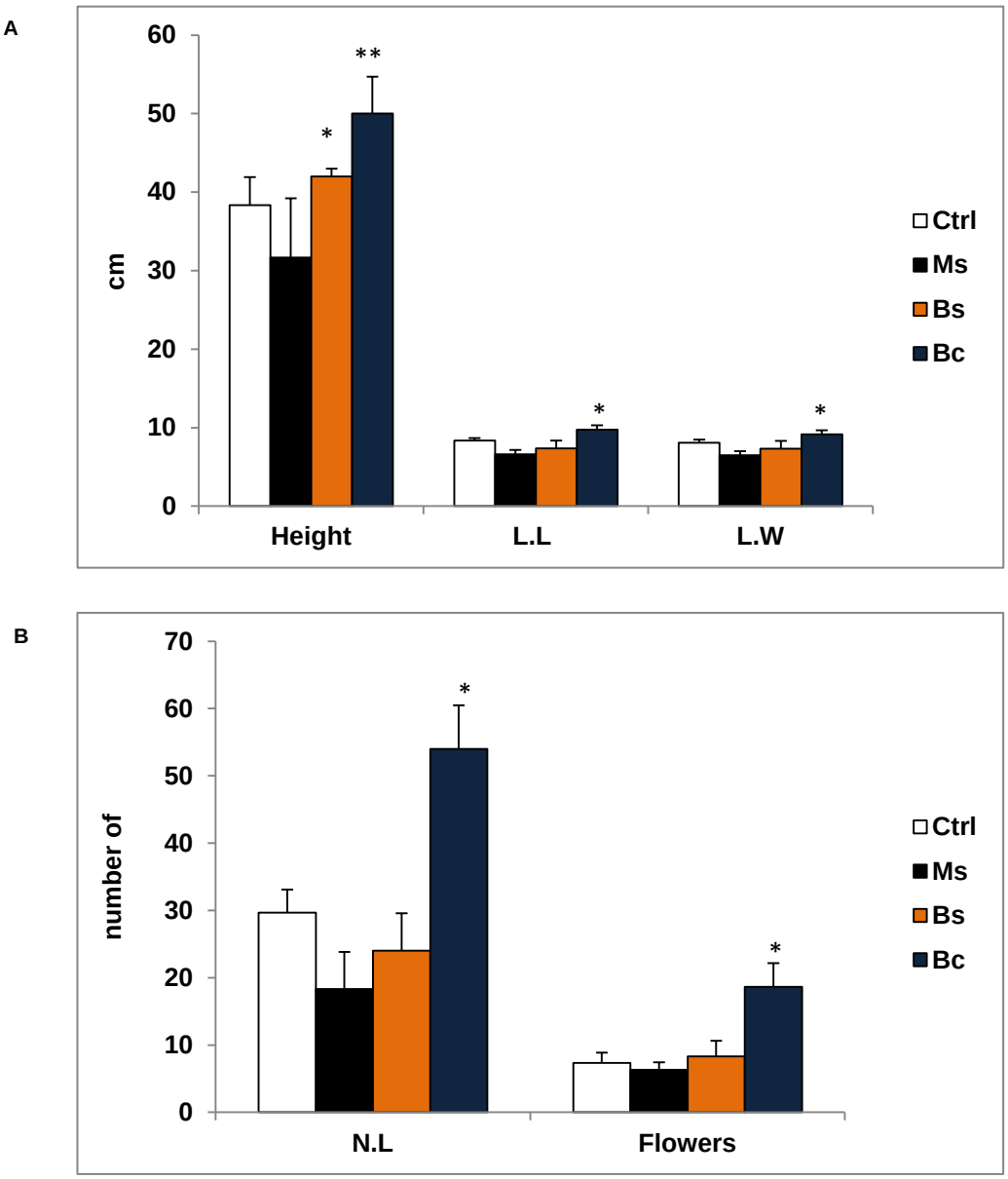


Figure 5.1 Morphological traits evaluated in E1 plants from different treatments. Control, Ms, Bs and Bc treated plants grown in greenhouse during the 2015 year (composite of Spring, late Summer and Fall/Winter data) in 12 week old plants. A) Measurements of Height, leaf length (L.L), leaf width (L.W). B) Number of leaves (N.L) and number of flowers (N.F). Means were separated using Dunnett's test ($p < 0.05$). Asterisks denote significant differences.

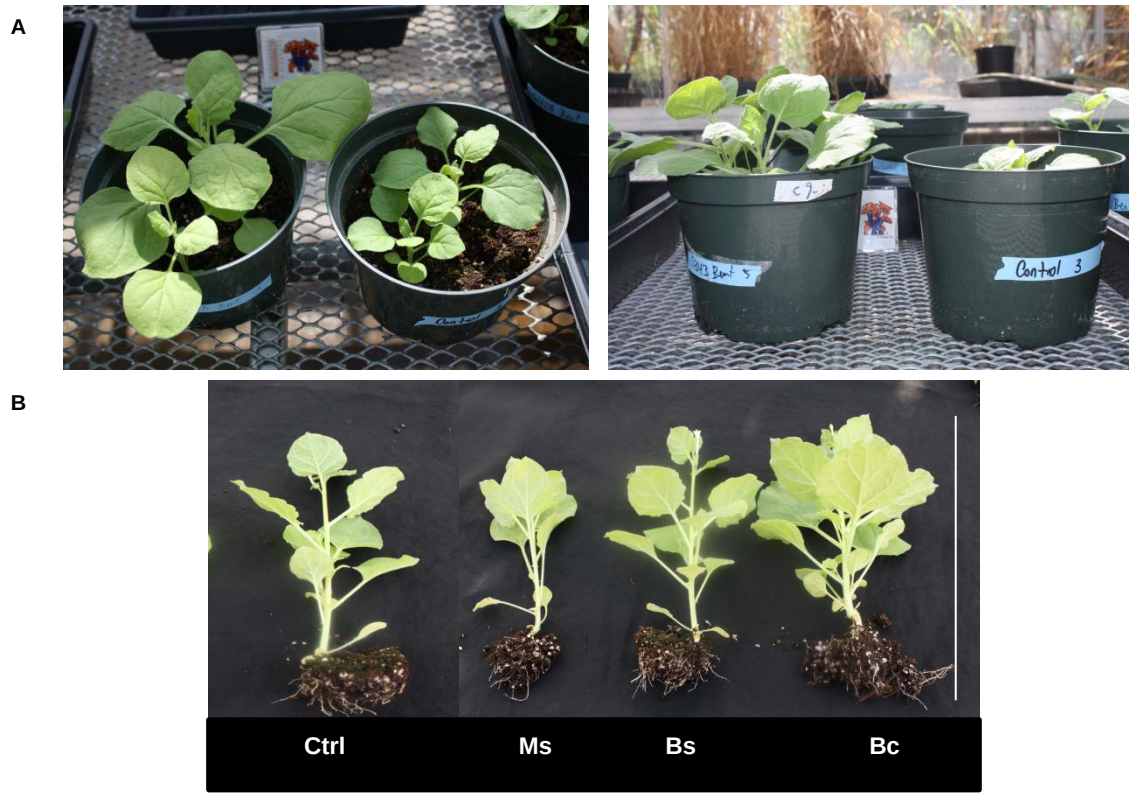


Figure 5.2 Plants grown under greenhouse conditions at week 5. A) Aerial and side view of progeny of treated plants evaluated under greenhouse conditions without application of bacterial seed treatment. B) Comparison of control plants vs Ms/E1, Bs/E1 and Bc/E1 during weeks 5-6. Scale bars represent 18cm.

Based on our findings, we decided to isolate through culturing dependent techniques the microbial organisms that inhabit in the seeds of the E1 (which showed a similar phenotype to their mother). We believed that if no treatment was being applied again to the seeds before placing them in the soil, and still during early development we were seeing a consistency among the phenotype before transitioning to flowering, some sort of “memory” or organisms selected by the plant under the original environmental conditions that plants were growing, had to be transmitted to the descendants in order to only showed in early stages of development.

We obtained a total of 12 different strains for E1 seeds from all treatments. Because of the difficulty of isolating from seeds being placed directly in agar plates, we used a liquid media as a start point for isolation and all cultures were later plated continually in order to generate pure cultures (Figure 5.3).

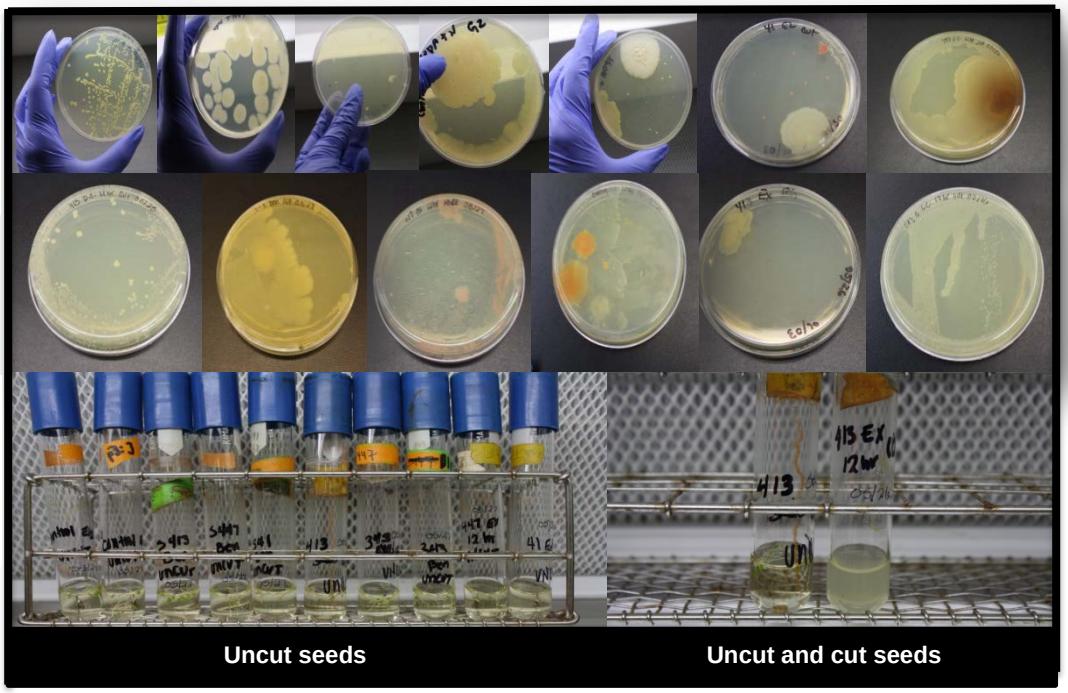


Figure 5.3 Isolated organisms from seeds belonging to E1 progeny of the treated mother plants. A) A total of 12 differential organisms were cultured and purified. B) Pictures show uncut seeds in media tubes vs cut seeds, showing that organisms isolated were coming from the inside of the seed.

The data shown in this chapter is very preliminary, but it has led us towards asking more questions that could explain how striking changes induced in mother plants are able to be transfer to a following generation. We hypothesize that presence and production of metabolites from the organism originally used as an inoculum in the mother plants, determined the conditions and the information transferred to the seedlings in the new progeny, as well as the organisms selected.

In the figure 5.4, we layout the steps that may be involve in the selection and transferring of organisms to following generations. Using this as our guideline, we have establish a list of future studies to be developed in this area.

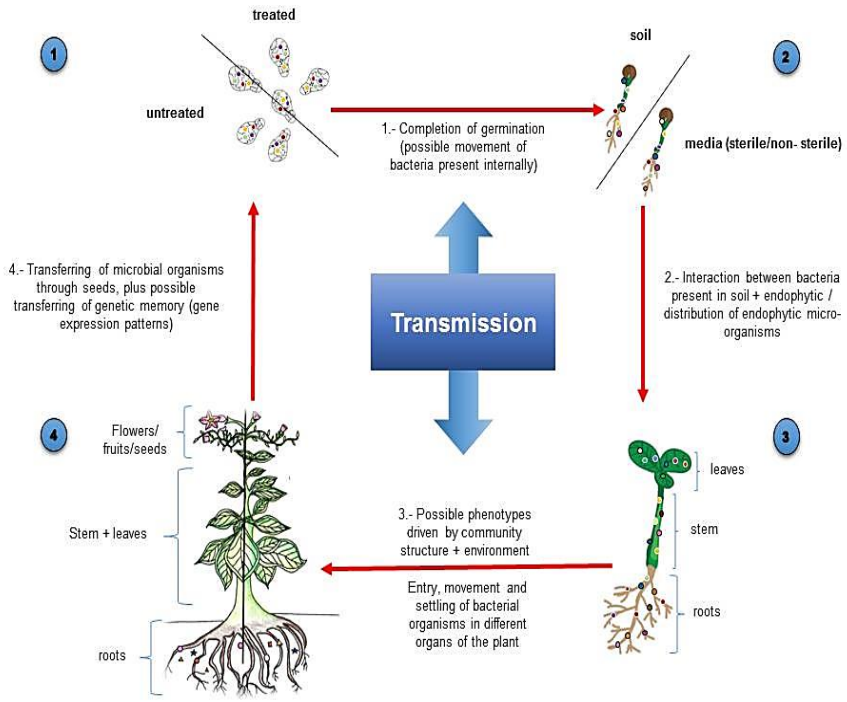


Figure 5.4 Transmission of bacterial organisms during the different stages of growth and development of plants. 1) Represent the germination process between treated and untreated seeds with a bacterial inoculum. 2) The environment where plants develop will have an effect on the final selection of bacterial organisms selected or interacting with the plant. Here, we see a seedling grown on media (sterile/non-sterile vs soil), which will ultimately allow endophytic organisms in the seed to be possibly more or less competitive in the presence of other organisms. 3) Phenotypes and organs where endophytes can reside or enter in the plant. 4) Movement and selection of organisms necessary to be transferred to new progeny.

Currently, we are evaluating the seed composition variations among all treatments. Variables like: fatty acid profile, starch and cellulose were some of the selected characteristics to determine if any impact occurs at this level. From these characteristics, we are interested in the fatty acid profile and starch content, because tobacco seeds are known for being high in lipid reserves, and starch is considered to be one of the key factors in allowing transferring of organisms (Mano *et al.*, 2006).

Inoculation of seeds from the E1 group with the original inoculums used in the mother plants (Ms, Bs and Bc), have been established under greenhouse and in vitro conditions. Ultimately, besides the evaluation of the morphology changes possibly induced by treating the E1 seeds, we hope to make crosses among treated vs non-treated E1, to test the persistence or loss of the phenotypes previously identified.

Lastly, all seeds from supernatants and cell treated plants have also been and will continue to be evaluated for effects in germination and morphology. Our final goal will be to try to describe as much as possible the effects that bacterial inoculants have had in the plant as a whole, and how some of these consistent responses also affect the development of the descendants.

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4. PUBLICATIONS

- Matos, Ángela; Sánchez, Andrea; Cervantes, Armando. Effects of the use of colchicine as an inducer of polyploidy in plant *Aloe* (*Aloe vera* L.) in vivo and *in vitro.* Bulletin of Latin American and Caribbean Society of cacti and other succulents. Volume 7, No. 2. May-Agust 2010.
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