Diversity and plant growth promoting potential of microbiota associated with sugarcane in Pakistan

Mirza Nabeel Baig

Propositions

1. Plant growth promoting bacteria play an important role in supporting growth of Pakistani crops.
   (This thesis)
2. The rhizosphere is more important than the phyllosphere as home for a broad diversity of plant growth promoting bacteria.
   (This thesis)
3. *Akkermansia muciniphila* can protect and improve human health.
4. Sponges and their associated microorganisms are important sources for novel antimicrobials.
5. Humanity makes a person complete.
6. A good rest is the best for a test – and for a PhD.

Propositions belonging to the thesis, entitled

Diversity and plant growth promoting potential of microbiota associated with sugarcane in Pakistan

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This thesis is dedicated to my parents, my baji jan and my family.

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Chapter 1

General Introduction and Thesis Outline

Introduction

The worldwide population continues to increase, and as a result global food availability is expected to become more limited in the near future, resulting in a situation where it is difficult to feed every person in the world [1, 2]. It is therefore mandatory to increase agricultural production in the coming years, yet without losing sustainability goals out of sight [3]. To this end, agricultural practices are increasingly moving towards more sustainable and environmentally friendly approaches [4]. Such approaches include, amongst others, increased use of transgenic plants developed by modern technology as well as the application of plant growth promoting bacteria (PGPB) [5, 6]. Sugarcane is an important cash and industrial crop in various regions worldwide, and different parts of the plant are being used. Research described in this thesis focuses on agricultural practices in Pakistan, where sugarcane is mostly grown in two seasons. Importantly, agricultural soils in Pakistan have salinity and drought problems – in fact most of the land is wasted by salinity and drought [7]. To sustainably increase the yield and production of sugarcane and the utilization of wasted land, there is an urgent need for adapting new technologies. Conventional breeding has been instrumental for the improvement of sugarcane crop yield in last century but it is not very successful towards increasing abiotic stress tolerance [8]. To this end, the multigenic nature of abiotic stress responses in sugarcane and the complex nature of abiotic stresses and variability in plants, impair the prospects for conventional breeding. Hence, plant breeders started thinking towards the implementation of transgenic techniques [9, 10]. Conventional breeding supported by genetic engineering can help in development of new varieties with high yield, biomass production and resistance against abiotic stress [11]. Genetic engineering relying on the insertion of new genes in the host plant genome can be used to develop new phenotypic characteristics such as increased resistance against environmental stresses like drought and salinity [12]. The impact of new technologies and transgenic plants, however, on the environment, is still largely unknown. To this end, microbial communities related to sugarcane are an important, yet largely neglected research area. PGPB present in the rhizosphere as well as in the phyllosphere have a great importance to agriculture. PGPB are used as biofertilizer to increase the fertility of soil and provide a more environmentally friendly alternative to chemical fertilizers [13]. Such chemical fertilizers are not only expensive, but also have adverse and harmful effects on crops as well as on the environment. The beneficial effects of PGPB rely on different mechanisms, including more direct as well as indirect effects on plant growth. Direct effects mostly include the ability of PGPB to perform biological nitrogen fixation (BNF), phosphorus solubilisation (PSB) or production of phytohormones such as indole acetic acid (IAA) to increase plant growth [14].

More indirect effects include the role of PGPB as biocontrol agent against different plant pathogens. Also, root exudates are very important chemicals released by the plant root, particularly with respect to the strong relationship between plants roots and bacteria and especially PGPB. Different methods are used for the assessment of bacterial diversity present in the plant rhizosphere and phyllosphere. To this end, both cultivation-based as well as biomolecular, cultivation-independent methods are being employed. Cultivation based methods can provide important information about phenotypic traits, however, in general only capture a minor fraction of the overall microbial diversity present in microbial ecosystems. In turn, molecular methods, particularly those targeting the 16S ribosomal RNA (rRNA) gene of bacteria, are key to the identification of cultured bacteria. Furthermore, sequence analysis of 16S rRNA gene fragments PCR amplified directly from DNA extracted from environmental samples can provide more comprehensive information about bacterial diversity and community composition [15]. Different types of next generation sequencing (NGS) techniques are used for measuring bacterial diversity. Currently, Illumina (e.g. HiSeq) sequencing is most routinely used for amplicon sequencing and metagenomic analysis of bacterial communities [16]. In this introduction I will provide an overview of the current state of the art regarding bacterial diversity present in the rhizosphere and phyllosphere of sugarcane by using cultivation and molecular techniques. Furthermore, I will describe the current knowledge regarding PGPB and their effect on plant growth, including details of PGPB application as biofertilizer. Finally, I will introduce the general aim of the research described in this thesis and give an overall outline.

Sugarcane

Sugarcane (*Saccharum officinarum* spp.) is an annual crop classified into the Kingdom Plantae, Phylum Magnoliophyta, Order Poales, Family Poaceae, Genus *Saccharum*. It is one of the world's top ten food crops cultivated in tropical and sub-tropical areas and composed of six species [17]. All varieties of sugarcane are hybrids of the genus *Saccharum*. Four cultivated species are *S. officinarum L., S. barberi Jeswiet, S. sinense Roxb.,* and *S. edule Hassk* and two wild species, *S. spontaneum L. and S. robustum* [18, 19].

All commercial canes grown today are inter-specific hybrids. All commercial hybrid sugarcane cultivars are crosses between *S. officinarum L*. and *Saccharum spontaneum L*. that have been developed through selective breeding [20].

Sugarcane is a tall perennial plant growing erect up to 5 or 6 m and producing multiple stems. The plant is composed of four principal parts, including the root system, stalk, leaves and inflorescence. Sugarcane is a C4 plant having high efficiency in converting light energy to biomass, and especially sucrose. Sugarcane has essentially four growth phases, including (1) germination, (2) tillering, (3) grand growth, and (4) maturity and ripening [21].

Research described in this thesis focuses on agricultural practices in Pakistan, where sugarcane is cultivated in two seasons during spring and autumn. Sugarcane is among the most important

crops after wheat and rice grown worldwide and cultivated in 105 countries [22]. Among these countries, Pakistan is the fifth largest sugarcane producing country [23], and eighth biggest consumer of sugar [24, 25]. The sugar industry is the second largest agro-based industry in Pakistan comprising 89 sugar mills operational [26]. Sugarcane production accounts for 3.7% of the country's value-added agriculture and 0.8% of its Gross domestic product (GDP) in 2021-22. During 2021-22, sugarcane was cropped on 1,260 thousand ha in Pakistan. To date, the highest sugarcane crop production was recorded in Pakistan at 88.651 million tonnes during 2021-22 (taken from (http://finance.gov.pk/survey/chapters_19/Economic_Survey_2021_22.pdf).

Globally, sugarcane production amounted to 1.5 billion tons in 2007, increasing to 1.9 billion tonnes in 2019. Comparing the two most important crops used for sugar production, sugarcane production was 1.9 billion tonnes in 2021 as compared to 270 million tonnes of sugar beet in 2021. Other sugar crops are less important and play a minor role with the production of less than one million tonnes in 2021 as compared to sugarcane and sugar beet. Based on global sugarcane production data from 2019, Brazil was the most important sugarcane producer, accounting for 38% of the total world sugarcane production (Table 1). After that India produced 22%, and China, Pakistan and Thailand all produced 4-6% each. Different factors affects the sugarcane production, including climate change, price fluctuation and WHO guidelines that cause a reduction of sugarcane consumption.

Table 1. Sugarcane production worldwide in 2019

Country	Production (tons)	Production per person (kg)	Acreage (ha)	Yield (kg/ha)
Brazil	746,828,157	3,564.251	10,042,199	74,369
India	376,900,000	282.009	4,730,000	79,682.9
China	108,718,971	77.998	1,414,973	76,834.7
Thailand	104,360,867	1,508.472	1,372,169	76,055.4
Pakistan	67,173,975	332.755	1,101,946	60,959.4
Mexico	56,841,523	455.688	785,905	72,326.2

Data taken from: https://www.atlasbig.com/en-gb/countries-by-sugarcane-production

Each part of sugarcane has significant economic importance. Its cane stem is used in the manufacturing of gur, shakar, (brown sugar) and cane tops as fodder for animals [27]. The industrially important products of sugarcane include bagasse, molasses, filter cake, straw and wax. Bagasse is used for the manufacturing of paper, paper board products, hardboard, as raw material for chemicals and as livestock feed. Bagasse is a renewable resource that is also used for the production of biofuel such as ethanol and electricity, and it is used as fuel for boilers and lime kiln to generate steam which is used to run the sugar mills. Bagasse has also been shown to be an effective bio-sorbent for pollutants such as chromium, cadmium, nickel and dyes in synthetic wastewater. Therefore, it has potential in wastewater management [28].

Sugarcane is the main source of table sugar (sucrose), with 80% of sucrose being produced from sugarcane worldwide with the remaining 20% coming from sugar beet. An overview of the material flow from sugarcane and its by-products juice, bagasse and straw, and their role in sugarcane industries is provided in Fig. 1 [29].

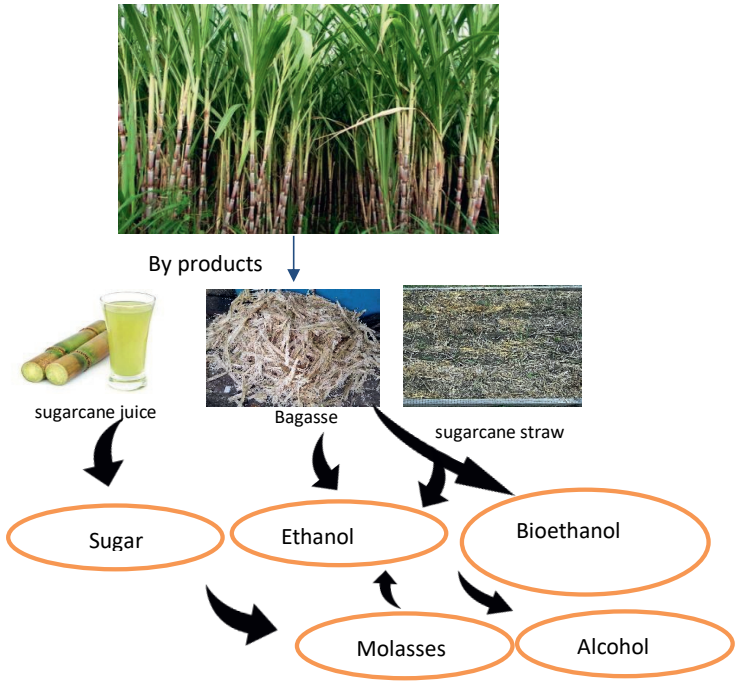

Figure 1. General overview of material flow from sugarcane to derived products

Current challenges faced by agriculture in Pakistan

Utilization of land is more efficient nowadays with increasing yield of sugarcane by using modern technology as compared to traditional agriculture, also because each part of sugarcane is used. Nevertheless, various types of abiotic stresses have gradually led to sugarcane yield losses by as much as 70%. Among various abiotic stresses, plants mostly encounter temperature extremes, drought, and increased salinity. Globally, about 22% of the agricultural land is saline, and drought is expected to further increase in the future [30].

Sugarcane is almost a yearlong crop, and its cultivation requires a large quantity of water till it matures. Globally water stress is the most important limiting factor in crop production. The response and adaptation of species to water stress are very critical for their success in any environmental niche and for productivity in several major food producing countries, with far reaching impact on global food production. According to a recent estimate, around 28% of the

world's land is too dry to support vegetation and up to 45% of the agricultural land is subject to continuous or frequent drought, wherein 38% of the world human population resides [31].
Increased crop production requires more consumption of water. As a consequence, particularly developing countries are confronted by challenges with severe risks to food security. To face these challenges there is an urgent need for stress tolerant crops, especially against drought, to meet the future global food needs. That's why drought tolerance has come to the front of agronomic research in recent years due to decreasing irrigation assets and increased costs associated with irrigation application [32].
The total geographical area of Pakistan is 79.61 Mha, of which 21.99 Mha are cultivated while 9.31 Mha remain cultivable wasteland. This cultivable wasteland is an arid to semiarid land mass and is primarily used as rangeland but with very low productivity. Pakistan is located in a semiarid region of the world with an average annual rainfall of under 240 mm. According to the Faulenmark indicator (a benchmark water scarcity indicator), Pakistan's estimated current water availability per capita of around 1066 m^3 places it in the "highest water stress category [33].
Increasing incidents of drought, desertification, deforestation, and soil erosion are presenting a serious threat to arid and semiarid regions in Pakistan [34].
Salinity is the second major problem for agriculture after drought stress and associated yield loss. Salinity decreases the plant growth affecting 20% of the world's irrigated lands. The harmful effects of salt on plants are a result of (1) water shortfall that results from the relatively high solute concentrations in soil and (2) excessive sodium ion (Na+) concentrations in the cytoplasm. Excessive concentrations of salt in plants change many biochemical processes and result in yield loss in the end. In Pakistan, large areas of land have a high concentration of salt and can no longer be used for agriculture [30, 35-37].
To meet the population's sugar requirement, it is necessary to expand sugarcane cultivation to marginal areas usually affected by abiotic stresses such as extreme temperatures, drought, salinity or chemical toxicity. Among these environmental stresses, water deficit, salinity and frost are the main factors that influence sugarcane productivity as they directly affect tillering and culm height resulting in decreased sucrose production. Stress also influences many biochemical and physiological processes, such as photosynthesis, leaf cell size, transpiration, water potential, growth rate and stomatal closure [38].

Genetically modified crops

To increase the yield of crops, previously traditional technology or conventional breeding was used. New varieties were evolved mostly through import of cane fuzz, however, the selection breeding and flowers are only produced in a specific region in Pakistan, which makes conventional breeding for sugarcane difficult [39].
To this end, new technologies based on genetic engineering are being developed to increase crop yield, for example through increased salt, drought and herbicide resistance [40].

Most genetically modified plants (GMPs) developed by 2008 can be categorized along the following phenotypes: (1) herbicide resistance, (2) resistance to pests, (3) resistance to pathogens, (4) environmental stress resistance, (5) alteration of root exudates, (6) alteration of plant composition, (7) production of compounds relevant to pharmaceutical or chemical industry (8) bioremediation of polluted environments [41]. In 1982, the first GMP that was produced was an antibiotic resistant tobacco plant [42]. The first field trials with GMPs occurred in France and the USA in 1986 when herbicide resistant tobacco plants were engineered [43, 44]. In 1987, Plant Genetic Systems (Ghent, Belgium) was the first company to develop genetically engineered (tobacco) plants with insect tolerance by expressing genes encoding for insecticidal proteins from *Bacillus thuringiensis* (Bt) [45]. The first GMP approved for sale in the U.S., in 1994, was the Flavr Savr Tomato which was modified for its longer shelf life [46].

In 2010, eight insect resistant GMPs expressing Bt and one hybrid cotton variety were officially approved by the government of Pakistan. In Pakistan, 2014 was the fifth year of commercialization of Bt crop with an area of 2.9 Mha .

Transgenic plants have been produced for years with improved agronomic characteristics. Improved and reproducible systems of gene transformation remains the main focal point in plant genetic engineering. Improvement of currently grown sugarcane cultivars through conventional breeding is difficult due to its complex genome and low fertility while genetic engineering has emerged as a potential tool to introduce desirable traits directly into commercially cultivated varieties from any endogenous and exogenous sources [47].

With the increase in land area under transgenic crops, concerns have also been raised over the potential detrimental effects of GMPs on human health, environment and non-target organisms including soil microbial communities. The major concerns are the possibility of creating invasive plant species, the unintended consequences of transgene flow to indigenous plants and microorganisms and development of super pests [48]. Nevertheless, understanding of environmental risks of genetically modified crops and their impact on soil and plant associated microbial communities, particularly those residing in the rhizosphere, remains limited. Such understanding is furthermore complicated by the fact that microbial communities are not only affected by the GMP, but also by many other factors [49, 50].

Plant-associated microbial communities

Microorganisms are the most important component of the rhizosphere and contribute to the ecological fitness of their host plant. Soil microbes are involved in important processes that might occur in the rhizosphere, including that of sugarcane, as outlined in more detail in the following sections [51, 52].

The structure and function of the plant microbiome are generally regulated by several biotic and abiotic factors such as host genotype, developmental stage of host plants, the health status of plant hosts, and different environmental parameters. Based on the involvement of biotic or

abiotic factors in plant microbe interactions, distinct microbial communities are observed and distributed in three parts of the plant, namely the rhizosphere, the phyllosphere, and the endosphere [53, 54]. These three different parts are colonized by different types of microbial communities, which is also reflected in the different genetic make-up of these communities [55, 56].

The rhizosphere

The rhizosphere is the interface between roots and bulk soil and is affected by the roots of the plant. The rhizosphere is rich in nutrients compared to the bulk soil, and many biological and chemical activities and interactions between roots as well as root-associated micro- and macrobiota take place in this region [57-65].

Hiltner was the first scientist to introduce the concept of the rhizosphere as a narrow zone of soil surrounding the roots where microbial populations are stimulated by root activities [66]. The original rhizosphere concept has changed as it is now known that many physical, chemical and biological activities take place in the roots, including bacteria that cause increased root growth [67]. Different types of microorganisms live in the rhizosphere such as bacteria, fungi, protozoa and algae. Among these, bacteria comprise the largest fraction. Plants select bacteria, which increase plant growth, by the synthesis, accumulation and secretion of a diverse array of organic compounds also referred to as root exudates [68, 69]. Bacteria are the most abundant microorganisms in the rhizosphere creating, together with the plant and other microorganisms, a selective environment where diversity is lower compared to the surrounding bulk soil. Bacteria in the rhizosphere can affect plant physiology to a great degree, especially considering their competitiveness in root colonization [68, 70].

Bacteria which live in the rhizosphere are often referred to as rhizobacteria, and their roles in supporting plant physiology include a broad range of functions such as mechanical support and facilitating water and nutrient uptake.

The compounds secreted by plant roots through root exudates act as chemical attractants for a vast number of heterogeneous, diverse and actively metabolizing soil microbial communities. Root exudates affect the chemical and physical properties of the soil and affect the structure of soil microbial communities in the rhizosphere. The composition of root exudates is dependent on the physiological status and species of plants and microorganisms. Root exudates promote beneficial plant microbe interactions and inhibit the growth of competing plant species [71, 72]. A portion of the plant derived small organic molecules contained in the exudates is further metabolized by microorganisms in the vicinity as carbon and nitrogen sources, and some of these microbial metabolites are subsequently taken up by plants for growth and development [71].

Carbon fluxes are most important for rhizosphere function. By the process of root exudation approximately 5–21% of photosynthetically fixed carbon is transported to the rhizosphere [73].

Three most important interacting components of the rhizosphere are the rhizosphere (soil), the rhizoplane, and the root. The rhizosphere is the zone of soil affected by roots through the release of substrates that affect microbial activity. The rhizoplane is the root surface containing attached soil particles, whereas the root is also colonized by endophytic microorganisms [74]. Microbial colonization of the rhizoplane and root tissues is referred to as root colonization, whereas the colonization of the soil affected by the root is called rhizosphere colonization [75-77].

Microorganisms are essential for germination and healthy growth of plants, and important microorganisms are vertically transmitted. Roots of important crops like rice, wheat and sugarcane are colonized by PGPB belonging to genera *Acetobacter*, *Arthrobacter*, *Azoarcus, Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella* and *Zoogloea*. These bacteria are present in the rhizosphere and also in the interior regions of the plant roots and stem and usually do not cause any disease symptoms. In the past two decades, molecular tools have been extensively employed to identify and further characterize PBPB, and these bacteria not only help in plant growth promoting traits, but also produced different signalling compounds which improving the plant growth and tolerance against biotic and abiotic stresses [78-84].

Endophytic bacteria are bacterial species that live within plant tissues without exhibiting harmful effects on the plant. Endophytic bacteria have more intricate and efficient interactions with their host plant then rhizosphere bacteria. Having said that, the rhizosphere is occupied by many potential bacterial endophytes, which is not surprising considering that the rhizosphere is an ecosystem that closely interacts with plant roots. Bacterial endophytes generally colonize the internal tissues of plants and are found in nearly every plant worldwide. Some endophytes are able to promote the growth of plants. Similar to rhizosphere PGPB, endophytic PGPB can act to facilitate plant growth in agriculture, horticulture and silviculture as well as in strategies for environmental clean-up (i.e., phytoremediation) with similar mechanisms. Genome comparisons between bacterial endophytes and PGPB are starting to point towards potential genetic factors involved in an endophytic lifestyle, which should facilitate a better understanding of the functioning of bacterial endophytes [85, 86]. PGPB and their interaction with plant roots and PGPB traits and their function helping in plant growth are visualized in Fig. 2 [87, 88].

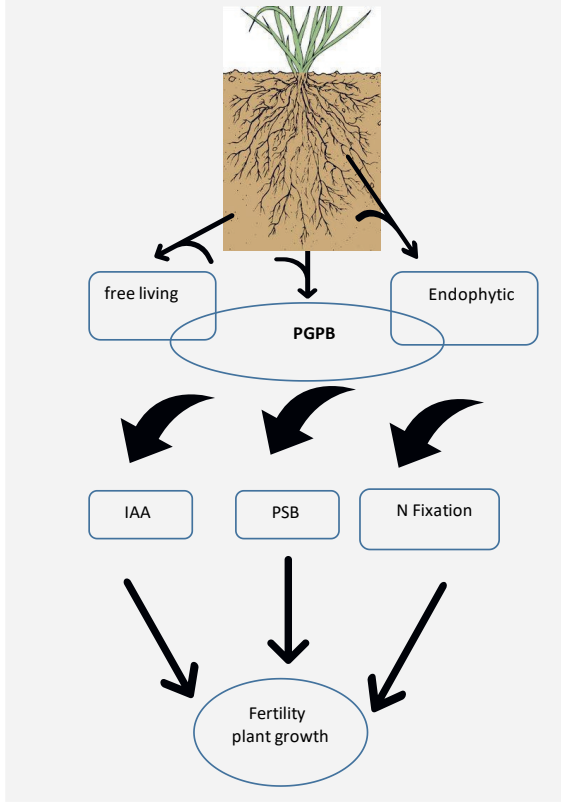

Figure 2. The rhizosphere and different types of interactions with PGPB. Different types of interaction between rhizosphere and bacteria are indicated, including the traits of these bacteria that contribute to plant growth. Major PGPB describe in this figure are: IAA, indole acetic acid producing bacteria; PSB, phosphate solubilizing bacteria; N Fixation, nitrogen fixing bacteria.

The phyllosphere

The region above the soil, including the aerial habitat colonized by microbes is termed phyllosphere, and the bacteria present in this region are called epiphytes. The above ground parts of plants are normally colonized by a variety of bacteria. Few microbial species can be isolated from within plant tissues, whereas some are recovered from the surfaces of healthy plants [89, 90].

The phyllosphere can be divided into different regions including the caulosphere (surface of stems), asthenosphere (surface of flowers), carposphere (surface of fruits), and phylloplane (surface of leaves). All of these phyllospheric components are diversified from each other in terms of their significantly different microbial communities [91].

The most important part of the phyllosphere are leaves as compared to buds, stem and flowers. The bacterial abundance associated with leaves averages 10^6 to 10^7 cells/cm^2 (up to 10^8 cells/g) that colonize the leaf surface [92, 93]. The total global lower and upper surface area of leaves has been estimated with 10^9 km^2 in which 10^{26} bacterial cells are present [94]. The microbial communities of leaves are very diverse and include a large number of different bacterial genera that colonize the phyllosphere. Epiphytic bacterial communities differ significantly in size among and within plants of the same species in close proximity and over short time scales as well as over the growing season [95, 96].

The phyllosphere microbiome is characterized by poor nutrient conditions when compared to for example the rhizosphere and has a high species richness. At the phylum level the phyllosphere bacterial communities are composed mainly of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, with a predominance of the classes *Alphaproteobacteria* and *Gammaproteobacteria*. Further analysis of community composition at the genus level showed consistent presence of genera *Pseudomonas, Sphingomonas, Methylobacterium, Bacillus, Massilia, Arthrobacter, and Pantoea* in the phyllosphere of different plant species [97, 98].

Epiphytic bacterial colonization depends upon availability of carbon and nitrogen nutrients. In the phyllosphere of the plant leaf surface, carbon sources such as glucose, fructose, and sucrose are spatially heterogeneous, which facilitates the development of distinct microbial communities in the phyllosphere. Nutrient availability as well as environmental factors such as temperature, moisture, radiation during day and night, pollution, pH, host genotype and geographic location also affect the microbiome of the phyllosphere [99, 100]. The diversity of bacterial communities present in the phyllosphere also depends on plant morphology, the position and height of leaves as well as leaf age and leaf structure [101, 102].

Small amounts of nutrients can be washed from leaves due to different factors. Simple sugars such as glucose, fructose, and sucrose are the dominant carbon sources on plant leaves and are thought to simply leach from the interior of the plant [103, 104]. Populations of culturable aerobic bacteria on leaves are dominated by a few genera. Variations in the microbial communities of leaves over multiple temporal and spatial scales have provided important and detailed knowledge about the identity and the ecology of bacterial leaf inhabitants [105].

Our knowledge of phyllosphere microbiomes has in the past been hampered by problems associated with culture-based methods regarding the cultivability and failure of many phyllosphere bacteria to grow under *in vitro* conditions, but also by the fact that these microbiomes have received less attention as compared to rhizosphere microbiomes. As outlined earlier, however, the phyllosphere is among the most important niches for microbial communities on earth [106], where microbes that live in the phyllosphere exhibit different types of interactions with each other and the host, which have to be understood [107, 108].

Phyllosphere bacteria have important effects on plant health [109]. Phyllosphere bacteria play an important role in plant growth traits such as

the production of phytohormones, nitrogen fixation and the degradation of organic pollutants. Phyllosphere microorganisms also play important roles in maintaining population sizes of deleterious organisms and some are a potential source of pharmaceuticals and biological control agents [110-114].

As indicated already, phyllosphere microbiomes play a vital role in the cycling of elements as saprophytes and in remediating residual pesticides and atmospheric hydrocarbon pollutants, and bear significance for plant development and health as biofertilizers, phytostimulators and biopesticides to protect against plant pests and pathogens [109, 115, 116]. The diverse phyllosphere microbial communities mediate foliar functional traits, influence plant fitness, and contribute to several ecosystem functions including nutrient and water cycling [117], and they impact significantly on the ability of incoming organisms to successfully colonize the leaf surface [118].

Finally, microorganisms also colonize the endosphere that consists of inner plant tissues, inhabited by microorganisms intimately interacting with the host plant. The endosphere is composed of the internal root tissue (endorhizosphere), internal shoot and leaf tissue (endophyllosphere), internal plant reproductive tissue, and the internal seed tissue [53, 119, 120].

Plant growth promoting (rhizo)bacteria

PGPB are native soil bacteria that colonize the rhizosphere, plant roots or other (aerial) parts of the plant, resulting in stimulation of plant growth either directly and/or indirectly. PGPB have three major properties to live near soil and roots: (1) most proficient to colonize the root surface, (2) ability to survive in the competitive rhizosphere environment, (3) plant growth promotion and protection traits as also shown in Fig. 2 [76, 121-123].

PGPB are classified by different ways. First they can be classified based on their functional activities: (1) increasing the availability of nutrients to the plant, (2) production of phytohormones (hormonal plant growth promotion), (3) biodegradation (degrading organic pollutants) and (4) biocontrol (controlling diseases, mainly by the production of antibiotics and antifungal metabolites) [123]. PGPB are also classified on the basis of their location: extracellular PGPB (ePGPB), i.e. existing in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex. Some examples of ePGPB include members of the genera *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia, Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* [124].

In turn, intracellular PGPB (iPGPB) exist inside root cells generally in specialized nodular structures. iPGPB are also referred to as endophytic bacteria. Some examples of iPGPB are members of the family *Rhizobiaceae*, including the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* [125].

PGPB are also classified on the basis of their shape, including mostly Gram-negative rods, with a lower proportion being Gram positive rods, cocci or pleomorphic in shape. Numerous actinomycetes constitute a major part of rhizosphere microbial communities displaying extensive plant growth beneficial traits [126]. These include members of the genera *Micromonospora*, *Streptomyces*, *Streptosporangium* and *Thermobifida* that have been shown to act as biocontrol bacteria against different root pathogens [52].

PGPB finally include epiphytic bacteria that live associated with the outer surface of plants mostly in the phyllosphere, and that help in plant growth by using different mechanisms.

Mechanisms of plant growth promotion

PGPB have received worldwide importance for agricultural benefits, and a diverse array of bacteria including species of *Rhizobium, Agrobacterium, Pseudomonas, Azospirillum, Azotobacter, Bacillus, Klebsiella*, and many others have been shown to facilitate plant growth both under greenhouse and field conditions.

The use of PGPB is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements. There is a high diversity of PGPB present in the rhizosphere of sugarcane, yet there is a need to further characterize the diversity of PGPB associated with sugarcane, as well as the diverse mechanisms by which these bacteria promote plant growth [127, 128].

PGPB-mediated plant growth promotion occurs by the alteration of the whole microbial community in the rhizosphere niche through the production of various substances [129].

Plant beneficial microbiomes affect plant health by different mechanisms, including the production of hormones such as IAA, nutrient supply, fixation of nitrogen, and the mobilization of phosphorus and minerals such as iron, resistance against biotic stress factors (pathogen and parasite defence), resistance against abiotic factors (water deficiency, salt and drought stress), and production of other bioactive metabolites [130, 131].

Nitrogen fixation

Nitrogen (N) is the most important nutrient and occupies the highest requirement position in plant nutrition compared to the other essential nutrients. Nitrogen is required for cellular synthesis of vital biomolecules including proteins, nucleic acids (DNA and RNA) and chlorophyll for normal plant growth [52].

Nitrogenous fertilizers have increased crop yields and food availability worldwide, but also adverse effects on the environment [132].

The earth's atmosphere contains 78% nitrogen in the form of dinitrogen (N_2). N_2 cannot be used directly by plants as source of nitrogen, however, it is converted into ammonia by biological N fixation (BNF) by microorganisms. These microorganisms use a complex enzyme system called nitrogenase for BNF [133], and play an important role in sustainable agriculture [134]. A overview of relevant nitrogen cycle reactions is shown in Fig. 3 [135].

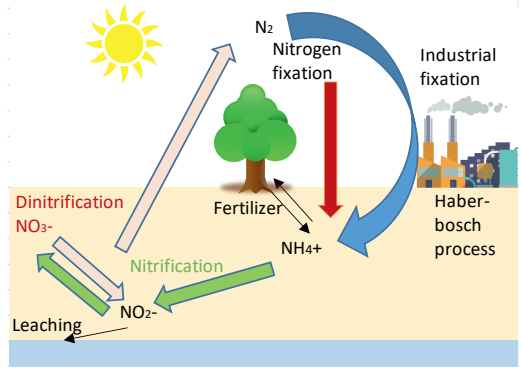

Figure 3. Most relevant reactions of the nitrogen cycle showing the conversion of atmospheric nitrogen into bioavailable forms. Blue arrows indicate nitrogen fixation including biological and industrial processes. Green arrows indicate microbial nitrification processes. Pink arrows indicate denitrification. Black arrows indicate the flow of different compounds in the soil.

Nitrogen fixing bacteria are categorized into two types based on their lifestyle. The first type, symbiotic nitrogen fixing bacteria, have an interaction with plant roots, and more specially the roots of leguminous plants [131]. Symbiotic nitrogen fixing bacteria within the alpha-proteobacterial family of *Rhizobiaceae* infect and establish a symbiotic relationship with the roots of leguminous plants. The establishment of the symbiosis involves a complex interplay between host and symbiont that results in the formation of root nodules within which the rhizobia colonize as intracellular symbionts [136].

The second type of nitrogen fixing bacteria is referred to as non-symbiotic or free-living bacteria which also fix nitrogen using a nitrogenase. Non-symbiotic nitrogen fixing bacteria provide only a small fraction of the fixed nitrogen that the bacterially associated host plant requires [52]. Most of the genera of PGPB play a major role in BNF such as *Pseudomonas, Azospirillum, Azotobacter, Bacillus* and *Klebsiella* [137-140].

The nitrogenase complex catalyses biological N_2 reduction to ammonium and is composed of two highly conserved proteins, including the iron protein also known as nitrogenase reductase (encoded by the *nifH* gene) and the molybdenum iron protein also known as dinitrogenase (encoded by the *nifDK* genes). The *nifH* genes encoding the iron-protein component of nitrogenase are evolutionarily conserved and highly valuable for phylogenetic analysis for the detection and identification of nitrogen fixing bacteria [141, 142]. This gene has therefore become the marker gene of choice for researchers studying the phylogeny, diversity, and abundance of nitrogen-fixing microorganisms [143, 144].

Nitrogen fixing bacteria are ecologically important as they provide input of fixed nitrogen (N) in many terrestrial and aquatic habitats. They have been studied in a variety of ecosystems using multiple approaches to measure rates and characterize diazotroph populations. N_2 fixation is fundamentally important because it makes atmospheric N_2 available to relieve ecosystem N limitation [145, 146].

A number of nitrogen-fixing diazotrophs have been isolated from the rhizosphere and interior of sugarcane plants and have been demonstrated to bear great potential to fix N_2 associated with sugarcane plants. Hence, BNF may help farmers to maintain sugarcane yields under reduced N-fertilization [147].

Many plant-associated diazotrophic bacteria have been shown to promote plant growth. Analysing the transcription of the *nifH* gene in different plant tissues can provide new insights into the active nitrogen-fixing bacteria. High resolution genome-based approaches are needed to trace diazotrophs that are inoculated into plant tissues because they may be very similar to indigenous endophytic strains that already reside within the plant and with which they have to compete [148-151].

Nif genes are typically found in a cluster of around 20–24 kb with seven operons encoding 20 different proteins. Phylogenetic analyses of *nifH* gene sequences revealed five basic clusters of genes homologuous to *nifH*. Cluster I contains sequences of aerobic nitrogen fixers including members of the phyla *Proteobacteria* and *Cyanobacteria*, as well as the genera *Frankia* and *Paenibacillus*. Cluster II is generally thought of as the alternative nitrogenase cluster because it contains sequences from FeFe and FeV nitrogenases which differ from the conventional FeMo cofactor-containing nitrogenase. Cluster III consists of anaerobic nitrogen fixers from Bacteria and Archaea including *Desulfovibrionaceae*, *Clostridia*, *Spirochetes*, and *Methanobacteria*. Cluster IV and cluster V contain sequences that are paralogs of *nifH* and which are not involved in nitrogen fixation [152-154].

Many PCR primers have been developed to target the *nifH* gene with the purpose of amplifying this gene sequence from environmental samples. Use of *nifH* as a marker gene has allowed scientists to characterize the diversity and ecology of nitrogen fixing bacteria and archaea. *NifH* gene diversity analysis has indicated that nitrogen fixing microorganisms can be found in a broad range of habitats, including marine, terrestrial, extreme, anthropogenic, host-associated and agricultural environments, and that their diversity differs in different habitats [155-158].

BNF is a very energy demanding process, requiring at least 16 moles of ATP for each mole of reduced nitrogen, and hence it would be advantageous if bacterial carbon resources were directed toward oxidative phosphorylation, which results in the synthesis of ATP, rather than glycogen synthesis, which results in the storage of energy in the form of glycogen. In line with this, treatment of legume plants with rhizobia having a deleted gene for glycogen synthase resulted in a considerable increase in both the nodule number and plant dry weight with reference to treatment with the wild-type strain [159].

Phosphorus solubilisation

Phosphorus (P) is one of the essential macronutrients required for plant growth and development. Phosphorus is known to be involved in many functions associated with plant growth and metabolism. Several important cellular, metabolic and reproductive functions rely on sufficient phosphorus supply. Having said that, only about 25% of the phosphorus applied to the soil is available for crops and the rest becomes unavailable due to chemical fixation with aluminium and iron in acidic soils. Phosphate solubilization by soil bacteria that make the P available for plant growth is an important mechanism underlying the beneficial effects of PGPB [160-162].

Pakistani soils have low phosphorus status with 80–90% of soils being deficient. This low availability of phosphorous to plants is because the majority of soil P is found in insoluble forms, whereas plants are only able to take up phosphorous in two soluble forms, namely the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) ions. The insoluble P is present as an inorganic mineral such as apatite or as one of several organic forms including inositol phosphate (soil phytate), phosphomonoesters, and phosphotriesters. To overcome this deficiency, chemical phosphorus fertilizer is used, however, its cost is high to fulfil the requirements and most of the added P forms a complex with calcium (Ca), aluminium (Al), and iron (Fe), making phosphorus unavailable for plant uptake and unsustainable for plants and the environment. Furthermore, organic forms of phosphorus account for 30–50% of the total phosphorus in most soils. Inoculation with phosphate solubilizing bacteria (PSB) is a promising approach, which improves the deficiency of phosphorus. PSB combined with chemical fertilizer (superphosphate and rock phosphate) have been shown to reduce the chemical fertilizer dose requirement by 25–50% [52, 163, 164]. The composition of PSB that are present in soil varies between different soils. *Pseudomonas, Bacillus, Rhizobium* and *Enterobacter* are the most important genera of PSB, and increasing solubilization of fixed phosphorus produced high crop yields [165, 166].

The major mechanism of phosphate solubilization involves the production of organic acids that are released by PSB to lower the pH of their environment (Fig. 4). Due to the existence of an equilibrium between anions and protons, the protons are consumed in the dissolution of the phosphorus. Chelation of cations has also been implicated in phosphate solubilization [167].

Chelation involves the formation of two or more coordinate bonds between an anionic or polar molecule and a cation resulting in a ring structure complex. Organic acid anions with oxygen containing hydroxyl and carboxyl groups, have the ability to form stable complexes with cations such as Al^{3+}, Ca^{2+}, Fe^{2+} and Fe^{3+} that are often bound with phosphate in poorly solubilized forms. The organic acids convert tricalcium phosphate to di- and mono-basic phosphates

resulting in an enhanced availability of phosphorus to the plant. The type and quantity of organic acids produced differ for different genera of PSB [163, 168-173].

For sustainable agriculture the use of biofertilizers is very important, environmentally friendly and increases the yield [174]. The first use of PSB as biofertilizers dates back to the 1950's [175]. PGPB together with PSB can reduce phosphorus fertilizer application by 50% without any significant reduction in crop yield [176].

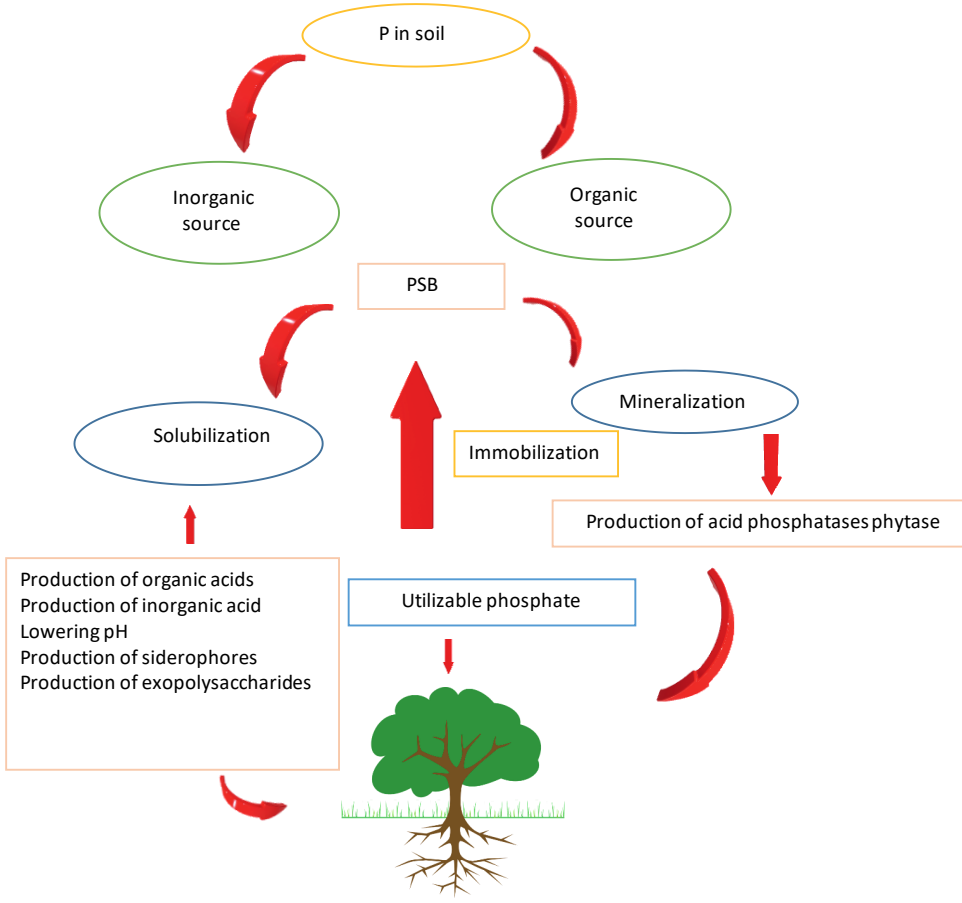

Figure 4. A schematic diagram of soil phosphorus (P) biogeochemical cycles. There are two sources, organic and inorganic, that phosphate solubilizing bacteria (PSB) make available for plants by solubilization and mineralization. The different types of products help in plant growth.

Indole acetic acid (IAA) production

Phytohormones or plant growth regulators are organic substances, which at low concentrations (< 1 mM), promote, inhibit, or modify growth and development of plants [177]. Microbial synthesis of the phytohormone auxin (indole-3-acetic acid/indole acetic acid/IAA) has been

known for a long time. It has been reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites [178, 179]. IAA also acts as a reciprocal signalling molecule affecting gene expression in several microorganisms. IAA plays a very important role in rhizobacteria plant interactions. Downregulation of IAA synthesis as signalling is associated with plant defence mechanisms against a number of phytopathogenic bacteria [180].

IAA produced by rhizobacteria affects the above physiological processes of plants by changing the plant auxin pool, biosynthesis of various metabolites, and resistance to stressful conditions. Bacterial IAA increases the root surface area and length and thus provides the plant with greater access to soil nutrients. IAA loosens plant cell walls and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria. IAA has also been identified as an effector molecule in plant microbe interactions both in pathogenesis and phytostimulation [181].

IAA is involved in multiple processes, including cell division, differentiation and vascular bundle formation. It increases the rate of xylem and root development, controls processes of vegetative growth, initiates lateral and adventitious root formation, mediates responses to light, gravity and fluorescence, affects photosynthesis as well as pigment formation. These three processes are also essential for nodule formation, and it has been shown that certain auxin levels in legume plants are necessary for nodule formation [131]. The role of IAA producing bacteria with respect to their plant growth promoting traits is shown in Fig. 5 [182].

The main precursor for IAA is tryptophan, which plays a role in modulating the level of IAA biosynthesis [164]. Tryptophan stimulates IAA production, whereas anthranilate, which is a precursor for tryptophan, reduces IAA synthesis. By this mechanism IAA biosynthesis is fine-tuned because tryptophan inhibits anthranilate formation by a negative feedback regulation of anthranilate synthase, resulting in an indirect induction of IAA production [183]. Supplementation of culture media with tryptophan increases the IAA production by most rhizobacteria [184].

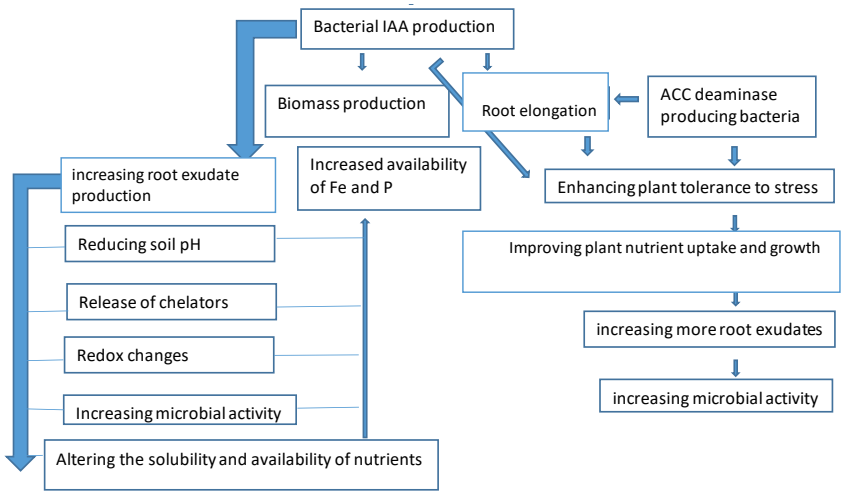

Figure 5. Function of bacterial IAA production, illustrating important roles of ACC deaminase and siderophore producing bacteria and PSB.

Biosynthesis of tryptophan starts from chorismate in a five-step reaction encoded by the *trp* genes. Chorismate is the precursor for the synthesis of aromatic amino acids and is synthesized starting from phosphoenolpyruvate and erythrose 4-phosphate in the shikimate pathway, which is a common pathway for the biosynthesis of aromatic amino acids and many secondary metabolites [178, 185, 186]. Starting with tryptophan at least five different pathways have been described for the synthesis of IAA, and most pathways show similarity to those described in plants although some intermediates can differ [186]. These pathways are (1) Indole-3-acetamide (IAM) pathway, (2) Indole-3-pyruvate (IPyA) pathway, (3) Tryptamine (TAM) pathway, (4) Tryptophan side-chain oxidase (TSO) pathway, and (5) Indole-3-acetonitrile (IAN) pathway [179].

Production of these hormones can be occasionally induced by certain microbial strains such as members of the PGPB genera *Enterobacter, Pseudomonas, Bacillus, Burkholderia, Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Ralstonia, Serratia* and *Rhizobium* [187].

Microbial isolates from the rhizosphere of various crops appear to have a greater potential to synthesize and release auxins as secondary metabolites because of the rich supply of substrates available in root exudates. Production of auxins by microbial isolates varies greatly among different species and strains of the same species and is also influenced by culture conditions, growth stage and availability of the substrate(s). IAA production by a number of *Acetobacter diazotrophicus* isolates from sugarcane was confirmed [188].

PGPB as Biofertilizers

Biofertilizers are defined as living microorganisms, which when applied to seed, plant surfaces, or soil, colonize the rhizosphere and/or internal and external parts of the plant, and increase growth by making essential nutrients more available to the plant. Biofertilizers are different from organic fertilizer – i.e. fertilizer containing organic compounds which directly, or by their decay, increase soil fertility. Unlike green manure, manure, intercropping, or compost and chemical fertilizer, biofertilizers are rhizosphere organisms, which increase availability of soil nutrients but cannot replace soil nutrients [189, 190]. Biofertilizers must contain living microorganisms which enhance plant growth by improving the nutrient status of the plant. Biofertilizers include PGPB, however, some also have fungi such as arbuscular mycorrhizae, which increase plant growth and are present in the rhizosphere [191, 192]. These fungi are not studied in the research described in this thesis.

Biofertilizers play a major role in the development of sustainable nutrient management systems with less adverse effects on the environment [193]. As outlined in detail earlier in this introduction, PGPB can be used to reduce the use of chemical fertilizers, pesticides, and supplements. Most PGPB significantly increase plant height, root length, and dry matter production of shoot and root of plants. Furthermore, PGPB can serve as biocontrol agent when inoculated to seeds and help in the control of plant pests and pathogens. Not all PGPB are biofertilizers, as some bacteria are only biopesticides which promote plant growth by controlling harmful organisms. Some PGPB promote growth by carrying both biofertilizer and biopesticide features. *Burkholderia cepacia* acts as biocontrol against *Fusarium* spp. and also increases plant growth by siderophore (iron solubilizer) production under low iron availability in soil [194].

PGPB have been used as biofertilizing agents in many crops such as, *Triticum aestivum* (wheat), *Zea mays* (maize), *Oryza sativa* (rice) and sugarcane, under different climatic and edaphic conditions [195], and have been inoculated with PGPB as biofertilizer in soils of different geographical regions [196]. Previously use of PGPB to increase crop yield was limited due to the variability and inconsistency of results between laboratories, and between greenhouse and field studies, and a major reason was the lack of reliable technology [197].

Methods to study soil microbial diversity

The biosphere is dominated by microorganisms but only 0.1-10% of all microorganisms are estimated to be culturable whereas many microorganisms remain uncultured [198]. Hence, the total microbial diversity and community structure of a specific environment cannot be described accurately without knowledge about non-cultured microorganisms [199].

Soil microbial diversity and community structure depend on various factors such as soil pH, temperature, moisture content, nature and amount of root exudates, soil nutrient status and crop rotation [200-202].

Bacteria are usually identified using phenotypic techniques that are mainly based on production of enzymes and metabolism of carbohydrates. A number of biochemical and morphological methods have been employed for identification of bacteria [203].

These methodologies are often based on chromogenic enzymatic reactions and are available in commercial kits (BioLog Inc, Hayward, CA, USA; API20E and API ZYM systems by Vitek, Inc, St. Lois, MO, USA). Furthermore, intrinsic antibiotic resistance, fluorescent antibody techniques, polyacrylamide gel electrophoresis of total protein and fatty acid analysis have been used for bacterial identification. DNA based genotypic methods including DNA-DNA hybridization, DNA-RNA hybridization, use of random or specific primers in PCR, RFLP (Restriction Fragment Length Polymorphism) have also been used successfully for identification of bacteria. Ribosomes are an important component of the protein synthesis machinery in all living cells including bacteria. A bacterial ribosome is composed of multiple ribosomal proteins and three ribosomal RNAs (rRNAs), i.e 23S rRNA, 16S rRNA and 5S rRNA. The genes that encode these rRNAs are organized in the genome as *rrn* operon, and often multiple *rrn* operons are encoded in a bacterial genome [204-207].

16S rRNA gene targeted methods for studying the diversity of culturable and non-culturable bacteria

In 1909, H. Joel Conn reported that cultivation of bacteria present in soil on artificial media only gave rise to the formation of colonies for 1.5% of all bacteria in soil [208]. Similarly, Vagn Jensen was the second scientist who concluded fifty years later that cells able to form colonies were not representative of the total bacterial diversity. Cultivation independent methods confirmed the presence of a much larger diversity of soil bacteria. Having said that, we should also not forget that the isolation and cultivation of many important soil bacteria allowed e.g. for the discovery of antibiotics isolated from microbial cultures, as well as for studies of bacterial physiology, genetics, and ecology [209-215].

The first full-length bacterial 16S rRNA gene containing 1524 nucleotides (GenBank accession no. J01859) was sequenced in 1972 by Ehresmann and his colleagues for *Escherichia coli* [216]. Application of molecular ecological methods in the 1990s such as based on surveys of genes after PCR amplification, has allowed cultivation-independent investigations of the microbial communities of soils. Different methods such as those that target functional genes, phylogenetically informative genes, or RNAs, have been developed from the early 1990s. Especially methods that target 16S rRNA and its gene have become powerful tools to study bacteria in different kinds of samples, including those derived from soils [70, 217-221].

Bacterial identification by sequencing of the 16S rRNA gene has become one of the standard bacterial identification methods. Many scientists have used this technology for measuring phylogenetic similarity between isolates for many years [222].

Nowadays 16S rRNA gene-based amplicon sequence analysis is widely used to determine the taxonomic composition of microbial communities. Phylogenetic tree analysis provides information about the evolutionary relationship between taxa by phylogenetic comparison and provides improved understanding of microbial community structure [223].

Bacterial 16S rRNA genes are ideal markers for phylogenetic studies due to the presence of both highly conserved regions that can be targeted by conserved probes and primers, as well as hypervariable regions as species-specific signature sequences. Ribotyping, i.e. fingerprinting of genomic DNA restriction fragments of 16S rRNA genes has been used extensively in the past [224, 225]. To this end, PCR conditions were developed for the *in vitro* amplification of bacterial 16S rRNA genes. Oligodeoxynucleotides which are complementary to conserved regions at the 5' and 3' termini of bacterial 16S rRNAs are used to prime DNA synthesis in PCR. Amplification products can then be used for comparison with sequences of previously reported rRNA genes that allows for the identification of isolates and for the characterization of the composition of mixed microbial communities. Currently, microbial community structure and composition is most commonly studied on the basis of 16S rRNA sequence analysis through next generation sequencing technologies such as those provided e.g. by Illumina [226, 227].

Based on sequence data generated by such sequencing technologies, microbial diversity can in general be defined as the number of individuals allocated to different taxa and their distribution between taxa [228]. Species diversity further consists of species richness that describes the total number of species present, and species evenness that measures the distribution of species [229]. The result of sequence analysis provides a full view of microbial community composition present in a specific environment [230]. Application of high throughput DNA sequencing is constantly growing due to the rapidly decreasing costs for DNA sequencing and breakthroughs in the bioinformatic analysis [231].

Sequence analysis of DNA purified from environmental samples enables taxonomic identification of the microbial communities present within them. Currently, two main methods are being employed. First, metataxonomic analysis is based on sequencing the 16S rRNA gene, which exhibits sufficient variation in the nine hypervariable regions (V1-V9) between taxa for identification. Second, shotgun metagenomic sequencing is increasingly being used in which whole genomes of all organisms that are present in a given sample are analysed [232].

Metataxonomic and metagenomic analysis has been widely used for a number of applications, such as identifying the human microbiome, classifying microbial populations within the ocean and even for the analysis of the bacterial communities that develop on coffee machines [233]. The introduction of next generation sequencing technologies resulted in greater sequencing throughput and output [234].

Research aim and thesis outline

The research described in this thesis aims to assess the bacterial diversity present in rhizosphere and phyllosphere of wild type and transgenic sugarcane crops by using complementary cultivation dependent and cultivation independent methods in conjunction with *in vitro* and *in vivo* experiments. To this end, bacterial diversity and composition is assessed using biomolecular approaches targeting taxonomic and functional marker genes, whereas culturable bacterial diversity is characterized by Sanger sequencing of 16S rRNA genes of isolates. We also aimed to identify genera and phyla of PGPB by using different techniques. Furthermore, these isolates are then tested for their potential as biofertilizer, replacing chemical fertilizers, by assessing their beneficial effect on plant health and root development in a number of *in vitro* and *in vivo* experiments (Fig. 6). Lastly in this thesis I provide an outlook towards future experiments to detail further study and application of these bacteria.

This thesis consists of six chapters.

In **Chapter 1** I provide a general introduction of the topics dealt with by the research described in this thesis. To this end, this chapter provides a comprehensive literature review of PGPB, and major traits of these microorganisms are discussed, including IAA production, phosphorus solubilization and nitrogen fixation. Furthermore, an account of the current state of the art with respect to the effects of transgenic crops on the bacterial diversity present in their rhizosphere and phyllosphere is given. Finally, I outline the general aims and give an outline of my thesis.

Chapter 2 describes the isolation of different bacterial strains belonging to the genera *Bacillus, Pseudomonas*, *Paenibacillus* and *Enterobacter* from sugarcane. Strains are characterized based on their biochemical properties and by identification on the basis of their 16S rRNA genes. The effect of the isolates on sugarcane with respect to growth and root development is assessed through several *in vivo* and *in vitro* experiments.

Chapter 3 describes the identification of three major groups of PGPB (*Bacillus, Pseudomonas* and *Burkholderia)* and their comparison using *in vitro* and *in vivo* experiments with wheat. The major aim of the research described in this chapter is to identify PGPB which can be further used as biofertilizer in the field.

Chapter 4 provides an account of the bacterial diversity of transgenic and non-transgenic sugarcane crop by using culture dependent methods and identification of isolates on the basis of 16S RNA genes by Sanger sequencing. The effect of transgenic sugarcane on culturable bacteria present in rhizosphere and phyllosphere of sugarcane is assessed.

Chapter 5 provides a cultivation-independent comparison of the bacterial diversity of the rhizosphere of transgenic and wild-type sugarcane, using Illumina HiSeq sequencing of PCR-amplified 16S rRNA gene fragments and downstream bioinformatic and statistical analyses as implemented in R.

Finally, in **Chapter 6**, I discuss the results obtained in this study in a broader context, which concludes with an outlook with respect to future perspectives.

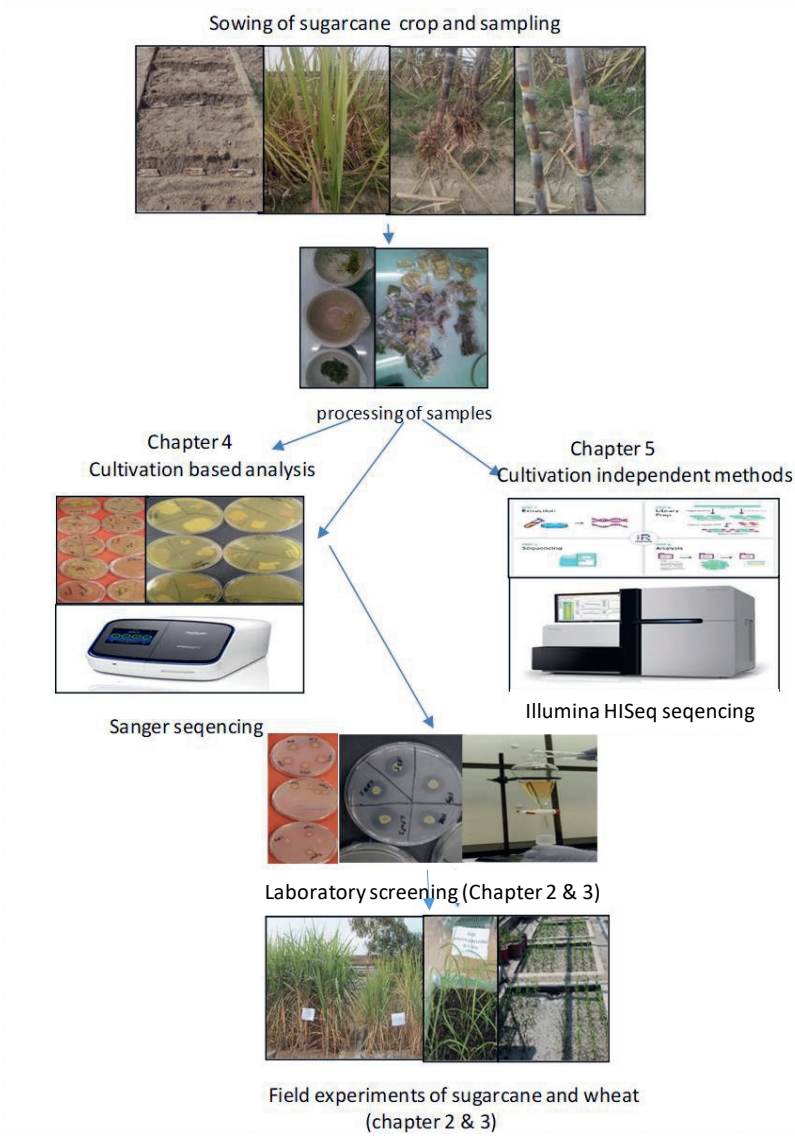

Figure 6. A brief overview of research and tools used in the research described in different chapters of the thesis.

References

1. Di Caracalla, V.d.T., The impacts on global food security and nutrition of the military conflict in Ukraine. 2022. FAO-CSF.
2. Agnusdei, G.P., et al., Towards circular economy in the agrifood sector: Water footprint assessment of food loss in the Italian fruit and vegetable supply chains. Ecological Indicators, 2022. 137: p. 108781.
3. Callens, K., et al., Microbiome-based solutions to address new and existing threats to food security, nutrition, health and agrifood systems' sustainability. Frontiers in Sustainable Food Systems, 2022. 6: p. 537.
4. Bomberg, M. and L. Ahonen, Geomicrobes: life in terrestrial deep subsurface. j. Frontiers in Microbiology, 2017. 8: p. 103.
5. Beed, F., et al., Climate change and micro-organism genetic resources for food and agriculture: state of knowledge, risks and opportunities,. Background Study Paper No. 57. Rome, Italy: Commission on Genetic Resources for Food and Agriculture (FAO).
6. Kumawat, K.C., S. Nagpal, and P. Sharma, Potential of plant growth-promoting rhizobacteria-plant interactions in mitigating salt stress for sustainable agriculture: A review. Pedosphere, 2022. 32(2): p. 223-245.
7. Patade, V.Y., S. Bhargava, and P. Suprasanna, Halopriming imparts tolerance to salt and PEG induced drought stress in sugarcane. Agriculture, Ecosystems & Environment, 2009. 134(1-2): p. 24-28.
8. Esmaeili, N., G. Shen, and H. Zhang, Genetic manipulation for abiotic stress resistance traits in crops. Frontiers in Plant Science, 2022. 13: p. 1011985.
9. Hu, H. and L. Xiong, Genetic engineering and breeding of drought-resistant crops. Annual Review of Plant Biology, 2014. 65: p. 715-741.
10. Kumar, K., et al., Genetically modified crops: current status and future prospects. Planta, 2020. 251: p. 1-27.
11. Verma, K.K., et al., Genetic engineering: an efficient approach to mitigating biotic and abiotic stresses in sugarcane cultivation. Plant Signalling & Behaviour, 2022. 17(1): p. 2108253.
12. Basra, S., N. Ahmad, and A. Khaliq, Water relation studies in water stressed sugarcane (*Saccharum officinarum L.*). International Journal of Agricultural Biology, 1999. 1(1/2): p. 1-4.
13. Li, J., et al., Application of microbial inoculants significantly enhances crop productivity: A meta-analysis of studies from 2010 to 2020. J. Sustainable Agriculture and Environment, 2022. 1(3): p. 216-225.
14. Wassermann, B., H. Müller, and G. Berg, An apple a day: which bacteria do we eat with organic and conventional apples. Frontiers in Microbiology, 2019. 10: p. 1629.
15. Sessitsch, A., N. Pfaffenbichler, and B. Mitter, Microbiome applications from lab to field facing complexity. Trends in Plant Science, 2019. 24(3): p. 194-198.
16. Lange, L., et al., Developing a sustainable and circular bio-based economy in EU by partnering across sectors, upscaling and using new knowledge faster, and for the benefit of climate, environment & biodiversity, and people & business. Frontiers in Bioengineering and Biotechnology, 2021: p. 1456.
17. Bower, R. and R.G. Birch, Transgenic sugarcane plants via microprojectile bombardment. The Plant Journal, 1992. 2(3): p. 409-416.
18. D'Hont, A., et al., Sugarcane: A Major Source of Sweetness, Alcohol, and Bio-energy, in Genomics of Tropical Crop Plants, Springer New York, NY.2008, p. 483-513.

19. Kumar, P. and S. Srivastava, From Conventional to Molecular Approaches Building Bridges for Sugarcane Genetic Improvement, in Sustainable Sugarcane Production. Apple Academic Press. 2018, p. 115-142.
20. Lee, S., Characterization of a major cluster of genes involved in nitrogen fixation and another required for indole-3-acetic acid biosynthesis in the sugarcane endophyte, *Acetobacter diazotrophicus*. 2001.
21. James, G. and B. Tate, Sugarcane.: Black Well Science, 2004. Wiley Online Library.
22. Shahid, A., et al., Economic Importance and Yield Potential of Sugarcane in Pakistan, in Sugarcane. Intech Open 2023, Rijeka. p. Ch. 4.
23. Raza, H., et al., Economic analysis of jaggery (Gur) production in tehsil shakargar. J. Global Innovations in Agricultural Science, 2018. 6: p. 69-73.
24. Ali,r.z., Advanced sugarcane production technology. Report 2012. https://www.scribd.com/document/371757688/03-Advance-Sugarcane-Production-Technology-ranazulfiqar-Ali
25. Iqbal, M.A. and A. Iqbal, Sugarcane production, economics and industry in Pakistan. American-Eurasian Journal of Agricultural & Environmental Sciences. 2014. 14(12): p. 1470-1477.
26. Pakistan, E., SOURCES: Pakistan Department of Tourism Annual Report, 1969; Pakistan Affairs 34 (no. 9): 2 (May 1, 1981); Asia Travel Trade (February 1984): 48. This is surprising. Superficially one might expect Pakistan to have a political tourism history rather like India, of which it. The Politics of Tourism in Asia, 2019: p. 153.
27. Qureshi, M.A. and S. Afghan, Sugarcane cultivation in Pakistan. Pakistan Society of Sugar Technologist, 2005.
28. Khan, M.A., et al., Evaluation of new sugarcane genotypes developed through fuzz. Correlation of cane yield and yield components. Pakistan Journal of Applied Science, 2003. 3(4): p. 270-273.
29. Arruda, P., Perspective of the sugarcane industry in Brazil. Tropical Plant Biology, 2011. 4: p. 3-8.
30. Rhoades, J. and J. Loveday, Salinity in irrigated agriculture. Agronomy, 1990(30): p. 1089-1142.
31. Kamburona,. C. K. Evaluating the genetic diversity and performance of peanut (*Arachis hypogaea L.*) lines. Diss. University of Pretoria, 2008.
32. Athar, H.R. and M. Ashraf, Strategies for Crop Improvement Against Salinity and Drought Stress: An Overview, in Salinity and Water Stress: Springer, 2009, p. 1-16.
33. Qureshi, A.S., et al., Challenges and prospects of sustainable groundwater management in the Indus Basin, Pakistan. Water Resources Management, 2010. 24(8): p. 1551-1569.
34. Farooq, M., et al., Plant drought stress: effects, mechanisms and management. Sustainable Agriculture, 2009: p. 153-188.
35. Zhu, J.-K., Regulation of ion homeostasis under salt stress. Current Opinion in Plant Biology, 2003. 6(5): p. 441-445.
36. Dionisio-Sese, M.L. and S. Tobita, Antioxidant responses of rice seedlings to salinity stress. Plant Science, 1998. 135(1): p. 1-9.
37. Maathuis, F.J. and A. Amtmann, K+ nutrition and Na+ toxicity: the basis of cellular K+/Na+ ratios. Annals of Botany, 1999. 84(2). p. 123-133.
38. Molinari, H.B.C., et al., Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.) osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiologia Plantarum, 2007. 130(2) p. 218-229.
39. Paisán-Ruíz, C., et al., Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron, 2004. 44(4): p. 595-600.

40. G.Raza, K.Ali., Z.Mukhtar, S.Mansoor, M.Arshad and S.Asad, The response of sugarcane *Saccharum officinarum_L*. African Journal of Biotechnology, 2010. 9(51): p. 8739-8747.
41. Tabashnik, B.E., et al., Insect resistance to transgenic Bt crops: Lessons from the laboratory and field. J. Economic Entomology, 2003. 96(4): p. 1031-1038.
42. Fraley, R.T., et al., Expression of bacterial genes in plant cells. Proceedings of the National Academy of Sciences, 1983. 80(15): p. 4803-4807.
43. James, C., Global review of commercialized transgenic crops. Current Science 2003. v. 84(3). p.303-307.
44. Daghan, H., Transgenic tobacco for phytoremediation of metals and metalloids in transgenic plant technology for remediation of toxic metals and metalloids. Elsevier. 2019, p. 279-297.
45. Vaeck, M., et al., Transgenic plants protected from insect attack. Nature, 1987. 328(6125): p. 33.
46. Martineau, B., First fruit: the creation of the flavr savr tomato and the birth of biotech food. McGraw-Hill New York. 2001, p. 269.
47. Rasul, F., et al., Enhanced transformation efficiency of *Saccharum officinarum* by vacuum infiltration assisted *Agrobacterium*-mediated transformation. International Journal of Agriculture and Biology, 2014. 16(6): p. 1147-1152.
48. Wolfenbarger, L.L. and P.R. Phifer, The ecological risks and benefits of genetically engineered plants. Science, 2000. 290(5499): p. 2088.
49. Dunfield, K.E. and J.J. Germida, Impact of genetically modified crops on soil and plant associated microbial communities. J. Environmental Quality, 2004. 33(3): p. 806-815.
50. Zhou, D., et al., Cry1Ac transgenic sugarcane does not affect the diversity of microbial communities and has no significant effect on enzyme activities in rhizosphere soil within one crop season. Frontiers in Plant Science, 2016. 7: p. 265.
51. Wolfenbarger, L.L. and P.R. Phifer, The ecological risks and benefits of genetically engineered plants. Science, 2000. 290(5499): p. 2088-2093.
52. Bhattacharyya, P.N. and D.K. Jha, Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology, 2012. 28(4): p. 1327-1350.
53. Rossmann, M., et al., Plant Microbiome: Composition and functions in plant compartments, in The Brazilian Microbiome. 2017, Springer. p. 7-20.
54. Philippot, L., et al., Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology, 2013. 11(11): p. 789.
55. Boon, E., et al., Interactions in the microbiome: communities of organisms and communities of genes. FEMS Microbiology Reviews, 2014. 38(1): p. 90-118.
56. Andreote, F.D., T. Gumiere, and A. Durrer, Exploring interactions of plant microbiomes. Scientia Agricola, 2014. 71: p. 528-539.
57. Majeed, A., et al., Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Frontiers in Microbiology, 2015. 6(198).
58. Kumar, A., B.R. Maurya, and R. Raghuwanshi, Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (*Triticum aestivum L.*). Biocatalysis and Agricultural Biotechnology, 2014. 3(4): p. 121-128.
59. Khan, N., A. Bano, and M.A. Babar, The root growth of wheat plants, the water conservation and fertility status of sandy soils influenced by plant growth promoting rhizobacteria. Symbiosis, 2017. 72(3): p. 195-205.

60. Zaheer, A., et al., Isolation, characterization, and effect of phosphate-zinc-solubilizing bacterial strains on chickpea (*Cicer arietinum L.*) growth. Saudi Journal of Biological Sciences, 2019.
61. Idris, E.E., et al., Tryptophan-dependent production of Indole-3-Acetic Acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Molecular Plant-Microbe Interactions, 2007. 20(6): p. 619-626.
62. Awais, M., et al., Isolation, characterization and association among phosphate solubilizing bacteria from sugarcane rhizosphere. Cytology and Genetics, 2019. 53(1): p. 86-95.
63. Modi, K. and P. Patel, Isolation and characterization of plant growth promoting rhizobacteria associated with *Saccharum officinarum L*. Current Synthetic and Systems Biology, 2017. 5: p. 2332-0737.
64. Muthukumarasamy, R., et al., Isolation of bacterial strains possessing nitrogen-fixation, phosphate and potassium-solubilization and their inoculation effects on sugarcane. 2017.
65. Prashar, P., N. Kapoor, and S. Sachdeva, Rhizosphere: its structure, bacterial diversity and significance. Reviews in Environmental Science and Bio/Technology, 2014. 13(1): p. 63-77.
66. Hiltner, L.T., Uber neuere Erfahrungen und Probleme auf dem Gebiet der Boden Bakteriologie und unter besonderer Berücksichtigung der Gründüngung und Broche. Arbeit. Deut. Landw. Ges. Berlin, 1904. 98: p. 59-78.
67. McCully, M. The rhizosphere: the key functional unit in plant/soil/microbial interactions in the field. Implications for the understanding of allelopathic effects. in Proceedings of the 4th World Congress on Allelopathy. 2005 v.20.
68. Barriuso, J., et al., Ecology, genetic diversity and screening strategies of plant growth promoting rhizobacteria (PGPR). Journal of Plant Nutrition, 2008: p. 1-17.
69. Walker, T.S., et al., Root Exudation and Rhizosphere Biology. Plant Physiology, 2003. 132(1): p. 44.
70. Marilley, L. and M. Aragno, Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Applied Soil Ecology, 1999. 13(2): p. 127-136.
71. Kang, B.G., et al., Use of plant growth-promoting rhizobacteria to control stress responses of plant roots. Plant Biotechnology Reports, 2010. 4(3): p. 179-183.
72. Nardi, S., et al., Soil organic matter mobilization by root exudates. Chemosphere, 2000. 41(5): p. 653-658.
73. Marschner, H., Mechanisms of regulation of mineral nutrition in higher plants. Bulletin - Royal Society of New Zealand, 1974.
74. Barea, J.-M., et al., Microbial co-operation in the rhizosphere. Journal of Experimental Botany, 2005. 56(417): p. 1761-1778.
75. Dessaux, Y., P. Hinsinger, and P. Lemanceau, Rhizosphere: so many achievements and even more challenges. Plant and Soil. 2009, p.1-3
76. Kloepper, J.W., Plant growth-promoting rhizobacteria (other systems). *Azospirillum*/plant associations, 1994. 187: p. 137-166.
77. Kloepper, J., et al., The Rhizosphere and Plant Growth. Keister, DL and Cregan, PB. Dordrecht, 1991.p.315.
78. Berg, G., et al., Plant microbial diversity is suggested as the key to future biocontrol and health trends. FEMS Microbiology Ecology, 2017. 93(5): p. fix050-fix050.
79. Calvo, P., L. Nelson, and J.W. Kloepper, Agricultural uses of plant biostimulants. Plant and Soil, 2014. 383(1): p. 3-41.

80. Bashan, Y., et al., Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant and Soil, 2014. 378(1-2): p. 1-33.
81. Schultz, N., et al., Inoculation of sugarcane with diazotrophic bacteria. Revista Brasileira de Ciência do Solo, 2014. 38(2): p. 407-414.
82. de Oliveira, A.L.M., et al., Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria. Plant and Soil, 2006. 284(1-2): p. 23-32.
83. Muñoz-Rojas, J. and J. Caballero-Mellado, Population dynamics of *Gluconacetobacter diazotrophicus* in sugarcane cultivars and its effect on plant growth. Microbial Ecology, 2003. 46(4): p. 454-464.
84. Shrivastava, U.P., Molecular diversity assessment of plant growth promoting rhizobacteria using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA Gene. 2017, 2017. 5(1): p. 9.
85. Contreras, M., et al., A glimpse of the endophytic bacterial diversity in roots of blackberry plants (*Rubus fruticosus*). Genetics and molecular research: GMR, 2016. 15(3).
86. Santoyo, G., et al., Plant growth-promoting bacterial endophytes. Microbiology Research, 2016. 183: p. 92-9.
87. de Souza, R., A. Ambrosini, and L.M.P. Passaglia, Plant growth-promoting bacteria as inoculants in agricultural soils. Genetics and Molecular Biology, 2015. 38: p. 401 - 419.
88. James, E.K. and F.L. Olivares, Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Critical Reviews in Plant Sciences, 1998. 17(1): p. 77-119.
89. Hirano, S.S. and C.D. Upper, Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a Pathogen, ice nucleus, and epiphyte. Microbiology and Molecular Biology Reviews, 2000. 64(3): p. 624-653.
90. Morris, C., Fifty years of phyllosphere microbiology: significant contributions to research in related fields. Phyllosphere Microbiology, 2002. p.365-375.
91. Farré-Armengol, G., et al., Bidirectional interaction between phyllospheric microbiotas and plant volatile emissions. Trends in Plant Science, 2016. 21(10): p. 854-860.
92. Andrews, J.H. and R.F. Harris, The ecology and biogeography of microorganisms on plant surfaces. Annual Review of Phytopathology, 2000. 38(1): p. 145-180.
93. Johnson, K. and V. Stockwell, Management of fire blight: a case study in microbial ecology. Annual Review of Phytopathology, 1998. 36(1): p. 227-248.
94. Vorholt, J.A., Microbial life in the phyllosphere. Nature Reviews Microbiology, 2012. 10: p. 828.
95. Thompson, I., et al., Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugar beet (*Beta vulgaris*). Plant and Soil, 1993. 150(2): p. 177-191.
96. Ercolani, G., Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. Microbial Ecology, 1991. 21(1): p. 35-48.
97. Bulgarelli, D., et al., Structure and functions of the bacterial microbiota of plants. Annual Review of Plant Biology, 2013. 64: p. 807-838.
98. Compant, S., et al., A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. Journal of Advanced Research, 2019.
99. Seasonal community succession of the phyllosphere microbiome. Molecular Plant-Microbe Interactions, 2015. 28(3): p. 274-285.

100. Rastogi, G., G.L. Coaker, and J.H.J. Leveau, New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS Microbiology Letters, 2013. 348(1): p. 1-10.
101. Wilson, M. and S.E. Lindow, Ecological similarity and coexistence of epiphytic ice-nucleating (Ice+) *Pseudomonas syringae* strains and a non-ice-nucleating (Ice-) biological control agent. Applied and Environmental Microbiology, 1994. 60(9): p. 3128-3137.
102. Wilson, M. and S.E. Lindow, Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Applied and Environmental Microbiology, 1994. 60(12): p. 4468-4477.
103. Tukey Jr, H., The leaching of substances from plants. Annual Review of Plant Physiology, 1970. 21(1): p. 305-324.
104. Mercier, J. and S. Lindow, Role of leaf surface sugars in colonization of plants by bacterial epiphytes. Applied and Environmental Microbiology, 2000. 66(1): p. 369-374.
105. Dickinson, C., B. Austin, and M. Goodfellow, Quantitative and qualitative studies of phylloplane bacteria from *Lolium perenne*. Microbiology, 1975. 91(1): p. 157-166.
106. Bringel, F. and I. Couée, Pivotal roles of phyllosphere microorganisms at the interface between plant functioning and atmospheric trace gas dynamics. Frontiers in Microbiology, 2015. 6(486).
107. Innerebner, G., C. Knief, and J.A. Vorholt, Protection of *Arabidopsis thaliana* against leaf-pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a controlled model system. Applied and Environmental Microbiology, 2011. 77(10): p. 3202-3210.
108. Knief, C., N. Delmotte, and J.A. Vorholt, Bacterial adaptation to life in association with plants – A proteomic perspective from culture to in situ conditions. Proteomics, 2011. 11(15): p. 3086-3105.
109. Ali, N., et al., The potential of epiphytic hydrocarbon-utilizing bacteria on legume leaves for attenuation of atmospheric hydrocarbon pollutants. Journal of Environmental Management, 2012. 93(1): p. 113-120.
110. Lindow, S.E. and J.H.J. Leveau, Phyllosphere microbiology. Current Opinion in Biotechnology, 2002. 13(3): p. 238-243.
111. Rico, L., et al., Community structures of N2-fixing bacteria associated with the phyllosphere of a Holm oak forest and their response to drought. Plant Biology, 2014. 16(3): p. 586-593.
112. Fürnkranz, M., et al., Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. The ISME Journal, 2008. 2: p. 561.
113. Manching, H.C., P.J. Balint-Kurti, and A.E. Stapleton, Southern leaf blight disease severity is correlated with decreased maize leaf epiphytic bacterial species richness and the phyllosphere bacterial diversity decline is enhanced by nitrogen fertilization. Frontiers in Plant Science, 2014. 5(403).
114. Sandhu, A., L.J. Halverson, and G.A. Beattie, Bacterial degradation of airborne phenol in the phyllosphere. Environmental Microbiology, 2007. 9(2): p. 383-392.
115. Lugtenberg, B.J., T.F. Chin-A-Woeng, and G.V. Bloemberg, Microbe–plant interactions: principles and mechanisms. Antonie Van Leeuwenhoek, 2002. 81(1-4): p. 373-383.
116. Delmotte, N., et al., Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proceedings of the National Academy of Sciences, 2009. 106(38): p. 16428.
117. Vacher, C., et al., The Phyllosphere: Microbial jungle at the plant–climate interface. Annual Review of Ecology, Evolution, and Systematics, 2016. 47(1): p. 1-24.

118. Hunter, P.J., et al., Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (Lactuca species) phyllosphere. Applied and Environmental Microbiology, 2010. 76(24): p. 8117-8125.
119. Carvalho, T., et al., Nitrogen signalling in plant interactions with associative and endophytic diazotrophic bacteria. Journal of Experimental Botany, 2014. 65(19): p. 5631-5642.
120. Yaish, M.W., I. Antony, and B.R. Glick, 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, 2015. 107(6): p. 1519-1532.
121. Kloepper, J.W. Plant growth-promoting rhizobacteria on radishes. in Proc. of the 4th Internet. Conf. on Plant Pathogenic Bacter, Station de Pathologie Vegetale et Phytobacteriologie, INRA, Angers, France, 1978. 1978.
122. Vessey, J.K., Plant growth promoting rhizobacteria as biofertilizers. Plant and soil, 2003. 255(2): p. 571-586.
123. Somers, E., J. Vanderleyden, and M. Srinivasan, Rhizosphere bacterial signalling: a love parade beneath our feet. Critical Reviews in Microbiology, 2004. 30(4): p. 205-240.
124. Kloepper, J. A review of mechanisms for plant growth promotion by PGPR. in 6th international PGPR workshop. 2003.
125. Figueiredo, M.d.V.B., et al., Plant growth promoting rhizobacteria: fundamentals and applications, in Plant growth and health promoting bacteria. 2010, Springer. p. 21-43.
126. Merzaeva, O. and I. Shirokikh, Colonization of plant rhizosphere by actinomycetes of different genera. Microbiology, 2006. 75(2): p. 226-230.
127. Rudorff, B.F.T., et al., Studies on the rapid expansion of sugarcane for ethanol production in São Paulo State (Brazil) using Landsat data. Remote Sensing, 2010. 2(4): p. 1057-1076.
128. Taulé, C., et al., The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum L.*), and the identification and characterization of part of the associated diazotrophic bacterial community. Plant and Soil, 2012. 356(1-2): p. 35-49.
129. Kloepper, J. and M. Schroth, Relationship of in vitro antibiosis of plant growth-promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology, 1981. 71(10): p. 1020-1024.
130. Antunes, J., et al., Diversity of plant growth-promoting bacteria associated with sugarcane. Genetics and Molecular Research, 2017. 16(2).
131. Glick, B.R., Plant growth-promoting bacteria: mechanisms and applications. Scientifica, 2012. 2012.
132. Rao, D.L.N., Recent advances in biological nitrogen fixation in agricultural systems. Proceedings of the Indian National Science Academy, 2014. 80(2): p. 359.
133. Nosrati, R., et al., Phosphate solubilization characteristics of efficient nitrogen fixing soil Azotobacter strains. Iran Journal of Microbiology, 2014. 6(4): p. 285-95.
134. Govindarajan, M., et al., Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkholderia vietnamiensis*. Plant and Soil, 2006. 280(1-2): p. 239-252.
135. Kenichi, I., et al., Ammonia accumulation of novel nitrogen-fixing bacteria, in Biotechnology, S. Reda Helmy, Editor. 2012, IntechOpen: Rijeka. p. Ch. 2.
136. Ahemad, M. and M.S. Khan, Effects of pesticides on plant growth promoting traits of Mesorhizobium strain MRC4. Journal of the Saudi Society of Agricultural Sciences, 2012. 11(1): p. 63-71.

137. Ahemad, M. and M. Kibret, Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University - Science, 2014. 26(1): p. 1-20.
138. Gouda, S., et al., Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. Microbiological Research, 2018. 206: p. 131-140.
139. Van Der Heijden, M.G.A., et al., Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. FEMS Microbiology Ecology, 2006. 56(2): p. 178-187.
140. Reed, S.C., C.C. Cleveland, and A.R. Townsend, Functional ecology of free-living nitrogen fixation: A Contemporary Perspective. Annual Review of Ecology, Evolution, and Systematics, 2011. 42(1): p. 489-512.
141. Kumar, V. and R. Gera, Isolation of a multi-trait plant growth promoting *Brevundimonas* sp. and its effect on the growth of Bt-cotton. 3 Biotech, 2014. 4(1): p. 97-101.
142. Bishop, P.E. and R.D. Joerger, Genetics and molecular biology of alternative nitrogen fixation systems. Annual Review of Plant Biology, 1990. 41(1): p. 109-125.
143. Rao, D. Recent advances in biological nitrogen fixation in agricultural systems. in Proc. Indian nation. Sci. Acad. 2014.
144. Rubio, L.M. and P.W. Ludden, Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase. Annual Review of Microbiology, 2008. 62(1): p. 93-111.
145. <Zehr_et_al-2003- Nitrogenase gene diversity ,Environmental_Microbiology.pdf>.
146. Thaweenut, N., et al., Two seasons' study on *nifH* gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum* spp. hybrids): expression of *nifH* genes similar to those of rhizobia. Plant and Soil, 2011. 338(1): p. 435-449.
147. Lin, L., et al., Plant growth-promoting nitrogen-fixing enterobacteria are in association with sugarcane plants growing in Guangxi, China. Microbes and Environments, 2012. 27(4): p. 391-398.
148. Fischer, D., et al., Molecular characterisation of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (*Saccharum* sp.). Plant and Soil, 2012. 356(1): p. 83-99.
149. Sevilla, M., et al., Comparison of benefit to sugarcane plant growth and 15N2 incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wildtype and Nif- mutant strains. Molecular Plant-Microbe Interactions, 2001. 3.
150. Franck. S and Rene. B. Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixer in soil. Research in Microbiology 2001, v 152. p.95-103.
151. Allen, R.S., et al., Expression of 16 nitrogenase proteins within the plant Mitochondrial Matrix. Frontiers in Plant Science, 2017. 8: p. 287.
152. <file.pdf>.
153. <595.full.pdf>.
154. Cury Fracetto et al. Free-living diazotrophs drive castor bean nitrogen input in tropical semiarid soils. Soil Science, 2019. 49.
155. Boddey, R., et al., Biological nitrogen fixation associated with sugar cane, in Nitrogen fixation. 1991, Springer. p. 105-111.
156. Döbereiner, J., J. Day, and P. Dart, Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum-Azotobacter paspali* association. Microbiology, 1972. 71(1): p. 103-116.
157. Döbereiner, J., J. Day, and P. Dart, Nitrogenase activity in the rhizosphere of sugar cane and some other tropical grasses. Plant and Soil, 1972. 37(1): p. 191-196.

158. Sajjad Mirza, M., et al., Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. Plant and Soil, 2001. 237(1): p. 47-54.
159. Marroquí, S., et al., Enhanced symbiotic performance by *Rhizobium tropici* glycogen synthase mutants. Journal of Bacteriology, 2001. 183(3): p. 854-864.
160. Poonguzhali, S., Munusamy Madhaiyan, and Tongmin Sa, <phosphors slubilization bacteria from cabbage.pdf>. Journal of Microbiology and Biotechnology, 2007. 4(18): p. 773–777.
161. Martins, M.R., et al., Impact of plant growth-promoting bacteria on grain yield, protein content, and urea-15 N recovery by maize in a cerrado oxisol. Plant and Soil, 2017: p. 1-12.
162. Kim, J. and D.C. Rees, Nitrogenase and biological nitrogen fixation. Biochemistry, 1994. 33(2): p. 389-397.
163. Khan, M.S., et al., Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environmental Chemistry Letters, 2009. 7(1): p. 1-19.
164. Zaidi, A., et al., Plant growth promotion by phosphate solubilizing bacteria. Acta Microbiologica et Immunologica Hungarica, 2009. 56(3): p. 263-284.
165. Poonguzhali. S et al., Isolation and identification of Phosphate solubilizing bacteria from chinese cabbage and their effect on growth and phosphorus utilization of plants. Microbiology and Biotechnology 2008. 18(4).p.773-777.
166. Vikram, A. and H. Hamzehzarghani, Effect of phosphate solubilizing bacteria on nodulation and growth parameters of greengram (*Vigna radiata L. Wilczek*). Research Journal of Microbiology, 2008. 3(2): p. 62-72.
167. Prathap, M. and K.B.D. Ranjitha, A critical review on plant growth promoting rhizobacteria. Journal of Plant Pathology & Microbiology, 2015. 6(4): p. 1-4.
168. Awais, M., et al., Isolation, characterization and inter-relationship of phosphate solubilizing bacteria from the rhizosphere of sugarcane and rice. Biocatalysis and Agricultural Biotechnology, 2017. 11(Supplement C): p. 312-321.
169. Rafique, M., et al., Enhancement of maize plant growth with inoculation of phosphate-solubilizing bacteria and biochar amendment in soil. Soil Science and Plant Nutrition, 2017: p. 1-10.
170. Clayton, A.S., et al., Evaluation methods used for phosphate-solubilizing bacteria. African Journal of Biotechnology, 2016. 15(34): p. 1796-1805.
171. <8 Baliah, Pandia&Kumar-f.pdf>.
172. <IJM-6-285.pdf>.
173. T. Nikitha, M.S., E.U.B.R. B. Sadhana, and S.S. Vani, Phosphorous and phosphate solubilising bacteria and their role in plant nutrition. International Journal of Current Microbiology and Applied Sciences, 2017. 6(4): p. 2133-2144.
174. Ahmad, I., et al., Effect of divalent anions on photodegradation kinetics and pathways of riboflavin in aqueous solution. International Journal of Pharmaceutics, 2010. 390(2): p. 174-182.
175. Krasilinikov, N., On the role of soil micro-organism in plant nutrition. Microbiologiya, 1957. 26: p. 659-672.
176. Khan, A.A., et al., Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. Journal of Agriculture & Biological Sciences, 2009. 1(1): p. 48-58.
177. Damam, M., et al., Plant growth promoting substances (phytohormones) produced by rhizobacterial strains isolated from the rhizosphere of medicinal plants. International Journal of Pharmaceutical Sciences Review and Research, 2016. 37(1): p. 130-136.

178. Santner, A., L.I.A. Calderon-Villalobos, and M. Estelle, Plant hormones are versatile chemical regulators of plant growth. Nature Chemical Biology, 2009. 5(5): p. 301.
179. Camerini, S., et al., Introduction of a novel pathway for IAA biosynthesis to rhizobia alters vetch root nodule development. Archives of Microbiology, 2008. 190(1): p. 67-77.
180. Patten, C.L. and B.R. Glick, Bacterial biosynthesis of indole-3-acetic acid. Canadian Journal of Microbiology, 1996. 42(3): p. 207-220.
181. Brandl, M.T. and S.E. Lindow, Environmental signals modulate the expression of an indole-3-acetic acid biosynthetic gene in *Erwinia herbicola*. Molecular Plant-Microbe Interactions, 1997. 10(4): p. 499-505.
182. Etesami, H., H.A. Alikhani, and H.M. Hosseini, Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. MethodsX, 2015. 2: p. 72-78.
183. Spaepen, S., J. Vanderleyden, and R. Remans, Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiology Reviews, 2007. 31(4): p. 425-448.
184. Spaepen, S. and J. Vanderleyden, Auxin and plant-microbe interactions. Cold Spring Harbor Perspectives in Biology, 2011. 3(4): p. a001438.
185. Merino, E., R.A. Jensen, and C. Yanofsky, Evolution of bacterial trp operons and their regulation. Current Opinion in Microbiology, 2008. 11(2): p. 78-86.
186. Dosselaere, F. and J. Vanderleyden, A metabolic node in action: chorismate-utilizing enzymes in microorganisms. Critical Reviews in Microbiology, 2001. 27(2): p. 75-131.
187. Ahemad, M. and M.S. Khan, Ecological assessment of biotoxicity of pesticides towards plant growth promoting activities of pea (*Pisum sativum*) specific *Rhizobium* sp. strain mrp1.. Emirates Journal of Food and Agriculture, 2012: p. 334-343.
188. Fuentes-Ramirez, L.E., et al., *Acetobacter diazotrophicus*, an indoleacetic acid producing bacterium isolated from sugarcane cultivars of Mexico. Plant and Soil, 1993. 154(2): p. 145-150.
189. Vessey, J.K. and T.J. Buss, *Bacillus cereus* UW85 inoculation effects on growth, nodulation, and N accumulation in grain legumes: controlled environment studies. Canadian Journal of Plant Science, 2002. 82(2): p. 282-290.
190. Okon, Y. and C.A. Labandera Gonzalez, Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biology and Biochemistry, 1994. 26(12): p. 1591-1601.
191. Bethlenfalvay, G.J., Mycorrhizae in the agricultural plant-soil system. Symbiosis, 1993. 14: p.413-425.
192. Mazid, M., et al., Significance of sulphur nutrition against metal induced oxidative stress in plants. Journal of Stress Physiology & Biochemistry, 2011. 7(3). p.165-184.
193. Malusà, E., F. Pinzari, and L. Canfora, Efficacy of biofertilizers: challenges to improve crop production, in Microbial inoculants in sustainable agricultural productivity. 2016, Springer. p. 17-40.
194. Bevivino, A., et al., Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. FEMS Microbiology Ecology, 1998. 27(3): p. 225-237.
195. Lucy, M., E. Reed, and B.R. Glick, Applications of free living plant growth-promoting rhizobacteria. Antonie Van Leeuwenhoek, 2004. 86(1): p. 1-25.
196. Hafeez, F.Y., et al., Plant growth-promoting bacteria as biofertilizer. Agronomy for Sustainable Development, 2006. 26(2): p. 143-150.
197. Mishustin, E. and A. Naumova, Bacterial fertilizers, their effectiveness and mode of action. Mikrobiologiya, 1962. 31: p. 543-555.

198. Handelsman, J., Metagenomics: application of genomics to uncultured microorganisms. Microbiology and Molecular Biology Reviews, 2004. 68(4): p. 669-685.
199. Schloss, P.D. and J. Handelsman, Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. Genome Biology, 2005. 6(8): p. 229.
200. Acosta-Martinez, V., et al., Predominant bacterial and fungal assemblages in agricultural soils during a record drought/heat wave and linkages to enzyme activities of biogeochemical cycling. Applied Soil Ecology, 2014. 84: p. 69-82.
201. Daniel, R., The metagenomics of soil. Nature Reviews Microbiology, 2005. 3(6): p. 470.
202. Zhou, Y., W. Liu, and H. Ye, Effects of pesticides metolachlor and S-metolachlor on soil microorganisms in aquisols. II. Soil respiration. Ying yong sheng tai xue bao= The Journal of Applied Ecology, 2006. 17(7): p. 1305-1309.
203. Iltchenco, J., et al., Microbial consortia composition on the production of methane from sugarcane vinasse. Biomass Conversion and Biorefinery, 2019: 10. p. 1-11.
204. Roberts, G.P., et al., Regulation and characterization of protein products coded by the nif (nitrogen fixation) genes of *Klebsiella pneumoniae*. Journal of Bacteriology, 1978. 136(1): p. 267-279.
205. Beynon, J., et al., Comparative organization of nitrogen fixation-specific genes from *Azotobacter vinelandii* and *Klebsiella pneumoniae*: DNA sequence of the nifUSV genes. Journal of Bacteriology, 1987. 169(9): p. 4024-4029.
206. Valdivia, B., M. Dughri, and P. Bottomley, Antigenic and symbiotic characterization of indigenous *Rhizobium leguminosarum* bv. tripolii recovered from root nodules of *Trifolium pratense L*. sown into subterranean clover pasture soils. Soil Biology and Biochemistry, 1988. 20(3): p. 267-274.
207. Miller, R. and J. Sirois, Relative efficacy of different alfalfa cultivar-rhizobium *meliloti* strain combinations for symbiotic nitrogen fixation. Applied and Environmental Microbiology, 1982. 43(4): p. 764-768.
208. Conn, H.J., Soil flora studies part1. Journal of Bacteriology, 1917. 2(1): p. 35-45.
209. Rychlik, T., et al., Application of the PCR-DGGE technique to the fungal community of traditional Wielkopolska fried ripened curd cheese to determine its PGI authenticity. j. Food Control, 2017. 73, Part B: p. 1074-1081.
210. Station, N.Y.S.A.E. and H. Conn, Microscopic study of bacteria and fungi in soil. 1918.
211. Melillo, J.M., J.D. Aber, and J.F. Muratore, Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology, 1982. 63(3): p. 621-626.
212. Jensen, V., The plate count technique. The Ecology of Soil Bacteria, 1968: p. 158-170.
213. Palleroni, N.J., Prokaryotic diversity and the importance of culturing. Antonie Van Leeuwenhoek. 1997. 72(1): p. 3-19.
214. Strohl, W.R., Antimicrobials, in Microbial diversity and bioprospecting. J. American Society of Microbiology. 2004, p. 336-355.
215. Zinder, S.H. and A.A. Salyers, Microbial ecology new directions, new importance, in Bergey's Mannual of Systematic Bacteriology. New York, NY.2001, p. 101-109.
216. Zimmermann, R.A., et al., Location of ribosomal protein binding sites on 16S ribosomal RNA. Proceedings of the National Academy of Sciences, 1972. 69(5): p. 1282-1286.
217. Borneman, J., et al., Molecular microbial diversity of an agricultural soil in Wisconsin. Applied and Environmental Microbiology, 1996. 62(6): p. 1935.
218. Embley, T.M. and E. Stackebrandt, Species in practice: exploring uncultured prokaryote diversity in natural samples. Systematics Association Special Volume, 1997. 54: p. 61-82.

219. Hugenholtz, P., B.M. Goebel, and N.R. Pace, Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology, 1998. 180(18): p. 4765.
220. Ludwig, W. and H.-P. Klenk, Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics, in Bergey's aMnual of Systematic Bacteriology. Springer. 2005, p. 49-66.
221. Ueda, T., Y. Suga, and T. Matsuguchi, Molecular phylogenetic analysis of a soil microbial community in a soybean field. J. European Journal of Soil Science, 1995. 46(3): p. 415-421.
222. Patel, J.B., 16S rRNA Gene sequencing for bacterial pathogen identification in the clinical laboratory. Molecular Diagnosis, 2001. 6(4): p. 313-321.
223. Mori, H., et al., Vitcomics2: visualization tool for the phylogenetic composition of microbial communities based on 16S rRNA gene amplicons and metagenomic shotgun sequencing. BMC Systems Biology, 2018. 12(2): p. 30.
224. Vartoukian, S.R., R.M. Palmer, and W.G. Wade, Strategies for culture of 'unculturable' bacteria. FEMS Microbiology Letters, 2010. 309(1): p. 1-7.
225. Zimmermann, R.A., et al., Location of ribosomal protein binding sites on 16S ribosomal RNA. Proceedings of the National Academy of Sciences, 1972. 69(5): p. 1282.
226. Roesch, L.F., et al., Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME , 2007. 1(4): p. 283.
227. Mocali, S. and A. Benedetti, Exploring research frontiers in microbiology: the challenge of metagenomics in soil microbiology. Research in Microbiology, 2010. 161(6): p. 497-505.
228. Kannepalli, S. and K.W. Farrish, Effectiveness of Various carbon amendments in the bioremediation of perchlorate contaminated soils. International Journal of Environmental Bioremediation & Biodegradation, 2016. 4(3): p. 68-79.
229. Lynch, J.M., et al., Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. Biology and Fertility of Soils, 2004. 40(6): p. 363-385.
230. Ward, D.M., R. Weller, and M.M. Bateson, 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community.1990. Nature, 345(6270): p. 63-65.
231. Corcoll, N., et al., Comparison of four DNA extraction methods for comprehensive assessment of 16S rRNA bacterial diversity in marine biofilms using high-throughput sequencing. FEMS Microbiology Letters, 2017. 364(14): p. fnx139-fnx139.
232. Zhou, J., et al., High-throughput metagenomic technologies for complex microbial community analysis: open and closed formats. MBio, 2015. 6(1): p. e02288-14.
233. Zafra, G., et al., Functional metagenomic analysis of the coffee (*Coffea arabica*) fermentation. Chemical Engineering Transactions, Chemical Engineering Transactions 2018. 64: p. 355.
234. Tennant, R.K., et al., Metagenomic analysis of silage. Journal of Visualized Experiments, 2017(119).

Chapter 2

Isolation, Characterization and Identification of *Pseudomonas, Bacillus, Paenibacillus*, and *Enterobacter* Strains from Sugarcane, and Screening for their Potential as Plant Growth Promoting Bacteria

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Abstract

The aim of the present study was to isolate and characterize potential plant growth promoting bacteria from the rhizosphere of sugarcane plants and their effects on plant growth. Four isolates (ATRS1, ATS1, PD3 and PD2) were obtained and initially characterized on the basis of indole acetic acid production, phosphate solubilization and predicted nitrogen fixation capacity as measured by amplification of the *nifH* gene. Isolates were identified based on 16S rRNA gene sequence analysis, and they were found to be most closely related to members of the genera *Bacillus*, *Pseudomonas*, *Paenibacillus* and *Enterobacter*. Isolates were subsequently tested as plant growth promoting bacteria in inoculation experiments performed with sugarcane plants in *in vitro* micropropagation experiments as well as in microplots under natural conditions. Inoculation with *Bacillus* sp. strain ATRS1 resulted in significant ($p \leq 0.05$) improvements as compared to control plants in the field and for micropropagated plants and produced higher biomass (fresh and dry weight), root and shoot length. Roots were further analyzed with a Rhizoscanner and image analysis data showed that strain ATRS1 significantly ($p \leq 0.05$) increased cumulative root length, surface area, tips and root volume of inoculated sugarcane plants as compared to other strains and non-inoculated control plants.

INTRODUCTION

Sugarcane (*Saccharum officinarum*) is an economically important crop cultivated in several countries, including, but not restricted to, countries in Latin America. Also in Pakistan, sugarcane is of great importance for the country's economy [1]. Sugarcane yields depend on availability of soil nutrients, mainly nitrogen (N) phosphorus (P) [2]. Biological nitrogen fixation (BNF) is one of the activities carried out by a group of bacteria commonly referred to as plant growth-promoting bacteria (PGPB), which can be found in several ecosystems, and establish symbiotic association with plants [3].The mechanisms used by PGPB during the promotion of plant growth are well known and include plant nutrient-acquisition strategies that modulate plant growth, such as through BNF, the production of plant hormones and phosphate solubilization [4]. Sugarcane is an exhaustive and extracting crop that can uptake a large amount of soil nutrients for its biomass production. In addition to micronutrient uptake, about 205 kg N fertilizers such as ammonia and urea, 55 kg P_2O_5, 275 kg K_2O, 30 kg S, 3.5 kg Fe, 1.2 kg Mn, 0.6 kg Zn and 0.2 kg Cu are removed from the soil for a target yield of 100 t/ha [5]. For sustained production of sugarcane, three major nutrients (N, P and K) are required, including 150 kg N/ha, for sugarcane crop, 220 kg N/ha for sugarcane ratoon and 60 kg of P_2O_5 and K_2O each for both sugarcane plant and ratoon. During the leaching process 16 and 45% of N is wasted through soil layers by heavy irrigation required for the cultivation of sugarcane [6, 7]. Similarly, out of the total P fertilizer applied to the crop, only 15–20% can be used, and the rest is fixed in the soil as phosphates of calcium, aluminum or iron, depending on the soil properties. Previous research showed that sugarcane without addition of fertilizers yielded about 40 t/ha of cane annually. The soil nitrogen reserve under this crop, however, increased by 50% of the initial value which clearly indicated that the root-associated diazotrophs contribute a significant quantity of nitrogen for sustained production of sugarcane [8]. Environmental protection and the need to enhance agricultural output have made research into new sustainable technologies necessary. In recent years, interest in soil microorganisms that can promote plant growth has risen [9]. Inoculation of N-fixing microbes to sugarcane has increased the cane yield by 5–15%, saved 25 kg fertilizer N/ha and also improved juice quality parameters, viz sucrose levels and purity [10]. Roots of sugarcane are frequently colonized by nitrogen fixing PGPB. These bacteria are present in the rhizosphere and in the interior regions of the plant roots and stem and usually don't cause any disease symptoms [11]. PGPB are very important worldwide and have direct and indirect effects on plant growth [12]. PGPB used as biofertilizers resulted in improved growth and increased yield of several cereal crops [13]. A number of different bacteria promote plant growth, including *Azotobacter* spp., *Azospirillum* spp., *Pseudomonas* spp., *Acetobacter* spp., *Enterobacter* spp., *Burkholderia* spp. and *Bacillus* spp. [14-17]. The principal mechanisms of growth promotion are: production of growth-stimulating phytohormones, nitrogen fixation, [9, 18, 19], mobilization of phosphate [20, 21], siderophore production [22], antibiotic production [23], and induction of plant systemic

resistance to pathogens [24]. The above mechanisms imply a direct contact between bacteria and the root surface or inner tissues between cells in the root cortex, sites where there is maximum bacterial activity due to the release of organic components, also referred to as root exudates [25]. Root colonization is considered essential for plant growth promotion by rhizobacteria [26]. Temperature, pH, soil type, plant genotype and the competence of indigenous microorganisms are some of the factors that can affect the colonization process [17,18]. The use of beneficial microbes has several advantages over chemicals as they do not accumulate in the food chain. Also, when chemicals are used to enhance the yield they also affect non-target plants, induce resistance and affect endogenous beneficial bacteria [27, 28]. Soil bacterial communities maintain the soil ecosystem and balance the natural system. Interactions between the plant rhizosphere and soil microbes have been shown to enhance the soil quality and promote plant health. Inoculation of bacteria to plants establishes relationships between plants and microbes [29]. Cultivation of tissue explants with beneficial bacteria increased plant growth during the initial development stage and plants became more healthy [30].

In the present study we first isolated bacterial strains from the sugarcane rhizosphere, followed by their identification based on 16S rRNA gene sequence analysis and initial screening with respect to their ability to produce indole acetic acid (IAA) and to solubilize phosphate in pure cultures. Furthermore, BNF capacity was assessed based on *nifH* gene amplification. The effect of bacterial inoculation of four isolates on plant growth was tested in micropropagation and microplot field experiments.

MATERIALS AND METHODS

Isolation of bacterial strains

The experiments were carried out in experimental plots of the National Institute for Biotechnology and Genetic Engineering (NIBGE, Faisalabad, Pakistan) under natural conditions during the growing season in 2016. The soil was clay loam and was not previously used for cultivation of plants. All conditions were maintained during growing seasons of sugarcane.

Soil and root samples were collected from the rhizosphere of sugarcane plants at a mature growth stage ten months after sowing. Samples were stored at 4°C and used for further study. Rhizosphere soil and root samples were used for preparation of 10× serial dilutions up to 10^{-6} in saline solution (0.89%NaCl). Hundred microlitres soil suspension from each dilution (10^{-3} to10^{-6}) was spread on LB (Luria-Bertani) agar plates. Briefly, the medium contained (per litre) 5 g yeast extract, 10 g peptone, 10 g NaCl, 12 g agar, and pH was adjusted to 7.0 before autoclaving [31, 32]. The plates were incubated at 30 °C for 5 days. Bacterial colonies were recorded on the bases of difference in their morphology (color, shape and size), and colony

forming units (cfu/g) of each dominant colony type were determined from LB agar plates. From Pikovskaya agar plates (containing per liter: 0.5 g yeast extract, 10 g dextrose, 5 g calcium phosphate, 0.5 g ammonium sulfate, 0.2 g potassium chloride, 0.1 g magnesium sulfate, 0.0001g manganese sulfate, 0.0001 g ferrous sulfate and 15 g agar, and pH was adjusted to 7 before sterilization) the colonies with a clear halo zone were considered positive for phosphate solubilization [33]. Representative bacterial colonies were transferred to fresh nutrient agar plates and pure cultures were obtained by repeated streaking on their respective medium, and colony color, shape and size were analyzed. Individual colony-derived cultures were also preserved as glycerol (20% v/v) stocks at -80 °C.

Phosphate solubilization

In order to confirm the phosphate solubilization phenotype, cultures derived from colonies grown on Pikovskaya agar plates were grown in LB broth medium at 30 °C for 48 h with shaking (300 rpm). From these cultures, 50 µL were spotted again on freshly prepared Pikovskaya agar plates that were incubated for one week at 30 °C. Formation of a halo zone was considered as confirmation for phosphate solubilization. For quantitative estimation of phosphate solubilization, strains were grown in LB broth (50 ml, pH 7) containing 5 g/L tri-calcium phosphate as insoluble P source [34]. These cultures were grown for 15 days at 28±2 °C on a shaker. The supernatant was obtained from bacterial cultures by centrifugation at 4000 × g for 15 min, and cell-free supernatant was filtered and processed for quantitative estimation of phosphate solubilization using the molybdate blue color method [35].

IAA production (indole-3-acetic acid) by bacterial isolates

For quantification of indol-3-acetic acid (IAA) production, bacterial strains were grown in LB broth for one week at 30 °C in a shaker. After incubation cell-free culture medium was obtained by centrifugation at 4000 × g for 15 min, pH was adjusted to 2.8 with HCl (1N) and further sample preparation and analysis by HPLC was done according to standard protocols [36, 37].

Extraction of total genomic DNA

Total genomic DNA from bacterial cultures was extracted by the CTAB method [38] with some modifications and further protocol for DNA extraction according to standard protocols [39].

Identification of bacterial isolates and phylogenetic analysis

For the identification of bacterial isolates, extracted DNA was used as template for PCR amplification of near full length bacterial 16S ribosomal RNA (rRNA) genes as reported previously [40].

For *nifH* gene amplification, the same thermal conditions were used for PCR, except that the annealing temperature was 48 °C and the primer set PolF/PolR was used for amplification of

nifH as reported previously [41]. PCR products were sent for Sanger sequencing with primer 27F at GATC Biotech (Cologne, Germany). The obtained 16S rRNA gene sequences were trimmed using Bio-Edit, and sequences were further identified through BLAST search at NCBI (National Centre for Biotechnology Information) (www.ncbi.nlm.nhi.gov). Sequences were deposited to Gene Bank at NCBI and accession numbers were obtained (see Table 1). Phylogenetic trees were constructed using 16S rRNA gene sequences of the bacterial isolates along with closely related sequences in the NCBI data base and type strain sequences. These sequences were subjected to multiple alignment by MUSCLE and phylogenetic analysis was performed using software MEGA 7 [42]. Distances were computed using the Poisson correction method, and phylogenetic tree were made by using the Neighbor-Joining method. After identification of isolates by comparative 16S rRNA gene sequence analysis, the four strains were tested as PGPB in inoculation experiments performed in microplot fields under natural conditions and sugarcane micropropagation experiments under controlled conditions as described in the following.

Micropropagation of sugarcane plantlets

Field grown sugarcane plants were used for *in vitro* culture studies. The explants were obtained from the apical portion of the stem of the field grown sugarcane plants collected from the sugarcane field that is maintained for research purposes at NIBGE. The outer layers of the leaf sheath were peeled off until the inner most leaves with a thickness of 5 mm were exposed. Explants of 3–4 mm in size were dissected by making cuts with sterilized tools under aseptic conditions. The sliced tissues were placed in magenta jars (Sigma) containing 25 ml modified MS medium [43]. The cultures were incubated in the dark at 25 ± 2 °C and the induced calli were maintained on the same medium for callus induction by serial transfers. Then 5–6-week-old embryogenic calli were placed on the MS regeneration medium for differentiation and regeneration at a photoperiod of 16 h [43]. The regenerated plantlets were then transferred to the MS rooting media for root induction for 3–4 weeks [43]. After four weeks roots of the plantlets were washed with sterilized water under aseptic conditions to remove the rooting medium and then transferred to a basket which contained sterilized peat moss or sand. For normal plant growth, Hoagland solution was given at half strength as nutrient source [44]. After one week of plant stabilization, bacterial cultures were prepared for inoculation of sugarcane plants. Cultures were grown in LB agar broth (50 ml) at 30 ºC for 48 h and centrifuged at 4000 g for 10 min. The resulting cell pellet was washed with 0.8% saline solution and re-suspended in 100 ml saline. Five hundred microlitres of bacterial culture was applied directly near to the root system.

A total of five treatments were prepared with nine plantlets per treatment. Treatments included four different bacterial isolates as well as a non-inoculated control. Plantlets were grown at 30 ± 2 ºC during the day and 25 ± 2 ºC during the night and harvested after eight weeks.

Harvested plant roots were washed to remove adhering sand and peat moss particles, and washed roots were dried with blotting paper. Fresh and dry weight, and root and shoot length, were recorded for three representative plants. The root system of each plant was separated and spread on a transparent glass water tank and placed on a rhizoscanner (EPSON Perfection V700 Photo, Suwa, Nagano Prefecture, Japan) equipped with Win RHIZO software (Regent Instruments Inc., Canada) that was used for the measurement of different root parameters and image analysis. The plant material was dried to a constant weight in an oven at 70 ºC for 72 h. The dried plant samples were ground to a fine powder and total %N and %P of these samples was determined by using a semi micro-Kjeldahl method based on wet combustion in a Rapid Kjeldahl System [45, 46].

Sugarcane microplot experiment

The same four bacterial strains were also used in inoculation experiments in experimental plots of NIBGE in 2018 under natural conditions during the growing season. The soil was clay loam not previously exposed to any plants. Sugarcane plants were sown in plots in a Randomized Complete Block Design (RCBD) with fifteen replicates. Control sugarcane plants were also sown in a separate plot. For inoculation of sugarcane plants, bacterial cultures were grown in LB broth (50 ml) at 30 ºC for 48 h and centrifuged at 4000 g for 10 min. The resulting cell pellet was washed with 0.8% saline solution and re-suspended in 100 ml saline. One ml of bacterial culture was applied directly near to the root system. Fifty percent of the recommended fertilizer dose of urea and DAP (250 kg/ha of DAP and 250 kg/ha urea) was applied to each plot and data of different agronomic parameters was recorded after harvesting plants at maturity. To this end, roots of each plant were washed. Plant fresh and dry weight was recorded, yield (number of canes per plant, average cane diameter, length of cane) and total plant weight was recorded for each plant.

Quantification of microbial populations

The bacterial population size (log cfu/g of soil) was estimated in sugarcane micropropagation and microplot experiments after harvest. Samples were collected from the rhizosphere of sugarcane plants of inoculated and non-inoculated plants. Bacterial population (cfu) was estimated by plating serial dilutions of soil suspensions in 0.8% saline solution on LB agar plates. Colonies were differentiated based on their morphology, and counted separately, with particular attention to the four different PGPB used in this study as well as other colony morphologies.

Statistical analysis

The effects of different bacterial isolates on different growth parameters of sugarcane in micropropagation and microplot experiments were determined through analysis of variance (ANOVA) using STATISTIX 8.1 software. Means were compared by applying least significant difference test (LSD) at alpha 0.05 on all the parameters.

RESULTS

Morphology, identification and phylogenetic analysis of bacterial isolates

Soil and root samples were collected from the rhizosphere of sugarcane plants in 2016, and serial dilutions in saline were plated on LB agar and Pikovskaya agar plates. Obtained colonies were categorized based on colony characteristics, and representative isolates were subjected to initial screening for IAA production, phosphate solubilization and nitrogen fixation, as well as phylogenetic identification. To this end, genomic DNA was extracted from the selected cultures for the amplification of 16S rRNA genes using universal bacterial primers targeting conserved regions.

The four best strains were selected on the basis of the initial screening (data not shown), further functionally and phylogenetically characterized in more detail as described in the following and used for *in vitro* and *in vivo* experiments in sugarcane.

Bacterial identification by 16S rRNA gene sequence analysis indicated that the four selected bacterial strains isolated from the rhizosphere of sugarcane were most closely related to representatives of four genera, namely *Bacillus, Pseudomonas*, *Paenibacillus* and *Enterobacter* (Table 1). The data showed 99% sequence identity of isolates ATRS1 and PD3 with the 16S rRNA gene of a *Bacillus* sp. and *Pseudomonas* sp., respectively, and 100% sequence identity of the PD2 sequence with the 16S rRNA gene of an *Enterobacter* sp. The sequence of isolate ATS1 showed 98% sequence homology with the 16S rRNA gene of a *Paenibacillus* sp.. Further phylogenetic analyses provided additional insight with respect to the phylogenetic relationship of isolated strains with closely related strains and type strains, respectively (Fig. 1A-D).

Table 1. Identification of bacterial isolates based on 16S rRNA gene sequence analysis and colony morphology**.**

Isolate code	Organism identified	Accession number 16S rRNA gene	Closest type strain in NCBI database	16S rRNA gene identity (%)	Colony morphology
ATRS1	*Bacillus* sp.	MK230981	*B. haynesii* (NR157609)	99	Off white, big
ATS1	*Paenibacillus* sp.	MK250490	*P. massiliensis* (NR115175)	98	Off white, small rounded
PD3	*Pseudomonas* sp.	MK230968	*E. ludwigii* (NR042349)	99	Off white rough
PD2	*Enterobacter* sp.	MK230966	*E. cloacae* (NR145647)	100	Off white big flate

Functional properties of bacterial isolates

In order to provide further evidence for the potential of the selected isolates as PGPB, relevant functional properties were evaluated *in vitro*. In order to test for the ability of the isolates to produce the phytohormone IAA, isolates were grown in LB broth medium supplemented with tryptophan as a precursor for IAA biosynthesis. Among the bacterial isolates *Bacillus* sp. strain ATRS1 showed the highest amount of IAA (14.68 mg/L) in growth medium followed by *Paenibacillus* sp. strain ATS1 (12.02 mg/L), *Pseudomonas* sp. strain PD3 (7.58 mg/L) and *Enterobacter* sp. strain PD2 (7.16 mg/L) (Table 2).

Phosphate solubilization by bacterial isolates was initially screened on Pikovskaya agar plates containing insoluble tri-calcium phosphate (TCP). Development of a halo zone was taken as indication of phosphate solubilization ability of bacteria and halo zones were observed for all four isolates (data not shown). Phosphate solubilization was quantified using photospectrometric analysis of liquid culture supernatants. Among the bacterial isolates highest P-solubilization activity was observed in *Pseudomonas* sp. strain PD3 (9.9 mg/L), followed by *Bacillus* sp. strain ATRS1 (9.5 mg/L), whereas lower P-solubilization activity was recorded for *Enterobacter* sp. strain PD2 (8.3 mg/L) and *Paenibacillus* sp. strain ATS1 (8.5 mg/L) (Table 2).

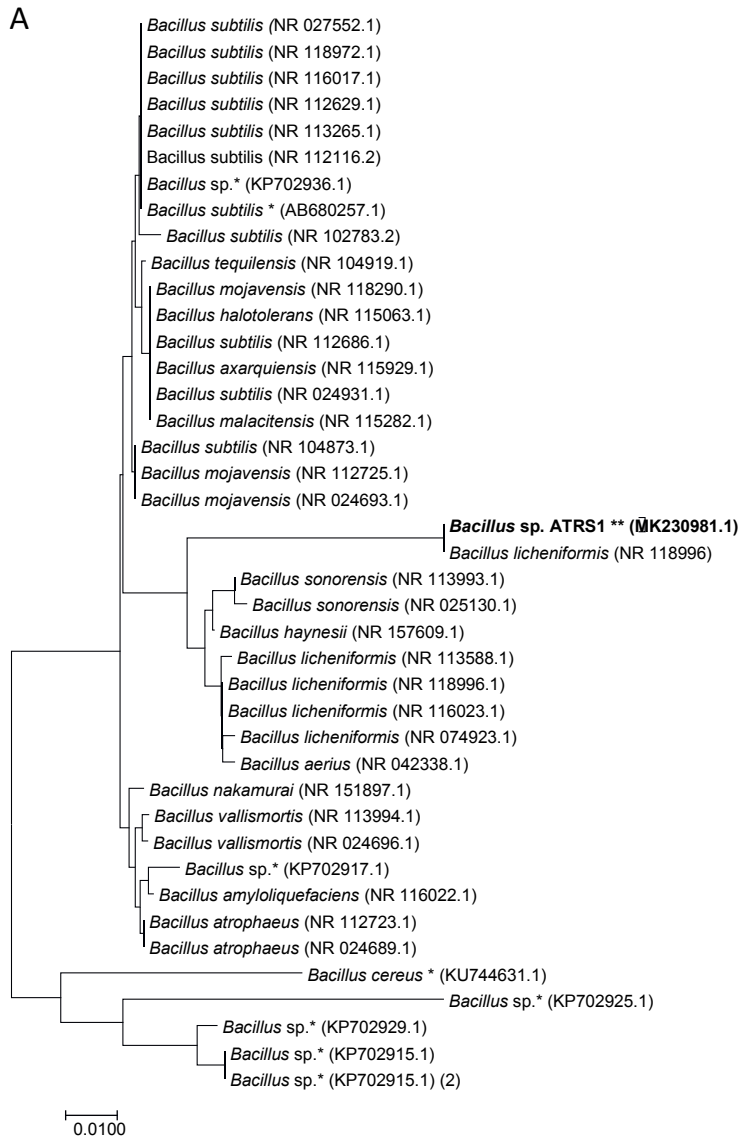
Finally, nitrogen fixation potential was predicted based on PCR amplification of the *nifH* gene, which tested positive for all four isolates.

Table 2. Production of IAA, phosphate solubilization and nitrogen fixation by the bacterial isolates from sugarcane.

*Bacterial cultures were grown in LB containing tryptophan as precursor of IAA biosynthesis; **Bacterial cultures were grown in

Bacterial strain	**IAA (mg/L)***	**Available Phosphorus (mg/L)****	**N fixation**
Bacillus sp. ATRS1	14.68 ± 0.76	9.5 ± 0.25	Positive
Paenibacillus sp. ATS1	12.02 ± 0.03	8.5 ± 0.11	Positive
Pseudomonas sp. PD3	7.58 ± 0.16	9.9 ± 0.20	Positive
Enterobacter sp. PD2	7.16 ± 0.29	8.3 ± 0.15	Positive

Pikovskaya medium containing TCP as insoluble phosphorous. ****nifH* gene amplification was taken as indication of N fixation potential.
The values given are an average of three replicates, with standard deviation.


A
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Bacillus subtilis (NR 118972.1)
Bacillus subtilis (NR 116017.1)
Bacillus subtilis (NR 112629.1)
Bacillus subtilis (NR 113265.1)
Bacillus subtilis (NR 112116.2)
Bacillus sp.* (KP702936.1)
Bacillus subtilis * (AB680257.1)
Bacillus subtilis (NR 102783.2)
Bacillus tequilensis (NR 104919.1)
Bacillus mojavensis (NR 118290.1)
Bacillus halotolerans (NR 115063.1)
Bacillus subtilis (NR 112686.1)
Bacillus axarquiensis (NR 115929.1)
Bacillus subtilis (NR 024931.1)
Bacillus malacitensis (NR 115282.1)
Bacillus subtilis (NR 104873.1)
Bacillus mojavensis (NR 112725.1)
Bacillus mojavensis (NR 024693.1)
Bacillus sp. ATRS1 ** (MK230981.1)
Bacillus licheniformis (NR 118996)
Bacillus sonorensis (NR 113993.1)
Bacillus sonorensis (NR 025130.1)
Bacillus haynesii (NR 157609.1)
Bacillus licheniformis (NR 113588.1)
Bacillus licheniformis (NR 118996.1)
Bacillus licheniformis (NR 116023.1)
Bacillus licheniformis (NR 074923.1)
Bacillus aerius (NR 042338.1)
Bacillus nakamurai (NR 151897.1)
Bacillus vallismortis (NR 113994.1)
Bacillus vallismortis (NR 024696.1)
Bacillus sp.* (KP702917.1)
Bacillus amyloliquefaciens (NR 116022.1)
Bacillus atrophaeus (NR 112723.1)
Bacillus atrophaeus (NR 024689.1)
Bacillus cereus * (KU744631.1)
Bacillus sp.* (KP702925.1)
Bacillus sp.* (KP702929.1)
Bacillus sp.* (KP702915.1)
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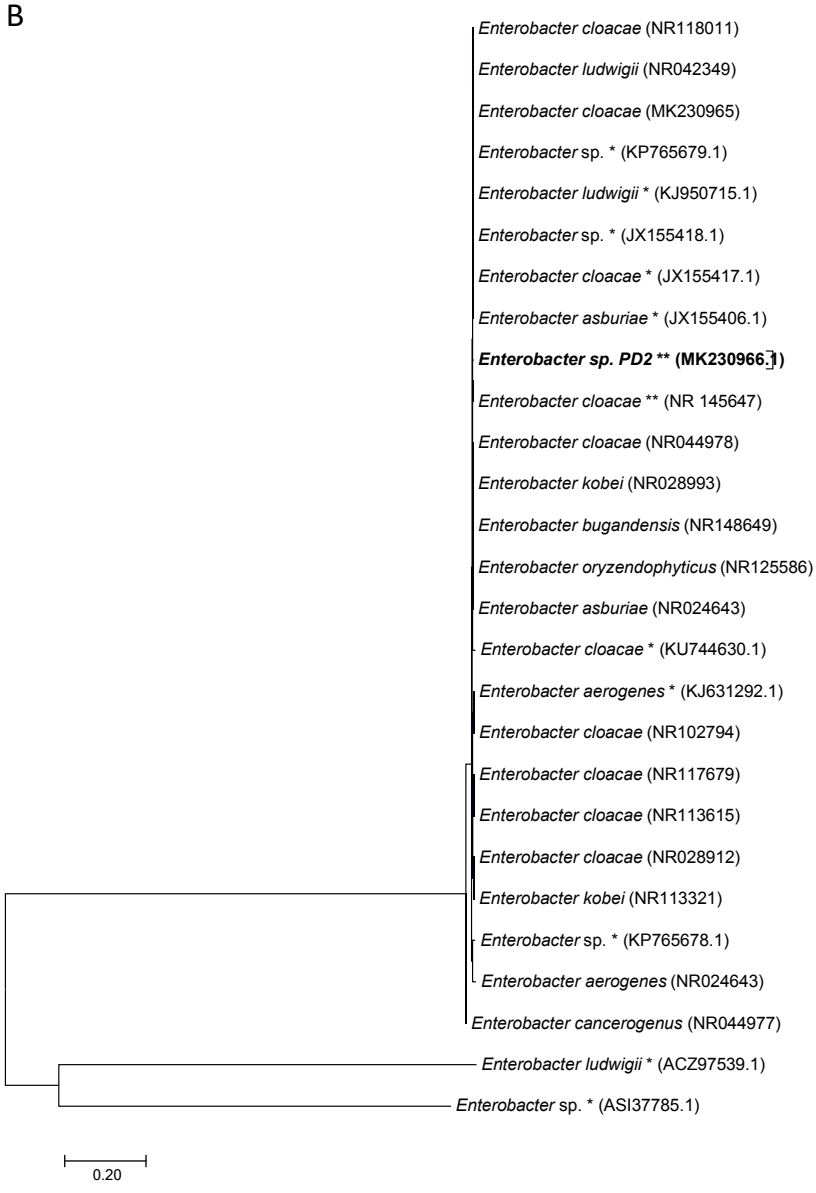
B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Enterobacter sp. * (KP765679.1)
Enterobacter ludwigii * (KJ950715.1)
Enterobacter sp. * (JX155418.1)
Enterobacter cloacae * (JX155417.1)
Enterobacter asburiae * (JX155406.1)
Enterobacter sp. PD2 ** **(MK230966.1)**
Enterobacter cloacae ** (NR 145647)
Enterobacter cloacae (NR044978)
Enterobacter kobei (NR028993)
Enterobacter bugandensis (NR148649)
Enterobacter oryzendophyticus (NR125586)
Enterobacter asburiae (NR024643)
Enterobacter cloacae * (KU744630.1)
Enterobacter aerogenes * (KJ631292.1)
Enterobacter cloacae (NR102794)
Enterobacter cloacae (NR117679)
Enterobacter cloacae (NR113615)
Enterobacter cloacae (NR028912)
Enterobacter kobei (NR113321)
Enterobacter sp. * (KP765678.1)
Enterobacter aerogenes (NR024643)
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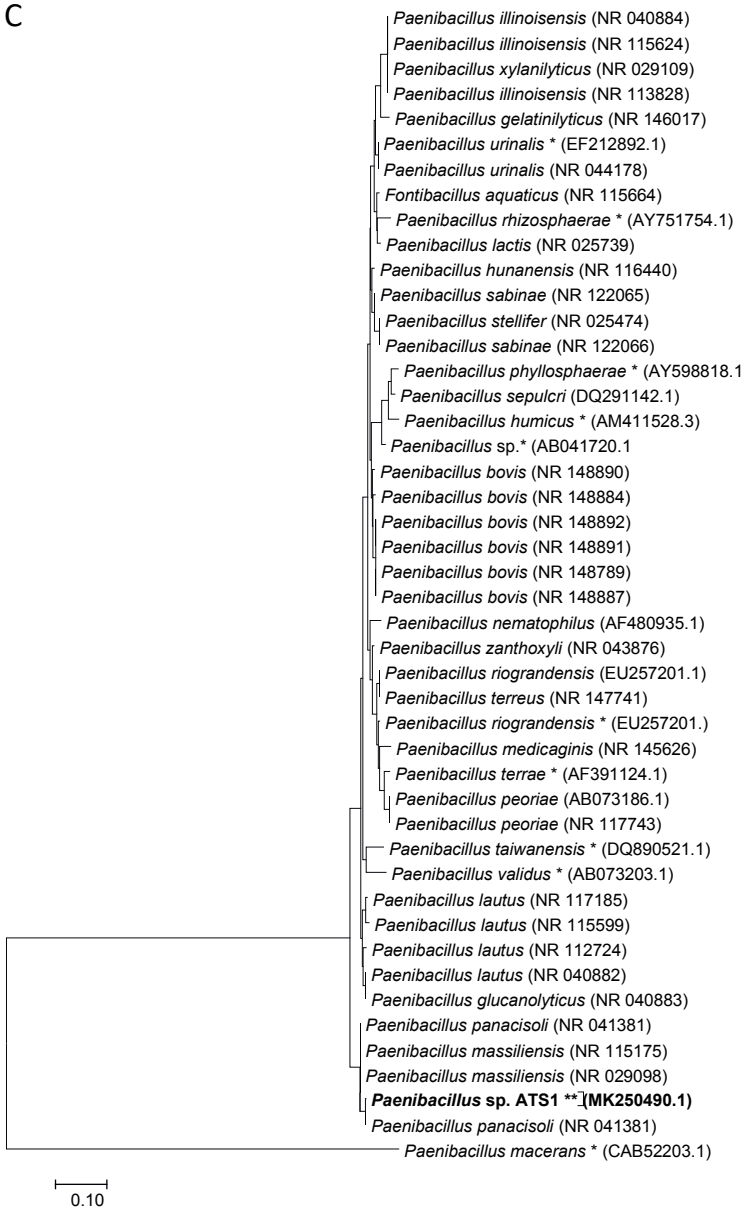
C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Paenibacillus xylanilyticus (NR 029109)
Paenibacillus illinoisensis (NR 113828)
Paenibacillus gelatinilyticus (NR 146017)
Paenibacillus urinalis * (EF212892.1)
Paenibacillus urinalis (NR 044178)
Fontibacillus aquaticus (NR 115664)
Paenibacillus rhizosphaerae * (AY751754.1)
Paenibacillus lactis (NR 025739)
Paenibacillus hunanensis (NR 116440)
Paenibacillus sabinae (NR 122065)
Paenibacillus stellifer (NR 025474)
Paenibacillus sabinae (NR 122066)
Paenibacillus phyllosphaerae * (AY598818.1)
Paenibacillus sepulcri (DQ291142.1)
Paenibacillus humicus * (AM411528.3)
Paenibacillus sp.* (AB041720.1
Paenibacillus bovis (NR 148890)
Paenibacillus bovis (NR 148884)
Paenibacillus bovis (NR 148892)
Paenibacillus bovis (NR 148891)
Paenibacillus bovis (NR 148789)
Paenibacillus bovis (NR 148887)
Paenibacillus nematophilus (AF480935.1)
Paenibacillus zanthoxyli (NR 043876)
Paenibacillus riograndensis (EU257201.1)
Paenibacillus terreus (NR 147741)
Paenibacillus riograndensis * (EU257201.)
Paenibacillus medicaginis (NR 145626)
Paenibacillus terrae * (AF391124.1)
Paenibacillus peoriae (AB073186.1)
Paenibacillus peoriae (NR 117743)
Paenibacillus taiwanensis * (DQ890521.1)
Paenibacillus validus * (AB073203.1)
Paenibacillus lautus (NR 117185)
Paenibacillus lautus (NR 115599)
Paenibacillus lautus (NR 112724)
Paenibacillus lautus (NR 040882)
Paenibacillus glucanolyticus (NR 040883)
Paenibacillus panacisoli (NR 041381)
Paenibacillus massiliensis (NR 115175)
Paenibacillus massiliensis (NR 029098)
***Paenibacillus* sp. ATS1 ** (MK250490.1)**
Paenibacillus panacisoli (NR 041381)
Paenibacillus macerans * (CAB52203.1)
0.10

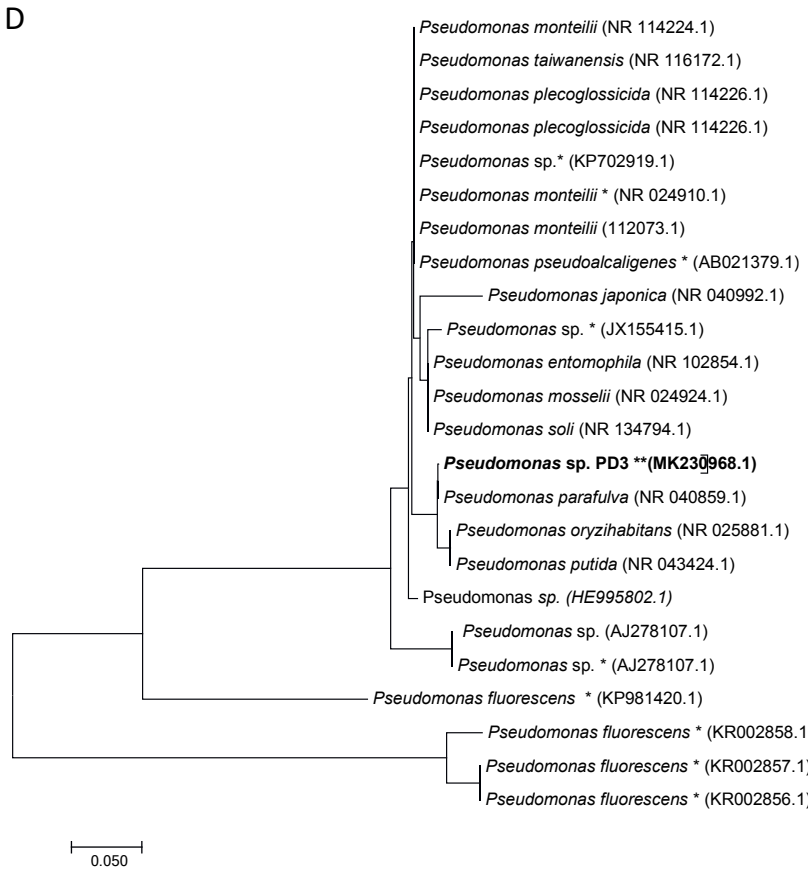

Figure 1. Phylogenetic trees showing the phylogenetic relationship of different strains of *Bacillus* (**A**)*, Enterobacter* (**B**), *Paenibacillus* (**C**) and *Pseudomonas* (**D**), on the basis of 16S rRNA gene sequences. Amplified 16S rRNA gene fragments from isolates were sequenced and initially analyzed using BLAST through NCBI. Sequences of closely related strains were downloaded from the NCBI database and aligned using MUSCLE. Trees were constructed using the neighbor joining method using software packages MEGA 7 and Fig tree. Distances were computed using the Poisson correction method. The reference bar indicates the fraction of nucleotide substitutions per position. *Type strains. **isolates obtained in this study.

Bacterial inoculation experiments

As described above, four isolates were selected on the basis of selected functional properties, including IAA production, phosphate solubilization and predicted capacity to fix molecular nitrogen. These four strains were then further tested for their growth promotion traits in experiments using (1) sterilized peat moss or sand under controlled laboratory conditions and (2) microplot experiments under field conditions. Experiments were performed on sugarcane plants with inoculation of isolates and a non-inoculated control.

Micropropagation

In the present study four strains were tested in micropropagated sugarcane plantlets cultivated either in peat moss or in sand. In the experiments in peat moss, a significant ($p \leq 0.05$) improvement in growth parameters of inoculated plants was recorded over non-inoculated control plants (Table 3). Among the bacterial strains *Bacillus* sp. strain ATRS1 showed significant ($p \leq 0.05$) improvements in growth parameters compared with control plants and highest values were observed in shoot length (60.00 cm), root length (12.17 cm), shoot dry weight (0.86 g), root dry weight (0.28 g), total root length (152.32 cm), root surface area (26.26 cm^2), root volume (0.36 cm^3), and tips (308.33) as compared to control plants and those inoculated with the other three strains. Inoculation with *Paenibacillus* sp. strain ATS1 also showed significant ($p \leq 0.05$) improvement in shoot dry weight (0.77 g), root dry weight (0.25 g), shoot length (59.33 cm), root length (9.83 cm), cumulative root length (113.67 cm) , surface are (23.93 cm^2), root volume, (0.40 cm^3), and tips (129.00) compared to non-inoculated control plants and plants inoculated with strains PD2 and PD3. Inoculation with *Pseudomonas* sp. strain PD3 showed improvement in shoot dry weight (0.74 g), root dry weight, (0.25 g), shoot length (48.66 cm), root length (9 cm), cumulative root length (87.25 cm), surface area (16.58 cm^2), root volume (0.25 cm^3), and tips (145.67) compared to control plants, whereas inoculation with *Enterobacter* sp. strain PD2 showed improvement in root dry weight (0.22 g) and tips (126.67) compared to control plants. Furthermore, total nitrogen and phosphorus content were determined from oven dried samples, showing higher %N content in *Bacillus* (3.77%), *Pseudomonas* (3.33%) and *Enterobacter* (3.87%), strains compared to non-inoculated control (3.07%) and *Paenibacillus*-treated (3.13%) plantlets. *Bacillus* (2.13%) *Paenibacillus* (1.91%), *Pseudomonas* (2.04%) and *Enterobacter* (2.33%) strains also showed higher % P content compared to the control (1.73%) (Table 3).

In micropropagation experiments in sand, *Bacillus* sp. strain ATRS1 showed significantly ($p \leq 0.05$) improved growth parameters as compared to non-inoculated control plants, with highest increase in shoot length (41.33 cm), root length (9.10 cm), shoot dry weight, (0.63 g), root dry weight (0.50 g), total root length (65.28 cm), root surface area (13.23 cm^2), root volume (0.16 cm^3) and tips (140.67) compared to controls and plantlets inoculated with the other three strains (Table 4). Inoculation with *Paenibacillus* sp. strain ATS1 also showed significant ($p \leq 0.05$)

improvement in shoot dry weight (0.55 g), root dry weight (0.43 g), shoot length (37.67 cm), root length (8 cm), cumulative root length (55.36 cm), surface area (9.14 cm^2), root volume (0.12 cm^3), diameter (0.43 mm) and tips (91) compared to non-inoculated control plants. Plants inoculated with *Pseudomonas* sp. strain PD3 showed improvement in shoot dry weight (0.52 g), root dry weight (0.33 g), shoot length (36.67 cm), root length (5.67 cm), cumulative root length (51.33 cm), surface area (5.38 cm^2), root volume (0.16 cm^3), nd tips (109) compared to control plants, whereas inoculation with *Enterobacter* sp. strain PD2 resulteda in improved shoot dry weight (0.48 g), root dry weight (0.30 g), shoot length (28 cm), root length (4.53 cm), cumulative root length (40.78 cm), root volume (0.093 cm^3), and tips (90) compared to control plants. Furthermore, total nitrogen and phosphorus content were determined from oven dried samples, showing higher %N and %P content in all inoculated plants compared to uninoculated controls (Table 3).

Visual differences in root development and root structure could also be seen from rhizoscanner images as shown in supplementary Fig. S1.

The four selected bacterial strains were also inoculated to sugarcane plants grown in microplots, again with non-inoculated plants as control. The plants were harvested at full maturity, ten months after sowing in 2018. During harvesting plants were properly uprooted with the complete root system and subsequently washed to remove mud. Roots were air-dried to remove excessive water. Significant ($p \leq 0.05$) differences were observed in the root system of inoculated plants as compared to non-inoculated control plants (Table 5). Inoculation of sugarcane with PGPB resulted in increased plant growth, including increased root length, dry weight of plants and yield (number of canes per plant, cane length and diameter). Among the four different treatments, plants inoculated with *Bacillus* sp. strain ATRS1 showed increased dry weight (366.67 g), root length (305.80 cm), and average diameter (3.06 cm^2) compared to the control. *Paenibacillus* sp. strain ATS1 increased the dry weight (353.33 g), root length (295.87 cm) and average diameter (2.61 cm^2). Plants inoculated with *Pseudomonas* sp. strain PD3 had increased total plant dry weight (300 g), root length (275.40 cm) and average diameter (2.21 cm^2). Inoculation with *Enterobacter* sp. strain PD2 resulted in increased dry weight (306 g) root length (259.73 cm) and average diameter (23.49 cm^2) as compared to the non-inoculated control. All treatments showed a significant increase in at least one parameter related to yield, i.e. with respect to cane length and/or average diameter (Table 5). To this end, inoculation with *Bacillus* sp. strain ATRS1 resulted in significantly increased ($p \leq 0.05$) cane length compared to the control as well as plants inoculated with the other three strains. %N and %P content were determined for oven dried field samples of roots and stems separately, which showed higher %N content in *Bacillus* (1.63% R, 1.60% S), *Paenibacillus* (1.50% R, 1.53% S), *Pseudomonas* (1.67% R, 1.70% S) and *Enterobacter* (1.13% R, 1.43% S) strains as compared to the control (0.90% R, 1.07% S) (Table 5). Similarly, also %P content was higher in roots and stems of plants inoculated with *Bacillus* (0.31% R, 0.32 %S), *Paenibacillus* (0.26% R, 0.24% S),

Pseudomonas (0.28% R, 0.28% S*)* and *Enterobacter* (0.27% R, 0.29% S) strains compared to the control (0.21% R, 0.21% S).
Visual differences between inoculated and non-inoculated plants grown in microplots are also shown in supplementary Figures S2 and S3 with respect to differences in root growth and cane development (Fig. S2) as well as concerning cane diameter and length (Fig. S3).

Rhizosphere bacterial population

In order to assess whether inoculated strains effectively colonized the rhizosphere of sugarcane plants in micropropagation and microplot experiments, rhizosphere soil suspensions were inoculated on LB agar plates. The visual inspection of bacterial colonies indicated that the rhizosphere of plants inoculated with specific strains was highly enriched in bacterial colonies typical for the respective inocula, whereas the plates inoculated with rhizosphere suspensions from control plants showed a more even distribution of different bacterial colony types.

Table 3. Effect of bacterial inoculation on root structure and growth of sugarcane plantlets grown in peat moss

Treatment	Root length (cm)	Shoot length (cm)	Root dry weight (g)	Shoot dry weight (g)	Total root length (cm)	Surface Area (cm^2)	Diameter (mm)	Root Volume (cm^3)	Tips	%N	%P
Bacillus sp. ATRS1	12.17^{A}	60.00^{A}	0.28^{A}	0.86^{A}	152.32^{A}	26.26^{A}	0.54^{B}	0.36^{A}	308.33^{A}	3.77^{A}	2.13^{B}
Paenibacillus sp. ATS1	9.83^{AB}	59.33^{AB}	0.25^{AB}	0.77^{AB}	113.67^{AB}	23.93^{A}	0.67^{A}	0.40^{A}	129.00^{B}	3.13^{C}	1.91^{D}
Pseudomonas sp. PD3	9.00^{AB}	48.66^{BC}	0.25^{AB}	0.74^{B}	87.25^{AB}	16.58^{AB}	0.59^{AB}	0.25^{AB}	145.67^{B}	3.33^{B}	2.04^{C}
Enterobacter sp. PD2	7.50^{BC}	37.33^{CD}	0.22^{B}	0.69^{BC}	70.62^{BC}	11.78^{BC}	0.53^{BC}	0.11^{BC}	126.67^{B}	3.87^{A}	2.33^{A}
Control	4.00^{C}	26.33^{D}	0.16^{C}	0.42^{C}	24.71^{C}	4.16^{C}	0.58^{B}	0.070^{C}	30.00^{C}	3.07^{C}	1.73^{E}
SEM	1.76	6.15	0.02	0.05	24.44	4.76	0.04	0.07	36.24	0.05	0.01

Plants were grown in peat moss in randomized complete block design with three replicates and uprooted along with root system growth of eight weeks. The values are an average of three replicates. Least significant difference (LSD) test was applied at LSD ($p \leq 0.05$). Superscript letters indicate statistically different values per column. SEM, standard error of means.

Table 4. Effect of bacterial inoculation on root structure and growth of sugarcane plantlets grown in sand

Treatment	Root length (cm)	Shoot length (cm)	Root dry weight (g)	Shoot dry weight (g)	Total root length (cm)	Surface Area (cm^2)	Diameter (mm)	Root Volume (cm^3)	Tips	%N	%P
Bacillus sp. ATRS1	9.10^{A}	41.33^{A}	0.50^{A}	0.63^{A}	65.28^{A}	13.23^{A}	0.34^{B}	0.16^{AB}	140.67^{A}	3.23^{B}	1.92^{B}
Paenibacillus sp. ATS1	8.0^{B}	37.67^{AB}	0.43^{B}	0.55^{B}	55.36^{A}	9.14^{B}	0.43^{A}	$0.12A^{B}$	91^{A}	2.83^{C}	1.53^{C}
Pseudomonas sp. PD3	5.67^{C}	36.67^{B}	0.33^{C}	$0.52B^{C}$	51.33^{AB}	5.38^{C}	0.31^{B}	0.16^{AB}	109^{A}	2.87^{C}	1.44^{D}
Enterobacter sp. PD2	4.53^{D}	28^{C}	0.30^{C}	0.48^{C}	40.78^{B}	4.52^{CD}	0.31^{B}	0.093^{B}	90^{A}	3.37^{A}	1.97^{A}
Control	3.33^{E}	20^{D}	0.22^{D}	0.40^{D}	24.16^{C}	2.67^{D}	0.30^{B}	0.02^{C}	30^{B}	2.50^{D}	1.19^{E}
SEM	0.45	2.04	0.03	0.03	6.39	0.91	0.03	0.03	26.07	0.06	0.01

Plants were grown in sand in randomized complete block design with three replicates and uprooted along with root system growth of eight weeks. The values are an average of three replicates. LSD test was applied at LSD ($p \leq 0.05$). Superscript letters indicate statistically different values per column. SEM, standard error of means.

Table 5. Effect of bacterial inoculation on root structure and growth of sugarcane plants grown in micro plots

Treatment	Total plant dry weight (g)	Total root length (cm)	No of canes per plant	Average diameter (cm^2)	Long cane length (cm)*	Short cane length (cm)*	%N R	%N S	%P R	%P S
Bacillus sp. ATRS1	366.67^{A}	305.80^{A}	15.333^{A}	3.06^{B}	12.66^{A}	7.73^{A}	1.63^{A}	$1.60A^{B}$	0.31^{A}	0.32^{A}
Paenibacillus sp. ATS1	353.33^{AB}	295.87^{A}	17.067^{A}	2.61^{C}	10.20^{B}	6.51^{BC}	1.50^{B}	1.53^{BC}	0.26^{C}	0.24^{D}
Pseudomonas sp. PD3	300.00^{B}	275.40^{B}	16.333^{A}	2.21^{D}	12.66^{A}	6.62^{B}	1.67^{A}	1.70^{A}	0.28^{B}	0.28^{C}
Enterobacter sp. PD2	306.00^{B}	259.73^{B}	14.867^{A}	3.49^{A}	10.66^{B}	6.25^{C}	1.13^{C}	1.43^{C}	0.27^{C}	0.29^{B}
Control	180.00^{C}	142.60^{C}	15.467^{A}	1.72^{E}	10.7^{B}	6.35^{BC}	0.90^{D}	1.07^{D}	0.21^{D}	0.21^{E}
SEM	28.42	9.51	1.40	0.07	0.38	0.17	0.04	0.05	0.00	0.00

Plants were grown in sand in randomized complete block design with fifteen replicates and uprooted along with root system growth of eight weeks. The values are an average of three replicates. LSD test was applied at LSD ($p \leq 0.05$). Superscript letters indicate statistically different values per column. R, root; S, shoot. SEM, standard error of means. *Canes were devised into two length classes based on visual inspection for the purpose of this measure.

DISCUSSION

In the present study a number of potential PGPB were isolated from the rhizosphere of sugarcane plants, followed by their functional characterization, identification by 16S rRNA gene sequence analysis as well as their evaluation as biofertilizer through inoculation in experimental models of sugarcane. Functional characterization of isolates was based on assessment of their capacity to produce the plant hormone IAA, as well as their potential with respect to phosphate solubilization and nitrogen fixation. Isolation and characterization of *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Enterobacter* strains and their effects on plant growth as PGPB in sugarcane and wheat cultivation has previously been reported in various studies [36, 47]. In the current study, four representative isolates were obtained after cultivation of sugarcane rhizosphere material suspensions on nutrient agar media plates. These isolates were identified on the basis of comparative 16S rRNA gene sequence analysis with closely related, publicly available sequences. Bacterial strains belonging to the genera *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Enterobacter* were previously found to be common in sugarcane crop [48, 49].

Isolation of *Paenibacillus* spp. strains from the rhizosphere of various crops all over the world has been reported previously [50]. *Paenibacillus* spp. strains were mostly found in roots of plants [51]. Furthermore, it has previously been shown that *Paenibacillus* spp. strains increased root and shoot length after inoculation as PGPB [51-53]. Also, the importance of *Paenibacillus* spp. as phytohormone producing PGPB has been reported earlier [54]. Similarly, isolation of *Pseudomonas* spp. strains as the largest group of PGPB mostly present in the rhizosphere has been reported [55], and their role in supporting plant growth through IAA production and phosphate solubilization was shown before [55, 56]. Furthermore, isolation of *Bacillus* spp. as PGPB has previously been reported for many crops, particularly from plant roots [56-58]. Finally, *Enterobacter* spp. strains are well known for their nitrogen fixation capacity and have been isolated repeatedly as PGPB from different crops [59].

Production of IAA by the bacterial isolates from sugarcane obtained here was investigated in pure culture. IAA is a phytohormone which plays a central role in plant growth in rhizosphere and phyllosphere of many crops [60], through its role in development and regulation of many biological processes [61, 62]. IAA production is an important characteristic of PGPB, IAA production by PGPB in sugarcane was also investigated in a recent study, and it was found to enhance growth significantly in two sugarcane plants as compared to negative controls after inoculation [63]. In the present study, IAA production was detected in all four isolates of *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Enterobacter*. *Bacillus* sp. strain ATRS1 was found to produce the highest amount of IAA as compared to the other strains. Previously reported *Bacillus* spp. isolates obtained from different crops including, for example, maize, wheat,

soybean, corn, and tomato also showed IAA production in a range of 18 to 28 mg/L [64-67]. To this end, the IAA production capacity of strain ATRS1 reported here (14.56 mg/L) falls well within the same order of magnitude. Production of IAA by *Paenibacillus* strains was previously reported for wheat and cucumber [68, 69], and IAA production by strains of the genera *Pseudomonas and Enterobacter* has also been reported in various crops [70-75].

Phosphate solubilization by bacteria is an important mechanism for plant growth [76]. Phosphorus is an essential element for normal plant growth, and use of phosphate solubilizing bacteria (PSB) has been proposed as an environmental friendly approach for sustainable agricultural development in many crops such as sugarcane, cotton, rice or chickpea, and vegetable crops including e.g. tomato, brinjal, okra, and chili. Application of PSBs also provides an alternative route to reduce the cost of chemical phosphorus fertilizer [77-80]. Previous research showed that PSB promote the growth of sugarcane [81]. In the present study, phosphate solubilization activity of bacterial isolates was studied in Pikovskaya medium supplemented with tri-calcium phosphate as source of phosphorus. Among the tested strains, efficient phosphate solubilization was shown by *Pseudomonas* sp. strain PD3 (9.9 mg/L), *Bacillus* sp. strain ATRS1 (9.5 mg/L), *Paenibacillus* sp. strain AST1 (8.5 mg/L), and *Enterobacter* sp. strain PD2 (8.3 mg/L). These results confirm previous studies with respect to phosphate solubilization by isolates of *Paenibacillus* in wheat and cucumber [68, 82], *Pseudomonas* strains isolated from sugarcane, rice and chickpea (15.91 mg/L) [56, 77, 83, 84], *Enterobacter* [72-75] and *Bacillus* spp. isolates (10 to 18 mg/L) isolated from sugarcane and other crops [85, 86].

Nitrogen is a key nutrient for normal crop growth and required in large quantities. To this end, BNF by PGPB can help in the reduction of the need for chemical nitrogen fertilizer [87]. The role of PGPB in nitrogen fixation and promotion of sugarcane growth has been previously reported [2, 88-91]. Our study showed that all strains carried the *nif*H gene providing an indication of nitrogen fixation by these isolates. Members of the genera *Paenibacillus, Pseudomonas, Enterobacter and Bacillus* have previously been reported as nitrogen fixers in wheat, sugarcane, cucumber and other crops, in all cases supported by the detection the *nif*H gene [68, 70-75, 82].

Use of PGPB with the aim of improving nutrient availability and plant growth has been studied for several centuries [49]. In the present study four bacterial isolates showing high IAA and phosphate solubilization ability were used as inoculants for sugarcane in two experimental models, namely micropropagation and microplot experiments. The inoculated sugarcane plants showed significant improvement of most of the growth parameters recorded in this study. Previous research characterized a *Bacillus* sp. isolate as PGPB, which promoted sugarcane growth through an increase in root shoot length biomass (dry weight of root and shoot), P% and N% content [92]. In the present study the highest increase in root shoot length biomass

(dry weight of root and shoot), cumulative root length volume and tips of roots of sugarcane plants and phosphorus (P)% and nitrogen (N)% content was shown for plants inoculated with *Bacillus* sp. strain ATRS1 in both experimental models. Similar studies were reported previously, showing that among several strains tested, plants inoculated with *Bacillus licheniformis* showed the highest increase in root shoot length and root shoot dry weight, cumulative root length volume, tips of roots and P% and N% content [85, 86]. *Paenibacillus* isolates have great potential as PGPB as reported before [93]. In our study *Paenibacillus* sp. strain AST1 also showed good plant promoting traits in both models with respect to increased root and shoot dry weight, root and shoot length as well as other parameters as compared to non-inoculated control plants. Similarly, *Pseudomonas* spp. strains have been isolated as PGPB from sugarcane, rice and chickpea and were shown to stimulate plant growth by increasing biomass production (dry weight of root and shoot), root and shoot length and other parameters, including P% and N% content [56, 77, 83, 84]. Similarly, our study supported earlier findings with respect to growth promoting effects of *Enterobacter* spp. isolates in wheat and rice [94, 95].

In addition to the characterization of functional properties and effects on plant growth traits elicited by PGPB, effects on plant-associated bacterial diversity as well as PGPB population sizes in the rhizosphere are also important parameters. In the present study bacterial populations were quantified on agar plates with respect inoculated strains and compared with other bacterial colonies. Each treatment inoculated with one of the four specific strains showed highest bacterial cfu/g soil of the specific strain compared to all other bacterial colonies on plates, whereas non-inoculated control plants showed an equal distribution of all bacterial colony types, in line with earlier studies [96, 97]

Conclusions

In the present study, bacteria representative of several previously known PGPB like genera, including *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Enterobacter* were isolated from the rhizosphere of sugarcane and showed significant effects with respect to the promotion of plant growth and root development in different experimental sugarcane crop models. PGPB identified in the present study may be utilized as biofertilizer in sugarcane and other crops to make agriculture more sustainable and reduce the need for chemical fertilizer.

References

1. Pisa, G., et al., Diversity of 16S rRNA genes from bacteria of sugarcane rhizosphere soil. Brazilian Journal of Medical and Biological Research, 2011. 44(12): p. 1215-1221.
2. Taulé, C., et al., The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum L.*), and the identification and characterization of part of the associated diazotrophic bacterial community. Plant and Soil, 2012. 356(1-2): p. 35-49.

3. Moreira, F.M.d.S., et al., Bactérias diazotróficas associativas: diversidade, ecologia e potencial de aplicações. Comunicata Scientiae, 2010. 1(2): p. 74.
4. Antunes, J., et al., Diversity of plant growth-promoting bacteria associated with sugarcane. Genetics and Molecular Research: GMR, 2017. 16(2).
5. Anderson, T.-H. and K.H. Domsch, Ratios of microbial biomass carbon to total organic carbon in arable soils. Soil Biology and Biochemistry, 1989. 21(4): p. 471-479.
6. Singh, K.P., et al., Yield and soil nutrient balance of a sugarcane plant–ratoon system with conventional and organic nutrient management in sub-tropical India. Nutrient Cycling in Agroecosystems, 2007. 79(3): p. 209-219.
7. Yadav, R. and S. Prasad, Conserving the organic matter content of the soil to sustain sugarcane yield. Experimental Agriculture, 1992. 28(1): p. 57-62.
8. Suman, A., Biological nitrogen fixation in relation to improving sugarcane productivity. Summer School held at IISR, Lucknow, 2003. 15: p. 61-64.
9. Barazani, O. and J. Friedman, Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? Journal of Chemical Ecology, 1999. 25(10): p. 2397-2406.
10. Hari, K. Biofertilizers in sugarcane. in Lead paper presented in 10th sugarcane research and development workers' meeting for South Karnataka, Shimoga, Karnataka, India. 1995.
11. Sajjad Mirza, M., et al., Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. Plant and Soil, 2001. 237(1): p. 47-54.
12. Erturk, Y., et al., Effects of plant growth promoting rhizobacteria (PGPR) on rooting and root growth of kiwifruit (*Actinidia deliciosa*) stem cuttings. Biological Research, 2010. 43(1): p. 91-98.
13. Govindarajan, M., et al., Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkholderia vietnamiensis*. Plant and Soil, 2006. 280(1-2): p. 239-252.
14. Bashan, Y. and H. Levanony, Current status of *Azospirillum* inoculation technology: Azospirillum as a challenge for agriculture. Canadian Journal of Microbiology, 1990. 36(9): p. 591-608.
15. Brown, M.E., Seed and root bacterization. Annual Review of Phytopathology, 1974. 12(1): p. 181-197.
16. Kloepper, J., R. Lifshitz, and M. Schroth, *Pseudomonas* inoculants to benefit plant production. ISI Atlas of Science: Animal and Plant Sciences, 1988. 1(1): p. 60-64.
17. Kloepper, J.W., R. Lifshitz, and R.M. Zablotowicz, Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology, 1989. 7(2): p. 39-44.
18. Probanza, A., et al., The influence of native rhizobacteria on european alder (Alnus glutinosa (L.) Gaertn.) growth. Plant and Soil, 1996. 182(1): p. 59-66.
19. Gutiérrez-Mañero, F.J., et al., The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiologia Plantarum, 2001. 111(2): p. 206-211.
20. De Freitas, J., M. Banerjee, and J. Germida, Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus L.*). Biology and Fertility of Soils, 1997. 24(4): p. 358-364.
21. Vazquez, P., et al., Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. Biology and Fertility of Soils, 2000. 30(5-6): p. 460-468.
22. Raaska, L., L. Viikari, and T. Mattila-Sandholm, Detection of siderophores in growing cultures of *Pseudomonas* spp. Journal of industrial Microbiology, 1993. 11(3): p. 181-186.

23. Whipps, J.M., Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany, 2001. 52(suppl_1): p. 487-511.
24. Ramamoorthy, V., et al., Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Protection, 2001. 20(1): p. 1-11.
25. Nardi, S., et al., Soil organic matter mobilization by root exudates. Chemosphere, 2000. 41(5): p. 653-658.
26. Jjemba, P. and M. Alexander, Possible determinants of rhizosphere competence of bacteria. Soil Biology and Biochemistry, 1999. 31(4): p. 623-632.
27. Shen, D., Microbial diversity and application of microbial products for agricultural purposes in China. Agriculture, Ecosystems & Environment, 1997. 62(2-3): p. 237-245.
28. Triques, M.C., et al., The ecotoxicity of sugarcane pesticides to non-target soil organisms as a function of soil properties and moisture conditions. International Journal of Environmental Research, 2022. 16(4): p. 61.
29. Del Carmen Jaizme-Vega, M., A.S. Rodríguez-Romero, and M.S.P. Guerra, Potential use of rhizobacteria from the *Bacillus* genus to stimulate the plant growth of micropropagated bananas. Fruits, 2004. 59(2): p. 83-90.
30. Parray, J.A., et al., Interaction of rhizobacterial strains for growth improvement of *Crocus sativus L*. under tissue culture conditions. Plant Cell, Tissue and Organ Culture, 2015. 121(2): p. 325-334.
31. Maniatis, T., Molecular cloning. A Laboratory Manual, 1982.
32. Vincent, J.M., A manual for the practical study of the root-nodule bacteria. A Manual for the Practical Study of the Root-Nodule Bacteria., 1970.
33. RI, P., Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiologiya, 1948. 17: p. 362-370.
34. Pikovskaya, R., Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologiya, 1948. 17: p. 362-370.
35. Watanabe, F. and S. Olsen, Test of an ascorbic acid method for determining phosphorus in water and NaHCO3 extracts from soil 1. Soil Science Society of Merica Journal, 1965. 29(6): p. 677-678.
36. Tahir, M., et al., Isolation and identification of phosphate solubilizer'*Azospirillum*, *Bacillus'* and'*Enterobacter*'strains by 16SrRNA sequence analysis and their effect on growth of wheat ('*Triticum aestivum'L*.). Australian Journal of Crop Science, 2013. 7(9): p. 1284.
37. Arshad, M., et al., Comparison of rhizosperic bacterial populations and growth promotion of *AVP1* transgenic and non-transgenic cotton by bacterial inoculations. Journal of Animal & Plant Sciences, 2016. 26(5).
38. Wilson, K., Preparation of genomic DNA from bacteria. Current Protocols in Molecular Biology, 2001. 56(1): p. 2.4. 1-2.4. 5.
39. Qaisrani, M.M., et al., Isolation and identification by 16s rRNA sequence analysis of *Achromobacter*, *Azospirillum* and *Rhodococcus* strains from the rhizosphere of maize and screening for the beneficial effect on plant growth. Pakistan Journal of Agricultural Sciences, 2014. 51(1).
40. Versluis, D., et al., Recovery of previously uncultured bacterial genera from three Mediterranean sponges. Marine Biotechnology, 2017. 19: p. 454-468.
41. Ayyaz, K., et al., Isolation and identification by 16S rRNA sequence analysis of plant growth-promoting azospirilla from the rhizosphere of wheat. Brazilian Journal of Microbiology, 2016. 47(3): p. 542-550.

42. Kumar, S., G. Stecher, and K. Tamura, MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 2016. 33(7): p. 1870-4.
43. Murashige, T. and F. Skoog, A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 1962. 15(3): p. 473-497.
44. Hoagland, D.R. and D.I. Arnon, The water-culture method for growing plants without soil. Circular. California agricultural experiment station, 1950. 347(2nd edit).
45. Mirza, M.S., et al., Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. Plant and Soil, 2001. 237(1): p. 47-54.
46. Rowland, A.P. and H.M. Grimshaw, A wet oxidation procedure suitable for total nitrogen and phosphorus in soil. Communications in Soil Science and Plant Analysis, 1985. 16(6): p. 551-560.
47. Kruasuwan, W. and A. Thamchaipenet, Diversity of culturable plant growth-promoting bacterial endophytes associated with sugarcane roots and their effect of growth by co-inoculation of diazotrophs and *Actinomycetes*. Journal of Plant Growth Regulation, 2016. 35(4): p. 1074-1087.
48. Da Silveira, A.P.D., et al., Exploitation of new endophytic bacteria and their ability to promote sugarcane growth and nitrogen nutrition. Antonie van Leeuwenhoek, 2019. 112(2): p. 283-295.
49. Modi, K. and P. Patel, Isolation and characterization of plant growth promoting rhizobacteria associated with *Saccharum officinarum L*. Current Synthetic and Systems Biology, 2017. 5: p. 2332-0737.
50. Heulin, T., et al., *Bacillus polymyxa* and *Rahnella aquatilis*, the dominant N2-fixing bacteria associated with wheat rhizosphere in french soils. European Journal of Soil Biology (France), 1994.
51. Chanwav, C. and L. Nelson, Field and laboratory studies of *Triticum aestivum L*. inoculated with co-existent growth-promoting *Bacillus* strains. Soil Biology and Biochemistry, 1990. 22(6): p. 789-795.
52. Ryu, C. and C. Park. Enhancement of plant growth induced by endospore forming PGPR strain, *Bacillus polymyxa* E681. in Proceedings of the 4th International Workshop on Plant Growth-Promoting Rhizobacteria. 1997.
53. Sudha, S., R. Jayakumar, and V. Sekar, Introduction and expression of the *cry1Ac* gene of *Bacillus thuringiensis* in a cereal-associated bacterium, *Bacillus polymyxa*. Current Microbiology, 1999. 38(3): p. 163-167.
54. Lebuhn, M., T. Heulin, and A. Hartmann, Production of auxin and other indolic and phenolic compounds by *Paenibacillus polymyxa* strains isolated from different proximity to plant roots. FEMS Microbiology Ecology, 1997. 22(4): p. 325-334.
55. Srivastava, S., et al., Gene expression profiling through microarray analysis in Arabidopsis thaliana colonized by *Pseudomonas putida* MTCC5279, a plant growth promoting rhizobacterium. Plant Signaling & Behavior, 2012. 7(2): p. 235-245.
56. Tiwari, S., et al., Pseudomonas putida attunes morphophysiological, biochemical and molecular responses in *Cicer arietinum L*. during drought stress and recovery. Plant Physiology and Biochemistry, 2016. 99: p. 108-117.
57. Gamez, R., et al., Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (*Musa acuminata Colla*). Microbiological Research, 2019. 220: p. 12-20.
58. García, J.A.L., et al., Effect of inoculation of *Bacillus licheniformis* on tomato and pepper. Agronomie, 2004. 24(4): p. 169-176.

59. Mehnaz, S., Plant growth-promoting bacteria associated with sugarcane, in bacteria in agrobiology: crop ecosystems. 2011, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 165-187.
60. Shahzad, R., et al., Indoleacetic acid production and plant growth promoting potential of bacterial endophytes isolated from rice (*Oryza sativa L.*) seeds. Acta Biologica Hungarica, 2017. 68(2): p. 175-186.
61. Ghosh, P.K., et al., Role of IAA metabolizing enzymes on production of IAA in root, nodule of Cajanus cajan and its PGP *Rhizobium* sp. Biocatalysis and Agricultural Biotechnology, 2013. 2(3): p. 234-239.
62. Etesami, H., H.A. Alikhani, and H.M. Hosseini, Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. MethodsX, 2015. 2: p. 72-78.
63. Guo, D.-J., et al., Complete genome analysis of sugarcane root associated endophytic diazotroph *Pseudomonas aeruginosa* DJ06 revealing versatile molecular mechanism involved in sugarcane development. Frontiers in Microbiology, 2023. 14: p. 1096754.
64. Đorđević, S., et al., The use of bacterial indole-3-acetic acid (IAA) for reduce of chemical fertilizers doses. _ Hemijska Industrija. 2017. 71(3): p. 195-200.
65. Tabatabaei, S., et al., Indole-3-acetic acid (IAA) producing *Pseudomonas* isolates inhibit seed germination and α-amylase activity in durum wheat (*Triticum turgidum L.*). Spanish Journal of Agricultural Research, 2016. 14(1): p. 0802.
66. Pattnaik, S., et al., Indole acetic acid production and growth-promoting activity of *Methylobacterium extorquens* MP1 and *Methylobacterium zatmanii* MS4 in tomato. International Journal of Vegetable Science, 2017. 23(4): p. 321-330.
67. Yu, Z., et al., Isolation and characterization of indole acetic acid producing root endophytic bacteria and their potential for promoting crop growth. Journal of Agricultural Science and Technology. 2016. 18: p. 1381-1391
68. Wang, Y., et al., Phosphate solubilization of *Paenibacillus polymyxa* and *Paenibacillus macerans* from mycorrhizal and non-mycorrhizal cucumber plants. African Journal of Microbiology Research, 2012. 6: p. 4567-4573.
69. Guemouri-Athmani, S., et al., Diversity of *Paenibacillus polymyxa* populations in the rhizosphereof wheat (*Triticum durum*) in Algerian soils. European Journal of Soil Biology, 2000. 36(3): p. 149-159.
70. Ahmad, F., I. Ahmad, and M.S. KHAN, Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. Turkish Journal of Biology, 2005. 29(1): p. 29-34.
71. Liu, R., et al., Genomic and phenotypic analyses of *Pseudomonas psychrotolerans* PRS08-11306 reveal a turnerbactin biosynthesis gene cluster that contributes to nitrogen fixation. Journal of Biotechnology, 2017. 253: p. 10-13.
72. Lee, K.-E., et al., Isolation and characterization of the high silicate and phosphate solubilizing novel strain *Enterobacter ludwigii* GAK2 that promotes growth in rice plants. Agronomy, 2019. 9(3): p. 144.
73. Pramanik, K., et al., Characterization of a Cd2+-resistant plant growth promoting rhizobacterium (*Enterobacter* sp.) and its effects on rice seedling growth promotion under Cd2+-stress in vitro. Agriculture and Natural Resources, 2018. 52(3): p. 215-221.
74. Nutaratat, P., A. Monprasit, and N. Srisuk, High-yield production of indole-3-acetic acid by *Enterobacter* sp. DMKU-RP206, a rice phyllosphere bacterium that possesses plant growth-promoting traits. 3 Biotech, 2017. 7(5): p. 305.
75. Yi, Y., W. Huang, and Y. Ge, Exopolysaccharide: a novel important factor in the microbial dissolution of tricalcium phosphate. World Journal of Microbiology and Biotechnology, 2008. 24(7): p. 1059-1065.

76. Awais, M., et al., Isolation, characterization and association among phosphate solubilizing bacteria from sugarcane rhizosphere. Cytology and Genetics, 2019. 53(1): p. 86-95.
77. Rajwar, J., et al., Comparative phosphate solubilizing efficiency of psychrotolerant *Pseudomonas jesenii* MP1 and *Acinetobacter* sp. ST02 against chickpea for sustainable hill agriculture. Biologia, 2018. 73(8): p. 793-802.
78. Chung, H., et al., Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biology and Biochemistry, 2005. 37(10): p. 1970-1974.
79. Awais, M., et al., Isolation, characterization and inter-relationship of phosphate solubilizing bacteria from the rhizosphere of sugarcane and rice. Biocatalysis and Agricultural Biotechnology, 2017. 11: p. 312-321.
80. Baliah, N.T., G. Pandiarajan, and B.M. Kumar, Isolation, identification and characterization of phosphate solubilizing bacteria from different crop soils of Srivilliputtur Taluk, Virudhunagar District, Tamil Nadu. Tropical Ecology, 2016. 57(3): p. 465-474.
81. Lamizadeh, E., N. Enayatizamir, and H. Motamedi, Isolation and identification of plant growth-promoting rhizobacteria (PGPR) from the rhizosphere of sugarcane in saline and non-saline soil. International Journal of Current Microbiology and Applied Sciences, 2016. 5(10): p. 1072-83.
82. Cherchali, A., et al., Isolation and identification of a phosphate-solubilizing Paenibacillus polymyxa strain GOL 0202 from durum wheat (*Triticum durum Desf.*) rhizosphere and its effect on some seedlings morphophysiological parameters. Biocatalysis and Agricultural Biotechnology, 2019. 19: p. 101087.
83. Li, H.-B., et al., Genetic diversity of nitrogen-fixing and plant growth promoting *Pseudomonas* species isolated from sugarcane rhizosphere. Frontiers in Microbiology, 2017. 8(1268).
84. Nautiyal, C.S., et al., Plant growth-promoting bacteria *Bacillus amyloliquefaciens* NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress. Plant Physiology and Biochemistry, 2013. 66: p. 1-9.
85. Goswami, D., et al., Screening of PGPR from saline desert of Kutch: Growth promotion in Arachis hypogea by *Bacillus licheniformis* A2. Microbiological Research, 2014. 169(1): p. 66-75.
86. Jose Antonio Lucas García, et al., Effect of inoculation of *Bacillus licheniformis* on tomato and pepper. Agronomie, 2004. 24(4): p. 169-176.
87. Rao, D. Recent advances in biological nitrogen fixation in agricultural systems. in Proceedings of the Indian National Science Academy. 2014.
88. Quecine, M.C., et al., Sugarcane growth promotion by the endophytic bacterium *Pantoea agglomerans* 33.1.1. Applied and Environmental Microbiology, 2012. 78(21): p. 7511.
89. Cavalcante, J., et al., Members of the ethylene signalling pathway are regulated in sugarcane during the association with nitrogen-fixing endophytic bacteria. Journal of Experimental Botany, 2007. 58(3): p. 673-686.
90. Lin, L., et al., Plant Growth-promoting Nitrogen-fixing enterobacteria are in association with sugarcane plants growing in Guangxi, China. Microbes and Environments, 2009. advpub: p. 1204160379-1204160379.
91. Thaweenut, N., et al., Two seasons' study on nifH gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum* spp. hybrids): expression of nifH genes similar to those of rhizobia. Plant and Soil, 2011. 338(1): p. 435-449.

92. Chandra, P., P. Tripathi, and A. Chandra, Isolation and molecular characterization of plant growth-promoting *Bacillus* spp. and their impact on sugarcane (*Saccharum* spp. hybrids) growth and tolerance towards drought stress. Acta Physiologiae Plantarum, 2018. 40(11): p. 199.
93. Kim, C.-G., et al., *Paenibacillus panacihumi* sp. nov., a potential plant growth-promoting bacterium isolated from ginseng-cultivated soil. Archives of Microbiology, 2018. 200(7): p. 1049-1055.
94. Borham, A., et al., Phosphate solubilization by *Enterobacter cloacae* and its impact on growth and yield of wheat plants.. Journel of Sustaiable Agricural Sciences 2017 43 (2) p 89-103
95. Singh, M., Isolation and characterization of insoluble inorganic phosphate solubilizer rice rhizosphere strain *Enterobacter cloacae* BAU3. Journal of Applied and Natural Science, 2018. 10(4): p. 1204-1209.
96. Stets, M.I., et al., Quantification of *Azospirillum brasilense* FP2bacteria in wheat roots by strain-specific quantitative PCR. Applied and Environmental Microbiology, 2015. 81(19): p. 6700-6709.
97. Tahir, M., et al., Cultivation-based and molecular assessment of bacterial diversity in the rhizosheath of wheat under different crop rotations. PloS One, 2015. 10(6): p. e0130030.

Supplementary Material

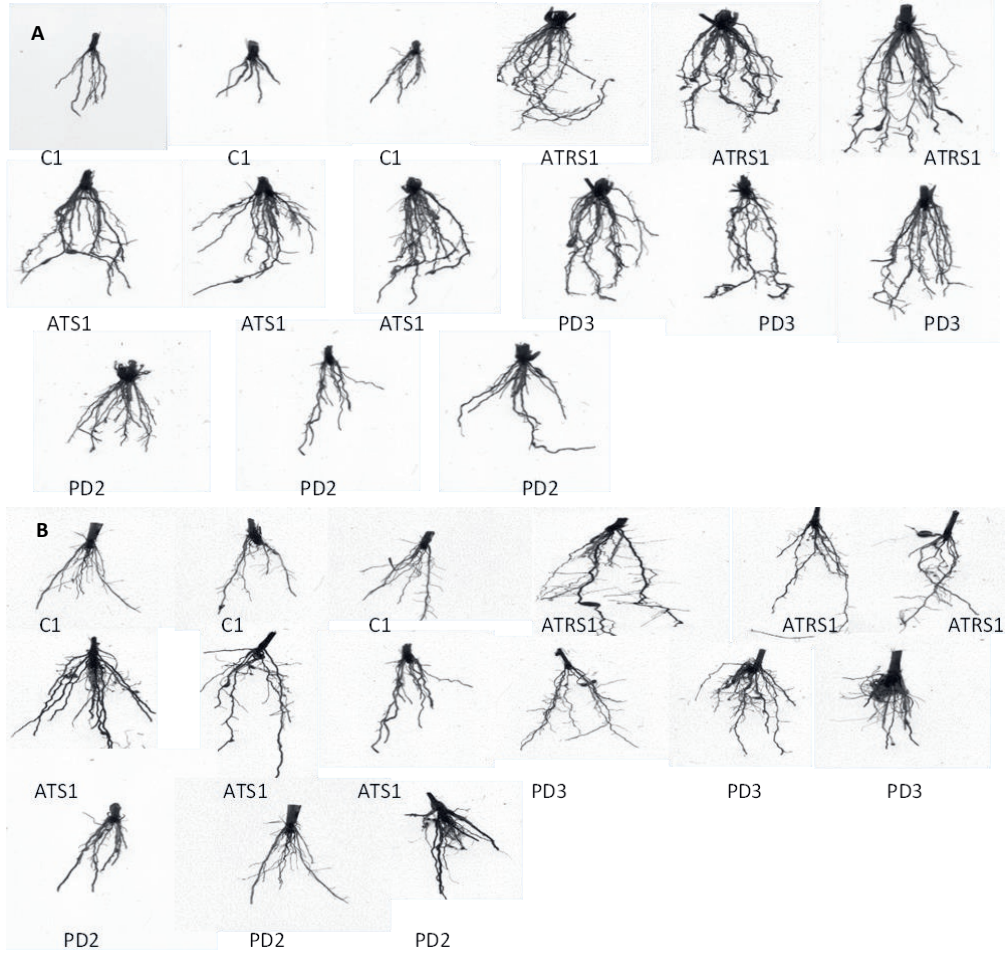

Figure S1. Root image analysis by Rhizoscanner (EPSON Perfection V700 Photo, Suwa, Nagano Prefecture, Japan) equipped with Win RHIZO software (Regent Instruments Inc., Canada). Images were taken of three replicates of each treatment of roots harvested after eight weeks of growth in sterilized peat moss (**A**) or sand (**B**). Treatments are indicated by the strain designations and C for the non-inoculated control.

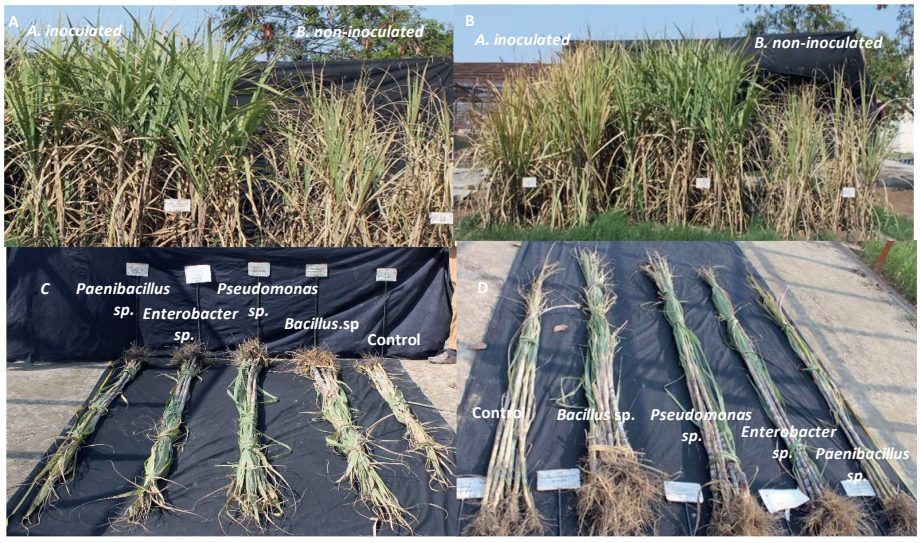

Figure S2. Images A & B illustrate the difference in growth between inoculated and non-inoculated sugarcane plants. Photographs show inoculated plants on the left side, whereas the plants on the right are non-inoculated control plants. **.** Images C &D illustrate the difference between root growth and structure of inoculated and non-inoculated sugarcane

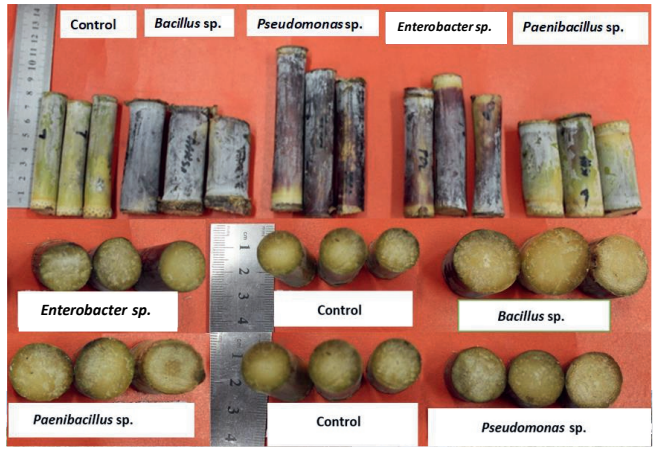

Figure S3. Images illustrating the differences in cane length and diameter of inoculated and non-inoculated sugarcane plants.

Chapter 3

Isolation, Identification and Characterization of Multi Trait Plant Growth Promoting Bacteria from the Rhizosphere of Sugarcane and Application in Wheat

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Abstract

The present study focused on the isolation and characterization of potential plant growth promoting bacteria from the rhizosphere of sugarcane plants and their impact on wheat growth. Fifteen strains belonging to three genera, i.e. *Pseudomonas*, *Burkholderia* and *Bacillus*, were isolated from the rhizosphere soil and roots of sugarcane. Isolates were initially characterized on the basis of indole acetic acid production, phosphate solubilization and biological nitrogen fixation potential, and subsequently screened for their ability to promote plant growth in wheat. Inoculation showed that all 15 tested stains significantly improved at least two of five plant growth parameters assessed in this study, including increased dry weight of root and shoot, root and shoot length, and yield with respect to tiller numbers as compared to non-inoculated controls. Comparison between members of the three genera showed that inoculation with *Burkholderia* spp. strains resulted on average in the highest increase of biomass production, yield, and root and shoot length in field experiments. Among the 15 isolates, inoculation with *Bacillus* sp. strain 4 (DR4) led to the highest increase in biomass production, yield and root and shoot length.

INTRODUCTION

Sugarcane (*Saccharum officinarum L.*) is one of the most important industrial agricultural crops of Pakistan and cultivated in more than 100 tropical and subtropical countries, providing 75% of the world sugar and being the main source of a range of additional products such as bio ethanol and some natural pharmaceutical products [1-4]. Plant growth promoting bacteria (PGPB) are free-living soil bacteria that successfully colonize the rhizosphere of plant roots, and enhance the growth and yield of plants when applied to the seed or crops roots [5, 6]. The rhizosphere of sugarcane plants has been shown to bear a diversity of PGPB, several of which have been isolated and inoculated in wheat, chickpea and other crops. Major bacterial taxa present in the sugarcane rhizosphere are *Bacillus*, *Pseudomonas*, *Enterobacter*, *Burkholderia*, *Acetobacter*, and *Azotobacter*. Several of these have been shown to act as PGPB with relevant multi-trait characteristics such as indole acetic acid production (IAA), phosphate solubilization and biological nitrogen fixation (BNF) [7-12]. The genus *Bacillus* is one of major groups of PGPB, with members being present in the rhizosphere of many crops and characterized for their capacity for P-solubilization, IAA production and major contribution to plant growth and development in many crops such as wheat, soyabean and cabbage [13-17]. Members of the genus *Burkholderia* comprise another major group of PGPB with multi-trait characteristics, and most studied strains have been shown to have positive effects on the growth of a broad variety of crops like tomato, onion, potato, grapevine, maize and Arabidopsis [18-22]. Together with previously mentioned genera, members of the genus *Pseudomonas* are among the best studied, most diverse and ecologically significant PGPB, being characterized as phosphate solubilizing bacteria (PSB), and with the capacity for IAA production, and several other activities with positive roles in increasing plant growth and yield in wheat and a range of other crops [23-25]. Phosphorus (P) is the 2nd major plant nutrient required for normal plant growth, however, Pakistani soils in general have only 80–90% of the P-content required for normal plant function [26, 27]. To this end, PSB can solubilize insoluble phosphate to make it available for plant roots to be absorbed [28]. PSB can be identified and characterized by halo zone formation in Pikovskaya agar medium [29]. IAA is an essential and versatile plant hormone required throughout the life of a plant, playing a major role in plant development, and the ability to produce IAA has a wide occurrence among PGPB [30-32]. Nitrogen (N) is another major nutrient required in large amounts. Various PGPB are able to fix molecular nitrogen. One of the characteristic molecular determinants of this activity is the *nifH* gene that is part of the gene cluster encoding the enzyme nitrogenase catalyzing the reduction of atmospheric nitrogen N_2 to ammonium (NH_4^+) [33, 34].

Rice, wheat, maize, and barley are the most important crops cultivated in the world in the tropical and subtropical zones but also in the temperate zones, and on a variety of soils, i.e., clay loam or loam-textured soil. More than 50% of the world population's energy intake occurs through these crops. Among these crops, wheat is the most important one. It is used in a broad

range of products for human consumption and contains 60%–68% starch, 8%–15% protein, 1.5%–2% fat, 2%–2.5% cellulose, and 1.5%–2% minerals [35-38]. In order to allow for the cultivation of wheat in a large area for satisfying the global dietary requirement, chemical fertilizer has been used to increase crop yield, with adverse effects on the environment. To this end, PGPB can provide an alternative, more sustainable means through a less adverse effect on the environment, reduction of the need for chemical fertilization and maintenance yield of wheat [39-43]. To this end, PGPB isolated from sugarcane have been shown to be effective in various other crops [43-47], and might thus also be of interest for wheat cultivation.

In the present study we therefore aimed to isolate PGPB from sugarcane having potential to act as biofertilizer based on initial screening for IAA production, phosphate solubilization and BNF potential. Subsequently selected strains were tested as inocula on wheat plants, assessing their effect in microplot field experiments under natural conditions.

MATERIALS AND METHODS

Isolation of bacteria from the rhizosphere of sugarcane

Soil samples were collected from the rhizosphere of sugarcane plants that were grown in microplots. Sampling was done after ten months of growth. Representative soil samples along with some fine roots from the rhizosphere were collected and stored at 4 °C and used for further studies. These rhizosphere samples were used for preparation of 10 × serial dilutions up to 10^{-6} in saline solution (0.89% NaCl). From each dilution, 100 µL were spread on LB and Pikovskaya agar plates as described previously (Chapter 2). The plates were incubated at 30 °C for one week, bacterial colonies were recorded on the bases of difference in their morphology (color shape and size), and colony forming units (cfu/g) of each dominant and different bacterial colony type were counted. Colonies on Pikovskaya agar plates with clear halo zone were considered positive for phosphate solubilization. Bacterial colonies were further purified by continuous streaking on fresh plates containing the respective growth medium. Isolates were also cryopreserved at -80 °C in the presence of 20% glycerol.

Quantitative estimation of phosphate solubilization by bacteria

In order to confirm their phosphate solubilization phenotype, bacterial isolates selected from Pikovskaya plates were grown in LB broth medium at 30 ºC for 48 h with shaking (300 rpm). From this culture 50 µL were spotted on Pikovskaya agar plates. Plates were sealed with para-film and incubated at 30 °C for 2 weeks. Bacterial colonies with clear halo zone formation were selected and considered as positive strains for phosphate solubilization. Quantitative estimation of phosphate solubilization by bacteria was done by the molybdate blue colorimetric method according to standard protocols [48].

IAA production by bacterial isolates

For quantification of IAA production, bacterial strains were grown in LB broth for one week at 30 °C in a shaker. After incubation for one week, cell-free culture medium was obtained by centrifugation at 4000 × g, for 15 min, pH was adjusted to 2.8 with HCl (1N), and further sample preparation and analysis on HPLC was followed according to standard protocols [48, 49].

DNA extraction from pure cultures of bacterial isolates

For identification of bacterial isolates by PCR amplified 16S rRNA gene sequence analysis, DNA from pure bacterial cultures was extracted by the CTAB method with minor modifications and DNA extraction was achieved according to standard protocols [49, 50].

Identification of bacterial isolates

For identification of isolates, their 16S rRNA gene was amplified by PCR using conserved primers PA and PH according to standard protocols [51]. PCR products were sent to GATC biotech (Cologne, Germany) for Sanger sequencing. The obtained partial 16S rRNA gene sequences were trimmed (Bio-Edit) and identified through BLAST search at NCBI (National Centre for Biotechnology Information; www.ncbi.nlm.nhi.gov). Sequences were deposited to Gen Bank and accession numbers are given in Table 1. Phylogenetic trees were constructed by using 16S rRNA gene sequences of bacterial strains from sugarcane. These sequences were subjected to multiple alignment with publicly available 16S rRNA gene sequences of closely related strains and type strains by MUSCLE and phylogenetic analysis was performed using software MEGA 7. Distances were computed using the Poisson correction method, and phylogenetic trees were constructed using the Neighbor-Joining method [52-54].

Wheat field experiments

After initial screening (IAA production, phosphate solubilization and BNF potential), 15 representative strains belonging to three genera, i.e. *Bacillus, Pseudomonas* and *Burkholderia*, were selected for inoculation experiments. The experiment was laid out in a Randomized Complete Block Design (RCBD) with 15 treatments that were done in duplicate. One treatment was a non-inoculated control. Seeds of wheat plants (Sehar) were surface-sterilized with 0.1% NaOCl for 5 min, followed by washing in sterilized distilled water. Seeds were sown in microplots (1.2 × 1.2 m) in a net house with ambient conditions with respect to temperature and moisture in their growing season. The plant to plant distance was kept at 18 cm, and a row to row distance of 36 cm was maintained. Less than half the recommended fertilizer dose of nitrogen was supplied as urea, and phosphorus and potassium were applied at 50% of recommended fertilizer doses (recommended doses for NPK are 120, 100 and 70 kg ha^{-1}, respectively) as single super phosphate (SSP) and KCl, respectively, to all treatments. For inoculation of plants (wheat) bacterial cultures were grown in LB agar broth (50 ml) at 30 ºC

for 48 h and centrifuged at 4000 g for 10 min. The resulting cell pellet was washed with 0.8% saline solution and re-suspended in 100 ml saline. One ml of bacterial suspension was applied directly near to the root system. After harvesting, data were recorded for each treatment as root length, shoot length, dry weight and yield.

Statistical analysis

The effect of different treatments on different growth parameters of wheat in the microplot experiment were determined through analysis of variance (ANOVA) using STATISTIX 8.1 software. Means were compared by applying least significant difference test (LSD) at alpha 0.05 for all parameters.

RESULTS

Morphology, identification and phylogenetic analysis

Samples were collected from the rhizosphere of sugarcane in 2016, and bacterial strains were purified in nutrient agar plates, followed by initial screening for plant growth promoting properties in the laboratory (data not shown). After that genomic DNA was extracted from pure cultures for the amplification of the 16S rRNA gene using conserved primers. Identification by 16S rRNA gene sequence analysis showed that bacterial strains isolated from the rhizosphere of sugarcane belonged to three major genera, namely *Bacillus*, *Pseudomonas*, and *Burkholderia*, with 16S rRNA gene sequence identities with type strains ranging between 98 – 100% (Table 1).

Furthermore, in order to verify the phylogenetic affiliation of obtained sugarcane rhizosphere isolates, phylogenetic trees were constructed using 16S rRNA gene sequences of the bacterial strains along with closely related sequences downloaded from NCBI, sequence of type and novel strains reported as PGPB (Fig. 1A-C).

Table 1. Identification of bacterial isolates based on 16S rRNA gene sequence analysis and colony morphology.

N0	Isolate code	Organism identified	Accession Number	Closest type strain in NCBI data base	16S rRNA identity (%)	Colony morphology
1	CDRS2	*Bacillus* 1	MK250054	*Bacillus licheniformis* NR_118996	98%	Off white Rough edge
2	CDRS3	*Bacillus* 2	MK230983	*Bacillus licheniformis* NR_118996	98%	Off white Smooth
3	DR3	*Bacillus* 3	MK250056	*Bacillus tequilensis* NR_10491	98%	Off white
4	DR4	*Bacillus* 4	MK250416	*Bacillus pumilus* NR_043242	100%	Off white
5	ATR1	*Bacillus* 5	MK250420	*Bacillus cereus* NR_074540	100%	Off white
6	PD4	*Pseudomonas* 1	MK230969	*Pseudomonas monteilii*	99%	Light off white

				NR_114224		
7	PD5	*Pseudomonas* 2	MK230970	*Pseudomonas monteilii* NR_112073	99%	Light off white
8	PAT1	*Pseudomonas* 3	MK255028	*Pseudomonas oryzihabitans* NR_115005	99%	Light off white
9	ATR4	*Pseudomonas* 4	MK230976	*Pseudomonas oryzihabitans* NR_114041	99%	Off white
10	ATL2	*Pseudomonas* 5	MK230978	*Pseudomonas oryzihabitans* NR_117269	99%	Off white
11	CPD1	*Burkholderia* 1	MK250047	*Burkholderia lata* NR_102890	98%	Whitish off white
12	CPD2	*Burkholderia* 2	MK250048	*Burkholderia contaminans* NR_104978	99%	Whitish off white
13	CPD5	*Burkholderia* 3	MK250039	*Burkholderia contaminans* NR_104978	99%	Whitish off white
14	PAT2	*Burkholderia* 4	MK250870	*Burkholderia gladioli* NR_113629	99%	Whitish off white
15	CPAT3	*Burkholderia* 5	MK256295	*Burkholderia gladioli* NR_117553	98%	Whitish off white

Phenotypic characterization of bacterial isolates

IAA production by the 15 bacterial sugarcane rhizosphere isolates was determined by growth in LB broth medium supplemented with tryptophan as a precursor for IAA biosynthesis and subsequent IAA analysis by HPLC. Among the five *Bacillus* strains, *Bacillus* sp. strain CDRS3 showed the maximum amount of IAA in the medium (10.6 µg/mL) in growth medium followed by *Bacillus* sp. strain DR4 (10.04 µg/mL), *Bacillus* sp. strain ATR1, (4.2 µg/mL), *Bacillus* sp. strain CDRS2 (1.6 µg/mL) and *Bacillus* sp. strain DR3 (1.3 µg/mL). IAA production was also observed in all five strains of *Pseudomonas* with strain PD5 producing the highest amount of IAA (6.92 µg/mL) in the medium, followed by strain ATR4 (6.3 µg/mL), strain ATL2 (5.8 µg/mL), strain PAT1 (4.4 µg/mL) and strain PD4 (1.2 µg/mL. Among the five strains of *Burkholderia,* strain CPD5 yielded the highest concentration of IAA in the medium (10.3 µg/mL), followed by strain PAT2 (7.6 µg/mL), strain CPD1 (6.4 µg/mL), strain CPAT3 (5.9 µg/mL) and strain 2 CPD2 (5.3 µg/mL) (Table 2).

Phosphate solubilization by bacterial strains was assessed on Pikovskaya agar plates containing insoluble tri-calcium phosphate (TCP). Halo zone formation was taken as indication of phosphate solubilization ability and was observed in all 15 strains. Phosphate solubilization was further quantified. Among the strains of *Bacillus,* strain CDRS3 showed highest activity (9.3 µg/mL), followed by strain DR4 (9 µg/mL), strain DR3 (6.2 µg/mL), strain ATR1 (5.2 µg/mL) and strain CDRS2 (4.3 µg/mL). Phosphate solubilization in members of the genus *Pseudomonas* ranged between 9 µg/mL for strain PD5 to 3.9 µg/mL for strain PAT1. Phosphate solubilization in *Burkholderia* spp. strains was highest for strain CPD1 (14.8 µg/mL) and lowest for strain CPD2 (5.4 µg/mL).

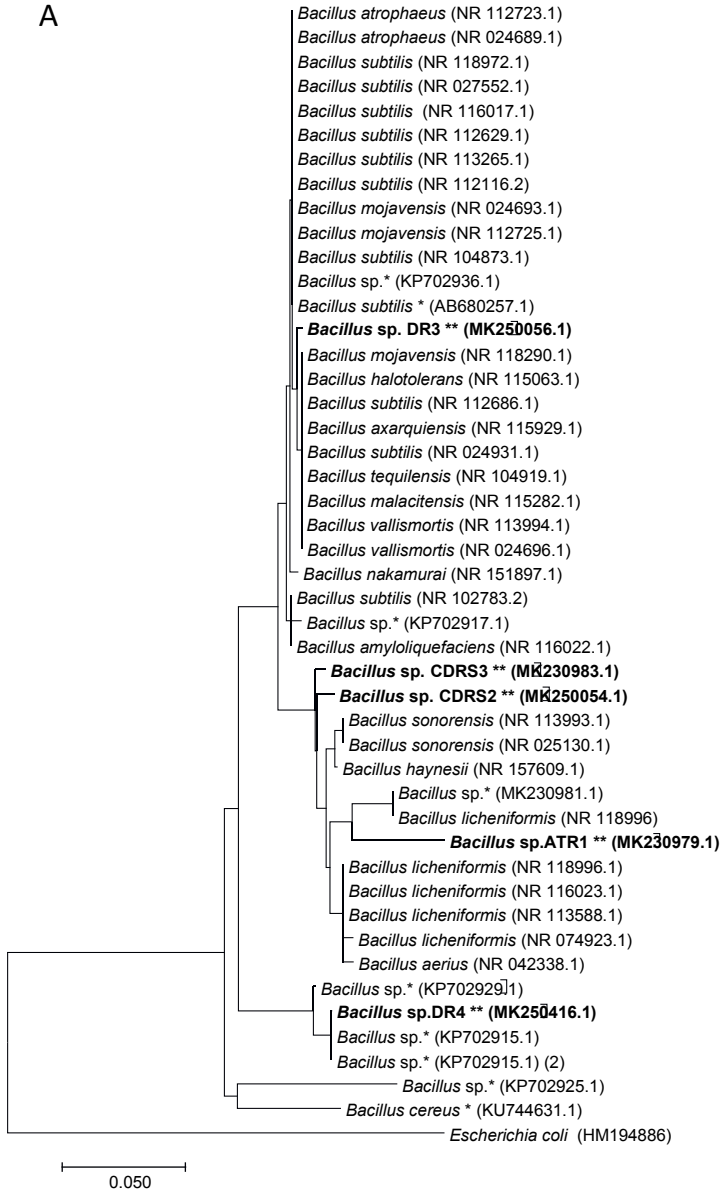
Molecular identification of BNF potential was achieved by PCR amplification of a fragment of approximately 360 bp of the *nifH* gene. Among the 15 isolates, a total of nine strains tested

positive, including three strains of *Bacillus* (CDRS2, CDRS3, DR4) and two strains of *Pseudomonas* (PD4, ATR4). Among the *Burkholderia* isolates, only strain CPD2 tested negative (Table 2).

Table 2. Production of IAA, phosphate solubilization and nitrogen fixation by the bacterial isolates from sugarcane.

No	Bacterial strain	IAA (µg/mL)*	Available Phosphorus (µg/mL)**	N fixation
1	*Bacillus* sp. CDRS2	1.6 ± 0.06	4.3 ± 0.41	Positive
2	*Bacillus* sp. CDRS3	10.6 ± 0.25	9.3 ± 0.5	Positive
3	*Bacillus* sp. DR3	1.3 ± 0.15	6.2 ± 0.2	Negative
4	*Bacillus* sp. DR4	10.04 ± 0.05	9 ± 0.3	Positive
5	*Bacillus* sp. ATR1	4.2 ± 0.2	5.2 ± 0.2	Negative
6	*Pseudomonas* sp. PD4	1.2 ± 0.3	6.3 ± 0.25	Positive
7	*Pseudomonas* sp. PD5	6.92 ± 0.02	9 ± 0.11	Positive
8	*Pseudomonas* sp. PAT1	4.4 ± 0.4	3.9 ± 0.15	Negative
9	*Pseudomonas* sp. ATR4	6.3 ± 0.1	7.4 ± 0.3	Negative
10	*Pseudomonas* sp. ATL2	5.8 ± 0.3	4.6 ± 0.2	Negative
11	*Burkholderia* sp. CPD1	6.4 ± 0.41	14.8 ± 0.3	Positive
12	*Burkholderia* sp. CPD2	5.3 ± 0.41	5.4 ± 0.4	Negative
13	*Burkholderia* sp. CPD5	10.3 ± 0.3	5.6 ± 0.2	Positive
14	*Burkholderia* sp. PAT2	7.6 ± 0.2	8.8 ± 0.3	Positive
15	*Burkholderia* sp. CPAT3	5.9 ± 0.3	8.2 ± 0.2	Positive

*Bacterial cultures were grown in LB containing tryptophan as precursor of IAA biosynthesis; **Bacterial cultures were grown in Pikovskaya medium containing g TCP as insoluble phosphorous and *nifH* gene amplification was taken as indicator for N fixation potential. The values given are an average of three replicates with standard deviation.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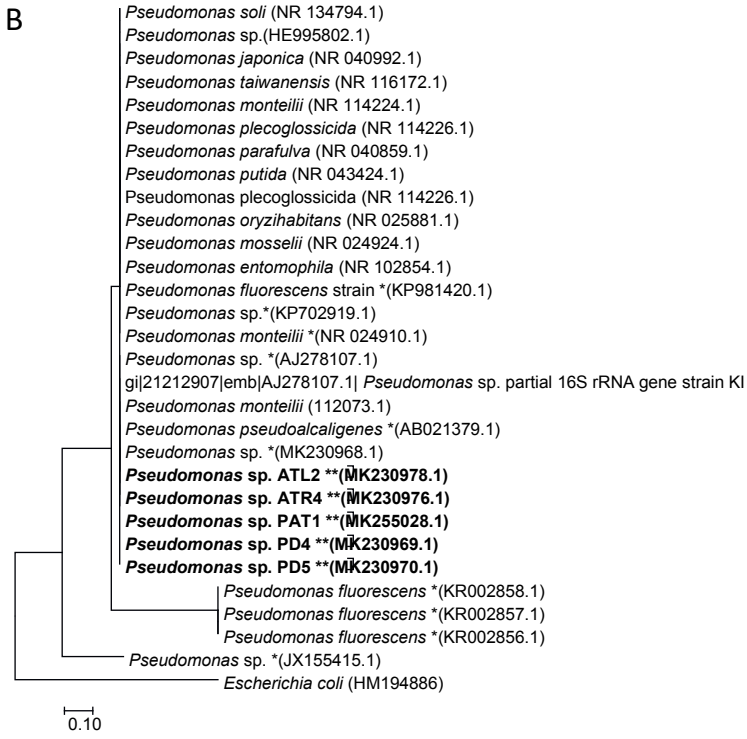
Bacillus atrophaeus (NR 024689.1)
Bacillus subtilis (NR 118972.1)
Bacillus subtilis (NR 027552.1)
Bacillus subtilis (NR 116017.1)
Bacillus subtilis (NR 112629.1)
Bacillus subtilis (NR 113265.1)
Bacillus subtilis (NR 112116.2)
Bacillus mojavensis (NR 024693.1)
Bacillus mojavensis (NR 112725.1)
Bacillus subtilis (NR 104873.1)
Bacillus sp.* (KP702936.1)
Bacillus subtilis * (AB680257.1)
Bacillus sp. DR3 ** (MK250056.1)
Bacillus mojavensis (NR 118290.1)
Bacillus halotolerans (NR 115063.1)
Bacillus subtilis (NR 112686.1)
Bacillus axarquiensis (NR 115929.1)
Bacillus subtilis (NR 024931.1)
Bacillus tequilensis (NR 104919.1)
Bacillus malacitensis (NR 115282.1)
Bacillus vallismortis (NR 113994.1)
Bacillus vallismortis (NR 024696.1)
Bacillus nakamurai (NR 151897.1)
Bacillus subtilis (NR 102783.2)
Bacillus sp.* (KP702917.1)
Bacillus amyloliquefaciens (NR 116022.1)
Bacillus sp. CDRS3 ** (MK230983.1)
Bacillus sp. CDRS2 ** (MK250054.1)
Bacillus sonorensis (NR 113993.1)
Bacillus sonorensis (NR 025130.1)
Bacillus haynesii (NR 157609.1)
Bacillus sp.* (MK230981.1)
Bacillus licheniformis (NR 118996)
Bacillus sp.ATR1 ** (MK230979.1)
Bacillus licheniformis (NR 118996.1)
Bacillus licheniformis (NR 116023.1)
Bacillus licheniformis (NR 113588.1)
Bacillus licheniformis (NR 074923.1)
Bacillus aerius (NR 042338.1)
Bacillus sp.* (KP702929.1)
Bacillus sp.DR4 ** (MK250416.1)
Bacillus sp.* (KP702915.1)
Bacillus sp.* (KP702915.1) (2)
Bacillus sp.* (KP702925.1)
Bacillus cereus * (KU744631.1)
Escherichia coli (HM194886)
0.050


B
Pseudomonas soli (NR 134794.1)
Pseudomonas sp.(HE995802.1)
Pseudomonas japonica (NR 040992.1)
Pseudomonas taiwanensis (NR 116172.1)
Pseudomonas monteilii (NR 114224.1)
Pseudomonas plecoglossicida (NR 114226.1)
Pseudomonas parafulva (NR 040859.1)
Pseudomonas putida (NR 043424.1)
Pseudomonas plecoglossicida (NR 114226.1)
Pseudomonas oryzihabitans (NR 025881.1)
Pseudomonas mosselii (NR 024924.1)
Pseudomonas entomophila (NR 102854.1)
Pseudomonas fluorescens strain *(KP981420.1)
Pseudomonas sp.*(KP702919.1)
Pseudomonas monteilii *(NR 024910.1)
Pseudomonas sp. *(AJ278107.1)
gi|21212907|emb|AJ278107.1| *Pseudomonas* sp. partial 16S rRNA gene strain KI
Pseudomonas monteilii (112073.1)
Pseudomonas pseudoalcaligenes *(AB021379.1)
Pseudomonas sp. *(MK230968.1)
Pseudomonas sp. ATL2 **(MK230978.1)
Pseudomonas sp. ATR4 **(MK230976.1)
Pseudomonas sp. PAT1 **(MK255028.1)
Pseudomonas sp. PD4 **(MK230969.1)
Pseudomonas sp. PD5 **(MK230970.1)
Pseudomonas fluorescens *(KR002858.1)
Pseudomonas fluorescens *(KR002857.1)
Pseudomonas fluorescens *(KR002856.1)
Pseudomonas sp. *(JX155415.1)
Escherichia coli (HM194886)
0.10

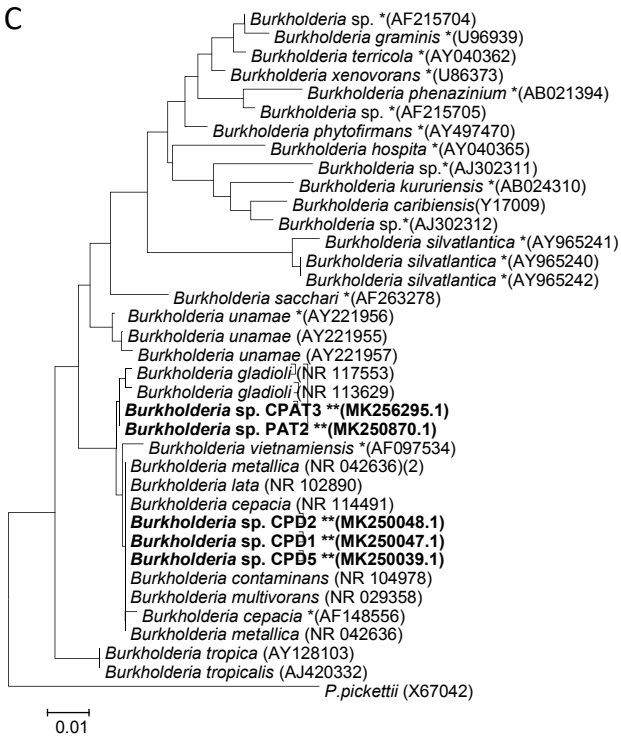


Figure 1. Phylogenetic trees showing the relationships of different strains of *Bacillus* (**A**), *Pseudomonas* (**B**), and *Burkholderia* (**C**), including strains isolated in this study (Bold **) as well as most closely related type stains (*) and other closely relative sequences available at NCBI, based on 16S rDNA sequence comparisons. 16S rRNA gene sequences were aligned by using MUSCLE W. The tree topology was inferred by the neighbour-joining method and the phylogenetic analysis was conducted by using MEGA version 7 and Fig tree.

Wheat growth promoting effects of sugarcane-derived bacteria

Microplot experiments were conducted to evaluate the effect of bacterial isolates on wheat plants. In the present study 15 isolates obtained from the rhizosphere of sugarcane and belonging to three major genera of PGPB, i.e. *Bacillus*, *Pseudomonas* and *Burkholderia*, were selected on the basis of initial phenotypic screening for IAA production, phosphate solubilization and BNF potential. These strains were then tested for their growth promotion traits in microplot experiments under natural conditions. Experiments were performed on wheat plants with inoculation of isolates, with non-inoculated plants as negative control. Wheat plants were harvested at maturity stage at four months after sowing. During harvesting plants were properly uprooted with their entire root system and washed to remove mud, and roots were air-dried to remove excessive water. Comparison of inoculated plants with non-inoculated control plants showed significant ($p \leq 0.05$) improvement in several plant growth parameters such as root length, shoot length, root dry weight, shoot dry weight and number of tillers per plants (Table 3).

Overall, highest increase in wheat plant growth parameters, except for root dry weight, was observed for plots inoculated with *Bacillus* sp. DR4, that resulted in increased shoot dry weight (3.07 g), shoot length (80.60 cm), root length (15.26 cm) and number of tillers (4.6). With respect to root dry weight, only plants inoculated with *Pseudomonas* sp. PD5 showed a significant increase as compared to non-inoculated controls (0,83 g vs. 0,61 g).

Inoculation with *Bacillus* sp. CDRS2 showed significant improvement ($p \leq 0.05$) in shoot dry weight (2.27 g), shoot length (75.66 cm) and root length (11.60 cm) as compared to control plants. Inoculation with *Bacillus* sp. CDRS3 showed significant improvement in in shoot dry weight (2.77 g), shoot length (80.13 cm), root length (14 cm) and number of tillers (4.86), whereas plants inoculated with *Bacillus* sp. DR3 showed increase in shoot dry weight (2.45 g) and root length (10.26 cm). Inoculation with *Bacillus* sp. ATR1 resulted in significant improvements in shoot dry weight (2.70 g), shoot length (77.46 cm) and root length (12.33cm) as compared to control plants.

Inoculation with *Pseudomonas* sp. PD4 showed significantly higher shoot dry weight (2.39 g) and root length (12.73 cm), whereas plants inoculated with *Pseudomonas* sp. PD5 showed increased shoot dry weight (2.79 g), dry root weight (0.83 g), shoot length (76.80 cm) and root length (13.73 cm). Inoculation with *Pseudomonas* sp. PAT1 resulted in higher shoot dry weight (2.35 g), root length (10.93 cm) and number of tillers (4.13) as compared to control plants. Treatment with *Pseudomonas* sp. ATR4 showed significant improvement in shoot dry weight (2.62g), shoot length (74.86 cm), root length (13 cm) and number of tillers (4.46), whereas wheat plants inoculated with *Pseudomonas* sp. ATL2 only showed improved in shoot dry weight (2.47 g) and root length (10.80 cm).

Amongst the five strains of *Burkholderia*, inoculation with *Burkholderia* sp. CPD1 showed highest improvement in shoot dry weight (2.63 g), shoot length (79.60 cm) and root length

(15.26 cm), as well as an increased number of tillers per plant (4.46) as compared to control plants. Inoculation with *Burkholderia* sp. CPD2 resulted in improved shoot length (75.40 cm), root length (11.33 cm) and number of tillers (4.20), whereas plants inoculated with strain CPD5 showed higher shoot dry weight (2.59 g), shoot length (79.26 cm), root length (12.93 cm), and number of tillers (4.66). Inoculation with *Burkholderia* sp. PAT2 lead to an increase in shoot dry weight (2.40 g), shoot length (78.40 cm) and root length (12.00 cm), while strain CPAT3 resulted in higher shoot dry weight (2.44g), root length (12.13 cm) and number of tillers (4.33) as compared to control plants.

Visual differences between inoculated and non-inoculated plants grown in microplots are also shown in supplementary Figure S1 with respect to differences in root growth and cane development.

Table 3. Effect of bacterial inoculation on root structure and growth of wheat plants.

No	Treatment	Shoot Dry weight (g)	Root dry weight (g)	Shoot length (cm)	Root length (cm)	Tillers per plant
1	*Bacillus* sp. CDRS2	2.27^{EF}	0.61^{B}	75.66^{CD}	11.60^{DEFG}	3.87^{DEFG}
2	*Bacillus* sp. CDRS3	2.77^{B}	0.73^{AB}	80.13^{AB}	14^{AB}	4.86^{A}
3	*Bacillus* sp. DR3	2.45^{CDE}	0.61^{B}	71.93^{EF}	10.26^{HI}	3.8^{DEFG}
4	*Bacillus* sp. DR4	3.07^{A}	0.79^{B}	80.60^{A}	15.26^{A}	4.6^{AB}
5	*Bacillus* sp. ATR1	2.70^{B}	0.63^{B}	77.46^{ABCD}	12.33^{CDE}	4.0^{CDEFG}
6	*Pseudomonas* sp. PD4	2.39^{DE}	0.67^{B}	71.26^{F}	12.73^{BCD}	3.20^{H}
7	*Pseudomonas* sp. PD5	2.79^{B}	0.83^{A}	76.80^{BCD}	13.73^{B}	4.00^{CDEFG}
8	*Pseudomonas* sp. PAT1	2.35^{E}	0.69^{B}	71.26^{F}	10.93^{FGH}	4.13^{BCDEF}
9	*Pseudomonas* sp. ATR4	2.62^{BC}	0.77^{B}	74.86^{DE}	13^{BC}	4.46^{ABC}
10	*Pseudomonas* sp. ATL2	2.47^{CDE}	0.63^{B}	71.13^{F}	10.80^{GH}	3.60^{FGH}
11	*Burkholderia* sp. CPD1	2.63^{BC}	0.74^{B}	79.60^{AB}	15.26^{A}	4.46^{ABC}
12	*Burkholderia* sp. CPD2	2.33^{EF}	0.65^{B}	75.40^{CD}	11.33^{EFGH}	4.20^{BCDE}
13	*Burkholderia* sp. CPD5	2.59^{BCD}	0.72^{B}	79.26^{AB}	12.93^{BC}	4.66^{AB}
14	*Burkholderia* sp. PAT2	2.4^{DE}	0.69^{B}	78.40^{ABC}	12^{CDEFG}	3.73^{EFGH}
15	*Burkholderia* sp. CPAT3	2.44^{CDE}	0.68^{B}	74.26^{DEF}	12.13^{CDEF}	4.33^{ABCD}
16	Control	2.12^{F}	0.61^{B}	71.07^{F}	9.13^{I}	3.46^{GH}
	SEM	0.10	0.17	1.71	0.66	0.30

Plants were grown in microplots in randomized complete block design with 15 replicates and uprooted along with root system after growth for four months. Values are an average of 15 replicates. Least significant difference (LSD) test was applied at LSD ($p \leq 0.05$). Superscript letters indicate statistically different values per column. SEM, standard error of means.

DISCUSSION

In the present study a number of different types of isolates belonging to three major genera were isolated from the rhizosphere of sugarcane, characterized in terms of phylogeny and plant growth promoting properties *in vitro*, after which their potential as biofertilizer of wheat plants was assessed in microplot experiments under field conditions. To this end, a total of 15 isolates belong to the genera *Bacillus, Pseudomonas and Burkholderia* were selected after initial screening. Similarly, several previous studies reported on the isolation and characterization of strains of these three genera, including their effect on the growth of wheat and other crops [11, 48, 55, 56].

Following the major aim of the present study, i.e. to find strains through *in vitro* and *in vivo* experimentation that can act as biofertilizer to make agriculture more sustainable and environment friendly, five strains of each of the three genera were selected for testing as potential PGPB in microplots of wheat under natural conditions. To this end, all strains showed significant improvement in at least two different plant growth parameters.

In line with observations shown here, previous research also reported on similar phenotypic traits of *Pseudomonas* spp. strains, including IAA production, phosphate solubilization and plant growth promotion traits, as well as the fact that such strains are often found in the rhizosphere [57, 58]. Similarly, previous studies also showed isolation of *Bacillus* spp. as PGPB, which colonized plant roots and possessed plant growth promoting traits in different crops [58-60]. Furthermore, *Burkholderia* spp. are versatile organisms that show multiple traits for plant growth promotion [61], and that have been found associated with sugarcane and a variety of other crops [62-64].

The present study showed that all 15 tested isolates were able to produce IAA in pure culture. IAA is a phytohormone, which plays a central role in plant growth in rhizosphere and phyllosphere of many crops, where it functions as a regulator of many biological processes, and IAA production has been reported as one of the important mechanisms of action of PGPB [65-68]. When comparing the three different genera tested here for their ability to produce IAA, *Burkholderia* spp. isolates produced on average the highest amount (7,1 ± 1,98; Table 2), followed by the other two genera *Bacillus* (3,8 × 3,99) and *Pseudomonas* (4,9 ± 2,28). This is in line with earlier studies that showed good production of IAA by members of the genera *Burkholderia* [69], *Bacillus* [70-73] and *Pseudomonas* [74-80]. In addition to IAA production, we also screen isolates for their ability to solubilize phosphate. It is well established that phosphate solubilization by bacteria is an important mechanism for plant growth [10]. Through the activity of PSB, phosphorus which is present in rhizosphere and soil near to the plant root in unavailable form can be made available to plants. Previous studies explored this idea and reported the use of PSB as environmental friendly approach for sustainable agricultural development in many field crops (sugarcane, cotton, rice, chickpea) and vegetable crops

(tomato, brinjal, okra, and chili), also as an alternative way to reduce costs related to the application of chemical phosphorus fertilizer [26, 28, 40, 68, 81, 82]. As for IAA production, also phosphate solubilization was observed for all tested strains, with members of *Burkholderia* showing highest average values (8,5 ± 3,80, Table 2) followed by strains of *Bacillus* (6,8 ± 2,55) and *Pseudomonas* (6,2 ±2,07). Previous results also showed that members of the genus *Burkholderia* act as PSB in various field crops [63, 64]. Similar results were previously reported for strains belonging to the genera *Bacillus* [83, 84] and *Pseudomonas* [40, 58, 85, 86]. As a third phenotypic characteristic relevant for plant growth promotion, in the present study we also aimed to predict BNF potential based on the molecular detection of *nifH* gene. A large body of literature reported that nitrogen is major element for normal growth of crops and required in large quantities. To this end, BNF by PGPB can aid in the reduction of nitrogen chemical fertilizer [87-93]. In the present study, the majority of isolates tested positive for the *nifH* gene, including two, three and four strains of *Pseudomonas, Bacillus* and *Burkholderia*, respectively. Previous studies showed that strains belonging to *Pseudomonas* and *Bacillus* act as nitrogen fixer in wheat, sugarcane, cucumber and a number of other crops [74-79, 94, 95]. Similarly, previous studies also showed that *Burkholderia* spp. isolates have the ability to fix nitrogen, promoting the growth of sugarcane, maize and other crops [96-98]. Use of PGPB with the aim of improving nutrient availability and plant growth has been studied from previous centuries [11]. In the present study 15 strains were selected and used as inoculants for wheat, and five complementary measures were taken to assess plant growth, including shoot and root dry weight, root and shoot length and the number of tillers per plant. Significant improvement of at least two of these measures of plant growth was observed for all 15 strains. To this end, our results are in line with those of earlier studies that also showed that members of the genus *Bacillus* and related strains promoted plant growth in sugarcane resulting in increased root shoot length and biomass (dry weight of root and shoot), and in other crops [83, 84, 99]. Similarly, previous studies showed PGPB activity in a number of crops for strains of *Pseudomonas* [40, 58, 85, 86] and *Burkholderia* [62-64, 96, 100, 101].

Conclusion

In the present study, a total of 15 bacterial strains were isolated and selected for downstream experiments based on *in vitro* functionality screening on the base of IAA production, phosphate solubilization and BNF potential. Strains were identified as members of the genera *Bacillus, Pseudomonas and Burkholderia* on the bases of their 16S rRNA gene sequences and confirmed as plant growth promoting bacterial inoculants in wheat experiments. PGPBs identified in the present study may be utilized as biofertilizer in sugarcane, wheat and other crops to make agriculture more sustainable through reduction of the need for chemical fertilizers.

References

1. Fischer, D., et al., Molecular characterisation of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (*Saccharum sp*.). Plant and Soil, 2012. 356(1-2): p. 83-99.
2. Rasul, F., et al., Enhanced transformation efficiency of *Saccharum officinarum* by vacuum infiltration assisted *Agrobacterium*-mediated transformation. International Journal of Agriculture and Biology, 2014. 16(6).
3. Nasir, I.A., et al., Herbicide-tolerant sugarcane (*Saccharum officinarum L*.) plants: an unconventional method of weed removal. Turkish Journal of Biology, 2014. 38(4): p. 439-449.
4. Goldemberg, J., S.T. Coelho, and P. Guardabassi, The sustainability of ethanol production from sugarcane. Energy Policy, 2008. 36(6): p. 2086-2097.
5. Majeed, A., et al., Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Frontiers in Microbiology, 2015. 6(198).
6. Kumar, A., B.R. Maurya, and R. Raghuwanshi, Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (*Triticum aestivum L*.). Biocatalysis and Agricultural Biotechnology, 2014. 3(4): p. 121-128.
7. Khan, N., A. Bano, and M.A. Babar, The root growth of wheat plants, the water conservation and fertility status of sandy soils influenced by plant growth promoting rhizobacteria. Symbiosis, 2017. 72(3): p. 195-205.
8. Zaheer, A., et al., Isolation, characterization, and effect of phosphate-zinc-solubilizing bacterial strains on chickpea (*Cicer arietinum L*.) growth. Saudi Journal of Biological Sciences, 2019.
9. Idris, E.E., et al., Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Molecular Plant-Microbe Interactions, 2007. 20(6): p. 619-626.
10. Awais, M., et al., Isolation, characterization and association among phosphate solubilizing bacteria from sugarcane rhizosphere. Cytology and Genetics, 2019. 53(1): p. 86-95.
11. Modi, K. and P. Patel, Isolation and characterization of plant growth promoting rhizobacteria associated with *Saccharum officinarum L*. Current Synthetic and Systems Biology, 2017. 5: p. 2332-0737.
12. Muthukumarasamy, R., et al., Isolation of bacterial strains possessing nitrogen-fixation, phosphate and potassium-solubilization and their inoculation effects on sugarcane. Indian Journal of Experimental Biology. 2017. 55(3): p. 161-70..
13. Tiwari, S., V. Prasad, and C. Lata, Chapter 3 - *Bacillus*: Plant growth promoting bacteria for sustainable agriculture and environment, in new and future developments in Microbial Biotechnology and Bioengineering, J.S. Singh and D.P. Singh, Editors. 2019, Elsevier. p. 43-55.
14. Akinrinlola, R.J., et al., Evaluation of bacillus strains for plant growth promotion and predictability of efficacy by in vitro physiological traits. International Journal of Microbiology, 2018. 2018: p. 11.
15. Nguyen, M.L., et al., Biostimulant effects of *Bacillus* strains on wheat from in vitro towards field conditions are modulated by nitrogen supply. Journal of Plant Nutrition and Soil Science. 2019. 182: p. 325-334.
16. Ku, Y., et al., Root colonization and growth promotion of soybean, wheat and Chinese cabbage by *Bacillus cereus* YL6. PLOS ONE, 2018. 13(11): p. e0200181.

17. Tahir, M., et al., Regulation of antioxidant production, ion uptake and productivity in potato (*Solanum tuberosum L.*) plant inoculated with growth promoting salt tolerant *Bacillus* strains. Ecotoxicology and Environmental Safety, 2019. 178: p. 33-42.
18. Naveed, M., et al., Drought stress amelioration in wheat through inoculation with Burkholderia phytofirmans strain PsJN. Plant Growth Regulation, 2014. 73(2): p. 121-131.
19. Frommel, M.I., J. Nowak, and G. Lazarovits, Growth enhancement and developmental modifications of in vitro grown potato (*Solanum tuberosum* spp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiology, 1991. 96(3): p. 928.
20. Tallapragada, P., R. Dikshit, and S. Seshagiri, Influence of *Rhizophagus* spp. and *Burkholderia seminalis* on the growth of Tomato (*Lycopersicon esculatum*) and Bell Pepper (*Capsicum annuum*) under drought stress. Communications in Soil Science and Plant Analysis, 2016. 47(17): p. 1975-1984.
21. Fan, X., et al., Soil inoculation with *Burkholderia* sp. LD-11 has positive effect on water-use efficiency in inbred lines of maize. Plant and Soil, 2015. 390(1): p. 337-349.
22. Su, F., et al., *Burkholderia phytofirmans* PsJN reduces impact of freezing temperatures on photosynthesis in Arabidopsis thaliana. Frontiers in Plant Science, 2015. 6(810).
23. Yadav, S., et al., Genetic and functional diversity of fluorescent *Pseudomonas* from rhizospheric soils of wheat crop. Journal of Basic Microbiology, 2014. 54(5): p. 425-437.
24. Ul Hassan, T. and A. Bano, Construction of IAA-Deficient Mutants of *Pseudomonas* moraviensis and their comparative effects with wild type strains as bio-inoculant on wheat in saline sodic soil. Geomicrobiology Journal, 2019. 36(4): p. 376-384.
25. Hassan, T.U. and A. Bano, Role of carrier-based biofertilizer in reclamation of saline soil and wheat growth. Archives of Agronomy and Soil Science, 2015. 61(12): p. 1719-1731.
26. Lamizadeh, E., N. Enayatizamir, and H. Motamedi, Isolation and identification of plant growth-promoting rhizobacteria (PGPR) from the rhizosphere of sugarcane in saline and non-saline soil. International Journal of Current Microbiology and Applied Sciences, 2016. 5(10): p. 1072-83.
27. Rafique, M., et al., Enhancement of maize plant growth with inoculation of phosphate-solubilizing bacteria and biochar amendment in soil. Soil Science and Plant Nutrition, 2017: p. 1-10.
28. Awais, M., et al., Isolation, characterization and inter-relationship of phosphate solubilizing bacteria from the rhizosphere of sugarcane and rice. Biocatalysis and Agricultural Biotechnology, 2017. 11: p. 312-321.
29.
30. Abel, S., Auxin is surfacing. ACS Chemical Biology, 2007. 2(6): p. 380-384.
31. Park, J.-E., et al., GH3-mediated auxin homeostasis links growth regulation with stress adaptation response in *Arabidopsis*. Journal of Biological Chemistry, 2007. 282(13): p. 10036-10046.
32. Kazan, K., Auxin and the integration of environmental signals into plant root development. Annals of Botany, 2013. 112(9): p. 1655-1665.
33. Poly, F., L.J. Monrozier, and R. Bally, Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Research in Microbiology, 2001. 152(1): p. 95-103.
34. Qian, H., et al., Improved primers and a pyrosequencing approach recover wide range of nitrogen reductase genes from peatlands.

35. Cassman, K.G., Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. Proceedings of the National Academy of Sciences, 1999. 96(11): p. 5952-5959.
36. Baumann, P., A. Kirchhofer, and U. Schälchli, Sanierung Schwall/Sunk–Strategische Planung. Ein Modul der Vollzugshilfe Renaturierung der Gewässer. Bundesamt für Umwelt, Bern. Umwelt-Vollzug, 2012. 1203: p. 126.
37. Thrupp, L.A., Linking agricultural biodiversity and food security: the valuable role of agrobiodiversity for sustainable agriculture. International Affairs, 2002. 76(2): p. 265-281.
38. Zaidi, A. and S. Khan, interactive effect of rhizotrophic microorganisms on growth, yield, and nutrient uptake of wheat. Journal of Plant Nutrition, 2005. 28(12): p. 2079-2092.
39. Alori, E.T., B.R. Glick, and O.O. Babalola, microbial phosphorus solubilization and its potential for use in sustainable agriculture. Frontiers in Microbiology, 2017. 8: p. 971.
40. Rajwar, J., et al., Comparative phosphate solubilizing efficiency of psychrotolerant Pseudomonas jesenii MP1 and Acinetobacter sp. ST02 against chickpea for sustainable hill agriculture. Biologia, 2018. 73(8): p. 793-802.
41. Govindarajan, M., et al., Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkholderia vietnamiensis*. Plant and Soil, 2006. 280(1): p. 239-252.
42. Hirel, B., et al., The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. Journal of Experimental Botany, 2007. 58(9): p. 2369-2387.
43. Calvo, P., L. Nelson, and J.W. Kloepper, Agricultural uses of plant biostimulants. Plant and Soil, 2014. 383(1): p. 3-41.
44. Bashan, Y., et al., Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant and Soil, 2014. 378(1-2): p. 1-33.
45. Schultz, N., et al., Inoculation of sugarcane with diazotrophic bacteria. Revista Brasileira de Ciência do Solo, 2014. 38(2): p. 407-414.
46. De Oliveira, A.L.M., et al., Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria. Plant and Soil, 2006. 284(1-2): p. 23-32.
47. Muñoz-Rojas, J. and J. Caballero-Mellado, Population dynamics of *Gluconacetobacter diazotrophicus* in sugarcane cultivars and its effect on plant growth. Microbial Ecology, 2003. 46(4): p. 454-464.
48. Tahir, M., et al., Isolation and identification of phosphate solubilizer *Azospirillum*, *Bacillus* and *Enterobacter* strains by 16SrRNA sequence analysis and their effect on growth of wheat ('*Triticum aestivum'L*.). Australian Journal of Crop Science, 2013. 7(9): p. 1284.
49. Qaisrani, M.M., et al., Isolation and identification by 16S rRNA sequence analysis of *Achromobacter*, *Azospirillum* and *Rhodococcus* strains from the rhizosphere of maize and screening for the beneficial effect on plant growth. Pakistan Journal of Agricultural Sciences, 2014. 51(1).
50. Arshad, M., et al., Comparison of rhizospheric bacterial populations and growth promotion of avp1 transgenic and non-transgenic cotton by bacterial inoculations. Journal of Animal and Plant Sciences, 2016. 26(5): p. 1284-1290.
51. Versluis, D., et al., Recovery of previously uncultured bacterial genera from three Mediterranean sponges. Marine Biotechnology, 2017. 19: p. 454-468.

52. Thompson, J.D., D.G. Higgins, and T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 1994. 22(22): p. 4673-4680.
53. Saitou, N. and M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 1987. 4(4): p. 406-425.
54. Kumar, S., et al., MEGA2: molecular evolutionary genetics analysis software. Bioinformatics, 2001. 17(12): p. 1244-1245.
55. Kruasuwan, W. and A. Thamchaipenet, Ddversity of culturable plant growth-promoting bacterial endophytes associated with sugarcane roots and their effect of growth by co-inoculation of diazotrophs and *Actinomycetes*. Journal of Plant Growth Regulation, 2016. 35(4): p. 1074-1087.
56. Da Silveira, A.P.D., et al., Exploitation of new endophytic bacteria and their ability to promote sugarcane growth and nitrogen nutrition. Antonie van Leeuwenhoek, 2019. 112(2): p. 283-295.
57. Srivastava, S., et al., Gene expression profiling through microarray analysis in Arabidopsis thaliana colonized by *Pseudomonas putida* MTCC5279, a plant growth promoting rhizobacterium. Plant signaling & behavior, 2012. 7(2): p. 235-245.
58. Tiwari, S., et al., *Pseudomonas putida* attunes morphophysiological, biochemical and molecular responses in *Cicer arietinum L*. during drought stress and recovery. Plant Physiology and Biochemistry, 2016. 99: p. 108-117.
59. Gamez, R., et al., Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (*Musa acuminata Colla*). Microbiological Research, 2019. 220: p. 12-20.
60. García, J.A.L., et al., Effect of inoculation of *Bacillus licheniformis* on tomato and pepper. Agronomie, 2004. 24(4): p. 169-176.
61. Rombola, T.H., et al., Identification and enzymatic characterization of acid phosphatase from *Burkholderia gladioli*. BMC Research Notes, 2014. 7(1): p. 221.
62. Castro-González, R., et al., High diversity of culturable *Burkholderia* species associated with sugarcane. Plant and Soil, 2011. 345(1): p. 155-169.
63. Bernabeu, P.R., et al., Colonization and plant growth-promotion of tomato by *Burkholderia tropica*. Scientia Horticulturae, 2015. 191: p. 113-120.
64. Tagele, S.B., et al., Effectiveness of multi-trait *Burkholderia contaminans* KNU17BI1 in growth promotion and management of banded leaf and sheath blight in maize seedling. Microbiological Research, 2018. 214: p. 8-18.
65. Shahzad, R., et al., Indoleacetic acid production and plant growth promoting potential of bacterial endophytes isolated from rice (*Oryza sativa L.*) seeds. Acta Biologica Hungarica, 2017. 68(2): p. 175-186.
66. Ghosh, P.K., et al., Role of IAA metabolizing enzymes on production of IAA in root, nodule of Cajanus cajan and its PGP Rhizobium sp. Biocatalysis and Agricultural Biotechnology, 2013. 2(3): p. 234-239.
67. Etesami, H., H.A. Alikhani, and H.M. Hosseini, Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. MethodsX, 2015. 2: p. 72-78.
68. Glick, B.R., Plant growth-promoting bacteria: mechanisms and applications. Scientifica, 2012. 2012.
69. Pereira, A.P.d.A., et al., Salinity influence on the growth and production of indole acetic acid by endophytic *Burkholderia* spp. from sugarcane. Bioscience Journal, 2012. 28(1, Suppl. 1): p. 112-121.

70. Đorđević, S., et al., The use of bacterial indole-3-acetic acid (IAA) for reduce of chemical fertilizers doses. _ HEMIJSKA INDUSTRIJA, 2017. 71(3): p. 195-200.
71. Tabatabaei, S., et al., Indole-3-acetic acid (IAA) producing *Pseudomonas* isolates inhibit seed germination and α-amylase activity in durum wheat (*Triticum turgidum L.*). Spanish Journal of Agricultural Research, 2016. 14(1): p. 0802.
72. Pattnaik, S., et al., Indole acetic acid production and growth-promoting activity of *Methylobacterium extorquens* MP1 and *Methylobacterium zatmanii* MS4 in tomato. International Journal of Vegetable Science, 2017. 23(4): p. 321-330.
73. Yu, Z., et al., Isolation and characterization of indole acetic acid producing root endophytic bacteria and their potential for promoting crop growth. Journal of Agricultural Science and Technology. 2016. 18(5): p. 1381-1391
74. Ahmad, F., I. Ahmad, and M.S. KHAN, Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. Turkish Journal of Biology, 2005. 29(1): p. 29-34.
75. Liu, R., et al., Genomic and phenotypic analyses of *Pseudomonas psychrotolerans* PRS08-11306 reveal a turnerbactin biosynthesis gene cluster that contributes to nitrogen fixation. Journal of Biotechnology, 2017. 253: p. 10-13.
76. Lee, K.-E., et al., Isolation and characterization of the high silicate and phosphate solubilizing novel strain *Enterobacter ludwigii* GAK2 that Promotes Growth in Rice Plants. Agronomy, 2019. 9(3): p. 144.
77. Pramanik, K., et al., Characterization of a Cd2+-resistant plant growth promoting rhizobacterium (*Enterobacter* sp.) and its effects on rice seedling growth promotion under Cd2+-stress in vitro. Agriculture and Natural Resources, 2018. 52(3): p. 215-221.
78. Nutaratat, P., A. Monprasit, and N. Srisuk, High-yield production of indole-3-acetic acid by *Enterobacter* sp. DMKU-RP206, a rice phyllosphere bacterium that possesses plant growth-promoting traits. 3 Biotech, 2017. 7(5): p. 305.
79. Yi, Y., W. Huang, and Y. Ge, Exopolysaccharide: a novel important factor in the microbial dissolution of tricalcium phosphate. World Journal of Microbiology and Biotechnology, 2008. 24(7): p. 1059-1065.
80. Yong, J., et al., Analyses of Indole Compounds in Sugar Cane (*Saccharum officinarum L.*) Juice by High Performance Liquid Chromatography and Liquid Chromatography-Mass Spectrometry after Solid-Phase Extraction. Separations, 2017. 4(1): p. 7.
81. Chung, H., et al., Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biology and Biochemistry, 2005. 37(10): p. 1970-1974.
82. Baliah, N.T., G. Pandiarajan, and B.M. Kumar, Isolation, identification and characterization of phosphate solubilizing bacteria from different crop soils of Srivilliputtur Taluk, Virudhunagar District, Tamil Nadu. Tropical Ecology, 2016. 57(3): p. 465-474.
83. Goswami, D., et al., Screening of PGPR from saline desert of Kutch: Growth promotion in Arachis hypogea by *Bacillus licheniformis* A2. Microbiological Research, 2014. 169(1): p. 66-75.
84. Jose Antonio Lucas García, et al., Effect of inoculation of *Bacillus licheniformis* on tomato and pepper. Agronomie, 2004. 24(4): p. 169-176.
85. Li, H.-B., et al., Genetic diversity of nitrogen-fixing and plant growth promoting *Pseudomonas* Species isolated from sugarcane rhizosphere. Frontiers in Microbiology, 2017. 8(1268).
86. Nautiyal, C.S., et al., Plant growth-promoting bacteria *Bacillus amyloliquefaciens* NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress. Plant Physiology and Biochemistry, 2013. 66: p. 1-9.

87. Rao, D. Recent advances in biological nitrogen fixation in agricultural systems. in Proceedings of the Indian National Science Academy. 2014. 80(2): p. 359
88. Compant, S., et al., Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology, 2005. 71(9): p. 4951.
89. Taulé, C., et al., The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum L.*), and the identification and characterization of part of the associated diazotrophic bacterial community. Plant and Soil, 2012. 356(1-2): p. 35-49.
90. Quecine, M.C., et al., Sugarcane growth promotion by the endophytic bacterium *Pantoea agglomerans* 33.1 Applied and Environmental Microbiology, 2012. 78(21): p. 7511.
91. Cavalcante, J., et al., Members of the ethylene signalling pathway are regulated in sugarcane during the association with nitrogen-fixing endophytic bacteria. Journal of Experimental Botany, 2007. 58(3): p. 673-686.
92. Lin, L., et al., Plant Growth-promoting nitrogen-fixing *Enterobacteria* rre in association with sgarcane plants growing in guangxi, china. Microbes and Environments, 2009. advpub: p. 1204160379-1204160379.
93. Thaweenut, N., et al., Two seasons' study on nifH gene expression and nitrogen fixation by diazotrophic endophytes in sugarcane (*Saccharum* spp. hybrids): expression of nifH genes similar to those of rhizobia. Plant and Soil, 2011. 338(1): p. 435-449.
94. Cherchali, A., et al., Isolation and identification of a phosphate-solubilizing Paenibacillus polymyxa strain GOL 0202 from durum wheat (*Triticum durum* Desf.) rhizosphere and its effect on some seedlings morphophysiological parameters. Biocatalysis and Agricultural Biotechnology, 2019. 19: p. 101087.
95. Wang, Y., et al., Phosphate solubilization of *Paenibacillus polymyxa* and *Paenibacillus macerans* from mycorrhizal and non-mycorrhizal cucumber plants. African Journal of. Microbiology Research, 2012. 6: p. 4567-4573.
96. Perin, L., et al., Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. Applied and Environmental Microbiology, 2006. 72(5): p. 3103-3110.
97. Caballero-Mellado, J., et al., *Burkholderia unamae* sp. nov., an N2-fixing rhizospheric and endophytic species. International Journal of Systematic and Evolutionary Microbiology, 2004. 54(4): p. 1165-1172.
98. Gillis, M., et al., Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N2-fixing isolates from rice in Vietnam. International Journal of Systematic and Evolutionary Microbiology, 1995. 45(2): p. 274-289.
99. Chandra, P., P. Tripathi, and A. Chandra, Isolation and molecular characterization of plant growth-promoting *Bacillus* spp. and their impact on sugarcane (*Saccharum* spp. hybrids) growth and tolerance towards drought stress. Acta Physiologiae Plantarum, 2018. 40(11): p. 199.
100. Poupin, M.J., et al., Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. PLoS One, 2013. 8(7): p. e69435.
101. Naveed, M., et al., L-Tryptophan-dependent biosynthesis of indole-3-acetic acid (IAA) improves plant growth and colonization of maize by *Burkholderia* phytofirmans PsJN. Annals of Microbiology, 2015. 65(3): p. 1381-1389.

Supplementary material

Figure S1. Visual comparison of wheat plants inoculated with sugarcane rhizosphere-derived strains of *Bacillus* (top row; 1,2,3,4,5), *Burkholderia* (middle row; 1,2,3,4,5) and *Pseudomonas* (bottom row; 1,2,3,4,5) to non-inoculated control plants.

Chapter 4

Diversity of Culturable Endophytic Bacteria in Transgenic and Non-Transgenic Sugarcane

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Abstract

Sugarcane is an important crop for food, fodder and sugar industry. The present study explored bacterial endophytes present in sugarcane as a resource for sustainable strategies to enhance crop yield. Furthermore, this study served to evaluate potential differences in culturable endophytes between three different transgenic sugarcane varieties with increased abiotic stress tolerance, including AtNHX1 (cultivar CPF-246), AVP1 (CSSG-668) and Dreb1 (CPF-246) and corresponding wildtypes. Naturally occurring bacterial endophytes were isolated from rhizosphere and phyllosphere of transgenic and corresponding wildtype sugarcane. A total of 108 strains affiliated with 20 different genera were isolated from rhizosphere and phyllosphere. Obtained isolates were affiliated with three major phyla of plant growth promoting bacteria including *Proteobacteria, Firmicutes* and *Bacteroidetes,* and the bacterial diversity retrieved by cultivation from sugarcane rhizosphere and phyllosphere largely differed. The most common bacterial species isolated from the transgenic and wildtype sugarcane were most closely affiliated with well-known genera of endophytes such as *Pseudomonas*, *Burkholderia*, *Bacillus*, *Staphylococcus*, *Enterobacter, Paenibacillus* and *Pantoea,* with several minor differences being observed between the transgenic and non-transgenic sugarcane varieties studied here with respect to the diversity of cultured endophytes present in rhizosphere and phyllosphere of sugarcane.

INTRODUCTION

Sugarcane (*Saccharum officinarum)* is a cash crop that is mainly grown in warm temperate to tropical regions of Asia. It contributes 85% of world sugar production followed by sugar beet. Economically, sugarcane is the 2nd most important crop after cotton in Pakistan [1]. Sugarcane is not only economically important for sugar production, but sugarcane by-products are also used in different industries, promoting the country's economy in terms of its earning capacity [2, 3].

Sugarcane is also an important source of employment for millions of people in both the agricultural as well as industrial sector. In Pakistan between fifteen to twenty five thousand families are involved in sugarcane industries, while 0.98 million farmers are involved in cultivation of sugarcane crop. Overall around four million people are involved directly or indirectly in sugarcane related activities [4, 5], and local and national governments promote its growth and production, especially in the provinces Punjab and Sindh [6].

Despite its economic importance and share in the world market, sugarcane production suffers from many problems that are mainly caused by abiotic and climatic factors such as high sunlight intensity and high temperatures, dew, frost, drought and hailstorm, windstorm, salinity, humidity and sunburn [7].

In addition, numerous adverse biotic factors such as susceptibility to various diseases, insect pests and pathogen invasion are among the major yield limiting factors that mainly contribute towards low sucrose contents and recovery in cultivated varieties [8].

Improvement of currently grown sugarcane cultivars through conventional breeding is difficult due to its complex genome and low fertility while genetic engineering has emerged as a potential tool to introduce desirable traits directly into commercially cultivated elite varieties from any endogenous and exogenous sources [9]. To increase productivity and decrease cost, researchers are investing in new sugarcane varieties developed by classical breeding as well as genetically modified plants, searching to implement characteristics such as insect resistance, herbicide resistance and higher sugar content [10, 11].

Ways of improving sugarcane productivity are subject to intense investigation in Pakistan. There are different methods that can contribute to increasing crop production, including the generation of transgenic varieties through new genetic technology as well as the application of so-called plant growth promoting bacteria (PGPB). Both are sustainable approaches, which can help to increase the crop yield while limiting potentially harmful effects on the environment [12-14].

A potential source for new PGPB are bacterial endophytes which live inside or associated with plant tissues. The microbial community of the host plant can have a positive effect on the plant, caused mainly by those microorganisms that live in intimate contact with the plant tissues, such as endophytes or rhizoplane microbes [15].

Endophytes can be defined as microorganisms such as bacteria, archaea and fungi that live in plant tissues (root, stem, leaf) and complete at least one life cycle and cause no harmful effects on the host plant. Such endophytes have also been shown to modulate plant fitness and can survive under harsh environmental conditions [16].

Endophytic bacteria occupy internal tissues of plants without causing damage to their hosts [17]. Endophytic bacteria stimulate the plant growth by using different mechanisms, including indole acetic acid production (IAA), biological nitrogen fixation (BNF), phosphate solubilization, to just name a few. This group of bacteria bears potential for applications in sustainable agriculture and increase crop yield and health without adverse effects on plant growth [18-20]. Hence, it is important to identify these groups of bacteria, which can potentially be used as biofertilizer for sustainable agriculture [21]. Furthermore, establishing and understanding the diversity of plant associated bacteria and their role in plant development can contribute to the conservation of biodiversity [22, 23]. Bacterial endophytes have been found to colonize a broad variety of plants, such as sugar beet, prairie plants, agronomic crops such as different varieties of potato, wheat and rice, and have been shown to increase plant health [24-27].

The present study was conducted to characterize the microbial diversity of potential PGPB, present in rhizosphere and phyllosphere in different parts of wildtype and transgenic sugarcane by using cultivation. Sanger sequencing of 16S ribosomal RNA (rRNA) genes, as well as basic functional screening, of isolates was used for characterization of the cultivable bacterial diversity. With respect to transgenic varieties, we focused on three well-described lines developed at the National Institute for Biotechnology and Genetic Engineering (NIBGE), including cultivar CSSG-668 transgenic for the AVP1 gene for improved drought tolerance [28], cultivar CPF-246 transgenic for AtNHX1 resulting in increased salt tolerance and cultivar CPF-246 transgenic for DREB1A for improved frost resistance.

MATERIALS & METHODS

Field experiments and cultivars used

The experiments were carried out in experimental plots of NIBGE, Faisalabad, Pakistan, under natural conditions during the growing season in 2016. The soil was clay loam and not previously used for cultivation of any other plants. The climate of Faisalabad is semi-arid with hot and humid summers and dry and cool winters. Average summer temperatures range between 25 to 40 °C in June, while average winter lows are at 10 °C in January. The average annual rainfall lies at about 375 mm.

For this experiment, we used three different transgenic sugarcane varieties that are characterized by increased abiotic stress tolerance, as well as the corresponding non-transgenic cultivars CPF-246 and CSSG-668. As to the transgenic lines, we included *AtNHX1* (CPF-246),

AVP1 (CSSG-668) and *DREB1A* (CPF246). All different transgenic and non-transgenic lines were sown in the experimental plots. Transgenic plants of each line were grown in triplicate. Samples were collected at harvest after ten months of growth from the transgenic sugarcane and corresponding non-transgenic sugarcane plants from different parts, i.e. the phyllosphere (leaves, stem, cane) and the rhizosphere (root, rhizosphere soil and bulk soil). Sampling was done in three replicates of each part separately.

Sample processing, cultivation and characterization of isolates

Sampling was done by using sterilized tools, and samples were immediately transported to the laboratory. Root debris was removed by sieving through a 2 mm mesh. Leafs, stem, cane and roots were cut into small pieces with sterilized scissors and separately ground into power with liquid nitrogen using mortar and pestle. Powdered samples from rhizosphere and phyllosphere were used for preparation of 10 × serial dilutions up to 10^{-6} in saline solution (0.89% NaCl). 100 µl (10^{-3} to10^{-6}) from each dilution was spread on freshly prepared LB (Luria-Bertani) agar plates that were then incubated at 30 °C for 5 days. Bacterial colonies were recorded on the bases of differences in their morphology (colour shape and size), and colony forming units (cfu/g) of each dominant and different bacterial colony type were counted. In order to assess the ability of isolates to solubilize phosphate, Pikovskaya agar plates were prepared as described previously (Chapter 2), and bacteria which formed colonies with a clear halo zone were considered phosphate solubilizing bacteria (PSB). In addition, production of IAA and gene-based prediction of BNF was tested as described in Chapter 2 [29, 30].

Representative bacterial colonies were transferred to fresh LB agar plates, and pure cultures were obtained by repeated streaking, and colony morphology i.e. colour, shape and size were recorded. Bacterial cell morphology and motility were studied using a microscope (Microscope, LaboPhot 2 Nikon, Japan). Isolates were also preserved as 20% glycerol stocks at -80 °C.

Total genomic DNA was extracted from bacterial cultures by the CTAB method [31]. with some modifications and further protocol for DNA extraction as reported previously [32]. For the identification of bacterial isolates, extracted DNA was used as template for PCR amplification of near full length bacterial 16S rRNA genes as reported previously [32, 33]. For *nifH* gene amplification, the same thermal conditions were used for PCR, except that the annealing temperature was 48 °C and the primer set PolF/PolR was used for amplification of *nifH* as reported previously [34]. 16S rRNA gene amplicons generated from isolate cultures were sent for Sanger sequencing with primer 27F at GATC Biotech (Cologne, Germany). The obtained, partial 16S rRNA gene sequences were trimmed (Bio-Edit) and identified through BLAST search at NCBI (National Centre for Biotechnology Information; www.ncbi.nlm.nhi.gov). Sequences were deposited at Gene Bank at NCBI. For accession numbers, please refer to Table 1.

RESULTS

Samples were collected from transgenic varieties *AtNHX1* (CPF-246), *AVP1* (CSSG-668) and *DREB1* (CPF-246) and corresponding non-transgenic sugarcane cultivars CPF-246 and CSSG-668, from six different parts representing the phyllosphere (leaf, stem, cane), rhizosphere soil, bulk soil and roots in 2016. Pure cultures were obtained by serial dilution in saline and cultivation on LB agar plates. Obtained colonies were categorised based on colony characteristics, and representative isolates were subjected to initial screening for IAA production, phosphate solubilization and nitrogen fixation potential.

A total 108 strains were identified from rhizosphere and phyllosphere of six different parts of transgenic and corresponding non-transgenic sugarcane. Bacterial identification by 16S rRNA gene sequence analysis indicated that bacterial strains were most closely related to representatives of different major groups of known PGPB, including *Bacillus, Pseudomonas*, *Paenibacillus, Enterobacter* and others (Table 1). More specifically, isolates could be affiliated to 20 different bacterial genera. Among these, most frequently we isolated members of the genus *Bacillus* (34 strains), followed by *Staphylococcus* (15 strains), *Burkholderia* (12 strains), *Pseudomonas* (9 strains), and *Pseudoxanthomonas* and *Curtobacterium* (each 4 strains). Three isolates were obtained from each of the genera *Chryseobacterium*, *Ralstonia*, *Brevibacterium*, *Pantoea* and *Exiguobacterium*. Two isolates were obtained for genera *Enterobacter*, *Lysinibacillus*, *Acinetobacter, Paenibacillus*, *Kocuria* and *Sphingomonas*, while genera *Halobacillus*, *Brevundimonas* and *Planomicrobium*were represented by single isolates (Table 1).

When assessing the distribution and identity of isolates among the different isolation sources, particularly rhizosphere and phyllosphere-derived isolates were different. Whereas rhizosphere isolates were predominated by members of the genera *Bacillus*, *Burkholderia* and *Pseudomonas*, most predominant genera among phyllosphere isolates included *Bacillus* and *Staphylococcus*, with additional differences in the identity and abundance of minor taxa (Table 2). When comparing the distribution of isolates between transgenic and non-transgenic lines, less pronounced differences were observed. It should be noted, however, that in this study, only representative colonies were characterized, and overall abundance of each colony type was not recorded. To this end, the number of isolates obtained also did not allow differentiation of results for individual transgenic and non-transgenic lines, and thus, robust statistical analyses could not be included.

Table 1. Identification of bacterial isolates based on 16S rRNA gene sequence analysis and colony morphology.

No	Isolate code*	Source**	Accession Number	Closest type strain in NCBI database	16SrRNA gene identity (%)	Morphology	P#	IAA#	N#
1	TR1	DR1	MK250045	*Burkholderia* sp.	99	off white large and rough	+	+	+
2	TR2	DR2	MK230965	*Enterobacte*r sp.	99	off white large and rough	+	+	+
3	TR3	DR3	MK250040	*Burkholderia* sp.	99	off white large and rough	+	+	+
4	TR4	DRS1	MK250041	*Burkholderia* sp.	99	off white large and rough	+	+	+
5	TR5	DRS2	MK230963	*Chryseobacterium sp.*	99	off white large and rough	+	-	-
6	TR6	DRS3	MK231010	*Ralstonia* sp.	99	off white, rod	+	-	-
7	TR7	DBS1	MK231011	*Ralstonia* sp.	99	off white, rod	+	-	-
8	TR8	DBS2	MK250871	*Burkholderia* sp.	99	off white large and rough	+	-	-
9	TR9	DBS3	MK250815	*Brevibacterium* sp.	99	off white large and rough	-	+	-
10	TR10	ATR1	MK250051	*Bacillus* sp.	100	white, large thin rod motile	-	+	+
11	TR11	ATR2	MK250816	*Brevibacterium* sp.	98	off white small, motile rods	+	+	-
12	TR12	ATR3	MK250052	*Bacillus* sp.	99	medium white shiny thin rod	+	+	+
13	TR13	ATDRS1	MK250489	*Paenibacillus* sp.	99	cream short motile rods	-	+	+
14	TR14	ATDRS2	MK255030	*Kocuria* sp.	100	cream rounded	-	-	+
15	TR15	ATDRS3	MK255026	*Lysinibacillus* sp.	98	off white rod,non-motile	-	+	+
16	TR16	ATBS1	MK255056	*Acinetobacter* sp.	99	off white short rod non motile	+	+	+
17	TR17	ATBS2	MK250873	*Burkholderia* sp.	100	off white large and rough	+	+	+
18	TR18	ATBS3	MK255057	*Curtobacterium* sp.	99	off white rounded	+	+	+
19	TR19	AR1	MK230997	*Bacillus* sp.	100	medium white, shiny, thin rod	+	+	-
20	TR20	AR2	MK230998	*Bacillus* sp.	100	cream rod and motile	-	+	-
21	TR21	AR3	MK230973	*Pseudomonas* sp.	99	brown large, small cells motile	+	+	-
22	TR22	ARS1	MK230974	*Pseudomonas* sp.	99	brown large, small cells motile	-	-	+
23	TR23	ARS2	MK250049	*Burkholderia* sp.	99	off white large and rough	+	+	+
24	TR24	ARS3	MK250050	*Burkholderia* sp.	99	off white large and rough	+	+	+
25	TR25	ABS1	MK250817	*Brevibacterium* sp.	98	off white large and rough	-	+	-
26	TR26	ABS2	MK250489	*Kocuria* sp.	98	Cream rounded	-	-	-
27	TR27	ABS3	MK230969	*Pseudomonas* sp.	99	Light brown	+	+	-
28	NTR1	CDR1	MK250040	*Burkholderia* sp.	99	off white large and rough	+	+	+
29	NTR2	CDR2	MK250042	*Burkholderia* sp.	99	off white large and rough	+	+	-
30	NTR3	CDR3	MK230988	*Bacillus* sp.	99	cream rod and motile	+	+	-
31	NTR4	CDRS1	MK250043	*Burkholderia* sp.	99	off white large and rough	+	+	-
32	NTR5	CDRS2	MK250044	*Burkholderia* sp.	99	off white large and rough	+	+	+
33	NTR6	CDRS3	MK253229	*Pantoea* sp.	99	Yellow and rod shape	+	+	-

34	NTR7	CDBS1	MK250053	*Bacillus* sp.	98	white rough motile	+	+	+
35	NTR8	CDBS2	MK250488	*Paenibacillus* sp.	100	Cream, short, motile and rods	+	+	+
36	NTR9	CDBS3	MK250417	*Bacillus* sp.	99	White, rough and motile	-	+	-
37	NTR10	CATR1	MK250418	*Bacillus* sp.	100	light brown and egg shape	-	+	-
38	NTR11	CATR2	MK256212	*Acinetobacter* sp.	98	off white, short rod and non motile	+	+	+
39	NTR12	CATR3	MK250419	*Bacillus* sp.	100	medium white, shiny and thin rod	+	+	-
40	NTR13	CATRS1	MK253230	*Staphylococcus* sp.	99	off white rounded	+	+	-
41	NTR14	CATRS2	MK230980	*Bacillus* sp.	100	medium white, shiny and thin rod.	+	+	-
42	NTR15	CATRS3	MK231012	*Ralstonia* sp.	99	off white, rod	-	+	+
43	NTR16	CATBS1	MK255058	*Sphingomonas* sp.	100	white rod	-	+	+
44	NTR17	CATBS2	MK230977	*Pseudomonas* sp.	98	brown ,small, motile rods	-	+	-
45	NTR18	CATBS3	MK255057	*Curtobacterium* sp.	100	off white rounded	-	+	-
46	NTR19	CAR1	MK230999	*Bacillus* sp.	100	medium white shiny thin rod	+	+	+
47	NTR20	CAR2	MK256295	*Burkholderia* sp.	100	off white large and rough	+	+	-
48	NTR21	CAR3	MK250817	*Bacillus* sp.	100	cream rod	+	+	-
49	NTR22	CARS1	MK231000	*Bacillus* sp.	99	white, large thin rod motile	+	+	-
50	NTR23	CARS2	MK231001	*Bacillus* sp.	100	medium white shiny thin rod	-	+	-
51	NTR24	CARS3	MK255027	*Halobacillus* sp.	99	orange rod	+	+	-
52	NTR25	CBS1	MK230974	*Pseudoxanthomonas* sp.	98	Off white rough,	-	-	-
53	NTR26	CBS2	MK250416	*Bacillus* sp.	98	Whitish, rod			
54	NTR27	CBS3	MK250418	*Pseudoxanthomonas* sp.	98	Off white rough	-	-	-
55	TP1	DL1	MK255028	*Pseudomonas* sp.	99	yellow rod	-	+	-
56	TP2	DL2	MK256213	*Lysinibacillus* sp.	99	off white rod and non motile	+	+	+
57	TP3	DL3	MK250420	*Bacillus* sp.	99	medium white shiny and thin rod	-	+	+
58	TP4	DS1	MK256216	*Pantoea* sp.	99	Yellow and rod shape	-	+	-
59	TP5	DS2	MK255021	*Staphylococcus* sp.	98	off white and rounded	+	+	-
60	TP6	DS3	MK255023	*Staphylococcus* sp.	99	off white and rounded	-	+	-
61	TP7	DC1	MK250421	*Bacillus* sp.	99	white rough and motile	-	+	-
62	TP8	DC2	MK230983	*Bacillus* sp.	98	white rough and motile	+	+	+
63	TP9	DC3	MK253231	*Staphylococcus* sp.	98	off white and rounded	-	+	+
64	TP10	ATL1	MK253232	*Staphylococcus* sp.	99	off white and rounded	-	+	-
65	TP11	ATL2	MK253233	*Staphylococcus* sp.	100	off white rounded	-	+	-
66	TP12	ATL3	MK230985	*Bacillus* sp.	99	creamy rod	+	+	-
67	TP13	ATS1	MK230967	*Pantoea* sp.	99	yellow rounded	-	+	-
68	TP14	ATS2	MK230986	*Bacillus* sp.	99	cream rod	-	+	-
69	TP15	ATS3	MK256230	*Staphylococcus* sp.	99	off white and rounded	-	+	-
70	TP16	ATC1	MK230989	*Bacillus* sp.	99	white, large thin rod motile	+	+	+

71	TP17	ATC2	MK230990	*Bacillus* sp.	99	creamy rod	+	+	+
72	TP18	ATC3	MK230993	*Bacillus* sp.	99	creamy rod and motile	+	+	-
73	TP19	AL1	MK256227	*Staphylococcus* sp.	98	off white rounded	+	-	+
74	TP20	AL2	MK230994	*Bacillus* sp.	99	creamy rod and motile	-	+	-
75	TP21	AL3	MK230995	*Bacillus* sp.	100	creamy rod and motile	+	+	+
76	TP22	AS1	MK255059	*Sphingomonas* sp.	99	white rod	+	+	+
77	TP23	AS2	MK231002	*Bacillus* sp.	99	Creamy, rod	-	+	+
78	TP24	AS3	MK255029	*Chryseobacterium* sp.	98	Orange, rod	-	+	-
79	TP25	AC1	MK230979	*Bacillus* sp.	98	Creamy, rod	+	+	-
80	TP26	AC2	MK250056	*Bacillus* sp.	98	Cream, rod	+	+	-
81	TP27	AC3	MK255025	*Pseudoxanthomonas* sp.	99	Off white rough	-	-	-
82	NTP1	CDL1	MK256214	*Planomicrobium* sp.	99	light orange and rod	-	+	+
83	NTP2	CDL2	MK256215	*Staphylococcus* sp.	99	off white rounded	-	+	-
84	NTP3	CDL3	MK256729	*Exiguobacterium* sp.	98	off white irregular	-	+	-
85	NTP4	CDS1	MK230975	*Pseudomonas* sp.	99	brown large, small cells and motile	-	+	-
86	NTP5	CDS2	MK255022	*Exiguobacterium* sp.	99	orange and rounded	-	+	-
87	NTP6	CDS3	MK255024	*Staphylococcus* sp.	99	off white and rounded	-	+	-
88	NTP7	CDC1	MK255025	*Pseudomonas* sp.	99	light brown and egg shape	-	+	-
89	NTP8	CDC2	MK255060	*Curtobacterium* sp.	98	off white and rounded	-	+	-
90	NTP9	CDC3	MK230984	*Bacillus* sp.	98	white rough and motile	+	+	-
91	NTP10	CATL1	MK230964	*Chryseobacterium sp.*	99	Orange and rod	-	+	+
92	NTP11	CATL2	MK253234	*Staphylococcus* sp.	100	off white and rounded	-	+	-
93	NTP12	CATL3	MK256255	*Staphylococcus* sp.	100	off white and rounded	-	+	-
94	NTP13	CATS1	MK256256	*Curtobacterium* sp.	99	off white and rounded	-	+	-
95	NTP14	CATS2	MK253235	*Staphylococcus* sp.	98	off white and rounded	-	+	-
96	NTP15	CATS3	MK256730	*Exiguobacterium* sp.	99	orange and rounded	-	+	-
97	NTP16	CATC1	MK256731	*Brevundimonas* sp.	99	cream and circular	-	+	-
98	NTP17	CATC2	MK230987	*Bacillus* sp.	99	white, large thin rod motile	+	+	+
99	NTP18	CATC3	MK230991	*Bacillus* sp.	98	creamy and rod	+	+	-
100	NTP19	CAL1	MK230992	*Bacillus* sp.	99	creamy rod motile	-	+	-
101	NTP20	CAL2	MK256229	*Staphylococcus* sp.	99	off white rounded	-	+	-
102	NTP21	CAL3	MK256231	*Pseudomonas* sp.	99	light brown, egg shape	-	+	-
103	NTP22	CAS1	MK256228	*Staphylococcus* sp.	99	off white rounded	-	+	-
104	NTP23	CAS2	MK230996	*Bacillus* sp.	99	white, large thin rod and motile	+	+	+
105	NTP24	CAS3	MK230972	*Pseudomonas* sp.	98	brown large, small cells and motile	-	+	-
106	NTP25	CAC1	MK256231	*Pseudoxanthomonas* sp.	98	Off white, rough	-	-	-

107	NTP26	CAC2	MK230966	*Enterobacter* sp.	99	Off white, large rough	+	+	+
108	NTP27	CAC3	MK230981	*Bacillus* sp.	99	Creamy and rod	+	+	-

*Abbreviated codes used for isolate names: **TR** Transgenic rhizosphere, **NTR** Non transgenic rhizosphere, **TP** Transgenic phyllosphere, **NTP** non transgenic phyllosphere.

Abbreviated codes used for isolation source (from left to right): **Genes: D, DREB1A; **AT** AtNHX1; **A**, AVP1**. Rhizosphere parts: R**, Root; **RS**, Rhizosphere soil; **BS,** Bulk soil. **Phyllyosphere parts**: **L**, Leaf; **S**, Stem; **C**, Cane.

#**P**, phosphate solubilization; **IAA**, IAA production; **N**, nitrogen fixation potential

Table 2. Distribution of isolates across different isolation sources (please refer to Table 1 for abbreviations).

No	Genus	TR	NTR	TP	NTP	Total
1	*Burkholderia*	7	5	0	0	12
2	*Enterobacter*	1	0	0	1	2
3	*Chryseobacterium*	1	0	1	1	3
4	*Ralstonia*	2	1	0	0	3
5	*Brevibacterium*	3	0	0	0	3
6	*Bacillus*	4	11	13	6	34
7	*Paenibacillus*	1	1	0	0	2
8	*Kocuria*	2	0	0	0	2
9	*Lysinibacillus*	1	0	1	0	2
10	*Acinetobacter*	1	1	0	0	2
11	*Curtobacterium*	1	1	0	2	4
12	*Pseudomonas*	3	1	1	4	9
13	*Halobacillus*	0	1	0	0	1
14	*Pantoea*	0	1	2	0	3
15	*Sphingomonas*	0	1	1	0	2
16	*Staphylococcus*	0	1	7	7	15
17	*Planomicrobium*	0	0	0	1	1
18	*Brevundimonas*	0	0	0	1	1
19	*Exiguobacterium*	0	0	0	3	3
20	*Pseudoxanthomonas*	0	2	1	1	4
	Total	27	27	27	27	108

DISCUSSION

The present study was conducted to contribute to the development of sustainable agricultural systems through agricultural crops that incorporate useful traits. To this end, we set out to evaluate the culturable bacterial diversity of endophytes present in the rhizosphere and phyllosphere of transgenic and corresponding wild type sugarcane crop lines. A second aim of the present study was the determination of the potential of obtained isolates to serve as PGPB in the sugarcane's rhizosphere and phyllosphere.

There is an increase in the adoptability and cultivation of genetically modified crops [35]. However the implementation of such genetically modified crops requires well defined risk assessment prior to adoption in any agricultural system. These risks include the impact of genetically modified plants on soil-associated microbial communities as plants are known to have a profound effect on the abundance, diversity and activity of soil microorganisms living in close proximity with their roots in a soil zone called the rhizosphere [36, 37]. Comparison of culturable bacterial diversity can help to determine the structurally abundant, functionally viable and potentially valuable bacteria that can ultimately be used as inoculum to influence plant health in a positive manner. Limited studies are available regarding the bacterial diversity in sugarcane rhizosphere and phyllosphere. Knowledge of the diversity of endophytic bacteria is important for both ecological and biotechnological studies [38].

In the present study bacterial diversity was compared by using a culture-dependent approach. Overall, we observed 12 different genera in the rhizosphere of transgenic sugarcane, among which *Bacillus* spp. strains were predominant (four of 27 isolates). Members of the genus *Bacillus* have been previously reported as endophytes in many crops, including their beneficial effects on crop yield. Among these studies, the genus *Bacillus* was also found in the rhizosphere and phyllosphere of sugarcane as endophytes [39, 40]. Besides the genus *Bacillus*, also members of the genera *Burkholderia* (7/27) and *Pseudomonas* (3/27) were predominantly present in the rhizosphere of transgenic sugarcane. Active endophytes affiliated with the genus *Burkholderia* were previously reported to help against plant pathogens in sugarcane [40]. In our study other genera were also retrieved at lower frequency from the rhizosphere of transgenic sugarcane (Table 2). Many previous studies also showed that different genera of culturable bacteria are found as endophytes found in a broad range of crops, and which have positive impact on plant growth [24, 41-43]. In the present study we retrieved members of 12 different genera from the rhizosphere of non-transgenic sugarcane, most frequently affiliated with *Burkholderia* (5/27) and *Bacillus* (11/27). Some genera were only retrieved from the rhizosphere of transgenic and/or wild type sugarcane, including *Hallobacillus, Acinetobacterium, Paenibacillus, Brevibacter* and *Kocuria* and these genera were not observed in phyllosphere samples. A previous study also showed *Burkholderia* spp. in sugarcane as endophytes [43]. Members of *Bacillus* and *Pseudomonas* have been reported as endophytes that help in plant protection in many crops [44]. In our study we observed that the diversity of culturable bacteria in the rhizosphere of transgenic sugarcane is comparable to that of the corresponding wild type varieties. This is in line with a previous study that also showed no significant effect of transgenic sugarcane on bacterial diversity [45].

Bacterial strains was also isolated from the phyllosphere of transgenic and wild type sugarcane plants to observe the culturable bacterial endophyte diversity. The phyllosphere is also a very important region of sugarcane, and different types of endophytes have been reported from different parts [46-49]. Results obtained here showed the presence of 10 different of genera the phyllosphere of wildtype sugarcane, whereas members of eight different genera were retrieved from the transgenic lines (Table 2). From the phyllosphere of transgenic sugarcane, members of the genera *Bacillus* (13 of 27 isolates) and *Staphylococcus* (7/27) were retrieved at the highest frequency. This was similar to what was found for wild-type sugarcane with seven and six isolates from *Staphylococcus* and *Bacillus*, respectively, however, also members of the genera *Pseudomonas* (4/27), *Exigobacterium* (3/27) and *Curtobacterium* (2/27) were retrieved at relatively high frequency. Previous studies showed that *Curtobacterium* was present as endophytes in many crops [50-52]. In our study *Staphylococcus* was dominant in the phyllosphere of both transgenic and wild type sugarcane, in line with earlier findings that reported *Staphylococcus* to be associated with sweet pepper and other crops as endophytes and to promote plant growth [53-56]. In our study *Pseudomonas* was also present in the

phyllosphere of both transgenic and wild type sugarcane. A previous study showed that *Pseudomonas* present in the phyllosphere of wheat could act as antagonist against different bacterial pathogens [57]. In the present study, members of the genera *Bacillus* and *Pseudomonas* were present in rhizosphere and phyllosphere of transgenic and wild type sugarcane. This is at least in part in line with previous studies that also showed that endophytic bacteria such as *Pseudomonas, Bacillus* and *Burkholderia* are present in the rhizosphere and phyllosphere of sugarcane. The lack of detection of members of *Burkholderia* from the phyllosphere in our study might be due to the relatively limited number of isolates that were characterized. In our study a slightly lower number of genera was retrieved from the phyllosphere of transgenic varieties as compared to the wild type sugarcane plants, however, given the limited sample size, this should probably not be taken as a sign of adverse effects on the microbial diversity associated with the phyllosphere in transgenic plants. Most of the phyllosphere isolates obtained in our study tested positive for one or more plant growth promoting traits (Table 1). This is in line with previous studies on endophytes present in the phyllosphere with respect to the presence of beneficial endophytes [58-60]. In the present study, members of the genus *Enterobacter* were isolated from the rhizosphere and phyllosphere of transgenic and wild type sugarcane as endophytes, reinforcing results of previous studies [61-63]. In this study members of the genus *Pantoea* were isolated from the phyllosphere of transgenic plants and the rhizosphere of wildtype sugarcane. Previous study showed that *Pantoea* was found in sugarcane as endophytes, promoting plant growth [52].

To conclude, the present study was conducted to compare the culturable bacterial diversity of endophytes from the rhizosphere and phyllosphere of transgenic and corresponding non-transgenic sugarcane cultivars. Overall, only limited differences were observed between transgenic and non-transgenic sugarcane lines with respect to their associated, cultured rhizosphere and phyllosphere microbiota. Previous studies also reported limited effects of genetically modified plants on the associated microbial communities [64-72]. In line with our study, observed effects were minor in comparison with the effect caused by cultivar soil type, crop location and plant growth stage [73]. Further studies will be required, including more extensive, quantitative cultivation campaigns as well as molecular assessment of microbial diversity, in order to provide unequivocal information on the possible effect of different transgenic lines on plant-associated microbiomes and corresponding consequences for crop and soil health.

References

1. Gill, N.K., R. Gill, and S. Gosal, Factors enhancing somatic embryogenesis and plant regeneration in sugarcane (*Saccharum officinarum L.*). Indian Journal of Biotechnology, 2004. 3: p. 119-123.

2. Egan, B., C. Ryan, and R. Francki, Fiji disease. Diseases of sugarcane: major diseases., Elsevier 1989: p. 263-287.
3. Jackson, P.A., Breeding for improved sugar content in sugarcane. Field Crops Research, 2005. 92(2-3): p. 277-290.
4. Iqbal, M.A. and A. Iqbal, Sugarcane production, economics and industry in Pakistan. Journal of Agricultural and Environmental Sciences. 2014. 14(12): p. 1470-1477.
5. Afghan, S., et al., Sugarcane breeding in Pakistan. Sugar Tech, 2022. 24(1): p. 232-242.
6. Raza, H.A., et al., Sugarcane production and protection constraints faced by the growers of Punjab, Pakistan with special focus on the role of agricultural extension worker in related mitigation. Pakistan Journal of Agricultural Sciences, 2020. 57(6).
7. Gilbert, R., et al., Agronomic evaluation of sugarcane lines transformed for resistance to sugarcane mosaic virus strain E. Crop Science, 2005. 45(5): p. 2060-2067.
8. Raza, G., et al., The response of sugarcane (*Saccharum officinarum L*) genotypes to callus induction, regeneration and different concentrations of the selective agent (geneticin -418). African Journal of Biotechnology, 2010. 9.
9. Arruda, P., Genetically modified sugarcane for bioenergy generation. Current Opinion in Biotechnology, 2011. 23: p. 315-22.
10. Dal-Bianco, M., et al., Sugarcane improvement: how far can we go? Current Opinion in Biotechnology, 2012. 23(2): p. 265-270.
11. Wu, H. and F. Altpeter, Sugarcane (*Saccharum* Spp. Hybrids), in *Agrobacterium* Protocols. Springer New York, 2015. 2: p. 307-316.
12. Shameer, S. and T. Prasad, Plant growth promoting rhizobacteria for sustainable agricultural practices with special reference to biotic and abiotic stresses. Plant Growth Regulation, 2018. 84(3): p. 603-615.
13. Glick, B.R., Plant growth-promoting bacteria mechanisms and applications. Scientifica, 2012.
14. Pinto, L., et al., Simulation of agroforestry systems with sugarcane in Piracicaba, Brazil. Agricultural Systems, 2005. 86(3): p. 275-292.
15. Hardoim, P.R., et al., The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Review, 2015. 79(3): p. 293-320.
16. Dias, A.C., et al., Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World Journal of Microbiology and Biotechnology, 2009. 25: p. 189-195.
17. Santoyo, G., et al., Plant growth-promoting bacterial endophytes. Microbiological Research, 2016. 183: p. 92-99.
18. Hallmann, J., et al., Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology, 1997. 43(10): p. 895-914.
19. Dias, A.C., et al., Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World Journal of Microbiology and Biotechnology, 2009. 25(2): p. 189-195.
20. Gaiero, J.R., et al., Inside the root microbiome: bacterial root endophytes and plant growth promotion. American Journal of Botany, 2013. 100(9): p. 1738-1750.
21. De Santi Ferrara, F.I., et al., Endophytic and rhizospheric *enterobacter* isolated from sugarcane have different potentials for producing plant growth-promoting substances. Plant and Soil, 2012. 353(1-2): p. 409-417.
22. Germida, J.J., et al., Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus L.*) and wheat (*Triticum aestivum L.*). FEMS Microbiology Ecology, 1998. 26(1): p. 43-50.

23. Sturz, A., et al., Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil borne plant pathogens. Plant Pathology, 1999. 48(3): p. 360-369.
24. Dent, K.C., J.R. Stephen, and W.E. Finch-Savage, Molecular profiling of microbial communities associated with seeds of Beta *vulgaris* subsp. *Vulgaris* (sugar beet). Journal of Microbiological Methods, 2004. 56(1): p. 17-26.
25. Germida, J. and S. Siciliano, Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. Biology and Fertility of Soils, 2001. 33(5): p. 410-415.
26. Sessitsch, A., et al., Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomycetes*-specific PCR of 16S rRNA genes. FEMS Microbiology Ecology, 2002. 39(1): p. 23-32.
27. Sun, L., et al., Endophytic bacterial diversity in rice (*Oryza sativa L.*) roots estimated by 16S rDNA sequence analysis. Microbial Ecology, 2008. 55(3): p. 415-424.
28. Kumar, T., et al., Genetic improvement of sugarcane for drought and salinity stress tolerance using *Arabidopsis* vacuolar pyrophosphatase (AVP1) gene. Molecular Biotechnology, 2014. 56: p. 199-209.
29. Watanabe, F. and S. Olsen, Test of an ascorbic acid method for determining phosphorus in water and NaHCO3 extracts from soil. Soil Science Society of America Journal, 1965. 29(6): p. 677-678.
30. Tahir, M., et al., Isolation and identification of phosphate solubilizer'*Azospirillum*, *Bacillus*' and'*Enterobacter*'strains by 16SrRNA sequence analysis and their effect on growth of wheat ('*Triticum aestivum'L.*). Australian Journal of Crop Science, 2013. 7(9): p. 1284-1292.
31. Wilson, K., Preparation of genomic DNA from bacteria. Current Protocols in Molecular Biology, 2001. 56(1): p. 2.4. 1-2.4. 5.
32. Qaisrani, M.M., et al., Isolation and identification by 16s rRNA sequence analysis of *Achromobacter*, *Azospirillum* and *Rhodococcus* strains from the rhizosphere of maize and screening for the beneficial effect on plant growth. Pakistan Journal of Agricultural Sciences, 2014. 51(1).
33. Arshad, M., et al., Comparsion of rhizospheric bacterial populations and growth promotion of AVP1 transgenic and non transgenic cotton by bacterial inoculations. Journal of Animal & Plant Sciences, 2016. 26(5).
34. Ayyaz, K., et al., Isolation and identification by 16S rRNA sequence analysis of plant growth-promoting *azospirilla* from the rhizosphere of wheat. Brazilian Journal of Microbiology, 2016. 47(3): p. 542-550.
35. Yasmin, S., et al., Plant beneficial rhizobacteria for sustainable increased yield of cotton with reduced level of chemical fertilizers. Pakistan Journal of Biotechnology, 2013. 45(2): p. 655-662.
36. Dunfield, K.E. and J.J. Germida, Impact of genetically modified crops on soil and plant associated microbial communities. Journal of Environmental Quality, 2004. 33(3): p. 806-815.
37. Verbruggen, E., et al., Testing potential effects of maize expressing the *Bacillus thuringiensis Cry1Ab* endotoxin (Bt maize) on mycorrhizal fungal communities via DNA-and RNA-based pyrosequencing and molecular fingerprinting. Applied and Environmental Microbiology, 2012. 78(20): p. 7384-7392.
38. Biodiversity in Soils: Use of molecular methods for its characterization. Encyclopedia of Environmental Microbiology.
39. Pindi, P.K., T. Sultana, and P.K. Vootla, Plant growth regulation of Bt-cotton through *Bacillus* species. 3 Biotech, 2014. 4(3): p. 305-315.

40. Van Antwerpen, T., R. Rutherford, and J. Vogel, Assessment of sugarcane endophytic bacteria and rhizospheric *Burkholderia* species as antifungal agents. Proceedings of the Annual Congress. South African Sugar Technology Association, 2002. 76: p. 301-304.
41. Caballero-Mellado, J. and E. Martinez-Romero, Limited Genetic Diversity in the endophytic sugarcane bacterium *Acetobacter diazotrophicus*. Applied and Environmental Microbiology, 1994. 60(5): p. 1532.

43. Suman, A., et al., Molecular assessment of diversity among endophytic diazotrophs isolated from subtropical Indian sugarcane. World Journal of Microbiology and Biotechnology, 2001. 17(1): p. 39-45.
44. Fernando, W., et al., Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Protection, 2007. 26(2): p. 100-107.
45. Zhou, D., et al., *Cry1Ac* transgenic sugarcane does not affect the diversity of microbial communities and has no significant effect on enzyme activities in rhizosphere soil within one crop season. Frontiers in Plant Science, 2016. 7: p. 265.
46. Mashiane, R.A., et al., Metagenomic analyses of bacterial endophytes associated with the phyllosphere of a Bt maize cultivar and its isogenic parental line from South Africa. World Journal of Microbiology and Biotechnology, 2017. 33(4): p. 80.
47. Vacher, C., et al., The phyllosphere: microbial jungle at the plant climate interface. Annual Review of Ecology, Evolution, and Systematics, 2016. 47: p. 1-24.
48. Lindow, S.E. and J.H. Leveau, Phyllosphere microbiology. Current Opinion in Biotechnology, 2002. 13(3): p. 238-243.
49. Valverde, A., et al. Preliminary Diversity Studies of Culturable Phyllosphere Bacteria on Chestnut (*Castanea sativa*). in III International Chestnut Congress 693. 2004.
50. Bell, C., et al., Endophytic bacteria in grapevine. Canadian journal of Microbiology, 1995. 41(1): p. 46-53.
51. Idris, R., et al., Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. Applied and Environmental Microbiology, 2004. 70(5): p. 2667.
52. Loiret, F., et al., A putative new endophytic nitrogen-fixing bacterium *Pantoea* sp. from sugarcane. Journal of Applied Microbiology, 2004. 97(3): p. 504-511.
53. Rasche, F., et al., Chilling and cultivar type affect the diversity of bacterial endophytes colonizing sweet pepper (*Capsicum anuum L.*). Canadian Journal of Microbiology, 2006. 52(11): p. 1036-1045.
54. Vendan, R.T., et al., Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. The Journal of Microbiology, 2010. 48(5): p. 559-565.
55. Srivastava, S., et al., Influence of inoculation of arsenic-resistant *Staphylococcus arlettae* on growth and arsenic uptake in *Brassica juncea* (*L.*) Czern. Var. R-46. Journal of Hazardous Materials, 2013. 262: p. 1039-1047.
56. Holguin, G. and Y. Bashan, Nitrogen-fixation by *Azospirillum brasilense* Cd is promoted when co-cultured with a mangrove rhizosphere bacterium (*Staphylococcus* sp.). Soil Biology and Biochemistry, 1996. 28(12): p. 1651-1660.
57. Müller, T., et al., Fluorescent pseudomonads in the phyllosphere of wheat: potential antagonists against fungal phytopathogens. Current Microbiology, 2016. 72(4): p. 383-389.
58. Valverde, A., et al., Culturable bacterial diversity from the chestnut (*Castanea sativa Mill.*) phyllosphere and antagonism against the fungi causing the chestnut blight and ink diseases. AIMS Microbiology, 2017. 3(2): p. 293-314.

59. Rico, L., et al., Community structures of N_2-fixing bacteria associated with the phyllosphere of a H olm oak forest and their response to drought. Plant Biology, 2014. 16(3): p. 586-593.
60. Fürnkranz, M., et al., Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. The ISME Journal, 2008. 2(5): p. 561.
61. Araújo, W.L., et al., Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Applied and Environmental Microbiology, 2002. 68(10): p. 4906.
62. Zinniel, D.K., et al., Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Applied and Environmental Microbiology, 2002. 68(5): p. 2198.
63. Kuklinsky-Sobral, J., et al., Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environmental Microbiology, 2004. 6(12): p. 1244-1251.
64. Singh, A.K., M. Singh, and S.K. Dubey, Changes in *Actinomycetes* community structure under the influence of Bttransgenic brinjal crop in a tropical agroecosystem. BMC Microbiology, 2013. 13(1): p. 122.
65. Singh, A.K., M. Singh, and S.K. Dubey, Rhizospheric fungal community structure of a Bt brinjal and a near isogenic variety. Journal of Applied Microbiology, 2014. 117(3): p. 750-765.
66. Rasche, F., et al., Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild-type potatoes, vegetation stage and pathogen exposure. FEMS Microbiology Ecology, 2006. 56(2): p. 219-35.
67. Dunfield, K.E. and J.J. Germida, Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. FEMS Microbiology Ecology, 2001. 38(1): p. 1-9.
68. Rasche, F., et al., Impact of transgenic potatoes expressing anti-bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. Journal of Applied Ecology, 2006. 43(3): p. 555-566.
69. Heuer, H., et al., Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. Applied and Environmental Microbiology, 2002. 68(3): p. 1325-1335.
70. Lamarche, J. and R.C. Hamelin, No evidence of an impact on the rhizosphere diazotroph community by the expression of *Bacillus thuringiensis Cry1Ab* toxin by Bt white spruce. Applied and Environmental Microbiology, 2007. 73(20): p. 6577-83.
71. Van Overbeek, L. and J.D. Van Elsas, Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum L.*). FEMS Microbiology Ecology, 2008. 64(2): p. 283-296.
72. Hannula, S.E., W. de Boer, and J. van Veen, A 3-Year Study Reveals that plant growth stage, season and field site affect soil fungal communities while cultivar and GM-trait have minor effects. Plos One, 2012. 7(4): p. e33819.
73. Singh, A.K., M. Singh, and S.K. Dubey, Changes in *Actinomycetes* community structure under the influence of Bt transgenic brinjal crop in a tropical agroecosystem. BMC Microbiology, 2013. 13(1): p. 122.

Chapter 5

Effect of Transgenic Sugarcane on the Composition of Rhizosphere and Phyllosphere Microbiota

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Abstract

Microorganisms associated with crops tightly interact with each other and their host in the rhizosphere and phyllosphere, and many members of these plant associated microbiomes contribute to the plant-microbiome holobiont with plant growth promoting traits. Complementary to this contribution of plant-associated microorganisms to plant growth, the generation of transgenic crops can provide additional means to improve production and to overcome adverse effects of environmental abiotic and biotic stress factors. Nevertheless, information regarding the effect of transgenic crops on the environment as well as plant- and soil associated microbial communities remains scarce. Therefore, in this study, we comprehensively compared microbial communities in rhizosphere and phyllosphere of transgenic and corresponding wildtype sugarcane varieties, using 16S ribosomal RNA gene amplicon Illumina sequencing. Sampling was done from six different plants sites of rhizosphere and phyllosphere. These included samples from roots, rhizosphere soil and bulk soil, as well as stem, leaf and cane parts as representative samples of the phyllosphere. Results of alpha and beta diversity analyses on the bases of unique amplicon sequence variants (ASVs) showed that there microbial communities associated with transgenic and non-transgenic sugarcane were highly similar. Comparison of relative abundance distributions also showed similar abundances of major microbial phyla in transgenic and wildtype sugarcane crops. In rhizosphere samples, high relative abundances of phyla *Proteobacteria*, *Parcubacteria*, *Bacteroidetes*, *Saccharibacteria*, *Actinobacteria*, *Firmicutes*, and *Chloroflexi* were seen in transgenic and wildtype sugarcane lines. In the phyllosphere, *Saccharibacteria*, *Actinobacteria*, *Firmicutes*, *Chloroflexi*, and *Proteobacteria* were most abundant in transgenic and wildtype sugarcane.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an important industrial crop and is cultivated in tropical and subtropical areas in more than 80 countries especially for sugar and biofuel production [1]. Sugarcane is an annual crop belonging to the genus *Saccharum*, family Poaceae, tribe Andropogoneae. Sugarcane is globally widely distributed and has many economic dimensions, especially for the sugar industry, in many tropical and subtropical countries. In many countries where sugarcane is cultivated, the general population likes to drink sugarcane juice as a delicious, refreshing, and popular beverage [2]. It is cultivated in two seasons in Pakistan during spring and autumn [3], however, sugarcane productivity in Pakistan is much lower than in most of the sugarcane growing countries of the world [4].

Factors causing low production

Environmental factors could reduce the yield of agricultural crops up to 70% on this earth as was already stated by J.S. Boyer 40 years ago [5]. The low production and yield of agricultural crops are due to a broad variety of abiotic factors such as heat, drought and salinity related to climate change and global warming. It has been proposed that in order to improve the yield of crops one needs to explore the genetic potential of agricultural crops [6, 7]. Approximately 10% of the world's land is arable land and characterized as stress free zone, whereas the remaining 90% of land is facing one or more of a broad range of different environmental stress factors for crop cultivation, affecting agricultural crops and reducing the yield, quality and quantity of products [8]. To this end, a range of different approaches are employed to improve agricultural production, and to deal with abiotic and environmental stresses. These approaches include, among others (1) conventional breeding, (2) tissue culture, (3) chemical priming and (4) genetically modified organisms (GMOs) [9, 10].

Abiotic stress is considered to be one of the primary causes of low crop production worldwide, reducing crop yields by more than 50% [11, 12]. Similarly, biotic stress factors pose major problems for agriculture crops. Crop plants are vulnerable to various biotic and abiotic stresses during their life cycle. Plants have different mechanisms to deal with these types of stress, including, e.g., signal transduction, up-regulation of stress-related genes, and production of specific proteins and metabolites. For example plants can easily deal with salt, cold and drought stress based on such mechanisms [13], however, the polygenic nature of mechanisms to respond to these stresses is not yet fully understood [14]. To this end, biomolecular approaches as well a range of complementary omics approaches are increasingly being employed to understand the molecular bases of the stress response and resistance of plants, in order to help in sustainably increasing crop yield and production [15]. In the era of biotechnology *Arabidopsis thaliana* as model plant has been instrumental for improvement of crops and other plants against abiotic stress using GMO technology. Different types of crops have been produced, with some of them being released as commercial products, while others are still in the phase of testing efficacy and

safety in green house and field [16]. Most of the studies with model plants were performed under specifically controlled conditions in the green house, however, field studies need to take varying environmental conditions into account, as well as the need to deal with multiple stresses [17].

Conventional breeding and genetic engineering

Conventional breeding comprises a process to develop a new variety by using natural methods and crossing two plants of the same species with different characteristics to generate offspring with improved characteristics by combining genes inherited from both parents. Conventional breeding helps in enhancing crop yield and production but has not proven very successful towards increasing abiotic stress tolerance [9, 18]. Plant breeders therefore started engaging in other ways towards manipulation of genetic material and thus phenotypes. To this end, genetically modified or transgenic crops may improve agricultural productivity and also sustainable technology (Fig. 1) [19]. Like many other crops, transgenic technology has been used to develop genetically modified sugarcane varieties in recent years. The World Health Organization (WHO) defined GMOs as organisms whose genetic material has been altered in an artificial way using recombinant DNA techniques also known as genetic engineering (IFBC, 1990) [20]. GMO technology is used worldwide in a broad range of different crops such as, e.g. soyabean, maize, cotton, and canola, with countries as U.S.A, Brazil, Argentina, Canada and India being at the top of list in generating transgenic plants as reported by the International Service for the Acquisition of Agro-Biotech Applications (ISAAA, https://www.isaaa.org/) [21].

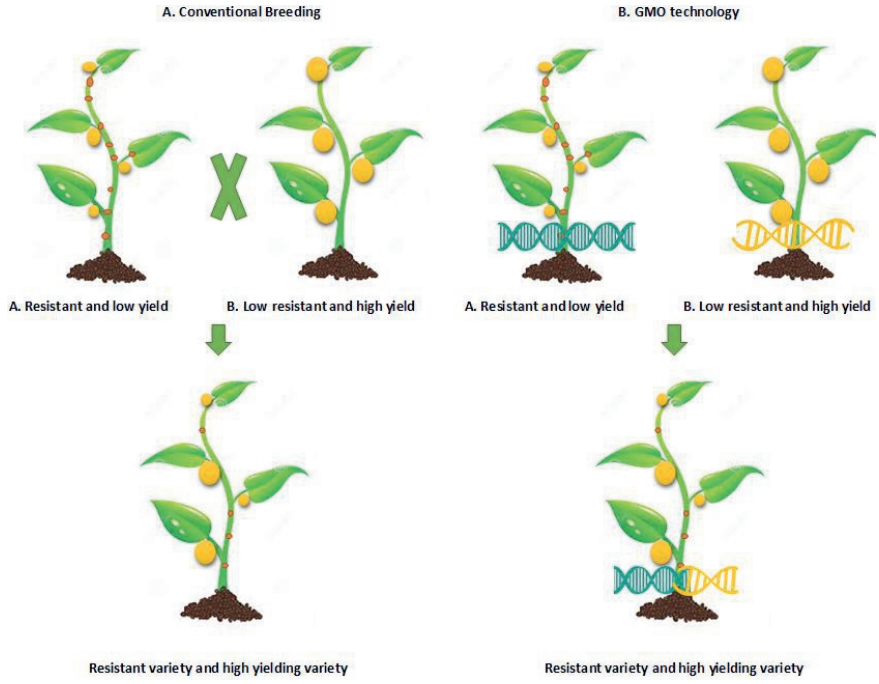


Figure 1. A simplified overview of conventional breeding (**A**) and GMO technology (**B**)

Stress-related targets for genetic engineering

Salinity is one of the major abiotic stress factors. Particularly the outermost layer of soil is affected by salinity, posing major threat to agricultural crops and world food security [22, 23]. Agricultural losses caused by salinity are difficult to assess but estimated to be substantial and expected to increase with time. Salinity limits the plant growth affecting 25% of the world's agricultural lands [7, 24-26]. In soils characterized by increased salinity, salts accumulate in the soil and create hurdles for roots to transport water through xylem tissue, resulting in reduction of growth and development of plants [27]. Overexpression of different stress related genes has been shown to reduce the uptake of Na^+ to the cytosol and to improve the salt tolerance in a variety of crops such as wheat [28], rice [29], sugarcane [30], alfalfa [29], cotton [31], and peanut [32]. One of the major genes used in this context is *atNHX1* [33].

Drought is another major abiotic stress factor, as severe loss of water faced by crops results in extensive yield loss [34-38]. Around one third of the land on this planet comprises arid and semi-arid regions, where drought is a severe problem and affects agriculture [23]. Thus, there is strong need to develop drought-tolerant crops, which grow under drought stressed conditions in water conflict areas. There is very complex mechanism for crops under drought stress

including different types of pathways. A large number of studies were conducted related to transgenic crops with improved drought tolerance. Monsanto U.S.A developed and first commercialized drought-tolerant transgenic corn in 2009 under the name of drought Gard MON87460, which expressed the shock protein B from *Bacillus subtilis* [39]. Different types of genes and mechanisms are involved in drought stress, which promote plant growth. The *DRED1* gene is a major gene that has previously been reported to help in drought tolerance in many crops. Drought related binding element (DRE) proteins such as DREB1 and DREB2 are transcriptional factors, which activate drought tolerance genes such as *RD29A*. Overexpression of these genes resulted in tolerance against abiotic stresses, and especially drought stress [40], and has been applied in a variety of crops including, e.g., wheat, rice, tomato [41, 42] and peanut [43, 44]. To this end, water is essential for sugarcane growth, and shortage of water affects all biochemical processes of sugarcane. Water stress results in the destruction of sugarcane parts, and thus, there is need to develop sugarcane, which can grow easily under water stress conditions without adverse effects on its yield and biomass production. Many studies also showed that multi-stress related genes are involved in salt and drought tolerance and have their own different mechanism, yet more research is needed to understand plant responses towards these genes under different environmental conditions [45].

Frost is a third major abiotic stress that affects plant biochemical processes, resulting in plant death during winter season during prolonged frost periods. Sugarcane is a perennial crop, which is harvested mostly in fall and winter season. It is a cold sensitive crop, when temperature and water levels are low and stalks containing a high amount of sugar. Frost stress negatively impacts sugarcane yield, reducing sugar content and ethanol production [46-49]. Hence, there is need to develop a sugarcane crop, which can tolerate different types of abiotic stresses.

A role for microorganisms

The use of genetically modified or transgenic plants has a great potential for future agriculture but asks for a well-defined risk assessment. These risks lead us to determine the environmental risks above and below the soil and also the impact of genetically modified plants on soil associated microbial communities. Plants are known to have a profound effect on the abundance, diversity and activity of soil microorganisms living in close proximity with their roots in a soil [50, 51]. Soil microbes play important roles in various aspects of the terrestrial ecosystem, such as soil fertility during plant growth and biogeochemistry of the cycling of organic and non-organic compounds throughout the entire ecosystem. Soil microbiota mediates or regulates a variety of functions essential for plant growth and productivity, soil resource structure, and ecosystem health. So-called plant growth promoting bacteria (PGPB) present in the rhizosphere are among the best characterized functional groups of microorganisms known for playing a significant role in plant health and development and being responsible for numerous functions including nutrient cycling and decomposition, which can significantly influence vegetation dynamics [52, 53].

Rhizosphere and phyllosphere

Plant roots release exudate chemicals, which are used by PGPB that live in the vicinity of the rhizosphere. Mostly these microorganisms are beneficial for plants as well for the environment by having different traits. As the first interaction of plants with microorganisms is mostly associated with the roots, there is the possibility that transgenic plants having new genes for new traits release root exudates that might affect microorganisms present in soil [54, 55]. Hence, it is necessary to assess the biosafety and impact of new transgenic crop varieties on environmental, human and animal health (FAO/WHO) [56-58]. The rhizosphere is the region of roots and soil attached to the roots, comprising a proper biological system in which different biological activities are occurring. The rhizosphere and its microbiome is characterized by different types of interactions [59-67]. Similar to the rhizosphere, the second major region characterized by plant-microbiome interactions is the phyllosphere, comprising all arial parts of the plant, and being a region with major activities and biochemical processes. The phyllosphere is furthermore characterized by exposure to harsh environmental conditions, which, among other factors, contribute to a lower microbial diversity as compared to the rhizosphere [68-74].

Microbial diversity

Microbial communities residing in the rhizosphere and phyllosphere differ in diversity, composition and functionality, and their characterization contributes to our understanding of rhizosphere and phyllosphere functioning [75].

There is a high diversity of PGPB associated with the rhizosphere and shoot of sugarcane, and the knowledge of the diversity of PGPB associated with sugarcane is an important step towards understanding the role bacteria play in promoting plant growth traits [76-78]. Microbial diversity in soil has been analysed by various methods including cultivation-dependent and molecular methods. Most of the active viable soil bacteria observed under a microscope are hard to cultivate on agar plates [79], and the need for the application of culture independent methods is now widely accepted [80]. The analysis of 16S ribosomal RNA (16S rRNA) genes has become an essential component of the microbial ecologist's tool kit to evaluate the microbial composition of diverse environments such as soils, oceans, and our own bodies [81]. To this end, the use of high throughput DNA sequencing technologies in particular the amplicon sequencing of the microbial 16S rRNA gene, has been established as a fundamental tool for understanding the structure and diversity of microbial communities [82]. Analysis of the resulting sequences provides a full view of the studied microbial community, including the vast majority of organisms that are hard to culture using standard protocols [83]. The applicability of high throughput DNA sequencing is constantly growing due to the rapidly decreasing costs for DNA sequencing and breakthroughs in the required downstream bioinformatic analysis [84]. Amplicon sequencing as well as the more comprehensive metagenomic shot-gun

sequencing of environmental DNA has been widely used for many applications, such as identifying the members of a given microbiome, classifying microbial populations within the ocean as well as for the analysis of the microbial communities associated with humans, animals and plants [85]. Plant microbe interaction studies give rise to knowledge with respect to the collection of microorganisms and their gene content which maintain a continuous relationship over the lifetime of the plant [86]. Elucidating nature and extent of these interactions offers significant opportunities for improving plant health, for example, through nutrient cycling, neutralizing toxic compounds, discouraging pathogens, and promoting resistance to abiotic stresses, generating significant impact on plant productivity [87].

Pakistan is facing many problems which affect sugarcane production, and thus, efforts are needed to sustain sugarcane production by conservation of available water, efficient use of water, land and other input resources. Such efforts include attempts towards genetic improvement or alteration in sugarcane varieties to sustain sugarcane yields under changing environmental conditions with increasing exposure to abiotic stresses, as well as the application of PGPB to sustain sugar production in cane stalks. Researchers continue to apply new approaches in the field of biotechnology to induce stress tolerance for economic production of sugarcane [88]. In this study, we set out to study the microbiome of different transgenic sugarcane lines previously developed at the National Institute for Biotechnology and Genetic Engineering (NIBGE, Faisalabad, Pakistan) for increased tolerance against salinity, drought and frost. Microbial communities present in rhizosphere and phyllosphere of transgenic and corresponding non-transgenic sugarcane cultivars were characterized by 16S rRNA gene amplicon sequencing.

MATERIALS AND METHODS

Sowing of sugarcane

The experiments were carried out in experimental plots of NIBGE, Faisalabad, Pakistan, under natural conditions during the growing season. The soil was clay loam and never previously planted with any plants. For this study, which is described in detail in Chapter 4 of this thesis, transgenic varieties AtNHX1 (CPF-246), AVP1 (CSSG-668) and DREB1A (CPF-246) and corresponding non-transgenic sugarcane cultivars CPF-246 and CSSG-668 were sown in these plots.

Sampling Design

Samples were collected from transgenic sugarcane and wildtype control plants from six different parts representing the phyllosphere and rhizosphere. Phyllosphere samples included healthy leaves, stem and cane, whereas rhizosphere samples included bulk soil, rhizosphere soil and roots. Sampling was done using sterilized tools, and samples were immediately transported

to the laboratory. Soil samples were taken from soil attached to the roots (rhizosphere) and from the space between two plants (bulk soil). The soil particles attached to roots were carefully collected after uprooting plants and mixed well. Root debris was removed by sieving through 2 mm mesh.

Surface sterilization and DNA extraction

To focus on endophytic microbial communities we removed as much as possible external surface born microbes from all plant samples. Each sample was surface sterilized by first washing for 30 s in sterile ultrapure double-distilled water to remove dirt or debris from surfaces, followed by 0.5% NaOCl (bleach) for 2 min and 70% ethanol for 4 min. After surface sterilization, air dried samples were cut into small portions with a sterile knife for genomic DNA extraction. First, each sample was ground separately with a sterile mortar and pestle under liquid N_2 followed by DNA extraction using the MP Biomedicals Soil DNA extraction kit for soil samples and MP Biomedicals DNA isolation kit for phyllosphere. The DNA quality was assessed by agarose gel electrophoresis, and the DNA purity and concentrations were determined by calculating the A260/A280 ratio using Nano Vue Plus TM (GE Healthcare). The final DNA concentrations were adjusted to 20 ng/µL.

PCR and sequence analysis

PCR was carried out in a 50 µL volume, including 5x HF Buffer (green), 10 µL barcoded primers 515F and 806R (10 µM), 1 µL 10 mM dNTPs (Promega, Madison, USA), 0,5 µL 2U/µL Phusion hot start DNA polymerase (Finnzymes, Vantaa, Finland), and 36.5 µL nuclease free water and 1 µL template DNA. PCR primers (515F, 5'-GTGYCAGCMGCCGCGGTA-3' and 806R, 5'-GGACTACNVGGGTWTCTAAT-3') targeting the V4 region of the 16S rRNA gene (approximately 290 nucleotides) for both archaea and bacteria were used [89-91]. PCR reactions were performed in triplicate including negative and positive control reactions. PCR conditions consisted of an initial denaturation at 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, annealing at 56 °C for 10 s, and elongation at 72 °C for 10 s, followed by a final extension at 72 °C for 7 min and hold at 4 °C. Triplicate reactions were pooled, and presence of PCR products of the correct size was verified by 2% agarose gel electrophoresis. PCR products were purified by CleanNA magnetic beads (CleanNA Waddinxveen, The Netherlands), and final elusion in 25 µL nuclease free water (Promega) and quantified using a Qubit 2.0 fluorometer (Invitrogen). Finally, samples were pooled in equimolar concentrations and sent to GATC Biotech (Konstanz, Germany; now part of Eurofins Genomics Germany GmbH) for sequencing on an Illumina Hiseq2500 instrument. Sequencing data was deposited in the NCBI Sequence Read Archive under BioProject XXXX.

Data analysis

Illumina sequence data was processed and analysed using the NG-Tax pipeline [92]. Briefly, paired-end libraries were combined, and only read pairs with perfectly matching primers and

barcodes were retained. To this end, both primers were barcoded to facilitate identification of chimeras produced during library generation after pooling of individual PCR products. Both forward and reverse reads were trimmed to 100 bp to avoid overlap, which would affect the quality filtering. Paired-end trimmed forward and reverse reads were concatenated to yield sequences of 200 bp that were used for subsequent sequence data processing. De multiplexing, amplicon sequence variant (ASV) picking, chimera removal and taxonomic assignment were done as implemented in NG-Tax. Reads were ranked per sample by abundance and ASVs were added to an initial ASV table for that sample starting from the most abundant sequence until the abundance was lower than 0.1%. The final ASV table was created by clustering the reads that were initially discarded as they represented ASVs <0.1% of the relative abundance with the ASVs from the initial ASV table with a threshold of 98.5% similarity [92]. Taxonomic assignment using the most abundant sequence of each ASV was done utilizing the UCLUST algorithm [93]. and the Silva 111 SSU Ref database [94, 95].

Microbial diversity (alpha and beta) analyses as well as statistical analyses were performed in R 3.5.1. software using different packages (https://www.r-project.org/) [96]. Alpha diversity (phylogenetic and non-phylogenetic) was calculated in R by using function plot richness in package phyloseq [97]. Phylogenetic diversity was calculated using package picante. Between-group differences in alpha diversity were assessed using a Kruskal-Wallis test, whereas a Wilcoxon rank-sum test was employed for pairwise comparisons. Beta diversity was also calculated in R by using unconstrained multivariate analysis based on different, complementary distance metrics calculated from ASV data. Principle coordinate analysis (PCoA) was performed using pairwise weighted and non-weighted UniFrac distance metrics, which are phylogenetic distance measures that do (weighted) or do not (unweighted) take abundance of individual taxa into account. Furthermore, non-metric multidimensional scaling (NMDS) was performed based on pairwise non-phylogenetically weighted Bray Curtis dissimilarities.

RESULTS

Naturally occurring microbial communities were accessed in transgenic and corresponding wildtype sugarcane varieties grown on native soil in different plots. Three different varieties of transgenic sugarcane with increased tolerance towards abiotic stress were grown on plots to access associated microbial community present in the rhizosphere as well in the phyllosphere. A total of 108 samples were analysed in this study, taken from six different parts of rhizosphere and phyllosphere of triplicate plants of each variety at maturity after 10-12 months of growth. Overall, a total of 32015788 high-quality sequence reads were obtained from all samples, including 13352488 and 18282077 reads from rhizosphere and phyllosphere samples, respectively. The minimum and maximum number of reads per sample were 171385 and 522149 reads, respectively, with an average number of reads per sample of 422149.

Alpha diversity

Microbial alpha diversity of each sample was calculated at ASV level using different metrics. Richness and diversity metrics were compared between transgenic and corresponding wildtype plants (Fig. 1).

To assess microbial richness, we evaluated a number of commonly used non-phylogenetically weighted metrics, including Observed and Chao1, as well as phylogenetic diversity. Furthermore, non-phylogenetically weighted Shannon and Simpson diversity indices were used. Overall, while rhizosphere microbial communities consistently showed higher alpha diversity than phyllosphere samples, no significant difference in alpha diversity was observed between transgenic and wildtype sugarcane, neither for rhizosphere nor phyllosphere microbial communities.

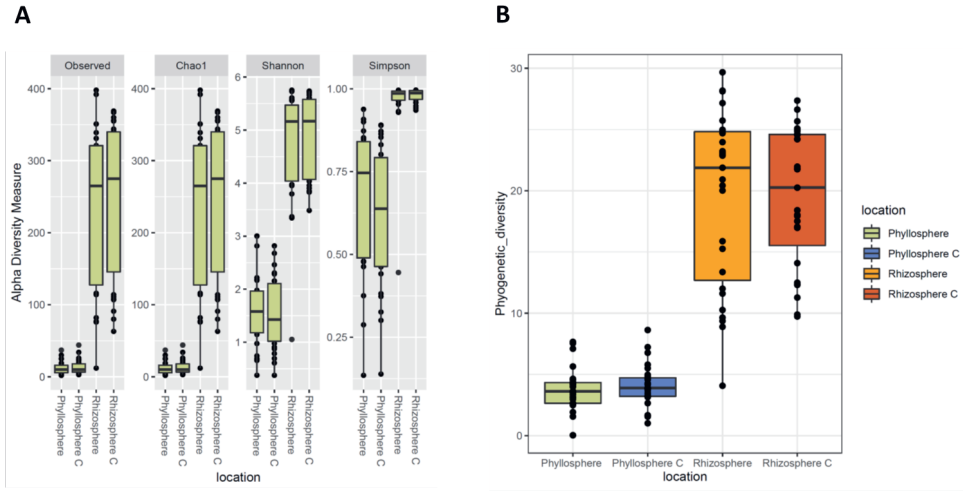
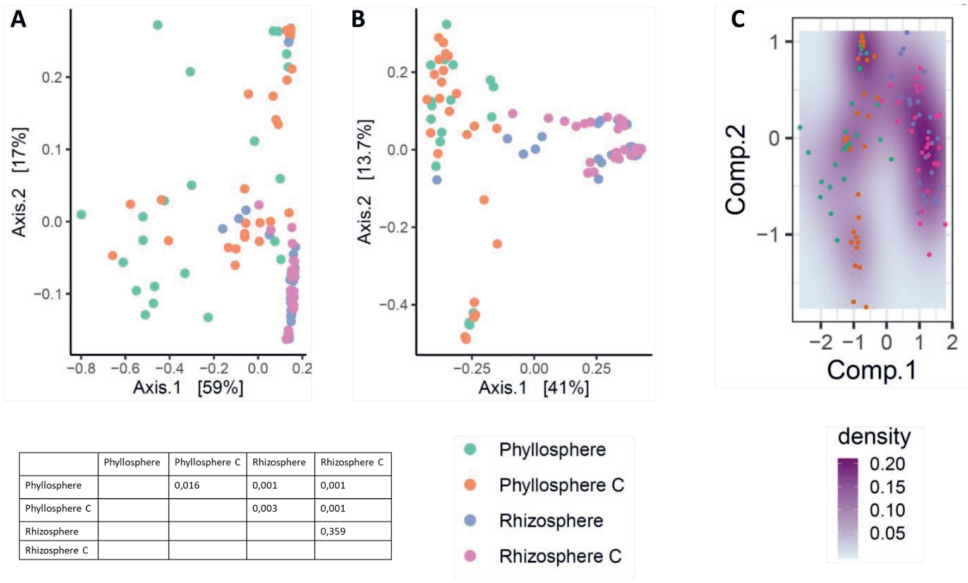


Figure 1. Alpha diversity of microbial communities based on different richness and diversity metrics, including Observed, Chao1, Shannon and Simpson index (**A**) and phylogenetic diversity (**B**) of rhizosphere and phyllosphere of transgenic and wildtype (indicated by C) sugarcane varieties. All analyses are based on triplicate samples.

Beta diversity

Beta diversity analysis (pairwise comparison of the microbial composition between samples) provides a measure of the distance or dissimilarity in composition between each sample pair. To this end, three complementary pairwise distance/dissimilarity metrics based on ASV level compositional data were used as input.

PCoA based on weighted and unweighted UniFrac matrices showed similar clustering between microbial communities in transgenic and wildtype sugarcane in both regions, i.e. rhizosphere and phyllosphere (Fig. 2A,B). Overall, for weighted UniFrac based PCoA, the first two axes together explained 63% of the observed variation in ASV-level microbial community composition (Fig. 2A), whereas this was somewhat lower (54,7%) in case of unweighted UniFrac distances (Fig. 2B). Furthermore, PERMANOVA of weighted UniFrac distances indicated a significant effect of location (rhizosphere vs. phyllosphere; $p < 0,01$). Also, composition of phyllosphere microbiota of transgenic vs. wildtype plants was found different ($p = 0,016$) (Fig. 2A). Similar results were obtained when clustering was done by NMDS based on Bray Curtis dissimilarities, including clear clustering of rhizosphere of transgenic and wild type sugarcane samples distinct from phyllosphere samples of both transgenic and wildtype plants, whereas no clear separation was observed between samples from transgenic and corresponding wildtype varieties (Fig. 2C).



	Phyllosphere	Phyllosphere C	Rhizosphere	Rhizosphere C
Phyllosphere		0,016	0,001	0,001
Phyllosphere C			0,003	0,001
Rhizosphere				0,359
Rhizosphere C				

Figure 2. Multivariate analysis of microbial communities was done by Principle Coordinate Analysis based on pairwise weighted (**A**) and unweighted (**B**) UniFrac distances and by Non-metric Multidimensional Scaling of Bray-Curtis dissimilarities (**C**) between samples taken from the rhizosphere and phyllosphere of transgenic and non-transgenic sugarcane. Percentages at the axes of PCoA plots indicate the variation in compositional data explained by the first two axes. The table down left indicates permuted p-values from PERMANOVA of weighted UniFrac based distances.

Composition at phylum and class level

In order to assess possible differences in presence and relative abundance of individual microbial groups at different levels of taxonomic resolution, we performed compositional analysis in R studio. At the phylum level, no obvious difference in microbial relative abundance was observed between samples taken from transgenic and wildtype plants, in both rhizosphere and phyllosphere. In total, in the present study, 26 major bacterial and archaeal phyla were observed (supplementary Fig. S1A).

In the phyllosphere of transgenic and wildtype sugarcane, the phyla *Saccharibacteria*, *Actinobacteria*, *Firmicutes*, and *Chloroflexi* were predominant. When analysing microbial composition for the different sample types, we observed that in leaves of transgenic and wildtype sugarcane, *Proteobacteria*, *Saccharibacteria*, *Firmicutes* and *Actinobacteria* were most predominant. Visual inspection of barplots of microbial composition at phylum level indicated that communities associated with leaves of transgenic and wildtype sugarcane plants were largely similar. Stems of both transgenic and wildtype sugarcane were predominated by *Proteobacteria*, *Firmicutes* and *Actinobacteria*, whereas canes were predominated by *Firmicutes* and *Proteobacteria*. More rigorous statistical analyses were unfortunately not feasible considering the low number of samples per sample type.

Rhizosphere samples derived from transgenic and wild type sugarcane were characterized by predominance of a larger number of phyla as compared to phyllosphere samples, including *Proteobacteria, Parcubacteria*, *Gemmatimonadetes*, *Bacteroidetes*, *Verrucomicrobia*, *Saccharibacteria*, *Actinobacteria*, *Firmicutes* and *Chloroflexi*. In root samples we mostly observed *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Armatimonodetes*, *Firmicutes* and *Parcubacteria* in both transgenic and wildtype sugarcane. In rhizosphere soil, *Saccharibacteria*, *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Parcubacteria*, *Gemmatimonadetes*, *Bacteroidetes*, *Verrucomicrobia Acidobacteria*, *Armatimonadetes*, *Tectomicrobia*, *Nitrospirae*, *Chlorobi*, and *Cyanobacteria* were most abundant in both transgenic and wildtype sugarcane, whereas in bulk soil, *Actinobacteria*, *Chloroflexi*, *Proteobacteria*, *Parcubacteria*, *Gemmatimonadetes*, *Bacteroidetes*, *Verrucomicrobia*, *Acidobacteria*, and *Armatimonadetes* were most predominant in both transgenic and wildtype sugarcane plants.

Relative abundance was also assessed at class level, showing the presence of 72 major bacterial and archaeal classes in microbial communities associated with both transgenic and wildtype sugarcane plants (Fig. S1B). No obvious differences were observed at class level between transgenic and wildtype sugarcane. The classes *Chloroflexia*, *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria*, *Actinobacteria* and *Fibrobacteria* were the most abundant classes in all parts of the phyllosphere, whereas rhizosphere samples of transgenic and wildtype sugarcane were characterized by predominance

of *Gammaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Actinobacteria, Alphaproteobacteria, Bacilli,* Marine_Group_I, Soil_Crenarchaeotic_Group(SCG), *Bacteroidia, and Acidimicrobiia*.

DISCUSSION

Did transgenic sugarcane change the microbial community in rhizosphere and phyllosphere ?

In the current study we assessed possible differences in microbial composition in rhizosphere and phyllosphere microbial communities when comparing transgenic and wildtype sugarcane plants. Previous studies also similarly reported on transgenic crops and their effects on microbial diversity present in rhizosphere and phyllosphere [98, 99].

One of the major observations of our study was that there were limited differences in the composition of microbial communities associated with the rhizosphere and phyllosphere of transgenic and wildtype sugarcane varieties. Previously several other studies have reported similar results regarding the lack of significant effects of crop transformation with *Bacillus thuringiensis* toxin (Bt) on diversity and population size of microbial communities [100-104].

The transgenic sugarcane varieties used in the present study carry different genes, *DREB1, AtNHX1* and *AVP1*, which confer improved tolerance towards abiotic stresses such as drought salt and frost. This research work was conducted to assess possible differences in the composition of microbial communities present in the rhizosphere and phyllosphere of transgenic and wildtype sugarcane. A large number of studies showed that use of genetically modified plants can have a great potential for sustainable agriculture. Transgenic crops are currently being cultivated at a commercial scale in many countries, and the area devoted to transgenic pest resistant varieties worldwide reached 13 million hectares already in 2019 [6]. However, an increase in the adoptability and cultivation of genetically modified crops demands well-defined risk assessment. These risk assessment studies are essentially required because some transgenic plants are known to change the rhizosphere environment through root exudates, which consequently affects the growth of microorganisms in the rhizosphere [105-109].

We observed that major phyla known to contain PGPB are present with the same abundance in both transgenic and wildtype sugarcane. Although the taxonomic resolution of 16S rRNA gene amplicon sequence data does not allow differentiation at strain level, this suggests that abundance of PGPB was similar in both transgenic and wildtype sugarcane, in line with results obtained by cultivation (Chapter 4). Similar results were also reported from other studies,

showing that transgenic crops had no harmful effects on microbial diversity and presence of PGPB in rhizosphere and phyllosphere [102, 110-114].

On the basis of different, complementary alpha diversity measures, we did not observe significant differences in the richness and diversity of microbial communities associated with the rhizosphere and phyllosphere of transgenic and wildtype sugarcane plants. This is in line with other studies, however, other studies also reported differences in transgenic plant associated microbial communities. A previous study showed that the expression of resistance genes in transgenic bananas had no consequences for non-target rhizobacteria and endophytes [115]. In turn, another study found that the presence of transgenes led to changes detected in the endophytic fungal community of maize leaves [115]. The root interior microbial communities of transgenic canola were less diverse and differed from those in non-transgenic plants [116, 117]. Similarly, transgenic tobacco also had altered rhizosphere/rhizoplane microbial communities, however, these effects were temporary, and the diversity of the community was restored to the original level after one cycle of plant cultivation [118]. Previous research work was also conducted in transgenic and non-transgenic poplar for ten years, which showed no significant difference in microbial communities [118-120].

In addition to alpha diversity, we also assessed beta diversity of the microbial communities associated with rhizosphere and phyllosphere of transgenic and wildtype sugarcane lines. Overall, we observed similar clustering patterns in both transgenic and wildtype sugarcane samples, with significant differences only being observed between transgenic and wildtype sugarcane phyllosphere microbiota in addition to the obvious differences between rhizosphere and phyllosphere associated microbial communities. Similar results were obtained in a different study that did not find differences between transgenic and non-transgenic cotton associated microbial communities [121, 122]. Previous research also showed that diversity of rhizosphere bacteria of transgenic, herbicide (glufosinate)-resistant corn was not different from that of the corresponding non-transgenic variety [123].

In light of such minor or even absent effects of genetic engineering on crop-associated microbial communities, it is good to recall that a broad range of different factors cause changes in microbial diversity, such as abiotic factors including pH of soil, soil quality, type of soil and temperature (cold and hot), and biotic factors including different types of interactions between plants and beneficial and pathogenic microbes [124-128].

Comparison of relative abundance and composition of transgenic and non-transgenic sugarcane microbiota

Our compositional analyses showed that there was a higher microbial diversity in the rhizosphere as compared to the phyllosphere in both transgenic and wildtype sugarcane. Previous studies also showed that highly rich and diverse rhizosphere microbiomes are present

in different parts of the rhizosphere of different transgenic crops, including corn, canola, soybean and maize [123, 129-132].

In line with the limited changes in microbial community composition observed here, a previous study also showed that *Bacillus thuringiensis* toxins present in transgenic corn rhizospheres did not change culturable microbial communities and their abundance. Similarly, it was shown that Bt toxins from both *Bacillus thuringiensis* and transgenic crops had no apparent effect on soil microorganisms such as bacteria, fungi, algae, earthworms, nematodes, and protozoa [133-135]. In turn, previous research work showed that soil enzyme activities, based on the substrate degrading ability of enzymes, in a fluvo-aquic soil planted with transgenic Bt and non-Bt cotton were found different in different growth periods. However, for each stage of the life cycle during cotton growth, few significant differences were found between Bt and non-Bt treatments, suggesting that Bt cotton had no harmful effects on soil enzyme activities [113, 136, 137].

Bacterial diversity and abundance was also accessed in the phyllosphere of transgenic and wildtype sugarcane. The phyllosphere of sugarcane is also very important, particularly considering that during the harvesting season, sugarcane phyllosphere leaves are used as fodder for animals, whereas the cane is used as juice. Hence, it is necessary to check for possible effects of transgenic sugarcane on the phyllosphere. To this end, we observed that microbial relative abundance and diversity was very similar in transgenic and wildtype sugarcane plants. This is in line with previous studies that showed that the phyllosphere is a unique and dynamic habitat densely colonized by microorganisms. Due to the fundamental role of phyllosphere microbial communities in several processes related to plant development, such as nitrogen fixation, protection against phytopathogens, and biosynthesis of phytohormones, knowledge about the composition of such communities has great importance for their functionality and correspondingly for normal functioning of plants [138-140].

We observed that most predominant phyla of the phyllosphere were *Saccharibacteria, Actinobacteria, Firmicutes, Chloroflexi,* and *Proteobacteria*. This is in agreement with other studies on the phyllosphere of transgenic crop, showing that rhizosphere and phyllosphere are dominated by *Proteobacteria* and *Actinobacteria* [99, 139, 141].

Overall we observed 26 phyla including predominantly *Proteobacteria, Firmicutes, Bacteroidetes, Acidobacteria, Actinobacteria,* and *Chloroflexi* in all parts of the rhizosphere of transgenic and wildtype sugarcane. This is in line with previous studies that reported on the dominance of members of these phyla in rhizosphere microbial communities [140, 142-146]. The detection of these phyla has also been reported through metagenomic studies in the rhizosphere of different plants [147, 148].

We observed that at the phylum level, *Actinobacteria* and *Proteobacteria* dominated the rhizosphere of transgenic and wildtype sugarcane as compared to phyllosphere samples. *Actinobacteria* have been reported to colonize any tissue or organ of the host plant. Different

tissues and parts of the plant can be colonized by different *Actinobacteria*, which might be determined by host-microbe interactions [149, 150].

Our study showed that the abundance of actinobacteria was higher in the rhizosphere than in the phyllosphere, in line with a previous study that showed that endophytic *Actinobacteria* are abundant in rhizosphere but occur moderately in the phyllosphere [150]. Dominant populations of *Actinobacteria* have also been reported for maize rhizosphere in a tropical region [151].

Several studies showed that members of the *Proteobacteria* phylum are metabolically versatile and genetically diverse, comprising the largest fraction of the microbial community in soil ecosystems including the rhizosphere [152]. It was also found that members of the *Bacteroidetes* constituted dominant populations in the rhizosphere of maize and canola. Members of this microbial group are capable of degrading complex macromolecules, thus contributing to the turnover of carbon, nitrogen and phosphorus [153].

In conclusion, this study showed that microbial diversity and relative abundance only differed to a limited extent between transgenic and wildtype sugarcane varieties. there are many other abiotic and biotic factors which affect the microbial diversity and relative abundance of microbial communities in rhizosphere and phyllosphere such as soil type, pH of water and soil, climate condition, and excess usage of chemical fertilizer to crops. Many previous studies showed that there are also many other factors such as plant cultivars, soil structure, and environmental factors (plant growth phase) to have more impact on soil microbial community structures as compared to transgenic crops [131, 154, 155], putting the observed minor effects of transgenic sugarcane varieties in a broader perspective.

References

1. Molinari, H.B.C., et al., Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiologia Plantarum, 2007. 130(2): p. 218-229.
2. Yong, J., et al., Analyses of indole compounds in sugarcane (*Saccharum officinarum L.*) Juice by high performance liquid chromatography and liquid chromatography mass spectrometry after solid phase extraction. Separations, 2017. 4(1): p. 7.
3. Iqbal, M.A.I.a.A., Sugarcane production, economics and industry in Pakistan. American Eurasian Agriculture and Environment Science, 2014. 14(12): p. 1470-1477.
4. Antunes, J., et al., Diversity of plant growth-promoting bacteria associated with sugarcane. Genetics and Molecular Research, 2017. 16(2).
5. Boyer, J.S., Plant productivity and environment. Science, 1982. 218 (4571): p. 443-448.
6. Esmaeili, N., et al., Co-overexpression of *AVP1* and *OsSIZ1* in *Arabidopsis* substantially enhances plant tolerance to drought, salt, and heat stresses. Scientific Reports, 2019. 9(1): p. 7642.

7. Wang, W., B. Vinocur, and A. Altman, Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 2003. 218(1): p. 1-14.
8. Dita, M.A., et al., Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. Euphytica, 2006. 147: p. 1-24.
9. Kumar, K., et al., Genetically modified crops: current status and future prospects. Planta, 2020. 251: p. 1-27.
10. Rivero, R.M., et al., Developing climate resilient crops: improving plant tolerance to stress combination. The Plant Journal, 2022. 109(2): p. 373-389.
11. Rattana, K., Callus induction, regeneration and transformation of sugarcane (*Saccharum officinarum L.*) with chitinase gene using particle bombardment. African Journal of Biotechnology, 2012. 11(24).
12. Ghulam Raza, K.A., Zahid Mukhtar, Shahid Mansoor, Muhammad Arshad and a.S. Asad, The response of sugarcane (*Saccharum officinarum L*). African Journal of Biotechnology, 2010. 9(51): p. 8739-8747.
13. Chinnusamy, V., J. Zhu, and J.-K. Zhu, Cold stress regulation of gene expression in plants. Trends in Plant Science, 2007. 12(10): p. 444-451.
14. Reguera, M., Z. Peleg, and E. Blumwald, Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops. Biochimica et Biophysica Acta - Gene Regulatory Mechanisms, 2012. 1819(2): p. 186-194.
15. Van Emon, J.M., The omics revolution in agricultural research. Journal of Agricultural and Food Chemistry, 2016. 64(1): p. 36-44.
16. Umezawa, T., et al., Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Current Opinion in Biotechnology, 2006. 17(2): p. 113-122.
17. Mittler, R., Abiotic stress, the field environment and stress combination. Trends in Plant Science, 2006. 11(1): p. 15-19.
18. Hu, H. and L. Xiong, Genetic engineering and breeding of drought-resistant crops. Annual Review of Plant Biology, 2014. 65: p. 715-741.
19. Sengar, K., Biotechnology to Enhance sugarcane productivity and stress tolerance. CRC Press, 2018.
20. Organization, W.H., Diet, nutrition, and the prevention of chronic diseases. Report of a joint WHO/FAO expert consultation, World Health Organization, 2003.916.
21. James, C., The activities of the international service for the acquisition of agribiotech applications (ISAAA) in crop biotechnology transfer. Journal of Science of Food and Agriculture, 2001. 81(9): p. 813-821.
22. Rao, K.M., A.S. Raghavendra, and K.J. Reddy, Physiology and molecular biology of stress tolerance in plants. Springer Science and Business Media, 2006.
23. Abdelraheem, A., et al., Progress and perspective on drought and salt stress tolerance in cotton. Industrial Crops and Products, 2019. 130: p. 118-129.
24. Pitman, M.G. and A. Läuchli, Global impact of salinity and agricultural ecosystems in salinity. Environment Plants Molecules, 2002. p. 3-20.
25. Rhoades, J. and J. Loveday, Salinity in irrigated agriculture. Agronomy, 1990(30): p. 1089-1142.
26. Dionisio-Sese, M.L. and S. Tobita, Antioxidant responses of rice seedlings to salinity stress. Plant Science, 1998. 135(1): p. 1-9.
27. Zhu, J.-K., Plant salt tolerance. Trends in Plant Science, 2001. 6(2): p. 66-71.
28. Lv, S., et al., H+-pyrophosphatase from S alicornia europaea confers tolerance to simultaneously occurring salt stress and nitrogen deficiency in *Arabidopsis* and wheat. Plant, Cell and Environment, 2015. 38(11): p. 2433-2449.

29. Kim, J.-J., et al., Overexpression of a proton pumping gene *OVP1* enhances salt stress tolerance, root growth and biomass yield by regulating ion balance in rice (*Oryza sativa L.*). Environmental and Experimental Botany, 2020. 175: p. 104033.
30. Kumar, T., et al., Genetic improvement of sugarcane for drought and salinity stress tolerance using *Arabidopsis* vacuolar pyrophosphatase (AVP1) gene. Molecular Biotechnology, 2014. 56: p. 199-209.
31. Pasapula, V., et al., Expression of an *Arabidopsis* vacuolar H+-pyrophosphatase gene (AVP1) in cotton improves drought-and salt tolerance and increases fibre yield in the field conditions. Plant Biotechnology Journal, 2011. 9(1): p. 88-99.
32. Qin, H., et al., Expression of the Arabidopsis vacuolar H+-pyrophosphatase gene *AVP1* in peanut to improve drought and salt tolerance. Plant Biotechnology Reports, 2013. 7: p. 345-355.
33. Yarra, R. and P. Kirti, Expressing class I wheat NHX (*TaNHX2*) gene in eggplant (*Solanum melongena L.*) improves plant performance under saline condition. Functional and Integrative Genomics, 2019. 19: p. 541-554.
34. Carr, M. and J.W. Knox, The water relations and irrigation requirements of sugar cane (*Saccharum officinarum*): a review. Experimental Agriculture, 2011. 47(1): p. 1-25.
35. Flexas, J. and H. Medrano, Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. Annals of Botany, 2002. 89(2): p. 183-189.
36. Turner, N.C. and P.l.J. Kramer. Adaptation of plants to water and high temperature stress. Library of Congress Cataloging in Publication Data, 1980.
37. Brown, K.F., et al., Effect of drought on growth and water use of sugar beet. The Journal of Agricultural Science, 1987. 109(3): p. 421-435.
38. Dos Santos, C.M., et al., Water relations and osmolite accumulation related to sugarcane yield under drought stress in a tropical climate. International Journal of Plant Production, 2019.
39. Liang, C., Genetically modified crops with drought tolerance: achievements, challenges, and perspectives. Drought stress tolerance in plants. Molecular and Genetic Perspectives, 2016. 2: p. 531-547.
40. Shinozaki, K. and K. Yamaguchi-Shinozaki, Gene networks involved in drought stress response and tolerance. Journal of Experimental Botany, 2007. 58 (2): p. 221-227.
41. Ravikumar, G., et al., Stress-inducible expression of *AtDREB1A* transcription factor greatly improves drought stress tolerance in transgenic indica rice. Transgenic Research, 2014. 23: p. 421-439.
42. Bhatnagar-Mathur, P., et al., Stress-inducible expression of *At DREB1A* in transgenic peanut (*Arachis hypogaea L.*) increases transpiration efficiency under water-limiting conditions. Plant Cell Reports, 2007. 26: p. 2071-2082.
43. Pellegrineschi, A., et al., Stress-induced expression in wheat of the *Arabidopsis thaliana DREB1A* gene delays water stress symptoms under greenhouse conditions. Genome, 2004. 47(3): p. 493-500.
44. Hsieh, T.-H., et al., Tomato plants ectopically expressing *Arabidopsis CBF1* show enhanced resistance to water deficit stress. Plant Physiology, 2002. 130(2): p. 618-626.
45. Zandalinas, S.I., et al., Plant responses to climate change: metabolic changes under combined abiotic stresses. Journal of Experimental Botany, 2022. 73(11): p. 3339-3354.
46. Rudorff, B.F.T., et al. Frost damage detection in sugarcane crop using MODIS images and SRTM data. IEEE International Geoscience and Remote Sensing Symposium. 2012.
47. Li, Y., et al., Survey of frost and cold damage on sugarcane production in Guangxi in 2010/2011 milling season and countermeasures. Journal of Southern Agriculture, 2011. 42(1): p. 37-42.

48. Shah, S.H., et al., Piercing and incubation method of in planta transformation producing stable transgenic plants by overexpressing *DREB1A* gene in tomato (*Solanum lycopersicum Mill.*). Plant Cell, Tissue and Organ Culture, 2015. 120(3): p. 1139-1157.
49. Beck, E.H., R. Heim, and J. Hansen, Plant resistance to cold stress: mechanisms and environmental signals triggering frost hardening and dehardening. Journal of Biosciences, 2004. 29(4): p. 449-459.
50. Pinton, R., Z. Varanini, and P. Nannipieri, The rhizosphere as a site of biochemical interactions among soil components, plants, and microorganisms, in The Rhizosphere. CRC Press, 2000. p. 17-34.
51. Fang, M., et al., Bacterial diversity in rhizospheres of nontransgenic and transgenic corn. Applied and Environmental Microbiology, 2005. 71(7): p. 4132-6.
52. Kent, A.D. and E.W. Triplett, Microbial communities and their interactions in soil and rhizosphere ecosystems. Annual Reviews in Microbiology, 2002. 56(1): p. 211-236.
53. Siddiqui, Z.A. and M. Sayeed Akhtar, Effects of antagonistic fungi, plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi alone and in combination on the reproduction of Meloidogyne incognita and growth of tomato. Journal of General Plant Pathology, 2009. 75(2): p. 144.
54. Kang, B.G., et al., Use of plant growth-promoting rhizobacteria to control stress responses of plant roots. Plant Biotechnology Reports, 2010. 4(3): p. 179-183.
55. Nardi, S., et al., Soil organic matter mobilization by root exudates. Chemosphere, 2000. 41(5): p. 653-658.
56. Margetts, B., FAO/WHO launch expert report on diet, nutrition and prevention of chronic diseases. Public Health Nutrition, 2003. 6(4): p. 323-325.
57. Kok, E.J. and H.A. Kuiper, Comparative safety assessment for biotech crops. Trends in Biotechnology, 2003. 21(10): p. 439-444.
58. Sharma, K.K., et al., Development and deployment of transgenic plants: biosafety considerations. In Vitro Cellular and Developmental Biology Plant, 2002. 38(2): p. 106.
59. Majeed, A., et al., Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Frontiers in Microbiology, 2015. 6(198).
60. Kumar, A., B.R. Maurya, and R. Raghuwanshi, Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (*Triticum aestivum L.*). Biocatalysis and Agricultural Biotechnology, 2014. 3(4): p. 121-128.
61. Khan, N., A. Bano, and M.A. Babar, The root growth of wheat plants, the water conservation and fertility status of sandy soils influenced by plant growth promoting rhizobacteria. Symbiosis, 2017. 72(3): p. 195-205.
62. Zaheer, A., et al., Isolation, characterization, and effect of phosphate-zinc-solubilizing bacterial strains on chickpea (*Cicer arietinum L.*) growth. Saudi Journal of Biological Sciences, 2019. 26(5): p.1061-1067.
63. Idris, E.E., et al., Tryptophan dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Molecular Plant Microbe Interactions, 2007. 20(6): p. 619-626.
64. Awais, M., et al., Isolation, characterization and association among phosphate solubilizing bacteria from sugarcane rhizosphere. Cytology and Genetics, 2019. 53(1): p. 86-95.
65. Modi, K. and P. Patel, Isolation and characterization of plant growth promoting rhizobacteria associated with *Saccharum officinarum L*. Current Synthetic and Systems Biology, 2017. 5: p. 2332-0737.

66. Muthukumarasamy, R., et al., Isolation of bacterial strains possessing nitrogen-fixation, phosphate and potassium-solubilization and their inoculation effects on sugarcane. Indian Journal of Experimental Biology. 2017. 55(3): p. 161-70.
67. Prashar, P., N. Kapoor, and S. Sachdeva, Rhizosphere: its structure, bacterial diversity and significance. Reviews in Environmental Science and Biotechnology, 2014. 13(1): p. 63-77.
68. Rossmann, M., et al., Plant Microbiome: Composition and functions in plant compartments. The Brazilian Microbiome, 2011. p. 7-20.
69. Turner, T.R., E.K. James, and P.S. Poole, The plant microbiome. Genome Biology, 2013. 14(6): p. 209.
70. Rastogi, G., et al., Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. The ISME Journal, 2012. 6(10): p. 1812.
71. Bringel, F. and I. Couée, Pivotal roles of phyllosphere microorganisms at the interface between plant functioning and atmospheric trace gas dynamics. Frontiers in Microbiology, 2015. 6: p. 486.
72. Berg, G., et al., The plant microbiome explored: implications for experimental botany. Journal of Experimental Botany, 2015. 67(4): p. 995-1002.
73. Peñuelas, J. and J. Terradas, The foliar microbiome. Trends in Plant Science, 2014. 19(5): p. 278-280.
74. Aleklett, K., M. Hart, and A. Shade, The microbial ecology of flowers: an emerging frontier in phyllosphere research. Botany, 2014. 92(4): p. 253-266.
75. Omori, W.P., et al., Influence of vinasse application in the structure and composition of the bacterial community of the soil under sugarcane cultivation. International Journel Microbiology, 2016. : p. 2349514.
76. Kim, K., et al., An overview of different techniques on the microbial community structure, and functional diversity of plant growth promoting bacteria. Korean Journal of Soil Science and Fertilizer, 2016. 49(2): p. 144-156.
77. Berg, S., et al., Ecological communities are vulnerable to realistic extinction sequences. Oikos, 2015. 124(4): p. 486-496.
78. Knief, C., Analysis of plant microbe interactions in the era of next generation sequencing technologies. Frontiers in Plant Science, 2014. 5: p. 216.
79. Ueda, T., Y. Suga, and T. Matsuguchi, Molecular phylogenetic analysis of a soil microbial community in a soybean field. European Journal of Soil Science, 1995. 46(3): p. 415-421.
80. Osman, J.R., G. Fernandes, and M.S. DuBow, Bacterial diversity of the rhizosphere and nearby surface soil of rice (*Oryza sativa*) growing in the Camargue (France). Rhizosphere, 2017. 3: p. 112-122.
81. Haas, B.J., et al., Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Research, 2011. 21.
82. Stoeck, T., et al., Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Molecular Ecology, 2010. 19 (1): p. 21-31.
83. Ward, D.M., R. Weller, and M.M. Bateson, 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature, 1990. 345(6270): p. 63-65.
84. Corcoll, N., et al., Comparison of four DNA extraction methods for comprehensive assessment of 16S rRNA bacterial diversity in marine biofilms using high-throughput sequencing. FEMS Microbiology Letters, 2017. 364(14): p. fnx139-fnx139.
85. Tennant, R.K., et al., Metagenomic analysis of silage. Journel of Visulized Experiments, 2017(119).

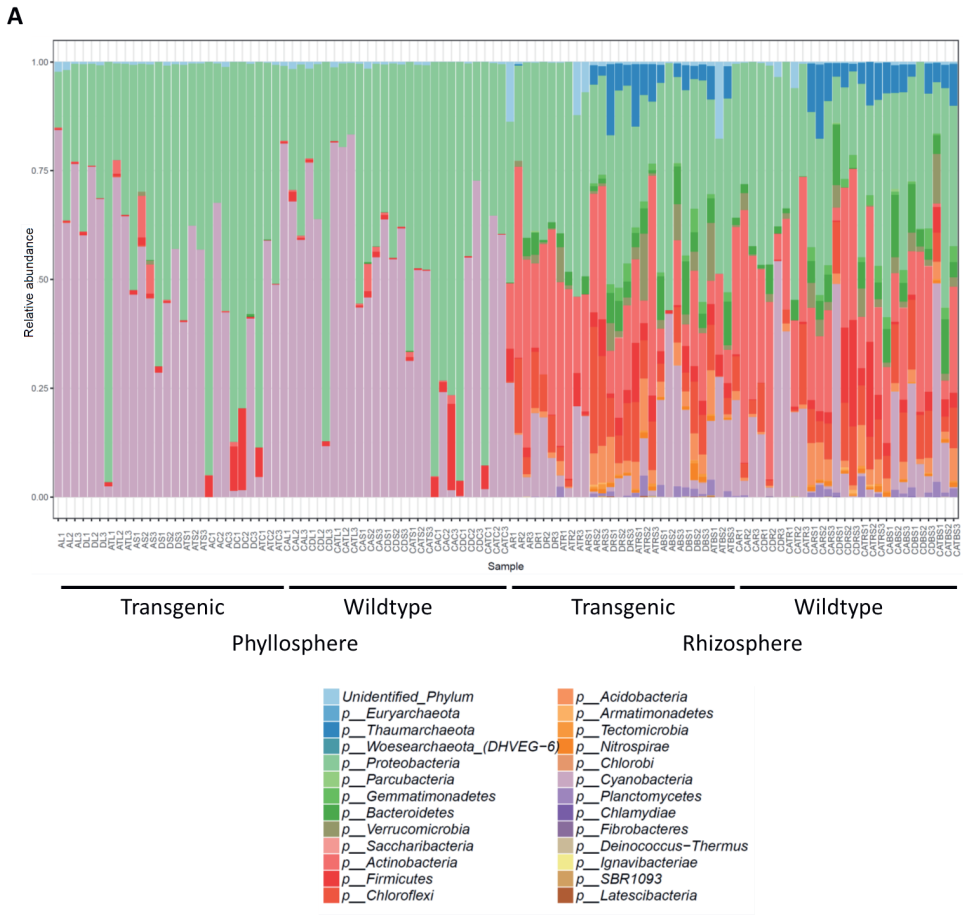
86. Vacher, C., et al., The Phyllosphere: Microbial jungle at the plant–climate interface. Annual Review of Ecology, Evolution and Systematics, 2016. 47(1): p. 1-24.
87. Li, H.-B., et al., Genetic diversity of nitrogen-fixing and plant growth promoting *Pseudomonas* species isolated from sugarcane rhizosphere. Frontiers in Microbiology, 2017. 8: p. 1268.
88. Malik, K.B., Global warming and its impact on cane production under Pakistan conditions. Pakistan Sugar Journal, 2018. 33(4).
89. Caporaso, J.G., et al., Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences, 2011. 108(1): p. 4516-4522.
90. Apprill, A., et al., Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton.Aquatic Microbial Ecology 2015. 75: p. 129-137.
91. Apprill, A., et al., Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquatic Microbial Ecology, 2015. 75: p. 129-137.
92. Ramiro-Garcia, J., et al., NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. F1000 Research, 2016. 5: p. 1791-1791.
93. Edgar, R.C., Search and clustering orders of magnitude faster than BLAST. Bioinformatics, 2010. 26(19): p. 2460-2461.
94. Yilmaz, P., et al., The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Research, 2013. 42(D1): p. D643-D648.
95. Dat, T.T.H., et al., Archaeal and bacterial diversity and community composition from 18 phylogenetically divergent sponge species in Vietnam. Peer J, 2018. 6: p. e4970.
96. Team, R.C., R-A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing, 2014.
97. McMurdie, P.J. and S. Holmes, phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. Plos One, 2013. 8(4): p. e61217.
98. van Wyk, D.A.B., et al., Ecological guild and enzyme activities of rhizosphere soil microbial communities associated with Bt-maize cultivation under field conditions in north west province of south africa. Journal of Basic Microbiology, 2017. 57(9): p. 781-792.
99. Mashiane, R.A., et al., Metagenomic analyses of bacterial endophytes associated with the phyllosphere of a Bt maize cultivar and its isogenic parental line from South Africa. World Journal of Microbiology and Biotechnology, 2017. 33(4): p. 80.
100. Prischl, M., et al., Genetically modified Bt maize lines containing *cry3Bb1*, *cry1A105* or *cry1Ab2* do not affect the structure and functioning of root-associated endophyte communities. Applied Soil Ecology, 2012. 54: p. 39-48.
101. Sun, C., et al., No adverse effects of transgenic maize on population dynamics of endophytic Bacillus subtilis strain B916-gfp. Microbiology Open, 2017. 6(1): p. e00404.
102. Da Silva, D.A.F., et al., Endophytic microbial community in two transgenic maize genotypes and in their near-isogenic non-transgenic maize genotype. BMC Microbiology, 2014. 14(1): p. 332.
103. Giovannetti, M., C. Sbrana, and A. Turrini. The impact of genetically modified crops on soil microbial communities. Biology Forum/Rivista di Biologia. 2005.
104. Sarkar, B., A. Patra, and T. Purakayastha, Transgenic Bt-cotton affects enzyme activity and nutrient availability in a sub-tropical inceptisol. Journal of Agronomy and Crop Science, 2008. 194(4): p. 289-296.

105. Mina, U., et al., An approach for impact assessment of transgenic plants on soil ecosystem. Applied Ecology and Environmental Research, 2008. 6(3): p. 1-19.
106. Fontes, E.M., et al., The environmental effects of genetically modified crops resistant to insects. Neotropical Entomology, 2002. 31(4): p. 497-513.
107. Domingo, J.L., Toxicity studies of genetically modified plants: a review of the published literature. Critical Reviews in Food Science and Nutrition, 2007. 47(8): p. 721-733.
108. Craig, W., et al., An overview of general features of risk assessments of genetically modified crops. Euphytica, 2008. 164(3): p. 853-880.
109. Fresco, L.O. Genetically modified organisms in food and agriculture: where are we? where are we going? Conference on Crop and Forest Biotechnology for the Future, Royal Swedish Academy of Agriculture and Forestry, Faikenberg, Sweden, 2001. 16.
110. Rosenblueth, M. and E. Martínez-Romero, Bacterial endophytes and their interactions with hosts. Molecular Plant Microbe Interactions, 2006. 19(8): p. 827-837.
111. Ma, B., et al., Shifts in diversity and community structure of endophytic bacteria and archaea across root, stem and leaf tissues in the common reed, *Phragmites australis*, along a salinity gradient in a marine tidal wetland of northern China. Antonie Van Leeuwenhoek, 2013. 104(5): p. 759-768.
112. Devare, M., C. Jones, and J. Thies, Effect of *Cry3Bb* transgenic corn and tefluthrin on the soil microbial community. Journal of Environmental Quality, 2004. 33(3): p. 837-843.
113. Shen, R.F., H. Cai, and W.H. Gong, Transgenic Bt cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. Plant and Soil, 2006. 285(1): p. 149-159.
114. Saxena, D. and G. Stotzky, *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. Soil Biology and Biochemistry, 2001. 33(9): p. 1225-1230.
115. Nimusiima, J., et al., Transgenic banana plants expressing *Xanthomonas* wilt resistance genes revealed a stable non-target bacterial colonization structure. Scientific Reports, 2015. 5: p. 18078.
116. Dunfield, K.E. and J.J. Germida, Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica* napus. FEMS Microbiology Ecology, 2001. 38(1): p. 1-9.
117. Siciliano, S. and J. Germida, Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic B. napus cv. Excel and B. rapa cv. Parkland. FEMS Microbiology Ecology, 1999. 29(3): p. 263-272.
118. Andreote, F.D., et al., Transgenic tobacco revealing altered bacterial diversity in the rhizosphere during early plant development. Antonie Van Leeuwenhoek, 2008. 93(4): p. 415-424.
119. Wang, Y., et al., Endophytic communities of transgenic poplar were determined by the environment and niche rather than by transgenic events. Frontiers in Microbiology, 2019. 10(588).
120. Zhang, L., et al., Analysis of extracellular polymeric substances (EPS) and ciprofloxacin-degrading microbial community in the combined Fe-C micro-electrolysis-UBAF process for the elimination of high-level ciprofloxacin. Chemosphere, 2018. 193: p. 645-654.
121. Zak, J.C., et al., Functional diversity of microbial communities: a quantitative approach. Soil Biology and Biochemistry, 1994. 26(9): p. 1101-1108.

122. Garland, J.L. and A.L. Mills, Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Applied and Environmental Microbiology, 1991. 57(8): p. 2351-2359.
123. Schmalenberger, A. and C.C. Tebbe, Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and comparison to its non transgenic cultivar Bosphore. FEMS Microbiology Ecology, 2002. 40(1): p. 29-37.
124. Dunfield, K.E. and J.J. Germida, Impact of genetically modified crops on soil and plant associated microbial communities. Journal of Environmental Quality, 2004. 33(3): p. 806-815.
125. Brimecombe, M.J., F.A. De Leij, and J.M. Lynch, The effect of root exudates on rhizosphere microbial populations. The rhizosphere, biochemistry and organic substances. Soil Plant Interface (eds R. Pinton, Z. Varini, P. Nannipieri), 2000. p. 95-140.
126. Lozupone, C.A. and R. Knight, Global patterns in bacterial diversity. Proceedings of the National Academy of Sciences, 2007. 104(27): p. 11436-11440.
127. Thiem, D., et al., How does salinity shape bacterial and fungal microbiomes of Alnus glutinosa roots? Frontiers in Microbiology, 2018. 9: p. 651.
128. Yaish, M.W., et al., Impact of soil salinity on the structure of the bacterial endophytic community identified from the roots of caliph medic (Medicago truncatula). Plos One, 2016. 11(7): p. e0159007.
129. Hardoim, P.R., L.S. van Overbeek, and J.D. van Elsas, Properties of bacterial endophytes and their proposed role in plant growth. Trends in Microbiology, 2008. 16(10): p. 463-471.
130. Compant, S., C. Clément, and A. Sessitsch, Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry, 2010. 42(5): p. 669-678.
131. Fang, M., et al., Bacterial diversity in rhizospheres of nontransgenic and transgenic corn. Applied and Environmental Microbiology, 2005. 71(7): p. 4132-4136.
132. Montanari-Coelho, K.K., et al., Endophytic bacterial microbiome associated with leaves of genetically modified (*AtAREB1*) and conventional (BR 16) soybean plants. World Journal of Microbiology and Biotechnology, 2018. 34(4): p. 56.
133. Koskella, J. and G. Stotzky, Larvicidal toxins from *Bacillus thuringiensis* subspp. kurstaki, morrisoni (strain tenebrionis), and israelensis have no microbicidal or microbiostatic activity against selected bacteria, fungi, and algae in vitro. Canadian Journal of Microbiology, 2002. 48(3): p. 262-267.
134. Liu, B., et al., Effects of transgenic plants on soil microorganisms. Plant and soil, 2005. 271(1-2): p. 1-13.
135. Motavalli, P., et al., Impact of genetically modified crops and their management on soil microbially mediated plant nutrient transformations. Journal of Environmental Quality, 2004. 33(3): p. 816-824.
136. Weinert, N., et al., Effects of genetically modified potatoes with increased zeaxanthin content on the abundance and diversity of rhizobacteria with in vitro antagonistic activity do not exceed natural variability among cultivars. Plant and Soil, 2010. 326(1-2): p. 437-452.
137. Balachandar, D., et al., Impact of transgenic Bt-cotton on the diversity of pink-pigmented facultative methylotrophs. World Journal of Microbiology and Biotechnology, 2008. 24(10): p. 2087-2095.
138. Andreote, F.D., T. Gumiere, and A. Durrer, Exploring interactions of plant microbiomes. Scientia Agrícola, 2014. 71(6): p. 528-539.

139. Redford, A.J., et al., The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environmental Microbiology, 2010. 12(11): p. 2885-2893.
140. Knief, C., et al., Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. The ISME Journal, 2012. 6(7): p. 1378-1390.
141. Dohrmann, A.B., et al., Importance of rare taxa for bacterial diversity in the rhizosphere of Bt-and conventional maize varieties. The ISME Journal, 2013. 7(1): p. 37.
142. Philippot, L., et al., Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology, 2013. 11(11): p. 789.
143. Barriuso, J., J.R. Valverde, and R.P. Mellado, Effect of *Cry1Ab* protein on rhizobacterial communities of Bt-maize over a four-year cultivation period. Plos one, 2012. 7(4): p. e35481.
144. Newman, M.M., et al., Glyphosate effects on soil rhizosphere-associated bacterial communities. Science of the Total Environment, 2016. 543: p. 155-160.
145. Vorholt, J.A., Microbial life in the phyllosphere. Nature Reviews Microbiology, 2012. 10(12): p. 828.
146. Dini-Andreote, F., et al., Dynamics of bacterial community succession in a salt marsh chronosequence: evidences for temporal niche partitioning. The ISME Journal, 2014. 8(10): p. 1989.
147. Bhandari, V. and R.S. Gupta, Molecular signatures for the phylum (class) *Thermotogae* and a proposal for its division into three orders (*Thermotogales, Kosmotogales* ord. nov. and *Petrotogales* ord. nov.) containing four families (*Thermotogaceae*, *Fervidobacteriaceae* fam. nov., *Kosmotogaceae* fam. nov. and *Petrotogaceae* fam. nov.) and a new genus *Pseudothermotoga* gen. nov. with five new combinations. Antonie van Leeuwenhoek, 2014. 105(1): p. 143-168.
148. Strickland, M.S., et al., Testing the functional significance of microbial community composition. Ecology, 2009. 90(2): p. 441-451.
149. Dinesh, R., et al., Endophytic actinobacteria: diversity, secondary metabolism and mechanisms to unsilence biosynthetic gene clusters. Critical Reviews in Microbiology, 2017. 43(5): p. 546-566.
150. Nimnoi, P., N. Pongsilp, and S. Lumyong, Endophytic *actinomycetes* isolated from Aquilaria crassna Pierre ex Lec and screening of plant growth promoters production. World Journal of Microbiology and Biotechnology, 2010. 26(2): p. 193-203.
151. Li, X., et al., Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar. Soil Biology and Biochemistry, 2014. 68: p. 392-401.
152. Xie, W.-J., J.-M. Zhou, and H.-Y. Wang, [Influence of Cu2+, Cd2+ and cypermethrin on microbial functional diversity in different fertilization soils]. Huan Jing ke Xue= Huanjing kexue, 2008. 29(10): p. 2919-2924.
153. García-Salamanca, A., et al., Bacterial diversity in the rhizosphere of maize and the surrounding carbonate-rich bulk soil. Microbial Biotechnology, 2013. 6(1): p. 36-44.
154. Baumgarte, S. and C.C. Tebbe, Field studies on the environmental fate of the *Cry1Ab* Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. Molecular Ecology, 2005. 14(8): p. 2539-2551.
155. Shen, R.F., H. Cai, and W.H. Gong, Transgenic Bt cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. Plant and Soil, 2006. 285(1-2): p. 149-159.

Supplementary material



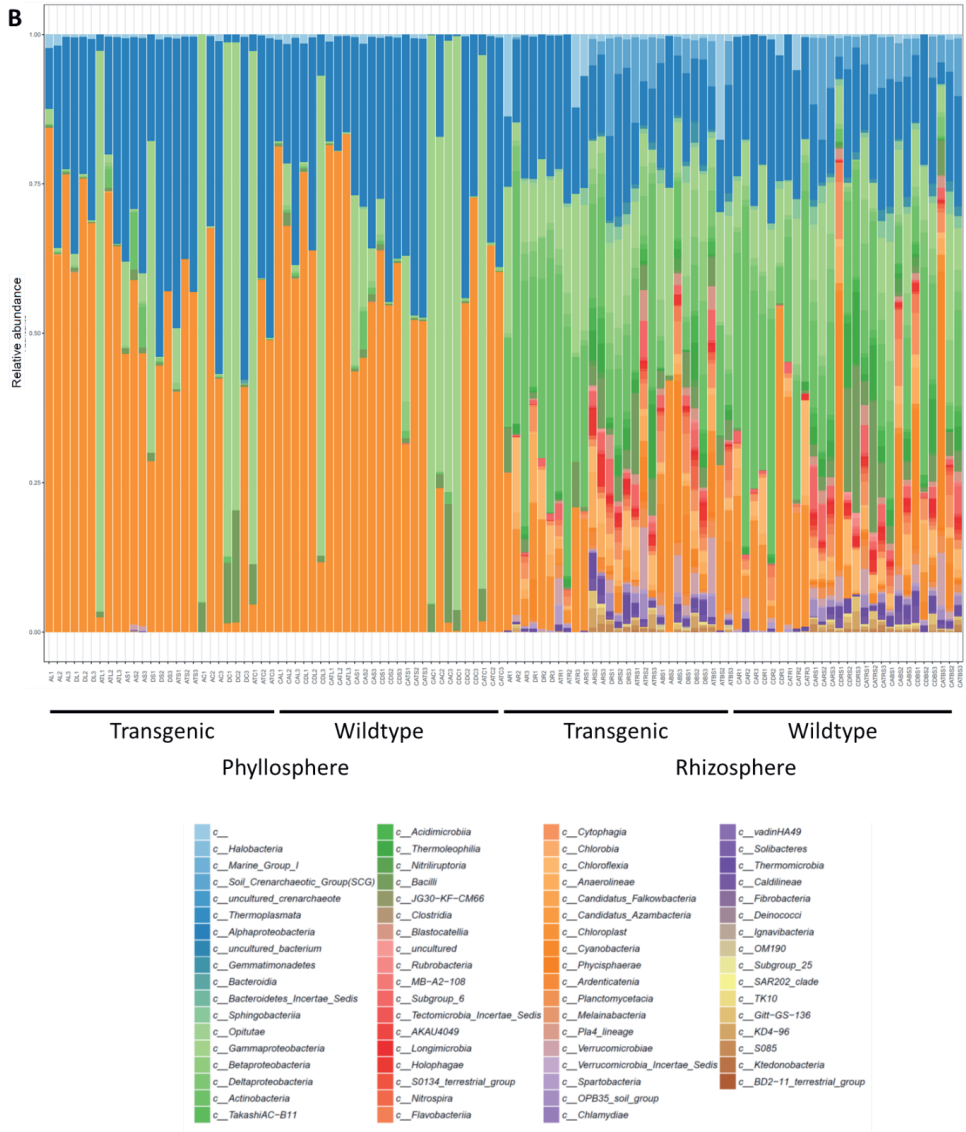


Figure S1. Relative abundance bar plots of microbial composition at phylum (**A**) and class (**B**) level of phyllosphere and rhizosphere samples from transgenic and non-transgenic sugarcane plants. For sample names, abbreviated codes were used according to the isolation source. "C" in the left-most position indicates control (wildtype) plants. **Genes: D**, DREB1A; **AT** AtNHX1; **A**, AVP1**. Rhizosphere parts: R**, Root; **RS**, Rhizosphere soil; **BS,** Bulk soil. **Phyllyosphere parts**: **L**, Leaf; **S**, Stem; **C**, Cane.

Chapter 6

General Discussion

In Pakistan sugarcane is a very important crop from an agricultural, industrial and societal perspective. The research described in this thesis was conducted to address current problems in sugarcane production, and to provide new knowledge that can potentially be leveraged to increase sugarcane yield in the framework of more sustainable agriculture. In Pakistan sugarcane production is currently facing many problems, and both the rural farming community and the industry are striving for more sustainable existence and growth. There are a number of reasons for lower cane yield, and among those are the cultivation of low yield varieties and abiotic stress. Major abiotic stress factors that affect plant growth and that e.g. include salinity, drought and frost, resulted in the wasting of a major area of Pakistan. This also continues to lead to increasingly reduced areas available to agriculture, and as a consequence, increased the hunger and unemployment for many farmers. To this end, the development of new varieties plays a key role in both increasing sugar yield as well as sustainability of cane production, whereas the use of unapproved, inferior quality cane varieties affect sugarcane production and environmental health. Many efforts are being made in Pakistan to increase cane production by introducing high yielding varieties and adoption of improved crop production through application of genetic engineering techniques. Recent years have seen a rapid adoption of genetically modified (GM) crops by farmers in Pakistan, however, public discussions and controversies remain with respect to risks and benefits of these crops. Therefore, the research described in this thesis was conducted to contribute to biosafety studies of transgenic crops and their effects on soil organisms, as well as to isolate and characterize microorganisms that can be used for improving plant health and sustainable production.

In this thesis we discussed that sustainable agricultural practices are the answer to multifaceted problems. One of the main objectives of the present study was the risk assessment of the soil environment, and in particular associated microbial communities, by comparing the diversity of microbiota from rhizosphere and phyllosphere of transgenic and their wild type sugarcane plants by using cultivation and molecular techniques (chapters 4 and 5). Furthermore, research described in this thesis focused on bacteria that can act like biofertilizer without harmful effects on the environment and as alternative for chemical fertilizers (chapters 2 and 3). We isolated and characterized a number of bacterial strains that showed positive effects on crop growth and thus can be considered as biofertilizer of sugarcane.

Numerous independent academic groups and regulatory bodies have reviewed the evidence about risks, concluding that commercialized GM crops are safe for human consumption and the environment [1, 2]. There is a large body of studies showing that GM crops cause benefits in terms of higher yields and cost savings in agricultural production and welfare gains among

adopting farm households [2-5]. In countries where GMO technology has been accepted, the land area planted for commercial production of transgenic plants is increasing every year [6]. Nevertheless, the effects of transgenic plants on the soil and ecosystem function should be carefully evaluated before the release of any transgenic plant variety [7-10]. Previous studies showed that plants have a profound effect on the abundance, diversity and activity of soil microorganisms living in close proximity with their roots in a soil zone defined as the rhizosphere. Among these, plant growth promoting bacteria (PGPB) represent one of the best characterized functional groups of rhizobacteria known for playing a significant role in plant health and plant development [11-13]. Previous studies showed that plant genetic transformation might alter these fundamental microbial processes. To this end, rhizobacteria have been defined as good indicator organisms and have been studied to assess the general impact of GMO plants on the soil environment [14-18]. In previous studies possible impacts on rhizosphere microbial communities were a major concern because little is known of diversity-function relationships of microorganisms with transgenic plants in the soil environment [19, 20].

Research described in chapters 2 and 3 focused on the isolation, identification and functional characterization of potential PGPB and their role in plant growth and sustainability. Strains were isolated from the rhizosphere of sugarcane on nutrient media, initially characterized on the basis of indole acetic acid production (IAA), phosphate solubilization and biological nitrogen fixation (BNF) in the laboratory, identified based on their 16S ribosomal RNA (rRNA) gene sequence, and tested as plant growth promoting bacteria using *in vitro* and *in vivo* inoculation experiments.

Use of PGPB with the aim of improving nutrient availability and plant growth promotion has been studied during the past few decades. PGPB are specific microorganisms, which benefit plant growth with minimally adverse effects on the environment. Plant growth promoting traits that were considered included IAA production. IAA is an important hormone for root proliferation and root hair formation, as well as for growth of young leaves. IAA synthesized by bacteria plays a major role in root growth, cell elongation, tissue differentiation, plant growth promotion and responses to light and gravity. Phosphate solubilization by bacteria is another important mechanism utilized for plant growth promotion [21]. Phosphate solubilization is one of the major traits in rhizosphere bacteria. Phosphorus is one of the essential macronutrients for crops and usually applied as phosphate fertilizer. Finally, microorganisms capable of BNF are a vital group of PGPB which help in biological nitrogen fixation from the atmosphere. In BNF nitrogenase enzyme converts nitrogen from air to ammonia.

In chapter 2 we selected the four best strains after initial screening. Strains were affiliated to four different genera well known for containing members that act as PGPB, including *Bacillus*, *Pseudomonas*, *Paenibacillus* and *Enterobacter*. We could confirm their plant growth promoting traits *in vitro* in micropropagation experiments and *in vivo* in micro plot experiments. All four strains could be shown to enhance plant growth parameters such as root length, shoot length,

biomass, fresh and dry weight of roots and shoots and root development of sugarcane plantlets after they were inoculated with one of the four strains.

While in chapter 2, isolates were tested for their plant growth promoting traits in sugarcane, in chapter 3, bacterial strains that were isolated from sugarcane were tested as inoculum in wheat crop. A total of 15 strains were selected and were identified as members of three major genera of PGPB, including *Bacillus*, *Pseudomonas* and *Burkholderia*. All 15 strains were shown to enhance at least two of five measured parameters of plant growth, with *Bacillus* sp. strain DR4 showing best results. Overall, *Pseudomonas* spp. strains showed lower plant growth improvement as compared to strains affiliated with the two other genera. Results of our research also well fits with other previous studies. Previous studies also showed that production of IAA is a natural ability of some bacteria. IAA can also be a signalling molecule in bacteria and therefore can have a direct effect on plant physiology [22]. It has been estimated that more than 80% of bacteria isolated from the rhizosphere can produce the plant growth regulator IAA [23, 24]. Similarly, recent studies also showed that phosphate solubilizing bacteria (PSB) are a good alternative to chemical phosphorus fertilizer [25]. Previous studies are also in line with our findings with respect to the identity of isolates with plant growth promoting traits. To that end, species of *Bacillus* are common inhabitants among the resident microbiota of various species of plants including sugarcane, where they play an important role in plant protection and growth promotion [26, 27]. In another study sugarcane and some other crops were cultured after inoculation with a commercial product of *Bacillus* effective against soil borne pathogens [28]. *Pseudomonas* spp. strains are ubiquitous bacteria in agricultural soils and have many traits that make them well suited as PGPB. Specific strains of *Pseudomonas* spp. used as seed inoculants on crop plants (potato, radish, sunflower and sugar beet) caused statistically significant yield improvement in field tests [29].

Whereas in chapter 2 & 3 research focused on potential PGPB and their effect on plant growth as biofertilizer and comparison between different groups of PGPB, the experiments described in chapters 4 & 5 mainly addressed the bacterial diversity of transgenic and wild type sugarcane varieties by using both cultivation and molecular techniques, in order to assess possible effects of transgenic crops on rhizosphere and phyllosphere. Such information is relevant to risk assessment of transgenic crops, which is essential for safeguarding the adoptability and cultivation of genetically modified crops [30]. A previous study showed that risk assessment studies are essentially required before releasing any GMO variety because some transgenic plants (e.g., insect resistant) are known to affect the rhizosphere environment through root exudates, which consequently affects the growth of microorganisms in the rhizosphere [31].

In chapter 4 we investigated the identity of bacteria isolated from different parts of the rhizosphere and phyllosphere of transgenic and wild type sugarcane using cultivation on nutrient agar. A total of 108 isolates were obtained and could be classified as members of 20 different genera, including, among others, members of the genera *Bacillus*, *Staphylococcus*, *Burkholderia*, *Pseudomonas*, *Pseudoxanthomonas*, *Paenibacillus*, *Chryseobacterium*,

Ralstonia, *Brevibacterium*, *Curtobacterium*, *Pantoea* and *Enterobacter* in both transgenic and wild type sugarcane crop. Overall, members of the genus *Bacillus* were most frequently isolated. Only minor differences were observed with respect to cultured endophyte diversity when comparing transgenic and non-transgenic sugarcane varieties. This study also relates to many previously conducted studies, including the types of potential PGPB that can be isolated from the rhizosphere of wheat, rice and sugarcane [32]. Bacterial strains of the genus *Bacillus* colonize the plant root zone and affect plant growth by employing different mechanisms [33, 34]. The present study suggested that *Bacillus* spp. strains are more frequent colonizers in the rhizosphere of transgenic and wild type sugarcane plants more frequently as compared to other rhizosphere bacteria. Occurrence of *Bacillus* in the rhizosphere of sugarcane has been reported in Pakistani soil, and several studies reinforced the presence and abundance of *Bacillus* spp. in the rhizosphere of various crops [35, 36]. Studies on bacterial diversity of transgenic tobacco showed that *Bacillus* spp. were commonly present in transgenic and wild type rhizosphere [37]. Presence of bacterial strains belonging to the genus *Paenibacillus* has been shown in the rhizosphere of different crops [38, 39]. High efficiency in host root colonization of plant rhizosphere by *Pseudomonas* spp. has been reported resulting in improved crop yield [40, 41]. A previous study also showed that *Burkholderia* is also a common colonizer in the rhizosphere of sugarcane as PGPB identified by cultivation [42, 43]. Nevertheless, we have to keep in mind that cultivation-based approaches remain biased with respect to culturability of different members of environmental microbiomes. To this end, culture-independent molecular methods are being applied for the more comprehensive assessment of microbial diversity by amplifying and sequencing specific marker genes such as the 16S rRNA gene, from environmental DNA samples [44, 45]. This notion was reinforced by the fact that we observed fewer genera by cultivation (chapter 4) as compared to molecular characterization of rhizosphere and phyllosphere microbial communities (chapter 5). In the rhizosphere of transgenic and wild type sugarcane crop, we observed *Proteobacteria, Parcubacteria, Bacteroidetes, Saccharibacteria, Actinobacteria, Firmicutes* and *Chloroflexi* as most predominant among the detected phyla. A lower number of predominant phyla was observed in the phyllosphere of transgenic and wild type sugarcane, including *Saccharibacteria, Actinobacteria, Firmicutes, Chloroflexi,* and *Proteobacteria*. Previous research supports this study as detection of these phyla in different soils has been reported through amplicon sequence analysis [46, 47]. Major phyla detected in chapter 5 have been reported in a variety of environments. *Proteobacteria* include metabolically versatile and genetically diverse strains, comprising the largest fraction of the bacterial community in soil ecosystems including the rhizosphere [48]. Dominant populations of *Actinobacteria* have been reported on maize roots in a tropical soil [49]. It was also found that members of the *Bacteroidetes* constituted dominant populations in the rhizosphere of maize and canola. Members of these bacterial groups are capable of degrading complex macromolecules, thus contributing to the turnover of carbon, nitrogen and phosphorus [50]. It has been reported that the changes in the microbial community structure associated with genetically modified plants were temporary and did not persist into the next season [51], in line with the limited

differences between microbial communities associated with the phyllosphere microbiota of transgenic and wildtype sugarcane varieties observed in our study.

Concluding remarks and future perspective

In the studies described in chapters 4 and 5 we found that transgenic sugarcane had no adverse effect on microbial diversity and relative abundance of different populations present in rhizosphere and phyllosphere of transgenic and wild type sugarcane. This further opens avenues for the application of transgenic varieties with increased tolerance towards different abiotic stress factors prevalent in areas with high concentrations of salts, water deficiency and frost in Pakistan. Thus, these transgenic sugarcane varieties can help in (re-)utilizing affected land areas. We also identified major groups of PGPB by using cultivation based approaches, initial screening and characterization as PGPB. Strains of these major groups of PGPB such as *Bacillus, Pseudomonas* and *Burkholderia,* and some other strains such as *Enterobacter* and *Paenibacillus* showed promising results in micropropagation and field experiments. Future studies may focus on detailed experiments in laboratory and field experiments under different condition and different titres to support approval and application of these PGPB as efficient and beneficial alternatives to chemical fertilizers. Future studies should also focus on direct comparison of these PGPB with chemical fertilizer to check out their abilities and efficiency in fields for calculation of yield production. Such studies should also include metagenomic and other omics analyses in order to facilitate the generation of new knowledge on strain-resolved involvement of individual microbial populations in plant growth promoting traits.

References

1. DeFrancesco, L., How safe does transgenic food need to be? Nature Biotechnology, 2013. 31(9): p. 794.
2. Pray, C.E., et al., Five years of Bt cotton in China–the benefits continue. The Plant Journal, 2002. 31(4): p. 423-430.
3. Qaim, M. and S. Kouser, Genetically modified crops and food security. Plos One, 2013. 8(6): p. e64879.
4. Huang, J., et al., Genetically modified rice, yields, and pesticides: assessing farm-level productivity effects in China. Economic Development and Cultural Change, 2008. 56(2): p. 241-263.
5. Qaim, M. and G. Traxler, Roundup Ready soybeans in Argentina: farm level and aggregate welfare effects. Agricultural Economics, 2005. 32(1): p. 73-86.
6. Khush, G.S., Genetically modified crops: the fastest adopted crop technology in the history of modern agriculture. Agriculture and Food Security, 2012. 1(1): p. 1-2.
7. Jepson, P., B. Croft, and G. Pratt, Test systems to determine the ecological risks posed by toxin release from *Bacillus thuringiensis* genes in crop plants. Molecular Ecology, 1994. 3(1): p. 81-89.
8. Akhtar, M., M. Ashraf, and M. Akhtar, Sugarcane yield gap analysis: Future options for Pakistan. Science Technology and Development I, 2003: p. 38-48.

9. Chattha, M., Ehsanullah (2003) Agro-quantitative and qualitative performance of different cultivars and strains of sugarcane (*Saccharum officinarum, L.*). Pakistan Sugar Journal, 1806. 18(6): p. 2-5.
10. Mian, A. Sugarcane variety composition in Pakistan. in Proceedings of Seminars on Agriculture. Organized by Pakistan Society of Sugar Technologists held at Faisalabad on. 2006. p.107-121.
11. Pinton, R., Z. Varanini, and P. Nannipieri, The rhizosphere: biochemistry and organic substances at the soil-plant interface. CRC Press, 2007.
12. Kent, A.D. and E.W. Triplett, Microbial communities and their interactions in soil and rhizosphere ecosystems. Annual Reviews in Microbiology, 2002. 56(1): p. 211-236.
13. Siddiqui, Z.A. and M.S. Akhtar, Synergistic effects of antagonistic fungi and a plant growth promoting rhizobacterium, an arbuscular mycorrhizal fungus, or composted cow manure on populations of *Meloidogyne incognita* and growth of tomato. Biocontrol Science and Technology, 2008. 18(3): p. 279-290.
14. Dunfield, K.E. and J.J. Germida, Impact of genetically modified crops on soil-and plant-associated microbial communities. Journal of Environmental Quality, 2004. 33(3): p. 806-815.
15. Chattha, A., et al. Prospects of sugar industry & sugarcane cultivation in Punjab. In Proceedings of 41th Annual Convention of Pakistan Society of Sugar Technologists held at Rawalpindi on. 2006. p.173-181.
16. Bashir, G.M., Physio-agronomic studies on flat virus pit plantation of autumn and spring sugarcane (*Saccharum QlficinolUm L.*). University of Agriculture Faisalabad Pakistan, 1995.
17. Gilbert, N., Case studies: A hard look at GM crops. Nature News, 2013. 497(7447): p. 24.
18. Andreasen, M., GM food in the public mind–facts are not what they used to be. Nature Biotechnology, 2014. 32(1): p. 25.
19. Angle, J.S., Release of transgenic plants: biodiversity and population-level considerations. Molecular Ecology, 1994. 3(1): p. 45-50.
20. Bruinsma, M., G. Kowalchuk, and J. Van Veen, Effects of genetically modified plants on microbial communities and processes in soil. Biology and Fertility of Soils, 2003. 37(6): p. 329-337.
21. Liu, B., et al., *Bacillus wuyishanensis* sp. nov., isolated from rhizosphere soil of a medical plant, Prunella vulgaris. International Journal of Systematic and Evolutionary Microbiology, 2015. 65(Pt_7): p. 2030-2035.
22. Spaepen, S., J. Vanderleyden, and R. Remans, Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiology Reviews, 2007. 31(4): p. 425-448.
23. Glick, B.R., Beneficial plant-bacterial interactions. Heidelberg Springer, 2015. p.383.
24. Chen, Y., et al., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Applied Soil Ecology, 2006. 34(1): p. 33-41.
25. Rosa, P.A.L., et al., Inoculation with plant growth-promoting bacteria to reduce phosphate fertilization requirement and enhance technological quality and yield of sugarcane. Microorganisms, 2022. 10(1): p. 192.
26. Berg, G., Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology, 2009. 84: p. 11-18.
27. Lehman, R.M., et al., Understanding and enhancing soil biological health: the solution for reversing soil degradation. Sustainability, 2015. 7(1): p. 988-1027.

28. Kavitha, K., et al., Development of bioformulations of antagonistic bacteria for the management of damping off of Chilli (*Capsicum annuum L*). Archives of Phytopathology and Plant Protection, 2005. 38(1): p. 19-30.
29. Vovides, A.G., et al., Nitrogen fixation in preserved, reforested, naturally regenerated and impaired mangroves as an indicator of functional restoration in mangroves in an arid region of Mexico. Restoration Ecology, 2011. 19(201): p. 236-244.
30. Lin, L., et al., Effects of seasonal snow cover on soil nitrogen transformation in alpine ecosystem: A review. Yingyong Shengtai Xuebao, 2011. 22(8).
31. Mina, U., et al., An approach for impact assessment of transgenic plants on soil ecosystem. Applied Ecology and Environmental Research, 2008. 6(3): p. 1-19.
32. Mehnaz, S., et al., Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. Canadian Journal of Microbiology, 2001. 47(2): p. 110-117.
33. Zhang, H., et al., Knockout of ituD gene of *Bacillus subtilis* S44 strain and impact of its biocontrol effect to cotton rhizoctoniosis. Plant Pathology Journal (Faisalabad), 2014. 13(2): p. 125-132.
34. Guo, Q., et al., Fengycin produced by *Bacillus subtilis* NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. Microbiological Research, 2014. 169(7-8): p. 533-540.
35. Upadhyay, S.K., D.P. Singh, and R. Saikia, Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline condition. Current Microbiology, 2009. 59: p. 489-496.
36. Yasmin, S., et al., Plant-beneficial rhizobacteria for sustainable increased yield of cotton with reduced level of chemical fertilizers. Pakistan Journal of Botany, 2013. 45(2): p. 655-662.
37. Fernando, W., et al., Biological control of Sclerotinia sclerotiorum (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Protection, 2007. 26(2): p. 100-107.
38. Nguyen, X.H., et al., Isolation of butyl 2, 3-dihydroxybenzoate from *Paenibacillus elgii* HOA73 against *Fusarium oxysporum f*. sp. lycopersici. Journal of Phytopathology, 2015. 163(5): p. 342-352.
39. Pindi, P.K., T. Sultana, and P.K. Vootla, Plant growth regulation of Bt-cotton through *Bacillus* species. 3 Biotech, 2014. 4: p. 305-315.
40. Joshi, P. and A. Bhatt, Diversity and function of plant growth promoting rhizobacteria associated with wheat rhizosphere in North Himalayan region. International Journal of Environmental Sciences, 2011. 1(6): p. 1135-1143.
41. Khalid, A., M. Arshad, and Z. Zahir, Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. Journal of Applied Microbiology, 2004. 96(3): p. 473-480.
42. Malviya, M.K., et al., Comparative analysis of sugarcane root transcriptome in response to the plant growth-promoting *Burkholderia anthina* MYSP113. Plos One, 2020. 15(4): p. 0231206.
43. Morgado González, A., D. Espinosa Victoria, and F.C. Gómez Merino, Efficiency of plant growth promoting rhizobacteria (PGPR) in sugarcane. Terra Latinoamericana, 2015. 33(4): p. 321-330.
44. Collavino, M.M., et al., nifH pyrosequencing reveals the potential for location-specific soil chemistry to influence N 2-fixing community dynamics. Environmental Microbiology, 2014. 16(10): p. 3211-3223.

45. Falcón, L.I., E. Escobar-Briones, and D. Romero, Nitrogen fixation patterns displayed by cyanobacterial consortia in Alchichica crater-lake, Mexico. Hydrobiologia, 2002. 467: p. 71-78.
46. Bhandari, V. and R.S. Gupta, Molecular signatures for the phylum (class) *Thermotogae* and a proposal for its division into three orders (*Thermotogales, Kosmotogales* ord. nov. and *Petrotogales* ord. nov.) containing four families (*Thermotogaceae*, *Fervidobacteriaceae* fam. nov., *Kosmotogaceae* fam. nov. and *Petrotogaceae* fam. nov.) and a new genus *Pseudothermotoga* gen. nov. with five new combinations. Antonie Van Leeuwenhoek, 2014. 105: p. 143-168.
47. Walters, K.E. and J.B. Martiny, Alpha-, beta-, and gamma-diversity of bacteria varies across habitats. Plos One, 2020. 15(9): p. e0233872.
48. Xie, W.-J., J.-M. Zhou, and H.-Y. Wang, Influence of Cu2+, Cd2+ and cypermethrin on microbial functional diversity in different fertilization soils. Huan Jing ke Xue= Huanjing Kexue, 2008. 29(10): p. 2919-2924.
49. Li, X., et al., Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar. Soil Biology and Biochemistry, 2014. 68: p. 392-401.
50. García-Salamanca, A., et al., Bacterial diversity in the rhizosphere of maize and the surrounding carbonate-rich bulk soil. Microbial Biotechnology, 2013. 6(1): p. 36-44.
51. Dunfield, K.E. and J.J. Germida, Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (*Brassica napus*). Applied and Environmental Microbiology, 2003. 69(12): p. 7310-7318.

Summary

The major aim of the research described in this thesis was to assess the bacterial diversity in the rhizosphere and phyllosphere of wildtype and transgenic sugarcane, and to evaluate to what extent members of the sugarcane microbiota can be employed as biofertilizers for sustainable agriculture as plant growth promoting bacteria (PGPB). PGPB help in plant growth and root development with minimally adverse effects to the environment as compared to chemical fertilizer applications. With the aim of development of new sugarcane varieties characterized by improved tolerance against abiotic stresses, transgenic crops can help in utilization of stress-affected land with concomitant increase in sugarcane yield and sustainability of production processes. Nevertheless, risk assessment of transgenic sugarcane with respect to its surrounding environment, and particularly the rhizosphere, is also necessary before releasing new varieties.

In **chapter 1**, I provided a general introduction based on a literature review of the current state of the art with respect to transgenic crops and their effects on rhizosphere and phyllosphere microbial communities. To this end, both cultivation dependent and cultivation independent, biomolecular approaches can be employed. Furthermore, I introduced the concept of PGPB, including their characterization on the bases of plant growth promoting traits. Three major traits were discussed in chapter 1, including indole acetic acid (IAA) production, phosphate solubilization and biological nitrogen fixation (BNF).

In **chapter 2**, laboratory and field experiments focused on the effect of four strains of PGPB, isolated from sugarcane and affiliated with *Bacillus*, *Paenibacillus*, *Pseudomonas* and *Enterobacter,* on sugarcane growth and root development. After initial *in vitro* screening of pure culture isolates on the bases of IAA production, phosphate solubilization, and BNF potential on the bases of *nifH*-targeted PCR, plant growth promoting traits were tested in short term experiments in sand, peat moss, and in microplots. *Bacillus* sp. strain ATRS1 was most efficient among the four tested strains in micropropagation as well as in microplot experiments.

In **chapter 3**, we isolated and selected 15 strains affiliated with three different major genera of PGPB, i.e. *Bacillus, Burkholderia* and *Pseudomonas*, on the bases of the plant growth promoting traits mentioned above and that were assessed using *in vitro* screening. *In vivo* plant growth promotion was then tested in microplot experiments, where strains were inoculated to wheat plants. All tested strains stimulated at least two of five measures of wheat growth, with *Bacillus* sp. strain DR4 being overall the most effective strain.

In **chapter 4** as well as **chapter 5**, experiments focused on the assessment of possible risks related to the introduction of transgenic sugarcane, particularly concerning the microorganisms associated with rhizosphere and phyllosphere. In **chapter 4**, microorganisms were studied by using cultivation. A total of 108 strains affiliated with 20 different bacterial genera were isolated from the rhizosphere and phyllosphere of transgenic and corresponding wild type sugarcane plants, and strains were characterized based on their 16S rRNA gene sequences as well as IAA production, phosphate solubilization and BNF potential. While the cultured bacterial diversity

from sugarcane rhizosphere and phyllosphere largely differed, only minor differences were observed between the transgenic and non-transgenic sugarcane varieties regarding the diversity of cultured endophytes.

In **chapter 5**, samples from the same experiment were subjected to molecular, cultivation independent analysis of microbial community composition by high throughput amplicon quenchingng of PCR-amplified 16S rRNA gene fragments. Analyzed samples included material from roots, rhizosphere soil and bulk soil, as well as stem, leaf and cane parts as representative samples of the phyllosphere. There was no significant difference in alpha diversity of rhizosphere and phyllosphere microbiota of transgenic and wild type sugarcane plants. Furthermore, beta diversity analysis showed similar clustering patterns in both transgenic and wildtype sugarcane samples, with significant differences only being observed between transgenic and wildtype sugarcane phyllosphere microbiota in addition to the expected, clear differences between rhizosphere and phyllosphere associated microbial communities.

Finally, in **chapter 6** I discussed the general findings from these experimental chapters. Based on the observations reported here, future research should focus on extensive metagenomic analysis in combination with well-controlled field experiments for detailed comparison of transgenic and non-transgenic sugarcane associated microbiome structure and function, including mechanistic aspects of PGPB functionality, as a basis for new microbiome management tools towards sustainable sugarcane production.

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Colophon

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