Maize endophytes – diversity, functionality and application potential

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To my Parents (Late) .....

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Chapter 1

Abstract

There is a growing worldwide awareness for the need to increase food production to feed the rapidly expanding global human population. The search for microorganisms that improve soil fertility and enhance plant nutrition has continued to attract attention due to the increasing cost of chemical fertilizers and their negative environmental impacts. The use of biological inoculants based on PGPB appears to be a promising alternative to chemicals fertilizers and holds immense potential for sustainable agriculture and environment owing to their environment friendly traits. These bacteria are known to enhance growth and yield of plants by fixing atmospheric nitrogen, solubilization of phosphate, production of phytohormones and siderophores, biocontrol activity as well as reducing the level of stress ethylene in host plants. The key aspects in successful application of PGPB inoculant technology are the use of a proper formulation of inoculant preparations, the selection of an adequate carrier and the design of correct delivery methods.

We evaluated the growth promotion potential and colonization capacity of five endophytic bacterial strains (FA13, FF34, FC42, FB12 and FD17) for enhancing growth and yield of different maize cultivars. A range of different lab assays in regard to potential plant growth promotion was performed and strains were further evaluated for improving growth of five maize cultivars under axenic and natural soil conditions. We found that the inoculant strains had the potential to improve maize seedling growth under axenic conditions. In the containment trial, FD17 inoculation significantly increased plant biomass and grain yield up to 39 and 42%, respectively, as compared to the un-inoculated control. Inoculation also improved the photochemical efficiency of photosystem II (PSII)

of maize plant and reduced the time needed for flowering. We confirmed that strain FD17 is efficiently colonizes the rhizosphere, roots and stems of maize plant. Based on rigorous testing *Enterobacter* sp. strain FD17 showed highest potential to promote growth and health of maize grown under natural soil conditions. This study suggested that *in vitro* plant growth promoting traits and potential of maize seedling growth promotion by bacterial endophytes could be used for the selection of potential inoculant strains subjected for further testing as bio-inoculant under field conditions.

The second part of the study aimed to assess the drought stress resilience of maize and wheat through endophytic colonization by *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17 in the pot and field trials. Results of pot trial revealed that bacterial inoculation minimized the drought stress-imposed effects significantly increasing shoot biomass, root biomass, relative water content, photosynthesis and photochemical efficiency of PSII up to 66, 70, 30, 75 and 10%, respectively, compared to the un-inoculated control. The inoculant strains efficiently colonized maize seedlings and were recovered from roots, shoots and leaves of both irrigated and stressed plants. The potential of PsJN inoculation was further evaluated to ameliorate the effects of drought stress on growth and yield of wheat under natural field conditions. The plants were exposed to drought stress at tillering and flowering growth stage by skipping the respective irrigation. PsJN inoculation gave better response to wheat at the tillering stage and resulted in significant increase in plant biomass, photosynthesis and grain yield compared to the control. Inoculation increased grain yield up to 21 and 18%, respectively, at both stages over the un-inoculated control. Similarly, PsJN inoculated plants showed higher antioxidant activity and nutrient (NPK) contents compared to

control under stress conditions. These studies suggested that endophytic bacteria could be efficiently used to reduce the effects of drought stress on growth and yield of maize and wheat.

In the third phase of study, we evaluated the L-TRP-dependent response of PsJN inoculation to maize growth and auxin biosynthesis under pot conditions. *In vitro* data revealed that PsJN produced auxin (IAA equivalents) without L-TRP addition (0.84 µg mL^{-1}), however, IAA equivalents substantially increased when the medium was supplemented with L-TRP (11.78 µg mL^{-1}). PsJN inoculation supplemented with L-TRP (10^{-5} M) significantly increased root biomass and shoot biomass up to 62 and 55%, respectively, compared to the un-inoculated control. The inoculant strain colonized more efficiently maize seedlings in the presence of exogenously applied L-TRP. The results imply that L-TRP-derived IAA biosynthesis in the rhizosphere by PsJN inoculation could be a useful approach for improving the growth and yield of maize.

Keywords: Endophyte, *Burkholderia phytofirmans* PsJN, *Enterobacter* sp. FD17, photosynthesis, antioxidant activity, nutrient content, drought stress, L-tryptophan, precursor-inoculum interaction, endophytic colonization, maize

Zusammenfassung

Das schnelle Wachstum der menschlichen Bevölkerung lässt uns das Augenmerk auf die Frage legen, wie der gesteigerte Bedarf nach Lebensmitteln gedeckt werden könnte. Pflanzenwachstumsfördernden Mikroorganismen stellen eine Alternative zu kostspieligen und umweltschädigenden chemischen Düngern und Pestiziden dar. Diese Bakterien besitzen beispielsweise die Fähigkeit, die Versorgung der Pflanze mit Mikronährstoffen zu fördern, indem sie Luftstickstoff binden, gebundenes Phosphat aus dem Boden lösen und der Pflanze verfügbar machen, Pflanzenhormone und Siderophoren produzieren. Weiters können sie Gesundheit der Pflanzen schützen, indem sie Pathogene biologisch bekämpfen und das Stresshormon Ethylen abbauen. Die Schlüsselfaktoren einer erfolgreichen technologischen Anwendung von pflanzenwachstumsfördernden Bakterien sind die Sicherstellung einer hohen Überlebensrate und einer effektiven Besiedelung der Wirtspflanzen. Hierfür notwendig ist die Entwicklung geeigneter Formulierungen und Aufbringungstechniken.

In unserer Studie evaluierten wir das Wachstumspotenzial und die Kolonisierungsfähigkeit von fünf Endophytenstämmen (FA13, FF34, FC42, FB12 und FD17) um das Wachstum und den Ertrag von verschiedenen Maissorten zu erhöhen. Unterschiedliche Labormethoden wurden angewandt, um die Stämme auf ihre pflanzenwachstumsfördernde Eigenschaften zu prüfen. Die direkte Wirkung auf Pflanzen wurde unter axenischen und natürlichen Bodenbedingungen untersucht. Dabei wurde festgestellt, dass die Bakterien das Pflanzenwachstum unter keimfreien Bedingungen fördern. FD17-Inokulierung bewirkte in einem Containment Versuch eine Steigerung der Pflanzenbiomasse von 39% sowie eine Ertragsteigerung von 42%, verglichen mit der

nicht inokulierten Kontrolle. Darüber hinaus verbesserte die Behandlung mit dem Baktreium auch die Effizienz des Photosystem II (PSII) der Maispflanze und verringerte die Wachstumsdauer bis zum Erreichen des Blühstadiums. Unsere Versuche bestätigten die erfolgreiche Kolonisierung von Rhizosphäre, Wurzel und Stamm der Maispflanze mit FD17. Unsere Tests zeigten, das *Enterobacter* sp. FD17 das höchste Potential besitzt, Pflanzenwachstum unter natürlichen Bodenbedingungen zu fördern und dass *in vitro* Tests von bakteriellen Endophyten dazu geeignet sind, bestimmte Stämme für die Anwendung in Feldversuchen zu selektieren.

Der zweite Teil der Studie beschäftigte sich mit erhöhter Trockenstresstoleranz von Mais und Weizen durch die endophytische Besiedelung durch *Burkholderia phytofirmans* PsJN und *Enterobacter* sp. FD17 in Topf- und Feldversuchen. Die Resultate der Topfversuche zeigten eine signifikante Reduktion der Trockenstresssymptome verglichen mit der nicht inokulierten Kontrolle. Dies zeigte sich in der Erhöhung der Stammbiomasse (+ 66%), der Wurzelbiomasse (+70%), des relativen Wassergehalts (+30%), der Photosyntheserate (+75%) sowie der photochemischen Effizienz von PSII (+ 10%). Die Bakterienstämme kolonisierten sehr effizient die Maissetzlinge und konnten in den Wurzeln, im Stamm und den Blättern sowohl von nicht gestressten als auch gestressten Pflanzen nachgewiesen werden. Der Trockenstress wurde durch das Aussetzen der Bewässerung in den Zeiträumen der Ausläuferbildung und der Blütenbildung hervorgerufen. In Weizen zeigte der Stamm PsJN eine größere Wirkung bei der Inokulierung während der Sprossungsphase und resultierte in einer signifikanten Steigerung der Pflanzenbiomasse, der Photosyntheserate und des Ernteertrags, verglichen mit der Kontrolle. Im Detail bewirkte die Inokulation eine Ertragssteigerung

von 18 -21% gegenüber der nicht inokulierten Kontrolle. Außerdem zeigten PsJN-inokulierte Pflanzen unter Stresse eine höhere Antioxidationsrate sowie einen höheren Nährstoffgehalt im Vergleich zur nicht inokulierten Kontrolle. Unsere Resultate lassen vermuten, dass endophytische Bakterien verwendet werden können, um die Effekte von Trockenstress während des Wachstums von Mais und Weizen zu vermindern.

In der dritten Phase der Doktorarbeit evaluierten wir die L-TRP-abhängige Wirkung von *B. phytofirmans* PsJN auf das Wachstum von Maispflanzen so wie die Auswirkung auf die Produktion von Auxinen in Topfversuchen. Unsere *in vitro* Studien zeigten, dass PsJN ohne Zugabe von L-TRFP (0,84 µg mL^{-1}), Auxin (IAA Äquivalente) produzierte. Durch Zugabe von L-TRP (11.78 µg mL^{-1}) wurde die Produktion von IAA Äquivalenten weiter erhöht. Die kompinierte Anwendung von PsJN und L-TRP (10^{-5} M) bewirkte eine Steigerung der Sprossbiomasse zwischen 55 und 62% , verglichen mit der nicht-inokulierten Kontrolle. Der Bakterienstamm kolonisierte die Maissetzlinge effizienter in der Anwesenheit von exogenem L-TRP. Die Resultate lassen vermuten, dass eine Inokulation mit PsJN bei gleichzeitiger L-TRP Zugabe einen sinnvollen Beitrag zur Ertragssteigerung von Mais bewirken könnte.

Keywords: Endophyten, Pflanzenwachstumsförderung, *Burkholderia phytofirmans* PsJN, *Enterobacter* sp. FD17, Photosynthese, Nährstoffgehalt, Trockenstress, L-tryptophan, Kolonisierung mit Endopyhten, Mais, Weizen

Chapter 1

General Introduction

1.1. Background, objectives and scope of the research

Despite a significant growth in food production over the past-half century, one of the most important challenges facing society today is how to feed an expected population of approximately nine billion by the middle of the twentieth century. However, it will be challenging over the next 50 years to increase crop productivity further in order to meet an expanding population, due to a range of issues such as decreasing arable land, increasing water scarcity, rapid global climate change, and the use of biomass for biofuels production. To meet the expected demand for food without significant increases in price, it has been estimated that we need to produce 70-100 percent more food in light of the increasing impacts of global climate change and concerns over energy security (FAO, 2010; Godfray et al., 2010). Higher cereals grain production has only been possible with high inputs of inorganic or synthetic chemical (NPK) fertilizers. However, the intensive use of agricultural resources, particularly water and fertilizer, now and in the foreseeable future is exceedingly expensive and/or environmentally damaging, and cannot remain indefinitely, because of overwhelming demand for water for non-agricultural residential uses and the skyrocketing energy cost of fertilizer production (Ghimire and Craven, 2013).

There has been a steady rise in agricultural production since the green revolution, but the scope of high-input agriculture with current technologies and available crop plants will not be sufficient to feed the rapidly growing world population in the context of a

dwindling supply of agricultural inputs (Den Herder et al., 2010). This is particularly true in developing countries, where population growth will be the greatest but access to agronomic inputs will be the most limited. In intensive cropping systems, supplementing soil nutrients by the use of chemical fertilizer is considered inevitable for obtaining optimum yield of crops. To acquire optimum crop yield potential, adequate plant acquisition of nutrients is necessary. However, even when nutrients are provided externally, their utilization by plants is highly dependent upon the physical, chemical and biological conditions in the soil, which is located in the immediate vicinity of plants' roots, known as the rhizosphere (a term first introduced by Hiltner, 1904). This thick layer (a few millimeters) of soil is intimately and continuously affected by roots' metabolic processes, creating a zone of intense activity comparatively different from the surrounding bulk soil. The rhizosphere's contribution to soil fertility and sustainability and, thus to optimum plant growth, is all out of proportion to its physical volume (Römheld and Neumann, 2006).

Achieving further increments in agricultural productivity with a reduction in agrochemical use for economic or environmental reasons will need a new generation of technologies. Although great efforts have been made over the last three decades to increase the crop productivity by introducing new varieties and better farm management approaches, sufficient production of these crops using external inputs (e.g. agro-chemicals) is still a distant target. Moreover, it is well established now that continuous use of chemical fertilizers subverts soil ecology, disrupts the environment, degrades soil fertility and consequently may show harmful effects on human health (Ayala and Rao, 2002) and also may contaminate ground water (Joshi et al., 2006).

Soil is a rich storehouse of diverse communities of microorganisms with multifaceted metabolic activities. A number of diverse phylogenetic groups of microbes in nature have been described, which increase crop productivity by various plant growth-promoting mechanisms (Dutta and Podile, 2010; Hayat et al., 2010).

The use of biological fertilizers based on plant growth-promoting bacteria (PGPB) appears to be a promising alternative to chemicals. Consequently, over the years, the utilization of PGPB, usually either rhizosphere bacteria or endophytes, as bio-fertilizers and/or bio-pesticides has received increasing attention. These microorganisms may not only ensure the availability of essential nutrients to plants but also enhance nutrient use efficiency (Khalid et al., 2009). Mechanisms by which PGPB may stimulate plant growth and nutrition include biological nitrogen fixation (BNF), synthesis of phytohormones, environmental stress relief, synergism with other bacteria-plant interactions, inhibition of plant ethylene synthesis, as well as increasing availability of nutrients like phosphorus, iron and other micro-elements, and growth enhancement by volatile compounds (Hardoim et al., 2008; Mitter et al., 2013). Numerous investigators have developed microbial inoculants that confer beneficial properties such as such as BNF, uptake of phosphorus (P) and other mineral nutrients, biocontrol and plant hormone production for increasing the crop productivity (Dodd and Ruiz-Lozano, 2012; Miransari, 2011). Development of stable, efficacious and eco-friendly microbial formulations that contain phylogenetically diverse and naturally occurring soil microbes with multiple complementary functions designed to enhance the productivity of a broad spectrum of crops with reduced input of nitrogen (N) and P fertilizers and pesticides is the ideal goal. It is encouraging that some real progress is being made in this direction, and perhaps

better microbial formulations for increasing crop productivity may become available soon (Reddy and Saravanan, 2013).

The term endophyte is applied to microorganisms that live inside plants for at least part of their life cycle without being pathogenic. In contrast, some endophytes confer to the host benefits such as stress reduction, increased root growth and nutrient availability (Hardoim et al., 2008; Mitter et al., 2013). Endophytic bacteria may in future be even more important than rhizosphere bacteria in promoting plant growth promotion and nutrition, because they escape competition with rhizosphere microorganisms and achieve a more intimate contact with plant tissues. Endophytes have the capacity to colonize the plant interior and may mediate more consistent effects, particularly when applied as bio-fertilizers. Extensive utilization of endophytic microbes, particularly those that are readily cultivable on minimal resources to produce large amounts of plant inoculum, could play a significant role in feeding an ever-burgeoning world population.

1.2. Different Niches for PGPB Colonization

The beneficial effects of PGPB (rhizobacteria and endophyte) on plant growth depend on an intimate association between these bacteria and the host plant. However, the degree of relationship can vary depending on where and how PGPB colonizes the host plant. It can be categorized into two levels of interaction: rhizospheric and endophytic.

1.2.1. Rhizospheric Niches

The rhizosphere is the compartment of the soil around the roots that is under plant influence (Kennedy, 2005). Plant growth-promoting rhizobacteria (PGPR) represent a

wide variety of soil bacteria which, when grown in association with a host plant, result in stimulation of growth and yield of their host (Vessay, 2003). Some scientists adopt a more expanded definition for rhizospheric PGPRs, including bacteria from the rhizoplane, which is the surface of plant roots and soil particles strongly adhered to it (Antoun and Prevost, 2005; Figure 1). Plant roots offer a niche for the proliferation of PGPRs that thrive on root exudates and lysates. Population densities of bacteria in the rhizosphere may be up to 100-fold higher than in bulk soil and up to 15% of the root surface may be covered by micro-colonies of different bacterial strains. These bacteria utilize the nutrients that are released from the host for their growth; they also secrete metabolites into the rhizosphere that can act as signaling compounds (van Loon, 2007).

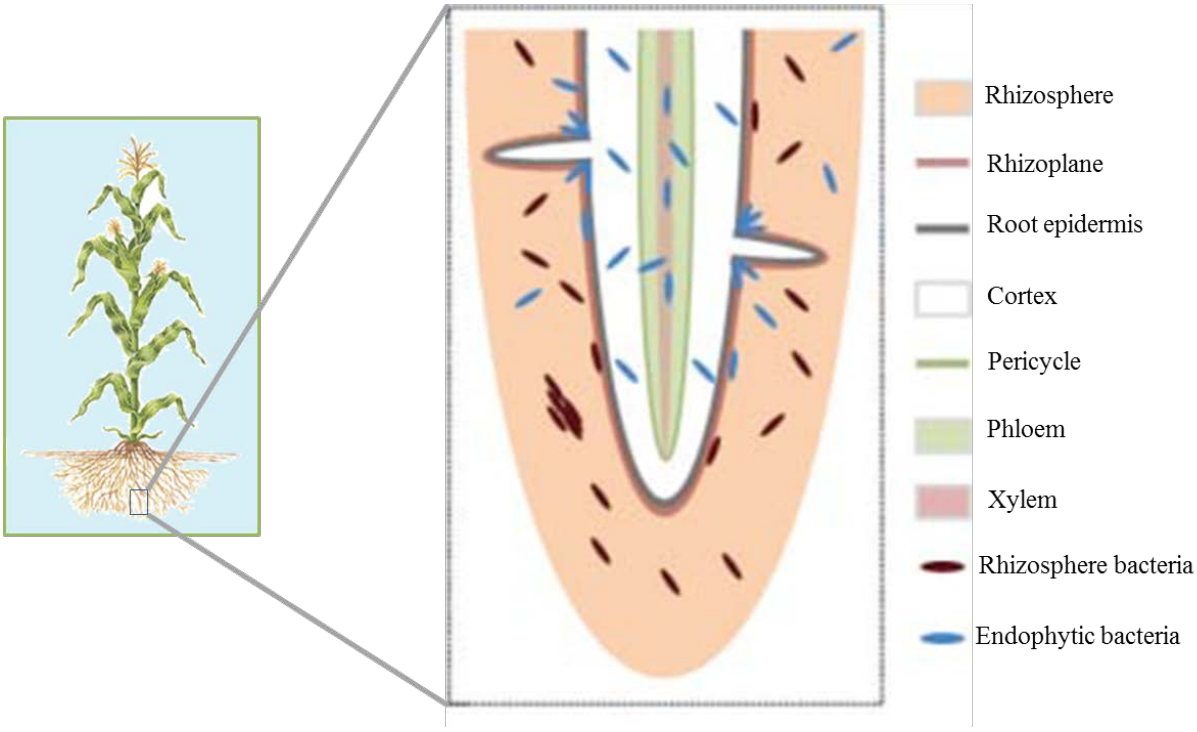

Figure 1. Root niches for PGPB colonization. Rhizospheric PGPRs (garnet cells) colonize rhizosphere soil area and roots surface (rhizoplane), but they can not invade internal plant tissues. Endophytic bacteria (blue cells) colonize any region within the epidermis of the plant root, and they can reside in apoplastic intercellular spaces and

xylem vessel apoplast. The endophytes, in general, invade the internal plant tissues through sites of injury in the epidermis, root tips and root cracks formed at the sites of lateral root emergence (adapted from Carvalho et al., 2013).

Plants modify the physico-chemical properties and biological composition of the rhizosphere through a variety of mechanisms, which can affect the ability of PGPRs to colonize the plant rhizosphere. This includes changes in water potential, pH, salinity, partial pressure of oxygen and mineral and organic composition due to plant exudation (Hasegawa et al., 2005). Along with these changes, root exudates directly influence nutrient availability and uptake or have indirect effects through interaction with beneficial soil microorganisms. A remarkable feature of the rhizosphere is that rhizodeposition and root turnover account for up to 20-40% of the carbon input into soil and clearly is the major driver for soil biological processes (Morgan and Whipps, 2001; Jones et al., 2009). There has been long-standing recognition of the importance of root exudates as sources of nutrients and carbon for promoting and sustaining beneficial soil microflora. The main component of root exudation is the mucilage, which contains polysaccharides, organic acids, vitamins and amino acids, therefore an excellent substrate for microbial growth and their proliferation. Mucilage adheres to water, helping to form a highly hydrated environment for roots and rhizosphere microbial communities. These exudates principally affect microbial communities in two ways. Firstly, they provide rich and relatively readily available source of nutrients and energy. Secondly, there is growing evidence of a diverse range of chemical signals from plant roots to microorganisms and vice versa that influence microbial community structure and functions. This generates a functionally complex community with a high level of competition for colonization by

bacteria that may be beneficial, neutral and or pathogenic toward plants (Dick, 2012; Carvalho et al., 2013).

There is an increasing body of evidence that the biology of the rhizosphere could be exploited by manipulating root and microbial interactions to improve the productivity and sustainability of agricultural systems. These rhizospheric microorganisms have shown the potential to increase nutrient availability and uptake to plants, stimulate growth and protect plants from disease causing pest and pathogens (Dick, 2012). This would be particularly valuable toward the development of sustainable and biologically based agricultural systems because this has potential for reduced or no external inputs.

1.2.2. Endophytic Niches

The term endophyte is applied to microorganisms that live within plant tissues for all or part of their life cycles and cause no apparent infections or symptoms of disease (Bacon and White, 2000; Saikkonen et al., 2004). This concept has been further extended to encompass all bacteria that can be isolated from surface-sterilized plant tissues and do not visibly harm host plants (Hallmann et al., 1997). According to their life strategies, bacterial endophytes can be classified as "obligate" or "facultative" (Hardoim et al., 2008). Obligate endophytes are severely dependent on the host plant for their growth and survival. While facultative endophytes have a phase in their life cycle in which they survive outside host plants.

Two of the most frequently raised questions in regarding with endophytic bacteria are what is the origin of endophytes and how do they enter plant tissues in nature? To answer the first question, endophytic bacteria appear to originate from seeds (Pleban et al., 1995; Adams and Kloepper, 1996), vegetative planting material (Dong et al., 1994),

rhizosphere soil (Hallmann et al., 1997; Mahaffee and Kloepper, 1997) and the phylloplane (Beattie and Lindow, 1995). Endophytic sites comprise any region within the plant, although the vascular system is sometimes considered separately (Figure 1). With the exception of seed-originated bacteria, which are already present in the plant, potential endophytes must first colonize the root surface prior to entering the plant materials. The initial processes of colonization of plant tissue by endophytic bacteria can be via stoma, lenticels, areas of emergence of lateral roots and germinating radicles (Hallmann et al., 1997). However, the main entry for endophytic bacteria appears to be through wounds and cracks that naturally occur as a result of plant growth, or through root hairs and at epidermal conjunctions (Sprent and de Faria, 1988). Several authors have reported colonization of the secondary root emergence zone by bacterial endophytes (Wiehe et al., 1994; Mahaffee et al., 1997; Mattos et al., 2008).

The endophytic niches require special recognition because of its unique habitat and potential to affect plants growth. Endophytic bacteria can colonize the apoplastic and interacellular locations in roots without causing harmful effects on the host plant (Schulz and Boyle, 2006). Although endophyte populations vary in different plants according to many factors, bacterial populations are generally larger in roots and smaller in stems and leaves (Lamb et al., 1996). Additionally, the population density of endophytic bacteria found in plants depends on the plant species, genotype, and tissue; the growth stage and specialization of the bacteria; differences in colonization pathway; and mutual exclusion of different bacterial populations (Sturz et al., 1997; Strobel and Daisy, 2003).

Because of their intimate contact or location within roots, they may more protected from adverse changes in the environment than bacteria in the rhizospher and

would be expected to interact closely with their host and face less competition for nutrients and spaces (Beattie, 2006; Rosenblueth and Martínez-Romero, 2006). The endophytic bacteria, which are usually present in plant tissues are able to enhance plant growth and hence final produce (yield). Interestingly, various research works had indicated the applications of natural and genetically modified endophytic bacteria for the alleviation of stresses (both abiotic and biotic). The endophytic bacteria can also significantly contribute to enhancing plant growth and nutrient uptake, and hence can be assumed a source of fertilization for the host plant (Luo et al., 2011; Miransari, 2011; Mitter et al., 2013).

Microscopic examination revealed that PGPRs do not live within healthy host cells. The few reports that propose an intracellular localization are controversial and could characterize a saprophytic colonization (Bellone et al., 1997; Cocking et al., 2006). The microorganisms that reach the intercellular regions must compete with the host plant defense system, which is activated when bacteria enters in the plant environment. Remarkably, bacteria colonize tissues of most organs of the infected plant, without causing pathogenic effects and any visible disease symptoms (Hardoim et al., 2008; de Carvalho et al., 2011). There is growing interest in finding bacterial strains with biological control or plant growth-promoting capabilities. If these bacteria can be found in internal plant tissues, as they can in the rhizosphere, these bacteria may have the unique capacity to elicit beneficial effects from within the plants. As new beneficial bacterial strains are identified, appropriate delivery of these strains to specific plant tissues will be needed. To use endophytic bacteria in practical agronomic production, reliable and practical methods of inoculation must be developed. Several delivery

systems have been reported for endophytic bacteria (Hallmann et al., 1997; Bressan and Borges, 2004).

1.2.3. Mechanisms of bacterial plant growth promotion

Plant growth promotion by bacteria in an agrobiology system consists of two levels: rhizospheric and endophytic (Bhattacharyya and Jha, 2012). PGPB are (e.g. rhizobacteria and endophyte) isolated from the rhizo- and or endosphere, which when inoculated into the seed or soil stimulate plant growth through one or more of several functional mechanisms. The growth stimulation by PGPB can be a consequence of nitrogen fixation (BNF), production of phytohormones, such as indole-3-acetic acid (IAA), cytokinins and gibberellins, biocontrol of phytopathogens through the production of antifungal or antibacterial agents, siderophore production, nutrient competition and induction of acquired host resistance, or enhancing the bioavailability of minerals (Hallmann et al., 1997; Rosenblueth and Martinez-Romero, 2006; Mitter et al., 2013). Several studies have indicated that endophytic colonization can also result in increased plant vigor, and it confers tolerance to biotic and abiotic stresses (Hallmann et al., 1997; Azevedo and Araujo, 2003), enhanced drought tolerance (Arachevaleta et al., 1989), and improved phosphorus utilization (Verma et al., 2001; Wakelin et al., 2004). Although the interaction between endophytic bacteria and their host plants is not fully understood, many isolates showed beneficial effects on their hosts and may play an important role in the physiology and growth of these plants. PGPB may use more than one of these mechanisms to enhance plant growth (Figure 2; Table 1) as experimental evidence suggests that the plant growth stimulation is the net result of multiple mechanisms that

may be activated simultaneously (Martinez-Viveros et al., 2010). Recently, biochemical and molecular approaches are providing new insight into the genetic basis of these biosynthetic pathways, their regulation and importance in plant growth promotion and biological control (Joshi and Bhatt, 2011). However, many well-known

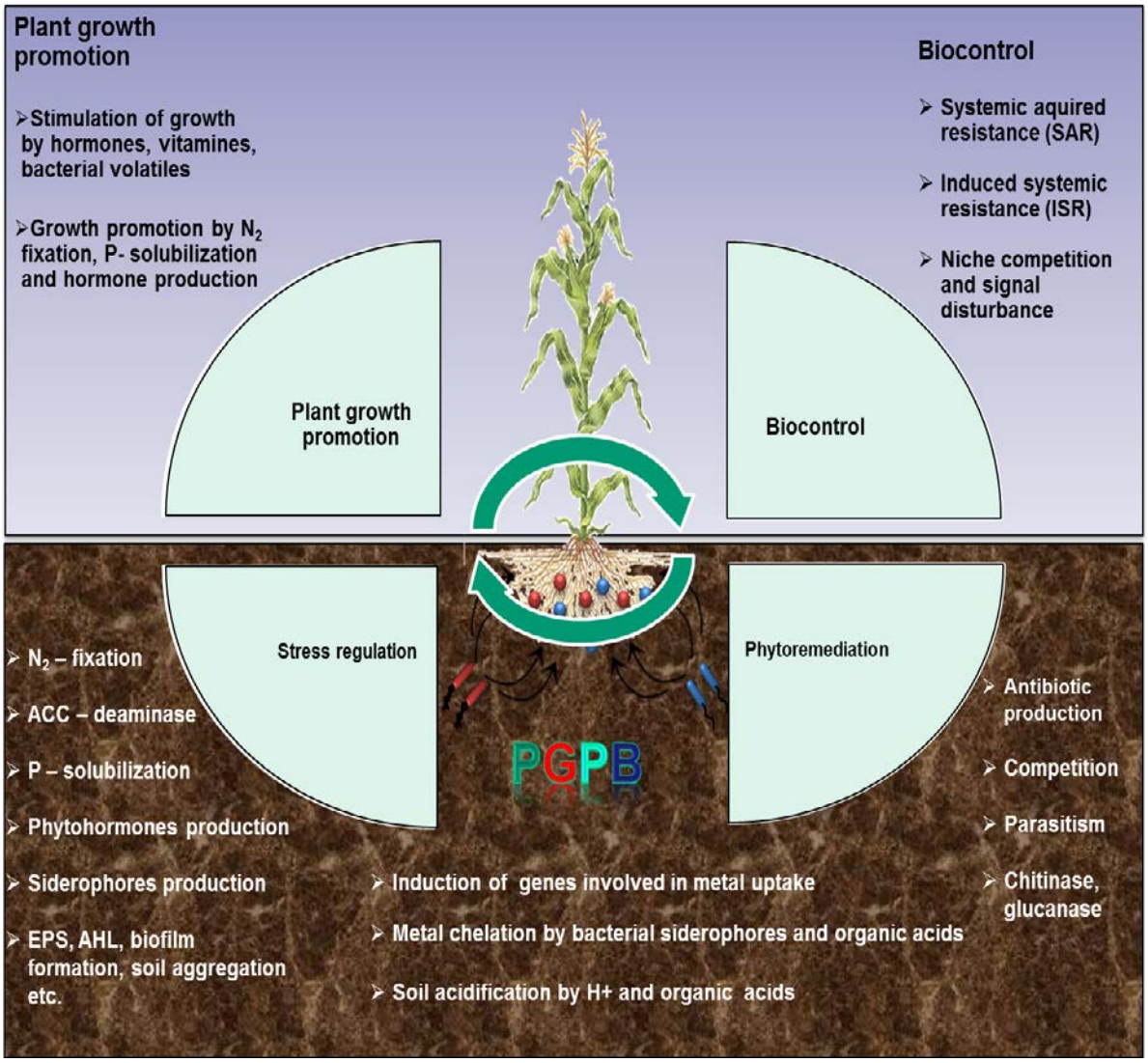

Figure 2. Potential beneficial effects of plant-associated bacteria on plant growth and soil health.

Table 1. Observed effects of plant growth promotion by PGPB inoculation in various crops

Test crop	Bacteria	Experimental conditions	Proposed mechanism(s)	Plant response to PGPR / endophyte inoculation	Reference
Arabidopsis thaliana	*Bacillus* sp. L254, L266, L272a	Petri plate assay	Volatile organic compounds (VOCs) production	Rhizobacterial inoculation increased plant biomass by 2 fold	Gutierrez-Luna et al. (2010)
Medicago sativa	*Arthrobacter agilis* UMCV2	Axenic trial	VOCs production	Inoculation resulted in 40% increase in plant biomass	Velazquez-Becerra et al. (2011)
Peppermint	*Pseudomonas fluorescens, Bacillus subtilis*, *Azospirillum brasilense*	Petri dish assay	VOCs production	Essential oil content was increased by 2-fold	Santoro et al. (2011)
Pearl millet	*Pseudomonas*, *Citrobacter*, *Acinetobacter*, *Serratia*, *Enterobacter* spp.	Pot trial	P- solubilization	Increased root length (45-75%), shoot length (5-68%) and biomass (64-88%)	Misra et al. (2012)
Wheat	*Pseudomonas, Bacillus, Azospirillum*	Axenic / pot trials	IAA production	Increase in spike length (33%), number of tillers (71%) and seed weight (39%)	Hussain and Husnain (2011)
Cucumber	*Ochrobactrum haematophilum* H10	Pot trial	IAA production, P - solubilization	Increased leaf length (27%) and root length (58%)	Zhao et al. (2012)
Tomato	*Gluconacetobacter diazotrophicus* PAL 5 and UAP 5541	Greenhouse experiment	N_2-fixation	Increased total fruit number (18%) and weight upto 14%	Luna et al. (2012)
Canola	*Methylobacterium fujisawaense* strains CBMB 20, CBMB 10	Gnotobiotic	ACC deaminase activity	Increased root length up to 78%	Madhaiyan et al. (2008)
Clusterbean	*Bacillus coagulans*	Pot trial	P- solubilization	Improvement plant biomass (25%), root length (28%), P content (22%) and seed yield	Yadav and Tarafdar (2012)

				(19%)	
Strawberry	*Paenibacillus polymyxa* RC05, *Bacillus* spp. RC23	Field trial	IAA production	Increased fruit weight (19%) and quality fruit ratio (32%)	Erturk et al. (2012)
Neem plant	*Streptomyces* strains AzR-010, 049, 051	Controlled	IAA production	Improved germination (39%), root length (30%) and shoot length (31%)	Verma et al. (2011)
Black pepper	*Bacillus tequilensis* NII-0943	Pot trial	IAA production, P-solubilization and ACC deaminase	Increased Root length (77%) and shoot length (112.5%)	Dastager et al. (2011)
Sugar beet	*Acinetobacter johnsonii* strain 3–1	Pot trial	IAA production and P- solubilization	Increased plant dry weight (69%) and yield (37%)	Shi et al. (2011)
Muskmelon	*Bacillus subtilis* Y-IVI	Pot trial	IAA, Siderophore production	Increased shoot dry weight (100%) and length (34%)	Zhao et al. (2011)
Rice	*Pseudomonas* sp. PAC,	Glass tube assay	P- solubilization	Increased plant height and shoot P content	Nico et al. (2012)
	Bacillus sp. SVPR30, *Paenibacillus polymyxa* ATCC 10343	Greenhouse	IAA production	Inoculation produced 39% increase in plant dry biomass	Beneduzi et al. (2008)
Maize	*Acinetobacter rhizosphaerae* BIHB 723	Pot trial	P- solubilization	Increased shoot height (19%), shoot biomass (32%) and P uptake (83%)	Gulati et al. (2010)
Sorghum	*Azospirillum brasilense* SM	Axenic	IAA production	Increased shoot length (28%) and and dry (62%)	Malhotra and Srivastava (2009)
Mungbean	*Pseudomonas*, *Escherichia*, *Micrococcus*, *Staphylococcus* sp.	axenic	IAA production	Significantly enhanced shoot length (48%) and biomass (44%)	Ali et al. (2010)
Apple	*Bacillus* OSU-142, *Bacillus* M-3, *Burkholderia* OSU-7, *Pseudomonas* BA-8	Field trial	IAA production, cytokinin production	Increased shoot length by 59, 18, 7 and 14% and fruit yield by 116, 88, 138 and 74%, respectively	Aslantas et al. (2007)

plant pathogens may also be typical endophytic bacteria that normally cause no visible disease symptoms (Kobayashi and Palumbo, 2000) but become pathogenic under certain conditions or within different host genotypes (Misaghi and Donndelinger, 1990). The major mechanisms action of PGPR involved in the improvement of plant growth and development are discussed in the following sections.

1.3.1. Phytohormone production

Phytohormones (also known as plant growth regulators) are low molecular-weight natural products that act at micromolar concentrations to regulate essentially all physiological and developmental process during a plant's life cycle (Chiwocha et al., 2003). Auxins, gibberellins, cytokinins, abscisic acid and ethylene are the best known plant hormones (Zahir et al., 2004; Khalid et al., 2006). The production of auxins, cytokinins, gibberellins and abscisic acid is considered a common characteristic of PGPB (Frankenberger and Arshad, 1995; Khalid et al., 2004; Spaepen et al., 2007) and is suggested as one of the most plausible mechanisms of action affecting plant growth and development positively (Zahir et al., 2004).

There are a number of reports, which advocate the effectiveness of these growth regulators (PGRs) for enhancing plant growth and development (Glick et al., 2007; Lugtenberg and Kamilova, 2009). The ability to synthesize phytohormones is widely distributed among plant-associated bacteria, and in some studies 80% of the bacteria associated with plants were able to produce IAA (Patten and Glick, 1996; Khalid et al., 2006). The root-growth-promoting hormone auxin, present in root exudates, is usually synthesized from the exudate amino acid tryptophan. The tryptophan concentration in

exudates differs strongly among plants (Kravchenko et al., 2004). Many studies have described the ability of plant-associated bacteria to produce phytohormones, such as auxin (Vessey, 2003; Spaepen et al., 2007), and the ability to produce IAA is considered to be responsible for plant growth promotion by beneficial bacteria, such as *Azospirillum*, *Alcaligenes faecalis*, *Klebsiella*, *Enterobacter*, *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Costacurta and Vanderleyden, 1995).

The importance of auxin production in PGPB ability to promote plant growth has been demonstrated through inoculation studies with bacteria mutants (Barbieri and Galli, 1993; Patten and Glick, 1996). Seeds bacterization with the auxin-generating *P. fluorescens* WCS365 did not result in an increase in the root or shoot weight of cucumber, sweet pepper and tomato, but led to a significant increase in the root weight of radish (Kravchenko et al., 2004). Patten and Glick (2002) observed 35–50 % longer roots in canola inoculated with wild-type GR12-2 compared to IAA-deficient mutant and un-inoculated control.

Similarly, many *Pseudomonas*, *Bacillus* and *Azospirillum* spp. produce cytokinin and gibberellins, and positive effects on plant biomass have been reported by these hormones (Spaepen et al., 2009; Gamalero and Glick, 2011). Steenhoudt and Vanderleyden (2000) demonstrated that the main mechanism used by *Azospirillum* for enhancing plant growth is the production of phytohormones. Very recently, auxin (IAA) production have been described to be putatively involved in plant growth-promotion in efficient colonization of *Arabidopsis thaliana* by strain PsJN (Zúñiga et al., 2013). Although commercially available phytohormones are also used for promoting plant growth, microbially produced phytohormones are more effective due to continuous slow

release, whereas the balance between inhibitory and stimulatory levels of chemically produced hormones is low is challenging.

1.3.2. Plant growth enhancement through increase in nutrients availability

In addition to plant growth promotion through the production of phytohormones, some PGPB can improve plant nutrition by providing specific nutrients for plants, especially N, P, iron (Fe) and zinc (Zn) (Glick et al., 1999; Podile and Kishore, 2006).

Nitrogen is an essential nutrient to plants, which can be easily lost in the soil by leaching, volatilization and bacterial denitrification (Vance, 2001). An important feature of some PGPB is the ability to supply N to host plants through BNF. This is one of the most important biological processes required for plant growth and is being performed by diazotrophic bacteria, which are able to reduce atmospheric N into a form available to plants. The symbiotic relationship between legumes and N_2-fixing bacteria and nitrogen fixation by free-living bacteria without forming association is a source of N for plant (Carvalho et al., 2010). Co-inoculation of PGPB with rhizobia caused positive effect on nitrogen fixation, plant biomass, and grain yield in various leguminous crops like alfalfa, soybean and mungbean (Tilak et al., 2006; Zahir et al., 2011). Similarly, *Azospirillum* sp. have the potential to increase nitrogen fixation (Rai and Hunt, 1993), which can contribute about 70% of the total nitrogen requirement of the host plant (Malik et al., 1997). The presence of such bacteria also enhances ability of plant to use nitrogen efficiently and minimizes its leaching and denitrification losses.

Phosphorous is a major essential macronutrient-promoting plant growth and development, and low levels of soluble phosphate can limit the growth of plants. An estimated 40% of the crop yields on the world's agriculture land are limited by P

availability. This low availability of P to plants is because of the vast majority of soil P is found in insoluble form, while the plants can only absorb it in two soluble forms, the monobasic ($H_2PO_4^-$) and the di basic (HPO_4^{2-}) ions (Glass, 1989). Several phosphate solubilizing microorganisms are now recorded to convert the insoluble form of P to soluble form through acidification, secretion of organic acids or protons (Richardson et al., 2009) and exchange reactions (Hameeda et al., 2008). Many plant-associated phosphate-solubilizing microbes belong to species of genera *Bacillus, Pseudomonas* and *Rhizobium* (referred to as powerful P-solubilizers) as well as strains of *Enterobacter*, *Klebsiella*, *Proteus*, *Burkholderia*, *Serratia, actinomycetes, Agrobacterium, Micrococcus, Flavobacterium* and various fungi such as *Aspergillus* and *Penicillium* spp. use phosphatase for mineralizing organic/inorganic phosphates in the soil to a soluble plant-usable form. Phosphate fertilizers are second only to nitrogen fertilizers in terms of costs to the farmer and represent a major cost for agricultural production worldwide (Richardson, 2007; Reddy and Saravanan, 2013).

The production of low-molecular-weight ferric-chelating compound siderophores may directly increase the iron availability for plant and may indirectly protect the plant from pathogenic organisms (Etchegaray et al., 2004; Siddiqui, 2005; Singh et al., 2010). Some bacteria produce hydroxamate-type siderophores, and others produce catecholate-types (Neilands and Nakamura 1991). Siderophores play an important role in iron nutrition of plants (Jin et al., 2006). Vansuyt et al. (2007) reported that the Fe–pyoverdine complex synthesized by *Pseudomonas fluorescens* C7 was efficiently taken up by *Arabidopsis thaliana* resulting in enhanced iron content in plant tissue and better growth.

Due to high price and certain environmental concerns about chemical fertilizers, the use of such inoculants would be consistent with sustainable agriculture while at the same time increasing the monetary benefits to the farmer by lowering of the quantities of agrochemical fertilizers applied to the field and potential increases in crop yields. Moreover the use of PGPB in combination with inorganic fertilizer can increase the availability of nutrients to the crops (Kumar et al., 2009) and therefore could be useful for increasing the efficiency of fertilizers.

1.3.3. Decreasing plant ethylene level by ACC deaminase activity

Ethylene is a plant hormone that is involved in the regulation of many physiological responses (Reid, 1995). Many plant species require ethylene for seed germination. Usually, its rate of production rises during germination and seedling growth (Abeles et al., 1992). Generally, ethylene shows enhancement in root initiation and growth at low level, but higher levels can lead to suppression in root elongation (Esashi, 1991; Jackson, 1991). The level of ethylene production is generally increased under stress conditions.

ACC deaminase is an enzyme present in certain microorganisms that can hydrolyze 1-aminocyclopropane-1-carboxylate deaminase (ACC) into ammonia and α-ketobutyrate (Glick et al., 1998). Hence, PGPB containing ACC deaminase can decrease the amount of ACC, as well as ethylene, outside the germinating seeds, which eliminates the potential inhibitory effect of higher ethylene concentrations (Glick et al., 1998). Holguin and Glick (2001) demonstrated that the release of ACC deaminase by various PGPB in the rhizosphere could increase root elongation and plant growth by reducing ethylene synthesis.

The plants inoculated with PGPB containing ACC deaminase can have longer roots (Glick et al., 1999) and can be better able to resist the inhibitory effects of ethylene stress imposed by heavy metals (Burd et al., 2000), pathogens (Wang et al., 2000), drought (Zahir et al., 2008), salinity (Mayak et al., 2004a) and flooding (Grichko and Glick, 2001). Besides, treatment of plant seeds or roots with bacteria containing ACC deaminase typically reduces ACC and ethylene levels about 2–4 fold (Penrose and Glick, 2001). The role of ACC deaminase enzyme activity in plant growth promotion has been clearly demonstrated in the symbiosis of *Burkholderia phytofirmans* PsJN and canola. Sun et al. (2009) constructed a knock-out mutant of *B. phytofirmans* strain PsJN lacking ACC deaminase activity. The PsJN mutant was no longer able to promote the elongation of the roots of canola seedlings. Concisely, PGPB containing ACC deaminase could be used as successful inoculant because of having an effective strategy for improving growth and yield of crops via adjusting ethylene level in plants.

1.3.4. Biocontrol activity

Plant diseases are responsible for annual crop yield losses at a total value of more than 200 billions (Agrios, 2005). Resistant plants and chemicals are often used to control plant disease. However, resistance does not exist against all diseases and the breeding of resistant plants takes many years (5-10 years). Likewise, acceptance of genetically engineered resistance is still a sensitive issue in the some parts of the world. The use of agrochemicals (e.g. fungicides, insecticides and pesticides) is negatively perceived by consumers and supermarket chains. Biocontrol is a mechanism in which microorganisms promote the growth of plants indirectly by inhibiting the growth of pathogens due to the secretion of secondary metabolites, i.e. antibiotics (Raaijmakers et al., 2002), phenazines

(Mavrodi et al., 2006), 2,4-diacetyl phloroglucinol (Phl) (Dunne et al., 1998), pyoluteorin (Nowak-Thompson et al., 1999), pyrrolnitrin (Kirner et al., 1998), Zwittermycin A (Emmert et al., 2004), kanosamine (Milner et al., 1996) and HCN (Chandra et al., 2007). Siderophore production in Fe stress conditions provides microorganism an additional advantage, resulting in the exclusion of pathogens due to Fe starvation (Arora et al., 2001). Generally, PGPB control phyto-pathogens via antagonism of the pathogen or by changing the host plant susceptibility. Bacteria can antagonize soil borne pathogens through various mechanisms such as competition, antibiosis and orparasitism (Handelsman and Stabb, 1996; Mehboob et al., 2009; Mitter et al., 2013).

1.3.5. Enhancement of photosynthetic activity

Photosynthesis is considered as one of the very important reactions in plant growth and development. Under stress environment, reduction in photosynthesis occurs that might be due to decrease in leaf expansion, premature leaf senescence, impaired photosynthetic machinery, and associated reduction in food production (Wahid and Rasul, 2005). PGPB may enable the plants to maintain their growth by causing positive effect on photosynthesis. Shi et al. (2010) reported significantly increased the photochemical efficiency and total chlorophyll content in leaves of sugar beet by inoculation with *Bacillus pumilus* and *Acinetobacter johnsonii*, respectively. Bacterization of *Vitis vinifera* L. cv. Chardonnay (grapevine) with *Burkholderia phytofirmans* PsJN resulted in a 1.3 times higher CO_2-fixation rate and a 2.2 times higher O_2 evolution as compared to noninoculated plants (Ait Barka et al., 2006). Xie et al. (2009) demonstrated that enhanced photosynthetic activity in *Arabidopsis* by volatile emission from *Bacillus subtilis* might be due to accumulation of iron, because iron is often a limiting ion in

photosynthesis. They also observed that when bacterial volatile signal was withdrawn, the photosynthetic capacity and iron content returned to untreated levels. The importance of Fe has already been documented by Spiller and Terry (1980), who demonstrated that biogenesis of the photosynthetic apparatus is associated with a high demand of iron availability. Very recently, Fernandez et al. (2012) monitored various photosynthesis parameters such as net photosynthesis, intercellular CO_2 concentration, stomatal conductance, activity of photosystem II, and total chlorophyll content in cold-stressed grapevine plantlets inoculated with *B. phytofirmans* PsJN as compared to non-bacterized controls. The authors clearly showed that the increase in plant photosynthetic activity was not due to a modulation of stomata conductance in grapevine colonized by strain PsJN. Thus, the mechanism underlying the stimulation of plant photosynthesis by *B. phytofirmans* PsJN remains elusive.

1.3.6. Growth enhancement through vitamins

Vitamins are organic nutritional factors that influence the growth of living microorganisms. In addition to the vitamins present in root exudates as a source for bacterial growth (Mozafar and Oertli, 1993), certain bacterial species also produce vitamins (Dahm et al., 1993). Like other growth promoting traits of PGPB, the production of vitamins also causes positive effect on plant growth and development (Azaizeh et al., 1996; Dakora, 2003). More root colonization ability of vitamin producing *Pseudomonas fluorescens* has been observed by Marek-Kozaczuk and Skorupska (2001). Similarly, co-inoculation of vitamin-producing *P. fluorescens* and *Rhizobium* stimulated the growth and symbiotic nitrogen fixation in clover plants (Marek-Kozaczuk et al., 1996).

1.4. Drought stress and crop productivity

Plants are limited to protect themselves against environmental stresses including drought stress. Thus, plants develop a wide range of strategies to cope with stress situations. Under conditions of water deficiency, drought escape and drought tolerance are two important strategies to ensure plant growth and health. There is limited reported information dealing with the role of microbes on the improvement of drought tolerance.

1.4.1. Effect of drought stress on the physiology of plants

Drought is a situation that lowers plant water potential and turgor to the extent that plants face difficulties in executing normal physiological and biochemical functions. Water stress changes plant physiology and biochemistry (Abdullah et al., 2011). Fundamental changes that occurred as a result of dehydration include changes in physiological and biochemical processes (Sangtarash, 2010), membrane structure and ultrastructure of subcellular organelles (Yordanov et al., 2003) and water relations (Gorai et al., 2010). Plant growth under drought stress is influenced by loss of turgor, stomatal closure, inhibition in cell growth and enlargement, altered photosynthesis, changes in plant metabolites (Bartels and Sunkar, 2005), respiration, carbohydrates, growth promoters, ion uptake, nutrient metabolism (Farooq et al., 2008) and nutrient uptake (Akinci and Losel, 2010). However, its impact depends on the duration and intensity of stress (Chaves et al., 2009), genetically determined plant sensitivity and capacity (Valladares et al., 2007), developmental stage and species of plant (Jaleel et al., 2008), soil type and climate (DaMatta and Ramalho, 2006).

Summarily, under the condition of mild water deficit the plant could adapt through changes in molecular and physiological mechanisms but have to pay the price in the form of reduced biomass yields (Boutraa and Sanders, 2001). Severe deficit of water may result in the arrest of photosynthesis, reduction in turgor, water potential, solutes concentrations in the cytosol and increase of extra-cellular matrices and also leads to the inhibition in cell enlargement (Bhatt and Srinivasa Rao, 2005). Subsequently, continuous accumulation of abscisic acid (ABA) and compatible osmolytes, overproduction of reactive oxygen species (ROS) result in wilting and finally plant death (Jaleel et al., 2008).

1.5. Strategies for improving drought stress tolerance in plants

Drought is worldwide limiting crop productivity and leads to increase in desertification and food insecurity. Hence, the employment of strategies capable of improving the plant potential for higher productivity while ameliorating the adverse effects of drought are needed (see Figure 3). Some of the strategies that proved highly effective in improving tolerance in plants against drought stress are illustrated below.

1.5.1. Germplasm screening

Living material bearing heredity from which new plant can grow is called germplasm such as seeds, rootstock, or leaf plant tissue. In a germplasm screening strategy the superior germplasm is collected by selection of some lines with higher levels of drought tolerance from crop well adapted to harsh conditions from different climatic areas. The selected germplasm undergoes series of screenings under greenhouse and field conditions. The germplasm with recorded high levels of drought tolerance is evaluated

under greenhouse conditions to determine the levels of drought tolerance. The germplasm finally selected is propagated for multiplication and distribution to farming communities. It is a lengthy and time-consuming procedure.

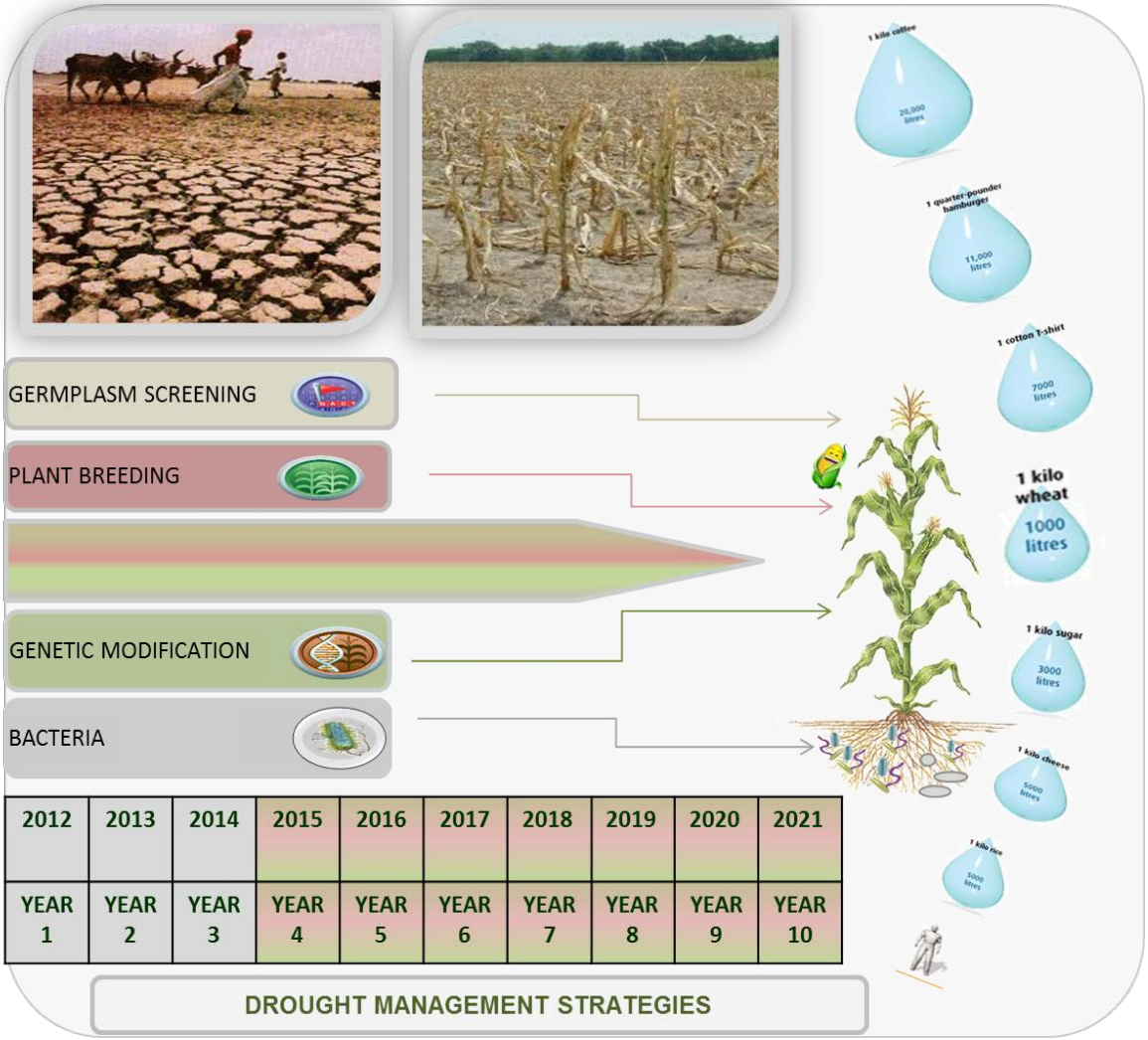

Figure 3 . Schematic view of d ifferent d rought management s trategies o ver th eir time periods

Establishment of proper, practical, reliable, cheap and fast selection methods and multiplication system are the prerequisite of this strategy. Large and expensive glasshouse and field trials are required to screen out drought-tolerant desired plant genotypes. Multidisciplinary approaches to measure the effect of drought stress on the physiology, biochemical and morphology of the plants. Though increases in drought stress tolerance through this strategy are feasible but to a small extent.

1.5.2. Breeding drought tolerance genotypes

Plant breeding could be used to develop tolerant genotypes that are resistant to drought. It appears a competent and lucrative way of manipulating crops to enable them to grow efficiently in drought-prone environments. The contribution of traditionally plant breeding towards tackling the challenges of global food security is enormous since from the last century (Rajaram, 2005). Considerable drought tolerant cultivars/lines of important food crops have been developed during last century. In it new crop varieties or lines with desirable traits are produced through deliberate crossing of closely or distantly related individuals.

Traditionally, manipulation via plant breeding is done through controlled pollination. Plant breeders, at first step deliberately generate genetic diversity that would not exist in nature. Then to generate new plant varieties, they cross and re-cross plants over several generations, followed by artificial selection of progeny with desired traits. The selected resistant plants are then evaluated for their level of drought resistance. The high yielding resistant plants are than multiplied and distributed for field cultivation under drought stressed environments. Presently, chickpea FLIP 87-59C (Singe t al., 1996), common bean SEA5 (Sing et al., 2001), peanut ICGV 87354 (Reddy et al., 2001),

soybean R 01-416F (Chen e t a l., 2007), w heat N E01643 (Baenziger e t a l., 2008) sunflower Morlin (Bergman et al., 2001) are amongst the world prominent cultivar/lines developed through traditional breeding approach.

However, i n t his m ethod onl y pl ants of the same species ar e used to in troduce traits. B reeding has had l imited s uccess i n improving dr ought t olerance i n c rops du e to the multigenic n ature o f th e d rought to lerance tra its. A lso, it c ould r esult in inbreeding and can d eteriorate the b reed an d the p lant m ay b ecome m ore p rone t o d isease o r mutations. Undesirable t raits c an a lso be f ixed t hrough p lant br eeding uni ntentionally. This results in narrow genetic diversity, and a potential loss of some indigenous species. Plant br eeding t hrough selection involves l arge i nvestments of t ime, l abor a nd c ost-intensive and doe s not always w ork (Ashraf, 2010). H ybrids formed d o not br eed true, creating offspring with different traits, thus causing traits obtained to be lost.

1.5.3. Transgenic approach

Plants r esulting f rom a dding a foreign gene ar e o ften r eferred t o as t ransgenic p lants. Plant b reeders ove r the globe a re p ursuing genetic m odification more actively i n t hese days t o ev olve s tress t olerant cu ltivars/lines o f v arious crops (Ashraf et al., 2008). Because us ing t his a pproach t he d esired traits c ould be m ore c arefully i ntroduced f rom either a different variety of the same crop plants or a different plant species while making it easy to exclude less appealing traits. The prospects of improving drought tolerance in crops t hrough genetic e ngineering seem v ery promising because it i s p ossible t o incorporate only t he s pecific c loned ge nes a nd avoid the t ransfer o f u ndesirable g enes. Through genetic engineering it is possible to pyramid genes with similar effects (Gosal et al., 2009). Genetically m odified plants designed t o r esist d rought ha ve t he pot ential t o

withstand more strongly and produce higher yields under water limited conditions. Salient crops, which have been improved for drought tolerance through successful incorporation of genes include soybean (Ronde et al., 2004), peanut (Bhatnagar-Mathur et al., 2009), wheat (He et al., 2011), rice (Zhou et al., 2009), maize (Quan et al., 2004), tobacco (Karim et al., 2007) and tomato (Kalamaki et al., 2009). However, in most cases the performance of transgenic cultivars/lines developed have been tested under controlled conditions and few lines are on the market.

1.5.4. Applying microorganisms to promote drought tolerance in plants

This approach involves the use of biological products or substances, which contain living microorganisms to manage plant stress. Some studies have shown that the inhibitory effects of drought stress could be alleviated by the use of appropriate tolerant plant growth-promoting bacteria (Mnasri et al., 2007). Bacteria have been isolated that can enhance the ability of crops to withstand water stress by increasing seedling vigor, root elongation and various crop physiological and biochemical responses (Alverez et al., 1996; Zahir et al., 2008). These microorganisms can survive under drought stress conditions through various mechanisms such as the production of EPS (Nocker et al., 2012), biofilm formation (Chang et al., 2007) and osmolytes production in order to avoid cell water loss (McNeil et al., 1999). Besides, microorganisms can also offer plant protection against desiccation through the maintenance of a moist environment and conducive to root growth and development, supply of nutrients, hormones, also acting as plant growth promoters (Kavamura et al., 2013). Molecular mechanisms underlying the plant-stimulating activity of bacteria under drought stress has been summarized in Table

2. Figure 4 shows PGPB mediating growth promotion mechanisms under various abiotic stresses.

Drought stress causes oxidative damage at the cellular level. To overcome oxidative damage, plants produce antioxidant enzymes [catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD)] that scavenge free radicals (Simova-Stoilova et al., 2008). Interestingly, Kohler et al. (2008) described that *Pseudomonas mendocina* inoculation improved lettuce (*Lactuca sativa* L.) performance by enhancing CAT under severe drought stress. Creus et al. (2004) reported that inoculation with *Azospirillum brasilense* improved relative leaf water content, growth and yield of wheat (*Triticum aestivum* L.). Plants treated with bacteria producing EPS increased resistance to water stress because EPS is responsible for biofilm formation on the surface of potato roots, thus increasing yield (Bensalim et al., 1998). Bacterized seedlings revealed improved soil aggregation and root adhering soil and higher relative water content in the leaves of maize plants (Sandhya et al., 2010). The work of Mayak et al. (2004b) shows that a bacterial strain (*Achromobacter piechaudii*) containing ACC deaminase conferred tolerance to water deficit in tomato and pepper. Ethylene production was reduced in bacterial treated plants, resulting in significant increase in fresh and dry biomass compared to un-inoculated controls. Zahir et al. (2008) reported that *Pseudomonas* spp. improved the growth of pea (*Pisum sativum*) under drought stress in axenic conditions as well as in potted soil. They concluded that inoculation might have reduced the ethylene synthesis, which led to better root growth and total plant biomass under drought stress.

Bacterial-induced drought resistance is a potentially important technology for arid and semiarid regions of the world, and that have crops that frequently prone to drought

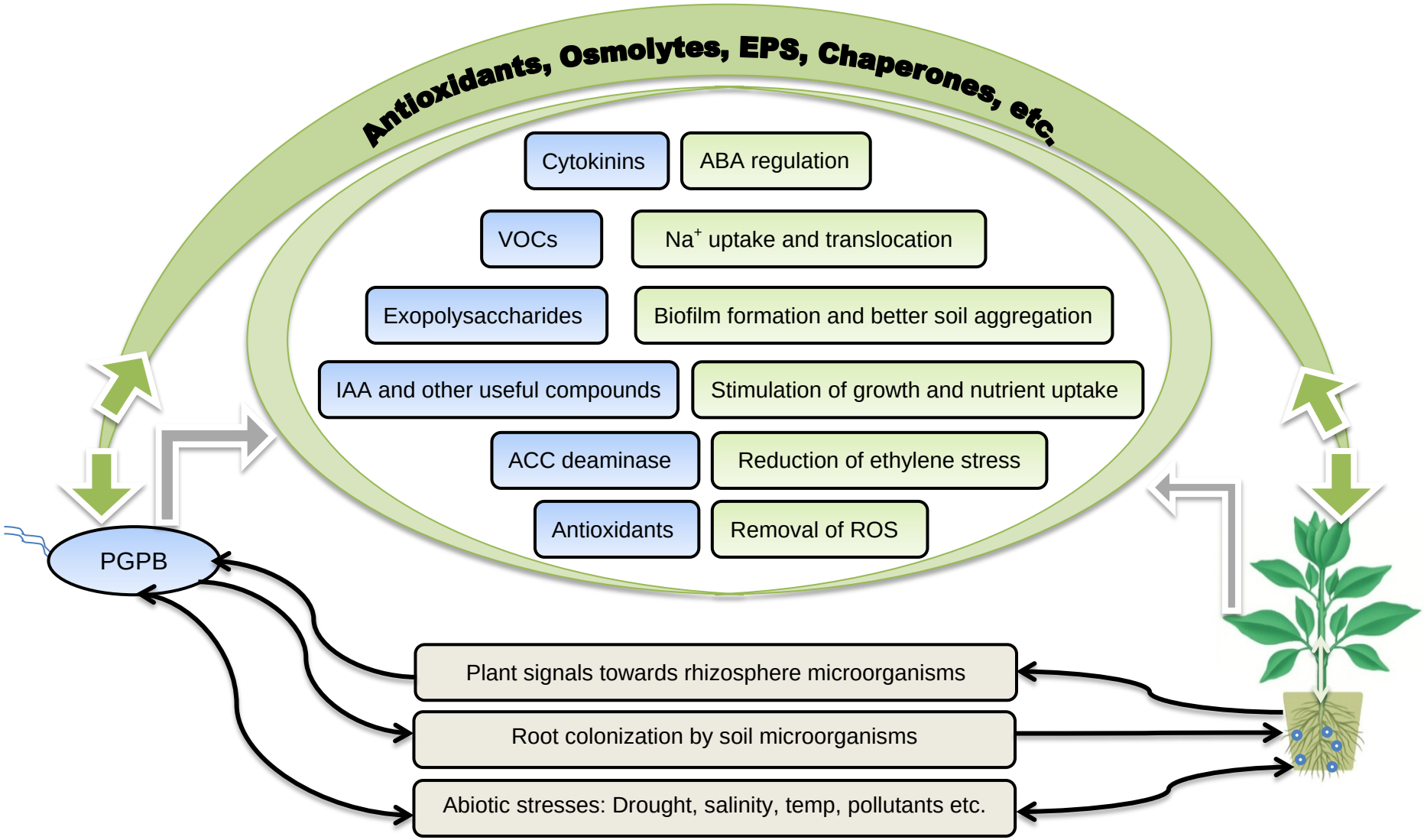

Figure 4. Potential mechanisms of PGPB mediated growth promotion of plants under abiotic stress

Table 2. Plant growth promotion by PGPR/endophyte inoculation under drought stress conditions

Test crop	Beneficial bacteria	Experimental conditions	Proposed mechanism(s)	Plant response	Reference
Capsicum annuum	*Achromobacter*, *Klebsiella*, *Citrobacter* sp.	Pot trial	ACC deaminase	Increased t oot l ength (20%) a nd fresh weight (60%)	Marasco e t al . (2012)
Vigna unguiculata	*Bacillus* sp. RM-2	Pot trial	ACC d eaminase, IAA pr oduction, P - solubilization	Significant i ncrease i n seed germination, s hoot bi omass a nd pod yield	Minaxi et al. (2012)
Triticum aestivum	*Bacillus amyloliquefaciens* 5113, *Azospirillum brasilense* NO40	Axenic	Homeostasis	Plants s howed at tenuated transcript l evels s uggesting improved ho meostatic mechanisms	Kasim et al . (2013)
	Bacillus safensis W10, *Ochrobactrum pseudogregnonense* IP8	Pot trial	Antioxidants / osmoprotectant	Increased r oot a nd s hoot bi omass, yield, a nd c hlorophyll b eside higher antioxidant activity	Chakraborty e t al. (2013)
	Streptomyces sp. DE07, DE10, DE27	Axenic/field trial	Production of phytohormones	Improved s eedlings vi gour a nd yield upto 88%	Yandigeri et al. (2012)
Helianthus annuus	*Achromobacter xylosoxidans* SF2, *Bacillus* sp. SF3, SF4	Axenic/pot trial	Phytohormone production	Higher s hoot-root d ry m atter, a nd salicylic ac id/abscisic acid w as observed	Castillo e t a l. (2013)
	Pseudomonas sp. strain GAP-P45	Pot trial	Exopolysaccharides production	Increased total d ry b iomass u p t o 65%	Sandhya e t a l. (2009)
Vigna radiata	*P. fluorescens* Pf1, *B. subtilis* strains EPB 5, EPB 22, EPB 31	Pot trial	Catalase / peroxidase enzyme	Higher cat alase an d peroxidase was observed in stressed plants	Saravanakumar et al. (2012)
Cicer arietinum	*Paenibacillus lentimorbus* B-30488	Pot trial	Biofilm formation	Increased in shoot le ngth (30%), 100 s eed weight (9%) a nd d ry weight (20%)	Khan et al . (2011)

Ornamental species	*Variovorax paradoxus* 5C-2	Pot trial	ACC deaminase	Lowered ethylene emission from mature leaves consequently reduced abscission of the leaves	Sharp et al. (2011)
Saccharum officinarum cv. M 1176/77 and R 570	*Azospirillum* isolates, Azo 195, Azo 249, Azo 274	Pot trial	Auxins production	Increased shoot height (15%) and root dry mass (75%) in cv M 1176/77 whereas cv R 570 responded negatively	Moutia et al. (2010)
Zea mays L.	*Pseudomonas* sp. BV-P13, GRF HAP-P14, GAP-P45, GRFHYTP52, WAPP53	Pot trial	Antioxidant enzymes	Increased plant biomass, proline, sugars, free amino acids, while protein and starch content was reduced	Sandhya et al. (2010)
Trifolium repens	*Pseudomonas* sp., *P. putida*, *B. megaterium*	Pot trial	IAA production	Increased shoot and root biomass, and water content	Marulanda et al. (2009)
Pisum sativum	*Pseudomonas* sp. ACC-5, ACC-14, Q-7	Axenic/pot trial	ACC deaminase activity	ACC5 increased dry weight (150%), root length (92%), shoot length (45%), and water use efficiency (147%)	Zahir et al. (2008)
Solanum tuberosum	*Bacillus* sp. DH-11, 40	Pot trial	ROS-scavenging enzymes	Enhanced mRNA expression levels of the various ROS scavenging enzymes and higher proline content	Gururani et al. (2013)

stress. However, research on the interactions of endophytic microorganisms with plants, environmental changes and drought resistance is in its infancy.

1.6. Inoculant technology - formulation and commercialization

The application of PGPB for improving crop production is becoming an emerging technology owing to their environment friendly traits. To take advantage of the demonstrated beneficial effects of various soil microbial groups in increasing plant growth and yields, many different types of microbial inoculants (biofertilizers) have been in use for a long time. Biofertilizers are biological preparation containing live or latent cells of microorganisms or their metabolites, which when inoculated to seed, soil or roots of seedlings, promote plant growth and enhance harvestable yield. Biofertilizers, generally marketed, contain microbes capable of N_2-fixation, phosphate solubilization/mineralization, phytohormone production and biocontrol. For example, bacteria belonging to *Azotobacter* and *Azospirillum* have been applied to enhance cereal growth (as biofertilizers), and mainly *Bacillus* and *Pseudomonas* have been applied for biocontrol (as biopesticides) of plant diseases (Fravel, 2005; Bravo et al., 2011). Table 3 shows some selected commercially available microbial inoculants with their producers/trade name. The development of techniques for the large scale production of pure inoculants, with high infectivity potential, is the main issue to be tackled in order to allow a wide use of biofertilizers. The key aspects in PGPB inoculation (biofertilizers) technology are the use of a proper formulation of inoculant preparations, the selection of an adequate carrier, and the design of correct delivery methods.

Table 3 . E xamples o f co mmercial p roducts f or p lant growth-promotion us ing symbiotic / free-living bacteria

Bacterial ingredient	Product	Intended Crop	Company
Rhizobia	VAULT® HP plus INTEGRAL®	Soybeans	Becker U nderwood Corporate, USA
Azotobacter chroococum, Bacillus megaterium, Bacillus firmans	Biogreen	Fields crops	AgroPro, L LC, Volo, IL
Undisclosed	Sumagrow®	Field crops	Bio S oil E nhancers, Inc. H attiesburg, MS
Bacillus pumilus GB34	Yield Shield	Soybean	Bayer Crop Science, LP, North Carolina
Diazotrophs	TwinN	Field crops	Mapleton A gri Biotec P ty L td. Australia
Delftia acidovorans and *Bradyrhizobium*	BioBoost	Canola	BrettYoung S eeds, Ltd., Canada
Rhizobium	SeedQuest®	Legume	Soygro (Pty) L td., South Africa
Bacillus subtilis MBI 600	Subtilex	Field crops	MicroBio G roup Ltd., USA
Rhizobium sp.	Legumefix	Legume crops	Legume Technology L td. UK
Undisclosed	Agri-Buffa®	Field crops	Agrichem, Australia
Pseudomonas syringae	Bio-save	Citrus, pome fruit, potato, a pples, pears and cherries	JET H arvest Solutions, Longwood, FL

Undisclosed	Accele-Grow-M	Field crops	Accelegrow Technologies, I nc. Atlanta, GA
Bacillus subtilis GB03	Companion®	Turf, gr eenhouse, nursery c rops, ornamental	Growth pr oducts, Ltd., Australia
Bacillus subtilis and *Bradyrhizobium japonicum*	HiStick N /T, Turbo-N	Soybean	Becker U nderwood Corporate, USA
Bacillus subtilis and *Bradyrhizobium japonicum*	Patrol N/T	Soybean	United A gri Products (U AP) Canada, Inc.
Bacillus amyloliquefaciens strain FZB42	RhizoVital® 42	Field crops	ABiTEP G mbH, Berlin, Germany
Pseudomonas chlororaphis strain MA342	Cedomon®	Barley, o ats, wheat, rye	Lantmännen BioAgri AB, Sweden

To use PGPB in practical crop production, reliable and practical methods of inoculum delivery must be developed. A variety of methods exist for the delivery of bacteria to crops in the field. Bacteria may be delivered as wet inoculants, peat-based inoculants, adsorbed onto inert materials or encapsulated within other materials. Wet inoculants generally have good stability and reasonable lifetimes; however, unless they are produced locally, cell suspensions need to be shipped, often long distances. Peat-based inoculants are quite common, are relatively inexpensive and offer good stability; however, peat may be quite variable in terms of quality, availability, nutrients and the presence of other organisms. Adsorption of bacteria onto inert materials like talc, kaolinite, lignite, vermiculite has also been used with some success. For carriers that shall be used for seed coating, a good adhesion to seeds is also important (Hegde and Brahmaprakash, 1992). Bacteria can also be stored by lyophilization, which allows achieving high survival rates, without any carrier. However, during the process a cryoprotectant must be added, which is essential for protecting the bacterial cell membrane and cytoplasm against dehydration. Lyophilized microbial cultures can be incorporated into a solid carrier or utilized directly. A promising approach, which has been addressed in the recent years, is bio-encapsulation or micro-encapsulation of microbial cells leading to increased shelf-life and microbial activity (John et al., 2011). Nowadays, research focuses on the creation of new carriers that can have all of these characteristics, and can provide positive results for the application of biological inoculants. Thus, a good carrier may not have all the characteristics cited, but it is recommended that it has as many as possible (Bashan, 1998).

Application methods for the delivery of PGPB to crops in the field are relatively limited. Farmers are not keen on purchasing specialized equipment to be used for microbial-based products. Formulated inoculants should be readily applied using standard farming machinery and straightforward methods. In general, methods developed for application of biological inoculants in the rhizosphere and phyllosphere are also valid for endophytic bacteria (Hallmann et al., 1997). Inoculation can be done through application to the plant material or to the soil. The latter method can be more convenient for the farmer because of less time required, but generally a higher amount of inoculant is then needed (Malusa et al., 2012). Soil inoculation can be done either with solid or liquid formulations. Application of liquid inoculant is often more convenient for the farmer because of less time required, ease of application, and the equipment routinely used on a farm can be used. The development of inexpensive and efficient technologies for the efficient delivery of inocula, probably by modification of sprayers and sprinklers, could facilitate use of PGPB formulations. Normally, the carrier is mixed with the inoculum in the factory, but it could be mixed by the farmer prior to application, especially when liquid formulations are used. Depending on the particular inoculant formulation, the inoculant can be used for seed coating, for dipping seedlings, direct application to the furrow, or as foliar application (Reddy and Saravanan, 2013).

Over the last three decades, numerous miracles PGPB were proposed, never formulated to any product and practically forgotten apart from the paper describing their discovery. Despite the limited understanding of PGPB-plant interactions, a few numbers of these bacteria are nevertheless used commercially as adjuncts to agricultural production (Lucy et al., 2004). Very recently, Glick (2012) addressed a number of issues

for the extensive commercialization of PGPB strains such as (i) screening of those traits that are most important for efficient functioning and subsequent selection of PGPB strains with appropriate biological characteristics, (ii) consistency among regulatory agencies in different countries regarding what strains can be applied to the soil environment, (iii) a better understanding of the pros and cons of using rhizospheric vs. endophytic bacteria, (iv) selection of efficient PGPB strains that properly deliver under specific environmental conditions (e.g., cool and warm weather, saline and drought conditions), (v) development of effective delivery methods of PGPB to plants in various settings (e.g. in the field vs. in the greenhouse), (vi) a better understanding of the potential interactions between the host plant, PGPB and AM fungi.

1.7. Outline of the thesis

This thesis is structured in six chapters. Chapter 1 describes the background and objectives of the research and provides a general introduction on plant growth-promoting bacteria (PGPB), their colonizing niches and mechanisms of actions and their application under normal and drought stress conditions. This chapter ends with the practical aspects of bacterial formulation and commercialization, and with some commercially available products.

Chapter 2 shows results from experiments addressing the capability of plant growth promotion and colonization by selected bacterial endophytic strains isolated from maize roots. A range of different lab assays in regard to potential plant growth promotion was performed and strains were evaluated for improving growth of five maize cultivars

under axenic and natural soil conditions. We found that strains had the potential to improve maize seedling growth under axenic conditions. *Enterobacter* sp. strain FD17 showed highest potential to promote growth and yield of maize grown under natural conditions. This study suggested that *in vitro* plant growth-promoting traits and the potential of maize seedling growth promotion by bacterial endophytes could be used for the selection of potential inoculant strains subjected for further testing as bio-inoculant under field conditions.

Chapters 3 and 5 describe the effect of inoculation of bacterial endophytes *Burkholderia phytofirmans* strain PsJN and *Enterobacter* sp. FD17 on growth, water status and photosynthetic activity of two maize cultivars under drought stress imposed at vegetative stage and flowering stage, respectively. In these chapters plant growth promotion and colonization potential of selected endophytic strains (PsJN and FD17) on two maize cultivars were evaluated. The inoculant strains efficiently colonized maize seedlings and were recovered from root, shoot and leaves of both irrigated and stressed plants. Results revealed that bacterial inoculation minimized the drought stress-imposed effects significantly increasing shoot biomass, root biomass, leaf area, chlorophyll content, photosynthesis, and photochemical efficiency of PSII. In chapter 3, bacterized seedlings showed higher leaf relative water content (30%) compared to control, whereas 43% higher leaf damage in terms of relative membrane permeability was observed in non-inoculated plants under drought stress. Our data suggest that endophytic bacteria could be efficiently used to reduce the effects of drought stress on growth and photosynthesis of maize.

In Chapter 4, we investigated the potential of endophytic bacteria *Burkholderia phytofirmans* PsJN to ameliorate the effects of drought stress on growth, physiology and yield of wheat (*Triticum aestivum* L.) under natural field conditions. The plants were exposed to drought stress at different stages of growth (tillering stage and flowering stage) by skipping the respective irrigation. Inoculation of wheat with PsJN significantly diluted the adverse effects of drought on relative water contents and CO_2 assimilation rate thus improving the photosynthetic rate, water use efficiency and chlorophyll content over the un-inoculated control. Inoculation resulted in better grain yield (up to 21 and 18% higher, respectively) than the respective un-inoculated control. Based on our results we conclude that application of PsJN is effective to improve physiology, relative water content and biomass of wheat under reduced irrigation. The improved plant physiological and antioxidant activity ultimately leads to enhanced crop yield and quality.

It is described that the L-tryptophan (L-TRP) - dependent biosynthesis of indole-3-acetic acid (IAA) improves plant growth promotion and colonization of maize by *Burkholderia phytofirmans* PsJN in Chapter 6. Results revealed that PsJN inoculation supplemented with L-TRP (10^{-5} M) gave the most promising results and significantly increased plant height, photosynthesis, chlorophyll content, root biomass and shoot biomass compared to control. The inoculant strain colonized more efficiently to maize seedlings in the presence of exogenously applied L-TRP. The results imply that substrate (L-TRP) - derived IAA biosynthesis in the rhizosphere by PsJN inoculation could be a useful approach for improving the growth, photosynthesis and nutrient content of maize.

In Chapter 7, a summary is given of the major findings of the thesis. Concluding remarks and future prospects complete this chapter.

1.8. Objectives of the thesis

This Ph.D. thesis focuses on the characterization of beneficial endophytic bacteria with their potential to promote plant growth and abiotic (drought) stress tolerance. Aims were as follows:

- Characterization of potential plant-beneficial traits of the isolated endophytes
- Screening of selected endophytic strains for plant growth promotion and colonization capacity to different maize cultivars under gnotobiotic conditions.
- Evaluation of the most promising isolate for improving growth and yield of maize cultivars under natural soil conditions
- To evaluate the potential of endophytic strains *Burkholderia phytofirmans* PsJN and *Pantoea* sp. FD17 for improving growth, photosynthesis and water content of two maize cultivars under drought stress conditions.
- To evaluate the potential of *Burkholderia phytofirmans* PsJN for improving physiology, antioxidants, growth and yield of wheat under drought stress in the field.
- To study the synergistic effect of the amendment of L-TRP and the associated IAA synthesis by *Burkholderia phytofirmans* PsJN for improving growth and the strain's colonization of maize plants.
- Developing inoculation strategies for efficient colonization by selected bacteria (previously found highly effective for enhancing crop yield) for the prosperity of agriculture industry.

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Chapter 2

The endophyte *Enterobacter* sp. FD17: a maize growth enhancer selected based on rigorous testing of plant beneficial traits and colonization characteristics

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Running Title: Beneficial effect of endophytic bacteria on maize growth and yield

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Abstract

With the aim to select powerful microbial strains to be used for the enhancement of maize yield and resistance to abiotic and biotic stresses, we tested five endophytic bacterial strains previously isolated from maize roots. A range of different lab assays in regard to potential plant growth promotion was performed and strains were further evaluated for improving growth of five maize cultivars under axenic and natural soil conditions. Endophytic colonization was an additional component in our selection process as it is of high importance for an inoculant strain to efficiently colonize the plant environment. All strains had the potential to improve maize seedling growth under axenic conditions. *Enterobacter* sp. strain FD17 showed both the highest growth promoting activity under axenic conditions as well as colonization capacity. FD17 was therefore selected for further plant tests in a net house, in which two different maize cultivars were grown in large pots until ripening and subjected to out-door climatic conditions. Results showed that inoculation significantly increased plant biomass, number of leaves plant^{-1}, leaf area, and grain yield up to 39, 14, 20, 42%, respectively, as compared to the un-inoculated control. Similarly, inoculation also improved the photochemical efficiency of photosystem II (PSII) of maize plant and reduced the time needed for flowering. We also confirmed that strain FD17 is able to colonize the rhizosphere, roots and stems. Based on rigorous testing *Enterobacter* sp. strain FD17 showed highest potential to promote growth and health of maize grown under natural conditions. This study suggested that *in vitro* plant growth promoting traits and potential of maize seedling growth promotion by bacterial endophytes could be used for the selection of potential inoculant strains subjected for further testing as bio-inoculant under field conditions.

Key Words: *Enterobacter* sp. FD17, PGP traits, plant growth, plant colonization, maize

Introduction

The exponential growth of the world´s population led to an increasing demand on food production, which is accompanied by an increasing demand on fertilizers. This has not only caused an extreme rise in the price of fertilizers, but will lead to an unavoidable world shortage of e.g. phosphate within decades (Vance 2011). We face the same problem for water and global warming makes the situation even worse. Innovative agricultural management systems are needed to fulfill the future global demand for food, feed, and fiber products while minimizing negative atmospheric, soil, and water quality impacts (Hayati et al. 2011; Lambin and Meyfroidt 2011).

The use of biological inoculants based on plant growth-promoting bacteria (PGPB) appears to be a promising alternative to chemicals. Consequently, over the years, the utilization of PGPB, usually either rhizosphere bacteria or endophytes, as bio-fertilizers and/or bio-pesticides has received increasing attention. These microorganisms may not only ensure the availability of essential nutrients to plants but also enhance nutrient use efficiency (Khalid et al. 2009). Endophytic bacteria may in future be even more important than rhizosphere bacteria in promoting plant growth promotion and nutrition, because they escape competition with rhizosphere microorganisms and achieve a more intimate contact with plant tissues.

Proposed mechanisms by which PGPB may stimulate plant growth and nutrition include biological nitrogen fixation (BNF), synthesis of phytohormones, environmental stress relief, synergism with other plant-microbe interactions, inhibition of plant ethylene

synthesis, as well as increasing availability of nutrients like phosphorus, iron and other micro-elements, and growth enhancement by volatile compounds (Hardoim et al. 2008; Compant et al. 2010; Mitter et al. 2013). However, the expression of such bacterial activities under laboratory conditions does not necessarily guarantee the same beneficial effects in association with plants grown under natural conditions (Fuentes-Ramirez and Caballero-Mellado 2005; Smyth et al. 2011).

The identification and selection of effective PGPB, which are effective when applied onto plants grown in natural soils, can be time consuming, laborious and costly. The selection procedure usually involves the collection of plant-associated bacteria and subsequent screening under various environmental conditions. Usually screenings are performed in a bottom-up approach, starting with multiple lab assays and tests performed under gnotobiotic conditions. Selected strains are then tested in the greenhouse followed by field evaluation (Khalid et al. 2004; Hynes et al. 2008). In many studies bacteria have been isolated and screened for few plant growth-promoting capacities in the lab, typically for IAA, ACC deaminase, P solubilization or antibiotic production (Penrose and Glick 2003; Khalid et al. 2004; Hynes et al. 2008; Bashan et al. 2013). However, many of these strains then failed to be successful when applied onto plants grown in non-sterile soil. Strains may either fail to successfully colonize or beneficial activities may not be expressed in association with plants grown under greenhouse or under field conditions (Smyth et al. 2011).

The aim of the present study was to select a bacterial strain, which has the potential to enhance plant performance in the field and which potentially works with different plant genotypes. We therefore tested five bacterial strains, which were

previously isolated as root endophytes from maize (Prischl et al. 2012) and subjected them to a rigorous testing in the lab addressing a multitude of characteristics, gnotobiotic plant tests with five maize varieties as well as plant colonization characteristics. *Enterobacter* sp. strain FD17 was selected as most promising strain and further tested with two maize cultivars grown in a net house, in which plants were exposed to natural, climatic conditions.

Materials and methods

Endophyte strains used in this study

Previously bacterial endophytes were isolated from the maize roots by Prischl et al. (2012). Based on IAA production (qualitative test) and ACC-deaminase activity, five isolates - *Caulobacter* sp. FA13, *Pantoea* sp. FF34, *Sphinogobium* sp. FC42, *Pseudomonas* sp. FB12, and *Enterobacter* sp. FD17, were characterized in this study and tested in detail in regard to plant growth promotion and colonization. To confirm the genus assigned based on 16S rRNA gene analysis (Prischl et al. 2012), strain *Enterobacter* sp. FD17 was further characterized by partial sequence analysis of the *gyrB* gene (Yamamoto and Harayama 1995; Brady et al. 2008). Sequencing was done making use of the Sanger sequencing service of the company AGOWA (Berlin, Germany). Retrieved sequences were visualized with sequence alignment editor package of BioEdit and identified by BLAST analysis.

Nucleotide sequence accession number

The sequence determined in this study was deposited in the GenBank database with the accession number KF147850.

Functional characterization of the endophytic strains

Phenotypic and physiological characterization

Bacterial strains from overnight grown cultures in TSA broth were streaked on TSA agar plates and incubated at 30°C. After 24 h, the color and shape of colonies were noted. Cell motility and shape of single colony was observed under light microscope (Nikon, Japan).

The pH limits for bacterial growth was determined adjusted to pH values between 5 and 12 in triplicates. The dependence of bacterial growth on different salt concentrations was determined in the same medium containing 1-6% NaCl. Furthermore, the ability to growth in methanol/ethanol as sole C source was analyzed.

Bacterial capacity to aggregate formation may positively affect their dispersal and survival in the plant environment and adsorption to plant roots. The extent of aggregation formation was measured in six replicates following the method of Madi and Henis (1989) with some modifications. Aliquots of liquid culture containing aggregates were transferred to glass tubes and allowed to stand for 30 min. Aggregates settled down to the bottom of each tubes, and the suspension was mostly composed free of cells. The turbidity of each suspension was measured at 540 nm (ODs) with a microplate reader (Synergy 5; BioTek Instrument Inc., Winooski, USA). Cultures were then dispersed with a tissue homogenizer for 1 min and the total turbidity (OD) was measured. The percentage of aggregation was estimated as follows:

% aggregation = (ODt – ODs) x 100 / ODt

Motility assays (swimming, swarming and twitching) were performed following the methods of Rashid and Kornberg (2000). Swim plates (LB media contained 0.3% agarose) were inoculated in triplicates with bacteria from an overnight culture on TSA

agar plates grown at 30°C with a sterile toothpick. For swarming, plates (NB media contained 0.5% agar and glucose) were inoculated with a sterile toothpick. Twitch plates (LB broth containing 1% Difco granular agar) were stab inoculated with a sharp toothpick to the bottom of petri dish from an overnight grown culture in TSA agar plates.

Biofilm formation was analyzed using overnight grown bacterial culture in 96 well microtiter plates by staining with 1% crystal violet (CV) for 45 min. To quantify the amount of biofilm, CV was destained with 200 µl of 100% ethanol. The absorbance of 150 µl of the destained CV, which was transferred into a new microtiter plate was measured at 595 nm (modified from Djordjevic et al. 2002).

Biochemical characterization

Biochemical tests such as oxidase, catalase, gelatin hydrolysis and casein hydrolysis of the selected strains were performed. Oxidase and catalase activities were tested with 1% (w/v) tetramethyl-p-phenylene diamine and 3% (v/v) hydrogen peroxide solution, respectively. Gelatin and casein hydrolysis was performed by streaking bacterial strains onto a TSA plates from the stock culture. After incubation, trichloroacetic acid (TCA) was applied to the plates and made observation immediately for a period of at least 4 min (Medina and Baresi 2007).

Quantification of auxin production

Auxin production by bacterial isolates both in the presence and absence of L-tryptophan (L-TRP) was determined colormetrically and expressed as IAA equivalent (Sarwar et al. 1992). Two days old bacterial cells grown (28°C at 180 rpm) in TSA broth supplemented with 1% L-TRP solution were harvested by centrifugation (10, 000 g for 10 min). Three

mL of the supernatants were mixed with 2 mL Salkowski´s reagent (12 g L^{-1} $FeCl_3$ in 429 ml L^{-1} H_2SO_4). The mixture was incubated at room temperature for 30 min for colour development and absorbance at 535 nm was measured using spectrophotometer. Auxin concentration produced by bacterial isolates was determined using standard curves for IAA prepared from serial dilutions of 10-100 µg mL^{-1}.

Assays for phosphorus solubilization and siderophore production

Bacterial strains were evaluated for their ability to solubilize phosphates (organic/inorganic P). Aliquots (10 µL) of overnight bacterial growth culture in TSA medium were spot inoculated on to NBRI-PBP (Mehta and Nautiyal 2001) and calcium/sodium phytate agar medium (Rosado et al. 1998). Solubilization of organic/inorganic phosphates was detected by the formation of a clear zone around the bacterial growth spot. Phosphate solubilization activity was also determined by development of clear zone around bacterial growth on Pikovskaya agar medium (Pikovskaya 1948). Bacterial isolates were assayed for siderophores production on the Chrome azurol S (CAS) agar medium described by Schwyn and Neilands (1987) as positive for siderophore production.

Assays for exopolysaccharide, NH_3 and HCN production

For exopolysaccharide (EPS) activity (qualitative), strains were grown on Weaver mineral media enriched with glucose and production of EPS was assessed visually (modified from Weaver et al. 1975). The EPS production was monitored as floc formation (fluffy material) on the plates after 48 h of incubation at 28 ± 2°C. Strains were tested for the production of ammonia (NH_3) in peptone water as described by

Cappuccino and Sherman (1992). The bacterial isolates were screened for the production of hydrogen cyanide (HCN) by inoculating King's B agar plates amended with 4.4 g L^{-1} glycine (Lorck 1948). Filter paper (Whatman no. 1) saturated with picrate solution (2% Na_2CO_3 in 0.5% picric acid) was placed in the lid of a petri plate inoculated with bacterial isolates. The plates were incubated at 28 ± 2°C for 5 days. HCN production was assessed by the colour change of yellow filter paper to reddish brown.

Assays for poly-hydroxybutyrate (PHB) and n-acyl-homoserine lactone (AHL) production

The bacterial isolates were tested for PHB production (qualitative) following the viable colony staining methods using Nile red and Sudan black B (Juan et al. 1998; Spiekermann et al. 1999). The LB plates with overnight bacterial growth were flooded with 0.02% Sudan black B for 30 min and then washed with ethanol (96%) to remove excess strains from the colonies. The dark blue coloured colonies were taken as positive for PHB production. Similarly, LB plates amended with Nile red (0.5 µL mL^{-1}) were exposed to UV light (312 nm) after appropriate bacterial growth to detect PHB production. Colonies of PHA-accumulating strains showed fluorescence under ultraviolet light. The bacterial strains were tested for AHL production following the method modified from Cha et al. (1998). The LB plates containing 40 µg ml^{-1} X-Gal were plated with reporter strains (*A. tumefaciens* NTL4.pZLR4). The LB plates were spot inoculated with 10 µL of bacterial culture and incubated at 28 ± 2 °C for 24 h. Production of AHL activity is indicated by a diffuse blue zone surrounding the test spot of culture. *Agrobacterium tumefaciens* NTL1 (pTiC58ΔaccR) was used as positive control and plate without reporter strain was considered as negative control.

Enzyme hydrolyzing activities

Bacterial hydrolyzing activities due to amylase, cellulase, chitinase, lipase, pectinase, protease and xylanase were screened on diagnostic plates after incubation at 28°C. Amylase activity was determined on agar plates following the protocol Männistö and Häggblom (2006). Formation of an opaque halo around colonies indicated lipase activity. Cellulase and xylanase activities were assayed on plates containing (per liter) 5 g of carboxymethyl cellulose or birch wood xylan, 1 g of peptone and 1 g of yeast extract. After 10 days of incubation, the plates were flooded with gram's iodine staining and washing with 1M NaCl to visualize the halo zone around the bacterial growth (modified from Teather and Wood 1982). Chitinase activity of the isolates was determined as zones of clearing around colonies following the method of Chernin et al. (1998). Protease activity was determined using 1% skimmed milk agar plates, while lipase activity was determined on peptone agar medium. Formation of halo zone around colonies was used as indication of activity (Smibert and Krieg 1994). Pectinase activity was determined on nutrient agar supplemented with 5 g L^{-1} pectin. After 1 week of incubation, plates were flooded with 2% hexadecyl trimethyl ammonium bromide solution for 30 min. The plates were washed with 1M NaCl to visualize the halo zone around the bacterial growth (Mateos et al. 1992).

Antagonistic activities against plant pathogenic bacteria, fungi and oomycetes

The antagonistic activities of bacterial isolates were screened against plant pathogenic bacteria (*Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Streptococcus pneumoniae*), fungi (*Fusarium caulimons*, *Fusarium graminarium*, *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani*, *Thielaviopsis basicola*) and oomycetes

(*Phytophthora infestans*, *Phytophthora citricola*, *Phytophthora cominarum*). For antibacterial assays, the bacterial isolates and pathogen were cultivated in TSA broth at 30°C for 24 h. The bacterial isolates were spot-inoculated (10 µL aliquots) on TSA plates pre-seeded with 100 µL tested pathogen. The plates were incubated at 28°C for 48 h and clear zones of inhibition were recorded.

Antagonistic activity of the bacterial isolates against fungi and oomycetes was tasted by the dual culture technique on potato dextrose agar (PDA) and yeast malt agar (YMA) media (Dennis and Webster 1971). A small disk (5 mm) of target fungus/oomycetes was placed in the center of petri dishes of both media. Aliquots of 10 µL of overnight bacterial cultures grown in TSA were spotted 2 cm away from the center. Plates were incubated for 14 days at 24°C and zones of inhibition were scored.

Effect of endophytic strains on maize germination

Inoculants of the selected strains were prepared in 50 mL TSA broth in 100 mL Erlenmeyer flasks and incubated at 28 ± 2 °C for 48 h in the orbital shaking incubator (VWR International, GmbH) at 180 r min^{-1}. The optical density of the broth was adjusted to 0.5 measured at 600 nm using spectrophotometer (Gene Quant Pro, Gemini BV, The Netherlands) to obtain a uniform population of bacteria (10^8 - 10^9 colony-forming units (CFU) mL^{-1}) in the broth at the time of inoculation.

Maize seeds were surface-sterilized with 70% ethanol (3 min), treated with 5% NaOHCl for 5 min, and followed by washing 3 times with sterile distilled water (1 min each time). The efficacy of surface sterilization was checked by plating seed, and aliquots of the final rinse onto LB plates. Samples were considered to be successfully sterilized

when no colonies were observed on the LB plates after inoculation for 3 days at 28°C. Surface-disinfected seeds of different maize cultivars (Helmi, Morignon, Pelicon, Peso and Cesor) were immersed in the bacterial suspensions for 30 min. The bacterized seeds were deposited onto soft water-agar plates (0.8%, w/v agar) and plates were placed in the dark at room temperature (24 ± 2°C). After 96 hrs the percentage of germinated seeds was scored. Surface-sterilized seeds, but not bacterized (treated in TSA broth), served as the germination control.

***In vitro* screening of efficient strains under axenic conditions**

A growth chamber experiment was conducted on maize to screen the selected strains for their growth promoting activity under gnotobiotic conditions. We used specially designed glass tubes with beaded rim (Duran group, DURAN GmbH, Mainz, Germany) for the experiment. The glass tubes were covered with lid to generate fully axenic conditions (no exposure to any environmental factors). Bacterial inoculant production and seed treatment were done as described above. As control, seeds were treated with sterilized TSA broth. Treated seeds were placed onto water-agar plates for germination. After 5 days, germinated seedlings (3-5 cm long) were transferred in the sterilized glass tubes containing sterilized 20 ml MS (Murashige and Skoog) medium (Duchefa Biochemie, The Netherlands) (4.8 g L^{-1}) and placed at 25 ± 2 °C set at a 16 h light and 8 h dark period, with a light intensity of 350 µmol m^{-2} s^{-1}. Data regarding shoot / root length and biomass were recorded after 24 days. Colonization of inoculant strains was scored by re-isolation of endophytes. One g of plant shoot was homogenized with a pestle and mortar in 4 ml of 0.9% (w/v) NaCl solution. The number of cultivable endophytes in maize

shoot, expressed in CFU per gram (fresh weight), was determined by spreading serial dilution up to 10^{-4} (0.1 mL) of homogenized surface-sterilized plant material onto TSA (DIFCO Laboratories, Detroit, Michigan) agar medium. Four replicates for each treatment were spread on the agar plates and incubated for 5 days at 28°C. Twenty colonies per treatment were randomly selected and their identity with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer (IGS) region (Reiter et al. 2002).

Net house experiment

On the basis of the results from tests performed under axenic conditions, strain FD17 was selected for further evaluation in a pot trial, in which plants were grown in large containers exposed to natural environmental conditions.

Maize plants were grown in soil collected from agricultural (maize) fields in Fischamend, Lower Austria, Austria. The soil was silty clay loam and had the following characteristics: 12% sand, 61% silt, 27% clay, pH 6.5, 3.3% total carbon, 0.18% total nitrogen, 0.13 mg g^{-1} available phosphorus, 0.066 mg g^{-1} extractable potassium.

Surface-disinfected seeds of two maize cultivars (Morignon and Peso) were immersed in bacterial suspension (prepared as described above) for 1 h. For the un-inoculated control, seeds were treated with sterilized TSA broth. Seeds were sown in a plastic tray (wiped with ethanol) and 12 days old seedlings were transferred into containers filled with 45 kg soil (2 plants in each container) and placed in a net house and exposed to natural environmental conditions.

Weather conditions i.e. precipitation, temperature and relative humidity were recorded by 'Zentralanstalt für Meteorologie und Geodynamik' (ZAMG) during the crop growth period and described in Figure 1. There were three replicated and the pots were arranged in a completely randomized design. Recommended dose of NPK fertilizers (160-100-60 kg ha $^{-1}$) were applied in each container and tap water was applied to the container for irrigation whenever needed.

Data of photochemical efficiency of PSII was recorded at flowering stage using handy PEA (Hansatech Instruments Ltd. England) in the mid of July where day time temperature varied from 30-35^{o}C. The PSII efficiency in terms of F_v/F_m was calculated from the data. Growth and yield contributing parameters were recorded at maturity. The plants were harvested 140 days after planting.

Rhizosphere and endophytic colonization of roots, stems and leaves by the inoculant strain were determined by plate counting using TSA plates. Root, stem and leave samples were washed, surface sterilized (as described above) and used for inoculant strain recovery (colonization). For this, samples were crushed in 0.9 % (w/v) NaCl solution, shaked with a pulsifier (Microgen Bioproducts Ltd., UK) for 30 sec and different dilutions were spread on TSA plates. Bacterial colonies were counted after 4 days of incubation at 28 ± 2^{o}C. The selected colonies were identified and confirmed by IGS region-based RFLP analysis.

Statistical analyses

The data of plant growth parameters and colonization were subjected to analyses of variance. The means were compared by Least Significant Difference (LSD) test ($p <$

0.05) to detect statistical significance among treatment (Steel et al. 1997). All of the statistical analyses were conducted using SPSS software version 19 (IBM SPSS Statistics 19, USA).

Results

Gyrase B sequence analysis of strain FD17

The partial nucleotide sequences of *gyrB* gene of strain FD17 revealed 99% identities with the Genbank entry CP002886.1, *Enterobacter cloacae* (987 bp).

Functional characterization of endophyte isolates based on lab assays

A range of features known to contribute to plant growth promotion, stress tolerance or biocontrol was tested. The results of characterization are summarized in Table 1. All strains showed IAA production (ranging from 1.83 to 10.33 $\mu g\ mL^{-1}$ IAA-equivalent), NH_3 and siderophore production (qualitative) but with variable degree of efficacy. Only strain FB12 was able to produce AHL. Three strains (FF34, FB12 and FD17) showed P-solubilization and PHB production, whereas only FB12 was able to produce HCN. Likewise, strain FB12 showed positive for all biochemical characters mentioned in the Table 1. Strain FA13 produced EPS more efficiently compared to FC42 and FD17. All strains showed lipase activity, while none of the strain produced amylase and protease activities. Pectinase activity was observed with strains FF34, FB12 and FD17. All strains were positive for cellulase and xylanase activity except strain FF34. Chitinase was produced by strains FB12 and FD17. Strain FD17 exhibited better aggregate stability, chemotaxis and biofilm formation as compared to other strains. Strains FB12 and FD17

showed antagonistic activity against various bacterial pathogens as compared to other strains. All strains showed antagonism against different fungal pathogens and oomycetes but with variable degree of efficacy. Strain FD17 showed highest antagonism against *F. caulimons*, *F. solani* and *P. citricola*.

Effect on maize germination

Inoculation of maize seeds with endophytic bacteria increased the germination rate of all cultivars by 20-40% compared to the un-inoculated control (Fig. 2). Maximum increase was observed by inoculation with strain FD17 (40%) in maize cv. Morignon followed by strains FF34, FA13, FB12 and FC42. The minimum increase was recorded by inoculation with strain FB12 and strain FC42 in the maize cultivars Helmi and Cesor, respectively.

***In vitro* screening of efficient strains under axenic conditions**

All strains significantly increased the seedling growth compared to the control (Table 2). For three cultivars (Peso, Morignon, Cesor), strains FD17 performed best in regard to root and shoot length production (Table 2), while for Helmi and Pelicon higher values were recorded with other strains. The maximum root length formation was found with cultivar Peso and strain FD17 (27.8 cm; control 19.25 cm). Strain FB12 inhibited root formation of cultivar Helmi, but promoted root formation of other cultivars. The maximum shoot length formation was observed with strain FD17 and cultivar Morignon (34.75 cm; control 27.75 cm). Again, some strains did not promote shoot formation of some cultivars, but had a positive effect on others (Table 2). All strains significantly promoted biomass production (Table 3). Strain FD17 was beneficial for all cultivars,

whereas other strains showed more cultivar-specific responses. Highest shoot biomass was found with Morignon and strain FD17 (increase by 96% compared to control treatment). Generally, cultivar Helmi showed low response to bacterial inoculation (Table 3).

Figure 3 shows that bacterial strains efficiently colonized all maize cultivars; viable cell numbers ranged from 1.20×10^4 to 2.93×10^7 cells g^{-1} fresh plant material. Maximum colonization was recorded by inoculation with strain FD17 in the shoot interior of Morignon followed by Peso. Strain FD17 was able to colonize all cultivars quite efficiently, whereas other strains showed more variable colonization characteristics. Peso was the only cultivar well colonized by all strains (Fig. 3).

Net house experiment

Based on the results obtained under axenic conditions, strain FD17 was selected for further evaluation for its performance under conditions close to those encountered in the field. The net house is a wire construct without glass roof, but is covered by a net only, which is permeable for rain. Plants encountered natural climate conditions. Data of climate conditions (Fig. 1) revealed precipitation ranges of 0 to 42.9 mm, temperature ranges of 4.6 to 34.6 ^oC and relative humidity values of 50.1 to 95.9 during the crop growth period.

Inoculation with strain FD17 led to a significant increase in leaf area of both cultivars (20% and 13%, respectively, Table 4). Similarly, biomass (leaf dry weight) was increased by 27% and 23% in the cultivars Peso and Morignon, respectively, as compared to the control. Similarly root and plant dry biomass and plant height were

significantly enhanced as well as cob weight (35% and 42% increase in Peso and Morignon, respectively, as compared to control).

Strain FD17 affected plant physiological characteristics such as increase of chlorophyll fluorescence (maximum increase of 9% in cultivar Peso) and reducing the time needed for the onset of flowering (up to 10 days in cultivar Peso) (Table 4).

Strain FD17 efficiently colonized rhizosphere, root, shoot and leaf interior of both maize cultivars and no significant differences in the viable cell number were encountered. Higher colonization was found in the rhizosphere and root interior as compared to the shoot and leaf interior (Table 5).

Discussion

The use of PGPB is currently gaining worldwide interest as a promising alternative to the use of potentially polluting chemical fertilizers and pesticides, particularly in organic production. PGPB may employ different mechanisms to enhance seed germination, root development or to improve mineral nutrition and water utilization (Dobbelaere et al. 2003; Mitter et al. 2013). Generally, a bottom-up approach is employed to select strains, which are promising for field application. Initially, lab-based tests of various activities potentially involved in plant growth promotion are used, followed in greenhouse studies and in some cases by field tests (Khalid et al. 2004; Smyth et al. 2011; Bulgarelli et al. 2013). Often only few characteristics are tested in the lab and strains selected in the lab have in many cases proven not to show the expected success when tested on plants grown in non-sterile soil (Barret et al. 2011). This might be due to the fact that an inoculant strain needs to be able to establish in a highly competitive environment, a characteristic,

which is rarely addressed in initial screenings. They also might be poor plant colonizers. Furthermore, several mechanisms involved in plant growth promotion might be needed to confer the beneficial effects and/or the desirable activities (observed under lab conditions) might only be expressed under specific conditions (reviewed for *Azospirillum* sp. by Bashan and de-Bashan 2010).

In this study we rigorously tested several strains previously isolated as endophytes from maize roots (Prischl et al. 2012). Based on IAA production and ACC-deaminase capability, five bacterial strains (used in the present study) were selected for rigorously testing their plant growth promotion potential in lab and plant assays as well as their plant colonization capacities (Table 1). Based on these numerous characteristics, we selected *Enterobacter* sp. strain FD17 and confirmed its plant growth-promoting effects with two maize cultivars grown in regular (maize) field soil and subjected to natural climatic conditions.

The five endophyte strains showed highly variable growth-promoting traits and plant growth promotion effects when tested in lab trials and plant assays. All of the isolates were able to produce IAA equivalent, but with variable efficiency in the presence and absence of L-TRP. Under axenic conditions, inoculation improved root-shoot length and biomass of different maize cultivars. It has been described that root growth can be stimulated or inhibited depending on the concentration of IAA produced by bacteria (Zúñiga et al. 2013). This is also in line with our results, where strain FF34 showed highest IAA production, but induced less root biomass than other strains producing less IAA. However, we have to be aware that we only have results of lab assays and have no information on *in planta* activities. Glick et al. (1998) introduced a model in which IAA

synthesis also plays a critical role in ACC synthesis, the immediate precursor of ethylene. Therefore, high concentrations of IAA lead to high levels of ethylene, decreasing root length (Strader et al. 2010). Recently, Bhattacharjee et al. (2012) observed an increase in plant biomass of rice cultivars due to IAA and ACC deaminase production by PGPB upon inoculation. The role of ACC deaminase in decreasing ethylene levels by the enzymatic hydrolysis of ACC into α-ketobutyrate and ammonia has been documented as one of the major mechanisms of PGPR in promoting root and plant growth (Glick et al. 1998). In our study, all strains produced ACC deaminase, which was most active in *Enterobacter* sp. strain FD17 and might be needed to confer beneficial effects.

Phosphorus is very important for normal plant growth and development. The inoculation with the microorganisms that have the ability to solubilize P could enhance the quantity of effective P and increase crop yields (Yao 2004). The strains used in this study have the ability to solubilize tricalcium phosphate and dicalcium phosphate and, therefore, could be effective for improving crop yield under natural conditions. This can be attributed to the release of different organic compounds in the rhizosphere by microorganisms may be important in the solubilization of various inorganic P compounds (Scervino et al. 2010). However, contrary to this, Collavino et al. (2010) reported that *in vitro* P solubilization by microorganisms was not necessarily associated to the promotion of plant growth. Very recently, Bashan et al. (2013) suggested that tricalcium phosphate (TCP), is relatively weak and unreliable as a universal selection factor for isolating and testing phosphate-solubilizing bacteria (PSB) for enhancing plant growth. Out of five strains, three were positive for EPS and PHB production. Again, strain FD17 showed highest activities. Bacterial EPS and PHB have been shown to provide protection from

such environmental insults as desiccation, predation, and the effects of antibiotics (Gasser et al. 2009; Staudt et al. 2012). They also contribute to bacterial aggregation, surface attachment, and plant–microbe symbiosis (Laus et al. 2005).

Bacterial survival and colonization in the plant environment are necessary for plant growth and yield. Recently, Zúñiga et al. (2013) described that the cell-to-cell communication (QS) system mediated by AHL is implicated in rhizosphere competence and colonization of *Arabidopsis thaliana* by *B. phytofirmans* PsJN. Chemotaxis (Motility), aggregate stability, and biofilm formation are important traits for root surface colonization (Danhorn and Fuqua 2007). It has been suggested that a prerequisite for the effective colonization of roots is positive chemotaxis towards root exudates (Bhattacharjee et al. 2012). A positive chemotaxis of selected strains therefore suggests that maize seed and root exudates release compounds that attract bacteria towards the plant leading to colonization. In the present study, only FB12 showed AHL-based QS signaling, however, other communications might be involved. Aggregation and biofilm formation were common traits in all tested strains. In the case of motility, four strains were positive for swimming while FD17 only showed swarming.

Volatile compounds such as ammonia and HCN produced by a number of rhizobacteria were reported to play an important role in biocontrol (Brimecombe et al. 2001). Production of ammonia was commonly detected in all selected isolates, however, only *Pseudomonas* sp. strain FB12 was able to produce HCN. Another important trait of PGPB, that may indirectly influence the plant growth, is the production of siderophores. They bind to the available form of iron Fe^{3+} in the rhizosphere, thus making it unavailable to the phytopathogens and protecting the plant health (Ahmad et al. 2008).

Siderophores are known for mobilizing Fe and making it available to the plant. In the present investigation, all isolates showed multiple PGP activities including siderophore production and antifungal activities against one or more test fungi. Additionally, strains FD17 and FB12 produced chitinase and various other hydrolytic enzymes, which might help bacterial cells for plant colonization as well as for antifungal activities. Furthermore, during plant invasion, bacterial cells may produce hydrolytic enzymes that are recognized by the plant, triggering the plant's resistance system (Trotel-Aziz et al. 2008).

Based on our rigorous testing, taking various potential plant growth-promoting mechanisms into account as well as different plant genetic backgrounds and colonization ability, we selected strain FD17, which also performed well under conditions resembling the situation in the field. Soil is a complex system and various biotic and abiotic factors may influence the behavior of particular strains in this environment. Further, it is well known that various stress factors frequently impact the plant and thus alter the allocation of photosynthates in the rhizosphere that may lead to changes in below-ground microbial communities and their interaction with the plant (Compant et al. 2010). Flavonoids are found within the plants that constitute a large part of root exudates (Cesco et al. 2012). Plant root colonization by the bacteria is considered as a primary step towards the successful initiation of the plant–microbe interaction (Bais et al. 2006). Colonization in turn depends on bacterial motility towards the plant root release or exudates. Root exudates are a rich source of nutrients for PGPB that undergo chemotaxis and colonize the plant root surface (Bhattacharjee et al. 2012). Strain FD17 was able to successfully compete with the natural microflora and successfully colonized the plant environment in addition to promoting plant growth.

Endophytic bacteria have been isolated from roots, leaves, stems and fruits by a number of researchers. In the present study, endophytic bacteria have been isolated from the maize roots. However, limited knowledge is available on plant seed as a source of endophytic bacteria, and their beneficial effects on plant growth rarely been described under natural conditions. Bacterial endophytes can be transferred from the plant environment to the seeds and may act as natural biofertilizers to the new plantlets early from seed germination (Puente et al. 2009; Ruiza et al. 2011).

Microbial inoculants on the basis of plant growth promoting or biocontrol agents have a great potential for sustainable crop production and ecofriendly environment management (Berg 2009). In our investigation, the endophytic strain FD17 increased growth and yield parameters of both maize cultivars under natural conditions. Maize, one of the most important crop species (C4), is known to be susceptible to moderate drought and relatively high temperature, and C4 photosynthetic efficiency declines with temperature at or above 35°C (Maiti 1996). In the present study, photosynthesis (F_v/F_m) was measured in the mid of July when temperature reached up to 35°C and observed significant differences between the inoculated treatment and the control. It is likely that endophytic bacteria while living inside plant tissues evoked various physiological processes to help plants to sustain photosynthesis and plant growth. Remarkable was also the ability of strain FD17 to influence the time needed for flowering again indicating the interaction between plant and microbe in regard to plant physiology.

The agricultural resources that commonly limit crop yield increases include water and nutrients, especially nitrogen (N) and phosphorous (P) (Sinclair and Rufty 2012). The interactions between AM fungi and bacteria in soil are of significant importance. Perhaps

fortuitously, the mycorrhizal fungi that are known to form associations with more than 80% of plant species, often enhance nutrient and water uptake. PGPR are able to increase AM fungal development by affecting root colonization as well as by enhancing plant N and P uptake (Richardson et al. 2009). Production of EPS by PGPB significantly enhanced the attachment of bacteria to mycorrhizal roots and AM fungal structures that influence the movement of bacteria in the rhizosphere (Bianciotto et al. 2001). Soil microbes are able to produce products that enhance the amounts of root exudates resulting in the activation of AM hyphae and hence higher rate of root colonization (Barea et al. 2005). However, further research is needed on the more detailed illustration of interactions between the host plant, AM fungi and soil bacteria by using different molecular techniques to enhance ecosystem productivity.

In this study, all the cultivars tested responded differently to inoculation with different endophyte isolates. Interestingly, one cultivar, Peso, was highly colonized by all strains, but plant growth promotion was only to a limited extent correlated with high colonization. However, strain FD17 was very efficient in colonizing different varieties and was also the most efficient plant growth promoter. Efficient colonization of cv. Morginon by strain FD17 indicates the specific cultivar colonizing capacity of the bacteria. Variety-specific effects on bacterial colonization are only poorly understood and the genetic basis for these effects need to be elucidated. Different crop species and varieties might produce different types of root exudates, which could trigger specific strains or taxa (Bais et al. 2006; Andreote et al. 2010). Also Pillay and Nowak (1997) and Montañez et al. (2012) reported that bacterization benefits depend on plant species, the cultivar and growth conditions. Also, in the present study, the TSA was not entirely

removed from the bacterial suspension as previously described by Pillay and Nowak (1997). It might influence the indigenous bacterial community and, thus indirectly had effects on plant.

In conclusion, *Enterobacter* sp. strain FD17 has high potential to confer beneficial effects to field-grown maize and it can be assumed that plant growth promotion is due to a combination of IAA, ACC deaminase and siderophore production and other nutrient providing activities. However, multi-sites field trials with strain FD17 need to be performed combined with the development of an appropriate formulation and application technology to warrant successful performance in the field.

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Table 1 Physico-chemical and growth-promoting characteristics of maize endophytes

Characteristics	*Caulobacter* sp. (FA13)	*Pantoea* sp. (FF34)	*Sphinogobium* sp. (FC42)	*Pseudomonas* sp. (FB12)	*Enterobacter* sp. (FD17)
Phenotypic and physiological characterization					
Colony colour	Gray	Yellow	Yellow	Gray	Creamy white
Colony morphology	Round	Round	Round	Round	Round
Bacterial growth conditions[a]					
NaCl					
2%	+	+	+	+	+
6%	-	+	-	-	+
pH					
5	+	+	+	+	+
12	+	-	-	+	+
Motility / chemotaxis[b]					
Swimming	+	+	-	++	+++
Swarming	-	-	-	-	+
Twitching	+	+	-	+	+
Biofilm formation					
OD (600 nm)	0.92±0.04	0.59±0.02	0.95±0.08	0.57±0.08	0.95±0.04
Biofilm (595 nm)	0.23±0.02	0.22±0.03	0.08±0.01	0.08±0.04	0.83±0.06
Aggregate stability (%)	35.91±2.57	26.07±0.88	32.61±2.13	36.38±1.48	40.22±1.99
Biochemical characterization[a]					
Catalase	+	+	+	+	+
Oxidase	-	-	-	+	-
Casein	-	-	-	+	-
Gelatin	-	+	-	+	+
Methanol	+	-	-	+	-
Ethanol	+	-	-	+	-
Growth promoting characterization[b]					
ACC-deaminase	+	+	+	++	+++
Auxin production (IAA equivalent, µg mL^{-1})					
without L-TRP	1.74 ±0.18	10.33 ±0.35	4.89 ±0.78	1.63 ±0.65	7.54 ±1.02
with L-TRP	16.13 ±1.05	95.34 ±2.14	38.41 ±1.78	7.26 ±1.05	12.30 ±0.98
P-solubilization (inorganic/organic P)					
$Ca_3(PO_4)_2$	-	++	-	+	+++
$CaHPO_4$	-	++	-	+	+++
$Ca(H_2PO_4)_2$	-	++	-	++	+++
Ca-phytate	-	++	-	++	+++
Na-phytate	-	++	-	++	+++
Exopolysaccharide	++	-	+	-	+
HCN production	-	-	-	+	-
NH_3 production	+	+	+	+	+
Siderophore production	+++	+	+	++	+++

AHL	-	-	-	+	-
PHB	-	+	-	+	+
Enzyme hydrolyzing activity[b]					
Amylase	-	-	-	-	-
Cellulase	+	-	+	+	++
Chitinase	-	-	-	+	+
Lipase	++	+	+	+++	++
Pectinase	-	+	-	+	+
Protease	-	-	-	-	-
Xylanase	+	-	+++	+	++
Antagonistic activities against plant pathogenic bacteria, fungi and oomycetes[b]					
Anti-bacterial activity					
A. tumefaciens	-	-	-	++	+
P. syringae	-	-	-	+++	+
S. aureus	-	-	-	+	-
Anti-fungal activity					
F. caulimons	++	+	+	++	+++
F. graminarium	+	+	+	+	++
F. oxysporum	+	++	+	++	++
F. solani	++	+	++	++	+++
R. solani	+	+	+	++	++
T. basicola	+	+	+	++	+
Anti-oomycete activity					
P. infestans	+	+	+	++	++
P. citricola	+	+	+	++	+++
P. cominarum	+	+	+	++	++

Results are obtained from 4-6 replicates

[a] -, absent; +, present

[b] **+,** low efficiency; ++, medium efficiency; +++, high efficiency

Table 2 Effect of inoculation with selected bacterial endophyte on root and shoot length of different maize cultivars under axenic conditions

Strain / Maize cv.	Root length (cm)					Shoot length (cm)				
	Helmi	Peso	Pelicon	Morignon	Cesor	Helmi	Peso	Pelicon	Morignon	Cesor
Control	18.52 $^{i-k}$	19.25 $^{g-i}$	15.50 $^{l-n}$	16.50 $^{k-m}$	14.25 ^{n}	14.77 ^{n}	25.12 ^{jk}	24.25 ^{kl}	27.75 ^{hi}	23.25 ^{lm}
Caulobacter sp. FA13	26.37^{a-c}	25.25 $^{b-d}$	25.75 $^{a-d}$	19.25 $^{g-i}$	20.00 ^{gh}	26.50 ^{ij}	33.75 $^{a-d}$	31.50 $^{e-g}$	32.92 $^{b-e}$	30.50 ^{g}
Pantoea sp. FF34	26.55 ^{ab}	22.50 ^{ef}	16.50 $^{k-m}$	16.75 $^{j-m}$	17.25 $^{i-l}$	28.60 ^{h}	28.87 ^{h}	28.32 ^{h}	32.12 $^{d-g}$	32.50 $^{c-f}$
Sphinogobium sp. FC42	18.67 $^{h-j}$	26.75 ^{ab}	27.50 ^{a}	17.50 $^{i-l}$	15.50 $^{l-n}$	22.50 ^{m}	31.15 ^{fg}	33.92 $^{a-c}$	32.15 $^{d-g}$	32.40 $^{c-f}$
Pseudomonas sp. FB12	14.75 ^{mn}	24.00 ^{de}	24.37 $^{c-e}$	21.00 ^{fg}	21.40 ^{fg}	15.25 ^{n}	32.45 $^{c-f}$	33.75 $^{a-d}$	32.50 $^{c-f}$	32.27 $^{c-f}$
Enterobacter sp. FD17[a]	24.67 $^{b-d}$	27.85 ^{a}	22.50 ^{ef}	21.57 ^{fg}	21.45 ^{fg}	26.35 ^{ij}	34.37 ^{ab}	32.15 $^{d-g}$	34.75 ^{a}	32.82 $^{c-f}$

Means sharing similar letter(s) in a column for each parameter do not differ significantly at P = 0.05

The data is average of 4 replicates

[a]Efficient strain selected for containment experiment

Table 3 Effect of inoculation with selected bacterial endophyte on root and shoot biomass of different maize cultivars under axenic conditions

Strain / Maize cv.	Root biomass (g)					Shoot biomass (g)				
	Helmi	Peso	Pelicon	Morignon	Cesor	Helmi	Peso	Pelicon	Morignon	Cesor
Control	0.81 ^{ij}	0.86 ^{gh}	0.68 ^{k}	0.76 ^{jk}	0.57 ^{l}	0.77 ^{p}	1.16 ^{kl}	0.87 ^{no}	1.01 ^{m}	0.79 ^{op}
Caulobacter sp. FA13	1.08 ^{g}	1.28 ^{ef}	1.04 ^{g}	1.26 ^{ef}	1.06 ^{g}	1.05 ^{m}	1.63 ^{e}	1.62 ^{e}	1.74 ^{d}	1.36 ^{hi}
Pantoea sp. FF34	1.39 ^{cd}	1.56 ^{a}	0.95 ^{h}	0.95 ^{h}	0.89 ^{gh}	1.30 ^{ij}	1.77 ^{cd}	1.09 ^{lm}	1.44 ^{gh}	1.34 ^{i}
Sphinogobium sp. FC42	1.24 ^{ef}	1.24 ^{ef}	1.20 ^{f}	1.10 ^{g}	1.07 ^{g}	1.08 ^{lm}	1.69 ^{de}	1.59 ^{ef}	1.75 ^{d}	1.51 ^{fg}
Pseudomonas sp. FB12	0.91 ^{h}	1.46 ^{bc}	1.10 ^{g}	1.29 ^{ef}	1.06 ^{g}	0.91 ^{n}	1.76 ^{d}	1.32 ^{ij}	1.92 ^{ab}	1.52 ^{fg}
Enterobacter sp. FD17[a]	1.46 ^{bc}	1.57 ^{a}	1.31 ^{de}	1.49 ^{ab}	1.26 ^{ef}	1.24 ^{jk}	1.86 ^{bc}	1.63 ^{e}	1.98 ^{a}	1.52 ^{fg}

Means sharing similar letter(s) in a column for each parameter do not differ significantly at P = 0.05

The data is average of 4 replicates

[a]Efficient strain selected for containment experiment

Table 4 Effect of inoculation with endophytic strain FD17 on physiology, growth parameters and yield of two maize cultivars grown in pots in field soil and exposed to natural climatic conditions (net house experiment)

Parameters / Treatment	Peso		Morignon	
	Un-inoculated	Inoculated with FD17[a]	Un-inoculated	Inoculated with FD17[a]
Fv/Fm	0.69 ^{c}	0.75 ^{b} (8.69)	0.73 ^{bc}	0.79 ^{a} (8.22)
Time to onset of flowering (days)	65.33 ^{b} (18.78)	55.00 ^{a}	70.67 ^{d} (6.54)	66.33 ^{bc}
Plant height (cm)	192.33 ^{d}	208.00 ^{ab} (8.15)	196.69 ^{cd}	213.68 ^{a} (8.64)
No. of leaves plant^{-1}	12.33 ^{c}	14.00 ^{ab} (13.54)	13.17 ^{bc}	14.67 ^{a} (11.39)
Leaf area (cm^{2})	494.26 ^{d}	556.27 ^{bc} (12.55)	512.39 ^{cd}	617.11 ^{a} (20.44)
Leaf dry weight (g)	22.21 ^{c}	28.16 ^{b} (26.79)	28.09 ^{b}	34.56 ^{a} (23.03)
Plant dry biomass (g)	114.18 ^{c}	153.77 ^{b} (34.67)	160.46 ^{b}	223.14 ^{a} (39.06)
Root dry biomass (g)	17.26 ^{c}	24.34 ^{b} (41.02)	19.73 ^{c}	28.28 ^{a} (43.34)
Cob weight (g)	115.28 ^{c}	155.83 ^{b} (35.18)	123.71 ^{c}	176.23 ^{a} (42.45)

Means sharing similar letter(s) in a row for each parameter do not differ significantly at P = 0.05

The data are based on 3 replicates

Values in brackets are % increase over control

[a]*Enterobacter* sp. strain FD17

Table 5 Colonization of strain FD17 in rhizosphere, root, stem and leaves of two maize cultivars (net house experiment)

Maize cv. / Plant compartment	Rhizosphere (cfu g^{-1} dry wt)	Root interior (cfu g^{-1} dry wt)	Shoot interior (cfu g^{-1} dry wt)	Leaf interior (cfu g^{-1} dry wt)
Peso	4.07×10^4 a	3.39×10^4 a	1.63×10^3 b	1.16×10^2 c
Morignon	9.85×10^4 a	8.59×10^4 a	3.72×10^3 b	6.23×10^2 bc

Means sharing similar letter(s) in a column/row do not differ significantly at P = 0.05

The data are based on 3 replicates

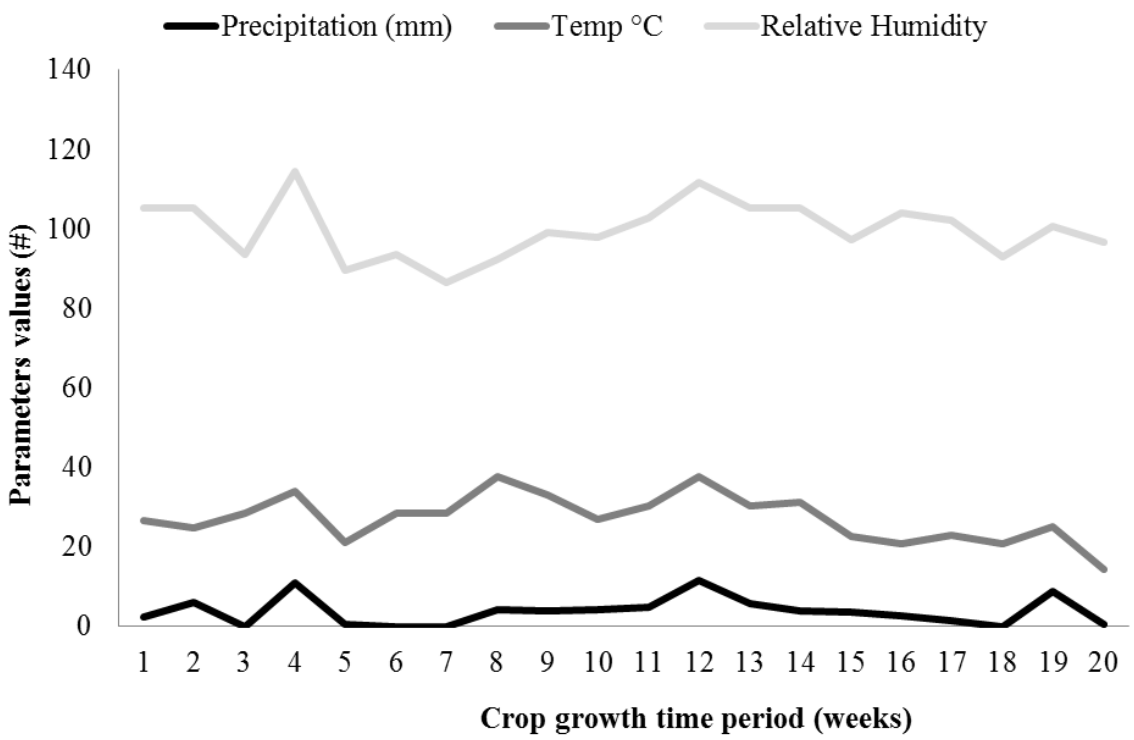

Figure 1 Weather conditions data during the crop growth period obtained from ZAMG, Austria

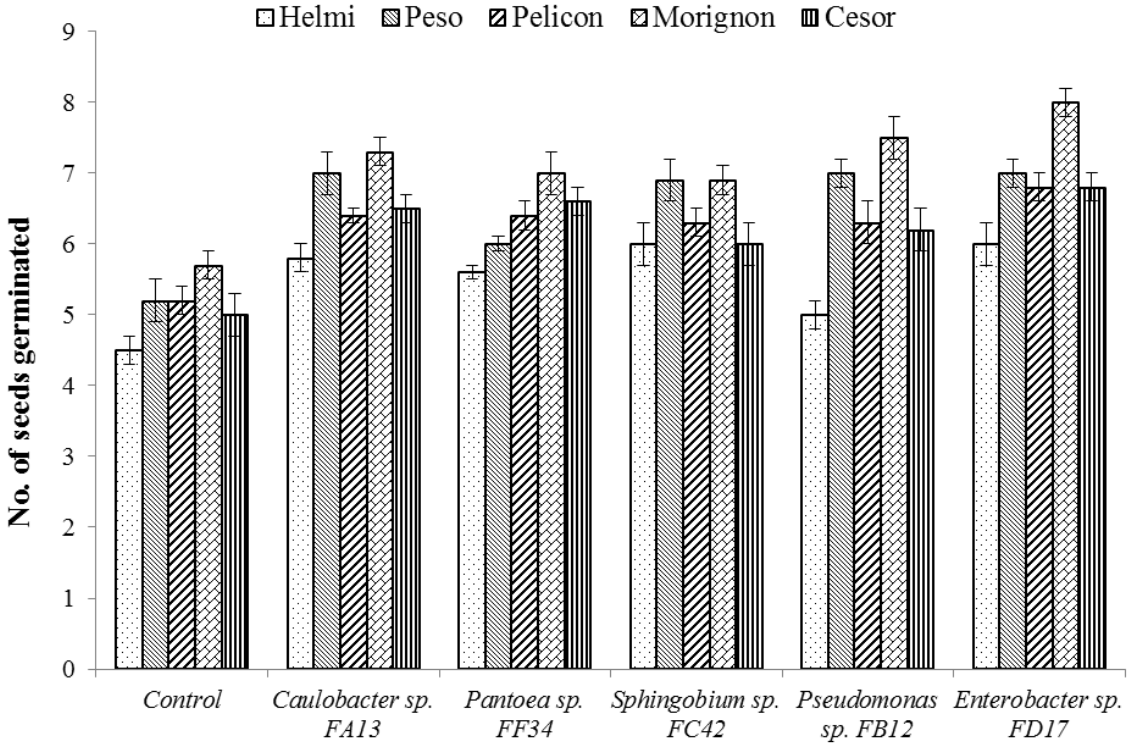

Figure 2 Effect of i noculation w ith s elected bacterial e ndophyte on ge rmination of different maize cultivars under axenic conditions. Data is average of three replicate ± SE

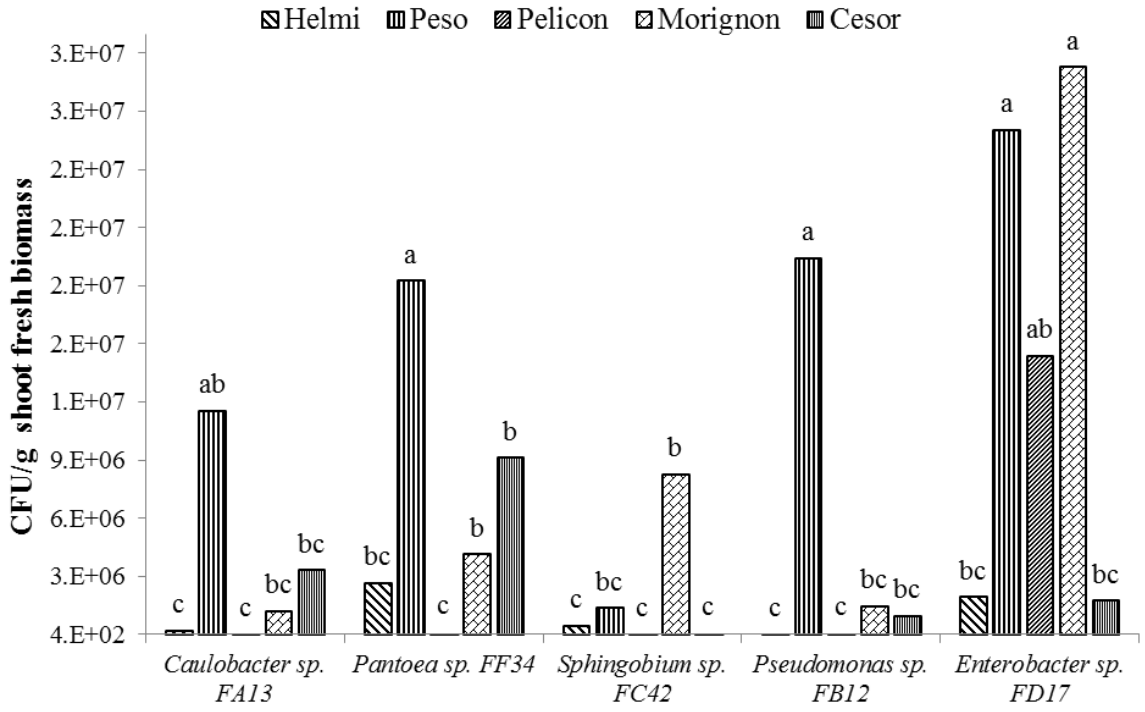

Figure 3 Persistence of selected endophytic strains in the shoot interior of different maize cultivars under axenic conditions. Bars sharing similar letters do not differ significantly at *P = 0.05*

Chapter 3

Increased drought stress resilience of maize through endophytic colonization by *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17

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Abstract

Drought is one of the major environmental stresses that adversely affects crop growth and productivity worldwide. The effect of inoculation of two bacterial endophytes *Burkholderia phytofirmans* strain PsJN and *Enterobacter* sp. FD17 on growth, water status and photosynthetic activity of two maize cultivars under drought stress conditions was investigated. Plants were exposed to drought stress by withholding irrigation at vegetative growth stage (45 days after planting). The inoculant strains efficiently colonized maize seedlings and were recovered from root, shoot and leaves of both irrigated and stressed plants. Drought stress had drastic effects on growth, leaf water content and photosynthesis of maize seedlings. Our results revealed that bacterial inoculation minimized the drought stress-imposed effects significantly increasing shoot biomass, root biomass, leaf area, chlorophyll content, photosynthesis, and photochemical efficiency of PSII. Similarly, bacterized seedlings showed higher leaf relative water content (30%) compared to control, whereas 43% higher leaf damage in terms of relative membrane permeability was observed in non-inoculated plants under drought stress. Strain PsJN was more efficient than FD17 in terms of influencing growth and physiological status of the seedlings under drought stress. Our data suggest that maize plants can be protected from inhibitory effects of the drought stress by the harbored bacterial endophytes. Although the degree of protection depends on the type of the bacterial strain and the plant genotype.

Key words: Endophyte, *Burkholderia phytofirmans*, *Enterobacter*, drought stress, maize

1. Introduction

Plants face various biotic and abiotic stresses in hostile environmental conditions. Among these, drought is a major abiotic factor that adversely affects crop growth and productivity worldwide. Drought is expected to cause serious plant growth problems for more than 50% of the arable lands by 2050 (Vinocur and Altman, 2005). Global warming will increase the severity and frequency of drought in the future leading to a possible decrease in global food production. At the same time a steadily increasing human population which could hit 9 billion by 2050 demands an increase in food supplies. The situation will in future be even more severe as desertification will further increase and the current amount of annual loss of arable area may double by the end of the century because of global warming (IPCC, 2007).

Modern agro-biotechnological strategies are being tested to enhance drought stress tolerance in plants such as the generation of transgenic plants with introduced novel genes or with altered expression levels of the existing genes (Lu et al., 2013). Development of drought-tolerant varieties through genetic engineering and plant breeding, coupled with natural resource management is also a promising and effective approach to improve agricultural productivity and water use efficiency against drought and water shortage (Warren, 1998). However, the complexity of abiotic stress tolerance mechanisms makes the task of introducing new tolerant varieties very difficult (also a long drawn procedure), and genetically modified plants are not well accepted in some regions of the world (Wahid et al., 2007).

On the one hand, plants possess natural protection systems that act against different stresses, but on the other hand, they also interact with a variety of soil

microorganisms that can alleviate the stress symptoms (Marulanda et al., 2006). Microbial communities are able to develop a range of activities that are very important in maintaining biological balance and sustainability in soil particularly under stress conditions (Kennedy et al., 1995; Kavamura et al., 2013). In stressed areas, plants are more dependent on microorganisms that are able to enhance their metabolic activity to combat stress (Kavamura et al., 2013). Rhizobacteria that exert beneficial effects on plant growth and development are referred to as plant growth promoting rhizobacteria (PGPR). PGPR are beneficial native soil bacteria that colonize the rhizosphere or plant roots and result in increased plant growth and yield (Kloepper et al., 1989). PGPR are adapted to adverse conditions and protect plants from the deleterious effects of drought stress, thus increasing crop productivity in arid or semiarid areas (Marulanda et al., 2007; Kavamura et al., 2013; Kasim et al., 2013). Several PGPR are reported to induce drought stress tolerance in some plants such as wheat, maize, sunflower, sugarcane and green gram (Sandhya et al., 2009, 2010; Moutia et al., 2010; Vardharajula et al., 2011; Saravanakumar et al., 2011; Kasim et al., 2013). Endophytic bacteria may in future be even more important than rhizosphere bacteria, because they escape competition with rhizosphere microorganisms and achieve more intimate contact with plant tissues.

Maize (*Zea mays* L.) is the third most important food crop globally in terms of sources of energy and protein in human nutrition. It is a C4 crop with a high rate of photosynthetic activity, leading to high grain and biomass yield. Climate change and the use of marginal land for crop production require the development of innovative management systems adapted to stressful environments, particularly drought stress. Annual yield losses due to drought average around 15% of potential yield (Edmeades,

2008). Climate change and population growth suggest that the production of major crops (maize, barley, wheat etc.) will move to marginal areas, mainly with water deficit (Edmeades, 2008).

We therefore evaluated the potential of two endophytic bacterial strains, *Burkholderia phytofirmans* strain PsJN and *Enterobacter* sp. FD17, for improving physiology and growth of maize under drought stress. *B. phytofirmans* PsJN is among the best studied plant growth promoting endophytes. It colonizes the rhizosphere and endosphere, and promotes growth, and enhances abiotic and biotic stress tolerance in a variety of crops and vegetables (Mitter et al., 2013). Recently, we found that *B. phytofirmans* PsJN efficiently colonizes maize plants upon seed inoculation and enhances germination, growth and flower onset (unpublished data). *Enterobacter* sp. FD17, was previously isolated from maize by Prischl et al. (2012), is able to improve germination, growth and yield of different maize cultivars under axenic and natural soil conditions (Naveed et al., 2013). Our results suggest that microbial inoculation assuaging stresses in plants can be utilized in agriculture in an environmentally friendly manner.

2. Materials and methods

2.1. GUS labeling of Enterobacter *sp. FD17*

The *Enterobacter* sp. FD17 was tagged with the glucuronidase A (*gus*A) gene following the protocol described by Wilson et al. (1995) and using the construct pCAM110 in which *gus*A is under control of the ptac promoter. Briefly, wild-type strain FD17 and *E. coli* (pCAM110 plasmid) was grown in 5 ml LB medium at 28 ± 1°C until the optical density of 0.6, at λ 600 nm. One mL bacterial cells were pelleted by centrifugation (14,

000 rpm, 10 min), washed three times with ice-cold distilled water, and resuspended in 100 and 1000 µL of saline buffer (0.85% NaCl). The cell suspension 100 µL of each was mixed and the mixture was spread on the selective plate and incubated overnight at 28 ± 1°C. Bacterial colonies carrying the *gus*A marker were selected on M9 medium [11 g $Na_2HPO_4.12H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.24 g $MgSO_4$, 11.1 mg, 1 ml Fe-EDTA solution, 1 ml trace elements solution (Alef, 1994) containing succinate, acetate and citrate (SAC), each at a concentration of 2 g, dissolved in one litre], amended with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 µg mL^{-1}), isopropyl-β-D-galactopyranoside (IPTG) (100 µg mL^{-1}) and spectinomycin (100 µg mL^{-1}) (Sigma, St. Louis, Mo.). Then the bacteria were examined by using an optical stereomicroscope (model SZCTV; Olympus) and an optical microscope (model BH2; Olympus).

Burkholderia phytofirmans PsJN is one of the best studied bacterial endophyte so far, originally isolated from surface-sterilized *Glomus vesiculiferum*-infected onion roots, and reported for growth promotion of various horticultural crops (Frommel et al., 1991; Nowak et al., 1995).

2.2. Labeling stability and bacterial growth comparison

Stability of the chromosomal integration of the *gus*A marker in strain FD17 was determined by growing in LB liquid medium for over 10 generations and then plating a dilution series on LB medium with or without the appropriate antibiotic. Furthermore, the colony and cell morphologies and growth patterns of the genetic derivatives were compared to those of the FD17 wild-type strain in LB medium and M9 minimal medium with 5% glucose (Sambrook et al., 1989).

2.3. Inoculum preparation and bacterial growth

Strains FD17::*gusA*10 and PsJN::*gusA*10 (Compant et al., 2005) were cultured in 250 mL LB broth containing spectinomycin [100 µg mL^{-1}] at 28 ± 1°C for 48 h in an orbital shaking incubator (VWR International GmbH, Austria) at 180 rpm in^{-1}. The optical density of the culture was measured at λ 600 nm using a spectrophotometer (Gene Quant Pro, Gemini BV, The Netherlands) and adjusted to 0.5 to obtain a uniform population of bacteria [10^8 - 10^9 colony forming units (CFU) mL^{-1}] for inoculation.

2.4. Plant material and growth conditions

A pot experiment was conducted in the greenhouse at the AIT campus in Tulln/Austria [altitude (174 m) and latitude (48° 19′ N] to compare the effectiveness of selected bacterial strains for promoting growth and yield of maize under drought stress conditions. Maize plants were grown in agricultural field soil collected from Tulln in Lower Austria, Austria. Soil used in the pots had the following physic-chemical characteristics: sand, 32%; silt, 38%; clay, 30%; pH, 7.28; total carbon, 2.4%; total nitrogen, 0.23%; available phosphorus, 40 mg 100 g^{-1}; extractable potassium, 19 mg 100 g^{-1}.

Maize seeds were surface-sterilized with 70% ethanol (3 min), treated with 2% sodium hypochlorite (NaClO) (5 min), and followed by repeated washing with sterile distilled water (3 times for 1 min). The efficacy of surface sterilization was checked by plating shoot and root, and aliquots of the final rinse onto LB plates. Seeds were considered to be successfully sterilized when no colonies were observed on the LB plates after inoculation for 3 days at 28 ± 1 °C. Surface-disinfected seeds (cvs. Mazurka and Kaleo, DOW AgroSciences, Vertriebsges.m.b.H Neusiedl am See, Austria) were

incubated in bacterial suspension [prepared as described above, (10^8 - 10^9 CFU mL^{-1})] for 2 hours. For the control, seeds were treated with sterilized LB broth. Three inoculated seeds (10^8 bacteria per seed) were sown in pots with cylindrical shape with diameter 27 cm and height 25 cm (Plastic Moram, China) containing 15 kg of soil and thinned to one plant after one week of germination. The experiment was conducted during the period of May to July 2011 in the greenhouse. The average maximum temperature was 20.6 - 27.6 ^oC (day) and 10.7 - 15.7 ^oC (night). Average relative humidity in the greenhouse chamber was 30%. The photoperiod in the chamber was set to a 16 h light and 8 h dark. Pots were arranged in a chamber of the greenhouse using a completely randomized design with three replications of each treatment. Recommended doses of N, P, and K fertilizers (160-100-60 kg ha^{-1}) were applied to each pot and equal amounts of tap water was applied to the pots to maintain optimal soil moisture depending on plant and soil conditions (upto 1000 ml). Drought stress was applied by stopping irrigation after 45 days of planting. After stopping irrigation plants were observed for signs of wilting. When shrinkage of leaves and stem were clearly visible plants were harvested and soil moisture content of both normal and reduced irrigated pots was determined.

2.5. Plant growth promoting trait measurement

Plants were harvested 66 days after sowing and the data of growth and physiology parameters were recorded before and after harvesting the pots.

Plant physiological parameters were recorded at midday (between 10:00 and 13:00) of both irrigated and drought-stressed pots.

2.5.1. Gaseous exchange measurement

Gaseous-exchange measurements i.e. [photosynthetic rate (net-rate of CO_2 assimilation at light saturation) (Asat)], stomatal conductance (g_s), transpiration rate (E) and vapor pressure deficit (VpdL) were measured with a Li-6400 portable photosynthesis system (Li-Cor, Inc. Lincoln, NE, USA) equipped with a CO_2 cartridge to adjust and maintain a constant CO_2 level of 400 µ mol mol^{-1} air within the leaf cuvette. Gas exchange was measured from the top third, fully developed leaf of each plant at the ambient light of stressed and non-stressed plants.

2.5.2. Chlorophyll fluorescence

Maximum photochemical efficiency of photosystem II (PSII (F_v/F_m)) was calculated from chlorophyll fluorescence data using handy PEA (Hansatech Instruments Ltd. England). Leaves were dark adapted for 30 min before the measurement.

2.5.3. Leaf area and chlorophyll content

Leaf area (3rd leaf from top) of irrigated and drought stressed plants was recorded using LI-3100C Area Meter (Li-Cor, Inc., Lincoln, NE, USA). Leaf chlorophyll content was determined by using Chlorophyll Meter (SPAD 502 Plus, Minolta, Japan). Each leaf sample was measured in at least six different areas.

2.5.4. Relative water content and membrane permeability

Flag leaves were used for measuring the relative water content (RWC) and relative membrane permeability (RMP). Leaves were cut, sealed within plastic bags and transferred to laboratory. After measuring fresh weights, leaves were placed in distilled water for 24 h at 4ºC in darkness. After soaking, leaves were carefully blotted with tissue paper and fully turgid weight was measured. Dry weight was measured after oven drying

the leaf samples at 72°C for 24 h. Relative water contents were determined following the equation described by Teulat et al. (2003).

$$\text{RWC (\%)} = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Fully turgid weight} - \text{Dry weight})} \text{ X 100}$$

For the RMP measurement, the leaves were cut into equal pieces and transferred to test tubes containing 20 ml of deionized distilled water. The test tubes were vortexed for 10 s and the solution was assayed for initial electrical conductivity (EC0). These tubes were kept at 4 ^{o}C for 24 h and then assayed for EC1. The same samples were autoclaved at 121^{o}C for 20 min to determine EC2. Percent RMP was calculated as following the formula described by Yang et al. (1996)

$$\text{RMP (\%)} = \frac{(\text{EC1} - \text{EC0})}{(\text{EC2} - \text{EC0})} \text{ X 100}$$

2.5.5. Agronomic parameters measurement

Plant agronomic parameters such as shoot and root fresh weight were record after harvest. Plant biomass (above and below ground) was determined after drying the whole plants at 72°C for 72 hours.

2.6. Detection and enumeration of inoculant strains

2.6.1. Rhizosphere colonization

Rhizosphere soil was obtained by agitating roots and sampling the soil still attached to the roots after plant harvesting. For the isolation of rhizosphere bacteria, soil slurry was prepared by mixing 5 g rhizosphere soil with 15 mL of 0.85% (w/v) NaCl solution and agitation (180 rpm) for 60 min at 30 ^{o}C. After sedimentation of soil particles, serial dilutions up to 10^{-4} were plated onto selective LB medium containing spectinomycin (100 µg mL^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 µg mL^{-1}), and

isopropyl-β-D-galactopyranoside (IPTG) (100 µg mL^{-1}) as described by Afzal et al. (2012). Plates were incubated at 28 ± 1°C for 3-4 days and blue colonies were counted to determine the average colonization value.

2.6.2. Endophyte colonization of root and shoot tissues

For the isolation of endophytes, 3 g of surface-sterilized roots were homogenized in 15 mL 0.85% NaCl (w/v) solution using a sterile mortar and pestle. Similarly, 5 g shoots of each treatment were homogenized in 15 mL 0.85 % NaCl (w/v) solution. The homogenized material was put in sterile plastic bags and subjected to oscillation in a pulsifier (Microgen Bioproducts Ltd., UK) for 45 sec at room temperature. After settling of the solid material, serial dilutions up to 10^{-3} were spread on selective LB medium. Plates were incubated at 28 ± 1°C for 48 hours and then transferred to 4°C for three days. Blue colonies were counted on each plate. Thirty blue colonies of each treatment were randomly picked and the identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S r RNA intergenic spacer region (IGS) (Reiter et al., 2002). Isolates and applied inoculant strains had identical restriction patterns.

2.6.3. Microscopy of endophytic colonization by **Enterobacter** *sp. FD17 and* **B. phytofirmans** *PsJN*

Fresh plant organs (roots, fourth internodes, and fifth leaves) removed from three six plantlets inoculated with either *gusA* marked strains PsJN and FD17, or a control (LB) were collected 60 days after inoculation. Samples were prepared for microscopy analysis as described by Compant et al. (2005), with some modifications. Briefly, plant organs (stem and leaves) were incubated in staining solutions containing IPTG at 37°C for 48 h.

The samples were de-stained with 70% (v/v) ethanol solution to stop the reaction. The samples were immersed in ethanol at room temperature until the removal of tissues chlorophyll. Stem sections of different treatments were cut with a microtome (LeicaVT1000S; Leica, Nussloch, Germany), collected on glass slides, examined with an inverted microscope (Axiovert 200 M, Zeiss, http://www.zeiss.com/) with an integrated camera (AxioCam MRc5, Zeiss, http://www.zeiss.com/).

2.7. Hydrogen peroxide (H_2O_2) localization in leaf tissues

Hydrogen peroxide (H_2O_2) generation in leaves was qualitatively detected by the diaminobenzidine tetrahydrochloride (DAB) staining method as described by Jambunathan (2012). Leaves were collected and incubated with 1 mg mL^{-1} DAB solution pH 3.8, followed by vacuum infiltration of the leaves at nearly 100–150 mbar for 1-2 min. Leaves were then incubated in plastic box for 5-6 h under high humidity conditions till brown precipitates were observed. The leaf chlorophyll was removed and de-stained with ethanol (96% v/v) under heating at 40°C. Leaf sections were cut, collected on glass slides, examined with Binocular microscope (Olympus, Japan), and photographed.

2.8. Statistical analysis

Data analyses for plant growth parameters and bacterial densities were done using SPSS software package version 19 (IBM SPSS Statistics 19, USA). Comparisons between treatments were carried out by one-way analysis of variance (ANOVA). Duncan's test was applied for ANOVA after testing homogeneity of variance (Steel et al., 1997).

3. Results

3.1. Plant physiological parameters

Inoculation with endophytic strains, PsJN and FD17, significantly increased photosynthetic rate (Asat) compared to the respective control in two maize cultivars, Mazurka and Kaleo, under both irrigated and stress conditions (Table 1). Maximum response up to 75% compared to control was recorded by PsJN inoculation in case of cv. Mazurka under drought stress. Inoculation with strain FD17 gave 53% (Mazurka) and 41% (Kaleo) increase in photosynthesis of both cultivars under drought stress conditions. Minimum response - 19% increase over control - was achieved by FD17 inoculation in Kaleo under normal irrigation. PsJN inoculated plants had higher stomatal conductance upon exposure to stress; 87% increase in Mazurka and 60% in Kaleo, compared to the non-inoculated control. Also FD17 inoculation resulted in increased stomatal conductance, i.e. 44% in Mazurka compared to the non-inoculated control under drought stress (Table 1). Minimum response – 14 and 19% increase in Kaleo and Mazurka, respectively over control - was achieved by FD17 inoculation under normal irrigation. Inoculation with strain PsJN under stress conditions increased transpiration rate up to 84% in Mazurka and 53% in Kaleo compared to the control. Similarly, FD17 treatment gave 50% (Mazurka) and 44% (Kaleo) increase in transpiration rate under drought stress compared to the control (Table 1). The data presented in Table 2 show that inoculation increased the vapor pressure deficit (VpdL) compared to control in both cultivars under stress conditions. In general, Mazurka showed minimum response to FD17 inoculation compared to control under irrigated and stress conditions. In case of Kaleo, FD17

inoculation resulted in 1.7 and 7% increase in VpdL compared to the control under irrigated and drought stress, respectively.

The maximum PSII efficiency was observed in Mazurka when inoculated with PsJN under normal condition compared to control (Table 2). PsJN inoculation increased PSII efficiency up to 10% in Mazurka compared to control under stress conditions. Kaleo showed minimum response to FD17 inoculation in regard to PSII efficiency compared to control under normal irrigation. Likewise, inoculation significantly increased chlorophyll content of both cultivars compared to control (Table 2). Inoculation with strain PsJN gave the highest increase in chlorophyll content, i.e. 22 and 19 % in Mazurka and Kaleo, respectively, compared to control in both cultivars under stress. FD17 inoculation resulted in 16 and 13% increase in chlorophyll content of Mazurka and Kaleo, respectively, compared to control under the same conditions. Minimum increase – 11 and 12% in chlorophyll content of Kaleo and Mazurka, respectively over control - was achieved by FD17 inoculation under normal irrigation.

3.2. Relative water content and membrane permeability

Data in Figure 1 shows the gravimetric soil moisture content in both normal (26-28% in Kaleo and Mazurka, respectively) and reduced irrigation (12-13% in Mazurka and Kaleo, respectively) at the time of harvest. Inoculation significantly improved RWC of both cultivars under normal and reduced irrigation (Table 2). PsJN inoculation resulted in maximum increase in RWC of Mazurka (30%) compared to control under drought stress. While 27% increase in RWC of Kaleo, was observed by PsJN inoculation compared to control under same conditions. FD17 inoculation increased RWC i.e. 27 and 20% of Mazurka and Kaleo, respectively compared to control under drought stress. The data in

Table 2 show that PsJN inoculation decreased relative membrane permeability (RMP) ranges from 38-43% in Mazurka and 29-41% in Kaleo, respectively, compared to control under normal and reduced irrigation. Its maximum decrease (43%) was observed after PsJN inoculation in Mazurka under stress conditions compared to control. Inoculation with strain FD17 resulted in decrease RMP from 21% (Kaleo) to 40% (Mazurka) compared to control under normal irrigation. FD17 also decreased RMP i.e. 41% (Mazurka) and 33% (Kaleo) compared to control under drought stress.

3.3. Agronomic trait measurement

Inoculation of maize seeds with endophytic bacteria increased the number of leaves per plant, leaf area, shoot and root dry weight both under normal and reduced irrigation (Table 3). Inoculation with strain PsJN increased the number of leaves in Mazurka (24%) and Kaleo (16%), respectively, compared to control under drought stress. While FD17 resulted in a 17 and 9% increase in number of leaves of both Mazurka and Kaleo, respectively, compared to control under drought stress conditions. Likewise, inoculation increased the leaf area of both cultivars compared to non-inoculated control under normal and reduced irrigation (Table 3). PsJN inoculation increased leaf area i.e. 21 and 20% in Kaleo and Mazurka, respectively compared to control under drought stress. FD17 resulted in 20 and 13% increase in the leaf area of both Mazurka and Kaleo, respectively, compared to control under drought stress. Data in Table 3 show that inoculation significantly increased plant biomass compared to the control. However, the inoculation response was more pronounced under water stress conditions compared to the un-inoculated control. Increased (48 – 66%) plant biomass was recorded in Mazurka when treated with strain PsJN compared to control under normal and stress conditions,

respectively. Similarly, PsJN inoculation gave 24-46% increase in Kaleo compared to control under same conditions. FD17 inoculation resulted 42 and 32% increase in plant biomass, respectively, in Mazurka and Kaleo compared to control under drought stress. Likewise, bacterial inoculation increased root biomass of both cultivars significantly compared to control (Table 3). PsJN inoculation increased root mass, i.e. 70 and 58% in Mazurka and Kaleo, respectively, compared to control in both cultivars under stress conditions. Likewise, PsJN inoculation resulted in 47 and 40% increase in Mazurka and Kaleo, respectively, compared to control under normal irrigation. FD17 inoculation increased root mass i.e. 64% (Mazurka) and 34% (Kaleo), respectively, compared to control under stress conditions. The minimum response (29-36% increase) was recorded in Kaleo and Mazurka, respectively, treated with FD17 compared to control under normal irrigation.

3.4. Enumeration and microscopic localization of endophytic bacteria and ROS in plant tissues

The inoculant strains efficiently colonized rhizosphere, root and shoot interior of both maize cultivars, Mazurka and Kaleo, under normal and reduced irrigation (Fig. 2). Higher titers of PsJN (CFU g^{-1} dry weight) were recovered the rhizosphere (5.86×10^5), root interior (5.44×10^5), and shoot interior (9.36×10^4) of Mazurka under normal conditions (Fig. 2 -4), and compare to Kaleo. In the case of FD17, 3.28×10^5 CFU g^{-1} DW rhizosphere, 3.06×10^5 CFU g^{-1} DW root interior and 5.97×10^4 CFU g^{-1} DW shoot interior were recorded under normal conditions. However, relatively less CFU of both strains were recorded in both cultivars under drought stress conditions. The percent viable cell numbers of PsJN decreased in the rhizosphere, root interior and shoot interior of

Mazurka (2-26%) and Kaleo (5-23%) by drought stress. In the case of FD17, the decrease in viable cell numbers was more pronounced than with strain PsJN. Figures 3-4 show the localization of the inoculated strains in different tissues of maize plant. Figure 5 shows the localization of H_2O_2 in the inoculated and control plants under normal and reduced irrigation. Under drought conditions more H_2O_2 was found in control than in inoculated plants.

4. Discussion

In the present study, two bacterial endophytic strains, *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17 were evaluated for improving growth and physiological parameters of two differentially adapted maize cultivars under drought stress conditions. PsJN colonizes the rhizosphere and endosphere, and promotes growth, and enhances abiotic and biotic stress tolerance in a variety of horticultural crops, e.g. potatoes, tomato and grapevines (Mitter et al., 2013). Very recently, Naveed et al. (2013) reported that FD17 efficiently colonizes the different cultivars of maize and enhances their growth and yield.

Endophytes live inside plants for at least part of their life cycle without being pathogenic. In contrast, some endophytes confer benefits to their plant host such as stress reduction, increased root growth and nutrient availability (Hardoim et al., 2008). Plant growth and development may be reduced in stress conditions due to impaired biochemical and physiological mechanisms. Such stresses may be relieved to some extent by the application of microbial inoculants, which evoke various natural mechanisms to help plants to sustain their growth under stress conditions (Yang et al., 2008;

Vardharajula et al., 2011). In the present study, we observed that endophyte inoculation improved maize plant growth under drought stress conditions, which resulted in better survival, root/shoot biomass and water content compared to the non-inoculated control (Tables 2–3). Increase in the total root system is the most commonly reported plant response mediated by PGPB inoculation in various plant species (Lucy et al., 2004). This can be caused by microbial hormone production, which is considered as the most plausible mechanism in controlling root growth and development. Firstly, bacterial production of the hormone auxin in the root zone using tryptophan as a precursor from root exudates is responsible for root architecture. Bacterial-induced alterations in root architecture might lead to an increase in total root surface area, consequently improved nutrient and water uptake, which may have positive effects on plant growth as a whole (Somers et al., 2004). Secondly, under drought stress conditions, the plant hormone ethylene endogenously regulates plant homeostasis and results in reduced root and shoot growth. However, degradation of the ethylene precursor ACC by bacterial ACC deaminase releases plant stress and rescues normal plant growth (Mayak et al., 2004). Both strains used in the present study produce ACC deaminase, it is likely that the stress-induced accelerated synthesis of ethylene was reduced by inoculant strains having ACC deaminase activity resulted in longer roots, which might be helpful in the uptake of relatively more water from deep soil (Reid and Renquist, 1997; Dodd et al., 2004). Mayak et al. (2004) also reported that inoculation with PGPR containing ACC deaminase confers resistance against drought stress in tomatoes and peppers.

The inoculation of maize plants with the bacterium *B. phytofirmans* PsJN resulted in higher plant biomass production, physiology and vitality in both varieties when

compared to *Enterobacter* sp. FD17 and the un-inoculated control under normal and reduced irrigation. Strain PsJN improved plant biomass and photosynthetic rate of the cultivar Mazurka up to 48 and 45%, respectively, compared to the control under normal irrigation. From numerous reports, it is evident that *B. phytofirmans* PsJN is a highly efficient plant beneficial bacterium promoting growth in a wide variety of plants (Mitter et al., 2013), however, there is evidence for plant genotype specific differences in the intensity of the effects (Pillay and Nowak, 1997; Da et al., 2012). Interestingly, this also can be seen from the present data as cultivar Mazurka responded better to PsJN inoculation than cultivar Kaleo. The data of not stressed plants indicated a correlation between growth stimulation and number of viable PsJN cells in both cultivars. It is likely that bacterial ability to promote plant growth and to establish endophytic populations is very often dependent on the plant genotype (cultivar) and developmental stage. Nowak et al. (2007) assumed that plant genotype specific differences in the plant stimulating effects are due to differences in PsJN titers in highly and poorly responsive varieties.

The response of plants to water deficit has been evaluated based on genetic, biochemical, and morpho-physiological traits. Among others, the leaf gas exchange, relative water content (RWC), photochemical efficiency of PSII, chlorophyll content, and regulation of the electron transport have been used as indicators of plant stress (Golding and Johnson, 2003; Hura et al., 2007; Maccaferri et al., 2011; Bürling et al., 2013). In the present study, bacterial colonization improved physiological traits of both maize cultivars under drought stress compared to the control. PsJN inoculation improved photosynthesis (net-rate of CO_2 assimilation under light saturation) (75%), chlorophyll content (22%) and efficiency of PSII (10%) of the cultivar Mazurka compared to the control treatment.

These observations are in accordance with previous reports on the potential of endophytic bacteria having multiple beneficial traits in improving plant productivity and to enhance drought tolerance in plants (Sandhya et al., 2010; Vardharajula et al., 2011). Plant-associated bacteria may also exudate osmolytes such as proline, glycine betaine, and trehalose in response to the stress, which along with other PGP attributes can possibly act synergistically with plant-produced osmolytes and stimulate the plant growth even under stressed conditions (Paul and Nair, 2008).

The relative water content is a good indicator of water stress (Fisher, 2000) and in this study, we observed that drought caused a reduction in relative water content in both inoculated and un-inoculated plants, however, inoculation significantly increased the relative water content compared to the un-inoculated controls. This may be due to a reduction in the inhibitory effect of drought on roots and the development of a more effective root system in the inoculated plants (Dodd et al., 2004). Drought stress accelerated relative membrane permeability (RMP) in the inoculated and un-inoculated seedlings compared to normal irrigation. However, bacterial inoculation helped seedlings to maintain the RMP and reduced 43% leaf damage compared to un-inoculated seedlings under drought stress. A positive correlation between drought stress sensitivity and membrane damage (EL) were observed by Vardharajula et al. (2011) and Sandhya et al. (2010), and the bacterial inoculation reduced the membrane damage in plants stressed by drought. In addition, reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) that cause lipid peroxidation of membranes (Sgherri et al., 2000) are produced during abiotic stresses. In our study, the inoculation reduced the H_2O_2 induced damage compared to control in both cultivars

under drought stress (Fig. 5). It is most probable that bacterial colonization augmented plant defense enzymes such as catalase, peroxidase, superoxide dismutase or phenolic compounds, to alleviate the oxidative damage elicited by drought.

Soil texture is the only factor affecting the moisture content at permanent wilting. The soil moisture content at the time of permanent wilting might conceivably be affected by the plant species, environmental conditions and the soil texture. Various researchers described the soil moisture relation to plant growth, and reported that moisture content at permanent wilting vary from 1% in sand to 25% in clay and even higher in soils containing much organic matter (see Kramer, 1994; Zotarelli et al., 2010). In the present study, we observed soil moisture content in the range of 12-13% of the drought stressed pots demonstrating the moisture wilting stage (Fig. 1). At harvesting time, the soil moisture content was in the range of permanent wilting point, although the plants were not dead. It is likely that some water was available to plants even though the soil was at the permanent wilting percentage.

The occurrence and activity of microbial inoculants are affected by a variety of environmental factors faced by the plant. In the present study, endophytic populations were more suppressed and viable cell numbers decreased in Mazurka than in Kaleo under stress conditions, while in the latter cultivar the viability of endophytic bacteria were affected only to a limited extent (Fig. 2). The numbers of viable bacterial cells in stressed plants of Mazurka were far below those in the cultivar Kaleo, but at the same time the relative increase in plant growth and vitality under drought was much higher. Plants undergo a number of metabolic and physiological alterations resulting in changed root

exudation during stress acclimation, which may influence the performance of an inoculant strain (Bais et al., 2006).

Out of the two endophytic strains used in this study, *B. phytofirmans* PsJN performed relatively better. This may be attributed to its intensive root/shoot colonization ability (Fig. 2-4) compared to *Enterobacter* sp. FD17, which made it more competitive. Similar findings were also obtained in other studies where strains having good root/shoot colonization showed more promising results than others (Fernandez et al., 2012; Yandigeri et al., 2012).

5. Conclusion

We provide evidence that endophytic colonization of bacteria may induce better drought stress tolerance in maize. Based on our results we conclude that application of *Burkholderia phytofirmans* strain PsJN is more efficient to improve physiology, relative water content and biomass of maize under drought conditions than *Enterobacter* sp. strain FD17. The improved plant physiology ultimately leads to enhanced crop yield and quality. Thus, endophytic bacteria could be efficiently used to reduce the effects of drought stress on growth and photosynthesis of maize.

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Table 1
Effect of endophyte inoculation on physiological parameters of two maize cultivars under drought stress conditions

Treatment	Mazurka		Kaleo		Mazurka		Kaleo	
	NH_2O[a]	DH_2O[b]	NH_2O	DH_2O	NH_2O	DH_2O	NH_2O	DH_2O
	CO_2 assimilation rate (Asat) (μmol CO_2 m^{-2} s^{-1})				Stomatal conductance (g_s) (mol H_2O m^{-2} s^{-1})			
Control	21.46±2.65^{cd}	10.87±0.69^{g}	22.80±2.18^{c}	14.77±1.77^{f}	0.120±0.02^{bc}	0.024±0.01^{g}	0.093±0.01^{cd}	0.045±0.02^{fg}
PsJN	31.11±1.15^{a}	19.06±1.61^{de}	28.49±1.15^{ab}	23.87±0.84^{c}	0.160±0.01^{a}	0.045±0.01^{ef}	0.115±0.02^{b}	0.073±0.01^{de}
FD 17	28.05±1.34^{ab}	16.63±1.38^{ef}	27.14±3.95^{b}	20.75±0.83^{cd}	0.143±0.01^{ab}	0.034±0.01^{fg}	0.106±0.01^{c}	0.062±0.01^{ef}
	Transpiration rate (E) (mmol H_2O m^{-2} s^{-1})				Vapor pressure deficit (kPa)			
Control	2.14±0.50^{c}	0.67±0.09^{f}	2.06±0.42^{c}	1.16±0.39^{e}	2.43±0.08^{bc}	2. 35±0.22^{bc}	2.32±0.20^{bc}	2.25±0.09^{c}
PsJN	3.30±0.17^{a}	1.23±0.16d^{e}	2.99±0.15^{ab}	1.77±0.21^{c}	2.54±0.08^{ab}	2.72±0.07^{a}	2.45±0.05^{bc}	2.55±0.02^{ab}
FD 17	2.72±0.31^{b}	1.00±0.12^{e}	2.70±0.32^{b}	1.67±0.18^{cd}	2.47±0.16^{bc}	2.50±0.11^{b}	2.36±0.16^{bc}	2.40±0.12^{bc}

Means sharing similar letter(s) in a column for each parameter do not differ significantly at P = 0.05

Data are average of three replicates ± standard deviation (SD)

[a]Normal irrigation

[b]Reduced water application

Table 2
Effect of endophyte inoculation on relative water content, relative membrane permeability, photochemical efficiency of PSII, and chlorophyll content of two maize cultivars under drought stress conditions

Treatment	Mazurka		Kaleo		Mazurka		Kaleo	
	NH_2O[a]	DH_2O[b]	NH_2O	DH_2O	NH_2O	DH_2O	NH_2O	DH_2O
	Relative water content (%)				Relative membrane permeability (%)			
Control	54.83±3.87^{e}	43.50±1.62^{f}	60.29±1.86^{cd}	54.96±2.17^{e}	10.52±1.79^{b}	16.85±1.71^{a}	10.20±1.37^{b}	14.60±1.31^{a}
PsJN	60.31±1.09^{cd}	56.66±2.59^{e}	74.21±1.96^{a}	69.88±2.32^{b}	6.55±1.12^{d}	9.55±0.92^{bc}	7.29±1.92^{cd}	8.62±1.35^{bcd}
FD17	61.32±2.56^{c}	55.31±3.73^{de}	70.02±1.92^{b}	64.68±2.64^{c}	6.29±1.20^{d}	9.95±1.51^{bc}	8.07±1.44^{bcd}	9.74±0.80^{bc}
	Maximum photochemical efficiency (F_v/F_m)				Chlorophyll content (spad value)			
Control	0.80±0.01^{cd}	0.74±0.02^{e}	0.80±0.02^{cd}	0.76±0.03^{d}	39.40±1.45^{e}	36.27±0.40^{f}	41.60±2.30^{de}	38.07±1.05^{ef}
PsJN	0.84±0.01^{a}	0.82±0.02^{bc}	0.83±0.01^{ab}	0.82±0.02^{bc}	46.40±1.01^{b}	44.13±0.85^{c}	48.13±1.26^{a}	45.13±0.68^{bc}
FD17	0.83±0.02^{ab}	0.81±0.02^{c}	0.82±0.01^{bc}	0.81±0.02^{c}	44.23±1.49^{c}	42.83±1.04^{d}	46.10±1.31^{b}	43.17±0.86^{cd}

Means sharing similar letter(s) in a column for each parameter do not differ significantly at P = 0.05

Data are average of three replicates ± standard deviation (SD)

[a]Normal irrigation

[b]Reduced water application

Table 3
Effect of endophyte inoculation on no of leaves per plant, leaf area, shoot and root dry weight of two maize cultivars under drought stress conditions

Treatment	Mazurka		Kaleo		Mazurka		Kaleo	
	NH_2O[a]	DH_2O[b]	NH_2O	DH_2O	NH_2O	DH_2O	NH_2O	DH_2O
	No. of leaves per plant				Leaf area (cm^2)			
Control	10.66±0.54^{cd}	9.67±0.58^{d}	11.33±0.57^{bc}	10.67±0.58^{cd}	332.60±7.34^{c}	309.94±7.24^{d}	315.77±3.47^{d}	294.10±5.63^{e}
PsJN	13.00±1.00^{a}	12.00±0.43^{abc}	13.00±1.00^{a}	12.33±0.57^{ab}	379.90±6.20^{a}	370.57±6.40^{a}	369.04±5.84^{a}	356.38±7.53^{b}
FD17	12.33±1.52^{ab}	11.33±0.58^{bc}	12.67±0.57^{ab}	11.67±1.15^{abc}	377.46±9.23^{a}	370.79±6.64^{a}	348.91±3.19^{b}	331.91±6.35^{c}
	Shoot dry matter (g)				Root dry matter (g)			
Control	24.07±1.93^{ef}	18.40±1.77^{g}	26.63±1.83^{cd}	21.70±1.57^{f}	2.49±0.11^{d}	1.41±0.11^{f}	2.46±0.22^{d}	1.55±0.12^{f}
PsJN	35.60±1.93^{a}	30.57±1.66^{b}	33.98±1.87^{ab}	31.60±1.68^{b}	3.66±0.23^{a}	2.40±0.15^{d}	3.45±0.12^{ab}	2.45±0.08^{d}
FD17	32.00±1.91^{b}	26.03±1.98^{cd}	30.70±2.09^{b}	28.67±1.77^{c}	3.38±0.21^{bc}	2.31±0.10^{d}	3.16±0.15^{c}	2.08±0.09^{e}

Means sharing similar letter(s) in a column for each parameter do not differ significantly at P = 0.05

Data are average of three replicates ± standard deviation (SD)

[a]Normal irrigation

[b]Reduced water application

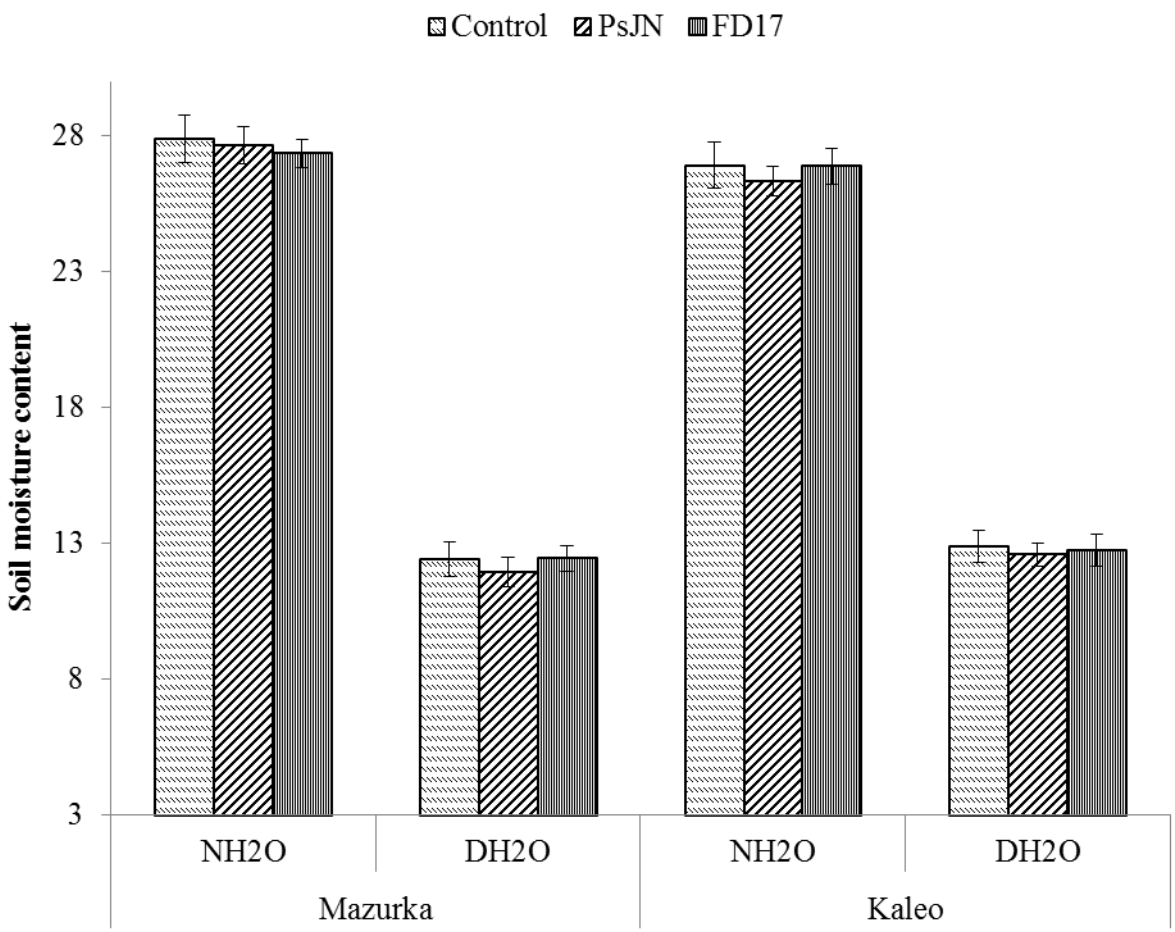

Figure 1 . Gravimetric so il m oisture co ntent (mass b asis %) o f n ormal an d r educed irrigation at the time of harvest, (NH_2O; normal irrigation, DH_2O; drought stress), data are average of three replicates ± standard deviation (SD)

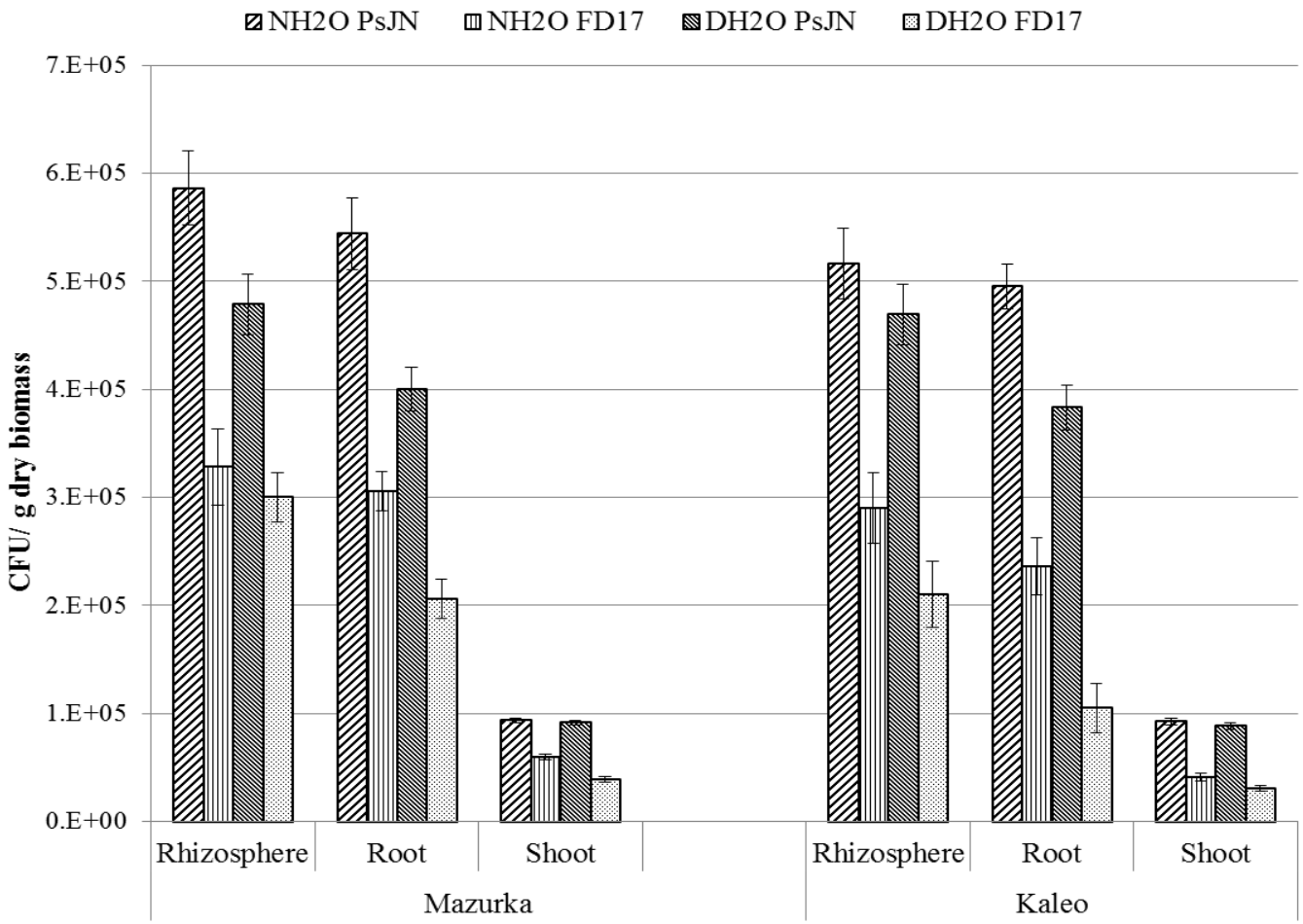

Figure 2. Persistence of selected endophytic strains in the rhizosphere, root and shoot interior of different maize cultivars under normal and reduced irrigation (NH_2O; normal irrigation, D H_2O; drought stress), data are average of three replicates ± standard deviation (SD)

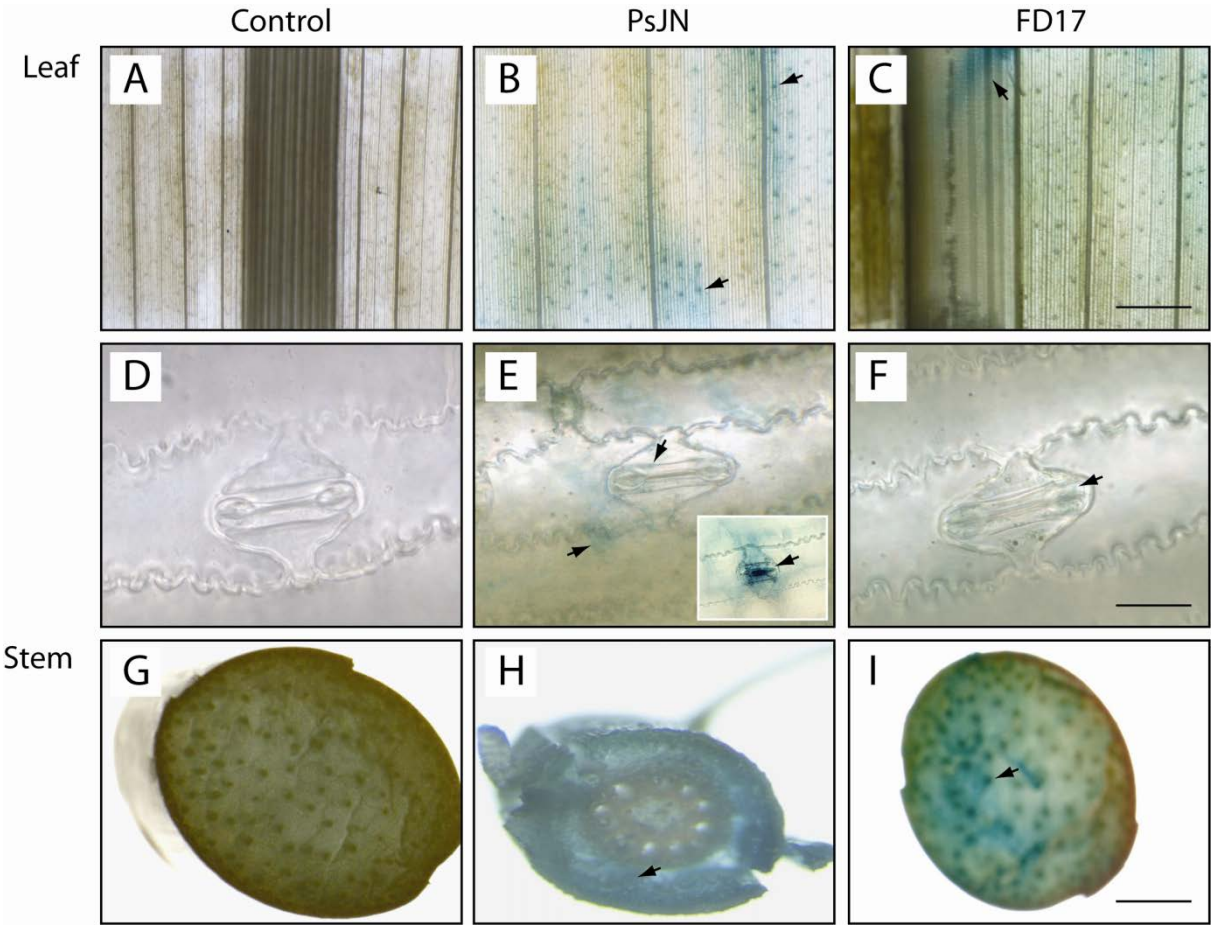

Figure 3. Photographs of the sixth internode and leaf internal tissue of *Zea mays* L. plants inoculated with PsJN::*gusA*10 and FD17::*gusA*10. (A and G) Photographed of the sixth leaf and stem of un-inoculated control or (B and H) inoculated with PsJN::*gusA*10, and (C and I) FD17::*gusA*10 inoculated plant showing the blue color in veins due to *gusA*-marked cells (arrowheads). Inverse microscope image of the leaf stomata of un-inoculated control (D) or inoculated with (E) PsJN::*gusA*10, and (F) FD17::*gusA*10 inoculated plant, showing bacteria in the stomata and guard cell. (A-C) bars = 400 µm; (D-F) bars = 20 µm; (G-I) bars = 400 µm

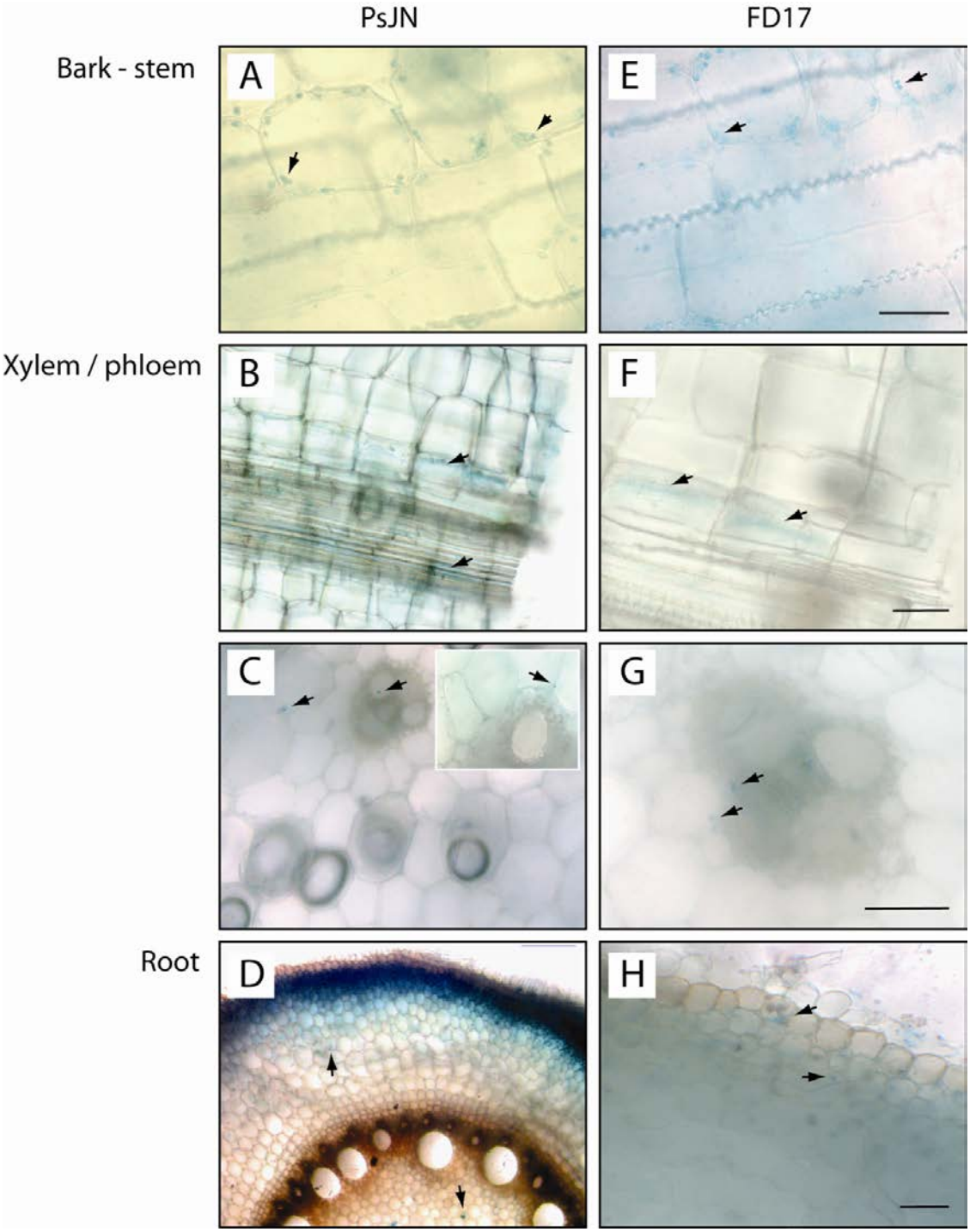

Figure 4. Photographs of the sixth internode, leaf internal tissue and root section of PsJN::*gusA*10 and FD17::*gusA*10 inoculated *Zea mays* L. plants. (A-D) Inverse microscope image of the stem, xylem/phloem and root of PsJN::*gusA*10 inoculated plants showing blue color due to *gusA*-marked cells (arrowheads). (E-H) Inverse microscope image of the stem, xylem/phloem and root of FD17::*gusA*10 inoculated plant showing blue color due to *gusA*-marked cells (arrowheads). (A-C) bars = 20 µm; (D and H) bars = 200 µm; (E-G) bars = 20 µm

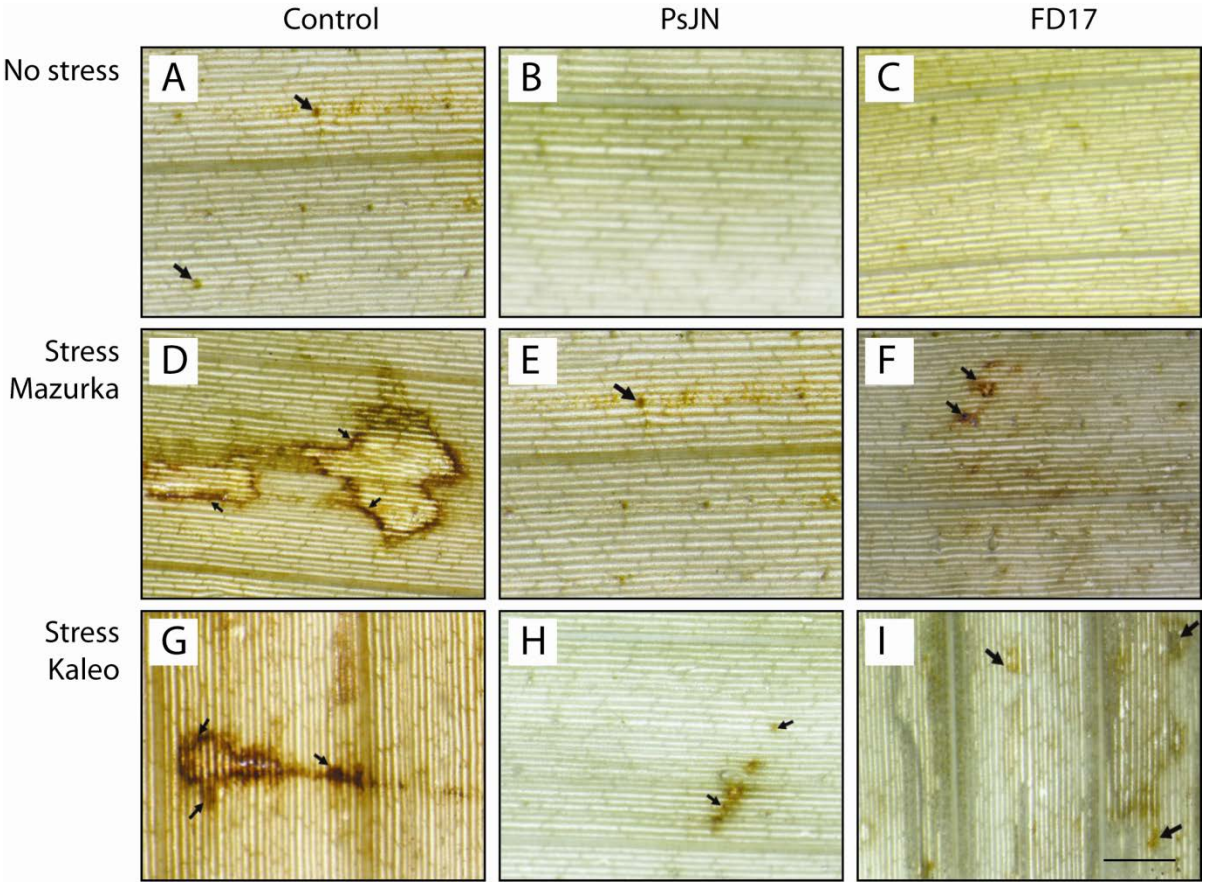

Figure 5. Photographs of the sixth leaf internal tissue of PsJN::*gusA*10 and FD17::*gusA*10 inoculated *Zea mays* L. plants. (A, D and G) Photographed of un-inoculated control showing the ROS (H_2O_2) production under normal and drought stress (arrowheads). (B, E and H) Photographed of cv. Mazurka and Kaleo of PsJN::*gusA*10 inoculated showing H_2O_2 production under normal and drought stress (arrowheads). (C, F and I) Photographed of cv. Mazurka and Kaleo of FD17::*gusA*10 inoculated showing H_2O_2 production under normal and drought stress (arrowheads). (A-I) bars = 500 µm

Chapter 4

Drought stress amelioration in wheat through inoculation with *Burkholderia phytofirmans* strain PsJN

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Running Title: Inducing drought tolerance in wheat through PsJN inoculation

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Abstract

Plant growth promoting endophytic bacteria *Burkholderia phytofirmans* PsJN was used to investigate the potential to ameliorate the effects of drought stress on growth, physiology and yield of wheat (*Triticum aestivum* L.) under natural field conditions. Inoculated and uninoculated (control) seeds of wheat cultivar Sahar 2006 was sown in the field. The plants were exposed to drought stress at different stages of growth (tillering stage and flowering stage) by skipping the respective irrigation. The results showed that drought stress adversely affected the physiological, biochemical and growth parameters of wheat seedlings. It decreased the CO_2 assimilation, stomatal conductance, relative water content, transpiration rate and chlorophyll contents in wheat. Inoculation of wheat with PsJN significantly diluted the adverse effects of drought on relative water contents and CO_2 assimilation rate thus improving the photosynthetic rate, water use efficiency and chlorophyll content over the un-inoculated control. Grain yield was also decreased when plants were exposed to drought stress at the tillering and flowering stage, but inoculation resulted in better grain yield (up to 21 and 18% higher, respectively) than the respective uninoculated control. Similarly, inoculation improved the ionic balance, antioxidant levels, and also increased the nitrogen, phosphorus, potassium and protein concentration in the grains of wheat. The results suggested that *Burkholderia phytofirmans* strain PsJN could be effectively used to improve the growth, physiology and quality of wheat under drought conditions.

Key words: Physiology, growth stages, *Burkholderia phytofirmans* PsJN, drought stress, wheat

Introduction

Plants are constantly exposed to a wide range of environmental stresses which limit plant productivity. Sustaining agricultural production under adverse environmental conditions, such as drought and high salinity, represents a major challenge. Drought is expected to cause serious plant growth problems for more than 50 % of the arable lands by 2050 (Vinocur and Altman 2005). Moreover, with global climate change, i.e., rising temperature and altered soil moisture, there is potential for long-lasting droughts across the globe in the near future (Overpeck and Cole 2006). Beneficial soil microorganisms such as bacteria and/or AM fungi can adapt to specific environmental conditions and develop tolerance to stressful conditions. The role of these microorganisms in plant abiotic stress tolerance (such as drought stress) is known and has been studied in the context of providing a biological understanding of the adaptation of living organisms to extreme environments (Marulanda et al. 2009).

In order to maintain or increase crop productivity, it has become necessary to evolve efficient low-cost technologies for abiotic stress management. Several strategies have been suggested for controlling the negative effects of drought stress in plants where breeding for tolerant varieties and genetic engineering are the most explored approaches (Warren 1998) along with resource management practices (Venkateswarulu and Shanker 2009). However, most of these techniques are time consuming and cost-intensive besides being not accepted well in some regions (Wahid et al. 2007). An alternative strategy is to induce stress tolerance by using beneficial microorganisms. Soil microorganisms with a potential for alleviation of abiotic stresses in combination with plant growth promotion would be extremely useful tools in sustainable agriculture.

Plant growth promoting rhizobacteria (PGPR) and/or endophytes that colonize the rhizosphere or plant interior enhance the plant growth by a variety of mechanisms. They can exert a beneficial effect on plant growth and nutrition probably due to fixation of atmospheric nitrogen (BNF), synthesis of phytohormones, synergism with other bacteria-plant interactions, inhibition of plant ethylene synthesis, as well as increasing availability of nutrients like phosphorus, iron and other micro-elements, and growth enhancement by volatile compounds (Compant et al. 2008; Mitter et al. 2013). It is accepted that the role of PGPR in contributing to plant establishment, growth, and drought tolerance when growing under water-stress conditions is the result of the sum of nutritional, physiological, and cellular effects (Vardharajula et al. 2011; Saravanakumar et al. 2011; Kasim et al. 2013).

Burkholderia phytofirmans PsJN is one of the most studied bacterial endophyte so far and is able to establish rhizospheric and endophytic populations associated with a variety of genetically unrelated plants. Originally isolated from surface-sterilized *Glomus vesiculiferum*-infected onion roots (Frommel et al. 1991), strain PsJN has been shown to colonize a wide variety of plants [(e.g. potato, tomato, peat moss and grapevines (Compant et al. 2008)] and it stimulates plant growth and vitality in many of its host plants under lab and greenhouse conditions. However, little is known about the inoculation response of PsJN to host plant under field conditions. Present study was conducted to evaluate the potential of *Burkholderia phytofirmans* strain PsJN for improving physiology, growth and yield of wheat under drought stress applied at different growth stages in the field conditions.

Materials and methods

Bacterial inoculum and plant bacterization

The bacterial inoculum was produced by transferring a loop of *B. phytofirmans* strain PsJN to 200 mL of LB liquid medium in a 500-mL Erlenmeyer flask incubated for 48 h at 28ºC and 150 rpm. The optical density of the broth was adjusted to 0.5 measured at λ 535 nm using spectrophotometer (Gene Quant Pro, Gemini BV, The Netherlands) to obtain a uniform population of bacteria (10^8 - 10^9 colony-forming units (CFU) mL^{-1}) in the broth at the time of inoculation. For inoculation, the obtained suspension of inoculum was mixed with sterilized peat (200 ml kg^{-1} peat) and incubated for 24 h at 28 ± 1°C before being used for seed coating (seed to peat ratio 1.25:1 w/w). Wheat seed dressing was done with the inoculated peat mixed with 10% sterilized sugar (sucrose) solution in 10:1 ratio. In the case of the non inoculated control, the seeds were coated with the sterilized peat treated with sterilized broth and 10% sterilized sugar solution.

Wheat (*Triticum aestivum* L. cv. Saher) seeds were provided by the Wheat Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan. The *B. phytofirmans* strain PsJN was obtained from the Culture Collection Section / Bioresource Unit, AIT - Austrian Institute of Technology GmbH, 3430-Tulln, Austria.

Field experiment under drought stress

A field trial was conducted at the Experimental Farm, Institute of Soil and Environmental Sciences, University of Agriculture (UAF), Faisalabad to assess the efficacy of *B. phytofirmans* strain PsJN for improving growth and yield of wheat (*Triticum aestivum* L.)

under drought stress during October to April 2011-12. The soil samples from the field were collected for analysis of various physicochemical characteristics. The soil was sandy clay loam (Typic Haplocambid) having pH 7.8; ECe, 2.11 dS m^{-1}; organic matter, 0.84%; total nitrogen, 0.06%; available phosphorus, 6.9 mg kg^{-1}; extractable potassium, 102 mg kg^{-1} and 36% saturation percentage.

Seeds of wheat were inoculated (coated) with bacterial culture-injected peat based-slurry. Inoculated and non-inoculated seeds were sown in the well-prepared field at 120 kg ha^{-1} with a plot size of 10 m^2. The seed was sown with Rabi drill and treatments were arranged in randomized complete block design with two factor factorial settings and four repeats. Recommended doses of NPK fertilizers at 120-90-60 kg ha^{-1} were applied as urea, di ammonium phosphate, and muriate of pot ash, respectively. Phosphorus and potassium were applied as a basal dose, while nitrogen was applied in splits (at tillering and booting stage). Weather conditions i.e. precipitation and temperature were recorded by "Meteorological department, UAF" during the crop growth period and described in Figure 1. Field was irrigated with canal water and rainfall contributed only 24.2 mm from October 2011 to April 2012. There were five irrigations applied (normal irrigation; IN) during the crop growth period. The drought stress was applied by skipping irrigation at tillering (IST) and flowering (ISF) growth stages of the crop.

Data of plant physiology, water relations and antioxidant contents of flag leaves were recorded from normal irrigation (IN) and reduced irrigation (IST & ISF) plots. Growth and yield contributing parameters were recorded after maturity. Grain and straw samples were analyzed for nitrogen, phosphorus and potassium content (Ryan et al.

2001).

Physiological and biochemical traits of plant

Physiological measurements

The plant physiological parameters were recorded at midday (between 10:00 and 14:00) of both irrigated and drought-stressed plots. Portable infra-red gas analyzer [IRGA (LCA-4) Germany] was used (at 1200-1400 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density) to measure transpiration rate (E), stomatal conductance (g_s), CO_2 assimilation rate (A) and sub-stomatal CO_2 concentration (C_i). Fully expanded flag leaves were selected for gaseous exchange measurements. Chlorophyll content was measured using SPAD-502 meter (Konica-Minolta, Japan). Readings were recorded with four repeats from each treatment. Water use efficiency (WUE) was derived by dividing photosynthetic rate (A) with transpiration rate (E).

Relative water contents and electrolyte leakage

Flag leaves were used for measuring the relative water content (RWC) and percentage of electrolyte leakage. After measuring the fresh weights, leaves were placed in distilled water for 24 h at 4ºC in darkness and the turgid weight was recorded. Dry weights were obtained after oven drying the leave samples at 72°C for 24 h. Relative water contents were determined following the equation 1 described by Teulat et al. (2003). For electrolyte leakage, leaf discs were transferred in 5 mL deionized water and EC (R1) was recorded with EC meter (Jenway Conductivity Meter Model 4070) after 4 h incubation at

28 ± 1ºC and 100 rpm in orbital shaking incubator (Firstek Scientific, Tokyo, Japan). The same samples were autoclaved at 121°C for 20 min to determine EC (R2). Electrolyte leakage (EL) was measured following the protocol described by Jambunathan (2010) using equation 2.

$$\mathbf{RWC} = \frac{(\mathbf{Fresh\ weight} - \mathbf{Dry\ weight})}{(\mathbf{Fully\ turgid\ weight} - \mathbf{Dry\ weight})} \quad \text{Equation 1}$$

$$\mathbf{\%EL} = \frac{\mathbf{EC\ before\ autoclaving\ (R1)}}{\mathbf{EC\ after\ autoclaving\ (R2)}} \times 100 \quad \text{Equation 2}$$

Analysis of stress-related metabolites

Total soluble sugars were measured by anthrone reagent (0.2%) following Sadasivam and Manickam (1992). A reaction mixture (10 mL) consisting of 200 µL leaf extract (1 g leaf homogenized in de-ionized water), 1800 µL DI water and 8 mL anthrone reagent was heated for 10 minutes in boiling water and cooled in ice bath to stop the reaction. Absorbance was measured at 630 nm and total soluble sugar concentration (µg mL^{-1}) was calculated using glucose standard curve.

Proline content was measured following Bates et al. (1973). A reaction mixture consisting of leaf extract (1 g leaf homogenized with 3% sulphosalicylic acid), ninhydrin acid and glacial acetic acid was heated at 100°C for 1 h in water bath. The reaction was stopped by cooling in ice bath and absorbance was recorded at 520 nm after mixture extraction with toluene. Proline content (µg mL^{-1}) was calculated following standard curve of L-proline.

Bradford (1976) method was followed to measure protein contents in green

leaves. A reaction mixture consisting of 200 µL leaf extract (1 g leaf homogenized in de-ionized water), 1800 µL DI water and 2 mL of Bradford reagent was incubated at room temperature for 10-20 minutes and spectrophotometric absorbance was measured at 595 nm afterwards. Bovine serum albumin standard curve was used to calculate the protein concentration (µg mL^{-1}).

Enzymatic / non enzymatic antioxidant activity assays

The enzymes were extracted by homogenizing frozen fresh leaf material in ice-cold solution containing potassium phosphate buffer (0.2 M, pH 7) having 0.1 mM EDTA.

For glutathione reductase (GR) activity, increase in spectrophotometer absorbance at 412 nm was observed due to reduction of DTNB (5,5´-dithiobis (2-nitrobenzoic acid)) into TNB (2-nitro-5-thiobenzoic acid). Thirty microliters of enzyme extract [extracted in potassium phosphate buffer (50 mM, pH 7.8) with 2 mM EDTA] was resuspended in three milliliters reaction mixture containing 0.75 mM DTNB, 0.1 mM NADPH and 1 mM GSSG (oxidized glutathione). GR activity was calculated in µmol TNB min^{-1} g^{-1} leaf fresh weight at 25 ± 2 °C following Smith et al. (1988). Ascorbate peroxidase (APX) activity was determined by tracking the ascorbate reduction through H_2O_2 with decrease in spectrophotometer absorbance at 290 nm (Nakano and Asada 1981). Two milliliters reaction mixture consisting of 20 µL crude leaf extract, 660 µL ascorbic acid solution, 660 µL potassium phosphate buffer (pH 7.0, 50 mM) and 660 µL H_2O_2 was used to measure APX activity. Decrease in absorbance was monitored for three minutes just after the addition of H_2O_2. Enzyme activity was calculated in the form of µmol ascorbate min-

^{1} g^{-1} leaf fresh weight. For catalase (CAT), 2 mL of 200 times diluted enzyme extract in potassium phosphate buffer (50 mM, pH 7.0) and 1 mL of 10 mM H_2O_2 was used following the method Cakmak and Marschner (1992). Decrease in absorbance at 240 nm due to H_2O_2 loss was observed for 3 min. CAT activity was calculated in µmol H_2O_2 min^{-1} g^{-1} fresh weight at 25 ± 2°C.

Lipid peroxidation or malondialdehyde (MDA) concentration was determined following the method described by Jambunathan (2010). A reaction mixture (2.5 mL) consisting of leaf extract (0.5 mL, extracted in 0.1% TCA), trichloroacetic acid (20%) and thiobarbituric acid (0.5%) was heated at 95ºC for 30 minutes in a fume hood and cooled in ice bath. Then absorbance was measured by spectrophotometer at 600 and 532 nm. The concentration of MDA was calculated using Beer and Lambert's law following the difference in absorbance (Abs at 532 – Abs 600). The concentration was expressed as µmol g^{-1} fresh weight of leaf. For total phenolics, 2 mL reaction mixture consisting of 20 µL leaf extract, 300 µL Na_2CO_3 (1 N), 1580 µL DI water and100 µL Folin Ciocalteu's reagent (0.25 N) was incubated in the dark at room temperature for 2 h. Then absorbance was recorded at 760 nm and concentration in µg mL^{-1} was calculated following gallic acid standard curve (Singleton et al. 1999).

Mineral nutrients measurement

Grain and straw (dry) samples (0.1 g) were ground and digested with sulphuric acid and hydrogen peroxide (2:1 ratio) following Wolf (1982), and final volume made upto 50 mL with deionized water. Nitrogen content was determined with Kjeldhal method. For

phosphorus, the extracted material (5 mL) was mixed in 10 ml of Barton reagent and total volume was made 50 mL. The samples were kept for half an hour and phosphorus contents were measured by spectrophotometer (Shimadzu, Japan) at λ 420 nm using standard curve. The Barton reagent was prepared as described by Ashraf et al. (1992). Potassium content was recorded by flame photometer using a standard curve (Ryan et al. 2001).

Enumeration of *B. phytofirmans* PsJN in the rhizosphere and root

The persistence of PsJN in the rhizosphere and root of wheat seedlings was monitored in a pot experiment. We used a genetic variant of the bacterial strain *B. phytofirmans* PsJN::gusA that carries a beta-glucuronidase reporter gene (*gus*A) that allows specific visualization of bacterial cells upon color formation. Wheat seeds were surface-sterilized with 70% ethanol (3 min), treated with 2% sodium hypochlorite (NaClO) (5 min), followed by repeated washing with sterile distilled water (3 times for 1 min). The PsJN inoculated seeds were sown in the pot having 1 kg soil (collected from the natural field). Four moisture levels were used i.e. normal water, 75% FC, 50% FC and 25% of field capacity in the pots. The plants were harvested 25 days after sowing and rhizosphere / root colonization was recorded.

For the isolation of bacteria, 3 g rhizosphere soil and 1 g of surface-sterilized root material was homogenized in 5 mL of 0.85% (w/v) NaCl solution. The material was shaken for 30 min at room temperature. After settlement of the material, serial dilutions up to 10^{-4} were plated onto selective LB medium containing spectinomycin (100 µg mL^{-}

^{1}), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 µg mL^{-1}), and isopropyl-β-D-galactopyranoside (IPTG) (100 µg mL^{-1}). The plates were incubated at 28 ± 1°C for 48 hours and then transferred to 4°C for three days. Blue colonies were counted on each plate and survival efficiency was calculated.

Statistical analysis

Two way analysis of variance (ANOVA) was used to analyze the data and Tukey's test was used to compare treatment means using Statistix 8.1 software (Copy right 2005, Analytical Software, USA). The means and standard errors were calculated using Microsoft Excel 2010.

Results

Growth physiology and agronomic yield

Skipping irrigation at the tillering (IST) or flowering (ISF) stage of wheat disturbed the growth and yield of the crop either inoculated or un-inoculated (Table 1). However, improvement in the growth has been observed with dilution of stress impact on the crop due to *B. phytofirmans* PsJN inoculation. A significant improvement in CO_2 assimilation rate (A) was recorded with PsJN inoculation compared to respective control at IST growth stage whereas "A" was similar between respective inoculated and un-inoculated plants under normal irrigations (IN) or ISF. Inoculations showed increment in the transpiration rate (E) of the crop during IN and skipped irrigation (IST or ISF) situations up to 21, 27 and 21%, respectively, over their respective controls. When water use efficiency (WUE) was calculated from A and E of the plants, there was no

significant difference between inoculation and control. Sub-stomatal CO_2 concentration (C_i) changed due to water deficit stress and PsJN inoculation, where 23, 16 and 18% increase was recorded due to inoculation at IN, IST and ISF stages, respectively, in contrast to the un-inoculated control. Inoculation of PsJN improved stomatal conductance (g_s) significantly about 52 and 24%, respectively, during water deficit at IST and ISF stages with respect to the corresponding non-inoculated controls. Although, "g_s" was similar at unstressed and water deficit stressed treatments with the inoculation of *B. phytofirmans* PsJN. More chlorophyll content in wheat plants was observed due to PsJN inoculation but water deficit reduced it in both inoculated and un-inoculated plants (Fig. 2). About 11, 3 and 14%, increase in chlorophyll content was recorded with PsJN inoculation at IN, IST and ISF growth stages, respectively, compared to the respective un-inoculated controls.

As for as agronomic yield of the wheat crop under reduced water system, inoculation of the plant growth promoting endophyte *B. phytofirmans* PsJN increased 1000 grain weight, straw and grain yields of the crop (Table 1). Under normal irrigation, inoculation enhanced up to 7, 19 and 18%, the 1000 grain weight, straw and grain yields respectively, over control. Water deficit at IST reduced the grain yield approximately with 28 and 26% in un-inoculated and PsJN inoculated crops, respectively. On the other hand, with water limitation through ISF, PsJN inoculation caused 18, 16 and 23% increase in grain yield, 1000 grain weight and straw yield respectively, over respective control treatment.

Enzymatic and non-enzymatic antioxidant activity

Increase in activity of glutathione reductase (GR) was observed due to inoculation of endophyte PsJN (Table 2). Maximum increase (115%) in GR was recorded with PsJN inoculation under no water deficit conditions compared to respective control whereas in conditions of drought at IST stage, inocculation resulted in 77% more GR in the plants in contrast to the respective un-inoculated control. Stress at the ISF stage was relieved due to inoculation of PsJN with a 58% increase in GR activity with respect to the corresponding un-inoculated control. Similarly, catalase activity was improved due to PsJN inoculation at normal as well as skipped irrigation systems compared to respective control treatments. However, maximum increase (about 78%) in catalase activity due to inoculation compared to control was measured at ISF stage. Ascorbate peroxidase activity was 3.2 fold higher in PsJN inoculated plants compared to control when drought was applied at the tillering growth stage of the crop (IST). Lipid peroxidation increased under stress and inoculation further improved malodialdehyde contents up to 81 and 40% at IST and ISF with respect to corresponding non-inoculated controls.

Accumulation of osmolytes (non-enzymatic anti-oxidants) in flag leaf changed due to the inoculation of *B. phytofirmans* PsJN and application of water deficit stress during tillering or flowering growth stage of wheat crop (Table 2). Although changes in total soluble sugars (TSS) remained statistically non-significant for drought or inoculation treatments, even so, drought increased TSS and inoculation decreased TSS compared to the respective non stressed or un-inoculated controls. Protein concentration in leaves was enhanced due to endophyte inoculation and PsJN also eliminated

the impact of drought on the protein contents. Almost 11% more proteins were recorded in leaves of inoculated plants compared to respective control at ISF whereas 16% increased protein content with inoculation was noted in IST treatment compared to respective control. Proline and total phenolic contents were highest in both un-inoculated and inoculated plant leaves at IST in contrast to respective non-stressed plants. However, inoculation reduced the total phenolic and proline contents up to 15 and 41% respectively during this stage and about 33 and 34% respectively at ISF over respective un-inoculated controls.

Mineral nutrition

Improvement in the mineral nutrition of wheat was recorded due to *B. phytofirmans* PsJN inoculation in normal as well as water limited conditions (Table 3). Both wheat straw and grain acquired high amounts of nitrogen, phosphorus and potassium due to water deficit and endophyte inoculation but the drought treatment results remained almost similar for inoculated and un-inoculated plants with respect to their corresponding controls. However, maximum grain N, K and P contents of 1.70, 0.19 and 0.21% respectively, were found in plants inoculated with PsJN and minimum values of 0.99, 0.14 and 0.14%, respectively, in the grains of non-inoculated crop plants. Straw nitrogen content was high (0.93%) in the inoculated plant subjected to ISF but maximum straw potassium (0.44%) was observed in PsJN treated plant subjected to IST. Maximum phosphorus in wheat straw was collected from the crop inoculated with PsJN and treated with IST.

Water relations

Relative water contents RWC demonstrate the genetic capability of the crop plants to combat or abide under water limited conditions where inoculation can be helpful (Fig. 3). Under normal irrigated conditions inoculated *B. phytofirmans* PsJN improved the RWC about 9% compared to un-stressed control. Water deficit tre atments, IST and ISF, reduced the RWC of un-inoculated control from 0.87 to 0.73 and 0.78 respectively, but inoculated plant leaves demonstrated no change with respect to inoculated and unstressed plants. An increase in electrolyte leakage (Fig. 4) was observed due to IST and ISF treatment of wheat but inoculation of *B. phytofirmans* PsJN rescued plant growth under stress and reduced the electrolyte leakage up to 5, 7 and 8% after IN, IST and ISF treatment respectively, compared to the respective un-inoculated controls.

Detection and enumeration of PsJN in the rhizosphere and root

The inoculant strain (PsJN) efficiently survived and colonized the rhizosphere and root interior of wheat seedlings (Fig. 5). The persistence of PsJN in the rhizosphere and root differed non significantly at normal water and at 75% of the field capacity. However, relatively less CFU (colony forming unit) of PsJN was recorded at 50% of field capacity conditions compared to normal watering. The lowest CFU was recovered at 25% of field capacity from the rhizosphere of wheat seedlings. Overall, as the moisture stress increased the inoculant strains preferred to colonize in the roots compared to soil.

Discussion

In the changing climate, plants are constantly exposed to abiotic stress, such as drought, which is one of the most serious problems associated with plant growth and development affecting agricultural demands. Inoculation with PGPR has been found effective under drought stress environment (Chanway and Holl 1994) to increase productivity. Beneficial plant–microbe (PGPR) interactions, impact of microbial inoculation on plant growth and differential mechanisms underlying growth promotion under stress conditions have been documented by various researchers (Saravanakumar et al. 2011; Kasim et al. 2013).

In the present investigation, the potential of the endophytic bacteria *Burkholderia phytofirmans* strain PsJN for improving physiology, antioxidant activity, growth and yield of wheat was evaluated under drought stress applied at different growth stages in field conditions. PsJN was originally isolated as a contaminant from *Glomus vesiculiferum*-infected onion roots (Frommel et al. 1991). It stimulates plant growth in many of its host plants. Metabolic activities suggested to be involved in these functions include phytohormone production, ACC deaminase activity and siderophore production (Sessitsch et al. 2005). Impact assessment of endophytic bacteria on plant growth promotion and underlying physiological and biochemical mechanisms is scarcely documented. Therefore, an understanding of the interactions between host plant and endophytic bacteria having influence on plant growth, physico-chemical changes, yield and drought stress tolerance is required.

The effect of inoculation with PsJN on the growth of wheat plants exposed to drought stress applied at different growth stages (tillering and flowering stage) was

studied. The results revealed that in general, drought stress applied at any stage of growth had strong negative effects on the growth of uninoculated wheat plants, but the magnitude of severity varied with the growth stage. Drought stress applied at the tillering stage had relatively stronger negative effects on shoot biomass and grain yield whereas drought stress applied at the flowering stages had a more negative effect on relative water content (RWC), electrolyte leakage (EL) and chlorophyll content. Similarly, changes in different plant physiological and biochemical processes were observed due to drought stress that might have contributed to the growth and development processes of the wheat plants. This premise was supported by the fact that plants showed variable responses to water deficit faced in their various development periods (Mogensen et al. 1985; Gupta et al. 2001). Wheat, one of the most important crop species, is known to be susceptible to even mild or moderate drought particularly at the booting stage; however, unfavorable soil water conditions at the beginning of the plant growth may also dramatically limit the biomass production and the photosynthetic ability of leaves and thus indirectly negatively affect the formation of reproductive organs and yield parameters (Mogensen et al. 1985; Gupta et al. 2001; Kettlewell et al. 2010). In the present study, PsJN inoculations gave better response to wheat at tillering stage and resulted in significant increase in plant biomass, photosynthesis and grain yield compared to control as was evident from the data documented in Tables 1–2. This might be because of the suppression of stress-induced accelerated synthesis of ethylene by the ACC-deaminase activity of PsJN in the inoculated plants. Sharp increases in ACC levels and consequently, ethylene synthesis in plants under drought stress conditions have been frequently reported (Mayak et al. 2004;

Zahir et al. 2008). Also, PsJN was previously reported as a potent root colonizers (also observed in our lab study, Fig. 5); it is highly probable that PsJN while living inside plant tissues evoked various physiological and metabolic processes to help the plants to sustain their growth under stress conditions (Vardharajula et al. 2011; Theocharis et al. 2012; Fernandez et al. 2012).

The beneficial influence of bacterial inoculations was also apparent in terms of improved physiological and biochemical changes. Under drought stress, the photosynthetic activity in term of CO_2 assimilation rate (A) was markedly reduced in the un-inoculated and PsJN treated plants without disruption of stomatal conductance (g_s) while in the latter case, the photosynthetic rate was effected only to a limited extent. The cessation of growth resulting from drought stress reduces the capacity for energy utilization which, in turn, probably results in feedback inhibition of photosynthetic rate (Wang et al. 2003). The PsJN inoculation increased photosynthetic parameters e.g. CO_2 assimilation, transpiration rate, and stomatal conductance under drought stress compared to control. Furthermore, bacterization affected chlorophyll content but not the water use efficiency (Fig. 2, Table 1). Effects on photosynthesis parameters have been described in the literature for other beneficial plant–microbe interactions (Sandhya et al. 2010; Vardharajula et al. 2011; Yandigeri et al. 2012).

Drought stress is accompanied by the formation of reactive oxygen species (ROS) such as O_2, H_2O_2, and OH (Mittler 2002), which damage membranes and macromolecules. Plants have developed several antioxidation strategies to scavenge these toxic compounds. Enhancement of antioxidant defense in plants can thus increase

tolerance to different stress factors. Antioxidants (ROS scavengers) include enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins. Additional compounds, such as carbohydrates, sugars and phenolics, can also function as ROS scavengers (Wang et al. 2003; Theocharis et al. 2012; Fernandez et al. 2012). In our study, PsJN inoculation showed higher antioxidant activity of plants compared to control under drought stress. However, phenolics and sugars contents decreased in the bacterized plants compared to control. Very recently, Chakraborty et al. (2013) reported increased antioxidant levels in drought-stressed wheat plants inoculated with beneficial bacteria. Contrary to our study, Fernandez et al. (2012) reported higher sugars content in PsJN treated grapevine plantlets under stress conditions.

Drought-stressed plants accumulate various molecules such as proline, glycine, betaine etc., thereby protecting enzyme activity (Saravanakumar et al. 2011). Proline, the best-characterized stress-responsive molecule, is often synthesized by plants in response to diverse abiotic or biotic stresses. Moreover, the accumulation of a compatible solute (e.g. proline) is an energy-consuming process in addition to the already existing metabolic costs. We found that the proline concentration in plant leaves increased with drought but that inoculation with PsJN under drought stress decreased the proline content. In the present study, the proline content was higher in the PsJN-inoculated plantlets than in the non-bacterized plantlets under stress exposure; in accordance with conclusions by Theocharis et al. (2012) that increase in proline content enhances the

stress tolerance of PsJN treated grapevine plantlets.

The relative water content (RWC) and lower electrolyte ion leakage (EL) in plants exposed to drought, has been considered indicative of a relative tolerance to water stress (Fisher 2000; Pereyra et al. 2006). In our study, RWC and EL declined in both inoculated and uninoculated seedlings under drought stress compared to normal irrigation. However, bacterial inoculation did help plants to maintain their RWC and EL during drought stress periods. The relatively higher water content and low EL as evident from Figures 3 and 4 in the inoculated seedlings compared to control under drought stress, indicate that endophyte inoculation gives tolerance to plants under reduced irrigation. This may be due to a reduction in the inhibitory effect of drought on roots and the development of a more effective root system for water uptake in the inoculated plants (Dodd et al. 2004; Zahir et al. 2008). Similarly, a positive correlation between drought stress sensitivity and membrane damage (EL) were observed by Vardharajula et al. (2011) and Sandhya et al. (2010), and the bacterial inoculation reduced the membrane damage in drought stressed plants compared to control.

The bacterial inoculations were also effective in improving the nitrogen, phosphorus, potassium, and protein content of various plant components. Under stress conditions, nutrient (NPK) contents of plant tissues were increased in response to inoculation, most likely due to increased root growth that exploited more soil volume for efficient uptake of nutrients by the plants, resulting in more biomass production. Enhanced nutrient concentrations in plant tissues were reported by bacterial inoculation under stress conditions (Vivas et al. 2003; Nadeem et al. 2006).

Soil is a complex system and various biotic and abiotic factors may influence the behavior of particular strains in this environment. In our pot study, we used non-sterilized soil and observed that endophytic population was more suppressed and the viable cell number dropped more drastically in soil than in root at lower moisture levels while at higher moisture levels, viability of endophytic bacteria seemed hardly affected (Fig. 5). It is well known that various stress factors frequently impact the plant and thus alter the allocation of photosynthates in the rhizosphere that may lead to changes in below-ground microbial communities and their interaction with the plant (Compant et al. 2008). Strain PsJN was able to successfully compete with the natural microflora and successfully colonized the plant environment (Fig. 5) in addition to promoting plant growth. In our field investigation, the PsJN inoculation improved physiology and growth parameters of wheat under natural field conditions. It is likely that bacteria colonization (inside plant tissues) evoked various physiological processes to help the plants to sustain photosynthesis and plant growth under natural soil condition.

In conclusion, *B. phytofirmans* inoculation modulates biochemical and physiological parameters of wheat seedlings under drought stress conditions. Based on our results we conclude that application of PsJN is effective to improve physiology, relative water content and biomass of wheat under reduced irrigation. The improved plant physiological and antioxidant activity ultimately leads to enhanced crop yield and quality. Thus, inoculation with *B. phytofirmans* strain PsJN could be efficiently used to partially or completely eliminate the effects of drought stress on growth and yield of wheat.

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Table 1 Growth physiology and yield of wheat with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition

Drought stress	Uninoculated	PsJN inoculated	Uninoculated	PsJN inoculated
	Photosynthetic rate (A) (μmol CO_2 m^{-2} s^{-1})		Transpiration rate (E) (mmol H_2O m^{-2} s^{-1})	
IN	14.95±0.41 a	15.62±0.96 a	6.05±0.89 bcd	7.29±0.36 a
IST	9.48±0.24 c	12.94±0.46 b	5.40±0.11 d	6.83± 0.12 abc
ISF	12.19± 1.29 b	13.16±1.19 b	5.78±0.09 cd	7.00±0.25 cd
HSD	1.31		1.16	
	Water use efficiency (WUE = A/E)		Substomatal CO_2 content, (Ci)(vpm)	
IN	2.47±0.14 a	2.15±0.13 ab	247±4.38 bc	304±5.29 a
IST	1.76±0.05 b	1.90±0.08b	230±5.33 c	267±2.94 b
ISF	2.14±0.04 ab	1.89±0.10 b	227±3.35 c	265± 4.91b
HSD	0.46		24.91	
	Stomatal conductance (g_s) (mol H_2O m^{-2} s^{-1})		1000 grain weight (g)	
IN	0.32±0.06 ab	0.38±0.02 a	39.22±1.13 b	42.05± 1.02a
IST	0.25±0.01 c	0.38±0.02 a	34.56±0.46 c	39.68±0.80 b
ISF	0.29±0.02 bc	0.36±0.01 a	35.37± 0.82c	41.08± 0.59ab
HSD	0.07		1.90	
	Straw yield (Mg ha^{-1})		Grain yield (Mg ha^{-1})	
IN	5.16±1.05 b	6.16± 1.06a	3.31±1.02 b	3.91±0.95 a
IST	4.20±1.13 c	5.60±1.09 ab	2.38±0.82 c	2.87±0.87bc
ISF	4.98±1.14 b	6.12±1.02 a	2.43±0.92 c	2.87±0.65 bc
HSD	0.71		0.60	

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.01$

IN, Ir rigation n ormal; I ST, Irr igation s kipped a t tillering; IS F, I rrigation skipped a t flowering; HSD, Tukey's Honestly Significant Difference

Data are average of four replicates ± Standard error (SE)

Table 2 Antioxidant activity (enzymatic and non-enzymatic) of wheat with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition

Drought stress	Uninoculated	PsJN inoculated	Uninoculated	PsJN inoculated
	Glutathione reductase (GR) (μmol TNB min^{-1} g^{-1} fw)		Catalase (CAT) (μmol H_2O_2 min^{-1} g^{-1} fw)	
IN	4.00±0.23 e	8.61±0.29 cd	190±6.12 d	232±8.42 d
IST	7.36±0.52 d	13.07±0.32 b	317±7.43 c	482±6.54 b
ISF	9.79±0.85 c	15.48±0.98 a	332±5.98 c	590±6.72 a
HSD	2.21		49	
	Ascorbate peroxidase (APX) (μmol ascorbate min^{-1} g^{-1} fw)		Malondialdehyde (MDA) (μmol MDA g^{-1} fw)	
IN	46±0.36 e	63±0.63 de	1.44± 0.05d	3.03±0.03 c
IST	95±1.08 d	405±1.22 a	3.66±0.13 c	6.63±0.22 a
ISF	154± 1.17c	241± 0.98b	4.55±0.29 b	6.35±0.32 a
HSD	42		0.71	
	Protein contents (μg g^{-1})		Proline contents (μg g^{-1})	
IN	1.40±0.04 c	1.68±0.06 a	0.50±0.05 c	0.39±0.03 d
IST	1.39±0.05 c	1.61±0.06 a	0.71±0.08 a	0.42±0.04 cd
ISF	1.48±0.03 b	1.64±0.05 a	0.61±0.02 b	0.40±0.04 d
HSD	0.08		0.09	
	Total phenolics (μg g^{-1})		Total soluble sugars (μg g^{-1})	
IN	90.77±5.12 e	62.83±5.62 f	3.41±0.15 a	3.31±0.13 a
IST	177.53±7.61 a	151.75±6.52 c	3.99±0.24 a	3.58±0.31 a
ISF	162.50±5.28 b	109.57± 4.98d	4.03±0.54 a	3.45±0.43 a
HSD	7.44		1.54	

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.01$

IN, Irrigation normal; IST, Irrigation skipped at tillering; ISF, Irrigation skipped at flowering; HSD, Tukey's Honestly Significant Difference

Data are average of four replicates ± Standard error (SE)

Table 3 Mineral nutrition of wheat straw and grain with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition

Drought stress	Uninoculated	PsJN inoculated	Uninoculated	PsJN inoculated
	Grain nitrogen (%)		Straw nitrogen (%)	
IN	1.29±0.09 b	1.64±0.08 a	0.26±0.05 c	0.79±0.06 b
IST	0.99±0.14 c	1.56±0.12 a	0.72±0.02 b	0.80±0.07 b
ISF	1.19±0.08 b	1.70±0.10 a	0.74±0.02 b	0.93±0.05 a
HSD	0.15		0.09	
	Grain potassium (%)		Straw potassium (%)	
IN	0.14±0.01 c	0.20±0.02 a	0.27±0.01 d	0.37±0.02 ab
IST	0.17±0.01 bc	0.19±0.01 ab	0.34±0.01 bc	0.44±0.03 a
ISF	0.15±0.02 bc	0.18±0.01 ab	0.29±0.02 cd	0.41±0.01 a
HSD	0.04		0.07	
	Grain phosphorus (%)		Straw phosphorus (%)	
IN	0.14±0.01 b	0.21±0.02 a	0.15±0.01 c	0.20±0.02 bc
IST	0.15±0.01 ab	0.19±0.01 ab	0.17±0.03 c	0.26±0.01 a
ISF	0.16±0.02 ab	0.21±0.02 a	0.17±0.01 bc	0.21±0.02 ab
HSD	0.07		0.05	

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.01$

IN, Irrigation normal; IST, Irrigation skipped at tillering; ISF, Irrigation skipped at flowering; HSD, Tukey's Honestly Significant Difference

Data are average of four replicates ± Standard error (SE)

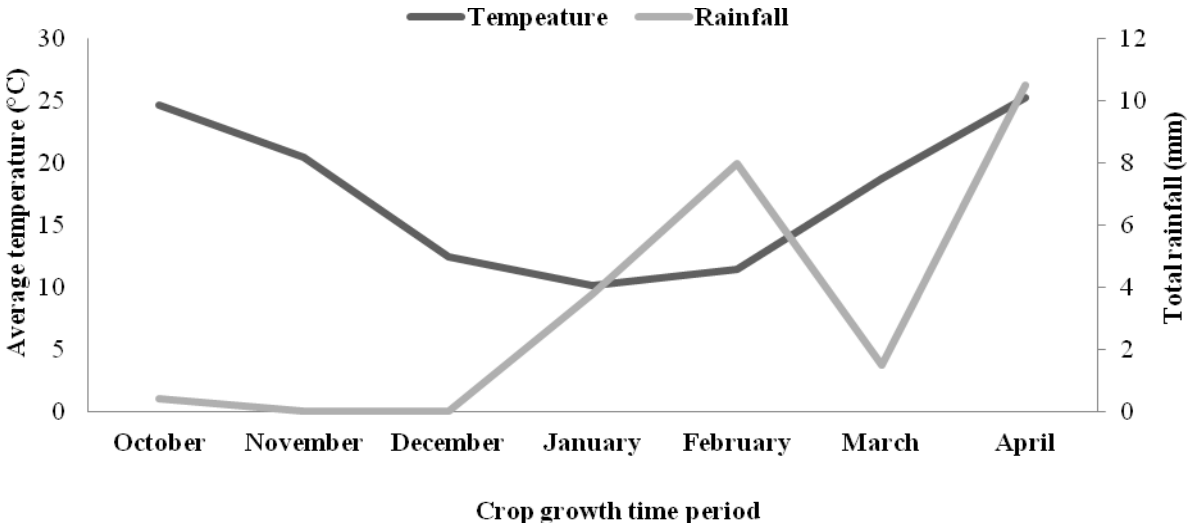
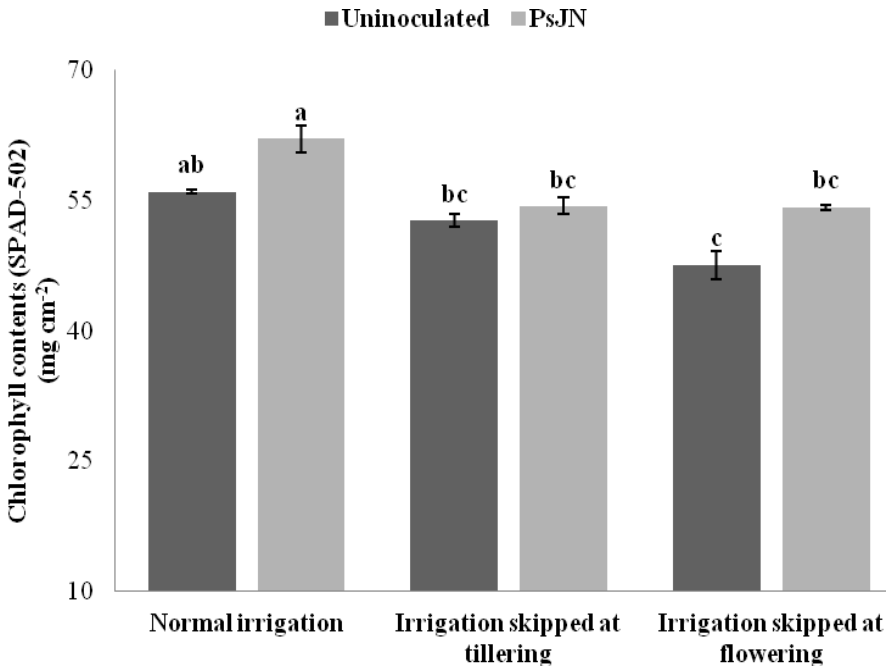
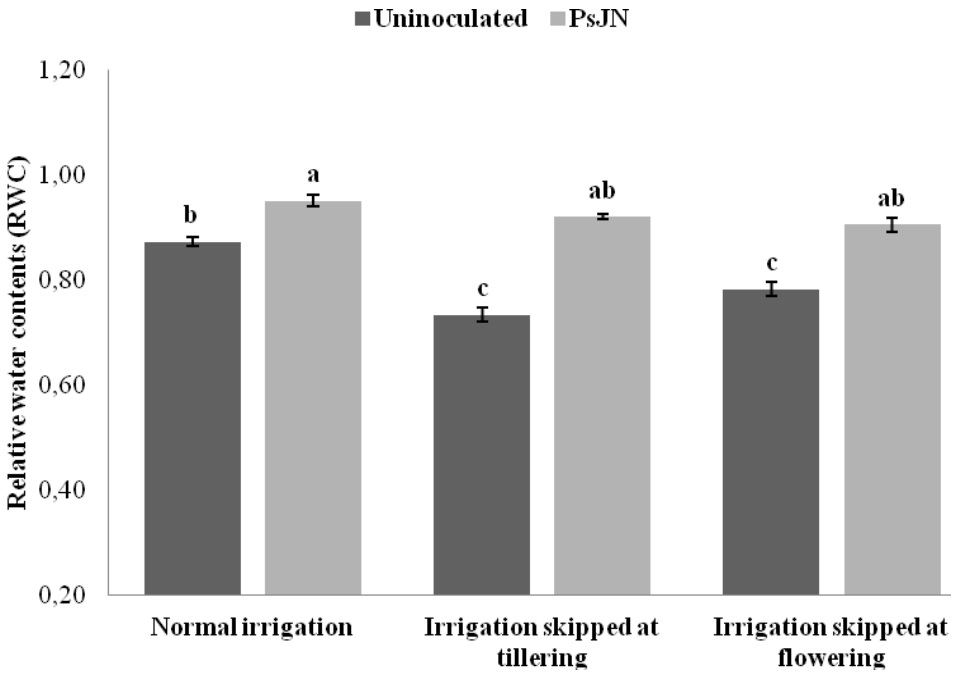


Figure 1 Weather conditions data during the crop growth period obtained from "Meteorological department" UAF, Pakistan



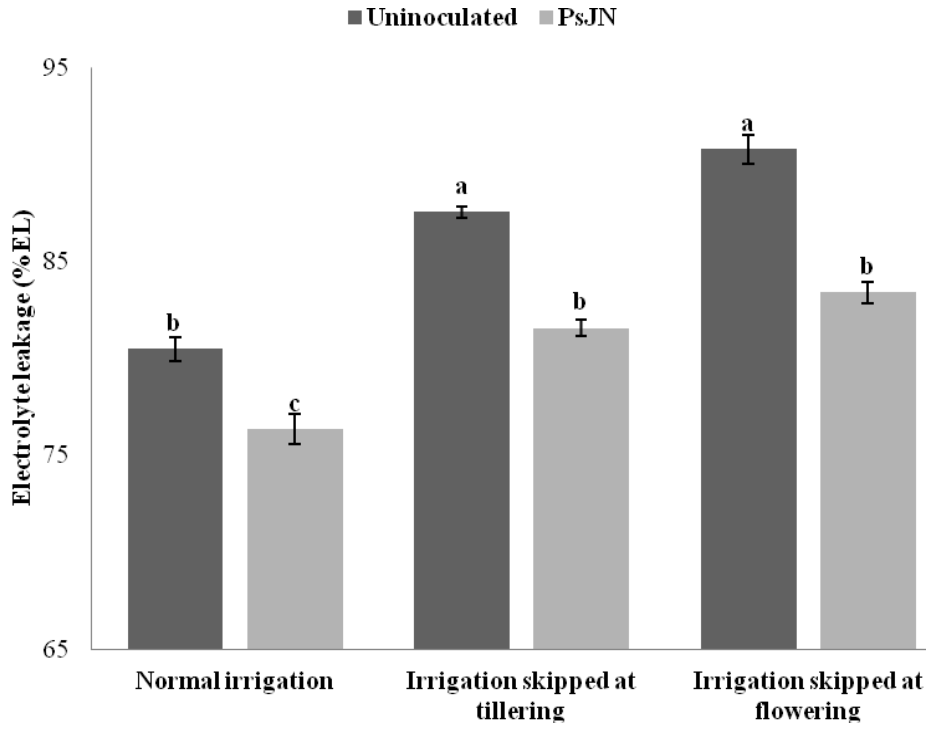
Note. Bars sharing similar letters are statistically similar at $p \leq 0.01$.

Figure 2 Chlorophyll contents of wheat flag leaf with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition



Note. Bars sharing similar letters are statistically similar at $p \leq 0.01$.

Figure 3 Relative water contents of wheat flag leaf with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition



Note. Bars sharing similar letters are statistically similar at $p \leq 0.01$.

Figure 4 Electrolyte leakages of wheat flag leaf with or without PsJN inoculation when irrigations were not skipped (IN) or skipped at tillering (IST) or flowering (ISF) growth stages in field condition

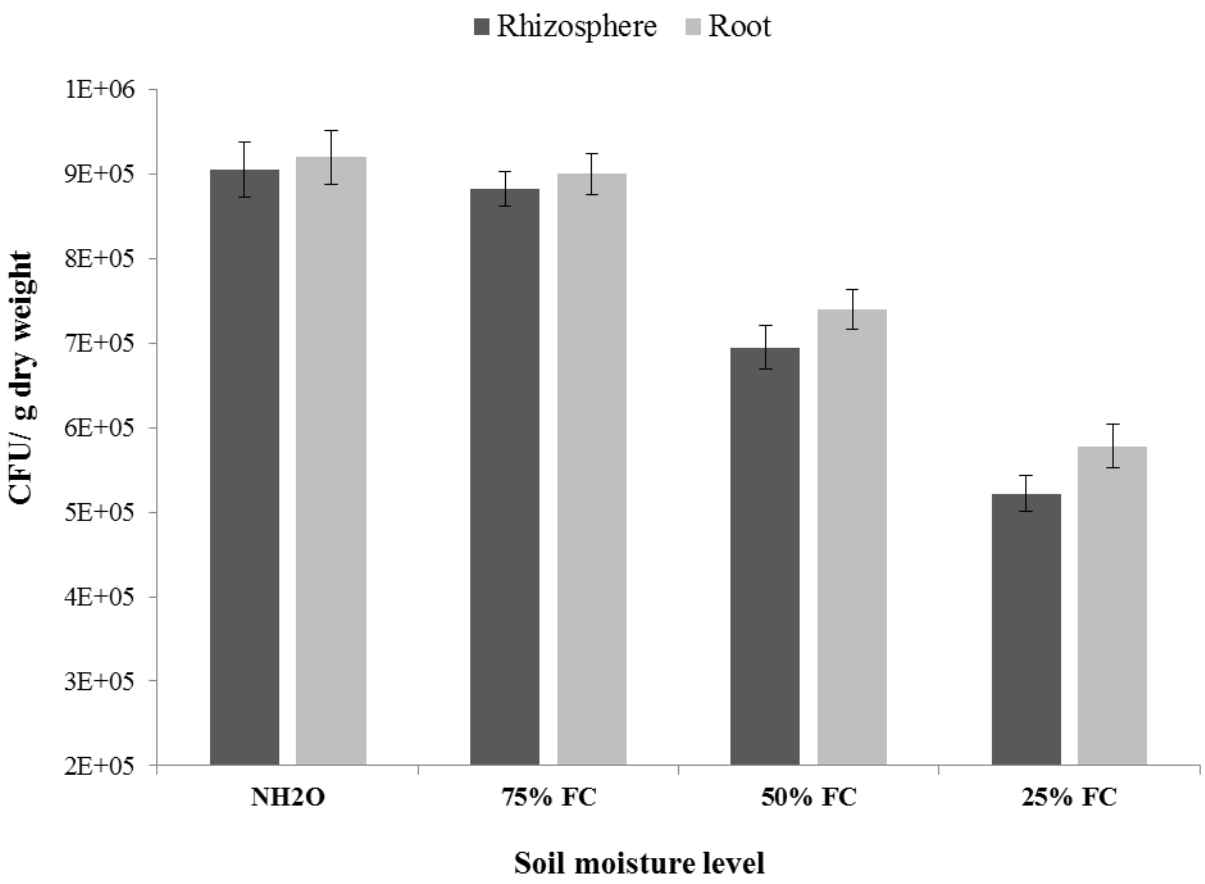


Figure 5 Survival efficiency of PsJN in the rhizosphere and root interior of wheat seedlings (NH_2O, normal watering; FC, field capacity), data are average of three replicates ± standard deviation (SD)

Chapter 5

Endophytic colonization of *Burkholderia phytofirman* strain PsJN induce drought-stress tolerance in maize

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Running Head: PsJN colonization induced drought tolerance

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Abstract

Drought stress is one of the major constraints hampering agricultural production owing to its impact on plant water status and photosynthetic pigments. The effect of inoculation of a plant-growth promoting bacterium *Burkholderia phytofirmans* strains PsJN on growth, water status and photosynthetic activity of two maize cvs under drought stress conditions was investigated. Drought stress induced by withholding irrigation had drastic effects on growth and photosynthesis of maize seedlings. However, seed bacterization of maize with *B. phytofirmans* PsJN improved plant (root/shoot) biomass, leaf chlorophyll contents and relative water status upto 62, 61, 21 and 29% over control under drought stress conditions. Similarly, PsJN inoculation significantly increased photochemical efficiency of PSII and photosynthetic activity upto 9 and 68% of cv Mazurka compared to control under stressed conditions. Contrary to this, inoculation decreased electrolyte leakage compared to uninoculated seedlings under drought stress. The inoculant strain efficiently colonized maize seedling and recovered from root, shoot and leaves of irrigated and stressed plants. In conclusion, our study clearly demonstrates that bacterial inoculants could be used to minimize the negative effects of drought stress on growth and photosynthesis of maize.

Key words: Endophytic bacteria, plant growth promotion, photosynthesis, drought tolerance, maize

1. Introduction

Drought is a potential major constraint to maize production in all areas where it is grown. Global warming, deforestation, and urbanization will all increase the severity and frequency of drought in the future, leading to a possible decrease in global food production at the same time that a steadily increasing human population which could hit 9 billion by 2050 demands an increase in food supplies. In spite of limited arable land coupled with the rising consumer's demand of high quality food, free from chemicals, food production is one of the major global challenges. Therefore, it has become obligatory to investigate the ways to mitigate the adverse effect of drought stress and increase crop productivity within a finite natural resource basis.

During the past couple of decades, plant growth-promoting rhizobacteria (PGPR) have received worldwide importance and acceptance in agricultural practice and are promising alternatives to agrochemicals (fertilizers and pesticides). In the late 1970s Kloepper and Schroth introduced the term "plant growth promoting rhizobacteria (PGPR)" to describe bacteria that colonize plant roots after seed inoculation and that stimulate plant growth (Kloepper and Schroth, 1978). The PGPR are reported to influence the growth, yield, and nutrient uptake by an array of mechanisms. Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth (Yasmin et al., 2007).

Endophytes are per definition microorganisms – bacteria or fungi – that colonize living plant tissue without being pathogenic to the plant. Endophytic bacteria may in

future be even more important than rhizosphere bacteria in promoting plant growth because they escape competition with rhizosphere microorganisms and achieve more intimate contact with the plant tissues. In addition, inherent nature of certain endophytes to potentially colonize plants in a systematic manner provides a novel approach as a delivery system to plant for various beneficial traits (Döbereiner, 1992; Kobayashi and Palumbo, 2000; Fuentes-Ramirez and Caballero-Mellado, 2005).

Burkholderia phytofirmans strain PsJN is one of the best studied bacterial endophytes. Originally isolated from surface-sterilized *Glomus vesiculiferum*-infected onion roots (Frommel et al., 1991), strain PsJN has been shown to colonize a wide variety of plants [(e.g. potato, tomato, peat moss and grapevines (Compant et al., 2008)] and that it stimulates plant growth and vitality in many of its host plants.

Therefore, the aim of this study was to investigate the effect of *B. phytofirmans* strain PsJN inoculation on growth, relative water status, chlorophyll fluorescence, and photosynthetic pigments of maize (*Zea mays*) under drought stress conditions.

2. Materials and Methods

The effect of *Burkholderia phytofirmans* strain PsJN inoculation on the physiology and growth parameters of maize cultivars Mazurka and Kaleo was tested under greenhouse conditions. We used a genetic variant of the bacteria strain *B. phytofirmans* PsJN::gusA (Compant et al., 2005) that carries a beta-glucuronidase reporter gene (*gus*A) that allows specific visualization of bacterial cells upon color formation. Maize seeds were kindly provided by DOW AgroSciences Vertriebsges.mbH Neusiedl am See, Austria.

2.1. Green house experiment and growth conditions

Plants were grown in pots filled with local field soil. The soil was ground, passed through a 2 mm sieve and analyzed for various physico-chemical characteristics, i.e., sand, 32%; silt, 38% ; c lay, 30% ; p H, 7.28; t otal c arbon, 2.4%; total ni trogen, 0 .23%; a vailable phosphorus, 40 mg/100 g; extractable potassium, 19 mg/100 g soil. Each pot was filled with 15 kg soil receiving nutrient inputs of NPK at 160, 100, 60 kg ha^{-1}, respectively.

For s eed i noculation, s urface-sterilized seed s w ere t reated w ith bacterial suspension of s train P sJN (10^8-10^9 CFU m L^{-1}) unde r l ab c onditions. I n t he cas e o f uninoculated c ontrol, seeds w ere treated w ith s terilized LB br oth. I noculums were prepared by gr owing *Burkholderia phytofirmans* strain P sJN in 2 50 mL E rlenmeyer flasks containing LB media amended with spectinomycin [100µg mL^{-1}]. The broth was inoculated with a single cell and incubated at 28 ± 2°C for 72 h i n a shaking incubator (VWR International, GmbH, Austria) at 180 r min^{-1}.

Five maize seeds either inoculated with strain PsJN or broth only were sown in each pot at equal distance. The pots of each treatment were arranged randomly, with three repeats at a mbient l ight an d t emperature i n a g reenhouse. T ap w ater w as u sed f or irrigation. After germination, uniform plant population was maintained by thinning upto one plants pot^{-1}. The drought stress was applied by w ithdrawing water after 60 days of planting (flowering stage). The irrigation was first reduced and then completely stopped for a period of one week.

2.2. Plant growth measurements

Data of plant growth parameters including plant height, shoot biomass and root biomass were recorded. Plant height was measured before harvesting. Shoot and root biomass was recorded by uprooting the plant and drying at 72^{o}C after harvesting.

2.3. Plant ecophysiology measurements

The plant physiological parameters were recorded at midday (between 11:00 and 13:30) of fully expanded leaves near the top of both irrigated and drought-stressed plants. Photosynthetic pigments of 3rd leaf from top were measured using a portable gas exchange system (Li-Cor 6400, Lincoln, NE, USA). During measurements, the leaves were illuminated with lamp light (1500 µ mol m^{-2} s^{-1} and maintained at a relative air humidity of 20 ± 2% and a leaf temperature 25 ± 2 ^{o}C. Chlorophyll fluorescence was measured of irrigated and drought-stressed plants using a portable PEA handy (Hansatech Instruments Ltd. England). Leaves were dark adapted for 30 min before measurement and the maximum photochemical efficiency of PSII (Fv/Fm) was calculated from chlorophyll fluorescence data.

The leaf chlorophyll content was determined by using Chlorophyll Meter (SPAD 502 Plus). Each leaf sample was measured at least six different areas of each treatment with three replicates.

2.4. Electrolyte leakage and relative water content

Electrolyte leakage (EL) was measured following the protocol Jambunathan (2010), and relative water contents were determined following the equations described by Mayak et al. (2004).

EL (%) = EC1 / EC2 x 100

RWC (%) = (Fresh weight - Dry weight) / Fully turgid weight - Dry weight x 100

2.5. Persistence of B. phytofirmans PsJN in the rhizosphere, root and shoot interior

For the isolation of bacteria, 5 g rhizosphere soil and 3 g of surface-sterilized root/shoot materials was homogenized in 15 mL of 0.85% (w/v) NaCl solution. The homogenized material was shaken with pulsifier for 45 sec at room temperature. After the settlement of materials, serial dilutions up to 10^{-4} were plated onto selective LB medium containing spectinomycin (100 µg mL^{-1}), 5 -bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 µg mL^{-1}), and isopropyl-β-D-galactopyranoside (IPTG) (100 µg mL^{-1}). The plates were incubated at 28 ± 2 ^{o}C for 48 hours and then transferred to 4 ^{o}C for three days. Blue colonies were counted on each plate. Thirty blue colonies of each treatment were randomly picked and the identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S r RNA intergenic spacer region (IGS) (Afzal et al. 2012).

2.6. Microscopy of endophytic colonization in plant tissues

Fresh plant organs (roots and leaves) were removed from inoculated and non-inoculated plant. Samples were then prepared for microscopy analysis as described by Compant et al. (2005), with some modifications. Briefly, plant samples were dipped in staining solutions containing IPTG (100 µg mL^{-1}) at 37 ^{o}C for 48 h. The samples were de-stained with 70% (v/v) ethanol solution to stop the reaction. Leaves and stem sections of plant

were cut with a microtome (LeicaVT1000S; Leica, Nussloch, Germany), collected on glass slides, examined with an inverted Microscope (Axiovert 200 Carl Zeiss, Germany), and photographed.

2.7. Statistical analysis

Data of plant growth parameters and bacterial densities were subjected to analysis of variance (ANOVA) using SPSS software package version 19 (IBM SPSS Statistics 19, USA). The treatment means were compared by Duncan's multiple range test at 5% probability. The means and standard errors were calculated using Microsoft Excel 2010.

3. Results

Inoculation of maize seeds with *B. phytofirmans* PsJN increased plant height of both cultivars ranging from 14-24% as compared to control under irrigated and drought stress conditions (Table 1). Maximum increase was observed by inoculation (24%) in maize cv. Mazurka as compared to the respective control under drought stress.

Inoculation increased chlorophyll content significantly as compared to control. Up to 21% increase was observed by PsJN inoculation in cv. Mazurka under stress conditions. The poorest response to bacterial inoculation was observed in cv. Kaleo under normal culture conditions (Table 1).

Likewise, inoculation increased the chemical efficiency of PSII as compared to control. Significant increase in PSII efficiency was observed in cv. Kaleo under drought stress (Table 1). Inoculation with strain PsJN increased photosynthesis activity of plants

with the increase ranging from 18-68% as compared to control under normal and stressed conditions (Table 1). The maximum increase in photosynthesis (38%) was recorded in cv. Mazurka under stressed conditions. The weakest response (18%) to bacterial inoculation was observed in cv. Kaleo under normal culture conditions (Table 1).

The data in Table 2 revealed that inoculation with strains PsJN increased relative water content (RWC), electrolyte leakage (EL) and plant biomass as compared to the controls. Maximum RWC was observed in cv. Kaleo under normal conditions. However, inoculation with strain PsJN resulted in the highest increase in RWC (29%) as compared to control in cv. Mazurka under stressed conditions. The lowest increase in RWC was recorded in cv. Kaleo under normal conditions.

Bacterial inoculation decreased EL in both cultivars (Table 2). The strongest decrease was observed in cv. Kaleo under normal and stressed conditions.

Inoculation with strain PsJN significantly increased plant biomass (23-61%) of both maize cultivars over respective control under normal and stressed conditions. The strongest response to inoculation was observed in cv. Mazurka i.e. 43 and 61% increased biomass, respectively as compared to control under both conditions. The poorest response was observed in cv. Kaleo i.e. 23% increase in shoot biomass over control under normal conditions.

Similarly, PsJN inoculation increased root biomass of both cultivars i.e. 38-62% as compared to control under normal and stressed conditions (Table 2). Cv. Mazurka grown under drought stress conditions showed strongest response (62%) to the bacterial inoculation, whereas plants of cv. Kaleo grown under normal water regime showed the

lowest increase in root biomass as compared to the as compared to the corresponding control.

B. phytofirmans PsJN efficiently colonized rhizosphere, root, shoot and leaf interior of both maize cultivars, Mazurka and Kaleo (Fig. 1, 2). However, an about ten times higher viable cell number (CFU/g dry weight) was recovered for the rhizosphere and roots as compared to shoot tissue (Fig. 1). In general, we recorded a higher viable cell number in plants of cv. Mazurka than in cv. Kaleo. In stressed plants of both cultivars the number of viable PsJN cells was remarkably lower than in plants treated with a normal water regime.

4. Discussion

In changing climate, plants are constantly exposed to abiotic stress, such as drought, which is one of the most serious problems associated with plant growth and development affecting agricultural demands. The management of drought-affected soils is essential to meet the ever increasing food demands. Inoculation with plant growth promoting bacteria (PGPB) has been found effective under drought stress environment (Chanway and Holl, 1994) to increase productivity. Growth promotion by the PGPB may be attributed to multifarious mechanisms such as production of PGP hormones and other PGP activities (Glick, 1995). In the present investigation, plant growth-promoting endophytic bacterium was evaluated on the growth and photosynthesis of maize cvs under drought stress conditions.

The inoculation of maize plants with the bacterium *B. phytofirmans* PsJN stimulated plant biomass production, physiology and vitality in both varieties. From numerous reports it is evident that *B. phytofirmans* PsJN is a highly efficient plant beneficial bacterium promoting growth in a wide variety of plants (Compant et al., 2008), however, there is evidence for plant genotype specific differences in the intensity of the effects (Da et al., 2012; Trognitz et al., 2008). Interestingly, this can be seen also from the present data. Cultivar Mazurka responded stronger to inoculation with strain PsJN than cultivar Kaleo in both drought stress and normal water conditions, and this was more pronounced in stressed plants. Nowak and colleagues assumed that plant genotype specific differences in the plant stimulating effects is due to differences in PsJN titers in highly and poorly responsive varieties, which reach much higher levels in highly-responsive genotypes (Nowak et al., 2007). The data of not drought stressed plants indicate a correlation between stimulation and PsJN titers as we recorded a higher number of viable PsJN cells in Mazurka than in Kaleo. However, in drought stressed plants strain PsJN was more suppressed and the viable cell number dropped more drastically than in Kaleo, while in the latter cultivar the viability of strain PsJN seemed hardly effected. The number of viable PsJN cells in stressed plants of cv. Mazurka was far below that of cv. Kaleo but at the same time the relative increase in plant growth and vitality under drought was much higher.

By comparing the performance of the two maize cultivars under drought stress we saw that cv. Kaleo was less negatively affected than cv. Mazurka. Overall biomass production, photosynthesis and water content were higher and electrolyte leakage lower in plants of cv. Kaleo, indicating that cv. Kaleo was more resistant towards drought stress

in our experiment as compared to cv. Mazurka. In general, inoculation with *B. phytofirmans* PsJN significantly minimized the negative effects of drought on maize biomass production and physiology. Similar effects were observed when *B. phytofirmans* PsJN was tested for its ability to enhance chilling resistance in *Vitis vinifera* L. cv. Chardonnay (Ait Barka et al., 2006; Fernandez et al., 2012). The higher tolerance of PsJN colonized grapevine plantlets to chilling were related to alterations in photosynthesis and sugar metabolism. Recently, Theocharis and colleagues (2012) showed that cold stress-related gene transcripts and metabolites increased earlier and faster, and reached higher levels in PsJN-colonized Chardonnay grapevine plantlets than in control plants.

In conclusion, *B. phytofirmans* PsJN efficiently colonized maize plants and stimulated plant growth in both cultivars tested. Our study clearly demonstrates that bacterial inoculants could be used to minimize the negative effects of drought stress on growth and photosynthesis of maize.

Acknowledgment

We thank Anton Grahsl for help with the greenhouse experiment and Mr. Amjad Abbas from the Institute of Plant Pathology at the Department of Crop Sciences, University of Natural Resources and Applied Sciences, Vienna for their help and guidance in microscopy.

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Table 1. Effect of *Burkholderia phytofirmans* strain PsJN on plant height, chlorophyll content, chlorophyll fluorescence and photosynthesis of maize under drought stressed conditions

	Plant height (cm)				**Chlorophyll content (SPAD value)**			
	Mazurka		Kaleo		Mazurka		Kaleo	
	N H_2O[†]	D H_2O[‡]	N H_2O	D H_2O	N H_2O	D H_2O	N H_2O	D H_2O
Control	182.53±3.50c	167.67±2.52d	179.98±3.82c	164.33±2.08d	39.03±1.53de	34.93±1.55f	40.60±2.31cd	36.90±1.31ef
PsJN	208.67±3.05a	207.33±3.52a	205.33±2.30ab	202.33±2.07b	45.80±1.51a	42.10±0.85bc	44.47±0.93ab	44.13±0.68ab
	Chlorophyll fluorescence (FV/Fm)				**Photosynthesis (µmoles CO_2 cm^{-2} s^{-1})**			
Control	0.780±0.01b	0.739±0.01c	0.796±0.02ab	0.754±0.01c	18.81±2.45c	10.73±1.12d	22.13±2.65b	17.44±2.26c
PsJN	0.823±0.01a	0.802±0.02ab	0.817±0.01a	0.811±0.01 a	26.11±1.48a	18.03±1.56c	25.99±1.56a	23.54±1.19ab

*Means sharing similar letter(s) in a column for each parameter do not differ significantly at P = 0.05

Data are average of three replicates ± Standard Deviation (SD)

[†]Normal irrigation

[‡]Reduced water application

Table 2. Effect of *Burkholderia phytofirmans* strain PsJN on relative water content, electrolyte leakage, shoot dry matter and root dry matter on maize under drought stressed conditions

	Relative water content (%)				**Electrolyte leakage (%)**			
	Mazurka		Kaleo		Mazurka		Kaleo	
	N H_2O[†]	D H_2O[‡]	N H_2O	D H_2O	N H_2O	D H_2O	N H_2O	D H_2O
Control	52.17±2.67d	44.50±1.62e	64.96±2.06b	58.29±1.86c	8.06±1.74bc	12.06±1.33a	7.76±1.65bc	10.15±1.14ab
PsJN	58.31±1.12c	57.31±2.59c	70.88±1.45a	67.88±2.32ab	6.29±1.21d	7.95±0.55bc	5.79±0.89d	7.02±1.47cd
	Shoot dry matter (g)				**Root dry matter (g)**			
Control	21.13±2.01de	18.40±1.77g	57.63±1.82de	22.07±1.50f	2.48±0.09b	1.53±0.11c	2.46±0.23b	1.63±0.07c
PsJN	37.38±1.20a	29.57±1.66cd	33.98±1.47b	32.34±1.79bc	3.50±0.18a	2.47±0.13b	3.39±0.08a	2.56±0.08b

*Means sharing similar letter(s) in a column for each parameter do not differ significantly at P = 0.05

Data are average of three replicates ± Standard Deviation (SD)

[†]Normal irrigation

[‡]Reduced water application

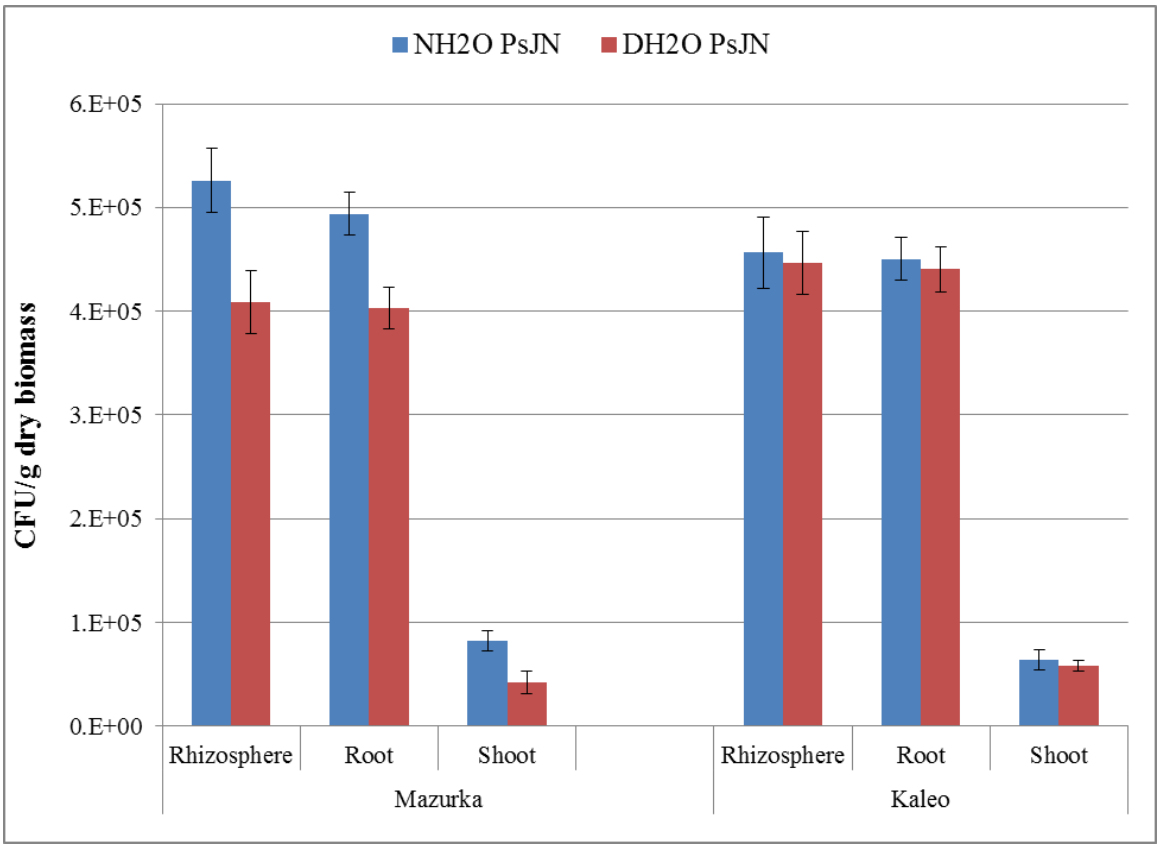

Figure 1. Persistance of *Burkholderia phytofirmans* strain PsJN in the rhizospher, root interior and shoot interior of two maize cultivars under normal and water stressed conditions

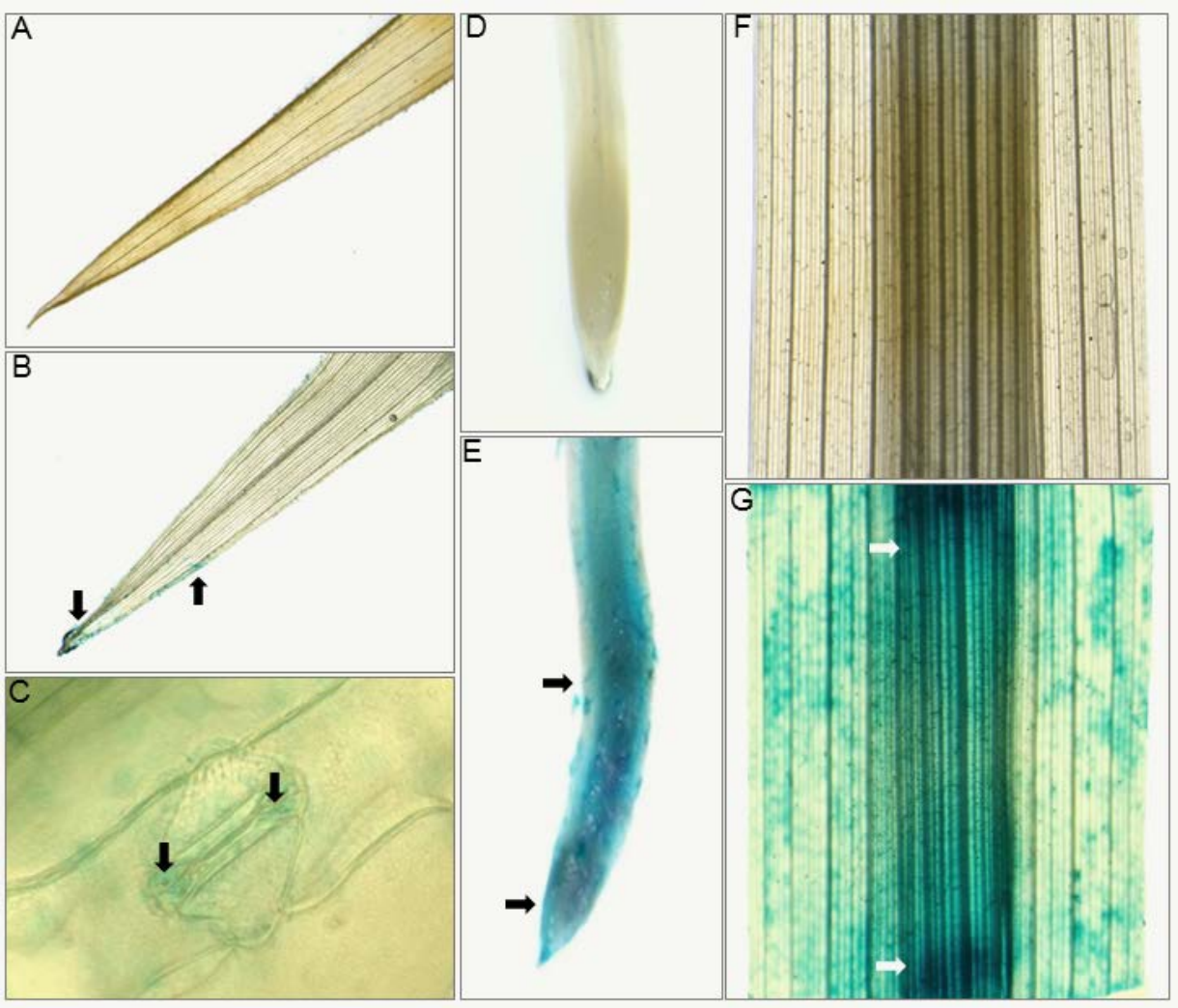

Figure 2. Photographs of the fourth leaf internal tissue of PsJN inoculated *Zea mays* L. plants. (A, B, F and G) Photographed (binocular microscope; Olympus, Japan) of the fourth leaf (tip and middle) and root of uninoculated control (A, D and F) or inoculated with PsJN: gusA10 (B, E and G), showing the blue color in veins due to gusA-marked cells (arrowheads). Inverse microscope (Axiovert 200M; Zeiss, Hallerbergmos, Germany) image of the leaf stomata of PsJN gusA inoculated plant, showing bacteria in the stomata and guard cell.

Chapter 6

L-Tryptophan dependent biosynthesis of indole-3-acetic acid (IAA) improves plant growth promotion and colonization of maize by *Burkholderia phytofirmans* PsJN

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Running Title: PsJN and L-Tryptophan interaction to improve maize growth

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Abstract

Burkholderia phytofirmans PsJN is a well-known plant growth-promoting bacterium, which establishes rhizospheric and endophytic colonization in different plants. PsJN inoculation promotes growth of different horticultural crops. L-Tryptophan (L-TRP) application may further improve its effectiveness due to substrate (L-TRP)-dependent inoculum (PsJN)-derived auxins in the rhizosphere. In the present study, substrate (L-TRP) dependent response of PsJN inoculation to maize growth and auxins biosynthesis was evaluated under pot conditions. *In vitro* auxin biosynthesis by PsJN was determined in the absence and presence of L-TRP, a physiological precursor of auxins. Surface-disinfected seeds were treated with peat-based inoculum and L-TRP solutions (10^{-4} and 10^{-5} M). Results revealed that L-TRP and PsJN inoculation when applied alone significantly increased the growth parameters of maize compared to untreated control. However, PsJN inoculation supplemented with L-TRP (10^{-5} M) gave the most promising results and significantly increased plant height, photosynthesis, chlorophyll content, root biomass and shoot biomass up to 18, 16, 45, 62 and 55%, respectively, compared to the un-inoculated control. Similarly, higher values of N, P and IAA content were observed with precursor (L-TRP) – inoculum (PsJN) interaction. The inoculant strain efficiently colonized maize seedlings and recovered from rhizosphere, root and shoot of plants. The results imply that substrate (L-TRP) - derived IAA biosynthesis in the rhizosphere by PsJN inoculation could be a useful approach for improving the growth, photosynthesis and nutrient content of maize plant.

Keywords: *B. phytofirmans* PsJN, L-tryptophan, precursor-inoculum interaction, endophytic colonization, maize

Introduction

Beneficial plant-microbe interactions in the rhizosphere are primary determinants of plant health and soil fertility (Jeffries et al. 2003). The rhizosphere represents a highly dynamic space for interactions between plant roots and beneficial soil microorganisms (Bais et al. 2006). In the rhizosphere, molecular communication between microorganisms and their plant hosts plays a fundamental role in pathogenesis and in the establishment of beneficial interactions (Mark et al. 2005). Endophytes colonizing plants internally without harming their host may have pronounced positive effects on plant growth and health (Hardoim et al. 2008; Mitter et al. 2013a). Over the years, the utilization of plant growth-promoting bacteria (PGPB), usually either rhizosphere bacteria or endophytes, as bio-fertilizers and/or bio-pesticides has received increasing attention and is becoming popular for agricultural production. These microorganisms may not only ensure the availability of essential nutrients to plants but also enhance nutrient use efficiency (Khalid et al. 2009).

Plant growth regulators (PGRs) play a vital role in controlling plant growth and development. Auxins are an important class of hormones controlling many aspects of root development and architecture, such as primary root growth, lateral root formation, and root hair development (Fukaki and Tasaka 2009). Despite the fact that plants are capable to synthesize auxins, they respond to exogenously applied auxins during certain growth phases (Frankenberger and Arshad 1995; Zahir et al. 2010a).

Plant growth-promoting bacteria (PGPB) produce beneficial effects on plant growth through several mechanisms such as nitrogen fixation, improved nutrient uptake, phytohormone production, and induction of systemic resistance (ISR) (Compant et al.

2008; Mitter et al. 2013a). It is likely that plant growth-promoting effects exerted by plant-beneficial bacteria are due to the bacterial production of plant hormones such as indole-3-acetic acid (IAA), cytokinins and gibberellins (Bottini et al. 2004; Lugtenberg and Kamilova 2009). About 80% of bacteria from rhizosphere are able to produce indole acetic acid (IAA), indicating a possible role in interaction with the plant (Patten and Glick 1996; Spaepen et al. 2007). L-Tryptophan (L-TRP) is considered an efficient physiological precursor of auxins in higher plants as well as for microbial biosynthesis of auxins (Davies 2004; Khalid et al. 2006). *In vitro* studies have demonstrated that some microorganisms can produce small amounts of auxins in the absence of L-TRP, however, in its presence, the microbiota produce much greater quantities of auxins (Khalid et al. 2004a; Zahir et al. 2010a). Exogenous application of L-TRP to soils has also been shown to stimulate synthesis of auxins, influencing plant growth and development positively (Khalid et al. 2004b; Zahir et al. 2010a, b).

Burkholderia phytofirmans strain PsJN, an efficient plant growth-promoting bacterium was isolated from onion roots and reported for growth promotion of horticultural crops e.g. potatoes, tomato and grapevines (Frommel et al. 1991; Nowak et al. 1995; Ait Barka et al. 2000). In addition *B. phytofirmans* PsJN colonization enhanced protection against *Verticillium* sp. in tomato (Sharma and Nowak 1998), *Botrytis cinerea* and *Pseudomonas syringae* in grapevine (Ait Barka et al. 2002; Bordiec et al. 2011). So far, molecular mechanisms responsible for plant growth-promotion in PsJN such as due to the reduction of the plant ethylene hormone levels by 1-aminocyclopropane-1-carboxylic acid (ACC) has been described (Sun et al. 2009). Very recently, auxin (IAA) production and quorum sensing have been described to be putatively involved in plant

growth-promotion and cell-to-cell communication in efficient colonization of *Arabidopsis thaliana* by strain PsJN (Zúñiga et al. 2013). The objective of this study was to study, whether the amendment of L-TRP and the associated IAA synthesis by strain PsJN affect plant growth and the strain's colonization of maize plants.

Materials and methods

The effect of *Burkholderia phytofirmans* strain PsJN inoculation on the physiology and growth parameters of maize was tested under wirehouse conditions. We used a genetic variant of the strain *B. phytofirmans* PsJN::*gusA* (Compant et al. 2005) that carries a beta-glucuronidase reporter gene (*gusA*) that allows specific visualization of bacterial cells upon color formation. Maize (cv. Neelam) seeds were provided by the Maize Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan.

Measurement of auxin production by PsJN

Auxin production by *B. phytofirmans* PsJN, both in the presence and absence of L-TRP (Sigma, St. Louis MO), was determined colorimetrically in terms of IAA equivalents produced (Sarwar et al. 1992). Two days old bacterial cultures grown (28 ± 2°C at 180 rpm) in LB broth supplemented with sterilized 1% L-TRP solution were harvested by centrifugation. Control cultures, which did not receive L-TRP were included. Three milliliters supernatants were mixed with 2 mL Salkowski's reagent (12 g L^{-1} $FeCl_3$ in 429 mL L^{-1} H_2SO_4). Mixture were incubated at room temperature for 30 min for colour development and absorbance was measured at 535 nm using a spectrophotometer (Nicolet Evolution 300 LC, Thermo Electron Corp., Madison, WI). Auxin concentration

produced by bacterial isolates was determined using standard curve for IAA prepared from serial dilutions of 10-100 µg mL^{-1}.

Bacterial inoculum and growth conditions

Inoculum of the *gusA* marked strain PsJN was prepared in 250 mL Erlenmeyer flasks in LB broth containing spectinomycin antibiotic (100 µg mL^{-1}). The broth was inoculated with strain PsJN::*gusA* and incubated at 28 ± 2°C for 48 h in the orbital shaking incubator (VWR International, GmbH, Austria) at 180 r min^{-1}. The optical density of the broth was adjusted at 0.5 measured at λ 600 nm using spectrophotometer (Gene Quant Pro, Gemini BV, The Netherlands) to obtain a uniform population of bacteria (10^8 - 10^9 colony-forming units (CFU) mL^{-1}) in the broth at the time of inoculation.

Seed bacterization and phytohormone treatment

The carrier material was collected from the Changa Manga forest soil, Pakistan, sterilized at 20 psi pressure and 121°C temperature for half an hour and inoculated with broth culture. Peat based inoculum was incubated at 28 ± 2°C by adding 10% sugar solution to increase the microbial populations. For inoculation, the desired suspension of inoculum (10^8 - 10^9 CFU mL^{-1}; 250 mL kg^{-1} peat) was mixed with sterilized peat and incubated for 24 h at 28 ± 2ºC before being used for seed coating (seed to peat ratio 1.25:1 w/w). Maize seed dressing was prepared with the inoculated peat mixed with 10% sterilized sugar (sucrose) solution in 10:1 ratio. In the case of non-inoculated control, seeds were coated with the sterilized peat treated with sterilized broth and 10% sterilized sugar solution. For the phytohormone treatment, maize seeds were treated with L-TRP solutions (10^{-4} and 10^{-}

^{5} M) mixed slurry. For the combined treatment, L-TRP solution was mixed with bacterial culture at the time of inoculation.

Wirehouse experiment

A pot experiment was conducted in the wirehouse, Soil Bacteriology Section, Ayub Agricultural Research Institute, Faisalabad, Pakistan, to observe the efficacy of PsJN inoculation along with two levels of L-TRP (10^{-4} and 10^{-5} M) for improving growth and photosynthesis of maize. Soil used for the experiment was collected from the field, air dried, thoroughly mixed, passed through 2 mm sieve and analyzed for various physical and chemical characteristics. The soil was sandy clay loam having pH, 7.86; EC, 1.39 dS m^{-1}; organic matter, 0.76%; total nitrogen, 0.035%; available phosphorus, 7.76 mg kg^{-1} and extractable potassium, 119 mg kg^{-1}.

Maize seeds were surface-sterilized by dipping in 70% ethanol for 2 min and treated with 5% NaClO for 5 min followed by washing 3 times with sterile distilled water (1 min each time). The efficacy of surface sterilization was checked by plating seeds and aliquots of the final rinse ont o LB plates. The experiment contained the following treatments: 1) Control, 2) PsJN inoculation, 3) L-TRP solution (10^{-4} M), 4) L-TRP solution (10^{-5} M), 5) PsJN and L-TRP (10^{-4} M), 6) PsJN and L-TRP (10^{-5} M). Surface-disinfected maize seeds were coated with PsJN / L-TRP treated slurry (described above). For the control, seeds were coated with sterilized LB broth treated slurry. Three seeds were sown in each pot containing 16 kg of soil and thinned to one plant after one week of germination. Pots were arranged in the wirehouse using a completely randomized design with three replications of each treatment. Recommended doses of N, P, and K fertilizers (160-100-60 kg ha^{-1}) were applied to each pot and equal amount of tap water was applied

to the pots whenever needed. The crop was harvested after 45 days and various physiological and agronomic parameters were recorded.

Measurement of Physiology and growth parameters

Physiological measurements

Plant physiological parameters were recorded at midday (between 10:00 and 14:00) of both treated and untreated plants. Portable infrared gas analyzer [IRGA (CI-340) Germany] was used (at 1200-1400 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density) to measure transpiration rate (E), photosynthetic rate (A) and photosynthetically active radiation (PAR). Fully expanded flag leaves were selected for measurements. Leaves were crushed in acetone for determination of chlorophyll content (a and b) and absorbance at λ 645 nm and 663 nm was noted on spectrophotometer after centrifuging at 1000 rpm for 10 minutes (Arnon 1949).

Agronomic trait measurement

Plant agronomic parameters such as plant height, flag leaf length / width, shoot biomass and root biomass were recorded after harvesting the maize plants. Plant biomass (above and below ground) was obtained after drying the whole plants at 65°C for 72 hours.

Post experiment soil analysis

IAA equivalents from the rhizosphere soil of maize were determined after 15 and 30 days after planting (DAP) using Salkowski's reagent as described by Sarwar et al. (1992). Post-harvest soil and plant samples were analyzed for extractable P and soil N (Ryan et al. 2001).

Persistence of *B. phytofirmans* PsJN::gusA in the rhizosphere, root and shoot interior

Rhizosphere soil was obtained by agitating roots and sampling the soil still attached to the roots after harvesting. For rhizosphere colonization, soil slurry was prepared by mixing 5 g rhizosphere soil with 15 mL of 0.85% (w/v) NaCl solution and agitation (180 rpm) for 30 min at 28°C. After sedimentation of soil particles, serial dilutions up to 10^{-5} were plated onto selective LB medium containing spectinomycin (100 µg mL^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGlcA) (100 µg mL^{-1}), and isopropyl-β-D-galactopyranoside (IPTG) (100 µg mL^{-1}). The plates were incubated at 28 ± 2°C for 3-4 days and blue colonies were counted to determine the colonization value.

For root / shoot colonization, 2 g of surface-sterilized roots were homogenized in 10 mL 0.85% NaCl solution by using a sterile mortar and pestle. Similarly, 3 g shoots of each treatment were homogenized in 15 mL 0.85% NaCl solution. The homogenized material was placed in a shaker for 30 min at 28°C. After settling the solid material, serial dilutions up to 10^{-4} were spread on selective LB medium. The plates were incubated at 28 ± 2°C for 48 hours and then transferred to 4°C for three days. Blue colonies were counted on each plate and colonization was calculated.

Microscopy of endophytic colonization in plant tissues

Fresh plant organs (root and leaves) removed from three plantlets inoculated with strain PsJN::*gusA* and uninoculated plants were collected 30 days after inoculation. Samples were prepared for microscopy analysis as described by Compant et al. (2005), with some modifications. Briefly, plant organs (root and leaves) were dipped in staining solutions

containing IPTG at 37°C for 48 h. The samples were de-stained with 70% (v/v) ethanol solution to stop the reaction and the removal of tissues chlorophyll. Root and leaf sections of different treatments were cut, collected on glass slides, examined with a binocular microscope (Olympus, Japan) and an inverted microscope (Axiovert 200 Carl Zeiss, Germany) and photographed.

Statistical analysis

The data of plant growth parameters and colonization were subjected to analyses of variance. The means were compared by Least Significant Difference (LSD) test ($p < 0.05$) to detect statistical significance among treatment (Steel et al. 1997). All of the statistical analyses were conducted using SPSS software version 19 (IBM SPSS Statistics 19, USA). The percent increases in growth parameters as well as colonization were correlated against IAA equivalent using the Excel 2010.

Results

Results revealed that exogenously applied L-TRP and PsJN::*gusA* inoculation significantly increased the physiology and growth of maize when tested in separate treatments. Combined application of PsJN::*gusA* inoculation and L-TRP further increased the growth of maize (Table 1-3).

Agronomic parameters

Data in Table 1 show that PsJN::*gusA* inoculation significantly increased the plant height, leaf length and width over control. PsJN inoculation along with L-TRP further increased

the plant height, leaf length and leaf width by 18, 22 and 23%, respectively, compared with control (Table 1). The combined treatment of PsJN::*gusA* and L-TRP (10^{-5}M) resulted in a maximum increase, i.e. 55%, in shoot dry biomass compared to control. Up to 33 and 31% increase over control in dry biomass was observed by separate application of PsJN::*gusA* and L-TRP (10^{-5} M), respectively. PsJN::*gusA* inoculation increased root biomass up to 44% compared to the control treatment (Table 1). Maximum increase 62% in root biomass was observed by PsJN::*gusA* inoculation and L-TRP (10^{-5}M) treatment compared to control. Minimum increase in root biomass, i.e. 20% compared to control, was observed by L-TRP (10^{-4} M) treatment.

Physiological parameters

Data (Table 1) showed that PsJN::*gusA* inoculation and L-TRP (10^{-5} M) application increased photosynthesis by 5 and 11%, respectively, compared to control. Combined application of PsJN::*gusA* and L-TRP (10^{-5} M) gave maximum increase in photosynthesis (16%) compared to control, whereas application of PsJN::*gusA* alone resulted in a 22% increase compared to control (Table 2). Maximum increase in transpiration up to 34% was observed by PsJN::*gusA* inoculation supplemented with 10^{-5} M L-TRP compared to control. In the case of photosynthetically active radiation (PAR), 15 % increase was observed by combined treatment of PsJN::*gusA* and L-TRP (10^{-5} M) compared to control (Table 2). Separate PsJN::*gusA* inoculation and L-TRP (10^{-5} M) application increased the PAR by 7 and 8% compared to control, respectively. Data in Figure 1 show that combination of PsJN::*gusA* and L-TRP significantly increased the chlorophyll (a, b) content of maize plants than when applied individually. Again, a maximum increase by 17 and 45%, respectively, was observed by the combined application of PsJN::*gusA* and

L-TRP (10^{-5}M). PsJN::*gusA* inoculation resulted in a 15 and 28% increase in chlorophyll a and b compared to the uninoculated control.

IAA biosynthesis and mineral plant and soil analysis

In vitro data of IAA biosynthesis (Table 3) showed that PsJN produced auxin (IAA equivalents) without L-TRP addition, however, IAA equivalents substantially increased when the medium was supplemented with L-TRP. PsJN produced IAA equivalents (11.78±1.99 µg mL^{-1}) when L-TRP was amended. Similarly, data of the pot trial (Fig. 2) showed that PsJN::*gusA* inoculation and L-TRP treatment individually increased *in vivo* IAA concentration in the plant rhizosphere soil compared to the untreated control. PsJN::*gusA* inoculation showed 22 and 16% increase in IAA content 15 and 30 DAP compared to the control, respectively. L-TRP application increased IAA content up to 39 and 31% at both 15 and 30 DAP compared to control. Combined application of PsJN::*gusA* and L-TRP (10^{-5}M) gave maximum increase in IAA equivalents, which was 55 and 50% at 15 and 30 DAP, respectively, compared to the control.

Data regarding the plant mineral contents revealed that separate PsJN::*gusA* inoculation or L-TRP increased N and P contents (Table 2). Maximum increase in shoot N and P contents up to 10 and 26% was achieved by combined application of PsJN::*gusA* and L-TRP (10^{-5} M). Likewise, PsJN::*gusA* supplemented with L-TRP (10^{-5} M) increased soil N and P contents by 16 and 8%, respectively, as compared to the untreated control (Table 2).

A significantly positive correlation (r values) was observed between soil IAA-equivalent and maize plant growth promotion caused by PsJN inoculation and L-TRP amendment (Table 4).

Enumeration and microscopic localization of PsJN::gusA in the rhizosphere, root and shoot interior

The inoculant strain efficiently colonized the rhizosphere and interior of maize roots and shoots (Table 3). However, when supplemented with L-TRP, persistence of PsJN::*gusA* increased as compared to inoculation treatment alone in the rhizosphere and plant tissues. In the inoculation treatment, 2.30×10^5 CFU g^{-1} rhizosphere soil, 1.05×10^5 CFU g^{-1} root interior and 8.21×10^3 CFU g^{-1} shoot interior were recovered. However, more CFU of the inoculant strain g^{-1} dry weight were recovered from the rhizosphere (9.20×10^5), root interior (5.83×10^5), and shoot interior (4.06×10^4) in the presence of L-TRP (10^{-5} M). Figure 3 shows the localization of the inoculated strain in different tissues i.e. root and leaf of maize plants.

Discussion

Plant-associated microorganisms are known to play a key role for plant nutrition and health (Compant et al. 2008) and might be important components of future fertilizers and pesticides. Endophytes have the capacity to colonize the plant interior and may mediate more consistent effects, particularly when applied as bio-fertilizers. *B. phytofirmans* strain PsJN is a plant growth-promoting endophytic bacterium, which colonizes the rhizosphere and internal tissues of its plant hosts, and promotes growth and yield of different crops through multifarious mechanisms (Sessitsch et al. 2005; Mitter et al. 2013b). In the present study, we demonstrated that L-tryptophan dependent biosynthesis of IAA by strain *B. phytofirmans* PsJN improves its ability to promote plant growth and colonization.

Endophytes live inside plants for at least part of their life cycle without being pathogenic. In contrast, some endophytes confer to the host benefits such as increased root growth and nutrient availability (Hardoim et al. 2008). In the present study, it was observed that PsJN inoculation improved maize plant growth and physiology, which was observed as better survival, root/shoot biomass and nutrient content compared to the uninoculated control (Tables 1–3). Increase in the total root system is the most common reported plant response mediated by PGPB inoculation in various plant species (Lucy et al. 2004). Several bacterial mechanisms have been proposed and hormone production is considered as the most plausible mechanism in controlling root growth and development (Mantelin and Touraine 2004). Production of phytohormones such as auxins in the root zone using L-tryptophan as a precursor from the root exudates by bacteria is responsible for root architecture (Ludwig-Müller 2011). Bacterial-induced alterations in root architecture might lead to an increase in total root surface area, consequently improved nutrient and water uptake, which may have positive effects on plant growth as a whole. This premise is further supported by our results showing that IAA production in strain PsJN is mostly dependent on the presence of L-tryptophan. When tryptophan is not added, only very low levels of IAA are produced in the culture medium (Table 3). It has been reported that increasing amounts of L-tryptophan stimulate the secretion of IAA by PGPB, which is responsible for the phytostimulatory effect of bacteria on plants (Omay et al. 1993). Similarly, our results strongly indicate that the improvement of plant growth and development is, at least partly, due to auxin production, particularly when L-tryptophan is amended. A significantly positive correlation (r) was observed between plant growth promotion and soil IAA-equivalent (Table 4). These findings are supported

by the work of other researchers who elucidated the effect of PsJN inoculation on the growth and development of various crops (Nowak et al. 1995; Ait Barka et al. 2000). A direct link between the production of IAA by *Azospirillum* and improvement in wheat growth was demonstrated by Dobbelaere et al. (1999).

In the present study, IAA secreted by a bacterium PsJN promoted root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. ACC deaminase produced by *B. phytofirmans* PsJN (Sessitsch et al. 2005) is involved in the stimulation of root elongation, which might be helpful in the uptake of relatively more water and nutrients from deep soil. ACC deaminase hydrolyzes plant ACC, the immediate precursor of ethylene, and thereby prevents the production of plant growth-inhibiting levels of ethylene (Sun et al. 2009). It is likely that IAA and ACC deaminase production by PsJN stimulate root elongation. Exogenous IAA is known to increase transcription and activity of ACC synthase, which catalyzes the production of ACC in plants (Patten and Glick 2002). ACC production in plant stimulates ACC deaminase activity in bacteria (Li and Glick 2001).

Auxins are an important class of hormones controlling many aspects of plant development and root morphology. L-Tryptophan is considered an efficient physiological precursor of auxins and its exogenous application to soils has been shown to influence plant growth and development positively (Arshad and Frankenberger 1997). As majority of rhizosphere microflora (≥80%) are able to produce phytohormones e.g. auxins, which directly enhance plant growth. There is the exciting possibility that most bacteria are capable of producing growth regulators continuously, provided that precursors of phytohormones are available in the rhizosphere (Bais et al. 2006). Root exudates could

supply the pool of precursors for rhizosphere bacteria to biotransform. Because appreciable levels of IAA are not produced unless an external source of tryptophan is supplied to the rhizosphere bacteria, endogenous levels of tryptophan are not sufficient for IAA production. There is likely a high demand for tryptophan by bacteria, as it is used to produce many essential compounds such as proteins and vitamins (Martens and Frankenberger 1993). In the present study, the exogenously applied L-tryptophan at 10^{-5} M proved to be more effective in improving the growth parameters of maize compared to 10^{-4} M L-tryptophan and the untreated control. The effect in modifying plant growth and development observed by L-tryptophan in our study was concentration-dependent. The mechanism of action of L-tryptophan on plant growth may be attributed to direct uptake of these compounds by plant roots, a change in the rhizosphere microflora discouraging root pathogens or by microbial conversion of other plant-associated microorganisms into metabolites (such as IAA) resulting in a beneficial rhizosphere for plant growth (Sarwar and Frankenberger 1994; Khalid et al. 2006).

Under natural soil conditions, it is assumed that concentration dependent effects of exogenous IAA on bacterial growth may serve as a signaling system in regulating the growth of plant-associated bacteria and IAA production by them (Kulkarni et al. 2013). The microbial production of auxin might have involved in communication with host plants and alter auxin biosynthesis in the host (Zúñiga et al. 2013). This implies that the microbial auxin (IAA) is not only synthesized and secreted, but it also enters host root cells in sufficient quantities as to alter normal plant growth and development. In the present study, combined application of PsJN and L-tryptophan was found to be more effective and showed maximum increase of plant growth. The importance of auxin

production in ability of bacteria to promote plant growth has been demonstrated through inoculation studies using bacterial mutants (Barbieri and Galli 1993). Fewer studies have described the role of microbial auxin (IAA) in plant growth promotion by using their mutants and molecular tools to understand its role in the beneficial plant interactions (Patten and Glick 2002; Idris et al. 2007; Spaepen et al. 2008). However, the involvement of auxin in root growth promotion and colonization of *Arabidopsis thaliana* by using a PsJN mutant has been described recently (Zúñiga et al. 2013). Bacterial-induced root growth in the presence of IAA causes several changes in physical and chemical properties of the soil, which can affect the ability of bacteria to colonize the rhizosphere (Table 3). In our study, it is assumed that improvement in the plant growth might be due to L-tryptophan dependent IAA biosynthesis by PsJN in the rhizosphere, which might optimize the endogenous suboptimal plant hormone level, or improve mineral uptake by plant roots. Zahir et al. (2010a, b) reported the synergistic response of L-tryptophan and PGPB inoculation for improving growth and yield of mungbean as compared to separate application.

PGPB maintain the symbiotic relationship with plants through their roots. Bacteria principally utilize amino acids and other nutrients released from plant roots as exudates. L-tryptophan present in the root exudates or applied exogenously serves as the precursor for IAA biosynthesis (Kulkarni et al. 2013). From the results of present study, it is likely that stimulated bacterial IAA production in the presence of the IAA precursor L-tryptophan in the rhizosphere (Fig. 2) might be involved in plant growth promotion, suggesting a close symbiotic relationship between the plants and colonizing PsJN. The ability to colonize plant roots may depend to some degree on the capability of the

bacterium to synthesize IAA. Moreover, it has been proposed that bacterial IAA synthesis contributed to enhanced rhizosphere competence and plant interior colonization (Fig. 3, Table 3) by stimulation of the release of plant exudates (Lambrecht et al. 2000).

We provide the evidence that L-tryptophan dependent IAA biosynthesis by *B. phytofirmans* PsJN affects its ability to promote plant growth and colonization of maize plants. Based on our results we conclude that combined application of PsJN and L-tryptophan is more effective to improve plant photosynthesis and biomass than their separate application. Overall, this study implies that the combined application of L-tryptophan and strain PsJN is an attractive approach for improving the growth and nutrient content of maize plants. However, further field investigations are needed to confirm its potential under natural soil environment.

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Table 1 L-tryptophan dependent response of PsJN inoculation on the growth parameters and photosynthesis of maize

Treatment	Plant height (cm)	Flag leaf width (cm)	Flag l eaf l ength (cm)	Shoot dr y biomass (g pot^{-1})	Root dr y biomass (g pot^{-1})	Photosynthesis (µ mole m^{-2} s^{-1})
Control	70.3 d*	4.53 d	42.83 d	75.7 e	2.31 e	66.20 d
PsJN Inoculation	76.0 c	4.87 c	48.17 c	100.3 c	3.32 bc	73.53 b
L-TRP (10^{-4} M)	75.0 c	4.57 d	47.83 c	89.7 d	2.78 d	68.33 cd
L-TRP (10^{-5} M)	75.3 c	4.90 c	48.83 bc	99.5 c	3.15 c	69.83 c
PsJN + L-TRP (10^{-4} M)	79.0 b	5.20 b	49.83 b	109.0 b	3.52 b	73.93 ab
PsJN + L-TRP (10^{-5} M)	83.0 a	5.53 a	52.50 a	117.4 a	3.74 a	76.47 a
LSD	2.94	0.255	1.186	7.165	0.205	2.89

*Means sharing similar letter(s) in a column for each parameter do not differ significantly at *P = 0.05*

Table 2 L-tryptophan dependent response of PsJN inoculation on the physiology parameters and nutrient concentration of maize

Treatment	Transpiration (m mole m^{-2} s^{-1})	Photosynthetically active radiation (µ mole m^{-2} s^{-1})	Plant N-content (%)	Plant P-content (%)	Soil N (%)	Available P (mg kg^{-1})
Control	5.63 d*	670.97 d	1.117 e	0.208 d	0.032 d	7.55 d
PsJN Inoculation	6.87 bc	716.53 c	1.177 c	0.225 c	0.035 bc	7.90 bc
L-TRP (10^{-4} M)	6.03 d	705.40 c	1.153 d	0.224 c	0.034 c	7.79 c
L-TRP (10^{-5} M)	6.73 c	721.63 c	1.173 c	0.225 c	0.035 bc	7.92 bc
PsJN + L-TRP (10^{-4} M)	7.30 ab	745.47 b	1.200 b	0.249 b	0.036 ab	8.04 ab
PsJN + L-TRP (10^{-5} M)	7.53 a	770.40 a	1.230 a	0.262 a	0.037 a	8.13 a
LSD	0.469	16.43	0.017	0.010	0.001	0.143

*Means sharing similar letter(s) in a column for each parameter do not differ significantly at *P = 0.05*

Table 3 Persistence of PsJN in the rhizosphere, root and shoot interior of maize plant and i*n vitro* auxin biosynthesis by PsJN

Treatment	Rhizosphere (CFU g^{-1} dry soil)	Root interior (CFU g^{-1} dry weight)	Shoot interior (CFU g^{-1} dry weight)	*In vitro* auxin production by PsJN (IAA equivalent µg mL^{-1})	
				Without L-TRP	With L-TRP
PsJN	2.30×10^5 b*	1.05×10^5 b	8.21×10^3 d	0.84±0.33†	11.78±1.99
PsJN + L-TRP (10^{-4}M)	7.10×10^5 a	3.95×10^5 b	3.04×10^4 c		
PsJN + L-TRP (10^{-5}M)	9.20×10^5 a	5.83×10^5 ab	4.06×10^4 c		

*Means sharing similar letter(s) in a column for each parameter do not differ significantly at $P = 0.05$.

Data is average of three replicate ± SD

Table 4 Correlation (*r*) between growth parameters of maize and their percent increases and soil IAA equivalent values

Plant Parameter	IAA production		IAA production	
	15 DAP	30 DAP	15 DAP	30 DAP
Plant height (cm)	0.90†	0.93†	0.79‡	0.87‡
Flag leaf width (cm)	0.81	0.89	0.78	0.87
Flag leaf length (cm)	0.95	0.95	0.84	0.91
Shoot dry biomass (g pot^{-1})	0.90	0.93	0.74	0.84
Root dry biomass (g pot^{-1})	0.85	0.88	0.61	0.73
Photosynthesis (μ mole m^{-2} s^{-1})	0.74	0.79	0.47	0.60
Transpiration (m mole m^{-2} s^{-1})	0.84	0.88	0.64	0.75
PAR (μ mole m^{-2} s^{-1})	0.93	0.96	0.85	0.92
Chlorophyll (a)	0.87	0.82	0.61	0.73
Chlorophyll (b)	0.92	0.92	0.76	0.85
Plant N content (%)	0.90	0.93	0.76	0.85
Plant P content (%)	0.89	0.94	0.86	0.92
Soil N content (%)	0.92	0.94	0.77	0.86
Available P (mg kg^{-1})	0.93	0.95	0.79	0.88
Rhizosphere colonization (CFU/ g dry weight)	0.99	0.99	-	-
Root colonization (CFU/ g dry weight)	0.98	0.99	-	-
Shoot colonization (CFU/ g dry weight)	0.98	0.99	-	-

†Correlation coefficient (*r*) between growth parameters / colonization of maize plants and IAA equivalent

‡Correlation coefficient (*r*) between % increase in growth parameters over control and IAA equivalent

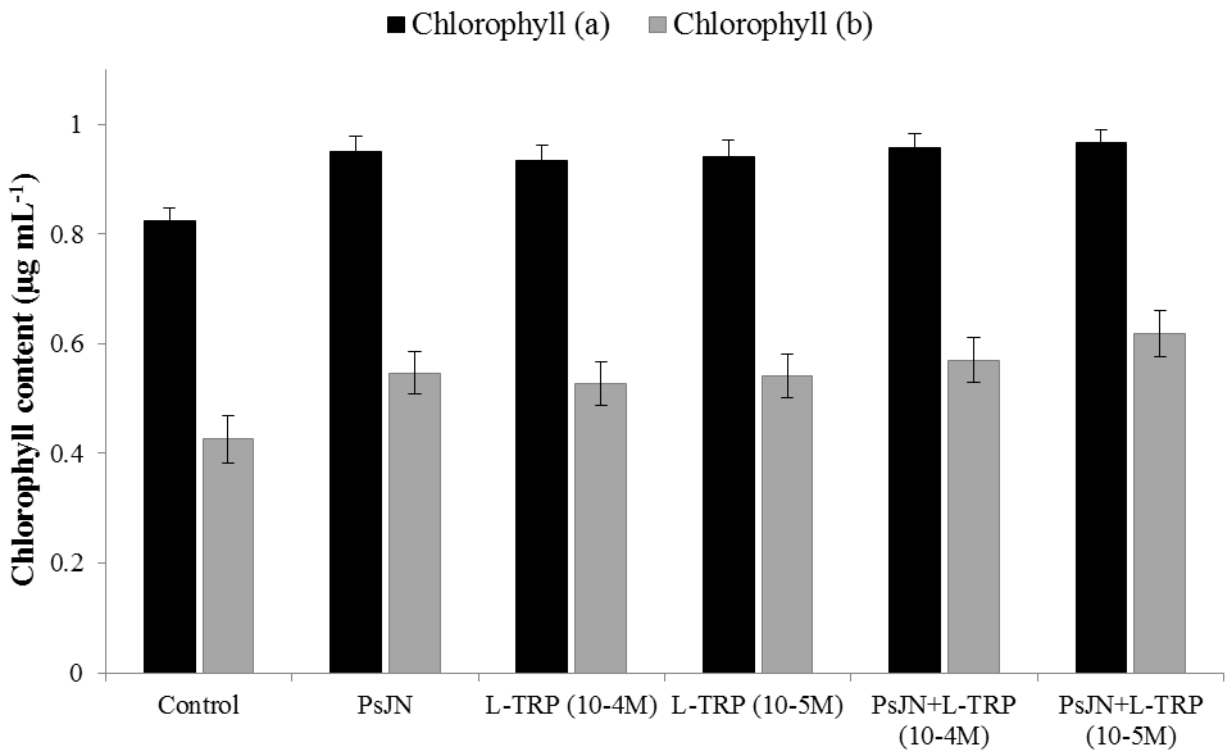

Figure 1. L-tryptophan dependent response of PsJN inoculation on the chlorophyll a and b content of maize plant, Data is average of three replicate ± SE

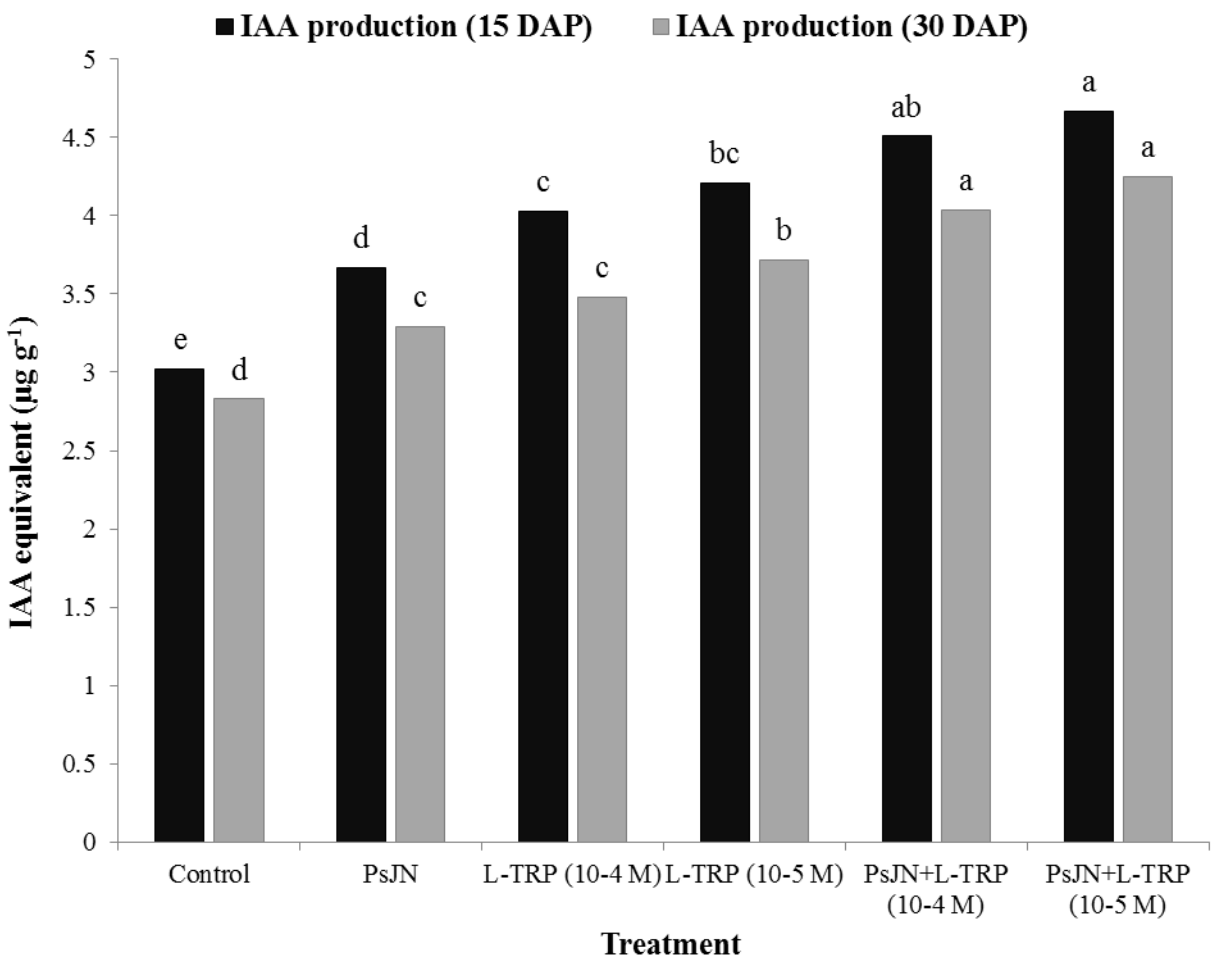

Figure 2 . L-tryptophan de pendent IAA pr oduction by PsJN i noculation in th e rhizosphere s oil of ma ize plant [15 a nd 30 d ays a fter p lanting (D AP)]. Bars sh aring similar letters for each parameter do not differ significantly at *P = 0.05*

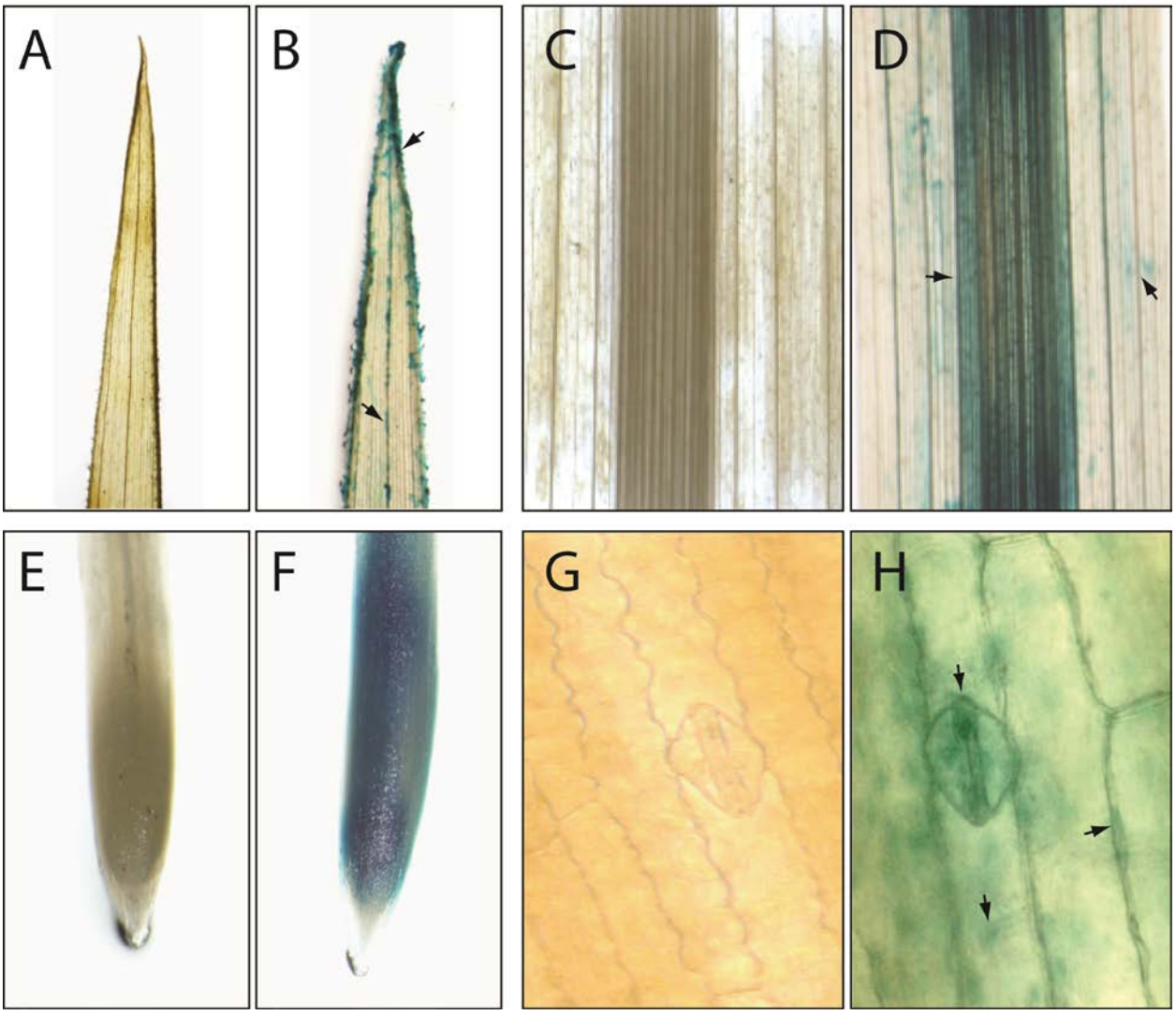

Figure 3. Photographs of the fourth leaf internal tissue of PsJN inoculated *Zea mays* L. plants. (A, C and E) Photographed (binocular microscope) of the fourth leaf (tip and middle) and root of uninoculated control or inoculated with PsJN::*gusA* (B, D and F), showing the blue color in veins due to gusA-marked cells (arrowheads). (G and H) Inverse microscope image of the leaf stomata of control and PsJN *gusA* inoculated plant, showing bacteria in the stomata and guard cell

Chapter 7

General Conclusions

There is a growing worldwide awareness for the need to increase food production to feed the rapidly expanding global human population. The conventional approach to increase agricultural productivity through massive inputs of chemical fertilizers is not sustainable because of high costs and concerns about global warming, environmental pollution and safety concerns. Also, there is a considerable resistance in some areas of the world in using genetically engineered crops for increasing food production. The search for microorganisms that improve soil health and enhance plant nutrition has continued to attract attention due to the increasing cost of fertilizers and their negative environmental effects. The use of biological inoculants based on PGPB appears to be a promising alternative to chemicals. PGPB strains that are often used (singly or in mixture) include species of genera *Bacillus, Pseudomonas, Azospirillum, Azotobacter, Trichoderma* and *Glomus;* species of other genera are used but somewhat less frequently. Various researchers reported 5–20% increase in yield of different crops by these inoculants. Endophytes have the capacity to colonize the plant interior and may mediate more consistent effects, particularly when applied as bio-fertilizers. Therefore, extensive utilization of endophytic microbes for increasing productivity of food crops is an attractive ecofriendly, cost-effective and sustainable alternative, which could play a significant role in feeding an ever-burgeoning world population.

The first work phase in this PhD study was aimed on the identification of a bacterial strain that has the potential to enhance plant performance in the field and

efficiently interacts with different plant genotypes. We selected five endophytic bacterial strains (FA13, FF34, FC42, FB12 and FD17) and a range of different lab assays in regard to potential plant growth promotion was performed and strains were further evaluated for improving growth of five maize cultivars under axenic and natural soil conditions. Endophytic colonization was an additional component in our selection criteria (which is rarely addressed in initial screenings) as it is of high importance for an inoculant strain to efficiently colonize the plant environment. The results showed that the inoculant strains had the potential to improve maize seedling growth under axenic conditions. Strain FD17 showed both the highest growth promoting activity under axenic conditions as well as colonization capacity. Results of containment study revealed that FD17 inoculation significantly increased plant biomass and grain yield up to 39 and 42%, respectively, as compared to the un-inoculated control. Inoculation also improved the photochemical efficiency of photosystem II (PSII) of maize plant and reduced the time needed for flowering. Bacterial survival and colonization in the plant environment are necessary for plant growth and yield. In this study, all the cultivars tested responded differently to inoculation with different endophyte isolates. Interestingly, cultivar Peso was highly colonized by all strains, but plant growth promotion was only to a limited extent correlated with high colonization. However, strain FD17 was very efficient in colonizing different varieties and was also the most efficient plant growth promoter. Our findings further indicated that efficient colonization in the rhizosphere as well as in the root/shoot interior of cultivar Morginon by strain FD17 in the containment trial indicated the specific cultivar colonizing capacity of the bacteria. The study suggested that *in vitro* plant growth promoting traits and potential of maize seedling growth promotion by

bacterial endophytes could be used for the selection of potential inoculant strains subjected for further testing as biofertilizers under field conditions.

Impact assessment of endophytic bacteria on plant growth promotion and underlying physiological and biochemical mechanisms is scarcely documented under abiotic stress. Therefore, an understanding of the interactions between host plant and endophytic bacteria having influence on plant growth, physico-chemical changes, yield and drought stress tolerance is required.

The second work phase was intended to assess the drought stress resilience of maize and wheat through endophytic colonization by *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17 in the pot and field trials. Results of our pot trial revealed that bacterial inoculation minimized the drought stress-imposed effects significantly increasing shoot biomass, root biomass, leaf area, chlorophyll content, photosynthesis and photochemical efficiency of PSII up to 66, 70, 21, 22, 75 and 10%, respectively, compared to the un-inoculated control. Bacterized seedlings showed higher leaf relative water content (30%) compared to control, whereas 43% higher leaf damage in terms of relative membrane permeability was observed in non-inoculated plants under drought stress. The inoculant strains efficiently colonized maize seedlings and were recovered from roots, shoots and leaves of both irrigated and stressed plants. The data of not stressed plants indicated a correlation between growth stimulation and number of viable PsJN cells in both cultivars. Our findings also indicated that endophytic populations were more suppressed and viable cell numbers decreased in Mazurka than in Kaleo under stress conditions. It is likely that bacterial ability to promote plant growth and to establish

endophytic populations is very often dependent on the plant genotype and developmental stage.

Burkholderia phytofirmans PsJN was used to investigate the potential to ameliorate the effects of drought stress on growth, physiology and yield of wheat under natural field conditions. The plants were exposed to drought stress at different stages of growth (tillering stage and flowering stage) by skipping the respective irrigation. PsJN inoculation gave better response to wheat at the tillering stage and resulted in significant increase in plant biomass, photosynthesis and grain yield compared to the control. Inoculation increased grain yield up to 21 and 18%, respectively, at both stages over the un-inoculated control. Drought stress is accompanied by the formation of reactive oxygen species (ROS), which damage membranes and macromolecules. Plants have developed several strategies to cope with oxidative stress. Enhancement of antioxidant defense in plants can thus increase tolerance to different stress factors. The findings of our study revealed that PsJN inoculated plants showed higher antioxidant activity compared to control under stress conditions. It is most probable that bacterial colonization augmented plant defense enzymes such as catalase, peroxidase, superoxide dismutase or phenolic compounds, to alleviate the oxidative damage elicited by drought. Likewise, under stress conditions, nutrient (NPK) contents of plant tissues were increased in response to inoculation, most likely due to increased root growth that exploited more soil volume for efficient uptake of nutrients by the plants, resulting in more biomass production.

These studies suggested that endophytic bacteria could be efficiently used to reduce the effects of drought stress on growth and yield of maize and wheat.

L-Tryptophan is considered an efficient physiological precursor of auxins and its exogenous application to soils has been shown to influence plant growth and development positively. In the third work phase, we evaluated the L-TRP-dependent response of PsJN inoculation to maize growth and auxin biosynthesis under pot conditions. *In vitro* data revealed that PsJN produced auxin (IAA equivalents) without L-TRP addition (0.84 µg mL^{-1}), however, IAA equivalents substantially increased when the medium was supplemented with L-TRP (11.78 µg mL^{-1}). In this study PsJN inoculation supplemented with L-TRP (10^{-5} M) significantly increased photosynthesis, chlorophyll content, root biomass and shoot biomass up to 16, 45, 62 and 55% , respectively, compared to the un-inoculated control. The inoculant strain colonized more efficiently maize seedlings in the presence of exogenously applied L-TRP. The ability to colonize plant roots may depend to some degree on the capability of the bacterium to synthesize IAA. Moreover, we proposed that bacterial IAA synthesis contributed to enhanced rhizosphere competence and plant interior colonization by stimulation of the release of plant exudates. The results imply that L-TRP-derived IAA biosynthesis in the rhizosphere by PsJN inoculation could be a useful approach for improving the growth, photosynthesis and yield of maize.

Future prospects and recommendations

PGPB commercial products are increasingly being used in sustainable agriculture, but there is an urgent need for investigation in order to understand more in depth the molecular bases of PGPB-plant interactions. Exploitation of PGPB–plant interactions can result in the promotion of plant health and soil fertility, which can play a significant role in low-input sustainable agriculture applications for both food and nonfood crops. An understanding of the mechanisms enabling these beneficial bacteria to interact with host plants will be essential to fully achieve the biotechnological potential of efficient plant-bacterial partnerships for a wider range of applications. For a more comprehensive development and utilization of microbial inoculants, there are several issues that need to be resolved in future research:

- Rapid screening and selection of effectively and competitively multifunctional bacterial strains, which can be used in a variety of crops and efficiently interact with different plant genotypes.
- As our understanding of the complex environment of the rhizosphere, mechanisms action of PGPB, and practical aspects of inoculant formulation and delivery increases, we expect new PGPB products. The success of bio-inoculants will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms. Rhizosphere management will require attention of soil and crop cultural practices as well as inoculant formulation and delivery methods.
- Understanding of the ecology of PGPB improves, it should be possible to obtain a better understanding of the mechanisms that are involved in plant growth

promotion and identify situations in which bioaugmentation with soil inoculants may be useful for increasing crop yields.

- The lack of consistency in PGPB inoculation results in the field remains a major challenge for the use of these bacteria as biological fertilizers. Advances in the understanding of the molecular basis of these interactions may help in the establishment of successful plant-PGPB associations.
- Genetic improvement of PGPB strains to enhance colonization and effectiveness may involve addition of one or more traits associated with plant growth promotion and development. Genetic manipulation of host crops for root-associated traits to enhance establishment and proliferation of beneficial microorganisms is being pursued.
- Follow-up of the dynamics of bacterial colonization of plants. In this regard, an important question that must be resolved for most systems is whether PGPB are continuously colonizing and promoting growth of the developing roots or alternatively, the major effects are at the early stages of root development.
- Assessment of optimal bacterial concentration. Bacterial numbers in the rhizosphere and on the root surface/ or root interior are very important. Relatively high numbers (10^6–10^7 CFU per g of plant tissue) are needed when the beneficial effects are seeking as biofertilizers [nutrients (N, P, Zn and Fe) fixation /solubilization /uptake]. On the other hand, relatively lower colonization numbers (10^3–10^5 CFU per g plant tissue) are necessary if the main mechanism of plant growth promotion is derived from the production of plant growth substances

(phytostimulation). This is an issue that should be further verified for the different PGPB-plant systems.

- The relevance of endophytic vs. rhizosphere colonization. There is a need to deeply investigate the relative advantages of endophytic colonization and rhizosphere / root surface colonization.
- Signal exchange between the bacterium and the plant. In recent years, the use of high-throughput techniques contributed with significant advances in the elucidation of signal molecule exchange between bacterial symbionts and their plant hosts.
- Understanding the reasons for the failures in greenhouses and in the field conditions may lead to the isolation of improved strains. Application of PGPB for improving nutrition in crop plants is strongly connected to our better understanding of bacterial diversity, host specificity, mode of action, appropriate formulation and methods of application.
- Recent advances on whole genome sequencing of several PGPB strains and of plants of agronomic importance will provide future basis for better understanding of PGPB–plant interactions and development of improved strains as effective biofertilizer for eco-friendly low-input sustainable agriculture.
- Advanced understandings are needed in the area of PGPB-plant interactions at different stages. Research is needed to understand which genes are turned on or turned off at the various stages of the interaction, by both the bacteria and the host plants. This knowledge could contribute with new ideas as to which traits could be enhanced or reduced for more efficient plant growth promotion and yield.

- We know relatively little about the phytohormone production in PGPB. Biosynthetic pathways, enzymes and genes responsible for synthesis must be considered as the initial step to understand the potential of bacterially produced phytohormones to regulate the plant growth and development in agricultural conditions. Only the physiological and molecular functionality of auxins (IAA) has been described in both, chemically defined medium and plant–microbe interaction. For others e.g. cytokinins, gibberellins, ABA, ethylene, JAs, polyamines and nitric oxide, we only know about the ability of several microorganisms to produce it under defined conditions and only in few cases in plant–microbe interactions. Integration of both, microbial and plant models could be the beginning of a new understanding about the natural process potentially leading to a better understanding of the "plant-microbe physiology".
- PGPB survival properties. Investigating the traits (PHAs, EPS, LPS, aggregate stability and biofilm formation etc.) that contribute to bacterial survival under adverse conditions during inoculant production, storage, seeds inoculation and colonization of plants is also very important. It is also necessary to carry out studies to assess inoculum performance at different time points during storage.
- Quality control of bio-inoculants. This is a very critical issue that should be assessed and demands collaborative efforts between the producing companies and research institutions. Since these products (biofertilizers / biopesticides) are composed of living organisms, it is very important to confirm the bacterial strain at all stages of the production and to ensure that high bacterial numbers are maintained until the product reaches the farmer's hands.

- Future research is also needed to improve the endophyte - host plant interaction through endophyte molecular breeding. Compared with crop genetic engineering, the engineering of endophytic bacteria should be a much easier process. The genetic modification of endophytes with useful genes will impart new traits into host plants inoculated with these bacteria.

Effective harnessing of the power of beneficial microorganisms for alleviating human food needs appears feasible and the expanding research involving microbial physiology/ecology/biochemistry, biotechnology, genomics and proteomics and applications of beneficial microorganisms should yield rich dividends in the future agriculture in a sustainable way.

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Awards / Honors / Scholarships:

- The Gregor Mendel Society award **"Visionary ideas on genetics"** The future of the global supply of food and biomass resources, 2012. Gregor-Mendel Society, Vienna, Austria.
- Third Place for Outstanding Poster Presentation in **AIT Poster Awards** 2012, Vienna, Austria.
- Overseas Ph.D. Scholarship by Higher Education Commission (HEC), Islamabad, Pakistan.
- Higher Education Commission allowance in M. Sc. during 2004-2006.
- Board Scholarship at University of Agriculture Faisalabad during 2000-2004.

Publications (Research articles/reviews articles/book chapters, during dissertation):

A. Research articles

1. **Naveed, M**., B. Mitter, S. Yousaf, M. Pastar, M. Afzal and A. Sessitsch. 2013. The endophyte *Enterobacter* sp. FD17: a maize enhancer selected based on rigorous testing of plant beneficial traits and colonization characteristics. **Biology and Fertility of Soils DOI: 10.1007/s00374-013-0854-y.**
2. **Naveed, M**., B. Mitter, T. G. Reichenauer and A. Sessitsch. 2013. Endophytic colonization of *Burkholderia phytofirman* strain PsJN induce drought-stress tolerance in maize**. Acta Horticulturea (In press).**
3. **Naveed, M**., B. Mitter, T. G. Reichenauer, W. Krzysztof and A. Sessitsch. 2013. Increased drought stress resilience of maize through endophytic colonization by Burkholderia phytofirmans PsJN and Enterobacter sp. FD17. **Environmental and Experimental Botany (Revision Submitted).**
4. **Naveed, M**., M. B. Hussain, Z. A. Zahir, B. Mitter and A. Sessitsch. 2013. Drought stress amelioration in wheat through inoculation with *Burkholderia phytofirmans* strain PsJN. **Plant Growth Regulation (Submitted).**
5. **Naveed, M**., M. B. Hussain, Z. A. Zahir, B. Mitter and A. Sessitsch. 2013. L-Tryptophan dependent production of indole-3-acetic acid (IAA) improves plant growth promotion and colonization of maize by *Burkholderia phytofirmans* PsJN. **Applied Soil Ecology (Submitted).**
6. **Naveed, M**., B. Mitter, M. Puschenreiter and A. Sessitsch. 2012. Characterization of seed endophytic bacteria and their growth promotion effect on different maize cultivars **(In preparation)**.

B. Review articles

1. Nadeem, S.M., Z.A. Zahir, **M. Naveed** and M. Asharf. 2010. Microbial ACC-deaminase: Prospects and applications for inducing salt tolerance in plants. **Critical Reviews in Plant Sciences 29(6): 360 - 393.**

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C. Book chapters

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D. PATENT

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