Development of remediation technique utilizing plant-microbe synergy on persistent organic pollutants contaminated soil

University of Yamanashi, Graduate School Department of Education

Integrated Graduate School of Medicine, Engineering and Agricultural Sciences PhD Program

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Ahmad Mahmood

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CHAPTER 1 INTRODUCTION

1.1. Background

Continuous rise in world population asks for increase in food production which has been achieved using input-intensive agriculture. However, the food production is facing certain challenges including those of plant nutrition and soil pollution. Plant nutrition has issues regarding widely used sole-application of chemical fertilizers which are: increase in fertilizer prices, exhaustion of raw materials, environmental pollution, and decreasing soil fertility status (Mahmood and Kataoka, 2018). Another important and emerging issue is the effect of such chemicals on functionality of natural plant growth nutrition factories; the microbes (Berg and Koskella, 2018). Therefore, it is necessary to search for sustainable plant nutrition solutions, which can not only sustain crop production, but also maintain soil health and productivity.

Another emerging issue is that of soil degradation, which is threatening food security worldwide. This degradation is of several types among which magnitude of soil salinization and organic pollutants contamination is amongst the largest contributors. Soil salinity is intimidating crop production around the globe and is affecting approximately 20% of irrigated land (Qadir et al., 2014). Under the changing climate and rising sea levels, it is foreseen that coastal areas would face the problem of salinization (Dasgupta et al., 2015). Similarly, irrigation water has been among major causes of salinity in some parts of the world (Abbas et al., 2013). The salinization of soil impacts livelihood of farmers due to severe effects on crop yields besides damaging global food production. Similarly, soil pollution is another major cause of soil degradation encompassing different pollutants, where persistent organic pollutants (POPs) have been termed alarmingly dangerous due to their effect on human and animals health. POPs were categorized in 2001 under Stockholm Convention and until today 32 pollutants have been categorized aimed at a) elimination and b) restriction of their use and production, besides c) reducing their unintentional release (Secretariat of the Stockholm

Convention). These POPs not only pose threat of entry into food chain (Namiki et al., 2013), but also affect plant growth (Zhang et al., 2017). Therefore, soils facing issues of salinity and POPs-contamination need to be cleaned for better and safe crop production.

Sustainable agriculture and utilization of degraded soils hold the answer to above questions. Both the approaches can be augmented with the use of microorganisms which through several mechanisms help plants grow better, overcome stresses and/or thrive in stress environments. These microbes have been termed as plant growth promoting microorganisms. Other classes of microbes have also been found which degrade pollutants and help plants ameliorate the salinity stress. These microbes constitute epiphytes and endophytes where endophytes have gained priority due to certain merits over those of free-living microbes. Such microbes can thus be utilized in addressing the issues like those of crop nutrient requirement, soil salinity and organic pollutants.

1.2. Endophytic bacteria and their diversity

The bacteria which spend at least part of their life within the plant body without showing symptoms of disease are termed as endophytic bacteria. The endophytic bacteria are ubiquitous in nature and colonize all type of plants (Santoyo et al., 2016). The reports from of isolation of such bacteria from different plants bring forward the concept of interactions between plants, and microbes beyond just rhizosphere (reviewed by Hardoim et al., 2015). The endophytes bacteria help the plant grow better, like that of rhizosphere microbes, through several mechanisms encompassing production, and competition (Mahmood and Kataoka, 2019). The widely accepted direct mechanisms include release of certain hormones, fixing nitrogen, solubilizing phosphorus, and sequestering iron. Among the indirect mechanisms; physical, and chemical competition with the pathogenic microbes, induced systemic resistance, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and siderophore production can be reported (Santoyo et al., 2016).

The endophytic environment provides stability as compared to phyllosphere, and rhizosphere, thus ensuring stress-free functionality of the microbes. The soil microbes, on the other hand, must cope with the competition posed by indigenous microbial community (Mahmood et al., 2016), thus the endophytes have a plus. For instance, the diversity of soil environments; be it polluted with organics, or inorganics, or the hurdles of carbon to nitrogen ratios limit the functionality of rhizosphere microbes. Simultaneous problems of desiccation, and exposure to extreme climatic parameters limit their functioning and survival so the endophytes become advantageous.

The plants and endophytic bacteria evolved simultaneously and comprise certain kinds of relationships among themselves. These synergic and/or antagonistic associations constitute several functions, helping or damaging one or both organisms (Lundberg et al., 2012; van der Lelie et al., 2009). The plants being the employer in most cases look to recruit the desired functional microbes (Lemanceau et al., 2017), and consequently, they search for the best candidates in the available pools; rhizosphere (Schulz and Boyle, 2005) and phyllosphere in endophytic instances. The frequently changing rhizosphere as compared to endosphere proposes difficulties to the microbial community triggering certain evolutionary mechanisms. Besides, physical transport of microbes also leads to much diverse soil microbial community pool (Hooper et al., 2008). The temporal and spatial changes in plant endophytic community are therefore apparent. The extent of diversity among microbes can be enormous, as around 3000 new genera have been published in last 40 years (Parte, 2018), yet major part remains undiscovered.

Diversity derives the survival of the organisms. The microbial survival leads to upper trophic levels' survival thus the formers' density and diversity holds salient position. The changing climate scenarios having mixed effect on functionality and plant-associations of the microbes (Compant et al., 2010b) are joined by varying crop husbandry and soil conditions.

Thus, under such circumstances it is need of the time to look for microbes which can help plants sustain under diverse and adverse environments besides tolerating such instances. Similarly, the increasing role of biofertilizers in sustainable agriculture asks for suitable candidates for inoculation. This suitability varies with the objectives, however single strain contributing to several plant growth-promoting (PGP) abilities would be preferable. Similarly, the soil contamination getting complex with every day, especially in developing countries, poses survival and functionality hurdles for the microbes in rhizosphere. Eevers et al. (2016) reported the alteration in endophytic community due to organic pollutant incidence. So, the microbes tolerant to such sites besides the targeted function can be chosen. Finally, the microbes are source of novel metabolites (reviewed by Demain and Sanchez, 2009) and pathways. This characteristic of microbes can be used conventionally besides their potential for biotechnology.

The search for best suitable microbes asks for thorough study of microbial pools. If the sampling stage, for example, does not coincide with the targeted functional microbes, then they would be seldom found. Thus, exploring the diversity of microbes linked with the growth stages stands vital. Past studies chasing microbial diversity bring forward different aspects studied. Microbial community is mainly determined by the genotype and environmental factors (Andrew et al., 2012). The bacterial community in *Arabidopsis* was found to be influenced by soil type and the host (Bulgarelli et al., 2012). Another instance from *Arabidopsis* explored the effect of organs too besides the habitat, and found both factors structuring the bacterial community within roots and leaves (Bodenhausen et al., 2013). Among the edaphic factors, the shifts in bacterial community corresponded to changes in pH (Hardoim et al., 2012). Another study documents the prominent effect of microbe-microbe interaction determining the endophytic community besides the abiotic and genotypic factors (Agler et al., 2016).

Except for the habitat- and genotype-related community structuring, the rhizosphere bacterial community determines the endophytic community when it comes to temporal diversity follow up. In grapevine, different organ-associated microbes emerged mainly from that of rhizosphere (Zarraonaindia et al., 2015). Specifically, culturable endophytic bacterial diversity checked in sugarcane resulted in 32 strains belonging mainly to Enterobacteriaceae, Bacilli, Microbacteriaceae and Pseudomonadaceae (Magnani et al., 2010). Another study exploring the cultivable and non-cultivable endophytic bacteria concluded the mixed trend before transplanting in the field where diversity increased (Miguel et al., 2016). Correa-Galeote et al. (2018) checked the maize endophytic bacteria diversity and found profound effect of continuous cropping over fallow soil. Ulrich et al. (2008) explored the culturable endophytic bacteria diversity in field grown poplar and found the effect of plant genotype on bacterial community. The studies observing temporal diversity include a rhizosphere study in canola bringing forward the effect of environment (Farina et al., 2012), yet the endophytic diversity, particularly with respect to growing stages is seldom studied to best of our knowledge. Another diversity-check gap remains in plants as the only such study has been carried out in eucalyptus tree (Miguel et al., 2016).

1.3. Plant growth promoting bacteria

The environmental, and sustainability factors are major motives behind the preference of biological or organic fertilizers over the chemical ones. The biological fertilizers also termed as biofertilizers include certain microorganisms, plant growth promoting microorganisms (PGPM) including plant growth promoting bacteria (PGPB), fungi (PGPF) and cyanobacteria (PGPC). Potential of beneficial microorganisms in enhancing crop productivity and combating stress conditions has been widely discussed and reviewed (Ahemad and Kibret, 2014; Glick, 2012; Goswami et al., 2016; Hayat et al., 2010; Santoyo et al., 2016; Souza et al., 2015b). PGPB includes the bacteria showing positive effects on the plants, either free living in the

rhizosphere, or endophytic; living within the plants without showing symptoms of any damage, and enhance the plant growth and stress tolerance through different mechanisms viz. symbiotic and non-symbiotic nitrogen fixation, facilitation of nutrient uptake including phosphorus, potassium and iron, release of certain metabolites involved in plant growth promotion and stress tolerance, and remediation of organic and inorganic pollutants (Ahemad and Kibret, 2014; Meena et al., 2015; Santoyo et al., 2016). They have been used in wide plants and positive effects have been studied (reviewed by Berg et al., 2016; Saha et al., 2016; Yakhin et al., 2017). However, increase in stress environments like those of saline, polluted and drought affected etc. are increasing throughout the world and plant survival is at stake in most environments. These challenges are posing difficulties for conventional methods, thus PGPM come as attractive alternative. Similarly, application of such plant beneficial microbes is usually studied for their effect on plant growth rather the crop yield. Hence, there are gaps how the application can help plants grow better in field as applied inoculum has to survive and compete. Also, long-term application of plant growth promoting bacteria is hypothesized to reduce the chemical fertilizer requirement which needs to be investigated in real scale.

1.4. Bacteria-assisted remediation of problem soils

1.4.1. Soil salinity and rhizosphere bacteria

Soil salinity is among major abiotic stresses and severely affects plant growth and yield because most of the crop plants are sensitive to salt (Shrivastava and Kumar, 2015). Therefore, soils with even slightest concentrations of salts are unable to be cultivated or perform poorly when cultivated. Changing climate, mismanagement and other factors are making it worse. Utilization of such soils is thus needed which will not only enhance the productivity but also can improve the soil conditions.

Plant-microbe interaction as discussed above has developed certain shapes including antagonism, and synergism, where the plants, besides providing space, and food, also utilizes

such relationships for coping biotic, and abiotic stresses. Same is the case in the salt-loving plants or halophytes which in combination with epiphytic and endophytic microbes cope the stress more efficiently (Ruppel et al., 2013). The microbes in the discussion include fungi, bacteria, and archaea; which have different extent of salinity tolerance and ameliorating the stress. Instances of such microbes from extreme environments, and their adaptation to the particular locale (Imhoff, 2017; Siliakus et al., 2017), especially isolations from saline habitats indicate that the microbes have promising ability to counter such stress.

Microbes associated with saline habitats or halophytes can be categorized as halotolerant, and halophilic. The former can, occasionally, tolerate up to 25% sodium chloride (NaCl), and among the latter; microbes needing salts for their growth. Additionally, the non-halophiles require less than 1% NaCl, slight halophiles grow in 1-3% NaCl, moderate halophiles grow in 3-15% NaCl, and the extreme halophiles can grow in an environment containing 15-25% NaCl concentrations (Margesin and Schinner, 2001; Ventosa et al., 2008). The heterotrophic nature of the microbes contrasting to the autotrophs has enabled the formers with adaptation towards certain environments, as of saline, resulting in an abundance of microbes even in saturated environments. The tolerance and utilization mechanisms bring forward the prospects of such microbes in saline and hypersaline soils.

Wide range of habitats has resulted in halophilic and/or halotolerant microbes, for instance, salt lakes (Hedi et al., 2009), sea coasts (Kumar et al., 2012), arctic terrains (Yukimura et al., 2009), salt mines (Enache et al., 2014), plants pickled in salt solution (Abou-Elela et al., 2010), soil (Orhan and Gulluce, 2015), and endophytic environments (Zhao et al., 2013). Several examples of isolation from habitats mentioned above and application to other crops have shown salinity tolerance, along with enhancing the plant growth.

Halotolerance and halophilicity show different responses and adapt to such circumstances differently, offering the manipulation of such interactions for enhancing

required, and ever-sought better plant growth. The diversity of halotolerant bacteria has been reported from rhizosphere soil, and endophytic environment and genera like *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, and *Pseudomonas* have been reported as enhancing salt stress tolerance in diverse kind of plants (Paul and Lade, 2014). The isolations of endophytic bacteria from *Medicago truncatula*, followed by sequencing analysis revealed similarities of the isolates with reported halotolerant, and halophilic bacteria (Yaish et al., 2016). The study further documented the alteration of the bacterial community due to salinity, and PGP mechanisms. Another instance showed the effect of salinity on the metabolism of rhizobacteria (Szymańska et al., 2016a), suggesting the hinderance in physiology, thus leading the bacteria towards tolerance and utilization of salts. Such circumstances lead towards the biochemical, and genetic adaptation of bacterial communities towards adverse environments (van der Meer, 2003), such as salinity. The bacterial interaction with the halophytes therefore offers potential of ameliorating the salinity along with enhancing the growth of plants.

Common ice-plant (*Mesembryanthemum crystallinum* L) being a halophyte, can grow in up to 400 mM salinity level (Agarie et al., 2007; Hasanuzzaman et al., 2014; Herppich et al., 2012), and can be used as food, feed, and phytoremediation agent besides its exploration for associated bacteria. Besides plants' adaptation to saline environments, they also recruit bacteria adapted to such environments help the former grow better (Mahmood et al., 2019a). The bacteria thus help the plants thrive in extreme environments through plant growth promotion as discussed above. Other specific mechanisms can be activation of defense mechanisms like cell wall restructuring, and release of exopolysaccharides (Rampelotto, 2010). Additionally, the problem of nutrient fixation within the soil, phosphate as an example when applied in the saline soils is quickly immobilized, and bacteria capable of solubilization can help increase

availability leading towards better plant growth (Goldstein, 2009). Potential of such bacteria from saline habitats thus stands important for remediation of soils and thriving of plants.

1.4.2. Persistent organic pollutants and bacteria

Persistence of pesticides has led to widespread environmental pollution, which was not foreseen at the time of advent of such chemicals. Such chemicals were extensively used when first introduced, and were applied to not only the plants, but also human, and animals. The massive, and uncontrolled use has led to pesticide pollution in all the habitats, where soil persistence remains prominent. The pesticides in discussion include a wide variety, however, organochlorines; although banned decades ago yet have been reported widely from soil (Camenzuli et al., 2016; Wang et al., 2007a). The dichlorodiphenyltrichloroethane (DDT) has been used, and persisted so long, and so wide that it has even been reported in human milk (Smith, 1999), and devastatingly in developed countries too, putting the extent of pollution in developing countries so at risk. Similar is the case with pentachlorophenol (PCP) which was also developed simultaneously, banned later on but still being used and is toxic to living beings (Proudfoot, 2003).

Both DDT and PCP have been banned during 1970s and 1990s in developed and developing countries, respectively. And even decades later, the reports of DDTs and PCP from soil seldom stop. Considering the consequences of such pollution, the remediation procedures started immediately after the ban, but the initial techniques used included usually chemical methods, hydrolysis, redox, and photolysis, yet the lack of sustainability, limited efficiency, secondary pollutants issue, and high costs restricted such methods. Under these circumstances, biological methods gained importance for their ease, efficiency, and sustainability. The plants, among the biological methods, used as phytoremediation agents; prefer conjugation, and sequestration of the pollutants, but some microbes on the other hand, have evolved themselves,

and are able to transform such persistent chemicals to the simplest, and nontoxic end products i.e. carbon dioxide and water.

The transformation, and remediation capability of microbes depends on the microorganism type, type, and level of pollutant, and prevailing environmental conditions. The bioaugmentation, and bio-stimulation have been found increasing the remediation, however the diversity of the environment, and co-contamination often restricts the function of even potential strains. Therefore, the synergism between phytoremediation, and bioremediation gained importance, which in other words can be simplified as microbe assisted phytoremediation. The microbes can help the plant better adapt, sequester, or transform the pollutants which have been up-taken by the plants with the collective or individual objective of reducing the toxicity, eliminating the water potential dynamics for better moisture uptake, and/or helping the symbionts. There are chances of subsequent *in planta* degradation as well. Although rhizosphere microbes have shown potential for bioremediation of several pollutants (Matsumoto et al., 2009), the interest in endophytic microbes increased which have the advantage of stable environment on their side opposite to the free living, or generally referred to as rhizosphere microbes. The ideal culture conditions provided by the plants usually correspond to those of the laboratory, which include single pollutant, optimum temperature, and pH. This synergism can lead to better remediation even in co-contaminated sites, and higher level of the pollutants.

Endophytic bacteria have been isolated, and characterized for diverse kind of pollutants including phenol (Chen et al., 2017), trichloroethylene (Doty et al., 2017), chlorpyrifos (Feng et al., 2017), and diuron (Wang et al., 2017b) recently. On the other hand, the remediation of effluents, as an example of co-contaminations, the tannery effluent (Ashraf et al., 2017), have been reported. Similar cases from the heavy metal pollutants have also been observed, where fungal endophytes have enhanced tolerance (Gong et al., 2017; Li et al., 2016; Yamaji et al.,

2016), and phytoremediation, and bacterial endophytes have also showed increased tolerance (Román-Ponce et al., 2016), and enhanced phytoremediation (Mesa et al., 2017; Montalbán et al., 2017).

The resistance, tolerance, metabolism, and co-metabolism of DDT (Lin et al., 2014; Wang et al., 2010; Zhu et al., 2012) and PCP (Ito et al., 2018) by bacteria including those of rhizosphere bacteria have been reported. However, not much has been explored been carried out by endophytic bacteria in case of DDTs, while there is one report of PCP degradation by endophytic bacteria (Marihal et al., 2014). The gaps thus remain in our understanding about endophytic degradation of such pollutants.

This study was thus planned with the objectives of investigating:

- the endophytic bacterial diversity in the leaf-stalk of cucumber at two different locations with various management practices,
- cucumber plant growth promoting ability of endophytic bacteria and their application in real scale field experiment,
- the potential of endophytic and rhizosphere bacteria in amelioration of salinity stress,
- the capability of endophytic bacteria in biodegradation of persistent organic pollutants,
- enhancement of plant growth in persistent organic pollutants-contaminated soil,
- uptake of persistent organic pollutants by cucumber plant applied with plant growth promoting,
- and developing endophytic bacteria assisted phytoremediation for persistent organic pollutants.

CHAPTER 2 DIVERSITY OF ENDOPHYTIC BACTERIAL COMMUNITIES IN CUCUMBER (*CUCUMIS SATIVUS* L.)

2.1. Introduction

Cucumber (*Cucumis sativus* L.) is majorly consumed worldwide in the daily diet. Its global production is approximately 80.6 million tons (Food and Agriculture Organization of the United, 1998), where China leads with 67.4% (Burton, 2017). The supply–demand competition in several countries requires continuous increase in the cucumber yield. Furthermore, it absorbs persistent organic pollutants from the soil (Otani et al., 2007). Thus, cucumber is an essentially used in daily diet and also plays a vital role in environmental conditions. Due to the increasing demand of cucumber in Japan, there is a shortage of cultivable land area (35,800 ha in 1966 to 10,800 ha in 2017) and overall production (1,089,000 t in 1979 to 559,500 t in 2017) (Ministry of Agriculture, 2017). The cucumber yield per unit area can be increased by application of microbes.

The endophytic bacteria reside in the internal plant tissues without causing any pathogenesis, and thus help the plant growth through various functions such as release of certain chemicals, biomass production, bioavailability and activation mechanisms (Hardoim et al., 2015; Santoyo et al., 2016). The endophytes have been reported to suppress diseases (Malfanova et al., 2013; Sun et al., 2013) and enhance growth (Ozaktan et al., 2013) in cucumber plants. A meta-analysis revealed the abiotic stress mitigation in several crops by diverse endophytic bacteria and fungi (Rho et al., 2018). The microorganisms present in the plants compared to those in the rhizosphere have a closer interaction with their host due to their ability of colonizing the plant tissues. The endophytic microbes besides having superiority over rhizosphere microbes due to stability of endosphere also have plants' preference. Consequently, endophytic bacteria are comparatively less prone to the environmental adversities, xenobiotics and nutrient limitation, which are quite prevalent in soil. These characteristics make

endophytes preferable candidates. Nevertheless, information of endophytic bacterial diversity in cucumber, regarding the stages of plant growth, is insufficient.

In contrast, plants commonly face several stresses throughout their lifecycle and adapt to such conditions via various strategies including the association with microbes during their growth. The knowledge of endophytic bacterial diversity enables better crop management. Besides, the follow-up of non-culturable endophytic bacteria using high throughput sequencing has enabled to monitor the whole endophytic community rather than a small culturable fraction. This endophytic community mainly derives from rhizosphere as observed in grapevine (Zarraonaindia et al., 2015). In this context, it is essential to periodically evaluate diversity and endophytic bacterial count, nevertheless the multi-phasic endophytic bacterial diversity in the above-ground plant parts, in particular leaf-stalk has been rarely studied. This study was thus conducted to reveal the endophytic bacterial diversity in the leaf-stalk of cucumber. We compared the endophytic bacterial diversity at two different locations with various management practices.

2.2. Materials and methods

2.2.1. Plant material

Two locations were selected for the experiment: a commercial cucumber farmer's greenhouse (Site 1: N35.573616, E138.486816) and University of Yamanashi Research Farm (Site 2: N35.604073, E138.578506). For Site 1, the cucumber (*Cucumis sativus* L.) was purchased as pumpkin–cucumber (rootstock–scion) grafted nursery commercially. The first sampling was carried out immediately after transplantation (Nursery Stage). For Site 2, six un-grafted seedlings were purchased commercially, of which three were used for isolation and three were planted under field conditions. Both locations were sampled for leaf stalk (from three plants) after every month making a total of four samples Stage 1) Nursery Stage, Stage 2) Flowering Initiation Stage: 30 days after transplanting, Stage 3) Fruit Development Stage: 60 days after

transplanting, and Stage 4) Maturity Stage: 90 days after transplanting. The lowest healthy leaves were selected every time and only the leaf stalk was considered for isolation. The samples as a whole were transferred to the sampling bags, moved to laboratory, and were subjected to manipulation approximately within 1 h of the sampling.

Crop husbandries at both sites differed, as for the Site 1, drip irrigation system was used once in two days for almost 30 min. Conventional intensive agriculture was practiced: including pesticide application (fungicides having active ingredient Manzeb (20.0%), Flutianil (5.0%) and 2,4,5,6-tetrachloroisophthalonitrile (TPN) 40.0% among others were applied 12 times, and insecticide having active ingredients Emamectin benzoate (1.0%), Tolfenpyrad (15%), Flonicamid (10.0%), Buprofezin (25.0%) were applied 7 times) and fertilizer and growth enhancers' application (9 doses making a total of 189 kg ha^{-1} nitrogen, 38.5 kg ha^{-1} phosphorus, 185.5 kg ha^{-1} potassium, 208 kg ha^{-1} magnesium, 742 kg ha^{-1} calcium, 0.7 kg ha^{-1} boron, and foliar spray of a liquid formulation including nitrogen, phosphorus, potassium, magnesium, manganese, molybdenum, boron, iron, copper, zinc and silicic acid). Manure was also applied before transplanting the nursery. At Site 2, irrigation was applied once every day to maintain the field capacity and no other inputs were used. Both locations offered shoot support to the plants. In the Site 1, two cucumber crops per year have been being processed for more than 30 years; however, Site 2 is used for experimental purpose otherwise is fallow.

2.2.2. Sample preparation for endophytic bacteria

The stalk samples were washed and cut into 2–3 mm discs with aseptic scissors which were surface sterilised by thorough washing with 70% ethanol for 1 min and 1% NaOCl (Sodium Hypochlorite) for 10 min. Discs were then washed and rinsed with sterilised distilled water (SDW) for several times (at least 5 times). For the first samplings, the disinfected discs were pressed against the agar plates to check the success of surface sterilization, and no signs of microbial presence were observed. Half of the samples were subjected to isolation as explained

in the next section and the remaining half were stored at −80 °C for next generation sequencing (NGS) for non-culturable bacteria.

2.2.3. Culturable endophytic bacteria

2.2.3.1. Isolation

The surface disinfected discs were crushed with 5 mL of SDW using sterilised mortar and pestle under aseptic conditions. An aliquot of 50 µL was spread on Reasoner's 2A agar (R2A) (Oxoid Limited, Hampshire, UK) and was replicated 2–3 times using disposable spreader. The plates were incubated for 6–7 days at 25 °C and appearing colony forming units (CFUs) were enumerated. Even slightly distinct colonies were considered and streaked until a single colony per plate was obtained. These colonies were stored at −80 °C in 20% glycerol (in 0.8% NaCl w/v).

2.2.3.2. DNA Extraction and PCR amplification

The DNA of bacterial strains was extracted via ZR Fungal/Bacterial DNA MiniPrep Kit™ (Zymo Research Corp., CA, USA). The 16S rRNA gene was amplified by a T100™ Thermal Cycler (Bio-rad, CA, USA), comprising cycles as: 95 °C for 5 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; and a final extension at 72 °C for 7 min. A total of 25 µL polymerase chain reaction (PCR) mixture was used, with 1 µL of sample, and 10 mM primers, 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′), 9.5 µL of nuclease free water and 12.5 µL of GoTaq® Green Master Mix. Differential primer selections for culturable and non-culturable endophytic bacteria were selected based on suitable long base pairs for each analysis. The success of DNA extraction was confirmed by gel electrophoresis.

2.2.3.3. PCR-Restriction Fragment Length Polymorphism (RFLP)

The isolated strains were further screened for their genotype using two restriction enzymes *Hinf*I and *Alu*I (New England Biolabs Japan Inc., Tokyo). About 4.5 µL of amplified nucleotide

sample of each strain was mixed with one of the enzymes (0.5 µL), buffer (1 µL) and SDW (4 µL) in 1.6-mL Eppendorf Tubes. The tubes were incubated for 30 min at 37 °C. The incubated-samples were later loaded into 2% agarose gel (in TAE buffer), run for 23 min and compared amongst and with co-run 100 bp ladder, after staining.

2.2.3.4. 16S rRNA gene sequencing and submission of sequences

The 16S rRNA gene was amplified as explained above. The direct sequencing method was employed to obtain the sequences of the amplified nucleotides. The obtained sequences were cross-compared with those of the DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) and the nearest species was noted. Then the sequences corresponding to the identified isolates were submitted to DDBJ under the accession numbers LC440722–LC440763 (42 isolates) for Site 1, and LC441044-LC44111 (68 isolates) for Site 2 (Table 2.1).

2.2.4. Non-culturable endophytic bacteria

The stored surface sterilised leaf-stalk samples were subjected to DNA isolation using FastDNA™ Spin Kit for Soil (MP Biomedicals Europe, Japan). The concentration of DNA was checked using a nano-spectrophotometer and DNA was diluted to 1 ng μl^{-1} using sterile water accordingly. The V4 region of 16S rRNA gene was amplified using specific primers; 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The PCR product quantification and qualification were done by mixing same volume of 1× loading buffer (containing SYB green) with PCR products and operating electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400–450 bp were chosen for further experiments. PCR products were mixed in equal density ratios. Thereafter, the mixed PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries, 250 bp paired-end reads, generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina

and quantified via Qubit and Q-PCR, were sequenced on an Illumina HiSeq 2500 platform. In order to maintain the reliability of the data, quality control was performed at each step of the procedure. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). Quality filtering on the raw tags was performed under specific filtering conditions to obtain high-quality clean tags (Bokulich et al., 2013) according to the Qiime (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) quality controlled process (Caporaso et al., 2010). The tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar et al., 2011) to detect the chimera sequences (http://www.drive5.com/usearch/manual/chimera_formation.html). Next, the chimera sequences were removed (Haas et al., 2011), and the effective tags were finally obtained. Sequence analysis were performed via Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/) using all the effective tags (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was used against the SSU rRNA database of SILVA Database (http://www.arb-silva.de/) (Wang et al., 2007b) for species annotation at each taxonomic rank (Threshold:0.8~1) (Quast et al., 2013). To obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/) was used for rapid comparison of multiple sequences (Edgar, 2004). OTUs abundance information was normalised using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analyses were all performed based on this output normalised data.

The reads were submitted to DDBJ Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index-e.html) under Bioproject and are available under accession number DRA007980 (In sequence of Site 1: stage 1–4 and Site 2: stage 1–4).

2.2.5. Data Analysis

The data was aligned on the basis of different stages for both sites. The Shannon–Weiner and Simpson diversity indices were calculated (Keylock, 2005; Spellerberg and Fedor, 2003).

2.3. Results

2.3.1. Number of culturable isolates and non-culturable operational taxonomic units

The culturable isolates and non-culturable OTUs observed in the four stages of cucumber from two different sites have been compiled in Table 2.1. The culturable bacteria presented a continuous increase at Site 1. Four culturable strains were isolated in 1st sampling, which increased seven times in the 2nd sampling and other five-times in the 3rd sampling. The fourth sampling provided 166 isolates. Culturable endophytic bacteria also increased at Site 2 but starting from Flowering Initiation Stage onwards, as the Nursery Stage revealed numerous isolates than that of Flowering Initiation Stage and Fruit Development Stage. This trend observed at Site 2 was different from that of Site 1; moreover, the extent of increase also differed from that observed at Site 1. The Nursery Stage of Site 2 presented 76 isolates, which reduced by 2.7 times in Flowering Initiation Stage. From the second stage onwards, there was continuous increase in the number of isolates. The increase from Flowering Initiation Stage to Fruit Development Stage was observed 1.5 times and from Fruit Development Stage to Maturity Stage, it was 2.1 times. In contrast, the non-culturable endophytic bacteria evidenced a varying trend than that of the culturable ones, which also varied between the locations. For the Site 1, non-culturable endophytic bacteria increased until the first three stages, and decreased in Maturity Stage; however; this count in Maturity Stage was higher than that of Flowering Initiation Stage. For Site 2, the Nursery Stage presented maximum number of OTUs,

a decrease in the following stage which increased later on. The number of OTUs differed on big scale among both sites as Site 2 indicated on-average less number when compared with Site 1.

Table 2.1 Comparison of culturable isolates and non-culturable operational taxonomic units (OTUs) observed, Shannon-Weiner and Simpson Diversity Indices of both locations. Stage 1: Nursery Stage, Stage 2: Flowering Initiation Stage at 30 days after transplanting, Stage 3: Fruit Development Stage at 60 days after transplanting, and Stage 4: Maturity Stage at 90 days after transplanting

		Culturable			Non-culturable		
	Sampling	**No. of Isolates**	**Shannon**	**Simpson**	**OTUs**	**Shannon**	**Simpson**
Site 1	Stage 1	4	0.69	0.50	310	1.68	0.47
	Stage 2	28	1.71	0.81	431	2.10	0.53
	Stage 3	149	2.13	0.86	643	2.46	0.55
	Stage 4	166	2.02	0.86*	506	2.22	0.54
Site 2	Stage 1	76	0.12	0.05	125	1.44	0.43
	Stage 2	28	2.04	0.85	46	1.21	0.47
	Stage 3	42	2.26	0.87	49	1.33	0.52
	Stage 4	89	2.37	0.89	100	1.46	0.54

*Not rounded off value =0.856

2.3.2. The diversity across different stages

The diversity of endophytic bacteria was compared considering the Shannon–Weiner and Simpson diversity indices (Table 2.1). The Shannon–Weiner diversity index of culturable endophytic bacteria from Site 1 increased for the first three stages and then decreased. The Simpson index, presenting the same trend differed slightly (Fruit Development Stage = 0.860 > Maturity Stage = 0.856). For Site 2, both Shannon–Weiner and Simpson Diversity Indices revealed increase in the growth of cucumber plants. Although the number of isolates was significantly higher in Nursery Stage, it presented decreased diversity.

The non-culturable endophytic bacterial diversity changed in a similar pattern to that of culturable endophytic bacteria; however, the extent of change was comparatively lower. Site 1 revealed increase in both the diversity indices until Fruit Development Stage and then they decreased. This extent of increase was more evident in Shannon–Weiner index, where Simpson

index also presented similar but smaller changes. In contrast, Site 2 revealed higher Shannon–Weiner index in the Nursery Stage, which further decreased in Flowering Initiation Stage but increased later. The Simpson index showed a constant increase since Nursery Stage, opposite to that of Shannon–Weiner index. The extent of increase of both diversity indices was almost similar.

The culturable endophytic bacteria were classified based on RFLP analysis, where a total of 18 types in Site 1 and 30 types in Site 2 were observed (Table 2.2). The DNA sequences of each RFLP type resulted in a total of 11 genera (not included unidentified = 4) from Site 1, which belonged to three phyla: Firmicutes, Proteobacteria and Actinobacteria. Similarly, 21 genera (not included unidentified = 4) appeared from Site 2 and belonged to five phyla: Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Deinococcus-Thermus (Figure 2.1). Excluding the unidentified genus and comparing among the sampling stages, only one genus appeared in Nursery Stage at Site 1. The Flowering Initiation Stage, Fruit Development Stage and Maturity Stage presented 5, 7 and 8 genera, respectively. For Site 2 as well, only 1 genus appeared in Nursery Stage, and thereafter, an 8-fold increase in the genera was observed in Flowering Initiation Stage. Subsequently, 10 and 11 genera were observed in Fruit Development and Maturity Stages, respectively.

The non-culturable endophytic bacterial analysis revealed overall 320 genera from Site 1 and 63 genera from Site 2 (excluding unidentified genera). There were instances of several genera shared by different stages which is explained in later parts. Furthermore, at Site 1, 141 (44.1% among the total genera observed) different genera were observed in Nursery Stage, which further increased to 172 (53.8% among the total genera observed) different genera in the Flowering Initiation Stage, 280 (87.5% among the total genera observed) genera in Fruit Development Stage and later decreased to 230 (71.9% among the total genera observed) genera in Maturity Stage (90 days after transplanting). In contrast, at Site 2, 48 (76.2% among the total

genera observed) genera appeared in Nursery Stage (maximum in all the stages sampled), which further decreased to 13 genera (20.6% among the total genera observed) in Flowering Initiation Stage, further increasing to 17 (27% among the total genera observed) genera in Fruit Development Stage, followed by 42 (66% among the total genera observed) genera (2.5 times than that of Fruit Development Stage) in Maturity Stage. Thus, taxonomically at the phylum level, 36 phyla appeared at Site 1, whereas only 7 phyla were observed at Site 2 (excluding unidentified phyla). Proteobacteria being the most abundant at both sites was followed by Firmicutes and Bacteroidetes, respectively (Figure 2.2a & 2.2b).

Table 2.2 Frequency of culturable endophytic bacterial isolates; their nearest genus and accession numbers from Site 1 and 2 based on RFLP. The isolates sharing same RFLP number across different stages and sites do not represent the same species. S1 (Stage 1): Nursery Stage, Stage 2: Flowering Initiation Stage at 30 days after transplanting, Stage 3: Fruit Development Stage at 60 days after transplanting, and Stage 4: Maturity Stage at 90 days after transplanting.

	Site 1					Site 2			
	RFLP-types	**Genus**	**Accession No.**	**Frequency %**	**RFLP-types**	**Genus**	**Accession No.**	**Frequency %**
Stage 1	RFLP-1	*Bacillus* sp. 1	LC440722	25	S1 RFLP-1	*Curtobacterium* sp. 1	LC441044	97.4
	RFLP-2	*Bacillus* sp. 2	LC440723	25	RFLP-2	*Curtobacterium* sp. 2	LC441045	2.6
	RFLP-3	Unidentified 1		25	Stage 2 RFLP-1	*Sphingomonas* sp. 1	LC441046	3.6
	RFLP-4	Unidentified 2		25	RFLP-2	*Xanthomonas* sp. 1	LC441047	3.6
Stage 2	RFLP-1	*Pseudomonas* sp. 1	LC440724	7.1	RFLP-3	*Arthrobacter* sp. 1	LC441048	7.1
	RFLP-2	*Agrobacterium* sp. 1	LC440725	3.6	RFLP-4	*Sphingomonas* sp. 2	LC441049	3.6
	RFLP-3	*Nocardioides* sp. 1	LC440726	10.7	RFLP-5	*Xanthomonas* sp. 2	LC441050	10.7
	RFLP-4	*Microbacterium* sp. 1	LC440727	7.1	RFLP-6	*Xanthomonas* sp. 3	LC441051	3.6
	RFLP-5	*Agrobacterium* sp. 2	LC440728	3.6	RFLP-7	*Rhizobium* sp. 1	LC441052	10.7
	RFLP-6	*Methylobacterium* sp. 1	LC440729	7.1	RFLP-8	*Arthrobacter* sp. 2	LC441053	3.6
	RFLP-7	*Methylobacterium* sp. 2	LC440730	3.6	RFLP-9	*Bacillus* sp. 1	LC441054	3.6
	RFLP-8	*Microbacterium* sp. 2	LC440731	7.1	RFLP-10	*Microbacterium* sp. 1	LC441055	3.6
	RFLP-9	*Agrobacterium* sp. 3	LC440732	7.1	RFLP-11	*Sphingomonas* sp. 3	LC441056	3.6
	RFLP-10	*Microbacterium* sp. 3	LC440733	7.1	RFLP-12	*Bacillus* sp. 2	LC441057	3.6
	RFLP-11	*Agrobacterium* sp. 4	LC440734	10.7	RFLP-13	*Arthrobacter* sp. 3	LC441058	7.1
	RFLP-12	*Pseudomonas* sp. 2	LC440735	7.1	RFLP-14	*Aeromicrobium* sp. 1	LC441059	7.1
	RFLP-13	Unidentified 1		17.9	RFLP-15	*Aeromicrobium* sp. 2	LC441060	3.6
Stage 3	RFLP-1	*Pseudomonas* sp. 1	LC440736	12.8	RFLP-16	*Paenibacillus* sp. 1	LC441061	3.6
	RFLP-2	*Sphingomonas* sp.1	LC440737	2	RFLP-17	Unidentified 1		17.9
	RFLP-3	*Sphingomonas* sp. 2	LC440738	1.3	Stage RFLP-1	*Sphingomonas* sp. 1	LC441062	7.1
	RFLP-4	*Aureimonas* sp. 1	LC440739	4.7	RFLP-2	*Frigoribacterium* sp. 1	LC441063	7.1
	RFLP-5	*Agrobacterium* sp. 1	LC440740	10.7	RFLP-3	*Paenibacillus* sp. 1	LC441064	2.4

	RFLP-6	*Pseudomonas* sp. 2	LC440741	7.4
	RFLP-7	*Microbacterium* sp. 1	LC440742	6
	RFLP-8	*Methylobacterium* sp. 1	LC440743	6
	RFLP-9	*Microbacterium* sp. 2	LC440744	6
	RFLP-10	*Curtobacterium* sp. 1	LC440745	4
	RFLP-11	*Microbacterium* sp. 3	LC440746	6
	RFLP-12	*Methylobacterium* sp. 2	LC440747	6
	RFLP-13	*Sphingomonas* sp. 3	LC440748	7.4
	RFLP-14	*Agrobacterium* sp. 2	LC440749	8.7
	RFLP-15	Unidentified 1		3.4
	RFLP-16	Unidentified 2		2
	RFLP-17	Unidentified 3		3.4
	RFLP-18	Unidentified 4		2
Stage 4	RFLP-1	*Pseudoclavibacter* sp. 1	LC440750	10.8
	RFLP-2	*Brachybacterium* sp. 1	LC440751	7.2
	RFLP-3	*Curtobacterium* sp. 1	LC440752	15.7
	RFLP-4	*Pseudomonas* sp. 1	LC440753	5.4
	RFLP-5	*Agrobacterium* sp. 1	LC440754	3.6
	RFLP-6	*Sphingomonas* sp. 1	LC440755	1.8
	RFLP-7	*Methylobacterium* sp. 1	LC440756	2.4
	RFLP-8	*Microbacterium* sp. 1	LC440757	7.2
	RFLP-9	*Agrobacterium* sp. 2	LC440758	3
	RFLP-10	*Agrobacterium* sp. 3	LC440759	17.5
	RFLP-11	*Methylobacterium* sp. 2	LC440760	6.6
	RFLP-12	*Methylobacterium* sp. 3	LC440761	8.4
	RFLP-4	*Sphingomonas* sp. 2	LC441065	9.5
	RFLP-5	*Agrobacterium* sp. 1	LC441066	2.4
	RFLP-6	*Paenibacillus* sp. 2	LC441067	2.4
	RFLP-7	*Phycicoccus* sp. 1	LC441068	2.4
	RFLP-8	*Rhizobium* sp. 1	LC441069	2.4
	RFLP-9	*Agrobacterium* sp. 2	LC441070	2.4
	RFLP-10	*Microbacterium* sp. 1	LC441071	2.4
	RFLP-11	*Massilia* sp. 1	LC441072	2.4
	RFLP-12	*Lysinimonas* sp. 1	LC441073	4.8
	RFLP-13	*Microbacterium* sp. 2	LC441074	2.4
	RFLP-14	*Frigoribacterium* sp. 2	LC441075	2.4
	RFLP-15	*Frigoribacterium* sp. 3	LC441076	2.4
	RFLP-16	*Pseudomonas* sp. 1	LC441077	2.4
	RFLP-17	*Sphingomonas* sp. 3	LC441078	2.4
	RFLP-18	*Pseudomonas* sp. 2	LC441079	2.4
	RFLP-19	*Lysinimonas* sp. 2	LC441080	2.4
	RFLP-20	*Methylobacterium* sp. 1	LC441081	2.4
	RFLP-21	*Massilia* sp. 2	LC441082	2.4
	RFLP-22	*Rhizobium* sp. 2	LC441083	2.4
	RFLP-23	*Massilia* sp. 3	LC441084	2.4
	RFLP-24	*Frigoribacterium* sp. 4	LC441085	2.4
	RFLP-25	Unidentified 1		23.8
Stage 4	RFLP-1	*Frigoribacterium* sp. 1	LC441086	3.4
	RFLP-2	*Hymenobacter* sp. 1	LC441087	1.1
	RFLP-3	*Sphingomonas* sp. 1	LC441088	3.4

RFLP-13	*Microbacterium* sp. 2	LC440762	3	RFLP-4	*Sphingomonas* sp. 2	LC441089	1.1
RFLP-14	*Methylobacterium* sp. 4	LC440763	5.4	RFLP-5	*Microbacterium* sp. 1	LC441090	3.4
RFLP-15	Unidentified 1		1.8	RFLP-6	*Microbacterium* sp. 2	LC441091	1.1
				RFLP-7	*Microbacterium* sp. 3	LC441092	1.1
				RFLP-8	*Microbacterium* sp. 4	LC441093	1.1
				RFLP-9	*Variovorax* sp. 1	LC441094	2.2
				RFLP-10	*Microbacterium* sp. 5	LC441095	1.1
				RFLP-11	*Flavobacterium* sp. 1	LC441096	1.1
				RFLP-12	*Aureimonas* sp. 1	LC441097	1.1
				RFLP-13	*Methylobacterium* sp. 1	LC441098	7.9
				RFLP-14	*Sphingomonas* sp. 3	LC441099	2.2
				RFLP-15	*Frigoribacterium* sp. 2	LC441100	7.9
				RFLP-16	*Frigoribacterium* sp. 3	LC441101	1.1
				RFLP-17	*Sphingomonas* sp. 4	LC441102	3.4
				RFLP-18	*Deinococcus* sp. 1	LC441103	3.4
				RFLP-19	*Rhizobium* sp. 1	LC441104	4.5
				RFLP-20	*Sphingomonas* sp. 5	LC441105	1.1
				RFLP-21	*Sphingomonas* sp. 6	LC441106	3.4
				RFLP-22	*Sphingomonas* sp. 7	LC441107	2.2
				RFLP-23	*Sphingomonas* sp. 8	LC441108	5.6
				RFLP-24	*Frigoribacterium* sp. 4	LC441109	7.9
				RFLP-25	*Lysinimonas* sp. 1	LC441110	7.9
				RFLP-26	*Deinococcus* sp. 2	LC441111	1.1
				RFLP-27	Unidentified 1		12.4
				RFLP-28	Unidentified 2		1.1
				RFLP-29	Unidentified 3		3.4
				RFLP-30	Unidentified 4		2.2

2.3.3. Dynamic change in endophytic bacteria

The identification of the culturable isolates revealed different genera among various stages of the cucumber plant. *Bacillus* from Site 1 and *Curtobacterium* from Site 2, observed in Nursery Stage, were not observed in the later stages (Figure 2.1). Four genera, namely *Agrobacterium*, *Methylobacterium*, *Pseudomonas* and *Microbacterium*, were observed commonly in the Flowering Initiation, Fruit Development and Maturity Stages at Site 1. Frequencies of these genera from all the culturable bacteria at that stage revealed a different trend, wherein only *Methylobacterium* indicated continuous increase (Figure 2.1). For Site 2, three genera, namely *Microbacterium*, *Sphingomonas* and *Rhizobium* persisted temporarily for Flowering Initiation, Fruit Development and Maturity Stages. Percentage of *Microbacterium* indicated a decline in Fruit Development Stage compared to that of the pervious growth stage (Flowering Initiation Stage), but again increased in later Maturity Stage. *Sphingomonas*, however, constantly increased; whereas, *Rhizobium* increased in Fruit Development Stage and later decreased in Maturity Stage. *Microbacterium* spp. was interestingly prevalent in all the three stages; Flowering Initiation, Fruit Development and Maturity Stages, at both sites; however, the trend was different (Figure 2.1).

Except for the genera prevalent over the last three stages, few were observed only in two stages. *Curtobacterium* and *Sphingomonas* were observed in Fruit Development and Maturity Stages at Site 1, whereas four genera, namely *Methylobacterium*, *Frigoribacterium*, *Lysinimonas* at Fruit Development and Maturity Stages, and *Paenibacillus* (Flowering Initiation and Fruit Development Stages) were observed incident in two temporal stages at Site 2. *Curtobacterium* from Site 1 indicated decrease in the Maturity Stage from that of Fruit Development Stage, whereas *Sphingomonas* was increased. For Site 2, all the above-mentioned

genera presented an increase in the subsequent stages except for *Lysinimonas*, which remained constant.

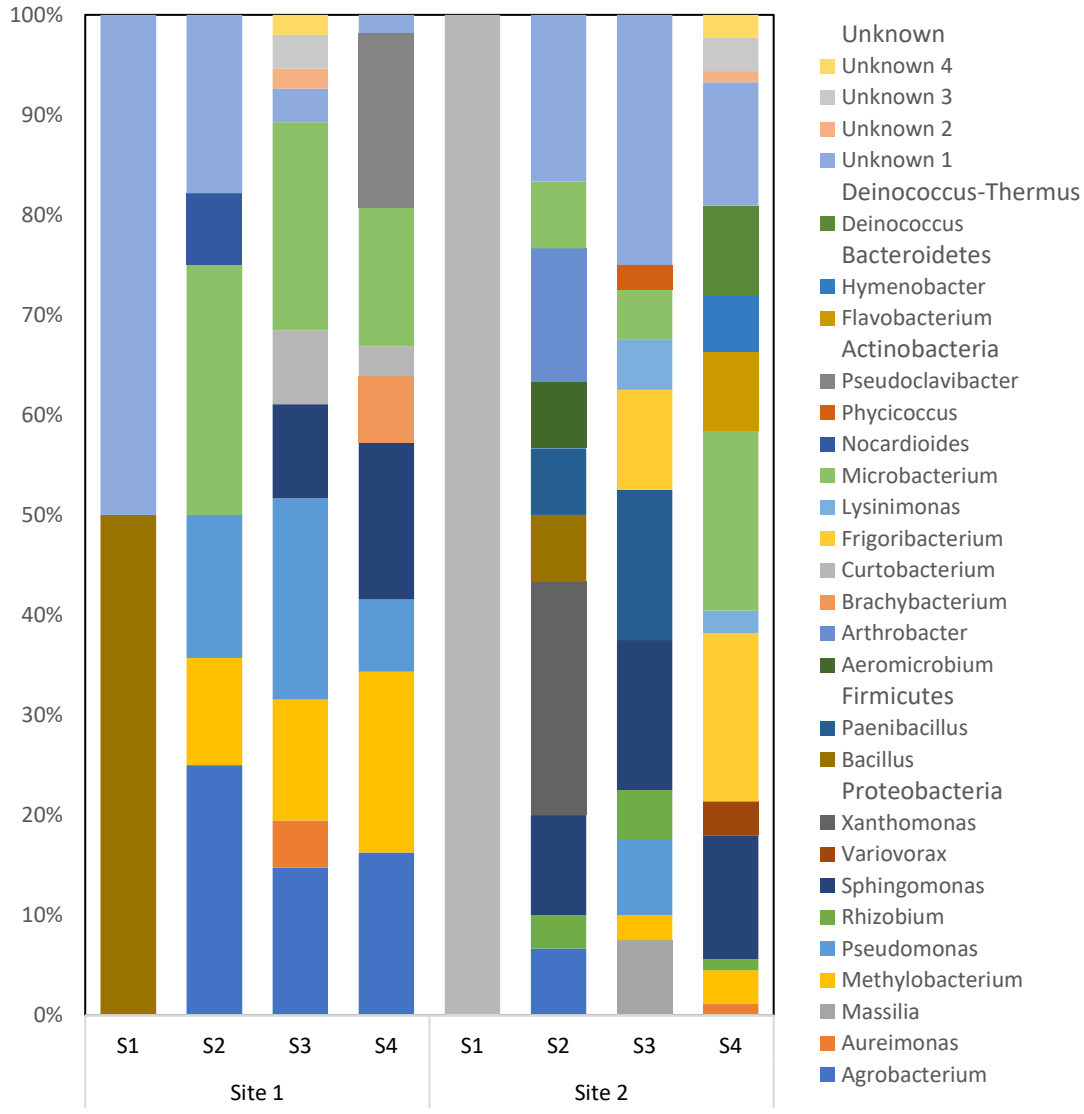


Figure 2.1 Distribution of different culturable bacterial genera from Site 1 and Site 2. The unknown genera across stages and sites don't necessarily represent same genus. S1: Nursery Stage, S2: Flowering Initiation Stage at 30 days after transplanting, S3: Fruit Development Stage at 60 days after transplanting, and S4: Maturity Stage at 90 days after transplanting.

For the non-culturable endophytic bacteria at Site 1, 93 (29.1% among the total genera observed) of the genera indicated existence among all of the stages sampled. Similarly, 72 (22.5% among the total genera observed) genera were appeared among three stages; whereas, 79 (24.7% among the total genera observed) of the distinct genera were appeared among two different stages. Remaining 76 (23.8% among the total genera observed) genera were observed only in one stage (excluding unidentified ones, classified as others). For Site 2, only 6 (9.5% among the total genera observed) genera were appeared in all the 4 stages of sampling, and 12 (19% among the total genera observed) distinct genera were appeared in 3 different stages and 14 (22.2% among the total genera observed) were by two distinct stages. Remaining 31 (49.2% among the total genera observed) genera were observed solely in any one stage of sampling.

The multiphasic heatmap of different phyla from Site 1 and Site 2 presented the dynamics of non-culturable endophytic bacteria in cucumber (Figure 2.2a, 2.2b). Certain phyla, in particular, Proteobacteria, Bacteroidetes and Actinobacteria were abundant at Site 1 and increased gradually. Few other phyla also indicated an increasing trend; however, some of these were intriguingly abundant only in Fruit Development Stage of sampling corresponding to similar results in the culturable endophytic bacteria (Table 2.1, Figure 2.1). Firmicutes were more abundant in Fruit Development Stage. The density of certain phyla decreased with the age of plant. Site 2 presented more phyla in culturable endophytic bacteria when compared with Site 1; however, it only revealed seven non-culturable phyla excluding the unidentified ones, classified as others (Figure 2.2b). The number of non-culturable endophytic bacterial phyla at Site 2 unlike Site 1 (Table 2.1) revealed different trend, and maximum density was observed in Nursery Stage except for Proteobacteria; which indicated increasing trend until Fruit Development Stage and then decreased further.

Firmicutes and Bacteroidetes decreased once after Nursery Stage and further tended to increase in the later plant growth phases.

(a)

Phyla	S1	S2	S3	S4
Proteobacteria				
Firmicutes				
Bacteroidetes				
Chloroflexi				
Actinobacteria				
Synergistetes				
Tenericutes				
Spirochaetes				
Planctomycetes				
Armatimonadetes				
Acidobacteria				
Euryarchaeota				
Aminicenantes				
Thermotogae				
Fusobacteria				
Nitrospirae				
Verrucomicrobia				
Chlorobi				
Gemmatimonadetes				
Deinococcus-Thermus				
Ignavibacteriae				
Atribacteria				
WS1				
Lentisphaerae				
Thermomicrobia				
Deferribacteres				
Hydrogenedentes				
Cloacimonetes				
Latescibacteria				
BRC1				
Saccharibacteria				
SR1_(Absconditabacteria)				
RBG-1_(Zixibacteria)				
TM6_(Dependentiae)				
TA06				
Caldiserica				
Others				

(b)

Phyla	S1	S2	S3	S4
Proteobacteria				
Firmicutes				
Bacteroidetes				
Actinobacteria				
Planctomycetes				
Spirochaetes				
Verrucomicrobia				
Others				

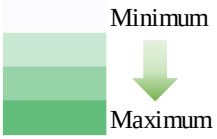
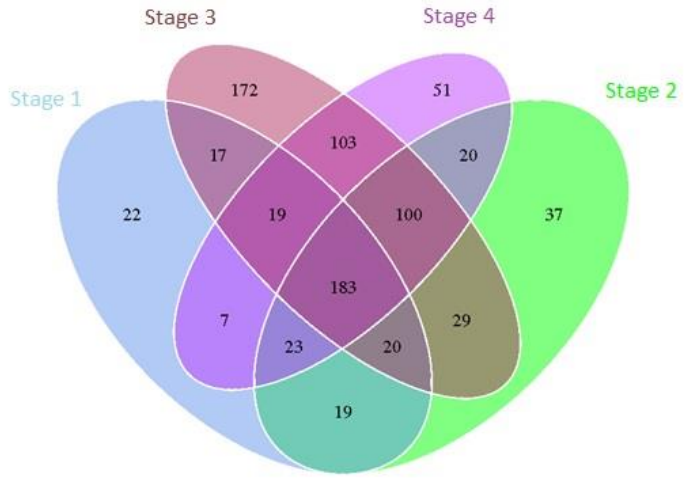


Figure 2.2 Heatmap illustrating the trend of operational taxonomic units (OTUs) of non-culturable endophytic bacteria from each phylum from Site 1 (a) and Site 2 (b) across different stages. Color from light to dark represents the increase in incidence of OTUs in each phylum. S1: Nursery Stage, S2: Flowering Initiation Stage at 30 days after transplanting, S3: Fruit Development Stage at 60 days after transplanting, and S4: Maturity Stage at 90 days after transplanting.

The Venn diagrams from Site 1 (Figure 2.3a) and Site 2 (Figure 2.3b) depict the division of non-culturable OTUs in each stage and their overlapping across different stages. Both the sites revealed differential response and differed in number of OTUs (compiled in Table 2.1). Results revealed that 183 (22.3%) OTUs from Site 1 and 30 (19.3%) from Site 2 were common among all the stages sampled. For Site 1, the number of OTUs belonging specifically to each stage and not found in other stages presented an increasing trend with the age of plant until Fruit Development Stage. From Fruit Development Stage onwards, the number decreased by almost three times. The OTUs' incidence indicated that Nursery Stage had 22 (2.7%) of the total strains, which were not observed in any other stage. Fruit Development Stage stood with the highest sole possession of 172 (20.9%); whereas, Flowering Initiation Stage and Maturity Stage exhibited 37 (4.5%) and 51 (6.2%) of the total OTUs, respectively, as not observed in any other stage. Moreover, Nursery Stage shared 19 (2.3%) OTUs with Flowering Initiation Stage, 17 (2.1%) with Fruit Development Stage and only 7 (0.9%) OTUs with Maturity Stage. The stages 2 and 3 shared maximum number of OTUs with their subsequent stages, respectively. For Site 2, the stage-specific non-culturable endophytic bacteria presented a variant response where Nursery Stage comprised 44 (28.4%) of the total OTUs. Furthermore, Flowering Initiation Stage presented 3 (1.9%) and Fruit Development Stage showed only 1 (0.6%) of the strains solely attributed to the corresponding stage. The last stage revealed 21 (13.5%) OTUs only observed in this particular stage. The distribution at Site 2 as depicted in Venn diagram (Figure 2.2b), revealed preference for Nursery Stage except for the OTUs observed commonly in all stages, and sole attribution of Maturity Stage. Interestingly, there was no sharing of non-culturable OTUs among the first three stages. Similarly, stages 2 and 3 shared none of the strains between themselves.

(a)



(b)

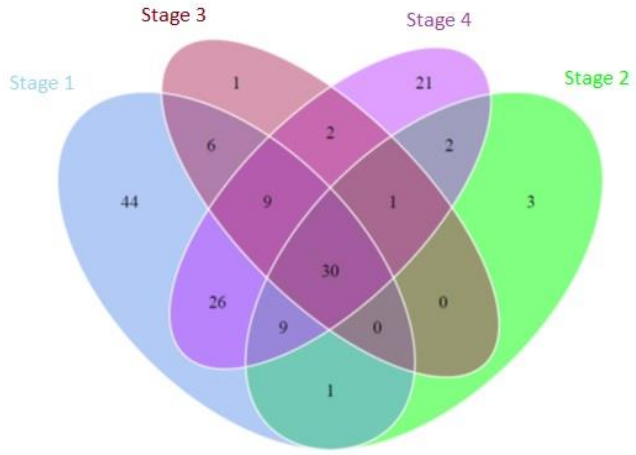


Figure 2.3 Venn-diagram of non-culturable operational taxonomic units (OTUs) from Site 1 (a) and Site 2 (b). Stage 1: Nursery Stage, Stage 2: Flowering Initiation Stage at 30 days after transplanting, Stage 3: Fruit Development Stage at 60 days after transplanting, and Stage 4: Maturity Stage at 90 days after transplanting

2.3.4. Comparison of endophytic bacteria between both geographical locations

Of all the 25 culturable genera isolated from both sites, only 8 (32%) genera were observed common at both locations (Figure 2.1). Different extents of similarity were found between the locations as *Microbacterium* spp. existed in the six different sampling stages across both locations (Flowering Initiation, Fruit Development and Maturity Stages at both Sites). *Sphingomonas* spp. (Fruit Development and Maturity Stages at Site 1 and Flowering Initiation, Fruit Development and Maturity Stages at Site 2) and *Methylobacterium* spp. (Flowering Initiation, Fruit Development and Maturity Stages at Site 1 and Fruit Development and Maturity Stages at Site 2) followed as they were found at five different sampling stages across the locations. Similarly, *Agrobacterium* spp. and *Pseudomonas* spp. were present at four different sampling stages (Flowering Initiation, Fruit Development and Maturity Stages at Site 1 and only Flowering Initiation Stage at Site 2), *Curtobacterium* spp. at three different sampling stages (Fruit Development and Maturity Stages at Site 1 and only Nursery Stage at Site 2), and *Aureimonas* spp. (Fruit Development Stage at Site 1 and Maturity Stage at Site 2) and *Bacillus* spp. (Nursery Stage at Site 1 and Flowering Initiation Stage at Site 2) at two different stages across both sites.

The non-culturable endophytic bacterial analysis presented 47 (14% of the total distinct genera observed which were 336) genera, which were common at both locations with varying levels. There were 3 (7.3% of the shared 47 genera) genera which were observed common in all the stages at both locations. Similarly, 7 (14.9%) genera were present at seven stages, 8 (17%) were present in six stages and 9 (19.1%) in five stages across the locations. The number declined further presenting 8 (17%) genera in four stages, 6 (12.8%) in three stages, and also 6 (12.8%) genera in two different stages at both sites. Site 1 showed exclusive possession of 289 (86% of the total distinct genera=336) genera, whereas Site 2 only showed that of 16 (4.8% of the total distinct genera=336) genera.

2.3.5. Differences between culturable and non-culturable endophytic bacteria

Certain differences among the culturable and non-culturable endophytic bacteria from cucumber were observed. Site 1 revealed more differences among the OTUs observed in the culturable and non-culturable bacteria as compared to Site 2. Comparing the culturable and non-culturable endophytic bacteria genera from both sites (Table 2.1), Site 1 showed major differences compared to Site 2. Site 1 presented a total of 11 culturable genera (+ 4 unidentified) while 320 non-culturable endophytic bacterial genera, indicating a difference of 29.1-fold. For Site 2, however, the difference accounted for only 3-fold. The density also differed as observed in CFU and OTU count (Table 2.1). Although several endophytic bacteria were present at Site 1, only a fraction was found to be culturable. Site 2, corresponding to genera-based discussion, presented better cultivability results.

2.4. Discussion

This study revealed the dynamic change of culturable and non-culturable endophytic bacteria in the leaf-stalk of cucumber. As listed in Table 2.1, the continuous increase in the culturable and non-culturable endophytic bacteria presented the increasing interaction of host with its internal microbiome. Bourceret et al. (2018) observed the rhizosphere microbial community changes due to the presence of plants which reflects the influence on endophytic community later. Chi et al. (2005) observed the recruitment of root endophytic bacteria from soil, which eventually move to the aboveground parts of plants. Ju and Zhang (2015) also reported that the shifts of microbial community have been established based on the environment. The environment inside the plant might be stable compared to the outside environment; however, endophytic bacteria might undergo certain changes with increasing environmental stresses on the plant. Circumstantially, the plants recruit microbes from the soil reservoir as observed in grapevine (Zarraonaindia et al., 2015).

Actinobacteria, Firmicutes, Proteobacteria and other phyla as observed in culturable and non-culturable endophytes in this study (Table 2.2, Figs. 21, 2.2a, and 2.2b) are well known for their diverse functions and promote plant growth (Gontia-Mishra et al., 2017) besides their abundance in the endosphere (reviewed by Liu et al., 2017). The presence of these phyla in cultivation dependent techniques has previously been observed in cucumber (Mahaffee and Kloepper, 1997) and common bean (Costa et al., 2012).

In this study, the endophytic bacterial count from Site 1 was comparatively higher than that of Site 2. This can be related to the continuous fertilizer application and irrigation practices being followed at Site 1. The irrigation and fertilizer applied, activate the microbes in soil, which with the help of certain plant exudates are able to colonise the plants. Both the culturable and non-culturable approaches indicated major differences in the endophytic bacterial diversity among various sampling stages and across the two sites explored (Table 2.1). At Site 1, the diversity of culturable endophytic bacteria revealed lower values in the Nursery and Maturity Stages compared to Site 2, which indicated a continuous increase. The cucumber plants at Site 1 were grafted using pumpkin rootstock, a common practice among farmers of the area; consequently, higher endophytic bacterial richness and diversity was observed after the Flowering Initiation Stage. Thus, endophytic bacteria detected in the Nursery Stage may have been derived from the rootstock and they disappeared in Flowering Initiation Stage due to their adaptation ability. A previous study reported a strong effect of breed type on the endophytic microbial community in variant rootstock–scion grafted apple (Liu et al., 2018). Similarly, the effect of rootstock on the endophytic bacterial community has been observed (Poudel et al., 2018); however, the of microbial functions such as plant growth promoting ability was not linked to the rootstock source (Marasco et al., 2018).

Persistence of a few genera both in the culturable and non-culturable groups was observed. The plants look for possible microbial endophytic candidates in horizontal and vertical transmission. The vertical transfer (reviewed by Frank et al., 2017) presents the possibility of mutualism between plant and microbes which leads to persistence of certain bacteria over different stages and generations of the plant. The *Bacillus* spp., *Methylobacterium* spp., *Microbacterium* spp., *Pseudomonas* spp., *Rhizobium* spp. and *Sphingomonas* spp. have widely been reported for their role in PGP; thus, the plants need them. Interestingly, culturable *Agrobacterium* spp. also prevailed over the stages in Site 1, which has been reported from hemp plants and was found to produce siderophores (Scott et al., 2018). The tenacity of endophytic bacteria across stages can thus be linked with the possible functional support by the microbes, which leads to their continuous presence within the plant tissues, also in following generations. Moreover, the instances of certain genera disappearing in the following stages (Figure 2.3a and 2.3b) indicate trimming when necessary. Similar results were observed in wheat where abundance of certain lactic acid bacteria revealed changes during the plant growth and later processing (Minervini et al., 2015). The microbial diversity in plants can help them tackle various problems. The plants thus recruit the microbes from soil or the vertically transmitted ones as discussed above.

In addition, commercial farming system also can be linked to endophytic bacterial diversity with frequently used fertilizers and pesticides (explained in Materials and Methods section). Campisano et al. (2014) reported that the endophytic bacteria were affected by the pest management practices. Additionally, *Mesorhizobium* abundance where no chemical fertilizers were used, was also observed (Campisano et al., 2014). Similar phenomenon was also observed in the present study. The cucumber plants at Site 1 were continuously treated with chemicals and revealed none of Rhizobiales, however *Rhizobium* spp. was observed in 3 different stages at Site

2. Similarly, although the number of culturable isolates was higher at Site 1, the diversity was limited when compared with Site 2 (Tables 2.1 and 2.2). This suggests that plants alter the endophytic bacteria according to their needs affecting both the number and diversity of microbes.

In relation to the cultivability of endophytic bacteria; we observed only a fraction in the case of Site 1 and a higher percentage in Site 2 of culturable endophytic bacteria as compared to those of non-culturable ones. In this sense, it was reported by Stewart (2012) that minute fractions can be cultivated axenically; however certain non-culturable microbes keep on contributing chemically towards plant growth, yet research in these aspects remains in initial stages.

2.5. Summary

The endophytic bacteria residing in the leaf-stalk of cucumber were observed tending to increase with age of the host. However, bacterial number was discovered decreasing at site when the plant moved from fruit development stage to maturity phase. Furthermore, culturable bacterial diversity and number was considerably lesser compared to those of non-culturable. It was observed that Fruit Development Stage (2 months after transplanting) at Site 1 and Maturity Stage (3 months after transplanting) at Site 2 were microbially the most diverse stages of cucumber plants.

CHAPTER 3 PLANT GROWTH-PROMOTING ABILITY OF ENDOPHYTIC BACTERIA

3.1. Introduction

Epiphytes, which are free-living, and endophytic plant growth-promoting (PGP) microorganisms have recently gained importance in crop production. Endophytic microbes, which live within plant tissues without causing disease symptoms, are preferred over rhizosphere microbes, in part because of the stability of the endosphere. PGP endophytic bacteria have been used because of their role in plant growth enhancement and stress tolerance (Santoyo et al., 2016). For example, endophytic isolates from different sources improved *Pisum sativum* growth through mechanisms like gluconic acid production and phosphate solubilization (Otieno et al., 2015). Furthermore, endophytic bacteria from date palm enhanced root growth of canola under normal and saline conditions (Yaish et al., 2015). Another report of diazotrophic *Paenibacillus* spp. showed that its inoculation improved plant morphological parameters (Puri et al., 2016). Thus, endophytic bacteria show PGP abilities, as demonstrated in the aforementioned examples, and they can be used for better crop growth.

Certain direct and indirect functions of endophytic bacteria in enhancing plant growth and/or improving soil quality have been reported. The direct mechanisms of PGP endophytic bacteria include nitrogen fixation, phosphorus and potassium solubilization, and hormone and siderophore production; indirect mechanisms include lowering of ethylene, biocontrol of certain pathogens, iron chelation, and inducing systemic resistance to the plants (Bulgarelli et al., 2013; Christie and Nowak, 2000; Compant et al., 2010a; Hallmann et al., 1997; Hardoim et al., 2008; Ryan et al., 2008). Endophytic microbe utilization in remediation of organic contaminant- and metal-polluted soils has also demonstrated multiple functions of such microbes (Rosenblueth and Martínez-

Romero, 2006). Similarly, endophytic bacteria are also a source of novel metabolites and pathways (Gao et al., 2018; Jiao et al., 2016; Singh et al., 2017). Endophytic microbes' contribution to plant health at different levels can be studied using advanced technologies, such as whole plant metabolomics. The endophytic microbiome regulates the quantity of certain metabolites within the endosphere (Del Giudice et al., 2008; Lòpez-Fernàndez et al., 2016) and also biotransforms certain plant-produced metabolites (Fu et al., 2017) and stimulates the production of other chemicals (Gao et al., 2015; Li et al., 2012).

Endophytic bacteria similar to that of rhizosphere bacteria have also shown promising results in enhancing plant growth (Borah et al., 2019; Egamberdieva et al., 2017). However, usual studies focus on growth of the plants rather the crop yields which are sought in real scale. The survival of plants and their growth in field depends on long-term supply of nutrients. Furthermore, axenic conditions are quite conflicting to the circumstances prevalent in the field. This contrast can sometimes lead to difficulties of potential isolates adapting and contributing to plant growth. Similar instances have been found where Gange and Gadhave (2018) postulated the compatibility issues of applied inoculum with that of indigenous microbial population. Likewise, host specificity of plant growth promoting bacteria has been reported (Long et al., 2008). Therefore, the application to functioning of applied inoculum is a complex process and can work if all the circumstances are favorable (Martínez-Viveros et al., 2010). This asks for thorough experimentation, especially under practical conditions which can bring forward the effect of such inoculations. Similarly, the potential of isolates must be checked towards achieving yield if not enhancing as compared to that of chemical fertilizers.

Cucumber (*Cucumis sativus* L.) is a widely consumed vegetable worldwide. Continuous rise in its demand requires more yield (Burton, 2017; Food and Agriculture Organization of the United, 1998), for which fertilizer-intensive agriculture is practiced. However, chemical fertilizers have certain issues concerning excessive application, such as burden on nonrenewable resources viz. raw forms of nutrients, energy requirements for manufacturing, and concerns regarding effects on plant and microbial functions (Berg and Koskella, 2018; Kaminsky et al., 2018). These circumstances necessitate sustainable solutions for enhancing cucumber yield per unit area. Utilization of PGP endophytic bacteria is a potential alternative. Crop yield response to application of plant growth promoting bacteria remains least explored as there are reports from rice (Banik et al., 2019; Yanni and Dazzo, 2010), tomato (Sarma et al., 2011), sugar beet (Shi et al., 2011), chickpea (Nautiyal et al., 2002), maize (Riggs et al., 2001) and sugarcane (Chauhan et al., 2012), yet there is lack of information in vegetable crops like that of cucumber and most of the studies focus on plant growth rather productivity. So, this study was planned to evaluate the response of cucumber plants to application of plant growth promoting endophytic bacteria in comparison to those of no treatment and chemical fertilizer application. Furthermore, to the best of our knowledge only two previous reports of endophytic bacterial isolation from cucumber have been reported, and those studies investigated disease suppression and/or plant growth enhancement (Akbaba and Ozaktan, 2018; Ozaktan et al., 2013). Thus, gaps remain in our understanding of the extent of incident endophytic bacteria in cucumber, their PGP activities, and their effects on the biochemical and physiological responses of the plants. The aims of this study were to identify cucumber growth-promoting endophytic bacteria and document the metabolomic response of cucumber plants after application of potential endophytic bacteria.

3.2. Materials and methods

3.2.1. Isolation of endophytic bacteria

Information on plant materials used for isolation of endophytic bacteria is compiled in Table 3.1. Plant growth conditions were similar to those of sampled for the first study (Section 2.2.1). The plants already growing at N35.573616, E138.486816 (Greenhouse cucumber farm), N35.604073, E138.578506 (University of Yamanashi Research Farm), and N35.665889, E138.462967 (Cucumber farm) at the age explained in Table 3.1 were sampled. The two lowest healthy leafstalks (petioles) per sample were cut from the plant and moved to the laboratory in plastic bags. Bacterial isolation from the material was carried out within approximately 1 h of sampling. Isolation was carried out (explained in section 2.2.3.1) and mixture was spread on R2A (Oxoid Limited, Hampshire, UK) and potato dextrose agar (PDA, pH adjusted to 7.0).

3.2.2. PGP screening

Endophytic bacterial application to the seeds was carried out by seed biopriming. Each strain was grown in 1 mL of Reasoner's 2A broth (R2B) broth with one surface-sterilized cucumber seed (NaOCl for 30 s, 70% ethanol for 1 min, and rinsed with SDW) using a test tube covered with a silicon lid. The tubes were incubated with shaking (200 rpm) at 25°C for 23 h and, right before radicle emergence, incubation was stopped, and the seeds were sown. Five replicates of R2B with seeds but no inoculation were treated as the control. The seeds were sown in polypropylene tubes with lids (exterior dimensions: 4 cm × 11 cm, volume: 120 mL) filled with 60 g of the sand–soil mixture (1:1) (with 12 mL of distilled water added) and autoclaved (121°C) for 1 h. The tube with the broth was emptied into the growth tube, and the seed was immersed almost 1-cm deep in the soil. The growth tubes were moved to the growth chamber (25°C; 16-h day, 8-h night). The experiment continued for 35 days; then, the plant roots were washed, and length was measured.

Five strains (two from the first batch, and one from each subsequent batch) were selected for further analysis based on root length (Table 3.1).

Table 3.1 Description and identification of endophytic bacterial strains used.

Strain No.	Location	Plant age	Nearest neighbor	Accession No.
4	Commercially purchased nursery	2-true leaf stage	*Curtobacterium* spp.	LC504252
72	Commercially purchased nursery	2-true leaf stage	*Brevibacillus* spp.	LC504253
167	Greenhouse cucumber farm	3 months	*Paenibacillus* spp.	LC504254
193	University of Yamanashi Research Farm	1 month	*Bacillus* spp.	LC504255
227	Cucumber farm	2 months	*Microbacterium* spp.	LC504256

3.2.3. Characterization and 16S rRNA sequencing-based identification of selected strains

Indole-3-acetic acid (IAA) production, phosphate solubilization, siderophore production, and 1-aminocyclopropane-1-carboxylate (ACC)-deaminase and *nif*H genes were detected for the selected strains using standard procedures. For IAA production, the strains were grown for 96 h at 25°C in IAA production media (30 g of glucose, 2 g of beef extract, 3 g of $CaCO_3$, pH 7, and 1 L of SDW) with and without 1 mM tryptophan. The cultures were harvested by centrifugation at 10,000 *g* for 10 min and 300 µL supernatant was mixed with 1.2 mL of Salkowski's reagent (Acuña et al., 2011; Patten and Glick, 2002). The presence of the color pink was estimated by a spectrophotometer at 535 nm.

Pikovskaya (1948) media, which contained tri-calcium phosphate, was used to grow each strain and incubated for 7 days at 25°C to investigate phosphorus solubilization. The clear zone formation was recorded, and results were compiled as: -, no clear zone; +, detectable but weak clear zone; ++, very obvious clear zone.

For siderophore production assay, chrome azurol S (CAS) medium (Pérez-Miranda et al., 2007; Schwyn and Neilands, 1987) was used to grow the strains and was covered with overlay media (6.04 mg of CAS, 7.3 mg of hexadecyltrimetyl ammonium bromide, 3.04 g of piperazine-1,4-bis (2-ethanesulfonic acid), and 1 mL of 1-mM $FeCl_3 \cdot 6H_2O$). The color change of CAS from blue to light orange or yellow was observed and categorized as: -, no color change; +, color change; ++, color change throughout the media.

Extracted DNA (Section 2.2.3.2) was amplified under conditions: one cycle at 94°C for 4 min; 30 cycles at 94°C for 60 s, 54°C for 60 s, and 72°C for 2 min; and a final extension at 72°C for 7 min for ACC; and one cycle at 94°C for 5 min; 30 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 2 min; and a final extension at 72°C for 5 min for *nif*H. The PCR mixture included 1.0 µL each of 10-mM primer, which included PolF (5′-TGCGAYCCSAARGCBGACTC-3′) and PolR (5′-ATSGCCATCATYTCRCCGGA-3′) (Poly et al., 2001) for *nif*H, and ACCf (5′-GCCAARCGBGAVGACTGCAA-3′) and ACCr (5′-TGCATSGAYTTGCCYTC-3′) (Jha et al., 2012) for ACC-deaminase, 1 µL of extracted nucleotide, 9.5 µL nuclease free water, and 12.5 µL of GoTaq® Green Master Mix. Subsequently, gel electrophoresis was performed, and the bands were stained and observed.

The identified sequences were submitted to DDBJ (accession numbers are compiled in Table 3.1).

3.2.4. Pot experiments

There were two pot experiments carried out; pot experiment 1 and pot experiment 2 conducted with different growth conditions. Soil mixture (1:1 mixture of sand and soil) was used for both pot experiments. The soil analysis of the sand–soil mixture is compiled in Table 3.2. For the pot experiment 1, 450 g of mixture was weighed in the pot (100-cm^2 high-density polystyrene plastic

Neubauer pot, Fujiwara Seisakusho, Ltd., Tokyo, Japan). The seeds were bio-primed as described above, grown under aseptic conditions in between sterilized filter papers for 48 h, and then transplanted (one seedling per pot). Control and fertilizer control seeds were also treated using R2B under similar conditions. The fertilizer control treatment received the recommended dose of liquid fertilizer (a formulation of 6% N, 10% P, and 5% K). The pots were placed in the aforementioned growth chamber under the same conditions. The plants were irrigated daily at 60% of maximum water holding capacity. The experiment ran for 42 days and the plants were then harvested. Root length, and root fresh and dry weight were recorded.

A larger scale pot experiment, pot experiment 2, using 5 kg of the sand–soil mixture was carried out in glass-house conditions. Each pot (500-cm^2 high-density polystyrene plastic Wagner pot, Fujiwara Seisakusho, Ltd., Tokyo, Japan) was transplanted with one seedling following the above-described procedure. The fertilizer control treatment received the recommended dose of fertilizer (9.6 mg of nitrogen, phosphate, and potassium). The plants were irrigated daily and harvested 62 days after transplant. At harvest, the plants were subjected to similar parameters as in the first pot experiment.

Table 3.2 Analysis of the sand–soil mixture used for tube and pot experiments.

Soil Properties	**Values**
pH (Soil: H_2O)	7.4
EC (mS cm^{-1})	0.16
CaO (mg kg^{-1})	21.5
MgO (mg kg^{-1})	4.5
K_2O (mg kg^{-1})	1.97
Ca: Mg	3.43
Mg: K	5.33
Ca: K	18.3
Cation exchange capacity (CEC) (mg kg^{-1})	1.0
P_2O_5 (mg kg^{-1})	0.99
NH_4-N (mg kg^{-1})	0.03
NO_3-N (mg kg^{-1})	0.33

3.2.5. Field experiments

A 2-year field experiment was carried out at Research Farm of University of Yamanashi (N35.604073, E138.578506). Bacterial (same bacterial strains as used for pot experiments i.e. strains 4, 72, 167, 193 and 227) application to the seeds was performed as explained in previous sections. Following the application, the seeds were germinated between sterilized filter papers and transplanted to the field in randomized complete block design (RCBD). Distance of row × row and plant × plant was approximately 1 m. The blocks were managed according to slope of the field. Irrigation was applied immediately after transplantation and was applied every two days using sprinkler irrigation system. For the yield, number of fruits was counted. On harvest, whole plant was measured for shoot length and plant weight at harvest.

3.2.6. Metabolome analysis

Another pot experiment was carried out using two potential PGP strains, 4 and 227, against the uninoculated control for metabolome analysis. The sand–soil mixture (1:1) was autoclaved at 121ºC for 1 h, and 450 g was placed into the pot (100 cm^2). Bio-primed seeds were transplanted as explained above. Plant growth continued for 45 days undergrowth conditions explained in previous section. The stalk of the 4^{th} leaf of five replicates was cut with sterilized scissors and dried in a freeze drier (FDU-12AS, AS One Corporation, Japan). The freeze-dried samples were crushed using Micro Smash (TOMY SEIKO Co. Ltd., Japan) and weighed before analysis using Capillary Electrophoresis Time-of-Flight Mass Spectrometry (CE-TOFMS) in two modes for cationic and anionic metabolites. Then, 200 metabolites (137 metabolites in cation mode and 63 metabolites in anion mode) were detected based on the standard library of Human Metabolome Technologies (HMT) (Yamagata, Japan). Subsequently, 31.9 mg from the control, 46.8 mg from strain 4, and 35.9 mg from strain 227 were separately mixed with 600 μL of methanol containing

internal standards (50 μM) and homogenized with a homogenizer (1,500 rpm, 120 sec × 1 times). Then, chloroform (600 μL) and Milli-Q water (240 μL) were added to the homogenates, mixed thoroughly, and centrifuged (2,300 × g, 4ºC, 5 min). The water layer (200 μL) was filtrated through a 5-kDa cut-off filter (ULTRAFREE-MC-PLHCC, HMT) to remove macromolecules. The filtrate was centrifugally concentrated and resuspended in 50 μl of ultrapure water immediately before measurement.

Peaks detected in CE-TOFMS analysis were extracted using automatic integration software (MasterHands 2.17.1.11, developed at Keio University) to obtain peak information, including m/z, migration time (MT), and peak area. The peak area was then converted to relative peak area by following Equation 1. The peak detection limit was determined based on a signal–noise ratio of 3.

Equation 1:

$$Relative\ Peak\ Area = \frac{Metabolite\ Peak\ Area}{Internal\ Standard\ Peak\ Area \times Sample\ Amount}$$

Putative metabolites were then assigned from HMT's standard library and the known–unknown peak library based on m/z and MT. The tolerance was ± 0.5 min for MT and ± 10 ppm (Equation 2) for m/z. If several peaks were assigned the same candidate, the candidate was assigned separate branch numbers.

Equation 2:

$$Mass\ error\ (ppm) = \frac{Measured\ Value - Theoretical\ Value}{Measured\ Value} \times 10^6$$

Absolute quantification was performed for target metabolites. All metabolite concentrations were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and using standard curves, which were obtained by single-point (100 μM) calibrations.

3.2.7. Data analysis

The means of PGP parameters were compared using Tukey's HSD test. Similarly, analysis of variance (ANOVA) was carried out to determine the statistical effect of treatments in Statistix 8.0 (Analytical Software, FL, USA).

3.3. Results

3.3.1. Isolation and screening of isolates

Around 300 endophytic bacterial strains were isolated from cucumber leaf-stalks, and initial screening was carried out for potential PGP strains. Four batches of 80 strains each were compared with five replicates (for each media; i.e., R2A and PDA) of the control. Root elongation to different extents was observed (data not given), and five strains with the greatest root lengths were selected (strains 4, 72, 167, 193, and 227). The root lengths of plants with these strains compared with the control are available in Table 3.3. Treatment with all strains showed, on average, three-fold increase in root length compared with the control ($p<0.001$); treatment with strain 72 showed an average root length of 24.4 cm, followed by strain 4 (22.7 cm), strain 227 (21.3 cm), strain 167 (20.8 cm), and strain 193 (20.7 cm). However, treatment with all strains produced statistically similar results (Tukey's HSD, Table 3.3).

3.3.2. Pot experiments

The two pot experiments followed the initial screening with different soil quantities and conditions (Table 3.3). Belowground parts of the plants were observed; thus, root length, and fresh and dry weight were monitored, which were significantly affected by treatment application ($p<0.05$) in pot experiment 1. Similar to the initial screening, strain 72 produced a maximum root length of 36.2 cm, which was statistically similar to all other strains and fertilizer control treatment (Tukey’s HSD). Control treatment followed and produced a minimum mean root length of 21.8 cm. The root fresh and dry weight trends differed slightly from that of root length, and maximum root fresh

weights were observed in plants applied with strains 227 and 4, but were statistically the same as all other treatments except the control (Tukey's HSD), which showed the lowest value ($p<0.05$). Root dry weight was also significantly affected by treatment application ($p<0.05$) and showed a similar pattern to fresh weight, in which strains 4- and 227-applied plants had maximum values of 0.9 and 1 g, respectively.

In the second pot experiment, the trends of the parameters studied were similar to that of the first pot experiment, but differences were more evident. The parameters observed were significantly ($p<0.001$) affected by the application of fertilizer or endophytic bacteria (Table 3.3). The first parameter, root length, was significantly affected by application of different treatments ($p<0.001$). Maximum root length was statistically the same between plants in which strains 227, 4, and 167 were applied. The root length produced by treatment with strain 193 (31.2 cm) was statistically the same as that produced by treatment with strain 167; treatment with strain 72 produced a mean root length of 28.2 cm, and this was not statistically different from the fertilizer control, which had a mean root length of 25.3 cm. Control treatment showed the statistically smallest root length (20.6 cm).

Root fresh and dry weight were also significantly affected by application of different treatments ($p<0.001$). Root fresh weight was highest in strain 4-applied plants but was statistically the same as root fresh weight of strain 227-applied plants. Strain 227-applied plants showed a root fresh weight of 8.5 g, which was statistically the same as those of strains 167- and 193-applied plants. Strains 167 and 193 produced statistically similar values to strain 72 and chemical fertilizer. However, the control treatment showed the minimum root fresh weight, which was statistically the same as that of the fertilizer control treatment. Maximum root dry weight was observed in strain 4-applied treatment (2.8 g) and was statistically similar to that of strain 227-applied

treatment. Treatments with strains 167 and 193 followed, with root dry weights of 2.4 g and 2.3 g, respectively, which were statistically similar. Treatment with strain 72 produced a root dry weight of 2.1 g, which was statistically the same as that of the fertilizer control (1.8 g). The control treatment had the lowest root dry weight (1.5 g), which was statistically the same as the fertilizer control treatment.

Table 3.3 Response of root parameters to application of different treatments in screening and pot experiments. Treatments with the same letter are statistically similar based on Tukey's HSD at 5% level of significance. '±' indicates the standard deviation among three replications for the tube experiment, five for pot experiment 1, and three for pot experiment 2.

	Tubes	**Pot experiment 1**			**Pot experiment 2**		
	Root Length (cm)	Root length (cm)	Root Fresh Weight (g)	Root Dry Weight (g)	Root Length (cm)	Root Fresh Weight (g)	Root Dry Weight (g)
Control	7.8±0.8 b	21.8±2 b	1.4±0.2 b	0.4±0.2 b	20.6±1.2 e	4.2±0.2 d	1.5±0.1 d
Fertilizer Control	-	25.2±3.5 ab	2.0±0.6 ab	0.7±0.3 ab	25.3±0.6 d	5.9±0.3 cd	1.8±0.1 cd
Strain No. 4	22.7±2.4 a	33.5±10.5 ab	3.0±0.9 a	0.9±0.2 a	35.9±0.9 a	9.0±0.2 a	2.8±0.03 a
Strain No. 72	24.4±3.2 a	36.2±6.4 a	2.3±0.9 ab	0.6±0.1 ab	28.2±1.7 cd	6.6±1.1 c	2.1±0.2 c
Strain No. 167	20.8±0.7 a	34.1±2.2 ab	2.8±0.5 ab	0.7±0.1 ab	34.2±3.0 ab	6.9±1.1 bc	2.4±0.5 bc
Strain No. 193	20.7±5.8 a	34.7±9.3 ab	2.4±1.6 ab	0.6±0.2 ab	31.2±1.4 bc	7.1±0.3 bc	2.3±0.2 bc
Strain No. 227	21.3±0.2 a	35.6±9.8 ab	3.1±0.3 a	1.0±0.2 a	36.0±0.8 a	8.5±0.5 ab	2.7±0.1 a

3.3.3. PGP trait characterization of selected strains

Qualitative analysis of different PGP traits was carried out, and results are provided in Table 3.4. All selected strains possessed certain traits, but not all of the explored traits. For example, IAA production and phosphate solubilization were observed in strains 4, 167, 193, and 227 at different concentrations, whereas strain 72 did not show IAA production and P-solubilization. The siderophore production trends also differed, as strains 4 and 227 did not show siderophore production, but strains 72 and 193 showed substantial production. Strain 167 also showed siderophore production, although less than strains 72 and 193. The ACC-deaminase and *nif*H gene amplification showed that three strains (4, 167, and 227) had ACC-deaminase activity, whereas only two strains (167 and 193) had the nitrogen-fixation activity.

Table 3.4 Plant growth-promoting traits of selected strains. The color change extent for IAA, P-solubilization, and siderophore production was recorded as: -, no color change; +, color change; ++, color change throughout the media. Detection of amplified ACC and *nif*H genes using corresponding primers is marked as +; absence is marked as -.

Strain No.	IAA	P	Siderophore	ACC	*nif*H
4	+	++	-	+	-
72	-	-	++	-	-
167	++	+	+	+	+
193	+	+	++	-	+
227	++	++	-	+	-

3.3.4. Field experiments

Plant growth and number of fruits were recorded, and effect of treatments was noted. Analysis of variance (ANOVA) showed that all the parameters: shoot length and fresh weight at harvest and number of fruits, recorded were significantly affected by application of different endophytic bacteria ($p<0.05$), however, number of fruits in year 1 was found to be non-significant ($p>0.05$).

Vegetative parameters including the shoot length at year 1 ($p<0.01$) and year 2 ($p<0.001$) and fresh shoot weight at year 1 ($p<0.01$) and year 2 ($p<0.05$) at harvest were found

significantly affected by application of treatments. In first year, shoot length at harvest was found maximum in strain 4-inoculated plants which was followed by strain 227 and strain 193, and only all three of these stood significant over that of control treatment (Student t-test, Figure 3.1). Furthermore, only strain 4 and 227 applied plants differed significantly compared to that of control plants (Tukey's HSD Test). The strain 227 applied plants, however, shared significance with those of fertilizer control plants hence share same letter. The remaining three endophytic bacterial strains encompassing strain 72, 167 and 193 although showed similarity with that of strain 4 and strain 227-inoculated plants, yet they also stood same with the control treatment plants. The results of shoot length were more evident in year 2 ($p<0.001$), and all the treatments including that of fertilizer control were found to be significant compared to that of control (Student t-test, $p<0.05$). Comparison of treatment means showed similar trend to that of first year, where only strain 4 and 227 were statistically superior to that of control treatment plants (Tukey's HSD Test). However, the strain 4 shared significance with other endophytic bacterial treatments besides the fertilizer control treatment.

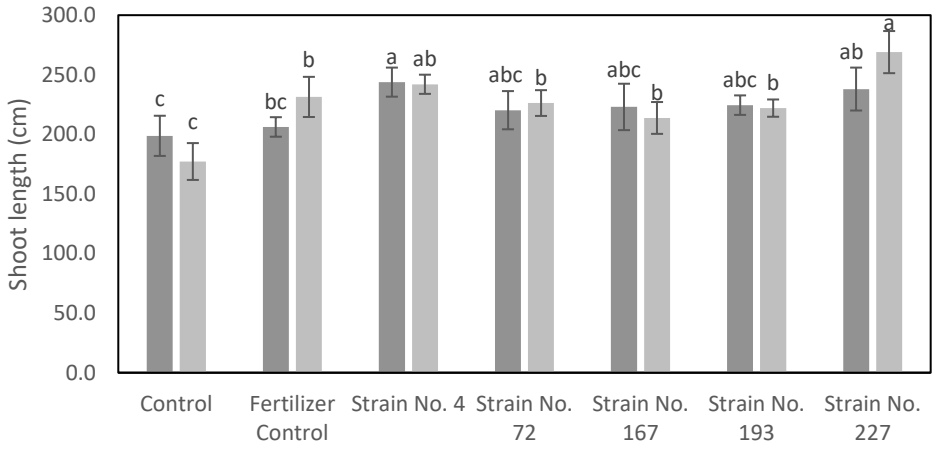


Figure 3.1 Response of shoot length (cm) at harvest to application of different treatments in both years. Dark colored bars indicate year 1 while light-colored bars present year 2. The error bars show the standard deviation among the replications, while the treatments with the same letter are statistically similar based on Tukey's HSD at 5 % level of significance.

Fresh shoot weight at harvest was also found to be significantly affected by application of the treatments in year 1 ($p<0.01$) and year 2 ($p<0.05$, Figure 3.2). All the endophytic bacteria-applied plants showed statistically more fresh shoot weight compared to that of fertilizer control and uninoculated control in year 1 while only strain 227 showed significance in year 2 (Student t-test, $p<0.05$). Pairwise comparison of the treatment means from year 1 confirmed the previous statement along with showing superiority of strain 72-inoculated plants, while all other strains were statistically same with that of fertilizer control treatment (Tukey's HSD Test). Furthermore, strains 4, 227, 167 and fertilizer control treatments were not significantly different from control treatment plants. In the second year, pairwise comparison of shoot weight at harvest showed that strain 227 led with average value of 379.3 g but was statistically superior to that of control treatment plants but same with those of other bacteria-inoculated and fertilizer control plants (Tukey's HSD Test). Moreover, except for strain 227, all other treatments showed similar statistical level with that of control plots.

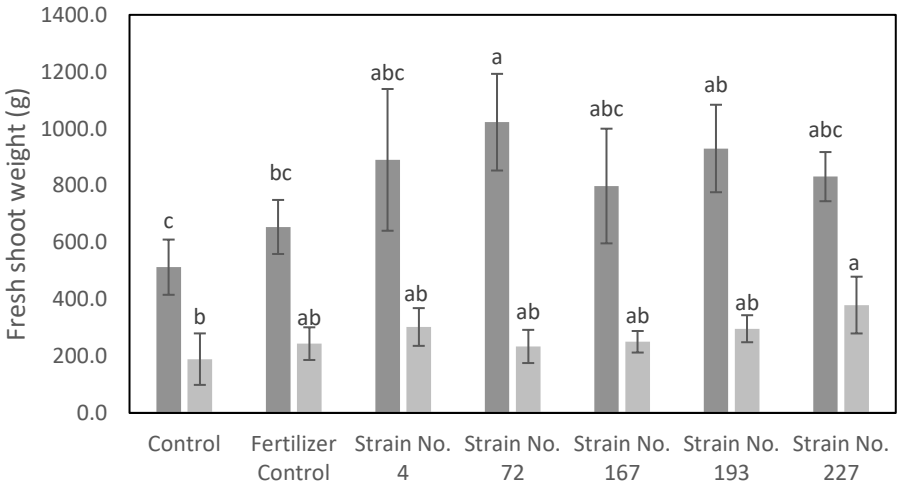


Figure 3.2 Response of shoot weight (g) at harvest to application of different treatments in both years. Dark colored bars indicate year 1 while light-colored bars present year 2. The error bars show the standard deviation among the replications, while the treatments with the same letter are statistically similar based on Tukey's HSD at 5 % level of significance.

Number of fruits was recorded to bring forward the effect of application of endophytic bacteria on cucumber yield potential (Figure 3.3). Number of fruits had non-significant effect of treatments in year 1, however, in the year 2; only strain 4 and 227 significantly contributed to number of fruits when compared with control treatment (Student t-test, $p<0.05$, Figure 4). Comparison of treatment means showed that strain 227 was leading with average fruit number of 15, but shared statistical significance with those of strain 4, 193 and fertilizer control. Following were the strains 72 and 167 which were same with that of control treatment plants (Tukey's HSD Test).

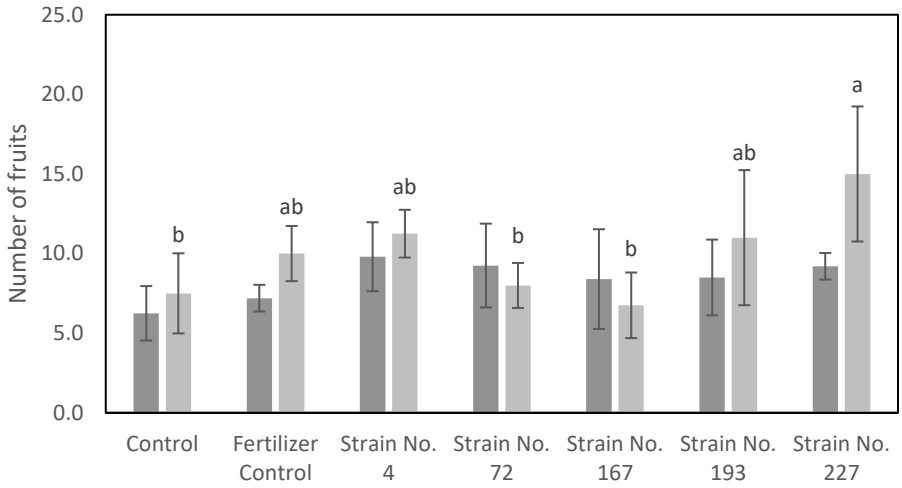


Figure 3.3 Response of number of fruits at harvest to application of different treatments in both years. Dark colored bars indicate year 1 while light-colored bars present year 2. The error bars show the standard deviation among the replications, while the treatments with the same letter are statistically similar based on Tukey's HSD at 5 % level of significance.

3.3.5. Metabolome analysis

Corresponding to the isolated part of the plants, the metabolome of the leaf-stalk from CE-TOFMS measurement showed 200 peaks (137 in cation mode and 63 in anion mode) that were annotated based on HMT's standard library and the known–unknown peak library. Selected data are provided in Figures 3.4, 3.5, and 3.6. As shown in Figure 3.4, the relative area of all observed metabolites differed among the three treatments. Of all 200 metabolites explored, 42.5% had higher concentrations and quantities in both endophytic bacteria-applied cucumber

plants compared with the control. Another 20% of metabolites increased in one bacterial application but decreased in another compared with the control. Similarly, 20% of the metabolites were lower in both bacteria-applied treatments compared with the control. Furthermore, 11.5% of the chemicals were not detected in the control treatment but were observed in either of the bacteria-treated cucumber plants. Similarly, 5% of the metabolites were not detected in either of the bacterial application treatments but present in the control treatment. Moreover, 1% of chemicals were not detected in either of the bacteria-applied treatments but were produced in control plants.

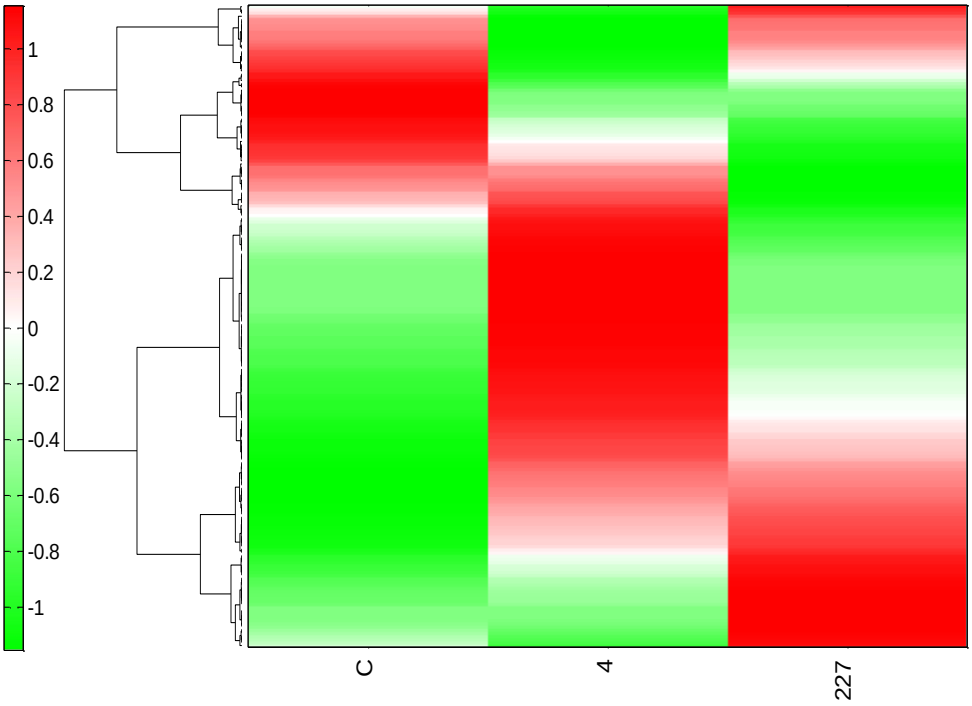


Figure 3.4 Heatmap of all the metabolites based on relative peak area in the control and strains 4- and 227-applied cucumber plants. The horizontal axis and vertical axis show sample names and peaks, respectively. The distances between peaks are displayed in the tree diagram. Green (low) to red (high) represents the increase in relative area of each peak among the three treatments.

A few important plant metabolites, based on previous literature (Li et al., 2014), were compiled based on standardized relative area (Figure 3.5). The alcohol and sugar alcohol metabolites increased when endophytic bacteria were applied. Polyamines were also higher in bacteria applied plants, except for urea and spermidine, and urea was only detected in the

control treatment. The extent of variance differed among metabolites and was correlated with bacterial strain. Amino acids and derivatives included 26 different metabolites, among which 18 were lower in the control compared with either of the bacteria-applied treatments (Figure 3.5). Furthermore, N6-acetyllysine, 1-aminocyclopropane-1-carboxylic acid homoserinelactone, N-acetylmethionine, S-adenosylmethionine, S-methylmethionine, N5-ethylglutamine, and Nw-methylarginine, were observed more in the control treatment than the bacterial treatments. Similarly, the fatty acid 4-acetamidobutanoic acid remained undetected in the control, but showed values of 0.38 and 0.76 when treated with strains 4 and 227, respectively. Hormones and nucleosides included 11 different metabolites, of which five were lower in the control treatment compared with treatments with either of the bacterial strains. Strain 4 treatment yielded higher values for four metabolites, whereas strain 227 treatment showed higher values for three metabolites. Organic acids were dominated by strain 4, with a few exceptions of metabolites that were also present with strain 227. Among a total of 12 organic acids, treatment with strain 4 showed higher values in 10 compared with the other two treatments (i.e., the control and strain 227). Treatment with strain 227 also produced four values were higher than those of the control and strain 4. Control treatment showed increased values of three organic acids over treatment with either of the strains. Similarly, the concentration of sugars and their derivatives also differed based on application of endophytic bacteria; however, two sugars showed more accumulation in the control. Treatment with strain 4 showed increased amounts of three sugars and their derivatives; in contrast, treatment with strain 227 did not chemically contribute to this group.

Groups	Metabolites	C	4	227
Alcohol and sugar alcohol	Glycerol			
	Glycerol 3-phosphate			
Polyamines	N1-Acetylspermidine			
	Spermidine			
	Spermine			
	N-Acetylputrescine			
	Putrescine			
	Urea			
Amino acids and derivatives	Carboxymethyllysine			
	N-Acetyllysine			
	Trimethyllysine			
	N6-Acetyllysine			
	Citrulline			
	Homoserine			
	S-Adenosylhomocysteine			
	Threonic acid			
	N,N-Dimethylglycine			
	N-Acetylmethionine			
	S-Adenosylmethionine			
	S-Methylmethionine			
	Methionine sulfoxide			
	N-Methylproline			
	5-Oxoproline			
	cis-4-Hydroxyproline			
	Hydroxyproline			
	N5-Ethylglutamine			
	Nw-Methylarginine			
	L-Tryptophan			
	N-Acetylserine			
	N-Acetylornithine			
	Ornithine			
	N-Acetylalanine			
Fatty acids	4-Acetamidobutanoic acid			
Organic acids	Argininosuccinic acid			
	trans-Glutaconic acid			
	Phenylpyruvic acid			
	Pyruvic acid			
	Glucuronic acid Galacturonic acid			
	Shikimic acid			
	3-Dehydroshikimic acid			
	Citric acid			
	Isocitric acid			
	Citramalic acid			
	Gluconic acid			
	2-Oxoglutaric acid			
Sugars and derivatives	Fructose 6-phosphate			
	Glucose 6-phosphate			
	UDP-glucose UDP-galactose			
	Ascorbate 2-glucoside			
	Ascorbic acid			

Figure 3.5 Heatmap of major metabolite groups based on relative peak area in the control and strains 4- and 227-applied cucumber plants. Green (low) to red (high) represents the increase in relative area of each peak among the three treatments.

Nutrient deficiency-linked metabolites showed a decreasing trend in control plants compared with endophytic bacteria-applied plants. Among the nitrogen-related metabolites, the relative area of 2-oxoglutaric acid was 0.035 in strain 227-applied plants, but was 0.029 in control plants. Similarly, a two-fold increase in citric acid was observed in both strains 4- and 227-applied plants compared with the control. Isocitric acid showed increases of 1.2- and 1.3-fold in strains 4- and 227- applied plants, respectively, compared with the control plants. The fumaric acid results also validated that there was nutrient stress in control plants; strain 4-applied plants showed 1.4 times more fumaric acid, but strain 227-applied plants had only slightly more compared with control plants. Of the phosphorus-related metabolites, glycerol 3-phosphate increased in both bacteria-applied treatments; however, the difference between strain 227-applied plants and the control was lower compared with that of strain 4-applied and control plants. An interesting result was observed in urea quantification in control plants, which was not detected in the other two treatments. Similarly, significant deposition of uridine diphosphate-glucose in control plants was 1.9-fold greater than in strains 4- and 227-applied plants. For tryptophan, the precursor of IAA was increased under both bacteria-applied treatments. Strains 4- and 227-applied plants showed 1.1- and 1.6-fold increases in tryptophan over the control, respectively, which corresponded to the axenic IAA production by the strains (Table 3.4).

Furthermore, the metabolites that remained undetected in control plants but were observed in either or both of the endophytic bacteria-applied plants were associated with histidine metabolism (methylimidazoleacetic acid), fatty acid and lipid metabolism (itaconic acid, 2-hydroxy-3-methylpentanoic acid, 2-hydroxyvaleric acid, (R)-3-hydroxybutyric acid), protein and/or amino acid metabolism or biosynthesis (carboxymethyllysine, N-Ethylmaleimide, Symmetric dimethylarginine), polyamine metabolism (creatine, ornithine, 4-trimethylammoniobutanoic acid), tryptophan metabolism (L-kynurenine), and membrane

stability (4-acetamidobutanoic acid), and most of them also serve as an energy source. Similarly, either or both of the strains contributed to production of certain metabolites not detected in control plants, including an amino acid (L-carnitine), sugar (glucaric acid), antibiotic (metronidazole), isoquinoline (2(N)-methyl-norsalsolinol), alkaloid (piperidine), antifungal compound (propionic acid), and organosulfur compound (trimethyl sulfonium).

Both of the strains tested showed differential responses between themselves and affected tricarboxylic acid (TCA) and urea cycles in addition to the metabolism of fatty acids and amino acids. Specifically, strain 4 showed a prominent effect on urea and TCA cycles, whereas strain 227 influenced carnitine and amino acid production and metabolism. For the urea cycle (Figure 3.3), L-arginine was highest in strain 4-applied plants, with a relative area of 0.03, followed by strain 227-applied plants (0.03) and then control treatment (0.02). Although L-arginine continued to form urea in the control treatment, there was no urea detected in either of the bacteria-applied treatments. However, arginine changed to ornithine when strain 4 was applied, whereas ornithine was not detected in control and 227-applied plants. Additionally, citrulline was observed more in strain 227-applied plants (0.0008), whereas the control (0.0005) and strain 4-applied plants (0.0005) showed nearly equal relative areas. Strain 4-applied plants again showed increased values of citrulline conversion to argininosuccinate compared with control plants and strain 227-applied plants. Similarly, conversion of argininosuccinate to fumaric acid was also higher in strain 4-applied plants followed by 227-applied plants and then control plants. Interestingly, the level of argininosuccinate was lower in strain 227-applied plants compared with control plants, but the level of fumaric acid showed an opposite trend. Strain 4-applied plants also performed better in the TCA cycle with a few exceptions. Pyruvic acid was highest in strain 4, followed by control and strain 227-applied plants. The anaerobic glycolysis from pyruvate to lactate and S-lactoylglutathione was also observed more in strain 4-applied plants compared with control and strain 227-applied plants. In the citric acid cycle,

citric acid concentration was lowest in the control treatment. Strain 227-applied plants showed more citric acid than strain 4-applied plants. However, cis-aconitic acid was recorded more in strain 4-applied plants followed by strain 227-applied and control plants. Isocitric acid was also observed more in strains 4- and 227-applied plants compared with control plants. Finally, 2-oxoglutaric acid was highest in strain 227-applied plants, followed by control and strain 4-applied plants.

The effect of strain 227 was more prominent in fatty acid metabolism, especially that of carnitine; trimethyllysine was higher in both strain-applied plants compared with control plants, and actinine and carnitine were only detected in strain 227-applied plants. Similarly, L-kynurenine, a metabolite of the amino acid L-tryptophan, was only observed in strain 227-applied plants. Tryptophan, however, was increased in both bacteria-applied treatments but was greater in strain 227-applied plants, which corresponded to IAA production by both strains (Table 3.4).

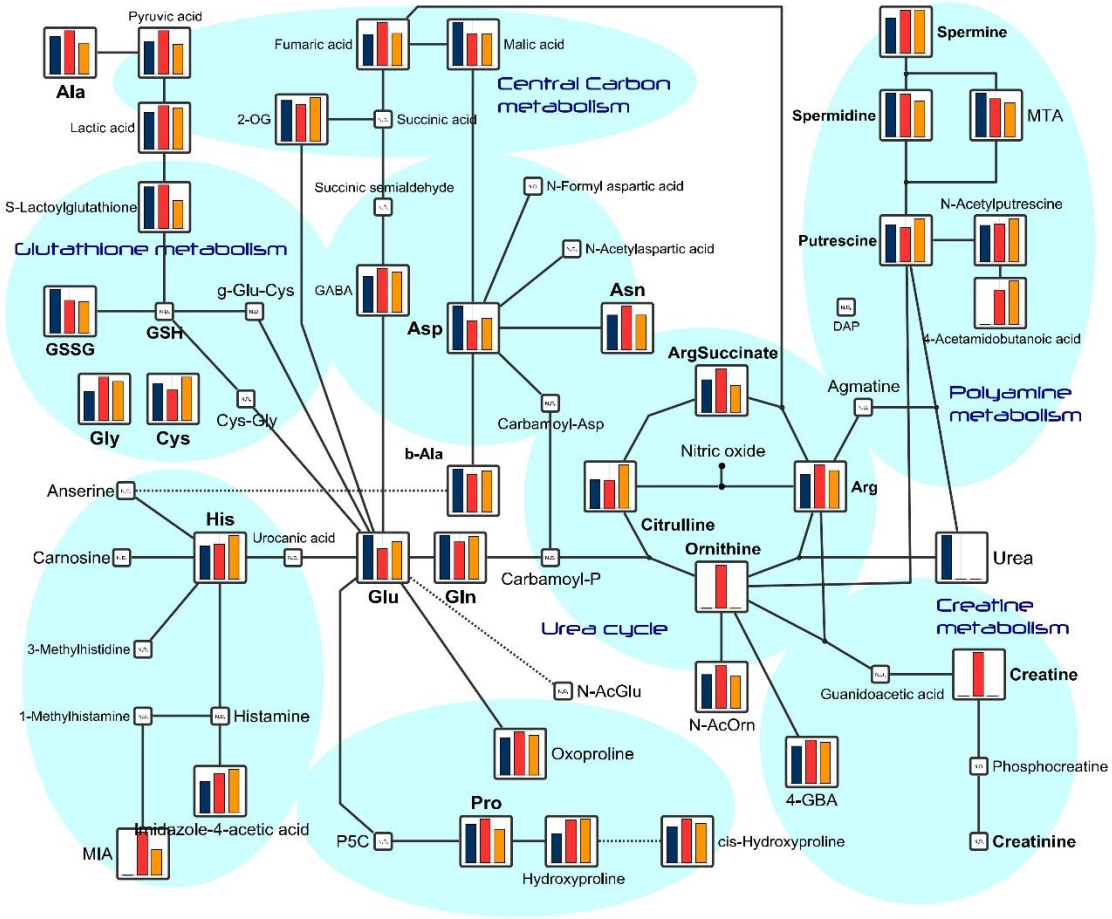


Figure 3.6 Pathway map of detected metabolites in this study according to the Human Metabolome Technologies (HMT) Standard Metabolites Library. The bars/lines represent relative areas of each metabolite in the control (blue) and treatments with strains 4 (red) and 227 (orange). N.D.: not detected. Ala: L-Alanine, 2-OG: 2-Oxoglutaric acid, MTA: 5'-Deoxy-5'-methylthioadenosine, GSSG: Glutathione (GSSG)_divalent, GSH: Glutathione, GABA: Gamma-aminobutyric acid, Asp: L-Aspartic acid, Asn: L-Asparagine, Gly: Glycine, Cys: L-Cysteine, ArgSuccinate: Argininosuccinic acid, b-Alanine: β-Alanine, Arg: L-Arginine, His: L-Histidine, Glu: L-Glutamic acid, Gln: L-Glutamine, NN-AcOrn: N-Acetylornithine, MIA: 1-Methyl-4-imidazoleacetic acid, Pro: L-Proline, 4-GBA: 4-Guanidinobutyric acid.

3.4. Discussion

3.4.1. PGP ability of endophytic bacteria

The plant-associated microbiome has been explored for possible roles in crop production and is increasingly important for development of environment-friendly and resource-efficient agricultural approaches. In this study, we explored the endosphere of different-aged cucumber plants from a variety of sampling locations to identify PGP endophytic bacteria. The roles of the selected endophytic bacteria were assessed for plant growth and productivity enhancement and metabolomic response (Table 3.3, Figures 3.1-3.6), and PGP traits were checked (Table 3.4).

Plant growth was enhanced by the endophytic bacterial strains to varying degrees. In the initial screening, an approximately three-fold increase in root length was observed in bacteria-inoculated plants compared with control plants. This root biomass enhancement has been observed in different crop plants when applied with PGP bacteria (Devi et al., 2017; Etesami and Alikhani, 2016; Khalifa et al., 2016). The effect of these bacteria on root architecture has been linked to phytohormone production and signaling (reviewed by Vacheron et al., 2013). IAA production has been reported to enhance root length (Aloni et al., 2006), but it was interesting to see that strain 72-applied plants showed the highest average root length but did not show IAA production (Table 3.4); however, these plants showed siderophore production, which may be one of the reasons underlying enhanced root length, as similar results were observed by a siderophore-producing bacterial strain in maize (Sah et al., 2017). Another report in rice was documented in which an actinomycete capable of siderophore production enhanced root biomass compared with the control and strains that lacked siderophore production (Rungin et al., 2012). Furthermore, there may be other mechanisms underlying the plant growth enhancement caused by strain 72 that need to be explored.

The subsequent pot experiments showed different extents of plant growth promotion compared with the control and fertilizer control (Table 3.3). The pot experiment 1 showed that strains 4- and 227-applied plants had the greatest root fresh and dry weight, which significantly differed from the results of the other endophytic bacterial strains. For root length, however, only strain 72-applied plants showed significant increase compared with the control. Additionally, although the strains had the capability, as shown in the screening process, and greater root length was expected, complex interactions between the host, endophytic bacteria, and environmental factors can affect the strain's functionality. Another reason may be the size of pots used in experiment 1 (100 cm^2). The roots grew well until the roots could no longer grow in search of nutrients. The second pot experiment revealed more clear differences, and strains 4 and 227 enhanced plant parameters better than other strains and the control. Both of these strains showed IAA production, phosphate solubilization, and ACC-deaminase activity (Table 3.4). It is notable that other strains also had similar PGP traits, although to different extents, yet plant growth enhancement varied.

These results showed increased importance of phosphate solubilization among the checked traits. The phosphorus solubilization capability has been considered the most important characteristic for plant growth enhancement under moderate fertility conditions (Chabot et al., 1996). For example, strain 167 also shared statistical significance for root length with strains 4 and 227 in pot experiment 2; besides having IAA, P-solubilization, and ACC-deaminase activity similar to strain 4 and 227, strain 167 also possessed siderophore production and the *nif*H gene. Siderophore production and *nif*H, however, may not have contributed to root fresh and dry weight as they did for root length.

3.4.2. Plant growth promotion by endophytic bacteria in the field experiment

The plant growth promoting ability of bacteria under saline conditions has been reviewed (Numan et al., 2018), as experiential in this study. The climatic and soil dynamics

significantly affect the microbial functionality before their adaptation to the environment (Schimel et al., 2007). Stress adapted microbes; salinity tolerant as explored in this study can be used, and help plants alleviate the stress (Gopalakrishnan et al., 2015; Singh et al., 2015). The ability of microbes conferring salt tolerance to the plants is well known, besides enhancing the latter's growth. The mechanisms in doing so involve genetic and proteomic responses besides certain functional support by the inoculated microbes as reviewed (Ilangumaran and Smith, 2017; Kumar and Verma, 2018; Liu and Zhang, 2015), and discussed above.

A two-year field trial employing same treatments as those of pot experiments i.e. five strains 4, 72, 167, 193 and 227 were compared with those of control and fertilizer control. Field experiment was conducted aimed at evaluating the response of number of fruits of cucumber to application of endophytic bacteria which has not been reported to best of our knowledge. Previous reports of endophytic bacteria from cucumber checked plant growth and pathogen biocontrol (Akbaba and Ozaktan, 2018; Ozaktan et al., 2013). Although there are studies evaluating the yield of rice (Banik et al., 2019; Yanni and Dazzo, 2010), tomato (Sarma et al., 2011), sugar beet (Shi et al., 2011), chickpea (Nautiyal et al., 2002), maize (Riggs et al., 2001) and sugarcane (Chauhan et al., 2012) in response to endophytic microbes, yet there is lack of information in vegetable crops like that of cucumber and most of the studies focus on plant growth rather productivity.

Vegetative growth parameters showed significant changes when applied with endophytic bacterial and fertilizer treatments, however, it was interesting to see that fertilizer control treatment showed no significant effect as compared to that of control treatment except for shoot length in 2nd year (Figure 3.1-3.3). It can be corresponded to utilization of available nutrients for fruit development, and the applied fertilizer may have been diverted to fruit rather vegetative growth. Also, usual practice of fertilizer application to cucumber crop in vicinity of the experimental field is regular and intensive application (Mahmood et al., 2019b), which was

not the case in this study as we applied the fertilizer only once in the start of growing season. It presents that periodic and continuous fertilizer application is required for better yields from cucumber.

In addition, shoot weight at harvest was higher in year 1 as compared to that of year 2 (Figure 3.2). it can be linked with the weather conditions in second year, which were quite different to those of year 1, and shorter periods of sunlight hours may have contributed to increase in shoot length, but all of the focus must have been on fruit development. It has been observed that seedlings having ample sunlight resulted in better shoot weight than those of which did not have sufficient sunlight (United States Forest Service, 1980). It was interesting to see that only strain 227 enhanced fresh shoot biomass significantly in year 2 of the experiment (Figure 3.2). Similar results were obtained where inoculation of *Burkholderia amfibaria* enhanced fresh shoot biomass compared to control and other bacterial strains (Larsen et al., 2017). It can be correlated with IAA production by strain 227 which was higher than other strains except for strain 167 (Table 2.3).

Number of fruits somehow cleared the picture where strain 227 was the only significant treatment (Figure 3.3). It is noticeable here that the number of fruits for strain 227 applied plants was 2-folds as compared to that of control. The co-application of endophytic bacteria with chemical fertilizer may have answers, because if the endophytic bacterial application may have led to increase in number of fruits, the size or weight of the fruits directly depended on the nutrients available. The application of chemical fertilizer can thus be suggested to enhance the crop yields.

3.4.3. Metabolomic analysis

A wealth of information on the role of PGP bacteria is available and has been explored in various crops (reviewed by Pii et al., 2015; Santoyo et al., 2016; Souza et al., 2015a). However,

little is known about the biochemical interface or level of interaction between beneficial microbes and plants. Thus, besides exploring growth enhancement by endophytic bacterial strains, this study also investigated the possible role of applied bacteria in plant metabolite balance, particularly in the leaf-stalk, from which these bacteria were originally isolated. Previous studies that observed the effect of plant beneficial bacteria on plant metabolomics have concluded significant regulation of a wide variety of metabolites (Li et al., 2014; Maggini et al., 2017; Scherling et al., 2009). Endophytes can regulate production of certain metabolites, which was evident from the varying concentrations observed in this study (Figures 3.4-3.5); this may be related to production and influencing mechanisms of endophytic bacteria.

Key plant metabolites, as shown in Figure 3.2, showed dominance in strain 4-applied plants followed by strain 227-applied and then control plants; however, control treatment had the highest amounts of some metabolites. Interestingly, spermidine was highest in control plants, immediately followed by strain 4-applied plants. The spermidine in control plants may have come from 5'-deoxy-5'-methylthioadenosine, which was also higher in control plants. Alternatively, strain 4 may have produced spermidine. A similar mechanism of spermidine production and plant growth promotion has been observed (Xie et al., 2014). Furthermore, urea was only detected in control plants. Similar results were observed in barley under phosphorus-deficient conditions (Huang et al., 2008). Urea accumulation may correspond to the higher levels of allantoate observed in control plants, which shows increased accumulation in response to diverse kinds of stresses in plants (Takagi et al., 2016). Additionally, plants store nitrogen in the form of arginine, which later transforms to amino acids and urea for utilization by the plants. Arginine concentration, as observed in this study (Figure 3.5), was comparatively higher in endophytic bacteria-applied plants and may be related to enhanced nitrogen availability to the plants facilitated by bacteria. Both bacteria, however, lacked the *nif*H gene, which indicates that enhanced nitrogen assimilation and/or increased nitrogen availability to plants may occur

through mechanisms other than nitrogen fixation. Arginine catabolism (Esteban et al., 2016) to produce urea in control plants, ornithine production by strain 4, and citrulline in strain 227 demonstrate different mechanisms within each plant. Bypassing of urea production in bacteria-applied plants may have led to polyamine production (Figure 3.5). Alternatively, urea accumulation in control plants may be linked to increased nitrogen requirements for better growth of bacteria-inoculated plants, which may have exhausted the nitrogen quicker. Similarly, the transformation of arginine to ornithine and citrulline by the bacteria (except to urea) is another potential explanation. Ornithine production, as shown by strain 4, was previously observed in *Burkholderia* through upregulation of the anaerobic arginine deiminase pathway in sugarcane plants (Paungfoo-Lonhienne et al., 2016).

Amino acids and their derivatives were generally lower in control plants compared with either or both of the endophytic bacteria-applied treatments, with the exceptions of N6-acetyllysine, N-acetylmethionine, S-adenosylmethionine, and N5-ethylglutamine (Figure 3.2). Enhanced amino acid concentrations have also been documented in rice when inoculated with *Herbaspirillum seropedicae* (Curzi et al., 2008). The decrease of amino acids in the control plants may be correlated with nutrient deficiency, especially of nitrogen, as observed in tomato plants (Fernie and Urbanczyk-Wochniak, 2004). Similarly, evidence of enhanced amino acid availability to the plants leading to better plant growth has been found (Nautiyal et al., 2000; Różycki et al., 1999). For example, inoculation of *Arabidopsis* plants with *Pseudomonas putida* resulted in upregulation of amino acid synthesis (Srivastava et al., 2012). Therefore, PGP endophytic bacteria may enhance the supply of amino acids to the plants, which may have led to a greater quantity of amino acids and their derivatives within the inoculated plants compared with non-treated plants. Armengaud et al. (2009) also observed accumulation of non-acidic amino acids in plants facing potassium deficiency, which was also observed in this study in the cases of N6-acetyllysine, homoserine, N5-ethylglutamine, and Nw-methylarginine (Figure 3.2).

Few of the methionine compounds were observed in greater concentrations in control plants, which may be because of metabolite production by endophytic bacteria that inhibited methionine synthesis (Okazaki et al., 2007).

Organic acids within the endosphere of cucumber plants were also higher in quantity and concentration in endophytic bacteria-applied plants (Figure 3.2). A lower quantity and concentration of organic acids, particularly of 2-oxoglutaric acid, citric acid, isocitric acid, and fumaric acid in control plants, indicates nitrogen deficiency in these plants, and this corresponds to the findings of Stitt and Fernie (2003). However, Stitt and Fernie (2003) also observed decreased malic acid, which was not observed in this study. Abiotic stress on plants has been observed to decrease citric acid and/or increase malic acid concentrations (Martinez et al., 1994; Timpa et al., 1987). Additionally, involvement of the plant exudate malate in acquiring phosphorus has also been debated (Schulze et al., 2002). The higher concentration of malic acid in control plants may be because plant-produced malate tended to be transported to soil to enhance phosphorus availability. Furthermore, glycerol-3-phosphate concentration was greater under strain 4-applied treatment. These results were contrary to the findings of Morcuende et al. (2007) but similar to those of Misson et al. (2005), who observed varying responses of glycerol-3-phosphate to phosphorus deprivation. It is noticeable that both strains showed phosphorus-solubilizing capabilities (Table 3.4), which may have enhanced the quantity of glycerol-3-phosphate in bacteria-applied plants. A plant-specific response similar to that of malate; accumulating amino acids, carbohydrates, and organic acids under phosphorus deficiency may explain the increased glycerol-3-phosphate quantity (Obata and Fernie, 2012), because both strains showed phosphorus solubility that leads to enhanced phosphorus availability for endophytic bacteria-applied plants.

Similarly, lack of potassium in plants can be linked to increased soluble sugars concentration (Armengaud et al., 2009), which was observed in the case of uridine diphosphate

glucose in this study (Figure 3.2). Furthermore, the higher ascorbate concentration in strain 4-applied plants may have been produced to counter oxidative damage (Smirnoff and Wheeler, 2000), whereas the same function may have been served by nicotinic acid in strain 227 (Berglund et al., 2017), because nicotinic acid was higher in strain 227-applied plants. It can thus be concluded that endophytic bacterial strain-specific response may prevail, and production or induction of certain metabolites can perform specific functions that enhance plant growth.

The endophytic bacterial strains led to different classes of metabolite production or induction that were not detected in control plants. Endophytes have been explored to produce or induce the production of certain metabolites, and their diverse backgrounds have been linked to such mechanisms (Fernandez et al., 2012; Ludwig-Müller, 2015; Venugopalan and Srivastava, 2015). Production, however, varied between both PGP endophytic bacteria (i.e., strains 4 and 227 used in this study), which may be related to the genetic makeup of the microbes. A variety of microbes have been observed to produce diverse kind of metabolites that benefit plants (Firáková et al., 2007).

Strain 227, besides sharing certain metabolite-production trends with strain 4, showed an exclusive role in carnitine metabolism through actinine and carnitine production. Carnitine has been linked to fatty acid metabolism (Bourdin et al., 2007) and accumulated in treatments in which PGP bacteria and plant growth regulators were applied (Khan et al., 2019). Jacques et al. (2018) suggested that rhizosphere microbes provide carnitine to plants in addition to other functions, such as transportation of metabolites and cell maintenance. Furthermore, 6-N-trimethyllysine was 1.6- and 1.5-fold higher in strains 4- and 227-applied plants, respectively, compared with the control plants, and γ-butyrobetaine was only detected in strain 227-applied plants. 6-N-trimethyllysine and γ-butyrobetaine are precursors of L-carnitine in plants (Rippa et al., 2012), which was also observed in this study. However, the role of γ-butyrobetaine seems

to be more important in carnitine biosynthesis of plants; varying quantities of 6-N-trimethyllysine were observed in all the three treatments, but carnitine was only observed in the strain 227-applied plants, which demonstrates the importance of γ-butyrobetaine in carnitine biosynthesis.

The metabolomic study also revealed that very little is known about the interaction of endosymbionts with their hosts, which led to not even a single metabolite concentration similarity among each of two treatments (Figure 3.4); this indicates that, although strains 4 and 227 enhanced plant growth in the pot experiments (Table 3.3) to similar extents, there was substantial contrast among the metabolites detected within the plant tissues. Consequently, each endophytic bacterium has a different role in enhancing plant growth; thus, solely studying the axenic PGP traits may not be useful for screening of isolates, but real plant growth experiments are needed. Another important metabolite, oxidized glutathione, was lower in both bacteria-applied plants compared with the control. Glutathione accumulation has been documented in response to pathogenic infection (Noctor et al., 2012); thus, it can be postulated that neither endophyte was pathogenic toward the host plants. Endophytic interactions with host plants can thus be considered a complex process, and applied endophytic bacteria can produce or induce the production of certain metabolites that enhance plant growth.

3.5. Summary

Plant growth promoting endophytic bacteria were isolated from cucumber, and significant contributions towards root length were observed. This growth enhancement by selected endophytic bacterial strains viz. strain 4, 72, 167, 193, and 227 was later confirmed by different molecular and genetic traits responsible for growth and number of fruits' increase. The subsequent two-year field trial was carried out with the same 5 endophytic bacterial strains, which cleared the results gained in pot experiments. The applied endophytic bacteria not only contributed to vegetative growth, but also strains 4 and 227 significantly affected number of

fruits when compared with that of non-treated plants. These two strains thus can be identified as potential candidates for improving cucumber growth and yield. In later trials, both these strains i.e. strain 4 and 227 were evaluated for their role in plant metabolomics and considerable regulation of metabolites was observed. It can thus be concluded that plant growth promoting endophytic bacteria can be attractive approach for sustainable crop production.

CHAPTER 4

APPLICATION OF BACTERIA IN AMELIORATION OF SALINITY

4.1. Introduction

Soil salinity is a worldwide problem and is attributed to natural and human induced salinization. Natural factors attribute to parent material, soil characteristics, and climatic parameters. While, irrigation with saline water, poor drainage practices, and excessive irrigation are among the anthropogenic factors. Such salt-affected soils hinder the plant growth and are on the rise throughout the world and are estimated to cover an area of 260 Mha worldwide (Cherlet et al., 2018). Thus, under the increasing food requirement and decreasing land area circumstances, utilization of salt affected soils is needed for at least bringing them under vegetation, besides opportunities of fodder production (Tlili et al., 2017).

Plants adapt to salinity stress through various mechanisms; expulsion of sodium (Na^+) ions, reducing their uptake, and limiting the concentration in the cytoplasm by storing it in other organelles (Arzani and Ashraf, 2016). The roots being the first to face salinity stress adapt to the stress through twisting and curling, besides thickening of the cell walls. Roots also develop specified Casparian strips to regulate the influx of salts (Karahara et al., 2004). However, the crop plants being glycophytic in nature are sensitive to salinity stress (Al Hassan et al., 2016), and salinity level up to 100-200 mM can inhibit the growth of such plants (Newell, 2013). Zhu (2001) postulated that glycophytes must adapt to the salinity slowly, either through natural, or artificial breeding. It is also put that genes from halophytes can help improve the halotolerance in glycophytes (Newell, 2013). Breeding for complex salt tolerance trait been met least success (Flowers, 2004; Hanin et al., 2016), thus an integration of conventional breeding, and genetic engineering can make it fruitful (Fita et al., 2015). Additionally, the increased uniform cropping caused the loss of wide variety of cultivars forever (Hammer and

Khoshbakht, 2005), limiting the genepool. Such low variation genetic pools are attributed to limited success in building salt tolerant varieties (Turan et al., 2012). Thus, vegetation of such salt affected soils requires bypassing new variety development and utilize present resources. The microbe assisted tolerance of such stresses seems attractive, as rhizosphere microbes besides tolerating stress also confer tolerance to the plants in addition to promoting the latter's growth (Banik et al., 2018; Jha and Subramanian, 2016).

Microbes in discussion encompass rhizosphere bacteria which enhance the plant growth under saline conditions through different processes, viz. ion homeostasis (Ilangumaran and Smith, 2017), influencing the genetics, and proteomics (Paul and Nair, 2008), antioxidant enzymatic activity (Kim et al., 2014), release of exopolysaccharides bonding with the sodium (Upadhyay et al., 2011), accumulation of inorganic solutes like K^+, Na^+, and Mg^+ (Egamberdiyeva and Islam, 2008) and reducing the ethylene level through 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Mayak et al., 2004; Siddikee et al., 2010). The synergism between plant and microbes helps the plants adapt to their specific habitat, as reported in case of thermotolerance (Redman et al., 2002), and halotolerance (Rodriguez et al., 2008). Correspondingly, potential of microbiome associated with halophytes, epiphytic or endophytic, has vast potential of enhancing the plant growth under saline conditions. For instance, application of salt-tolerant *Serratia marcescens* regulated the osmoprotectants, and antioxidant activity conferring salinity tolerance to wheat plants (Singh and Jha, 2016). Similarly, *Bacillus pumilus* and *Pseudomonas pseudoalcaligenes*, when applied to salt-sensitive variety of rice under the saline conditions, significantly reduced the lipid peroxidation and superoxide dismutase activity. Additionally, the inoculation enhanced the cell viability through decreased reactive oxygen species (ROS) toxicity (Jha and Subramanian, 2014). In the soil, glomalin related soil proteins (GRSP) were reported increased with the inoculation of plant growth promoting rhizobacteria PGPR (Kohler et al., 2010). Paul

and Nair (2008) concluded that PGPR can be successfully employed as crop inoculants under saline conditions.

Mesembryanthemum crystallinum L. (Common ice-plant); native to Africa, inhabits terrestrial and coastal area of Americas, Australia, and Europe, and is being utilized throughout the world (Atzori et al., 2017; You et al., 2015; Zhang et al., 2018). It has evolved to survive in saline environments through shifts in metabolism pathways, compatible solutes accumulation within the cytosol (Libik et al., 2004), and developing bladder cells (Trofimova et al., 2003). Such mechanisms help the plant withstand the stress through osmotic regulation. Consequently, the Common ice-plant can be grown in higher salinity levels (Stetsenko et al., 2009), even irrigated with seawater (Atzori et al., 2017), which will help revegetation of such saline habitats.

Growing the plants in such habitats through seed, or nursery is hindered due to higher levels of salinity and salt-sensitivity of the plants. Thus, growing the nursery under low NaCl conditions and transplanting seems possible solution. Besides, microbe inoculation can help plants withstand such saline conditions (Jha and Subramanian, 2014; Singh and Jha, 2016). On the other hand, survival and functionality of microbes is also limited due to salinity, so tolerant microorganisms are needed to be explored. Aiming so, the rhizosphere of Common ice-plant was explored for incident salt-tolerant bacteria having plant growth promoting traits.

4.2.Materials and methods

4.2.1. Soil and Plant Material

For the common ice-plant, soil was sampled from two different locations viz. University of Yamanashi Research Farm as Soil-I, and coastal area as Soil-II, where in the latter; two locations viz. 2 m from sea (Soil-IIa), and 20 m (Soil-IIb) from sea were selected (N35°32’31.5’’, E134°12’35.3’’). 1 kg of soil from the first location was simultaneously used

to grow three Common ice-plant plants in a pot with salinity level of 5 g kg^{-1} using NaCl (Nacalai Tesque, Inc., Kyoto, Japan) and was later used for isolation of bacteria as Soil-III.

4.2.2. Isolation of rhizosphere bacteria

The isolation was carried out from all three soils through serial dilution plate technique. 0.5 g of soil was mixed with sterilized distilled water (SDW), and for each dilution 0.5 mL of suspension was transferred to the next tube containing 4.5 mL of SDW. Each suspension was mixed thoroughly using Vortex Mixer, and from the 3rd tube, an aliquot of 50 µL was spread on Potato Dextrose Agar (PDA) media (Eiken Chemical Co. Ltd., Tochigi, Japan), adjusted to pH 7, and R2A agar media (Oxoid Limited, Hampshire, UK) using single-use plastic spreader. The colonies appeared after 96 hours were picked and streaked until single colony per plate was obtained. 20% Glycerol stock (in 0.8% NaCl w/v) was prepared for all of the isolates and stored at -80ºC.

4.2.3. Screening and characterization of rhizosphere bacteria

Salinity tolerance of the isolates was carried out in liquid culture, modified from (Mendpara et al., 2013) of Potato Dextrose Broth (PDB, pH 7) and R2A broth with 513 mM of salinity compared with control without NaCl (0 mM). The culture was grown for 72 h at 25ºC, and the bacterial growth was measured using spectrophotometer at 660 nm at the end of incubation.

Following, the growth of two strains selected later, based on their salinity tolerance and PGP potential, were also grown in PDB at 250, 500, 750, 1000, and 1250 mM NaCl concentration compared with 0 mM in Erlenmeyer flasks. The experiment consisted of 3 replications. After 2 weeks of incubation, the growth of both strains was to be measured at 660 nm, however the optical density was observed only in PR-6, whereas due to the floc formation by strain PR-3, the later suspension was harvested using vacuum fitted stainless steel strainer, and dry weight of the pellet was measured post drying for 48 h at 60ºC.

Moreover, all the strains having salinity tolerance were tested for production of indole acetic acid (IAA) as explained in previous sections.

4.2.4. Assay for plant growth promoting ability and PGP traits of selected strains

Plant growth promoting ability of the strains selected on the basis of production of IAA was tested using vertical petri plate technique, where five surface sterilized seeds of Common ice-plant were placed on the upper side of the agar petri plate, and bacteria was streaked on the bottom side [modified from (Liu et al., 2014)]. The length of root was monitored and measured after 7 days while comparing with control having no bacterial inoculation.

Phosphate solubilization, siderophore production, ACC deaminase and nitrogen fixation activities were checked for all the endophytic and rhizosphere strains (Section 3.2.3).

4.2.5. 16S rRNA analysis of selected bacteria

To identify the selected microorganisms (PR-3 and PR-6), the nucleotide sequences of their 16S rDNA were investigated using molecular techniques as explained in section 2.3.2.2. The nucleotide sequences determined were compared with those in the DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/). All sequence data, including newly obtained and retrieved sequences, were aligned using the computer program BioEdit (available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Distance-based phylogenetic trees were generated using the model of Jukes and Cantor (1969) and a neighbor-joining algorithm (Saitou and Nei, 1987). The topology of phylogenetic trees was evaluated by bootstrap resampling (1,000 replicates). Clustal W, provided by the DNA Data Bank of Japan (available at http://www.ddbj.nig.ac.jp/Welcome-j.html), was used for the analyses.

4.2.6. Pot experiment for plant growth promoting ability of selected strains

Similarly, a pot experiment was conducted using the potential strains; PR-3 and PR-6 under saline conditions. The pots were filled with 500 g of soil and one seed per pot was planted. Each strain as the bacterial suspension, grown under incubation shaking for 48 hours at 25ºC,

in potato dextrose broth (PDB) was inoculated into the soil surrounding the Common ice-plant, 18 days after germination. Same volume of PDB as used for the treatments was used control treatment, which did not include any bacteria. The stress was applied using NaCl solution; 100 mM for first 2 days, and 300 mM for next 2 days (total 4.73 g of NaCl kg^{-1} soil), followed by irrigation with distilled water once every day starting from 25^{th} day of germination. The experiment consisted of 5 replications. After 32 days, the plants were harvested and were subjected to measurement of root length, fresh and dry weight of stem and leaves.

4.2.7. Statistical Analysis

The means were calculated for the replications, and statistical analysis was carried out using student *t*-test between treatments and control.

4.3. Results

4.3.1. Isolation, and characterization of rhizosphere bacteria

All soil materials collected were analyzed for incident bacteria using the respective media, and it was observed that each site resulted into varying number of strains. The isolation carried out from all the locations resulted in 152 strains in total; where 64 strains were isolated from university research farm (Soil-I), 64 from coastal area (Soil-II) 1, 24 from coastal area 2 (Soil-II), and 16 from the rhizosphere of Common ice-plant grown in soil (Soil-III).

The functionality of microbes depending upon their adaptation to the target habitat was tested through exploring the potential of isolates to grow under saline environment. For the purpose, the salinity tolerance screening consisting of 513 mM concentration of NaCl was carried out, compared with control, where 80 out of 152 strains (52.6%) were observed tolerant, and could grow well on the salinity level applied, and significant differences were observed between the absorbance of control, and saline cultures of the respective strain. More than half of the strains showed high salinity tolerance in relation to their natural habitats. Corresponding

to the media used to grow the strains, only 36.4% of the strains were found salt tolerant from PDA media, whereas 50% were observed tolerant growing on R2A media.

The two selected strains, PR-3, and PR-6; isolated from rhizosphere of Common ice-plant were further evaluated for their salinity tolerance, and it was observed that the growth of bacteria showed linear relationship with the NaCl concentration except for the 1000 mM concentration (Figure 4.1). The pellet dry weight for PR-3, and OD_{600} for PR-6 showed similar trend. The rhizosphere of Common ice-plant resultantly provides the evidence of recruiting function-specific strains, helping the plant grow better in the salinity stress, however the incidence of the salinity tolerance can be rare trait.

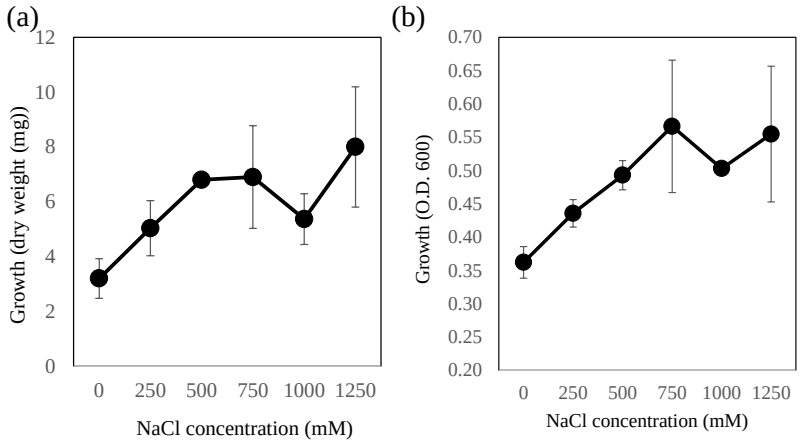


Figure 4.1 The salinity tolerance ability of strain PR-3; (a) presents the dry weight of the pellet harvested after 2 weeks of incubation, and PR-6 (b); illustrates the measurement of optical density at 600 nm. The values are mean of three replications and error bars show the standard deviation among the replications.

The IAA production by the microbes being important for initial plant growth, and influencing the root architecture, hence was selected as a candidate property for observing the PGP potential of isolates. The IAA assay resulting in the emergence of pink color of the media was examined, and both the strains i.e. PR-3, and PR-6 were found positive for the IAA production (Table 4.1) with tryptophan. In the initial experiments, all of the isolates were explored, where 53 out of 80 strains showed color change, compared among the strains, and against control. Both the strains in discussion showed higher activity among the tested isolates.

Table 4.1 Plant growth promoting traits in salinity tolerant strains

	IAA	P solubilization	N fixation	ACC deaminase	Siderophore
Streptomyces sp. PR-3	+	+	-	+	-
Bacillus sp. PR-6	+	-	-	+	+

+ indicates the possession of following trait where – indicates the lack of the trait. Both the strains showed NaCl tolerance up to 1250 mM.

4.3.2. Assay for plant growth promoting ability

The PGP traits are worth mentioning if the plant growth is enhanced significantly, and vice versa. For this purpose, plant growth promotion potential of the isolates was evaluated based on the clear changes in root architecture occurred in petri plates with and without inoculation. From all the isolates tested, the 2 strains; PR-3, and PR-6 were found enhancing the root length of Common ice-plant when compared to control, however only PR-6 had significant effect ($P<0.05$). The fresh weight of root was significantly increased in both treatments against control ($P<0.05$) (Figure 4.2).

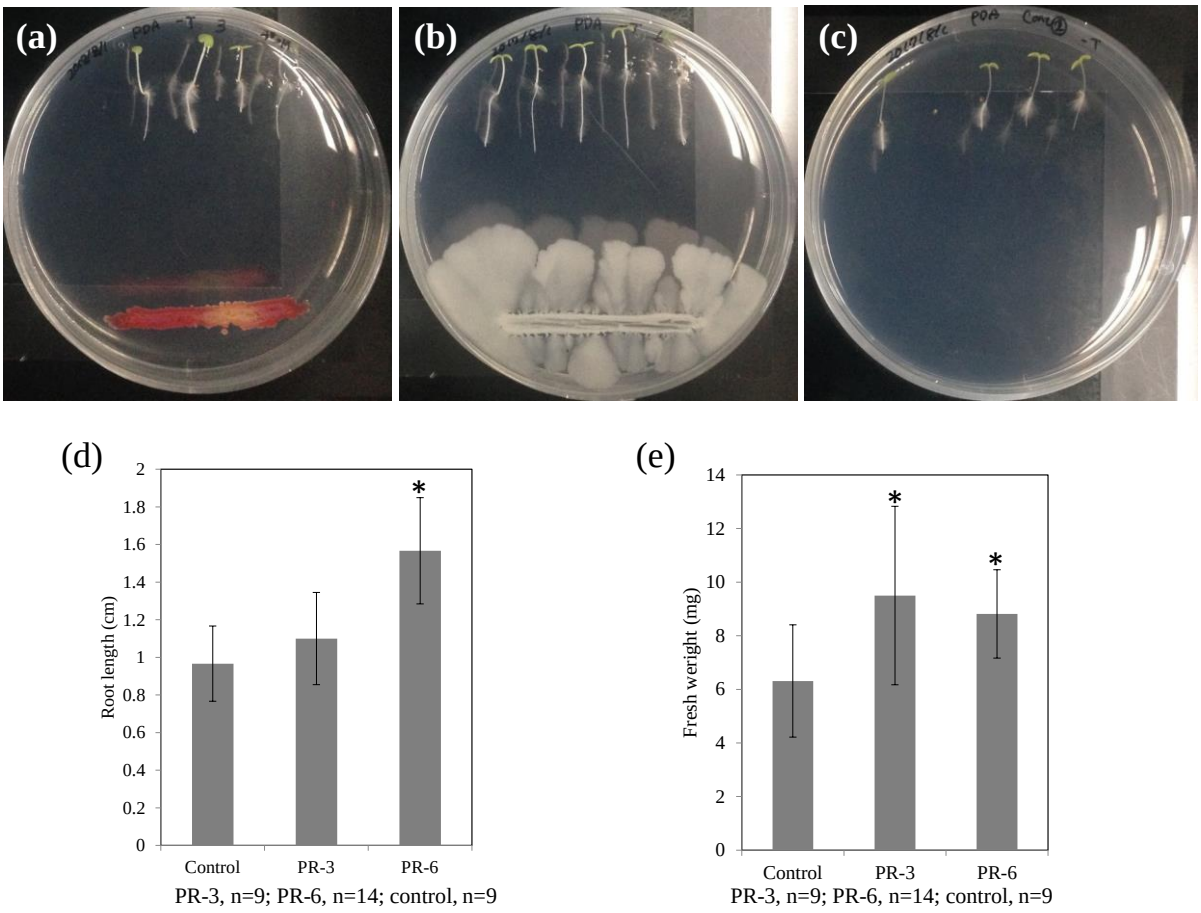


Figure 4.2 The effect of bacterial strains on root growth as compared with Control **(a)** The growth of Common ice-plant as affected by strain PR-3, **(b)** The growth of Common ice-plant as affected by strain PR-6, **(c)** The growth of Common ice-plant as affected by no inoculation, **(d)** The root length measured following the experiment as affected by Control, PR-3, and PR-6, **(e)** The fresh weight of the root following the experiment as affected by Control, PR-3, and PR-6. The bars show the standard deviation among the replications, where * indicates the statistical significance of the treatments when compared with control treatment using student t-test ($P<0.05$).

4.3.3. 16S rRNA analysis of selected bacteria

The 16S rRNA gene sequences acquired post-DNA extraction for both strains were compared with those of present, and already reported in the GenBank. The length of DNA base pairs was observed at 610 bp. The genus level identification was acquired, and the phylogenetic dendrogram showed that the strains PR-3, and PR-6 were closely related to *Streptomyces diastaticus* strain WZ902, and *Bacillus subtilis* subsp. inaquosorum strain LM03-B (Figure 4.3). Both the strains were submitted to GenBank with accession numbers as LC390202 for PR-3,

and LC390203 for PR-6. Identification and accession numbers for strains 4 and 227 are listed in Table 3.1.

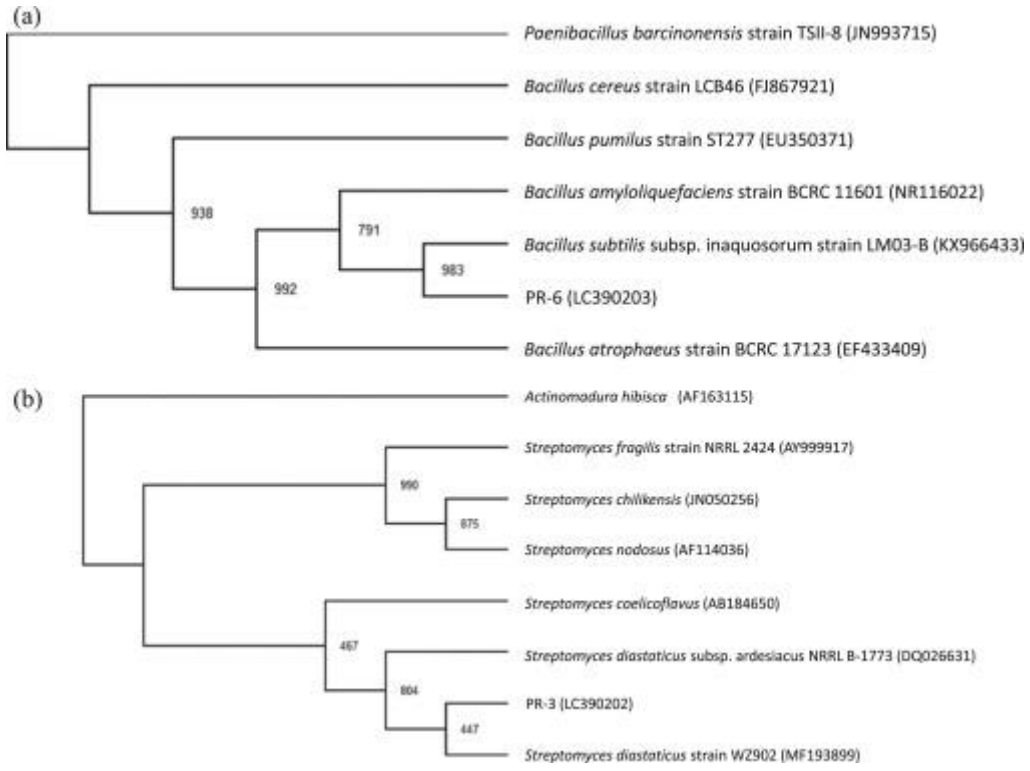


Figure 4.3 The distance-based phylogenetic tree of strain PR-6 (a) and PR-3 (b) used in the study constructed using the neighbor-joining algorithm.

4.3.4. Assay for PGP traits of selected strains

The strain PR-3 showed halozone on Pikovskaya agar petri plates when compared with control showing the P-solubilization ability. PR-6 did not show clear zone.

Further, gene amplification for the *nif*H, and ACC deaminase was carried out for the confirmation of the corresponding traits, and gene amplification assays indicated that none of the isolates were capable to fix nitrogen, but both of them were ACC deaminase positive advocating the presence of stress related characteristics needed for survival in saline environments. The incidence of ACC-deaminase activity among the isolates ensuring the stress tolerance by microbes, as well as providing the plant with reduced ethylene concentration was expected.

Finally, the ability of strains to produce siderophores; enhancing the availability of iron both for microbial, and plant cells, besides providing the pathogen control, was checked. The siderophore production was reciprocal to that of phosphorus solubilization, and only PR-6 showed the ability. The full, or partial color changes were observed, and where PR-3 showed no color change given the '-', the PR-6 showed a '+' with complete change of the color to yellow when compared with control, and other non-siderophore producing strains.

4.3.5. Pot experiment for plant growth promoting ability of selected strains

The pot experiment, after investigation of PGP traits through laboratory techniques, was conducted (Figure 4.4), and in parallel to axenic assays, the root length results obtained from the pot experiment were in the order of control being the lowest, PR-3 and PR-6. However, only PR-6 showed a significant increase in root length comparing to the control ($P<0.05$). Both strains showed substantial increases in fresh root weight and above ground biomass, over the control treatment, but only PR-3 differed in root weight ($P<0.05$), where both the bacterial applications differed significantly for fresh weight of above-ground parts ($P<0.05$). The control treatment resulted in less than half of the contributions made by the treatments in both the weight parameters.

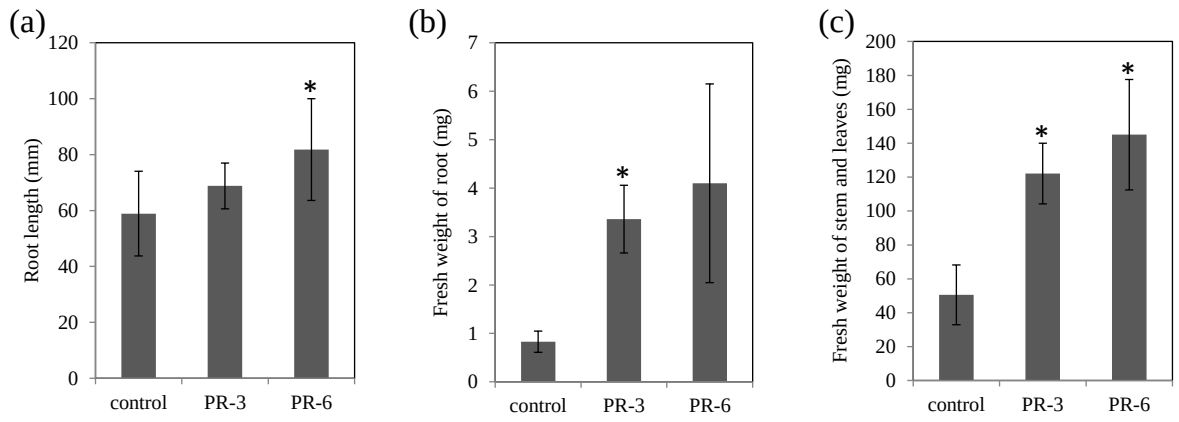


Figure 4.4 (a) The root length measured following the pot experiment as affected by Control, PR-3, and PR-6, **(b)** The fresh weight of the root following the pot experiment as affected by Control, PR-3, and PR-6, **(c)** The fresh weight of above ground plant parts following the pot experiment as affected by Control, PR-3, and PR-6. The bars show the standard deviation among the replications, where * indicates the statistical significance of the treatments when compared with control treatment using Dunnett's test ($P<0.05$).

4.4. Discussion

The sites explored for incident bacteria resulted into 152 strains in total, corresponding to similar results by different studies (Grobelak et al., 2015; Rahman et al., 2017; Zhao et al., 2018). Similar instances of halotolerant bacteria have been reported to enhance the plant growth besides conferring salinity tolerance (Table 4.2). The plants influence the rhizosphere community (Hubbard et al., 2017), and are selective for the population dynamics of the microbes for target functions. The function here was the growth of plants under salinity stress, and as discussed above almost 52% strains not only showed tolerance to salinity, two of them even promoted plant growth under such circumstances. Relatedly, rhizosphere of other halophytes explored; *Salicornia europaea* (Szymańska et al., 2016a) and *Suaeda salsa* (Yuan et al., 2016), has shown potential benefits of salinity tolerance, and plant growth enhancement. The genetic, and biochemical adaptation in the stress environments also enables bacteria with the mechanisms needed for coping such conditions. Instances of salt tolerance of rhizosphere bacteria (Table 4.2) advocate the synergism of plant and microbes against biotic and abiotic

stresses. The augmented salt tolerance of bacteria is a consequence of regulation of ions (Vreeland, 1987), transporters (Zhang et al., 2008), and genes.

Table 4.2 Salt tolerance level of different rhizosphere bacteria

Inoculated to	**PGPR genera**	**Salinity tolerance**	**Reference**
Ice plant	*Streptomyces* spp. *Bacillus* spp.	<1250mM	This study
Tall fescue	*Enterobacter*	<2600 mM	(Kapoor et al., 2017)
Wheat	*Dietzia*	<1000 mM	(Bharti et al., 2016)
Soybean	*Stenotrophomonas*	<770 mM	(Egamberdieva et al., 2016; Wolf et al., 2002)
Mungbean	*Pantoea*	<1200 mM	(Panwar et al., 2016)
Wheat	*Serratia*	<1027 mM	(Singh and Jha, 2016)
Rice	*Bacillus* *Citrobacter*	<1027 mM	(Habib et al., 2015)
Wheat	*Klebsiella*	<1027 mM	(Singh et al., 2015)
Groundnut	*Ochrobactrum*	<300 mM	(Paulucci et al., 2015)
Galega	*Pseudomonas*	<500 mM	(Egamberdieva et al., 2010)

Wide extent of sampling, along with modification of the soil habitat resulted into high proportion of salinity tolerance, although limited in some, while higher in the other strains. The salinity tolerance shown by the strains PR-3, and PR-6 in this study is in accordance with many studies, as examples of salinity tolerance by rhizosphere bacteria (Table 4.2) isolated from non-saline and saline habitats have been reported (Bharti et al., 2013; Kataoka et al., 2017; Park et al., 2017; Ramadoss et al., 2013; Shukla et al., 2012). The range of salinity tolerance corresponds to the habitat, and halophilic bacteria can grow even near the saturation point having 26.4% salinity (Banwart, 1989), so the diversity of microbes, and their tolerance ability can vary as observed in this study.

The plant growth promoting ability of the isolates, PR-3, and PR-6, was observed, which in later biochemical and molecular characterization was confirmed. First, the aforementioned isolates had the ability of producing IAA. Similar results have also been obtained in certain studies; where plant growth promotion, IAA production, phosphorus solubilization, and ACC deaminase activity have been reported from halophytic endophytic

and epiphytic bacteria (Szymańska et al., 2016b). Bacteria isolated from diverse environments have been found capable of producing IAA with a high probability of production by the isolates from extreme environments (Mapelli et al., 2013; Qin et al., 2015). Similarly, the induction of IAA production by the salt stress has also been reported (Dong et al., 2017). The plant growth under salinity stress being limited, asks for recruitment of PGP bacteria which enhance root growth through production of indole acetic acid (Kloepper et al., 2007; Yang et al., 2009), and other mechanisms.

The strain PR-3 was found to have the phosphorus solubilizing potential between the two investigated. Different cases of phosphorus solubility have been reported by various microbes, and the genus *Streptomyces* as shown in this study; among actinomycetes has this ability as reviewed by (Alori et al., 2017a). The production of various organic acids has been stated playing role in solubilizing phosphorus (Alori et al., 2017b; Wei et al., 2018). The tendency of bacteria to produce specific organic acid according to the phosphorus source has also been investigated (Chen et al., 2016), which suggests the flexibility in the bacterial capabilities.

No strain was found positive for presence of *nif*H gene. Likewise, limited diazotrophic association with plants is also known (Dahal et al., 2017), which can be a reason behind no nitrogen fixation capacity of investigated bacteria.

Both the strains were found positive for ACC deaminase activity, as reported (Qin et al., 2014), also identifying the potential role of halophyte-associated bacteria in ameliorating the salinity stress (Navarro-Torre et al., 2017; Qin et al., 2014).

Furthermore, the siderophore production shown by the *Bacillus* spp. strain PR-6 in this study, has been reported widely in the same genus (Yu et al., 2011), where examples of siderophore producing bacteria from halophytes have also been published (Kataoka et al., 2017; Sgroy et al., 2009). The competition for the iron acquisition, and the potential of

siderophores against biocontrol of pathogens leads towards the siderophore production, thus helping the plants withstand with the stress.

4.5. Summary

Streptomyces sp. PR-3 and *Bacillus* sp. PR-6 from this study were selected as salt tolerant (<1200 mM NaCl) bacteria having Common ice-plant growth promoting ability. Either or both strains also showed genetic, and functional traits of plant growth promotion including indole acetic acid, phosphorus solubilization, ACC deaminase activity, and siderophore production. However, neither of the two had nitrogen fixation ability. Such salt tolerant PGPR can assist the phytoremediation of saline soils.

CHAPTER 5 PERSISTENT ORGANIC POLLUTANTS (POPS) DEGRADING BACTERIA

5.1. Introduction

Persistent organic pollutants (POPs) as categorized under Stockholm Convention 2001 have long been used in controlling pests of animals, and crop plants. In the initial years of development, such chemicals were used extensively and sometimes directly to animal bodies. The case of dichlorodiphenyltrichloroethane (DDT) can be quoted as an example, and although banned in most of the countries, it still persists in soil besides being produced and used in some parts of the world (van den Berg et al., 2017). Similar is the case with pentachlorophenol (PCP) which serves as leather and wood preservative, however is toxic to animals when exposed. Production of PCP although has been banned in most parts of the world, yet it is still being produced and used (van der Zande, 2010). The main problems with these POPs are their persistence, long-range transportation and bioaccumulation potential. Their persistence in soil is cause of concern due to chances of their entry into the food chain besides runoff towards water bodies. Certain plant groups can uptake such pollutants, and their residues in fruits have pose a continuous threat (Namiki et al., 2013), and can eventually gain entry to the food chain. Food contaminated with such pollutants can lead to several health problems in animals (U.S. Environmental Protection Agency, 1999), thus contaminated sites need to be cleaned. Similarly, biological damage to plants growing in POPs-contaminated soils has been known in terms of reduced germination, decreased biomass and chlorophyll contents (Hanano et al., 2014).

The reclamation of such polluted soil resources can be carried out through physical, chemical and biological methods (Yang et al., 2018). The mechanical and chemical methods have certain issues encompassing those of being: costly, lesser public acceptable, and not environment friendly besides compromising the soil quality (Lim et al., 2016). Biological methods; phytoremediation and bioremediation, on the other hand can be termed comparatively

more sustainable and applicable to contaminated sites. The phytoremediation although has been successful in removing the POPs from soil, but later handling issues, phytovolatilization, and uncontrolled transformation limit its use (reviewed by Khan and Doty, 2011). Bioremediation or the use of microorganisms in removal of such pollutants from soil can be an attractive alternative. Similarly, endophytic microbe-assisted phytoremediation also seems striking option because of the potential of microbes in degrading the pollutants after uptake by the plants (Khan and Doty, 2011). Plant groups like those of cucurbits have been observed accumulating organochlorine pesticides (Clostre et al., 2014), thus can be explored for incident endophytic bacteria which can have possible role in degradation of such contaminants.

The microbes evolve to the incident xenobiotics quickly and can degrade such chemicals to minimum possible metabolites (Haiser and Turnbaugh, 2013; Top and Springael, 2003). Microorganisms also have the potential for novel pathways and genetic capabilities in degrading such pollutants. Another issue with respect to pollutant-uptake by plants; including those of the accumulator plants is the availability of the pollutant. It can be hypothesized that if endophytic microbes can enhance the bioavailability of such pollutants, their uptake can be improved which can later be attacked by the degrading-microbes residing within the endosphere. The rhizosphere microbes' functionality is also countered by co-contamination which is the case for many polluted sites worldwide. Here again, the endophytes may have merits over those of free-living microbes.

Endophytic bacteria-assisted phytoremediation can thus be used for removal of DDTs and PCP from soil besides the bioremediation of the remaining pollutants within the soil. There are instances where pollutant degrading microbes have been isolated from soil, and have been employed to degrade DDT and PCP, both in axenic and field conditions. For instance, *Rhodococcus wratislaviensis* was isolated from soil with DDT application history, and was introduced to same soil under laboratory condition and DDT degradation was observed

(Egorova et al., 2017). Another report of DDT degrading bacterium from DDT contaminated site was reported, and the degradation capability of the bacterium *Ochrobactrum* spp. was confirmed by gene presence (Pan et al., 2017). A degrading bacterium from organochlorine pesticides-contaminated soil was observed degrading DDT in pot experiment, and lower metabolites were observed (Qu et al., 2015). Several other instances of degrading bacteria from contaminated soil have been observed as well (Bajaj et al., 2014; Lovecka et al., 2015; Wang et al., 2011; Xie et al., 2011). Incidence of PCP in soil ecosystem and its transportation to water bodies has also raised problems. Similar to that of DDTs, PCP-degrading bacteria have been explored for remediation of PCP from the environment (Lopez-Echartea et al., 2016). In addition, El-Bialy et al. (2019) explored paddy soil and found two promising PCP degraders.

Endophytic bacteria-assisted phytoremediation has been explored for various kind of pollutants including those of heavy metals as well as the organic pollutants (Afzal et al., 2014; Gatheru Waigi et al., 2017). Heavy metal accumulation was observed increased when a consortium of bacteria and fungi was applied along with Salicaceae trees (Guarino et al., 2018). For the organic pollutants, an interesting report presented that mulberry roots accumulated the organic pollutants and when the roots were dead, were attacked by polychlorinated biphenyls degrading bacteria (Leigh et al., 2002). Similarly, collaboration of an endophytic fungus with rice resulted in removal of polycyclic aromatic hydrocarbons from rice seedlings (Fu et al., 2018). The literature thus suggests the potential of microbiome associated to polluted soils in remediation of such soils. The endophytic microbes can also be used for removal of pollutants from soil. The endophytes can be applied before sowing the phytoremediation agents which can exert quick colonization of the endosphere, and this symbiosis can subsequently remediate the contaminated soil. The bioaugmentation is necessary for quick degradation as observed (Egorova et al., 2017).

Furthermore, a DDT producing factory located in north west of Pakistan KPK (34° 00' 24" N and 71° 56' 04" E) operated between 1963-1994 (Khwaja et al., 2006). Vegetation, soil and water in the surrounding areas had concentration of DDT or its metabolites up to 500-4500 µg kg^{-1} and 7.5-2841 µg g^{-1}, 0.07-0.4 µg ml^{-1}, respectively (Jan et al., 2009; Younas et al., 2013). These studies indicate that monitoring of residues over the time has been carried out, but the remediation efforts are lacking. This study was thus planned to revisit the site and look for locally adapted microbes which can degrade the DDT residues and can be used for *in situ* remediation of the site. Also, endophytic bacteria were explored for their role in degradation of DDT and PCP, which would be used for remediation of contaminated soil sites.

5.2. Materials and Methods

5.2.1. Site description, soil and plant material

An abandoned dichlorodiphenyltrichloroethane (DDT) producing factory located at Amangarh, Nowshera, Pakistan (34°00′24″ N and 71°56′04″ E) was sampled in March 2017. The soil samples were collected from 20 different points (each point approximately 2-3 m in diameter) starting from the very location of the factory production unit and spreading to each direction. Rhizosphere and bulk soil were sampled, and each sample weighed approximately 50 g. The location was observed being transformed to residential area, and was different to earlier reports (Younas et al., 2013). Animal gazing and children playing was also observed. Uncontrolled sewerage water from nearby houses was flowing close to the vicinity.

The soil samples were imported to Japan under permission from Department of Plant Protection of Pakistan (Permit No. 454611), and Kawasaki Branch, Yokohama Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries, Japan (Permit No. 29Y362). The imported soil was handled according to the Plant Protection Departments' guidelines, labelled and was kept in separation in cold storage facility (4 °C) of Department of Environmental

Sciences, University of Yamanashi until further use. The soil was subjected to manipulation within a year of sampling.

For the endophytic bacteria, diverse kind of Cucurbitaceae plants were used for isolation. In first set of experiments, cucumber, zucchini, pumpkin and bitter gourd were grown at University of Yamanashi Research Farm and sampling was carried out followed by isolation (Section 2.2.3.1). All of the cucurbits were later grown in DDT contaminated soil imported from Pakistan under growth conditions mentioned in Section 3.2.2. Explanation regarding isolation sources is given below:

- Cucumber stem, leaf stalk and roots
- Zucchini stem and roots
- Pumpkin stem and roots
- Bitter gourd stem and roots
- *Luffa acutangular* (Hechima/Luffa) stem and roots
- *Lonicera tschonoskii* (Hyoutan) stem and roots
- *Benincasa hispida* (Tougan/Wax gourd) stem and roots
- Wild-type plants
    - Cucumber from two different locations
    - Pumpkin
    - Weed plants
- Grown in Pakistan soil; contaminated with DDT
    - Cucumber stem, leaf stalk and roots
    - Zucchini
    - Pumpkin
    - Bitter gourd
    - *Luffa acutangular* (Hechima/Luffa)
    - *Lonicera tschonoskii* (Hyoutan)
    - *Benincasa hispida* (Tougan/Wax gourd)
- Grown in artificially contaminated soil (soil was collected from four different sources in order to enhance the rhizosphere microbial pool)
    - Cucumber

5.2.2. Chemicals and measurement of DDTs from soil

Dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene (DDE) dichlorobenzophenone (DBP) and organic solvents including those of acetonitrile, acetone, and hexane were purchased from Dr. Ehrenstorfer GmbH, Germany. 500 mg L^{-1} stock solution of all the chemicals was prepared using high performance liquid chromatography- (HPLC) grade acetone. 500 mg L^{-1} stock solution of DDT in acetone was provided by National Agriculture and Food Research Organization (NARO) Division of Hazardous Chemicals, Institute for Agro-Environmental Sciences, Tsukuba, Japan.

For measurement of DDT and its derivatives, 4 g of soil from composite sample (explained in next section) was weighed in 50 ml-falcon tube, put to sonication for 30 min at 60 °C after mixing with 20 ml of 50% acetonitrile aqueous solution. The sonication was followed by horizontal shaking for 30 min at 200 shakings per min. The falcon tubes were then centrifuged for 10 min at 2500 rpm. 1 ml of the supernatant was transferred to 1.5-ml Eppendorf tube and centrifuged again at 13500 rpm for 10 min. 800 µl of the supernatant was transferred to glass-vial and measured using HPLC against a set of standards. HPLC was fitted Inertsil® ODS-SP (4.6mm×250mm) column (GL Sciences Inc., Tokyo, Japan). The samples were injected with 70% acetonitrile in 0.1% trifluoroacetic acid aqueous solution at flow rate of 1 ml min^{-1}.

5.2.3. Isolation and identification of bacteria

5.2.3.1. Isolation from DDT-contaminated soil from Pakistan

Composite sample was prepared by mixing the soil from five sampled points (closest to production unit) and used for isolation of bacteria through dilution plate technique (Section 4.2.3). All the isolates were subjected to degradation test (explanation follows), and a DDD degrading strain 885C was identified using 16S rRNA sequencing.

5.2.3.2. Isolation from stem and root endosphere

For the isolation of endophytic bacteria, same procedure as explained in chapter 1 (Section 2.2.3) was followed. For the root endosphere, the roots were thoroughly washed with distilled water followed by suspension in the same to remove all the soil particles. Once the roots were clean and free of soil, they were subjected to isolation as for the stem.

Additionally, endophytic fungi were also isolated from cucumber and zucchini to evaluate their DDD and DDE degradation ability. Unlike that of bacteria, fungi were isolated using the Rose Bengal.

5.2.4. Identification of bacteria

Identification of bacteria was carried out as explained in section 2.2.3.4 and 4.2.6.

5.2.5. DDD, DDE and PCP degradation assays and metabolites detection

5.2.5.1. Petri-plate assay

For screening from enormous isolates, petri-plate assay was deemed quicker as compared to that of degradation test. For the petri-plate assay, 5 mg L^{-1} of DDD and DDE mixture was prepared using dimethyl ether which makes crystalline surface when sprayed. Following, isolation was carried out as explained in previous sections. DDD and DDE were sprayed using manual sprayer, and the plates were incubated until halozone was observed. The isolates showing halozones were picked, streaked and subjected to degradation test in broth culture.

5.2.5.2. Degradation test in broth culture

All the isolates were grown in 5 ml of respective broth i.e. R2B and PDB for 24 hours (25 °C, 250 shakings per minute) using 20-ml glass tubes with glass lid. Three tubes without inoculation were treated as control. DDD and DDE were added (from 500 mg L^{-1} stock solution in acetone) to all the tubes making final concentration of 5 mg L^{-1}. PCP degradation test was carried out with a final concertation of 2 mg L^{-1}. The test tubes were grown under

aforementioned conditions, and after 14 days, 5 ml of acetonitrile was added to each tube. The tubes were shaken for 5 minutes, and 1 ml of the solution was taken into 1.5-ml Eppendorf tubes, and centrifuged for 15 minutes at 15000 rpm. The supernatant was transferred to glass vials and measured using HPLC against a set of standards.

After the confirmation of degradation of DDD by a strain 885C, the 14-day regrown PDB culture was mixed with 30 ml ethyl acetate in a separation flask and shaken. The broth culture was separated, and ethyl acetate was harvested in round bottom flask. Ethyl acetate harvest was performed thrice, and all of it was evaporated using a vacuum evaporator (Nihon Buchi K. K., Tokyo, Japan). 3 ml hexane was then added to round-bottom flask, shaken well and taken into glass vial. The metabolites were detected using Gas Chromatography–Mass Spectrometry (GC-MS) fitted with a GC-2010 gas chromatograph with a 30 m column (MIGHTY CAP ENV-8MS; Kanto Chemical Co., Inc.) interfaced with a mass selective detector (GCMS-QP2010 Plus). The oven temperature was programmed to increase from 140 to 200°C at 10°C/min, 200 to 270°C at 2°C/min and 270 to 300°C at 30°C/min. The inlet temperature was set at 250°C.

Similarly, confirmation of PCP degradation by an endophytic strain (strain 14) isolated from cucumber was carried out and degradation was confirmed.

5.2.6. Time-course degradation of DDD and PCP by strains 885C and 14

Four batches of strain 885C was grown in PDB for 48 hours under conditions mentioned previously, and DDD was added making a final concentration of 5 mg L^{-1}. 3 replications of 885C inoculated tubes along with uninoculated were used for remaining DDD in the media at days 0, 14, 21 and 28 and was checked using HPLC as explained in previous section.

For the strain 14, similar conditions experiment was conducted, however, the concentration used was 2 mg L^{-1} and the experiment continued for 7 days. Sampling was done on day 1, 2, 3 and 7.

5.2.7. Biomass determination of strain 885C

Biomass of the strain 885C was checked alongside the time-course degradation because of difficulties in measurement of optical density due to growth habit of the strain. The strain was grown in test tubes with silicon lids for 48 hours (25 °C, 250 shakings per minute), and the biomass for day 0 was checked. Following the day 0, 3 batches consisting of 3 replications added DDD (concentration 5 mg L^{-1}) and 3 replications as control were grown. The biomass from each batch was checked on 14, 21 and 28 days after initial shaking.

5.3. Results

5.3.1. DDTs in Pakistan soil

The DDT producing factory in discussion worked for almost 31 years, and after its closure, a lot of manipulation has been observed be previous studies besides incidence of dichlorodiphenyltrichloroethane (DDTs), however, the locality has been subjected to certain developments. During the sampling for this study, the site was observed being manipulated for new construction. Soil was dug for approximately 1 m for building of walls.

The soil samples collected from the very site of production unit were mixed to make a composite sample and analyzed for measurement of DDTs. Thorough mixing and sonication of the soil was performed and checked for the presence of DDTs. Results from HPLC revealed that soil had small concentrations of DDT, dichlorodiphenyldichloroethylene (DDE) and 4,4-dichlorobenzophenone (DBP) as compared to that previous studies. It was observed that total concentration of DDTs was at 0.7 mg kg^{-1} soil, where DDT was found at 0.4 mg kg^{-1} soil. Following was DDE which was 0.2 mg kg^{-1} and then DBP which was observed at 0.1 mg kg^{-1}.

5.3.2. Isolation of bacteria and screening for degradation of DDD, DDE and PCP

A total of 24 isolates (18 on PDA, and 6 on R2A) were observed after the isolation from DDT-contaminated soil through dilution plate technique at 3-5 days incubation. All the isolates were subjected to screening for DDD and DDE degradation under 14-day incubation, and only one strain 885C was observed having the capability of DDD degradation. In this screening, the strain 885C showed 48.6% degradation of DDD in 14 days incubation in PDB medium. The initial confirmation of DDD-degradation was followed by DDT degradation test to investigate the transformation of DDT. In the DDT-degradation test by strain 885C, DDT was added as the substrate after 48 h incubation and harvested after 14 days. It was observed that 84.5% of the DDT was transformed to DDD in 14 days of incubation (Figure 5.1). The retention time for DDD, DDE and DDT were 6.5, 10.9 and 9.1, respectively. Subsequent 16S rRNA-based identification of the strain 885C was carried out and it was identified to be *Streptomyces* spp. (Figure 5.2).

For PCP, a total of 15 endophytic strains were checked for their degradation ability among which 8 strains showed degradation (13.8-83.6%) in 14-day incubation. Three strains namely strains: 14, 15 and 16 showed promising results and were selected for further analysis. Strain 14 among the three was selected for time-course degradation.

Endophytic degradation of DDD and DDE was carried out for around 800 bacterial and fungal strains but none of the tested strains showed degradation. The petri-plate assay although showed chances of degradation, yet degrading strains could not be harvested through any means.

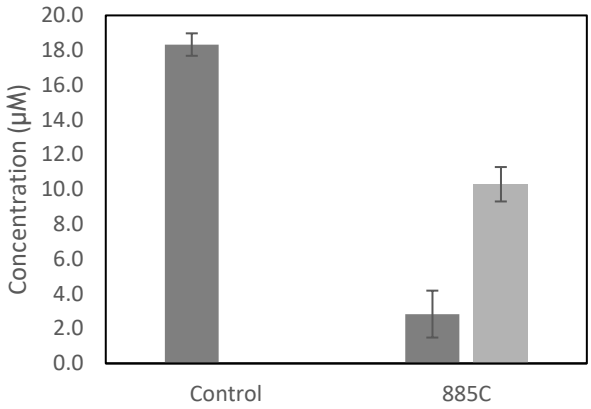


Figure 5.1 DDT transformation to DDD by strain 885C in 14-days incubation. The dark colored bars represent the concentration of DDT and light-colored bar shows quantity of DDD in the media for uninoculated control and strain 885C inoculated PDB media over 14-days incubation. The error bars show standard deviation among the replications.

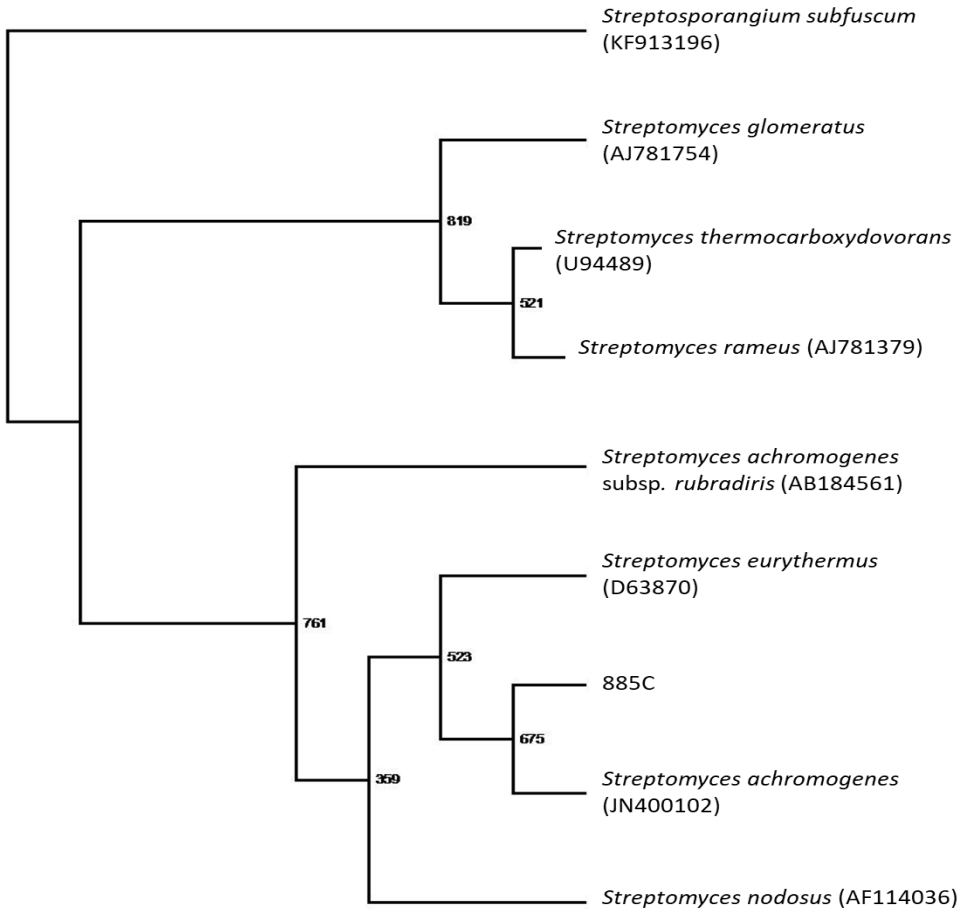


Figure 5.2 Phylogenetic relationships of strain 885C isolated in this study and related species. The phylogenetic tree of 16S rRNA region sequences was generated by the neighbor-joining method. The tree was tested for support by performing bootstrap re-sampling (1000 replicates). Bootstrap values are given at branch points, and the accession numbers of each sequence employed are in parentheses.

5.3.3. Time course degradation of DDD and PCP

Time course degradation test was performed to follow the degradation trend, and the results revealed significant degradation of DDD ($p<0.05$) with the time while increase in bacterial growth/mass was observed (Figure 5.3). In first 14 days of incubation, 37.9% degradation of DDD was observed by strain 885C, resulting in 3.2 mg L^{-1} remaining in the medium. The concentration showed slight change until day 21 which showed 2.9 mg L^{-1} remaining in the media. For the day 28, however, the degradation rate increased up to 56% and remaining concentration of DDD in strain 885C inoculated medium was observed at 2.1 mg L^{-1} compared to 4.7 mg L^{-1} in control.

The biomass of strain 885C with and without DDD addition was observed alongside the time course degradation test. Bacterial growth/mass in PDB can be said to increase with the incubation when DDD as a substrate was added compared to that of no addition of DDD (Figure 5.3). It was observed that biomass of strain 885C was higher in first 14 days where there was no addition of DDD. It showed a slight decrease in day 21, and further decrease in day 28. On the other hand, the 885C-inoculated medium with the addition of DDD showed an increase until day 21 and remained at same level until day 28.

The PCP time-course degradation by strain 14 showed decrease from the 2nd day of incubation (Figure 5.4), while there was slight degradation in first day of sampling (degradation percentage 5.6%). In the 2nd day of sampling, strain 14 showed up to 88.1% degradation while remaining quantity of PCP in the media was 0.9 µM as compared to that of control which had 7.5 µM. The degradation continued for until day 7 where strain 14 showed a degradation percentage of 97.5%.

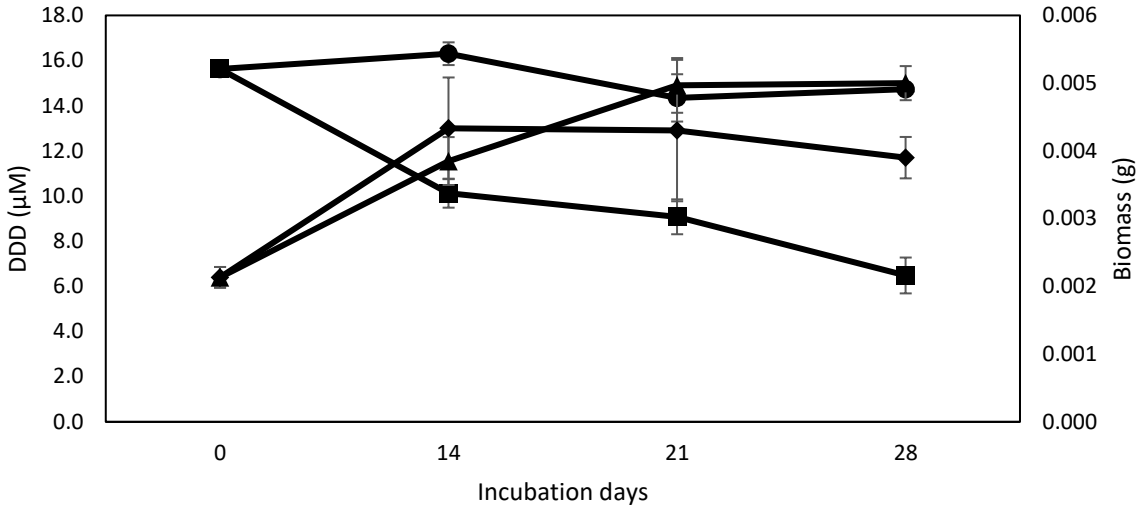


Figure 5.3 Time-course degradation of DDD by strain 885C. The lines represent the concentration of DDD in uninoculated control (●), and strain 885 inoculated PDB media (■), and bacterial biomass of strain 885C without (◆) and with DDD (▲) over the 28 days incubation period. The error bars show standard deviation among the replications.

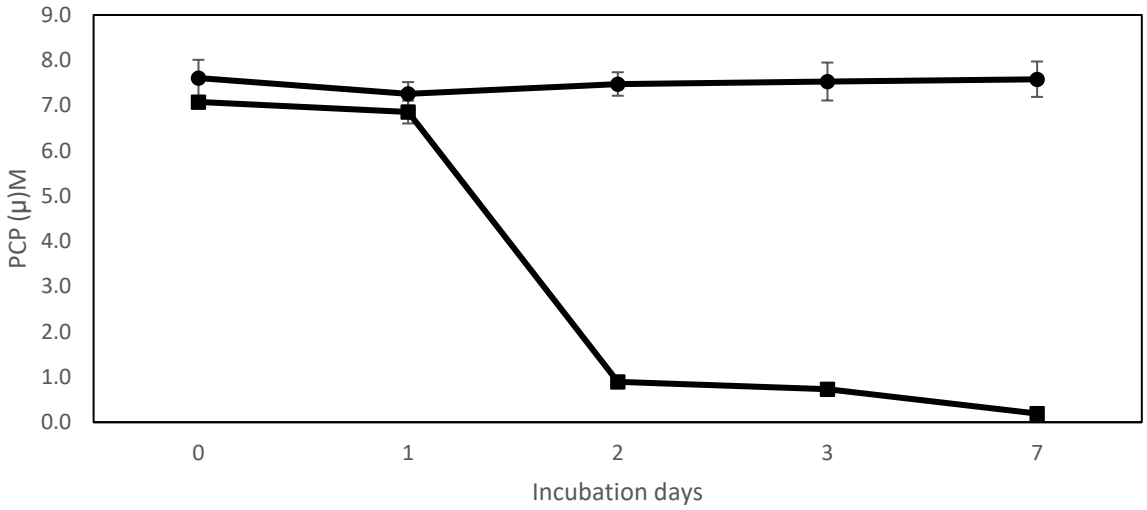


Figure 5.4 Time-course degradation of PCP by strain 14. The lines represent the concentration of PCP in uninoculated control (●), and strain 14 inoculated PDB media (■) over 7 days incubation period. The error bars show standard deviation among the replications.

5.3.4. Putative DDD degradation pathway

Intermediate metabolite detection from 14-day culture of 885C with DDD as substrate was carried out by GC-MS and two metabolites were identified compared with control. The unknown metabolites were refereed to those of known compounds of DDT, and DDOH (mass

ion at m/z) and DBP (mass ion at m/z) (Figure 5.5) were identified. Retention times for DDOH and DBP were, respectively. The incidence of both the metabolites indicated that DDT is transformed to DDD by trichloromethyl group-dechlorination by the strain 885C. The DDD further underwent hydroxylation resulting into (2,2-bis(p-chlorophenyl) ethanol) DDOH, and consequently decarboxylation resulting into formation of DBP (Figure 5.5). Mass spectra of the metabolites detected is given in Figure 5.6.

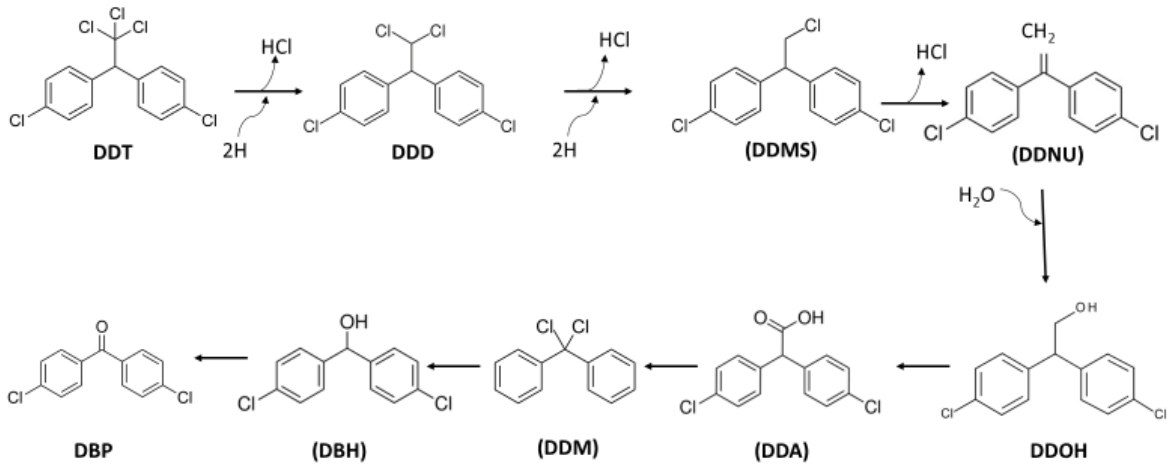


Figure 5.5 Proposed degradation pathway of DDT by strain 885C. The metabolites indicated with parenthesis are putative metabolites. Adapted from (Aislabie et al., 1997). DDT = 1,1,1-trichloro-2,2-bis(p-chloropenyl)ethane; DDD = l,l-dichloro-2,2-bis(p-chlorophenyl)ethane; DDMS = 1 -chloro-2,2-bis(p-chlorophenyl)ethane; DDNU = 2,2-bis(p-chlorophenyl)ethylene; DDOH = 2,2-bis(p-chlorophenyl)ethanol; DDA = bis(f>-chlorophenyl)-acetic acid; DDM = bis(pchlorophenyl) methane; DBH=4,4'-dichlorobenzhydrol; DBP = 4,4' dichlorobenzophenone.

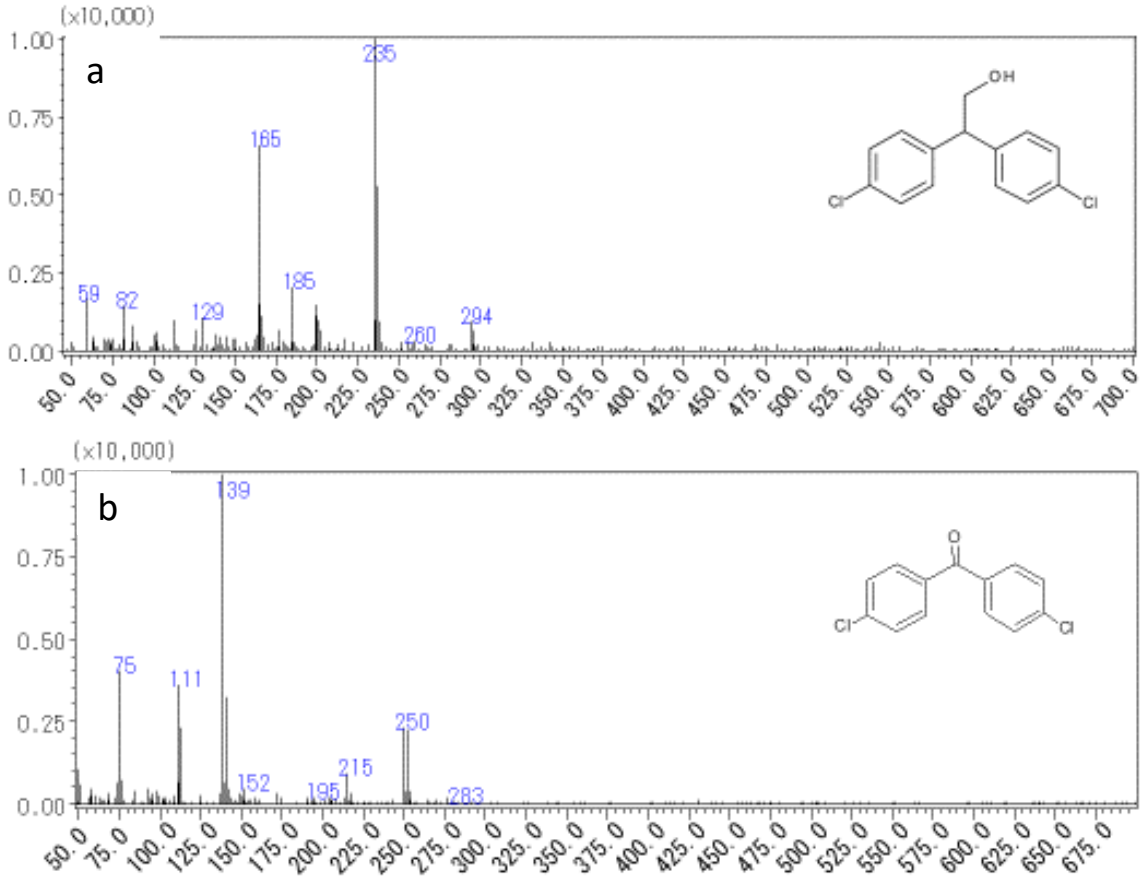


Figure 5.6 Mass spectra of metabolites detected DDOH (a) and DBP (b) by gas chromatography-mass spectrometer (GC-MS) in 14-day incubation using strain 885C. DDOH = 2,2-bis(p-chlorophenyl) ethanol; DBP = 4,4' dichlorobenzophenone.

5.4. Discussion

5.4.1. Degradation of DDD by strain 885C

Production of DDT started in Pakistan in 1962 and there were two DDT production units working in Pakistan; Amangarh (the site sampled in this study), and Kala Shah Kaku (31°44'27", 74°16'07"). DDT was banned in Pakistan in 1993, and both the units stopped working in the following year. However, surroundings and location of both the sites have been observed having considerable amounts of DDTs (Table 5.1). The DDT was also observed in this study even after thorough manipulation of the site (Khwaja et al., 2006). This presents distribution of large amounts of residues, as well as the persistence of DDT along with other persistent organic pollutants (POPs) in the soil. Although DDT is transformed to other

derivatives, DDD and/or DDE depending on the degrading microbes and mode of respiration, but the former chemical is still detected in soils in considerable quantity. This study was thus focused on finding potential bacteria for remediation of the very site of factory, which has not yet been discovered to best of our knowledge. Although the isolation resulted in 24 bacterial strains, only one strain was capable of degrading DDD. The degradation can be said a rare trait among the incident bacteria, as observed by (Wang et al., 2017a), where they observed only two strains were responsible for more than 50% degradation of DDTs among 12 isolates. Similar results have also been obtained in other organochlorine pesticides (Qu et al., 2015) and other pesticide pollutants (Cai et al., 2015).

Table 5.1 Concentration of DDTs in soil, plant and water samples from former DDT production sites in Pakistan. Amangarh was sampled during this study.

Metabolites	**Site**	**Soil (µg g^{-1})**	**Plant (µg g^{-1})**	**Water (µg ml^{-1})**	**Reference**
DDTs	Amangarh	Up to 806 (surface soil) Up to 817 (subsurface soil) Up to 918 (deep horizon soil)			(Ullah et al., 2019)
DDTs	Amangarh	0.0002-0.062 (dust from nearby area)			(Sohail et al., 2018)
DDTs	Amangarh	2.8-651.3	50-450		(Younas et al., 2013)
DDTs	Amangarh	247-9157			(Ahad et al., 2010)
DDT	Amangarh	0.01-11.3 (surroundings)			(Khwaja, 2008)
DDT	Amangarh	242.3-1858		0.1-0.4	(Khwaja et al., 2006)
DDTs	Kala Shah Kaku	0.8-1.8			(Syed and Malik, 2011)
DDTs	Kala Shah Kaku			0.002-7.9 nearby nullah	(Tehseen et al., 1994)

The DDT transformation to DDD was observed in this study, which was further degraded to DBP as terminal metabolite (Figure 5.4). The transformation of DDT to its initial derivatives i.e. DDD or DDE depends mainly on mode of respiration and degrading microbes. It is interesting to see that strain 885C converted DDT to DDD, however, DDD was not detected in the soil while traces of DDE were observed. It can thus be postulated that the soil may inhabit certain other microbes capable of converting DDT to DDE. The occurrence of DDE can be linked with the aerobic dechlorination of DDT (Holland, 1996).

The terminal metabolite observed in this study was DBP. Several other reports have also found DBP as terminal metabolite among few microbes, however there are also instances of DBP breakdown (reviewed by Aislabie et al., 1997). This puts forward the need to look for DBP degrading bacteria from the said location. The degradation of such pollutants particularly that of DDT has been observed by microbial consortium (Bidlan and Manonmani, 2002), therefore, it can be expected that there can be DBP degrading bacteria in the soil which can be utilized in complete breakdown of DDT. The sampled location still has DDTs which pose a threat to local population, thus needs urgent cleanup, declaration of the contaminated zone and seizure of construction at and around the site. For the cleanup of the soil, locally adapted bacterial strain, 885C, as isolated in this study can be used besides more research into DBP degrading bacteria. Further, other microbes can be searched which may degrade DDT completely.

5.4.2. Degradation of PCP by strain 14

Pentachlorophenol has been used mainly in preservation and pest control purposes since its development. However, its toxicity to animals emerged and it was also categorized in Stockholm Convention and has been banned in most of the countries. However, its use is still allowed in few parts of the world mainly for wood preservation. Also, it is being produced in Mexico and the United States.

The emerging issues with PCP like those of toxicity, pollution of soil, uptake by the plants, and contamination of water bodies have asked for its remediation. Under these circumstances, PCP bio-degradation has been explored and instances of degradation or biotransformation have been reported (reviewed by Lopez-Echartea et al., 2016). The reports however mostly belong to those of rhizosphere bacteria, except for the one which documents the presence of endophytic bacteria capable of degrading PCP in axenic assays (Marihal et al., 2014). In this study, we could isolate 8 strains capable of degrading PCP in laboratory tests. Among these 8, three were quite promising and showed up to more than 95% degradation in 14-day incubation. The incidence of PCP degradation in endophytic bacteria presents that once the plants uptake the contaminant, the bacteria residing inside the plant help degrade rather normal sequestration pathway. Examples of PCP bioaccumulation by plants have brought forward small amounts of uptake (Bellin and O'Connor, 1990; Hechmi et al., 2015). Interestingly, we studied that axenic PCP degradation was possible up to 3 mg L^{-1}, however, at higher concentrations like those of 4 and 5 mg L^{-1}, although the bacteria grew well but was unable to degrade within 7 days. It can be postulated that maybe more incubation time is required for higher concentrations or inability of the endophytes in degrading such higher quantity.

5.4.3. Endophytic bacterial degradation of DDTs

Although DDTs degradation has been explored by rhizosphere bacteria (Egorova et al., 2017; Pan et al., 2017; Qu et al., 2015), yet there are no instances of degradation by endophytic bacteria except for an uptake experiment which also postulated no uptake enhancement or degradation (Eevers et al., 2018). This presents incidence of DDTs degradation by sole endophytic bacterium may not be possible. Although there are reports of DDTs accumulation by cucumber, more in roots than shoots (Namiki et al., 2013), yet it can be put forward that fate of accumulated DDTs can be: a) sequestration by plants, b) transformation by non-culturable

endophytic bacteria and/or c) phyto-transformation. The uptake can be the escape strategy of plants, which with the help of secondary metabolites would be able to uptake the pollutants and store within the plant body to avoid stress to roots. The secondary metabolites like those of surfactants can lead to enhanced bioavailability of pollutants. On the other hand, certain microbes have also been explored for production of surfactants (Desai and Banat, 1997). It can thus be said that some microbial and plant synergy may have led to accumulation of DDTs in plant. Lack of DDT degrading endophytic bacteria asks for exploring plants which have originally been exposed to such contamination for considerable period of time. The evolution of microbes towards degradation of contaminants is a complex process (van der Meer, 2003), thus more vast sampling points, diversity of plants and enrichment techniques are encouraged to look for biodegradation of DDTs by endophytic bacteria.

5.5. Summary

A bacterial strain 885C was isolated from DDT contaminated soil and could degrade DDT until DBP in laboratory assays. Time course-degradation showed up to 56% degradation in 28 days. On the other hand, no endophytic bacterial or fungal strains were observed capable of degrading DDTs in axenic assays. PCP degrading endophytic bacteria were, however, discovered, and strain 14 among them could degrade PCP. The degradation percentage in 7 days incubation was observed at 97.5%.

CHAPTER 6 ENDOPHYTIC BACTERIA-ASSISTED PHYTOREMEDIATION

6.1. Introduction

Soil pollution especially that of organic pollutants poses continuous threat to crop production and food security particularly in developing countries. The pollution sources can be irrigation water, applied pesticides, leakage and mismanagement. This soil pollution not only affects the crop production, but also leads to contamination of water bodies through runoff. These circumstances ask for remediation of soil and for that different approaches have been employed. Microbe-assisted phytoremediation comes handy due to enhanced uptake, *in planta* degradation, and decreased phytovolatilization. The uptake if exceeds the degradation ability of the incident degrading microbes, the later handling can however be problematic similar to that of phytoremediation.

Cucurbits have the potential of accumulating organochlorine pesticides (Namiki et al., 2013). However, the uptake of such pollutants is affected by different factors particularly those of bioavailability of the pollutants, co-contamination of soil, and capability of plants to uptake such contaminants among others. Similarly, the bioremediation and phytoremediation when used solely face challenges like: survival of the microbes, survival of the plants, and level of contaminants etc. The microbes assisted phytoremediation thus comes as an attractive alternative.

Different studies have found cumulative effect of applied microbes on removal of pollutants from soil. For instance, *Bacillus safensis*; an endophytic bacteria was isolated from *Chloris virgate* and resulted in enhanced degradation of hydrocarbons in soil (Wu et al., 2019). Similarly, Iqbal et al. (2019) isolated an endophyte *Pseudomonas* sp., inoculated it to *Lolium perenne* and *Arabidopsis thaliana* and observed removal of total petroleum hydrocarbon from soil. There are other instances of hydrocarbon (Baoune et al., 2019; Mesa-Marín et al., 2019) and other related pollutants' (reviewed by He et al., 2019) removal from soil through synergy

of endophytes with plants. However, there is lack of information on pesticidal pollutants particularly organochlorines. Organochlorines are an emerging threat due to their accumulation potential, and increased use. As discussed above, developing countries face a lot of challenges regarding such pollutants. Persistence of such pollutants in soil and water ecosystems pose a continuous threat to life. This study was thus planned to look for possible endophytic bacteria capable of enhancing uptake of DDT and its derivatives and PCP. Further, cucumber being a cucurbit was targeted to check if endophytic bacteria could synergize the uptake from soil followed by subsequent degradation.

6.2. Materials and methods

6.2.1. Isolation and characterization of endophytic bacteria

Bacteria were isolated and characterized as explained in section (Section 2.2.3). Plant growth promoting endophytic bacteria which showed potential in PGP experiments, strains 4 and 227, were selected for PGPE-enhanced phytoremediation assay of DDTs and PCP. Both bacterial strains were applied to the seeds as explained in section 3.2.2.

6.2.2. Plant-uptake assay

30 g of soil collected from University of Yamanashi Research Farm was weighed in polypropylene tubes with lids (exterior dimensions: 4 cm × 11 cm, volume: 120 ml), and was autoclaved at 121 C for 1 h after addition of distilled water at 60% field capacity. Later, pollutant was applied with dichotomous earth (washed) and thoroughly mixed with soil. DDD and DDE were added together making a final concentration of 5 mg kg^{-1} each, while PCP was added to make it to 2 mg kg^{-1}. The tubes were left to aging at room temperature for 2 weeks. Three replications of each i.e. DDD and DDE mixed soil, and PCP mixed soils were used for checking the concentration in the soil before the start of experiment. Seeds for 5 replications of each treatment viz. control, strain 4 and strain 227 were grown as explained above and sown in the tubes. Two batches for PCP- (1 month and 4 months after transplantation) and three for

DDD and DDE-uptake (1 month, 3 months and 4 months after transplantation) by the cucumber plants were checked. Soil from same batches was also checked for its remaining quantity in the soil.

6.2.3. Quantification of pollutants' in soil and plant

Quantification of PCP and DDTs from soil was performed as explained in section 3.2.2. For the plant, whole plant was homogenized in 150 ml acetone and mixture was passed through glass fiber filter and was evaporated until 5-10 ml at 40 °C using vacuum evaporator (Nihon Buchi K. K., Tokyo, Japan). The remaining extract was washed through diatomite column (InterSep K-Solute 10-ml, GL Sciences Inc. Tokyo) with 100 ml of hexane and again evaporated using aforementioned evaporating conditions. Following the complete evaporation, 3 ml of acetonitrile was added, mixed thoroughly and collected in glass vial through graphite column. The harvested extract was measured using HPLC against a set of standards.

6.3. Results

6.3.1. PCP uptake by cucumber plants

PCP accumulation by cucumber plants was checked and compiled in figure 6.1 and 6.2. it was observed that the effect of applied endophytic bacteria i.e. strain 4 and 227 on uptake of PCP per plant was significant in the plants grown for 4 months unlike that of 1-month old plants (Student t-test). In 4-month old plants, the applied bacteria not only significantly affected plant biomass, but also the uptake of PCP ($P<0.05$). However, the concentration of PCP remaining in the soil was found to be non-significant. It is noteworthy that remaining quantity of PCP in soil was lowest in strain 4-applied treatments, while uptake was higher in strain 227-applied pots. In the 1-month old plants, although differences were observed in plant biomass and PCP uptake but were statistically non-significant ($P>0.05$).

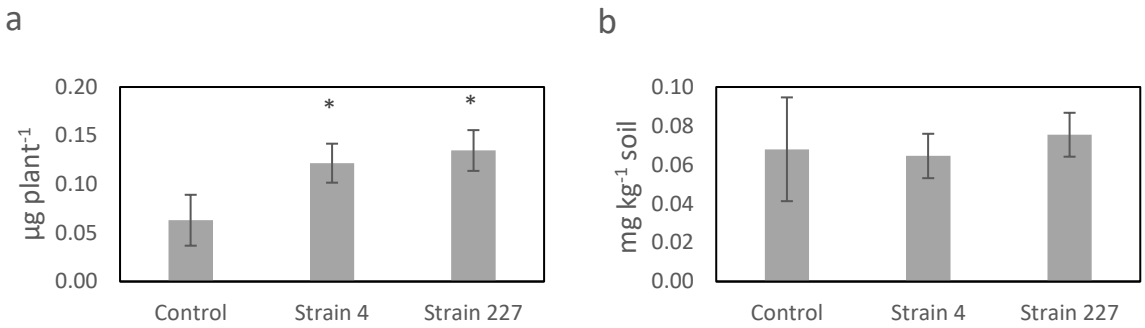


Figure 6.1 Concentration of PCP in plant (a) and soil (b) after growing the plants for 4 months. The error bars show standard deviation among the replications, where * indicates the statistical significance of each treatment when compared with control treatment using student t-test ($P<0.05$). n=3 except for control treatment where n was 5.

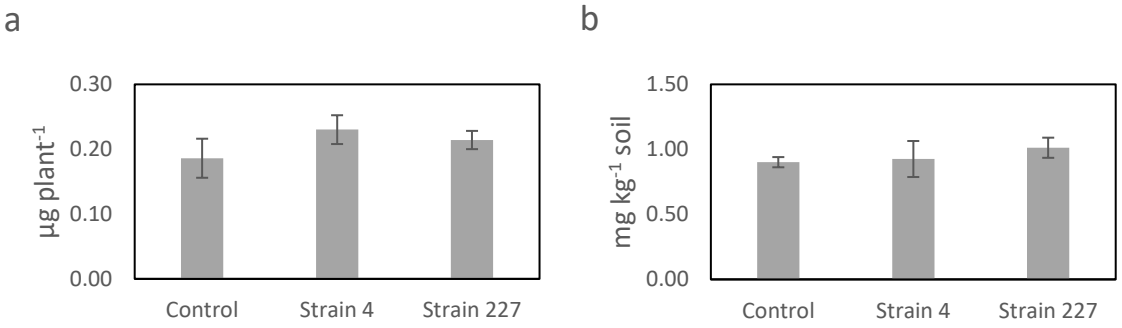


Figure 6.2 Concentration of PCP in plant (a) and soil (b) after growing the plants for 1 month. The error bars show standard deviation among the replications. The effect was found to be non-significant when compared using student t-test. n=3.

6.3.2. DDD and DDE uptake by cucumber plants

Uptake of DDD and DDE by cucumber plants was found to be non-significant in all three batches ($P>0.05$, Table 6.1). Corresponding to the pollutant, higher tendency of DDE uptake was observed by cucumber plants against that of DDD. Furthermore, age of plant, it was observed that highest accumulation of DDD was in 3-month old plants (significant as compared to that of 1-month old plants), while no uptake in 4-month old plants was observed. The uptake of DDE also depicted similar case with respect to significance and uptake, however, uptake of DDE was also observed in 4-month old plants (Table 6.2).

Table 6.1 Concentration of DDD and DDE in plant after growing the plants for 1, 2 and 4 months. n=3 except for control treatment plants in 4-month old batch where n was 5. ± indicates the standard deviation among replications.

	DDD (µg plant^{-1})			DDE (µg plant^{-1})		
Age of plants	**Control**	**Strain 4**	**Strain 227**	**Control**	**Strain 4**	**Strain 227**
1-month	0.01±0.0042	0.001±0.0002	0.001±0.0003	0.17±0.06	0.14±0.10	0.15±0.01
3-months	0.33±0.03	0.30±0.08	0.34±0.12	0.77±0.10	0.73±0.10	0.74±0.03
4-months	-	-	-	0.04±0.011	0.04±0.006	0.05±0.007

Table 6.2 Concentration of DDD and DDE in soil after growing the plants for 1, 2 and 4 months. n=3 except for control treatment plants in 4-month old batch where n was 5. ± indicates the standard deviation among replications.

	DDD (mg kg^{-1} soil)			DDE (mg kg^{-1} soil)		
Age of plants	**Control**	**Strain 4**	**Strain 227**	**Control**	**Strain 4**	**Strain 227**
1-month	1.88±1.38	0.88±0.14	1.08±0.33	0.73±0.08	0.67±0.07	0.74±0.05
3-months	0.64±0.07	0.80±0.12	0.72±0.10	0.73±0.06	0.85±0.08	0.80±0.08
4-months	0.39±0.33	0.51±0.19	0.65±0.44	0.44±0.15	0.64±0.14	0.74±0.29

6.4. Discussion

Plant growth promoting endophytic bacteria have been employed in vast areas of research encompassing biocontrol, plant growth and yield enhancement, and biodegradation of pollutants (Compant et al., 2019; He et al., 2019; Santoyo et al., 2016; White et al., 2019). Although a lot has been debated about endophytes-assisted phytoremediation, yet exploitation of this synergy has not been fully explored except for two reports which could not find significant evidence of PGPE enhanced DDE uptake by the plants (Eevers et al., 2016, 2018). Similar is the case of PCP, where Nakamura et al. (2004) could analyze its degradation in rhizosphere rather the uptake. It was thus hypothesized that if plant growth enhancing endophytic bacteria can help grow the plant better, can they also enhance its growth in polluted soil besides increasing the uptake and subsequent degradation. The degradation, however, is linked with incidence of degrading bacteria. In this study, it was observed that PGPE enhanced the growth of cucumber plants in polluted soils, however the results in case of DDD and DDE contaminated soil were not different. Instances of heavy metal tolerant plant growth promoting

microorganisms have been discussed to ameliorate the deleterious effect of contamination and enhance plant growth (Mishra et al., 2017). The mutual relationship of microbes with plants thus holds the key to growing plants in contaminated sites. Plant survival sometimes is an issue in such soils (Mishra et al., 2017), therefore if microbes can help plants withstand such stress, it can be used as a plus. Consequently, the remediation of such sites can be performed if accumulator plants are joined with plant growth promoting microorganisms. Another point here is the survival and functionality of rhizosphere microbes, which gets complicated in co-contaminated sites. These circumstances thus ask for endophytic bacteria as we used here, which if successfully colonize the endosphere have lesser issues as compared to those of epiphytes (Mahmood et al., 2019b).

Enhanced plant growth was hypothesized to enhanced uptake of persistent organic pollutants. This hypothesis was found to be true in case of PCP uptake by cucumber plants (Figure 6.1), however it was not the case in DDD and DDE accumulation (Table 6.1). Uptake of organochlorines has been observed by cucurbits (Namiki et al., 2013), which can be the case in accumulation of PCP, DDD and DDE in cucumber plants. The case of DDD and DDE was, however, contrary to findings of Otani et al. (2007) who observed considerable uptake of dieldrin by cucumber. However, it has been observed that accumulator-grafted cucumber plants may have the potential for increased uptake of such pollutants (Otani and Seike, 2007). Such grafting can thus help build better phytoremediation agents as compared to those of non-grafted cucumber plants.

The dynamics of pollutant concertation in plant and soil present another picture. The DDD and DDE experiment was conducted at 5 mg kg^{-1}, and PCP was carried out at 2 mg kg^{-1} initial concentration. The recovery of pollutants from soil as well as plants, however does not represent the complete quantity of pollutants applied. The initial recovery after ageing for two weeks showed that quantity of PCP was 0.8±0.05 mg kg^{-1} while it was applied at 2 mg kg^{-1}.

Similarly, the quantity of DDD and DDE was 2.7±0.43 mg kg^{-1} and 2.7±0.09 mg kg^{-1}, respectively while the application was done to maintain a concentration of 5 mg kg^{-1}. It can be said that either the pollutants were volatilized during the course of study, or there is a possibility of degradation *in planta*. This can be observed in DDD and DDE uptake experiment where 3-month old plants had significantly higher concentration than that of 4-month old. The uptake of the pollutants by plants, if it had happened, may have gone under biodegradation by endophytic bacteria vertically transmitted through seeds. We used autoclaved soil, and surface-sterilized seeds for the experiment, however there are chances of endophytic bacteria in the seeds (Shahzad et al., 2018) which may have ability of degradation of such pollutants. On the other hand, wide isolation sources could not yield culturable DDTs-degrading bacteria (see chapter 3). It can therefore be linked with non-culturable bacteria, who may have been helping the plants. The non-culturable endophytic bacterial community constitutes bigger proportion than culturable bacteria (see chapter 1). Similar case can be corresponded to PCP, where for instance, although strain 227-inoculated plants showed more accumulation than strain 4-applied plants, but quantity of PCP in soil was also higher in the former treatment. Therefore, there is possibility of incidence of endophytic bacteria apart from those applied which remains least explored.

Spatial dynamics of plant uptake of PCP propose another factor, where 1-month old plants in control treatment showed higher accumulation compared to that of 4-month old plants. It can be postulated that the plants reached at a threshold level even after one month of growing, but as the plant biomass increased with the age of the plants, the plants could not uptake more PCP. On the other hand, the plants inoculated with plant growth promoting endophytic bacteria continued to accumulate PCP with their growing biomass.

6.5. Summary

Plant growth promoting endophytic bacteria enhanced plant growth under PCP contaminated conditions besides enhancing the uptake by the plants. This was however only true for plants grown for 4 months. The case of DDD and DDE contaminated site was different and growth of plants as well as uptake were not significantly affected by application of endophytic bacteria. The DDD and DDE uptake was higher in 3-months old plants against those of 1-month and 4-month old plants. The concentration of pollutants in the soil was not recovered fully which may have undergone volatilization, *in planta* degradation by unknown microbes, or degradation within the soil manipulated by plants.

CHAPTER 7 CONCLUSIONS

In first part, diversity of culturable and non-culturable endophytic bacteria from cucumber was followed. The diversity of endophytic bacteria in leaf-stalk of cucumber plants at Site 1 increased with the age of plants up to Fruit Development Stage which tended to decrease later in Maturity Stage. The number of endophytic bacteria at Site 1 kept on increasing. For the Site 2, however, there was a continuous increase in endophytic bacterial diversity, while the bacterial number also increased with the exception of Nursery Stage which showed more number than that of Flowering Initiation and Fruit Development Stages. Plants chose certain bacteria to stay throughout their lifecycle, and bacterial community may also have undergone pruning when necessary, as some endophytic bacterial genera prevailed regardless of plant age and cultivability, but others disappeared or reduced in number. The non-culturable endophytic bacteria revealed variation but tended to be more diverse and richer than the culturable ones. Our results suggested that Fruit Development Stage (2 months after transplanting) at Site 1 and Maturity Stage (3 months after transplanting) at Site 2 were microbially the most diverse stages of cucumber plants, which should be considered for sampling in future studies. Future research should be focused on following the diversity from seed to seed besides comparison of other similar or distant crop species, which will help in understanding the endophytic bacterial diversity better.

Secondly, plant growth promoting potential of culturable isolates from cucumber was explored. Around 300 endophytic bacterial strains were isolated from leaf-stalks of cucumber and investigated for their PGP potential. PGP was a dominant characteristic among the isolates, from which five strains were selected and further investigated using pot experiments. Strains 4 and 227 were dominant and confirmed to increase number of fruits as well as growth in simultaneous field experiment. Both the strains were subsequently explored using a whole metabolomic approach that compared the concentrations of 200 chemicals against control

treatment. All of the metabolites tested differed among the treatments, around 43% of which increased in endophytic bacteria-treated plants. The levels of contributions from both bacteria varied with regard to enhancing plant growth or metabolite release, either by the plant or bacteria. Therefore, although both strains had common PGP traits, the underlying mechanisms may differ. Future research should separately investigate the metabolites that originate from microbes and plants to elucidate the contribution of applied microbes. Similarly, metabolomic comparison between endophytic and rhizosphere microbes may improve our understanding of interactions among microbes and their hosts.

Following, rhizosphere of common ice plant; a halophyte, was explored for incident plant growth promoting bacteria and two strain PR-3 and PR-6 showed positive effects. Both the strains also possessed molecular traits responsible for plant growth promotion, and enhanced plant growth under salinity stress. It is postulated that these two isolates can be helpful in utilization of saline soils for cultivation. Also, practical application of promising endophytic bacterial strains from previous pot experiments, strain 4, 72, 167, 193 and 227, were evaluated for their contribution to cucumber productivity in a 2-year field experiment. It was observed that all the strains helped plant growth and number of fruits as a yield parameter, however, strains 4 and 227 showed maximum advantage. Both the strains can thus be recommended for sustainable production of cucumber in the area.

Succeeding, occurrence of persistent organic pollutants encompassing DDTs and PCP was investigated. Firstly, DDT contaminated soil was explored, and it yielded 24 bacterial isolates, among which one strain 885C could degrade DDD. The strain 885C showed significant degradation of DDD over the course of time and 55.9% degradation was observed in 28 days incubation. The putative degradation pathway showed that DDT was transformed to DDD, which was further degraded to DDOH and DBP. Secondly, endophytic bacteria were investigated for degradation of DDD and DDE and PCP. No endophytic bacteria were observed

capable of degrading DDTs, however, promising bacteria for biodegradation of PCP were isolated. The strain 14 among potential isolates showed 97.5% degradation of PCP in 7 days.

Lastly, plant growth promoting endophytic bacteria were checked if they could enhance growth of plants in contaminated soil and uptake of such contaminants. It was observed that although plant growth was enhanced by applied bacteria (strains 4 and 227) in both DDD+DDE and PCP contaminated soil, only PCP was significantly accumulated by plants supported by applied bacteria. Therefore, it can be put forward that strains 4 and 227 not only enhanced growth and number of fruits of plants in noncontaminated site, but also showed potential of enhancing plant growth in contaminated soil. These two strains can thus be used for sustainable cucumber production and removal of persistent organic pollutants from soil particularly that of PCP. For the DDTs, the strain 885C can be used for remediation of polluted site due to its adaptation to local climate and conditions.

It can thus be postulated that application of endophytic bacteria can enhance plant growth and biomass which can improve the uptake of pentachlorophenol. PCP can later be attacked by strain 14, which can lead to biodegradation of this pollutant. However, there is not sufficient evidence of enhanced DDD and DDE uptake by the plants, therefore the DDD and/or DDT can be degraded by strain 885C. The synergy of plants with bacteria can thus be manipulated for enhanced pollutant removal from soil (Figure 7.1).

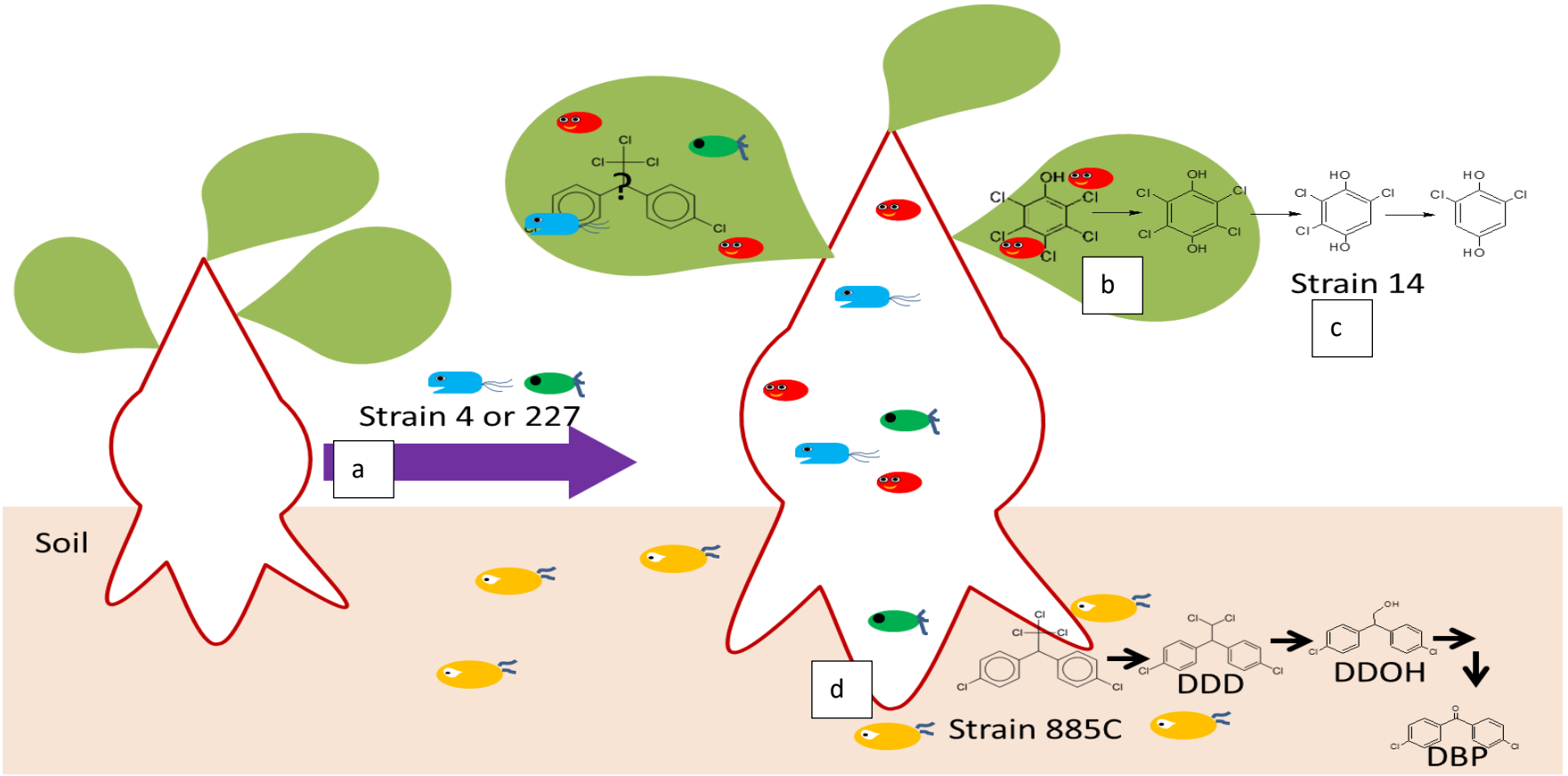


Figure 7.1 Schematic representation of integrated pollutants management using plant-microbe synergy. Step a: application of strain 4 and/or 227 can enhance growth and biomass of the plants, followed by step b: where enhanced biomass can result into enhanced uptake of pentachlorophenol and step c: subsequent degradation of PCP can occur in endosphere of the plants. For the dichlorodiphenyltrichloroethane (DDTs), as we could not find evidence of enhanced uptake (?), so that can be degraded in 'step d' by strain 885C.

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REFERENCES

Abbas, A., Khan, S., Hussain, N., Hanjra, M. A., and Akbar, S. (2013). Characterizing soil salinity in irrigated agriculture using a remote sensing approach. *Physics and Chemistry of the Earth, Parts A/B/C* **55-57**, 43-52.

Abou-Elela, S. I., Kamel, M. M., and Fawzy, M. E. (2010). Biological treatment of saline wastewater using a salt-tolerant microorganism. *Desalination* **250**, 1-5.

Acuña, J., Jorquera, M., Martínez, O., Menezes-Blackburn, D., Fernández, M., Marschner, P., Greiner, R., and Mora, M. (2011). Indole acetic acid and phytase activity produced by rhizosphere bacilli as affected by pH and metals. *Journal of soil science and plant nutrition* **11**, 1-12.

Afzal, M., Khan, Q. M., and Sessitsch, A. (2014). Endophytic bacteria: Prospects and applications for the phytoremediation of organic pollutants. *Chemosphere* **117**, 232-242.

Agarie, S., Shimoda, T., Shimizu, Y., Baumann, K., Sunagawa, H., Kondo, A., Ueno, O., Nakahara, T., Nose, A., and Cushman, J. C. (2007). Salt tolerance, salt accumulation, and ionic homeostasis in an epidermal bladder-cell-less mutant of the common ice plant Mesembryanthemum crystallinum. *Journal of Experimental Botany* **58**, 1957-1967.

Agency, U. S. E. P. (1999). "Integrated Risk Information System (IRIS) on Pentachlorophenol.National Center for Environmental Assessment."

Agler, M. T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S.-T., Weigel, D., and Kemen, E. M. (2016). Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. *PLOS Biology* **14**, e1002352.

Ahad, K., Mohammad, A., Khan, H., Ahmad, I., and Hayat, Y. (2010). Monitoring results for organochlorine pesticides in soil and water from selected obsolete pesticide stores in Pakistan. *Environmental Monitoring and Assessment* **166**, 191-199.

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University - Science* **26**, 1-20.

Aislabie, J. M., Richards, N. K., and Boul, H. L. (1997). Microbial degradation of DDT and its residues—A review. *New Zealand Journal of Agricultural Research* **40**, 269-282.

Akbaba, M., and Ozaktan, H. (2018). Biocontrol of angular leaf spot disease and colonization of cucumber (Cucumis sativus L.) by endophytic bacteria. *Egyptian Journal of Biological Pest Control* **28**, 14.

Al Hassan, M., Pacurar, A., López-Gresa, M. P., Donat-Torres, M. P., Llinares, J. V., Boscaiu, M., and Vicente, O. (2016). Effects of salt stress on three ecologically distinct Plantago species. *PloS one* **11**, e0160236.

Aloni, R., Aloni, E., Langhans, M., and Ullrich, C. I. (2006). Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. *Annals of botany* **97**, 883-893.

Alori, E. T., Glick, B. R., and Babalola, O. O. (2017a). Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture. *Frontiers in Microbiology* **8**, 971.

Alori, E. T., Glick, B. R., and Babalola, O. O. (2017b). Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture. *Frontiers in Microbiology* **8**.

Andrew, D. R., Fitak, R. R., Munguia-Vega, A., Racolta, A., Martinson, V. G., and Dontsova, K. (2012). Abiotic Factors Shape Microbial Diversity in Sonoran Desert Soils. *Applied and Environmental Microbiology* **78**, 7527-7537.

Armengaud, P., Sulpice, R., Miller, A. J., Stitt, M., Amtmann, A., and Gibon, Y. (2009). Multilevel analysis of primary metabolism provides new insights into the role of potassium nutrition for glycolysis and nitrogen assimilation in Arabidopsis roots. *Plant physiology* **150**, 772-785.

Arzani, A., and Ashraf, M. (2016). Smart Engineering of Genetic Resources for Enhanced Salinity Tolerance in Crop Plants. *Critical Reviews in Plant Sciences* **35**, 146-189.

Ashraf, S., Afzal, M., Naveed, M., Shahid, M., and Zahir, Z. A. (2017). Endophytic bacteria enhance remediation of tannery effluent in constructed wetlands vegetated with Leptochloa fusca. *International Journal of Phytoremediation*, 00-00.

Atzori, G., de Vos, A. C., van Rijsselberghe, M., Vignolini, P., Rozema, J., Mancuso, S., and van Bodegom, P. M. (2017). Effects of increased seawater salinity irrigation on growth and quality of the edible halophyte Mesembryanthemum crystallinum L. under field conditions. *Agricultural Water Management* **187**, 37-46.

Bajaj, A., Mayilraj, S., Mudiam, M. K. R., Patel, D. K., and Manickam, N. (2014). Isolation and functional analysis of a glycolipid producing Rhodococcus sp. strain IITR03 with potential for degradation of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT). *Bioresource Technology* **167**, 398-406.

Banik, A., Dash, G. K., Swain, P., Kumar, U., Mukhopadhyay, S. K., and Dangar, T. K. (2019). Application of rice (Oryza sativa L.) root endophytic diazotrophic Azotobacter sp. strain Avi2 (MCC 3432) can increase rice yield under green house and field condition. *Microbiological Research* **219**, 56-65.

Banik, A., Pandya, P., Patel, B., Rathod, C., and Dangar, M. (2018). Characterization of halotolerant, pigmented, plant growth promoting bacteria of groundnut rhizosphere and its in-vitro evaluation of plant-microbe protocooperation to withstand salinity and metal stress. *Science of The Total Environment* **630**, 231-242.

Banwart, G. J. (1989). Control of Microorganisms by Retarding Growth. *In* "Basic Food Microbiology", pp. 545-650. Springer US, Boston, MA.

Baoune, H., Aparicio, J. D., Acuña, A., El Hadj-khelil, A. O., Sanchez, L., Polti, M. A., and Alvarez, A. (2019). Effectiveness of the Zea mays-Streptomyces association for the phytoremediation of petroleum hydrocarbons impacted soils. *Ecotoxicology and Environmental Safety* **184**, 109591.

Bellin, C. A., and O'Connor, G. A. (1990). Plant Uptake of Pentachlorophenol from Sludge-Amended Soils. *Journal of Environmental Quality* **19**, 598-602.

Berg, G., Rybakova, D., Grube, M., and Köberl, M. (2016). The plant microbiome explored: implications for experimental botany. *Journal of experimental botany* **67**, 995-1002.

Berg, M., and Koskella, B. (2018). Nutrient- and Dose-Dependent Microbiome-Mediated Protection against a Plant Pathogen. *Current Biology* **28**, 2487-2492.e3.

Berglund, T., Wallström, A., Nguyen, T.-V., Laurell, C., and Ohlsson, A. B. (2017). Nicotinamide; antioxidative and DNA hypomethylation effects in plant cells. *Plant Physiology and Biochemistry* **118**, 551-560.

Bharti, N., Pandey, S. S., Barnawal, D., Patel, V. K., and Kalra, A. (2016). Plant growth promoting rhizobacteria Dietzia natronolimnaea modulates the expression of stress responsive genes providing protection of wheat from salinity stress. *Scientific Reports* **6**, 34768.

Bharti, N., Yadav, D., Barnawal, D., Maji, D., and Kalra, A. (2013). Exiguobacterium oxidotolerans, a halotolerant plant growth promoting rhizobacteria, improves yield and content of secondary metabolites in Bacopa monnieri (L.) Pennell under primary and secondary salt stress. *World Journal of Microbiology and Biotechnology* **29**, 379-387.

Bidlan, R., and Manonmani, H. K. (2002). Aerobic degradation of dichlorodiphenyltrichloroethane (DDT) by Serratia marcescens DT-1P. *Process Biochemistry* **38**, 49-56.

Bodenhausen, N., Horton, M. W., and Bergelson, J. (2013). Bacterial Communities Associated with the Leaves and the Roots of Arabidopsis thaliana. *PLOS ONE* **8**, e56329.

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A., and Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature methods* **10**, 57-59.

Borah, A., Das, R., Mazumdar, R., and Thakur, D. (2019). Culturable endophytic bacteria of Camellia species endowed with plant growth promoting characteristics. *Journal of Applied Microbiology* **127**, 825-844.

Bourceret, A., Leyval, C., Faure, P., Lorgeoux, C., and Cébron, A. (2018). High PAH degradation and activity of degrading bacteria during alfalfa growth where a contrasted active community developed in comparison to unplanted soil. *Environmental Science and Pollution Research* **25**, 29556-29571.

Bourdin, B., Adenier, H., and Perrin, Y. (2007). Carnitine is associated with fatty acid metabolism in plants. *Plant Physiology and Biochemistry* **45**, 926-931.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F. O., Amann, R., Eickhorst, T., and Schulze-Lefert, P. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* **488**, 91.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Themaat, E. V. L. v., and Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. *Annual Review of Plant Biology* **64**, 807-838.

Burton, J. (2017). The World Leaders In Cucumber Production. https://www.worldatlas.com/articles/the-world-leaders-in-cucumber-production.html.

Cai, Z., Zhang, W., Li, S., Ma, J., Wang, J., and Zhao, X. (2015). Microbial Degradation Mechanism and Pathway of the Novel Insecticide Paichongding by a Newly Isolated Sphingobacterium sp. P1-3 from Soil. *Journal of Agricultural and Food Chemistry* **63**, 3823-3829.

Camenzuli, L., Scheringer, M., and Hungerbühler, K. (2016). Local organochlorine pesticide concentrations in soil put into a global perspective. *Environmental Pollution* **217**, 11-18.

Campisano, A., Antonielli, L., Pancher, M., Yousaf, S., Pindo, M., and Pertot, I. (2014). Bacterial Endophytic Communities in the Grapevine Depend on Pest Management. *PLOS ONE* **9**, e112763.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**, 335.

Chabot, R., Antoun, H., and Cescas, M. P. (1996). Growth promotion of maize and lettuce by phosphate-solubilizing Rhizobium leguminosarum biovar. phaseoli. *Plant and Soil* **184**, 311-321.

Chauhan, H., Bagyaraj, D. J., and Sharma, A. (2012). Plant growth-promoting bacterial endophytes from sugarcane and their potential in promoting growth of the host under field conditions. *Experimental Agriculture* **49**, 43-52.

Chen, J., Zhang, L., Jin, Q., Su, C., Zhao, L., Liu, X., Kou, S., Wang, Y., and Xiao, M. (2017). Bioremediation of phenol in soil through using a mobile plant–endophyte system. *Chemosphere* **182**, 194-202.

Chen, W., Yang, F., Zhang, L., and Wang, J. (2016). Organic Acid Secretion and Phosphate Solubilizing Efficiency of Pseudomonas sp. PSB12: Effects of Phosphorus Forms and Carbon Sources. *Geomicrobiology Journal* **33**, 870-877.

Cherlet, M., Hutchinson, C., Reynolds, J., Hill, J., Sommer, S., and Von Maltitz, G. (2018). "World Atlas of Desertification: Rethinking Land Degradation and Sustainable Land Management," Publications Office of the European Union.

Chi, F., Shen, S.-H., Cheng, H.-P., Jing, Y.-X., Yanni, Y. G., and Dazzo, F. B. (2005). Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Applied and environmental microbiology* **71**, 7271-7278.

Christie, B. R., and Nowak, J. (2000). Bacterial Endophytes: Potential Role in Developing Sustainable Systems of Crop Production AU - Sturz, A. V. *Critical Reviews in Plant Sciences* **19**, 1-30.

Clostre, F., Letourmy, P., Turpin, B., Carles, C., and Lesueur-Jannoyer, M. (2014). Soil Type and Growing Conditions Influence Uptake and Translocation of Organochlorine (Chlordecone) by Cucurbitaceae Species. *Water, Air, & Soil Pollution* **225**, 2153.

Compant, S., Clément, C., and Sessitsch, A. (2010a). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry* **42**, 669-678.

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *Journal of Advanced Research* **19**, 29-37.

Compant, S., Van Der Heijden, M. G. A., and Sessitsch, A. (2010b). Climate change effects on beneficial plant–microorganism interactions. *FEMS Microbiology Ecology* **73**, 197-214.

Convention, S. o. t. S.

Correa-Galeote, D., Bedmar, E. J., and Arone, G. J. (2018). Maize Endophytic Bacterial Diversity as Affected by Soil Cultivation History. *Frontiers in Microbiology* **9**.

Costa, L. E. d. O., Queiroz, M. V. d., Borges, A. C., Moraes, C. A. d., and Araújo, E. F. d. (2012). Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (Phaseolus vulgaris). *Brazilian Journal of Microbiology* **43**, 1562-1575.

Curzi, M. J., Ribaudo, C. M., Trinchero, G. D., Curá, J. A., and Pagano, E. A. (2008). Changes in the content of organic and amino acids and ethylene production of rice plants in response to the inoculation with Herbaspirillum seropedicae. *Journal of Plant Interactions* **3**, 163-173.

Dahal, B., NandaKafle, G., Perkins, L., and Brözel, V. S. (2017). Diversity of free-Living nitrogen fixing Streptomyces in soils of the badlands of South Dakota. *Microbiological Research* **195**, 31-39.

Dasgupta, S., Hossain, M. M., Huq, M., and Wheeler, D. (2015). Climate change and soil salinity: The case of coastal Bangladesh. *Ambio* **44**, 815-826.

Del Giudice, L., Massardo, D. R., Pontieri, P., Bertea, C. M., Mombello, D., Carata, E., Tredici, S. M., Talà, A., Mucciarelli, M., Groudeva, V. I., De Stefano, M., Vigliotta, G., Maffei, M. E., and Alifano, P. (2008). The microbial community of Vetiver root and its involvement into essential oil biogenesis. *Environmental Microbiology* **10**, 2824-2841.

Demain, A. L., and Sanchez, S. (2009). Microbial drug discovery: 80 years of progress. *The Journal Of Antibiotics* **62**, 5.

Desai, J. D., and Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. *Microbiology and molecular biology reviews : MMBR* **61**, 47-64.

Devi, K. A., Pandey, G., Rawat, A. K. S., Sharma, G. D., and Pandey, P. (2017). The Endophytic Symbiont—Pseudomonas aeruginosa Stimulates the Antioxidant Activity and Growth of Achyranthes aspera L. *Frontiers in Microbiology* **8**.

Dong, R., Zhang, J., Huan, H., Bai, C., Chen, Z., and Liu, G. (2017). High Salt Tolerance of a Bradyrhizobium Strain and Its Promotion of the Growth of Stylosanthes guianensis. *International Journal of Molecular Sciences* **18**, 1625.

Doty, S. L., Freeman, J. L., Cohu, C. M., Burken, J. G., Firrincieli, A., Simon, A., Khan, Z., Isebrands, J. G., Lukas, J., and Blaylock, M. J. (2017). Enhanced Degradation of TCE on a Superfund Site Using Endophyte-Assisted Poplar Tree Phytoremediation. *Environmental Science & Technology* **51**, 10050-10058.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research* **32**, 1792-1797.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods* **10**, 996.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics (Oxford, England)* **27**, 2194-2200.

Eevers, N., Hawthorne, J. R., White, J. C., Vangronsveld, J., and Weyens, N. (2016). Exposure of Cucurbita pepo to DDE-contamination alters the endophytic community: A cultivation dependent vs a cultivation independent approach. *Environmental Pollution* **209**, 147-154.

Eevers, N., Hawthorne, J. R., White, J. C., Vangronsveld, J., and Weyens, N. (2018). Endophyte-enhanced phytoremediation of DDE-contaminated using Cucurbita pepo: A field trial. *International Journal of Phytoremediation* **20**, 301-310.

Egamberdieva, D., Berg, G., Lindström, K., and Räsänen, L. A. (2010). Co-inoculation of Pseudomonas spp. with Rhizobium improves growth and symbiotic performance of fodder galega (Galega orientalis Lam.). *European Journal of Soil Biology* **46**, 269-272.

Egamberdieva, D., Jabborova, D., and Berg, G. (2016). Synergistic interactions between Bradyrhizobium japonicum and the endophyte Stenotrophomonas rhizophila and their effects on growth, and nodulation of soybean under salt stress. *Plant and Soil* **405**, 35-45.

Egamberdieva, D., Wirth, S. J., Shurigin, V. V., Hashem, A., and Abd_Allah, E. F. (2017). Endophytic Bacteria Improve Plant Growth, Symbiotic Performance of Chickpea (Cicer arietinum L.) and Induce Suppression of Root Rot Caused by Fusarium solani under Salt Stress. *Frontiers in Microbiology* **8**.

Egamberdiyeva, D., and Islam, K. R. (2008). Salt-Tolerant Rhizobacteria: Plant Growth Promoting Traits and Physiological Characterization Within Ecologically Stressed Environments. *In* "Plant - Bacteria Interactions: Strategies and Techniques to Promote Plant Growth" (I. Ahmad;, J. Pichtel; and S. Hayat, eds.), pp. 263.

Egorova, D. O., Farafonova, V. V., Shestakova, E. A., Andreyev, D. N., Maksimov, A. S., Vasyanin, A. N., Buzmakov, S. A., and Plotnikova, E. G. (2017). Bioremediation of soil contaminated by dichlorodiphenyltrichloroethane with the use of aerobic strain Rhodococcus wratislaviensis Ch628. *Eurasian Soil Science* **50**, 1217-1224.

El-Bialy, H. A., Khalil, O. A. A., and Gomaa, O. M. (2019). Bacterial-mediated biodegradation of pentachlorophenol via electron shuttling. *Environmental Technology* **40**, 2416-2424.

Enache, M., Neagu, S., and Cojoc, R. (2014). Extracellular hydrolases of halophilic microorganisms isolated from hypersaline environments (salt mine and salt lakes). *Scientific Bulletin. Series F Biotechnol* **18**, 20-25.

Esteban, R., Ariz, I., Cruz, C., and Moran, J. F. (2016). Review: Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science* **248**, 92-101.

Etesami, H., and Alikhani, H. A. (2016). Rhizosphere and endorhiza of oilseed rape (Brassica napus L.) plant harbor bacteria with multifaceted beneficial effects. *Biological Control* **94**, 11-24.

Farina, R., Beneduzi, A., Ambrosini, A., de Campos, S. B., Lisboa, B. B., Wendisch, V., Vargas, L. K., and Passaglia, L. M. P. (2012). Diversity of plant growth-promoting rhizobacteria communities associated with the stages of canola growth. *Applied Soil Ecology* **55**, 44-52.

Feng, F., Ge, J., Li, Y., Cheng, J., Zhong, J., and Yu, X. (2017). Isolation, Colonization, and Chlorpyrifos Degradation Mediation of the Endophytic Bacterium Sphingomonas Strain HJY in Chinese Chives (Allium tuberosum). *Journal of Agricultural and Food Chemistry* **65**, 1131-1138.

Fernandez, O., Theocharis, A., Bordiec, S., Feil, R., Jacquens, L., Clément, C., Fontaine, F., and Barka, E. A. (2012). Burkholderia phytofirmans PsJN Acclimates Grapevine to Cold by Modulating Carbohydrate Metabolism. *Molecular Plant-Microbe Interactions* **25**, 496-504.

Fernie, A. R., and Urbanczyk-Wochniak, E. (2004). Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (Solanum lycopersicum) plants. *Journal of Experimental Botany* **56**, 309-321.

Firáková, S., Šturdíková, M., and Múčková, M. (2007). Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia* **62**, 251-257.

Fita, A., Rodríguez-Burruezo, A., Boscaiu, M., Prohens, J., and Vicente, O. (2015). Breeding and Domesticating Crops Adapted to Drought and Salinity: A New Paradigm for Increasing Food Production. *Frontiers in Plant Science* **6**.

Flowers, T. J. (2004). Improving crop salt tolerance. *Journal of Experimental Botany* **55**, 307-319.

Food, and Agriculture Organization of the United, N. (1998). FAOSTAT statistics database. [Rome?] : FAO, 1998-.

Frank, A. C., Saldierna Guzmán, J. P., and Shay, J. E. (2017). Transmission of Bacterial Endophytes. *Microorganisms* **5**, 70.

Fu, W., Xu, M., Sun, K., Hu, L., Cao, W., Dai, C., and Jia, Y. (2018). Biodegradation of phenanthrene by endophytic fungus Phomopsis liquidambari in vitro and in vivo. *Chemosphere* **203**, 160-169.

Fu, Y., Yin, Z.-H., and Yin, C.-Y. (2017). Biotransformation of ginsenoside Rb1 to ginsenoside Rg3 by endophytic bacterium Burkholderia sp. GE 17-7 isolated from Panax ginseng. *Journal of Applied Microbiology* **122**, 1579-1585.

Gange, A. C., and Gadhave, K. R. (2018). Plant growth-promoting rhizobacteria promote plant size inequality. *Scientific Reports* **8**, 13828.

Gao, H., Li, G., and Lou, H.-X. (2018). Structural Diversity and Biological Activities of Novel Secondary Metabolites from Endophytes. *Molecules (Basel, Switzerland)* **23**, 646.

Gao, Y., Liu, Q., Zang, P., Li, X., Ji, Q., He, Z., Zhao, Y., Yang, H., Zhao, X., and Zhang, L. (2015). An endophytic bacterium isolated from Panax ginseng C.A. Meyer enhances growth, reduces morbidity, and stimulates ginsenoside biosynthesis. *Phytochemistry Letters* **11**, 132-138.

Gatheru Waigi, M., Sun, K., and Gao, Y. (2017). Sphingomonads in Microbe-Assisted Phytoremediation: Tackling Soil Pollution. *Trends in Biotechnology* **35**, 883-899.

Glick, B. R. (2012). Plant Growth-Promoting Bacteria: Mechanisms and Applications. *Scientifica* **2012**, 15.

Goldstein, A. H. (2009). Bacterial solubilization of mineral phosphates: Historical perspective and future prospects. *American Journal of Alternative Agriculture* **1**, 51-57.

Gong, B., Liu, G., Liao, R., Song, J., and Zhang, H. (2017). Endophytic fungus Purpureocillium sp. A5 protect mangrove plant Kandelia candel under copper stress. *Brazilian Journal of Microbiology* **48**, 530-536.

Gontia-Mishra, I., Sapre, S., Kachare, S., and Tiwari, S. (2017). Molecular diversity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing PGPR from wheat (Triticum aestivum L.) rhizosphere. *Plant and Soil* **414**, 213-227.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L. L., and Krishnamurthy, L. (2015). Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech* **5**, 355-377.

Goswami, D., Thakker, J. N., Dhandhukia, P. C., and Tejada Moral, M. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. *Cogent Food & Agriculture* **2**, 1127500.

Grobelak, A., Napora, A., and Kacprzak, M. (2015). Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. *Ecological Engineering* **84**, 22-28.

Guarino, C., Paura, B., and Sciarrillo, R. (2018). Enhancing Phytoextraction of HMs at Real Scale, by Combining Salicaceae Trees With Microbial Consortia. *Frontiers in Environmental Science* **6**.

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., Ciulla, D., Tabbaa, D., Highlander, S. K., Sodergren, E., Methé, B., DeSantis, T. Z., Human Microbiome, C., Petrosino, J. F., Knight, R., and Birren, B. W. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome research* **21**, 494-504.

Habib, S. H., Kausar, H., Saud, H. M., Ismail, M. R., and Othman, R. (2015). Molecular Characterization of Stress Tolerant Plant Growth Promoting Rhizobacteria (PGPR) for Growth Enhancement of Rice. **18**, 184-191.

Haiser, H. J., and Turnbaugh, P. J. (2013). Developing a metagenomic view of xenobiotic metabolism. *Pharmacological research* **69**, 21-31.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology* **43**, 895-914.

Hammer, K., and Khoshbakht, K. (2005). Towards a 'red list' for crop plant species. *Genetic Resources and Crop Evolution* **52**, 249-265.

Hanano, A., Almousally, I., and Shaban, M. (2014). Phytotoxicity effects and biological responses of Arabidopsis thaliana to 2,3,7,8-tetrachlorinated dibenzo-p-dioxin exposure. *Chemosphere* **104**, 76-84.

Hanin, M., Ebel, C., Ngom, M., Laplaze, L., and Masmoudi, K. (2016). New Insights on Plant Salt Tolerance Mechanisms and Their Potential Use for Breeding. *Frontiers in Plant Science* **7**, 1787.

Hardoim, P. R., Hardoim, C. C. P., van Overbeek, L. S., and van Elsas, J. D. (2012). Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth Stages. *PLOS ONE* **7**, e30438.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., and Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary

considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews* **79**, 293-320.

Hardoim, P. R., van Overbeek, L. S., and Elsas, J. D. v. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology* **16**, 463-471.

Hasanuzzaman, M., Nahar, K., Alam, M. M., Bhowmik, P. C., Hossain, M. A., Rahman, M. M., Prasad, M. N. V., Ozturk, M., and Fujita, M. (2014). Potential Use of Halophytes to Remediate Saline Soils. *BioMed Research International* **2014**, 12.

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology* **60**, 579-598.

He, W., Megharaj, M., Wu, C.-Y., Subashchandrabose, S. R., and Dai, C.-C. (2019). Endophyte-assisted phytoremediation: mechanisms and current application strategies for soil mixed pollutants. *Critical Reviews in Biotechnology*, 1-15.

Hechmi, N., Ben Aissa, N., Abdenaceur, H., and Jedidi, N. (2015). Uptake and Bioaccumulation of Pentachlorophenol by Emergent Wetland Plant Phragmites australis (Common Reed) in Cadmium Co-contaminated Soil. *International Journal of Phytoremediation* **17**, 109-116.

Hedi, A., Sadfi, N., Fardeau, M.-L., Rebib, H., Cayol, J.-L., Ollivier, B., and Boudabous, A. (2009). Studies on the Biodiversity of Halophilic Microorganisms Isolated from El-Djerid Salt Lake (Tunisia) under Aerobic Conditions. *International Journal of Microbiology* **2009**, 731786.

Herppich, W. B., Huyskens-Keil, S., and Schreiner, M. (2012). Effects of saline irrigation on growth, physiology and quality of Mesembryanthemum crystallinum L., a rare vegetable crop. *2012* **82**, 8.

Holland, P. (1996). Pesticide residues-finding out more and more about less and less. *Chemistry in New Zealand* **60**, 10-25.

Hooper, S. D., Raes, J., Foerstner, K. U., Harrington, E. D., Dalevi, D., and Bork, P. (2008). A Molecular Study of Microbe Transfer between Distant Environments. *PLOS ONE* **3**, e2607.

Huang, C. Y., Roessner, U., Eickmeier, I., Genc, Y., Callahan, D. L., Shirley, N., Langridge, P., and Bacic, A. (2008). Metabolite Profiling Reveals Distinct Changes in Carbon and Nitrogen Metabolism in Phosphate-Deficient Barley Plants (Hordeum vulgare L.). *Plant and Cell Physiology* **49**, 691-703.

Hubbard, C. J., Brock, M. T., van Diepen, L. T. A., Maignien, L., Ewers, B. E., and Weinig, C. (2017). The plant circadian clock influences rhizosphere community structure and function. *The Isme Journal*.

Ilangumaran, G., and Smith, D. L. (2017). Plant Growth Promoting Rhizobacteria in Amelioration of Salinity Stress: A Systems Biology Perspective. *Frontiers in Plant Science* **8**.

Imhoff, J. F. (2017). Anoxygenic Phototrophic Bacteria from Extreme Environments. *In* "Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects" (P. C. Hallenbeck, ed.), pp. 427-480. Springer International Publishing, Cham.

Iqbal, A., Mukherjee, M., Rashid, J., Khan, S. A., Ali, M. A., and Arshad, M. (2019). Development of plant-microbe phytoremediation system for petroleum hydrocarbon degradation: An insight from alkb gene expression and phytotoxicity analysis. *Science of The Total Environment* **671**, 696-704.

Ito, K., Takagi, K., Matsushima, Y., Iwasaki, A., Tanaka, N., Kanesaki, Y., Martin-Laurent, F., and Igimi, S. (2018). Identification of the novel hcbB operon catalyzing the dechlorination of pentachlorophenol in the Gram-positive bacterium Nocardioides sp strain PD653. *Journal of Pesticide Science* **43**, 124-131.

Jacques, F., Rippa, S., and Perrin, Y. (2018). Physiology of L-carnitine in plants in light of the knowledge in animals and microorganisms. *Plant Science* **274**, 432-440.

Jan, M. R., Shah, J., Khawaja, M. A., and Gul, K. (2009). DDT residue in soil and water in and around abandoned DDT manufacturing factory. *Environmental Monitoring and Assessment* **155**, 31-38.

Jha, B., Gontia, I., and Hartmann, A. (2012). The roots of the halophyte Salicornia brachiata are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential. *Plant and Soil* **356**, 265-277.

Jha, Y., and Subramanian, R. B. (2014). PGPR regulate caspase-like activity, programmed cell death, and antioxidant enzyme activity in paddy under salinity. *Physiology and Molecular Biology of Plants* **20**, 201-207.

Jha, Y., and Subramanian, R. B. (2016). Rhizobacteria enhance oil content and physiological status of Hyptis suaveolens under salinity stress. *Rhizosphere* **1**, 33-35.

Jiao, J., Ma, Y., Chen, S., Liu, C., Song, Y., Qin, Y., Yuan, C., and Liu, Y. (2016). Melatonin-Producing Endophytic Bacteria from Grapevine Roots Promote the Abiotic Stress-Induced Production of Endogenous Melatonin in Their Hosts. *Frontiers in Plant Science* **7**.

Ju, F., and Zhang, T. (2015). Bacterial assembly and temporal dynamics in activated sludge of a full-scale municipal wastewater treatment plant. *The ISME journal* **9**, 683-695.

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. *Mammalian protein metabolism* **3**, 132.

Kaminsky, L. M., Thompson, G. L., Trexler, R. V., Bell, T. H., and Kao-Kniffin, J. (2018). Medicago sativa has Reduced Biomass and Nodulation When Grown with Soil Microbiomes Conditioned to High Phosphorus Inputs. *Phytobiomes Journal* **2**, 237-248.

Kapoor, R., Gupta, M. K., Kumar, N., and Kanwar, S. S. (2017). Analysis of nhaA gene from salt tolerant and plant growth promoting Enterobacter ludwigii. *Rhizosphere* **4**, 62-69.

Karahara, I., Ikeda, A., Kondo, T., and Uetake, Y. (2004). Development of the Casparian strip in primary roots of maize under salt stress. *Planta* **219**, 41-47.

Kataoka, R., Güneri, E., Turgay, O. C., Yaprak, A. E., Sevilir, B., and Başköse, I. (2017). Sodium-resistant plant growth-promoting rhizobacteria isolated from a halophyte, Salsola grandis, in saline-alkaline soils of Turkey.

Keylock, C. J. (2005). Simpson diversity and the Shannon–Wiener index as special cases of a generalized entropy. *Oikos* **109**, 203-207.

Khalifa, A. Y. Z., Alsyeeh, A.-M., Almalki, M. A., and Saleh, F. A. (2016). Characterization of the plant growth promoting bacterium, Enterobacter cloacae MSR1, isolated from roots of non-nodulating Medicago sativa. *Saudi Journal of Biological Sciences* **23**, 79-86.

Khan, N., Bano, A., and Babar, M. D. A. (2019). Metabolic and physiological changes induced by plant growth regulators and plant growth promoting rhizobacteria and their impact on drought tolerance in Cicer arietinum L. *PLOS ONE* **14**, e0213040.

Khan, Z., and Doty, S. (2011). Endophyte-assisted phytoremediation. *Plant Biol* **12**, 97-105.

Khwaja, M. A. (2008). POPs Hot Spot: Soil Contamination due to a Demolished Dichlorodiphenyltrichloroethane (Persistent Organic Pollutant) Factory, Nowshera, NWFP, Pakistan. *Annals of the New York Academy of Sciences* **1140**, 113-120.

Khwaja, M. A., Jan, M. R., and Gul, K. (2006). Physical verification and study of contamination of soil and water in and surrounding areas of abandoned persistent organic pollutant (DDT) factory in North West Frontier Province (NWFP) Pakistan. *Sustainable Development Policy Institute SDPI, Islamabad, Pakistan*, 54.

Kim, K., Jang, Y.-J., Lee, S.-M., Oh, B.-T., Chae, J.-C., and Lee, K.-J. (2014). Alleviation of salt stress by Enterobacter sp. EJ01 in tomato and Arabidopsis is accompanied by up-regulation of conserved salinity responsive factors in plants. *Molecules and cells* **37**, 109.

Kloepper, J. W., Gutiérrez-Estrada, A., and McInroy, J. A. (2007). Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. *Canadian Journal of Microbiology* **53**, 159-167.

Kohler, J., Caravaca, F., and Roldán, A. (2010). An AM fungus and a PGPR intensify the adverse effects of salinity on the stability of rhizosphere soil aggregates of Lactuca sativa. *Soil Biology and Biochemistry* **42**, 429-434.

Kumar, A., and Verma, J. P. (2018). Does plant—Microbe interaction confer stress tolerance in plants: A review? *Microbiological Research* **207**, 41-52.

Kumar, S., Karan, R., Kapoor, S., S.P, S., and S.K, K. (2012). Screening and isolation of halophilic bacteria producing industrially important enzymes. *Brazilian Journal of Microbiology* **43**, 1595-1603.

Larsen, J., Pineda-Sánchez, H., Delgado-Arellano, I., Castellano-Morales, V., Carreto-Montoya, L., and Villegas-Moreno, J. (2017). Interactions between microbial plant growth promoters and their effects on maize growth performance in different mineral and organic fertilization scenarios. *Rhizosphere* **3**, 75-81.

Leigh, M. B., Fletcher, J. S., Fu, X., and Schmitz, F. J. (2002). Root Turnover: An Important Source of Microbial Substrates in Rhizosphere Remediation of Recalcitrant Contaminants. *Environmental Science & Technology* **36**, 1579-1583.

Lemanceau, P., Blouin, M., Muller, D., and Moënne-Loccoz, Y. (2017). Let the Core Microbiota Be Functional. *Trends in Plant Science* **22**, 583-595.

Li, J., Zhao, G.-Z., Varma, A., Qin, S., Xiong, Z., Huang, H.-Y., Zhu, W.-Y., Zhao, L.-X., Xu, L.-H., Zhang, S., and Li, W.-J. (2012). An Endophytic Pseudonocardia Species Induces the Production of Artemisinin in Artemisia annua. *PLOS ONE* **7**, e51410.

Li, K., Pidatala, V. R., Shaik, R., Datta, R., and Ramakrishna, W. (2014). Integrated Metabolomic and Proteomic Approaches Dissect the Effect of Metal-Resistant Bacteria on Maize Biomass and Copper Uptake. *Environmental Science & Technology* **48**, 1184-1193.

Li, X., Li, W., Chu, L., White, J. F., Xiong, Z., and Li, H. (2016). Diversity and heavy metal tolerance of endophytic fungi from Dysphania ambrosioides, a hyperaccumulator from Pb–Zn contaminated soils. *Journal of Plant Interactions* **11**, 186-192.

Libik, M., Pater, B., Elliot, S., Ślesak, I., and Miszalski, Z. (2004). Malate Accumulation in Different Organs of Mesembryanthemum crystallinum L. Following Age-dependent or Salinity-triggered CAM Metabolism. *In* "Zeitschrift für Naturforschung C", Vol. 59, pp. 223.

Lim, M. W., Lau, E. V., and Poh, P. E. (2016). A comprehensive guide of remediation technologies for oil contaminated soil — Present works and future directions. *Marine Pollution Bulletin* **109**, 14-45.

Lin, S.-Y., Hameed, A., Liu, Y.-C., Hsu, Y.-H., Lai, W.-A., Huang, H.-I., and Young, C.-C. (2014). Novosphingobium arabidopsis sp. nov., a DDT-resistant bacterium isolated from the rhizosphere of Arabidopsis thaliana. *International Journal of Systematic and Evolutionary Microbiology* **64**, 594-598.

Liu, H., Carvalhais, L. C., Crawford, M., Singh, E., Dennis, P. G., Pieterse, C. M. J., and Schenk, P. M. (2017). Inner Plant Values: Diversity, Colonization and Benefits from Endophytic Bacteria. *Frontiers in Microbiology* **8**.

Liu, J., Abdelfattah, A., Norelli, J., Burchard, E., Schena, L., Droby, S., and Wisniewski, M. (2018). Apple endophytic microbiota of different rootstock/scion combinations suggests a genotype-specific influence. *Microbiome* **6**, 18.

Liu, W., Hou, J., Wang, Q., Ding, L., and Luo, Y. (2014). Isolation and characterization of plant growth-promoting rhizobacteria and their effects on phytoremediation of petroleum-contaminated saline-alkali soil. *Chemosphere* **117**, 303-308.

Liu, X.-M., and Zhang, H. (2015). The effects of bacterial volatile emissions on plant abiotic stress tolerance. *Frontiers in Plant Science* **6**.

Long, H. H., Schmidt, D. D., and Baldwin, I. T. (2008). Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PloS one* **3**, e2702-e2702.

Lopez-Echartea, E., Macek, T., Demnerova, K., and Uhlik, O. (2016). Bacterial Biotransformation of Pentachlorophenol and Micropollutants Formed during Its Production Process. *International journal of environmental research and public health* **13**, 1146.

Lòpez-Fernàndez, S., Compant, S., Vrhovsek, U., Bianchedi, P. L., Sessitsch, A., Pertot, I., and Campisano, A. (2016). Grapevine colonization by endophytic bacteria shifts secondary metabolism and suggests activation of defense pathways. *Plant and Soil* **405**, 155-175.

Lovecka, P., Pacovska, I., Stursa, P., Vrchotova, B., Kochankova, L., and Demnerova, K. (2015). Organochlorinated pesticide degrading microorganisms isolated from contaminated soil. *New Biotechnology* **32**, 26-31.

Ludwig-Müller, J. (2015). Plants and endophytes: equal partners in secondary metabolite production? *Biotechnology Letters* **37**, 1325-1334.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Rio, T. G. d., Edgar, R. C., Eickhorst, T., Ley, R. E., Hugenholtz, P., Tringe, S. G., and Dangl, J. L. (2012). Defining the core Arabidopsis thaliana root microbiome. *Nature* **488**, 86.

Maggini, V., De Leo, M., Mengoni, A., Gallo, E. R., Miceli, E., Reidel, R. V. B., Biffi, S., Pistelli, L., Fani, R., Firenzuoli, F., and Bogani, P. (2017). Plant-endophytes interaction influences the secondary metabolism in Echinacea purpurea (L.) Moench: an in vitro model. *Scientific Reports* **7**, 16924.

Magnani, G., Didonet, C., Cruz, L., Picheth, C., Pedrosa, F., and Souza, E. (2010). Diversity of endophytic bacteria in Brazilian sugarcane. *Genet Mol Res* **9**, 250-258.

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics (Oxford, England)* **27**, 2957-2963.

Mahaffee, W. F., and Kloepper, J. W. (1997). Temporal Changes in the Bacterial Communities of Soil, Rhizosphere, and Endorhiza Associated with Field-Grown Cucumber (Cucumis sativus L.). *Microbial Ecology* **34**, 210-223.

Mahmood, A., and Kataoka, R. (2018). Potential of Biopriming in Enhancing Crop Productivity and Stress Tolerance. *In* "Advances in Seed Priming" (A. Rakshit and H. B. Singh, eds.), pp. 127-145. Springer Singapore, Singapore.

Mahmood, A., and Kataoka, R. (2019). Application of Endophytes Through Seed Priming. *In* "Priming and Pretreatment of Seeds and Seedlings: Implication in Plant Stress Tolerance and Enhancing Productivity in Crop Plants" (M. Hasanuzzaman and V. Fotopoulos, eds.), pp. 509-521. Springer Singapore, Singapore.

Mahmood, A., Kataoka, R., Turgay, O. C., and Yaprak, A. E. (2019a). Halophytic Microbiome in Ameliorating the Stress. *In* "Ecophysiology, Abiotic Stress Responses and Utilization of Halophytes" (M. Hasanuzzaman, K. Nahar and M. Öztürk, eds.), pp. 171-194. Springer Singapore, Singapore.

Mahmood, A., Takagi, K., Ito, K., and Kataoka, R. (2019b). Changes in endophytic bacterial communities during different growth stages of cucumber (Cucumis sativus L.). *World Journal of Microbiology and Biotechnology* **35**, 104.

Mahmood, A., Turgay, O. C., Farooq, M., and Hayat, R. (2016). Seed biopriming with plant growth promoting rhizobacteria: a review. *FEMS Microbiology Ecology* **92**, fiw112-fiw112.

Malfanova, N., Kamilova, F., Validov, S., Chebotar, V., and Lugtenberg, B. (2013). Is l-arabinose important for the endophytic lifestyle of Pseudomonas spp.? *Archives of Microbiology* **195**, 9-17.

Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., Ouzari, I., Daffonchio, D., and Borin, S. (2013). Potential for Plant Growth Promotion of Rhizobacteria Associated with Salicornia Growing in Tunisian Hypersaline Soils. *BioMed Research International* **2013**, 13.

Marasco, R., Rolli, E., Fusi, M., Michoud, G., and Daffonchio, D. (2018). Grapevine rootstocks shape underground bacterial microbiome and networking but not potential functionality. *Microbiome* **6**, 3-3.

Margesin, R., and Schinner, F. (2001). Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles* **5**, 73-83.

Marihal, A., Jagadeesh, K., and Sarita, S. (2014). Biodegradation of pentachlorophenol by endophytic bacteria isolated from PCP-tolerant plant species. *Journal of Pure and Applied Microbiology* **8**, 1283-1288.

Martínez-Viveros, O., Jorquera, M. A., Crowley, D. E., Gajardo, G., and Mora, M. L. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of soil science and plant nutrition* **10**, 293-319.

Martinez, V., Nuñez, J. M., Ortiz, A., and Cerda, A. (1994). Changes in amino acid and organic acid composition in tomato and cucumber plants in relation to salinity and nitrogen nutrition. *Journal of Plant Nutrition* **17**, 1359-1368.

Matsumoto, E., Kawanaka, Y., Yun, S.-J., and Oyaizu, H. (2009). Bioremediation of the organochlorine pesticides, dieldrin and endrin, and their occurrence in the environment. *Applied Microbiology and Biotechnology* **84**, 205-216.

Mayak, S., Tirosh, T., and Glick, B. R. (2004). Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiology and Biochemistry* **42**, 565-572.

Meena, V. S., Maurya, B. R., Verma, J. P., Aeron, A., Kumar, A., Kim, K., and Bajpai, V. K. (2015). Potassium solubilizing rhizobacteria (KSR): Isolation, identification, and K-release dynamics from waste mica. *Ecological Engineering* **81**, 340-347.

Mendpara, J., Parekh, V., Vaghela, S., Makasana, A., Kunjadia, P. D., Sanghvi, G., Vaishnav, D., and Dave, G. S. (2013). Isolation and characterization of high salt tolerant bacteria from agricultural soil. *Eur. J. Exp. Biol* **3**, 351-358.

Mesa-Marín, J., Barcia-Piedras, J. M., Mateos-Naranjo, E., Cox, L., Real, M., Pérez-Romero, J. A., Navarro-Torre, S., Rodríguez-Llorente, I. D., Pajuelo, E., Parra, R., and Redondo-Gómez, S. (2019). Soil phenanthrene phytoremediation capacity in bacteria-assisted Spartina densiflora. *Ecotoxicology and Environmental Safety* **182**, 109382.

Mesa, V., Navazas, A., González-Gil, R., González, A., Weyens, N., Lauga, B., Gallego, J. L. R., Sánchez, J., and Peláez, A. I. (2017). Use of endophytic and rhizosphere bacteria to improve phytoremediation of arsenic-contaminated industrial soils by autochthonous Betula celtiberica. *Applied and Environmental Microbiology*.

Miguel, P. S. B., de Oliveira, M. N. V., Delvaux, J. C., de Jesus, G. L., Borges, A. C., Tótola, M. R., Neves, J. C. L., and Costa, M. D. (2016). Diversity and distribution of the endophytic bacterial community at different stages of Eucalyptus growth. *Antonie van Leeuwenhoek* **109**, 755-771.

Minervini, F., Celano, G., Lattanzi, A., Tedone, L., De Mastro, G., Gobbetti, M., and De Angelis, M. (2015). Lactic Acid Bacteria in Durum Wheat Flour Are Endophytic Components of the Plant during Its Entire Life Cycle. *Applied and Environmental Microbiology* **81**, 6736-6748.

Ministry of Agriculture, F. a. F. (2017). 平成 30 年産春植えばれいしょの作付面積、収穫量及び出荷量. *In* "作況調査（野菜）", Vol. 2019. Ministry of Agriculture, Forestry and Fisheries, Government of Japan.

Mishra, J., Singh, R., and Arora, N. K. (2017). Alleviation of Heavy Metal Stress in Plants and Remediation of Soil by Rhizosphere Microorganisms. *Frontiers in microbiology* **8**, 1706-1706.

Misson, J., Raghothama, K. G., Jain, A., Jouhet, J., Block, M. A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., Doumas, P., Nacry, P., Herrerra-Estrella, L., Nussaume, L., and Thibaud, M.-C. (2005). A genome-wide transcriptional analysis using Arabidopsis thaliana Affymetrix gene chips determined plant responses to phosphate deprivation. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 11934-11939.

Montalbán, B., Thijs, S., Lobo, M., Weyens, N., Ameloot, M., Vangronsveld, J., and Pérez-Sanz, A. (2017). Cultivar and Metal-Specific Effects of Endophytic Bacteria in Helianthus tuberosus Exposed to Cd and Zn. *International journal of molecular sciences* **18**, 2026.

Morcuende, R., Bari, R., Gibon, Y., Zheng, W., Pant, B. D., Bläsing, O., Usadel, B., Czechowski, T., Udvardi, M. K., Stitt, M., and Scheible, W.-R. (2007). Genome-wide reprogramming of metabolism and regulatory networks of Arabidopsis in response to phosphorus. *Plant, Cell & Environment* **30**, 85-112.

Nakamura, T., Motoyama, T., Suzuki, Y., and Yamaguchi, I. (2004). Biotransformation of pentachlorophenol by Chinese chive and a recombinant derivative of its rhizosphere-competent microorganism, Pseudomonas gladioli M-2196. *Soil Biology and Biochemistry* **36**, 787-795.

Namiki, S., Otani, T., and Seike, N. (2013). Fate and plant uptake of persistent organic pollutants in soil. *Soil Science and Plant Nutrition* **59**, 669-679.

Nautiyal, C. S., Bhadauria, S., Kumar, P., Lal, H., Mondal, R., and Verma, D. (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. *FEMS Microbiology Letters* **182**, 291-296.

Nautiyal, C. S., Johri, J. K., and Singh, H. B. (2002). Survival of the rhizosphere-competent biocontrol strain Pseudomonas fluorescens NBRI2650 in the soil and phytosphere. *Canadian Journal of Microbiology* **48**, 588-601.

Navarro‐Torre, S., Barcia‐Piedras, J. M., Mateos‐Naranjo, E., Redondo‐Gómez, S., Camacho, M., Caviedes, M. A., Pajuelo, E., Rodríguez‐Llorente, I. D., and Papen, H. (2017). Assessing the role of endophytic bacteria in the halophyte Arthrocnemum macrostachyum salt tolerance. *Plant Biology* **19**, 249-256.

Newell, N. (2013). Effects of Soil Salinity on Plant Growth. *Plant Physiology*.

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., Marquez-Garcia, B., Queval, G., and Foyer, C. H. (2012). Glutathione in plants: an integrated overview. *Plant, Cell & Environment* **35**, 454-484.

Numan, M., Bashir, S., Khan, Y., Mumtaz, R., Shinwari, Z. K., Khan, A. L., Khan, A., and Al-Harrasi, A. (2018). Plant growth promoting bacteria as an alternative strategy for salt tolerance in plants: A review. *Microbiological Research* **209**, 21-32.

Obata, T., and Fernie, A. R. (2012). The use of metabolomics to dissect plant responses to abiotic stresses. *Cellular and Molecular Life Sciences* **69**, 3225-3243.

Okazaki, S., Sugawara, M., Yuhashi, K.-i., and Minamisawa, K. (2007). Rhizobitoxine-induced chlorosis occurs in coincidence with methionine deficiency in soybeans. *Annals of botany* **100**, 55-59.

Orhan, F., and Gulluce, M. (2015). Isolation and Characterization of Salt-Tolerant Bacterial Strains in Salt-Affected Soils of Erzurum, Turkey. *Geomicrobiology Journal* **32**, 521-529.

Otani, T., and Seike, N. (2007). Rootstock control of fruit dieldrin concentration in grafted cucumber (Cucumis sativus). *Journal of Pesticide Science*, 0706230007-0706230007.

Otani, T., Seike, N., and Sakata, Y. (2007). Differential uptake of dieldrin and endrin from soil by several plant families and Cucurbita genera. *Soil Science and Plant Nutrition* **53**, 86-94.

Otieno, N., Lally, R., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K., and Dowling, D. (2015). Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. *Frontiers in Microbiology* **6**.

Ozaktan, H., Gül, A., Çakır, B., Yolageldİ, L., Akköprü, A., Fakhraeİ, D., and Akbaba, M. (2013). Isolation optimization of bacterial endophytes from cucumber plants and evaluation of their effects on growth promotion and biocontrol. *In* " Endophytes for plant protection: the state of the art. Proceedings of the 5th International Symposium on Plant Protection and Plant Health in Europe, Humboldt University Berlin, Berlin-Dahlem, Germany, 26-29 May, 2013" (C. Schneider, C. Leifert and F. Feldmann, eds.), pp. 262-268. Deutsche Phytomedizinische Gesellschaft e.V. Verlag, Braunschweig, Germany.

Pan, X., Xu, T., Xu, H., Fang, H., and Yu, Y. (2017). Characterization and genome functional analysis of the DDT-degrading bacterium Ochrobactrum sp. DDT-2. *Science of The Total Environment* **592**, 593-599.

Panwar, M., Tewari, R., and Nayyar, H. (2016). Native halo-tolerant plant growth promoting rhizobacteria Enterococcus and Pantoea sp. improve seed yield of Mungbean (Vigna radiata L.) under soil salinity by reducing sodium uptake and stress injury. *Physiology and Molecular Biology of Plants* **22**, 445-459.

Park, H. G., Lee, Y. S., Kim, K. Y., Park, Y. S., Park, K. H., Han, T. H., Park, C. M., and Ahn, Y. S. (2017). Inoculation with Bacillus licheniformis MH48 Promotes Nutrient Uptake in Seedlings of the Ornamental Plant Camellia japonica grown in Korean Reclaimed Coastal Lands. *Horticultural Science & Technology* **35**, 11-20.

Parte, A. C. (2018). LPSN – List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. *International Journal of Systematic and Evolutionary Microbiology* **68**, 1825-1829.

Patten, C. L., and Glick, B. R. (2002). Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. *Applied and environmental microbiology* **68**, 3795-3801.

Paul, D., and Lade, H. (2014). Plant-growth-promoting rhizobacteria to improve crop growth in saline soils: a review. *Agronomy for Sustainable Development* **34**, 737-752.

Paul, D., and Nair, S. (2008). Stress adaptations in a Plant Growth Promoting Rhizobacterium (PGPR) with increasing salinity in the coastal agricultural soils. *Journal of Basic Microbiology* **48**, 378-384.

Paulucci, N. S., Gallarato, L. A., Reguera, Y. B., Vicario, J. C., Cesari, A. B., García de Lema, M. B., and Dardanelli, M. S. (2015). Arachis hypogaea PGPR isolated from Argentine soil modifies its lipids components in response to temperature and salinity. *Microbiological Research* **173**, 1-9.

Paungfoo-Lonhienne, C., Lonhienne, T. G. A., Yeoh, Y. K., Donose, B. C., Webb, R. I., Parsons, J., Liao, W., Sagulenko, E., Lakshmanan, P., Hugenholtz, P., Schmidt, S., and Ragan, M. A. (2016). Crosstalk between sugarcane and a plant-growth promoting Burkholderia species. *Scientific Reports* **6**, 37389.

Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L. S., and Fernández, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. *Journal of Microbiological Methods* **70**, 127-131.

Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., and Crecchio, C. (2015). Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biology and Fertility of Soils* **51**, 403-415.

Pikovskaya, R. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* **17**, 362-370.

Poly, F., Ranjard, L., Nazaret, S., Gourbière, F., and Monrozier, L. J. (2001). Comparison of nifH Gene Pools in Soils and Soil Microenvironments with Contrasting Properties. *Applied and Environmental Microbiology* **67**, 2255-2262.

Poudel, R., Jumpponen, A., Kennelly, M. M., Rivard, C. L., Gomez-Montano, L., and Garrett, K. A. (2018). Rootstocks shape the rhizobiome: Rhizosphere and endosphere bacterial communities in the grafted tomato system. *Applied and Environmental Microbiology*, 1718-1765.

Proudfoot, A. T. (2003). Pentachlorophenol poisoning. *Toxicological reviews* **22**, 3-11.

Puri, A., Padda, K. P., and Chanway, C. P. (2016). Evidence of nitrogen fixation and growth promotion in canola (Brassica napus L.) by an endophytic diazotroph Paenibacillus polymyxa P2b-2R. *Biology and Fertility of Soils* **52**, 119-125.

Qadir, M., Quillérou, E., Nangia, V., Murtaza, G., Singh, M., Thomas, R. J., Drechsel, P., and Noble, A. D. (2014). Economics of salt-induced land degradation and restoration. *Natural Resources Forum* **38**, 282-295.

Qin, S., Miao, Q., Feng, W.-W., Wang, Y., Zhu, X., Xing, K., and Jiang, J.-H. (2015). Biodiversity and plant growth promoting traits of culturable endophytic actinobacteria associated with Jatropha curcas L. growing in Panxi dry-hot valley soil. *Applied Soil Ecology* **93**, 47-55.

Qin, S., Zhang, Y.-J., Yuan, B., Xu, P.-Y., Xing, K., Wang, J., and Jiang, J.-H. (2014). Isolation of ACC deaminase-producing habitat-adapted symbiotic bacteria associated with halophyte Limonium sinense (Girard) Kuntze and evaluating their plant growth-promoting activity under salt stress. *Plant and Soil* **374**, 753-766.

Qu, J., Xu, Y., Ai, G.-M., Liu, Y., and Liu, Z.-P. (2015). Novel Chryseobacterium sp. PYR2 degrades various organochlorine pesticides (OCPs) and achieves enhancing removal and complete degradation of DDT in highly contaminated soil. *Journal of Environmental Management* **161**, 350-357.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* **41**, D590-D596.

Rahman, S. S., Siddique, R., and Tabassum, N. (2017). Isolation and identification of halotolerant soil bacteria from coastal Patenga area. *BMC Research Notes* **10**, 531.

Ramadoss, D., Lakkineni, V. K., Bose, P., Ali, S., and Annapurna, K. (2013). Mitigation of salt stress in wheat seedlings by halotolerant bacteria isolated from saline habitats. *SpringerPlus* **2**, 6.

Rampelotto, P. H. (2010). Resistance of microorganisms to extreme environmental conditions and its contribution to astrobiology. *Sustainability* **2**, 1602-1623.

Redman, R. S., Sheehan, K. B., Stout, R. G., Rodriguez, R. J., and Henson, J. M. (2002). Thermotolerance Generated by Plant/Fungal Symbiosis. *Science* **298**, 1581-1581.

Rho, H., Hsieh, M., Kandel, S. L., Cantillo, J., Doty, S. L., and Kim, S.-H. (2018). Do Endophytes Promote Growth of Host Plants Under Stress? A Meta-Analysis on Plant Stress Mitigation by Endophytes. *Microbial Ecology* **75**, 407-418.

Riggs, P. J., Chelius, M. K., Iniguez, A. L., Kaeppler, S. M., and Triplett, E. W. (2001). Enhanced maize productivity by inoculation with diazotrophic bacteria. *Functional Plant Biology* **28**, 829-836.

Rippa, S., Zhao, Y., Merlier, F., Charrier, A., and Perrin, Y. (2012). The carnitine biosynthetic pathway in Arabidopsis thaliana shares similar features with the pathway of mammals and fungi. *Plant Physiology and Biochemistry* **60**, 109-114.

Rodriguez, R. J., Henson, J., Van Volkenburgh, E., Hoy, M., Wright, L., Beckwith, F., Kim, Y.-O., and Redman, R. S. (2008). Stress tolerance in plants via habitat-adapted symbiosis. *The Isme Journal* **2**, 404.

Román-Ponce, B., Ramos-Garza, J., Vásquez-Murrieta, M. S., Rivera-Orduña, F. N., Chen, W. F., Yan, J., Estrada-de los Santos, P., and Wang, E. T. (2016). Cultivable endophytic bacteria from heavy metal(loid)-tolerant plants. *Archives of Microbiology* **198**, 941-956.

Rosenblueth, M., and Martínez-Romero, E. (2006). Bacterial Endophytes and Their Interactions with Hosts. *Molecular Plant-Microbe Interactions* **19**, 827-837.

Różycki, H., Dahm, H., Strzelczyk, E., and Li, C. Y. (1999). Diazotrophic bacteria in root-free soil and in the root zone of pine (Pinus sylvestris L.) and oak (Quercus robur L.). *Applied Soil Ecology* **12**, 239-250.

Rungin, S., Indananda, C., Suttiviriya, P., Kruasuwan, W., Jaemsaeng, R., and Thamchaipenet, A. (2012). Plant growth enhancing effects by a siderophore-producing endophytic streptomycete isolated from a Thai jasmine rice plant (Oryza sativa L. cv. KDML105). *Antonie van Leeuwenhoek* **102**, 463-472.

Ruppel, S., Franken, P., and Witzel, K. (2013). Properties of the halophyte microbiome and their implications for plant salt tolerance. *Functional Plant Biology* **40**, 940-951.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiology Letters* **278**, 1-9.

Sah, S., Singh, N., and Singh, R. (2017). Iron acquisition in maize (Zea mays L.) using Pseudomonas siderophore. *3 Biotech* **7**, 121-121.

Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S., and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. *Environmental Science and Pollution Research* **23**, 3984-3999.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.

Santoyo, G., Moreno-Hagelsieb, G., del Carmen Orozco-Mosqueda, M., and Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiological Research* **183**, 92-99.

Sarma, M. V. R. K., Kumar, V., Saharan, K., Srivastava, R., Sharma, A. K., Prakash, A., Sahai, V., and Bisaria, V. S. (2011). Application of inorganic carrier-based formulations of fluorescent pseudomonads and Piriformospora indica on tomato plants and evaluation of their efficacy. *Journal of Applied Microbiology* **111**, 456-466.

Scherling, C., Ulrich, K., Ewald, D., and Weckwerth, W. (2009). A Metabolic Signature of the Beneficial Interaction of the Endophyte Paenibacillus sp. Isolate and In Vitro–Grown Poplar Plants Revealed by Metabolomics. *Molecular Plant-Microbe Interactions* **22**, 1032-1037.

Schimel, J., Balser, T. C., and Wallenstein, M. (2007). Microbial stress-response physiology and its implications for ecosystem function. *Ecology* **88**, 1386-1394.

Schulz, B., and Boyle, C. (2005). The endophytic continuum. *Mycological Research* **109**, 661-686.

Schulze, J., Tesfaye, M., Litjens, R. H. M. G., Bucciarelli, B., Trepp, G., Miller, S., Samac, D., Allan, D., and Vance, C. P. (2002). Malate plays a central role in plant nutrition. *Plant and Soil* **247**, 133-139.

Schwyn, B., and Neilands, J. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical biochemistry* **160**, 47-56.

Scott, M., Rani, M., Samsatly, J., Charron, J.-B., and Jabaji, S. (2018). Endophytes of industrial hemp (Cannabis sativa L.) cultivars: identification of culturable bacteria and fungi in leaves, petioles, and seeds. *Canadian Journal of Microbiology* **64**, 664-680.

Service, U. S. F. (1980). "Root characteristics of some important trees of eastern forests : a summary of the literature," Eastern Region, Forest Service, U.S. Dept. of Agriculture, [Milwaukee, Wis.?] :.

Sgroy, V., Cassán, F., Masciarelli, O., Del Papa, M. F., Lagares, A., and Luna, V. (2009). Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte Prosopis strombulifera. *Applied Microbiology and Biotechnology* **85**, 371-381.

Shahzad, R., Khan, A. L., Bilal, S., Asaf, S., and Lee, I.-J. (2018). What Is There in Seeds? Vertically Transmitted Endophytic Resources for Sustainable Improvement in Plant Growth. *Frontiers in Plant Science* **9**, 24.

Shi, Y., Lou, K., and Li, C. (2011). Growth promotion effects of the endophyte Acinetobacter johnsonii strain 3-1 on sugar beet. *Symbiosis* **54**, 159-166.

Shrivastava, P., and Kumar, R. (2015). Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi Journal of Biological Sciences* **22**, 123-131.

Shukla, P. S., Agarwal, P. K., and Jha, B. (2012). Improved Salinity Tolerance of Arachis hypogaea (L.) by the Interaction of Halotolerant Plant-Growth-Promoting Rhizobacteria. *Journal of Plant Growth Regulation* **31**, 195-206.

Siddikee, M. A., Chauhan, P., Anandham, R., Han, G.-H., and Sa, T. (2010). Isolation, characterization, and use for plant growth promotion under salt stress, of ACC deaminase-producing halotolerant bacteria derived from coastal soil. *J Microbiol Biotechnol* **20**, 1577-1584.

Siliakus, M. F., van der Oost, J., and Kengen, S. W. M. (2017). Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. *Extremophiles* **21**, 651-670.

Singh, M., Kumar, A., Singh, R., and Pandey, K. D. (2017). Endophytic bacteria: a new source of bioactive compounds. *3 Biotech* **7**, 315-315.

Singh, R. P., Jha, P., and Jha, P. N. (2015). The plant-growth-promoting bacterium Klebsiella sp. SBP-8 confers induced systemic tolerance in wheat (Triticum aestivum) under salt stress. *Journal of Plant Physiology* **184**, 57-67.

Singh, R. P., and Jha, P. N. (2016). The Multifarious PGPR Serratia marcescens CDP-13 Augments Induced Systemic Resistance and Enhanced Salinity Tolerance of Wheat (Triticum aestivum L.). *PLOS ONE* **11**, e0155026.

Smirnoff, N., and Wheeler, G. L. (2000). Ascorbic acid in plants: biosynthesis and function. *Critical reviews in plant sciences* **19**, 267-290.

Smith, D. (1999). Worldwide trends in DDT levels in human breast milk. *International Journal of Epidemiology* **28**, 179-188.

Sohail, M., Eqani, S. A. M. A. S., Podgorski, J., Bhowmik, A. K., Mahmood, A., Ali, N., Sabo-Attwood, T., Bokhari, H., and Shen, H. (2018). Persistent organic pollutant emission via dust deposition

throughout Pakistan: Spatial patterns, regional cycling and their implication for human health risks. *Science of The Total Environment* **618**, 829-837.

Souza, R. d., Ambrosini, A., and Passaglia, L. M. P. (2015a). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and molecular biology* **38**, 401-419.

Souza, R. d., Ambrosini, A., and Passaglia, L. M. P. (2015b). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and Molecular Biology* **38**, 401-419.

Spellerberg, I. F., and Fedor, P. J. (2003). A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon–Wiener' Index. *Global Ecology and Biogeography* **12**, 177-179.

Srivastava, S., Chaudhry, V., Mishra, A., Chauhan, P. S., Rehman, A., Yadav, A., Tuteja, N., and Nautiyal, C. S. (2012). Gene expression profiling through microarray analysis in Arabidopsis thaliana colonized by Pseudomonas putida MTCC5279, a plant growth promoting rhizobacterium. *Plant signaling & behavior* **7**, 235-245.

Stetsenko, L. A., Rakitin, V. Y., Shevyakova, N. I., and Kuznetsov, V. V. (2009). Organ-specific changes in the content of free and conjugated polyamines in Mesembryanthemum crystallinum plants under salinity. *Russian Journal of Plant Physiology* **56**, 808.

Stewart, E. J. (2012). Growing Unculturable Bacteria. *Journal of Bacteriology* **194**, 4151-4160.

Stitt, M., and Fernie, A. R. (2003). From measurements of metabolites to metabolomics: an 'on the fly' perspective illustrated by recent studies of carbon–nitrogen interactions. *Current Opinion in Biotechnology* **14**, 136-144.

Sun, Z.-B., Yuan, X.-F., Zhang, H., Wu, L.-F., Liang, C., and Feng, Y.-J. (2013). Isolation, screening and identification of antagonistic downy mildew endophytic bacteria from cucumber. *European Journal of Plant Pathology* **137**, 847-857.

Syed, J. H., and Malik, R. N. (2011). Occurrence and source identification of organochlorine pesticides in the surrounding surface soils of the Ittehad Chemical Industries Kalashah Kaku, Pakistan. *Environmental Earth Sciences* **62**, 1311-1321.

Szymańska, S., Płociniczak, T., Piotrowska-Seget, Z., and Hrynkiewicz, K. (2016a). Endophytic and rhizosphere bacteria associated with the roots of the halophyte Salicornia europaea L. – community structure and metabolic potential. *Microbiological Research* **192**, 37-51.

Szymańska, S., Płociniczak, T., Piotrowska-Seget, Z., Złoch, M., Ruppel, S., and Hrynkiewicz, K. (2016b). Metabolic potential and community structure of endophytic and rhizosphere bacteria associated with the roots of the halophyte Aster tripolium L. *Microbiological Research* **182**, 68-79.

Takagi, H., Ishiga, Y., Watanabe, S., Konishi, T., Egusa, M., Akiyoshi, N., Matsuura, T., Mori, I. C., Hirayama, T., Kaminaka, H., Shimada, H., and Sakamoto, A. (2016). Allantoin, a stress-related purine metabolite, can activate jasmonate signaling in a MYC2-regulated and abscisic acid-dependent manner. *Journal of experimental botany* **67**, 2519-2532.

Tehseen, W. M., Hansen, L. G., Wood, S. G., and Hanif, M. (1994). Assessment of chemical contaminants in water and sediment samples from Degh Nala in the province of Punjab, Pakistan. *Archives of Environmental Contamination and Toxicology* **26**, 79-89.

Timpa, J. D., Burke, J. J., Quisenberry, J. E., and Wendt, C. W. (1987). Effects of Water Stress on the Organic Acid and Carbohydrate Compositions of Cotton Plants. *Plant Physiology* **83**, 228-228.

Tlili, A., Tarhouni, M., Cardà, A., and Neffati, M. (2017). Use of local pastoral species to increase fodder production of the saline rangelands in southern Tunisia. *In* "EGU General Assembly Conference Abstracts", Vol. 19, pp. 1493.

Top, E. M., and Springael, D. (2003). The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Current Opinion in Biotechnology* **14**, 262-269.

Trofimova, M. S., Zhestkova, I. M., Kholodova, V. P., Andreev, I. M., Sorokin, E. M., Kruglova, A. G., and Kuznetsov, V. V. (2003). Osmotic water permeability of cell membranes from Mesembryanthemum crystallinum leaves: effects of age and salinity. *Physiologia Plantarum* **118**, 232-239.

Turan, S., Cornish, K., and Kumar, S. (2012). Salinity tolerance in plants: breeding and genetic engineering. *Australian Journal of Crop Science* **6**, 1337.

Ullah, S., Faiz, P., Aamir, M., Sabir, M. A., and Mahmood, Q. (2019). Occurrence and spatio-vertical distribution of DDT in soils of abandoned DDT factory area, Amangarh, Pakistan. *SN Applied Sciences* **1**, 817.

Ulrich, K., Ulrich, A., and Ewald, D. (2008). Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiology Ecology* **63**, 169-180.

Upadhyay, S. K., Singh, J. S., and Singh, D. P. (2011). Exopolysaccharide-Producing Plant Growth-Promoting Rhizobacteria Under Salinity Condition. *Pedosphere* **21**, 214-222.

Vacheron, J., Desbrosses, G., Bouffaud, M.-L., Touraine, B., Moënne-Loccoz, Y., Muller, D., Legendre, L., Wisniewski-Dyé, F., and Prigent-Combaret, C. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Frontiers in Plant Science* **4**.

van den Berg, H., Manuweera, G., and Konradsen, F. (2017). Global trends in the production and use of DDT for control of malaria and other vector-borne diseases. *Malaria journal* **16**, 401-401.

van der Lelie, D., Taghavi, S., Monchy, S., Schwender, J., Miller, L., Ferrieri, R., Rogers, A., Wu, X., Zhu, W., Weyens, N., Vangronsveld, J., and Newman, L. (2009). Poplar and its Bacterial Endophytes: Coexistence and Harmony. *Critical Reviews in Plant Sciences* **28**, 346-358.

van der Meer, J. R. (2003). Evolution of Metabolic Pathways for Degradation of Environmental Pollutants. *In* "Encyclopedia of Agrochemicals". John Wiley & Sons, Inc.

van der Zande, A. (2010). Exploration of management options for Pentachlorophenol (PCP) Paper for the 8th meeting of the UNECE CLRTAP Task Force on Persistent Organic Pollutants. Montreal.

Ventosa, A., Mellado, E., Sanchez-Porro, C., and Marquez, M. C. (2008). Halophilic and Halotolerant Micro-Organisms from Soils. *In* "Microbiology of Extreme Soils" (P. Dion and C. S. Nautiyal, eds.), pp. 87-115. Springer Berlin Heidelberg, Berlin, Heidelberg.

Venugopalan, A., and Srivastava, S. (2015). Endophytes as in vitro production platforms of high value plant secondary metabolites. *Biotechnology Advances* **33**, 873-887.

Vreeland, R. H. (1987). Mechanisms of Halotolerance in Microorganisms. *CRC Critical Reviews in Microbiology* **14**, 311-356.

Wang, B., Liu, W., Liu, X., Franks, A. E., Teng, Y., and Luo, Y. (2017a). Comparative analysis of microbial communities during enrichment and isolation of DDT-degrading bacteria by culture-dependent and -independent methods. *Science of The Total Environment* **590-591**, 297-303.

Wang, F., Jiang, X., Bian, Y.-r., Yao, F.-x., Gao, H.-j., Yu, G.-f., Munch, J. C., and Schroll, R. (2007a). Organochlorine pesticides in soils under different land usage in the Taihu Lake region, China. *Journal of Environmental Sciences* **19**, 584-590.

Wang, G.-l., Bi, M., Liang, B., Jiang, J.-d., and Li, S.-p. (2011). Pseudoxanthomonas jiangsuensis sp. Nov., a DDT-Degrading Bacterium Isolated from a Long-Term DDT-Polluted Soil. *Current Microbiology* **62**, 1760-1766.

Wang, G., Zhang, J., Wang, L., Liang, B., Chen, K., Li, S., and Jiang, J. (2010). Co-metabolism of DDT by the newly isolated bacterium, Pseudoxanthomonas sp. wax. *Brazilian Journal of Microbiology* **41**, 431-438.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007b). Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology* **73**, 5261-5267.

Wang, Y., Li, H., Feng, G., Du, L., and Zeng, D. (2017b). Biodegradation of diuron by an endophytic fungus Neurospora intermedia DP8-1 isolated from sugarcane and its potential for remediating diuron-contaminated soils. *PLOS ONE* **12**, e0182556.

Wei, Y., Zhao, Y., Shi, M., Cao, Z., Lu, Q., Yang, T., Fan, Y., and Wei, Z. (2018). Effect of organic acids production and bacterial community on the possible mechanism of phosphorus solubilization during composting with enriched phosphate-solubilizing bacteria inoculation. *Bioresource Technology* **247**, 190-199.

White, J. F., Kingsley, K. L., Zhang, Q., Verma, R., Obi, N., Dvinskikh, S., Elmore, M. T., Verma, S. K., Gond, S. K., and Kowalski, K. P. (2019). Review: Endophytic microbes and their potential applications in crop management. *Pest Management Science* **75**, 2558-2565.

Wolf, A., Fritze, A., Hagemann, M., and Berg, G. (2002). Stenotrophomonas rhizophila sp. nov., a novel plant-associated bacterium with antifungal properties. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1937-1944.

Wu, T., Xu, J., Liu, J., Guo, W.-H., Li, X.-B., Xia, J.-B., Xie, W.-J., Yao, Z.-G., Zhang, Y.-M., and Wang, R.-Q. (2019). Characterization and Initial Application of Endophytic Bacillus safensis Strain ZY16 for Improving Phytoremediation of Oil-Contaminated Saline Soils. *Frontiers in Microbiology* **10**.

Xie, H., Zhu, L., Xu, Q., Wang, J., Liu, W., Jiang, J., and Meng, Y. (2011). Isolation and degradation ability of the DDT-degrading bacterial strain KK. *Environmental Earth Sciences* **62**, 93-99.

Xie, S.-S., Wu, H.-J., Zang, H.-Y., Wu, L.-M., Zhu, Q.-Q., and Gao, X.-W. (2014). Plant Growth Promotion by Spermidine-Producing Bacillus subtilis OKB105. *Molecular Plant-Microbe Interactions* **27**, 655-663.

Yaish, M. W., Al-Lawati, A., Jana, G. A., Vishwas Patankar, H., and Glick, B. R. (2016). Impact of Soil Salinity on the Structure of the Bacterial Endophytic Community Identified from the Roots of Caliph Medic (Medicago truncatula). *PLoS ONE* **11**, e0159007.

Yaish, M. W., Antony, I., and Glick, B. R. (2015). Isolation and characterization of endophytic plant growth-promoting bacteria from date palm tree (Phoenix dactylifera L.) and their potential role in salinity tolerance. *Antonie van Leeuwenhoek* **107**, 1519-1532.

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in Plant Science: A Global Perspective. *Frontiers in Plant Science* **7**.

Yamaji, K., Watanabe, Y., Masuya, H., Shigeto, A., Yui, H., and Haruma, T. (2016). Root Fungal Endophytes Enhance Heavy-Metal Stress Tolerance of Clethra barbinervis Growing Naturally at Mining Sites via Growth Enhancement, Promotion of Nutrient Uptake and Decrease of Heavy-Metal Concentration. *PLoS ONE* **11**, e0169089.

Yang, J., Kloepper, J. W., and Ryu, C.-M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science* **14**, 1-4.

Yang, L., Chen, X., She, Q., Cao, G., Liu, Y., Chang, V. W. C., and Tang, C. Y. (2018). Regulation, formation, exposure, and treatment of disinfection by-products (DBPs) in swimming pool waters: A critical review. *Environment International* **121**, 1039-1057.

Yanni, Y. G., and Dazzo, F. B. (2010). Enhancement of rice production using endophytic strains of Rhizobium leguminosarum bv. trifolii in extensive field inoculation trials within the Egypt Nile delta. *Plant and Soil* **336**, 129-142.

You, X. D., Park, J. E., Takase, M., Wada, T., and Tojo, M. (2015). First report of Pythium aphanidermatum causing root rot on common ice plant (Mesembryanthemum crystallinum). *New Disease Reports* **32**, 36.

Younas, A., Hilber, I., ur Rehman, S., Khwaja, M., and Bucheli, T. D. (2013). Former DDT factory in Pakistan revisited for remediation: severe DDT concentrations in soils and plants from within the area. *Environmental Science and Pollution Research* **20**, 1966-1976.

Yu, X., Ai, C., Xin, L., and Zhou, G. (2011). The siderophore-producing bacterium, Bacillus subtilis CAS15, has a biocontrol effect on Fusarium wilt and promotes the growth of pepper. *European Journal of Soil Biology* **47**, 138-145.

Yuan, Z., Druzhinina, I. S., Labbé, J., Redman, R., Qin, Y., Rodriguez, R., Zhang, C., Tuskan, G. A., and Lin, F. (2016). Specialized Microbiome of a Halophyte and its Role in Helping Non-Host Plants to Withstand Salinity. *Scientific Reports* **6**, 32467.

Yukimura, K., Nakai, R., Kohshima, S., Uetake, J., Kanda, H., and Naganuma, T. (2009). Spore-forming halophilic bacteria isolated from Arctic terrains: Implications for long-range transportation of microorganisms. *Polar Science* **3**, 163-169.

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., Bokulich, N. A., Mills, D. A., Martin, G., Taghavi, S., van der Lelie, D., and Gilbert, J. A. (2015). The Soil Microbiome Influences Grapevine-Associated Microbiota. *mBio* **6**.

Zhang, C., Feng, Y., Liu, Y.-w., Chang, H.-q., Li, Z.-j., and Xue, J.-m. (2017). Uptake and translocation of organic pollutants in plants: A review. *Journal of Integrative Agriculture* **16**, 1659-1668.

Zhang, H., Kim, M.-S., Sun, Y., Dowd, S. E., Shi, H., and Paré, P. W. (2008). Soil Bacteria Confer Plant Salt Tolerance by Tissue-Specific Regulation of the Sodium Transporter HKT1. *Molecular Plant-Microbe Interactions* **21**, 737-744.

Zhang, J., Wang, P., Tian, H., Jiang, H., Wang, Y., and Yan, C. (2018). Identification of interior salt-tolerant bacteria from ice plant Mesembryanthemum crystallinum and evaluation of their promoting effects. *Symbiosis* **76**, 243-252.

Zhao, D., Zhao, H., Zhao, D., Zhu, X., Wang, Y., Duan, Y., Xuan, Y., and Chen, L. (2018). Isolation and identification of bacteria from rhizosphere soil and their effect on plant growth promotion and root-knot nematode disease. *Biological Control* **119**, 12-19.

Zhao, S., Zhou, N., Wang, L., and Tian, C.-Y. (2013). Halophyte-Endophyte Coupling: A Promising Bioremediation System for Oil-Contaminated Soil in Northwest China. *Environmental Science & Technology* **47**, 11938-11939.

Zhu, J.-K. (2001). Plant salt tolerance. *Trends in Plant Science* **6**, 66-71.

Zhu, Z.-q., Yang, X.-e., Wang, K., Huang, H.-g., Zhang, X., Fang, H., Li, T.-q., Alva, A. K., and He, Z.-l. (2012). Bioremediation of Cd-DDT co-contaminated soil using the Cd-hyperaccumulator Sedum alfredii and DDT-degrading microbes. *Journal of Hazardous Materials* **235-236**, 144-151.