

Agave seed endophytes: Ecology and impacts on root architecture, nutrient acquisition and cold stress tolerance

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Abstract

The genus *Agave* comprises plants that are a source of nutrients for humans and animals and can support their ecosystems. *Agave* extinction may impact a long list of organisms including plants, pollinators, animals and soil microorganisms. *Agaves* have an extraordinary adaptability to arid, semiarid environments. Physiological and morphological strategies allow them to survive under extreme conditions such as drought and high temperature (up to 61°C). In recent decades it has been discovered that bacterial and fungal communities in plants are not simple passengers, and this is especially true for microbial communities of seeds. Seed-transmission of endophytic microbes appears to be important in shaping the endophyte community in the mature plant and consequently acts as the initial inoculum for the plant microbiota. Those microbes participate in seedling growth, favors intake of nutrients and resistance to abiotic and biotic stress and, in some extreme cases, can be used as “food” for the plants.

Key words: *Agave angustifolia*, *Agave tequilana*, *Agave marmorata*, *Bacillus tequilensis*, Chilling stress, Endophytes, *Enterobacter*, MALDI-IMS, Metabolomics, Nutrient acquisition, *Pseudomonas aeruginosa*, Root Architecture, Root-Hairs, ROS.

0.1 Introduction

Seeds represent one of the most crucial stages of a plant’s life history. In natural ecosystems, a seed not only serves to initiate the life cycle and reproduce plant species, but also facilitates dispersal, adaptation and persistence in new environments (Truyens et al., 2015). Seeds as well as roots, leaves and flowers have evolved in association with diverse microbial communities, which we know as “microbiome”, and plays a decisive role in the growth and health of plants. There are two types of microorganisms associated with seeds: ‘seed-borne’ and ‘seed-transmitted’, this difference is based mainly on their location. The seed-borne microbes are found on the surface of the seed and represents transient colonizers of seed habitat. On the other hand, seed-transmitted microbes are persistent members of embryo and/or endosperm (Barret et al., 2016). Both types are important for the evolution of the microbial community of the seedling. Seed-transmitted endophytes are bacteria, fungi, viruses and Oomycetes, within other tissues (Nelson, 2018).

Endophyte persistence depends on their ability to colonize the aerial parts of the seedling and to compete with the native microbes of the soil, releasing secondary metabolites, which results in an area of high microbial activity around the germinated seed, called “spermophere”. That microbes are conserved across evolutionary and ecological boundaries, suggests that plants select microbes from the environment with beneficial properties to the host plants (Jonston-Monje and Raizada, 2011). Therefore, seed-microbiome ecologically represents the end point of a microbial community assembled in the seed, and the beginning of the assembly of the microbiome of the new seedling. Additionally, it is possible that seed-endophytic bacteria use seeds as a vehicle of dispersion towards different plants or environments.

Another interesting issue concerning the ecology of the seed microbiome, is microbial dynamics during germination, and emergence as far as seedling development. Barret et al., (2015) showed that seed germination of a large range of the species in family *Brassicaceae* does not affect microbial diversity. However,

changes in the microbial community composition were observed mainly during emergence. Microbial diversity decreases during the transition from the germinated seed to the seedling, which indicates a strong selection exerted by the seedling on the microorganisms of the seed. In arid and semi-arid environments that microbial colonization is essential for the establishment of plants (Coleman-Derr et al., 2014). Microorganisms increase the availability of water and nutrients for the plant, and this is important to survive drought stress. In both *Mammillaria fraileana* and in the giant cardon cactus *Pachycereus pringlei*, seed endophytic bacteria help seedlings establish and grow on barren rock, but when bacteria were eliminated from seeds, the development of seedlings stopped (Puente et al., 2009, Lopez et al., 2011).

Notwithstanding the sharp increase of publications focused on dissecting the seed endophyte microbiome observed in the last 5 years, according to our knowledge, few studies highlighted the structure of the endophyte microbiome in wild and domesticated agave plants. The published studies (Coleman-Derr et al., 2014, Desgarenes et al., 2014) emphasize the composition of bacteria and fungal communities, microbial diversity in function of nutrients in soil (low nitrogen content), farm management, biogeography and environmental conditions (e.g.: drought). Furthermore, only one publication shows cultivable endophytic bacteria with growth promotion in blue agave (Martinez-Rodriguez et al., 2014). But, until now, we don't have any information about seed-transmitted microbial communities and their ecology and functionality on agave plants.

In this chapter, we present our most recent advances in seed-endophyte research in three *Agave* species. We use culture-dependent methods to characterize bacterial communities that were identified by ribosomal profiles using MALDI-TOF mass spectrometry and 16SADNr sequencing. Also, we will show microbial dynamics during emergence and formation of the seedlings of one wild *Agave*. As well as microbiological and “omic’s” approaches to understand their functionality in nutrition, growth and resistance to chilling stress in blue agave.

0.2 Ecological importance of genus *Agave*.

Agave plants are part of the arid and semi-arid landscapes of central and northern Mexico and the southwestern United States. The genus *Agave* belongs to order *Asparagales* with more than 211 species. Mexico is considered to be the center of origin of *Agave*, because at least 159 species (75%) are endemic of the country (García-Mendoza, 2018). *Agave* is paraphyletic to the genera *Manfreda*, *Polianthes*, and *Prochnyanthes*, and the entire clade has been termed *Agave sensu lato* (Good-Avila et al., 2006). Agaves have CAM metabolism (Crassulacean Acid Metabolism), a specialized mode of photosynthesis that evolved from ancestral C_3 photosynthesis in response to water and CO_2 limitations. The CAM pathways enable agaves to colonize semi-arid environments where water is scarce and soil surfaces temperatures often exceed $60^{\circ}C$ (Abraham et al., 2016, Stewart, 2015). Moreover, the succulent and fiber-rich nature of agave leaves allows for the continuation of CO_2 fixation and other vital biochemical reactions during extended periods of drought, which can last up to 7 years or more (Stewart, 2015). Leaves, accumulate cutin, waxes and anthocyanins on their surfaces because they dissipate excessive sunlight (acting as photochemical quenchers) and control temperature

that it is important as key control of plant metabolic rates; in addition they create a barrier against uncontrolled gas exchange from the external surface of the epidermal cells and impermeable to water (Monja-Mio et al., 2015, Wright et al., 2017). The rosette arrangement of agave leaves allows maximal absorption of photosynthetically active radiation, and the funnelling of water to their superficial root system, that can shrink in response to drying soil, minimizing water loss. Plants generate fine roots to permit rapid water uptake after short-lived rain (Matiz et al., 2013, Campos et al., 2014). Dead leaves accumulate at the base of agaves also called “piña” (because it resembles a pineapple), buffering living tissue from high soil surface temperatures, reduced evaporation and increased soil organic matter content (Stewart, 2015). Other traits that have made agaves suitable as crops include caudices (starch-rich stems), semelparous flowering, long-lived perennial habit (with average of 25 years, but some may live far longer) and natural proclivity to grow on rocky, infertile soils of hillsides at coastal zones (Huerta-Lovera et al., 2018).

Depending on the agave species, sexual, asexual or both reproduction strategies have been observed in this genus. Asexual propagation through young rhizomatous suckers appear to be the most common and successful natural establishment strategy by dependence on the mother plant. Another asexual propagation method involves the production of thousands aerial plantlets or bulbils at the bracteoles (on the inflorescence), this comes into play when sexual reproduction is inefficient or unsuccessful, and ensures asexual reproduction even in absence of other flowering individuals, pollinators or in adverse environmental conditions (Arizaga and Ezcurra, 1995). It was discovered that bulbil induction in *A. tequilana* involves expression of several transcription factors as KNOX, MADS and, LEAFY regulated by auxin in combination of polarized auxin efflux transporters known as PIN-proteins (Abraham-Juarez et al., 2015).

For sexual reproduction, plants produce an inflorescence or “quiate” only once at the end of life cycle and this takes from 4 to 25 years depending on the Agave species. Sugars that were stored during plant growth are used for quiate development. Once initiated, quiate is covered with bracts, undergoes a period of rapid growth until reaching a height of 6m. At a height of 4m, lateral branches or umbels begin to form on the inflorescence. Each umbel is capable of producing hundreds of flowers (Arizaga and Ezcurra, 2002). Flowers are protandric and composed of green tepals with purple tips and 6 anthers joined to filaments at the base of tepals. In general, floral traits of *Agave* suggest adaptation to bat pollination or chiropterophily. Once the flowering begins, flowers produce protein-rich pollen and abundant nectar, mainly at night, which smells like ripening fruit and ammonia (Slauson, 2000, Borbón-Palomares et al., 2018). This aroma is attractive to hawkmoths, bees, hummingbirds, and the pollinating bats (Trejo-Salazar et al., 2015). The reproductive ecology of agaves is linked to a guild of pollinators; this is of great importance as destruction of the natural habitats of animal species who serve as pollinators and whose survival depends on consumption of agave nectar. The inflorescence contains fruits (capsules) with several thousand of viable and sterile seeds. Therefore, agave plants could spread sexually by seeds. Agave has light, small, flattened seeds, giving the ability to be dispersed by wind or animals. In agaves seed production varies widely among species; average seed numbers range from 777 in *A. angustifolia* to 780,000 in *A. palmeri* (Huerta-Lovera et al., 2018).

0.2.1 Why is agave propagation ecologically important?

Currently, production of *Agave* species, such as *A. americana*, *A. inaequidens*, *A. maximiliana*, *A. tequilana*, *A. potatorum*, and *A. salmiana*, is subject to domestication, and crops are grown in large extensions of land, harbouring millions of plants with commercial purposes. Propagation is by young rhizomatous suckers. This has caused a loss of genetic variability, so this practice agro-culture has induced fungal (*Fusarium oxysporum*) and bacterial (*Pectobacterium carotovora*) diseases in the agave plantations, hence reducing the number of useful plants (Aquino-Bolaños et al., 2011, Vega-Ramos et al., 2013, Ramirez-Ramirez et al., 2017). Another important aspect resulting in the loss of agaves as biotic resources, has been the exploitation of wild species for mezcal production resulting in reduction of their genetic variability, and the extinction of some species. *Agave marmorata* (Roetzl) became extinct because of its use for mezcal production. However, plants are not the only biotic resource that is being lost in the *Agave* ecosystems. For tequila and mezcal production, farmers avoid inflorescence development, since their formation causes a loss of the sugar concentration, which impacts the production of distillate beverages, so pollination dynamics in many species are disrupted. In addition, removal of inflorescences affects the relationship established hundreds of years ago between agaves and nectar-feeding bats *Leptonycteris yerbabuenae* (Martinez & Villa), *L. nivalis* (Saussure), and *Choeronycteris Mexicana* (Tshudi). These three bats species are migratory and are under protection in two categories (Threatened and Endangered). Diverse studies recorded in wild species have identified bats as the main pollinators (Trejo-Salazar et al., 2016). A co-evolution process has resulted in specialization that occurs between agaves and bats, because bat tongue morphology is adapted to flower structure, providing food and facilitating pollination (Ornelas et al., 2002). Nectarivorous bats forage in the areas of both continuous habitats and fragments; thus migratory bats help to maintain genetic connectivity between the different habitat fragments and plant populations visited. As strategy to protect bats, producers of *A. cupreata*-based mezcal adopted the practice of allowing about of 10% of the plants to flower, so that they obtain seeds to plant the next generation of agaves (Trejo-Salazar et al., 2016).

In examining recent publications that evaluate microbial communities of nectarivorous and other bats (Galicia et al., 2014, Carrillo-Araujo et al., 2015, Banskar^a et al., 2016, Banskar^b et al., 2016), we found a linkage with endophytic bacterial communities residing in agave seeds (Beltran-Garcia, unpublished results). This may suggest that loss of agave-bats interaction affects the structure of the seed microbiomes that directly or indirectly alters plants as a function of their resistance to various types of abiotic and biotic stress in semi-arid environments.

0.3 Biotechnological importance of agaves: From prebiotics to biofuels.

The *Agave* genus is one of the natural resources with social, economic and agro-ecological importance in Mexico. Traditionally, agave plants have been used for the production of alcoholic beverages such as: "Tequila", a distillate product derived from the fermentation of *A. tequilana*; "Mezcal", obtained from *A. angustifolia*, *A.*

potatorum, *A. salmiana*; “Bacanora” from *A. angustifolia*; “Raicilla” from *A. maximiliana* and *A. inaequidens*; and the pre-hispanic “Pulque”, using *A. atrovirens*, *A. ferox*, *A. mapisaga* and *A. salmiana* (Nava-Cruz et al., 2014). Pulque is a typically fermented but not distilled traditional drink, whose origin is prehispanic and which is made from the fermentation of mucilage-popularly known as aguamiel (Escalante et al., 2016). Pulque is a traditional alcoholic, non-distilled, fermented beverage produced by the fermentation of the sap known as “aguamiel” extracted from several *Agave* species such as *A. salmiana*, *A. atrovirens* and *A. mapisaga*. Aguamiel and pulque have traditionally been considered as healthy beverages due to their nutrient content. Scientific evidence supporting the relationship between the microbial diversity present in aguamiel and pulque has shown the antimicrobial effects of both the sap used as substrate and the final fermented product against pathogenic bacteria such as *Salmonella enterica* serovar *Typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Shigella flexneri* and *S. sonnei*. Giles-Gomez et al. (2016) reported the *in vitro* and *in vivo* probiotic assessment of *Leuconostoc mesenteroides* isolated from pulque. The genome analysis showed a pre-bacteriocin coding gene and six peptidoglycan hydrolase enzymes, probably involved in the antimicrobial activity.

Besides agave will be used as a food. A growing market has emerged from agave as sweetener (agave syrup or agave nectar) used as a healthy alternative to sucrose, honey and maple syrup. Candies, tortillas and stews also can be used as human food. In addition some agaves are a niche for insect larvae better known as “maguey worms” that are highly valued in the Mexican gastronomy. Currently the inulin-type fructans, known as ‘agavins’, the major carbohydrate found in agaves, which is being used as a source of insoluble fiber and prebiotics (Huazano-Garcia and Lopez, 2018). Enzymes in digestive tract cannot hydrolyze β -glucosidic bonds in inulin-fructans, which consequently promotes the growth of bifidobacteria in mammalian intestines (Cuevas-Juarez et al., 2017). Such microbial growth can lead numerous health benefits, including resistance to gut infections, improved lipid metabolism, higher calcium absorption and control of diabetes and dyslipidemia (Franco-Robles and Lopez, 2016). Other end products of *Agave* are also warranted, including their incorporation into dairy products and bread.

Agaves show potential as bioenergy crops due to their relative productivity in nutrient poor soils, with low consume of water. In addition the long history of producing agaves for alcoholic beverages, which could be capitalized upon as bioenergy crops. Also agave contain low amounts of lignin, considered a barrier to synthesis of lignocellulosic fuels, and this results in easy decomposition of the fibers, so the conversion of bagasse to ethanol is easier. Actually *A. tequilana* is grown as a rain-fed crop in the Australian seasonally dry tropics where summers are hot and wet and winters are cooler and dry. However, there are many possible matches between species of *Agave* with commercial potential and the range of climates and soils in Australia.

Insert Figure 1

0.4 The *Agave* microbiome...until now

Drylands take up 41.3% of the land surface and are found worldwide from mid-tropical to high latitudes. Drylands refer to hyper-arid (desert, 6.6%), arid (semi-desert, 10.6%), semi-arid (grassland, 15.2%) and dry-subhumid (rangeland 8.7%). Plant growing in drylands must resist drought, high UV radiation exposure, extreme changes in temperature and nutrient-limited soils. Under these hostile environment conditions *agaves* flourish, and consequently are considered as major biotic resources in drylands. For two Mexican research groups, the study of the *Agave* microbiome could explain the behaviour of *Agave* species in various ecosystems where they grow.

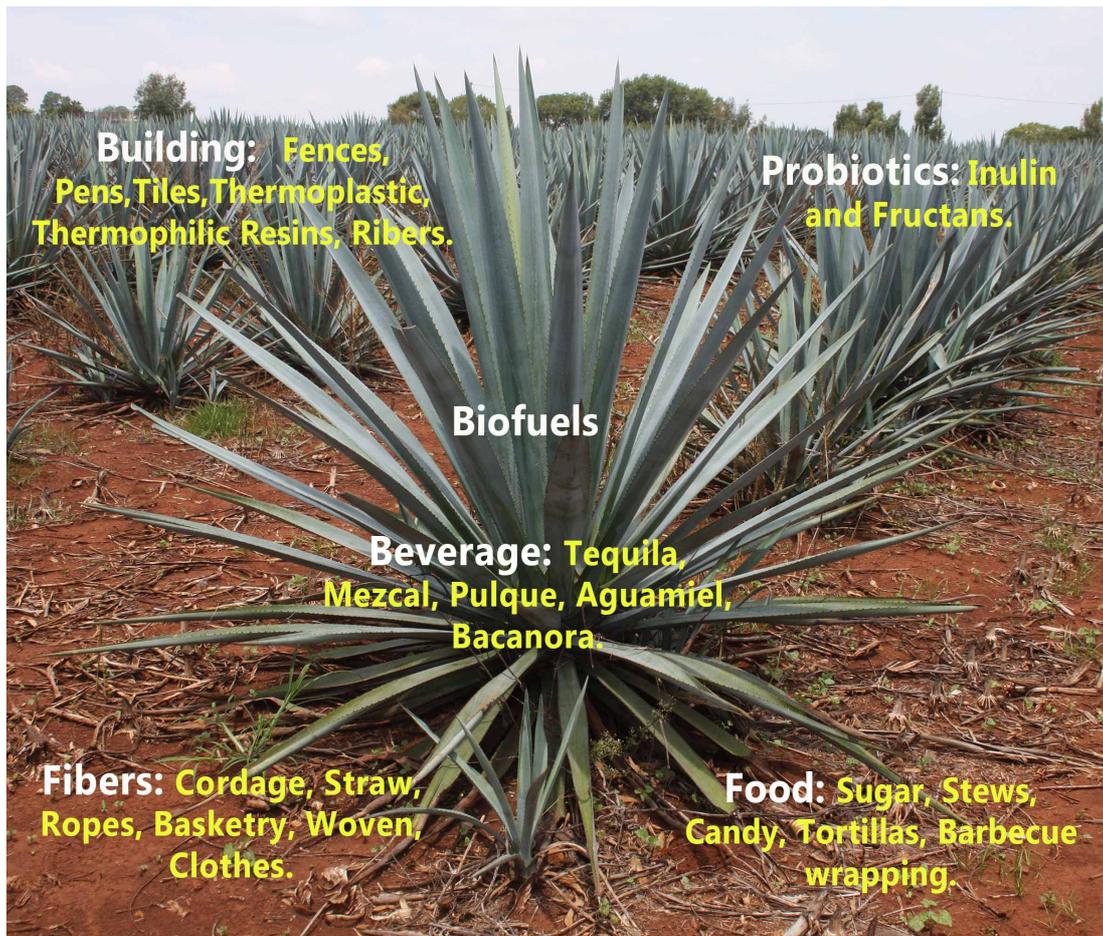


Figure 1. Historical, current and biotechnological end uses and products of *Agave* species.

The Mexican research group of Laila Partida-Martinez from CINVESTAV Unit at Irapuato, study *Agave* and cacti microbiomes. They are using metagenomic and *in silico* functional analysis to analyze the composition and diversity of microbial communities in wild and domesticated species of *Agave* (Fonseca-Garcia et al., 2018). The dominant bacteria belong to phyla such as Proteobacteria, Actinobacteria and Acidobacteria, and their diversity is modified in soils with low nitrogen content. In addition domestication of *A. tequilana* has influenced its microbiome diversity, represented more by bacteria and archea than by fungi

(Desgarenes et al., 2014, Coleman-Derr et al., 2015). Members of the Proteobacteria, Firmicutes, Actinobacteria and bacteroides comprised >80% of the plant associated communities. In the cultivated agave, Enterobacteriales represent nearly 43% of the rhizosphere, and Flavobacteriales 27.6% of the phyllosphere. Drought apparently does not influence the microbial communities, since they are similar between the species, so it was suggested that these microorganisms participate in plant fitness (Coleman-Derr et al., 2015). These reports suggest that adaptation of *Agave* species to arid areas, extreme environments and nutritionally limited soils, could be mediated not only by their inherent characteristics (CAM metabolism, morphology adapted to arid environments), but also by their symbiotic microbes.

0.4.1 The culturables microbes.

Martinez-Rodriguez et al. (2014) reported cultivable endophytic bacteria and evaluated their agronomic potentials and antifungal effects. Three hundred cultivable endophytic bacteria were isolated from leaf bases of *A. tequilana*. In plant tissue bacteria occurred at mean population densities of 3 million CFU/g of fresh plant. Bacteria were grouped into eight different taxa that shared high homology with other known sequences and were proved to be PGPB (Plant Growth Promoting Bacteria) for their ability to fix N, producing auxins, solubilizing phosphates and possessing antagonistic activity against a pathogenic strain of *Fusarium oxysporum* AC132, demonstrating that endophytic bacteria are a promising alternative as biofertilizers for agave cultures, or to re-plant microorganisms lost in agave seedlings originated by micropropagation where native endophytes may have been eliminated. Endophytic microbes become excellent candidates as bio-inoculants to improve the productivity of this domesticated *Agave*. In the table 1, we summarize those endophytic bacteria identified as part of the *Agave* microbiome using independent and dependent-culture methods.

0.5 Core microbiome of *Agave* seeds: Who is there?

In recent decades it has been discovered that seeds bear microbial communities comprised of bacteria and fungi. Seed transmission of endophytic microbes appears to be important in shaping the endophyte community in the mature plant and consequently acts as the initial inoculum for the plant microbiota (Barret et al., 2015). Seed-associated microbes should be regarded as very intimate microbial partners of higher plants, with the potential to connect successive plant generations (Shade et al., 2017). Those microbes participate in seedling growth, favor intake of nutrients and resistance to abiotic and biotic stress and, in some cases, can be used as “food” for the plants (Beltran-Garcia et al., 2014).

It is well known that endophytes transmitted by seed, were first discovered in rye grass in 1898, and the importance of seed bacteria has gradually been realized in recent decades. One hundred and thirty genera from 4 different phyla of 25 different plants species have been reported as naturally occurring seed endophytes (Truyens et al., 2015). The most predominant seed endophytes belong to the γ -proteobacteria class, Proteobacteria phyla, followed by the Actinobacteria, Firmicutes and Bacteroidetes phyla. In general, *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea* and *Acinetobacter* are often detected in seeds (Zhang et al., 2018). To investigate endophytic and seed-

endophytic populations researchers used the culture-dependent and culture-independent methods. Currently the newly developed high-throughput sequencing technique has been applied to assess plant seed microbiomes. Currently, one of the difficulties to studying plant endophytic microbiomes is the ability to determine a plant's core microbiome (Zhang et al., 2018). Discovery of the core microbiome is important for understanding the stable, consistent components across complex microbial assemblages.

A core microbiome is typically defined as the suite of members shared among microbial consortia from similar habitats, and is represented by the overlapping areas of circles in Venn diagrams, in which each circle contains the membership of the sample or habitat being compared (Shade and Handelsman, 2011). Yet, their cultivation in the laboratory is required to appreciate microbial physiology and to conduct experiments to evaluate the role that microbes play in plant fitness.

Table 1. Endophytic bacteria isolated or characterized from agave plants.

Agave species	Bacterial Genera (Isolates or *OTUs)
	Endophytic
<i>A. tequilana</i>	<i>Acinetobacter</i> sp., <i>A. baumannii</i> , <i>A. bereziniae</i> , <i>Acidobacteria Gp4</i> , <i>Alcaligenes faecalis</i> , <i>Acidovorax facilis</i> , <i>Achromobacter spanius</i> , <i>Arthrobacter roseus</i> , <i>Bacillus</i> sp., <i>B. litoralis</i> , <i>B. tequilensis</i> , <i>B. mycoides</i> , <i>B. oshimensis</i> , <i>B. safensis</i> , <i>B. siralis</i> , <i>Brevundimonas diminuta</i> , <i>Burkholderia caribensis</i> , <i>B. gladioli</i> , <i>B. phenazinium</i> , <i>Cellulosimicrobium cellulans</i> , <i>Citrobacter freundii</i> , <i>Clostridium</i> , <i>Cronobacter sakazakii</i> , <i>Chryseobacterium gleum</i> , <i>Corynebacterium glutamicum</i> , <i>Enterobacter</i> , <i>E. cancerogenus</i> , <i>E. cloacae</i> , <i>E. cowani</i> , <i>E. hormaechei</i> , <i>Enterococcus casseliflavus</i> , <i>Erwinia pyrifoliae</i> , <i>Gluconobacter oxydans</i> , <i>Klebsiella oxytoca</i> , <i>K. pneumonia</i> , <i>Lactobacillus fructivorans</i> , <i>L. paracasei</i> , <i>L. plantarum</i> , <i>Leuconostoc mesenteroides</i> , <i>Micrococcus luteus</i> , <i>Myroides odoratus</i> , <i>Pseudomonas</i> sp., <i>P. aeruginosa</i> , <i>P. libanensis</i> , <i>P. stutzeri</i> , <i>P. syringae</i> , <i>Reynarella</i> , <i>Solibacillus silvestris</i> , <i>Staphylococcus capitis</i> , <i>S. warneri</i> , <i>Streptomyces</i> , <i>Stenotrophomonas</i> sp. , <i>S. maltophila</i> , <i>S. acidaminiphila</i> , <i>Rhizobium radiobacter</i> , <i>Paenibacillus amylolyticus</i> , <i>Pantoea agglomerans</i> , <i>P. terreus</i> , <i>Ochrobactrum gallinifaciens</i> , <i>O. grignonense</i> , <i>Weissella halotolerans</i> .
<i>A. americana</i>	<i>Rhizobium</i> , <i>Pseudomonas</i>
<i>A. salmiana</i>	<i>Acidobacteria Gp4</i> , <i>Acidobacteria Gp6</i> , <i>Streptomyces</i> , <i>Clostridium</i> , <i>Reynarella</i> , <i>Enterobacter</i> , <i>Stenotrophomonas</i> , <i>Bacillus</i> ,

*Bacterial genera presented in bold were identified by Independent-Culture Method.



Figure 2. Seeds of *Agave* species. Seeds are flat and black in color and showed a considerably variation in size: shiny *A. marmorata* seeds (0.5 cm width x 0.5 cm length), Opaque *A. tequilana* seeds (0.9 cm width x 0.7 cm length) and opaque *A. angustifolia* (0.8 cm width x 0.7 length). Seeds were collected directly from fruits of plants located in Mexican states of Oaxaca, Jalisco and Sonora, respectively.

Three *Agave* species were selected under an ecological criterion to show the composition of the cultivable seed microbiome, according to domestication state and the economic tendency to create a monoculture. 1) *A. tequilana* Weber or “blue agave, was the first domesticated species, and it is no longer found in a wild condition. *A. tequilana* is in monoculture in five Mexican states that make up the appellation of origin “Tequila”. The crop shows low genetic variability; its cultivation depends on heavy chemical fertilization applications and high use of pesticides to control insects, and pathogens. In addition, it is susceptible to low temperatures; b) *A. angustifolia* Haw, also know as “agave espadin” is semi-domesticated and in progress towards being a monoculture, but it is still possible to find wild varieties in seasonal dry scrubs and forests of *Quercus*-*Pinus*, from Mexico to Central America. Its maturation takes from 8 to 12 years and is the source of production of the “Bacanora” and Mezcal in the states of Sonora and Oaxaca; its considered as a ancestor of both blue agave and henequen agave; c) *A. marmorata* Roezl, is an endemic species of lowland forests and scrub ends of the states of Oaxaca and Puebla. However, it is already in the process of domestication. Reports indicate that it is an endangered agave. “Mezcal tepextate” is produced exclusively from wild individuals that take 18-25 years to mature. It is found on sunny and rocky hillsides, sometimes clinging directly to the rock, on the top of a rock, or on almost vertical slopes with minimal or no topsoil to sustain it, however its roots extend affirming and avoiding erosion in rainy seasons. There are no protection programs for this species to safeguard its genetics and populations.

0.5.1 What microbes are inside agave seeds?

The colony-forming unit (CFU) of bacteria recovered from each *Agave* seed ranged from Log₁₀ 3.36-6.54 CFU per gram of seeds, where seeds of *A. angustifolia* had the highest number of bacteria recovered, and *A. tequilana* the lowest bacterial abundance. Significant difference was observed between *A. tequilana* and the other *Agave* species, but these differences were not significant between semi-domesticated *A. angustifolia* and wild *A. maximiliana* and *A. marmorata*. Colonies of

different morphologies and colors were picked for isolation and identification. Using MALDI-TOF mass spectrometry as the first option for ID, all bacteria were grouped. Two isolates of each group were selected and then identified by the partial sequencing analysis of 16SrDNA. A total of 500 seed-bacteria were identified. The number of seed-endophyte bacterial genera was diverse among *Agave* species. Microbial isolates of *A. maximiliana* was grouped in 8 genera, 16 in *A. angustifolia*, 14 genera in *A. tequilana* and 19 genera in *A. marmorata*. Further classification showed that bacteria belonged to four phyla: Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. The most commonly seed-endophytic bacteria on *Agave* belong to the phylum Firmicutes and constituted 54%, 50%, 47% and 44% of the total species types of *A. angustifolia*, *A. tequilana*, *A. maximiliana* and *A. marmorata* respectively (Figure 3). Species of the phylum Firmicutes included: *Bacillus altitudinis*, *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. endophyticus*, *B. galactosilyticus*, *B. gibsonii*, *B. licheniformis*, *B. megaterium*, *B. mojavensis*, *B. nealsoni*, *B. pseudomycoides*, *B. pumilus*, *B. rhizosphaerae*, *B. safensis*, *B. siralis*, *B. sonorensis*, *Bacillus* sp., *B. subtilis*, *B. tequilensis*, *B. thuringiensis*, *B. vallismortis*, *B. weihenstephanensis*, *Enterococcus casseliflavus*, *Lactobacillus crispatus*, *L. kalikensis*, *Listeria grayi*, *Lysinibacillus fusiformis*, *Solibacillus silvestris*, *Staphylococcus chromogenes*, *S. cohnii*, *S. epidermidis*, *S. haemolyticus*, *S. hominiis*, *S. pasteurii*, *S. warneri*, *S. xylosum*, *Paenibacillus* sp. and *P. taiwanensis*. Other phyla identified included Proteobacteria, Actinobacteria and Bacteroidetes. Proteobacteria was the second most abundant phylum and contained 28 species including *Acinetobacter radioresistens*, *A. lwoffii*, *Achromobacter denitrificans*, *A. spanius*, *Aeromonas molluscorum*, *Alcaligenes faecalis*, *Brevundimonas diminuta*, *Enterobacter ausburiae*, *E. cloacae*, *E. cowanni*, *E. hormachei*, *E. kobei*, *Klebsiella pneumoniae*, *K. variicola*, *Kosakonia oryzae*, *Novosphingobium aromaticivorans*, *Pantoea agglomerans*, *P. terra*, *Pseudomonas aeruginosa*, *P. lutea*, *P. monterlii*, *P. putida*, *Pseudomonas* sp., *P. stutzeri*, *Sphingomonas thalpophilum*, *Stenotrophomonas acidaminiphila*, *S. maltophila* and *S. rhizophila*. The phylum Actinobacteria included 9 genera such as *Brevibacterium*, *Curtobacterium*, *Kytococcus*, *Kocuria*, *Microbacterium*, *Micrococcus*, *Rhodococcus*, *Streptomyces* and *Tsukamurella*, and finally the phylum bacteroidetes had the lowest abundance and included 13 isolates of *Sphingobacterium* from *A. angustifolia* seeds.

Interestingly, the distribution pattern within seed-endophytic isolates of four agaves showed that 14 bacterial species comprised 48% of the total isolates, with the most abundant being *B. pumilus*, *B. subtilis*, *A. faecalis* and *E. cloacae* all of them with 28 isolates; 26 isolates of *B. safensis*. The second most abundant species were *Sphingobacterium thalpophilum*, from Bacteroidetes phylum that solely appear in *A. angustifolia* seeds (13), *P. aeruginosa* (10), *E. cowanni* (10), and *S. maltophila* (10). The third group including *K. marina* (7), *B. thuringiensis* (7), *B. sonorensis* (7), and *B. tequilensis* (7).

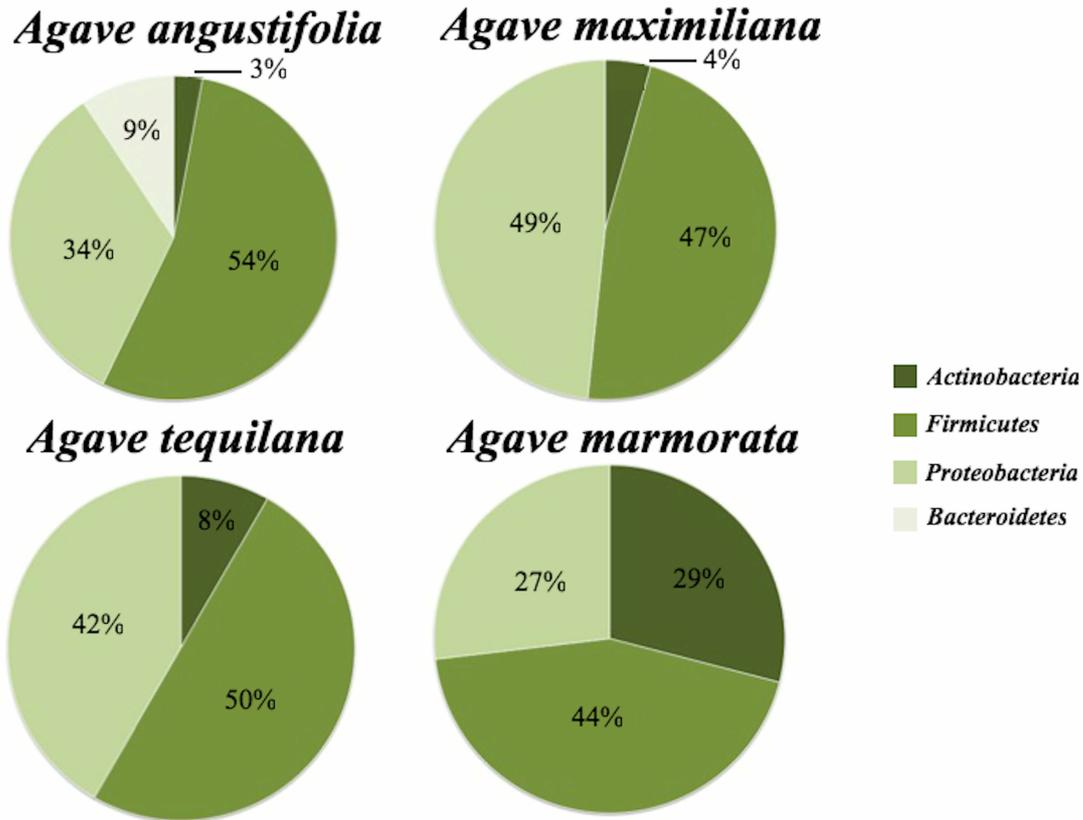


Figure 3. Relative abundance of cultured seed-endophytes of the *Agave* species.

The concept of core microbiome was firstly established for human microbiome, and further expanded to other host associated microbiomes such as plants (Shade and Handelsman, 2011). The composition and function of plant core microbiomes have been achieved for several model plants, such as *Arabidopsis*, maize, and rice (Johnston-Monje and Raizada, 2011, Lundberg et al., 2012, Edwards et al, 2015). The bacterial communities identified of the four *Agave* species were used to create the Venn diagram shown in the figure 4. We found enriched core taxa (genera) overlap among these agave seeds, which include bacterial genera *Bacillus* (Firmicutes), *Staphylococcus* (Firmicutes), *Micrococcus* (Actinobacteria), *Acinetobacter* (γ -proteobacteria), *Enterobacter* (γ -proteobacteria), *Pantoea* (γ -proteobacteria), *Pseudomonas* (γ -proteobacteria) and *Stenotrophomonas* (γ -proteobacteria). These findings suggested an interesting coevolution among those bacterial taxa and *Agave*. Besides the major shared microbial phylum of the agave seeds reported here, they also have been found in other plant seeds such as maize, bean, rice, *Brassica*, *salvia* and soybean. These bacterial taxa and their host seeds are summarized in table 2.

Table 2. Endophytic microbes found in seeds of different plant species.

Taxa	Plant species	Function
<i>Bacillus</i>	<i>Triticum aestivum</i> , <i>Oryza sativa</i> , <i>Lycopersicum esculentum</i> , <i>Tylosema esculentum</i> , <i>Zea mays</i> , <i>Curcubita pepo</i> , <i>Vitis vinifera</i> , <i>Glycine max</i> , <i>Coffea arabica</i> , <i>Brassica napus</i> , <i>Medicago sativa</i> ,	Plant growth promotion, antifungal, IAA production, metabolite production, osmotic stress tolerance,
<i>Staphylococcus</i>	<i>Z. mays</i> , <i>V. vinifera</i> , <i>Phaseolus vulgaris</i> , <i>M. sativa</i> ,	Plant growth promotion, antimicrobial,
<i>Micrococcus</i>	<i>O. sativa</i> , <i>C. arabica</i> , <i>M. sativa</i> ,	Plant growth promotion
<i>Acinetobacter</i>	<i>Z. mays</i> , <i>G. max</i> , <i>P. vulgaris</i> , <i>O. sativa</i>	Phytate solubilizing, Plant growth promotion, ACC deaminase
<i>Enterobacter</i>	<i>Z. mays</i> , <i>Nicotiana tabacum</i> , <i>Salvia miltiorrhiza</i> , <i>O. sativa</i> , <i>T. aestivum</i>	Plant growth promotion,
<i>Pantoea</i>	<i>Tylosema esculentum</i> , <i>Z. mays</i> , <i>O. sativa</i> , <i>C. arabica</i> , <i>T. aestivum</i> , <i>Hordeum vulgare</i>	Antibiotic production, IAA production, Antifungal, Plant growth promotion
<i>Pseudomonas</i>	<i>Phragmites australis</i> , <i>O. sativa</i> , <i>C. pepo</i> , <i>N. tabacum</i> , <i>B. napus</i> , <i>H. vulgare</i> , <i>M. sativa</i> ,	Plant growth promotion, antifungal, IAA production, metabolite production, mitigating metal toxicity, protease production
<i>Stenotrophomonas</i>	<i>O. sativa</i> , <i>N. tabacum</i> , <i>C. arabica</i> , <i>M. sativa</i> ,	Antifungal, Plant growth promotion, mitigating biotic and abiotic stress,

References: Rybakova et al., 2017, Verma et al, 2017, White et al, 2018, Yang et al., 2017, Rahman et al., 2018, Shazad et al., 2018, Chen et al, 2018.

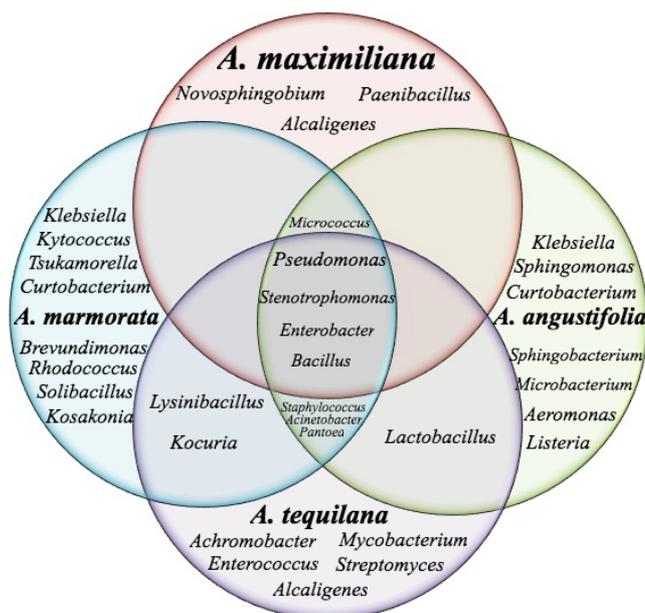


Figure 4. Venn diagram of the culturable seed-endophyte core microbiome based on shared composition of bacterial taxa of 3 *Agave* species. The major shared genera were *Pseudomonas*, *Enterobacter*, *Bacillus* and *Stenotrophomonas*. Different circles represent different *Agave* species, and the intersections of the circles showed the shared bacterial genera in their seeds.

As we show in table 2, the abundant taxa of seed-bacteria of agave are also conserved in other plant species. Functionality of *Bacillus* and *Pseudomonas* taxa range from plant growth promotion to disease protection. These genera in the seed microbiomes are likely to be important reservoirs of rhizosphere or endosphere microbiome. The ability of *Enterobacter* to increase seed germination and seedling growth has been reported (Shazad et al., 2018). In recent years, some studies found *Pantoea* spp. isolated from rice, maize, wheat and *Brassica* seeds, showed antagonistic activities against pathogens and plant-growth promoting traits (Ribakova et al., 2017). However, *Pantoea* is well-known as a plant pathogen that can be transmitted by seed (Barret et al., 2016). Therefore, the function of *Pantoea* as seed-associated microbe needs to be further evaluated.

In conclusion, contrary to other seed-microbiomes, the Firmicutes phylum dominates over other bacterial phyla, with predominance of *Bacillus* spp. In the next section, we will discuss this finding in relation to functionality of Firmicutes.

0.6 Dynamics of seed-endophytic bacteria during emergence until formation of the seedling: the case of wild *Agave marmorata*.

Seeds represent the initial microbial inoculum of the plant microbiome and could subsequently have a significant impact on plant health and productivity. Indeed, the composition of the seed microbiome can contribute to seed preservation, release of seed dormancy and increase or decrease in the germination rate. Moreover, seed transmission of phytopathogenic agents is a major means of dispersal, and is therefore significant in the emergence of any disease. Few studies have focused on community diversity and dynamic succession of endophytic bacteria during different seed developmental stages (Johnston-Monje and Raizada, 2011, Liu et al., 2013, Barret et al, 2015, Pitzschke, 2018).

Microbial community dynamics of the wild *A. marmorata* was assessed from seed to mature seedlings using MALDI-TOF/16S rDNA sequencing, and we found significant variability in the seeds and the subsequent stages of emergence at 10, 20, and 60 days, which include seedling development up to a one-year-old plant. In the “**intact seeds or stage 0**”, 95 strains were isolated and grouped into 20 genera and 35 species. The phylum Firmicutes (44%), dominated the number of total isolates of the seed, followed by Actinobacteria (30%) and phylum Proteobacteria (26%). The largest number of isolates belonged to the genera *Bacillus*, *Staphylococcus*, *Kocuria*, *Stenotrophomonas* and *Micrococcus*, which are commonly recognized as beneficial.

An interesting question to be answered is, why do *Agave* seeds host Firmicutes and Actinobacteria? To our knowledge, Firmicutes include bacteria that are generally more resistant to drying and UV radiation, which might provide an advantage in their capacity to survive on/in seeds. *Bacillus* is an aerobic endospore former; endospore formation may protect bacteria from changes within seeds (to tolerate storage, desiccation, seed maturation, seed germination). Also, *Bacillus* spp. use a wide range of substrates and have a large number of enzymes that

degrade complex polysaccharides such as extracellular cellulases. It has been hypothesized that plants have selected *Bacillus* as part of their core seed microbiome to assist with proteases, nitrogen uptake and assimilation following their migration to roots (Khalaf and Raizada, 2016). As we will show later, *Bacillus* species alter root architecture of agave seedlings and serve as “food” in nutrient limited soils.

Bacillus safensis, a seed-endophyte of maize upregulates genes involved in remodeling cell walls, the antioxidant responses and the inorganic N-uptake in the maize roots (Prieto et al., 2017). Recently, Irizarry and White (2018) showed how cotton seedling roots respond to inoculation with *B. amyloquefaciens*. They observed an overexpression of genes involved in auxin transport (WAT1), auxin homeostasis (IAA synthetase GH3.1) and lateral root formation by IAA14 gene expression. Thus, that up-regulation impacts root architecture. Also, they found the up-regulation of genes encoding enzymes involved for carbohydrate metabolism, nitrogen acquisition and genes to prevent fungal disease. In seeds, reactive oxygen species (ROS) production is beneficial for seed germination and seedling growth by regulating cellular growth and has important roles for endosperm degradation, mobilization of seed reserves, and protection against pathogens. Some *Bacillus* act as part of an antioxidant system indirectly by expression of genes encoding catalases, peroxidases and SOD, which work to prevent oxidative damage to the seedlings.

Micrococcus luteus (Actinobacteria) was identified as a seed-endophyte of agaves; this microbe has been considered to be an Rpf-producer (resuscitation promoting factors). The Rpf is a 16-19 KDa protein with muralytic activity, which can facilitate cell division and regrowth at very low picomolecular concentrations by remodeling the cell envelope of VBNC (viable but not culturable) cells. The release of Rpf by an actively growing individual into the environment wakes up neighboring cells, resuscitating them from a dormant state. Because Rpf is released outside of the cell, it has been proposed that it can potentially wake up neighboring cells from different lineages, increasing the competition for new available resources. The abundance of Firmicutes and Actinobacteria in agave seeds at stage 0, and the information published of their endophytic members, create a possible scenario of the function of these microbes in agave and maybe other plants from arid and semi-arid environments. Recently, we found strains of *M. luteus* producing Rpf in our collection derived from agave seeds. However, further studies are necessary to prove microbial functionality on seeds.

Independently on the initial composition of the seed’s microbiota, germination affects both microbial diversity and the number of isolates. Ten days after radicle appearance, seedlings undergo dramatic shifts in their microbial composition. Proteobacteria were the most abundant phylum at this stage (57%), indeed *Gammaproteobacteria*, becomes the most abundant bacterial class associated to seedlings, but alpha- and β -Proteobacteria also appear (Figure 5). Actinobacteria was the second most abundant (35%) and the Firmicutes decay up to 8%. As was previously reported, during germination the dominant taxa decrease with a marked increase in relative abundance of Proteobacteria (Barret et al., 2015, Torres-Cortes et al., 2018). During emergence the embryo takes nutrient released from endosperm, so the disposition of amino acids and simple carbohydrates may select for fast-growing microorganisms. However, the difference in growth rate is

not the only fact that can argue the abundance of Proteobacteria and the exclusion of Firmicutes. According to plant growth promotion activities of the abundant taxa such as *Stenotrophomonas*, *Pseudomonas*, *Achromobacter* and *Ochrobactrum*. We can suggest that microbes will be selected because of their influence in seedling growth and development, including nutrient acquisition, suppression of pathogenic microorganisms and promotion of resistance to biotic and abiotic stress (Singh and Jha, 2017, Zhang et al., 2018, White et al., 2018, Rahman et al., 2018). The predominance of phylum Proteobacteria is maintained until 60 days (60% vs Actinobacteria 20% and Firmicutes 20%), reaching its maximum level at 20 days (74%). New bacterial genera such as *Aeromonas*, *Sphingomonas*, *Alcaligenes* and *Burkholderia* appear in seedling tissues (Figure 5).

After 60 days, seedlings were transferred from phytigel to a microcosm with sand free of organic material for plant support. Seedlings were incubated for 10 months under conditions of 12h light-12h darkness at 32°C, watered with 1 ml of H₂O every 5 days. The composition of *A. marmorata* microbiome returned to have a greater abundance of the Phylum Firmicutes (60%) while Proteobacteria was 35% and Actinobacteria had the lower abundance (5%). The Log₁₀ was 5.204 CFU/gram of tissue. Strains of *B. pumilus*, *B. safensis*, *B. barcinonensis*, *L. fusiformis* and *Staphylococcus*. From phylum Proteobacteria we found species such as *A. xylosooxydans*, *A. insolitus*, *Burkholderia gladioli*, *S. maltophilia*, *A. faecalis*. As has been proposed by Barret et al. (2016), seeds disperse pathogenic microorganisms. Here we found phytopathogenic strains of *Pseudomonas caricapapayae*, *P. stutzeri* and *P. monteilii*. Some endophytes leave roots to colonize the surrounding soil, establishing a communication that benefits dispersion and colonization of largest areas.

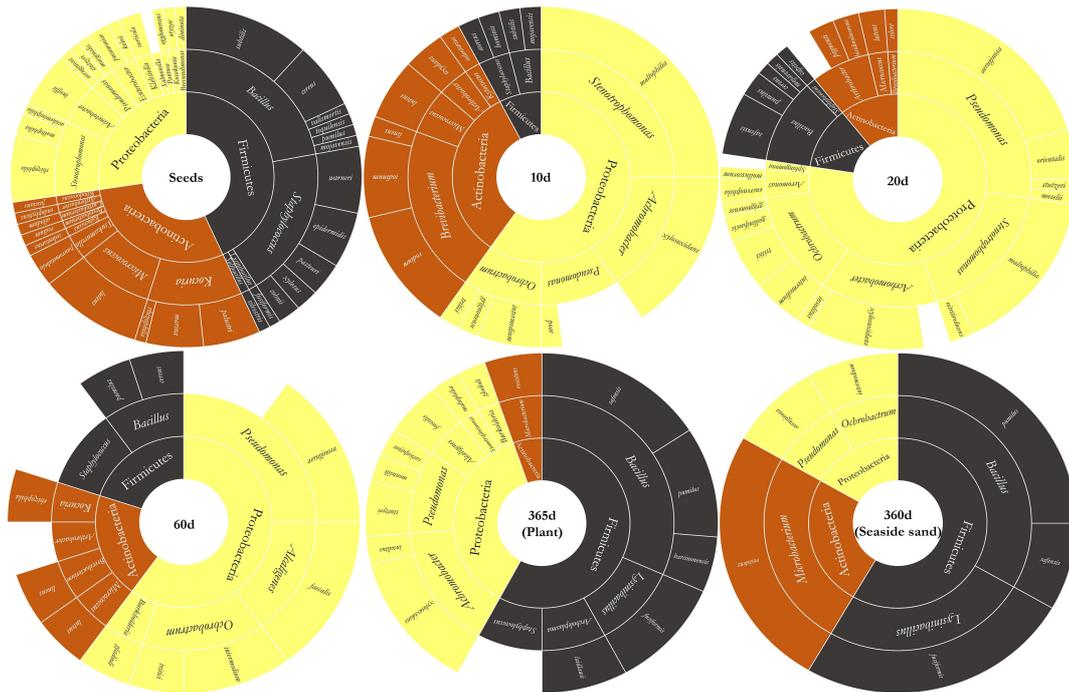


Figure 5. Ring charts showing composition endophytic bacteria and their dynamics from seed of *A. marmorata* to seedlings. Seeds contain a selected core of microbes whose functions are indispensable for seedling emergence. The ring shows phyla, genus and species and colors represent: Firmicutes (black), Actinobacteria (orange) and Proteobacteria (yellow).

0.7 Agave ‘eats’ microbial endophytes to survive in soils without nitrogen

Diazotrophic endophytes could provide nutrients to plants even though they lack nodules, under a process called associative nitrogen fixation (Carvalho et al., 2016). Some work has been done to explain how the bacterial nitrogen moves to the plant directly from microbes. Paungfoo-Lonhienne et al., (2010) showed how plants consume microbes internalized into root cells. They named this microbe consumption process ‘rhizophagy’ since in the process roots consumed microbes (Lonhienne et al., 2014). White et al. (2012) proposed oxidative nitrogen scavenging (ONS) as a mechanism for transfer of organic nitrogen from microbe to plant (White et al., 2014). ONS involves plant secretion of reactive oxygen species (e.g. H_2O_2) onto microbes and their secreted enzymes; microbes and their protein content is oxidatively degraded; later plants secrete proteases that further degrade denatured enzymes into peptides that may be absorbed by plants and associated bacteria. We also observed that in some plants intracellular bacterial endophytes in root epidermis cells exit plant roots at the tips of developing root hairs to acquire additional soil nutrients and then re-enter the root at the root tip meristem and are subjected to reactive oxygen as the root cells differentiate (Figure 6). Because of the cyclic nature of this process it has been denominated as ‘rhizophagy cycle’ and has been suggested to sustainably provide nutrients (micronutrients) from the symbiotic rhizobacteria (Prieto et al., 2017). We hypothesize that nutrients like iron and other difficult-to-acquire soil micronutrients may be obtained by plants from microbes that have siderophores

to sequester micronutrients and transport them back to plant roots where they may be oxidatively extracted and absorbed by root cells in the rhizophagy cycle. In plant tissues reactive oxygen secreted onto endophytic microbes may induce electrolyte leakage from bacteria that results in loss of electrolytes from bacterial cells. Electrolytes, including macro- and micro-nutrients, may then be absorbed by root cells.

Beltran-Garcia et al. (2014) conducted isotopic nitrogen tracking experiments to evaluate nitrogen transfer from bacterial cells into the plant. A seed-endophytic *B. tequilensis* was labeled with ^{15}N by cultivation in M9 broth containing ^{15}N -labeled nitrogen. ^{15}N -labeled bacteria were applied to plants of *Agave tequilana* over multiple months. ^{15}N -labeled nitrogen was measured in chlorophyll molecules using mass-spec analysis. Detection of ^{15}N in plant molecules demonstrated that nitrogen in the bacteria passed to the plant. In a second experiment comparing absorption of ^{15}N -labeled live bacteria to absorption of nitrogen in labeled but heat-killed bacteria, it was found that more nitrogen moved into the plants when live bacteria were used than when heat-killed bacteria were used. This suggested that movement of nitrogen from microbe to plant was more efficient from living endophytic microbes and thus may not simply be the result of mineralization of bacterial proteins in soils around plant roots.

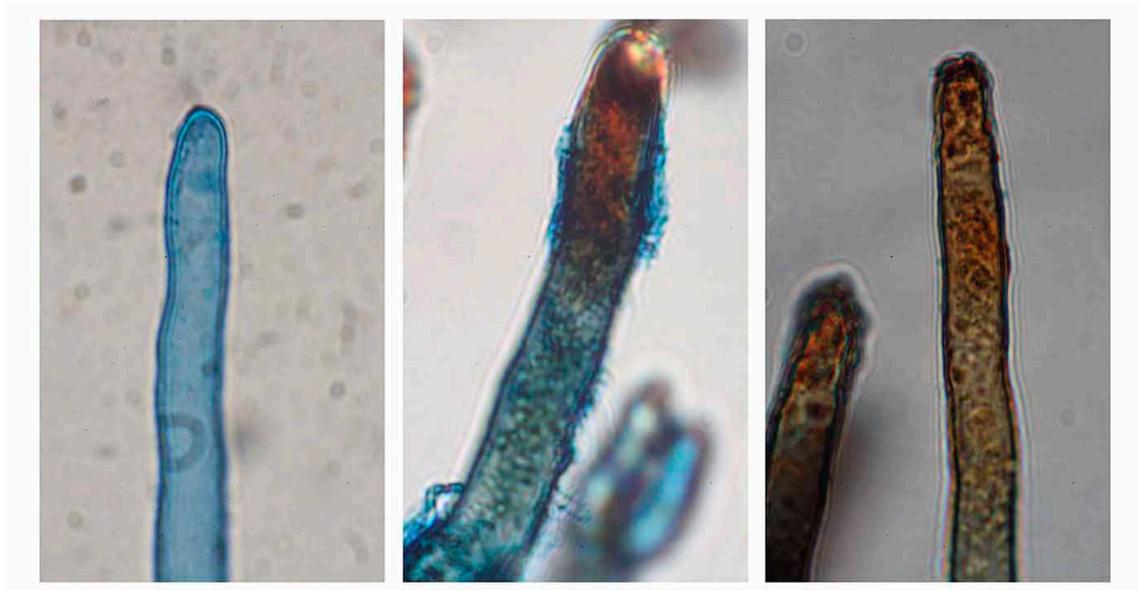


Figure 6. Plants without bacterial inoculation did not show intracellular bacteria in the root hairs (left-side). Two-days after inoculation with the *A. tequilana* seed-endophyte *Enterobacter cloacae*, presence of H_2O_2 (brown coloration) is observed in the tips of root hairs. Brown-spots within root hairs indicate sites of microbial degradation/exposure by reactive oxygen (right-side).

0.7.1 Seed-endophytic fungi may also transfer organic nitrogen.

Behie et al. (2012) showed the potential role of fungal endophytes for organic nitrogen transfer to plants. They used *Metarhizium robertsii* a soil-dwelling insect-pathogenic fungi and are able to form close symbiotic associations with plants as endophyte. To probe the movement of nitrogen, an insect larva was enriched with

$^{15}\text{NH}_4\text{Cl}$ and then infected with mycelia in a plant microcosm. They traced the ^{15}N incorporation in the amino acids of the two plant species. The fungus had the ability to infect larva and colonize roots at the same time, creating a bridge where the insect-derived nitrogen is translocated to plants. In our lab we isolated *Diaporthe* sp. as endophytic fungus from seeds of *A. tequilana*. *Diaporthe* (*Phomopsis*) species have often been reported as plant pathogens, non-pathogenic endophyte or saprobes, commonly isolated from a wide range of hosts. Agave plantlets were inoculated with *Diaporthe* and irrigated with water (MMN) medium as negative and positive source of nutrients respectively. Leaves of water-irrigated plants were chlorotic, developed a reddish-black pigmentation and then dried; plants irrigated with MMN, produced new leaves and appeared new roots, but at the end of the experiment biomass decreased. Fungal-treated plants developed more new leaves than other treatments and also increased root size, but finally did not influence the growth of the plants, senescence was delayed and no plants died. To evaluate ^{15}N transfer to plants, fungal mycelium was grown in modified Czapek-Dox broth enriched with $^{15}\text{NH}_4\text{Cl}$ and the inoculated into plant microcosm. The incorporation of the ^{15}N label into plants inoculated with ^{15}N Diaporthe is consistent with a scenario where N is transferred to plants when plants are nutrient limited. According to figure 7, our results show incorporation of ^{15}N into pheophytin, a molecule derived from plant chlorophylls. The percentage of relative abundance of isotopomers shows ^{15}N label into some of the four nitrogen atoms of tetrapyrrole (1N=873.57, 2N=874.57, 3N=875.57 and 4N=876.57).

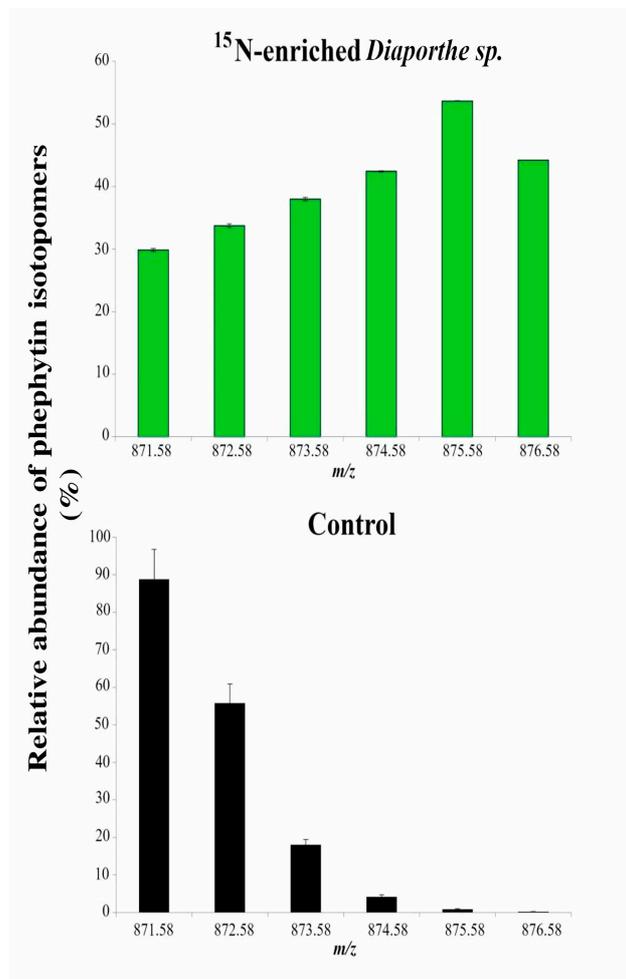


Figure 7. The Endophytic fungus *Diaporthe* sp. transfers Organic-N to Agave. Plants were watered monthly with ^{15}N -labeled *Diaporthe* sp. for a three-months period. We tracked incorporation of ^{15}N into pheophytin by mass-spec analysis, demonstrated that fungal cells passed nitrogen plants. We observed an increment of isotopomers at 874.58, 875.58 and 876.58 m/z, compared to nutrient solution as control. This indicates incorporation of ^{15}N into the four nitrogen atoms of phaeophytin. However, *B. tequilensis* was most effective, because of 871.58 m/z (theoretical value of pheophytin) was not totally reduced to negative values as we showed previously (Beltran-Garcia et al., 2014). Also, we could not recover *Diaporthe* cells from within agave roots, suggesting that the fungus was degraded to obtain nutrients to favor plant growth in absence of

nutrients.

0.7.2 Seed-endophytes can shape root architecture

Root architecture as often defined as the spatial configuration of the root system in growth media, and determines the 3-dimensional distribution of different root types in the root system across the soil profile. Variation in root system architecture plays a key role in crop nutrient efficiency (Prieto et al., 2017). The plant root system is obviously essential for plant growth and serves a wide range of functions, including nutrient and water acquisition, anchorage, and symbiosis with beneficial microbiota for enhancing the efficiency of nutrient absorption. Many endophytic and rhizospheric bacteria can indeed synthesize ethylene, gibberellins, cytokinins and auxins (Lopez-Bucio et al., 2007). Among these phytohormones, auxins, and in particular indole 3-acetic acid (IAA) coordinate modifications of root-system architecture, including functionality and morphology (e.g. root formation, apical dominance, and tropism). Root architecture influences on the ability of the plant to acquire nutrients and water, which in turn also impacts the development of organs that grow above ground. IAA production by microbes varies greatly between different species and strains (Sukumar et al., 2013). *Bacillus safensis*, a seed-transmitted bacteria isolated from maize, are able to alter root morphology and architecture in host seedlings, including accumulation of N-transporters, including organic N in the membranes of roots (Prieto et al., 2017). Here, seed-endophytes from agave were inoculated in corn seedlings to evaluate their functionality. We used maize plantlets, because agaves are slow-growing plants. We did not find significant difference in plant weight, leaves number, length and roots number compared with controls (sucrose 0.05% and 50% Murashige and Skoog solution). Slight significant differences were observed in parameters as roots number and plant height caused by *B. subtilis*, *B. altitudinis* and *B. pumilus* from *A. angustifolia* and *E. cloacae* of *A. tequilana* (figure 8). Not all bacteria are auxin-producers, but have in common the production of ACC deaminase and fixing nitrogen, therefore agave bacteria cannot be considered as biofertilizers for corn plants.

It is well demonstrated those endophytic bacteria play an important role for plant growth promotion by production of auxins and IAA-like molecules. Figure 9 shows the effect on root architecture of two selected endophytes of *A. tequilana* (*B. tequilensis* and *Enterobacter cloacae*) and *P. aeruginosa* from *A. marmorata*; the *Bacillus* and *Pseudomonas* are IAA-producers. It is well known that IAA at low concentration causes an elongation of roots, where as a high concentration reduces root length, increases root diameter, triggers lateral root emergence and increases root hair density. Each endophyte is associated with a different level of alteration of the primary root defined as size, root hair number, and lateral root formation (figure 8). More specifically, *E. cloacae* induced longer roots. Agave roots included in *B. tequilensis* and *E. cloacae* treatments, showed root length and lateral roots comparable to the non-inoculated plantlets respectively. Nevertheless, the emergence of lateral roots was greatest in *P. aeruginosa* followed by *B. tequilensis* (Figure 9). A strong increase in the length and number of root hairs was induced by *P. aeruginosa* in which plantlets displayed longer and more branched roots, similar results were founded by Zamioudis et al., (2013) using *Arabidopsis-Pseudomonas* spp. model; The highest number of root hairs was observed in plantlets treated with *B. tequilensis*, however these root hairs were shorter than those of the control. The *E. cloacae* treatment showed the most dramatic change, because seedlings developed shorter and smaller root hairs.. Finally, the colonization of root hairs apparently was highest in plants-treated with *E. cloacae* and was less in plants treated with *P. aeruginosa*; nevertheless it was difficult to distinguish clearly, mainly

due to root exudates. The appearance of root hairs shown in figure 9, is similar to the hairs presented in figure 6. At the end of our analysis on changes in root architecture induced by auxin-producers endophytes, we may discern promising candidates as potential growth promoters derived from agave seeds.

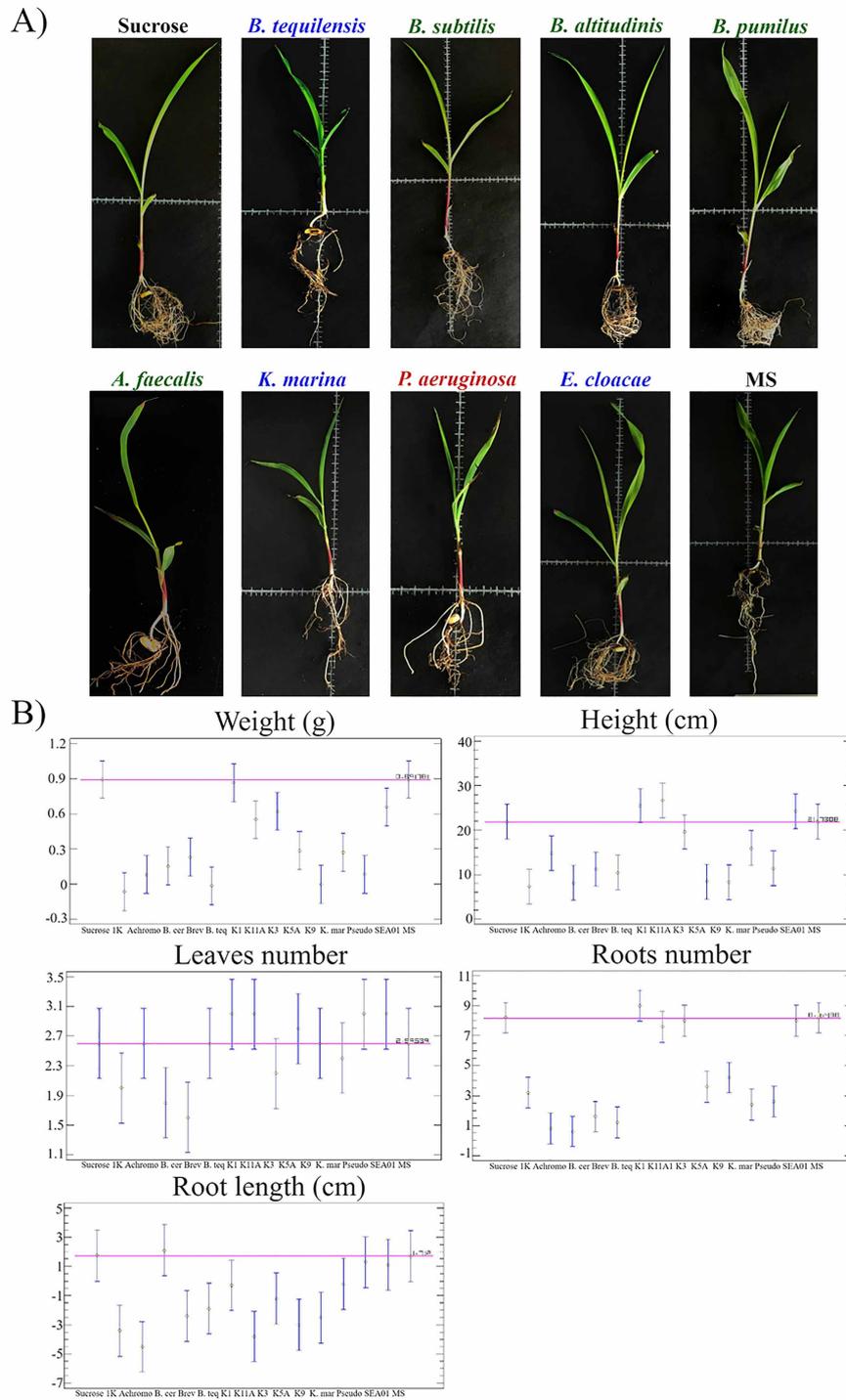


Figure 8. Agave bacteria in maize seedlings. A) Maize plantlets (after two weeks of growing) were inoculated with seed-endophytes from *A. tequilana* (letters in blue), *A. marmorata* (red) and *A. angustifolia* (green). B) Statistical analysis of one of each growth parameter.

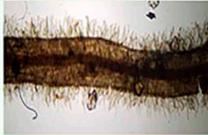
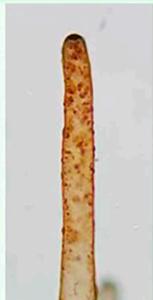
	Control	<i>Bacillus tequilensis</i>	<i>Enterobacter cloacae</i>	<i>Pseudomonas aeruginosa</i>
Primary root				
Numbers of root hair	469	791	283	712
Size of root hairs (µm)	500	250-500	250-500	700
Secondary roots	1	1	1	2
Colonization				

Figure 9. Seed-endophytic bacteria change root architecture. *A. tequilana* roots were stained with DAB-aniline blue after 30 days post-inoculation to evaluate colonization. The lower four microphotograph show root hairs with internal oxidizing bacteria in bacteria-treated plants.

0.8 Seed-endophytes confer fitness to cold stress on agave plantlets: A metabolomic approach.

Temperature is an important environmental factor that determines plant growth and development. Plants are continuously exposed to changes in diurnal or seasonal temperatures, and consequently must adjust their metabolism and physiology to improve or maintain their performance at the new growth temperature. When the temperature deviates from the range of optimal survival values, plants can experience a severe degree of physiological, cellular, metabolic, and molecular dysfunction that can lead to growth cessation and ultimately to death. Cold stress, which occurs annually with winter, is among the most intimidating forms of abiotic stress. Cold stress can be classified as either chilling stress (0-15°C or freezing stress (<0°C). For agave, little or nothing is known on the responses to cold stress. The knowledge that we have is totally empirical. Agave farmers mentioned that chilling temperatures provoke plant death if plants are less than 3-years-old. However, agave plants 4-years-old are slightly more resistant. Plants have developed mutualistic symbiosis with diverse fungal and bacterial endophytes that increase their fitness by conferring abiotic and biotic stress tolerance. Redman et al. (2001) mentioned that the effect of endophytic fungus could confer fitness to salinity as abiotic stress via habitat adapted symbiosis. The authors suggest that fungal symbiosis can mitigate the impact of climate change. This turns out to be very interesting, due the capacities of the agaves to grow in extreme environments. An increasing number of researches strengthen the proposal to use *Agave* species as an

option for climate change mitigation, including bioengineering of CAM metabolism as an alternative to transforming economically valuable C₃ crops (Abraham et al., 2016). To gain a better understanding of the functionality of agave seed-endophytic bacteria on the enhancement of chilling tolerance (4°C), we subjected plants-inoculated with *B. tequilensis*, *E. cloacae* and *P. aeruginosa* to chilling stress (for simplicity, that condition we call **+cold stress**), and were compared to non-inoculated plants (plant control). Also, we used plants-inoculated, but without chilling stress (**-cold stress**). Plant growth generally declines under chilling conditions, resulting in reduced capacity for energy utilization that leads to feedback inhibition of photosynthesis. Chilling-induced biomass reduction was attenuated in *B. tequilensis* and *P. aeruginosa* inoculated plants but not in *E. cloacae*. In figure 10, we show a selection of differentially expressed metabolites induced by chilling.. For this metabolomics analysis, we used Imaging Mass Spectrometry (IMS). One of the accumulated metabolites identified was IAA auxin in roots of **+cold stress**. It was reported that cold acclimation involves a decrease in endogenous IAA levels in Arabidopsis (Shibasaki et al., 2009). However, here an increased IAA accumulation was observed in root plantlets. The PI-(O-18:0-13:0) also known as 1-octadecyl-2-tridecanoyl-glycero-3-phospho-(1'-myo-inositol) is a bioactive glycerophosphoinositol (GPI), diffusible phosphoinositide (PI) metabolite of phospholipase A2. GPI biosynthesis involves the activity of myo-inositol-1-phosphate synthase (MIPS) that catalyzes the conversion of glucose 6-phosphate to MIP molecules (Abreu et al., 2007). This lipid molecule was detected only in roots with **+Cold stress**, especially in *B. tequilensis* and *P. aeruginosa*. There is also accumulating evidence that PI-derived signals are involved in plant stress response. Salt, cold, and osmotically signal stressed plants accumulate phosphatidyl inositols (PdtIns). Inositol phospholipids play a vital role in membrane trafficking and signaling pathways, auxin storage and transport, phytic acid biosynthesis, cell wall biosynthesis and production of stress-related molecules (Almaguer et al., 2004; Li et al., 2018). Thus, that lipid accumulation suggests that endophytic microbes can confer protection against chilling by activating signal transduction-dependent on PdtIns metabolism. Another component of cellular membrane was the sphingolipid C-8 ceramide; this metabolite was detected only in **+Cold stress**, with a relative abundance of 75% for *P. aeruginosa*, being the maximum value for this molecule. Besides playing structural roles in cellular membranes, sphingolipids function as bioactive signaling molecules in the regulation of abiotic stress responses, and their metabolism is associated with the reactive oxygen species scavenging system in plants (Dutilleul et al., 2015, Zhou et al., 2016, Hou et al., 2016). Ceramides are implicated in plant response to chilling and has been associated to ABA-dependent stomata closure in response to drought (Michaelson et al., 2016). [6]-Gingerdiol, 3, 5-diacetate belongs to the class of organic compounds known as fatty alcohol esters; this compound has antioxidant activity and is primarily located in the membranes in ginger rhizomes. Finally, the ubiquinone and NADH were reduced in all plantlets exposed to cold stress, suggesting a deviation of oxidative metabolism in mitochondria to maintain the cellular redox state to control ROS generation by means of energy-dissipating systems (Popov et al., 2001, Pastore et al., 2007).

0.8.2 Secondary metabolites were also detected under chilling stress and were potentially induced by endophytic bacteria. We found the flavonoids compounds orientin and diosmetin; these metabolites tend to accumulate in plants as part of the adaptation to low temperature. Flavonoid molecules also work as antioxidants, reducing ROS generated by low temperatures (Janmohammadi M. 2012, Sinha et al., 2015). These molecules are only accumulated in **+Cold stress** (Figure 10). Also, we observed

the accumulation of alkaloids solanidine and plakinamine B. The major abundance (75%) of the alkaloid plakinamine B was detected in plants +**Cold stress** with *B. tequilensis* endophyte. The presence of alkaloids in agave roots might be caused in response to ROS production under cold stress. Lanugone A is a cyclic terpene that was abundant in all +**Cold stress**, where *B. tequilensis* treatment was the major inducer of that molecule. Terpenoids are a broad class of lipophilic secondary metabolites synthesized in plants from isoprene units. These natural products exhibit a positive effect against biotic and abiotic stress conditions, and show antioxidant activity that may play an important role in the stabilization of the lipid membrane and improve environmental stress tolerance.

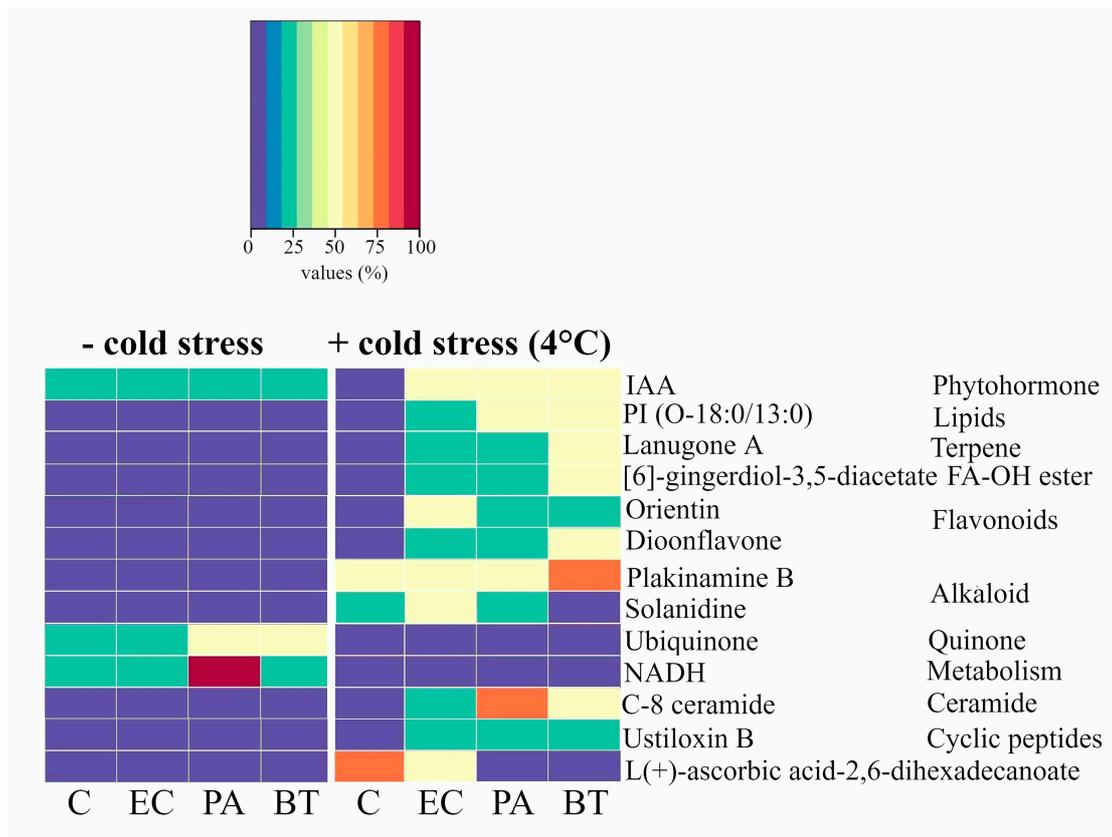


Figure 10. Elucidating seed-bacterial effects against chilling stress by Imaging Mass Spectrometry (IMS). Heat map of metabolites induced by chilling stress in plants with and without bacterial inoculations.

0.9 Conclusions

Agave is to the drier parts of the world what bamboo is to its wetter zones. Hundreds of species of agave that inhabit arid and semiarid environments, for thousand years have been sources of food, fiber, medicine, sugar, and prebiotics. Our Mexican iconic plant agave provides beverages that include tequila, mezcal and pulque. Currently, the agaves, especially the *A. tequilana* have been proposed as bioenergy crops due to their relative productivity in nutrient poor soils, with low consumption of water. In such manner, agave plants are considered to be the species of choice for mitigation of climate change.

We are convinced that the special physiological and metabolic features are not the only factor that determines the survival of agaves in arid environments. Microorganisms associated with agave plants are important not only to understand plant biology and fitness in dry lands, but also for future biotechnological development of agave to help create a sustainable agriculture for arid and non-fertile environments. In this chapter we focused on the microbiota transmitted by seeds, especially endophytes. Here we show predominant bacterial taxa and propose functions linked to survival and fitness strategies of agave. Our findings in part correlate with the low diversity data and content of endophytes presented by the research group lead by Dr. Laila Partida using plant tissues. We want to highlight, the decrease in diversity and content of seed-transmitted endophytes in the domesticated *A. tequilana* and the differences with wild agaves. This must open the eyes of the producers and the industry since in monoculture cultivation of agaves plants suffer diseases and depend of chemical fertilizers for growth. A motivation to study seed microbiomes is to better understand how these microbes work in plants, and understand the molecular mechanisms that influence agave resilience. We present preliminary data in terms of the metabolomic response to chilling stress in plants inoculated/colonized by those seed endophytic bacteria, where *B. tequilensis* was shown to protect plants and diminish the impact of cold stress. Problems caused by insects, diseases, low temperatures, and bud rot (Pudrición del cogollo) may destroy complete plantations of blue agave. It is clear that results shown here will generate more questions, but open extensive opportunities to investigate in topics with little information. Our studies suggest that production of the traditional and iconic Mexican beverage tequila may be improved through application of agave seed endophytes that have been lost through years of cultivation.

Acknowledgments

The Mexican group gratefully acknowledges the financial support from CONACYT: Proyectos de Desarrollo Científico para atender Problemas Nacionales (CONACYT 212875); Project 207400 of Bilateral Cooperation Mexico-Brazil funded by CONACYT and CNPq (Brazil, No. 490440/2013-4); Apoyo al fortalecimiento y desarrollo de la infraestructura científica y tecnológica convocatoria 2016-269607. A.M-R G.M-R and A.H-R thanks CONACYT for PhD fellowships # 720754, #256660 and #414739 respectively. Paolo Di Mascio thanks PFAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, No. 2012/12663-1, CEPID Redoxoma No. 2013/07937-8), CNPq [Conselho Nacional para o Desenvolvimento Científico e Tecnológico, No. 301307/2013-0, No. 159068/2014-2. James F. White is also grateful for support from USDA-NIFA Multistate Project W3157, and the New Jersey Agricultural Experiment Station.

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