BENEFICIAL MICROBES ALLEVIATE CLIMATIC STRESSES IN PLANTS

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BENEFICIAL MICROBES ALLEVIATE CLIMATIC STRESSES IN PLANTS

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EDITORIAL
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Editorial: Beneficial Microbes Alleviate Climatic Stresses in Plants

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The Editorial on the Research Topic

Beneficial Microbes Alleviate Climatic Stresses in Plants

Global climate change accelerates the concurrence of a variety of abiotic (e.g., drought, salinity, heavy metals, and extreme temperatures) and biotic stresses (e.g., phytopathogens), thus considerably affecting agricultural productivity and bioremediation efficiency, even forest ecosystems. In this scenario, plant growth promoting microorganisms (PGPM) are receiving increasing attention of agronomists and environmentalists as candidates to develop an effective, eco-friendly, and sustainable alternative to conventional agricultural (e.g., chemical fertilizers and pesticide) and remediation (e.g., chelators-enhanced phytoremediation) methods employed to deal with these climate change-induced stresses (Ma et al., 2011, 2016, Ma et al.). Research on PGPM [e.g. (plant growth promoting bacteria (PGPB), rhizobia, arbuscular mycorrhizal fungi (AMF)] have shown great potential in the management of various agricultural and environmental problems, however, to date, a collective database on the role of PGPM in alleviating various climatic stresses in plants is not available. Therefore, this research topic was launched to advance the knowledge of the mechanisms underlying plant-microbe interactions, review recent progress and address some of the challenges, thus providing the opportunities to translate basic knowledge into sustainable applications.

Salinity is a major abiotic stress limiting the growth and productivity of plants worldwide. It has been proved that harnessing the potential of plant growth promoting rhizobacteria (PGPR) is an alternative strategy to improve plant stress tolerance. Ilangumaran and Smith provide a comprehensive review of major research advances on physiological, biochemical, and molecular mechanisms attributed by PGPR regulating plant adaptation and tolerance to salinity stress. They summed up the principal mechanisms including (1) improvement of water and nutrient uptake, photosynthesis, and source-sink relationships; (2) activation of antioxidant activity, osmolyte accumulation, proton transport machinery, salt compartmentalization, and nutrient status; (3) modulation of phytohormone status, gene expression, protein function, metabolite synthesis, and secretion of signaling molecules and initiation of stress-responsive pathways. The systems biology perspective on plant-microbe interactions in response to salinity opens up new prospects of understanding the regulatory networks of PGPR induced plant salt tolerance, suggesting application of PGPR could serve as a promising measure to alleviate salt stress and improve global food production.

Among the mechanisms involved in plant-microbe interactions, microbial phytohormone production plays a crucial role in the stimulation of plant defense response against abiotic stress. A comprehensive review of the function, aspects and production of microbial phytohormones [e.g., auxins, cytokinins, abscisic acid (ABA), gibberellic acid, and salicylic acid] involved in the improvement of abiotic stress tolerance and defense response in crop plants under hostile environments (e.g., drought, salinity, nutrient deficiency, or heavy metal contamination) was provided by Egamberdieva et al.

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As an important component of global climate change, increasing nitrogen (N) deposition and its ecological consequences are of serious concern, since it may profoundly influence the structure and function of the forest ecosystem. Zhao et al. performed a field trial in a mixed deciduous forest of China with canopy addition of N and water for 4 years and found that the effect of increased N deposition and precipitation on AMF community composition was time-dependent, mediated by soil factors, and possibly related to the sensitivity and resilience of forest ecosystem to global changes.

The further understanding of how successful PGPR colonization can improve the growth and defense response of the plants against fungal pathogens under salinity stress implies the diverse physiological and biochemical mechanisms used by PGPR to confer both abiotic and biotic stress tolerance of host plants. This was what Jha and Singh exemplified to us in a study of the potential of PGPR *Stenotrophomonas maltophilia* SBP-9 to promote the growth of *Triticum aestivum* under biotic (*Fusarium graminearum*) and abiotic (salt) stresses.

Additionally, Egamberdieva et al. described a detailed study of the functional role of endophytic PGPB colonizing root tissues in enhancing the growth performance and controlling root rot in *Cicer arietinum* under saline soil conditions. They demonstrated the importance of endophytic bacterial colonization for the improved symbiotic performance of legume host plants with rhizobia, with increased root growth, nutrient bioavailability and faster osmotic adjustment, and reduction in H_2O_2 production and pathogen infection under saline soil conditions.

The emission of volatile organic substances (VOCs) of microorganism can increase disease resistance and abiotic stress tolerance, and thereby helping plants control a wide range of pathogens. Notably, Rybakova et al. combined *in vitro* and in planta methods with the study of the mode of interaction between PGPB and phytopathogens via their VOCs. They highlighted the identification of several antimicrobial and plant growth promoting VOCs and several other antimicrobial volatile substances that were produced by both *Paenibacillus polymyxa* and *Verticillium longisporum* as a reaction to one another's VOCs, and the regulation of their general metabolic activities. The findings contribute to a better understanding of the mechanisms of VOCs underlying the interaction between pathogens and their natural antagonists.

Common mycorrhizal networks (CMNs) by AMF interconnect plants, which play a crucial role in redistributing nutrients and draining carbon (C) from the different plant partners at different rates (Walder and van der Heijden, 2015). Rezáčová et al. established an intercropping experiment of two similar *Panicum* species with contrasting carbon (C_3 and C_4) metabolism. The plants were subjected to two different temperature regimes and inoculated with or without a consortium of AMF. A root-free compartment (RFC), but AMF-accessible, was added that was comprised of ^{15}N-labeled clover residues, which could allow direct assessment of the benefit of AMF on the plants in term of N derived from the organic biomass. Also, the isotopic C signature of the plants enabled the determination of which plant species were the main donor of C to the AMF. The authors found that the C_3 species continued feeding the CMNs by using the specific C signature of the plant, AMF biomass/specific lipids in the root compartments and in the RFC. Other important results are that the inoculation of AMF reduced the plant biomass production, but increased plant phosphorus uptake under both temperature regimes.

One example of novel applications for the sustainable use of diazotrophic bacterial and yeast endophytes in modulating stomatal behavior and increasing plant water relations was shown by Rho et al. The authors analyzed the effects of endophyte inoculation (multiple or single strain) on leaf water potential, whole-plant water use, and stomatal responses of *Oryza sativa* under CO_2 enrichment and water deficit. They found that the ABA production by endophytes contributed significantly to the stomatal reactions and the resulting plant physiological benefits.

Scientists from different fields of research, from basic science to applied science, are indeed contributing to the understanding of the molecular, cellular and physicochemical mechanisms underlying plant-microbe interactions under various environmental stresses, which will certainly contribute to generating novel solutions for the development of PGPM-based sustainable applications in agricultural and forestry systems.

AUTHOR CONTRIBUTIONS

YM draft the editorial text. YM, MV, and HF revised and approved the final version of the editorial text.

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Plant Growth Promoting Rhizobacteria in Amelioration of Salinity Stress: A Systems Biology Perspective

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Salinity affects plant growth and is a major abiotic stress that limits crop productivity. It is well-understood that environmental adaptations and genetic traits regulate salinity tolerance in plants, but imparting the knowledge gained towards crop improvement remain arduous. Harnessing the potential of beneficial microorganisms present in the rhizosphere is an alternative strategy for improving plant stress tolerance. This review intends to elucidate the understanding of salinity tolerance mechanisms attributed by plant growth promoting rhizobacteria (PGPR). Recent advances in molecular studies have yielded insights into the signaling networks of plant–microbe interactions that contribute to salt tolerance. The beneficial effects of PGPR involve boosting key physiological processes, including water and nutrient uptake, photosynthesis, and source-sink relationships that promote growth and development. The regulation of osmotic balance and ion homeostasis by PGPR are conducted through modulation of phytohormone status, gene expression, protein function, and metabolite synthesis in plants. As a result, improved antioxidant activity, osmolyte accumulation, proton transport machinery, salt compartmentalization, and nutrient status reduce osmotic stress and ion toxicity. Furthermore, in addition to indole-3-acetic acid and 1-aminocyclopropane-1-carboxylic acid deaminase biosynthesis, other extracellular secretions of the rhizobacteria function as signaling molecules and elicit stress responsive pathways. Application of PGPR inoculants is a promising measure to combat salinity in agricultural fields, thereby increasing global food production.

Keywords: salinity stress, plant tolerance, rhizobacteria, phytohormones, signaling

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INTRODUCTION

Climate change has exacerbated the severity of environmental stressors and affects crop production worldwide as part of the present Anthropocene Era. At the same time, there is a need to maintain food security for a growing global population through increases in crop production, while also forging agriculture more sustainable. Going forward, the quality of land and water will be critically pivotal for agriculture. Excess salt concentration in soil and water resources declines agricultural productivity, turns fertile fields to marginal lands, and leads to their abandonment. The Food and Agriculture Organization estimates that salinity has affected more than 6% of land area.

Much of this land is not under cultivation but, a substantial proportion of the cultivated land, which constitutes 45 million ha of irrigated land (20% of total) and 32 million ha under dryland agriculture (about 2% of total) has been affected (Munns and Tester, 2008). The proportion of salinized land area might increase owing to climate change conditions conducive for salt accumulation (Othman et al., 2006).

Soluble salts deteriorate the fertility of soil by causing adverse effects on plant growth and development (Munns and Tester, 2008). Osmotic stress is the immediate impact of salinity (occurs within minutes) due to hypertonic conditions and ion toxicity (occurs over several hours to days and weeks) is the result of toxic ions (Na^+ and Cl^-) accumulating in the cells. Perturbed water balance and ion homeostasis affect hormonal status, transpiration, photosynthesis, translocation of nutrients, and other metabolic processes (Munns, 2002a). Beneficial soil microbiota enhance soil-water-plant relations through intricate mechanisms and subtle signaling cues that are not yet well-understood. A widely-proven notion is that the ability of soil microbes to manipulate phytohormonal signaling and trigger several other mechanisms to work in an integrated fashion contribute to enhanced stress tolerance in plants (Dodd and Perez-Alfocea, 2012). Inoculation of crop plants with beneficial microbes is gaining agronomic importance since they facilitate cultivation under saline-prone conditions by improving salt tolerance and hence, restoring yield (Lugtenberg et al., 2013). Bacteria isolated from extreme environments such as deserts and oceans have been shown to induce salt tolerance in crop plants. For example, a *Pseudomonas fluorescens* strain isolated from date-palm rhizosphere in Saharan region promoted root growth in maize (*Zea mays*) seedlings under salt stress (Zerrouk et al., 2016). Wheat plants (*Triticum aestivum*) inoculated with *Serratia* sp. Sl-12, a halophilic bacterium isolated from a salt lake showed improved salt tolerance and increased shoot biomass (Singh and Jha, 2016).

This review focuses on the evaluation of plant growth promoting rhizobacteria (PGPR) within the context of systems biology approaches for the alleviation of salinity stress with a brief overview of the causes for salinity and courses of plant tolerance. Recent advances in 'omics' technologies deliver a holistic understanding of the regulatory networks of stress responses modulated by the PGPR. Further, the reader may refer to comprehensive reviews on utilization of other beneficial microorganisms including arbuscular mycorrhizal fungi (AMF), endosymbionts, halotolerant, and phyllosphere bacteria to alleviate salinity stress (Yang et al., 2009; Dodd and Perez-Alfocea, 2012; Glick, 2012; Vorholt, 2012; Egamberdieva and Lugtenberg, 2014).

SALINITY

Salinity is one of the major abiotic stressors that undermines plant growth and development (Pitman and Lauchli, 2002). Soil salinization is caused by natural or human activities that increase the concentration of dissolved salts, predominantly sodium chloride in the soil. Primary salinity is caused by natural processes, leading to significant salt accumulation in soil and groundwater over extended periods of time, which result in the formation of salt lakes, salt marshes, marine sediments, and salt scalds in the landscape. Sources of primary salinity may arise from weathering of rocks and minerals that releases soluble salts, precipitation that washes these salts downstream, wind-borne salts from oceans and sand dunes that are deposited inland, and influx of seawater followed by subsequent retreat (Pitman and Lauchli, 2002; Rengasamy, 2002).

Cultivation operations such as land clearing, excessive irrigation, and inadequate drainage are the reasons for secondary salinity. Native vegetation sustains the water table below the subsoil zone with deep roots in semi-arid and arid regions. Replacing perennial species with shallow rooted annual crops and long fallows increases water table leakage and groundwater recharge, which consecutively raises the water table level. Salt is deposited in the topsoil as the water evaporates, resulting in dryland salinity and may eventually form a salt scald. Salinity effects can be more detrimental when the groundwater table is high, as prominent in arid and coastal areas where only salt-tolerant plants (halophytes) grow (Doering and Sandoval, 1981; Rengasamy, 2002). Irrigated lands are more prone to salinity than drylands because irrigation water deposits salt behind, year after year. Secondary salinization has degenerated vast tracts of irrigated lands to the point that they are no longer economical for cultivation. Plants are often supplied with more water than they can utilize during evapotranspiration. For example, irrigation coupled with instances of heavy rainfall accelerates infiltration and groundwater recharge rates that raise the water table faster than it can drain. As the water table rises, it mobilizes dissolved salts from underground rocks close to the root zone. When the water table is within two meters of the soil surface in clay soils (less than a meter in sandy soils), there is a high probability of salt accumulation in the topsoil and salt stress to plants. Salt is also discharged and redistributed by surface runoff or leached down into soil profile by rainfall and then move laterally to watercourses (Sharma and Prihar, 1973; Pitman and Lauchli, 2002).

Poorly drained soils also suffer from waterlogging in irrigated areas. Clay soil (fine-textured) is less permeable than loam (medium-textured) and sandy soil (coarse-textured) and hence, it has high water holding capacity with low infiltration rate. Water can be stored and used by plants for a long time in clay soil but will not quickly transmit salt away from the root zone. The low porosity of clay soil acts as an impervious layer, causing inadequate drainage (Nassar and Horton, 1999). Inefficient irrigation and drainage systems lead to poor water distribution, resulting in over-irrigated waterlogged areas or under-irrigated water deficit areas, both causing salt accumulation. Waterlogging aggravates salinity stress by limiting aeration and nutrient supply to plants while proper grading and installation of drains to carry excess water and dissolved salts away from water stagnant areas may solve these problems. Groundwater mounds can develop in irrigated areas and force saline groundwater into waterways. Irrigation with salt-rich water increases salt being added to the soil and requires more water to leach out salts to prevent them from accumulating in the topsoil. Leaching reduces salinity levels when there is sufficient drainage and the groundwater table is

deep. Conservation farming practices recommend appropriate methods to improve soil structure and irrigation efficiency (Shalhevet, 1994; Bauder and Brock, 2001).

The amount of salt stored in the soil also depends on soil type, with sandy soil having low and high capacity for clay loam minerals due to Na^+ bound to negatively charged clay particles. Soil with ECe (electrical conductivity of saturated paste extract) of 4 dS m^{-1} is defined as saline by the USDA salinity laboratory. Most crop species are affected by ECe of less than 4 dS m^{-1} and thus, saline soil inhibits the yield of crops. Salinity caused by irrigation schemes has been recognized as a serious problem around the world since irrigated land is, on average, twice as productive as rain-fed land and produces about one-third of global food (Munns and Tester, 2008). Because salinity and water are inextricably linked, climate changes drive extreme consequences on agriculture when drought or flooding hit vulnerable regions. Salinization management has focused on improving irrigation water quality and soil drainage to strategically increase salt acclimation in crops (Pitman and Lauchli, 2002).

SALT TOLERANCE IN PLANTS

Salinity tolerance in plants is dependent on its physiological mechanisms, duration of exposure to saline conditions, concentration of salt around roots, local soil–water relations, and microclimate conditions (temperature, humidity, etc.). Salt tolerance is usually quantified over a given period as survival, vegetative growth, or harvestable biomass at different physiological stages of the plant in saline versus non-saline conditions (Munns, 2002b). Crop yield decreases when salt concentration is above the threshold salinity level due to salt affecting the development of reproductive structures or translocation of nutrient reserves. There is a great diversity in salt tolerance between species and each species has a specific threshold salinity. Environmental adaptations and inherent genetic traits regulate salinity tolerance mechanisms in glycophytes and halophytes (Munns, 2002b). The majority of the plants are glycophytes (sensitive to salt) and tend to exclude the salts from roots, delaying salinity stress (Zhu, 2007). Halophytes grow in saline conditions and therefore, possess enhanced tolerance to high salt levels. They accumulate salts, carry through the xylem stream and precipitate them on leaves. Some species have evolved specialized cells called salt glands in shoots to excrete salt on its surface, which is then removed by water or wind. Few attempts have been made to introduce halophyte genes in crop plants and cultivate halophytes for food, forage, or fuel (Flowers et al., 1986; Flowers and Colmer, 2015).

Salinity impairs plant growth by causing osmotic imbalance and ion toxicity. The first osmotic phase occurs immediately when salt concentration increases above a threshold level around the roots. The osmotic stress induces water deficit in roots and shoot growth is arrested within minutes of exposure, but then recovers over several hours to a slow steady rate of growth. The second phase develops with time and is driven by the toxicity of excess Na^+/Cl^- ions that accumulate in the cytoplasm. When the salt concentration exceeds the rate of exclusion by roots or cellular ability to compartmentalize salts in the vacuoles, it builds up in the cytosol and disrupts cellular structures and functions (Munns, 2002b). Hence, all salinity tolerance in plants is directed towards maintaining osmotic balance and ion homeostasis. Even though the loss of cell turgor after the immediate osmotic shock is transient, reduction of cell elongation and cell division rates in root tips and young leaves over time lead to growth inhibition (Passioura and Munns, 2000). Osmotic stress affects shoot and reproductive development, for instance, younger leaves emerge slowly, lateral buds remain quiescent and flowering starts earlier. The growth regulating mechanisms are speculated to be long-distance signals of hormones and their precursors from roots to shoots. Phytohormone signaling is essential for regulation of cell division and differentiation, thereby controlling plant developmental morphogenesis (Santner and Estelle, 2009). The integrated signaling pathways are crucial in plant protection and adaptation mechanisms during abiotic and biotic stresses. In addition to five classical phytohormones, auxin, gibberellin, cytokinin, abscisic acid, and ethylene, other molecules including salicylic acid, jasmonic acid, nitric oxide, brassinosteroids, and strigolactones have been known to function as plant growth regulators. Phytohormone status is interdependent and both negative feedback and positive stimulation of synthesis have been reported. Many of the proteins including some transcription factors and protein kinases involved in plant hormone signaling have been elucidated. Phytohormone signaling cascades influence osmotic balance and other salt tolerance mechanisms (discussed below) and regulate plant acclimatization to salinity (reviewed in detail by Waśkiewicz et al., 2016). The plant roots encounter salinity first and root elongation rate recovers after initial exposure to salt but root architecture undergoes transition over time and high salt concentration represses formation of lateral roots. The aboveground symptoms of salinity induced osmotic stress overlap to that of drought stress, including leaf senescence and stunted growth (Munns, 2002a).

Osmotic stress affects stomatal conductance instantly due to perturbed water balance and abscisic acid (ABA) synthesis in guard cells, causing stomatal closure. Over the next several hours, transpiration rate is stabilized to a new reduced rate and ABA levels *in situ* are established (Fricke et al., 2006). Increased osmotic tolerance results in greater leaf expansion and stomatal conductance, which is beneficial only when there is sufficient soil water for transpiration losses (Munns and Tester, 2008). Photosynthesis rate decreases not only because of reduced leaf area and lesser gas exchange but also due to feedback inhibition of unused photosynthates, after exposure to salinity. The growth of sink tissues is constrained and carbohydrates accumulate in plant meristems and storage organs, which otherwise would be used in their proliferation and expansion. Modulating carbohydrate production in source leaves, phloem transport, and sink utilization downregulate the feedback photoinhibition and boost plant energy metabolism (Paul and Foyer, 2001; Perez-Alfocea et al., 2010). Reactive oxygen species (ROS) are constantly generated by cell organelles as a metabolic by-product and function as signaling molecules but their production is spiked under stressed environments. ROS including hydrogen peroxide,

superoxide, and free oxygen radical are profoundly reactive with cellular components and induces programmed cell death. ROS cause chlorophyll degradation and lipid peroxidation that affects photosynthesis and membrane permeability, respectively (Apel and Hirt, 2004).

Plants have developed antioxidant mechanisms involving enzymes (superoxide dismutase, glutathione reductase, catalase, and peroxidases) and molecules (carotenoids, flavonoids, and other phenolics) that prevent tissues from oxidative damages by quenching and detoxifying ROS (Gill and Tuteja, 2010). Upregulation of antioxidant enzyme activity and metabolite synthesis is coordinated by gene networks in response to initial low levels of ROS and other signaling events (Mittler et al., 2004). Antioxidant production and osmolyte accumulation are considered as sensitive physiological markers of salt and other abiotic stresses (Munns, 2002a). A common metabolic change in response to salinity is the synthesis of low molecular weight organic compounds including polyols (sorbitol, mannitol, inositol, or glycerol), amino acids (proline or glutamate), and betaines (glycine betaine) that function as osmolytes. They are compatible solutes and accumulate in the cytosol to maintain osmotic balance both inside and outside the cell. Osmolytes also function as osmoprotectants by preventing desiccation of membranes and stabilize dehydrated enzymes rather playing role in osmoregulation. They facilitate stabilization of subcellular structures and free radical scavenging and protect plants from osmotic stress induced dehydration (Rhodes et al., 2002). Synthesis of osmolytes is an energy-demanding process yet enables the plant to recover from adverse effects of salt stress (Raven, 1985).

Effects of ionic stress are determinant under prolonged exposure to high salinity levels and predominant in salt-sensitive species. Sodium ions are toxic to many plants, so are high concentrations of chlorine, specifically those that are poor excluders of Na^+ (ex: rice and beans) and sensitive to Cl^- (ex: soybean and citrus). The influx of Na^+ from roots is deposited in the xylem, carried through the transpiration stream and accumulated in the leaf blade rather than roots. Excluding Na^+ is a daunting task because a relatively small proportion is recirculated through phloem and most of it remains in the shoot, causing toxicity (Munns, 2002a; Tester and Davenport, 2003). Hence, active efflux of Na^+ from cells and retrieval of Na^+ from xylem is required throughout the plant and achieved by regulatory networks of sodium/proton antiporters and high-affinity potassium transporters (Tester and Davenport, 2003; Davenport et al., 2005). A Na^+/H^+ antiporter SOS1 (salt overly sensitive) localized on the plasma membrane is involved in the transport of Na^+ out of the cell and its activity is dependent on substrate (Na^+) concentration (Qiu et al., 2002). Excess Na^+ ion concentration affects low-affinity potassium uptake system because of the similar chemical nature of Na^+ and K^+ ions thereby, inhibiting K^+ uptake by the roots. Plants activate high-affinity K^+ transporters (HKT) to increase the uptake of K^+ ions over Na^+ ions and K^+ concentration relative to Na^+ in cytoplasm increases salinity tolerance (Rodriguez-Navarro and Rubio, 2006). Salt accumulation in intracellular spaces restrain enzymes involved in photosynthesis and respiration and interfere with vesicular trafficking (Baral et al., 2015; Jacoby et al., 2016). Cytosolic activities are inhibited under a high Na^+/K^+ ratio and cells need to effectively compartmentalize sodium into vacuoles, which further improves osmotic adjustments. Intracellular compartmentation of Na^+ is regulated by Na^+/H^+ antiporters and Na^+/H^+ exchangers (NHX) on the tonoplast, which are driven by a proton gradient (Halfter et al., 2000).

Plants with adequate calcium supply have demonstrated enhanced salt tolerance and supplemental Ca^{2+} stimulates rapid leaf elongation rate (Cramer, 1992). Calcium mediated signaling is important in maintaining Na^+/K^+ ratios by sustaining potassium transporters and suppressing non-selective cation channels and a rise in cytosolic Ca^{2+} levels is the first detectable response to sodium stress (Epstein, 1998). Membrane depolarization activates Ca^{2+} channels in cellular membranes that regulate Ca^{2+} oscillations in the cytosol and generate Ca^{2+} signals under salt stress. The calcium signal sensor, calcineurin B-like protein (CBL4, previously identified as SOS3) forms a complex with a CBL-interacting protein kinase (CIPK24, identified as SOS2) to phosphorylate SOS1, thus enabling its activation (Halfter et al., 2000; Zhu, 2002). Other sensor proteins are calcium dependent protein kinases (CDPKs), SOS3-like calcium binding proteins (SCaBPs), and calmodulins (CaMs) (Chinnusamy et al., 2006). Progressive accumulation of Cl^- is toxic to chloroplasts and mitochondria, and tolerance of high Cl^- concentrations requires compartmentalization and exclusion. The active influx of Cl^- is catalyzed by a $Cl^-/2H^+$ symporter but passive uptake also occurs under saline conditions and efflux takes place through Cl^- permeable channels (Yamashita et al., 1994). Transport of Cl^- to shoots is limited by reduced xylem loading of Cl^- via anion channels (downregulated by ABA) and Cl^- is actively retrieved from the xylem stream (Gilliham and Tester, 2005).

Biochemical analysis, gene expression and mutant studies conducted to investigate molecular functions of plants in response to salinity revealed that complex signal transduction pathways and gene regulatory networks exist to alleviate stress (Hasegawa et al., 2000). Breeding of salt-tolerant genotypes to improve crop production has been persevered by plant scientists but in spite of the advances, relatively few determinant genetic traits for salt tolerance in crop species have been identified to date (Munns and Tester, 2008). However, the acquired knowledge will lead to the development of tolerant cultivars and implementation of sustainable crop protection measures that are environmentally safe. Conventional breeding practices and genetic engineering techniques could be the most relevant but often time-consuming and cost-intensive strategies. Meanwhile, application of beneficial microbes to increase salt tolerance in plants is a feasible alternative to reclaim salinity prone lands under cultivation (Berg, 2009). A plant, together with its associated microbial community, the phytomicrobiome function as a holobiont. The physiology and metabolism of the host plant are influenced by the phytomicrobiome, facilitating its adaptation to the habitat. Members of the phytomicrobiome, which include PGPR, AMF and other facultative endosymbionts are inoculated as microbial consortia and this strategy has gained interest lately

to enhance crop productivity in stressed environments (Smith et al., 2015b).

SALT TOLERANCE MEDIATED BY PLANT GROWTH PROMOTING RHIZOBACTERIA

During the past century, research has continuously demonstrated numerous beneficial associations between plants and microbes, beginning with the classic legume–rhizobia symbiosis. The plant rhizosphere is enriched with nutrient sources excreted from roots that support the higher abundance of microbial population than the surrounding bulk soil (Lugtenberg and Kamilova, 2009). Free-living beneficial bacteria dwelling in the rhizosphere that exert beneficial activities are known as plant growth promoting rhizobacteria (PGPR). Some of them are facultative endophytes that further invade intercellular spaces of host tissues and thrive as endophytes to establish a mutually beneficial association. PGPR living outside the plant cell are differently associated with plant roots and directly relate to the underlying mechanisms of plant–microbe interactions. The majority of the PGPR colonize the root surface and thrive in spaces between root hairs and rhizodermal layers whereas, some are not physically in contact with the roots (Gray and Smith, 2005). Root exudates are an integral part of rhizosphere signaling events and regulate communication in beneficial plant–microbe interactions. Phenols, flavonoids, and organic acids secreted by roots have been known to act as chemical signals for bacterial chemotaxis, secretion of exopolysaccharides, quorum sensing and biofilm formation during rhizosphere colonization (Bauer and Mathesius, 2004; Badri et al., 2009; Narula et al., 2009). Isolated from rhizosphere soils, PGPR are screened *in vitro* for plant growth promoting characteristics and tested for beneficial effects in greenhouse and field trials prior to commercialization. PGPR promote plant growth and development through diverse mechanisms such as enhanced nutrient assimilation (biofertilizers) by biological nitrogen fixation, phosphorous solubilisation or iron acquisition (Rodriguez and Fraga, 1999; Steenhoudt and Vanderleyden, 2006; Sharma et al., 2013; Jin et al., 2014; Kuan et al., 2016), control pathogens by antagonism and competition (biocontrol agents) (Compant et al., 2005; Beneduzi et al., 2012; Chowdhury et al., 2015), degrade organic pollutants and reduce metal toxicity of contaminated soils (bioremediation), and facilitate phytoremediation (Divya and Kumar, 2011; Nie et al., 2011; Janssen et al., 2015; Weyens et al., 2015).

Inoculation with PGPR has been known to modulate abiotic stress regulation via direct and indirect mechanisms that induce systemic tolerance (Yang et al., 2009). Many PGPR have been investigated for their role in improving plant-water relations, ion homeostasis and photosynthetic efficiency in plants under salt stress (**Figure 1**); their amelioration mechanisms are intricate and often not well-understood. These mechanisms are regulated by a complex network of signaling events occurring during the plant–microbe interaction and consequently ensuing stress alleviation (Smith et al., 2017). Accumulating evidence using high-throughput techniques implies that understanding the dynamic function of PGPR in relation to stomatal conductance, ion transport, water and nutrient uptake, phytohormonal status, signal transduction proteins, antioxidant enzymes, and carbohydrate metabolism in plants is important for determining the induced systemic tolerance (**Figure 2**).

Osmotic Balance

Plant growth promoting rhizobacteria regulate water potential and stomatal opening by affecting hydraulic conductivity and transpiration rate. Maize plants inoculated with *Bacillus megaterium* showed increased root hydraulic conductivity compared to uninoculated plants when exposed to salinity (2.59 dS m^{-1}) and this was correlated with increased expression of two ZmPIP (plasma membrane aquaporin protein) isoforms (Marulanda et al., 2010). PGPR induce osmolyte accumulation and phytohormone signaling that facilitate plants to overcome initial osmotic shock after salinization. Enhanced proline synthesis in transgenic *Arabidopsis thaliana* with proBA genes derived from *Bacillus subtilis* conferred salt tolerance to the plants (Chen et al., 2007). Inoculation of salt tolerant *Bacillus amyloliquefaciens* SN13 onto rice (*Oryza sativa*) plants exposed to salinity (200 mM NaCl) in hydroponic and soil conditions increased plant salt tolerance and affected expression of 14 genes, of which, four (*SOS1*, ethylene responsive element binding proteins *EREBP*, somatic embryogenesis receptor-like kinase *SERK1* and NADP-malic enzyme *NADP-Me2*) were upregulated and two [glucose insensitive growth *GIG* and (SNF1) serine-threonine protein kinase *SAPK4*] were downregulated under hydroponic conditions whereas, only *MAPK5* (Mitogen activated protein kinase 5) was upregulated under greenhouse conditions. Genes involved in osmotic and ionic stress response mechanisms were modulated by SN13 inoculation (Nautiyal et al., 2013).

Beneficial microorganisms can stimulate carbohydrate metabolism and transport, which directly implicate source-sink relations, photosynthesis, growth rate and biomass reallocation. Seed inoculated *B. aquimaris* strains increased total soluble sugars and reducing sugars in wheat under saline (ECe = 5.2 dS m^{-1}) field conditions and resulted in higher shoot biomass, NPK accumulation, and Na reduction in leaves (Upadhyay and Singh, 2015). Higher plant dry matter accumulation after 36 days in pepper (*Capsicum annuum*) plants inoculated with *Azospirillum brasilense* and *Pantoea dispersa* under salinity was related to enhanced stomatal conductance and photosynthesis, but neither chlorophyll concentration nor photochemical efficiency of photosystem II was affected (del Amor and Cuadra-Crespo, 2012). Microbes exposed to osmolality fluctuations in their surrounding environment accumulate large quantities of osmoprotectants in their cytosol (Kempf and Bremer, 1998). Under such circumstances, biosynthesis of osmolytes including proline, trehalose, and glycine betaines by PGPR is most likely to be quicker than their associated host plants. The compatible solutes absorbed through plant roots aid in maintaining osmotic balance and preventing cellular oxidative damage under saline conditions. Co-inoculation of bean (*Phaseolus vulgaris*) with *Rhizobium tropici* and *Paenibacillus polymyxa* strain modified to overexpress

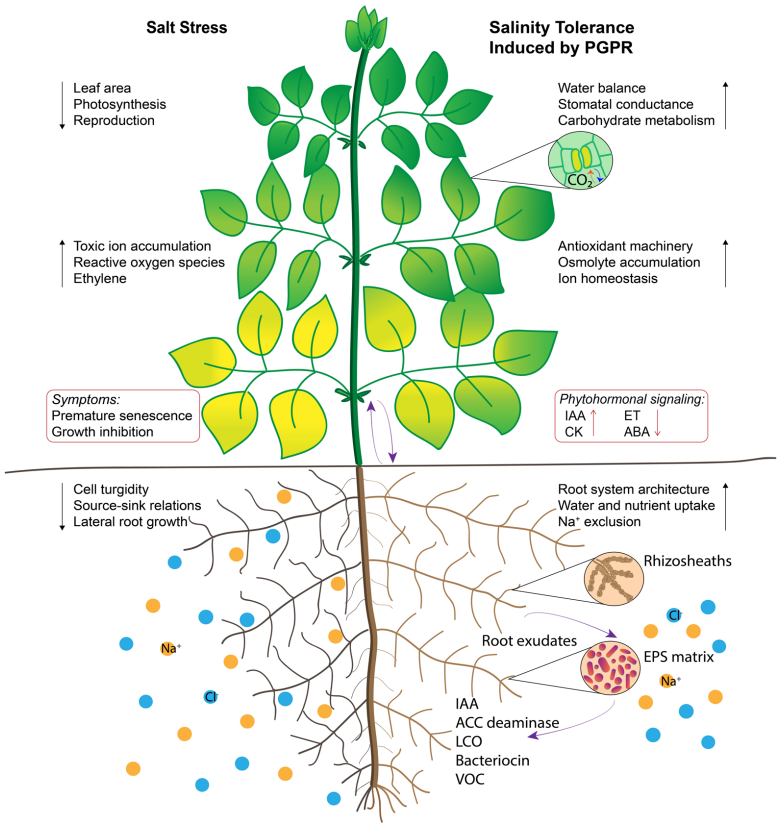

FIGURE 1 | Illustration of salt tolerance mechanisms induced by plant growth promoting rhizobacteria (PGPR). Root surfaces are colonized by PGPR and extracellular polysaccharide matrix acts as a protective barrier against salt stress. Some extracellular molecules function as signaling cues that manipulate phytohormonal status in plants. Enhanced root-to-shoot communication improves water and nutritional balance, source-sink relations and stomatal conductance. Stimulating osmolyte accumulation, carbohydrate metabolism and antioxidant activity delay leaf senescence, which inturn contribute to photosynthesis. Regulation of physiological processes are indicated by black arrows and signaling pathways are indicated by purple arrows.

trehalose 6-phosphate gene resulted in increased nodulation, N content and plant growth. A microarray analysis of nodules revealed upregulation of stress tolerance genes suggesting that extracellular trehalose, which functions as an osmoprotectant can induce salinity tolerance (Figueiredo et al., 2008).

Ion Homeostasis

Bacteria limit plant salt uptake by trapping cations in the exopolysaccharide matrix, altering root structure with extensive rhizosheaths, and regulating expression of ion affinity transporters. PGPR have been known to increase the mineral nutrient exchange of both macro and micronutrients and alleviate nutrient imbalance caused by the high influx of Na^+ and Cl^- ions. Microbial induced nutrient cycling (mineralization), rhizosphere pH changes (organic acids), and metal chelation (siderophores) increase plant nutrient availability (Dodd and Perez-Alfocea, 2012; Lugtenberg et al., 2013). PGPR help maintaining ion homeostasis and high K^+/Na^+ ratios in shoots by reducing Na^+ and Cl^- accumulation in leaves, increasing Na^+ exclusion via roots, and boosting the activity of high-affinity K^+ transporters. Inoculation of *Azotobacter* strains C5 (auxin producing) and C9 in maize plants under salt stress improved K^+ uptake and Na^+ exclusion. Chlorophyll, proline and polyphenol contents in leaves increased and PGPR inoculation enhanced plant stress responses (Rojas-Tapias et al., 2012). In a study conducted with *Arabidopsis thaliana* and *Burkholderia phytofirmans* PsJN to understand the spatiotemporal regulation of short and long-term salt stress, colonized plants exhibited higher tolerance to sustained salt stress. The expressional patterns of genes involved in ion homeostasis (*KT1*, *HKT1*, *NHX2*, and *SOS1*) were altered after stress and rapid molecular changes induced by PsJN may be linked to the observed salt tolerance (Pinedo et al., 2015). A halophyte grass, *Puccinellia tenuiflora* inoculated with *B. subtilis* GB03 showed less Na^+ accumulation and validated by upregulation of *PtHKT1* and *PtSOS1* genes but *PtHKT2* was downregulated in roots under high salt concentrations (200 mM NaCl) (Niu et al., 2016).

Phytohormone Signaling

Soil bacteria modulate plant hormone status by releasing exogenous hormones, metabolites, and enzymes that may contribute to increased salt tolerance. Besides, phytohormones and metabolites are synthesized *de novo* in the plants in response

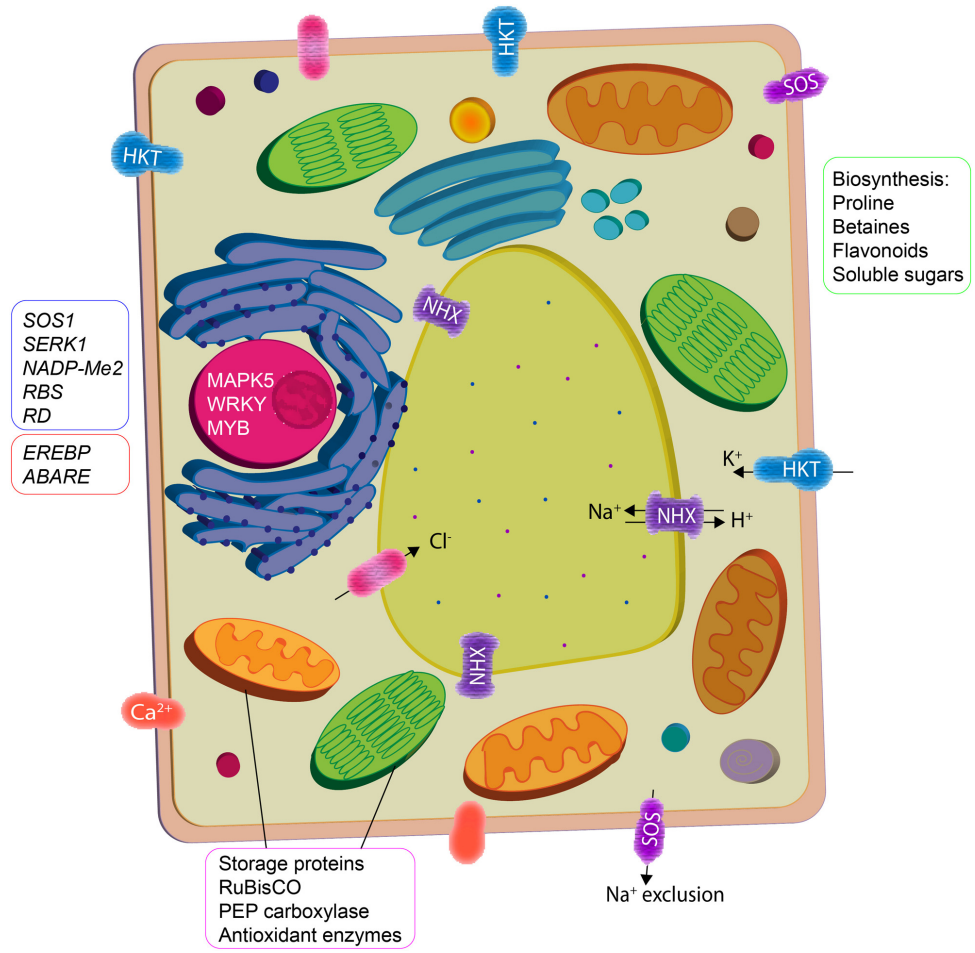

FIGURE 2 | Plant growth promoting rhizobacteria interaction mediate cellular activity in plants to ameliorate salinity stress. Osmotic imbalance and oxidative damage are reduced by enhanced biosynthesis of compatible solutes and antioxidants. Ion homeostasis is maintained by increase in activity of K^+ transporters (HKT) and H^+ exchangers (NHX) that facilitate salt compartmentalization/exclusion. PGPR also upregulate the expression of stress responsive genes (phytohormone signaling) and proteins (vegetative storage, photosynthesis, and antioxidant enzymes).

signaling events of plant–microbe interactions during stress (Dodd et al., 2010).

Auxin

Auxin biosynthesis occurs via multiple pathways in rhizobacteria and one is the utilization of tryptophan present in root exudates and its conversion into indole-3-acetic acid (IAA), which is absorbed by the plant roots. Together with the plant's endogenous IAA pool, an auxin signaling pathway is triggered and results in stimulation of cell growth and proliferation. IAA produced by PGPR is one of the most common and widely studied bacterial signaling molecules in plant–microbe interactions. The function of exogenous IAA is dependent on the endogenous IAA levels in plants. At optimal IAA concentration, acquisition of bacterial IAA may result in neutral, promotion or inhibition of plant growth (Dodd et al., 2010; Spaepen and Vanderleyden, 2011).

Bacillus amyloliquefaciens SQR9 enhanced salt stress tolerance (100 mM NaCl) of maize seedlings *in vitro* and bacterial inoculation increased chlorophyll and total soluble sugar contents, improved peroxidase and catalase activity, enhanced glutathione content, and K^+/Na^+ ratio. In addition, salinity induced ABA level was counteracted by SQR9 inoculation, which maintained it at the normal level. These physiological mechanisms to relieve salt stress were confirmed by the upregulation of genes *RBCS*, *RBCL* (encoding RuBisCo subunits), *H(+)-Ppase* (encoding H^+ pumping pyrophosphatase), *HKT1*, *NHX1*, *NHX2* and *NHX3*, and also the downregulation of *NCED* expression (encoding 9-*cis*-epoxycarotenoid dioxygenase) in inoculated seedlings (Chen et al., 2016). *Enterobacter* sp. EJ01 isolated from a halophyte plant, sea china pink (*Dianthus japonicus thunb*) improved plant growth and salt stress tolerance (200 mM) in Arabidopsis and tomato (*Solanum lycopersicum*) plants. Short-term treatment (6 h) with EJ01 increased expression of genes involved in salt stress response such as DRE-binding proteins *DREB2b*, Relative to Desiccation (*RD29A*, *RD29B*), late embryogenesis abundant (LEA) genes (*RAB18*), proline biosynthesis (*P5CS1*

and *P5CS2*), and stress-inducible priming processes (*MPK3* and *MPK6*) in Arabidopsis seedlings. GFP-tagged EJ01 displayed colonization of the bacteria in the rhizosphere and endosphere of Arabidopsis roots. In addition, ROS scavenging activities including antioxidant enzyme, ascorbate peroxidase were enhanced in inoculated tomato plants under salt stress (Kim et al., 2014).

The role of bacterial cytokinins in salt stress tolerance is largely unknown yet with relatively fewer studies. *Pseudomonas* strains (*P. aurantiaca* TSAU22, *P. extremorientalis* TSAU6 and *P. extremorientalis* TSAU20) enhanced growth up to 52%, compared to control plants and alleviated salinity (100 mM NaCl) induced dormancy of wheat seeds (Egamberdieva, 2009). Cytokinin producing *B. subtilis* inoculated onto lettuce seedlings under water deficit conditions increased accumulation of shoot biomass and shortened roots with only small effect on root biomass. Despite increased shoot cytokinins, the possible role in root-to-shoot signaling was latent seemingly hindered by shoot ABA (Arkhipova et al., 2007).

Ethylene

Synthesis of ethylene in response to stress may increase plant tolerance or expedite senescence (Morgan and Drew, 1997). Ethylene regulates plant adaptation to stress at the expense of growth and development. As ethylene levels increase under stress, transcription of auxin response factors is inhibited and it constraints plant growth. PGPR that secrete 1-aminocyclopropane-1-carboxylase (ACC) deaminase restrict ethylene biosynthesis in plants. The enzyme converts ACC, the precursor of ethylene to ammonia and α-ketobutyrate. Many studies have shown enhanced stress tolerance and growth promotion in plants conferred by soil bacteria producing ACC deaminase (Glick et al., 2007). The following examples illustrate some of the salt tolerance mechanisms induced by PGPR producing ACC deaminase.

Pseudomonas putida UW4 inoculated tomato (*Solanum lycopersicum*) seedlings showed increased shoot growth after 6 weeks in saline conditions up to 90 mM NaCl. The expression of *Toc GTPase*, a gene of the chloroplast protein import apparatus was upregulated, which may facilitate import of proteins involved as a part of stress response (Yan et al., 2014). A nutrient flow study of pea (*Pisum sativum* cv. Alderman) inoculated with *Variovorax paradoxus* 5C-2 under salt stress of 70 and 130 mM NaCl showed increased root to shoot K^+ flow and Na^+ deposition in roots, thereby increasing K^+/Na^+ ratio in shoots. Inoculation with PGPR also increased the photosynthesis rate and electron transport, while decreased stomatal resistance and xylem balancing pressure; overall improved the plant biomass (Wang et al., 2016). *Enterobacter* sp. UPMR18 inoculated okra (*Abelmoschus esculentus*) plants exhibited increase in antioxidant enzyme activities and transcription of ROS pathway genes when grown in 75 mM NaCl and showed enhanced salt tolerance (Habib et al., 2016). ACC deaminase producing strains of *Pseudomonas fluorescens* and *Enterobacter* spp. significantly improved maize yield in salt-affected fields. Higher K^+/Na^+ ratios and NPK uptake were also recorded in inoculated plants under salt stress (Nadeem et al., 2009).

Plant growth promoting rhizobacteria that produce both IAA and ACC deaminase can effectively protect plants from a range of stresses. IAA accumulation induces transcription of ACC synthase genes, which increases ACC concentration, leading to the production of ethylene. PGPR containing ACC deaminase may break down some of the excess ACC and lower plant ethylene levels during an advent of environmental stress and simultaneously allow IAA to promote plant growth (Glick, 2012). Endophytic bacteria (*Arthrobacter* sp. and *Bacillus* sp.) producing ACC deaminase and IAA increased proline content in sweet pepper (*Capsicum annuum*). The inoculated plants manifested downregulation of stress-inducible genes *CaACCO* (ACC oxidase) and *CaLTPI* (Lipid transfer protein) under mild osmotic stress (Sziderics et al., 2007). *Pantoea dispersa* PSB3 is a native bacterium in chickpea (*Cicer arietinum*) and produces IAA and ACC deaminase. Upon inoculation to chickpea cv. GPF2, it significantly improved plant biomass, pod number, pod weight, seed number, and seed weight in salt (150 mM NaCl) affected plants. The improved salt tolerance was associated with significant reduction of Na^+ uptake and electrolyte leakage and increase of relative leaf water content, chlorophyll content, and K^+ uptake (Panwar et al., 2016).

Abscisic Acid

There are relatively few studies on determining the role of exogenous ABA in plant–microbe interactions and whether bacterial ABA influences ABA status of plants under salt stress. However, PGPR modulate ABA biosynthesis and ABA-mediated signaling pathways that may contribute to the enhanced growth of salt-stressed plants. Halotolerant *Dietzia natronolimnaea* STR1 induced salinity (150 mM NaCl) tolerance mechanisms in wheat plants via modulation of an ABA-signaling cascade, validated by the upregulation of TaABARE (ABA-responsive gene) and TaOPR1 (12-oxophytodienoate reductase 1) leading to *TaMYB* and *TaWRKY* stimulation, followed by expression of stress response genes including upregulation of *TaST* (a salt stress-induced gene). Expression of SOS pathway related genes and tissue-specific responses of ion transporters were modulated. Gene expression of various antioxidant enzymes and proline content were increased, contributing to enhanced protection against salt stress in PGPR inoculated plants (Bharti et al., 2016). Cucumber (*Cucumis sativus*) plants inoculated with *Burkholderia cepacia* SE4, *Promicromonospora* sp. SE188 and *Acinetobacter calcoaceticus* SE370 had significantly higher biomass under salinity stress (120 mM NaCl). PGPR increased water potential and decreased electrolyte leakage. The inoculated plants showed down-regulation of ABA compared with control plants, while salicylic acid and gibberellin GA4 contents were increased (Kang et al., 2014a). Seed inoculation of cotton (*Gossypium hirsutum*) with *Pseudomonas putida* Rs-198 reduced ABA accumulation and increased plant biomass in salinized soil but the induced salt tolerance can also be attributed to regulated ionic balance and improved endogenous IAA content (Yao et al., 2010). Wheat plants inoculated with PGPR strains *Arthrobacter protophormiae* SA3 and *B. subtilis* LDR2 built up

IAA while conflicted the increase of ABA and ACC content under salt stress conditions (100 mM NaCl). The amelioration effect was further validated by the upregulation of *TaCTR1* (Serine/Threonine protein kinase – ethylene responsive) and *TaDRE2* (drought-responsive element) genes (Barnawal et al., 2017).

Extracellular Molecules

The extracellular secretions of PGPR including proteins, hormones, volatiles, polyamines, and other compounds have been determined to manipulate signaling pathways and regulatory functions that positively impact plant defense and development by stimulating growth, inducing disease resistance and eliciting stress tolerance (Barnawal et al., 2013; Kang et al., 2014b; Bhattacharyya et al., 2015; Smith et al., 2015a; Zhou et al., 2016).

Exopolysaccharides

Bacteria secrete exopolysaccharides (EPS) which are responsible for attachment, often along with other bacteria, to soil particles and root surfaces. EPS bind soil particles to aggregates, stabilizing soil structures, and increasing water holding capacity and cation exchange capacity (Upadhyay et al., 2011). EPS usually form an enclosed matrix of microcolonies, which confer protection against environmental fluctuations, water and nutrient retention, and epiphytic colonization (Balsanelli et al., 2014). They are also indispensable for mature biofilm formation and functional nodules in legume–rhizobia symbiosis (Stoodley et al., 2002; Skorupska et al., 2006). Inoculation of EPS producing *Pseudomonas mendocina* with an arbuscular mycorrhizal fungus, *Glomus intraradices* onto lettuce (*Lactuca sativa*) resulted in stabilization of soil aggregates under field conditions (Kohler et al., 2006). Inoculation with salt-tolerant *Halomonas variabilis* HT1 and *Planococcus rifietoensis* RT4 increased the growth of chickpea (*Cicer arietinum* var. CM-98) and soil aggregation with roots under high salt concentrations (up to 200 mM NaCl) (Qurashi and Sabri, 2012). Quinoa (*Chenopodium quinoa*) seeds inoculated with *Enterobacter* sp. MN17 and *Bacillus* sp. MN54 improved plant-water relations under saline irrigation conditions of 400 mM NaCl (Yang et al., 2016). EPS production and composition improve bacterial resistance to abiotic stress (Sandhya and Ali, 2015) but the role of EPS in plant salinity tolerance deserves further investigation.

Lipo-chitooligosaccharides

Legume–rhizobia symbiosis is affected by salt stress and high levels of salinity inhibit nodule formation and nitrogen fixation (Tu, 1981; Zahran, 1999). Lipo-chitooligosaccharides (LCOs) are secreted by rhizobia as Nod-factors (NFs) in response to flavonoids present in root exudates and initiate nodule formation. LCOs are conserved at the core but diverge in the *N*-Acetyl chain length, degree of saturation, and substitutions (glycosylation or sulfation), which are crucial in host specificity (Oldroyd, 2013). Nod-factors also act as stress response signals in legumes and NF synthesis is modulated by other PGPR and abiotic stresses. High salinity (100–200 mM NaCl) inhibited root hair deformation responses to increase in NF concentrations in Soybean (*Glycine max*) – *Bradyrhizobium japonicum* symbiosis (Duzan et al., 2004). Inoculation of IAA producing *Azospirillum brasilense* Cd into the *Rhizobium*-Bean (*Phaseolus vulgaris* cv. Negro Jamapa) symbiosis increased root branching and flavonoid synthesis under 50 mM NaCl. The co-inoculation also promoted *Nod*-genes expression in *R. tropici* CIAT899 and *R. etli* ISP42 grown in the presence of root exudates (Dardanelli et al., 2008). Free-living rhizobia are more resistant to salt stress than inside their legume hosts. *R. tropici* CIAT899 is highly tolerant to stress and high salt concentrations enhance *Nod*-gene expression, Nod-factor synthesis and diversity; 46 different NFs were identified compared to 29 NFs under control with only 15 NFs common to both (Estevez et al., 2009). Inoculation of *B. japonicum* 532C grown in genistein (a flavonoid) induced media significantly enhanced nodulation and growth of soybean under salinity levels (36 and 61 mM NaCl) and such positive effects become more evident with time (Miransari and Smith, 2009) and increased yield up to 21% under salinized field conditions in an earlier study.

Bacteriocins

Bacteriocins are small peptides secreted by rhizobacteria that are bactericidal or bacteriostatic against relative bacteria, thus providing a competitive advantage to the producer strain but might also promote microbial diversity in an ecologic niche (Kirkup and Riley, 2004). Application of thuricin 17, isolated from a soybean endosymbiont *Bacillus thuriengenesis* NEB 17 differentially altered the proteome of salt-stressed (250 mM NaCl) Arabidopsis plants. Expression of proteins involved in carbon and energy metabolism pathways were modulated by the bacterial signals. Proteins involved in photosynthesis including PEP carboxylase, RuBisCo-oxygenase large subunit, pyruvate kinase and proteins of photosystems I and II were upregulated along with other stress related proteins (Subramanian et al., 2016b). These bacterial signal compounds also induced similar changes in the proteome of soybean seeds at 48 h under 100 mM NaCl. In addition, isocitrate lyase and antioxidant glutathione-*S*-transferase were increased. These findings by shotgun proteomics suggested that thuricin 17 positively manipulate plant proteome profile and enhance physiological tolerance to salinity (Subramanian et al., 2016a).

Polyamines

Polyamines (PAs) are low molecular weight aliphatic amines with pronounced antioxidant activity that are ubiquitous in all living organisms and modulate ROS homeostasis by scavenging free radicals and stimulating antioxidant enzymes. The most abundant polyamines, spermidine, spermine, and putrescine are implicated in various developmental processes and stress responses in plants (Gupta et al., 2013). Application of exogenous polyamines increase abiotic stress tolerance but PGPR secretion of polyamines is largely unexplored. Spermidine from *Bacillus megaterium* BOFC15 increased cellular polyamine accumulation in Arabidopsis, thereby

TABLE 1 | Summary of PGPR interaction effects in crop plants under salinity stress from recent studies using systems biology approaches.

	PGPR	Crop species	Beneficial effects	Reference
1	*Bacillus amyloliquefaciens* SN13	*Oryza sativa*	Upregulation of *SOS1*, *EREBP*, *SERK1*, *NADP-Me2*	Nautiyal et al., 2013
2	*Bacillus amyloliquefaciens* SQR9	*Zea mays*	Upregulation of *RBCS*, *RBCL, HKT1*, *NHX1*, *NHX2,* and *NHX3*	Chen et al., 2016
3	*Bacillus megaterium*	*Zea mays*	Improved expression of two ZmPIP isoforms	Marulanda et al., 2010
4	*Bacillus thuriengenesis* NEB17	*Glycine max*	Upregulation of PEP carboxylase, RuBisCo-oxygenase large subunit, pyruvate kinase, and proteins of photosystems I and II, isocitrate lyase and antioxidant glutathione-*S*-transferase	Subramanian et al., 2016a
5	*Dietzia natronolimnaea*	*Triticum aestivum*	Modulation of ABA signaling cascade, SOS pathway related genes, tissue-specific responses of ion transporters	Bharti et al., 2016
6	*Enterobacter* sp. UPMR18 (ACC deaminase)	*Abelmoschus esculentus*	Increase antioxidant enzyme activities and upregulation of ROS pathway genes	Habib et al., 2016
7	*Pseudomonas putida* UW4 (ACC deaminase)	*Solanum lycopersicum*	Increased shoot growth and expression of *Toc GTPase*	Yan et al., 2014
8	*Pseudomonas simiae* AU	*Glycine max*	Upregulation of vegetative storage proteins, RuBisCO large chain proteins. Decrease in root Na^+ accumulation and increase in proline and chlorophyll content	Vaishnav et al., 2015

activating PA-mediated signaling pathways contributing to the osmotic stress tolerance of plants. The bacterial inoculation resulted in greater biomass, elevated photosynthetic capacity and higher antioxidant enzyme activity. Other tolerance mechanisms involved robust root system architecture and ABA dependent stress responses, which maintained water balance and stomatal conductance (Zhou et al., 2016).

Volatile Compounds

Volatile organic compounds (VOC) released from PGPR are known to stimulate plant growth, resulting in increased shoot biomass, and modulated stress responses. Perception of volatiles by plants and subsequently induced mechanisms require further research (Bailly and Weisskopf, 2012). *B. subtilis* GB03 VOCs mediated tissue specific regulations of Na^+ homeostasis in salt-stressed plants. Arabidopsis under 100 mM NaCl treated with VOCs decreased Na^+ accumulation by concurrently downregulating expression of *HKT1* in roots but upregulating it in shoots. Presumably, the induction of *HKT1* dependent shoot-to-root recirculation resulted in reduced Na^+ accumulation up to ∼50% throughout the plant. Treatment with VOCs increased leaf surface area, root mass, and total K^+ content when compared with controls whereas, inoculated *athkt1* mutants showed stunted growth. Exposure to VOCs reduced the total Na^+ level by 18% and enhanced shoot and root growth of *sos3* mutants in 30 mM NaCl (Zhang et al., 2008). A putative VOCs blend released from *Pseudomonas simiae* AU induced salt-tolerance in soybean (*Glycine max*) under 100 mM NaCl by decreasing root Na^+ accumulation and increasing proline and chlorophyll content. Protein expression analysis confirmed upregulation of vegetative storage proteins (Na^+ homeostasis), RuBisCO large chain proteins (photosynthesis) in exposed soybean seedlings (Vaishnav et al., 2015).

Paraburkholderia phytofirmans PsJN VOCs stimulate plant growth and induce salinity tolerance that have been demonstrated both *in vitro* (150 mM NaCl/15 mM $CaCl_2$) and in soil (200 mM NaCl/20 mM $CaCl_2$). Growth parameters of Arabidopsis plants measured as rosette area, fresh weight, and primary root length were higher than the control plants and exposure to VOCs showed parallel growth promoting effects of direct bacterial inoculation. The emitted compounds were analyzed and the plants were exposed to a blend of 2-undecanone, 7-hexanol, 3-methylbutanol molecules, which mimicked the effect of VOCs (Ledger et al., 2016). Genome wide mapping association of Arabidopsis accession lines revealed 10 genetic loci associated with growth stimulation in response to the presence of *P. simiae* WCS417r *in vitro*, which is partly caused by VOC produced by the bacterium. Even though the study was conducted to select lines for breeding strategies, it is interesting to note that the genotype variation of host plants has different interactions with the associated root microbiome (Wintermans et al., 2016).

CONCLUSION

Application of PGPR inoculants as biofertilizers and biocontrol agents is an integral component in organic farming practices (Babalola, 2010). With rising emphasis on sustainable agriculture, environmental protection, and food security, exploitation of beneficial soil microbiota is imperative. Abiotic stresses constraint yield and turn agriculture production systems fragile; in addition, persisting climate change intensify the frequency, degree, and resultant damage of stressful conditions. Plants have evolved complex mechanisms to tolerate abiotic stresses caused by various environmental factors, including salinity. Plant associated bacteria in soil mitigate the adverse effects of these

stresses in a more time-sensitive and cost-effective manner, where the development of tolerant cultivars has been somewhat overwhelmed. Research directed towards the application of PGPR in salt-affected fields encourages commercialization of inoculants for salinity tolerance. The systems biology of plant–microbe interactions in response to environmental stimuli such as salinity, opens up new prospects of understanding the regulatory networks of plant salt tolerance modulated by rhizosphere bacteria (**Table 1**). While the induced salt tolerance may be contributed by the release of extracellular compounds that function as chemical signals to the plant, improved soil properties that reduce the impact of salinity is another important benefit yet to be explored. Stress adaptation of plants are induced by associated microbiota and cutting-edge research as discussed above may be successfully applied to improve crop yield in saline prone regions. The potential application of PGPR to help plants deal with stress in agricultural fields seems vastly large, yet much is left to be utilized.

AUTHOR CONTRIBUTIONS

GI gathered literature and prepared the manuscript. DS provided feedback and oversaw progression of the manuscript.

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Phytohormones and Beneficial Microbes: Essential Components for Plants to Balance Stress and Fitness

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Plants are subjected to various abiotic stresses, such as drought, extreme temperature, salinity, and heavy metals. Abiotic stresses have negative impact on the physiology and morphology of plants through defects in the genetic regulation of cellular pathways. Plants employ several tolerance mechanisms and pathways to avert the effects of stresses that are triggered whenever alterations in metabolism are encountered. Phytohormones are among the most important growth regulators; they are known for having a prominent impact on plant metabolism, and additionally, they play a vital role in the stimulation of plant defense response mechanisms against stresses. Exogenous phytohormone supplementation has been adopted to improve growth and metabolism under stress conditions. Recent investigations have shown that phytohormones produced by root-associated microbes may prove to be important metabolic engineering targets for inducing host tolerance to abiotic stresses. Phytohormone biosynthetic pathways have been identified using several genetic and biochemical methods, and numerous reviews are currently available on this topic. Here, we review current knowledge on the function of phytohormones involved in the improvement of abiotic stress tolerance and defense response in plants exposed to different stressors. We focus on recent successes in identifying the roles of microbial phytohormones that induce stress tolerance, especially in crop plants. In doing so, this review highlights important plant morpho-physiological traits that can be exploited to identify the positive effects of phytohormones on stress tolerance. This review will therefore be helpful to plant physiologists and agricultural microbiologists in designing strategies and tools for the development of broad spectrum microbial inoculants supporting sustainable crop production under hostile environments.

Keywords: abiotic stress, plant microbiome, metabolites, phytohormones

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INTRODUCTION

The Food and Agricultural Organization has provided an estimate of the alarmingly increasing human population, expected to reach 8–9 billion by 2030 (FAO, 2010). As a result of increasing urbanization and industrialization, threats to the environment have increased, leading to the shrinkage of agricultural land on one hand and causing significant declines in crop growth on the

other hand. Abiotic stresses have the potential to restrict the growth of crop plants considerably, therefore leading to significant yield losses and posing a potential threat to global food security (Mahalingam, 2015).

Environmental stresses are detrimental to the growth of plants. Drought, salinity, heavy metal contamination, flooding, temperature (cold and high), and ultraviolet radiation are the key abiotic factors that modulate the growth of plants to the extent that a reduction in yield is a certain effect. Changes in the climate patterns of different regions have resulted in shifts in vegetation, and approximately 2,000 million hectares of land worldwide has been affected by increased water scarcity and salinization (El-Beltagy and Madkour, 2012). It is believed that approximately 25% of global agricultural land is affected by drought and approximately 5–7% is affected by salt (Ruiz-Lozano et al., 2012). Abiotic stresses inhibit plant growth by reducing water uptake and altering plant physiological and biochemical processes (Ahmad et al., 2010; Hashem et al., 2016). Heavy metals, including cadmium, lead, and mercury, are toxic and are mostly present in soils at low concentrations. However, due to their high mobility in the soil–plant system, they are readily taken up by plants and delivered to the shoot (Hart et al., 1998). Increases in metal concentrations cause retardation of growth, leading to necrosis, altered nutrient uptake, reduced enzyme activity and hence phytotoxicity (Groppa et al., 2012).

A better understanding of the different tolerance strategies for maintaining crop productivity through the manipulation of environmental conditions can be helpful for maintaining the maximum genetic potential of crops as much as possible. Phytohormones are important growth regulators synthesized in defined organs of the plant that have a prominent impact on plant metabolism (Kazan, 2013) and play an important role in the mitigation of abiotic stresses (Teale et al., 2006; Hu et al., 2013). However, abiotic stresses alter the endogenous levels of phytohormones, such as auxins, gibberellins, abscisic acid (ABA), jasmonic acid and salicylic acid (SA), which causes plant growth perturbations (Debez et al., 2001; Egamberdieva, 2009; Khan et al., 2014). Drought and salt stress have also been reported to inhibit phytohormone concentrations in plant tissue.

There has been enormous progress in research regarding crop improvement in hostile environments, and the role of some tools, such as microbial technology and genetic engineering, has been acknowledged. Accordingly, several strategies for improving plant stress tolerance by root-associated microbes, such as a low-input biotechnology, have been proposed (Khan et al., 2013). Plant-associated microbes live in plant tissue endophytically or symbiotically or they colonize the root surface and cooperate with each other by producing various metabolically active substances (Egamberdieva, 2011, 2012; Berg et al., 2013; Asaf et al., 2017). The stimulation of plant growth and nutrient acquisition by beneficial rhizobacteria has been correlated to the biosynthesis of plant growth regulators, including auxins (Etesami et al., 2015; Pereira et al., 2016), gibberellins (Khan et al., 2014), cytokinins (Kudoyarova et al., 2014), and ABA (Sgroy et al., 2009). The microbial regulators modulate plant hormone levels in plant tissue, and they have been found to have effects that are similar to exogenous phytohormone application (Egamberdieva, 2009; Turan et al., 2014; Shahzad et al., 2016). Based on the currently available studies on the effect of phytohormones on plant stress tolerance, this review attempts to improve the understanding of microbial phytohormones and their interactions with plants by assessing their influence on plant physiological and morphological properties. Based on important studies on the negative effect of abiotic stresses on plant growth regulators, we have also presented some potential traits of microbial phytohormones that can be used to increase plant growth and tolerance to stress factors. In this review, we will focus on the plant growth regulators synthesized by root-associated microbes, their diversity, physiology and their involvement in stress tolerance of plants to abiotic stresses including drought, salt, and heavy metals.

ROLE OF PHYTOHORMONES IN PLANT RESPONSE TO ABIOTIC STRESS

Auxins

Auxins are important phytohormones, and the auxin indole-3-acetic acid (IAA) was shown to promote several growth and developmental events, such as cell division, elongation, and differentiation (Asgher et al., 2015). IAA is synthesized from and chemically similar to tryptophan. Ljung (2013) produced strong evidence favoring auxin-mediated growth and developmental control through alterations in gene expression patterns. Many reports are available depicting varied modulations in the synthesis, transport, metabolism and activity of auxins after plant exposure to stresses (Ljung, 2013); however, plenty of research reports are available advocating the role of auxins in mediating and improving plant tolerance to abiotic stresses (Kazan, 2013). Rice plants exhibited a significant decline in IAA after exposure to salinity stress. In addition, this variation in IAA can induce growth modulation through an increase in other phytohormones, such as ABA, as reported by Iqbal and Ashraf (2013). Jung and Park (2011) found a link among auxin signaling and salt stress which developed through auxin involvement in modulating the membrane bound transcription factor NTM2. These involvements were further validated by over-expression studies on the IAA30 gene of NTM2 carried out by Park et al. (2011); however, the actual mechanism of IAA-induced mitigation of salinity is unclear.

Auxins have an important role, whether directly or indirectly, in promoting heavy metal tolerance, as Hu et al. (2013) observed that heavy metals have a negative effect on the biosynthesis of auxins. The toxic effect of lead (Pb) on sunflower plant growth was alleviated by the addition of a low concentration of IAA (10^{-10} M), which stimulated increases in root volume, surface area and diameter (Fässler et al., 2010). IAA induced an increase in shoot biomass and increased Pb and Zn accumulation in plant tissue, indicating the potential of auxins to enhance the phytoextraction of metals. Aluminum restricts root growth in *Medicago sativa* by reducing the transport and synthesis of IAA from shoot to root, which was confirmed after analyzing the expression of genes; however, exogenous application of IAA was observed to mitigate aluminum stress to some extent by

maintaining greater expression of the AUX1 and PIN2 genes (Wang S. et al., 2016). There was a positive effect after using auxins as priming sources. Iqbal and Ashraf (2007) have reported a significant mitigation of salt stress-induced hostile effects in wheat after seed priming with IAA, which resulted in ionic homeostasis and induction of SA biosynthesis. These studies indicate the existence of possible crosstalk between auxin and SA that mediates tolerance responses in plants. Salinity restricts the synthesis of IAA; however, the exogenous application of SA proved effective in mitigating hostile effects by causing significant alleviation of salinity-induced inhibition (Fahad and Bano, 2012).

Cytokinins

Cytokinins (CK), an important group of plant hormones are involved in the maintaining of cellular proliferation and differentiation and the prevention of senescence, therefore leading to the inhibition of premature leaf senescence (Schmulling, 2002). However, under stress conditions, particularly water stress at the grain-filling stage, it was observed that stay-green genotypes have the potential to exhibit increased tolerance, which was ascribed to an increased concentration of cytokinin in the xylem sap (Borrell et al., 2000). Zhang et al. (2010) demonstrated that cytokinin-over-expressing transgenic cassava exhibited greater tolerance to drought in comparison to wild-type plants. The genes involved in the biosynthesis of cytokinin have been over-expressed, and their role in stress tolerance has been validated. For example, the ipt gene has been validated in field analysis (Peleg and Blumwald, 2011). Reduced cytokinin leads to ABA-induced stomatal closure, thereby reducing carbon uptake and assimilation, and under stressful conditions, the up-regulation of cytokinin oxidase may also reduce carbon metabolism; work on this topic can be fruitful in improving the plant growth and yield. Mohapatra et al. (2011) demonstrated that cytokinin improves grain filling. Currently, exogenous application of cytokinin is being employed to optimize the internal concentrations of cytokinin. It has also been documented that heavy metals, such as zinc and lead, severely hamper the seedling growth of chickpea through the inhibition of GA_3 and Z concentrations in plant tissue (Atici et al., 2005). In an earlier report, the application of kinetin to chickpea stimulated plant growth and development under salt stress (Bozcuk, 1981), and in another report, kinetin alleviated cadmium stress in eggplant by enhancing its antioxidant potential (Singh and Prasad, 2014).

Abscisic Acid

Like other phytohormones, ABA is known to have an important role in plants by improving stress responses and adaptation. It is a naturally occurring sesquiterpenoid, which are a group of key phytohormones involved in the regulation of growth. There have been many reports advocating the role of ABA in integrating signaling during stress exposure with subsequent control of downstream responses (Wilkinson et al., 2012). Under abiotic stress the expression of stress responsive genes regulated by ABA-induced and -mediated signaling, leading to better elicitation of tolerance responses (Sah et al., 2016). In addition, ABA has been reported to control root growth and water content under drought stress conditions (Cutler et al., 2010). However, an abrupt increase in ABA concentrations during stress exposures can lead to growth retardation and can also modulate tolerance responses against stresses (Asgher et al., 2015). Nevertheless, there are reports suggesting the positive implication of exogenous ABA in reversing the ill effects of stresses, such as salinity (Gomez et al., 2002), chilling (Nayyar et al., 2005), drought (Bano et al., 2012), and cold stress (Li et al., 2014). Bano et al. (2012) demonstrated that exogenous application of ABA protected wheat from drought-induced oxidative damage by improving the antioxidant system and relative water content. Exogenous ABA application for improving stress tolerance has been proposed as an effective tool for stress mitigation. In *Solanum tuberosum*, Mora-Herrera and Lopez-Delgado (2007) observed that ABA application resulted in improved stress tolerance by reducing the production of free radicals through significant increases in the activity of the antioxidant enzyme peroxidase. Zhou et al. (2014) observed a significant alteration in the proteome of tea due to exogenous application of ABA under drought stress conditions, including changes in proteins involved in transport, carbon metabolism, and stress tolerance. It has been suggested that ABA maintains the levels of other hormones, such as ethylene, leading to the maintenance of shoot and root growth in *Zea mays* (Spollen et al., 2000). Upon stresses, ABA synthesis and accumulation in plant tissue increases. The most important role of ABA, in addition to its role in signaling, is its ability to act as an anti-transpirant after the induction of stomatal closure and reduction of canopy expansion (Wilkinson and Davies, 2002). Exogenous ABA application to rice seedlings exposed to drought led to the protection of photosynthesis by up-regulating the expression of the OsPsbD1, OsPsbD2, OsNCED2, OsNCED3, OsNCED4 and OsNCED5 genes, leading to improved photosynthetic capacity, and stomatal regulation under normal and stressed conditions, which suggests the involvement of these genes in photosystem II induction after exogenous ABA application. In plants exposed to stress conditions, ABA is involved in developing the deeper root system and causing other necessary root modifications to mediate optimal water and nutrient acquisition (Spollen et al., 2000; Vysotskaya et al., 2009). In addition, ABA maintains the hydraulic conductivities of shoot and root to better exploit soil water content, leading to the maintenance of tissue turgor potential and improved drought tolerance through up-regulation of the antioxidant system and the accumulation of compatible osmolytes (Chaves et al., 2003), which maintains the relative water content. In *Stylosanthes guianensis*, Zhou et al. (2005) demonstrated that ABA-induced antioxidant defense was mediated by improved nitric oxide synthesis. Guajardo et al. (2016) also reported improved activity of antioxidant enzymes after ABA treatment, which resulted in increased desiccation tolerance by reducing oxidative stress-induced damage. Cabot et al. (2009) reported that ABA applied exogenously inhibited accumulation of sodium and chloride in citrus plants with exogenously applied ABA. In another study, ABA treatment increased plant growth, nutrient uptake, and

nitrogen fixation in the common bean under salt stress (Khadri et al., 2006).

Gibberellic Acid

Another important plant growth regulator is gibberellin, which has a vital role in seed dormancy formation of floral organs, and lateral shoot growth (Olszewski et al., 2002). The available literature clearly reveals the ameliorative impact of gibberellic acid against salinity. Gibberellic acid was found to stimulate plant growth and development under various abiotic stress conditions (Ahmad, 2010). Enhanced plant water uptake and reduced stomatal resistance were observed in gibberellic acid-treated tomato plants grown under saline conditions (Maggio et al., 2010). Gibberellic acid induces efficient uptake and ion partitioning within the plant system, leading to enhanced growth and maintaining the metabolism of plants under normal and stress conditions (Iqbal and Ashraf, 2013). Under salt stress conditions, improved germination and growth due to gibberellic acid has been reported by several studies (Tuna et al., 2008; Ahmad, 2010; Manjili et al., 2012). In addition, gibberellins can exhibit crosstalk with other phytohormones, which elicits important responses and mediates tolerance mechanisms for enhancing stress tolerance. The synthesis of gibberellins can also be promoted through the application of other hormones, such as auxin (Wolbang et al., 2004). Enhanced synthesis of gibberellic acid leads to enhanced ABA catabolism. Moreover, gibberellic acid directly affects growth, yield, and mineral nutrition as well as nitrogen metabolism. Khan et al. (2004) reported increases in fruit yield, leaf area, and nitrogen, phosphorous, and potassium uptake in tomato due to the exogenous application of gibberellic acid. Moreover, an increase in osmotic components was reported in plants exposed to salt stress, and their content was further increased by gibberellic acid treatment. The endogenous application of GA resulted in amendment of osmotic stress in plants and maintenance of tissue water content (Ahmad, 2010). Such effects were observed by Manjili et al. (2012) for wheat and by Tuna et al. (2008) for maize. In addition, gibberellic acid enhanced antioxidant enzyme activity by lowering the levels of reactive oxygen species (ROS) which contributed to better growth under stress (Manjili et al., 2012). In addition, exogenous application of gibberellic acid mitigates salinity-induced effects on germination and growth in *Arabidopsis thaliana* by mediating enhanced synthesis of SA, which causes increased activity of isochorismate synthase 1 (Alonso-Ramirez et al., 2009). The same study also demonstrated that over-expression of the gibberellin-responsive gene from *Fagus sylvatica* enhanced the salt tolerance of *Arabidopsis*.

Salicylic Acid

Salicylic acid is another important phytohormone with a phenolic nature, and it has an important function in plant stress tolerance through modulation of antioxidative enzyme activities (Ahmad et al., 2011; da Silva et al., 2017). The alleviation of various abiotic stresses by application of SA was reported by Senaratna et al. (2000) for water stress, by Azooz et al. (2011) for salt stress and by Ahmad et al. (2011) for heavy metal stress. SA modulates several physiological processes involved in plant stress tolerance through stress activated signal pathways and response mechanisms (Ahmad et al., 2011; Janda et al., 2012; Khan et al., 2014). There are several reports on the alleviating effect of SA in plants, e.g., fava bean (Azooz et al., 2011), maize (Gunes et al., 2007), and wheat (Shakirova, 2007). Azooz et al. (2011) reported that the application of SA to sea water-treated *Vicia faba* plants not only ameliorated the negative effects on growth, biomass accumulation and antioxidant system but also caused efficient accumulation of organic osmolytes, such as proline and free amino acids. In salt-stressed *Vigna radiata*, L. Khan et al. (2014) reported a reduction in endogenous levels of ethylene due to SA application. The results published so far have shown that the application of SA promoted efficient sequestration and partitioning of deleterious ions, such as Na. Increased synthesis and accumulation of proline and ABA have been reported in salinity-stressed wheat seedlings, contributing to better growth and yield (Shakirova et al., 2003). In *Vigna radiata*, L. Khan et al. (2014) observed that treatment of seeds with SA helped to considerably mitigate salt stress-induced changes. SA-treated plants showed better growth in terms of biomass accumulation, promotion of cell division, and showed a higher photosynthetic rate and antioxidant enzyme activity (da Silva et al., 2017). In barley plants, salinity stress caused alterations in the rate of photosynthesis and membrane stability; however, these negative effects of salinity stress were ameliorated by the application of SA (Janda et al., 2012). Similar observations were reported by da Silva et al. (2017) in case of SA at 10^{-5} M which increased plant growth of sesame under drought stress (da Silva et al., 2017). The treatment of maize with SA reduced the accumulation of Na in plant tissue and mitigated salt-induced negative effects on plants (Gunes et al., 2007). In addition, SA inhibits lipid peroxidation, improves membrane stability (Azooz et al., 2011), sustains the transpiration rate, and decreases electrolyte leakage (Stevens et al., 2006). Tang et al. (2017) reported that SA application mitigates water stress by maintaining a lower ROS level. Several studies reported increased mitochondrial alternative oxidase (AOX) expression and activity by SA treatment; this enzyme plays an important role in tolerance to abiotic stresses (Zhang et al., 2010).

Altogether, these observations suggest that phytohormones play a vital role in plant tolerance to various abiotic stresses by modulating the physiological properties and defense system of plants. Since plants are closely associated with the microbes that live within plant tissues, microbial metabolites may have strong effect on plant physiological processes and metabolism. In earlier studies, Fulchieri et al. (1993) and Lucangeli and Bottini (1997) observed higher amounts of IAA and the gibberellin GA3 in the plant tissue of maize after the inoculation of plant growth promoting rhizobacteria (PGPR) strains. Similar observations were also reported by Fulchieri et al. (1993) in which *Azospirillum* increased levels of GA3 in maize seedlings. Thus, microbial phytohormones have vital importance in plant host metabolism and physiology under hostile environments.

ROOT-ASSOCIATED PHYTOHORMONE-PRODUCING MICROBES

Soils are sources of diverse organisms, including fungi, bacteria, and plants (Mendes et al., 2013). Plant roots are heavily colonized with microorganisms (compared to soil and other habitats) because of the rich nutrient component of root exudates (Schlaeppi and Bulgarelli, 2015; Hashem et al., 2016). The rhizosphere is a relatively nutrient-rich environment containing amino acids, sugars, fatty acids and other organic compounds, which attract microbes (Vorholt, 2012) that utilize the various nutrients released by the root. In turn, the microbes synthesize biologically active compounds, including phytohormones (auxins, cytokinins, gibberellins, and ABA), antifungal compounds, enzymes, and compatible solutes. These microbial metabolites play a vital role in plant growth, nutrition and development (Ruiz-Lozano et al., 2012; Sorty et al., 2016; Egamberdieva et al., 2017a). They can stimulate plant growth development, provide resistance to various abiotic and biotic stress factors, improve nutrient acquisition and protect plants from various soil-borne pathogens (Grover et al., 2013; Cho et al., 2015). The beneficial interactions of microbes in plants, their positive effect on plant growth and their improvement of stress tolerance under extreme environmental conditions have been extensively reviewed by Nadeem et al. (2014), and the mechanisms utilized by plant growth-promoting bacteria have been reviewed by Forni et al. (2017). There are several mechanisms of plant growth stimulation, plant protection and alleviation of salt stress by PGPR, such as nitrogen fixation; synthesis of osmoprotectants, exopolysaccharides, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, cell wall degrading enzymes, and phytohormones; modulation of antioxidant enzymes or nutrients; and solubilization of minerals, such as phosphorus, and potassium (Berg et al., 2013; Wang Q. et al., 2016; Mishra et al., 2017). The microbes mitigate stress responses by regulating the nutritional and hormonal balance in plants and inducing systemic tolerance to stress. One of the mechanisms of improvement of plant growth and stress tolerance by microbes is their phytohormone synthesizing ability in the rhizosphere or root tissue (Etesami et al., 2015). Microbial phytohormones affect the metabolism of endogenous growth regulators in plant tissue (Hashem et al., 2016; Sorty et al., 2016) and play a key role in changing root morphology upon exposure to drought, salinity, extreme temperature and heavy metal toxicity (Spaepen et al., 2008; Khan et al., 2011).

Root-associated microbes, including free living, symbiotic or endophytic microbes, can produce various type of phytohormones and belong to different genera and species (Sgroy et al., 2009). For example, Sorty et al. (2016) isolated diverse groups of organisms belonging to *Acinetobacter, Bacillus, Enterobacter, Pantoea, Pseudomonas, Rhizobium,* and *Sinorhizobium* from halotolerant weed (*Psoralea corylifolia* L.), and Egamberdieva et al. (2016) found *Arthrobacter, Bacillus, Enterobacter, Pseudomonas, Rhizobium, Brevibacillus, Cellulosimicrobium, Mycobacterium, Ochrobactrum, Paenibacillus,* and *Pseudoxanthomonas* associated with soybean root. The IAA-producing *Mycobacterium* species was observed in the rhizosphere of orchid (Tsavkelova et al., 2007), and *Azotobacter, Azospirillum, Cellulomonas, Mycoplana,* and *Rahnella* were found in the wheat rhizosphere (Egamberdiyeva and Hoflich, 2003; Egamberdieva et al., 2008). In other reports, *Pseudomonas* spp. (Lawongsa et al., 2008), *Arthrobacter* spp. (Piccoli et al., 2011), and *Enterobacter, Pseudomonas,* and *Stenotrophomonas* species were associated with plants that produced IAA (Khan and Doty, 2009). Piccoli et al. (2011) isolated the endophytic diazotrophic bacterium *Arthrobacter koreensis* which produce ABA, IAA, GA3 and jasmonic acid from the roots of the halophyte shrub *Prosopis strombulifera*. The endophytic strains of *Klebsiella* and *Enterobacter* isolates from sugar cane synthesize IAA (de Santi Ferrara et al., 2012). Mishra et al. (2017) isolated bacteria with IAA production ability from extreme environments, which were identified as *Pseudomonas* spp. and *Ochrobactrum* spp. In other studies *Halomonas desiderata*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis, Escherichia coli,* and *Pseudomonas fluorescens* G20-18 were reported to synthesize cytokinins (Salamone et al., 2001; Karadeniz et al., 2006; Großkinsky et al., 2016). Bacterial isolates from the rhizosphere of a vegetable (bitter gourd) belonging to genera *Bacillus*, *Klebsiella, Leifsonia,* and *Enterobacter* were able to produce IAA and improved maize growth in Cd-contaminated soil (Ahmad et al., 2016).

Naz et al. (2009) also observed cytokinin-producing species, such as *Arthrobacter, Bacillus, Azospirillum,* and *Pseudomonas*, that stimulated the root development of plants. ABA was also detected in root-associated microbes from various plants. Karadeniz et al. (2006) reported *Proteus mirabilis*, *Phaseolus vulgaris, Klebsiella pneumoniae, B. megaterium*, and *B. cereus* as ABA-producing bacteria. Species such as *Bacillus pumilus*, *Bacillus licheniformis*, *Acetobacter* sp., *Bacillus* sp., *Azospirillium* sp. were found among gibberellin-producing strains (Gutiérrez-Mañero et al., 2001; Bottini et al., 2004). Salomon et al. (2014) observed ABA-producing *B. licheniformis* Rt4M10 and *P. fluorescens* Rt6M10 in the rhizosphere of *Vitis vinifera*. *Achromobacter xylosoxidans* SF2, isolated from sunflower roots, was also able to produce ABA in minimal medium (Forchetti et al., 2007). Among IAA-producing bacteria associated with plants grown under saline soil, Rhizobia have also been shown to synthesize auxins, cytokinins and abscicic acids, increase plant growth and development and improve the yield of agricultural crops (Hayat et al., 2008). Actinobacteria have also been found to produce IAA, CK, GB-like substances (Shutsrirung et al., 2013; Vijayabharathi et al., 2016). Ruanpanun et al. (2010) found high IAA-producing nematophagous actinomycete and fungal isolates, such as *Aspergillus* and *Streptomyces*. In other studies, *Streptomyces* sp. Isolated from medicinal plant species *Taxus chinensis* and *Artemisia annua* showed IAA synthesis ability (Lin and Xu, 2013). Shutsrirung et al., 2013 reported IAA production in endophytic actinomycetes *Streptomyces, Nocardia, Nocardiopsis, Spirillospora, Microbispora,* and *Micromonospora* associated with mandarin.

The stress tolerance ability of bacterial strains provides important benefits to plants. The ability of root-associated microbes to synthesize phytohormones is typically not hampered by high salt concentrations (Egamberdieva and Kucharova, 2009). For example, phytohormone synthesis by endophytic actinobacteria *Streptomyces coelicolor* DE07 and *Streptomyces geysiriensis* DE27 was not inhibited under water stress (Yandigeri et al., 2012). The production of IAA by *A. brasilense* in osmotic stress conditions was higher than that of osmosensitive *A. brasilense* Sp7 (Nabti et al., 2007). In another study, *Pseudomonas putida, Pseudomonas extremorientalis, Pseudomonas chlororaphis,* and *P. aurantiaca* were able to produce IAA in a 4% NaCl conditions (Egamberdieva and Kucharova, 2009). *Pseudomonas* sp. and *Bacillus* sp. strains were able to produce IAA under high salt conditions (200–400 mM NaCl) and increased the plant biomass of *Sulla carnosa* under salt stress (Hidri et al., 2016).

The biosynthesis of phytohormones differs by bacterial strain. For example, *Bacillus* and *Pseudomonas* strains synthesized IAA concentrations up to 2.2 $\mu g\ mL^{-1}$, GA3 production by *A. xylosoxidans* and *B. halotolerans* was between 36.5 and 75.5 $\mu g\ mL^{-1}$ (Sgroy et al., 2009). In addition, ABA production was 0.3, 1.8, and 4.2 $\mu g\ mL^{-1}$ in the culture medium of *L. fusiformis* (Ps14), *B. subtilis* (Ps8), and *P. putida* (Ps30), respectively. In another study, *Bacillus amyloliquefaciens* associated with rice (*Oryza sativa* L.) synthesized gibberellins, and the quantities of GA differed, e.g., 17.8 ng mL^{-1} for GA20, 5.7 ng mL^{-1} for GA36, 5.6 ng mL^{-1} for GA24, 1.02 ng mL^{-1} for GA4, 0.7 ng mL^{-1} for GA53, 0.08 ng mL^{-1} for GA5, and 0.01 ng mL^{-1} for GA8 (Shahzad et al., 2016). Endophytic fungi *Aspergillus fumigatus* associated with soybean roots synthesized gibberellins, such as GA4 (24.8 ng mL^{-1}), GA9 (1.2 ng mL^{-1}), and GA12 (9.8 ng mL^{-1}) (Khan et al., 2011). Several studies reported SA production by root-associated bacteria, e.g., *B. licheniformis* MML2501 (18 $\mu g\ mL^{-1}$) (Shanmugam and Narayanasamy, 2008), and *Pseudomonas* sp. PRGB06 (6.8 $\mu g\ mL^{-1}$) (Indiragandhi et al., 2008).

MICROBIAL PHYTOHORMONES IN PLANT STRESS TOLERANCE

Microbes synthesize low amounts of phytohormones and improve stress tolerance and plant growth under various stress conditions, including salinity, heat, drought and metal toxicity, as reported in many studies (Sgroy et al., 2009; Egamberdieva et al., 2011, 2017b; Liu Y. et al., 2013). The beneficial effect of phytohormone-producing microbes on alleviating abiotic stress in plants was reported in numerous studies (**Figure 1**; Khan and Doty, 2009; Ngumbi and Kloepper, 2014; Hashem et al., 2016). Some examples of phytohormone-producing bacteria and their ability to mitigate abiotic stress are given in **Table 1**. Many studies have reported the positive effects of bacteria associated with plants and IAA production on plant growth stimulation under abiotic stress conditions. For example, bacterial strains *Curtobacterium flaccumfaciens* E108 and *Ensifer garamanticus* E110 isolated from *Hordeum secalinum* stimulated plant biomass and salt stress resistance in barley (Cardinale et al., 2015). The root-colonizing halotolerant bacterium *B. licheniformis* HSW-16 was able to mitigate salt stress-induced damage and stimulate the growth of wheat through the production of IAA under saline soil conditions (Singh and Jha, 2016). Similar observations were reported by Upadhyay et al. (2012) in which salt-tolerant bacterial strains *B. subtilis* and *Arthrobacter* sp. increased wheat biomass and total soluble sugars and reduced sodium concentration in plant tissue. Sorty et al. (2016) isolated salt-tolerant strain *Enterobacter* sp. NIASMVII from halotolerant weed (*Psoralea corylifolia* L.), which produces IAA (0.22 and 25.58 $\mu g\ mL^{-1}$) and enhances seed germination of wheat (*Triticum aestivum* L.). In another study, *Pseudomonas* spp. isolated from extreme environments (close to the sites of volcanos) synthesized IAA under salt stress (500 mM NaCl) and high temperature (40°C), and they were able to stimulate increases in the root and shoot biomass of maize (Mishra et al., 2017). According to Bianco and Defez (2009), protection of plants from negative effects of abiotic stress by IAA is related to enhanced cellular defense systems. Several salt-tolerant strains synthesizing IAA in culture medium, namely, *Serratia plymuthica* RR-2-5-10, *Stenotrophomonas rhizophila* e-p10, *P. fluorescens* SPB2145, *P. extremorientalis* TSAU20, and *P. fluorescens* PCL1751, improved cucumber biomass and yield in greenhouse conditions (9–24%) (Egamberdieva et al., 2011). Root-associated IAA-producing bacteria were found to improve drought stress in plants. Marulanda et al. (2009) observed increased plant biomass in clover (*Trifolium repens* L.) after seed treatment with *P. putida* and *B. megaterium* under drought, and they found a correlation between these changes and increased IAA. IAA-producing bacteria were also found to improve plant growth and development under nutrient-poor soil conditions. *Serratia* sp. isolated from chickpea nodules was found to produce IAA, which led to an increased grain yield of chickpea in nutrient-deficient soil (Zaheer et al., 2016). Many fungal species were also able to produce plant growth regulators and alter plant root system and physiology. Contreras-Cornejo et al. (2009) observed increased lateral root formation, root hair growth and modified root system architecture from *Trichoderma virens* inoculation, which resulted in increased plant biomass of *Arabidopsis thaliana*.

Microbial phytohormones also play an important role in metal-plant interactions, improving phytoextraction by plants. *A. xylosoxidans* Ax10 improved the root system of the *Brassica juncea* plant through IAA production activities, which increased copper phytoextraction (Ma et al., 2008). Similar results were observed by Zaidi et al. (2006) in which *B. subtilis* synthesizing IAA stimulated root growth and Ni accumulation in the Indian mustard plant (*B. juncea* L.). *B. megaterium* MCR-8, which produced auxin at a concentration of 68.5 mg 25 mL^{-1}, alleviated Ni stress in *Vinca rosea* and stimulated root and shoot growth. In addition, plant treatment with *B. megaterium* MCR-8 increased the accumulation of total phenols, flavonoids and defense-related enzymes, such superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), compared to uninoculated plants under Ni stress (Khan et al., 2017). In another study, Ahmad et al. (2016) observed inhibited seed germination and seedling growth of maize by Cd stress,

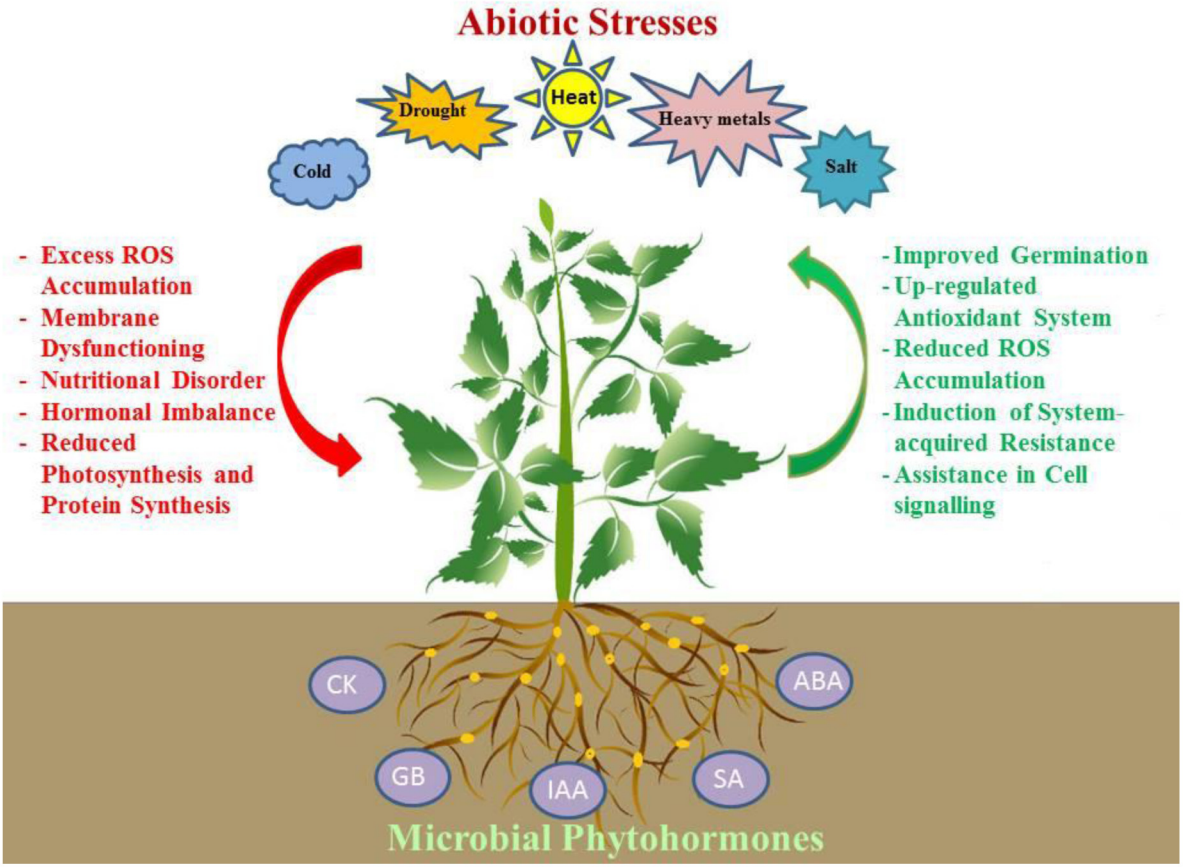

FIGURE 1 | An overview of mechanisms in microbial phytohormone-mediated plant stress tolerance. Several root associated microbes produce cytokinin (CK), gibberellin (GB), indole-3-acetic acid (IAA), salicylic acid (SA) and abscisic acid (ABA), which help plants to withstand stress by enhancing its antioxidant potential, by up-regulation of the antioxidant system and by accumulation of compatible osmolytes thus reducing oxidative stress-induced damage; improving photosynthetic capacity and membrane stability; promoting cell division and stomatal regulation; stimulating growth of root system, and acquisition of water and nutrients.

whereas Cd-tolerant and IAA-producing bacteria *Leifsonia* sp. and *Bacillus* sp. significantly increased shoot and root growth of maize in Cd-contaminated soil compared to controls. Similar observations were reported by Dourado et al. (2013) in which Cd-tolerant multi-tolerant bacteria *Burkholderia* sp. SCMS54 produced IAA and improved plant growth and stress tolerance of tomato to Cd stress. Islam et al. (2016) reported that Cr toxicity significantly inhibited maize growth, negatively affecting its physiological processes, such as photosynthetic pigment and carbohydrate metabolism, and increasing its levels of proline, H_2O_2, and MDA. In these conditions, Cr-resistant *P. mirabilis* isolates T2Cr and CrP450, combined with SA, mitigated the toxic effect of Cr, improved the root and shoot growth of maize and reduced oxidative stress in maize tissue by elevating its antioxidant activities.

The tripartite interaction of root-associated microbes with symbiotic microbes and the host plant is also a mutualistic interaction that improves plant growth under stress through the induction of osmoregulation, hormonal balance, biochemical processes and changes in metabolic interfaces among partners (Nadeem et al., 2014; Park et al., 2017). IAA-producing *B. subtilis* NUU4 in combination with *Mesorhizobium ciceri* IC53 stimulated root and shoot biomass and improved nodule formation in *chickpea* (*Cicer arietinum* L.) under salt stress, as compared to uninoculated plants and plants inoculated with *Mesorhizobium ciceri* IC53 alone (Egamberdieva et al., 2017c) (**Figure 2**).

The positive effect on root development by cytokinin-producing bacterial strains was also reported in many studies. For example, inoculation of maize with cytokinin-producing bacteria *Micrococcus luteus* chp37 isolated from the desert of Pakistan stimulated shoot and root biomass by 54% and modulated the physiological properties of the plant, including photosynthetic pigments, under drought conditions (Raza and Faisal, 2013). The cytokinin-producing root-associated bacteria strains *Arthrobacter, Bacillus, Azospirillum,* and *Pseudomonas* increased soybean shoot and root biomass as well as proline content in plant tissue under salt stress (Naz et al., 2009). A similar observation was reported by Liu F. et al. (2013) in which cytokinin-producing *B. subtilis* stimulated root biomass of *Platycladus orientalis* (oriental thuja) by 13.9% and increased cytokinin concentration of 47.52% in leaves relative to respective

TABLE 1 | Some examples of phytohormone-producing bacteria and their ability to mitigate abiotic stress.

Microorganisms	Phytohormone	Host plant, abiotic stress	Reference
Pseudomonas sp., *Bacillus* sp.	IAA	*Sulla carnosa* (Desf.), Salt stress	Hidri et al., 2016
Bacillus licheniformis	IAA	*Triticum aestivum* L., Salt stress	Singh and Jha, 2016
Bacillus subtilis, Arthrobacter sp.	IAA	*Triticum aestivum* L., Salt stress	Upadhyay et al., 2012
Pseudomonas putida, Bacillus megaterium	IAA	*Trifolium repens*, Drought stress	Marulanda et al., 2009
Marinobacterium sp., *Pseudomonas* sp., *Rhizobium* sp., *Sinorhizobium* sp.	IAA	*Triticum aestivum* L., Salt stress	Sorty et al., 2016
Serratia plymuthica, Stenotrophomonas rhizophila, Pseudomonas fluorescens, *Pseudomonas extremorientalis*	IAA	*Cucumis sativus,* Salt stress	Egamberdieva et al., 2011
Acinetobacter faecalis, Bacillus cereus, Enterobacter hormaechei, Pantoea agglomerans	IAA	*Triticum aestivum* L., Salt stress	Egamberdieva et al., 2008
Curtobacterium flaccumfaciens, Ensifer garamanticus	IAA	*Hordeum vulgare*, Salt stress	Cardinale et al., 2015
Streptomyces coelicolor, Streptomyces geysiriensis	IAA	*Triticum aestivum* L., Salt stress	Yandigeri et al., 2012
Bacillus subtilis	IAA	*Acacia gerrardii* Benth., Salt stress	Hashem et al., 2016
Pseudomonas sp.	IAA	*Zea mays,* Salt and heat stresses	Mishra et al., 2017
Serratia sp.	IAA	*Cicer arietinum* L., Nutrient stress	Zaheer et al., 2016
Achromobacter xylosoxidans	IAA	*Brassica juncea,* Cu stress	Ma et al., 2008
Pseudomonas putida	IAA	*Glycine max* (L.) Merr., Salt stress	Egamberdieva et al., 2017b
Leifsonia sp., *Bacillus* sp.	IAA	*Zea mays,* Cd stress	Ahmad et al., 2016
Burkholderia sp.	IAA	*Solanum lycopersicum* L., Cd stress	Dourado et al., 2013
Bacillus subtilis	IAA	*Brassica juncea* L., Ni stress	Zaidi et al., 2006
Bacillus megaterium	IAA	*Vinca rosea* L., Ni stress	Khan et al., 2017
Achromobacter xylosoxidans, Bacillus pumilus	SA	*Helianthus annuus*, Drought stress	Forchetti et al., 2010
Serratia marcescens	SA	*Zea mays*, Salt stress	Lavania and Nautiyal, 2013
Micrococcus luteus	CK	*Zea mays,* Drought stress	Raza and Faisal, 2013
Arthrobacter sp., *Bacillus* sp., *Azospirillum* sp.	CK	*Glycine max* (L.) Merr., Salt stress	Naz et al., 2009
Bacillus subtilis	CK	*Platycladus orientalis*, Drought stress	Liu F. et al., 2013
Aspergillus fumigatus	GA	*Glycine max* (L.) Merr., Salt stress	Khan et al., 2011
Azospirillum lipoferum	GA	*Triticum aestivum* L., Drought stress	Creus et al., 2004
Phoma glomerata, Penicillium sp.	GA	*Cucumis sativus,* Drought stress	Waqas et al., 2012
Bacillus amyloliquefaciens	ABA	*Oryza sativa* L., Salt stress	Shahzad et al., 2017
Bacillus licheniformis, *Pseudomonas fluorescens*	ABA	*Vitis vinifera* L., Water stress	Salomon et al., 2014
Trichoderma asperellum	IAA, GA, ABA	*Cucumis sativus,* Salt stress	Zhao and Zhang, 2015
Bacillus aryabhattai	IAA, GA, ABA	*Glycine max* (L.) Merr., Heat stress	Park et al., 2017

controls under water stress conditions. The higher content of cytokinin in plant tissue contributed to stomatal opening and alleviated some of the detrimental effects of water stress.

Aspergillus fumigatus produced gibberellins, such as GA4 (24.8 ng mL^{-1}), GA9 (1.2 ng mL^{-1}), and GA12 (9.8 ng mL^{-1}), which increased photosynthetic pigments, and shoot biomass of soybean under salt stress (Khan et al., 2011). *Azospirillum lipoferum,* which synthesizes GA, increased the stress tolerance of wheat to drought (Creus et al., 2004). Waqas et al. (2012) also reported improved salt and drought stress tolerance in cucumber plant by GA-producing endophytic fungi *Phoma glomerata* LWL2 LWL3, which produced GA1 (8.720 ng mL^{-1}), GA3 (2.420 ng mL^{-1}) and GA4 (0.220 ng mL^{-1}), and *Penicillium* sp., which produced GA1 (5.33 ng mL^{-1}) and GA3 (3.42 ng mL^{-1}) in culture filtrate. The fungal inoculation resulted in increased root and shoot growth and nutrient uptake, and reduced stress by down-regulating ABA and modifying SA and jasmonic acid concentrations in plant tissue. It is known that ABA and SA act as defense signaling constituents (Shinozaki and Yamaguchi-Shinozaki, 2007).

Salomon et al. (2014) observed ABA production by *B. licheniformis* and *P. fluorescens* that stimulated plant growth of grapevine under water stress by inducing ABA synthesis. Shahzad et al. (2017) reported ABA production by *Bacillus amyloliquefaciens* RWL-1 (0.32 ± 0.015–0.14 ± 0.030 ng mL^{-1}) under normal and saline conditions. Bacterial inoculation significantly increased root and shoot growth and the concentration of SA in plant tissue of rice under salt stress conditions. Park et al. (2017) isolated *Bacillus aryabhattai* strain SRB02 from the rhizosphere of soybean, and it significantly promoted the plant biomass and nodule number of soybean. The strains produced up to 2 ng mL^{-1} ABA in culture and increased the drought stress tolerance of soybean through stomatal closure under high temperatures (38°C) relative to control plants.

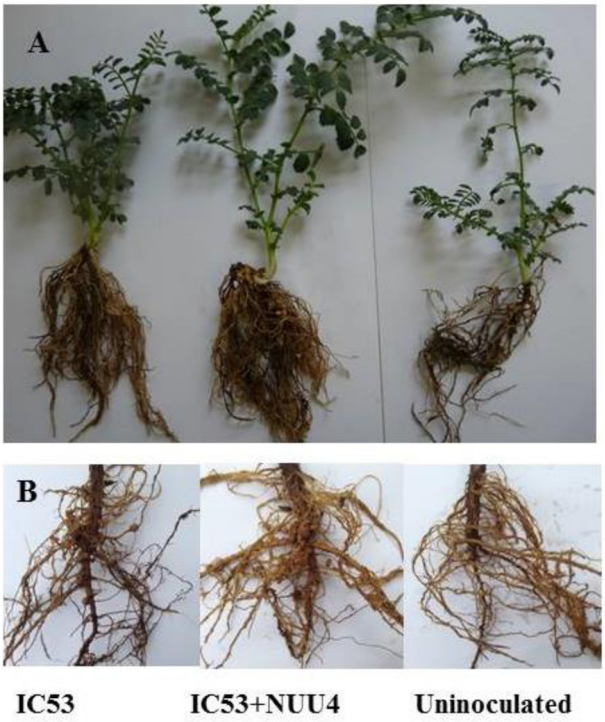

FIGURE 2 | Growth of chickpea in salinated soil after inoculation with *Mesorhizobium ciceri* IC53 alone or with the combination of *Mesorhizobium ciceri* IC53 and IAA-producing *Bacillus subtilis* NUU4 in pots **(A)** and under field condition **(B)** (Figure as originally published in Egamberdieva et al., 2017c).

Similar to the effects of other phytohormones, SA-producing endophytic bacteria *A. xylosoxidans* and *B. pumilus* also enhanced the biomass of sunflower seedlings under drought conditions (Forchetti et al., 2010). Similar observations were reported by Lavania and Nautiyal (2013) in which salt-tolerant SA-producing *Serratia marcescens* NBRI1213 stimulated root and shoot growth as well as nutrient acquisition by maize, and furthermore increased plant stress tolerance to salinity.

Some bacteria may produce several types of phytohormones in plant tissue that interact to modulate important physiological processes in plants, including hormonal balance. *Sphingomonas* sp. LK11 and *Serratia marcescens* TP1 produced 12.31 and 10.5 μM mL^{-1} of IAA in the culture broths, which stimulated root and shoot growth of soybean through increases in ABA and gibberellin and a decrease in jasmonic acid content compared to levels in the control plants (Asaf et al., 2017). *Trichoderma asperellum* Q1, which produces IAA, GA and ABA, stimulated the biomass fresh weight of cucumber seedlings under salt stress in comparison to untreated control plants (Zhao and Zhang, 2015). In addition, the concentration of phytohormones IAA, GA and ABA in cucumber leaves were also increased after application of *Trichoderma asperellum* Q1 under salt stress. Similar observations were reported by Park et al. (2017) for soybean inoculated with *Bacillus aryabhattai* SRB02, which produces IAA, GA, and ABA. The root and shoot growth and heat stress of soybean plants increased after bacterial inoculation. In addition, higher concentrations of IAA, JA, GA12, GA4, and GA7 were observed in plant tissue of *Bacillus aryabhattai* SRB02-treated plants. Similar observations were reported for maize inoculated with ABA-producing *Azospirillum lipoferum* and *A. brasilense* sp. 245 in which bacterial treatment resulted in an increased concentration of ABA in plant tissue (Cohen et al., 2015). These studies demonstrate the involvement of phytohormone modulation in plant tissue by plant-associated microbes that induce the stress tolerance of plants.

CONCLUSION AND FUTURE PROSPECTS

Overall, evidence was provided that the exogenous application of phytohormones of microbial origin is an important tool for increasing the abiotic and biotic stress tolerance of plants, providing potential practical applications under changing or extreme environmental conditions. The beneficial effects on plants mediated by microbes, such as the stimulation of plant growth, tolerance to abiotic stresses and resistance to pathogens, are based on the microbes' ability to produce auxins, gibberellins, SA, ABA, and cytokinins in plant tissues. Thus, plant-associated microbes hold the potential to modulate hormone levels and metabolism in plant tissue, especially in biochemical processes that can prevent the damaging effects of external stresses, such as drought, salinity, nutrient deficiency, or heavy metal contamination. Optimizing phytohormone balance in plant tissues under stress by beneficial microbes could be a crucial challenge in the development of sustainable approaches to crop production. More experimental studies on various plant species are needed to determine whether these are plant-specific traits and to better understand the mechanisms involved in the interactions between microbial metabolites and the host that help plants optimize their responses in hostile environments. More specifically, it can be worthwhile to employ loss-of-function or gain-of-function genetic mechanisms to explore the associated mechanisms or reveal the antagonistic or synergistic interactions of phytohormones. The identification of receptors leading to the expression of specific genes after the application of a microbial phytohormone is also an important topic. Furthermore, studies on the performance of phytohormone-producing microbes in field experiments are necessary, and they should include competition for nutrients and niches between the microbial inoculant and the indigenous microflora. Moreover, investigations of host-microbe-stress interactions and their involved mechanisms using omics-based approaches, such as proteomics, genomics, metagenomics, and metabolomics, are needed.

AUTHOR CONTRIBUTIONS

DE, SW, and EA designed and wrote the manuscript. AH and AA edited and helped in finalizing the manuscript.

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Influences of Canopy Nitrogen and Water Addition on AM Fungal Biodiversity and Community Composition in a Mixed Deciduous Forest of China

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Nitrogen (N) deposition and precipitation could profoundly influence the structure and function of forest ecosystems. However, conventional studies with understory additions of nitrogen and water largely ignored canopy-associated ecological processes and may have not accurately reflected the natural situations. Additionally, most studies only made sampling at one time point, overlooked temporal dynamics of ecosystem response to environmental changes. Here we carried out a field trial in a mixed deciduous forest of China with canopy addition of N and water for 4 years to investigate the effects of increased N deposition and precipitation on the diversity and community composition of arbuscular mycorrhizal (AM) fungi, the ubiquitous symbiotic fungi for the majority of terrestrial plants. We found that (1) in the 1st year, N addition, water addition and their interactions all exhibited significant influences on AM fungal community composition; (2) in the 2nd year, only water addition significantly reduced AM fungal alpha-diversity (richness and Shannon index); (3) in the next 2 years, both N addition and water addition showed no significant effect on AM fungal community composition or alpha-diversity, with an exception that water addition significantly changed AM fungal community composition in the 4th year; (4) the increment of N or water tended to decrease the abundance and richness of the dominant genus *Glomus* and favored other AM fungi. (5) soil pH was marginally positively related with AM fungal community composition dissimilarity, soil NH_4^+-N and N/P showed significant/marginal positive correlation with AM fungal alpha-diversity. We concluded that the effect of increased N deposition and precipitation on AM fungal community composition was time-dependent, mediated by soil factors, and possibly related to the sensitivity and resilience of forest ecosystem to environmental changes.

Keywords: nitrogen deposition, precipitation, AM fungi, forest ecosystem, community composition, climate change, canopy

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi can form mutualistic symbioses with the majority of terrestrial plants (Smith and Read, 2008) and provide vital ecological services such as improving plant mineral nutrition (Li et al., 2006; Subramanian et al., 2006), enhancing plant tolerance to biotic (Elsen et al., 2008; Affokpon et al., 2011) and abiotic stresses (e.g., flooding, high temperature) (Fougnies et al., 2007; Li et al., 2009; Zhu et al., 2011; Camprubi et al., 2012), altering the composition and diversity of plant communities and influencing the productivity, structure and stability of ecosystems (van Der Heijden et al., 1998, 2008; Jansa et al., 2008), and intensifying the resilience of ecosystem to global climate change (Martínez-García et al., 2017). In view of their ecological significance, investigation on AM fungal diversity and community assemblage has become hot topics in soil ecology in recent years. AM fungal community assembly could be predicted by both niche theory which assumes that the competition among species for limited resources and the differentiation of niche space across species allow species coexistence, emphasizing the importance of determined processes in structuring community assembly (Leibold and McPeek, 2006), and neutral theory which presumes that all species are ecologically equivalent, emphasizing the significance of stochastic processes and dispersal limitation depending on spatial scales (Hubbell, 2001). At global and regional scales, neutral theory weighs more than the ecological niche theory, and AM fungal distribution pattern is mainly shaped by geographical distance and climate factors. However, the ecological niche theory dominates at local scale and small scale, and the effects of host plants and the soil properties on AM fungal community become more important than geographical distance restriction (Chen et al., 2018).

In recent decades, global climate change driven by anthropogenic disturbance has been intensified and impacted the structure and function of multiple aquatic and terrestrial ecosystems (Marino et al., 2017). As an important component of global climate change, nitrogen (N) deposition and its ecological consequences have attracted serious concerns. At global scale, it is estimated that the N deposition rate has increased nearly 34 Tg N yr^{-1} in 1860–100 Tg N yr^{-1} in 1995 and may increase up to 200 Tg N yr^{-1} in 2050 (Galloway et al., 2004). Increased N deposition could cause negative effects on terrestrial ecosystems, such as biodiversity loss, soil acidification, productivity decline, nutrient imbalance and forest degradation (Vitousek et al., 1997; Magill et al., 2004; Hogberg et al., 2006; Bobbink et al., 2010; Lu et al., 2014). Most previous studies on N deposition are carried out in Europe and North America, while studies on N deposition and its ecological consequences in China are rather limited. As a fact, with the rapid development, China is also experiencing increasing N deposition, especially in its central and southeastern areas (Jia et al., 2014). The mean wet N deposition over China has increased nearly 25% from 1990s to 2000s (Jia et al., 2014), and the N deposition rate in China is predicted to continually increase in the coming decades (Liu et al., 2013).

Besides increased N deposition, changes in precipitation patterns is also an important component of global change. According to IPCC report, heavy precipitation events including the frequency and intensity of heavy precipitation over land regions increased markedly in recent years. In many mid-latitude regions, mean precipitation will gradually increase in the 21st century (Integrated Professional Competency Course [IPCC], 2013). Increased precipitation could change species richness and alter the plant community structure and aboveground net primary productivity (ANPP) in arid and semi-arid (water-limited) steppe ecosystems (Yang et al., 2011; Zeppel et al., 2014; Ren et al., 2015). Intensified precipitation can also elevate the risk of soil nutrient leaching (e.g., N, P) (Martínez-García et al., 2017). Moreover, under natural conditions, multiple global changes may occur simultaneously and interact with each other (Harpole et al., 2007). The impacts of N deposition on the ecosystem structure and function would be substantially altered by precipitation (Harpole et al., 2007; Yang et al., 2011; Araya et al., 2013). Previous studies showed that precipitation increment could alleviate the negative effects of increased N deposition by increasing the mobility and leaching of soil inorganic N (Li et al., 2016; Sun et al., 2017). More extensive studies on the interactive effects of N deposition and precipitation on ecosystems are still expected.

Up to date, very limited information is available as for the responses of belowground ecosystem especially for soil microbial communities, to climate changes (Li et al., 2016). Definitely more attention should be paid to the soil microorganisms which play important roles in nutrient cycling, organic matter decomposition, primary production, regulation of greenhouse emissions and other ecosystem functions (Philippot et al., 2013; Wagg et al., 2014; Delgado-Baquerizo et al., 2017). As an important functional group of soil microbes, AM fungi directly bridge up plant and soil, and are selected as model organism for studying belowground-aboveground interactions. It has been well documented that increased N deposition decreased the abundance (van Diepen et al., 2007, 2010; Camenzind et al., 2014, 2016), richness (Camenzind et al., 2014; Liu et al., 2014; Chen et al., 2017) and diversity (Chen et al., 2017), and altered the community composition (van Diepen et al., 2011; Chen et al., 2014; Zheng et al., 2014; Kim et al., 2015) of AM fungi. Precipitation increment can also decrease the abundance and alpha-diversity (Chen et al., 2017), and alter community composition (Gao et al., 2016; Chen et al., 2017) of AM fungi in semiarid (water-limited) steppe ecosystem. However, as far as we know, there were only limited reports on the interaction of N deposition and precipitation on AM fungal communities, and they all were carried out in water-limited steppe ecosystems (Li et al., 2015; Chen et al., 2017). The interaction could be different in humid forest ecosystem from arid/semi-arid steppe ecosystems. In addition, most previous studies on the ecological impacts of precipitation increment or N deposition on forest ecosystems largely ignored many canopy-associated ecological processes by understory addition of N or water. The canopy-associated processes may include N uptake by leaves, epiphytes and microbes; immobilization in decaying leaves or other dead organic matters; volatilization as water evaporates; and transformation of inorganic N to organic N (Zhang et al., 2015). Undoubtedly, canopy

processes are more important in forest than in grassland and should not be neglected. As seen in report, the percentage of retained N from N deposition by forest canopy could vary in different studies from 1∼5% to 10∼25%, depending on forest type and N deposition intensity (Zhang et al., 2015).

Furthermore, AM fungal community structure exhibits seasonal dynamics and interannual variability (Husband et al., 2002; Hazard et al., 2014). Husband et al. (2002) investigated the diversity and distribution of AM fungi colonizing tree seedling roots for 2 years in the tropical forest on Barro Colorado Island, Republic of Panama. They found that dominant AM fungal types in the first year were nearly entirely replaced by previously rare types in the following year; Hazard et al. (2014) investigated the effects of biosolids on AM fungal communities in grassland and arable agroecosystems and found that the effect of seasonality exceeded that of biosolids application. The AM fungal community compositions (using T-RFLP method) associated with *Lolium perenne* shifted with seasonality and year, some dominant AM fungi (e.g., T-RFs associated with *Rhizophagus irregularis*) were present in roots throughout and between years, others were only present seasonally (e.g., *HinfI*-HEX 422), and rarer species fluctuated in presence and frequency. However, many studies only made one sampling and overlooked the temporal dynamics of AM fungal community in response to environmental changes.

As a result, we conducted a field trial in a mixed forest in China's climate transition zone from subtropical to warm temperate climate to investigate the impacts of N deposition and precipitation increment on AM fungal community with canopy N and water addition. We carried out field investigation and collected soil samples every year since the experiment establishment in 2013, and analyzed AM fungal diversity and community structure by using the high throughput sequencing technology. We hypothesized that (1) canopy N or water addition would significantly decrease AM fungal richness, Shannon diversity index and change community composition; (2) N and water addition interactively shape AM fungal community; (3) the effects of N and water addition on AM fungal community would be time-dependent. To the best of our knowledge, this study for the first time investigated the interactive effects of increased N deposition and precipitation on AM fungal diversity and community composition in a forest ecosystem and is expected to allow better understanding of the impacts of climate changes on the forest ecosystems.

MATERIALS AND METHODS

Study Site

The study site was located in Jigongshan (JGS) National Nature Reserve (31°46′-31°52′ N, 114°01′-114°06′ E) representing a transitional zone from subtropical to warm temperate climate in China (Zhang et al., 2015). Dominant tree species in the forest are *Quercus acutissima* Carruth., *Quercus variabilis* Bl. and *Liquidambar formosana* Hance. Both oaks form ectomycorrhiza, while *Liquidambar formosana* Hance can form arbuscular mycorrhiza. In addition, there are some understory shrubs, such as *Lindera glauca* (Sieb.et Zucc.) Bl and *Rubus lambertianus* Ser., and herbs such as *Ophiopogon japonicas* (Linn. f.) Ker-Gawl. and *Phaenosperma globosa* Munro ex Benth. which also can form arbuscular mycorrhiza. The soil type is Ferri-Udic Argosols (yellow brown soils) and the pH value is 5.0-6.0.

According to the meteorological data from 1951 to 2011, the local mean annual temperature (MAT) is 15.2°C and the mean annual precipitation (MAP) is 1119 mm. 80% of the precipitation occurs in April- October. The average annual air humidity is 79%. The background N deposition rate in rainfall is 19.6 kg N $ha^{-1}yr^{-1}$, in which the NH_4^+/NO_3^- ratio is close to 1 (Zhang et al., 2015; Zhang et al., 2018). More information of JGS Reserve were described by Zhang et al. (2015).

Experimental Design

This experiment was set up as a fully randomized two-factor block design with four blocks, each block included four plots, and each plot was 17 m in radius. Within each block, each plot was randomly assigned with one of the four treatments: control (CK, ambient environment), canopy addition of N (CN), canopy addition of water (CW), and canopy additions of both N and water (CNW). In order to prevent the interference among treatments, a 20 m buffer zone was left between any two plots and a PVC isolation board with depth of 1 m was installed in the middle of the buffer zone.

The N application rate was 50 kg ha^{-1} yr^{-1}, which is predicted to occur in the near future in this region by Liu et al. (2013). The amount of water addition was 30% of the MAP (336 mm), and the magnitude was within the range of model predictions for future precipitation increment induced by warming in Northern Hemisphere by Yang et al. (2015). Nitrogen was added as NH_4NO_3 solution (7.7 mmol/L), both the solvent and water are from the local lake. Nitrogen and water were added during the growing season from April to October, and N was applied once a month (totally seven times per year), while water was added once a week (12 mm per week) to prevent surface runoff (Shi et al., 2018). The treatment dates were determined according to the phenology of the forest, i.e., the first time was conducted 1 week before all buds began to open (mid-April), and the last was conducted as leaf litter began to fall (mid-October) (Shi et al., 2016).

All the treatments except CK were realized with a forest canopy spraying system built in the center of the plots. This system can pump N solution or water to a height of 35 m (almost 5 m above the forest canopy) through PVC pipes (10 cm in diameter) which were fixed on the supporting tower. The N solution or water was evenly sprayed onto the forest canopy of the plot by four sprinklers with different spraying range and could be rotated 360° freely (Shi et al., 2018). The working rules and efficiency of the system were described in details by Zhang et al. (2015) and Shi et al. (2016, 2018).

Soil Sampling and Laboratory Analysis

We made sampling after the seventh treatment every year (mid or late November or December depending on the weather

condition). To minimize the impact of sampling position as far as possible, 5 dominant trees which are evenly distributed in the 20 × 20 m^2 core area of each plot were selected as target trees. Two soil cores (3 cm diameter, 10 cm depth) within the range of 2 m from each target tree, 10 soil cores in total per plot were taken and mixed into one soil sample. The fresh soil samples were transported to laboratory on ice bag, passed through a 2-mm sieve, and divided into 2 subsamples. One was freeze-dried for the DNA extraction, the other was air dried for analysis of soil physicochemical properties.

Soil moisture content was measured gravimetrically by oven-drying the fresh soil samples to constant weight at 105°C. Soil NH_4^+-N and NO_3^--N were extracted with 2 mol L^{-1} KCl (a soil to water ratio of 1: 5) and measured using a continuous flow analyzer (SAN++, Skakar, Breda, Holland). Soil available N (AN) was the sum of NH_4^+-N and NO_3^--N. Soil pH was determined in a soil/water suspension [1: 2.5 (w/v)] by PB-10 pH-meter (Sartorius, Göttingen, Germany). Soil organic carbon (SOC) was measured according to Walkley and Black (1934). Total N (TN) was determined on an element analyzer (Vario EL III, Elementar, Germany). Soil C/N ratio (C/N) was calculated based on SOC and TN. Soil available phosphorus (AP) was extracted with 0.5 M $NaHCO_3$ and measured using a colorimetric method (Murphy and Riley, 1962). Soil N/P ratio (N/P) was calculated based on AN and AP.

Soil DNA was extracted from 0.25 g freeze-dried soil sample by using the Power-Soil® DNA Isolation Kit (MO BIO Laboratories, San Diego, CA, United States) according to the manufacturer's instructions. Each soil DNA sample was diluted (1:5) with sterilized Milli-Q water. We conducted a nested PCR with primer pairs AML1/AML2 (Lee et al., 2008) and AMV4.5NF/AMDGR (Sato et al., 2005). The first PCR was conducted with a total volume of 25 μl, which contained 2.5 μl 10 × Ex Taq Buffer (Mg^{2+}+plus), 2.0 μl dNTP mixture, 0.25 μl Ex Taq (5U/μl)(TaKaRa, Dalian, China), 1.0 μl (10 mg/ml) BSA (TaKaRa, Dalian, China), 0.5 μl (10 μM) of each primer, 17.25 μl sterilized water and 1.0 μl DNA template. The PCR program of amplification are as follows: 94°C for 3 min; 35 cycles at 94°C for 45 s, 51°C for 40 s, 72°C for 1 min; followed by 72°C for 10 min and 16°C for 2 min. The first PCR products were diluted (1:10) with sterilized Milli-Q water, and then used as a template for the second PCR amplification (25 μl) under the following conditions: 94°C for 3 min; 35 cycles at 94°C for 40 s, 58°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min and 4°C for 2 min. The volume of the second PCR was the same as the first. An Eppendorf Mastercycler pro-thermocycler (Eppendorf, Hamburg, Germany) was used for PCR amplification. The PCR products were separated through a 1.5% agarose gel in 1×TAE, bands were excised, and purified with the 0.8×Agencourt AMPure XP Beads (Beckman Coulter Inc., Boulevard Brea, CA, United States). The amount of DNA in the purified PCR products was measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc., Hudson, NH, United States). The final products were mixed at equimolar concentrations and then subjected to sequencing on the Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Hanyu Biotech Co., Ltd.

Bioinformatics

The initial quality filtering and assembling of paired-end reads were performed by Shanghai Hanyu Biotech Co., Ltd. Raw sequence data were processed in Trimmomatic v 0.32 (Bolger et al., 2014). Main flows are as follows: (1) Remove the reads with N bases; (2) Remove the low-quality bases (Q value < 20); (3) Remove the reads itself and its pairing reads whose length is less than 50 bp. Paired-end reads were assembled by Mothur v.1.32.1 (Schloss et al., 2009) permitting 1 bp mismatches of primer bases. Sequences with maxhomop > 8, or shorter than 200 bp were removed. Chimeras were checked in Chimera.uchime v.4.2 (Edgar et al., 2011). OTUs clustering was achieved in Usearch v.9.0.2132_i86linux32 (Edgar, 2010) with a 97% identity threshold. Taxonomic assignment was performed by blasting the representative sequence of each OTU against NCBI GenBank and MaarjAM database (Öpik et al., 2010), a conservative approach was followed for the species identification: considering only identifications with > 97% similarity, >90% coverage and >200 BLAST score value (**Supplementary Table S1**). In order to further identify the taxonomic information for all AM fungal OTUs, we constructed a neighbor joining phylogenic tree (**Supplementary Figure S1**) in MEGA v5 (Tamura et al., 2011) with default parameters except that bootstrap replication was set at 1,000 with the Kimura 2 – parameter model. Representative sequences from each encountered AM fungal OTU have been deposited in GenBank (accession numbers MH205770 - MH205915).

Statistical Analysis

All statistical analyses were conducted in R (R Development Core Team). OTU tables are subsampled to the median according to de Ca'rcer et al. (2011) as the dataset of AM fungal community composition, and then used to calculate the richness and Shannon diversity index (H'). Shannon diversity index (H') was calculated using the function 'diversity' in R package 'vegan' (Oksanen et al., 2013). To analyze the effects of canopy N and water addition and their interactions on AM fungal richness, Shannon diversity index (H') and soil properties, two-way analysis of variance (ANOVA) was conducted, followed by Duncan's multiple range test. The significant difference was accepted at $P < 0.05$. Before evaluating the effects of canopy N and water additions and their interactions on AM fungal community composition, the data of AM fungal community composition was sqrt transformed, and then a two-way permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was performed using the function 'adonis2' in R package 'vegan' (Oksanen et al., 2013) with 9999 permutations. In order to further confirm the differences in the composition of AM fungal communities among treatments, we used the functions 'mrpp', 'adonis' and 'anosim' in R package 'vegan' (Oksanen et al., 2013) with 9999 permutations. To analyze the influence of soil properties (AP, pH, SOC, TN, C/N, moisture, NH_4^+-N, NO_3^--N, AN, N/P) on the AM fungal richness and Shannon diversity index (H'), we performed Pearson correlation analysis. To explore the relationship between AM fungal community composition

dissimilarity and soil properties, Mantel and partial Mantel test were carried out using functions 'mantel' and 'mantel.partial' in R package 'vegan' (Oksanen et al., 2013) with 9999 permutations. In all the analyses involved in AM fungal richness, Shannon diversity and soil properties, in order to discover outliers Dixon's *Q* test was used at 95% confidence level (Dean and Dixon, 1951) and to satisfy the assumption of normality, some soil properties were log or sqrt transformed: in the 1st year, C/N and N/P were log transformed; in the 2nd year, NH_4^+-N was sqrt transformed; in the 3rd year, SOC and N/P were log transformed, TN was sqrt transformed; in the 4th year, AP was sqrt transformed, SOC, C/N and AN were log transformed.

RESULTS

Effects of Canopy N and Water Addition on Soil Properties

In the 1st year, N addition significantly decreased soil pH. Significant interactive effect of N and water addition on soil C/N was observed: without water addition, N addition had negative effect on soil C/N; with water addition, the effect became positive. In the 2nd year, N addition significantly increased soil NH_4^+-N; water addition significantly increased soil AP, but decreased soil NH_4^+-N, AN and N/P. To the 3rd and 4th year, there were no significant effect of N or water addition on any soil properties, except that water addition significantly increased AN in the 3rd year (**Table 1** and **Supplementary Table S2**).

Overall Miseq-Sequencing Information and OTU Delineation

After filtering out chimeras, 624,153 sequences were kept and clustered into 567 OTUs. The sequences of AM fungi accounted for 74.90% (467,479/624,153) of the total sequences and the AM fungal OTUs accounted for 25.75% (146/567) of the total OTUs. Moreover, the majority of AM fungal sequences (62.65%, 292,883/467,479) and OTUs (64.38%, 94/146) belonged to genus *Glomus*, followed by *Acaulospora* sequences (32.58 %, 152,330/467,479) and OTUs (22.60 %, 33/146), while the others covered 7 genera from 6 families, 4 orders (**Figure 1**).

Effects of Canopy N and Water Addition on AM Fungal Alpha-Diversity and Community Composition

In the 1st year, both N and water addition significantly changed AM fungal community composition. There was also significant interactive effect of N and water addition on AM fungal community composition (**Tables 2**, **3**).

TABLE 1 | Effects of canopy additions of nitrogen and water on soil properties.

		d.f.		AP (mg/kg)	pH	SOC (g/kg)	TN (g/kg)	C/N	Moisture (%)	NH_4^+-N (mg/kg)	NO_3^--N (mg/kg)	AN (mg/kg)	N/P
1st year	CN	1	*F*	0.868	5.597	2.820	0.003	3.333	0.434	0.779	1.118	2.342	1.239
			p	0.376	**0.042**	0.132	0.958	0.105	0.527	0.400	0.318	0.160	0.298
	CW	1	*F*	0.151	0.140	0.387	0.649	2.985	0.079	0.559	0.057	0.092	0.928
			p	0.707	0.717	0.551	0.441	0.122	0.786	0.474	0.817	0.768	0.364
	CN × CW	1	*F*	0.054	0.579	0.653	1.957	7.602	0.650	0.020	0.898	0.859	0.343
			p	0.821	0.466	0.422	0.195	**0.025**	0.441	0.891	0.368	0.378	0.574
2nd year	CN	1	*F*	0.837	1.015	0.107	0.130	0.023	0.542	8.907	0.444	3.574	0.215
			p	0.387	0.343	0.751	0.727	0.882	0.480	**0.017**	0.522	0.096	0.657
	CW	1	*F*	5.688	0.232	0.144	0.311	0.735	0.044	22.221	0.113	12.148	7.881
			p	**0.044**	0.643	0.714	0.590	0.414	0.839	**0.002**	0.745	**0.008**	**0.026**
	CN × CW	1	*F*	1.227	0.027	0.025	0.299	0.010	0.441	3.903	1.792	3.837	0.024
			p	0.300	0.874	0.879	0.598	0.922	0.523	0.084	0.214	0.086	0.882
3rd year	CN	1	F	0.091	0.054	0.984	0.236	1.143	0.473	0.232	0.706	0.603	0.100
			p	0.769	0.822	0.347	0.639	0.313	0.509	0.641	0.423	0.457	0.759
	CW	1	*F*	0.387	0.410	3.495	4.076	0.470	0.930	5.089	3.715	7.030	1.961
			p	0.549	0.538	0.094	0.074	0.510	0.360	0.051	0.086	**0.026**	0.195
	CN × CW	1	*F*	0.344	0.005	0.094	1.138	0.403	0.682	0.263	0.467	0.360	0.700
			p	0.572	0.947	0.766	0.314	0.542	0.430	0.621	0.512	0.563	0.424
4th year	CN	1	*F*	0.000	2.186	0.167	0.115	0.018	1.132	1.626	0.500	1.210	0.946
			p	0.983	0.178	0.692	0.743	0.897	0.315	0.234	0.497	0.300	0.359
	CW	1	*F*	2.463	1.540	2.172	2.322	1.267	0.671	1.343	1.431	2.417	2.143
			p	0.155	0.250	0.134	0.162	0.290	0.434	0.276	0.262	0.154	0.181
	CN × CW	1	*F*	0.564	0.030	0.542	0.717	0.039	0.009	0.124	0.065	0.017	2.267
			p	0.474	0.867	0.480	0.419	0.848	0.928	0.733	0.804	0.898	0.171

F-values and p-values are presented, significant effects ($p < 0.05$) are highlighted in bold as determined by two-way ANOVA. N, nitrogen addition; W, water addition; N × W, the interaction.

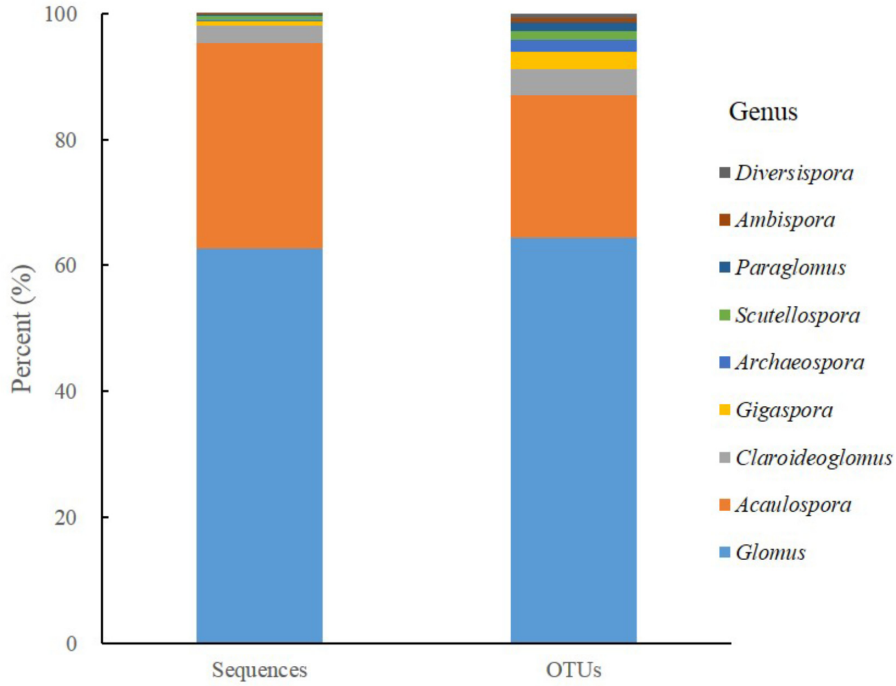

FIGURE 1 | The proportional distributions of sequences and derived AM fungal OTUs detected in all soil samples.

TABLE 2 | Effects of canopy addition of nitrogen and water on AM fungal community composition.

		1st year		2nd year		3rd year		4th year	
	d.f.	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
N	1	2.125	**0.037**	0.519	0.900	0.911	0.499	0.661	0.763
W	1	2.106	**0.037**	1.777	0.082	0.959	0.456	2.188	**0.029**
N × W	1	2.015	**0.047**	1.031	0.398	0.996	0.433	0.835	0.577

F-values and p-values are presented, significant effects ($p < 0.05$) are highlighted in bold as determined by PERMANOVA. N, nitrogen addition; W, water addition; N × W, the interaction.

TABLE 3 | The dissimilarity analysis of AM fungal community composition among different treatments.

	MRPP		ADONIS		ANOSIM	
	Statistic	*p*-value	Statistic	*p*-value	Statistic	*p*-value
AM fungal community of the 1st year	0.647	**0.008**	2.014	**0.005**	0.304	**0.004**
AM fungal community of the 2nd year	0.753	0.347	1.041	0.396	−0.003	0.465
AM fungal community of the 3rd year	0.756	0.645	0.824	0.690	−0.066	0.692
AM fungal community of the 4th year	0.773	0.281	1.154	0.277	0.046	0.303

Statistics and p-values are presented, significant effects ($p < 0.05$) are highlighted in bold.

However, no significant treatment effects were detected on AM fungal alpha-diversity (**Figure 2**). Moreover, N addition marginally decreased the relative abundance of *Glomus* (**Figure 3A**) but marginally increased *Acaulospora* relative abundance (**Figure 3B**). Simultaneously, water addition also marginally increased the relative abundance of *Acaulospora*, but showed no significant effect on *Glomus*. No significant interactions were found of N and water additions on the relative abundance of these two dominant genera.

In the 2nd year, water addition significantly decreased AM fungal richness and Shannon index (**Figure 2**), but did not change AM fungal community composition (**Tables 2**, **3**). By contrast, N addition showed no significant effect on AM fungal alpha-diversity or community composition (**Figure 2** and **Tables 2**, **3**). Also no significant interactions between N and water addition were observed on AM fungal alpha-diversity or community composition (**Figure 2** and **Table 2**). In addition, both N and water additions showed marginally positive effects to the richness of *Acaulospora*, in contrast, the marginally negative effects were found on *Glomus* richness (**Figures 3C,D**).

In the next 2 years, both N and water additions did not show significant effects on AM fungal alpha-diversity or community composition, with an exception that water addition significantly changed AM fungal community composition, accompanied by significant raise in *Acaulospora* abundance and significant decline in that of *Glomus* (**Figures 2**, **3A,B** and **Table 2**).

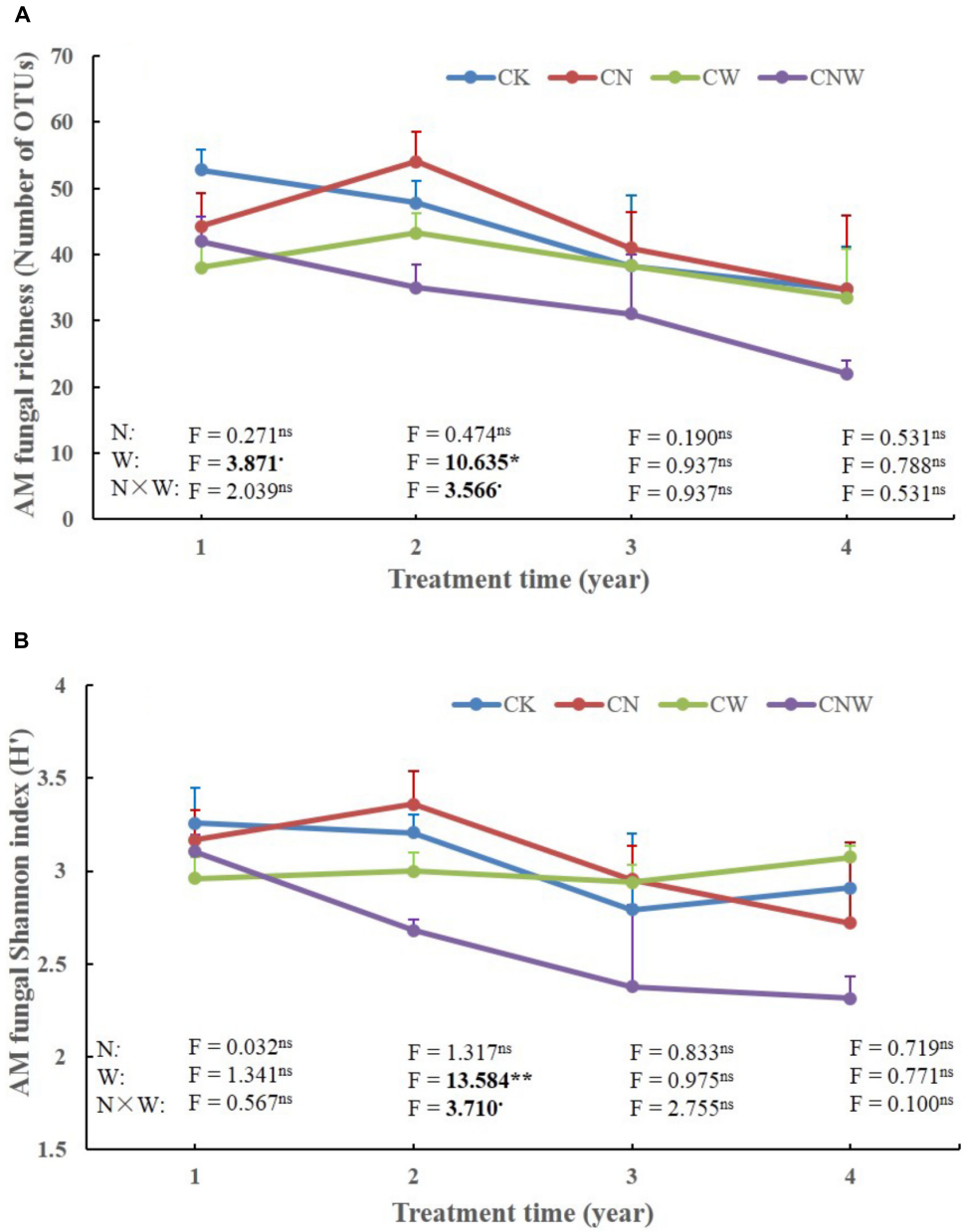

FIGURE 2 | Effects of canopy additions of N and water on AM fungal richness **(A)** and Shannon diversity index (H') **(B)** over time (4 years). CK, control; CN, canopy addition of N; CW, canopy addition of water; CNW, canopy additions of N and water. N, N addition; W, water addition; N × W, the results were the interaction. Significance of treatment effect was determined by two-way ANOVA. $^{**}p < 0.01$; $^{*}p < 0.05$; $^{\cdot}p < 0.1$; ns, not significant.

Correlation Between AM Fungal Richness, Shannon Index, Community Composition Dissimilarity and Soil Properties

Only soil NH_4^+-N and N/P were marginally positively correlated with AM fungal richness (**Table 4**), and significantly positively correlated with AM fungal Shannon index (**Table 4**). No other significant correlations were observed between AM fungal biodiversity and soil properties.

Mantel and partial Mantel test indicated that only soil pH showed marginally positive correlation with the dissimilarity among AM fungal community composition among different treatments (**Table 5**).

DISCUSSION

This study investigated the effects of canopy additions of N and water on AM fungal diversity and community composition

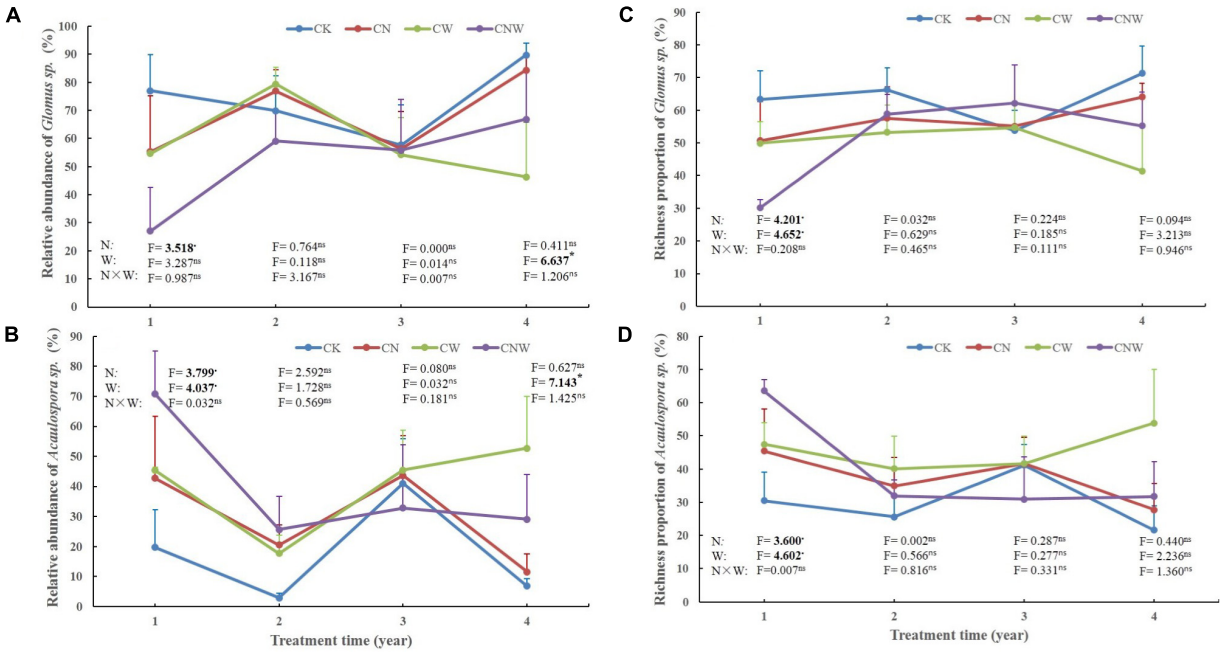

FIGURE 3 | Effects of canopy additions of N and water on the abundance and richness of *Glomus sp.* **(A,C)** and *Acaulospora sp.* **(B,D)** over time (4 years). CK, control; CN, canopy addition of N; CW, canopy addition of water; CNW, canopy additions of N and water. N, N addition; W, water addition; N × W, the interaction. Significance of treatment effect was determined by two-way ANOVA. $^{**}p < 0.01$; $^{*}p < 0.05$; $^{\cdot}p < 0.1$; ns, not significant.

TABLE 4 | Pearson correlation analysis between AM fungal richness, Shannon index (H') and soil properties in the 2nd year.

		Richness		Shannon index (H')	
	d.f.	r	p	r	p
AP (mg/kg)	12	−0.272	0.826	−0.434	0.939
pH	12	0.023	0.469	0.066	0.412
SOC (g/kg)	13	−0.041	0.558	−0.093	0.629
TN (g/kg)	13	0.279	0.157	0.150	0.297
C/N	13	−0.309	0.869	−0.267	0.832
Moisture (%)	13	0.044	0.438	0.079	0.390
NH_4^+-N (mg/kg)	12	0.443	0.056	0.499	**0.035**
NO_3^--N (mg/kg)	13	0.012	0.483	0.141	0.308
AN (mg/kg)	12	0.360	0.103	0.428	0.063
N/P	11	0.402	0.087	0.519	**0.035**

Correlation coefficients and p-values are presented, significant effects ($p < 0.05$) are highlighted in bold.

TABLE 5 | Mantel and partial Mantel test of AM fungal community composition with the matrices of soil properties in the 1st year.

	Mantel test		Partial Mantel test	
	r	p	r	p
AP (mg/kg)	−0.043	0.600	−0.074	0.731
pH	0.251	0.077	0.257	0.076
SOC (g/kg)	−0.05	0.601	−0.054	0.607
TN (g/kg)	0.002	0.474	0.000	0.480
C/N	−0.209	0.969	−0.218	0.976
Moisture (%)	0.082	0.235	0.081	0.251
NH_4^+-N (mg/kg)	−0.055	0.616	−0.066	0.663
NO_3^--N (mg/kg)	0.071	0.255	0.072	0.242
AN (mg/kg)	0.005	0.466	−0.004	0.493
N/P	0.019	0.397	0.007	0.438

Correlation coefficients and p-values are presented.

for consecutive 4 years and the results indicated that canopy N addition significantly changed AM fungal community composition, however, this effect was time-dependent, only occurred in the 1st year. While, the effect of water addition overwhelmed that of N addition, which not only changed the community composition, but also decreased the alpha-diversity of AM fungi, and these consequences were also time-dependent and only occurred in the earlier stages (1st/2nd year). In addition, the increment of N or water tended to decrease the abundance and richness of the most dominant genus *Glomus* and favored other AM fungi. The effects of N/water addition on AM fungal community composition were potentially mediated by soil properties, such as pH, NH_4^+-N and N/P.

Effects of Canopy N and Water Addition on Soil Properties

Nitrogen deposition usually leads to soil acidification (Lu et al., 2014; Tian and Niu, 2015; Chen et al., 2017), our study was not an exception (N addition significantly decreased soil pH in the 1st year), although we differently practiced canopy N addition.

Possible reasons for soil acidification resulting from N deposition include: (1) NH_4^+ ions are absorbed by plant roots, while H^+ will be released into soil, causing soil acidification (Smith and Read, 2008); (2) NH_4^+ ions are converted into nitrites and further converted into nitrates, producing H^+ leading to soil acidification (Azevedo et al., 2013); (3) NH_4^+ ions displacing base cations (Ca^{2+}, Mg^{2+}, K^+, Na^+) and the increasing loss of metal cations could reduce soil buffering capacity against acidification (Tian and Niu, 2015; Lucas et al., 2016). Following soil acidification, soil microbial community composition and activity could be changed (Wei et al., 2013). Soil acidification can also result in the loss of plant species across multiple ecosystems (Azevedo et al., 2013) and the suppression of plant growth and carbon (C) sequestration (Schulte-Uebbing and de Vries, 2018).

In the 2nd year, water addition significantly decreased soil NH_4^+-N and AN, which may attributed to significant leaching (Martínez-García et al., 2017) and runoff. The loss of N can cause negative impacts to the environment and human, such as eutrophication of water body and decline of crop productivity, which will likely be aggravated by intensive heavy precipitation events (Martínez-García et al., 2017). On the other hand, N addition significantly increased soil NH_4^+-N, consistent with many previous studies (Chen et al., 2014, 2017; Zhang et al., 2014). The increase of soil NH_4^+-N can increase the productivity of N-limited ecosystems such as grassland and forest in temperate zone (Aber et al., 1998; Bai et al., 2010); However, excessive N supply can also lead to the accumulation of reactive nitrogen in soil to a toxic level for plant (Wei et al., 2013) and other soil organisms, such as nematodes and fungi (Eno et al., 1955). In the study of Wei et al. (2013), NH_4^+ concentration showed negative relationships with plant composition; in Eno et al. (1955), the fungi and nematode numbers were decreased under all N addition levels. Compared to control, only 0.6% of the nematodes and 4.9% of the fungi survived under N addition level of 608 mg kg^{-1}.

Effects of Canopy N and Water Addition on AM Fungal Alpha-Diversity

In our study, N addition did not significantly decrease AM fungal richness and Shannon index, which failed to support our first hypothesis, also inconsistent with previous studies in forest (Camenzind et al., 2014), agriculture (Liu et al., 2014) and alpine meadow ecosystems (Zheng et al., 2014). In the study of Camenzind et al. (2014) and Liu et al. (2014), N addition significantly decreased AM fungal richness, while in the study of Zheng et al. (2014), N addition had significant positive effect on AM fungal alpha-diversity. How AM fungi respond to N addition is probably influenced by local environmental conditions, plant communities, intensity and frequency of N addition, experimental duration and other unknown factors (Porras-Alfaro et al., 2007; Wang et al., 2018). In this study, the ecosystem type is forest, which has higher species diversity and stability (strong resistance) than meadow, and agriculture ecosystems. More importantly, the mode of N application in our study was canopy spraying, different from Camenzind et al. (2014), in which N was directly added to the soil. As known, many canopy processes could substantially affect the consequences of N addition, however, the extent of the impact has not yet been clarified. In addition, although the total amount of N applied in Camenzind et al. (2014) was the same as this study, but the frequency of N application was different (7 times a year in this study, versus twice a year in Camenzind's). Low frequency with high rate could very likely over-estimate the effect of N deposition, as Zhang et al. (2014) confirmed the overestimation of plant species loss of N addition at high rates and low frequency in a temperate steppe.

In the present study, water addition significantly decreased AM fungal richness and Shannon diversity index in the 2nd year, in support of our first hypothesis, also consistent with Gao et al. (2016) and Chen et al. (2017) in steppe ecosystem. By Pearson correlation analysis, we found that NH_4^+-N and N/P were marginally positively correlated with AM fungal richness and significantly positively correlated with AM fungal Shannon diversity index. Meanwhile, NH_4^+-N and N/P were indeed significantly decreased after water addition, possibly due to run-off and leaching of N from soil (Martínez-García et al., 2017). The decrease of NH_4^+-N may have intensified the competition among species leading to the loss of AM fungal niche, while lost niche could finally lead to decrease of AM fungal diversity (Dickie, 2007; Gao and Guo, 2013). Moreover, water addition could affect soil nutrient balance including N/P ratio, which can largely affect AM fungal community composition (Verbruggen et al., 2015).

Effects of Canopy N and Water Addition on AM Fungal Composition

In the 1st year, N addition significantly changed AM fungal community composition, in support of our first hypothesis, and consistent with van Diepen et al. (2011) and Camenzind et al. (2014), although the mode of N addition were different. The underlying mechanisms for the N effects on AM fungal community composition could be: (1) N addition increased the availability of soil N and reduced the cost in uptake of N by plant, so the plant dependence on mycorrhizal fungi decreased, and the amount of C allocated to mycorrhiza also decreased, which finally strengthened the competition among AM fungal species, led to changes in AM fungal community composition (Huang et al., 2014). (2) N addition led to soil acidification, which can directly affect spore germination and mycelial development (Rousk et al., 2010). More importantly, different AM fungi prefer different optimum pH, so changes in soil pH may lead to changes in community composition of AM fungi (An et al., 2008). Soil acidification caused by N addition may have stronger direct influence on soil microbial community composition than indirectly through the changes in plant community (Wei et al., 2013). At the same time, AM fungal community composition was also significantly altered by water addition, supported our first hypothesis, and in agreement with Gao et al. (2016) and Chen et al. (2017) although their studies were carried out in water-limited ecosystems. Precipitation increment may directly change soil water status and affect the physiological activity of

AM fungi. Furthermore, increased precipitation can indirectly affect AM fungi via influencing the soil characteristics and plant communities. For example, in the study of Chen et al. (2017), changes in soil pH and plant species richness could shift AM fungal community composition. Gao et al. (2016) found that increased precipitation could alter fungal community composition through influencing soil moisture, NO_3^--N and root turnover.

The significant interactions between N and water addition on AM fungal community composition confirmed our second hypothesis, but inconsistent with Li et al. (2015) who observed no significant interactive effect of N and water increment in a semiarid grassland ecosystem after 8 years of experimental treatment. Chen et al. (2017) found that although there was no significant interactive effect of N and precipitation increment on AM fungal diversity, but significant interactive effect was observed on the relative abundance of some AM fungal OTUs. One possible explanation was experimental duration, as in our study the interaction was only observed after 1 year of treatment.

Moreover, we also confirmed that the effect of N and water addition on AM fungal community was time-dependent, in support of our third hypothesis. The results of Yang et al. (2016) demonstrated that under field conditions, AM fungal richness increased and community composition shifted after 15 days waterlogging. However, the time resolution in our study is year-based, therefore, more sampling at finer time scales is expected to test how quickly AM fungi respond to environmental changes. In addition, in a Mediterranean grassland, increased precipitation during rainy seasons significantly altered plant community and soil fungal community structure (Suttle et al., 2007; Hawkes et al., 2011), but in the dry season, fungal community did not respond to water addition in a different Mediterranean grassland (Barnard et al., 2013). Koyama et al. (2018) suggested that besides water amounts, timing of water manipulations can also be an important influencing factor, however, the present study did not involve the timing of water addition, which could be addressed in future research. In the meta-analysis by Wang et al. (2018), they found that N addition didn't change fungal richness significantly when the experimental duration was within 5 years or longer than 10 years, but had significant influence when the treatment duration was 5 ∼10 years. This study only lasted for 4 years, next we will continue to sample and study the long-term ecological effects of increased N deposition and precipitation.

Changes in the composition and structure of plant community may affect the amount and quality of C input to belowground thus affecting soil microbial biomass, activity, and community structure (Meier and Bowman, 2008; Treseder, 2008; Liu et al., 2016). For instance, in the meta-analysis of Liu et al. (2016), plant lignin, plant protein and soil lignin were significantly increased by 7.13, 25.94, 7.30%, respectively following N addition. On the one hand, the increase of litter quality could promote microbial growth and biomass accumulation; on the other hand, the increase of recalcitrant C compounds (e.g., lignin) could result in the decrease of C availability to soil microbes, inhibiting microbial growth and activity (Treseder, 2008). Moreover, community composition of some specific microbial groups can change under N additions, for example, the diversity of ectomycorrhizal fungi and the richness of fungal decomposers decreased after N fertilization or deposition (Treseder, 2008). It should be further noted that, as symbiotic fungi, AM fungi have host preference (Sanders, 2003; Croll et al., 2008), and their community composition and structure are closely linked to plant community characteristics (Öpik et al., 2010; Kivlin et al., 2011; Xu et al., 2016). At the regional scale, a significant relationship between AM fungal community composition and plant was observed by Xu et al. (2016). The results of Li et al. (2015) indicated that the AM fungal abundance and OTU richness were significantly correlated with the 7-year averaged ANPP and aboveground biomass of plant functional groups after 8-years N and water additions. In the study of Chen et al. (2017), significant correlation between plant species richness and AM fungal taxonomic composition was also recorded. Therefore, further research incorporating plant community data is still needed.

CONCLUSION

Increased N deposition and precipitation have significant interactive effect on AM fungal diversity and community composition, while precipitation increment have stronger effect on AM fungal community structure than increased N deposition in the forest ecosystem. The effect of N deposition and precipitation on AM fungal community composition was time-dependent, mediated by soil factors, and possibly related to the sensitivity and resilience of forest ecosystem to global changes. In the future, we will consider finer and broader time scales and take into account the plant data to achieve comprehensive understanding of AM fungal ecology in the forest ecosystem.

AUTHOR CONTRIBUTIONS

AZ conducted the experiments, analyzed the data, and drafted the manuscript. LL conceived the study, received financial support, and conducted parts of the experiments. BC, TX, and WX performed the data analysis. LS provided some basic data. WZ was responsible for the operation of this experimental platform. SF and HF designed and established this experimental platform. BC revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01842/full#supplementary-material

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The PGPR *Stenotrophomonas maltophilia* SBP-9 Augments Resistance against Biotic and Abiotic Stress in Wheat Plants

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Certain plant growth promoting bacteria have ability to ameliorate abiotic and/or biotic stressors, which can be exploited to enhance plant growth and productivity of the plants under stress conditions. Therefore, the present study aimed to examine the role of a rhizospheric bacterial isolate SBP-9 isolated from *Sorghum bicolor* (i) in promoting the wheat plant growth under salinity stress, and (ii) in enhancing the defense response in wheat against fungal pathogen "*Fusarium graminearum.*" The test isolate possessed plant growth promoting (PGP) traits including ACC deaminase (ACCD), gibberellic acid, indole acetic acid (IAA), siderophore, and inorganic phosphate solubilization. Under salt (NaCl) stress, inoculation of this isolate to wheat plant significantly increased plant growth in terms of various growth parameters such as shoot length/root length (20–39%), fresh weight/dry weight (28–42%), and chlorophyll content (24–56%) following inoculation of test isolate SBP-9. Bacterial inoculation decreased the level of proline, and malondialdehyde, whereas elevated the antioxidative enzymatic activities of superoxide-dismutase (SOD; 28–41%), catalase (CAT; 24–56%), and peroxidase (POX; 26–44%). Furthermore, it also significantly decreased the Na^+ accumulation in both shoot and roots in the range of 25–32%, and increased the K^+ uptake by 20–28%, thereby favoring the K^+/Na^+ ratio. On the other hand, the test isolate also enhanced the level of defense enzymes like β-1, 3 glucanase, phenylalanine ammonia lyase (PAL), peroxidae (PO), and polyphenol oxidase (PPO), which can protect plants from the infection of pathogens. The result of colonization test showed an ability of the test isolate to successfully colonize the wheat plants. These results indicate that *Stenotrophomonas maltophilia* SBP-9 has potential to promote the wheat growth under biotic and abiotic (salt) stressors directly or indirectly and can be further tested at field level for exploitation as bioinoculant.

Keywords: PGPR, ACC deaminase, ERIC-PCR, induced systemic tolerance, salt stress, osmolytes

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INTRODUCTION

Soil salinity is a naturally occurring problem in arid and semiarid regions and continuously increasing due to the exhaustive use of chemical fertilizers, and improper irrigation management (Bharti et al., 2013). It has reduced the world's irrigated area by 1–2% every year, thereby severely affecting the agriculture production (FAO, 2005). According to the UN (United Nations), at least 50% of the world's arable lands are subjected to salinity stress (Flowers and Yeo, 1995). The amount

of land affected by salt represents almost 20% of the world's cultivated area and it is increasing as a direct attribute of irrigation and agricultural malpractices (Glick, 2014). The plants growing under salinity and other stresses (both biotic and abiotic) suffer from a high level of ethylene termed as "stress ethylene" (Jackson, 1985) which adversely affects the growth and various metabolic processes leading to senescence in plants. Enhanced level of ethylene is one of the important markers for both abiotic and biotic stressors (Sheehy et al., 1991; Heidstra et al., 1997; Ma et al., 2003).

Certain PGPR (plant growth promoting rhizobacteria) strains equipped with enzyme ACCD can degrade the ACC to ammonia and α-ketobutyrate, and thereby minimize the level of stress ethylene (Mayak et al., 2004; Barnawal et al., 2012). Therefore, these PGPR reduce the inhibitory effect of stress ethylene generated under adverse environmental conditions, and reduce the endogenous level of ACC in plants. The effectiveness of bacterial isolate in plant growth stimulation and the alleviation of salt stress in salt-rich soils have been demonstrated in previous studies (Egamberdieva, 2008; Nadeem et al., 2010; Barnawal et al., 2014).

In addition to stress ethylene, increasing soil salinity affects the multitude of responses in plants including the several biochemical and physiological processes like synthesis of protein, lipid metabolism, photosynthesis, and ionic homeostasis (Parida and Das, 2005). It also restricts the water uptake and induces the toxicity of Na^+ (Ashraf et al., 2004; Mayak et al., 2004). PGPR can overcome the harmful effects of salinity by maintaining a favorable ratio of K^+/Na^+ ions amenable for plant growth under high salt levels (Mayak et al., 2004), accumulation of compatible solutes or osmolytes, stabilizing membrane lipids (Hincha et al., 2003; Bano and Fatima, 2009), maintenance of redox potential (Colmer et al., 1995; Yancey, 2005), free radicals scavenging (Smirnoff and Cumbes, 1989), binding to toxic metals (Geddie and Sutherland, 1993; Sharma and Dietz, 2006; Karthikeyan et al., 2007), and induction of transcription factors under stress responses (Gupta et al., 2012).

Among PGPR, plant growth promoting and biocontrol potential of *Stenotrophomonas* sp. has been reported in earlier studies which demonstrated that it can be used as an effective bioinoculants for plant growth promotion and controlling the wide range of plant pathogenic fungi and, therefore have great potential for biotechnology applications (Ryan et al., 2009; Berg et al., 2010). Plant growth promotion ability of *Stenotrophomonas rhizophila* strain DSM14405^T was observed in the high salt rich soils of Uzbekistan at levels up to 180% (Egamberdieva et al., 2011). Similarly, Singh et al. (2013) showed the Quorum quenching (QQ) activity against *Chromobacterium violaceum* CV026 and anti-biofilm activities of a rhizobacterium *Stenotrophomonas maltophilia* BJ01. Brooke et al. (2017) also encountered the multifarious approach of bacterium *S. maltophilia*. A previous report (Alavi et al., 2013) suggested that *S. rhizophila* possesses certain genes responsible for beneficial plant-microbe interaction, transport of osmoprotectants, biocontrol activity, and colonization. However, the detail characterization and mechanism of plant growth stimulated by *Stenotrophomonas* sp. under salinity stress conditions is still lacking.

Besides, rhizobial inoculants have also been reported to suppress disease by eliciting the induced systemic resistance (ISR) against a number of plant diseases (Kumari and Srivastava, 1999). The induction and increased production of defense-related enzymes during ISR are known to play a crucial role in host resistance (Chen et al., 2000; Ramamoorthy et al., 2002). Bacteria belonging to genera like *Pseudomonas* and *Bacillus* sp. have been known to induce resistance to bacterial and fungal pathogens. However, *S. maltophilia*-mediated elicitation of ISR in wheat is still unknown. A recent study has shown the biocontrol behavior of *S. maltophilia* (PD4560) against *Ralstonia solanacearum* by proteolytic enzyme production and through induction of pathogenesis related (PR) genes (Elhalag et al., 2016). The role of PR proteins in helping plants to counteract the stressed condition has been addressed in another study (Koike et al., 2002). However, the detailed characterization and its priming effect against fungal pathogen are at primary level.

The presence of salts in the soil is a major problem for agricultural crops like wheat that leads to a major drop in wheat grain yield in the range of 20–43%, with an overall average loss of 40%. The production of wheat in India is 80.2 million ton annually, which is about 12% of total world production (http://dacnet.nic.in). Therefore, there is an urgent need for an effective bioinoculant for its/their ability to promote wheat plant growth under saline stress. We hypothesized that halotolerant bacteria are able to ameliorate salinity stress and therefore, can be used as an effective tool for development of bio-formulations that can be tested in field trials. In this study, we made an attempt to explore the potential of a halotolerant ACC deaminase producing strain of *S. maltophila* for its multifarious PGP (plant growth promoting) traits to promote wheat plant growth under saline stress. For this, we examined the protective role of *S. maltophila* using diverse physiological and biochemical mechanisms, and to evaluate its efficacy to confer abiotic stress tolerance, particularly in wheat plant. In addition, the strain used in the present study was shown to induce ISR against fungal challenged wheat plants.

MATERIALS AND METHODS

Isolation of Bacteria

The bacterial strain was isolated from the rhizospheric soil of *Sorghum bicolor,* commonly growing in the arid region of Rajasthan, India. ∼1 g of soil sample was serially diluted (up to 10^{-9}) with sterile distilled water and 100 μl of the suspension was spread on the LB-agar medium. The plates were incubated for 48–72 h at 30°C. A total of 15 bacterial colonies with varying morphologies were selected and further cultured in minimal medium (DF) containing 3 mM ACC (Sigma-Aldrich, USA) (Dworkin and Foster, 1958). ACC utilizing bacterial isolates were screened for the ACC deaminase assay and other plant growth properties. Based on ability to utilize ACC as a nitrogen source, ACCD activity and other PGP features, isolate SBP-9 was selected for detailed study.

Biochemical Characterization and Identification of Strain SBP-9

The test isolate was characterized by various biochemical tests like methyl-red, Voges-Proskauer, Indole, citrate utilization, nitrate reductase, urease, oxidase, catalase, and gram staining using standard protocol (Harley and Prescott, 2002). Test of motility was also checked using standard procedure (Connelly et al., 2004). Test of carbohydrate utilization was performed using KB-009, carbohydrate utilization kit (Himedia, India). Antibiotic sensitivity test of isolate to standard antibiotics was evaluated using HTM-002, antibiotic sensitivity kit (Himedia, India). Antagonistic activity against certain fungal pathogens namely *Aspergillus flavus, Fusarium oxysporum*, *Fusarium moniliforme, Candida albicans, Penicillium citrium,* and *Fusarium graminearum* was determined by agar well-diffusion method. Molecular technology employing 16S rRNA amplification for identification of selected test organism was performed via polymerase chain reaction (PCR), following the standardized protocol (Singh et al., 2015). Taxonomic affiliation of SBP-9 was assigned (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and the phylogenetic relationship was established (Tamura et al., 2013).

Stress Tolerance Studies

The tolerance of the selected isolate toward various abiotic stressors like pH, temperature, and salinity was studied. Salt tolerance (1, 2, 4, 6, and 8% NaCl, w/v) was tested on DF-agar medium supplemented with ACC (3 mM). The strain was streaked on the solid-agar medium and visualized for the growth following incubation at 30°C for 2–3 days. Tolerance to varying temperatures was studied by streaking the isolate on tryptic-soy agar plates and incubated at different temperatures viz. 20–60°C. Tolerance to salt, pH, and temperature stress was also done by inoculating the strain in to tryptic soya broth medium and incubating at desired time interval. For pH studies, 100 µl of overnight grown culture (10^7 CFU ml^{-1}) was added to tryptic soya broth and pH of various ranges (5.0–10.0) was maintained by 2 N NaOH and 1 N HCl using the pH meter (Eutech, pH 1100). After 72 h, culture pellet was suspended in 2 ml of sterile water, and optical density (OD) was determined at 600 nm in a UV-Visible spectrometer (Jasco Corporation, Japan) to test the pH tolerance. Each culture was inoculated in triplicate sets.

Test for Plant Growth Promoting Features

ACC deaminase activity of isolate SBP-9 was tested by measuring the amount of α-ketobutyrate production, a cleavage product of ACC (Honma and Shimomura, 1978), and protein concentration were determined using the Bradford method (Bradford, 1976). The ACC deaminase activity was expressed in terms of nmol of α-ketobutyrate mg^{-1} protein. Test of phosphate solubilization was performed in NBRIP (National Botanical Research Institute's Phosphate) medium supplemented with insoluble tricalcium phosphate and quantified as per the standard protocol (Mehta and Nautiyal, 2001). A standard curve was prepared using various concentrations of K_2HPO_4 (Merck, India). Test of IAA production was done by using Salkowsky's reagent (Gordon and Weber, 1951), and optical density of the resulting solution was measured spectrophotometrically at 530 nm using a Jasco-630 UV-visible spectrophotometer. A standard curve of IAA was used for measuring the IAA concentration in test samples using un-inoculated medium as a control. Gibberellic acid production was tested by the spectrophotometric method (Holbrook et al., 1961). Test for siderophore production was evaluated on chrome azurole S-agar (CAS-agar) plates and observed for formation of color zone around the point inoculated colony (Schwyn and Neilands, 1987). Assay for ammonia production was tested using Nessler's reagent (Cappuccino and Sherman, 1992). A preliminary test for nitrogen fixation ability of SBP-9 was done by growing on $JNFb^-$ agar medium (Dobereiner, 1997). In addition, *nif* H gene was amplified using specific primers: Pol F (5′-TGCGAYCCSAARGCBGACTC-3′) and Pol R (5′-ATSGCCATCATYTCRCCGGA-3′) (Sigma–Aldrich), where Y = C/T, S = G/C, R = A/G, B = G/T/C.

Physiological Test of ACCD activity

The ACCD activity of SBP-9 was tested under various physiological conditions namely varying salt concentration, temperature, pH, and different incubation periods. To evaluate the activity under various salinity levels, different concentration of NaCl (2–8%) was supplemented in minimal medium containing 3 mM ACC, while for temperature assay SBP-9 was grown at different temperatures (25–45°C) in an incubator. Similarly, pH of the culture medium was adjusted with 2 N HCl and 1 M NaOH to attain pH 5.0 to 11.0. In addition, the enzymatic activity was also assessed under different incubation periods.

EVALUATION OF PLANT GROWTH PROMOTING TEST

Inoculum Preparation and Seed Treatment

Effect of the bacterial isolate SBP-9 on the growth of wheat plant (*Triticum aestivum* L.) under salinity stress was tested in a controlled environment of plant growth chamber. Soil used for pot study was analyzed for its various physicochemical properties using Atomic Absorption Spectrophotometer (AAS). The soil was autoclaved at 121°C for 1 h for 3 consecutive days to kill any microbial presence. Sterility of the soil was checked by standard serial dilution method. Physico-chemical characteristics of soil used in pot were as follows: pH 7.20 ± 0.05, EC 0.161 ± 0.03 ds m^{-1}, Olsen P 32.9 ± 1.7 mg kg^{-1}, Total N 57 ± 2.0 mg kg^{-1}, Total K 118.0 ± 3.1 mg kg^{-1}, Zn 0.221 ± 0.003 mg kg^{-1}, Cu 0.118 ± 0.003 mg kg^{-1}, Fe 2.88 ± 0.04 mg kg^{-1}, and Mn 0.916 ± 0.05 mg kg^{-1}. Preparation of bacterial inoculum (OD 0.15) and seed treatment was performed according to Penrose and Glick (2003). Briefly, wheat (*T. aestivum* L.) seeds were surface sterilized by treating with 70% ethanol followed by 2% sodium hypochlorite (NaOCl) solution for 3 min. The sterilized seeds were thoroughly washed using sterile water to remove all traces of sodium hypochlorite. The surface-sterilized seeds of wheat were kept in the bacterial suspension for 1 h. Surface sterilized seeds treated with 0.03 M $MgSO_4$ instead of bacterial suspension served as control. Twenty bacterized seeds were sown in each plastic pot (22 cm in height, 16 cm in diameter) filled

with sterilized soil (400 g) and grown with 16:8 photoperiods for 15 days after seed germination at 24 ± 2°C. For striking the salt stress of 150 mM (T-1) and 200 mM (T-2), NaCl was added in Hoagland medium to achieve the desired concentration for providing the nutrient as well as imposing the salt treatment to experimental plants. A set of control plants with 0 mM NaCl (T-0) was also taken for comparative analysis. Pots were arranged in completely randomized block design with three replications in each treatment.

For measuring growth (root/shoot length) and biomass (fresh/dry weight), five randomly selected plants from each replicate were used. To estimate the chlorophyll content, fresh leaf samples of 500 mg (0.5 g) were ground thoroughly with 80% acetone and centrifuged at 9,000 g for 10 min at 4°C. The absorbance of collected supernatant were read at 645 and 663 nm using a UV-Visible spectrometer (Jasco Corporation, Japan) to estimate total chlorophyll content (Moran and Porath, 1980). The same was calculated as follows:

$$\text{Chlorophyll} = [8.02 \times A_{633}] - [20.02 \times A_{645}]$$

Ionic Accumulation Analysis

To conduct an ionic analysis of plants treated with salt stress, roots were washed twice for eight to ten min in ice-cold 20 mM $CaCl_2$ to allow the exchange of cell wall bound K^+ and Na^+, and finally washed five to six times with autoclaved Milli-Q water. Roots and shoots were separated and oven dried at 70°C for 48 h. Afterward, 1 g plant tissue was ground in liquid N_2 and digested in a mixture of 30% H_2O_2, 65% HNO_3, and de-ionized water in a ratio of 1:1:1 at 120°C for 2 h to a final volume of 12 ml in a microwave digester. Ions namely Na^+, and K^+ were estimated by AAS (AAS 2380, Perkin Elmer, USA) at NHRDF (National Horticultural Research and Development Foundation; Nashik, India).

Antioxidant Assay

Plant leaves (0.5 g) were extracted in the buffer containing 5 ml of 50 mM phosphate buffer (pH 7.0) supplemented with 1% polyvinylpyrrolidone (PVPP). The crude extract was centrifuged at 10,000 g for 15 min at 4°C, and the obtained supernatant was used for the antioxidant assay. Superoxide dismutase assay, which is based on its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), was carried out as per the method of Beauchamp and Fridovich (1971) with minor modifications. The reaction mixture containing 100 µl of enzyme extract in 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, and 0.1 mM EDTA was made up to 3 ml. The assay mixture was incubated at room temperature under two fluorescent tubes (15 W) for 10 min to allow the development of purple color formazan which was then measured at 560 nm against the blank. One hundred microliters of distilled water was used as blank instead of enzyme extract. The reaction was stopped by switching off the light. The activity was measured in terms of inhibition of 50% of NBT photo-reduction at 560 nm and expressed as units per mg of protein.

Catalase test was determined by monitoring the reduction in the absorbance of H_2O_2 at 240 nm wavelength. The reaction mixture (3 ml) consisted of 100 µl enzyme extract with 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA and 12.5 mM H_2O_2. The activity was calculated based on an extinction coefficient of 0.04 mM^{-1} at 240 nm. The peroxidase (POD) activity in the extract was determined by the method of Kar and Mishra (1976) with minor modifications. The assay mixture consisted of 100 µl of enzyme extract with 0.1 M phosphate buffer, 0.1 mM pyrogallol, 5 mM H_2O_2 and incubated for 5 min at 25°C. For turning off the reaction 1.0 ml of 2.5 N H_2SO_4 was used and observed indigo color formed was read at 420 nm against blank containing water in place of enzyme extract.

Biochemical Analysis of Plant

Proline content in the leaves was determined following the standard protocol (Bates et al., 1973) with minor modifications. A 0.5 g of fresh leaves were homogenized in 3 ml of 5% (w/v) sulfosalicylic acid and centrifuged at 8,500 g for 10 min. Five hundred microliters of resulting supernatant was made up to 1 ml with sterile water and gently vortexed with 2 volumes of 2% ninhydrin. The mixture was boiled for 30 min at 100°C. After cooling, an equal volume of toluene was added to the mixture and upper aqueous phase was used for taking absorbance at 520 nm in a spectrophotometer (Jasco Corporation, Japan). The proline content was estimated by comparing with a standard curve of L-proline (Sigma-Aldrich, USA) as standard.

The extent of lipid peroxidation was calculated by measuring the malondialdehyde (MDA) content formed through thiobarbituric acid reaction following method of Hodges et al. (1999) with minor modification. The alcoholic extract (1 ml) of leaves was mixed with 1 ml of 0.5% thiobarbituric acid containing 20% trichloroacetic acid and heated up to 90°C for 30 min. Following cooling, the sample was centrifuged at 5,000 g for 5 min and the supernatant was read at 400, 532, and 600 nm. The MDA concentration was determined by its molar extinction coefficient (155 mM^{-1} cm^{-1}) and expressed as mmol MDA g^{-1} fresh weight (FW).

DEFENSE ASSAY

Inoculum Preparation and Plantlet Treatment

The wheat seeds were sterilized as per above mentioned protocol and left for germination in dark for about 4–5 days in a moist condition. The germinated seedlings were grown 9 days in controlled conditions with 16:8 photoperiod at 24 ± 2°C. Preparation of bacterial inocula was done as per above section. For fungus treatment, *F. graminearum* was grown in potato dextrose medium at 28°C for 3–4 days. After the growth, the culture was harvested at 7,000 g for 15 min. The obtained pellet was washed with sterile 1X phosphate buffer saline (PBS) and re-suspended in the buffer to attain 4,000 spores/ml of fungus. On the 9th day, germinated plants were challenged with the pathogen in bacterium primed plants and in control plants. A separate set of bacterium-inoculated and control plants were also taken for comparative analysis of defense enzymes during experimental study.

Plant samples were crushed in liquid nitrogen at every 24 h period for the next 6 days after inoculation. All the samples were assayed in triplicate sets. Each of the crushed plant material was aliquoted into four 1.5 ml eppendorf tubes (three for each PR protein assay) and stored at −70°C for later use. These tubes were then re-suspended in respective buffers with 0.5 g plant material being suspended in 1 ml buffer.

β 1, 3-Glucanase Assay

0.5 g of crushed plant tissue was extracted in 50 mM sodium acetate buffer (pH 5.0), and centrifuged at 12,000 g for 15 min at 4°C. The extract of 80 μl was mixed with 40 μl of 4% laminarin and kept at 40°C for 10 min. For stopping the reaction, 300 μl of dinitrosalicylic acid reagent was added in the mixture and heated for 10 min. To stabilize the color, 40 μl of 40% sodium potassium tartarate was added and diluted three times with distilled water to take its absorbance at 575 nm (Kurt, 1991).

Phenylalanine Ammonia Lyase Assay (PAL)

The crushed plant tissue (0.5 g) was re-dissolved in 50 mM Tris buffer (pH 8.8). The homogenate was centrifuged at 12,000 g for 15 min at 4°C, and supernatant was used for analysis. The reaction mixture consisted of 176 μl of 70 mM Tris pH 8.8, 70 μl of 10 mM phenylalanine, and 100 μl of enzyme extract. The reaction was allowed to proceed at 30°C for 60 min after which it was stopped by adding 200 μl of 2 N HCl. Finally, the reaction mixture was extracted with 200 μl of toluene by vortexing for 15 s, and the mixture was centrifuged at 2,000 g for 5 min to separate the phases. The upper phase was used for estimating the amount of cinnamic acid at 290 nm (Ramamoorthy et al., 2002).

Peroxidase Assay (PO)

The extract of plant tissue (0.5 g) was made in 10 mM sodium phosphate buffer (pH 6.0), and homogenate was centrifuged at 12,000 g for 15 min at 4°C. The reaction mixture consisted of 0.25% guaiacol, 10 mM sodium phosphate buffer, and 0.1 M H_2O_2 in 2.9 ml to which 0.1 ml of enzyme extract was added (Hammerschmidt et al., 1982).

Polyphenol Oxidase Assay (PPO)

0.5 g of crushed plant tissue was re-dissolved in 100 mM sodium phosphate buffer (pH 6.5), centrifuged at 12,000 g for 15 min at 4°C and supernatant was used for analysis. The reaction mixture consisted of 0.45 ml of 100 mM sodium phosphate buffer (pH 6.5), 50 μl of 0.01 M tert-butyl catechol, and 40 μl of enzyme extract (Mayer, 2006).

ROOT COLONIZATION

Root colonization of inoculated bacterium was determined on the 15th day of plant growth using serial dilution plating technique on NA-agar medium and number of viable cells was estimated as colony forming units (CFU) as described (Somasegaran and Hoben, 1994). Additionally, for confirming the identity of the colonized bacterium, ERIC-PCR (enterobacterial repetitive intergenic consensus) of recovered bacterial colonies from treated plants was performed as per standardized protocol (Singh et al., 2015). The treated plants were up-rooted and gently washed in sterile Milli-Q water to remove the soil particles and loosely bound bacteria from the roots. The g-DNA of the bacterial treated plant was isolated by bacterial DNA isolation kit. Pure culture of test isolate was also used as positive control.

STATISTICAL ANALYSIS

The experiment was conducted in completely randomized designs, and results were expressed as means ± standard errors of three independent replicates. The difference between means in each treatment was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range tests ($p = 0.05$, <0.05, <0.01) using by a DPS statistical software package (version 11.0).

RESULTS

Isolation and Primary Characterization of Bacteria

Based on the luxuriant growth on DF medium containing ACC, bacterial isolate SBP-9 was selected for further study. Continuous growth of SBP-9 on DF-ACC agar plate after several sub-culturing illustrated its ability to utilize ACC as a nitrogen source. It was found positive for the test of lipase, urease, and nitrate reductase, whereas negative for indole, methyl red, Voges-Proskauer, amylase, and catalase. In addition, SBP-9 also showed pectinolytic and cellulolytic (exoglucanase & endoglucanase) activities. It showed growth up to 50°C, while optimal temperature (based on OD) for the growth was 30°C. The isolate was able to tolerate salt concentration up to 8% NaCl, while the optimum growth was observed at 4% NaCl. Similarly, pH tolerance was found in a range of pH 6–11. Moreover, antibiotic sensitivity profiling of the isolate SBP-9 showed its resistance to kanamycin, ampicillin, tetracycline, gentamycin, whereas sensitivity to chloramphenicol, streptomycin, and erythromycin (**Table 1**). Among the tested carbon sources, SBP-9 utilized various carbon sources that have been summarized in Supplementary Table 1. The test isolate inhibited the growth of *F. oxysporum, F. graminearum,* and *P. citrium* and showed the swimming, swarming, and twitching motilities.

Identification and Phylogenetic Analysis

The PGPR isolate SBP-9 identified as *S. maltophilia* showed 100% identity with other reported gene sequences (16S rRNA) of *Stenotrophomonas* sp. (**Supplementary Figure 1**). Threshold of >98% sequence match with type strain was considered for identification. The sequence of resulting amplicon (580 bp) was submitted to the Genbank database under the accession number KJ950710.

Plant Growth Promoting Features

Quantitative value for ACC deaminase activity of isolate SBP-9 was determined as 362 ± 4.1 nmol of α-KB mg^{-1} protein h^{-1}. Formation of a clear zone around the streaked colony on media supplemented with an insoluble form of phosphate (tri-calcium

TABLE 1 | Biochemical characterization of isolate SBP-9.

Characteristic (s)	Activity
Gram test	–
Indole	–
MR	–
VP	–
Amylase	–
Lipase	+
Urease	+
Catalase	–
Nitrate reductase	+
Max. temperature tolerance (°C)	50
Salt (NaCl) tolerance(%)	8
pH tolerance	6–11
MOTILITY	
Swimming	+
Swarming	+
Twiching	+
ANTIBIOTIC RESISTANCE	
Chloramphenicol	+
Streptomycin	+
Erythromycin	+
Tetracycline	++
Kanamycin	++
Gentamycin	++
Ampicillin	++

+, sensitive; ++, resistant; +, positive; −, negative.

TABLE 2 | Plant growth promoting traits of strain SBP-9.

Plant growth promoting traits	Activity
ACCD activity (nmol of α-KB mg^{-1} Pr·hr^{-1})	362 ± 4.1
IAA production (μg/ml)	3.16 ± 0.12
Gibberellic acid (μg/ml)	5.40 ± 1.10
Phosphate solubilization (μg/ml)	10.73 ± 2.34
Siderophore index	+
HCN production	+
Ammonia production	+

±, standard deviation; +, positive.

phosphate) indicated mineral phosphate solubilizing activity. On quantification of phosphate solubilization, it solubilized 10.73 ± 2.34 μg ml^{-1}. Among the phytohormones, the isolate produced 3.16 ± 0.12 μg ml^{-1} indole-3-acetic acid, and 5.40 ± 1.10 μg ml^{-1} gibberellic acid. The appearance of orange-halo zone on the CAS-agar plate was considered as positive for siderophore production (**Supplementary Figure 2**). Continuous growth for several generations on N^- medium indicated an ability of the test isolate to fix atmospheric nitrogen. Moreover, amplification of the *nif*- H gene in *S. maltophilia* SBP-9 supports the nitrogen-fixing potential at the molecular level. The desired band of 300 bp corresponding to the *nif*-H gene was obtained by using universal primers for the *nif*-H gene (**Supplementary Figure 3**). In addition, it was also positive for ammonia, and HCN production (**Table 2**).

Physiological Enzyme Activities

The ACCD activity of the isolate SBP-9 was evaluated under various physiological conditions. Among different salt concentrations, highest ACCD activity of 365.38 ± 13.40 nmol α-KB mg^{-1} protein h^{-1} was observed in DF media supplemented with 4% of NaCl (**Figure 1A**). The increase in salinity from 2 to 4% increased the activity up to 59%, however, on further increase in NaCl concentration from 4 to 8%, activity was decreased up to 232%. Under varying temperature conditions, highest enzymatic activity was obtained at 30°C (370 ± 15 nmol α-KB mg^{-1} protein h^{-1}), a further decrease in activity was recorded with rise in temperature (**Figure 1B**). Assessment of ACCD activity under various pH values demonstrated that pH 8.0 (361.37 ± 13.0 nmol α-KB mg^{-1} protein h^{-1}) was optimum for enzymatic activity (**Figure 1C**). Higher enzymatic activity (360.90 ± 15.70 nmol α-KB mg^{-1} protein h^{-1}) was recorded after 48 h of incubation (**Figure 1D**).

Plant Growth in Response to Bacterial Inoculation

Physiochemical characteristics of soil used for plant growth study have been summarized in Supplementary Table. 2. S. *maltophilia* SBP-9 enhanced both shoot and root growth of wheat plant under tested salinity stress. SBP-9 inoculation significantly improved the shoot length by 39% ($P < 0.01$), and 19.66% ($P = 0.05$) in T-2, and T-1 treatments as compared to corresponding control (**Figure 2A**). In response to SBP-9, root length was increased by 28.81% ($P < 0.01$) and 21% ($P < 0.05$) in T-2 and T-1 treatments as compared to respective control (**Figure 2B**). Bacterial application increased the biomass of wheat plant under both non-saline and saline stress conditions. Shoot fresh weight (SFW) increased by 18.40% ($p = 0.05$), and 24% ($P < 0.05$) at T-1 and T-2 treatments as compared to respective control (**Figure 2C**). Compared to corresponding control, SBP-9 inoculation increased the shoot dry weight (SDW) by 16.5% ($P = 0.05$), 23% ($P < 0.05$), and 34.4% ($P < 0.01$) at T-0, T-1, and T-2 treatments (**Figure 2D**). Similarly improvement in root fresh weight (RFW) was 29% ($P < 0.01$), 35.4% ($P < 0.01$), and 59% ($P < 0.01$) at T-0, T-1, and T-2 treatments, as compared to their respective control (**Figure 2E**). Following bacterial inoculation, root dry weight (RDW) increased by 31% ($P < 0.01$), and 70% ($P < 0.01$) at T-1, and T-2 treatments, as compared to respective control (**Figure 2F**). In response to SBP-9 inoculation, total chlorophyll content also increased at various treatments. It is evident from **Figure 3** that highest increase in chlorophyll content of 55% ($P < 0.01$) was observed at treatment T-2, followed by 25% ($P < 0.01$) at treatment T-1, as compared to corresponding control.

Ionic Analysis in Response to *S. maltophilia* SBP-9

Change in ionic contents particularly Na^+ and K^+ in response to SBP-9 inoculation was tested under non-saline and at a salinity

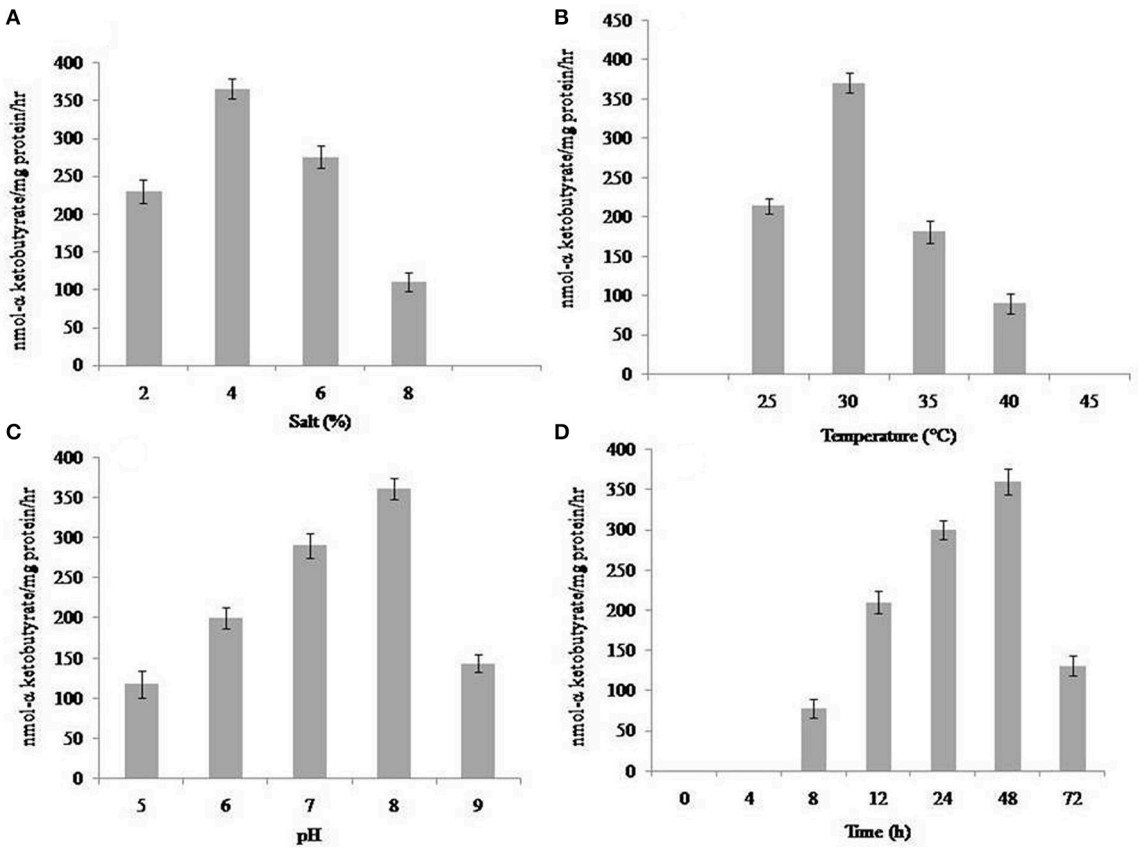

FIGURE 1 | Evaluation of ACC deaminase activity of isolate SBP-9 in various physiological conditions; **(A)** salt stress **(B)** different temperatures **(C)** varying pH **(D)** incubation time. Data represent mean ± SD of triplicate sets.

level of 0 (T-0), 150 (T-1), and 200 mM (T-2) of NaCl. Bacterial inoculation did not significantly affect the shoot and root Na^+ content under non-saline (T-0 treatment) condition. However, SBP-9 inoculation decreased the shoot Na^+ content by 25% ($P < 0.01$), and 32.28% ($P < 0.01$), as well as root Na^+ content by 30% ($P < 0.01$), and 24.5% ($P < 0.05$) in T-1 and T-2 treatments respectively, as compared to respective control (**Figures 4A,B**). Significant increase in shoot K^+ content was 22% ($P < 0.05$) and 32.5% ($P < 0.01$) at T-1 and T-2 treatments, as compared to respective control. Furthermore, SBP-9 inoculation significantly increased the root K^+ content by 29% ($P < 0.01$) and 35.8% ($P < 0.01$) at T-1 and T-2 treatments respectively, as compared to respective control (**Figures 4C,D**).

Proline and MDA content

The observed results indicated that SBP-9 reduced the proline and MDA content under both non-saline and saline stress conditions. Proline content was decreased by 21.45% ($P = 0.05$) under non-saline condition (T-0 treatment) as compared to corresponding control. The highest decrease in proline content was 45.94% ($P < 0.01$) followed by 32.13% ($P < 0.01$) at T-1 and T-2 treatments as compared to corresponding control (**Figure 5A**).

Inoculation with SBP-9 significantly reduced the MDA content under both non-saline and salt stress conditions. MDA content was decreased by 24.39% ($P < 0.05$) under non-saline condition (T-0 treatment) as compared to control. Similarly, SBP-9 inoculation reduced the MDA content by 36% ($P < 0.01$), and 30% ($P < 0.01$) at T-1 and T-2 treatments respectively (**Figure 5B**).

Antioxidative Activities

A significant difference in the antioxidative enzyme activities of SBP-9-inoculated and control plants was observed under salinity stress conditions. Inoculation with SBP-9 significantly increased the antioxidative (SOD, CAT, POX) activities to alleviate the salinity induced free radical damages. SBP-9 inoculation slightly increased the SOD activity (27.70%) at T 0 treatment. However, the maximum increase in activity was 40.81% ($P < 0.01$) and 39.58% ($P < 0.01$) at T-1 and T-2 treatment as compared to respective control plants (**Figure 6A**). Considering the CAT enzyme, higher activity 55.91% ($P < 0.01$) was observed at treatment T-1, followed by 39.33% ($P < 0.01$) and 24.35% ($P < 0.05$) at treatments T-2 and T-0 respectively, as compared to corresponding control plants (**Figure 6B**). The highest significant ($p = 0.05$) increase in POX activities was 38.23% ($P < 0.01$) and 34% ($P < 0.01$) at T-1 and T-0 treatment as compared to respective un-inoculated plants. In T-2 treatment, bacterial inoculation significantly ($P < 0.01$) increased the POX activity of 25.84% ($P < 0.05$) as compared to respective control (**Figure 6C**).

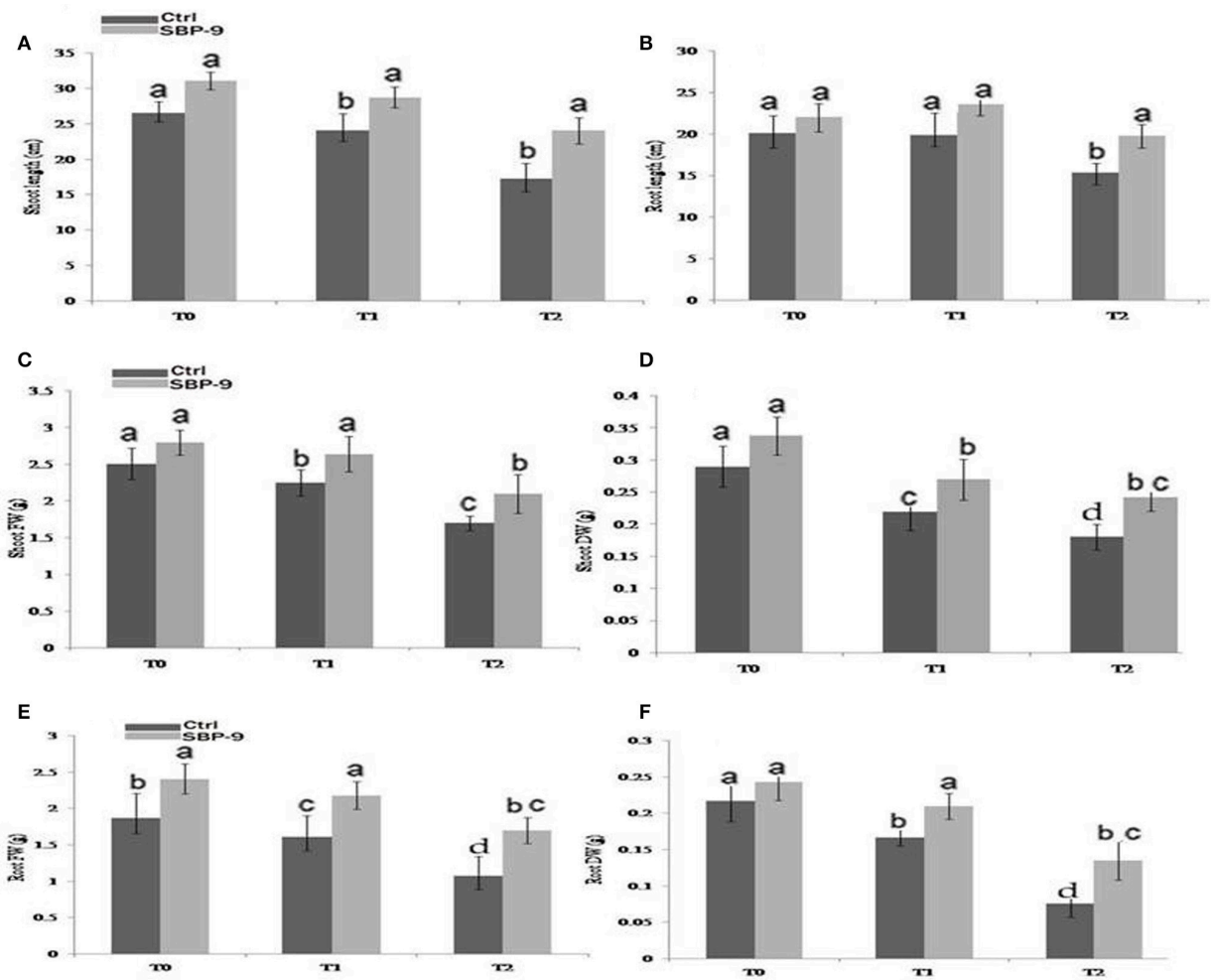

FIGURE 2 | Effect of inoculation of isolate SBP-9 on plant growth and biomass content under different treatments T-0 (0 mM NaCl), T-1 (150 mM NaCl), T-2 (200 mM NaCl); **(A)** Shoot length **(B)** Root length **(C)** Shoot fresh weight **(D)** Shoot dry weight **(E)** Root fresh weight **(F)** Root dry weight. Each data represent the mean ± SD of triplicate sets of five measurements ($n = 15$). Different letters on the bar in each column represent the significant difference.

Induction of Defense Response

In the plant growth experiment, the bacterial isolate significantly increased the defense enzymes in wheat plant challenged with the pathogen. Plants pre-treated with SBP-9 and challenge-inoculated with fungal pathogen showed a concurrent increase in β-1, 3 glucanase, PAL, PO, and PPO. Upon pathogen challenge in bacterized wheat plants, β-1, 3 glucanase activity started to increase up to 3rd day (332 ± 20 ng glucose/min/mg protein), thereafter declined gradually (**Figure 7A**). Similarly, wheat plant inoculated with pathogen in bacterium-primed plants also showed increase in the PAL activity. The higher induced activity was observed on 3rd day (23 ± 2.1 nmol of trans-cinnamic acid) (**Figure 7B**). Wheat plants treated with the isolate SBP-9 alone also had higher PAL activity, however the activity level was less during the first 3 days as compared to control plants challenged with a fungal pathogen. PO activity also increased in bacteria-treated plants challenged with the pathogen. The maximum activity was observed at 4th day after pathogen inoculation (**Figure 7C**). A similar pattern of increased PPO activity was recorded in bacterized wheat plants challenged with the pathogen. The plants of uninoculated control showed the lowest enzyme activity among all treatments (**Figure 7D**).

Colonization

Colonization efficiency of the bacterium was determined by plate counting after 15 days of plant growth. The associated bacterium was found in a range of 1.8×10^3 CFU g^{-1} of the root. No bacterial colonies were recovered from uninoculated control plants. In addition, for the ERIC-PCR profile obtained from total DNA of treated plants was identical to that of a pure culture of *S. maltophilia* SBP-9, which indicated that the bacterium had successfully colonized the plants (**Supplementary Figure 4**).

DISCUSSION

The present work is an attempt to evaluate the biotic and abiotic stress tolerance conferred by ACC-deaminase bacterium *S. maltophilia* SBP-9 in wheat plants. To the best

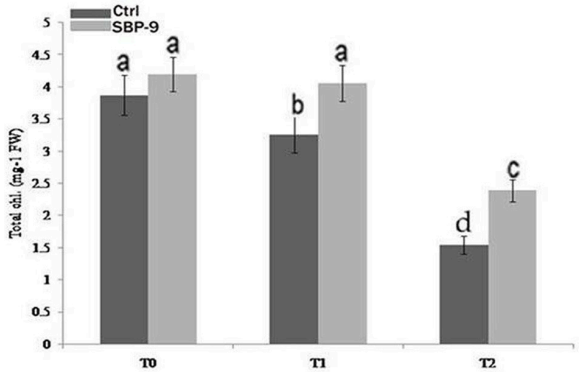

FIGURE 3 | Effect of inoculation of isolate SBP-9 on total chlorophyll content under different treatments (T-0, T-1, T-2). Significant difference in each column has been shown by different letters.

of our knowledge, the present study is the first to report *Stenotrophomonas* sp. having ACC deaminase activity and conferring induced systemic tolerance in wheat plant under salinity stressors. Plant rhizosphere is a preferred niche for soil microorganisms which is relatively rich in organic substrates, for stimulating microorganism growth and attracts diverse genera of microbial species. In response to saline stress, ACC is transported by the xylem to shoots where it is oxidized to ethylene Jackson, 1985). Meantime ACC is secreted by roots, which stimulates the proliferation inoculated ACCD producing bacteria (Grichko and Glick, 2001; Dobbelaere et al., 2003). From the previous finding, it was observed that exogenous application of ethylene or its precursor ACC decreased the root growth (Nukui et al., 2000), however bacteria equipped with ACC deaminase minimize the level of "stress ethylene" and thus confer resistance to various biotic and abiotic stresses. According to previous report (Glick et al., 1994) *AcdS*$^{-}$ mutant (lacking ACC deaminase activity) of PGPR strain *Pseudomonas putida* GR12-2 lost the ability to promote canola root elongation. Similarly, Sergeeva et al. (2006) proposed that ACC deaminase activity has a dual role in growth promotion and tolerance to high salt stress in transgenic canola plants.

Following above evidences, it is assumed that plant growth promotion by *S. maltophilia* SBP-9 in wheat seedlings exposed to salinity stress might be attributed to the ACC-deaminase activity, which in turn reduces the synthesis of ethylene. Isolate SBP-9 showed quite high ACCD activity, having >20 nmol of α-KB mg^{-1} h^{-1} which is enough to trigger systemic tolerance under stress conditions (Penrose and Glick, 2003). The strain was found to produce phytohormone IAA and solubilize phosphate that supports the plant growth stimulation under adverse condition like salt (Egamberdieva, 2009; Ramadoss et al., 2013). Imran et al. (2014) reported that bacterial isolates having multiple beneficial traits is better than having the single trait. Many of the rhizobacteria are known to release iron-chelating siderophores into the rhizosphere of its/their host plant that influence the uptake and availability of other metal like Zinc (Zn), Copper (Cu), and Iron (Fe) (Grichko et al., 2000; Egamberdiyeva, 2007; Dimkpa et al., 2009).

The ACCD activity of SBP-9 was characterized under different physiological conditions. Among different salt and temperature conditions, higher activity was recorded at 4% salinity and 30°C temperature. Significant plant growth promotion was achieved under salt stress on inoculation of isolate SBP-9 which could result from one or more growth promoting properties of the inoculated bacterium. Cheng et al. (2007) suggested that inoculation with ACCD bacterium *P. putida* UW4 enhances the various physiological parameters of *Brassica napus* under inhibitory level of salinity stressors. Likewise, significant increase in plant growth and number of leaves of *Limonium sinense* was also observed following inoculation of ACCD bacteria under salt stress (Sheng et al., 2014). In our study, SBP-9 was found as an efficient promoter of wheat plant especially under salt exposure (150 and 200 mM NaCl). In addition, salt stress also hampers the photosynthetic mechanism due to chlorophyll peroxidation (Tuna et al., 2008; Barry, 2009). However, inoculation of isolate SBP-9 significantly improved the leaf chlorophyll content as compared to uninoculated control under both non-saline and salinity conditions, illustrating the ability of the strain to counteract the salinity stressors. Our results are in concurrence with the previous study where ACCD producing bacteria stimulate the plant growth under varying salinity stressors (Barnawal et al., 2014). However, Contesto et al. (2008) suggested that *Arabidopsis* plants inoculated with ACCD mutant and its wild type counterpart did not show any difference in plant growth promoting effects under stress conditions. Thus, future work is required to establish the role of ACC deaminase in isolate SBP-9 by raising ACCD mutant of isolate SBP-9 and its effect on plant growth.

Exclusion of Na^+ and influx of K^+ are the plant's strategies for mitigating the salinity induced oxidative stress (Shabala and Cuin, 2008). In our study, inoculation with isolate SBP-9 significantly decreased the accumulation of Na^+ and increased K^+/Na^+ levels in both shoots and roots of the wheat plant under salinity stress. Our result is in congruence to a previous study where inoculation of ACCD bacteria significantly improved the K^+ content in tomato plants under salt stress (Mayak et al., 2004). Previous study of Zhang et al. (2008) suggested that *Arabidopsis* inoculation with *Bacillus subtilis* GB03 decreased the Na^+ content (54%) by down-regulating HKT1 expression in roots and up-regulating HKT1 expression in shoots to enhance shoot-to-root Na^+ recirculation. Therefore, correlating these changes in response to ACCD producing bacteria could provide the evidence of plant growth regulation in physiologically diverse conditions (Rodriguez-Rosales et al., 2008).

Alleviation of salinity-induced oxidative damages with the use of antioxidant enzymes is an important strategy of plants for increasing its tolerance to stress conditions. In the present work, increased activities of various antioxidant enzymes following bacterial inoculation illustrated that these enzymes play a crucial role in protection of plants under salinity like stresses. *A. xylosoxidans* increases the antioxidant activity in *Catharanthus roseus* (Karthikeyan et al., 2008) and in the *Solanum melongena* inoculated with *Pseudomonas* sp. DW1 (Fu et al., 2010). The bacterial SOD facilitates the removal of free radicals and plays an important role in their survival in the rhizosphere (Wang et al., 2007). The major breakdown product of SOD is H_2O_2, which is a

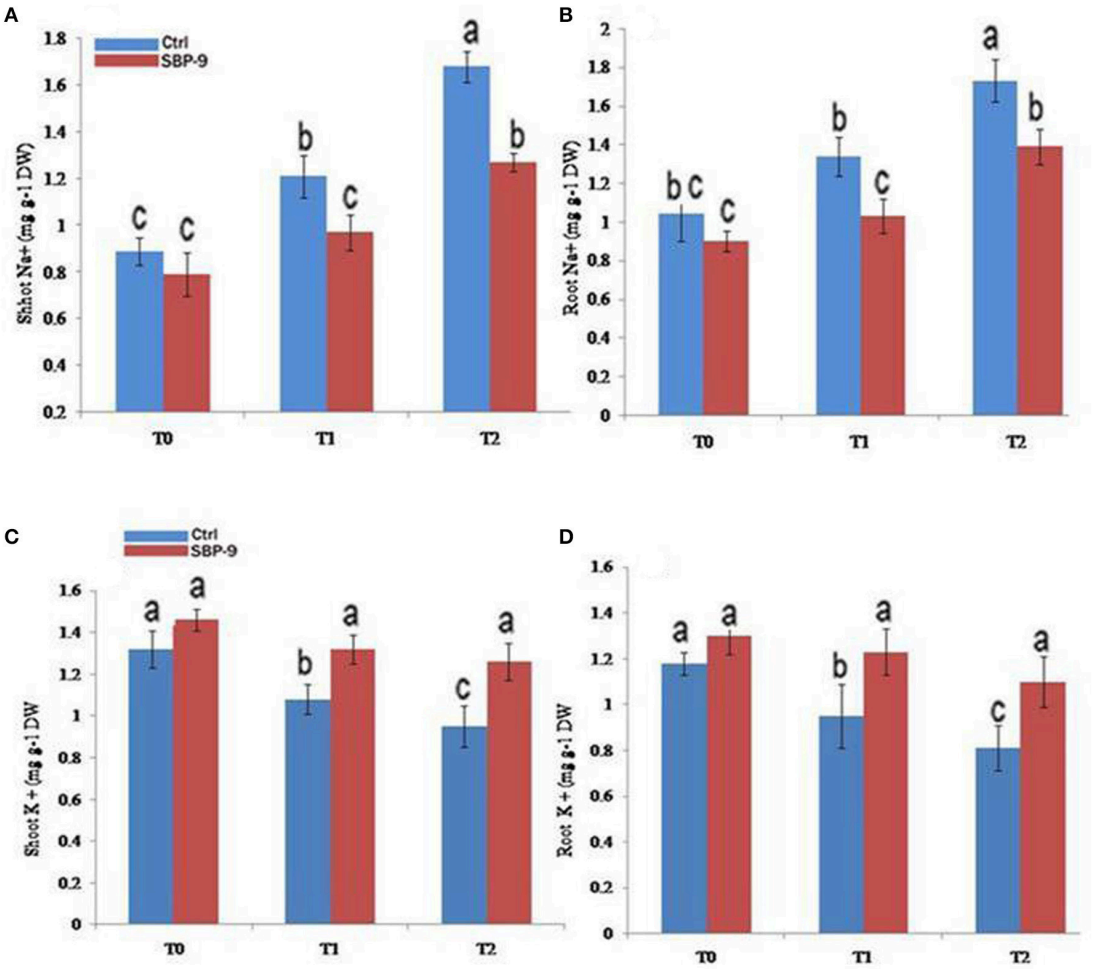

FIGURE 4 | Effect of NaCl and inoculation with SBP-9 on ionic uptake by plants under different treatments T-0, T-1, T-2; **(A)** Shoot Na^+ **(B)** Root Na^+ **(C)** Shoot K^+ **(D)** Root K^+. Values are mean ± SD of triplicate sets of five measurements in triplicates ($n = 15$). Different letters on the bar in each column represent the significant difference.

toxic lipid peroxidant, but can be eliminated by activities of CAT and POX antioxidant enzymes. The POX activity plays a major role in eliminating the stress induced H_2O_2 and malondialdehyde level, thus protecting the cell membrane integrity. Our data showed that activities of SOD, CAT, and POX enzymes in leaves of SBP-9 inoculated plants were higher compared to uninoculated plants under salinity stress. The increase in enzyme activities was probably due to the fact that bacterial inoculation stimulated the synthesis of these enzymes (Wang et al., 2010).

The accumulation of proline in response to salt stress protects the cell membrane, stabilizes the structure of the protein and scavenges the free hydroxyl radicals (Claussen, 2005). In the present study, the SBP-9-inoculated plants showed lower proline levels as compared to uninoculated control plants. This decrease in proline level in SBP-9-inoculated plants indicated that ACC deaminase producing bacterial-inoculated plants were less affected by salinity. Soil salinity increases the generation of ROS in plants which enhance the membrane lipid peroxidation and increase the MDA content (Koca et al., 2006; Yazici et al., 2007). Therefore, leaf MDA content is usually used to evaluate plant tolerance to salinity (Luna et al., 2000). The decrease in MDA content in SBP-9 inoculated plants indicates that bacterial inoculation protects the plants from the imposed salt stress.

Beneficial bacterial-mediated induced systemic resistance is associated with induction of various defense enzymes like β 1-3-glucanase, PAL, PPO, and PO (Meena et al., 2000). The previous investigation of Umamaheswari et al. (2010) showed the increased accumulation of these defense enzymes in watermelon plants confronted with *Alternaria alternate* in the presence of biocontrol microorganisms. However, in most of the study very little is known about the mechanisms governing the ISR response. The production of various defense enzymes in the presence of SBP-9 might illustrate its role in the generation of resistance to pathogen infection. The increased level of β 1-3-glucanase, PAL, PPO, and PO might play a key role in pathogen suppression in bacterium-primed plants challenged with the pathogen. The defense enzymes PAL and PO are involved in the biosynthesis of phytoalexins and phenolics that are primarily responsible for

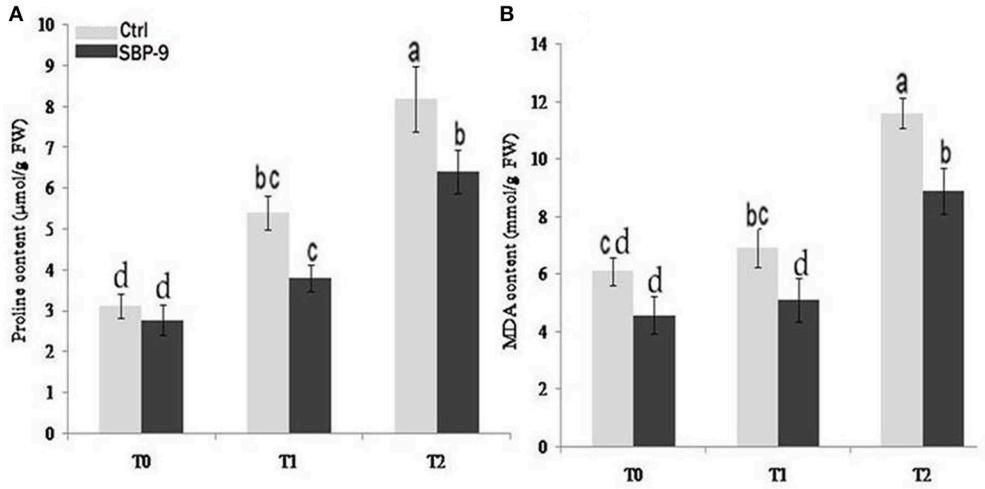

FIGURE 5 | Effect of SBP-9 inoculation on proline **(A)** and malondialdehyde content **(B)** under different treatments; T-0 (0 mM NaCl), T-1 (150 mM NaCl), T-2 (200 mM NaCl). Values are mean ± SD of triplicate sets of five measurements in triplicate sets ($n = 15$). Different letters on the bar in each column represent the significant difference.

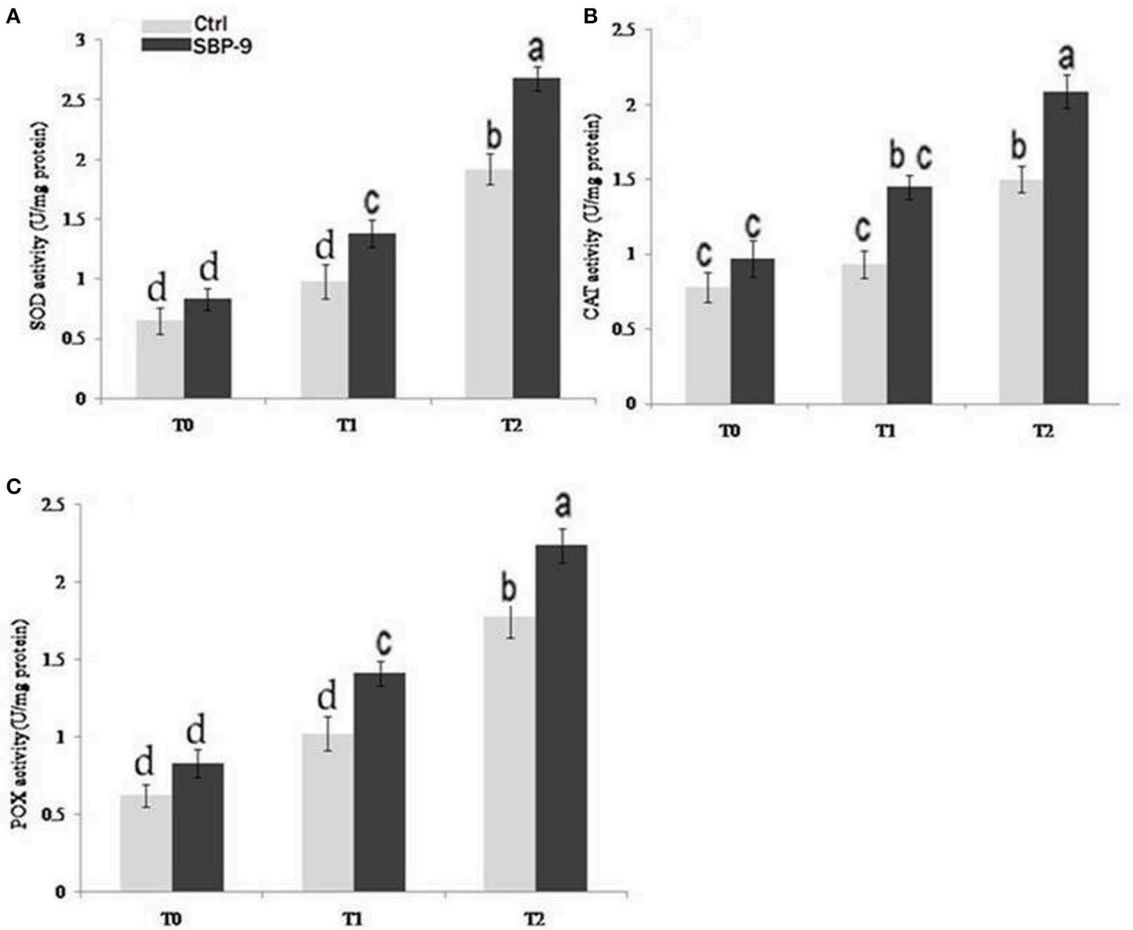

FIGURE 6 | Effect of NaCl and SBP-9 inoculation on the antioxidant activities under tested treatments T-0, T-1, T-2; **(A)** Super-oxide dismutase (SOD) **(B)** Catalase (CAT) **(C)** Peroxidase (POX). Each value is mean of three replicates ± SD. Different letters on the bar in each column represent the significant difference.

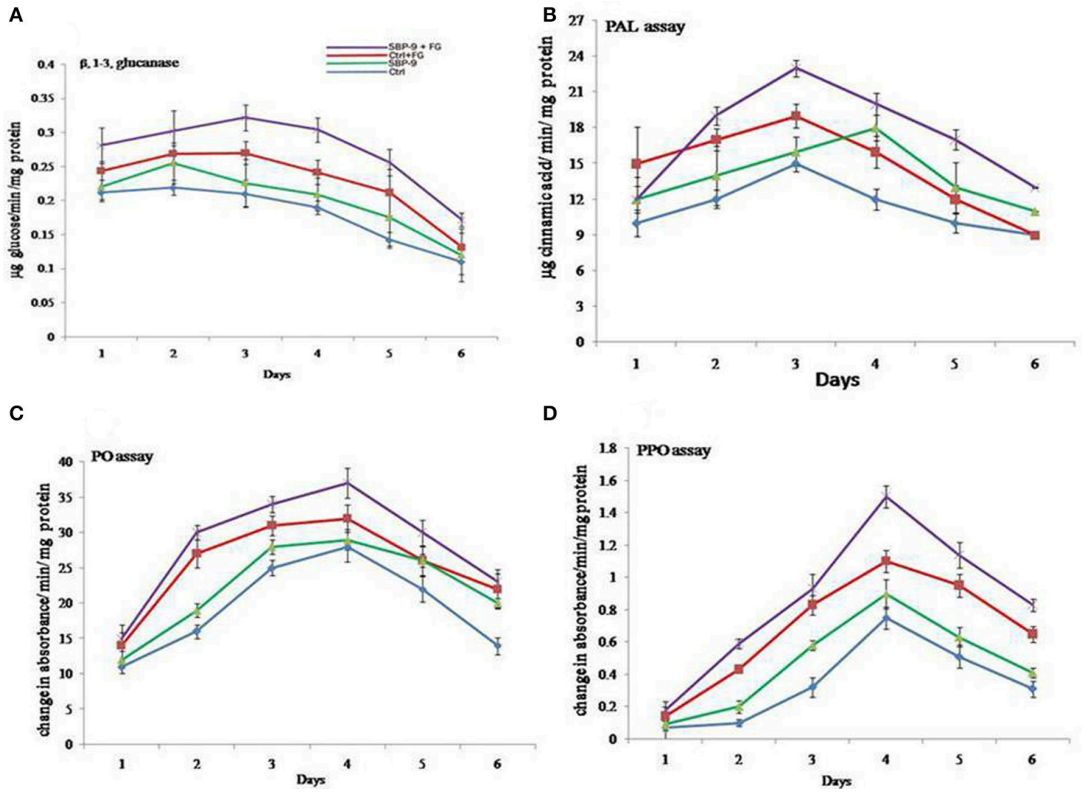

FIGURE 7 | Augmentation of defense response in wheat by *S. maltophila* SBP-9 against *Fusarium graminearum* under controlled conditions; **(A)** β 1, 3-glucanase assay **(B)** Phenylalanine ammonia lyase assay **(C)** Peroxidase assay **(D)** Polyphenol oxidase assay.

disease resistance (Daayf et al., 1997). PAL favors the formation of trans-cinnamic acid from phenylalanine, an intermediate in salicylic acid biosynthesis (Ryals et al., 1996). The activities of PO and PPO are linked to the generation of hydrogen peroxide and lignification during infection which inhibits phytopathogens directly or induce the generation of free radicals that inhibit the proliferation of pathogens (Silva et al., 2004). Besides, PPO oxidizes phenolics to toxic quinines and is involved in the terminal oxidation of diseased plant tissue (Kosuge, 1969).

Additionally the tested isolate SBP-9 showed all forms of motility which are required for the chemotactic responses and colonization (VandeBroek et al., 1998; Lugtenberg and Kamilova, 2009). Colonization ability of the isolate can provide the maximum benefits to plants as it establishes a close relationship with the plant host than rhizosphere and modulates the defense response to cope with adverse conditions. Effective colonizing bacterium encounters a protective environment where they could have a better survival and therefore more prolonged activity (Hardoim et al., 2008).

CONCLUSION

The observed results of present study demonstrates that use of the inoculation with multifarious plant growth promoting bacterium *S. maltophilia* significantly improves the growth, ionic balance and biochemical parameters of plants, thus allowing them to cope with imposed salinity stress. The effect of beneficial rhizobacteria on plant growth improvement are well-known, however the physiological and molecular mechanisms still need to be explored for better utilization of these microorganisms. The association of these microorganisms to plants at the genetic level will provide valuable insight for microbial mediated enhanced salinity tolerance and this may help to pave the way for the commercial application of the microorganism at field level. The selected strain in the present study should be tested for its capability to enhance plant growth in unsterilized soil and at field level also. Furthermore, the efficacy of strain can be tested on the other plants besides of wheat. Nevertheless, mechanistic studies between PGPR and plant are still in need to investigate the ways by which PGPR exert beneficial effects on plants.

AUTHOR CONTRIBUTIONS

RS performed the experiments and PJ mentored the research work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01945/full#supplementary-material

Supplementary Figure 1 | Phylogenetic tree showing the relationship of *S. maltophilia* SBP-9 to closely related bacteria. PCR amplified amplicon of partial 16S rRNA gene of SBP-9 was sequenced and used for the construction of a phylogenetic tree. The 16S rRNA gene sequence of closely related species was obtained from NCBI GenBank database. The tree was obtained using neighbor- joining method of software packages Mega version 6.0, at the bootstrap value of (*n*= 1,000).

Supplementary Figure 2 | Siderophore production by the test isolate *S. maltophila* SBP-9 on CAS-agar plate.

Supplementary Figure 3 | *nif*-H gene amplification in *S. maltophila* SBP-9.

Supplementary Figure 4 | Tracking of colonization of *S. maltophila* SBP-9 through ERIC-PCR profile of bacterium colonized on wheat plants and confirmation of its identity using profile of pure culture (Lane M: DNA ladder SM0311, Lane A: control DNA, Lane B: DNA of *S. maltophila* SBP-9 isolated from treated plant).

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Endophytic Bacteria Improve Plant Growth, Symbiotic Performance of Chickpea (*Cicer arietinum* L.) and Induce Suppression of Root Rot Caused by *Fusarium solani* under Salt Stress

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Salinity causes disturbance in symbiotic performance of plants, and increases susceptibility of plants to soil-borne pathogens. Endophytic bacteria are an essential determinant of cross-tolerance to biotic and abiotic stresses in plants. The aim of this study was to isolate non–rhizobial endophytic bacteria from the root nodules of chickpea (*Cicer arietinum* L.), and to assess their ability to improve plant growth and symbiotic performance, and to control root rot in chickpea under saline soil conditions. A total of 40 bacterial isolates from internal root tissues of chickpea grown in salinated soil were isolated. Four bacterial isolates, namely *Bacillus cereus* NUU1, *Achromobacter xylosoxidans* NUU2, *Bacillus thuringiensis* NUU3, and *Bacillus subtilis* NUU4 colonizing root tissue demonstrated plant beneficial traits and/or antagonistic activity against *F. solani* and thus were characterized in more detail. The strain *B. subtilis* NUU4 proved significant plant growth promotion capabilities, improved symbiotic performance of host plant with rhizobia, and promoted yield under saline soil as compared to untreated control plants under field conditions. A combined inoculation of chickpea with *M. ciceri* IC53 and *B. subtilis* NUU4 decreased H_2O_2 concentrations and increased proline contents compared to the un-inoculated plants indicating an alleviation of adverse effects of salt stress. Furthermore, the bacterial isolate was capable to reduce the infection rate of root rot in chickpea caused by *F. solani*. This is the first report of *F. solani* causing root rot of chickpea in a salinated soil of Uzbekistan. Our findings demonstrated that the endophytic *B. subtilis* strain NUU4 provides high potentials as a stimulator for plant growth and as biological control agent of chickpea root rot under saline soil conditions. These multiple relationships could provide promising practical approaches to increase the productivity of legumes under salt stress.

Keywords: chickpea, salinity, endophytes, rhizobia, symbioses, root rot

INTRODUCTION

Legumes are highly important crops in human and animal nutrition and are grown globally under a wide range of agro-climatic conditions as a cash crop and as a source of nitrogen assimilation via nitrogen fixation (Lüscher et al., 2011; Nyfeler et al., 2011). Legumes form important symbiotic relationships with rhizobia and are known as the most efficient system for biological nitrogen fixation (BNF) (Molla et al., 2001; Egamberdieva et al., 2013, 2016a; Santi et al., 2013). Numerous studies have shown that the symbiotic relationship between legumes and their rhizobia are susceptible to abiotic factors such as salinity, drought, and soil temperature, which can cause a failure in the infection and nodulation process (Slattery et al., 2001; Bouhmouch et al., 2005). Salt stress inhibits plant growth, nutrient uptake, and increases susceptibility of plants to soil-borne pathogens (Egamberdieva et al., 2011; Ahmad et al., 2015; Hashem et al., 2016). The susceptibility of plants to infection by soil borne pathogens was increased by salt stress, e.g., tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Triky-Dotan et al., 2005), and cucumber root rot caused by *Fusarium solani* (Egamberdieva et al., 2011). Nevertheless, microbes associated with a plant can have beneficial interactions, providing its partner organism biologically active compounds necessary for survival and proliferation (Marschner et al., 2001; Mercado-Blanco et al., 2004).

The interest in endophytic bacteria has increased, as they colonize the internal tissues of their host plants and improve plant tolerance to various abiotic stress factors and can protect plants from various pathogenic microbes (Malfanova et al., 2011; Hashem et al., 2016). Endophytic bacteria were found in different plants including crops, aromatic and medicinal plants, halophytes etc. (Azarias Guimarães et al., 2012; Sharma et al., 2012; Egamberdieva et al., 2017). Mutualistic associations between root associated microbes and plants bring benefits to the plant through an increased nutrient acquisition, altered metabolic interactions among the partners, alleviation of salt stress and improved symbiotic performance of legumes. The endophytic lifestyle may directly or indirectly assist during the infection and colonization processes of the rhizobium-host association and are coordinately involved in the adaptation of plants to stress tolerance (Hashem et al., 2016). The endophytes which effectively colonize plant tissue could be even more beneficial in a co-inoculation with rhizobia under salt stress as shown by previous work (Egamberdieva et al., 2016a). In addition, endophytic bacteria colonize root tissues and are capable to protect their hosts against invasion and damage by soil-borne pathogens (Mercado-Blanco et al., 2004; Rybakova et al., 2016). Endophytic bacteria colonizing internal plant tissue benefit plants using various traits, including synthesis of plant growth regulators (Beneduzi et al., 2012), osmoprotectants, exopolysaccharides (Berg et al., 2013), antifungal metabolites (Gond et al., 2015), and the modulation of plant physio-biochemical constituents (Hashem et al., 2016). However, despite the importance of the endophyte-plant relationship, our knowledge on the interactions between legumes, endophytes, and pathogens under hostile environmental conditions is still rather limited. Chickpea (*Cicer arietinum* L.) is a major food legume crop and an important source of protein in many countries, however its production is restricted by soil borne diseases and abiotic stress (Graham and Vance, 2003). A black root rot caused by *F. solani* is amongst the most serious fungal diseases of chickpea (Andrabi et al., 2011; Cabral et al., 2016).

The aims of the present study were to: (i) isolate and identify endophytic bacteria from the root nodule of chickpea with potential colonization patterns, (ii) assess their efficiency in improvement of plant growth, symbiotic performance of host plant, and alleviating salt stress, (iii) determine their biological control capability against *Fusarium* root rot on chickpea. Thus, our study intends to provide valuable information about interactions among rhizobia, endophytes, pathogens, and hosts under salt stress conditions.

MATERIALS AND METHODS

Isolation of Bacteria from Chickpea Nodules

Samples of healthy chickpea plants were collected from a field in the Syrdarya province of Uzbekistan, which is considered a salt affected region with summer daytime temperatures between 38 and 40°C. Soil is loamy sand and characterization was as follows: 43 ± 9 g sand kg^{-1}, 708 ± 12 g silt kg^{-1}, and 250 ± 13 g clay kg^{-1}, and had a cation exchange capacity of 23.6 ± 1 cmol kg^{-1}, with an exchangeable Na percentage of 4.41 and a Na absorption ratio of 0.32 (Egamberdieva et al., 2010). Electrical conductivity (EC) values of the saline soil were 7.2 dS m^{-1}. The organic matter content of the soil was 0.69% with total C, 2.5%; total N, 0.1%; Ca, 63.5 g/kg; Mg, 20.7 g/kg; K, 6.2 g/kg; P, 1.2 g/kg; Cl, 0.1 g/kg; Na, 0.7 g/kg; and the pH was 8.0 (Egamberdieva et al., 2010).

Three chickpea plants were randomly chosen from the field site and the whole plants including the root system were wrapped in plastic bags, brought to the laboratory and stored at 4°C until further processing. The root system of the collected plants was separated from the shoots and carefully washed under running tap water taking precaution to minimize root injury. Healthy, non-ruptured nodules were carefully collected from root and washed under running water. The nodules were surface sterilized by dipping in 95% ethanol for 1 min and then in 1% NaClO solution for 3 min, and rinsed in sterile distilled water. Sterilized nodules were weighed aseptically (1 g) and macerated in a mortar utilizing phosphate buffered saline (PBS) (20 mM sodium phosphate, 150 mM NaCl, pH 7.4). The nodule juice (1 ml) was placed in a tube containing 9 ml sterile PBS and shaken with a vortex for 1 min. The supernatant was collected, then serially diluted (10^1–10^5) and 100 μl aliquots from the appropriate dilutions were spread on Tryptic Soy Agar (TSA), nutrient agar (BD, Difco Laboratories, Detroit, USA), and King's B agar medium (King et al., 1954) in triplicate. The plates were incubated at 28°C and a representative number of colonies that displayed different colony morphologies were picked up from the plates and were re-streaked for the purification of the isolates. In total, 40 bacterial pure cultures were preserved on plates at −80°C.

Colonization Assay and Re-Isolation of Endophytic Isolates

The chickpea seeds (*C. arietinum* L., cv Uzbekistan-32) were obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA). Seeds were surface-sterilized for 5 min with 1% NaClO solution followed by 95% ethanol for 3 min, rinsed five times with sterile distilled water and germinated on 1% water agar in the dark at 28°C. The sterility of seeds was tested on TSA agar in incubation plates for 3 days at 28°C. No contaminants were found, indicating that the surface sterilization procedure was effective.

Forty bacterial isolates from the chickpea nodules were grown overnight in TSB broth, 1 ml of each culture was centrifuged, and cell pellets were suspended with PBS (cell density of 10^7 CFU/ml). Germinated seeds were dipped in bacterial suspension for 10 min, and were aseptically planted in a sterile sand column in the gnotobiotic system glass tubes (30 mm in diameter, 200 mm in length) as described by Simons et al. (1996). The tubes containing 100 g of sterilized washed sand were soaked with 10 ml of Hoagland's plant nutrient solution (Lynch et al., 1990), supplemented with 100 mM NaCl. The seedlings were grown in a growth cabinet with a 16-h light period at 22°C and an 8-h dark period at 16°C, one seed per tube with 10 replicates for each bacterial inoculant for 10 days. To re-isolate the bacteria from the root, the complete sand column was carefully removed from the tube and roots were surface-sterilized using 70% ethanol and 1% NaClO solution and were rinsed five times with sterile water to remove disinfectant. The root samples were macerated with a mortar and pestle, and macerated tissue extracts were serially diluted in PBS and 0.1 ml aliquots were spread on TSA plates and incubated at 28°C for 3–5 days to isolate bacterial endophytes. Of the inoculated isolates, 10 were re-isolated from the plants as endophytes and were characterized for plant growth promoting (PGP) traits.

Characterization of Bacterial Isolates

To test whether bacterial isolates were capable of stimulating plant growth, the seeds were surface-sterilized, and inoculated with the bacterial isolates as described above. Plants were grown in glass jars (1,000 ml). The jars were filled with a sterilized mixture of washed sand and vermiculite (1:1), and finally with Hoagland plant nutrient solution (Lynch et al., 1990) supplemented with 100 mM NaCl. The 10 seedlings per treatment in three replication were grown in a plant growth chamber with a 11-h light period at 24°C and an 8-h dark period at 18°C. After 3 weeks, the seedlings were removed from the sand and root and shoot dry weight was determined.

The determination of IAA (indole 3-acetic acid) was assayed as described by Bano and Musarrat (2003). The IAA concentration in culture was calculated using the calibration curve of pure IAA as a standard. The phosphate-solubilizing activity of the bacterial isolates was determined using Pikovskaya agar (Pikovskaya, 1948) containing precipitated tricalcium phosphate. The presence of clearing zones around the bacterial colonies was considered an indicator for positive solubilization activity. The production of chitinase enzymes was performed using colloidal chitin medium and protease activity was encouraged using sterile skim milk agar plates as described in Frändberg and Schnürer (1998), Dutta et al. (2015). The cellulose-degrading ability of the bacterial isolates was analyzed by streaking inocula on cellulose (Sigma-Aldrich, St. Louis, MO) Congo-Red agar media as described by Pratima et al. (2012). Lipase activity of the bacterial isolates was determined using the Tween lipase indicator assay (Howe and Ward, 1976). Furthermore, β-1,3 and β-1,4 glucanase activity was tested using the substrate lichenan (Sigma-Aldrich, St. Louis, MO) in top agar plates (Walsh et al., 1995). The production of HCN by bacterial isolates was determined following Castric (1975). The production of siderophores by the bacterial isolates was determined using chrome azurol S (CAS) agar media as described by Alexander and Zuberer (1991). The antagonistic abilities of selected isolates against pathogenic fungi *F. oxysporum*, *F. solani, Fusarium culmorum, Alternaria alternata*, and *Botrytis cinerea* were evaluated as described by Egamberdieva et al. (2016c). Briefly, fungal strains were grown in PDA plates for 5 days and small disks of agar piece with fungus were cut and replaced in the middle of fresh PDA plates. Holes (5 mm diameter) were made into PDA plates 2 cm away from fungal disc and 100 µl of bacterial cultures pregrown in TSB broth for 3 days were dropped into a hole. The plates were sealed with Parafilm® M and incubated at 28°C in darkness for 6 days. The growth inhibition zone of fungi was recorded.

Identification of Selected PGPR isolates

The isolation of DNA was carried out according to Töpper et al. (2010). A lysozyme solution [250 µl; 1 mg ml-1TE buffer (10:1, pH 7.4)] was used to re-suspend the filters and was added to a lysis buffer (250 µl; 20 µg proteinase K ml^{-1} 0.5% SDS). After incubation for 30 min at 55°C, 80 µl of 5 M NaCl, and 100 µl of preheated (55°C) CTAB (10% (w/v) hexadecyltrimethylammonium bromide in 0.7% NaCl) were added to the solution. After 10 min incubation at 65°C, 500 µl of chloroform: isoamyl alcohol (24:1) was added to the solution and centrifuged at 16,000 × g for 5 min. A TE buffer was used to re-suspend the DNA, which was precipitated with isopropanol. The 16S rDNA was amplified with polymerase chain reaction (PCR) using the universal forward 16SF: 5′-GAGTTTGATCCTGGCTCAG-3′ and reverse 16SR: 5′-GAAAGGAGGTGATCCAGCC-3′ primers (Yuwa-Amornpitak, 2012).

The PCR products were obtained following the procedure described in Hashem et al. (2016) and verified via gel electrophoresis on a 1.5% agarose gel stained with TAE (Tris-acetate-EDTA). Gels were visualized and digitized using the Fujifilm Imaging System. The PCR product was purified, and nucleotide sequences were determined using the automatic LI-COR DNA Sequencer 4000 L (Lincoln, USA). The nucleotide sequences were determined using a sequenator. The sequences were identified using the basic local alignment search tool (BLAST) and comparisons with the GenBank nucleotide data bank from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 was used to

create the phylogenetic tree of the selected bacterial isolates. The 16S rRNA gene sequences of the new isolates were registered in GenBank (NCBI) with the following accession numbers: *Bacillus cereus* isolate NUU1—KU975367.1, *Achromobacter xylosoxidans* isolate NUU2—KU975368.1, *Bacillus thuringiensis* isolate NUU3—KU975369.1, *Bacillus subtilis* isolate NUU4—KU975370.1. MEGAsoftware version 6.0 was used to create the phylogenetic tree of the selected bacterial isolates.

Plant Growth, Symbiotic Performance under Salinated Soil

Pot experiments were conducted to investigate the effect of endophytes on symbiotic performance of rhizobia with host and plant growth. The soil used for the pot experiments was described above. The treatments were as follows: (i) seeds inoculated with *Mesorhizobium ciceri* strain IC53 alone and (ii) *M. ciceri* IC53 combined with endophytic bacterial isolates. Bacterial inoculants were prepared and the germinated seeds were inoculated by immersing seeds in the cell suspensions as described above. *M. ciceri* IC53 was grown in yeast extract-mannitol (YM) (Difco) and endophytic bacteri in TSB medium. For co-inoculation, the cell suspension (cell density of 10^7 CFU/ml) of two bacterial isolates was mixed in a 1:1 ratio and vortexed vigorously to yield a homogenous suspension. One seed was sown per plastic pot, each pot containing 500 g of saline soil, at a depth of ∼1.5 cm. Each treatment contained six plants with three replications. The plants were grown in a shaded greenhouse for 30 days. The temperature ranged between 28 and 32°C during the day and between 18 and 22°C at night. At harvest, the shoot and root lengths and dry weight and nodulation were determined.

Isolation of Pathogenic Fungi

A chickpea field located in Syrdarya province, semi arid region of Uzbekistan was chosen to sample diseased plants. The field is characterized by high salinity (EC 7.5 dS/m), and a high share of plants showing stem root disease were found. The plants with external symptoms such as leaf browning, brown stems, and rooted taproot were collected and carried to the laboratory. The roots that showed disease symptoms were cut into 0.5 cm pieces, and were surface disinfected by dipping into 70% ethanol, then 1% NaOCl for 5 min. Thereafter, roots were washed with sterile distilled water three times and dried on sterile blotting paper. Sterile root pieces were placed on the surface of potato dextrose agar (PDA) (BD, Difco Laboratories, Detroit, USA) supplemented with chloramphenicol (Oxoid, UK) (150 ppm) and incubated at 25°C in the dark. After 8–10 days, three fungal colonies which differed morphologically were isolated, subcultured and purified. Morphological characteristics such as colony appearance were determined from fungal isolates grown on PDA medium after 5 days at 28°C. The formation of conidia, chlamidospores was examined microscopically using light microscope (Olympus BX50).

Plant Pathogenicity Test

For plant pathogenicity tests, three purified fungal isolates taken from diseased roots were grown on PDA medium at 25°C for 6 days. Sterile saline containing 0.1% (v/v) Tween 20 was poured onto plate cultures, gently washed with a sterile glass spreader and a suspension was collected in a sterile tube. The fungal suspension was filtered through glass wool to remove hyphal fragments and the suspension was centrifuged and resuspended with distilled sterilized water. The concentration of spores was counted using a hemocytometer and diluted to 10^8 spores ml^{-1}. For the pathogenicity assay, chickpea seeds were surface-sterilized with 1% NaClO solution followed by 95% ethanol for 5 min, then rinsed several times with sterile distilled water. The sterile seeds were germinated on 1% water agar in the dark at 28°C. The seedlings were dipped in a suspension of spores for 5 min, and then sown in sterile potting soil. Four treatments were used, seeds inoculated with three fungal isolates, and seeds without any treatment. Each treatment contained three replicated blocks (each block has 24 plants), making up a total of 72 plants for each treatment. Plants were grown under greenhouse conditions and were examined for root rot symptoms after 30 days. Among three isolates only one isolate showed root rot symptoms and was collected to re-isolate the fungus from root tissue as described above. Among three fungal isolates only one isolate caused brown, discolored taproots in chickpea. The isolation and purification of fungal isolates from affected tissues was performed as described above.

Identification of Fungal Isolates

The fungal isolates were grown on PDA agar plates at 25°C for 7 days. The fungal mycelium was collected from the surface of the agar with a sterile spatula and transferred to a sterile tube. The mycelium was washed in sterile tap water, centrifuged for 10 min at 4,000 × g, and then freeze-dried at −20°C. DNA was extracted by using the FastDNA Spin Kit (Qbiogene, CA, USA), following the manufacturer' instructions. PCR amplification of the rDNA ITS region was performed using fungal primer pairs ITS1 and ITS4 according to Abd-Elsalam et al. (2003). The PCR product was purified, and nucleotide sequences were determined using a DNA sequencer (4000 L, Lincoln, USA). The sequences of the fragments were identified using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/) with accession number KR528471.1 (*F. solani*).

Biological Control of Root Rot

Four endophytic bacterial isolates were tested for their ability to control root rot in chickpea caused by *F. solani* under greenhouse conditions. For the soil infestation, *F. solani* was grown in Chapek-Dox medium at 28°C for 4 days and the suspension was filtrated with sterile glass wool to remove the mycelium. The concentration of spores in the suspension was adjusted to 10^7 spores ml^{-1} and mixed thoroughly with the potting soil to obtain ∼10^7 spores kg^{-1} soil. The sterile seedlings were dipped in bacterial suspension of 1×10^8 CFU ml^{-1}. The inoculated seedlings were sown in plastic pots filled with natural saline soil infested with *F. solani* and each treatment contained four groups of 24 plants. The plants were grown under greenhouse conditions at a temperature range of 28–32°C day and 20–22 night. The plants were grown for 30 days and removed at harvest from soil, washed and examined for root rot symptoms as indicated

by browning and lesions. In healthy plants no disease symptoms were detected.

Field Experiment

Plant Growth, Nutrient Uptake, and Yield

The experimental design of the field trials in the salinated area of the Syrdarya province was initiated as described by Egamberdieva et al. (2014). The mean temperature of the growing season in 2014 was 17–19°C (April to May) and 36–38°C (June to July). The experimental plots (10 m^2) were arranged in a randomized block design with six replicates per treatment. The treatments were as follows: (i) un-inoculated control, (ii) seeds inoculated with *M. ciceri* strain IC53, and (iii) a combined inoculation of seeds with *M. ciceri* IC53 and *B. subtilis* NUU4. The bacterial inoculants were prepared as described above. *M. ciceri* was grown overnight in TY broth and the endophytic isolates were grown in TSB broth. For co-inoculation, cell suspensions of both strains (10^7 CFU/ml) were mixed in a 1:1 ratio. Chickpea (variety Uzbekistan-32) seeds were planted by hand in each plot in the beginning of April and irrigated by furrow irrigation. Six plants from each treatment were harvested after 2 months and plant shoots were separated from roots and dried to a constant weight at 100°C. The shoots were separated from the roots and dried in an oven at 75°C for 48 h and then powdered. Total nitrogen (Nt) was determined after dry combustion using a CNS elemental analyzer (LECO Corporation, St. Joseph, MI) according to DIN ISO 15178 (2001). The P, K, and Mg contents were analyzed according to DIN ISO 38414-S (1983). The number of pods and nodules per plant root were determined. Seed yields, taken from the two central rows of each plot (m^2 per plot), were estimated at maturity (3 months after sowing).

Plant Physiological Properties

To determine the chlorophyll content, leaf samples (0.5 g) were extracted in acetone (80%) and centrifuged at 10,000 × g for 10 min. The absorbance of the supernatant was recorded at 645 and 663 nm (T80 UV/VIS Spectrometer, PG Instruments Ltd, USA) against the solvent (acetone) (Arnon, 1949). To determine the soluble leaf proteins, the fresh leaves of each plant ($N = 5$) were frozen in liquid nitrogen, ground using a cold mortar, and macerated in 1.0 ml of 100 mM Tris buffer (pH 8.0). The extract was then centrifuged at 27,000 × g for 10 min at 4°C. The soluble leaf protein content was measured according to Bradford (1976).

Hydrogen peroxide (H_2O_2) content in chickpea leaves was evaluated as described by Mukherjee and Choudhuri (1983). The acetone and was used to extract leaf samples, and the supernatant (200 µl) was mixed with 0.04 ml of 0.1% TiO_2 and 0.2 ml NH_4OH (20%). The solution was mixed with 0.8 ml H_2SO_4, centrifuged for 15 min at 6,000 × g and the supernatant was read at 415 nm. The method described in Bates et al. (1973) was used for the estimation of the proline content in chickpea leaves. Briefly, leaf samples (0.5 g) m were extracted in sulfosalicylic acid (3%) and centrifuged for 30 min at 3,000 × g. The acid ninhydrin solution contains 1.25 g ninhydrin, 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid. The supernatant (2.0 ml) was mixed with the acid ninhydrin solution and glacial acetic acid and incubated for 10 min at 100°C. The reaction was stopped by placing the tubes in an ice container, and proline was separated with 4 ml toluene and finally, optical density was measured at 520 nm.

Statistical Analyses

Data obtained from the plant morphological and biochemical studies, as well as the number of diseased plants were subjected to analysis of variance (ANOVA) with SPSS software (version 15). The results are presented as average means and standard error (SE). The difference between means was compared by a high-range statistical domain (HSD) using Tukey's test. The treatment means were separated by the least significant difference (LSD) test at $P < 0.05$.

RESULTS

Isolation, Selection, and Characterization of Plant Growth Promoting Endophytic Bacteria

A total of 40 bacterial isolates were isolated from the surface-sterilized nodules of chickpea grown in saline soil from the Syrdarya province of Uzbekistan. The capability of 40 isolated endophytic bacteria to colonize and persist in plant hosts was tested by studies in a gnotobiotic sand system. Of these forty, 10 isolates colonized the plants at levels ranging from 3.01 to 5.4 $\log_{10}$ CFU/g (fresh weight) (**Table 1**). The endophytes EB2, EB6 EB9, and EB10 were able to colonize root tissue with higher densities than other isolates at titers between 4.60 and 5.45 $\log_{10}$ CFU/g (fresh weight).

Ten bacterial isolates were also screened for multiple plant growth promoting traits and for their plant growth promoting attributes under salt stress. Only four isolates EB2, EB6, EB9, and EB10 significantly stimulated root dry biomass of chickpea by 41, 38, 5, and 46%, whereas shoot dry biomass increased by 45, 5, 24, and 43%, respectively Other isolates did not show any significant impact on plant growth (**Table 1**).

Most of the bacterial isolates exhibited one or more plant growth-promoting activities (**Table 1**). The highest amount of IAA production was observed with EB10 isolate (8.6 µg ml^{-1}) and EB2 isolate (6.2 µg ml^{-1}). The isolates EB1, EB3, EB5, and EB9 did not show any IAA production. Six isolates, except EB1, EB3, EB4, and EB6, were able to produce one or more cell wall degrading enzymes. The isolates EB2 and EB10 *B* produced lipase, protease, cellulase, and chitinase emzymes. Antagonistic activity was recorded for endophytes against plant pathogenic fungi such as *F. oxysporum*, *F. solani, F. culmorum, A. alternata*, and *B. cinerea*. The isolate EB2 was highly effective against *Fusarium* pathogens, and only isolate EB10 showed antagonistic activity against all of the fungal pathogens (**Table 1**). HCN and siderophores were produced by two isolates, EB6 and EB10, and both were able to solubilize phosphate. Four selected bacterial isolates which showed best plant stimulating performance and beneficial traits were identified and chosen for further studies. Based on the nucleotide identity and phylogenetic analysis of the 16S rRNA gene sequences, EB2 (NUU1) was found to be 99% similar to *B. cereus* ATCC 14579 (NC_004722.1.), EB6 (NUU2) was *A. xylosoxidans* A8 (NC_014640.19, EB9 (NUU3)

TABLE 1 | Characterization of endophytic bacterial isolates.

Bacterial strains	Colonization (log10 CFU/g (root fw))[a]	Plant growth[b]		Exo-enzymes[c]								Antagonistic activity[c]				
		Shoot	Root	HCN[b]	PSB[b]	Siderophore[b]	IAA (μg/ml)[b]	Lipase	Protease	Cellulase	Chitinase	*F. oxysporum*	*F. solani*	*F. culmorum*	*A. alternata*	*B. cinerea*
EB1	3.01 bc	0.042 abc	0.016 b	−	−	−	−	−	−	−	+	−	−	−	−	−
EB2	4.60 abc	0.054 a	0.018 ab	−	−	−	6.2	+	+	+	+	+	+	+	−	−
EB3	4.43 abc	0.036 c	0.013 c	−	+	−	−	−	+	−	−	−	−	−	−	−
EB4	3.46 b	0.038 abc	0.014 bc	+	−	−	3.8	+	−	−	−	−	−	−	−	−
EB5	2.51 c	0.041 ab	0.015 abc	−	−	−	−	−	−	+	+	+	+	+	−	−
EB6	5.45 a	0.038 abc	0.018 ab	+	+	+	4.0	−	−	−	−	−	−	−	−	−
EB7	3.02 bc	0.037 bc	0.015 abc	−	−	−	5.1	−	+	+	+	+	+	+	−	−
EB8	2.89 bc	0.037 bc	0.015 abc	−	+	−	2.9	+	+	−	−	−	−	−	−	−
EB9	4.92 ab	0.046 ab	0.014 bc	−	−	−	−	−	+	−	+	−	+	+	−	−
EB10	5.01 ab	0.053 ab	0.019 a	+	+	+	8.6	+	+	+	+	+	+	+	+	+

a Plants were grown under gnotobiotic system for 10 days.
b Untreated plants with bacteria (control): shoot—0.37 and root—0.013 g/plant.
c All tests were conducted with the addition of 2% NaCl.
"+" positive. "–" negative.
Different letters in root colonization and plant growth data indicate significant differences based on Turkey's HSD test at $P < 0.05$.

was *B. thuringiensis* serovar konkukian str. 97-27 (NC_005957.1), and EB10 (NUU4) was *B. subtilis* subsp. *subtilis* str. 168 (NC_000964.3). **Figure 1** shows the phylogenetic tree of selected isolates (NUU1, NUU2, NUU3, and NUU4) created by using their 16S rRNA sequences.

Improvement of Plant Growth and Chickpea-Rhizobia Symbiotic Performance

The four selected isolates (*B. cereus* NUU1, *A. xylosoxidans* NUU2, *B. thuringiensis*, NUU3, and *B. subtilis* NUU4) were evaluated for their ability to improve plant growth and chickpea-rhizobia symbiotic performance in pots under saline soil conditions. A single-strain inoculation with the *M. ciceri* IC53 strain significantly improved the shoot height and nodule number compared to the un-inoculated plants. The shoot height increased 17% and the nodule number, on average, increased two-fold (**Table 2**). In comparison with un-inoculated chickpea, the endophytic isolates NUU1, NUU2, NUU3, and NUU4 increased the root dry weights by 24, 30, 30, 40% and shoot dry weights by 11, 13, 18, and 20% respectively. In comparison with the single-strain inoculation (*M. ciceri*), the co-inoculation of a *Mesorhizobium* symbiont with the endophytic isolate NUU4 further increased shoot and root weights and nodule number (**Table 2**, **Figure 2A**). The shoot height increased by 14%, the root and shoot dry weights increased 20 and 24% compared to single inoculated plants with *M. ciceri*, respectively. The co-inoculation of *M. ciceri* IC53 with *B. subtilis* significantly improved the nodulation of chickpea more than two-fold (**Table 2**).

Biological Control of Chickpea Root Rot under Saline Soil

The endophytic bacterial isolates were screened for the biocontrol of chickpea root rot under greenhouse conditions. The fungal isolate from chickpea roots with disease symptoms was found to be a causative agent of root rot after plant pathogenicity tests. The analysis of DNA sequences showed that the isolate revealed 99% sequence identity to *F. solani* strain QK1409120101 (GenBank accession number KR528471.1) and was therefore assigned to *F. solani*. The isolate was used in biological control assays to estimate biocontrol capability of endophytic isolates of chickpea against root rot under saline soil conditions.

The four selected isolates *B. cereus* NUU1, *A. xylosoxidans* NUU2, *B. thuringiensis* NUU3, and *B. subtilis* NUU4 were chosen to evaluate their ability to suppress chickpea root rot caused by *F. solani*. In non-infested soil, the portion of diseased plants was 6%, while in the presence of the pathogen, the portion of plants that displayed disease symptoms increased to 27%. The isolate *B. cereus* NUU1 demonstrated a disease reduction up to 17% compared to *Fusarium*-infected control plants that showed 27%. The isolates *A. xylosoxidans* NUU2 and *B. thuringiensis* NUU3 were not able to protect chickpea root against the fungal pathogen. *B. subtilis* NUU4 demonstrated the best performance reducing diseased plants by 8%, whereas Fusarium-infected control plants showed 27% diseased plants (Figure S1).

Plant Response to Bacterial Inoculants under Field Conditions

Plant Growth and Yield

The isolate *B. subtilis* NUU4, which demonstrated a positive effect on chickpea growth, symbiotic performance, and biological control of root rot in the preliminary pot experiments, was tested for efficiency under field conditions. The results indicate that a combined inoculation of *B. subtilis* NUU4 and *M. ciceri* IC53 was effective in terms of chickpea growth promotion, stress tolerance, nodulation, pod formation, and yield compared to the un-inoculated control and the treatment of IC53 alone. In

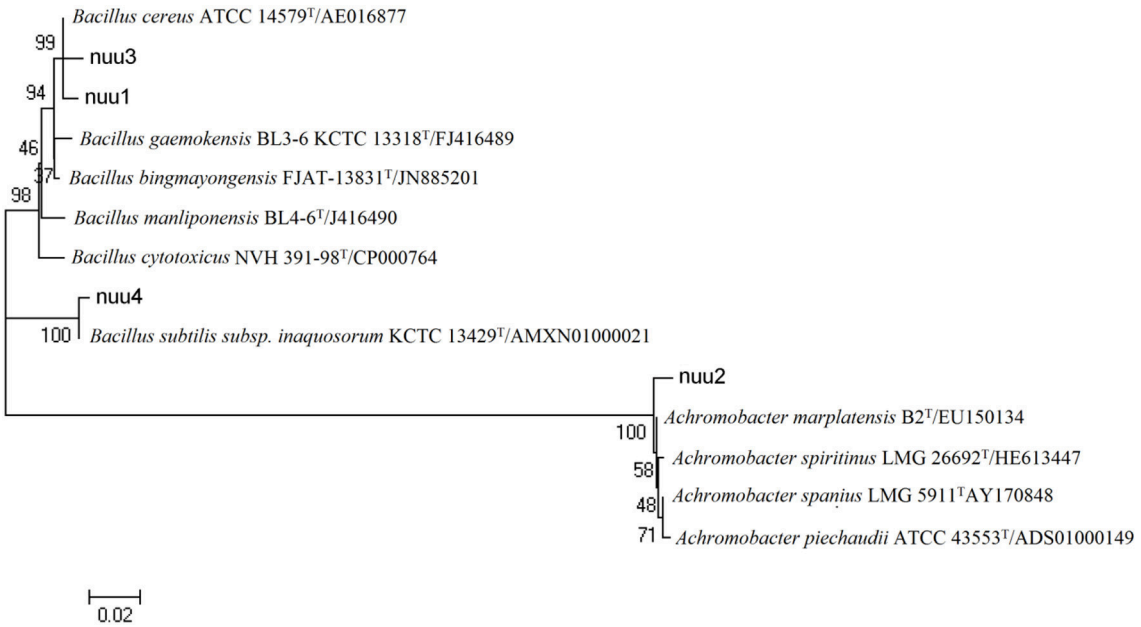

FIGURE 1 | Phylogenetic tree based on alignment of nucleotide sequences of the 16S rRNA genes among selected bacterial isolates isolated from chickpea root and related genera. The bacterial isolates are indicated as follows (*Bacillus cereus* strain, nuu1; *Achromobacter xylosoxidans* strain, nuu2; *Bacillus thuringiensis* strain, nuu3; *Bacillus subtilis* strain, nuu4).

TABLE 2 | The effect of endophytic bacteria alone and in combination with *Mesorhizobium ciceri* on chickpea shoot height (SH), nodule number (NN), root dry weight (RDW), and shoot dry weight (SDW) under saline soil conditions.

Treatments	SH (cm/plant)	NN (per/plant)	RDW (g/plant)	SDW (g/plant)
Control	13.7 cd ± 1.48	2.0 f ± 0.81	0.100 d ± 0.018	0.175 g ± 0.013
M. ciceri IC53	16.1 b ± 0.75	6.0 cd ± 0.82	0.120 cd ± 0.011	0.185 fg ± 0.013
B. cereus NUU1	13.3 d ± 1.09	2.7 ef ± 0.95	0.124 bc ± 0.007	0.195 dfg ± 0.011
B. cereus NUU1 + *M. ciceri* IC53	15.3 bc ± 1.10	6.0 cd ± 1.82	0.122 bcd ± 0.015	0.190 dfg ± 0.008
A. xylosoxidans NUU2	13.7 cd ± 1.23	3.7 def ± 0.95	0.130 bc ± 0.008	0.198 cdf ± 0.010
A. xylosoxidans NUU2 + *M. ciceri* IC53	16.6 b ± 1.65	9.0 b ± 1.90	0.135 abc ± 0.024	0.218 b ± 0.010
B. thuringiensis NUU3	13.7 d ± 1.47	3.8 cde ± 0.95	0.130 bc ± 0.013	0.208 bcd ± 0.017
B. thuringiensis NUU3 + *M. ciceri* IC53	16.6 b ± 1.04	9.0 bc ± 1.50	0.135 ab ± 0.025	0.215 bc ± 0.013
B. subtilis NUU4	14.2 cd ± 0.68	6.0 cd ± 1.82	0.140 abc ± 0.008	0.210 bcd ± 0.008
B. subtilis NUU4 + *M. ciceri* IC53	18.5 a ± 0.31	14.5 a ± 2.01	0.145 a ± 0.012	0.230 a ± 0.022

The plants were grown at a temperature range of 28–32°C in greenhouse condition for 30 days; each treatment contained six plants with three replications; different letters indicate significant differences based on Turkey's HSD test at $P < 0.05$.

the case of the single inoculation with IC53, the shoot height, nodule number, pod number, and yield were significantly ($p < 0.05$) increased, and there was no significant effect on root and shoot dry weights, (**Figures 3A–D**). The root dry weight of chickpea significantly increased ($P < 0.05$) by dual inoculation compared to control and single inoculation by 87 and 35% respectively (**Figure 3B**). For the dual inoculated chickpea seeds with IC53 and NUU4, plants contained 81 and 22% more nodules compared to the un-inoculated control and the single inoculation of IC53, respectively (**Figures 2B**, **3D**). The pod number and yield of inoculated chickpea with a combined inoculation were 39 and 13% higher compared to the un-inoculated plants, respectively, and 12 and 7% with the single inoculation of IC53 (**Figures 3E,F**).

Nutrient Acquisition

Under saline conditions, nitrogen (N), phosphorus (P), potassium (K), and magnesium (Mg) contents of roots and shoots in the un-inoculated plants were lower compared to single and dual inoculated chickpea with IC53 and NUU4 (**Figures 4A–D**). The nutrient content responded positively to both inoculation treatments, and the chickpea inoculated with

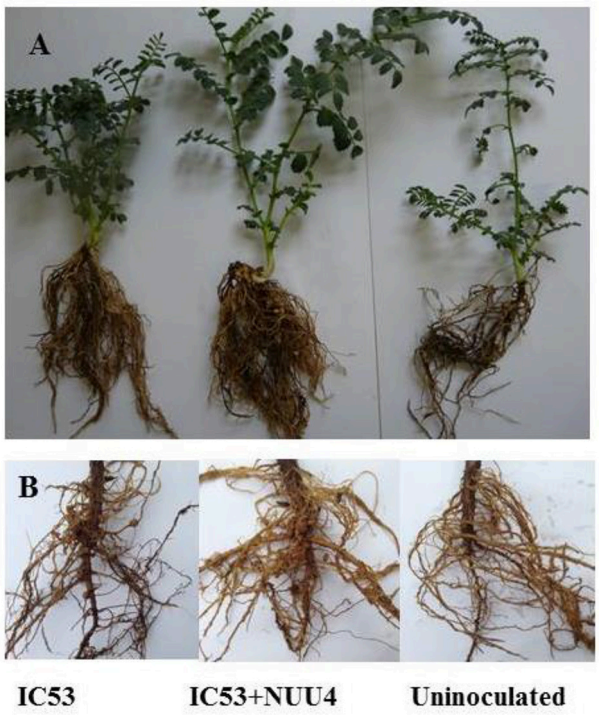

FIGURE 2 | Growth of chickpea plants after inoculation with *Mesorhizobium ciceri* IC53 alone or with the combination of *Mesorhizobium ciceri* IC53 and *Bacillus subtilis* NUU4 in pots **(A)** and under field condition **(B)**.

the IC53 strain and IC53 combined with NUU4 contained 12 and 31 and 5 and 26% more nitrogen in the shoots and roots compared to the un-inoculated plants, respectively (**Figure 4A**). The phosphorus content in the shoots of plants inoculated with both IC53 and IC53 combined with NUU4 showed no significant difference compared to the un-inoculated control (**Figure 4B**). However, the highest phosphorus content was detected from the root tissues inoculated with IC53 combined with NUU4 (36%) and with IC53 only (19%), compared to the un-inoculated control plants. A slightly similar phenomenon was detected for potassium content when chickpea was inoculated with IC53 combined with NUU4. The K content increased by 18% in the roots of chickpea treated with the dual inoculation of the microbes (**Figure 4C**). Magnesium content of chickpea shoots was not affected by both treatments, whereas plant roots contained significantly more (up to 29%) Mg after inoculation with IC53 and NUU4 compared to the un-inoculated plant tissues (**Figure 4D**).

Physiological Parameters

The chlorophyll content in chickpea leaves was lower in plants without the bacterial treatments. The plants inoculated with the combination of *M. ciceri* IC53 and *B. subtilis* NUU4 showed 51 and 26% higher photosynthetic pigments compared to the un-inoculated plants and the single inoculation with *M. ciceri* IC53, respectively (**Table 3**). The soluble leaf protein in chickpea also responded positively to both microbial inoculations. The combination of the two bacteria produced better results since co-inoculated chickpea tissues contained more leaf proteins (26%) than the un-inoculated tissues (**Table 3**).

Hydrogen peroxide and proline acts as stress-related signaling molecules that involved in the regulation of various abiotic and biotic stresses in plants. The content of hydrogen peroxide (H2O2) in the chickpea leaves was 7.87 (μM/g fresh weight). The single inoculation of plants with *M. ciceri* IC53 decreased H2O2 by 18% and a dual inoculation with *M. ciceri* IC53 and *B. subtilis* NUU4 by 29%. The plants grown under saline soil conditions had lower concentrations of proline (38.43 nM/g fresh weight) compared to the inoculated plants (49.79 nM/g fresh weight). Proline was increasingly produced by plants as a response to both the single and dual microbial inoculations. A combined inoculation of chickpea with *M. ciceri* IC53 and *B. subtilis* NUU4 significantly increased proline contents compared to the un-inoculated plants (29%), whereas a single inoculation with *M. ciceri* IC53 increased proline only by 11%.

DISCUSSION

We studied plant growth promoting endophytic bacteria isolated from chickpea to evaluate their role in biocontrol of root rot caused by *F. solani*, besides symbiotic performance and stress tolerance of chickpea under saline soil conditions. The endophytic bacteria which showed best plant beneficial properties were identified as *B. cereus*, *A. xylosoxidans*, *B. thuringiensis*, and *B. subtilis*. The species *A. xylosoxidans* was never previously observed as an endophytic bacterium associated with chickpea. This study reports the first detection of *A. xylosoxidans* in chickpea nodule tissue. Interestingly, some studies reported that *A. xylosoxidans* was able to form nodules in Mesquite (*Prosopis juliflora*) (Benata et al., 2008), cowpea (*Vigna unguiculata*) (Azarias Guimarães et al., 2012), and soybean (*Glycine max*) (Wedhastri et al., 2013). Several endophytic bacteria showed a potential for improving plant growth and stress tolerance (Hashem et al., 2016). These properties were also detected in the case of our endophytic bacterial isolates. The four selected isolates of *B. cereus* NUU1, *A. xylosoxidans* NUU2, *B. thuringiensis* NUU3, and *B. subtilis* NUU4 stimulated the root and shoot growth as well as the nodulation of chickpea. Similar observations were reported by Hashem et al. (2016), stating that *B. subtilis* stimulated root and shoot growth, nodulation and nutrient uptake of *Acacia gerrardii* under salt stress. Furthermore, the best plant growth promoting strain *B. subtilis* NUU4 showed good biocontrol capacity against chickpea root rot caused by *F. solani* under saline soil conditions. Chickpea root rot is a common disease in many countries of the world (Cabral et al., 2016). We isolated pathogenic fungi from infected chickpea roots and identified them as *F. solani.* This is the first report of *F. solani* causing chickpea root rot in a saline soil of Uzbekistan. Chemical plant protection agents are widely used to control various root pathogens, nonetheless they continue to spread and potentially harmful biocides accumulate in soils which are already impacted by other detriments such as salinity. Endophytic microbes are

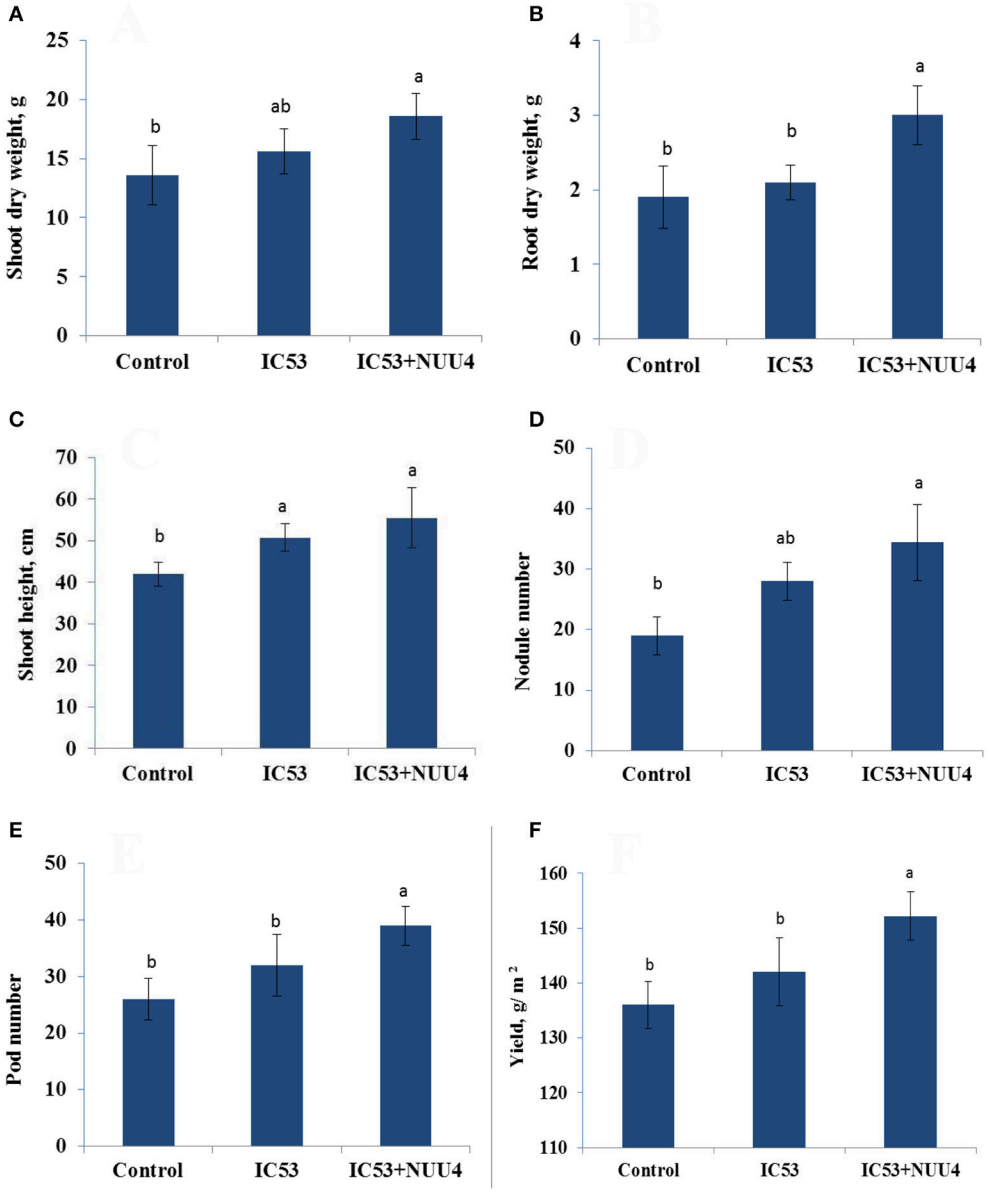

FIGURE 3 | Effects of seed inoculation with the combination of *Mesorhizobium ciceri* IC53 and *Bacillus subtilis* NUU4 and with *Mesorhizobium ciceri* IC53 strain alone on shoot **(A)** and root **(B)** dry weight, shoot **(C)** height, nodule number **(D)**, pod number **(E)**, and seed yield **(F)** grown under saline soil conditions. Columns represent the means of six plants ($N = 6$) and error bars show the standard error. Column means marked by different letters indicate significant differences based on Turkey's HSD test at $P < 0.05$.

known as potential biocontrol agents of soil borne diseases. The ability of endophytic bacteria colonizing internal plant tissues to protect host plants from soilborne pathogens was recently reviewed by Eljounaidi et al. (2016). For example, the endophytic bacterium *Pseudomonas fluorescens* PICF7 antagonistic against *Verticillum dahliae*, was found to be an effective biological control agent against verticillium wilt of olive (Mercado-Blanco et al., 2004). Several mechanisms behind the plant beneficial effects are reported, including the synthesis of plant growth regulators, antifungal compounds, cell wall degrading enzymes, and/or the modulation of the physio-biochemical processes in plants (Park et al., 2013; Cho et al., 2015; Parray et al., 2015). In our study, the isolate *B. subtilis* NUU4 produced IAA, HCN, siderophores, cell wall degrading enzymes, and demonstrated antagonistic activity against *F. oxysporum*, *F. solani*, *F. culmorum*, *A. alternate,* and *B. cinerea.* Those traits, alone or in combination, may result in an enhanced root growth, nutrient availability to the plants, and a reduction in pathogen infection.

The endophytic bacteria were also effective in chickpea rhizobia symbiotic interactions under saline arid soil conditions.

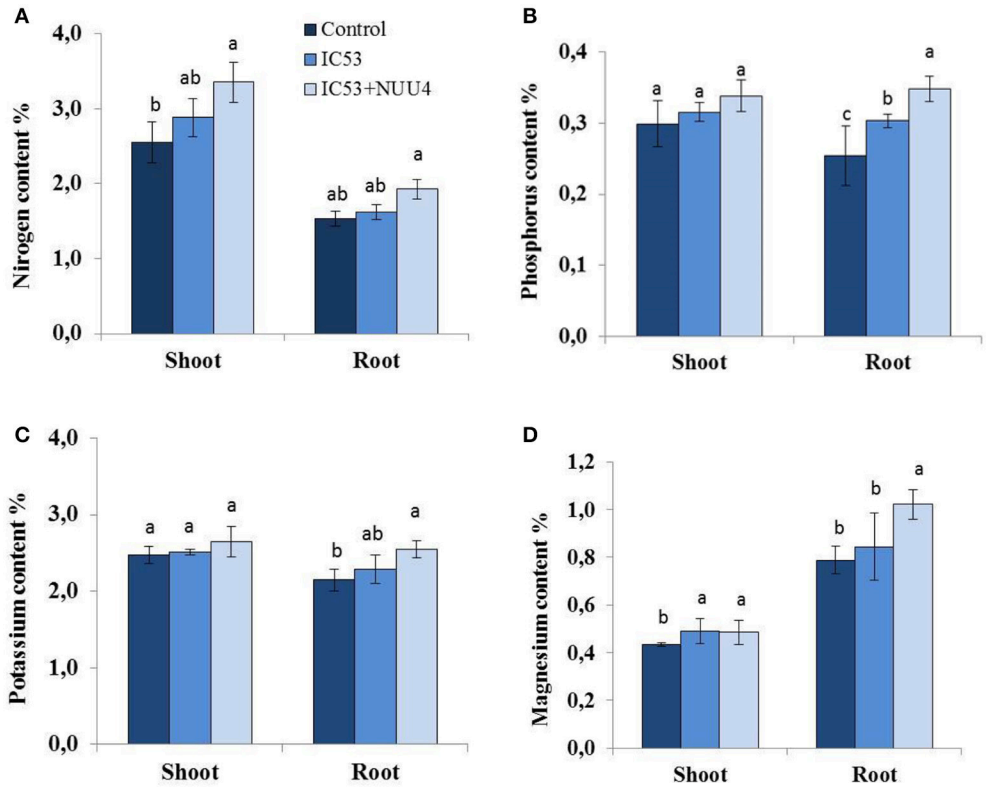

FIGURE 4 | Effects of seed inoculation with the combination of *Mesorhizobium ciceri* IC53 and *Bacillus subtilis* NUU4 and with *Mesorhizobium ciceri* IC53 strain alone on chickpea shoot and root **(A)** nitrogen, **(B)** phosphorus, **(C)** potassium, and **(D)** magnesium contents grown under saline soil conditions. Columns represent the means of six plants ($N = 6$) and error bars show the standard error. Column means marked by different letters indicate significant differences based on Turkey's HSD test at $P < 0.05$.

TABLE 3 | Effects of seed inoculation with *Mesorhizobium ciceri* IC53 alone and *Mesorhizobium ciceri* IC53 combined with *Bacillus subtilis* NUU4 on the contents of chlorophyll, protein, hydrogen peroxide, and proline of chickpea grown under saline soil conditions.

Treatment	Chlorophyll	Protein	Hydrogen peroxide	Proline
Control	1.32 ± 0.03 b	1.83 ± 0.02 b	7.87 ± 0.67 a	38.43 ± 3.1 bc
M. ciceri IC53	1.58 ± 0.02 ab	2.12 ± 0.01 ab	6.51 ± 0.63 b	42.87 ± 2.3 b
IC53 + *B. subtilis* NUU4	2.00 ± 0.01 a	2.31 ± 0.02 a	5.61 ± 0.87 c	49.79 ± 3.8 a

The plants were grown under field conditions for 60 days; different letters indicate significant differences based on Turkey's HSD test at $P < 0.05$. Chlorophyll, mg/g fresh weight; Protein, mg/g fresh weight; hydrogen peroxide, μM/g fresh weight; Proline, nM/g fresh weight.

It has been proposed that root associated plant beneficial bacteria living in a free or an endophytic lifestyle may directly or indirectly contribute to the infection and colonization processes of the rhizobium-host association (Egamberdieva et al., 2016b). In the soil-root system, endophytic bacteria do not interfere with the capability of rhizobia to form nodules in the plant roots, even though they may enhance nodulation and plant growth (Egamberdieva et al., 2016b). There are many reports on improved legume-rhizobia symbiotic performance by PGPR e.g., soybean (*G. max* L.) (Egamberdieva et al., 2016a), thal tree (*A. gerrardii*) (Hashem et al., 2016), chickpea (*C. arietinum* L.) (Panjebashi et al., 2012; Yadav and Verma, 2014), and peanut (*Arachis hypogaea*) (Badawi et al., 2011). Accordingly, we observed that the selected salt tolerant PGPR isolates improved the symbiotic performance of *M. ciceri* under saline soil conditions. The nodule number in single inoculated chickpea plants were slightly higher compared to untreated plants, which demonstrate evidence for the impact of salt stress on the symbiotic performance of rhizobia. Salinity above 3 dS m^{-1} was reported to inhibit nodulation in chickpea, except for salt tolerant genotypes which can nodulate salinity up to 6 dS m^{-1} (Rao et al., 2002). When colonizing plant tissues, endophytic bacteria produced various biological active metabolites, which resulted in improved root growth, higher stress tolerance, and the modulation of plant defense mechanisms (Bordiec et al., 2011). The endophytic bacteria have the capability to synthesize cell wall-degrading enzymes, such as cellulase, that are predicted to participate in the penetration of rhizobia into the root cortex and form nodules (Egamberdieva et al., 2013). In another study, Huang et al. (2011) found that the colonization of *Bacillus* and bacteroid formation inside plant cortical cells was similar compared to the infection of root hairs by rhizobia.

The nutrient acquisition in plants under salt stress is generally affected by the antagonistic impact of sodium (Attia et al., 2008) and a reduced root system (Egamberdieva et al., 2016b). We observed an improved N, P, K, and Mg uptake in chickpea inoculated with the combination of *M. ciceri* and *B. subtilis* under saline soil conditions. The stimulated root system induced by endophytic bacteria could explain the enhanced capacity of the plant to acquire and utilize more nutrients. Root associated microbes are also capable of solubilizing mineral nutrients and facilitating their availability to plants, increasing nutrient uptake (Setiawati and Mutmainnah, 2016). For example, phosphate-solubilizing *Pseudomonas* in combination with *Sinorhizobium ciceri* increased P uptake by chickpea (Messele and Pant, 2012). The isolate *B. subtilis* NUU4 was also able to solubilize phosphate, thus providing more phosphorus to chickpea plants.

The inoculation of chickpea with the endophytic isolates also affected several physiological properties of the plants. In our study, increased contents of chlorophyll pigments in chickpea leaves were observed in co-inoculated plants with *M. ciceri* and *B. subtilis*. Similar results were obtained by Heidari and Golpayegani (2012) for *Ocimum basilicum* grown under water stress, where a combined inoculation of *Pseudomonas* sp. and *Bacillus lentus* in plants stimulated chlorophyll synthesis as well as photosynthetic electron transport. Abiotic stress can increase hydrogen peroxide production in plants which is associated with membrane leakage (Ahmad et al., 2012). We have observed that hydrogen peroxide concentration in leaves of tomato grown in saline soil was decreased by bacterial inoculation compared to untreated plants. It is known that stress factors increase the production and accumulation of reactive oxygen species (ROS) while endophytic bacteria colonizing plant tissue reducing H_2O_2 synthesis may protect the membrane lipids from peroxidation. Hashem et al. (2016) also found that *B. subtilis* isolated from *A. gerrardii* plant tissue, reduced H_2O_2 production under salt stress conditions. Soluble proteins protect plants under stress, which result in an improved stress tolerance and reflect the availability of nitrogen for growth and development of plants (Andrews et al., 1999). Accordingly, we also observed higher soluble leaf protein concentrations in dual inoculated chickpea tissues with *M. ciceri* and *B. subtilis* compared to the control plants. A compatible osmolyte such as proline, glycine, or betaine plays an important role in plant tolerance to stress factors through osmotic adjustment (Hashem et al., 2015). We observed an increased proline content in chickpea plants inoculated with *M. ciceri* and *B. subtilis* that resulted in stress adaptation in saline soil. A similar observation was reported by Vardharajula et al. (2011) for maize, where *Bacillus* spp. improved plant growth and tolerance to drought stress via enhanced production of proline, amino acids, and soluble sugars.

In conclusion, we found first evidence of root rot of chickpea caused by *F. solani* in saline soils of Uzbekistan. The endophytic bacterial isolates with best PGP traits were capable to reduce the infection rate of root rot in chickpea and were effective in growth stimulation and resistance to salt stress. Furthermore, the mutualistic interaction of endophytic bacteria *B. subtilis* and *M. ciceri* improved the symbiotic performance of *M. ciceri* with host plants under saline soil conditions. Our findings demonstrated that endophytic bacteria which potentially colonize root tissue are effective biological control agents of chickpea root rot but also plant growth stimulators under saline soil conditions. Such multiple plant-microbial relationships could provide promising practical approaches to increase the productivity of legumes under salt stress.

AUTHOR CONTRIBUTIONS

DE and SW did experimental design work. DE and VS conducted experiments. AH and EA analyzed the data. DE, SW, and EA wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01887/full#supplementary-material

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Aerial Warfare: A Volatile Dialogue between the Plant Pathogen *Verticillium longisporum* and Its Antagonist *Paenibacillus polymyxa*

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Verticillium wilt caused by *Verticillium* spp. results in severe yield losses in a broad range of crops. *Verticillium* outbreaks are challenging to control, and exacerbated by increases in soil temperatures and drought associated with global warming. Employing natural antagonists as biocontrol agents offers a promising approach to addressing this challenge. *Paenibacillus polymyxa* Sb3-1 was proven to reduce the growth of *Verticillium longisporum* during *in vitro* experiments and was shown to promote the growth of oilseed rape seedlings infested with *V. longisporum*. Our novel approach combined *in vitro* and *in planta* methods with the study of the mode of interaction between Sb3-1 and *V. longisporum* EVL43 via their volatile organic compounds (VOCs). Volatile and soluble substances, produced by both microorganisms as a reaction to one another's VOCs, were detected by using both gas and liquid chromatography-mass spectrometry. *P. polymyxa* Sb3-1 continually produced antimicrobial and plant growth promoting VOCs, such as 2-nonanone and 3-hydroxy-2-butanone. Several other antimicrobial volatile substances, such as isoamyl acetate and durenol, were downregulated. The general metabolic activity of Sb3-1, including protein and DNA biotransformations, was upregulated upon contact with EVL43 VOCs. *V. longisporum* increased its production of antimicrobial substances, such as 1-butanol, and downregulated its metabolic activities upon exposure to Sb3-1 VOCs. Additionally, several stress response substances such as arabitol and protein breakdown products (e.g., L-Isoleucyl-L-glutamic acid), were increased in the co-incubated samples. The results obtained depict an ongoing dialog between these microorganisms resulting in growth inhibition, the slowing down of metabolism, and the cell death of *V. longisporum* due to contact with the *P. polymyxa* Sb3-1 VOCs. Moreover, the results indicate that VOCs make a substantial contribution to the interaction between pathogens and their natural antagonists and have the potential to control pathogens in a novel, environmentally friendly manner.

Keywords: biocontrol, Verticillium wilt, *Verticillium longisporum*, *Paenibacillus polymyxa*, volatile assay, VOCs, plant-microbe interactions, adaptation

INTRODUCTION

Verticillium wilt caused by *Verticillium* spp. is a serious fungal plant disease that affects up to 250 hosts, including many economically important crops such as potato, strawberry, alfalfa, oilseed crops, and a variety of tree species (Hiemstra, 1998; Pegg and Brady, 2002). While *V. dahliae* Kleb. (1913) and *V. albo-atrum* Reinke and Berthold (1879) attack a high number of host species, *V. longisporum* (C. Stark) Karapapa et al. (1997) has a more defined host range, primarily infesting cruciferous species (*Brassicaceae*). *V. longisporum* is the main contributor to Verticillium wilt in oilseed crops (Zeise and von Tiedemann, 2002; Depotter et al., 2016), and causes up to 50% of yield losses (Daebeler et al., 1988; Zeise and Steinbach, 2004). *Verticillium* spp. are the most challenging types of phytopathogens to control due to the extensive viability of their microsclerotia (resting structures), their broad host range, and the genetically heterogeneous and polyphyletic character of *Verticillium* isolates, as well as the absence of effective fungicide treatments (Fradin et al., 2009; Jiménez-Gasco et al., 2014). Due to the decrease in crop rotation time and global warming (Houghton et al., 2001), the number of disease incidents is expected to increase in the future (Heale and Karapapa, 1999; Siebold and von Tiedemann, 2012). *Verticillium* has an optimum temperature of 20°C and above for most of its life cycle stages (Siebold and von Tiedemann, 2012). In this context, global warming will increase the temperature of the soil's upper layers in which the fungal inoculum is found to be present (Zhang et al., 2005), causing the shifting of soil temperatures toward the biological optimum temperature for the fungus at locations where the disease is currently not a problem. Such temperature-related shifts, or 'fingerprints' in species distribution due to global warming have been observed within a large number of species (Root et al., 2003). In addition, the infection period can be extended at higher temperatures (Dunker et al., 2008). This concurs with recent observations that the wilt disease of the oilseed rape (OSR) has been occurring more frequently in recent, warmer years (Siebold and von Tiedemann, 2012). The Verticillium wilt of strawberry crops is also affected by temperature increases, as was shown by an increase of damage occurring during warm conditions (Schubert et al., 2009). Considering the current trend in plant disease control toward sustainable agriculture and the urgent need for agricultural adaptation to climate change (Howden et al., 2007), environmentally friendly solutions for Verticillium wilt problems, such as biological control, are especially desirable.

Several biocontrol agents (BCAs) such as *Serratia plymuthica* HRO-C48 (Müller and Berg, 2008), non-pathogenic *Verticillium* strains (França et al., 2013), as well as several strains of *Paenibacillus* and *Serratia* (Rybakova et al., 2016b) were proposed as potentially effective biocontrol agents against *V. longisporum*. Endophytic *Paenibacillus* strains are known for their plant growth promoting and biocontrol properties (Rybakova et al., 2016a). *P. polymyxa* Sb3-1 was shown to be particularly active against *V. longisporum in vitro*. This strain demonstrated a plant growth promoting effect on the oilseed rape seedlings in sterile soil, and was deleterious to the seedlings under sterile soil-free conditions (Rybakova et al., 2016b). Several modes of actions are suggested for *Paenibacillus* spp. as biocontrol agents. They are able to protect plants against pathogenic organisms in a variety of ways including the production of a variety of antimicrobials and insecticides and triggering a plant's defense system known as induced systemic resistance (Grady et al., 2016). *Paenibacillus* spp. can also produce a biofilm around plant roots that functions as a protective layer preventing pathogens from accessing plant tissue (Timmusk et al., 2005). Recently, the emission of antimicrobial volatile organic substances (VOCs) has been proposed as an important defense mechanism of *Paenibacillus* (Rybakova et al., 2016a). A multitude of interactions between organisms is based on the emission and perception of volatiles (Wenke et al., 2010). In fact, recent data suggests that the majority of all explored interactions is based on volatile compounds rather than on non-volatile ones (Kanchiswamy et al., 2015). Even plants can sense VOCs produced by neighboring plants (Baldwin et al., 2006; Dicke et al., 2009). Volatiles act as an important medium for interactions between bacteria and fungi below ground (Insam and Seewald, 2010; Effmert et al., 2012). In this context, it was shown that fungi emit a broad spectrum of VOCs with diverse ecological functions affecting both bacteria (Schmidt et al., 2015; Hacquard, 2017) and plants (Ditengou et al., 2015). Bacteria are known to produce highly diverse volatiles (Schulz and Dickschat, 2007). Such microbial VOCs can directly or indirectly mediate increases in the biomass of the host plant. They also increase disease resistance and abiotic stress tolerance, and thereby help plants to combat pathogens (Liu and Zhang, 2015). In terms of microbial interplay it is hypothesized that VOCs can be used either as infochemical molecules affecting the gene expression in the responding microorganism, or as competitive tools, providing an advantage by suppressing or eliminating potential enemies (Kai et al., 2007; Schmidt et al., 2015; Ossowicki et al., 2017). The diversity of soil bacteria and the presence of some less abundant soil bacteria, such as *Paenibacillus*, play important roles in the production of antifungal volatiles by soil bacterial communities (Hol et al., 2015; Schulz-Bohm et al., 2015). Diazine derivatives produced by several *Paenibacillus* spp., for example, are able to suppress the growth of bacteria, fungi and yeast (Cernava, 2012). Due to their great potential for application as a pathogen control in industrial environments a patent was filed for commercial utilization of novel bioactive compounds (Aichner et al., 2012). Currently, various tools are available to access bioactivity of microbial volatiles. These tools include pre-screening methods such as split-plate experiments and parallelized VOCs assays (Ryu et al., 2003; Cernava et al., 2015a). Further characterization of the substances involved requires an analytical approach. Highly concentrated volatiles can be captured with an air tight syringe (headspace method) and injected into a GC-MS system. Alternatively, headspace solid-phase microextraction (SPME) can also capture less abundant volatiles, which is beneficial when general VOC emissions of microorganisms are studied (Zhang and Pawliszyn, 1993).

While in recent years the diversity of microbial volatiles has been studied intensively, the ways in which they contribute to the mode of action of biocontrol agents, and their exact role in specific microbe-microbe interactions remain largely

unknown. In order to address this knowledge gap, we studied the interaction between plant pathogenic *Verticillium longisporum* EVL43 and the potential biocontol agent *Paenibacillus polymyxa* Sb3-1 via their volatiles *in vitro* and *in planta*. The main objective of the study was to understand the mode of action of the predicted biocontrol agent *P. polymyxa* Sb3-1 against plant pathogen *V. longisporum* via its volatiles. We hypothesized that the exchange of volatiles plays an important role in the interaction between these two microorganisms and is involved in the antagonistic effect of *P. polymyxa* Sb3-1 against Verticillium wilt.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The fungal pathogen used was *V. longisporum* (C. Stark) Karapapa et al. (1997) strains ELV25 and EVL43 from the collection TU Graz, Environmental Biotechnology, described in Messner et al. (1996). *V. longisporum* ELV25 was grown either on potato dextrose agar (PDA) or in Czapek Dox liquid culture (Sigma-Aldrich). *P. polymyxa* Sb3-1 (Köberl et al., 2013) as well as its rifampicillin resistant mutant *P. polymyxa* Sb3-1 rif^R (this study) were routinely grown on Standard I nutrient agar (NA, SIFIN, Berlin, Germany) at 30°C. When required, rifampicin was added at concentrations of 100 μg ml^{-1}. For the direct dual culture assays, Reasoner's 2A agar (R2A) (Roth, Karlsruhe, Germany), water yeast agar (WAY), and PDA were used.

Evaluation of the Plant Growth Promotion (PGP) and Biocontrol Effects of *P. polymyxa* Sb3-1 *In planta*

Oilseed rape (*Brassica napus* L. "Traviata H 605886"; KWS Saat Einbeck, Germany) seeds were treated with two concentrations of *P. polymyxa* Sb3-1 (log_{10} 7 and log_{10} 5 CFU ml^{-1}) applied to the seeds using the pelleting method according to the protocol described by Müller and Berg (2008). For the PGP studies, the experiment was performed in 5 replicates with 9 seedlings each (3 seeds per pot). The 2-week old seedlings were harvested and their fresh weight was compared to the untreated control. The bacterial abundance on the seeds and on the roots was estimated as described by Rybakova et al. (2016b). For the biocontrol studies, the 1-week old seedlings were inoculated with *V. longisporum* ELV25 using the root dipping method. The experimental setup included 15 pots per treatment with one plant per pot and replicate. The roots of the 1-week old seedlings were artificially injured using a scalpel. The seedlings were inoculated by dipping their roots for 30 min into 200 ml of Czapek-Dox broth with a 1-week old culture of *V. longisporum* ELV25 adjusted to 10^6 CFU ml^{-1}. Control treatments were treated the same way as the inoculated seedlings, however they were immersed in sterile water instead of *V. longisporum* ELV25 blastospore solution. Finally, all plants were transplanted back to the pots. After the appearance of the first disease symptoms, the disease reaction of plants was assessed based on the severity of symptoms as described by Müller and Berg (2008) at weekly intervals for the duration of 7 weeks after inoculation. Data on disease severity was used to calculate area under disease process curve (AUDPC) determined as $AUDPC = \Sigma((Si + Sti + 1)/2)*(ti + 1 - ti)$, where Si is the symptoms severity, and ti is the date of assessment of symptoms severity.

Volatile Metabolite Analyses with *P. polymyxa* Sb3-1 and *V. longisporum* ELV43

Antagonism of *P. polymyxa* Sb3-1 and *V. longisporum* ELV43 was tested with the "Two Clamp VOCs Assay" as described

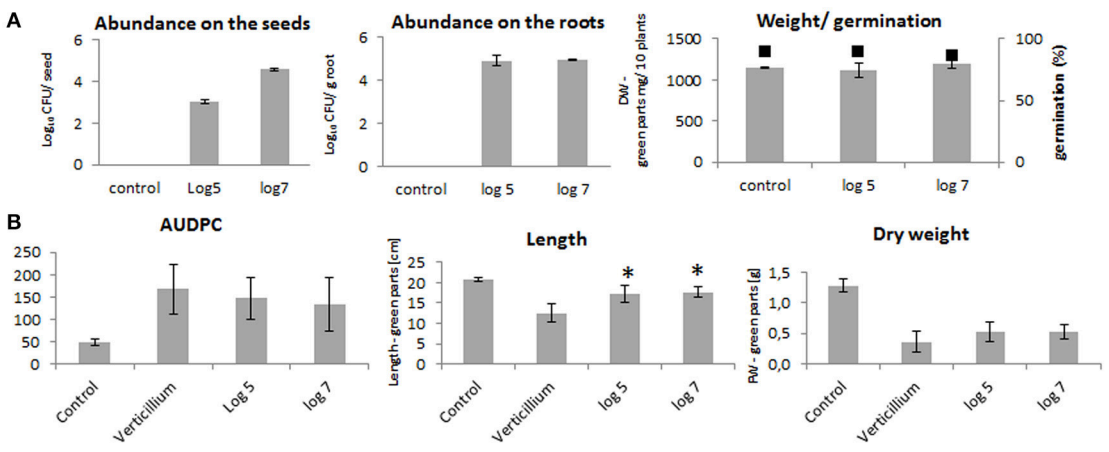

FIGURE 1 | PGP effect on the OSR seedlings and Verticillium wilt disease reduction due to the treatment of the OSR seeds with log_{10} 5 and log_{10} 7 CFU ml^{-1} of *P. polymyxa* Sb3-1. **(A)** Abundances of the Sb3-1 on the seeds (left panel) and on the roots (middle panel) as well as fresh weights and germination rates (black squares) of the of the 2-week old seedlings (right panel) treated with Sb3-1 compared to the untreated control (control). **(B)** The Verticillium wilt disease severity (AUDPC; left panel), and lengths and weights (middle and rights panels, respectively) of the 8 weeks old OSR seedlings infected with *V. longisporum*. The labels of the bars refer to the different concentrations of *P. polymyxa* Sb3-1 in the initial inoculant used for seed treatments: log 5, log_{10} 5 CFU ml^{-1} of Sb3-1; log 7, log_{10} 7 CFU ml^{-1} of Sb3-1; control, untreated control; *Verticillium*, untreated seedlings that were infected with *V. longisporum*. Error bars represent confidence interval ($P = 0.05$). The asterisk (*) denotes values that were significantly different from the non-treated control group values ($P < 0.05$) defined using pairwise *t*-test or non-parametric Mann–Whitney *U*-test, depending on the distribution of the samples.

in Cernava et al. (2015a). The assay was performed in nine replicates. Three plates with three wells each were used per replicate. The significance of the differences between zones of inhibition of *Verticillium* growth by different bacterial strains (**Table 2**) was calculated using one-way ANOVA and Tukey's HSD tests. For both analyses, the $P < 0.05$ were considered to be significant.

GC-MS headspace SPME experiments were carried out as described by Cernava et al. (2015a; Supplementary Figure 1). Identification of the volatile compounds was performed with the NIST MS Search 2.2 included in the Software-Package of the NIST 2014 database. Further verification was done by calculation of the covats index (CI) followed by comparisons to database entries of NIST Search 2.2 and the entries in the Online Database of NIST (http://webbook.nist.gov/). The raw data of all analyses is provided as Supplementary Data for this publication; file names include the analyzed microorganisms and the duration of the incubation prior to VOC sampling.

Soluble Metabolite Analyses of *P. polymyxa* Sb3-1 and *V. longisporum* ELV43

In the course of an LC-MS assay *V. longisporum* ELV43 and *P. polymyxa* Sb3-1 were co-incubated in order to exchange their VOCs without direct contact with one another. The assay was performed in three replicates. A 4-day old growth plate with *V. longisporum* ELV43 was placed on top of the *P. polymyxa* Sb3-1 plate and sealed to facilitate accumulation of VOCs. For the negative control, a fungal and a bacterial plate were incubated with a non-inoculated PDA plate. Cell lysis was performed using Ribolyser FastPrep-24 (MP Biomedicals, Santa Ana, California, USA) for two times 30 s at 6 m s^{-1} in 90% methanol. The cell free extract was stored at −70°C. The bacterial and fungal metabolite extracts were analyzed with a combined HPLC-hybrid quadrupole-orbitrap mass spectrometer (Q Exactive; Thermo Scientific, Bremen, Germany). A Luna 5u NH2 100A 250 × 4.6 column (Phenomenex, Aschaffenburg, Germany) was used to separate different metabolites from the cell extracts as described by Cernava et al. (2015b). Identification of the soluble compounds was performed with the XCalibur 2.2 and SIEVE 2.2 (Thermo Scientific, Bremen, Germany) and manual comparison of the spectra with corresponding spectra from literature as well as such from mzCloud (HighChem LLC, Bratislava, Slovakia).

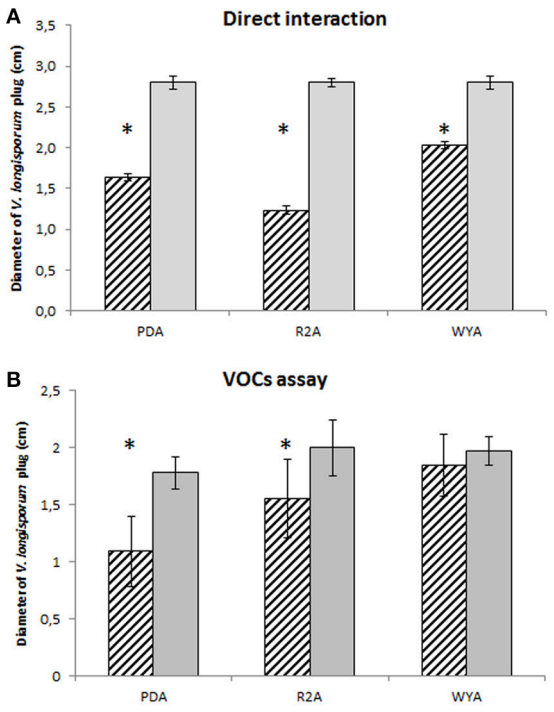

FIGURE 2 | Plate confrontation and VOCs assay of *P. polymyxa* Sb3-1 and *V. longisporum* ELV43 in three different growth media (PDA, R2A, and WYA) for nine days. Dashed columns show growth of *V. longisporum* ELV43 in the presence of *P. polymyxa* Sb3-1 **(A)** or its VOCs **(B)**, while the gray columns illustrate the growth of *V. longisporum* ELV43 without *P. polymyxa* Sb3-1 **(A)** or its VOCs **(B)**. The assay was performed in nine replicates. The asterisk (*) denotes values that varied significantly from control group values ($P < 0.05$) defined using pairwise *t*-test or non-parametric Mann–Whitney *U*-test, depending on the distribution of the samples.

Statistical Analysis

The PGP and antifungal effects of the Sb3-1 were statistically analyzed using the IBM SPSS program version 20.0 (IBM Corporation, Armonk, NY, USA). The significance of the differences in the assessed features (germination rate, plants' weights, diameter of the *Verticillium* plugs) between the control vs. each treatment group was calculated using a pairwise *t*-test with independent samples. The decision to make use of the non-parametric Mann–Whitney *U*-test as an alternative to the *t*-test was based an assessment of the distributions of variables (normal vs. non-normal).

RESULTS

PGP and Antifungal Effects of *P. polymyxa* Sb3-1 Applied to the Seeds of OSR

The seed treatments with $\log_{10}$ 5 and $\log_{10}$ 7 CFU ml^{-1} of *P. polymyxa* Sb3-1 rif^R resulted in recovery rates of $\log_{10}$ 3 ± 0.1 and $\log_{10}$ 4.6 ± 0.1 CFU $seed^{-1}$, respectively (**Figure 1A**). The concentration of Sb3-1 in the roots of the 2-week old seedlings was $\log_{10}$ 4.9 ± 0.3 CFU g $root^{-1}$, independent of the initial inoculum concentration. We observed no PGP effect or effect on the germination rate of the Sb3-1 treatment on the 2-week old OSR seedlings (**Figure 1A**). When the 1-week old seedlings were inoculated with *V. longisporum*, we observed a mild improvement in disease resistance and dry weight as well as a significant increase in the respective lengths of the seedlings treated with *P. polymyxa* Sb3-1 rif^R (**Figure 1B**). The observed increase in seedling length was 36 and 40% for the treatments with $\log_{10}$ 5 and $\log_{10}$ 7 CFU ml^{-1}, respectively, compared to the untreated control infested with *V. longisporum*.

P. polymyxa Sb3-1 and Its VOCs Inhibit Growth of *V. longisporum* ELV43 in the Plate Assay

In order to understand the mechanism of the antifungal effect of *P. polymyxa* Sb3-1 we designed a series of experiments

TABLE 1 | GC—MS headspace SPME identification of relevant VOCs produced by *P. polymyxa* Sb3-1 and *V. longisporum* ELV43 grown for 3 or 6 days in presence of each other volatiles.

RT (min)	Predicted compound[a]	Regulation[b]	Predicted function
VOCs PRODUCED BY Sb3-1 AFTER BOTH 3 AND 6 DAYS GROWTH IN PRESENCE OF *V. longisporum* VOCs			
8.521	1,3-Dioxolane, 2,2,4,5-tetramethyl-, trans-*	↑986%**	N.a.
12.238	Pentafluoropropionic acid, hexyl ester*	↓47,6%**	N.a.
19.248	**2-Nonanone***	–	Antibacterial (Schulz and Dickschat, 2007)
20.893	2-Decanone*	–	N.a.
20.980	2-Decanol*	–	N.a.
VOCs PRODUCED BY Sb3-1 ONLY AFTER 3 DAYS OF CO-INCUBATION WITH *V. longisporum* VOCs			
5.25	2,3- Butanedione*	–	PGP, antibacterial (Farag et al., 2006; Lee et al., 2012)
5.8	2-Methyl-1-Propanol*	–	Antifungal (Stotzky et al., 1976)
6.438	1-Butanol*	–	Antibacterial (Létoffé et al., 2014)
7.497	3-Hydroxy-2-butanone*	–	PGP, ISR (Ryu et al., 2003)
8.239	**2-Methyl-1-butanol***	–	Antifungal (Raza et al., 2015)
8.605	3-Methyl-2-pentanone*	–	N.a.
12.501	Isoamyl Acetate	↓56%	Antimicrobial (Ando et al., 2015)
13.335	2-Heptanol*	–	Antimicrobial (Orhan et al., 2012)
19.621	Decane, 2,6,7 trimethyl*	–	N.a.
20.773	**Hexadecanal**	–	Antifungal (Raza et al., 2015)
21.090	Decyl trifluoroacetate*	–	N.a.
23.091	Durenol	↓34.1%**	Possibly antimicrobial (Al Nomaani et al., 2013)
23.232	2-(2-Methylpropyl)-3- (1-methylethyl) pyrazine	–	Antimicrobial (Aichner et al., 2012)
26.556	Spathulenol	↓44%**	Plant metabolite (Pacciaroni et al., 2008)
24.686	2-Dodecanal*	–	N.a.
VOCs PRODUCED BY Sb3-1 ONLY AFTER 6 DAYS GROWTH IN PRESENCE OF *V. longisporum* VOCs			
10.25	2-Hydroxy-3-pentanone*	–	N.a.
12.25	Pentaflouropropionic acid, hexyl ester*	–	N.a.
15.75	2- Isopropylpyrazine	–	Antimicrobial (Aichner et al., 2012)
16.6	3(2H)-Thiononanoe	↑51.5%	N.a.
17.79	1,2-Butanediol, 1-phenyl	–	N.a.
18.74	p-cresol*	–	toxic for eukaryotic cells (Andriamihaja et al., 2015)
23.13	2- N-(2-methylpropyl) Benzothiazolamine	↓38.3%	N.a.
23.23	2-(2-Methylpropyl-)-3-(1-methylethyl) pyrazine	–	Antimicrobial (Aichner et al., 2012)
VOCs PRODUCED BY VL43 AFTER BOTH 3 AND 6 DAYS GROWTH IN PRESENCE OF *P. polymyxa* Sb3-1 VOCs			
5.80	Isobutanol*	–	Antifungal (Stotzky et al., 1976)
19.90	2-phenylethanol*	–	Antimicrobial (Liu and Zhang, 2015)
VOCs PRODUCED ONLY AFTER 3 DAYS OF CO-INCUBATION WITH *P. polymyxa* Sb3-1 VOCs			
7.65	Acetoin*	–	PGP, ISR (Ryu et al., 2003)
13.34	2-(4-Cyclohexyl-butanoylamino) -3-chloro-1,4-naphthoquinone	↑100%**	Putatively antifungal (Sasaki et al., 2002)
17.792	Bicyclo (2.2.1)-hepta-2,5-dien-7-ol; or 7-Hydroxynorbornadiene	↑100%**	N.a.
VOCs PRODUCED ONLY AFTER 6 DAYS GROWTH IN PRESENCE OF *P. polymyxa* Sb3-1 VOCs			
6.44	1-Butanol*	↑53.4%	Antimicrobial (Létoffé et al., 2014)
8.10	Isoamyl alcohol*	–	Antimicrobial (Ando et al., 2015)
9.23	1,2,4- Benzenetricarboxylic acid, 1,2 dimethyl ester	↑100%**	N.a.
17.793	Nα, Nω-Dicarbobenzoxy-L-arginine	↑100%**	N.a.
18.08	2-Nonanone*	–	Antifungal (Raza et al., 2015)
19.98	3-Phenyl-5-(benzylthio)isoxazole	↑17.9%	N.a.
25.84	5,6-Decadien-3-yne,5,7-diethyl	↑20%	N.a.

[a] *The selection of substances included in the Table was performed as followed: (1) only substances with match index with the NIST MS Search 2.2 included in the Software-Package of the NIST 2014 database over 500 were considered; (2) only the substances that were either verified using covats index [<15; labeled with asterisks (*)] or showed an up- or down-regulation due to the presence of the VOCs of the other microorganism were included in the Table. The predicted functions of the substances are highlighted in color as follows: the substances with predicted antimicrobial activity are shown in light red, substances with putatively plant growth regulating functions are light green and unknown substances are highlighted in gray.*

[b] *Regulation in presence of microorganism's VOCs (%). Arrow down (↓) indicates the significant down-regulation of the substance, while the arrow up (↑) represents the significant up-regulation of a substance. A minus sign ("–") denotes that no significant up- or downregulation of the substance in the presence of the VOCs was detected. The VOCs that were identified by Raza et al. (2015) as produced by P. polymyxa WR-2 are highlighted in bold.*

The double asterisk (**) denotes values that were significantly different from the non-treated control group values ($P < 0.05$) defined using pairwise t-test or non-parametric Mann–Whitney U-test, depending on the distribution of the samples.

TABLE 2 | Effect of *V. longisporum* ELV43 VOCs on *P. polymyxa* Sb3-1 metabolism detected by LC-MS.

3 days of co-incubation[a]	Putative function	Regulation[b]
Valine*	Protein component	↑
Glutamic acid*	Protein component	↑
Aminocaproic acid*	Lysine derivate/protein degradation	↓
6 days of incubation[a]	**Putative function**	**Regulation[b]**
Adenine*	DNA component	↑
Unknown structure ($C_{35}H_{47}NO_8$)*	n.a.	↓
Guanosine*	Lipoic acid metabolism	↑
Adenosine*	DNA component	↑
L-Threonyl-L-leucin*	Glycine, serine and threonine metabolism	↑
DL-Methionine*	Protein component	↑
DL-Glutamic acid*	Protein component	↑
Boc-L-glutamine (N∼2∼-(tert-Butoxycarbonyl)glutamin)	Glutamate derivate/protein degradation	↑
Citric acid*	General metabolism	↓
Bengamide derivate (2R,3R,4S,5R,6E)-3,4,5-Trihydroxy-2-methoxy-8-methyl-N-[(3S)-1-methyl-2-oxo-3-azepanyl]-6-decenamide)	Potentially cytotoxic, anticancer activity (Thale et al., 2001)	↑

[a]*Only verified substances that were up- or down-regulated by V. longisporum ELV43 VOCs are shown.*
[b]*Arrow up (↑) symbolizes that the corresponding substance is upregulated, while arrow down (↓) symbolizes a downregulation by V. longisporum ELV43 VOCs. N.a. (not available) means that a putative function could not be found. Asterisk (*) symbolized that the up- or downregulation was statistically significant ($p < 0.05$).*

TABLE 3 | Effect of *P. polymyxa Sb3-1* VOCs on *V. longisporum ELV 43* metabolism detected by LC-MS.

3 days of co-incubation[a]	Putative function	Regulation[b]
DL-Histidine*	Protein component	↓
DL-Proline*	Protein component	↓
DL-Glutamic acid*	Citric acid cycle/Protein component	↓
L-Isoleucyl-L-glutamic acid*	Protein breakdown product	↑
Cystathionine*	Cystein synthesis intermediate	↓
DL-Glutamine*	Protein component	↓
L-Saccharopine*	Lysine synthesis intermediate	↓
DL-2,6-Diaminopimelic acid	Cell wall component	↓
Pantothenic acid*	Vitamine	↓
6 days co-incubation[a]	**Putative function**	**Regulation**
Arabitol*	Sugar/response to cell stress	↑
L-Isoleucyl-L-glutamic acid	Protein breakdown product	↑
Methylglutaric acid*	n.a.	↑
Uridine*	RNA component	↑
N-Acetyl-L-Carnosine	Antioxidant	↓
Cystathionine*	Intermediate in the synthesis of cysteine	↓

[a]*Only verified substances that were up- or down-regulated by V. longisporum ELV43 VOCs are shown.*
[b]*Arrow up (↑) symbolizes that the corresponding substance is upregulated, while arrow down (↓) symbolizes a down regulation. n.a. (not available) means that a putative function could not be found. Asterisk (*) symbolized that the up- or downregulation was statistically significant ($p < 0.05$).*

demonstrating the interaction between *P. polymyxa* Sb3-1 and *V. longisporum* ELV43 *in vitro* on a dual-plate assay and using a specific VOCs assay. After 9 days of co-inoculation the mycelial growth of *V. longisporum* ELV43 was significantly inhibited by *P. polymyxa* Sb3-1 on all media tested (**Figure 2A**). The incubation of *V. longisporum* ELV43 with the VOCs of Sb3-1 resulted in the significant inhibition of mycelium growth only in combination with PDA and R2A as growth media (**Figure 2B**). The strongest inhibition of the mycelia growth by *P. polymyxa* VOCs was observed when both microorganisms were grown on PDA plates.

VOCs Involved in the Interaction between *P. polymyxa* Sb3-1 and *V. longisporum* ELV43

VOCs produced by *P. polymyxa* Sb3-1 after 3 and 6 days of co-incubation with *V. longisporum* ELV43 volatiles were analyzed using GC-MS headspace SPME, compared to database entries (NIST 2014 database), and verified using covats index (CI; **Table 1**). Several substances with strong antimicrobial effect, such as three alkyl-substituted pyrazines (Cernava, 2012) were found to be produced after either 3 or 6 days of bacterial growth regardless of the interaction with ELV43 VOCs. We found that higher quantities of known antimicrobial VOCs, such as 2-methyl-1-butanol, hexadecanal (Raza et al., 2015), and isoamyl acetate (Ando et al., 2015), were produced by Sb3-1 after 3 days of incubation than after 6 days of incubation (**Table 1**). The putatively antimicrobial substances isoamyl acetate and durenol were both downregulated upon contact with *V. longisporum* ELV43 VOCs. We also found two CI-verified substances, 2,3-butanedione and 3-hydroxy-2-butanone 1 within VOCs produced by Sb3-1 that are putatively involved in a bacterial PGP effect (Ryu et al., 2003; Farag et al., 2006). Neither of them was regulated by *V. longisporum* ELV43 VOCs (**Table 1**).

GC-MS headspace SPME analysis revealed that *V. longisporum* ELV43 produced several substances with

known antimicrobial properties such as isobutanol (Stotzky et al., 1976) and 2-phenylethanol (Liu and Zhang, 2015). These substances were detected after 3 and 6 days of *V. longisporum* growth. Acetoin, a substance with a known PGP effect that also induces systemic resistance and regulates auxin homeostasis in *A. thaliana* (Ryu et al., 2003), was detected in the volatile phase after 3 days of *V. longisporum* growth. Seven VOCs were upregulated in ELV43 during co-incubation with *P. polymyxa* Sb3-1 volatiles (**Table 1**). Two of them, a naphthoquinone-derivate and 1-Butanol, both exhibiting putative antimicrobial effect (Sasaki et al., 2002; Létoffé et al., 2014) were upregulated due to contact with Sb3-1 VOCs (100 and 53.4%, respectively).

Regulation of Soluble Metabolites Produced by *P. polymyxa* Sb3-1 and *V. longisporum* Elv43 as a Reaction to Each Other VOCs

The production of soluble metabolites by both microorganisms, *P. polymyxa* Sb3-1 and *V. longisporum* ELV43 in response to one another's VOCs was studied by means of high-resolution LC-MS analyses. More than 100 substances were detected in the *P. polymyxa* Sb3-1 samples (data not shown). Of the substances that we were able to be identified with a high degree of certainty (based on NIST 2014 database searches), several were differentially regulated due to the interaction with ELV43 VOCs. Two amino acids, valine and glutamic acid, were upregulated in the 3-day old culture of Sb3-1 due to its exposure to *V. longisporum* EVL43 volatiles, while one amino acid derivative, aminocaproic acid, was downregulated (**Table 2**). Six days after inoculation, several substances involved in the cellular metabolism, such as amino- and nucleic acids were upregulated. Only two substances, citric acid and an unidentified substance, were downregulated because of Sb3-1 exposure to ELV43 VOCs. Additionally, surfactin was detected in every sample of *P. polymyxa* Sb3-1 regardless of the EVL43 VOCs exposure. Surfactin is known for its biosurfactant and antibacterial properties (Pratap et al., 2013) and for its ability to activate the formation of biofilm (Timmusk et al., 2015). Spermidine, a stress protection agent (Kusano et al., 2008), was found after 6 days of Sb3-1 growth, while antimicrobial fusaricidin (Kajimura and Kaneda, 1996) was found after both 3 and 6 days of bacterial growth. Both components were not regulated by EVL43 VOCs. Interestingly, other well-known antimicrobial *Paenibacillus* metabolite polymyxin was not detected under the conditions tested in any of the samples.

Analysis of *V. longisporum* ELV43 metabolites showed that 3 days of incubation with *P. polymyxa* Sb3-1 VOCs lead to up-regulation of L-Isoleucyl-L-glutamic acid, an incomplete breakdown product of protein digestion. On the other hand, the concentration of several metabolites involved in cellular metabolism (e.g., several amino acids and intermediates in the amino acid synthesis), of a substance involved in the cell wall formation (DL-2,6-Diaminopimelic acid) as well as of pantothenic acid, decreased (**Table 3**). Interestingly, after 6 days of co-incubation with *P. polymyxa* Sb3-1 volatiles, the upregulation of detected substances prevailed over downregulation. We detected upregulation of arabitol, a carbohydrate involved in cell stress response, and several breakdown products of protein and RNA biosynthesis. On the other hand, N-Acetyl-L-Carnosine (known as an antioxidant) and cystathionine were downregulated following exposure to Sb3-1 VOCs.

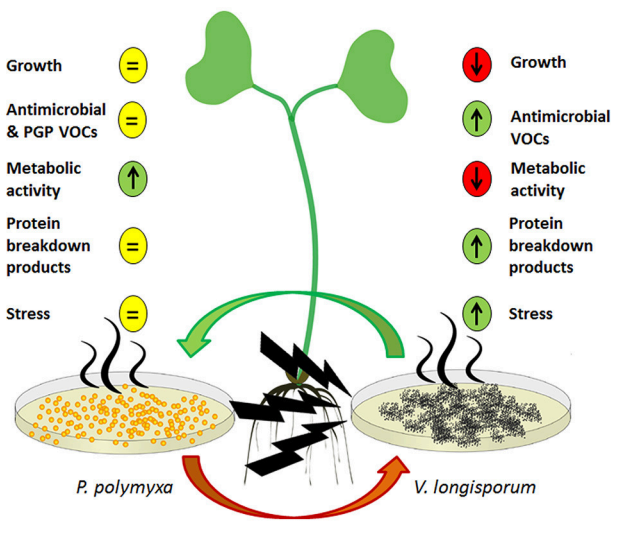

FIGURE 3 | A model of a dialog between *P. polymyxa* Sb3-1 (left) and *V. longisporum* (right) via their VOCs and their influence on the host plant (middle). The predicted up- and downregulated processes are shown as up- and down arrows, respectively. The non-regulated processes are depicted as an equal symbol.

DISCUSSION

The current study underpins the importance of volatiles as mediators of microbial interactions. Moreover, the results suggest that microbial VOCs from beneficial bacteria can induce substantial changes in target pathogens. *P. polymyxa* Sb3-1 was able to reduce the growth of *V. longisporum* ELV43 *in vitro* directly, and via its volatiles. The antifungal effect of Sb3-1 was confirmed *in planta* by a significant improvement in the lengths of the oilseed rape seedlings treated with Sb3-1 compared to the untreated control. The observed plant growth promotion effect was only detected when the seedlings were infested with *V. longisporum*. This suggests that the antagonistic properties of the *Paenibacillus* strain or its ability to induce resistance in the host plant were responsible for the beneficial effect on the plant growth. The production of a biosurfactant and activator of biofilm formation surfactin (Pratap et al., 2013; Timmusk et al., 2015) as well as the stress protection agent spermidine (Kusano et al., 2008) and antimicrobial fusaricidin by Sb3-1 may enhance the PGP effect under biotic stress conditions. The production of 2,3-butanedione and 3-hydroxy-2-butanone by Sb3-1 may also explain PGP properties of Sb3-1 observed in our greenhouse experiments and in previous *in planta* tests under sterile soil conditions (Rybakova et al., 2016b).

The majority of our results revealed contrasting effects with regards to the ways in which the two microorganisms reacted to one another's VOCs as illustrated by the model in **Figure 3**. While *P. polymyxa* Sb3-1 was not visually impaired in its growth due to ELV43 volatiles, the growth rate of *V. longisporum* ELV43 was significantly reduced. Both of the microorganisms constantly produced potentially antimicrobial volatiles; however the production of some antimicrobial VOCs by Sb3-1 was reduced upon contact with ELV43 VOCs, while ELV43 demonstrated upregulation of antimicrobial VOCs due to the interaction with Sb3-1. This may indicate that *P. polymyxa* does not "regard" *V. longisporum* as an enemy and slows its defense system down, while the fungus switches on some type of additional defense mechanism to counterattack the antimicrobial VOCs of Sb3-1. A similar effect was observed when we studied soluble metabolites of both interacting microorganisms. While *P. polymyxa* Sb3-1 demonstrated upregulation of some essential amino acids and DNA components, the opposite was found for *V. longisporum* ELV43. This finding may reflect a change in the metabolic rate of both microorganisms as a reaction to one another's VOCs. There are indications that the metabolism of *V. longisporum* ELV43 slows down due to the contact with damaging Sb3-1 VOCs as the cells undergo an apoptotic process. This was also confirmed by the observed accumulation of protein breakdown products and a specific carbohydrate indicating cellular stress (arabitol) by ELV43 exposed to Sb3-1 VOCs. In contrast, the metabolic activity of Sb3-1 seems to be activated by the ELV43 VOCs as is suggested by accumulation of protein and DNA components by ELV43.

Interestingly, out of 40 identified VOCs produced by *P. poly*myxa Sb3-1 only three (2-nonanone, 2-methyl-1-butanol, and hexadecanal) were identical with those identified by Raza et al. (2015) in *P. polymyxa* WR-2 VOCs. This strain was found to be active against *Fusarium oxysporum* f. sp. niveum *in vitro*. The variations in the composition of volatiles might be primarily due to differences in the growth media compositions, however the structure of *Paenibacillus*' "volatilomes" can also vary to a certain degree as highlighted by Rybakova et al. (2016a). In addition, it indicates a very specific pathogen-antagonist interaction. We first detected one putative PGP substance acetoin (3-hydroxy-2-butanone), which was also found to trigger induced systemic resistance in *Arabidopsis* (Rudrappa et al., 2010) produced by the plant pathogen *V. longisporum* ELV43. Although, this finding needs to be further investigated, it may also explain the plant-beneficial effect described for non-pathogenic *Verticillium* strains (França et al., 2013). Furthermore, it also supports the theory that microbial diversity is crucial for combating "microbiome diseases" such as Verticillium wilt because *Verticillium* species occur frequently in healthy plants and contribute to the functioning of the holo-biont (Berg et al., 2017).

In conclusion, our data indicates that *P. polymyxa* Sb3-1 and *V. longisporum* ELV43 are in a constant dialog with one another via their VOCs. This specific dialog results in the inhibition of cellular metabolism in *V. longisporum* ELV43 leading to the growth reduction of the fungus. This antagonistic effect in addition to the production of PGP volatiles leads to an overall positive effect on the growth of the infested host plant. This study contributes to the better overall understanding of the interactions between the potential BCA *P. polymyxa* Sb3-1 and the plant pathogen *V. longisporum*. It supports the effort to develop this beneficial bacterium into a viable BCA against Verticillium wilt, and offers a promising option in the progress toward realizing sustainable agriculture in the context of global warming.

AUTHOR CONTRIBUTIONS

GB and DR designed the study. UR carried out the *in vitro*, LC-MS and GC-MS assays and analyzed the GC-MS data. MS and DR carried out plant growth promoting and biocontrol assays. AS did the analytic for LC-MS assays. TC and AS consulted on the GC-MS assays and TC contributed to the VOCs-related parts of the final manuscript. DR wrote the final version of the manuscript with input from GB, TC, and UR. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01294/full#supplementary-material

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Little Cross-Feeding of the Mycorrhizal Networks Shared Between C_3-*Panicum bisulcatum* and C_4-*Panicum maximum* Under Different Temperature Regimes

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Common mycorrhizal networks (CMNs) formed by arbuscular mycorrhizal fungi (AMF) interconnect plants of the same and/or different species, redistributing nutrients and draining carbon (C) from the different plant partners at different rates. Here, we conducted a plant co-existence (intercropping) experiment testing the role of AMF in resource sharing and exploitation by simplified plant communities composed of two congeneric grass species (*Panicum* spp.) with different photosynthetic metabolism types (C_3 or C_4). The grasses had spatially separated rooting zones, conjoined through a root-free (but AMF-accessible) zone added with ^{15}N-labeled plant (clover) residues. The plants were grown under two different temperature regimes: high temperature (36/32°C day/night) or ambient temperature (25/21°C day/night) applied over 49 days after an initial period of 26 days at ambient temperature. We made use of the distinct C-isotopic composition of the two plant species sharing the same CMN (composed of a synthetic AMF community of five fungal genera) to estimate if the CMN was or was not fed preferentially under the specific environmental conditions by one or the other plant species. Using the C-isotopic composition of AMF-specific fatty acid (C16:1ω5) in roots and in the potting substrate harboring the extraradical AMF hyphae, we found that the C_3-*Panicum* continued feeding the CMN at both temperatures with a significant and invariable share of C resources. This was surprising because the growth of the C_3 plants was more susceptible to high temperature than that of the C_4 plants and the C_3-*Panicum* alone suppressed abundance of the AMF (particularly *Funneliformis* sp.) in its roots due to the elevated temperature. Moreover, elevated temperature induced a shift in competition for nitrogen between the two plant species in favor of the C_4-*Panicum*, as demonstrated by significantly lower ^{15}N yields of the C_3-*Panicum* but higher ^{15}N yields of the C_4-*Panicum* at elevated as compared to ambient temperature. Although the development of CMN (particularly of the dominant *Rhizophagus* and *Funneliformis* spp.)

was somewhat reduced under high temperature, plant P uptake benefits due to AMF inoculation remained well visible under both temperature regimes, though without imminent impact on plant biomass production that actually decreased due to inoculation with AMF.

Keywords: C_3 and C_4 photosynthesis, common mycorrhizal networks (CMNs), temperature, natural ^{13}C isotopic abundance, *Panicum* sp., arbuscular mycorrhiza, community, quantitative real-time PCR

INTRODUCTION

Arbuscular mycorrhizal fungi (subphylum Glomeromycotina; Spatafora et al., 2016) are obligate symbionts of a large majority (>60%) of land plant species (van der Heijden et al., 2015). AMF play a crucial role in nutrient uptake of the host plants (Smith and Read, 2008) as well as in improving their resistance to pathogens (Newsham et al., 1995; Vigo et al., 2000) and/or tolerance to drought and osmotic stresses (Aroca et al., 2007, Augé et al., 2014, 2015). Although efficient P transfer from the soil to the host plant mediated by AMF hyphae is frequently considered to be the major benefit of AM symbiosis for their host plants (Smith and Read, 2008; Smith et al., 2011), plant uptake of N via AMF mycelium also has been demonstrated (Bücking and Kafle, 2015 and references therein). Mycorrhiza-mediated N acquisition is particularly important when soil N is predominantly in organic forms or under moderate drought (Tobar et al., 1994; Hodge and Storer, 2015; Bukovská et al., 2018 and the references therein). In return for the supply of such mineral nutrients as P or N, the host plant provides the AMF with reduced C in the forms of simple carbohydrates and/or fatty acids (Lekberg et al., 2010; Roth and Paszkowski, 2017; Řezáčová et al., 2017a). The plant C allocation into the AMF hyphae ranges between 0.9 and 20% of its gross photosynthetic production (Jakobsen and Rosendahl, 1990; Bryla and Eissenstat, 2005; Konvalinková et al., 2017; Řezáčová et al., 2017a,b; Slavíková et al., 2017).

There is only a limited host specificity in AM symbioses. The same AMF species often colonizes several plant species simultaneously and, at the same time, roots of a single plant typically host multiple AMF species (Helgason et al., 2002; Vandenkoornhuyse et al., 2003). Thus, the shared AMF mycelium often interconnects two or more plant individuals of the same or different species, thereby establishing so-called CMNs. These CMN transport nutrients and C through soil ecosystems and redistribute symbiotic benefits and costs within a plant community (Walder and van der Heijden, 2015). CMN thus affect the survival, fitness, and competitiveness of their hosts, regulate plant coexistence (van der Heijden et al., 1998; Bever et al., 2010), and maintain plant community diversity (van der Heijden et al., 1998) and ecosystem stability. It has been documented that the rates of exchange of nutrients and C between the host plants and their fungal symbionts are variable depending on partner identity, age, AMF community composition (Jansa et al., 2008; Wagg et al., 2011), C supply of the neighboring plants interconnected through the CMN (Walder et al., 2012), and/or environmental conditions (e.g., Lekberg et al., 2013). There is accumulating experimental evidence that the CMN distribute nutrients among plant partners according to their C supply rates (Lekberg et al., 2010; Kiers et al., 2011). Similarly, a plant may distribute its C among fungal partners according to their mineral nutrient supply rates (Bever et al., 2009; Walder and van der Heijden, 2015). However, the supposed reciprocal control of the C-for-nutrients exchange whereby the most beneficial partner receives the most resources (Kiers et al., 2011) may not be valid under all circumstances (Walder and van der Heijden, 2015). The partitioning of mineral nutrients among neighboring plants acquired via CMN and the associated C costs are also likely to be influenced by both plant competition and facilitation (Weremijewicz et al., 2016). Nonetheless, the exact molecular mechanisms controlling the flows of nutrients and C through CMN are not yet precisely known (Bücking et al., 2016).

Global air temperatures are expected to rise in the years and decades to come (Latef et al., 2016) and the concomitant soil warming is expected to affect organisms living in soil, including AMF that interact with plants (Compant et al., 2010). Although the average expected temperature increase is not yet very high, it may be very significant in some regions as global warming shifts the temperature/precipitation distribution patterns across continents and also as hot and cold spells become more and less likely, respectively (Lobell and Gourdji, 2012). The crucial roles of AMF in soil C cycling, ecosystem primary productivity, soil C storage or soil C losses through decomposition, while exhibiting relatively low diversity within ecosystems (Mohan et al., 2014; Treseder, 2016), show that it is necessary to understand the impact of increased temperature on the nutrient-for-C exchange between plants and AMF. Increased soil temperature mostly increases the rate of C transfer to CMN and the fungal C respiration, thus promoting more extensive extraradical hyphal network development (Rillig et al., 2002; Heinemeyer et al., 2006; Hawkes et al., 2008; Compant et al., 2013). Elevated temperatures also affect plant growth directly, often reducing photosynthesis and respiration (e.g., Hawkes et al., 2008; Latef et al., 2016). Nevertheless, AMF can sometimes decrease the impact of temperature stress on their host plant (Hawkes et al., 2008; Zhu et al., 2011) and, because the symbiotic function is dependent upon the response of all partners to the environmental context, adaptation of either symbiont could alter the nature of the symbiosis (Bunn et al., 2009). Adaptation of one plant to a certain stress may therefore possibly mitigate the negative

Abbreviations: AM, arbuscular mycorrhiza; AMF, arbuscular mycorrhizal fungi; ANOVA, analysis of variance; C, carbon; C16:1ω5, AMF-specific fatty acid; CMNs, common mycorrhizal networks; M−, non-mycorrhizal control; M+, mycorrhiza inoculated; N, nitrogen; P, phosphorus; qPCR, quantitative real-time PCR; RFC, root-free compartment.

impact of high temperatures on the other (unadapted) plants interconnected via the CMN through maintaining the mutualistic nature of the symbiosis.

Vascular plants underwent a number of adaptations during their evolution, and the C_4 photosynthetic type is one of the most spectacular adaptive responses to high temperature, as well as to low CO_2, water, and mineral nutrient availabilities (Ehleringer and Monson, 1993). While C_3 plants provide the products of photosynthesis at lower metabolic costs under moderate temperatures, light levels, and water availabilities, C_4 plants are more effective under high light and arid conditions (Waller and Lewis, 1979; Voznesenskaya et al., 2001). In a study utilizing C_4-*Sorghum* and C_3-*Linum*, Walder et al. (2012) found that the C_4-*Sorghum* allocated large amounts of C to the CMN interconnecting the two plants compared to the C_3-*Linum*, but the C_3-*Linum* received a disproportionally large share of the isotopically (^{32}P- and ^{15}N-) labeled nutrient resources via the CMN. This imbalance in cost-benefit exchange, however, could be caused by the use of only one fungal species and thus absence of competition among different fungal species or incompatibility of the fungus with one or the other of the plant species. Wang et al. (2016), on the other hand, found that C_3-*Glycine* invested more C into CMN than did C_4-*Zea* when intercropped. Here, the results may also have been affected by what could be termed "cheating" by the sole fungal species used in the model experiment. Moreover, tripartite symbiosis – inoculation with AMF and rhizobia in parallel – may stand behind the stronger competitiveness of C_4-maize in that particular experiment, because maize, unlike soybean, would not bear any C costs connected with biological N_2 fixation. To predict the impact of elevated temperature on C and/or nutrient flows in plants interconnected by CMN, it is necessary to explore interactions of various plants through CMN constituted by more than one AMF species under various environmental conditions.

Toward that end, we conducted a C_3–C_4 plant co-existence (intercropping) experiment testing the role of AMF in the resource sharing and exploitation by a simplified plant community composed of congeneric C_3- (*Panicum bisulcatum*) and C_4- (*Panicum maximum*) grass species sharing CMN under ambient and at elevated temperatures. Further, we tested which of the hosts (the C_3- or the C_4-*Panicum*) bore the costs of C supply to the CMN under the different temperature regimes and whether nutritional benefits provided to the different plant hosts by the CMN changed upon altering the environmental conditions. To address these questions, we grew the plants in cultivation containers consisting of two plant compartments joined through a RFC. The rhizospheres of the two plants were thus spatially delimited from each other by a double root-penetration mesh barrier. To achieve a greater relevance to real field conditions as compared to majority of previous research on CMN, we inoculated the plants with a multispecies AMF community rather than, as previously, using a monospecific AMF inoculum (Walder et al., 2012; Weremijewicz et al., 2016). In the RFC, we supplied ^{15}N-labeled clover litter to trace N transfer from this material to the plants via the AMF hyphae. We used organic N source because it offered a neat possibility to provide isotopically labeled N in a spatially discrete manner (much less prone to diffusion than, e.g., NO_3^-), such patches have previously been reported to be efficiently explored by AMF hyphae, and the ^{15}N transfer from the organic amendments to plants could be used as a proxy of mycorrhizal nutrient benefits (Hodge and Fitter, 2010; Fellbaum et al., 2012; Bukovská et al., 2018).

We had expected that the C_4-*Panicum* would generally cover a larger share of the C supply to the CMN compared to the C_3-*Panicum*, because C_4 plants generally show higher mycorrhizal responsiveness than do C_3 plants (Hetrick et al., 1990; Hoeksema et al., 2010; Chandrasekaran et al., 2016) and C_4 plants are often larger in size and thus stronger C sources. Further, we expected that the C_4-*Panicum* would derive greater mycorrhizal benefits from the symbiosis compared to the C_3-*Panicum*, be these growth promotion or P and/or N uptakes. Based on the reciprocal reward concept sensu Kiers et al. (2011) we also expected the C_4-*Panicum* to cover a larger share of C supply to the CMN at elevated temperature than at ambient temperature. This is because the C_4 plants are better adapted to warmer conditions than are the C_3 plants.

MATERIALS AND METHODS

Experimental Design

The experiment was set up in a fully factorial design with two factors: mycorrhizal inoculation (mycorrhiza inoculated, M+, or not, M−), and cultivation temperature (ambient [25°C during daytime, designated as "low t" from this point on] or high [36°C during daytime, designated as "high t"]). There were four replicate cultivation containers established per each inoculation and temperature treatment combination. Individuals of two different host plant species (C_3 – *Panicum bisulcatum* and C_4 – *Panicum maximum*) were planted in the opposite compartments of each of the cultivation containers (see Supplementary Figure S1) as a "mixed culture." Thus, a total of 16 mixed-culture containers were set up at the beginning of the experiment. Later, 3 of these 16 containers had to be excluded due to poor growth of one or both of the plants, resulting finally in 13 mixed-culture containers retained for data analysis (yielding three replicates for both [M+ and M−] low t treatments and for the M− high t treatment). In addition, one monoculture container per each inoculation and temperature treatment combination was established with individuals of the same plant species in both compartments. The positions of the cultivation containers were completely randomized in the two growth chambers (high t or low t) and the positions were further re-randomized every week throughout the entire experiment.

Plants

Two grass species belonging to the genus *Panicum* L. were used in this study: *P. bisulcatum* Thunb. and *P. maximum* Jacq. These two plant species are well characterized in terms of their photosynthesis types, with *P. bisulcatum* being a typical C_3 plant and *P. maximum* having a C_4 (PCK subtype) type of photosynthesis (Pinto et al., 2014). Importantly, these two plant species display different C isotopic composition characteristic of their photosynthesis types, with the C_3 plants

being significantly more ^{13}C-depleted than the C_4 plants (see **Figure 1** for data). Seeds of both plant species were kindly donated by Dr. Oula Ghannoum, Hawkesbury Institute for the Environment, University of Western Sydney, Australia. The seeds were collected from small populations of glasshouse-grown plants of the respective species, derived originally from about 20 seeds of each species collected in the fields and propagated for at least seven generations under glasshouse conditions.

Cultivation Containers and Substrate

Plants were grown in compartmented containers consisting of two side compartments (10 cm × 10 cm × 11 cm; see **Figure 1** and Supplementary Figure S1), each planted with three plant seedlings belonging to one or the other grass species. The plants were interconnected through a middle, RFC (14 cm × 6 cm) separated from both of the plant compartments by 42 μm nylon meshes allowing AMF hyphae but not the roots to penetrate (**Figure 1**). There was a 30.5 ml labeling compartment (a tube with a diameter of 3.6 cm, 3 cm long) placed in the RFC, wrapped in a 200 μm nylon mesh on both sides (**Figure 1**), and filled with potting substrate supplemented with ^{15}N-labeled white clover biomass.

The substrate consisted of thoroughly mixed (volume-based) 10% γ-irradiated (>25 kGy) field soil from Litoměřice, Czechia (N50°31′54.53″ E14°06′7.10″), 45% autoclaved zeolite MPZ 1-25 from Zeopol[1] (grain size 1–2.5 mm), and 45% autoclaved quartz sand (grain size < 3 mm; for physicochemical properties, see Supplementary Table S1).

^{15}N labeling

To distinguish N uptake by plants via the mycorrhizal hyphae from that taken up directly via roots, the RFC was supplemented with ^{15}N-labeled biomass of white clover (*Trifolium repens* cv. Jura, Agrogen spol. s.r.o., Troubsko, Czechia) added to the labeling compartment (see above and **Figure 1**). The clover had been grown in a hydroponic tank (10 l) and fertilized with diammonium phosphate (3 g, 98% atom% ^{15}N) 2 weeks after sowing, in addition to a standard Long Ashton nutrient solution including the original (1.5 mM) P concentration (Hewitt, 1966). Clover shoots were harvested 22 days after sowing, dried at 65°C for 3 days and milled to a fine powder using an MM 200 ball mill (Retsch, Haan, Germany). The clover biomass contained 38% C, 2.64% N, and 0.30% P. Isotopic enrichment of N in the labeled clover biomass reached 42 atom% ^{15}N [corresponding to a $\delta^{15}N$ (vs. air N_2) = 186676]. Clover biomass was added at a rate of 1 g per 240 g of the potting substrate, mixed to homogenize and filled into the labeling compartments (40 g of the ^{15}N-enriched substrate per labeling compartment). Every labeling compartment thus received with the clover biomass approximately 4.4 mg N (out of which 1.84 mg was the ^{15}N), in addition to 63 mg C and 0.5 mg P. This resulted in nearly a duplication of the organic C concentration in the substrate of the labeling compartment (0.38% C as compared to 0.22% C in the rest of the cultivation containers, see also Supplementary Table S1) and in duplication of the N concentration in the substrate of the labeling compartments (0.02% N) as compared to the rest (0.01% N, see also Supplementary Table S1).

Mycorrhizal Inoculation

Half of the containers (M+) were supplemented with 133 g mycorrhizal inoculum per each plant compartment. The inoculum consisted of potting substrate containing root fragments of leek (*Allium porrum* L.), which had been used as a host plant in a previous pot culture containing either *Rhizophagus irregularis* (N. C. Schenck and G. S. Sm.) Schüßler and Walker (2010) – Chomutov (Krak et al., 2012), *Claroideoglomus claroideum* (N. C. Schenck and G. S. Sm.) Schüßler and Walker (2010) BEG 23, or *Funneliformis mosseae* (T. H. Nicolson and Gerd.) Schüßler and Walker (2010) BEG 95. The three monospecific inocula, respectively, were mixed in the following ratio: 34 g:85 g:14 g. BEG is an abbreviation for the International Bank for the Glomeromycota[2]. Further, the mycorrhizal inoculum was supplemented with 300 spores per plant compartment of *Acaulospora tuberculata* Janos and Trappe BEG 41 and 500 spores per plant compartment of *Gigaspora margarita* W. N. Becker and I. R. Hall BEG 34 (manually picked following wet sieving and decanting). The other half of the containers (M−) received 133 g non-mycorrhizal (mock) inoculum per each of the plant compartments. The mock inoculum consisted of potting substrate containing root fragments of leek from a previous pot culture grown under the same conditions and for the same period of time as the mycorrhizal pot cultures (above) but without any AMF. The inocula were provided as layers 4–5 cm beneath the surface of the potting substrate in the plant compartments.

Planting and Growth Conditions

Seeds of both plant species were pre-germinated in 15 cm Petri dishes on wet filter paper at 37°C for 12 h and then incubated at 35°C for 2 days, followed by incubation at 25°C for the next 2 days under ambient light. The Petri dishes with seeds were then kept in the greenhouse for another 3 days before transplanting them into the containers. Three seedlings of the respective plant species were planted in each of the plant compartments. The containers were then incubated in two identical growth chambers of the Institute of Microbiology, Prague, Czechia, providing a temperature control of ±0.5°C and light (high-pressure sodium vapor lamps combined with fluorescent tubes) intensity during the photoperiod of 300 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetically active radiation at plant level. For the first 26 days after planting, all plants were kept at the same growing conditions of 25/21°C average day (16 h photoperiod)/night temperatures. Thereafter, the temperatures were elevated for half of the containers to 36/32°C day/night, respectively (see Supplementary Figure S2). The containers were watered daily with deionized water to maintain approximately 85% water holding capacity of the substrate (assessed gravimetrically). From the fourth until the eighth week after planting, each plant compartment received weekly 65 ml Long Ashton mineral nutrient solution (Hewitt, 1966) with the P concentration reduced to 20% of the original

[1] www.zeolity.cz

[2] www.i-beg.eu

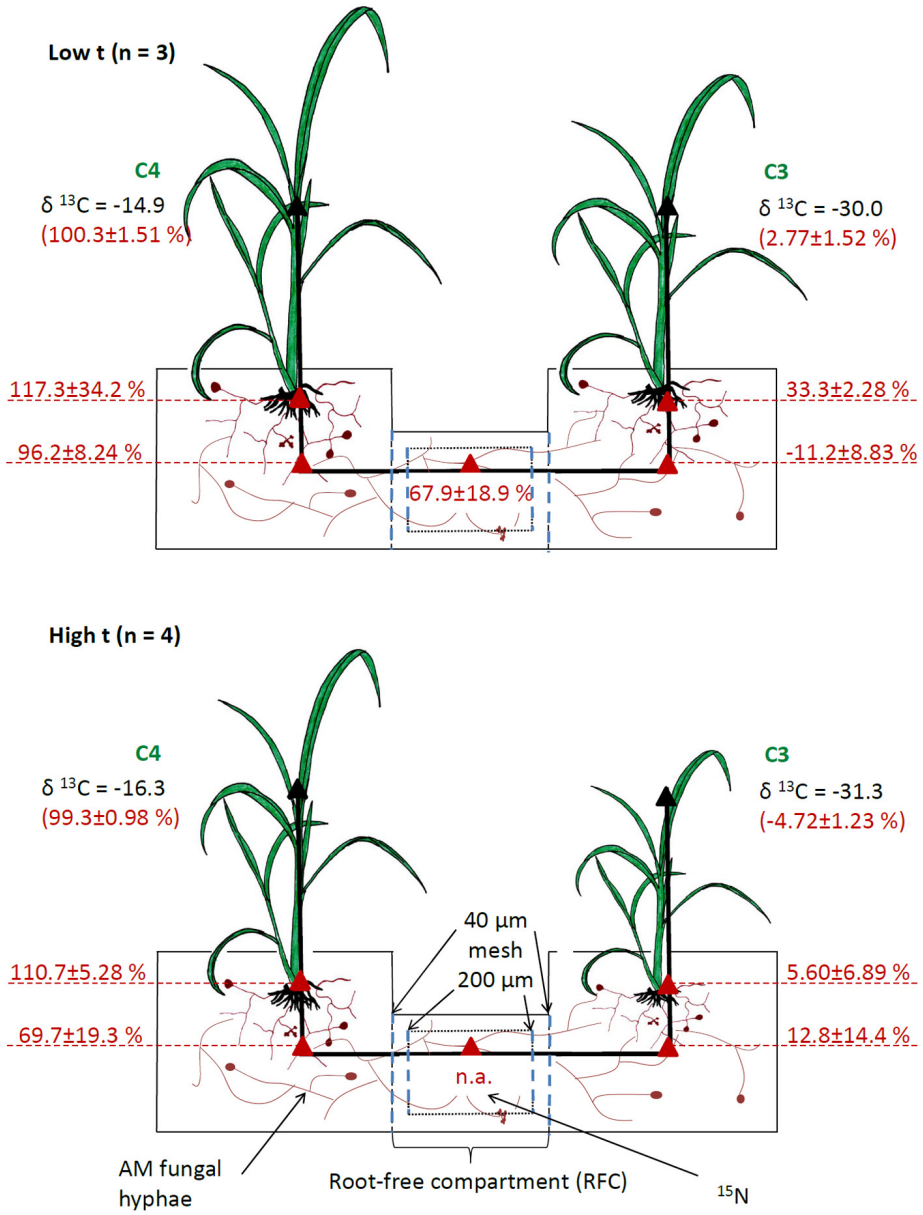

FIGURE 1 | Setup of experimental containers and indications of the measured carbon source of AMF-specific fatty acid (shown in red, on a scale from 0%, representing pure C_3-*Panicum* signature, to 100%, representing pure C_4-*Panicum* signature derived from monoculture cultivation containers) in mixed culture containers planted with C_4-*Panicum* on one side and C_3-*Panicum* on the other side, and with a root-free compartment (RFC) in the middle. Mean values ± standard errors are shown separately for ambient (low t) and elevated (high t) temperature regimes. The RFC was separated from the plant compartments by root-exclusion meshes and was thus accessible only to AMF hyphae. It contained ^{15}N-labeled clover biomass. The analyses were carried out using total lipid extracts from roots of both plants as well as substrate samples from both the plant and RFC compartments (red triangles). Calculations of the C source of AMF-specific fatty acid could not be carried for the RFC in the high t treatment because no significant amounts of the fatty acid were detected (n.a., not applicable). Numbers shown in black indicate mean $\delta^{13}C$ values (vs. VPDB standard) measured in plant shoots of the different plant species to indicate the range of $\delta^{13}C$ encountered in this research.

recipe. This resulted in total fertilization inputs equaling 54.6 mg N and 3.0 mg P per each plant compartment over the entire duration of the experiment.

Plant Harvest

The experimental plants were harvested 75 days after planting. The shoots were cut at the hypocotyl–root boundary and subsequently dried for 3 days at 65°C to determine the shoot dry weight. The roots were washed from the substrate under cold tap water and cut into 1 cm fragments. The roots were then divided into two parts and the fresh weights of both were recorded. One aliquot was immersed in 10% KOH (w:v) to determine the AMF colonization. The second aliquot was dried for 4 days at 65°C, weighed and the root dry weight of the whole

root system was calculated as described below. Fifty grams of representative (thoroughly mixed) substrate from each of the plant compartments and the RFC were dried at 65°C for 4 days. Dried shoot, root, and substrate samples were milled to a fine powder using an MM 200 ball mill before further elemental, isotopic, and molecular analyses.

Analyses and Calculations

Dry biomass of the roots was calculated using the fresh weight of the entire root system and dry-to-fresh biomass ratio of the roots measured on the root aliquot subjected to drying.

To determine the P concentration in plant tissues, 100 mg of a milled sample of each shoot and root were incinerated in a muffle furnace at 550°C for 12 h, the ashes were dissolved in 1 ml of concentrated (69%, w:v) HNO_3 and briefly boiled (250°C) on a hot plate. The extracts were then transferred into volumetric flasks (50 ml) through ashless filter paper (Whatman 41, P-lab, Prague, Czechia) and made up to 50 ml with ultrapure water. Orthophosphate concentration in the extracts was measured using the malachite green method (Ohno and Zibilske, 1991). The N and C concentrations and N and C isotopic compositions in shoots, roots, and substrate samples were measured using a Flash EA 2000 elemental analyzer coupled with a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States) as described earlier (Slavíková et al., 2017). Plant P and N contents were calculated from the measured nutrient concentrations in shoots and roots using the dry biomass data for the shoots and roots, respectively.

To calculate the amount of ^{15}N in the plant samples which had originated from the ^{15}N-labeled white clover (i.e., excess ^{15}N), the measured $\delta^{15}N$ values were first converted to *F*-ratios:

$$F = (0.0036782 \times (\delta/1000 + 1))/ (0.0036782 \times (\delta/1000 + 1) + 1),$$

where δ stands for $\delta^{15}N$ (‰) of the sample and 0.0036782 is the $^{15}N/^{14}N$ isotope ratio of the air.

The number of moles of N (n_N) in the sample was then calculated as follows:

$$n_N = m_N/(14.0030740048 + (15.0001088982 - 14.0030740048)^* F),$$

where m_N stands for the N content in the sample and 15.0001088982 and 14.0030740048 are the amounts (g) of 1 mol ^{15}N and ^{14}N, respectively.

Finally, the amounts of ^{15}N in the shoots added by labeling (excess ^{15}N, in moles) were calculated:

$$\text{excess}^{15}N = (Fs - Fu) \times n_N,$$

where Fs is the ^{15}N *F*-ratio in the sample, Fu is the average ^{15}N *F*-ratio in the corresponding samples of 10 unlabeled plants, and n_N is the number of N moles. Here, the N isotopic composition of plants from our previous experiment established using the same substrate and the same *Panicum* plants was used to subtract the natural abundance of ^{15}N.

Lipid extractions were carried out from the root and substrate samples of each of the plant compartments as well as from the RFC of each experimental container. It was performed according to the procedure described earlier by Frostegård et al. (1991) but with slight modifications: 0.1 g of dry roots or 10 g of substrate without roots was placed into a 50 ml centrifuge tube, then 15 ml of chloroform:methanol:sodium citrate buffer (0.15 M, pH 4.0) mixture (1:2:0.8, v:v: v) was added as well as 100 µl of the internal standard, nonadecanoic fatty acid (C19:0, Sigma-Aldrich, 1 µg μl^{-1} in hexane). Samples were incubated at room temperature overnight with occasional stirring, centrifuged for 10 min (750 × *g*, 16°C) and the liquid was then transferred into a new 50 ml centrifuge tube. Phase separation was achieved by adding 10 ml of the citrate buffer to each sample, intensive shaking, then centrifugation at 750 × *g* at 16°C for 2 min. The lower (organic) phase was collected with a 10 ml Hamilton syringe, filtered through a syringe-driven hydrophobic filter (Chromafil O-45/15 MS, Macherey-Nagel, Germany), then evaporated to dryness under N_2 flow at 35°C. Fatty acids were transmethylated using the trimethylchlorosilane approach (Welc et al., 2012) and the samples were then evaporated to dryness and dissolved in hexane (100 µl). The samples were next filtered through the hydrophobic filters and analyzed using a Trace 1310 gas chromatograph (Thermo Fisher Scientific) equipped with an Rtc-5 60 m/0.25 mm ID/1 µm coating column (Restek, Bellefonte, PA, United States) coupled to a Delta V Advantage mass spectrometer via GC Isolink (Thermo Fisher Scientific). This instrumentation allowed online conversion of all fatty acids in the eluate from the GC column directly to CO_2 and continuous measurement of the ^{13}C to ^{12}C ratios in the He (carrier gas)-CO_2 flow fed subsequently to the mass spectrometer (López-Mondéjar et al., 2018).

Identification of the different fatty acids in the chromatograms was carried out by comparison with qualitative GC standards. The concentrations of the individual fatty acids in the root and substrate samples were calculated using the sample weight and quantification of the internal standard (C19:0 fatty acid) in each individual sample.

Relative contribution of the C_3- and C_4-*Panicum* hosts to the C contained in the AMF biomass/hyphae in the roots and in the substrate filling the different compartments of the mixed-culture containers was assessed by analyzing the isotopic composition of AMF-specific fatty acid C16:1ω5 (Olsson, 1999; Voříšková et al., 2017) in the lipids extracted from the respective root and substrate samples and comparing them to the isotopic composition of the same fatty acids extracted from the containers planted with only a single plant species. Because the amounts of C16:1ω5 fatty acid in the RFC of the M+ containers did not differ significantly from those of the M− containers at high t (Supplementary Figure S3 and Supplementary Table S2), the origin of C in the AMF biomass in the RFC was only calculated for the containers at low t (although all samples were measured, see the raw data in Supplementary Table S5). The origin of C in the C16:1ω5 fatty acid was calculated as follows:

Initially, the $\delta^{13}C$ of C16:1ω5 was "cleaned off" the non-mycorrhizal background. This was done by subtraction of the isotopic contribution brought by the C16:1ω5 in the respective

sample (root or substrate) from the M− containers incubated under the identical conditions (low or high t). Thereafter, the origin of C (either from the C_3- or the C_4-host plant) in the C16:1ω5 was derived from the reference $\delta^{13}C$ values measured in the C16:1ω5 in the monoculture containers (at high t or low t, using average of two independent root samples or substrate samples from the two independent plant compartments or one RFC for each temperature regime). The contribution of the different plant species (C_3 or C_4) to the C detected in the C16:1ω5 was then expressed on a scale from 0% (corresponding to exclusive supply of C by the C_3-plant) to 100% (corresponding to exclusive C supply by the C_4-plant).

The extent of root length colonized by the AMF structures was assessed microscopically after staining the roots with Trypan blue according to Koske and Gemma (1989). We used the magnified intersection method according to McGonigle et al. (1990), scoring 100 magnified (×200) root intersections per sample.

The composition of AMF communities in the roots and in the different substrate samples was assessed on DNA extracted from the different samples using quantitative real-time PCR (qPCR) with taxon-specific markers (mt5, moss, clar, giga, and acau) targeting sequence-specific motifs of *Rhizophagus*, *Funneliformis, Claroideoglomus*, *Gigaspora*, and *Acaulospora*, respectively, in the mitochondrial (*Rhizophagus*) and nuclear (*Gigaspora*, *Funneliformis*, and *Claroideoglomus*) large ribosomal subunit RNA genes as described previously (Thonar et al., 2012; Couillerot et al., 2013). Abundance of *Acaulospora* in the samples from this particular experiment was quantified using a novel qPCR marker "acau" targeting the species-specific DNA motif in the nuclear large ribosomal subunit with forward primer 5′ GAG GAT TGCA GCG GAT G 3′, reverse primer 5′ CAA TCG TTA GCA AGC TAT CG 3′, and a fluorescent hydrolysis (TaqMan) probe FAM – TAG TCA CCT ACC TTC TG – BHQ1. This marker targets solely *A. tuberculata*, was designed in Allele ID version 6 software (Premier Biosoft International, Palo Alto, CA, United States), and generates an amplicon of 79 bp length. Primer annealing was set at 58°C for 30 s, in which conditions no significant cross-amplification was observed of the "acau" marker with the other AMF taxa included in our experiment, during cross-amplification testing prior to the AMF quantification in experimental samples (data not shown). Absence of cross-amplification of non-target AMF taxa included in this experiment with all the others taxon-specific markers have also been experimentally tested prior to the experiment (data not shown). The AMF taxa abundance data generated by the qPCR have been corrected for unspecific DNA losses using internal DNA standard spiked in each sample prior to DNA extraction, as described previously (Thonar et al., 2012). Solis Biodyne chemistry (5× HOT FIREPol Probe qPCR Mix Plus [ROX] master mix) was used for all qPCR assays according to manufacturer's recommendations in a StepOnePlus™ Real-Time PCR thermocycler (Applied Biosystems).

Statistical Analyses

Analysis of variance with $p < 0.05$ as the significance cutoff level were calculated in the R 3.2.1 statistical environment (R Core Team, 2013[3]) after checking for data conformity with ANOVA assumptions (i.e., normality and homogeneity of variances). The percentage data describing the AMF colonization in the roots (assessed by microscopy) were arcsine (square-root)-transformed before the analyses. Two-way ANOVAs with mycorrhizal inoculation and cultivation temperature as factors were performed on data summed per cultivation container (i.e., total plant biomass, plant P and N contents, ^{15}N excess in plant biomass, as well as the C16:1ω5 contents), and on the C_3 share (i.e., fraction of the total assignable to the C_3 plant contribution) in the per-container summed values of the total plant biomass, plant P and N contents, and the ^{15}N excess in plant biomass. Two-way ANOVA was also performed on C16:1ω5 contents in the RFC. Further, two-way ANOVA was carried out to separate effect of the plant photosynthesis type and the temperature regimes on the data describing the C source of C16:1ω5 fatty acid in the roots and in the plant compartments of the experimental containers. One-way ANOVAs with cultivation temperature as a factor were performed on microscopically recorded levels of mycorrhizal colonization of either C_3 or C_4 plants, and the AMF taxon abundances (measured by qPCR) as affected by temperature (separately for the C_3- and the C_4-plant compartments or for the RCF). For the qPCR data, levels of mycorrhizal colonization of roots, as well as data on C source of the C16:1ω5 fatty acid, only data were used from the M+ cultivation containers. This was because the M− plants showed no signs whatsoever of mycorrhizal colonization of roots and, regarding the qPCR data, only a single M− plant root sample showed a very low abundance of *Rhizophagus* (see Supplementary Table S5 for data). Two sample *t*-tests comparisons or one-way ANOVAs with least significance difference *post hoc* tests were performed to separate means of individual factor combinations to interpret significant interactions. Two-sample *t*-test comparisons and Wilcoxon tests were carried out to test the significances of differences in the C source of the C16:1ω5 fatty acid in the different compartments (i.e., roots, substrate from rooted compartment, and the RFC) of our experimental system. Mean values and standard errors per treatment combination were calculated and are presented here in the text and/or in the figures.

RESULTS

Whole Cultivation Container Level (i.e., C_3- and C_4-Plant Sides Summed Together)

Plant biomass production (i.e., the sum of both C_3 and C_4 plant biomass in the same cultivation container) was systematically suppressed by both AMF inoculation as well as by elevated temperature (Supplementary Table S2 and **Figure 2A**). These effects were independent, as no significant interaction between the two factors was recorded (Supplementary Table S2).

In contrast to the biomass production, AMF inoculation strongly promoted P uptake of the plants (i.e., sum of P content

[3]http://www.R-project.org/

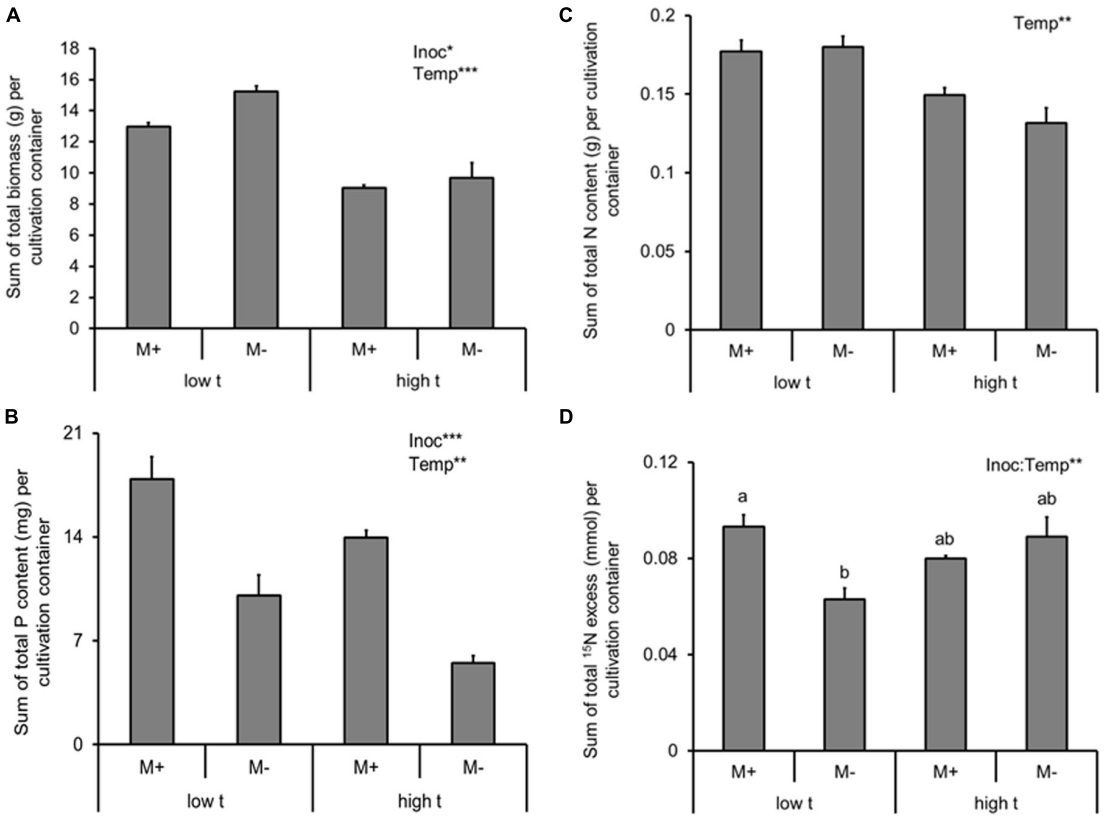

FIGURE 2 | Dry biomass **(A)**, phosphorus (P) content **(B)**, nitrogen (N) content **(C)** of the experimental plants (shoot and roots combined) and ^{15}N excess (i.e., ^{15}N derived from the isotopically labeled clover biomass added to the RFC), **(D)** detected in the plant biomass (shoot and roots combined), summed together for cultivation containers planted with both of the *Panicum* species (mixed cultures) as affected by mycorrhizal inoculation (M+, mycorrhizal inoculum added; M−, non-mycorrhizal control) and cultivation temperature (low t, 25°C during daytime; high t, 36°C during daytime). Mean values ± standard errors are shown ($n = 4$ for M+ high t treatment, $n = 3$ for all the other treatments). Significances of inoculation (Inoc), cultivation temperature (Temp), and their interaction are indicated (factors with $p \geq 0.05$ are not shown, ***$p < 0.001$, **$0.001 \leq p < 0.01$, *$0.01 \leq p < 0.05$). Different letters at the individual bars indicate significant differences between the means at $p < 0.05$.

of the C_3- and the C_4-plants growing side by side in the same cultivation container; Supplementary Table S2 and **Figure 2B**). P uptake of the model plant communities was suppressed by elevated temperature (Supplementary Table S2 and **Figure 2B**), and no significant interaction was recorded between the two factors (Supplementary Table S2).

Arbuscular mycorrhizal fungi inoculation (alone) had no significant effect on plant N acquisition (N content or excess ^{15}N; Supplementary Table S2 and **Figures 2C,D**), but elevated temperature decreased N uptake by the plants (summed together for both plant compartments of each cultivation container; Supplementary Table S2 and **Figure 2C**). Elevated temperature alone had, on the other hand, no significant effect on the excess ^{15}N in the plant biomass (i.e., the amount of N derived from organic fertilizer supplied to the RFC; Supplementary Table S2 and **Figure 2D**). Yet there was a significant interaction between AMF inoculation and temperature with respect to excess ^{15}N (Supplementary Table S2 and **Figure 2D**), with the AMF inoculation positively affecting the ^{15}N uptake by the plants at low t but having no effect at high t; **Figure 2D**).

When analyzing the effects of temperature and AMF inoculation on the total mass of AMF (as per the C16:1ω5 fatty acid content in all belowground compartments, i.e., roots in both of the plant compartments as well as in the substrate filling both of the plant compartments and the RFC), there was a strong effect recorded of both main factors (Supplementary Table S2 and **Figure 3**). Specifically, AMF inoculation strongly elevated the content of the C16:1ω5 fatty acid in the entire cultivation container as compared to the M− treatment, whereas temperature caused almost a three-fold decrease of the fatty acid content in the M+ containers at high t as compared to the M+ containers at low t (**Figure 3**). Only a small, though significant difference was recorded in the C16:1ω5 fatty acid content in the entire container between the M− treatments at low t and high t (**Figure 3**).

Competition for Resources Between the C_3- and the C_4-Plants

To address the competition between the two co-existing plant species, we calculated the fraction of plant biomass, P and N

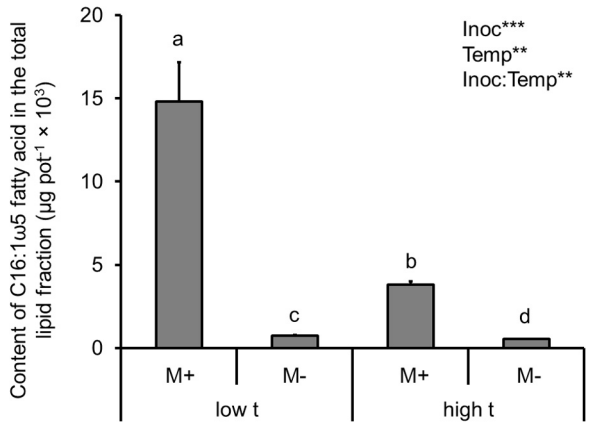

FIGURE 3 | Content of the AMF-specific fatty acid (C16:1ω5) in the total lipids extracted from the root and substrate samples, summed together per cultivation containers planted with both of the *Panicum* species (i.e., roots of both C_3- and C_4-*Panicum*, substrate in both of the plant compartments and the RFC), as affected by mycorrhizal inoculation (M+, mycorrhizal inoculum added; M−, non-mycorrhizal control) and cultivation temperature (low t, 25°C during daytime; high t, 36°C during daytime). Mean values ± standard errors are shown (n = 4 for M+ high t treatment, n = 3 for all the other treatments). Significances of inoculation (Inoc), cultivation temperature (Temp), and their interaction are indicated (factors with $p \geq 0.05$ are not shown, ***$p < 0.001$, **$0.001 \leq p < 0.01$). Bars with different letters above mean significant differences at $p = 0.05$. Different letters at the individual bars indicate significant differences between the means at $p < 0.05$.

yields, as well as the fraction of excess ^{15}N per cultivation container assignable to the C_3 plant (i.e., the share of resources diverted to the C_3 plant on a whole cultivation container basis, with the remaining part of the particular resource being assignable to the C_4 plant).

Based on these analyses, the C_3 plant always lost out in the competition for resources with the C_4 plant at high t (be it with respect to the biomass production, P uptake, N uptake, or excess ^{15}N; Supplementary Table S2 and **Figures 4A–C**). For none of the measured variables there was any effect of AMF inoculation (alone or in interaction with the temperature regime; Supplementary Table S2).

AMF Development

Roots of all M+ plants were highly colonized by the AMF, and the roots of M− plants remained free of AMF structures throughout the entire experiment. On average (± standard error), the hyphae, arbuscules, and vesicles occupied 65 ± 2%, 29 ± 3%, and 4 ± 1% of the root length of the M+ plants, respectively. The extent of root colonization of the C_3-*Panicum* was strongly reduced under elevated temperature, whereas no effect of temperature was recorded on the root colonization levels of the C_4-*Panicum* (Supplementary Table S3 and **Figure 5**).

Although the data on individual AMF taxa abundance in roots and in the potting substrate generated by the qPCR were quite noisy, there were a few cases of a significant reduction of abundance of *Rhizophagus* and *Funneliformis* (the two dominant taxa in our experiment) due to elevated temperature (**Figure 6**). No significant stimulation was observed of abundance of any of the AMF taxa in any of the experimental compartments by elevated temperature (**Figure 6**). Importantly, the abundance of none of the AMF taxa in C_4-plant roots was reduced by elevated temperature, whereas abundance of *Funneliformis* was significantly reduced upon elevated temperature in the C_3-plant roots as compared to ambient temperature (**Figure 6**). *Funneliformis* appeared to be most susceptible to elevated temperature in our experiment, whereas *Claroideoglomus*, *Gigaspora*, and *Acaulospora* (all three being not particularly abundant in our samples, though) did not indicate any change in abundance in any of the measured compartments due to the elevated temperature (**Figure 6**).

The C Source of CMN Interconnecting the C_3 and C_4 Plants

Elevated temperature alone did not affect the isotopic composition of C in the AMF-specific fatty acid in either roots or substrate from the plant compartments, whereas clear effect could be assigned to the photosynthesis type of the plant (C_3 vs. C_4) growing in the respective compartment (Supplementary Table S4). The data were thus pooled across both of the temperature regimes for all further statistical analyses (but displayed separately for low t and high t in **Figure 1**). Due to the constraints detailed above (i.e., no significant amounts of C16:1ω5 fatty acid detected in the RFC of the M+ containers, Supplementary Figure S3), any meaningful calculations could only be made for the RFC from the containers incubated at low t (for raw data, see Supplementary Table S5). Carbon isotopic composition of the C16:1ω5 fatty acid in the C_3-*Panicum* roots carried a clear C_3 imprint (on average across both temperature regimes 17.5 ± 6.2%, with 0% representing a pure C_3-isotopic signature), which was not significantly different ($p = 0.2$, t-test) from the isotopic imprint within the C16:1ω5 fatty acid in the substrate of the C_3 plant compartment (2.5 ± 9.0% across both temperature regimes). For numbers for each of the temperature regimes separately, see **Figure 1**, for raw data, see Supplementary Table S5.

Similarly, the C isotopic composition of the C16:1ω5 fatty acid in the roots and in the substrate of the C_4 plant compartments both carried a clear C_4-isotopic imprint (113.6 ± 12.3% and 81.0 ± 29.5%, respectively, **Figure 1**) and did not differ significantly from each other ($p = 0.1$). There was a significant difference in the C isotopic imprint within the C16:1ω5 fatty acid recorded in the RFC (at low t) and the substrate of the C_3 plant compartment analyzed at both high t and low t ($p < 0.01$), whereas no significant difference was recorded between the substrate beneath the C_4 plants (at both temperature regimes) and the RFC at low t ($p = 0.6$). The difference in C isotopic imprint of the C16:1ω5 fatty acid between the two plant compartments (C_3 vs. C_4) was highly significant ($p < 0.001$), and so it was between the roots of the C_3 and C_4 plants ($p < 0.001$).

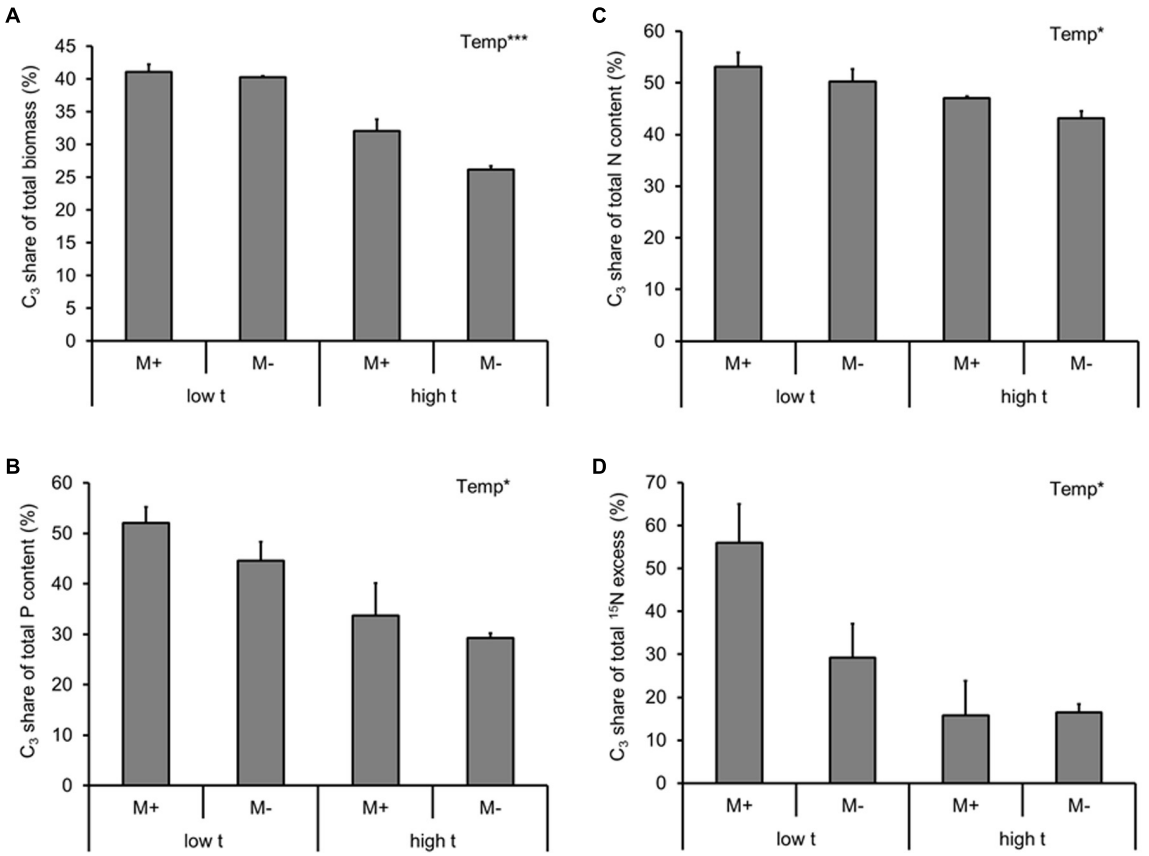

FIGURE 4 | Fraction of dry biomass **(A)**, phosphorus (P) content **(B)**, nitrogen (N) content **(C)** of the experimental plants (shoot and roots combined), and the ^{15}N excess (i.e., ^{15}N derived from the isotopically labeled clover biomass added to the RFC), **(D)** detected in the plant biomass (shoot and roots combined) per cultivation container assignable to the C_3 plant (i.e., the share of resources diverted to the C_3 plant on a whole cultivation container basis, with the remaining part of the particular resource being assignable to the C_4 species) as affected by mycorrhizal inoculation (M+, mycorrhizal inoculum added; M−, non-mycorrhizal control) and cultivation temperature (low t, 25°C during daytime; high t, 36°C during daytime) for the mixed cultures. Mean values ± standard errors are shown ($n = 4$ for M+ high t treatment, $n = 3$ for all the other treatments). Significances of inoculation, cultivation temperature (Temp), and their interaction are indicated (factors with $p \geq 0.05$ are not shown, $^{***}p < 0.001$, $^{*}0.01 \leq p < 0.05$).

DISCUSSION

Mycorrhizal Effects on Plant Biomass and Nutrient Uptake at Different Temperatures

Mycorrhizal inoculation strongly increased P content of the plants (nearly two-fold at low t and even more so under high t, **Figure 2B**), which confirmed that the symbiosis was fully functional under both temperature regimes in terms of P uptake improvement of the plants. Bit surprisingly though (but see also Řezáčová et al., 2017b, 2018), establishment of mycorrhizal symbiosis did not increase biomass production of our experimental plants (actually it did decrease it by about 10–15%, **Figure 2A**), most likely because of C limitation, due to temperature stress (of which the latter was, however, not measured directly, e.g., by assessing molecular stress markers or efficiency of photosynthetic C fixation) or other constraints (see also Johnson et al., 2015 for further discussion). N limitation of growth of the M+ plants due to competition for N between the plants and the AMF and/or their associated bacteria (such as described, e.g., by Saia et al., 2014 or Püschel et al., 2016) is not a likely explanation of the observed growth reduction because the N content of the plants was actually not affected at all by mycorrhiza formation (**Figure 2C**).

Elevated temperature strongly and negatively affected plant biomass, P and N contents of the mixed plant stands (**Figure 2**, most notable was the reduction of M− plant P content by more than 50% due to elevated temperature), eventually resulting in a significant suppression of the C_3 plant growth and nutrient uptake in the mixed culture containers (**Figure 4**). Yet there was no significant interaction between the factors of temperature and inoculation for any of the above parameters, indicating that mycorrhiza did not increase to any significant degree the tolerance (in terms of plant biomass production or mineral nutrition) of their host plants to elevated temperature. This result was quite surprising given other reports showing increased tolerance of the host plants to extreme temperatures due to formation of mycorrhizal symbiosis (e.g., Bunn et al., 2009).

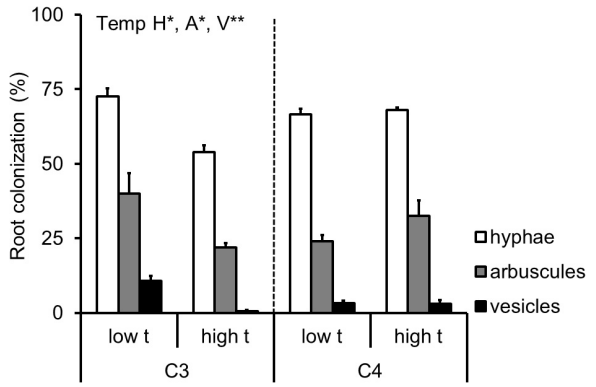

FIGURE 5 | Extent of root length colonized by the mycorrhizal fungal structures of the AMF-inoculated C_3- and C_4-*Panicum* grasses growing side by side in the mixed culture containers. Colonization levels are shown by the hyphae (H), arbuscules (A) and vesicles (V) as assessed microscopically following staining with Trypan blue, separately for the two grass species as affected by cultivation temperature (low t, 25°C during daytime; high t, 36°C during daytime). Mean values ± standard errors are shown (n = 4 for high t treatment and n = 3 for the low t treatment). Significance of temperature treatment is indicated for the C_3 plants only (*$0.05 > p \geq 0.01$, **$0.01 > p \geq 0.001$) because there was no statistically significant effect of temperature recorded for any of the parameters in the C_4 plants.

The biggest surprise was certainly the pattern of plant uptake of ^{15}N from the RFC (**Figure 2D**). Whereas M+ plants obtained significantly more ^{15}N than the M− plants from the organic N source at low t (and nearly significantly, p = 0.07, it was the C_3 plant that was preferentially served by the mycorrhizal networks with the ^{15}N at low t, as compared to M− conditions, **Figure 4D**), there was no significant difference between the M+ and M− plants at high t (**Figure 2D**). These results indicate that the decomposition of organic N amendment followed different trajectories at the different temperatures and that mycorrhiza was an important shaper of the decomposition and/or plant N uptake (consistent with earlier and recent reports by, e.g., Leigh et al., 2009; Hodge and Fitter, 2010; Bukovská et al., 2018) from the organic N source at low t only. At high t, in contrast, it seems that plant uptake of ^{15}N from the organic N source was mostly dominated by passive diffusion of inorganic N species toward the roots rather than N uptake via the mycorrhizal pathway (**Figure 2D**). This notion is also consistent with the lower abundance of AMF biomass in the RFC at high t as compared to low t (be it based on the AMF-signature fatty acid analyses, **Figure 3**, or by means of the qPCR analyses, **Figure 6**). It also seems that N decomposition was faster at high t (consistent with a report by Dessureault-Rompré et al., 2010 and references therein), resulting in nearly equal transfer of ^{15}N (in terms of its mass) to the plants at both temperature, whereas total N uptake actually decreased in response to elevated temperature (**Figure 2**).

Who Was Feeding the CMN at the Different Temperatures?

Based on the facts that C_3 and C_4 plants have very distinct isotopic composition ($\delta^{13}C$ values) of C in their biomass (**Figure 1**) and that these differences extend to the AMF hyphae associated with such host plants (Querejeta et al., 2003; Courty et al., 2015), we could disentangle the C source of the AMF hyphae in our experiment. Specifically, we could address the question, which of the plant species was preferentially feeding the CMN under the different temperature regimes. To this end, we employed stable isotopic analyses of the AMF-signature fatty acid (C16:1ω5) both in plant roots and in the potting substrate of the different compartments of our experimental containers populated with the same or different plant species. This concept had recently also been described in detail by Walder et al. (2013).

To our surprise, we were not able to obtain evidence for preferential feeding of the shared mycorrhizal network by the C_4 plant (at least under high t). Instead, the data showed that there were actually no changes in the pattern of feeding the CMN by one or the other plant species with increasing temperature, nor there was any overall asymmetry in feeding the CMN by the two different plant hosts. Our results thus demonstrate a very balanced investment of the two plants into the CMN and that this investment is not substantially affected by elevated temperature. Our data are thus in direct contrast to previous research showing that one or the other plant dominates the C supply to the CMN (Walder et al., 2012; Wang et al., 2016). The previous experiments had all used phylogenetically more distant plant species pairs, and it has been shown that plant size and/or presence of rhizobia could affect the C inputs into the CMN (Nakano et al., 1999; Wang et al., 2016; Weremijewicz et al., 2016). Because we used plant species closely related phylogenetically and of similar size and growth rates (although not necessarily so under all temperatures), such asymmetry in C feeding of the CMN as seen in previous experiments may not have developed in our case. Further, because for the first time to our knowledge in an experiment addressing C supply to CMN we used a synthetic community of the AMF instead of a single AMF isolate/genotype, the two different plant species could have preferentially associated with different AMF taxa, doing so with unknown consequences for the formation of the CMN. Nonetheless, molecular community analyses indicated that the plants did indeed share the same AMF taxa to a great extent (**Figure 6** and Supplementary Table S5). Further, both *Rhizophagus* and *Funneliformis*, which actually formed dominants of our realized AMF communities (**Figure 6**), are clearly capable of crossing the distances of several centimeters between the two root compartments (Jansa et al., 2005). Somewhat confounding, however, was the relatively low abundance of these dominant AMF taxa in the RFC at high t (particularly surprising was the apparent absence of *Funneliformis* there, **Figure 6**) and the concomitant low detection of the AMF-specific fatty acid in the RFC at high t. This might indicate rather low colonization of the RCF at high t by the AMF hyphae, provided the C16:1ω5 is a good AMF biomass marker at all temperatures (which may not in fact be the case [cf. Sumner et al., 1969]). What would be needed here is a direct proof of functional hyphal interconnection (or lack of it) between the two plants, which is currently not easy to provide. A further confounding factor may be that we actually

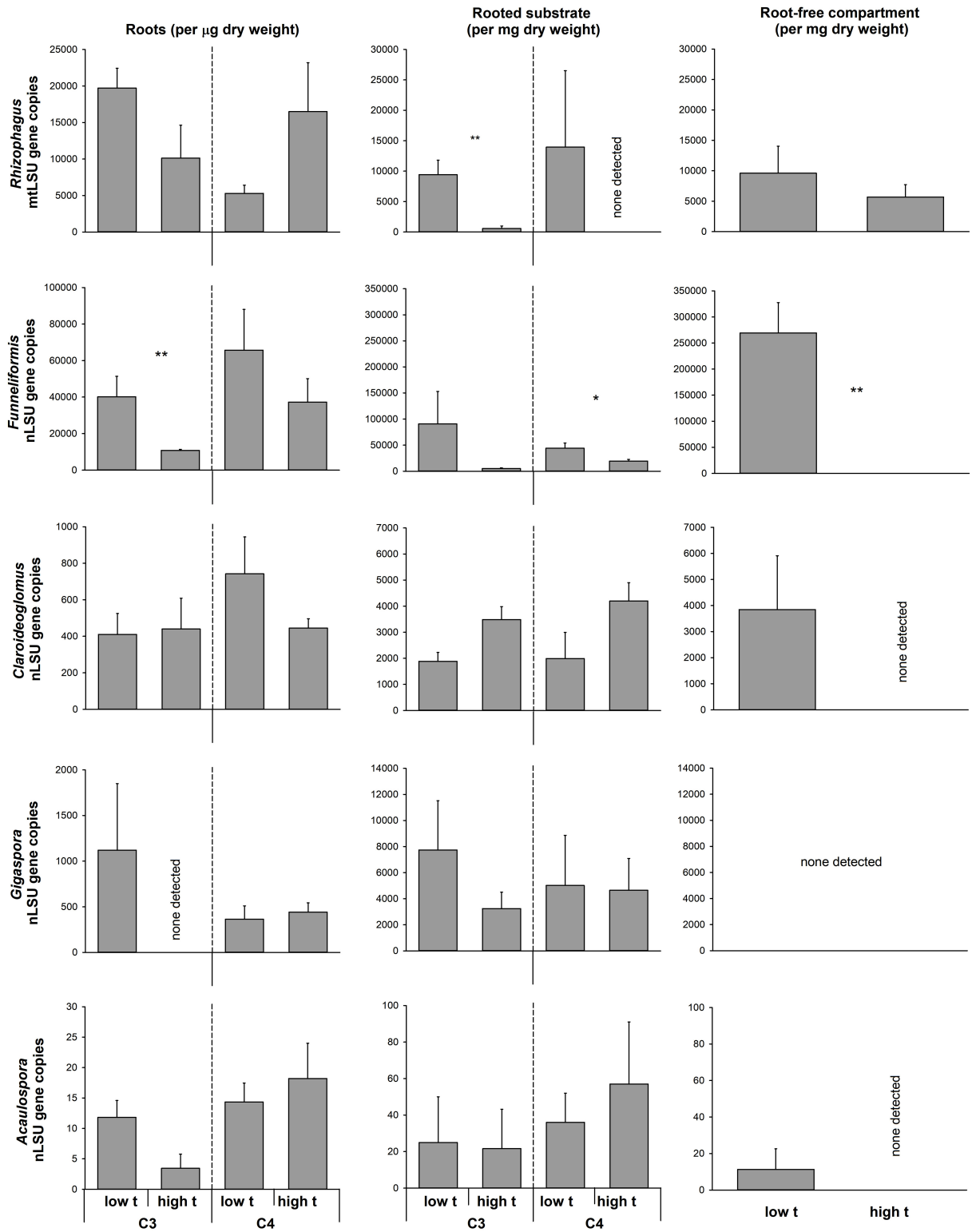

FIGURE 6 | Abundance of the different arbuscular mycorrhizal fungal taxa in the roots and substrate samples from the plant- and the RFCs of the mycorrhiza-inoculated cultivation containers containing both C_3 and C_4 plant species, as assessed by quantitative real-time PCR with taxon-specific markers for mitochondrial or nuclear large ribosomal subunit (mtLSU or nLSU, respectively). Statistically significant differences are shown between the different temperature regimes (low t, 25°C during daytime; high t, 36°C during daytime), separately for the C_3 and for the C_4 plants, if applicable. Mean values ± standard errors are shown (n = 4 for high t treatment and n = 3 for the low t treatment). *$0.05 > p \geq 0.01$, **$0.01 > p \geq 0.001$.

used the substrate collected from the labeling compartment as a proxy for the entire RFC to assess the AMF communities there both by the fatty acid and the qPCR approaches. The labeling compartment was enriched with organic N, but there was still some organic N-unenriched volume in the RFC (**Figure 1**), which we did not sample, however. Uncontrolled development of high t-specific microbial communities is at least theoretically possible in organic N-enriched patches (though the composition of saprotrophic microbes was not experimentally assessed here). And because not only synergistic, but also antagonistic interactions between certain soil microbes and AMF hyphae are known (e.g., Svenningsen et al., 2018), the observed suppression of some, though not all, AMF (**Figure 6**) in the RFC should be interpreted cautiously with respect to the CMN formation between the two side compartments of the cultivation containers.

Mycorrhizal Communities in and Outsides of Roots at the Different Temperatures

Interestingly, we observed a significant decrease in AMF colonization rates (as assessed microscopically) at high t as compared to low t in the roots of the C_3-*Panicum* (**Figure 5**). Because such a decrease was not observed in the C_4-*Panicum*, it seems that the AMF *per se* (at least those in the roots) were not particularly sensitive to elevated temperatures (probably with the only exception of *Funneliformis* in our experiment, **Figure 6**). This would be consistent with previous observations by others, e.g., Bunn et al. (2009), showing little sensitivity of both high temperature-adapted and -unadapted AMF genotypes to elevated temperatures. Further, these results indicate that the C_3-*Panicum* actively suppressed the development of the (dispensable) root symbionts (particularly *Funneliformis*, see **Figure 6**) at high t, where the plants were obviously severely stressed by the elevated temperature and/or presence of the C_4 neighbor (**Figure 4**). On the other hand, it remains to be explained why the root colonization by all of the AMF taxa was retained in the C_4 plants exposed to high t at levels comparable to those under the low t conditions. Most likely the C_4 plants, because of their better adaptation to elevated temperatures, were better able to continue benefitting (nutritionally) from the symbiosis at high t than the C_3-*Panicum* and thus they also returned larger amounts of their C back to their symbionts to maintain the high root colonization levels (Kiers et al., 2011).

In the substrate beneath the plants and particularly in the RFC, suppression of both *Funneliformis* and (occasionally) also of *Rhizophagus* (**Figure 6**) at high t was observed as compared to ambient temperature. This is interesting because these two AMF genera often dominate field AMF communities, particularly in the cropped soils (e.g., Jansa et al., 2014; Davison et al., 2015) and can confer great nutritional benefits to their host plants (Jansa et al., 2005, 2008). Reduction of their hyphal development at high t would possibly explain the lower amounts of AMF-specific fatty acid at high t detected in the experimental containers as compared to low t (**Figure 3**). We know from another experiment carried under ambient temperature that at least *Rhizophagus* hyphal development should be stimulated by the clover biomass added to the RFC (Bukovská et al., 2018). Indeed, both of the dominant AMF (*Funneliformis* and *Rhizophagus*) and at least two of the subdominants (*Claroideoglomus* and *Acaulospora*) developed decently in the RFC at low t, whereas *Gigaspora* was possibly not able to grow through the root-exclusion meshes (consistent with previous reports by Smith et al., 2004). Why the AMF except *Rhizophagus* actually disappeared from the RFC at high t (qPCR detection limit permitting, Thonar et al., 2012) remains unclear – but see discussion on the possible antagonistic biotic interactions above. It should also be tested in the future whether the capacity of the AMF to penetrate nylon meshes (which is generally very low for *Gigaspora* for instance) would be more restricted for the other AMF at high t than at low t (as our results suggest). It should also be tested whether hyphal development at large distance is restricted more at high t than at low t, e.g., due to larger metabolic costs of hyphal maintenance (Heinemeyer et al., 2006) or due to more severe antagonistic interactions with other soil biota (Svenningsen et al., 2018).

CONCLUSION

With respect to ongoing global changes and because CMN may reportedly mitigate at least some of the stresses experienced by their host plants (Lenoir et al., 2016; Millar and Bennett, 2016), it may become increasingly important to study the widespread effect of CMN on nutrient and C flows between plants and soil in complex plant and AMF communities subjected to various environmental conditions (Fitter et al., 2000; Heinemeyer et al., 2006). Not always the AMF help withstand stress (e.g., Pollastri et al., 2018), but sometimes (like here) at least the mineral nutrition of plants is improved upon symbiosis with AMF, if not the biomass production. Although we identified a number of technical issues that need further attention such as the need of direct proof regarding efficient formation of CMN, or the stability of C16:1ω5 synthesis under different temperatures, we clearly confirmed suitability of the AMF-signature fatty acid-based approach for tracking AMF C source to address the dynamics of C gains by the CMN supported by both C_3 and C_4 plants (first described by Walder et al., 2012). We also showed that extraradical AMF hyphae seem to be more susceptible to high temperatures than the AMF biomass in the roots. Our *Funneliformis* isolate (one of the dominant AMF in our experiment) was particularly strongly affected by elevated temperature, seemingly more than our *Rhizophagus* isolate or the other (subdominant) AMF genera – a path of research that certainly deserves more attention in the future. Particular attention should also be dedicated in the future to better description of the rates of organic N decomposition at different temperatures and how these (directly or interactively with other factors) affect the mycorrhizal N uptake pathway and nutrient-for-C trades at the symbiotic interface (sensu Fellbaum et al., 2014). Last but not least, more C_3–C_4 congeneric pairs should be employed as model hosts in similarly designed experiments as ours to allow generalizations of the results to a broader range of plant taxa (see Řezáčová et al., 2018 for further discussion).

AUTHOR CONTRIBUTIONS

JJ, LZ, OB, DP, TK, MH, and RS planned, designed, and carried out the experiments, including the different physicochemical and molecular analyses. VŘ and JJ analyzed the data and wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00449/full#supplementary-material

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Salicaceae Endophytes Modulate Stomatal Behavior and Increase Water Use Efficiency in Rice

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Bacterial and yeast endophytes isolated from the Salicaceae family have been shown to promote growth and alleviate stress in plants from different taxa. To determine the physiological pathways through which endophytes affect plant water relations, we investigated leaf water potential, whole-plant water use, and stomatal responses of rice plants to Salicaceae endophyte inoculation under CO_2 enrichment and water deficit. Daytime stomatal conductance and stomatal density were lower in inoculated plants compared to controls. Leaf ABA concentrations increased with endophyte inoculation. As a result, transpirational water use decreased significantly with endophyte inoculation while biomass did not change or slightly increased. This response led to a significant increase in cumulative water use efficiency at harvest. Different endophyte strains produced the same results in host plant water relations and stomatal responses. These stomatal responses were also observed under elevated CO_2 conditions, and the increase in water use efficiency was more pronounced under water deficit conditions. The effect on water use efficiency was positively correlated with daily light integrals across different experiments. Our results provide insights on the physiological mechanisms of plant-endophyte interactions involving plant water relations and stomatal functions.

Keywords: endophytes, rice, stomatal conductance, water potential, water relations, water use efficiency, water deficits, ABA

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INTRODUCTION

Climate change has become a great challenge in agriculture by reducing potential yield of crops as environmental stresses on crops increase (Cai et al., 2015). Growing human population will reach 9.6 billion by 2050 (United Nations Department of Economic and Social Affairs, 2015), which ominously implies the demand for crop production will concurrently increase substantially. As a result, two central themes to food security must be addressed: (1) how to increase crop plant resiliency with dynamically changing environmental conditions, and (2) how to improve crop yield with more sustainable methods that possibly lessen the burden of chemical and irrigation inputs used for both fertilizers and environment management (Flexas, 2016).

Amongst all of the resources in the agricultural industry, field irrigation water usage draws the most attention of scientists and the public alike. Currently, 53% of cereal production is met by irrigation. If this trend continues, agriculture will remain as the biggest player in draining freshwater globally by 2050 (Rosegrant et al., 2009). Moreover, considering the fact that climate change brings unstable precipitation, more frequent runoffs, and weather extremes such as the 2012–2014 drought in California, United States (Griffin and Anchukaitis, 2014), it will necessitate more efficient, innovative approaches to water use.

Recent efforts in achieving higher crop sustainability involve increasing plant water use efficiency (WUE), including but not limited to, genetic manipulation of plants to form less stomata (Franks et al., 2015), selection of drought tolerant genotypes through fast screening methods (Condon et al., 2004), and alteration of canopy structure to maximize light acceptance (Drewry et al., 2014). Nevertheless, these techniques are not readily available in the field, still under testing, or maybe unrealistic for larger scale applications.

Endophytes are microorganisms living in the intercellular spaces of plants, providing several benefits to hosts, in turn, receiving carbohydrate-based nutrients for their growth (Dobereiner, 1992). These are mutualistic symbionts that can enhance plant fitness and performance in terms of increasing host biomass under stressful conditions (Goh et al., 2013). For example, endophytes that confer water stress tolerance are well studied (Rho et al., 2018) and a number of research articles show their efficacy in plant fitness and plasticity under low water availability (Khan A.L. et al., 2015; Khan et al., 2016; Gagné-Bourque et al., 2016). Thus, a realistic aim is to supplant other current agriculture methods with endophytic symbioses to increase crop WUE and yields.

Doty et al. (2009) isolated diazotrophic – di-nitrogen fixing – endophytic bacteria and yeast from the Salicaceae family of plants – native poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) growing in primary substrate of natural riparian zones. In this environment the nutrient supply to the plants was severely limited due to frequent flooding. The isolates were characterized in the paper and subsequent publications clearly demonstrated their potential symbiotic traits in other host species across taxa including grass species such as rice (Khan et al., 2012; Knoth et al., 2013, 2014). A major goal of our past and current studies has been to test the effectiveness of the endophytes from Salicaceae hosts on improving the growth and mitigating stress of host plants from other taxa, especially crops. Since then, we have been using these isolates to find out their potential benefits in other agricultural crops and to explore their mechanistic level impacts. In line with these efforts, our recent publication, Kandel et al. (2015), demonstrated that this endophyte consortium significantly increased biomass of the same rice variety, leading to the increases in yield potential. However, Kandel et al. (2015) did not provide information on the physiological benefits and their underlying mechanisms. In this context, the present study delivers a mechanistic view of having endophyte symbiosis focusing on water relations of rice as a host.

Previous results have provided evidence for prospective impacts of the select endophytes on plant functional traits. The select endophytes were shown to have multiple potentially symbiotic traits including phytohormone production (indole-3-acetic acid in Xin et al., 2009), biological nitrogen fixation (Knoth et al., 2014; Doty et al., 2016), and cross-host biological nitrogen fixation capacity (Knoth et al., 2013; Kandel et al., 2015). In a recent study (Khan et al., 2016), it was demonstrated *in vitro* with liquid chromatography that select endophytic bacterial and yeast strains produce plant hormones, including abscisic acid (ABA) – a key player in stomatal control and development. In the article, the authors inoculated poplar cuttings to test stomatal response and photochemical efficiency under water deficit conditions, examining daytime stomatal conductance (g_s) and chlorophyll fluorescence (F_v/F_m). The results showed significant decreases in g_s over time and higher F_v/F_m in inoculated cuttings compared to controls. Multiple possible mechanisms based on microbial assays and genomic analysis were provided. Based on the findings of endophytic ABA production, we postulate that these symbionts can trigger stomatal closure and affect stomatal development of host plants, as potential mechanisms for the observed drought tolerance.

Rice (*Oryza sativa*) is currently the second most important staple crop worldwide following maize (FAOSTAT, 2015). Since rice is suggested to be an isohydric species (Parent et al., 2010), it is likely to be sensitive to daytime water demand as increasing transpiration rate aligns with rising air temperature and light intensity – both leading to an increase in vapor pressure deficits (VPD) between the air and leaf surface – during the daytime. The control of losing and blocking water vapor exchange on the surface of leaves is important for managing the resource as well as operating photosynthetic machinery by absorbing enough amount of CO_2 from the ambient air. Consequently, isohydric species have developed tighter governing of stomatal controls together with stable water potential adjustment to endure losing water during daytime, when gas exchanges of leaves occur most actively, and to survive in unfavorable water conditions (Tardieu and Davies, 1992).

Nonetheless, there are contradictory results about endophyte effects on stomatal control over numerous host plant species (Malinowski and Belesky, 2000). Some reported they facilitated the stomatal closure to conserve water inside, while others reported the opposite results; they assisted the hosts with opening stomata to absorb more CO_2 from the atmosphere to photosynthesize and eventually leading to more biomass gain. However, since the experiments were conducted in different contexts with unquestionably various combinations between the hosts and symbionts, clear conclusions cannot be drawn.

To date, multiple studies examined the impacts of endophytes on whole plant physiology related to water relations and leaf gas exchange (Richardson et al., 1993; Elmi and West, 1995; Morse et al., 2002; Rogers et al., 2012), but their complete mechanisms matching with inoculated endophytes' characteristics have not yet been demonstrated. To provide a comprehensive understanding of endophyte effects on host plant water relations, we conducted a series of greenhouse experiments to examine endophyte effects on physiological attributes of rice water relations: Stomatal responses, water potential, water use and biomass gain of rice plants upon endophyte inoculation. In this study, we tested

hypotheses: (1) the ABA-producing endophytes reduce diurnal g_s leading to reduced total water use and improved WUE, (2) the inoculation effects on plant water relations differ between different endophyte strains, and (3) the effect size of endophyte inoculation varies with environmental conditions such as water deficits, elevated CO_2, and light levels.

MATERIALS AND METHODS

Preparation of microbial materials for the four independent experiments shared the same protocols. All four experiments were conducted in a greenhouse in the Douglas Research Conservatory at the University of Washington (47°39′27″N, 122°17′21″W; 10 m elevation), Seattle, WA, United States. Details about the experimental settings are provided in **Table 1**.

Origins of Endophytes and Preparation for Inoculation

Nine different strains of diazotrophic endophytic bacteria and yeast were used in this study whose *in vitro* characteristics were identified and reported previously by Doty et al. (2009). WP1, WP5, WP9, WP19, WPB, WW5, WW6, and WW7C were isolated from wild black cottonwood (*P. trichocarpa*) and wild willow trees (*S. sitchensis*) at their native habitat, the Snoqualmie River, Western Washington, whereas PTD1 was isolated from hybrid poplar (*P. trichocarpa* × *P. deltoides)* (Doty et al., 2005). Details about the endophyte strains are provided in **Table 2**. All of these endophyte strains have been shown to be potentially diazotrophic by having positive amplification of *nifH* marker gene for nitrogenase reductase (Doty et al., 2009; Knoth et al., 2013). Their positive effects on biomass increase of different host plants were also demonstrated by several articles in consequent studies (Khan et al., 2012, 2016; Knoth et al., 2014; Kandel et al., 2015; Khan Z. et al., 2015). In addition, their colonization efficiency on host plants was established and reported in our previous work (Knoth et al., 2013; Kandel et al., 2015, 2017). We used the same inoculation method that was developed from the prior studies.

The selected endophytes were grown on N-limited combined carbon medium (NL-CCM, Rennie, 1981) for growth of endophytes to maintain their nitrogen fixation ability. All of them grew well on the media, visually identified after 48-h growth, and then cell suspension cultures were started in flasks containing NL-CCM broth. Three to five days later, optical density of the bacterial culture was measured using a spectrophotometer (UV-1700, Shimazu America Inc., Columbia, MD, United States). The final concentration of the bacterial solution for inoculation was adjusted to $OD_{600} = 0.1$ (equivalent to approximately 1×10^7 cells) using sterile deionized water and N-free liquid media (Doty et al., 2009). A mock-inoculum for the control group was prepared just with the N-free liquid media. All microbiological

TABLE 1 | Experiment designs in this study.

Category	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Growing season	Autumn to Spring (10/15/2014 – 3/28/2015)	Spring to Summer (3/21/2014 – 9/2/2014)	Summer to Autumn (7/14/2015 – 10/6/2015)	Spring to Summer (3/23/2017 – 7/20/2017)
Growing duration	158 days	165 days	84 days	119 days
Endophyte strains used	PTD1/WP5/WPB	WP5	*nifH* MIX (see **Table 2** for details)	*nifH* MIX (see **Table 2** for details)
Experimental settings	4 (CTRL/PTD1/WP5/WPB)	2 × 2 (CO2 × INOC)	2 × 2 (DRT × INOC)	2 (INOC)
Experimental environments	Greenhouse benches	Sunlit growing chambers	Greenhouse benches	Sunlit growing chambers
Nitrogen fertilization	1/4X N	1/4X N	1X N	1/4X N
Average RH (day/night)	48/54%	60/66%	57/71%	57/59%
Average air temperature (day/night)	22/19°C	23/19°C	29/20°C	21/17°C
Average instantaneous light intensity	174.0 μmol m^{-2} s^{-1}	176.3 μmol m^{-2} s^{-1}	313.7 μmol m^{-2} s^{-1}	201.7 μmol m^{-2} s^{-1}
Average daily light integral	9.5 mol m^{-2} d^{-1}	9.1 mol m^{-2} d^{-1}	35.8 mol m^{-2} d^{-1}	10.8 mol m^{-2} d^{-1}

TABLE 2 | List of the endophyte strains used in this study.

Endophyte	Closest rRNA Match	Source	Reference	Experiment
PTD1	*Rhizobium* sp.	Hybrid poplar (*Populus trichocarpa* × *P. deltoids)*	Doty et al., 2005	1, 3, and 4
WPB	*Burkholderia* sp.	Wild poplar (*P. trichocarpa*)	Doty et al., 2009	1, 3, and 4
WP1	*Rhodotorula* sp.	Wild poplar (*P. trichocarpa*)	Khan et al., 2012	3 and 4
WP5	*Rahnella* sp.	Wild poplar (*P. trichocarpa*)	Doty et al., 2009	1, 2, 3, and 4
WP9	*Burkholderia* sp.	Wild poplar (*P. trichocarpa*)	Doty et al., 2009	3 and 4
WP19	*Acinetobacter* sp.	Wild poplar (*P. trichocarpa*)	Doty et al., 2009	3 and 4
WW5	*Sphingomonas* sp.	Wild willow (*Sitka sitchensis*)	Doty et al., 2009	3 and 4
WW6	*Pseudomonas* sp.	Wild willow (*S. sitchensis*)	Doty et al., 2009	3 and 4
WW7C	*Curtobacterium* sp.	Wild willow (*S. sitchensis*)	Doty et al., 2009	3 and 4

The rRNA gene of each strain was compared with the BLAST NCBI database and identified as the closest match (Doty et al., 2009).

tasks were done in a sterile condition using proper aseptic sterilization techniques.

Experiment 1: A Greenhouse Study Using Multiple Endophyte Strains

Preparation of Plant and Microbial Materials

Three bacteria strains were used in this greenhouse study. WP5, WPB, and PTD1 were selected to compare their effects on water relations of the host plants with emphasis on stomatal behaviors and leaf water potential components. Experiment 1 was conducted from October 15th 2014 to March 28th 2015.

We used a very early to early maturing, semi-dwarf, Japonica variety M-206 rice (*O. sativa*) that was identified as the best responding rice variety to the endophyte inoculation in a previous study (Kandel et al., 2015). Rice seeds were surface-sterilized by imbibing them with 3% NaOCl for 4 h to remove any debris and contaminants on the seed coat. The seeds were rinsed with sterilized deionized water for four times to wash out remaining NaOCl. Although this surface sterilization technique does not guarantee the removal of all microbes in the seeds due to possible internal/inherent microbiome inside (Nelson, 2017), it is a widely used procedure to eliminate at least the ones on the surface. Thus, any differences in responses from the plants can be statistically interpreted as the effects of endophyte inoculation treatments.

The surface sterilized seeds were planted in 1-gal pots placed into plastic buckets. Horticultural root media (Sunshine Mix #2, Sun Gro Horticulture, Agawam, MA, United States) were used to grow the plants. There were four treatments groups, three with single strain inoculations (WP5/WPB/PTD1) and a mock-inoculated control (CTRL) with ten replicates in each treatment group. As such, the total number of pots was forty, and eight plants were planted in a pot. The subjects were placed in a greenhouse bench space where the air temperature and relative humidity (RH) are automatically controlled. To account for the potential environmental gradient along a greenhouse bench, we used a randomized complete block design with blocks placed across the bench. To further minimize location effects of the greenhouse, the position of pots within a block was reset every week. The average air temperature of the greenhouse environment during the experiment was 22/19°C, 16/8 h day/night supplemented with high pressure sodium light (400 W single phase bulbs, Phillips Electronics North America Corp., Andover, MA, United States) to compensate for the lack of sufficient sunlight during the winter-time. The average daily light integral (DLI) was 9.5 mol m^{-2} d^{-1} of photosynthetically active radiation (PAR). The air temperature and light intensity were recorded in pendant type data loggers (UA-002-08, Onset Computer Corporation, Bourne, MA, United States) at 30-min intervals during the experiment period. Relative humidity was 48/54% day/night in average (**Table 1**).

After inoculation, plants were given 200 mL of a N-free liquid nutrient solution adjusted at the quarter-strength modified Hoagland solution (Hoagland and Arnon, 1950). Deionized water was fully given until the water filled up to a 10-cm mark of the buckets to simulate the field growing conditions of paddy soil growing rice. The amount of water and fertilizer supplied was recorded weekly.

Gas Exchange Measurements

We refer to Moldenhauer et al. (2013)'s classification to specify the growth and development stages of rice plants hereafter.

Gas exchange measurements were taken three times during the experiment on day 58 (V3-4), 118 (V5), and 153 (R1-2) using portable photosynthesis systems equipped with IRGAs (LI6400XT, Li-Cor, Inc., Lincoln, NE, United States). The measurements were taken between 12 pm and 5 pm each time. 2-cm^2 leaf chamber fluorometers (6400-40, Li-Cor, Inc.) were set to measure photosynthetic assimilation rate (A), g_s, and real-time intrinsic WUE (A/g_s). Settings of the sensor heads were 1500 μmol m^{-2} s^{-1} PFD for saturating light intensity, 400 ppm of CO_2 concentration in the reference cell of the instruments, 25°C block temperature, 300 μmol s^{-1} flow rate, and 40–70% RH to optimize the microclimate for photosynthesis during the measurements. Before the measurements, a minimum of 3 min was given for the response time of the leaf samples.

Stomatal Conductance Measurements

Hundred and sixty-three days after germination, at about R3-4 stage of their growth, the diurnal changes of g_s were measured at 3-h intervals from 9 am to 6 pm using steady state leaf porometers (SC-1, Decagon Devices, Inc., Pullman, WA, United States). The instruments were calibrated on site before the first measurements taken at 9 am.

Leaf Water Potential Measurements

One plant per pot was randomly selected to measure destructive leaf water potential to analyze water relations of the hosts that may be influenced by the microorganisms. A pressure bomb (Model-1000, PMS, Albany, OR, United States) was used to measure midday (12 pm to 1 pm) leaf water potential of the samples. The second or third youngest leaf from the top was chosen and immediately after the readings, the rest of the plants were harvested for taking measurements of solute potential of plant extracts. This approach was used to parse out osmotic potentials for the entire leaf, assuming homogenized whole plant solute potential is the same as the leaf solute potential. The harvested leaves were transported to a −80°C deep freezer for breaking the cell walls to mix apoplastic and symplastic solutions. The frozen samples were loaded and sapped in a hydraulic plant sap press (Plant Sap Press #2720, Spectrum Technologies, Inc., Aurora, IL, United States). Four hundred microliter of the extracted plant solution was collected in sample cups of a thermocouple psychrometer (SC-10, Decagon Devices, Inc.) to measure osmotic potential of the solution. Soluble sugar content (SSC) of the remainders was assessed by a handheld refractometer (RHB-10/ATC, Horiba, Japan).

Calculation of Stomatal Density

Specimens for evaluating influences of the bacteria on stomatal development of the hosts were collected using a common stomatal imprint technique (Gay and Hurd, 1975). The sampling was done 2 days after g_s was measured on day 165. The imprints were collected from the abaxial side of the leaves using nail

polish. We counted stomata to calculate stomata density of the specimen. Three field of views were observed and the variables were counted for each sample. The triplicated observed variables were averaged to calculate the parameters. ImageJ program (Schneider et al., 2012) was used with an add-on package to count the numbers of stomata and epidermal cells of the specimen with 40X magnification from a standard compound microscope.

Verification of Colonization

General colonization characteristics of the endophytes and efficacy of the inoculation method used in the study are fully documented in Kandel (2016). Also, an extended review can be found in Kandel et al. (2017).

One rice plant per pot was harvested 99 days after germination to verify colonization of the endophytes. Leaf, stem, and root tissues were separately harvested and the surfaces of the tissues were sterilized by submerging them in 3% NaOCl for 3 min for leaf and stem tissues and 8 min for root tissues, followed by rinsing with sterilized deionized water four times to remove any remnants of NaOCl. The final rinsing water from each sample was collected to confirm the efficacy of the surface sterilization process. Approximately 100 mg of the sterilized tissues were transferred into 1.5-mL microtubes containing 400 μL NL-CCM solution and then they were ground with sterilized microtube pestles. The extracts were diluted into 10^{-4} using an aseptic serial dilution. The diluted solution was plated onto NL-CCM containing petri-dishes. After 48 h of incubation at room temperature, photos of the plates were taken on a photo stand. The photos were downloaded to further process colony forming unit (CFU) count. CFUs were counted with ImageJ program to compare the bacterial counts in the colonized tissues among the treatment groups. Total CFU count from the leaf, stem, root tissues combined was marginally higher for the inoculated plants compared to the control plants ($P = 0.085$).

Experiment 2: A CO_2 Enrichment Study Using a Single Endophyte Strain

A CO_2 enrichment study was designed and conducted to test stomatal responses affected by endophytes under two different atmospheric CO_2 concentration.

Preparation of Plant Materials

Experiment 2 was conducted from March 21st 2014 to September 2nd 2014. For each pot, surface sterilized four M-206 rice seeds were sown in a 3-gal plastic pot containing the same horticultural root media (Sunshine Mix #2, Sun Gro Horticulture) that was fully irrigated with tap water after seeding. Before placement in closed top chambers, the pots were nested in 4-gal plastic pails for easier measurements of fertilizer and water to be supplied. The watering and fertilizing were done through the gaps between the pots and the pails using a plastic funnel. The amount of water and fertilizer supplied was recorded weekly. A total of 32 pots were prepared; eight pots per chamber randomly assigned to receive control (E−) or endophyte (E+) treatments.

CO_2 Treatment and Inoculation

The experiment was a 2 × 2 factorial with eight replications; two levels of atmospheric CO_2 concentration – ambient CO_2 (AMB, approximately targeted to 400 ppm) and elevated CO_2 (ELE, app. 800 ppm) – and two levels of inoculation status – mock-inoculated control with surface sterilized seeds (E−) and diazotroph endophyte-inoculated treatment group (E+). Due to the nature of the CO_2 treatment with a chamber, a split plot design was applied to deploy the pots in two sets of chambers. For the detailed environmental controls and specifications of the chambers, refer to Kinmonth-Schultz and Kim (2011) and Nackley et al. (2016).

A total of four chambers were used in the experiment. Two were AMB chambers with ambient air supplied through air ducts from outside of the greenhouse and the other two were set to ELE chambers with pure CO_2 supplied through air tubing, of which concentration was controlled by flowmeters. Accordingly, a single chamber accommodated both four E− and four E+ plant pots; eight pots, plus a pot without plant samples for monitoring the amount of weekly evaporation from the soil surface by the airflow of the chambers.

The air temperature, RH, light intensity, and CO_2 concentration were monitored and recorded in a data logger (CR1000, Campbell Scientific, Logan, UT, United States). The average temperature during the experiment was 23/19°C and RH of the air in the chambers was 60/66% day/night. The light regime of the greenhouse was set to 16/8 h day/night (7 am – 9 pm as a photoperiod) and the average DLI from the sunshine and the supplementary lighting of the facility was 9.1 mol m^{-2} d^{-1} of PAR. The average atmospheric CO_2 concentrations of the two AMB chambers and the other two ELE chambers were 437/886 ppm, respectively. See **Table 1** for more details about the environmental settings.

In this experiment, one reference strain, WP5, was used. Seven days after sowing when 95% of the seeds were germinated, 2 mL of the prepared WP5 inoculum were added to each seedling. We pipetted the inoculum to the crown of the rice seedlings for the roots to easily absorb the solution. The other half of the plants were mock-inoculated with the same volume of the mock-inoculum for setting the control group.

As in Experiment 1, 200 mL of a quarter strength nitrogen with the nitrogen free medium was supplied and the plants were fully irrigated through the plastic pail to prevent any stress responses from water deficit.

Stomatal Conductance Measurements

At 128 days after germination around R3-4 stage of their growth, g_s of the youngest fully expanded leaves was measured at 3-h intervals to examine diurnal changes of the parameter using steady state leaf porometers (SC-1, Decagon Devices, Inc.). We applied the same measurement procedure described in Experiment 1.

Experiment 3: A Water Deficit Study Using Endophyte Consortia

The third experiment was designed to test the endophyte effects on long-term WUE under both well-watered and water

deficit conditions. The trial was conducted from July 14th to October 6th in 2015. The same plant material, M-206 rice, was prepared and used in this trial. The differences from the previous experiments were the endophyte treatment and the fertilization and irrigation conditions outlined below. Measured metrics were weekly pot-based transpiration and biomass allocation at harvest.

Preparation of Plant Materials and Inoculation/Water Deficit Treatments

The average air temperature recorded in data loggers (UA-002-08, Onset Computer Corporation) was 29/20°C in day/night. The average DLI was 35.8 mol m^{-2} d^{-1} of PAR with a 16/8 h day/night photoperiod of supplemental lighting. The air temperature and light intensity were recorded every 15 min. RH was 57/71% day/night in average (**Table 1**).

After the seed surface sterilization, four rice seeds were placed into 1-gal pots containing horticultural root media (Sunshine Mix #4, Sun Gro Horticulture). A total of 32 pots were prepared and placed into the same number of plastic buckets. Seven days after the germination, we inoculated the half of the samples with the prepared consortium of endophytes (**Table 2**). Targeted OD_{600} per strain was approximately 0.011, and so OD_{600} of the consortium of the selected nine strains was 0.1. Each plant received 2-mL of the consortium inoculum (MIX) using the inoculation technique outlined above. The other half was given the same volume of the mock-inoculum consisted of a N-free solution (CTRL).

Like Experiment 1, a randomized complete block design was applied to assign the pots on a bench in the greenhouse. Six pots without plants were located in the middle of the experimental design to collect weekly evaporation rate of the soil, and then the rate was used to calculate weekly transpiration during the experimental period. The total volumes of the water in the pots with plants were subtracted by the averaged volume of the water in the pots without plants every week to calculate weekly transpiration. Every week, 200 mL of the aforementioned fertilizer adjusted at a full strength nitrogen level of Hoagland was supplied and the pots were fully irrigated to 15-cm marks on the side of the buckets.

Six weeks after the germination, half of the pots were subjected to water deficit conditions. We stopped the weekly irrigation for these half (water deficit stressed, S), whereas continued full irrigation for the others (non-stressed, NS). From this point on, there were four treatment groups in the design: non-stressed control (NS_CTRL), non-stressed inoculated (NS_MIX), stressed control (S_CTRL), and stressed inoculated (S_MIX) with eight replications per each. To monitor the soil water status after the water deficit treatment, the soil water potential was measured by using a thermocouple psychrometer (SC-10, Decagon Devices, Inc.).

Water Use Efficiency Calculation

Weekly transpiration was recorded throughout the experimental period until the plants harvested after 4 weeks of induced water deficits. Total transpiration was calculated by adding up the weekly transpiration at harvest. Measured dry weights were divided by total transpiration after harvesting and 72-h drying at 70°C. The pot-based total transpiration and the measured dry weights were used to calculate long-term WUE.

$$\text{WUE of productivity} = \text{total biomass gain (g)} / \text{total transpiration (L/pot)}$$

Experiment 4: A Growth Chamber Study Using Endophyte Consortia

To provide support for a mechanistic understanding of the stomatal conductance responses, we repeated Experiment 2 in the previously used growth chamber of the same greenhouse facility from March 23rd 2017 to July 20th 2017. The objective of this experiment was to test the hypothesis that ABA produced endophyte consortia would increase *in vivo* ABA concentrations of the host plants.

Preparation of Plant Materials and Inoculation

For preparing the plant samples, the protocols from Experiment 2 were used. For preparing the microbial samples and inoculation process, the protocols from Experiment 3 were used. In short, a total of 32 rice plants were grown in 3-gal pots in four sunlit growing chambers. The half of the surface sterilized plants were inoculated by the *nifH* endophyte consortium 7 days after germination. They were grown under well-watered and nitrogen limited conditions until measurements and sampling taken over 104 days. The average air temperature recorded in a data logger (CR1000, Campbell Scientific) was 21/17°C in day/night. The average DLI was 10.8 mol m^{-2} d^{-1} of PAR with a 16/8 h day/night photoperiod of supplemental lighting. The air temperature and light intensity were recorded every 15 min. RH was 57/59% day/night in average. Further experimental details can be found in **Table 1**.

Stomatal Conductance Measurements

At 66 days after germination around V6-7 stage of their growth, g_s of the youngest fully expanded leaves was measured at 12 and 6 pm to examine diurnal changes of the parameter using steady state leaf porometers (SC-1, Decagon Devices, Inc.). We applied the same measurement procedure described in Experiments 1 and 2.

In Vivo ABA Assay

Diurnal *in vivo* ABA content was determined biochemically using the Phytodetek enzyme-linked immunosorbent assay (ELISA) kit (PDK 09347/0096, Agdia, Elkhardt, IN, United States). At 96 days after germination around R3-4 stage, the rice leaf samples were harvested at 12 pm and 6 pm. The fully expanded youngest leaves were immediately submerged into liquid nitrogen in centrifuge tubes. They were frozen and stored at −80°C until further analysis. The samples were ground into fine powder and approximately 100 mg of the powder of each was transferred into a microtube. ABA was extracted using 1 mL of 80% methanol at 4°C overnight. On the following day, the mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was collected in a new microtube. The pellet was resuspended and 1 mL of fresh 80% methanol was used to repeat the extraction process at 4°C overnight. Again, by

centrifuging the mixture at 10,000 rpm for 5 min and the supernatant was combined with the extracts from the previous day. The pooled supernatant was dried down using a vacuum concentrator until approximately 50 μL of liquid remained. Then, TBS buffer (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 3 mM NaN_3) was added up to a final volume of 500 μL of the extract. The buffered extract was then diluted 10-fold in TBS buffer. The diluted sample was used to further detect ABA, following the Phytodetek ELISA assay kit manual. The ABA concentrations were measured using a multichannel spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, United States). Each sample (CTRL/MIX) at each time point was replicated eight times.

Statistical Analysis

All physiology parameters measured in Experiments 1 through 4 were analyzed with R version 3.2.2 (R Development Core Team, 2015). For Experiment 1, the measures were subjected to contrast matrix to compare control vs. three single strain inoculated plants with blocking effect included in the model. For Experiment 2, the variables recorded were tested for the effects of a split-plotted CO_2 treatment with a proper statistical analysis. The chamber effect on variation was found not to be significant $\alpha = 0.05$ level. Consequently, two-way ANOVA was implemented to the variables corresponding to a two-way factorial design. Experiment 3 was designed with a 2×2 factorial structure with blocking effects on the experimental plot. Two-way ANOVA was applied to analyze the response variables. The data from Experiment 4 were analyzed using a simple *t*-test procedure at each time point to see significant differences between control vs. inoculated plants. The numbers of replication for Experiments 1, 2, 3, and 4 were eight, ten, eight, and eight, respectively.

RESULTS

Experiment 1: Greenhouse Study Using Multiple Endophyte Strains

Stomatal conductance (g_s) decreased during the daytime in E+ plants with multiple strains of the bacteria (PTD1/WP5/WPB). An average 27% decrease in g_s by multiple single strain endophytes was found with $P = 0.124$, 0.005, and <0.001 at 12, 3, and 6 pm from Experiment 1 (**Figure 1**).

Similar to g_s response, stomatal density also decreased by 12% ($P = 0.012$) in response to endophyte inoculation (**Figure 2**). That is, compared to 492 stomata/mm^2 in the control (CTRL), the average stomatal counts were 433 stomata/mm^2 in the inoculated plants (PTD1/WP5/WPB).

Intrinsic WUE, a proxy of short-term WUE, was not significantly changed in E+ plants ($P = 0.106$, **Figure 3**). Overall biomass and total transpiration over time did not respond to endophyte inoculation, suggesting little differences in long-term WUE in Experiment 1 (data not shown).

Leaf water potential decreased (21% more negative) in E+ plants regardless of the endophyte strains used ($P = 0.025$, **Figure 4A**). Osmotic potential, on the other hand, was increased (18% less negative) by the endophyte inoculation ($P < 0.001$, **Figure 4B**). This increase in osmotic potential was compensated by the 27% decrease in turgor pressure in the inoculated samples shown in **Figure 4C** ($P < 0.001$). No significant difference was found in soluble sugar content for PTD1/WP5/WPB (**Figure 4D**).

Experiment 2: CO_2 Enrichment Study Using a Single Endophyte Strain

Similar to Experiment 1, endophyte inoculation decreased g_s significantly during the daytime. An average 18% decrease in

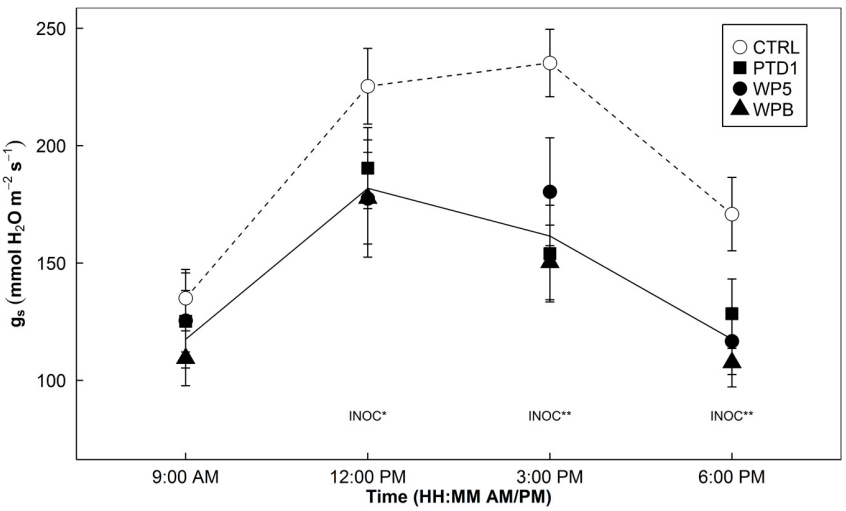

FIGURE 1 | Diurnal patterns of stomatal conductance (g_s) of rice leaves on 163 days after germination in a greenhouse bench experiment (Experiment 1). Open symbols indicate mean g_s of control groups, whereas closed symbols indicate mean g_s of single strain-inoculated groups (square/circle/triangle = PTD1/WP5/WPB, individually). Error bars of the means represent ± 1 SE of replicated samples ($n = 10$). Single strain endophyte inoculation effect (INOC) is provided at $P < 0.05$ (*), 0.01 (**) levels. Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison. Dotted and solid lines highlight mean responses of CTRL and INOC plants over time.

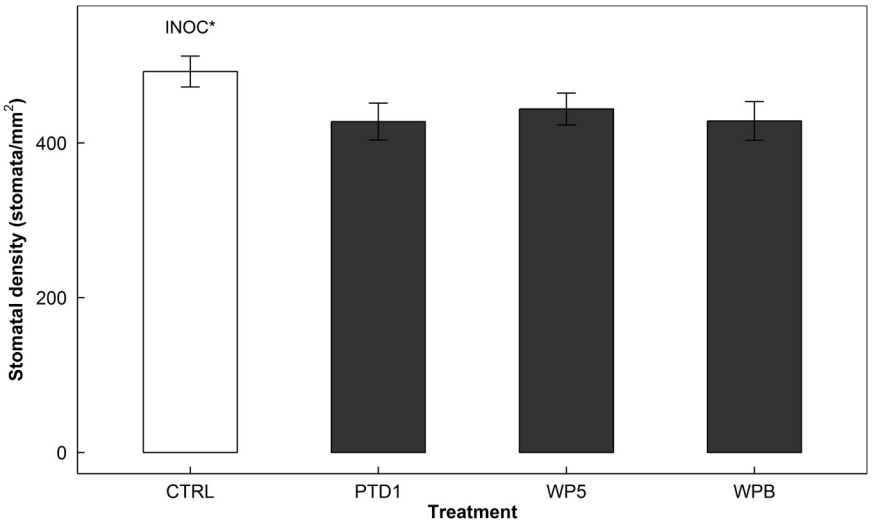

FIGURE 2 | Stomatal density of adaxial sides of rice leaf surfaces on 165 days after germination in a greenhouse bench experiment (Experiment 1). The stomatal imprints were collected from the same leaves that were used in stomatal conductance measurements in **Figure 1**. The bars present mean responses of control (CTRL, open) and the single strain inoculated (PTD1, WP5, WPB from left to right, closed) rice leaves. The error bars indicate ± 1 SE of the means (n = 10). Single strain endophyte inoculation effect (INOC) is provided at $P < 0.05$ (*) level. Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison.

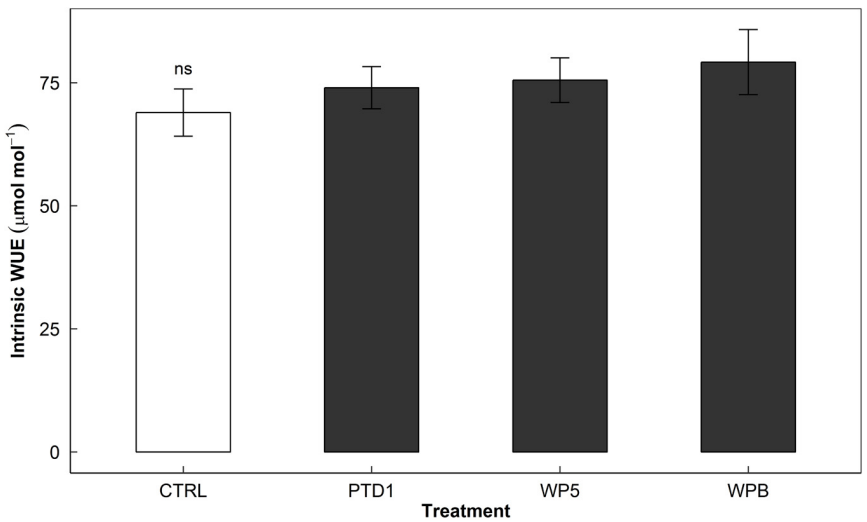

FIGURE 3 | Intrinsic water use efficiency (WUE), expressed as CO_2 uptake/H_2O loss in moles, of rice leaves measured on 58/118/153 days after germination in a greenhouse bench experiment (Experiment 1). The bars present aggregated mean responses of control (CTRL, open) and the single strain inoculated (PTD1, WP5, WPB from left to right, closed) rice leaves collected on the 3 days as the *time* effect on the measure were non-significant at $\alpha = 0.05$ level. The error bars indicate ± 1 SE of the means (n = 30). Single strain endophyte inoculation effect (INOC) was not significant (ns, $P = 0.106$). Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison.

g_s by a single strain endophyte was observed with $P = 0.037$, 0.013, and 0.081 at 12, 3, and 6 pm from Experiment 2 (**Figure 5**). No statistical differences were found in the values taken at other time points of the day between AMB and ELE conditions. There were no differences found in measurements made at 9 am between E− and E+ plants ($P = 0.195$ in **Figure 5**). During the peak time of photosynthetic gas exchange activities (12–3 pm), the differences in g_s became more pronounced, showing 20–21% decreases in E+ plants. High CO_2 lowered g_s by 29% across E− and E+ treatments (**Figure 5**).

Experiment 3: Water Deficit Study Using Endophyte Consortia

We found increases in biomass along with decreases in total transpiration over time and subsequent increases in WUE of productivity of rice plants under both non-stress (NS) and water deficit stress (S) conditions (**Figure 6**).

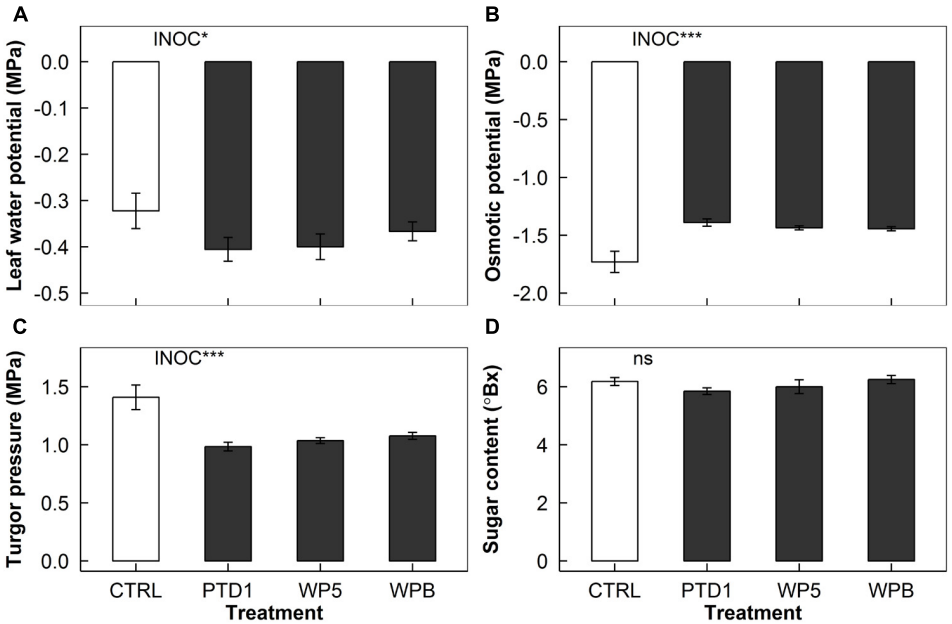

FIGURE 4 | Leaf water potential **(A)**, osmotic potential **(B)**, turgor pressure **(C)**, and soluble sugar content **(D)** of rice leaves on 165 days after germination grown in a greenhouse bench experiment (Experiment 1). The bars present mean responses of control (CTRL, open) and the single strain inoculated (PTD1, WP5, WPB from left to right, closed) rice leaves. The error bars indicate ± 1 SE of the means (n = 9). Single strain endophyte inoculation effect (INOC) is provided at $P < 0.05$ (*), 0.001 (***) level. No significance was found in soluble sugar content (ns). Contrast matrix was used to test CTRL vs. INOC (PTD1/WP5/WPB nested) comparison.

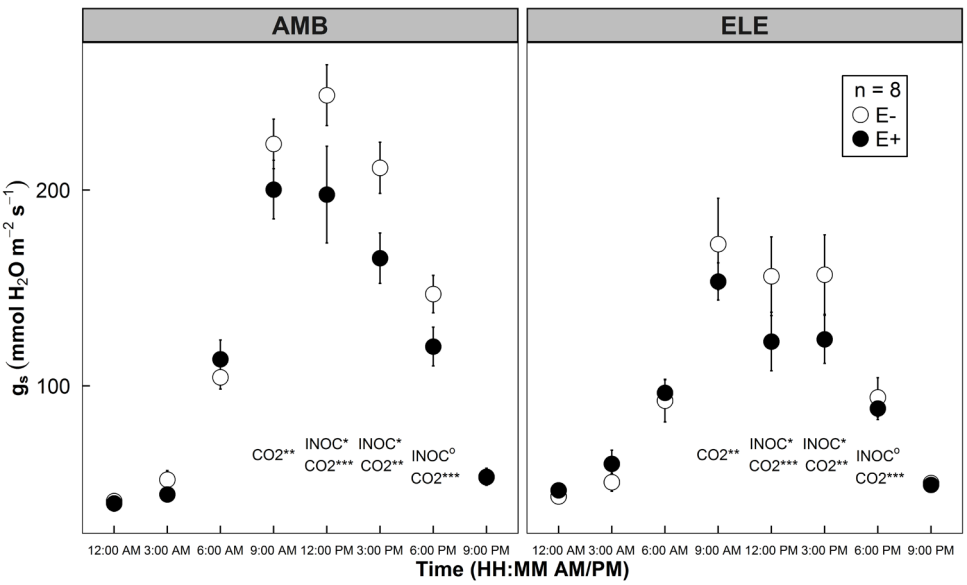

FIGURE 5 | Diurnal patterns of stomatal conductance (g_s) of rice leaves grown under two atmospheric CO_2 conditions: ambient (AMB, app. 400 ppm on the left panel) and elevated (ELE, app. 800 ppm on the right panel) in a sunlit chamber experiment (Experiment 2). Open symbols indicate mean g_s of control groups (E–), whereas closed symbols indicate mean g_s of WP5 inoculated groups (E+). Error bars of the means represent ± 1 SE of replicated samples (n = 8). Two-way ANOVA test results are indicated at each time point. CO_2 treatment effect (CO2) and endophyte inoculation treatment effect (INOC) are provided at $P < 0.10$ (°), 0.05 (*), 0.01 (**), 0.001 (***) levels.

The water deficit treatment affected all three measures significantly: a decrease in biomass by average 62% ($P < 0.001$, **Figure 6A**), a decrease in total transpiration by an average of 85% ($P < 0.001$, **Figure 6B**), and an increase in WUE of productivity by 221% on average ($P = 0.002$, **Figure 6C**) of the all plants (CTRL and MIX pooled). E+ plants showed an increase in biomass by

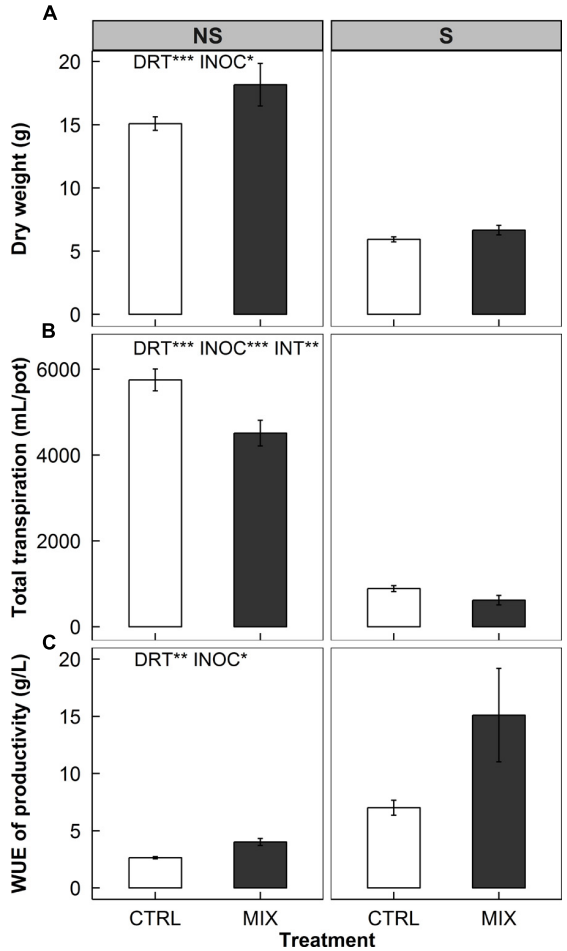

FIGURE 6 | Total biomass (top panels, **A**), total transpiration over time (middle panels, **B**), and water use efficiency (WUE) of productivity (bottom panels, **C**) of rice without (left panels, NS) and with (right panels, S) water deficits at harvest in a greenhouse bench experiment (Experiment 3). Open and closed bars indicate means of mock-inoculated controls (CTRL) and endophyte consortium-inoculated (MIX) plants, provided with error bars as ± 1 SE of the means (n = 8). Two-way ANOVA test results of the treatment effects are placed on each panel. Water deficit treatment effect (DRT), endophyte inoculation treatment effect (INOC), and interaction effect (INT = DRT x INOC) are provided at $P < 0.05$ (*), 0.01 (**), 0.001 (***) levels.

16% over E− plants across water deficit treatments (P = 0.039, MIX in **Figure 6A**). The endophyte effects on reducing total transpiration were greater under S than NS treatment (P = 0.009, the interaction effect – INT – in **Figure 6B**). The magnitudes of the decreases were 30 and 22% in S and NS treatment, respectively (P = 0.096 and <0.001).

The endophyte effects on WUE of the NS and S plants combined were significant (84% increase, P = 0.047, **Figure 6C**), and this was presumably derived from the decreases in total transpiration (26% decrease, $P < 0.001$, **Figure 6B**) rather than from the increases in biomass (16% increase, P = 0.039, **Figure 6A**). The endophyte treatment was more effective in S as the WUE increases were more than twofold compared to NS (116 vs. 52% in **Figure 6C**).

Under NS conditions, the remaining water in MIX was greater than one in CTRL while there was no significant difference in the soil water potential between CTRL and MIX (NS panels in **Supplementary Figure S1**). The water deficit treatment completely dried the remaining water in the buckets and the pots at harvest (S panels in **Supplementary Figure S1**).

Daily Transpiration Over Daily Light Integrals

As expected, daily transpiration increased with DLI over the growing periods in both E+ and E− plants (**Figure 7**). The interactions between DLI and endophyte inoculation on daily transpiration were highly significant (P = 0.006). This result suggests that reduction in whole-plant transpiration due to endophyte inoculation is more pronounced in high light conditions.

Experiment 4: *In Vivo* ABA Content Affected by Endophyte Consortia

There were no significant differences found in both g_s and *in vivo* ABA content between E− and E+ rice leaves at noon (**Figure 8**). In the afternoon at 6 pm, the decrease in g_s by the endophytes was found significant (P = 0.043, in **Figure 8A**). Also, the increase in *in vivo* ABA concentrations in E+ rice leaves was found at 6 pm (P = 0.006, in **Figure 8B**). The endophyte inoculation caused almost a threefold increase in *in vivo* ABA concentrations in rice leaves.

DISCUSSION

In the present study, we evaluated the effects of endophyte inoculation on water relations of a rice host. We tested the hypothesis that endophytes alter the host physiology to reduce transpiration of rice through stomatal regulations. A series of experiments conducted in this study illustrated that several changes in water relations took place when a rice plant was inoculated with diazotrophic endophytes. These physiological changes included significantly higher ABA concentrations, lower stomatal conductance, lower stomatal density, lower leaf water potential likely through a reduction in turgor pressure, and eventually an increase in WUE of the whole-plant as these responses accumulate over time and space.

Malinowski and Belesky (2000) reviewed plant physiological mechanisms of drought and mineral stress tolerance offered by cool-season grass endophytes, focusing on the impacts of fungal endophytes. The authors pointed out that some fungal species, even in the same fungal endophyte class, had opposite modes of actions on stomatal reactions and the following plants' water stress tolerance mechanisms. Similarly, some articles reported increases in g_s (Bae et al., 2009; Shukla et al., 2012; Gagné-Bourque et al., 2016), while others reported decreases in g_s (Turner, 1986; Richardson et al., 1993; Elmi and West, 1995), yet highlighting that either action both resulted in the hosts withstanding water deficit conditions.

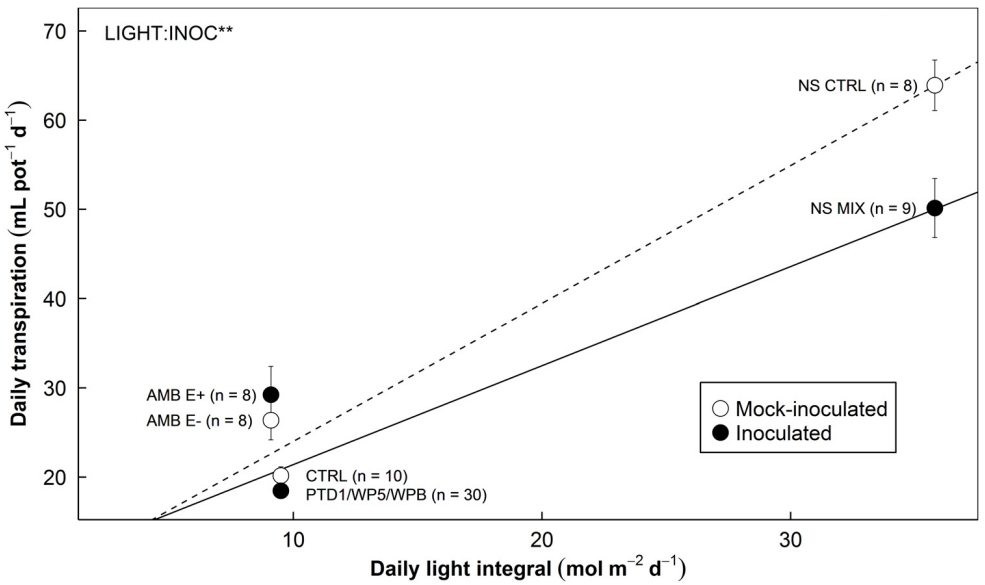

FIGURE 7 | The relationship between daily transpiration by rice plants and daily light integral during growing periods affected by endophyte inoculation in Experiments 1, 2, and 3 (left, middle, and right). Different endophyte and experimental settings were used (refer to **Table 1**). Open symbols (CTRL and E–) stand for the mean responses of mock-inoculated control groups. Closed symbols (PTD1/WP5/WPB, E+, and MIX) stand for the mean responses of endophyte-inoculated treatment groups. Error bars indicate ± 1 SE of the means. The sample sizes are provided in the parentheses. Dotted/solid line show the trends of the responses of control/inoculated groups. Data from elevated CO_2 in Experiment 2 and water deficit treatment in Experiment 3 are not included in this figure. The relationship is significantly affected by endophyte inoculation (INOC) at $P < 0.10$ level. There is an interaction effect (LIGHT:INOC) at $P < 0.01$ level.

Decreases in Stomatal Conductance During Daytime by Endophytes

In Experiments 1, 2, and 4, we observed clear patterns of stomatal behavior with decreases in g_s in the afternoon (**Figures 1**, **5**, **8A**). It is evident that stomatal movement of the host plants colonized by the endophytes is heavily dependent on time of day. These experiments performed under different environmental conditions revealed similar patterns of a decrease in g_s in the afternoon (**Figures 1**, **5**, **8A**).

Two potential mechanisms may explain this afternoon reduction of stomatal conductance: (1) hormonal influences from the endophytes – endogenous ABA production, or (2) recycling of microbial respiratory CO_2 in the Calvin cycle of the plants.

The first possibility of altered diurnal stomatal movement is the effects of ABA-production by endophytes. ABA is a key hormone of stomatal control and its diurnal fluctuation is well described by the dual source model (Tallman, 2004). The inoculated plants may have had a higher ABA level due to additional ABA provided by the endophytes, reaching the threshold faster to close stomata. Another related possibility is that endophytes might induce faster circadian clock responses to environmental cues fluctuating in a 24-h cycle (Seo and Mas, 2015). The two-fold increase in WUE by endophytes in Experiment 3 under water deficit conditions would be in line with this respect (**Figure 6C**). This implies the endophytes can cause the host to respond to the environmental changes more sensitively, enabling more efficient use of water as resources.

Stomatal control determines the efficiency of water relations over any other mechanisms, which is governed by ABA concentration (Tardieu and Davies, 1992). All the endophyte strains used in this study produced ABA ranging from 0.404 – 0.831 μg mL^{-1} in an *in vitro* assay from our earlier work (see Table 1 in Khan et al., 2016). Also, we directly showed the increases in *in vivo* ABA concentration along with the decreases in g_s in the afternoon in Experiment 4 (**Figure 8**). This confirms that the impacts of endophytes on ABA production and resulting decreases in g_s in rice were significant.

Another piece of evidence supporting ABA production by endophytes is the decreases in stomatal density of the inoculated plants. Together with the stomatal closure during the daytime, it is possible that endophyte-producing ABA affected stomatal development. ABA is also known to involve stomatal development under water deficit conditions (Chater et al., 2014). *In vitro* ABA production capacity of the select nine strains in Experiment 3 was assayed in our previous study, Khan et al. (2016) where the drought tolerance of the inoculated poplar trees was significantly enhanced. This decrease in stomatal density allowed the plants not only to conserve water during the daytime in response to the environmental change, but also may save the metabolic cost to build up guard cells rather than photosynthetic cells. Franks et al. (2015) demonstrated the genetically engineered *Arabidopsis* having less stomata, and therefore so lowered stomatal density, had an advantage over the wild type regarding WUE. Also, Gray et al. (2015) showed that the stomatal density lowered mutant consumed less water over time, displaying higher soil water content than the wild type. Both studies presented the importance of stomatal density as a key control parameter for WUE. Endophyte inoculation can be a

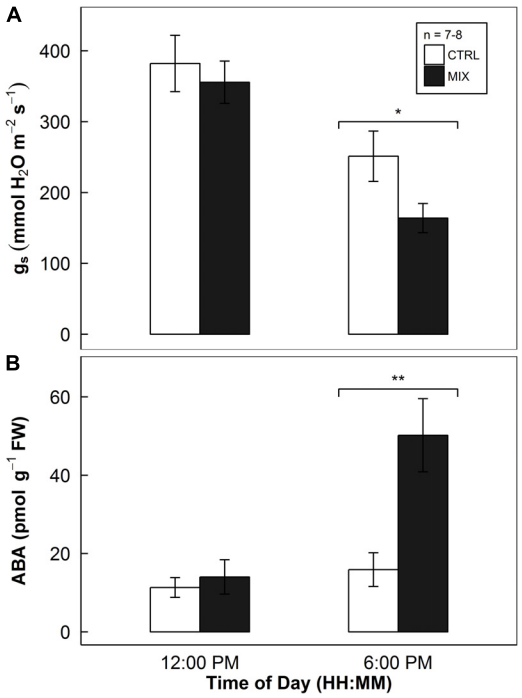

FIGURE 8 | Stomatal conductance of rice leaves at round V7-8 stage (top panel, **A**) and *in vivo* ABA concentrations (bottom panel, **B**) of rice leaves harvested at around R3–R4 stage in a greenhouse sunlit chamber experiment (Experiment 4). Open and closed bars indicate mean responses of mock-inoculated controls (CTRL) and endophyte consortium-inoculated (MIX) plants, respectively, provided with error bars as ± 1 SE of the means (n = 7–8). Endophyte inoculation treatment effect (INOC) is provided at $P < 0.05$ (*) and 0.01 (**) levels at each time point.

novel approach to modify stomatal density as a way to increase WUE.

Our second explanation is the microbial respiration and recycling of CO_2 molecules by the plants. As g_s decreased during the afternoon (**Figures 1**, **5**, **8A**), CO_2 supply from the atmosphere would drop in E+ plants. However, the increases in WUE (**Figure 6C**) suggest that somehow E+ plants could maintain the rate of photosynthetic CO_2 assimilation with less CO_2 through stomata. This implies that other source of CO_2 supply to the site of carboxylation may possibly contribute to the assimilation process. When carbohydrates are given to endophytes respiring in the intercellular spaces of the leaves, perhaps, this respired CO_2 is readily available for the Calvin cycle. This endophytic respired carbon would not have to travel through the diffusional pathway of CO_2, having advantage of the shorter travel distance. Indeed, Bloemen et al. (2013) reported that even CO_2 respired by root tissues was re-assimilated by the aboveground tissues up to 10–20% in poplar trees. Busch et al. (2013) also demonstrated that up to 24–38% of photorespired and respired CO_2 were re-assimilated, resulting in an increase of photosynthesis by 8–11% in rice and wheat. These two examples signify how important respired CO_2 sources from other parts of plants can be in the CO_2 assimilation process. Although challenging to experimentally prove, our hypothesis – the recycling endophytic respiratory CO_2 by the hosts' photosynthesis – holds a possibility.

As opposed to our results with bacterial and yeast endophytes, a meta-analysis from Augé et al. (2014) revealed an average 24% increase in g_s by mycorrhizae. They conducted the statistical analysis on 400+ individual studies including plants grown under water stressed conditions. Mechanistic differences exist between the two distinguished symbiotic styles. Mycorrhizae aid host plants in absorbing water from the rhizosphere, conferring drought tolerance likely by increasing relative water content of the plants. Conversely, endophytes – from this study – seem to assist host plants in conserving existing water mainly by decreasing g_s.

Decreases in Leaf Water Potential by Endophytes

Stomatal control comes first in importance before osmotic regulation of water potential in rice plants (Parent et al., 2010). We therefore examined leaf water potential components. The hypothesis behind this was that endophytes would reduce sugar content as they drain different forms of carbohydrates and organic acids from the hosts, leading to decrease in the ability to regulate osmolytes of the plants.

The endophyte inoculation resulted in a reduction in leaf water potential (**Figure 4A**). Interestingly, this reduction was mostly due to a reduction in turgor pressure whereas the osmotic potential increased with inoculation (**Figures 4B,C**). As the endophytes consume carbohydrates produced from the mesophyll cells and are involved in trafficking the transportable form of carbohydrates to the apoplast– mostly sucrose as a basic form (Lemoine et al., 2013) –, osmotic potential of the host cells was likely to be increased (i.e., less negative) (**Figure 4B**). The consumption of other osmolytes (e.g., organic acids) by endophytes can be another possibility of the increase in osmotic potential. As a consequence, the turgor pressure of the cells might have dropped further in part because lower sucrose and organic acid levels in the symplast as they are concurrently consumed and recycled by endophytes. Collectively, with lower g_s and water potential, the inoculated plants tend to have lower operational cost of water than the control plants.

Increases in Water Use Efficiency of Hosts

The alterations of stomatal development and diurnal behaviors accompanied with plasticity of cell water relations would offer host plants an advantage of saving water during the daytime, especially under high light and warmer conditions when evapotranspiration demand is high. Although stomata were closed and the CO_2 supply from the atmosphere to the intercellular, photosynthetic CO_2 assimilation was not affected by the endophyte inoculation. This advantage is likely to be cumulative over the entire growing period, implying that if there are more sunny days than cloudy and overcast days, the influences will be greater. The differences in our environmental conditions among Experiments 1 through 3 corroborate this point (**Table 1**). We did not find significant increases in biomass

and following increases in WUE of productivity in Experiments 1 and 2 (data not shown). Experiment 1 was conducted during mostly winter and early spring featuring the lack of accumulated light intensity and lower air temperature even in the controlled greenhouse environment. Experiment 2 was conducted in the naturally lit growth chambers where the frames of the chambers shaded the plants grown inside and lowered the actual air temperature of the chamber space. On the other hand, the plants grown in the summer season in Experiment 3 showed increases in biomass and long-term WUE (**Figure 6**). Experiment 3 was conducted under full sunlight condition – the average air temperature was approximately 8°C higher and the average DLI was around fourfold higher than the previous two experiments. The reduction in daily water use by endophytes also upholds this point (**Figure 7**). The magnitude of the reduction is positively correlated to DLI.

Another possibility is that a consortium of endophytes would benefit the plants more than a single strain of endophytes. This has to do with the collection of microbiota simulating the natural habitat would help the hosts more by interacting with each other (Bulgarelli et al., 2013). Knoth et al. (2014) also reported a consortium of multi-strain endophytes was more effective on increasing gain of final biomass of poplar clones in a greenhouse experiment. We used single strain endophytes in Experiments 1 and 2 where the decreases in g_s were observed, but no changes in WUE (**Figure 3**). In Experiment 3, however, where we used a consortium of nine endophyte strains, we found significant increases in long-term WUE (**Figure 6C**).

The decreases in the cumulative total transpiration of the inoculated plants were more significant under the water deficit conditions (**Figure 6B**). Endophytes helping plants under stress is reported in copious articles. Their beneficial effects on the host are thought to be augmented under various stress conditions because the endophytes inside plants signal stress response pathways before the stress is imposed and these microorganisms appear to turn on defense mechanisms of the plants (Pandey et al., 2012).

CONCLUSION

We showed that select bacteria and yeast endophytes decreased g_s suggesting that this stomatal response was the main reason for increases in WUE of the rice plants. The rice plants inoculated with multiple strains of the endophytes all showed the decrease in g_s and stomatal density. The decrease in g_s was also observed under a CO_2 enrichment condition. This stomatal response resulted in reduction of total transpiration of plants over growing periods. The effect size of the reduction was greater when DLI were higher and water supply to plants was limited under water deficit conditions. The reduction in total transpiration was the main reason for increases in long-term WUE in the rice plants. We suggest that the increases in ABA production by endophytes can be a possible mechanism for these stomatal reactions and the resulting whole plant physiological benefits.

AUTHOR CONTRIBUTIONS

Conceived idea and designed the experiments: HR and S-HK. Conducted the experiments: HR, NW, and VVE. Analyzed the data: HR and S-HK. Provided the materials and resources: SD and S-HK. Wrote the article: HR, VVE, SD, and S-HK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00188/full#supplementary-material

FIGURE S1 | The remaining water in the buckets (top panels, A) and water potential of the soil in the pots (bottom panels, B) without (left panels, NS) and with (right panels, S) water deficits at harvest. Open and closed bars indicate means of mock-inoculated controls (CTRL) and endophyte consortium-inoculated (MIX) plants, provided with error bars as ± 1 SE of the means (n = 8). Two-way ANOVA test results of the treatment effects are placed on each panel. Water deficit treatment effect (DRT), endophyte inoculation treatment effect (INOC), and interaction effect (INT = DRT x INOC) are provided at $P < 0.01$ (**) and 0.001 (***) levels.

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