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Use of plant growth promoting endophytic bacteria to alleviate the effects of individual and combined abiotic stresses on plants as an innovative approach to discover new delivery strategies for bacterial bio-stimulants

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Use of Plant Growth Promoting Endophytic Bacteria to Alleviate the Effects of Individual and Combined Abiotic Stresses on Plants as an Innovative Approach to Discover New Delivery Strategies for Bacterial Bio-stimulants

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Abstract

Bacterial endophytes are the organisms that live inside the plant for a full or a part of their life cycle. Endophytic bacteria have captured the interest of agriculture industry due to their plant beneficial properties, such as synthesis of phytohormones, solubilization of soil nutrients, and alleviation of biotic and abiotic stresses. Several studies have reported that stress tolerant endophytic bacteria can work with a similar performance as non-stressed conditions when inoculated to the plants under stressed conditions. Combination of abiotic stresses such as salinity, drought and low nitrogen stress can have additive or agonistic effects on bacterial and plant growth, and their interactions. However, very few studies have reported the impact of combined stress on endophytic bacterial assisted plant growth promotion. Therefore, understanding the underlying mechanisms of endophytic bacterial assisted plant's tolerance abiotic stresses may provide the means of better exploiting the beneficial abilities of endophytic bacteria in agricultural production. Thus, the aim of this thesis was to study the stress tolerance mechanisms, beneficial characteristics, and plant growth promotion characteristics of endophytic bacteria under individual and combined abiotic stresses. Transcriptome analysis of endophytic bacteria revealed that tolerance mechanisms to deal with one kind of stress is different than concurrent stresses. Salinity and drought stress largely modulated the genes involved in flagellar assembly and membrane transport, showing reduced motility under stress conditions to preserve the energy. Additionally, bacterial endophyte that can fix nitrogen was studied with maize plant growth promotion under drought and low nitrogen stress conditions. The results suggested that diazotrophic bacterial endophyte can promote plant growth under moderate individual and combined stress conditions. Plant growth promoting endophytic bacteria can be utilized as an efficient tool to increase crop production under individual and concurrent abiotic stresses.

Muhammad Aammar Tufail did this PhD within INTERFUTURE, a project funded by H2020 Marie Skłodowska-Curie Actions, Innovative Training Network, European Industrial Doctorate. For more than five years, he is exploring the potential of plant growth promoting endophytic bacteria to be used as bio-stimulants in order to increase crop (maize, tomato) production under extreme conditions such as salinity, drought, low nitrogen, and combined stress. He won several national and international awards in science communication and project idea competitions during his research career. His research aim is to build a world with more sustainable agriculture.

Table of Contents

List of Figures:

List of Tables:

Chapter 1-
Magic inside plant: Salt-loving endophytic bacteria ready to overcome plant salinity stress

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Abstract

Salinity stress is a global threat to crop production and gradually rising due to climate change. The conventional agronomic and breeding methods have always proven inadequate and time-consuming to mitigate the threat of salinity stress from agriculture production. Plant-associated microorganisms drive a growing focus on the on-spot improvements in plant tolerance to salinity stress. Plant-associated endophytic bacteria play a crucial role in increasing plant tolerance to several biotic and abiotic stresses and improving plant growth, health, and yield. Several studies have confirmed the functions of endophytic bacteria to the host plants including enhancing the availability of mineral nutrition, and regulating the production of phytohormones, enzymes, siderophores, antioxidants, and osmoprotectants. These scientific advances have helped the farming community to use endophytic bacteria to improve plants' ability to withstand in salinity stress and eventually contribute to the translation of the agriculture industry in salty environments. This article reviews endophytic bacteria with a brief description of the salinity stress and its tolerance mechanism in plants. Such knowledge should help increase understanding in the scientific community to research to gather data relating to the use of endophytic bacteria to reinforce salt stress plants that could improve farming practices and efficiency in saline environments.

1.1. Impact of soil salinity around the globe

Soil salinization is a term that includes sodic, alkaline, and saline soils, respectively defined as i)- high concentration of sodium (Na^+), ii)- high pH, and iii)- high concentration of salts, in the soil [1]. High levels of salinization may also result in the

depletion of existing soil resources, goods and services, affecting agricultural production and environmental health [2], ultimately becoming a socio-cultural and human health crisis that impedes economic and general well-being [3].

Soil salinization is one of the major threats to food security, limiting the crop yield and health [4]. The key natural processes of soil salinization are, climatic events such as rock and mineral weather, increased temperature fluctuations, changes in rainfall, and the addition of sea water in coastal areas. These mechanisms release salts to the earth and groundwater, which accumulate for a long time. The results of such mechanisms in arid and semi-arid regions are more evident [5]. Global temperatures are expected to increase by 1.5-5.8 ℃ and sea levels will increase by 1.9-5.89 mm/year by the end of the 21st century owing to climate change [6]. If the temperature increases, it contributes to more groundwater evaporation and more salt deposition per year in topsoil layer. In addition, excessive rainfall enhances the chances of leaching of salts from soil to ground water resources. Secondary salinization stems from human activity, such as irrigation with low quality water, field clearance, inadequate drainage, and other poor farming practices (Figure 1.1). In 1990, it was recorded that almost 20% of Earth's land seemed to be affected by salinity, and since then, the ratio kept on increasing. Every year, the saline lands are increased, and until now, more than half of total agricultural lands have been affected [7]. Around 831 Mha of land on the planet earth suffers from soil salinity. In comparison, anthropogenic activities affect around 76 Mha of land worldwide, a region greater than the whole Brazilian arable land [8]. In Europe, 30.7 Mha soil is saline and sodic [2] and Mediterranean countries are emerging hotspots of soil salinity sometimes coupled with soil alkalinity [4,9]. There is a long queue of countries where land degradation has started as a result of salinity [10]. Middle East countries showed that 20 Mha is impacted by soil salinity [11]. About 20 Mha in India and 1 Mha in Pakistan are on the verge of being unproductive and affected by soil salinity [12]. A recent study [13] reports that the yield of the major agricultural crops such as wheat, corn, rice, and barley has decreased by 70% because of salt stress. It is estimated that nearly 6% of the soil is heavily impacted by salinity stress, where 20% of the global irrigated areas and 2% of drylands have been destructed by salinity [14-16]. Moreover, inadequate management and irrigation methods combined with climate change in particularly arid and semi-arid areas have resulted in a substantial reduction in soil quality because of salinization, degradation, and loss of soil nutrients [17]. Salinized soils typically either have more than 15% of exchangeable sodium percentage (ESP) with pH> 8.5 and high concentration of carbonates and bicarbonates such as sodic soils or high osmotic potential with high electrical conductivity (EC > 4dSm^{-1}) called saline

soils or intermediate regarded as saline-sodic soils. These conditions in salt effected soils hinders crop growth [18,19].

Soil salinity puts substantial constraints on crop yields and represents more production losses than any other abiotic stress. The salinity threshold can vary from plant to plant. In general, cereals are sensitive to 4 dSm^{-1} EC (about 0.2 mPa osmotic pressure) while in plants with a low range of up to 2.5 dSm^{-1} EC, vegetables are sensitive. Natural and anthropogenic activities induce soil salinity. In comparison to drylands, irrigated soils are more vulnerable to accumulation of salts. Due to excessive irrigation and less tillage, soil water table rises. As this table rises, salts continue moving from the ground into the root region or accumulating in the topsoil. Irrigation with low quality (salt-rich) water further promotes salt deposition in topsoil. Therefore, irrigated salinity has been documented as a major issue, since irrigated land adds a big sum of food to the planet [5].

The deposition of salt degrades the physical properties of soil and increases alkalinity. Cations such as Ca^{2+} and Mg^{2+} found in the exchange sites of soil are replaced with Na^{+} ions during high salinity, which enhance the dispersion of soil structure. Salinity also increases the compression and reduces the hydraulic conductivity and oxygen supply in the rhizosphere. These soil changes cause nutrient inaccessibility and sodium toxicity in crop plants. The supply of nutrients for plants is highly affected by the alkaline environment. Major nutrients are accessible at neutral pH. As soil pH rises from the optimum range, most cations (K, Cu, Fe, and Zn) become less accessible to plants and soil (micro)organisms [5].

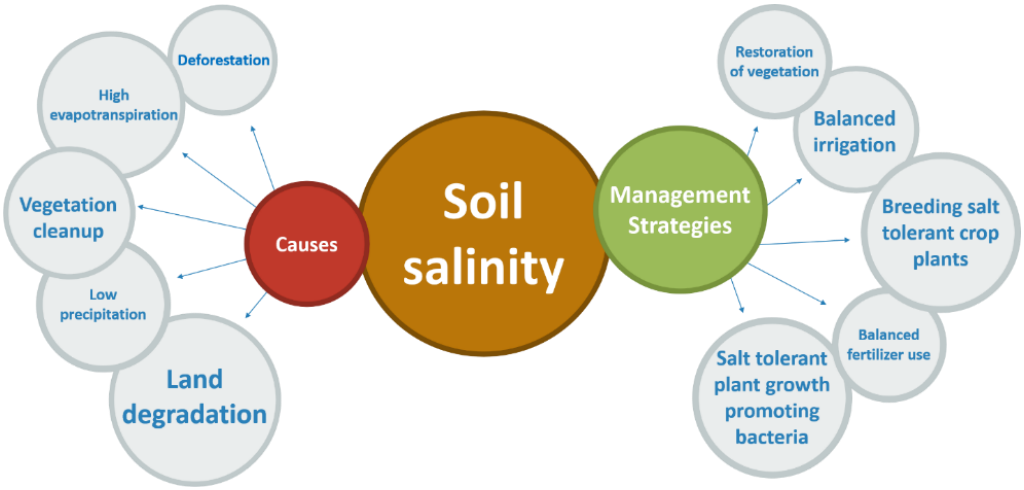

Figure 1. 1: Causes of soil salinity and management strategies to alleviate the effects of soil salinity on crop plants

1.2. How plants respond to soil salinity?

Soil salinity affects almost every part of the plant, but the young and growing parts are at high risk [20]. Plants are sessile and remarkably capable of dealing with various stress situations. Based on the ability to combat salinity stress, plants are divided into two groups: (i) glycophytes and (ii) halophytes. Glycophytes are salt-sensitive plants, while halophytes can withstand high salt concentrations, and most of the crops are from glycophyte group [21]. The effect of soil salinity is a blend of morphological and physical properties, and the processes like plant growth, nutrients uptake, and seed germination are altered. Both the vegetative and reproductive growth are affected by the soil salinity, and the plants are at risk of ion toxicity [22]. Due to the immaturity of protection and resistance mechanisms of plants, the seedling is the phase most sensitive to salt stress [23].

Excessive salt deposition affects plant growth in several ways as shown in (Figure 1.2) decreased plant growth, reduced photosynthetic rate, ion homeostatic mechanisms etc. [24,25]. The plants face two main stresses, osmotic and ionic stress, under high salinity according to [26] as shown in (Figure 1.3). Osmotic stress occurs shortly after exposure to salinity, leading to the formation of hypertonic conditions outside the cell. In contrast, after several days of exposure, ion stress emerges because of the aggregation of sodium (Na^+) and chloride (Cl^-) ion within the cell. Osmotic stress affects the water balance and decreases turgor pressure and elongation rates of the cell. Ion toxicity is built by the high accumulation of different nutrients in plants like Na and Cl. The excessive amount of sodium causes damage to plant cell walls and disturbs the osmotic balance. Ionic stress modifies ion homeostasis within the cell that induce changes in transpiration rate, translocations of nutrients, photosynthesis, and other metabolic processes [27]. Ionic stress contributes to excess sodium ion influx with the resulting cumulative potassium ion efflux. Osmotic stress contributes to dehydration and salt accumulation in the soil surrounding plant roots, negatively affecting cell elongation and lateral bud growth. Salinity stress contributes to deposition of toxic ions such as sodium in leaves. If these ions multiply above a threshold, they impede different essential physiological processes, including photosynthesis [26,28]. In general, changes in photosynthesis triggered by salinity are associated with changes in assimilatory pathways of nitrogen (N) and carbon (C), which eventually lead to reduced crop yields [29]. Excessive salts build-up hinders crop growth and mobilization of nutrient making plant more vulnerable to soil borne diseases [24,30,31].

The generation of reactive oxygen species (ROS) is another consequence of salinity stress on plants, that disrupt plant metabolism [32,33]. ROS production may be caused

not only by salinity, but also by other extremely stressors such as drought, flooding, or heavy metal toxicity, including mercury, arsenic, lead, and chromium [34,35]. Salinity stress causes the production of ROS such as superoxides, hydrogen peroxide, and hydroxyls, which has detrimental effects on plants triggered by oxidative stress, resulting in dysfunction and plant cell death [32,36].

The secondary DNA damage, such as loss of bases, interconnecting DNA protein and dual-stranded DNA breaks caused by ROS, are widely reported [37]. The plant must therefore react with enzymatic and non-enzymatic scavengers that reduce ROS-induced disruption to a stressed plant [36].

1.2.1. Tolerance mechanisms

Plants have their own immune system where various biochemical pathways function in a cascade and induce stress tolerance [35]. Crops show a prism of responses to salt stress that vary from situation to situation. Salt stress in plants triggers a variety of physiological reactions to interact with and withstand stress [35,38]. During osmotic stress, plants retain their moisture content by limiting cell division and cell elongation, limiting the growth of young leaves, branches, and lateral roots development and stomatal closure. In addition, plants retain a shoot-to-root ratio for continued existence under salinity stress since the heavier root collects higher levels of salts and does not facilitate the transition to the upper part of the plant [39].

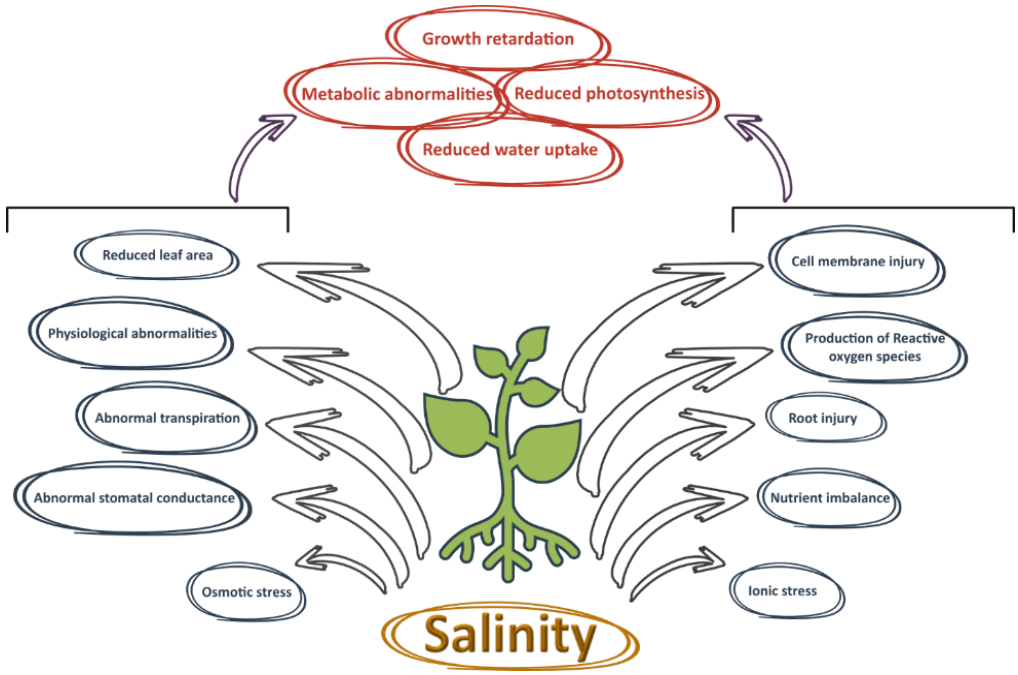

Figure 1. 2: Effects of salinity stress on plant growth and development

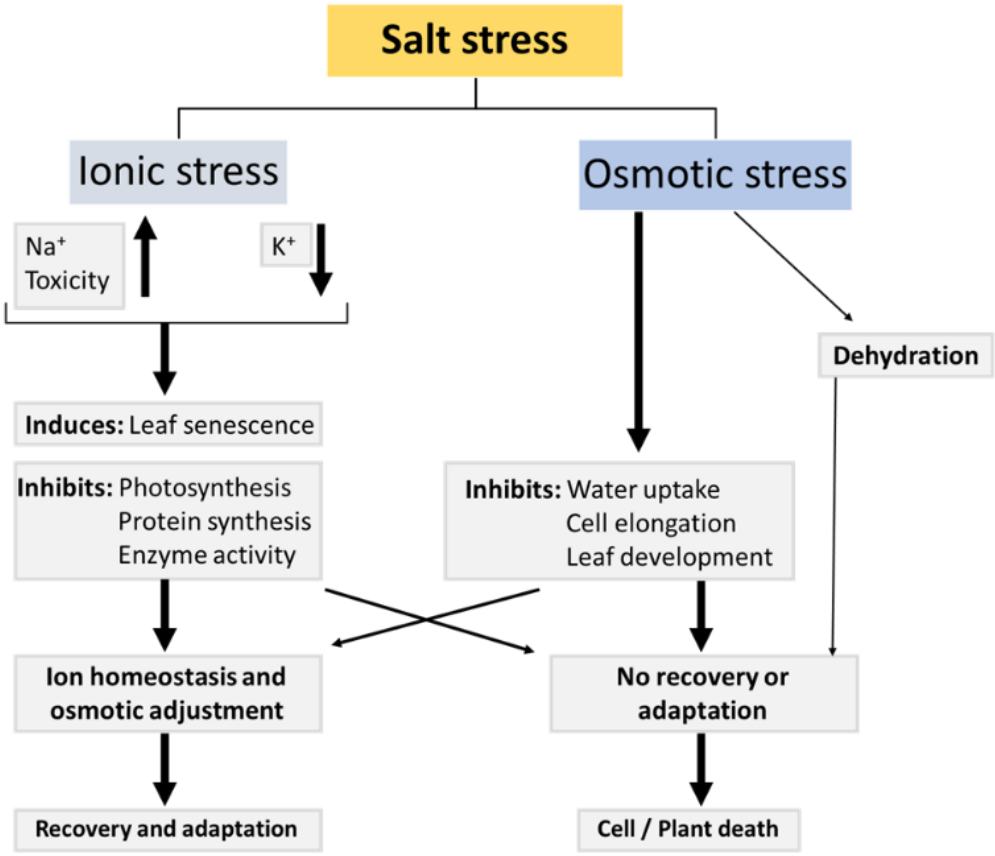

Figure 1. 3: Type and effects of salt stress on plant growth and development, modified from [26,40]

Salt-tolerant plants have a lower shoot-to-root ratio relative to sensitive plants. These phenotypic modifications in plants are mediated by various phytohormones, namely auxin, gibberellin (GBs), cytokinins (CKs), abscisic acid (ABA) and ethylene [41]. In order to offset the harmful effects of salinity stress, plants activate various kind of self-protection machineries to protect cells against oxidative damage. Different plant defence mechanisms for mitigating abiotic stresses such as salinity include, (i) accumulation of osmolytes, (ii) up-regulation of antioxidants and (iii) compartmenting toxic and lethal ions to less sensitive tissues [42,43]. The increased activity of anti-oxidative enzymes in plants has been documented as a tolerance mechanism under stress conditions [44]. The content of ascorbate and glutathione has also increased in response to salinity stress [44,45]. In addition, secondary metabolites of plants, such as carotenoids, tocopherols, flavonoids, polyamines, and phenolic compounds, perform defensive roles toward oxidative stress [46].

Plants are affected by ionic stress under extended exposure to salinity where a higher concentration of Na^+ induces cytotoxicity. To escape Na^+ toxicity, plants followed two separate mechanisms: increased intracellular or vacuolar sequestration and increased Na^+ extrusion [47]. The efficacy of these pathways provides plant with salt tolerance. Halophytes preserve the ionic toxicity of Na^+ by boosting the removal of Na^+ from cell by plasma membrane Na^+/H^+ antiporter, transfer of Na^+ to the xylem from roots and compartmentalize to the vacuole by tonoplast Na^+/H^+ antiporters [48]. As Na^+ is taken up by roots it builds up in the xylem and then passes through the transpiration stream and accumulated in leaf blades. Leaf tissue is more susceptible than any other tissue to ionic stress. Moreover, during recirculation from shoot to root, some proportion of Na^+ persists in the shoot [27,49]. An efflux of Na^+ from tissues and the recovery of Na+ from the xylem are therefore important to tolerating ionic stress and are accomplished by a regulatory network of many transporters [50]. Na+/H+ antiporter, a plasma-membrane-localized transporter, plays a major role in the Na^+ cell outflow [51]. SOS1 has been shown to have Na^+/H^+ antiporter activity in *Arabidopsis thaliana.* Furthermore, tonoplast antiporters perform ion compartmentalization functions in vacuoles. The Na^+/H^+ family is in this category in *A. thaliana* [52]. Plants also activate high-affinity transporters K^+ (HKT), which inhibit Na^+ root uptake. HKT increases K^+ ions absorption over Na^+ ions, resulting in an increase in the salt tolerance of plants [53].

At the cellular level, salt ions are sequestered inside vacuoles, thereby perturbing the osmotic equilibrium of the cell. As a result, the water exudes from the cytoplasm to the extracellular space, resulting in cell dehydration. To maintain certain osmotic pressure, plants accumulate low molecular weight organic compounds in the cytoplasm that are compatible with metabolic processes, known as compatible solutes, such as proline, trehalose, glycine betaine, mannitol and sucrose [21]. Compared with glycophytes (up to 10 mM), halophytes accumulate these compounds at higher concentrations, up to 40 mM [54]. While the advent of osmotic stress, the rate of photosynthesis reduces, more stomata are closed, and the leaf region is reduced to minimize water depletion, contributing to the build-up of accumulated carbohydrate providing input signals to reduce photosynthesis and strengthen metabolism processes [55]. ROS are continuously developed as metabolic by-products, due to the decrease in photosynthesis rate and an increase in energy metabolism. ROS is important as signalling molecules at low levels but cause oxidative damage at higher concentrations and affects membrane lipids, nuclear acids, and proteins [56]. Enzymes (e.g., catalase, glutathione reductase peroxidase and superoxide dismutase) or and secondary metabolites (e.g., ascorbic acid, tocopherol, glutathione) function in co-ordination, to shield plant cells from oxidative injury induced through the presence of ROS [57,58].

1.2.2. Plant microbe interaction

Microorganisms associated to plants contribute greatly to the promotion of plant growth and tolerance of salinity. These microbes increase soil-water-plant interactions, manage phytohormone signals and activate many other pathways that strengthen salt and drought stress tolerance in plants in an interconnected way [35,59]. The beneficial microbiota members are plant growth promoting bacteria, arbuscular mycorrhizae fungi and other endophytes. It is widely accepted that plant stress tolerance is related to their microbiome showing the importance of studying plant-microbe interaction under stress conditions [60-62].

To survive in their natural environments, plants establish associations with several members of their ecosystem. Microorganisms are among the most significant organisms that can establish beneficial relationships with plants [63]. Such plant-beneficial microorganisms, specifically bacteria, offer several advantages to their host plants and allow them to withstand various biotic and abiotic stresses that can have detrimental effects on their growth and development [64]. These bacteria may reside in their host plant externally or internally. Bacteria living outside their host plant are either rhizosphere, colonizing plant roots in the soil, or epiphytic, inhabiting plant leaves [65]. While bacteria that invade and colonize inside their host plant tissues are termed as endophytic bacteria [66]. Endophytic bacteria are part of the bacterial population of rhizosphere. The rhizosphere of plants is well known to serve as a source for a number of potential bacteria. Plant's endophytic bacteria (10^4-10^8 bacterial cells per g of root tissue) are less abundant than rhizosphere bacteria (10^6-10^9 bacterial cells per g of soil) [67].

Endophytic bacteria can be defined as bacteria isolated from plant tissues after surface sterilization, which cause no visible damage to their host plants [68]. Endophytes can also be defined as the microorganisms that reside inside plant tissue, especially leaves, branches and stem, at least a part of their life cycle, establishing an association with the host plants, without impacting its physiological processes [69] and without causing any apparent disease manifestation [70]. They form fundamental interactions with host plants through seed dissemination and support them through acquiring nutrients [71]. They improve vegetation by enhancing biological nitrogen fixation systems [72] and releasing certain plant growth promoting enzymes [73,74].

Endophytic microorganisms sustain plant health and fitness under extreme conditions. Several experiments have recently shown different pathways of endophytes to improve plant growth and development under various abiotic stress conditions [63,75]. But most endophytes have no favourable impact on plant growth under drought, salinity, heavy

metals, and harsh environments [76,77]. Genera of endophytic bacteria tested under the salt condition as a plant growth promoter along with their maximum ability to tolerate salts have been summarized in Figure 1.4. *Bacillus* is the highest salt tolerant genera that can tolerate up to 5000 mM NaCl [78], followed by *Pseudomonas* [79] 600 mM NaCl.

This review focused particularly on endophytic bacteria with a brief description of the salt stress and its tolerance mechanism in plant. To the best of our knowledge, there is no systematic study available on the exploitation of bacteria endophytes to fight against salt stress and how the endophytic bacteria improve the capacity of plants to grow better in saline environments. Such knowledge should therefore help to increase understanding in the scientific community to undertake research to gather data relating to the use of endophytic bacteria to reinforce salt stress plants that could improve farming practices and efficiency in saline environments.

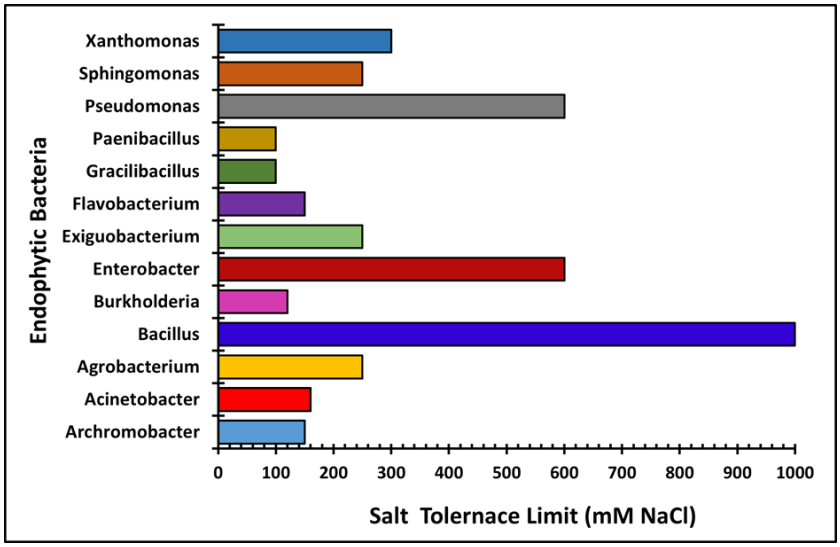

Figure 1. 4: Some documented genera of plant growth promoting endophytic bacteria with their maximum salt (NaCl) tolerance limits [57,78,80-96]

1.3. Role of endophytic bacteria to increase plant tolerance to saline stress

The mechanisms activated endophytic bacteria in the alleviation of salt stress are still an open subject of study [5,97]. Endophytes induce osmotic adjustment, detoxification, modulation of phytohormones, and acquisition of nutrients in plants to relieve the effects of salt stress (Figure 1.5). Inoculating plants with endophytic bacteria provide greater plant stabilization and development under salinity stress. It was observed that at 250 mM NaCl, plants inoculated with endophytic bacterial strains exhibited a substantial improvement in shoot length (17-68%), root length (52-127%), and leaf area (87-702%)

as compared to non-inoculated control plants [98]. Similarly, Yaish et al. [99] reported that canola seed pre-treated with the bacterial endophytes *Paenibacillus xylanexedens* PD-R6 and *Enterobacter cloacae* PD-P6, isolated from date palm tree, showed 27% increase in root elongation as compared to control plants at 100 mM NaCl stress. An overview of the various endophytic bacteria reported to ameliorate the effects of salinity stress on plants is provided in Table 1.1. Endophytic bacteria *Curtobacterium oceanosedimentum* SAK1, *Curtobacterium luteum* SAK2, *Enterobacter ludwigii* SAK5, *Bacillus cereus SA1*, *Micrococcus yunnanensis* SA2 and *Enterobacter tabaci* SA3 mitigated the effects of salt stress from rice plants in a pot experiment [100]. Another bacterial endophyte *Bacillus subtilis* BERA 71 has increased plant biomass and chlorophyll contents of *Cicer arietinum*, cv. Giza 1 and *Acacia gerrardii* Benth. Plants under salinity stress [57,101].

1.4. Mechanism of action

1.4.1. Antioxidants and osmolytes regulation

Endophytes may reduce the effect of salt stress on plants by accumulating osmolytes and antioxidants. The compounds are involved in osmotic improvement and stabilize free radical scavengers and cell components. Two strains of endophytic bacteria, *Bacillus* sp. and *Arthrobacter* sp., have been revealed to increase proline accumulation in pepper (*Capsicum annuum* L.) plants under osmotic stress conditions. Moreover, endophytization minimized the effects of stress by regulating the stress-inducing genes in pepper plants [102]. Jha and co-workers [103] reported that the endophytic bacterium *Pseudomonas pseudoalcaligenes* significantly increased the shoot biomass of rice seedlings grown under salinity stress by synthesizing large quantities of quaternary compounds like glycine betaine. They also found that relative to a single inoculation with the rhizobacterium *Bacillus pumilus* or *Pseudomonas pseudoalcaligenes*, their consortium provides increased protection by inducing elevated levels of antioxidants and osmoprotectants synthesis under salt stress. Shoot and root proteomics of canola plant inoculated with the endophytic bacterial strain *Pseudomonas putida* UW4 under salt stress revealed a higher presence of proteins related to antioxidative processes, photosynthesis, and membrane transportation [104]. Under salinity stress and infection of a pathogen *Sclerotium rolfsii*, the wheat endophytic bacterium *P. aeruginosa* PW09 inoculated in cucumber plants increased the accumulation of free phenolic and proline contents and even increased the activity of enzymes involved in plant defence mechanisms such as phenylalanine ammonia lyase [79]. Chickpea plants inoculated with the bacterial endophyte *Bacillus subtilis* (BERA 71) showed an increase in the biomass

of the plants and chlorophyll contents and decrease in the levels of ROS and lipid peroxidation when grown under saline stress conditions [57].

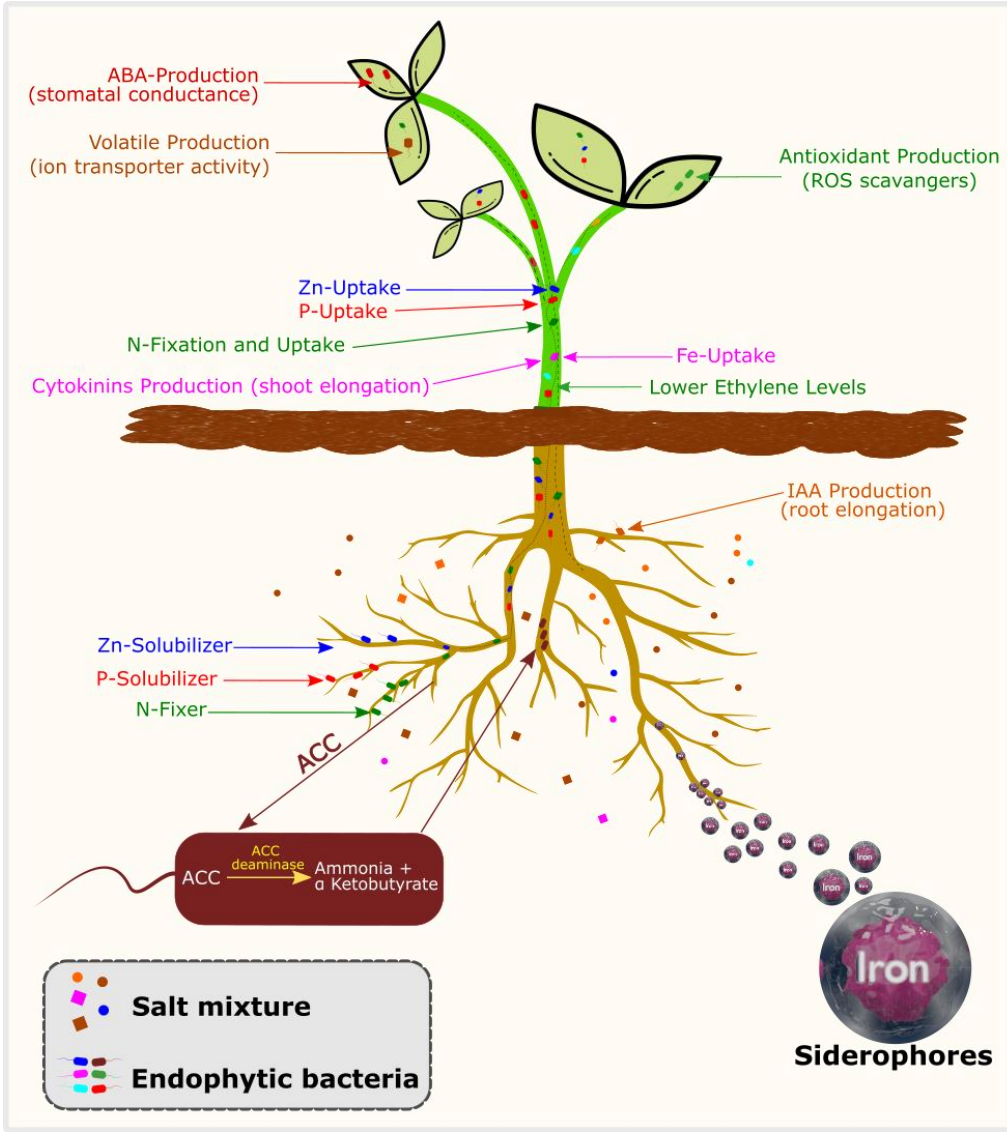

Figure 1. 5: Endophytic bacteria's function in plant salt tolerance. Endophytic bacteria with plant growth-promoting capacity counter-stress by eliminating toxic substances, releasing osmotic pressure, enhancing phytohormone production such as indole 3-acetic acid (IAA) and cytokinins (CKs) further enabling plants to cope with salinity stress and grow better. Boosted communication between root and shoot strengthens water and nutrient balance and stomatal conductance. Stimulating the accumulation of osmolytes, activities of antioxidant and metabolism of carbohydrates slows the leaf senescence, ultimately contributing to the rate of photosynthesis.

1.4.2. Ethylene toxicity reduction by 1-aminocyclopropane-1-carboxylic acid (ACC): deaminase activity

Ethylene is a gaseous plant hormone and is required by plants in very low quantities commonly less than 1.0 ppm (i.e., 1.0 μL L^{-1}) that is important in plant development. When produced at lower concentrations, ethylene can trigger germination of seeds, elongation of roots, initiation of flowering, and primordial development of root and leaf in roots and stems [105,106]. Under stress conditions, the levels of ethylene production by plants increases above critical threshold levels, although stress-induced production of ethylene typically inhibits plant growth [107].

Precursor of ethylene production is ACC that is converted into ethylene by an ACC oxidase [108]. Endophytic bacteria can influence the production of ethylene in plants through the action of ACC deaminase. This enzyme transforms ACC into ammonia and α-ketobutyrate in plants lowering the levels of ACC within plants. As a result, ACC deaminase reduces the levels of ethylene detrimental for plant growth under environmental stresses.

Rhizosphere bacteria producing ACC deaminase can effectively ameliorate the effects of biotic and abiotic stress on plant [109,110]. However, endophytic bacteria able to produce ACC-deaminase can be an excellent plant growth promoter under stress, as they limit the production of ethylene at any given site within the plant [63]. The first study of ACC deaminase activity in rhizobacteria was reported in 2003 [111]. Several reports are available on the isolation and characterization of ACC deaminase producing bacterial endophytes [99,112-117]. Noteworthy, many studies have suggested that the presence of ACC deaminase activity in PGPB is one of the keyways of promoting plant growth under normal and stress conditions [118-121].

Burkholderia phytofirmans PsJN, an endophytic bacterium assisted the growth promotion of canola seedling by ACC deaminase activities. The ACC deaminase function was confirmed by using a mutant strain of PsJN lacking ACC deaminase, which could not promote the same plant growth [47,122]. Endophytic bacteria *Achromobacter xylosoxidans* residing in *Catharanthus roseus* root have been recorded for mitigating the effects of salt stress (50, 100 and 150 mm NaCl) and promoted plant growth by developing low ethylene and rapidly activating antioxidants mechanisms [86]. Similarly, another report [82] showed the involvement of ACC deaminases in the ability of the endophytic bacteria *P. fluorescens* YsS6 and *P. migulae* 8R6 to promote tomato development and growth under two distinct NaCl gradients (165 and 185 mM). Endophytic bacterial strains *Isoptericola dokdonensis* KLBMP 4942, *Streptomyces pactum* KLBMP 5084, *Arthrobacter soli* KLBMP 5180 and *B. flexus* KLBMP 4941

increased seed germination and promoted growth of *Limonium sinense*, a halophyte plant, under salt stress conditions. Furthermore, it was observed that at 250 mM NaCl, plants inoculated with endophytic bacterial strains exhibited a substantial improvement in shoot length (17-68%), root length (52-127%), and leaf area (87-702%) as compared to non-inoculated control plants [98]. In a study, the salt tolerant endophytic bacterium *Brachybacterium paraconglomeratum*, isolated from *Chlorophytum borivilianum* roots, having ACC deaminase activity decreased osmotic and oxidative damage from the host plant caused by salt stress [123]. In another study, *Camelina sativa* plants treated with ACC deaminase producing endophytic bacterium *Pseudomonas migulae* 8R6 displayed an improved resistance to salinity, thus modulating the signalling of ethylene and abscisic acid. Moreover, *C. sativa* transgene lines expressing a bacterial *acdS* gene showed tolerance to salinity stress [124].

1.4.3. Regulation of phytohormones

Endophytic bacteria can also modulate important plant phytohormone levels that provide plant benefits and facilitate growth under stress conditions. ABA is a key phytohormone that helps plants to overcome the water loss via stomatal closure of leaves under osmotic stress conditions. It also helps plants withstand other stresses, such as salt and cold. the endophytic bacterial strain *Bacillus amyloliquefaciens* RWL$_{-1}$, able to produce ABA, improved the salt stress tolerance in rice plants. The bacterial strain inoculation contributed to substantially increase the biosynthesis of important amino acids and stimulated the plant's production of endogenous of salicylic acid, which helped rice plants survive under salt stress [92]. Auxin is another important phytohormone and IAA is a natural and active form of auxin produced by plants and endophytic bacteria. IAA contributes to cell division, stimulates seed germination, influences vegetative growth, helps in root elongation, induces pigment formation, photosynthesis, metabolites biosynthesis, and tolerance to various kinds of environmental stresses by plants [125]. Under salinity stress, an IAA and GBs producing endophytic bacterium *Leclercia adecarboxylata* MO1, when inoculated in *Solanum lycopersicum* plants, improved salinity tolerance, increased plant biomass, sugar and amino acids concentrations, and lowered ABA levels [119]. GBs play a key role in plant development processes, such as seed germination, stem elongation, leaf expansion, pollen maturation and fruit development [126]. The salinity tolerance of *Glycine max* cv. Pungsannamul was improved with the inoculation of a GBs producing endophytic bacterium *Curtobacterium oceanosedimentum* SAK1. Number of root tips increased in inoculated plants due to enhanced levels of endogenous GBs in plant under salinity stress [127]. Cytokinins are purine derivatives compounds known to be active in the

differentiation process of root callus and shoot development. By continuous use of CKs plant maintain their newly developed stem cells in shoot and root meristem [128,129]. Cytokinins regulate cell division, root growth and elongation, root hair formation, cell expansion, and tissue extension in plants [130]. Some genera of bacterial endophytes such as *Pseudomonas*, *Klebsiella*, *Bacillus*, and *Xanthomonas* have the ability to produce cytokinins that helps plant growth under salt stress [130,131]. *Methylobacterium oryzae* having ability to produce high levels of CKs increased the seed germination of maize and sorghum-sudan grass hybrid and improved salinity tolerance of plants [132]. Inoculation with the same strain improved *Lens culinaris* c.v. Medik tolerance to osmotic stress and improved physiological parameters, plant growth, plant endogenous cytokinins' levels [133].

1.4.4. Enhanced nutrient acquisition

A sufficient amount of one or more nutrients required for plant growth is typically deficient in soils. Plants need adequate supplies of essential macro (N, P, K, Ca, Mg etc.) and micro (Fe, Zn, B, Mo, Mn, Cu) nutrients for proper growth and development. As far as plant growth is concerned, N, P and K are the most limiting soil nutrients. Sadly, climate change entails abiotic stresses such as salinity, droughts and extreme temperatures that affect the biogeochemical transformation of these nutrients (N, P and K), rendering them less accessible for plant uptake [134-136]. In order for endophytic bacterial technology to be applicable in the context of soil salinity, it is important to recognize and use salt-tolerant endophytic bacterial strains. As biofertilizers, bacteria inoculants supply the plants with increased amount of essential nutrients for better plant growth, which if limited can inhibit the plant growth and show detrimental effects on plants ultimately leading to reduced crop production [137-139].

With the help of salt tolerant endophytic bacteria, plants can acquire increased amount of deficient plant nutrients including nitrogen, iron, phosphorus, potassium, and zinc which will help plants to grow better under stress conditions [91,131,140].

1.4.4.1. Nitrogen availability

Nitrogen (N) is the most limiting nutrient in the soil. N is an integral part of amino acids and proteins, enzymes, chlorophyll, and several vitamins. N improves the quality and quantity of plant biomass and grain yield [141]. Bacterial endophytes can increase the availability of nitrogen to their host plants. They provide fixed atmospheric N_2 to their host plants via nitrogenase enzyme activity [142]. In a recent study, a total of 316 nitrogen fixing and salinity tolerant endophytic bacteria were isolated from *Suaeda maritima* across 22 different location in Iran. These bacteria belong to phyla

Proteobacteria, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* and only *Zhihengliuella halotolerans* and *Brachybacterium* sp. belonging to Actinobacteria could fix-nitrogen at higher concentration of NaCl (6%). These two strains, when inoculated, increased the plant biomass and contributed to the total nitrogen pool of plant than uninoculated control under salinity stress [143].

1.4.4.2. Phosphorous solubilization

Phosphorous (P) is second most important essential nutrient for plants. P has a key role in photosynthesis, respiration, energy transfer as ADP and ATP (adenosine di- and triphosphate) and DPN and TPN (di- and triphosphopyridine nucleotide), DNA and RNA structure, root development, and flower initiation in plants [141]. P in soils is fixed and P-solubilizing endophytic bacteria utilize various mechanisms to solubilize phosphorous into plant available form including lower down the pH of the medium, chelation and exchange reactions on soil exchange sites, acidification of medium by releasing several kinds of organic acids such as oxalic, malonic, and glycolic acid, however, the type of acid produced vary specie to specie [144,145]. A study reported that P-solubilizers able to produce exopolysaccharides have greater potential to solubilize phosphorous than the isolates without EPS production activity [146]. In another study, Dias and coworkers [147] isolated two endophytic bacterial strains of *Bacillus* (*B. subtilis* and *B. megaterium*) which have the potential to solubilize phosphorous and promote plant growth. Six out of fifty-nine endophytic bacterial strains isolated from the roots of halotolerant plants including *Oenothera biennis* L., *Chenopodium ficifolium* Smith, *Artemisia princeps* Pamp, and *Echinochloa crus-galli* inhabiting sand dunes at Pohang beach in South Korea, were selected on the basis of their tolerance to salt stress, phosphate solubilization, production of phytohormones, siderophores and organic acid. These isolates, when inoculated in rice plants, enhanced the plant growth, sugar and chlorophyll contents as compared to uninoculated control, under salt stress [100].

1.4.4.3. Potassium solubilization

Potassium (K) is third most important essential macro nutrient required by plant for proper growth and development, photosynthesis, grain quality, nitrate reduction, protein, vitamin, and starch synthesis [148]. It gives plant resistance to biotic and abiotic stress. While significant quantities of total K are found in the soil, only a limited fraction is available for plant uptake. Like phosphorus, there are also insoluble forms of anthropogenic potassium deposits in soil. K-solubilizing endophytic bacteria improve the plant's nutrient intake and preserve the soil health and fertility [149]. The major role of K-solubilizing endophytic bacteria is to produce organic acid and helps in solubilizing the K entrapped in the K-minerals. Plant growth promoting bacteria have

been documented to generate polysaccharides, such as gluconate, increased quartz and albite dissolution [150]. In a study, exopolysaccharides produced by bacteria form a mucilage covering around bacterial cell, which increase the organic acid adsorption. These covering then strike with the silicate minerals and solubilize K while chelating silicates [151].

1.4.4.4. Zinc solubilization

Zinc is one of the micronutrients essential for plants at low levels and is harmful when present at high levels. Micronutrients are essential for plants for different processes, including photosynthesis, growth, reproduction and protein and chlorophyll synthesis. Zinc is an essential nutrient, and its deficiency affects not only crop yield but also human health. One of the key factors contributing to malnutrition symptoms in developing countries is zinc [152]. *Bacillus aryabhattai* inoculation into soybean and wheat root reportedly improved dry root weight, increased zinc uptake and auxin production [153]. Inoculation of two bacterial endophytes (*Sphingomonas* sp. SaMR12 and *Sphingomonas* sp. SaCS20) increased the Zn uptake and grain yield of rice plant under hydroponic and soil condition, as compared to uninoculated plants [154]. Another endophytic bacterium, *Bacillus amyloliquefaciens*, has the ability to solubilize P, K, and Zn at the same time [155]. Various genera of bacterial endophytes including, *Acinetobacter*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Paenibacillus*, and *Staphylococcus* isolated from *Phoenix dactylifera* plants have been reported to solubilize zinc [99]. *Bacillus subtilis* and *Arthrobacter* sp., when inoculated, increased the sum of Zn in grains of wheat by two folds through increasing the translocation and fortification of Zn into grains [156].

1.4.4.5. Siderophore production/Iron sequestration

Two species of endophytic bacteria *Pantoea* (*P. ananatis* and *P. agglomerans*) and *Pseudomonas* and *Burkholderia*, isolated from rice showed antagonistic activity and have been documented to produce siderophores [157]. Two strains of endophytic bacteria, *Burkholderia* sp. and *Enterobacter* sp., isolated from the roots of *Taxus wallichiana* Zucc., can tolerate up to 12% NaCl and can produce siderophores, ACC deaminase, IAA, and organic acids. Both strains, when inoculated on rice and soybean plants significantly increased the plant biomass, nodule biomass (soybean), reduced the pH of the soil (organic acids production) and enhanced the nitrogen, phosphorous and potassium contents of the soil [158].

1.5. Concluding remarks and future perspective

Salt stress adversely impact crop growth due to which agricultural production processes are likely to fail. Simultaneously, the researcher's emphasis has centred on using sustainable, friendly, and environmentally safe agriculture practices. However, several researchers have confirmed the potential of salt-tolerant endophytic bacteria to mitigate the harmful impacts of salinity stress in a more eco-friendly and cost-effective manner, even where salt-tolerant crop varieties and genotypes have not seen much success. Research projects aimed at leveraging bacterial endophytes to increase crop productivity in salt-affected agricultural fields provide compelling evidence for commercializing microbial formulations for improving salinity tolerance of crop plants. The further modulation of plant-endophyte interactions opens a new and prospective window to prevent plants from various abiotic stresses. The ability of endophytic bacteria to help plants cope with salinity stress in arable soils appears massive. Still, more developments are needed to be introduced in a real-life mass-adopted technology for salt-tolerant endophyte management. Future attention on the following points should be taken urgently:

1. Multi-crop and multi-location field studies of salt tolerance bacterial endophytes in salt affected soils are required to improve their usefulness further. Field performance of plant growth promoting bacteria depends on crop type (cultivar) [30] and colonization ability [159].;
2. In-depth research is required to explore the relationships between bacterial inoculants and indigenous native flora and fauna. It can open new windows for microbial technology. As reported by [160], microbiome population functions can be predicted from the additive functions of inoculated individuals.
3. Another important area of research is the genetic and molecular basis of halotolerant endophyte-mediated salinity tolerance in plants. Using proteomics, genomics, metabolomics, and nanotechnology methods, the dissection of network-web linked to the regulation of defense-related gene signaling and pathways to overcome salt stress in plants is needed.
4. Behavioural studies of endophytic bacteria inside plants having specific characteristics, using deficient mutants of same bacteria strain. As documented by [82,104], ACC deaminase deficient mutants of bacteria did not lower the ethylene levels of inoculated plants.

5. More research needed for K-solubilizing endophytic bacteria as K is third most important essential macronutrient required for better plant growth, a very little research has been done so far.
6. In future another important step will be to use consortium of endophytic bacteria with other plant growth promoting bacteria having multiple capabilities, and endophytic with arbuscular mycorrhizal fungi. According to [161] co-inoculation of plant growth promoting bacteria and arbuscular mycorrhizal fungi increased the maize growth under salt stress.
7. How plants recognize the helping endophytes? It is necessary to investigate the molecular factors that control the identification of salt-tolerant endophytes by plants. It would be essential to understand better the strain-specific activation of gene-mediated pathways in various crops.

Table 1. 1: List of recent scientific reports on improving salinity tolerance in plants using plant growth promoting endophytic bacteria

Endophytic bacteria	**Bacterial tolerance to salinity (NaCl)**	**Bacterial characterization**	**Experimental plant**	**Type of experiment**	**Intensity of salt (NaCl) stress on plant**	**Effects on plant growth under salinity**	**References**
Achromobacter xylosoxidans	5 mM	Motility, CAT[1] production, N-fixation	*Catharanthus roseus*	*In-vitro* and pot assay	50, 100 and 150 mM	ACCD activity reduced the ethylene production by plants, rapid regulation of antioxidants increased plant biomass	[86]

Pseudomonas fluorescens YsS6, *Pseudomonas migulae* 8R6	4%	ACCD[2] and PS[3] activity, IAA[4] and SID[5] production	*Solanum lycopersicum* H 72	Pot assay	165 and 185 mM	Inoculation with ACCD producing EB increased plant biomass, chlorophyll contents and number of flowers	[82]
Isoptericola dokdonensis KLBMP 4942, *Streptomyces pactum* KLBMP 5084, *Arthrobacter soli* KLBMP	0-13%	ACCD[2] activity	*Limonium sinense*	In-vitro assay	0-250 mM	Stimulated plant growth, influenced falvonoids accumulation	[98]

5180 and *B. flexus* KLBMP 4941							
Paenibacillus xylanexedens PD-R6 and *Enterobacter cloacae* PD-P6	50-200 mM	ACCD[2] activity and IAA[4] production	*Brassica campestris*	Gnotobiotic assay	100 mM	Modulated plant ethylene and IAA levels, increased nutrient uptake	[99]
Bacillus sp. EZB4 and *Arthrobacter* sp. EZB8	-	ACCD[2] activity, IAA[4] production	*Capsicum annuum*	In-vitro assay	-	Increased free proline contents in plants, mitigated the effects of osmotic stress	[102]

						induced by PEG-6000	
Pseudomonas pseudoalcaligenes	-	-	*Oryza sativa*	*In-vitro* and pot assay	0.5-2.5 g/kg of soil	Increased shoot biomass by synthesizing quaternary compounds like glycine betaine, induced elevated levels of antioxidants	[103]
Pseudomonas putida UW4	-	ACCD[2] activity,	*Brassica napus*	Hydroponic assay	250 mM	Alleviated salinity stress on plants, expression of antioxidative processes,	[104]

						photosynthesis, and membrane transportation-related proteins	
Pseudomonas aeruginosa PW09	2-8%	Biofilm formation, PS^3, antagonistic activity against *Sclerotium rolfsii*	*Cucumis sativus*	Pot assay	150 mM (EC 2.09 dSm^{-1} per pot)	Increased the accumulation of free phenolic and proline contents and the activity of enzymes involved in plant defence mechanisms under pathogen and salt stress	[79]

Bacillus subtilis (BERA 71)	1.5%	ACCD[2] activity, IAA[4] production and PS[3]	*Cicer arietinum*, cv. Giza 1 and *Acacia gerrardii* Benth.	Pot assay	200 mM	Increased plant biomass and chlorophyll contents, decreased ROS and lipid peroxidation in plants, enhanced enzymatic and non-enzymatic antioxidants' activity, decreased Na^+ concentration and increased	[57,101]

						nutrient uptake	
Brachybacterium paraconglomeratum SMR20	75-150 mM	ACCD[2] activity, IAA[4] production	*Chlorophytum borivilianum*	Pot assay	150 mM	Decreased osmotic and oxidative damage from plants, decreased lipid peroxidation, ethylene and ABA levels, enhanced IAA and chlorophyll contents and nutrient uptake of plants	[123]

Pseudomonas putida PIR 3C and *Raoultella terrigena* PCM 8	58-122 mM	ACCD[2] activity	*Glycine max*	In-vitro assay	58-122 mM	Increased plant biomass, reduced ethylene production and increased α-ketobutyrate, chlorophyll contents and germination percentage	[116]
Pseudomonas migulae 8R6	-	ACCD[2] activity	*Camelina sativa*	Pot assay	192, 213 mM NaCl or EC=15dSm^{-1}	Improved tolerance to salinity, modulation in ethylene and ABA levels,	[124]

						increased root biomass	
Bacillus amyloliquefaciens RWL-1	120, 250 mM	ABA[6], GBs[7], and essential amino acids (glutamic acid and proline) production	*Oryza sativa* 'Jin so mi'	Pot assay	120, 250 mM	Improved plant growth and biomass, upregulation of essential amino acids (glutamic acid, aspartic acid, phenylalanine, proline, and cysteine), reduced the levels of ABA and increased levels of SA,	[92]

Leclercia adecarboxylata MO1	120 mM	IAA[4] production and ACCD[2] activity	*Solanum lycopersicum*	Pot assay	120 mM	Improved salinity tolerance, increased plant biomass, sugar and amino acids concentrations, lowered ABA levels	[119]
Curtobacterium oceanosedimentum SAK1	100-400 mM	GBs[7], ABA[6], JA[8] and organic acid production and ACCD[2] activity	*Glycine max* cv. Pungsannamul	Pot assay	100, 200, 300 mM	Improved salinity tolerance, increased number of root tips due to GBs and IAA production,	[127]

						decreased ABA and JA contents, reduced ethylene levels, antioxidants' production	
Methylobacterium oryzae CBMB20	50, 100 mM	CKs[9] production, ACCD[2] activity, tolerance to drought, salinity, UV irradiation, heat, different temperature regimes, oxidative stress, starvation, biofilm formation,	*Zea maize* and sorghum-sudan grass hybrid	Gnotobiotic pouch assay	150 mM	Increased seed germination, improved tolerance to saline stress	[132,162,163]

		exopolysaccharides production, proline accumulation					
Curtobacterium oceanosedimentum SAK1, *Curtobacterium luteum* SAK2, *Enterobacter ludwigii* SAK5, *Bacillus cereus* SA1, *Micrococcus yunnanensis* SA2,	150 mM	PS[3], IAA[4], GBs[7] and SID[5] production	*Oryza sativa* 'Jin so mi'	Pot assay	150 mM	Improved plant tolerance to salinity, reduced ABA and glutathione contents, expression of flavin monooxygenase (*OsYUCCA1*) and auxin efflux carrier	[100]

Enterobacter tabaci SA3						(*OsPIN1*) genes	

[1] Catalase, [2] 1-aminocyclopropane-1-carboxylic acid deaminase, [3] Phosphate solubilizer, [4] Indole 3-acetic acid, [5] Siderophore, [6] Abscisic acid, [7] Gibberellins, [8] Jasmonic acid, [9] Cytokinins.

Chapter 2-

Can bacterial endophytes be a promising bio-inoculant for crop plants to combat salinity stress? A global meta-analysis of last decade (2011-2020)

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Abstract

Soil salinity is a major problem affecting crop productivity worldwide. There have been great re-search efforts in increasing the salt tolerance of plants through inoculation of endophytic bacteria, however, the comparative growth promoting effect under non-saline and saline stress conditions remains largely uncertain. Therefore, a global meta-analysis was conducted from forty-two peer-reviewed articles to quantify the growth promoting effects of endophytic bacterial inoculation and underlying mechanisms under non-saline and saline conditions. A total of seventy-seven ex-periments, including 25 different bacterial genera, were evaluated. On average, endophytic bacte-rial inoculation increased total fresh and dry biomass by 68% and 32%, respectively. The effect of endophytic inoculation on the growth-related attributes was always higher under salinity stress than non-saline condition. Under saline condition, the promoting effect of endophytic inoculation on germination rate, root biomass, root length and shoot length, was double or more than that under non-saline conditions. On morphological level, preferential improvement in plant biomass under salinity stress than non-saline condition was linked to preferential improvement in germination rate, shoot and root lengths and number of leaves. On physiological level, the relative better performance of the bacterial inoculants under saline condition was found associated with increase in total chlorophyll and chlorophyll b and lowering of ACC concentration. Moreover, under saline condi-tion, bacterial inoculation conferred significantly higher increase in root K^+ concentration and de-crease in leaf Na^+ concentration. In salt-sensitive (SS) plants, bacterial inoculation induced increase in chlorophyll-b and decrease in abscisic acid content was higher than salt tolerant (ST) plants. Under salinity stress, endophytic bacterial inoculation increased root K^+ concentration in both SS and ST plants, but decreased root Na^+ concentration only in ST plants. Overall, this meta-analysis suggests that endophytic bacterial inoculation is beneficial under both non-saline and saline con-ditions, but definitely the magnitude of benefit is higher under salinity stress and varies with the salt tolerance level of plants.

2.1. Introduction

Soil salinity is one of the biggest threats to agricultural production and food security. It was estimated that more than 6% of global land is currently salt affected[164]. In the event of climate change, irrational irrigation methods, improper application of fertilizers, and inadequate drainage networks, this situation is getting worst every day. It is estimated that 50% of arable land will be under serious salinity risk by 2050 [165-168]. The soil salinity imposes a blend of morphological and physical effects including impeded nutrients uptake, seed germination and overall plant growth. Short after exposure to salinity, plants face osmotic stress, which is followed by ion toxicity and

nutrient imbalance. Osmotic stress leads to the formation of hypertonic conditions outside the cell, act condition of pseudo water deficiency which make it impossible for plants to take up water. Ion toxicity is caused by the overaccumulation of sodium (Na^+) and chloride (Cl^-) ions within the cells. Excessive amounts of Na^+ and Cl^- damage plant cell walls, disturbs the osmotic balance, and modifies ion homeostasis within the cell, which ultimately induce changes in transpiration rate, translocations of nutrients, photosynthesis, and other metabolic processes [169]. In addition, soil salinity could indirectly minimize plant growth by hindering the activities of beneficial microbes living in the rhizosphere and reducing the accumulation of organic matter. To cope with salinity stress plants have evolved different physiological mechanisms including osmolyte aggregation, ion homeostasis, water absorption control, and antioxidants synthesis [170]. Moreover, plants establish interactions with a plethora of microorganisms that promote plant growth and mitigate plant stress [171]. Among those, endophytic bacteria showed to relive salt stress in plants by inducing osmotic adjustment, detoxification, modulation of phytohormones, and acquisition of nutrients [5]. Endophytic bacteria having 1-aminocylopropane,1-carboxylate (ACC) deaminase and indole-3 acetic acid production, nitrogen fixation, phosphate solubilization, siderophore production ability, had been concluded to promote the osmotic or ionic adaptation of the host plants [127,162,172,173].

Integrating the data across investigations through a meta-analysis might help to understand the extent and mechanisms of stress mitigation conferred by bacterial endophytes and would broader the use of endophytic bacteria in sustainable agriculture. A meta-analysis is a tool that synthesizes knowledge using a specific methodological procedure for data aggregation and analysis from various individual scientific studies [174]. It is particularly useful for answering study questions of great versatility and uncovering emergent properties within individual studies that would otherwise go undetected. The power of a meta-analysis becomes obvious when the outcomes of particular experiments vary in various laboratory conditions. Therefore, we used a meta-analysis for assessing the efficacy of endophytic bacterial inoculation in plant salinity stress mitigation. Although, a few meta-analyses have reported on the effect of inoculation of plant growth promoting rhizobacteria to improve abiotic stress tolerance of plants [168,175,176], however, the overall effect of endophytic bacterial inoculation to improve plant heavy metal tolerance have recently published by Franco-Franklin et al. [177]. Nevertheless, only Rho et al. (2018) have dedicated a small portion of their meta-analysis, consisting of a few studies, to the overall effect of bacterial endophytes to improve plant salinity tolerance. Moreover, there is no meta-analysis that has dissected the effects of endophytic bacteria on salt sensitive (SS) or salt tolerant (ST) host plants under salinity stress conditions as compared to non-saline conditions.

In this study, we hypothesized that (i) endophytic bacterial performance is better under salinity stress and (ii) salinity stress mitigation conferred by endophytic bacteria varies across SS and ST plants. To test our postulates, we extracted raw data from 42 articles

and conducted a meta-analysis. In addition to evaluate endophytic bacterial performance under salinity and non-saline condition, we classified the host plants into SS and ST groups, and then individually scored the effects sizes of each group to compare the bacterial effects on two types of host plants.

2.2. Materials and Methods

2.2.1. Database search and selection criteria

Metadata was obtained following PRISMA reporting guidelines [178,179]. A literature search was conducted in December 2020 using SCOPUS® (http://www.scopus.com) and Web of Science® (https://webofknowledge.com/) databases. Articles published in scientific journals in the English language only were retrieved using the following keywords combination: "plant growth promot*" AND "endophyt*" AND "bacteria*" AND ("salinity" OR "salt") AND "stress". The Boolean truncation ('*') character was included to ensure the variations of the words, such as promoting or promotion, endophyte or endophytic, and bacteria or bacterial. The logical operator ("AND") was used to refine articles that contained words written on both sides of the operator. The decision about the inclusion or exclusion of an article in study was made with mutual discussion between the authors.

2.2.2. Study selection

Research Metadata search from both databases yielded 227 articles, 150 of which were left after duplicate removal. To eliminate the publication bias, following eligibility criteria were predefined and adopted for the study selection:

1. The study should contain at least one bacterial endophyte irrespective of the colonization rate.
2. Bacterial inoculum should not include additives such as amino acid, humic acid, protein hydrolysate etc.
3. Both bacterial-inoculated and non-inoculated plants must have been evaluated under salinity-stress and no-stress conditions. If several levels of salinity stress are investigated in a study, the highest level shall be selected for this analysis.
4. One of the following parameters: biomass (yield and weight) or plant height, must have been reported in the study.
5. The results should have reported the means, standard errors/deviations, sample size and other relevant statistical information to calculate the effect size.

The studies that not fulfilling the above prescribed criteria were excluded from the analysis. If any of the traits was measured over time, the data only for the final date were considered. From the identified 150 article, only 42 met our selection criteria, and thus

proceeded to the analysis (Figure 2.1). The selected papers span last 10 years (2011-2020) (Figure 2.2).

2.2.3. Data extraction

Treatment means, sample size (number of replications [n]) and standard deviations were extracted from each study. If the standard error (SE) was given in a study, it was converted into standard deviation (SD) using following equation SE = SD ($n^{-1/2}$). The data given in the form of graphs were digitized using WebPlotDigitizer [180]. Since multiple experiments from one study do not increase the dependence of the meta-analysis on that study [181], different treatments or host/endophyte variants from the same article were regarded to be independent experiments and described in the study as a separate experiment. This technique increases the power of meta-analysis [182] and has been used in several meta-analyses [183-185].

Parameters related to plant biomass, photosynthetic rate, ion homeostasis, and metabolites (enzymatic and non-enzymatic) were collected from each study. To maintain the heterogeneity in each observation, parameters found in less than five data units were excluded from the study.

2.2.4. Meta-analysis

To estimate the effect sizes of bacterial endophytes under no-stress and salinity-stress conditions, log response ratios (ln*RR*) were calculated as the metrices of effect sizes using the following formula: ln*RR* = ln (V_i/V_c), where V_i is the mean of inoculated treatment and V_c is the mean of non-inoculated treatments [186]. Calculating ln*RR* as an effect size metric is appropriate because log transformation of the parameter(s) reported in different units among studies maintains symmetry within the analysis [21]. Furthermore, percent change (%Δ) can easily be calculated from ln*RR*, i.e. %Δ = (exp.(ln*RR*)-1*100). Pooled variances were calculated using the "escalc" function in the "metafor" (version 2.4-0) package of the R environment [187].

Prior to constructing the meta-analysis model, a heterogeneity test was performed to determine the choice of fixed or random/mixed effect model. Heterogeneity (Q) of the full dataset, including 1214 observation, was highly significant (Cochran's Q=164278, df=1213, $p < 0$), indicating that a random/mixed effects approach is guaranteed [188].

The data synthesis produced by this meta-analysis is balanced based on the weight of each study, to maintain their equal contribution to the results produced by the meta-analysis. In this study, the inverse variance method was used to assign the weights using meta and metafor packages in R. Estimated pooled effect sizes produced by the meta-analysis with their 95% confidence intervals (95% CI) were presented in forest plots.

The effect of inoculation with bacterial endophytes was considered significant if 95% CIs did not coincide with the zero line.

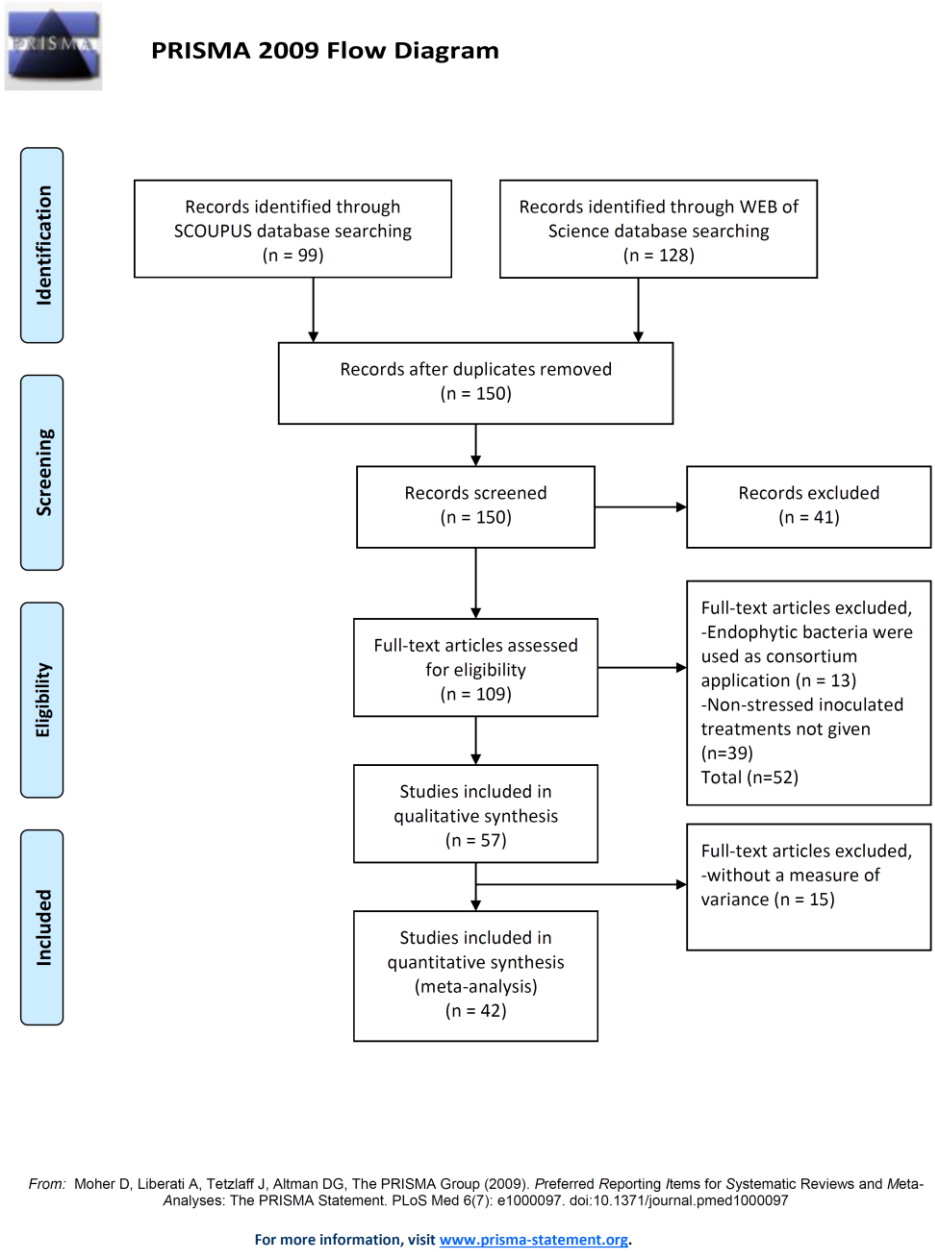

Figure 2. 1: Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram for the meta-analysis [179]

Overlaps on the zero line mean that there was no significant effect of inoculation and it is denoted by 'ns' [189]. A positive value indicates an increase and a negative value indicate a decrease in the effect size of plants inoculated with endophytic bacteria, which are denoted by percent change (±%). Statistical analyses were performed in the R environment (https://r-project.org/) using "metafor" [187], "meta" [190] and "ggplot" [191] packages.

2.3. Results

2.3.1. Metadata

Metadata was extracted from 42 peer-reviewed articles published in 21 countries between 2011 and 2020 (Figure 2.2 and 2.3). A total of 1214 observations (k) were obtained from a sum of 77 experiments. For each study, we used the uniform selection criteria, which involved endophytic bacterial inoculants and their usefulness for crop plants in both no stress and salinity stress conditions. Seed inoculation was used in 52% (k=632) observations, while seedling and soil inoculation methods were used in 26% (k=316) and 22% (k=266) observations, respectively (Figure 2.4a). The majority of the experiments (64% of the total) were conducted in pots, followed by *in-vitro* (27%), hydroponic (6%) and (3%) growth room trials (Figure 2.4b). In total, 24 bacterial genera of endophytes, 15-Gram negative and 9-Gram positive, were identified from the extracted meta data. Among gram-positive bacteria, *Bacillus* and *Streptomyces* were the most commonly used genera for inoculation. In case of gram-negative bacteria, *Pseudomonas*, *Pantoea*, *Burkholderia* and *Sphingomonas* were the notable genera used as inoculants in these experiments. Our extracted meta-data also identified miscellaneous bacterial genera, each with a contribution of <4% to the total experiments (Figure 2.4c).

2.3.2. Effects of endophytic bacterial inoculation on plant biomass and photosynthesis under salinity stress vs no-stress

In general, endophytic inoculation significantly enhanced all plant growth-related parameters except stem diameter (Figure 2.5a). This positive effect occurred in both no-stress as well as salinity stress conditions, although the effect size was larger when endophytic inoculation was carried out under salinity stress. The magnitude of plant growth promotion by endophytic inoculation ranged from 28 to 100% in salinity, and from 10 to 64% in no stress when compared to no-inoculated conditions. When summing the effect of growth conditions on endophytic performance, significant plant growth promotion effect was evident for total dry biomass, shoot length, root length,

root biomass and germination rate (Figure 2.5a). In both conditions, dry biomass and shoot length were by far the most responsive plant growth attributes to endophytic inoculation (Figure 2.6a; $p < 0.0001$).

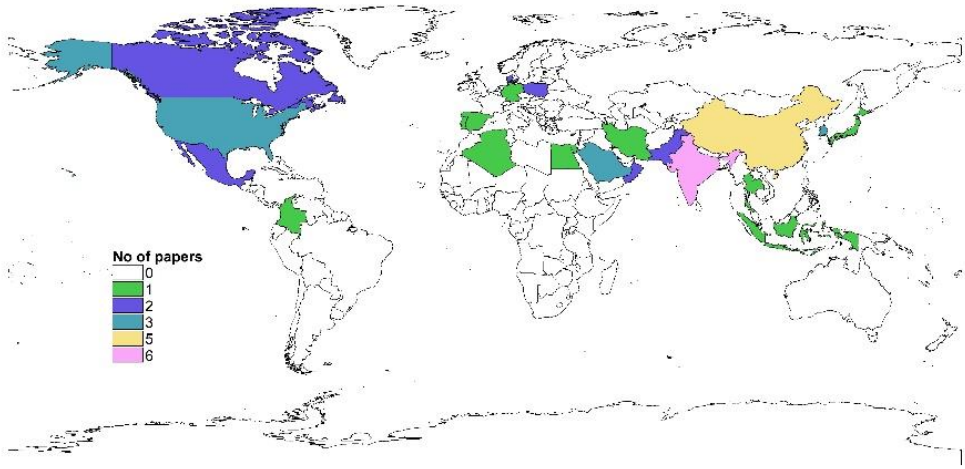

Figure 2. 2: Location of the experiments obtained from the selected studies (42) used in this meta-analysis. https://www.r-spatial.org/r/2018/10/25/ggplot2-sf.html

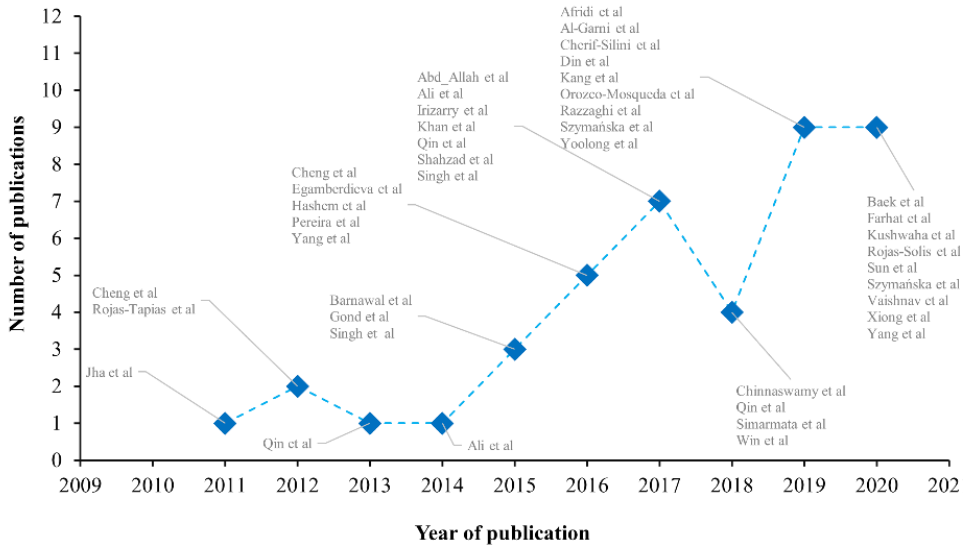

Figure 2. 3: The accumulated number of publications in this meta-analysis reported with in last 10 years (2011-2020). The data labels on each scatter point show the author names in that year

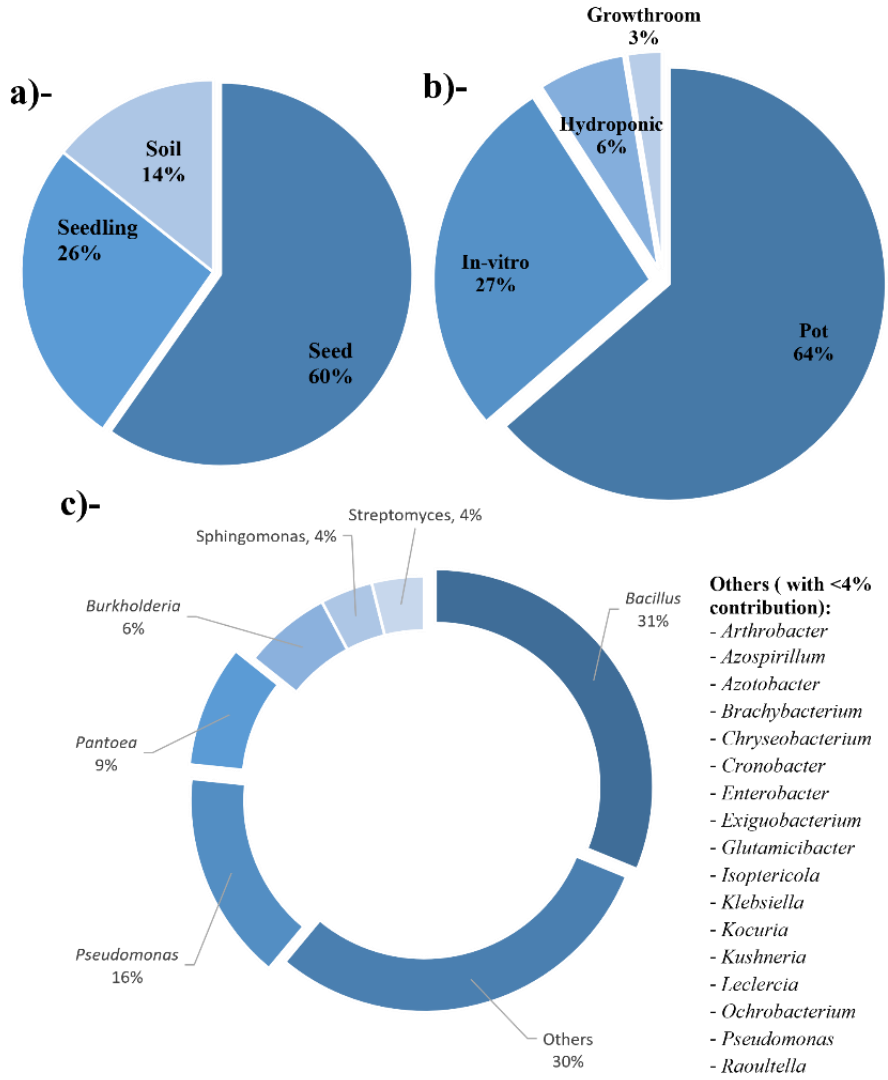

Figure 2. 4: General information about the 614 observations and 78 experiments obtained from 42 studies selected in this meta-analysis, a)- Inoculation method, b)- Experimental conditions, and c)- Genera of bacterial endophytes

The meta-analysis results showed that there was significant increment effect of endophytic inoculation on most of the plant photosynthetic attributes under salinity stress (Figure 2.5b). However, the rate of photosynthesis and carotenoids content followed the opposite pattern with endophytic inoculation, accounting for greater effects size under no stress conditions. Interestingly, endophytic inoculation resulted in significant decrease in leaf abscisic acid content, 28% in salinity stress and 24% in no

stress, when compared to uninoculated conditions. With regard to growth condition related efficiency of endophytes, significant differences were only detected in total chlorophyll, chlorophyll-b and leaves number of inoculated plants.

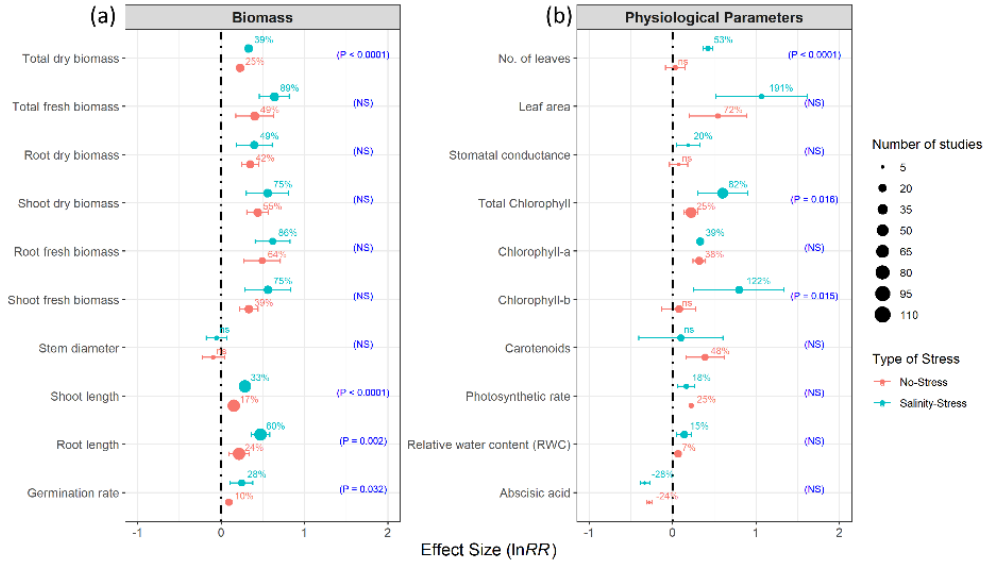

Figure 2. 5: Effect of endophytic bacterial inoculation on (a) Biomass, and (b) Physiological Parameters under no-stress and salinity-stress treatments. Error bars represent 95% confidence intervals (CIs). The inoculation effects were considered significant if the 95% CIs did not overlap the zero line. Size of the scatter point shows the number of experimental observations. P-values and 'NS' in parenthesis show the significant and non-significant differences, respectively, across the growth conditions (no-stress vs salinity stress)

2.3.3. Effect of endophytic bacterial inoculation on plant antioxidant enzymes, and ionic homeostasis under salinity-stress vs non-stress

Irrespective of plant stress types, response effect of POD activity and glutathione reductase to endophytic inoculation was statistically comparable (Figure 2.6a). In contrast, antioxidants activity (e.g., SOD and CAT) and proline contents were up-regulated by endophytic inoculation both under non-saline and saline conditions. Importantly, endophytic inoculation greatly decreased ACC-concentration (salinity stress 40% vs. no stress 17%) and MDA contents (salinity stress 30% vs. no stress 18%). Across all ionic homeostasis observations, root K concentration of salinity exposed plants was the most responsive ionic variables to endophytic inoculation, amounting to

39% higher root K localization than that of uninoculated plants (Figure 2.6b; $p < 0.0001$). Similarly, leaf analysis of inoculated plants had shown significant K accumulation by 27 and 32% for salinity stress vs. no stress conditions, respectively. With endophytic inoculation, leaf Na content in stressed plants was decreased by 23% compared to uninoculated conditions.

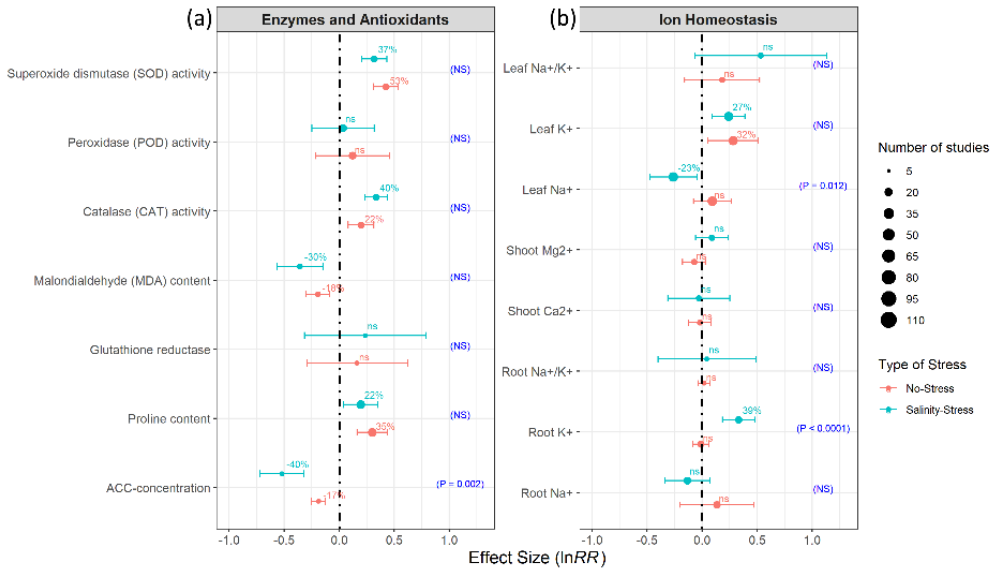

Figure 2. 6: Effect of endophytic bacterial inoculation on (a) Enzymes and Antioxidants, and (b) Ion Homeostasis under no-stress and salinity-stress treatments. Error bars represent 95% confidence intervals (CIs). The inoculation effects were considered significant if the 95% CIs did not overlap the zero line. Size of the scatter point shows the number of experimental observations. P-values and 'NS' in parenthesis show the significant and non-significant differences, respectively, across the growth conditions (no-stress vs salinity stress)

2.3.4. Comparative effects of endophytic bacterial inoculation on growth of salt sensitive- and salt-tolerant plants

In the context of salt sensitive plants, endophytic bacterial inoculation had the strongest influence on plant growth parameters, such as shoot and root length, total fresh and dry biomass, shoot fresh and dry biomass, root fresh biomass and germination rate, when plants were exposed to salinity stress (Figure 2.7a). When data was summed up for growing conditions, shoot length and total dry biomass were the most responsive to endophytic inoculation (Figure 2.7a; $p < 0.0001$). Moreover, root biomass and germination rate also showed greater degree of responsiveness to endophytic inoculation. In case of salt tolerant plants, effect size of endophytic inoculation for plant

biomass was relatively larger in salinity stress compared to no stress condition. In term of growth conditions, only root length demonstrated a substantially higher response effect to endophytic inoculation (Figure 2.7b; $p < 0.0001$).

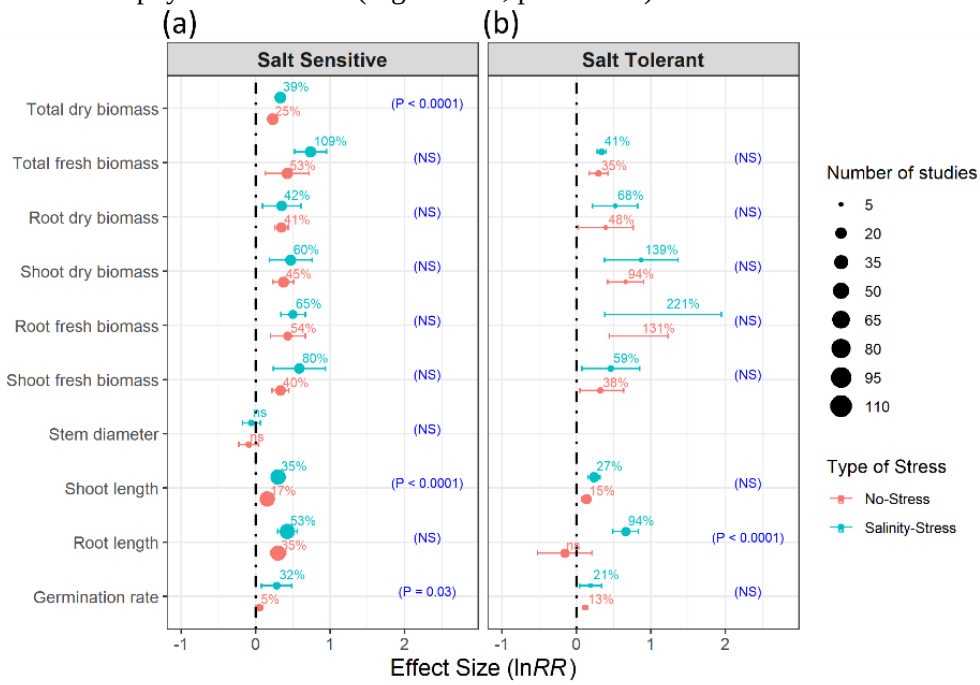

Figure 2. 7: Effect of endophytic bacterial inoculation on biomass related parameters of (a) Salt Sensitive, and (b) Salt Tolerant plants under no-stress and salinity-stress treatments. Error bars represent 95% confidence intervals (CIs). The inoculation effects were considered significant if the 95% CIs did not overlap the zero line. Size of the scatter point shows the number of experimental observations. P-values and 'NS' in parenthesis show the significant and non-significant differences, respectively, across the growth conditions (no-stress vs salinity stress)

Regarding photosynthetic pigmentation in salinity stress, total chlorophyll and chlorophyll-b had a greater response effect to endophytic inoculation in salt sensitive plant, while chlorophyll-a and carotenoids content showed significant improvement in salt tolerant plants (Figure 2.8a and b). Endophytic inoculation to both salt tolerant and sensitive plants displayed significant increase in the number of leaves. Despite the larger effect size of endophytic inoculation on the leaf area of salt tolerant plants, salinity stress exposure to salt sensitive plants induced significant expansion in the leaf area after endophytic inoculation compared to non-stressed plants.

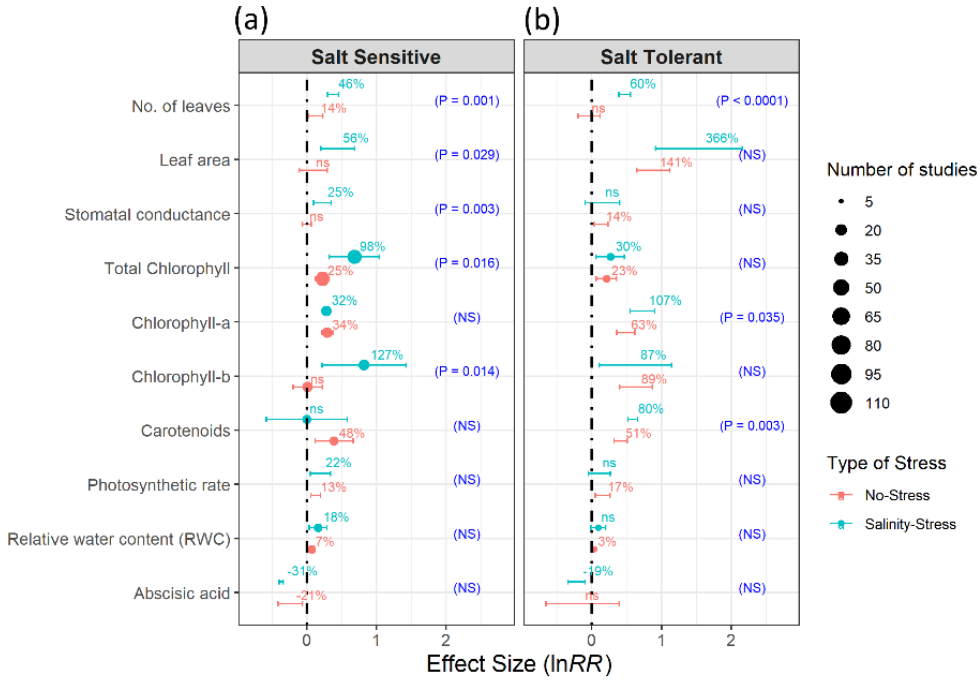

Figure 2. 8: Effect of endophytic bacterial inoculation on physiological parameters of (a) Salt Sensitive, and (b) Salt Tolerant plants under no-stress and salinity-stress treatments. Error bars represent 95% confidence intervals (CIs). The inoculation effects were considered significant if the 95% CIs did not overlap the zero line. Size of the scatter point shows the number of experimental observations. P-values and 'NS' in parenthesis show the significant and non-significant differences, respectively, across the growth conditions (no-stress vs salinity stress)

Notably, stomatal conductance, relative water content and photosynthetic rate showed greater response effect of endophytic inoculation in salt sensitive plants. For abscisic acid, both salt sensitive and tolerant plants demonstrated significant decrease following endophytic inoculation under salinity stress; however, this effect was insignificant compared to inoculant effectiveness across growth conditions. Overall, antioxidants activity and stress osmolytes synthesis in salt sensitive and tolerant plant were not significantly affected by endophytic inoculation when compared across growth conditions (Figure 2.9a, b). In contrast, significant up-regulation of SOD activity and lowering of ACC-concentration was exhibited by salt sensitive plants in response to endophytic inoculation, and this effect size was also noticeable across growth conditions (Figure 2.9a and b). For salinity related studies conducted on salt sensitive plants, on average, endophytic inoculation had pronounced effect on Na^+ (decrease by 22%) and K^+ (increase by 41%) ionic homeostasis across leaf and root tissues, respectively (Figure 2.10a). Moreover, these significant differences were also reflected across growth

conditions. In salt tolerant plants, a significant increase in root K^+ content and decrease in root Na^+ content prompted lower root Na^+/K^+ under salinity stress (Figure 2.10b). The endophytic inoculation also decreased leaf Na^+ content under both salinity and no stress conditions, although this effect was not significant across growth conditions.

Figure 2. 9: Effect of endophytic bacterial inoculation on enzymes and antioxidants of (a) Salt Sensitive, and (b) Salt Tolerant plants under no-stress and salinity-stress treatments. Error bars represent 95% confidence intervals (CIs). The inoculation effects were considered significant if the 95% CIs did not overlap the zero line. Size of the scatter point shows the number of experimental observations. P-values and 'NS' in parenthesis show the significant and non-significant differences, respectively, across the growth conditions (no-stress vs salinity stress)

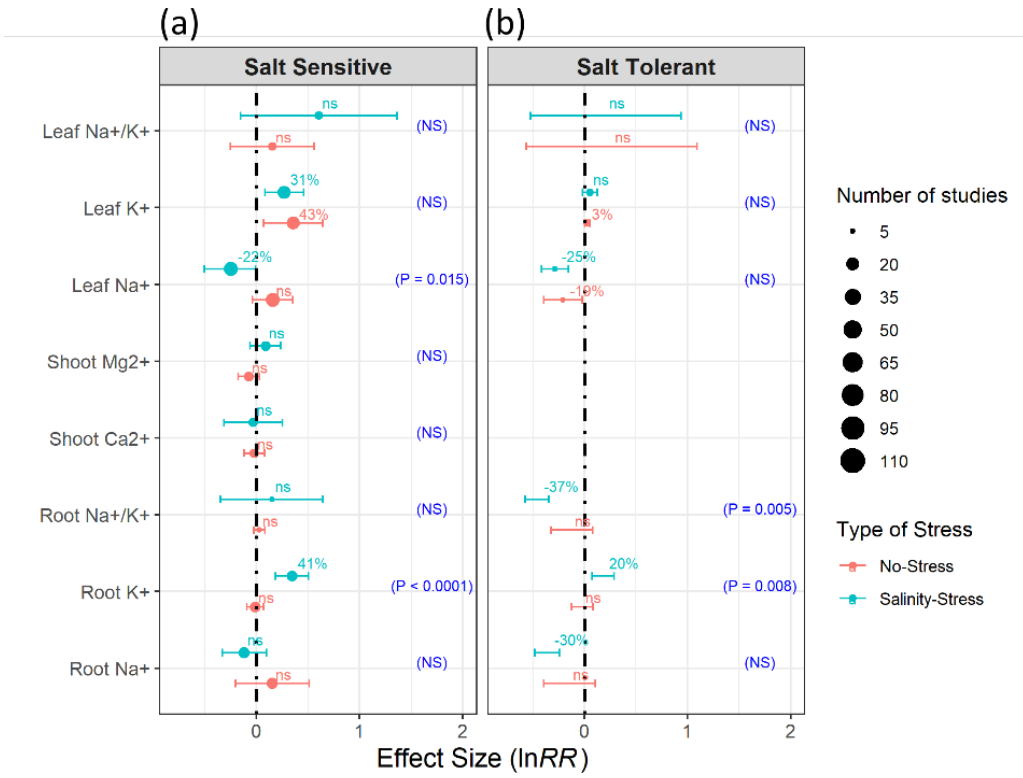

Figure 2. 10: Effect of endophytic bacterial inoculation on ion homeostasis of (a) Salt Sensitive, and (b) Salt Tolerant plants under no-stress and salinity-stress treatments. Error bars represent 95% confidence intervals (CIs). The inoculation effects were considered significant if the 95% CIs did not overlap the zero line. Size of the scatter point shows the number of experimental observations. P-values and 'NS' in parenthesis show the significant and non-significant differences, respectively, across the growth conditions (no-stress vs salinity stress)

2.4. Discussion

Agricultural intensification has resulted in higher crop yields over the last 50 years, but salinity stress remains a major threat to food security, severely limiting the growth and yield potential of crops worldwide [192,193]. In general, our meta-analysis on the subject-matter shows that salinity stress has garnered a great deal of attention in the last two decades for research and policy-framework to improve the productive potential of these marginal lands (Figure 2.1 and 2.3). The findings of this meta-analysis endorse that beneficial endophytic associations in crop plants can promote higher plant growth under salinity stress [194-196], as evidenced by improved germination rate, regulated Na/K homeostasis higher chlorophyll contents, and relative water content.

Identifying the peculiar biochemical characteristics of endophytic inoculant that effectively confer growth promoting benefits to crop plants under a variety of environmental conditions is critical if we are to develop these bioinoculants for successful field application. Our synthesized data demonstrate that the majority of endophytic inoculant used in the studies are gram positive bacteria (Figure 2.4). This is a significant finding as gram positive bacteria have previously been less documented in term of their application for plant growth promoter than gram negative bacteria [197]. Importantly, many gram-positive bacteria are spore-forming, and characterize for numerous bioactive compounds and secondary metabolites that could offer competence over other inoculants to enhance plant growth under stress agriculture [198]. Moreover, our meta-analysis revealed that seed inoculation was the most-commonly used method of introducing endophytic inoculum, and the results further reinforce that it is a relatively effective method, especially in salinity stress, when compared to seedling inoculation. Previous research has shown that assemblage of putative plant microbiomes from starting seed community are highly efficient root colonizer, a primary criterion for any suitable inoculant, and crucial in shaping up the early plant growth with some footprints of plant development in lateral stages [199,200]. Also, we found out that most of the endophytic inoculation related data is of pot experiments. This is again a stern reminder that use of bioinoculants as an appealing farm deliverable product for field scale application has yet to be realized.

The magnitude of plant adaptations to salinity stress is typically assessed by the gains in plant biomass [169]. The observed differences for seed germination, shoot and root biomass of inoculated plants in salinity stress are in agreement with previous work showing positive impact of endophytic inoculation on plant biomass such as plant colonization, auxins production and ACC-deaminase production [201,202]. According to the meta-analysis, the beneficial effects of endophytic bacterial inoculation on biomass production were higher under salinity stress than that under non-saline conditions and were associated with improved plant responses to enzymatic activities and ionic homeostasis under salinity stress. Plants grown in non-saline conditions are supposedly growing in optimal conditions with no growth constraints, where beneficial effects of the inoculation are driven by the additional supply of water and nutrients availability. However, under saline condition, the plants are under stressed and benefit from both from stress amelioration as well as of additional improvements in nutrients are and water uptake following bacterial inoculation. Hence, the higher improvements in plant biomass (root and shoot) with bacterial inoculation under salinity than non-saline condition are attributed to the physiological changes in plants, antioxidants

activity, photosynthesis, osmoregulation and ion homeostasis, whereby alleviating the salt stress [203,204].

Chlorophyll and carotenoids are important pigments for photosynthesis and prepare glucose which is needed for the growth of plants [205]. Sugars and carbohydrates play critical roles in signalling and defending stressed plants because they serve as the primary structural framework and energy supply for biomass processing and maintenance [206]. This meta-analysis showed that bacterial inoculation improved chlorophyll and carotenoid contents under both non-saline and saline conditions. Similarly, Yang et al. [207] found that photosynthetic rate of plant inoculated with endophytic bacteria was significantly increased for plants growing in non-saline and salinity-stress plants. The significantly higher increase in plant chlorophyll contents under salinity stress as compared to non-stressed conditions are also in line with recent findings [173,208].

Because of the lower osmotic potential, elevated salinity limits the water uptake by plant roots [170,209]. As a result , osmoregulation becomes an essential mechanism to overcome plant osmotic stress triggered by high salt concentration [210]. During osmoregulation, Plants expend bulk of the energy to accumulate and synthesize osmolytes at the expense of plant biomass [211,212]. In our study, proline accumulation was significantly increased in both stressed and non-stressed plants inoculated with bacterial endophyte (Figure 2.7). These findings are in accordance with the research outcomes that IAA, ACC deaminase and exopolysaccharides producing *Bacillus* strains enhanced seedling growth, germination rate and chlorophyll content under high levels of salinity stress and proline contents increased with bacterial inoculation under salinity [213,214].

Salinity stress can disrupt the equilibrium of ROS, resulting in oxidative damage to plants [215], destabilizing the membranes and impeding photosynthesis [216]. To prevent cells from ROS damages, plants synthesize ROS-scavenging enzymes, such as SOD and CAT, and reduce the levels of MDA [217]. In this meta-analysis showed that bacterial inoculation significantly increased CAT and SOD activities, and resultantly MDA contents were decreased (Figure 2.7a). In general, salinity-led oxidative stress decreases the photosynthesis by modifying photosynthetic pigments and reducing photosynthetic rate [218]. Thus, improved photosynthesis in inoculated plants may also be linked to improved antioxidant production within plants that counteracted ROS led destruction of chlorophylls and carotenoids (Figure 2.6b) [219,220].

The efficacy of a plant to regulate Na^+ absorption, distribution and compartmentalization depends on its salt resistance [221]. For binding sites, Na^+ competes with K^+ and thus interferes with a range of core physiological functions that depend on K^+ [222]. Importance of studying interaction of Na^+ and K^+ is widely accepted as a measure of plant tolerance to salt stress [170]. Significant increase in root and leaf K^+ and decrease in leaf Na^+ with endophytic bacterial inoculation under saline condition affirm it to be the key the mechanisms by which bacterial endophytes can ameliorate salinity stress (Figure 2.7b).

The growth promoting effect of endophytic bacterial inoculation was not limited to only salt sensitive plants, rather salt tolerant plant also exhibited substantially improvements in plant biomass, photosynthesis, antioxidants productions and ion homeostasis, both under non-saline and saline conditions. However, in salt-sensitive (SS) plants, bacterial inoculation induced increase in chlorophyll-b and decrease in abscisic acid content was higher than salt tolerant (ST) plants. This effect of endophytic bacteria may be regarded as the reversal of the negative effects of salinity which are more likely on salt sensitive than salt tolerant plants [223]. Under salinity stress, endophytic bacterial inoculation increased root K^+ concentration in both SS and ST plants, but decreased root Na concentration only in ST plants. Ultimately, in salt sensitive plants, root Na^+/K^+ ratio was not affected by endophytic inoculation. Salt-tolerant plants achieve salt tolerance either by excluding most of the Na^+ and Cl^- into the soil solution or by accumulating salt ions in the roots and root-stem junctions [224]. This indicates that Na exclusion is inherited trait and inoculation of endophytic bacteria failed to induce it in salt sensitive species. Thus, in slat sensitive species, endophytic bacteria induced salt tolerance only by increasing K uptake by roots. However, withdrawing such a definite conclusion is certainly realistic at this stage as only a few studies regarding salt stress induction by endophytic bacteria were found on ST plants.

2.5. Conclusions

This meta-analysis, including 42 articles, 77 experimental units and 1214 observations, spanning 10 years (2011-2020), suggests that endophytic bacteria induce changes in plant metabolism through increasing osmolyte accumulation (proline), K^+ acquisition, Na^+ exclusion, antioxidant enzymes regulation (CAT and SOD), photosynthesis improvements (chlorophyll-a, -b, photosynthetic rate), while decreasing MDA concentrations. Our finding from this meta-analysis suggests that endophytic bacterial inoculation is beneficially under both non-saline and saline condition, but definitely the magnitude of benefit is higher under salinity stress. Moreover, SS plants failed to

exclude Na^+ even with inoculation of endophytic bacteria and increase in K^+ uptake remains the main mechanism underlying bacterial induced salt-tolerance. Hence, this meta-analysis establishes that inoculation of plant growth promoting bacterial endophytes is an effective tool to improve plant growth under saline and non-saline conditions.

Articles analyzed in this meta-analysis: [104,123,173,201,202,207,208,213,214,220,225-256].

Chapter 3-

Transcriptomic comparison of plant growth promoting endophytic bacteria *Enterobacter ludwigii* and *Pantoea agglomerans* in response to individual and combined effects of salinity and drought stress

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Abstract

Abiotic stresses, such as salinity and drought are major threats to agricultural production. These environmental stresses not only limit plant growth but also inhibit the growth of bacteria. How plant growth promoting endophytic bacteria (PGPEB) *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G cope with salt and drought stress and regulate growth, and the genes responsible for tolerance mechanisms remain unknown. We applied RNA-Seq technology to determine the genes involved in salinity, drought, and combined stress tolerance and growth mechanisms of the PGPEB *E. ludwigii* 32A and *P. agglomerans* D7G. A total of 642 genes (257 down-regulated, 385 up-regulated) in *E. ludwigii* 32A and 1243 (552 down-regulated, 691 up-regulated) in *P. agglomerans* D7G were significantly regulated after the individual and combined stress treatments. Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes analysis revealed that the most enriched genes included those related to the membrane transport, cell motility, amino acid metabolism and general metabolic pathways. The quantitative real-time polymerase chain reaction data were similar to those obtained from RNA-Seq. The 32A and D7G strains maintained survival under individual and combined effects of salinity and drought stress, by regulating cellular and

metabolic processes. We highlighted the response mechanism of *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G dynamics of complex single or multiple stress-microbe interactions.

3.1. Introduction

Soil salinity is one of the biggest threats to agricultural production and food security. It was estimated that more than 6% of global land is currently salt affected[164]. In the event of climate change, irrational irrigation methods, improper application of fertilizers, and inadequate drainage networks, this situation is getting worst every day. It is estimated that 50% of arable land will be under serious salinity risk by 2050 [165-168]. The soil salinity imposes a blend of morphological and physical effects including impeded nutrients uptake, seed germination and overall plant growth. Short after exposure to salinity, plants face osmotic stress, which is followed by ion toxicity and nutrient imbalance. Osmotic stress leads to the formation of hypertonic conditions outside the cell, act condition of pseudo water deficiency which make it impossible for plants to take up water. Ion toxicity is caused by the overaccumulation of sodium (Na^+) and chloride (Cl^-) ions within the cells. Excessive amounts of Na^+ and Cl^- damage plant cell walls, disturbs the osmotic balance, and modifies ion homeostasis within the cell, which ultimately induce changes in transpiration rate, translocations of nutrients, photosynthesis, and other metabolic processes [169]. In addition, soil salinity could indirectly minimize plant growth by hindering the activities of beneficial microbes living in the rhizosphere and reducing the accumulation of organic matter. To cope with salinity stress plants have evolved different physiological mechanisms including osmolyte aggregation, ion homeostasis, water absorption control, and antioxidants synthesis [170]. Moreover, plants establish interactions with a plethora of microorganisms that promote plant growth and mitigate plant stress [171]. Among those, endophytic bacteria showed to relive salt stress in plants by inducing osmotic adjustment, detoxification, modulation of phytohormones, and acquisition of nutrients [5]. Endophytic bacteria having 1-aminocylopropane,1-carboxylate (ACC) deaminase and indole-3 acetic acid production, nitrogen fixation, phosphate solubilization, siderophore production ability, had been concluded to promote the osmotic or ionic adaptation of the host plants [127,162,172,173].

Integrating the data across investigations through a meta-analysis might help to understand the extent and mechanisms of stress mitigation conferred by bacterial endophytes and would broader the use of endophytic bacteria in sustainable agriculture. A meta-analysis is a tool that synthesizes knowledge using a specific methodological procedure for data aggregation and analysis from various individual scientific studies [174]. It is particularly useful for answering study questions of great versatility and uncovering emergent properties within individual studies that would otherwise go undetected. The power of a meta-analysis becomes obvious when the outcomes of particular experiments vary in various laboratory conditions. Therefore, we used a meta-

analysis for assessing the efficacy of endophytic bacterial inoculation in plant salinity stress mitigation. Although, a few meta-analyses have reported on the effect of inoculation of plant growth promoting rhizobacteria to improve abiotic stress tolerance of plants [168,175,176], however, the overall effect of endophytic bacterial inoculation to improve plant heavy metal tolerance have recently published by Franco-Franklin et al. [177]. Nevertheless, only Rho et al. (2018) have dedicated a small portion of their meta-analysis, consisting of a few studies, to the overall effect of bacterial endophytes to improve plant salinity tolerance. Moreover, there is no meta-analysis that has dissected the effects of endophytic bacteria on salt sensitive (SS) or salt tolerant (ST) host plants under salinity stress conditions as compared to non-saline conditions.

In this study, we hypothesized that (i) endophytic bacterial performance is better under salinity stress and (ii) salinity stress mitigation conferred by endophytic bacteria varies across SS and ST plants. To test our postulates, we extracted raw data from 42 articles and conducted a meta-analysis. In addition to evaluate endophytic bacterial performance under salinity and non-saline condition, we classified the host plants into SS and ST groups, and then individually scored the effects sizes of each group to compare the bacterial effects on two types of host plants.

3.2. Materials and Methods

3.2.1. Bacterial strains and culture conditions

Enterobacter ludwigii 32A (32A) and *Pantoea agglomerans* D7G (D7G) were conserved in 80% glycerol and routinely grown on Nutrient Agar (NA, Oxoid, Basingstoke, UK) dishes at 25°C ± 2°C. To prepare cell suspensions, 32A and D7G cells were grown in 5-ml nutrient broth (NB, Oxoid) in sterile 15-ml test tubes at 27°C ± 2°C in orbital shaker at 220 rpm. After 24 h, bacterial cell suspensions were centrifuged at 10,000 rpm and supernatants discarded. Pelleted cells were suspended in sterile 10 mM $MgSO_4.7H_2O$ solution and the final concentration was adjusted to 1×10^8 colony forming units (CFU)/ ml. Cell suspensions produced using this procedure were used throughout the experiments unless otherwise indicated.

3.2.2. Abiotic stresses tolerance assay

32A and D7G were grown in NB amended with 1%(**w/v**) NaCl (water potential -1.2 MPa) to induce salinity stress (SS) and 6.75% (**w/v**) polyethylene glycol (PEG; water potential -0.1 MPa) to induce drought stress (DS). The two stresses were combined (combined stress, CS) by amending NB with 1% NaCl + 6.75% PEG (water potential -1.3 MPa). NB alone was used as the untreated control. 32A and D7G were inoculated into sterile 15 ml tubes containing 5 ml of each medium and incubated at 28°C in an orbital shaker (200 rpm). After 24 h incubation, cell density was assessed by measuring

absorbance at 600 nm ($A_{OD600nm}$) using Synergy™ 2 (Biotek, Winooski, VT, USA). The effect size of growth modulation of the two PGPEB due to the exposure to stresses as compared to untreated conditions was calculated as log response ratios (lnRR) using the following formula: $lnRR = ln (X_t/X_c)$, where X_t is the mean cell density of stress treatment and X_c is the mean cell density of untreated control [186]. Percent change (%Δ) was calculated from lnRR, i.e. %Δ = (exp.(lnRR)-1*100). Pooled variances were calculated in the R environment (https://r-project.org/) using the "escalc" function in the "metafor" (version 2.4-0) package [187]. Three replicates (15 ml tubes) were used for each treatment and the experiment was repeated. Effect sizes (lnRR) with their 95% confidence intervals (95% CI) were presented in forest plots. The effect of stresses on PGPEB growth was considered significant if 95% CIs did not coincide with the zero line. Overlaps on the zero line mean that there was no significant effect of stresses on PGPEB growth and denoted by 'NS' [189].

3.2.3. Effect of abiotic stresses on indole 3-acetic acid production

The production of indole 3-acetic acid (IAA) was evaluated by slightly modifying the procedure described by [257]. Briefly, 500 ml of PGPEB cell suspensions (1×10^8 CFU/ml) in sterile saline solution (0.85% NaCl, ***w/v***), were inoculated into sterile 15 ml tube containing 5 ml Dworkin and Foster (DF) salt minimal broth (4 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7\ H_2O$, 2 g/l glucose, 2 g/l gluconic acid, 2 g/l citric acid) amended with 500 mg/l of L-Tryptophan (Sigma-Aldrich), as a nitrogen source. Moreover, 1% NaCl (-1.2 MPa), 6.75% PEG (-0.1 MPa) and 1% NaCl+6.75% PEG (-1.3 MPa), were added to respectively reproduce SS, DS and CS. DF salt minimal broth with L-Tryptophan (500 mg/l) was used as a control. Tubes were incubated at 25°C in an orbital shaker (200 rpm) for five days. After the incubation period, cultures were centrifuged at 5,000 rpm for 15 min at room temperature and 500 µl of each supernatant were transferred into sterile 2 ml microcentrifuge tubes containing 1 ml Salkowski's reagent (2.0 ml 0.5M $FeCl_3 \cdot 6\ H_2O$ and 98 ml 35% H_2SO_4 for 100 ml solution). After 30 min, 150 µl were transferred in wells (three replicates per tube) in 96-well polystyrene plate and the absorbance at 530 nm ($A_{OD530nm}$) was measured using Synergy™. The cell density of each bacterial suspension was also measure by measuring $A_{OD600nm}$. The IAA production was estimated from a standard IAA curve (Figure S3.1) and expressed as micrograms per millilitre and calculated as micrograms per 10^8 cfu. The modulation in IAA production of PGPEB due to the exposure to stresses as compared to untreated conditions was calculated as lnRR and %Δ as mentioned above in 2.2. Three replicates (15 ml tubes) were used for each treatment and the experiment was repeated.

3.2.4. Effects of abiotic stresses on endophytic colonization related features

3.2.4.1. Swimming and swarming ability

The ability of the PGPEB to move through swimming and swarming was evaluated according to [258] with few modifications. Briefly, swimming agar (10 g/l tryptone, 5 g/l NaCl, 3 g/l agar) and swarming agar (8 g/l nutrient broth, 5 g/l glucose, 5 g/l Bacto-Agar) were prepared. To reproduce salinity, drought and combined stress, the media reported above were respectively amended with 1% NaCl (-1.2 MPa), 3.4% Sorbitol (0.4 MPa) and 1% NaCl + 3.4% Sorbitol (-1.6 MPa), while media without any amendment were used as untreated controls. Ten µl bacterial suspensions prepared in 0.85% NaCl ($1x10^8$ cfu/ml) were spot inoculated in the center of the petri dishes. After incubation at 25°C for 48 h, the swimming and swarming motility were determined by measuring colony distribution on the medium in petri dishes by analyzing the picture made with gel/doc. Modulation in swimming and swarming ability of PGPEB in treated media with respect to untreated was calculated as lnRR and %Δ according to the formulae mentioned in 2.2. Three replicates (petri dishes) were used for each bacterial strain and the experiment was repeated two times.

3.2.4.2. Biofilm formation

The ability of PGPEB strains to form biofilm under stress conditions was evaluated on polystyrene microtiter dishes by slightly modifying the procedure described by Maddula et al. [259]. Briefly, a volume of 1.5 µl of each PGPEB cell suspension (1×10^8 cfu/ml) was inoculated into in 96-well polystyrene dishes containing 150 µl NB per well dishes. NB was amended with 1% NaCl, 6.75% PEG and 1% NaCl + 6.75% PEG to produce stress conditions, whereas NB without any amendment was used as untreated control. Dishes were incubated at 27°C for 48 h without shaking and final cell densities was determined ($A_{OD600nm}$) using Synergy™ 2. Unattached cells were removed by inverting the plate and tapping it onto absorbent paper. The remaining adherent bacterial cells were fixed to the dishes through an incubation at 50°C for 20 min and then stained with 150 µl of crystal violet solution (0.1% in sterile distil water (SDW)) per well. After 1 min incubation at room temperature, excess stain was removed by inverting the plate, then washing twice with distilled water (each wash 250 µl per well). Adherent cells were decolorized with an acetone/ethanol (20%/80%) solution (200 µl per well) for 5 min to release the dye into the solution. A volume of 100 µl was transferred from each well to another 96-well polysterene plate and the amount of dye (proportional to the density of adherent cells) was quantified by measuring the absorbance at 540 nm ($A_{OD540nm}$) using Synergy™ 2**.** A 96-well polystyrene plate was used for each treatment for cell density

and biofilm quantification. $A_{OD540nm}$ values (adherent cells) were divided by $A_{OD600nm}$ values (bacterial growth) in order to obtain the specific biofilm formation value (SBF). The effect size of modulation in SBF by PGPEB in treated NB with respect to untreated NB was calculated in five replicated of each bacterial strain in each treatment and the experiment was repeated two times.

3.2.5. Effects of abiotic stresses on endophytic colonization

The efficacy of PGPEB strains to colonize tomato plants under stress conditions was evaluated according to the procedure described by Belimov et al. [260] with slight modifications. Briefly, the Hoagland solution was amended with 1% NaCl, 6.75% PEG and 1% NaCl + 6.75% PEG to produce salinity, drought, and combined stress. Hoagland solution without any amendment was used as untreated control. Tomato seeds were sterilised and inoculated according to Sharma et al. [261]. For pre-germination, tomato seeds were placed in petri dishes (diameter 90 mm) containing a sterile filter paper and 5 ml of SDW. Petri dishes were wrapped with aluminium foil and incubated at 25°C for 48 h under dark condition. After incubation, sprouted seeds with same radical length (2 mm) were dipped in bacterial suspension (1×10^8 cfu/ml) prepared in 10 mM $MgSO_4.7H_2O$ for 10 sec. Inoculated seeds were then vertically placed in 30 mm test tube filled with 3 g sterile perlite and 10 ml Hoagland solution amended with NaCl and PEG with eight replications. 10 mM $MgSO_4.7H_2O$ solution without bacterial cells was used as a control treatment. Tubes were then placed in a growth chamber at 25°C with 16 h daylight period. After 21 days, each tomato plant was weighed and surface sterilised with ethanol 70% for 1 minute, 2% NaOCl for 3 min, and 70% ethanol for 30 sec, followed by three washes in SDW and dried on sterile filter paper. The success of surface sterilisation was checked by rolling the plantlets on the surface of R2A medium (Sigma-Aldrich, St. Louis, MO, USA) amended with cycloheximide (100 mg/l). Succeeded sterilisation was indicated by no bacterial growth on the medium after three days of incubation at 27°C. Once sterilised, each plantlet was transferred into sterile 2 ml microcentrifuge tube containing 1.5 ml of phosphate buffer (pH 7.1) and two sterile stainless-steel beads. Samples were macerated using a Mixer Mill Mm 200 Retsch® for 1 min at 25 rpm. Subsequently, the resulting solutions were serially diluted up to 10^{-7} and aliquots of 10 µl were spot inoculated on R2A medium amended with cycloheximide (100 mg/l) in triplicates. Petri dishes were incubated at 27°C and, CFU were counted after 48 h. The endophytic colonization was expressed as CFU/g of fresh tomato plant tissue. The effect size of modulation in endophytic colonization by PGPEB under stress conditions as compared to untreated control was calculated as lnRR and

%Δ according to the formulae mentioned above in 2.2. Eight plants were assayed for each bacterial strain and the experiment was repeated.

3.2.6. RNA Extraction, Library Construction, and Sequencing

The impact of SS, DS and CS on the transcriptome of 32A and D7G was assessed using a RNA-Seq analysis. To do that, 32A and D7G were grown in 5 ml of NB amended with 1% NaCl (SS), 6.75% PEG (DS) and 1% NaCl + 6.75% PEG (CS) contained into sterile 15 ml test tubes incubated at 27°C ± 2°C in an orbital shaker (200 rpm). NB alone was used as the untreated control. After 36 h growth, total RNA was extracted cell cultures by using Tri-Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Isolated RNA was purified using the RNeasy Mini Kit (Qiagen Sciences, Valencia, CA) and subjected to DNase treatment with an RNase-Free DNase set (Qiagen, Hilden, Germany) during RNA purification, following the manufacturer's instructions. Total RNA was quantified using a Qubit 3.0 Fluorometer (Invitrogen, Life Technologies, Waltham, MA) with Qubit RNA BR assay (Invitrogen, Life Technologies), and Tapestation 2200 (Agilent Technologies, Santa Clara, CA, USA) was used to check the RNA integrity. All treatments were performed in triplicates.

3.2.7. Illumina sequencing and mapping to the reference genomes

Library construction and Illumina Sequencing were carried out at Fasteris (Plan-les-Ouates, Switzerland). Each sample of total purified RNA was diluted with RNase-free water to a final concentration of 50 ng/µl. Ribosomal RNA (rRNA) depletion was performed using the Ribo-Zero™ rRNA Removal Kits (Bacteria) (Illumina, San Diego, California, USA). mRNA-Seq libraries were multiplexed (two libraries per lane) and sequenced with Illumina HiSeq High Output paired reads, according to manufacturer's instructions. Complementary DNA (cDNA) libraries were synthesized using TruSeq Stranded mRNA Library Prep (Illumina, San Diego, California, USA). Paired-end reads of 150 nucleotides were obtained using an Illumina HiSeq 4000 (Illumina, San Diego, California, USA). Raw sequences were deposited at the Sequence Read Archive of the NCBI (www.ncbi.nlm.nih.gov/sra) under accession number xxxx and BioProject number xxxx.

The subsequent sequence analysis was carried out using software included in Omicsbox 1.3.11, a platform of bioinformatics software (OmicsBox – Bioinformatics Made Easy, BioBam Bioinformatics, March 3, 2019, https://www.biobam.com/omicsbox). The Illumina HiSeq data was assessed for quality using FastQC [http://www.bioinformatics.babraham.ac.uk/projects/fastqc] [262]. Illumina paired-end (2 x 150 bp) reads for each sample were trimmed to increase overall quality using

Trimmomatic 0.38 [263]. The resulting reads of 32A and D7G were aligned to 32A (PRJEB8251 (EnVs6)) and D7G (PRJEB8258 (PaVv7)) genomes [264] using the STAR 2.7.5a. [265]. Read counts were extracted from STAR alignments using HTSeq [266].

3.2.8. Identification of differentially expressed genes and functional annotation

Genes with zero counts in all biological samples were excluded from the analysis and raw counts were normalized using the trimmed mean of M-values method [267]. Differentially expressed genes (DEGs) were identified using edgeR 3.28.0 [268]. A p-value < 0.01 and a log fold change (FC) of at least 1-fold upregulation/downregulation, in either condition, were chosen as cut off values for identifying significant genes. Venn diagrams summarizing the distribution of DEGs were drawn with an opensource software (https://www.draw.io/). Hierarchical clustering was done using the dist and hclust functions and heatmaps were created with 'pheatmap' package in the R environment.

The protein sequences of all predicted genes [264] were functionally annotated using Blast2Go (http://www.blast2go.org) [269]. Default settings were applied and a minimum E-value of 10^{-5} was imposed as cut off. DEGs were further annotated on the basis of the NCBI gene description, and they were classified in 20 functional classes [general metabolic pathways; carbohydrate metabolism; energy metabolism; lipid metabolism; nucleotide metabolism; amino acid metabolism; protein metabolism; secondary metabolism, DNA metabolism; RNA transcription and degradation; translation (ribosomes and tRNA); growth; oxidative stress; antagonistic activity; defence (detoxification, generic response to stress); transport, phosphotransferase systems and secretion; signal transduction and receptors; kinase/phosphatase; quorum sensing; and cell motility, chemotaxis and biofilm]. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways were functionally annotated using KAAS-KEGG automatic annotation server [270], https://www.genome.jp/kegg/kaas/ and KEGG mapper [271]. Scatter plots were made using 'ggplot' package in the R environment [272].

3.2.9. Validation of RNA-Seq

RNA-Seq was validated with qRT-PCR. First-strand cDNA was synthetized from 100 ng of purified RNA (previously used for RNA-Seq) with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) using random hexamers, according to manufacturer's instructions. qRT-PCR reactions were carried out with Platinium SYBR Green qPCR Super-Mix-UDG (Invitrogen, Carlsbad, California, USA) and specific primers **(Table. 1)**. Specific primers were designed using Primer3

software (http://bioinfo.ut.ee; [273]), and their specificity was assessed using PCR before gene expression analysis. qRT-PCR reactions were run for 50 cycles (95 °C for 15 s and 60 °C for 45 s) on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany). Each sample was examined in three technical replicates and dissociation curves were analysed to verify the specificity of each amplification reaction. The LightCycler 480 software, version 1.5 (Roche Diagnostics, Mannheim, Germany) was used to extract cycle treshold (Ct) values based on the second derivative calculation and the LinReg software, version 11.0, was used to calculate reaction efficiencies for each primer pair [274]. Relative expression levels were calculated according to the Pfaffl equation [275] using *E. ludwigii* 32A and *P. agglomerans* D7G growing in untreated-NB (without any stress) as the calibrator. The housekeeping gene *recA* was used for both 32A and D7G as constitutive gene for normalization. The linear relationship between the RNA-Seq $\log_2$FC values and the qRT-PCR $\log_2$FC values of selected genes was estimated by Pearson correlation analysis.

Table 3. 1: Specific primers of selected gene in *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G for qRT-PCR to validate RNA-Seq

***Enterobacter ludwigii* 32A Gene names**	**Forward primers**	**Reverse primers**
flgJ	AGTCACCGCTCGCCTTAATA	ACGACTTTATCGCCCAACTG
flil	CGCCGATTGAAGATGTGCTG	AATCACATCGGCCTTGGTGT
LuxR	TTCGCCACACCTTCAATCGA	CATGCCATCACAGCCGATTG
LexA	ATGCACCGCTAACAAATCGC	GCTGGCGCAGGAACATATTG
CopC	GACAAAGCGGTGGTGGAAAC	CGTTGAGTTTGGCTTTCCCG
recA	TGGAAATTTGTGACGCGCTG	AAGATCAGCAGCGTGTTGGA
***Pantoea agglomerans* D7G Gene names**		
ahpF	AAGCGCTGAACAAACGTGAC	CAGTTTCTGGCCTTCGGTCT
flgC	ACATCCACGTTCGGCATCTT	CTTACGTGGCAAAGCAGGTG
hpxA	CAGATCGCCATGTTTGCTGG	CGCTGTATTCAGGCGAAACG
GrxA	TGAACTGGCCGACAAACTGA	ATTCTCTTTGGTCCAGGCCG
GlpG	TTGCACGACCCTAACCATCC	AAACACGGCGATACAGAGCA
recA	TGGAAATTTGTGACGCGCTG	AAGATCAGCAGCGTGTTGGA

3.3. Results

3.3.1. Stress tolerance of PGPEB

In this study, significant differences were observed in PGPEB tolerance to salinity, drought, and combined stress. Drought (6.75% PEG), salinity (1% NaCl) and combined stress (1% NaCl+6.75% PEG) significantly modulated the growth of 32A by -5, -9 and -11%, respectively, as compared to untreated control. On the other hand, under salinity and drought stresses the growth of D7G was reduced by 10 and 12%, respectively, whereas no significant bacterial growth modulation was observed in D7G under combined stress than untreated (Figure 3.1).

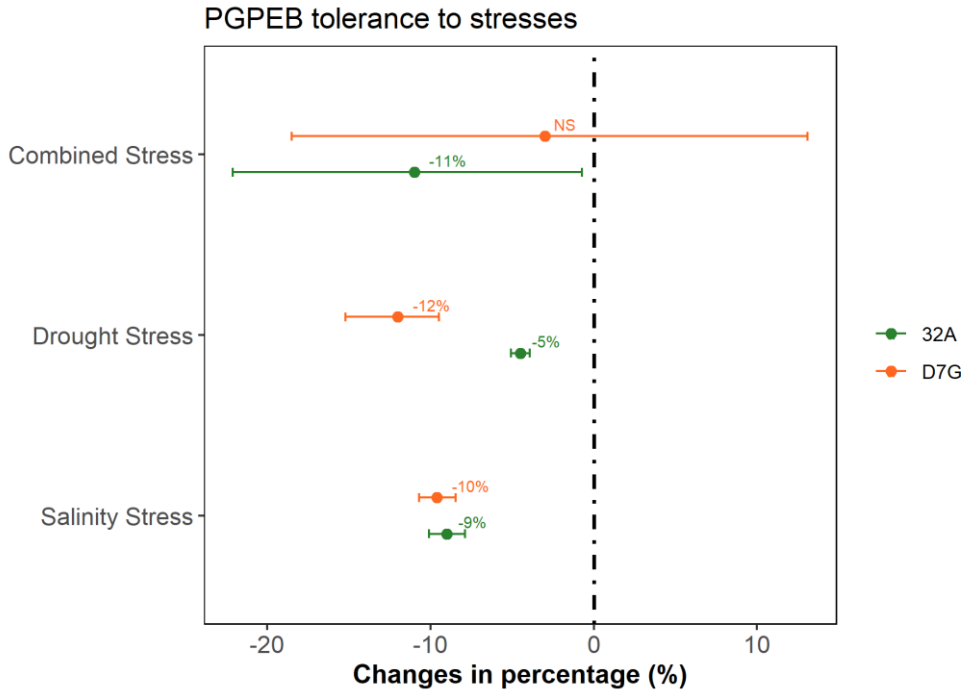

Figure 3. 1: Effect of salinity (1% NaCl), drought (6.75% PEG) and combined stress (1% NaCl+6.75% PEG) on growth of *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G. Error bars represent 95% Cl. Variables were considered significant if error bars did not overlap with zero line, which represents the untreated control treatment (without any stress)

3.3.2. Production of IAA by PGPEB under stress conditions

Indole 3-acetic acid (IAA) production was significantly increased (47%) by 32A under drought stress as compared to untreated control, while no significant modulation in IAA production was observed under salinity and combined stress. On the other hand, D7G produced significantly less (-48%) IAA under drought stress in comparison to the

untreated control. However, D7G produced similar amount of IAA under salinity and combined stresses as compared to the untreated control (Figure 3.2).

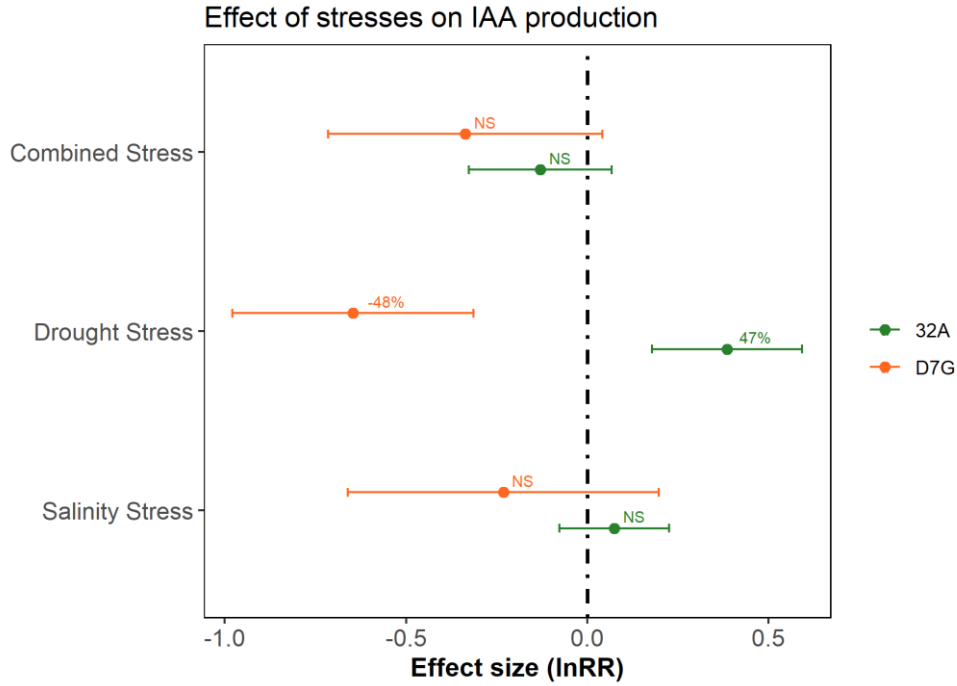

Figure 3. 2: Effect of salinity (1% NaCl), drought (6.75% PEG) and combined stress (1% NaCl+6.75% PEG) on IAA production by *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G. Error bars represent 95% Cl. Variables were considered significant if error bars did not overlap with zero line, which represents the untreated control treatment (without any stress)

3.3.3. PGPEB characteristics effected by salinity, drought and combiend stress

3.3.3.1. PGPEB motility

Drought stress increased the swimming motility of both PGPEB 32A and D7G by 25 and 131%, respectively, as compared to no-stress conditions. Swimming motility of 32A was negatively modulated under salinity stress by -61% and combined stress by -58% as compared to untreated condition. Swimming motility of D7G improved with drought stress, as compared to untreated control. On the other hand, no significant effects of salinity and combined stresses were observed on swimming motility of D7G (Figure 3.3a).

Swarming motility of 32A was stimulated under combined stress treatment by 11% (0.39±0.02 $\mu m^2 s^{-1}$) as compared to untreated control (0.35±0.01 $\mu m^2 s^{-1}$). However, no significant changes were observed in swarming motility of 32A under salinity and drought stresses in comparison to untreated condition. The highest negative modulation

in swarming motility was observed in D7G under drought condition (-34%). In addition, D7G significantly slowed down (-30%) its swarming motility under combined stress conditions. However, similar swarming motility was observed in D7G under salinity stress (Figure 3.3b).

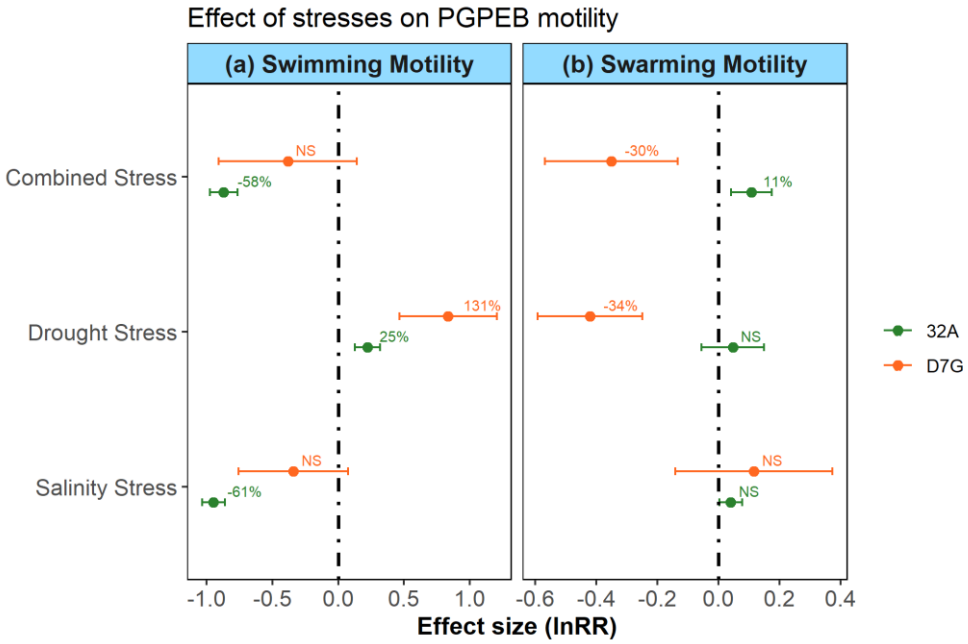

Figure 3. 3: Effect of salinity (1% NaCl), drought (3.4% Sorbitol) and combined stress (1% NaCl+3.4% Sorbitol) on (a) Swimming motility, and (b) Swarming motility of *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G. Error bars represent 95% Cl. Variables were considered significant if error bars did not overlap with zero line, which represents the untreated control treatment (without any stress)

3.3.3.2. Biofilm formation

In general, Specific biofilm formation (SBF) by both PGPEB ranged between 0.35-2.6, however, SBF by 32A was positively modulated by 45% (2.15±0.04) under salinity stress as compared to both D7G and untreated control (1.72±0.2). On the other hand, 32A formed significantly similar amount of SBF under drought and combined stress conditions. Likewise, no significant changes were observed in SBF by D7G under combined stress conditions than untreated condition. However, salinity and drought stresses significantly reduced the amount of SBF in D7G by -8% (0.46±0.01) and -23% (0.39±0.02), respectively, as compared to untreated control (0.50±0.01; Figure 3.4).

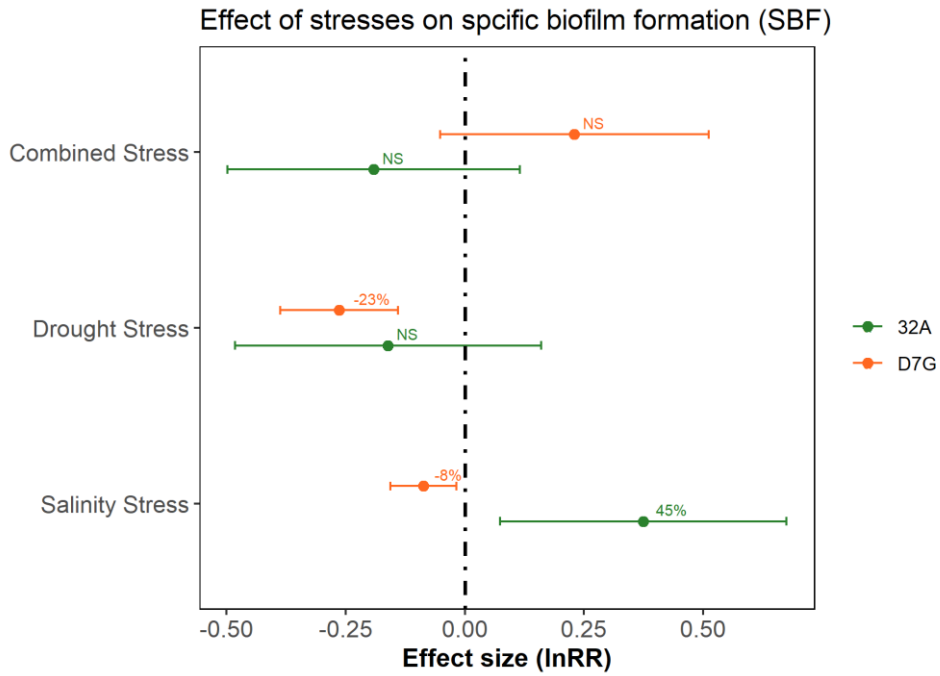

Figure 3. 4: Effect of salinity (1% NaCl), drought (6.75% PEG) and combined stress (1% NaCl+6.75% PEG) on specific biofilm formation (SBF) by *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G. Error bars represent 95% Cl. Variables were considered significant if error bars did not overlap with zero line, which represents the untreated control treatment (without any stress)

3.3.4. Effects of stresses on endophytic colonization rate of PGPEB strains

Overall, endophytic colonization rate decreased by 25-44% in all stress treatments as compared to the untreated control. However, endophytic colonization rate of 32A and D7G significantly lowered in saline conditions both by -44% (4.57±0.5 log10 cfu g^{-1} fresh weight) and -44% (4.67±0.2 log10 cfu g^{-1}), respectively, as compared to untreated 32A control (8.16±0.01 log10 cfu g^{-1}) and D7G control (8.37±0.21 log10 cfu g^{-1}). In drought stress condition, 32A and D7G colonization rate was reduced by -37% and -27%, respectively. Similarly, combined stress decreased the endothelization rate of 32A by -30% and D7G by -25%, in comparison to no stress conditions (Figure 3.5).

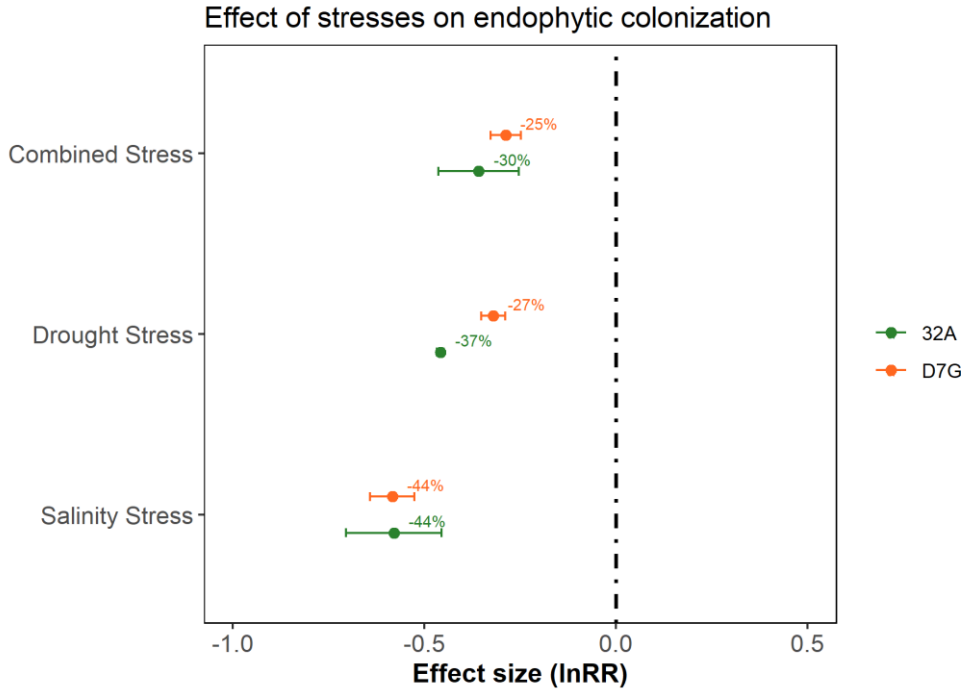

Figure 3. 5: Effect of salinity (1% NaCl), drought (6.75% PEG) and combined stress (1% NaCl+6.75% PEG) on endophytic colonization by *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G in tomato plants. Error bars represent 95% Cl. Variables were considered significant if error bars did not overlap with zero line, which represents the untreated control treatment (without any stress)

3.3.5. RNA-Seq analysis of Enterobacter ludwigii 32A and Pantoea agglomerans D7G response to salinity, drought, and combined stress

The transcriptomes of 32A and D7G were characterized after 36 h of exposure to salinity stress (SS), drought stress (DS), and combined stress (CS) treatments. In order to find the changes in the total transcriptomes of 32A and D7G, the data for stressed treatments were compared to their respective untreated control.

Significant changes were observed in a number of genes of both PGPEB strains, 32A and D7G. Differentially expressed genes (DEGs) in 32A and D7G were divided into two clusters (up- and down-regulated genes). A total of 642 DEGs in abiotic stress treated vs. control (untreated) cells of 32A were identified, which includes 257 repressed and 385 induced genes (Figure 3.6A and B). While 51.6% more DEGs were observed in D7G making a total of 1244 DEGs (552 down-regulated and 691up-regulated) after abiotic stress treatments for 36 h (Figure 3.6C and D). In 32A, a total of 190 genes

including 95 down-regulated and 95 up-regulated ones were modulated after SS, out of 193 total genes 61 down-regulated and 132 up-regulated after DS, and a total of 259 genes including 101 down-regulated and 158 up-regulated ones were observed after CS treatment (Figure 3.6A and B). While in D7G, out of 437 total modulated genes under SS, 237 were down-regulated and 200 genes were up-regulated. In drought stress, 44 genes were down-regulated and 104 up-regulated, making a total of 148 DEGs in D7G. After CS treatment, highest number of 658 DEGs was observed, including 387 up-regulated and 271 down-regulated genes (Figure 3.6C and D).

3.3.6. Gene ontology (GO) analysis of DEGs

The differentially expressed genes of 32A and D7G were subjected to functional analysis. All the predicted genes of 32A and D7G were functionally annotated using the Blast2GO program [269], and the DEG lists were subjected to enrichment analysis to retrieve over-represented GO categories. After GO enrichment analysis, all the DEGs were further annotated according to the NCBI gene description to increase the amount of information for genes not included in the first round of annotation using Blast2GO program. The outcome of this functional annotation approach resulted in a comprehensive description of the molecular mechanisms modulated in 32A and D7G after salinity, drought, and combined stress treatments (Figure 3.7 and 3.8). DEGs of 32A were mainly associated with functional categories of cell motility, membrane transport, RNA transcription and degradation, amino acid metabolism, and DNA metabolism (Figure 3.7).

In general, the 32A down-regulated genes included a large number of genes responsible for cell motility and membrane transport, while up-regulated genes included a large number of gene responsible for membrane transport and RNA transcription and degradation. Our data showed that majority of gene were annotated to biological processes, molecular functions, and cellular processes. GO analysis revealed that 17.37%, 7.89%, 7.89% and 7.37% of the modulated genes in 32A after 36 h of SS were involved in membrane transport, cell motility, DNA metabolism, and RNA transcription and degradation, respectively. Out of 193 DEGs involved in 22 biological functions identified in 32A under DS, 17.10%, 9.84%, 8.29%, and 7.77% genes were involved in membrane transport coupled with cell motility, amino acid metabolism, and RNA transcription and degradation, respectively. Under combined stress treatment, out of 259 DEGs of 32A, genes involved in membrane transport, RNA transcription and degradation, cell motility, amino acid metabolism and enriched with DEGs proportions of 16.22%, 8.11%, 7.34, and 6.95%, respectively (Figure 3.7). GO analysis showed that

17.39%, 10.53%, 6.41%, and 6.18% DEGs in D7G were involved in membrane transport, RNA transcription and degradation, amino acid metabolism, and general metabolic pathways, respectively, under SS. While, under DS, 21.62% 9.46% DEGs involved in membrane transport, and RNA transcription and degradation and under CS, out of 654 DEGs in D7G, 17.89%, 9.48%, 9.33% and 7.03% were involved in membrane transport, RNA transcription and degradation, carbohydrate metabolism and energy metabolism, respectively (Figure 3.8).

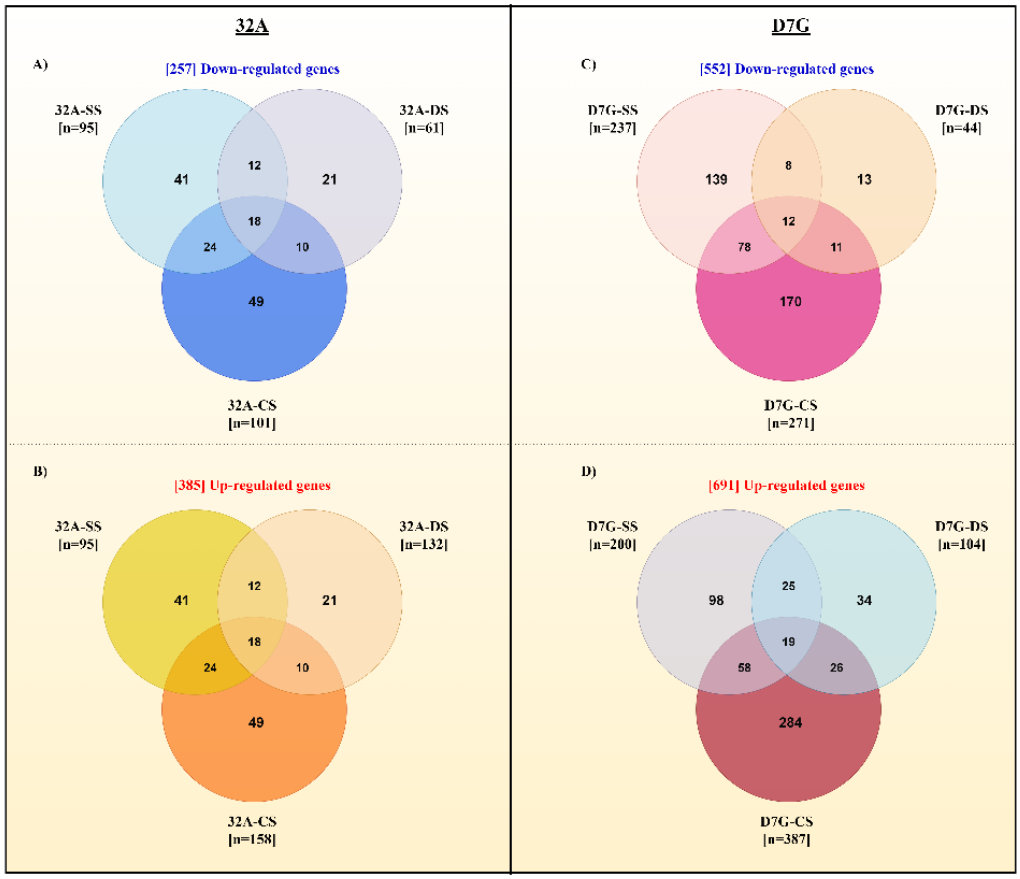

Figure 3. 6: Venn diagrams showing the number of significantly differentially expressed genes (DEGs) in *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G bacterial cells after 36 h of cultivation in response to the treatments: 1% NaCl salinity stress (SS), 6.75% PEG drought stress (DS) and 1% NaCl + 6.75% PEG combined stress (CS). A, C)- Number of Down-regulated, and B, D)- number of Up-regulated genes between samples at 36h of cultivation of 32A and D7G, respectively

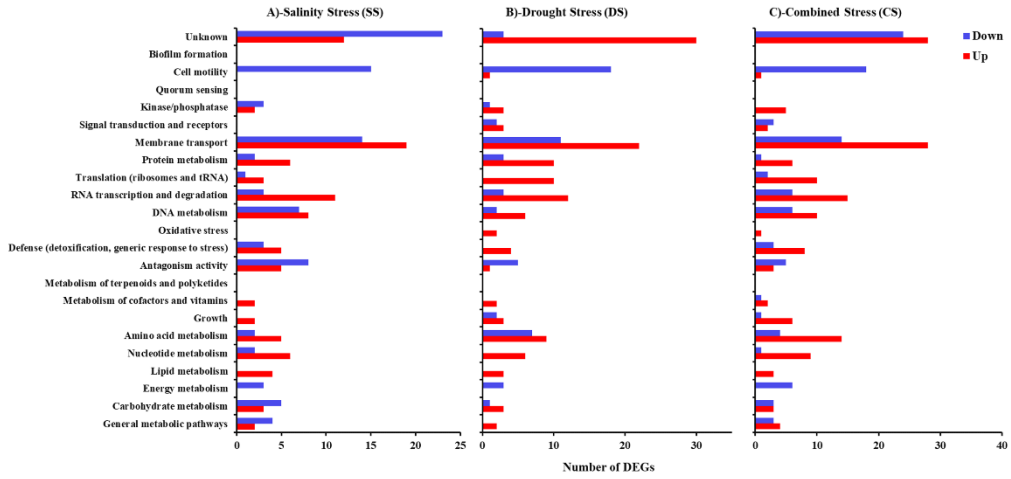

Figure 3. 7: Annotation of DEGs in *Enterobacter ludwigii* 32A after 36 h of 1% NaCl salinity stress (SS), 6.75% PEG drought stress (DS) and 1% NaCl + 6.75% PEG combined stress (CS). DEGs were classified in 15 functional classes on the basis of the NCBI gene description and Blast2GO description. The number of DEGs for each functional category is reported for *Enterobacter ludwigii* 32A genes modulated after 36 h of incubation at different stresses; blue, down-regulated DEGs; red, up-regulated DEGs. The total number of DEGs is shown for each functional class

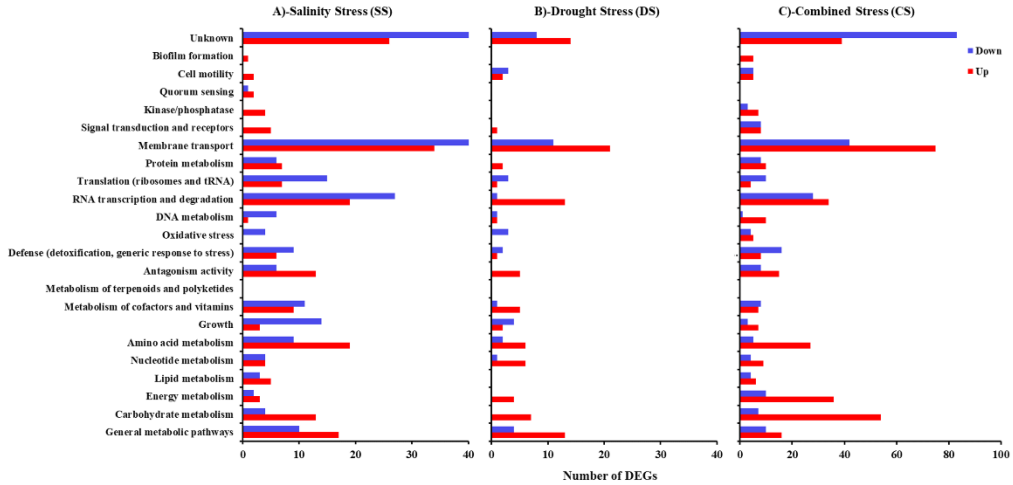

Figure 3. 8: Annotation of DEGs in *Pantoea agglomerans* D7G after 36 h of 1% NaCl salinity stress (SS), 6.75% PEG drought stress (DS) and 1% NaCl + 6.75% PEG combined stress (CS). DEGs were classified in 15 functional classes on the basis of the NCBI gene description and Blast2GO description. The number of DEGs for each functional category is reported for *Pantoea agglomerans* D7G genes modulated after 36 h of incubation at different stresses; blue, down-regulated DEGs; red, up-regulated DEGs. The total number of DEGs is shown for each functional class

3.3.7. KEGG analysis of DEGs

A total of 118 KEGG pathways matched to the whole genome of 32A. Whereas, the DEGs in 32A strain were matched to 58, 46 and 63 different pathway analysis under salinity, drought, and combined stress, respectively. Metabolic pathways, ABC transporters, and flagellar assembly were the most common pathways under salinity stress. A total of 626 genes including 23 DEGs were involved in metabolic pathways, 187 total genes and 16 DEGs were ABC transporters, and 43 total genes and 18 DEGs were involved in flagellar assembly, under salinity stress. Under drought stress, 33, 18, and 12 DEGs were involved in metabolic pathways, flagellar assembly, and ABC transporters, respectively. While, under combined stress 32, 18, and 16 DEGs were involved in metabolic pathways, flagellar assembly, and ABC transporters, respectively. A total of 283 genes and 9, 18, and 15 DEGs were involved in biosynthesis of secondary metabolites under salinity, drought, and combined stress, respectively. On the other hand, a total of 116 pathways matched to all annotated genes of D7G, whereas DEGs in D7G matched to 74, 39, and 94 pathways under salinity, drought, and combined stress, respectively. A total of 740, 312, 210, and 204 genes were involved in metabolic pathways, biosynthesis of secondary metabolites, ABC transporters, and microbial metabolism in diverse environments, respectively. Out of which, 76, 33, 36, and 21 DEGs (salinity stress), 30, 12, 21, and 5 DEGs (drought stress), and 161, 68, 57 and 56 DEGs were involved in metabolic pathways, biosynthesis of secondary metabolites, ABC transporters, and microbial metabolism in diverse environments, respectively. Moreover, 20, 10 and 38 DEGs in D7G were involved in quorum sensing pathway, under salinity, drought, and combined stress, respectively (Figure 3.9). The majority of the genes were up-regulated in both PGPEB 32A and D7G as shown in the clustering analysis (Figure 3.10).

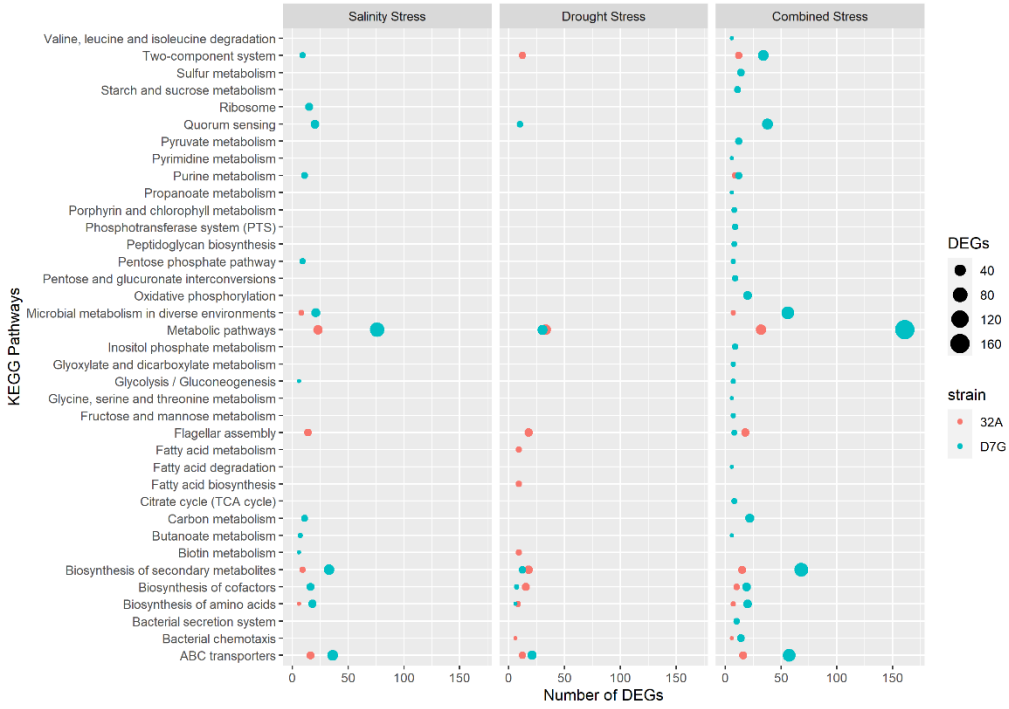

Figure 3. 9: Top pathways enriched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Size of the scatter point shows the number of DEGs in 32A and D7G

3.3.8. qRT-PCR validation

qRT-PCR analysis was performed to validate the RNASeq data. Five DEGs of each bacteria strain 32A and D7G were selected. Five genes of 32A including *FlgJ*, *Flil*, *LuxR*, *LexA* and *copC* and of D7G including *ahpF*, *flgC*, *hpxA*, *GrxA* and *GlpG* were selected, based on their activity in bacteria growth and movement under stress. All the selected genes were involved in biological processes to survive under stress conditions. In this study, given some variation in the relative fold change, the qRT-PCR results were consistent with those obtained from RNA-Seq analysis (Figure 3.11).

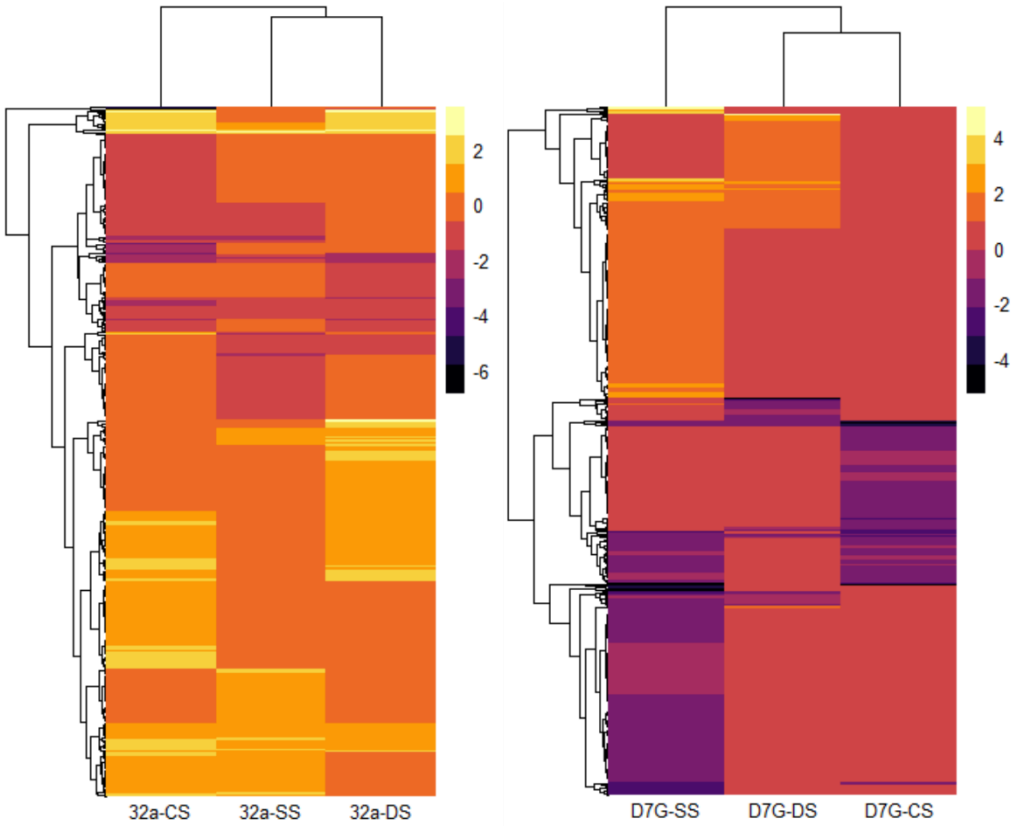

Figure 3. 10: Clustering heatmap of differentially expressed genes in *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G after 36 h of salinity stress (SS), drought stress (DS) and combined stress (CS)

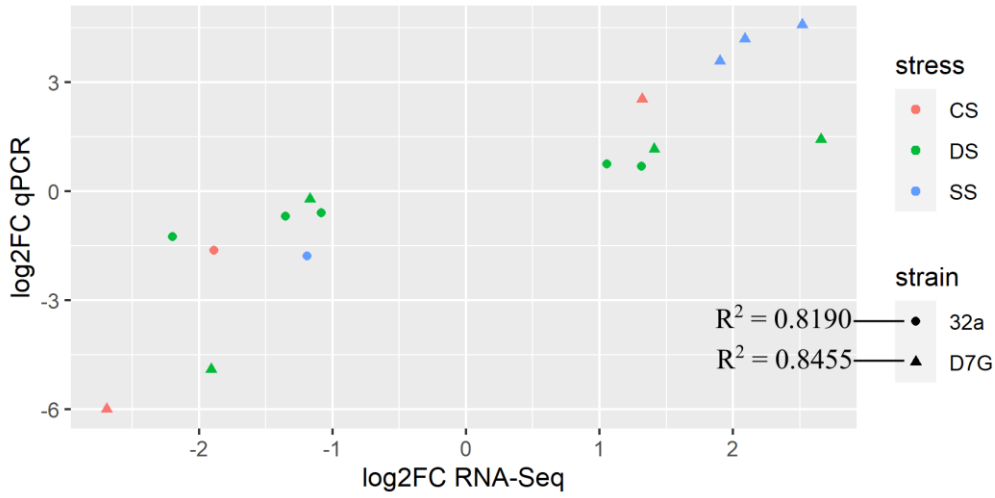

Figure 3. 11: Scatter plot of RNA-Seq and qRT-PCR relative expression levels. Pearson correlation test was applied to log2FC values assessed using the RNA-Seq and qRT-PCR of selected genes. Different colors in each graph correspond to Salinity, drought and combined stress donated as SS, DS, and CS, respectively

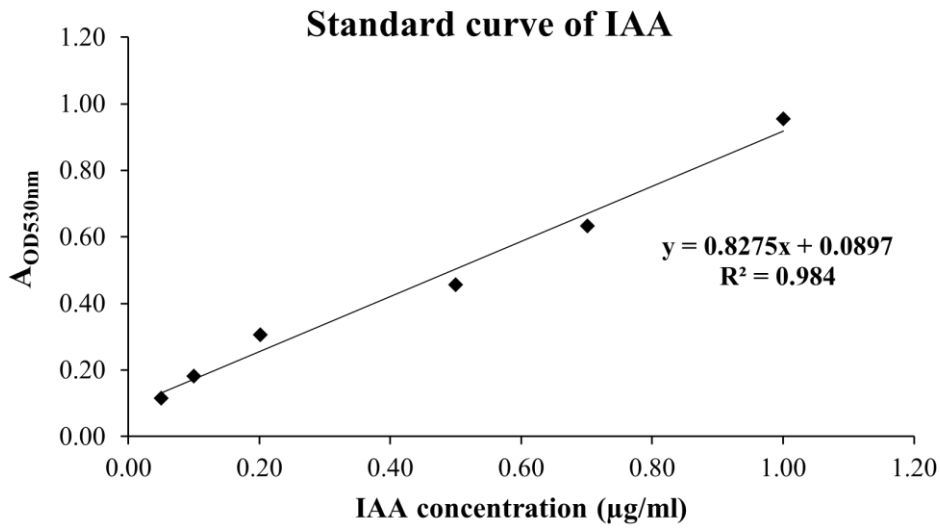

Figure S3. 1: Scatter plot showing standard curve of indole 3-acetic acid (IAA) concentration and optical density of solution at 530nm ($A_{OD530nm}$). Regression equation (y=0.8275x + 0.0897) was used to esmitate the amount of IAA production by bacteria

3.4. Discussion

The aim of this work was to understand the molecular functions underlying the endophytic bacterial tolerance to individual and combined effect of salinity and drought stress. Abiotic stresses such as salt and drought tolerant bacteria utilize many strategies to adapt to extreme conditions. While these mechanisms have been extensively researched for years, only a few experiments have demonstrated the variations on transcriptome level during bacterial growth under individual salinity or drought stress conditions. Bacterial transcriptome study under combined stress condition is extremely rare. In this work, the entire transcriptome of two bacteria strains *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G were analyzed after their exposure to individual and combined salinity and drought stress. These two bacterial strains (32A and D7G) performed better in stress tolerance, IAA and biofilm production, and endophytic colonization (being more motile) under individual and combined salinity, drought stress. Highlighting tolerance mechanism to individual and combined stresses, transcriptomic analysis of these bacteria showed significant changes in the expression levels of 642 and 1244 genes of 32A and D7G, respectively, after 36 h long exposure to salinity, drought, and combined stress. A total of 257 genes were down-regulated and 385 genes were up-regulated in 32A, while 552 and 691 genes were down- and up-regulated in D7G, respectively (Figure 3.6). Notably, analysis of DEGs with KEGG and GO terms revealed that, a significant variation occurs in the adaptation mechanisms of both 32A and D7G after their exposure to individual and combined stresses. Our results also illustrated that 32A regulated various pathways to cope with salt and drought stress. On the other hand, D7G regulated 47% more DEGs involved in various pathways under salinity and 54% more DEGs under combined stress treatment as compared to the drought stress. This might be due its significant tolerance to the drought stress (6.75% PEG, -0.1 MPa) applied in this study.

Several studies have confirmed the efficacy of PGPEB to induce salinity [276,277] and drought [278,279] tolerance in plants, which means that bacteria itself can survive under those extreme conditions, if they are helping the plants to improve their tolerance to abiotic stresses. For instance, it was observed that DEGs in both 32A and D7G were involved in common salinity and drought tolerance pathways including glycolysis/gluconeogenesis, glutathione metabolism, oxidative phosphorylation, etc. These common KEGG pathways also defines the tolerance of bacteria against combined salinity and drought stresses. Few studies confirmed that combined effects of abiotic stress may have an additive effect on plant growth with shared responses exhibiting

common physiological and molecular events, whereas physiological traits are unique to single stresses [280]. For example, co-occurrence of salt and drought stress severely impacted the stomatal conductance and photosynthetic rate of *Hordeum spontaneum* and enhanced oxidative damage. It also resulted in greater Na^+ accumulation in roots as compared to stem, shoot and leaves, while Na^+ in shoots increased under individual effect of salinity stress [281]. Similarly, plant open their stomata by transpiration to cool their leaves under heat stress, when it is combined with drought stress, this strategy of cooling down will lead to leaf wilting [282]. Similarly, our data indicates that bacterial response to individual and combined stresses have both additive and antagonistic pathways (Figure 3.9). For instance, number of up-regulated genes in 32A under combined stress is 30% less than the addition of up-regulated genes under both salinity and drought stress, showing an antagonistic effect. On the other hand, up-regulated genes in D7G after combined stress exposure are 32% more as compared to the sum of both salinity and drought stress treatments, showing an additive effect (Figure 3.6C and D).

Total number of DEGs significantly changed under stress treatment with PGPEB strains, showing their response towards individual and combined abiotic stresses, to retain their stable growth and development. Number of DEGs in our study was lower than a recent study on ice plant PGPEB *Halomonas* sp. MC1 exposed to 1.71 % NaCl induced salinity stress [283]. Based on the transcriptome profiling of 32A, biological process with highest enrichment included the membrane transport, cell motility, and RNA transcription and degradation (Figure 3.7). For D7G, highest enrichment included the membrane transport, RNA transcription and degradation, and amino acid metabolism (Figure 3.8). A study reported that, the higher number of gene in membrane bounded periplasmic space helps bacteria to thrive under stressful environments [284]. Our findings suggested that 32A modulated significantly large number of genes in combined stress treatment followed by salinity and drought (Figure 3.6A and B), similar trend was observed in D7G under combined stress treatment. Another study reported that membrane bound periplasmic spaces as one of the top four enriched terms in pollinator fig wasp in response to volatile organic compounds from its host figs [285]. Catabolic processes are crucial for PGPEB resistant to extreme conditions [286]. Bacterial catabolic genes are crucial not only in crops plant and agricultural soil but also vital in marine environments [287,288]. Our results showed that ion transmembrane transport genes were regulated under salt stress and combined stress treatments, suggesting that salt exposure stimulates the transport of ions in the membrane spaces. 32A and D7G also regulated the genes to build resistance against salt and drought stress

to equilibrate osmotic pressure. A study confirmed that salt stress regulates the ion diffusion in halotolerant bacteria's exoproteome [289].

Under ideal conditions, stress proteins are found in very low concentrations in the cell. However, as cells are subjected to stress that exceeds their resistance threshold, the expression of these proteins changes. Stress proteins, in collaboration with molecular chaperones, assist defectively folded proteins in regaining their proper structures, allowing them to sustain biological functions [290]. Such extreme hyper-saline conditions and maintenance of correct protein shape has been shown in a proteomic study on extreme halophilic archaeon *Halobacterium salinarum* [291]. Consistently in our research, up-regulation of many stress proteins coded genes (protein metabolism; Figure 3.7 and 3.8) have been observed under stress conditions. Microorganisms adopt another strategy to survive under salinity and drought stress by accumulating osmoprotactant solutes inside their bodies [292,293]. Our finding revealed that elevated level of salt and drought resulted in the expression of genes involved in pathways for microbial metabolism in diverse environments (*fadB*), glycine metabolism (*glyA*) and biosynthesis of amino acids (*glyA, dapE, dapE*), which play a part in synthesis and accumulation of osmoprotectants. Prokaryotes also regulates proline as a compatible solute under salinity or osmotic stress. The genes playing a role in proline regulation (*proHJ*) was up-regulated in *Bacillus subtillis* under elevated levels of salts [294]. Likewise, our findings for 32A exhibited the regulation of *proV* under drought, *proV* and *proW* under salinity and *proW* under combined stress. In case of D7G, *proV* was regulated in salinity stress while under combined stress *proV, proW* and *proX* and no proline synthesis gene was regulated under drought stress. On the other hand, sugars may also be used as osmoprotactants under salt stress [295]. The RNA-seq analysis of 32A and D7G exhibited that many genes were involved in the metabolism of sugars and carbohydrates. For instance, significantly more DEGs in D7G were involved in starch and sucrose metabolism pathway under combined stress as compared to salinity and drought stress, which might be due to the mixed effects of abiotic stresses resulting in the greater accumulation of starch and sugars. Similar trend was observed in D7G for DEGs involved in fructose and mannose metabolism pathways (Figure 3.9).

In the KEGG database, information is given concerning molecular interactions via known cellular and metabolic pathways [296]. In this study, DEGs in 32A matched to 58, 46 and 63 pathways in salinity, drought, and combined stress treatments. While 74, 39 and 94 pathways were enriched by DEGs in D7G under salinity, drought, and combined stress, respectively. This suggested that 32A and D7G maintained their

growth by regulating metabolic and cellular pathways. Our research showed that most significant pathway included metabolic pathways in both PGPEB strains under individual and combined stresses, followed by ABC transporters, flagellar assembly, and microbial metabolism in diverse environments. Metabolic pathways and microbial metabolism in diverse environments suggested the bacterial defense mechanism under these stresses. Highest number of DEGs (161) involved in metabolic pathways was observed in combined stress treatment in D7G. ABC transporter was among top five highly enriched pathways. ABC transporters are linked to the increase uptake of nutrients and excretion of cytotoxic compounds from bacterial cell [297]. We speculated that 32A and D7G regulated ABC transporters to thrive under salt stress. However, under drought stress exposure, ABC transporters in 32A were less enriched than the other factors including, biosynthesis of secondary metabolites, flagellar assembly, and biosynthesis of co-factors (Figure 3.9). Flagellar assemble pathway was also in the top enriched pathways. Flagellar assembly is not only linked to motility but also adhesion to surface. Other than motility, flagellar assembly pathways were also found to be involved in adhesion to the surfaces under environmental stresses [298]. For pathogenic bacteria to invade in the host, adhesion to mucus is an essential process which is controlled by flagellar assembly [299]. According to our finding, under both individual and combined stress conditions a large number of DEGs were involved in flagellar assembly pathways in both 32A and D7G, suggesting the motility and adhesion of bacterial strain for their survival. This might also be involved in the endophytic colonization of bacteria to the host plants for their better survival inside plant tissues. Several stress response genes in D7G were also expressed in drought and combined stress treatment such as *glpA* (glycerol-3-phosphate dehydrogenase), and *glpK* (glycerol kinase), involved in damage repairing mechanism of cell cause by environmental stresses [300].

High number of DEGs involved in outer membrane-bounded periplasmic space in the GO analysis suggested that 32A and D7G regulated salt tolerance mechanisms. A study reported that lipid bilayer in cell membrane is a regulated barrier in dealing with ionic stress in the presence of salt [301]. We consider that our findings are potentially useful in the study of other salt- and drought-tolerant bacteria and in their gene expression analysis under individual and combined stresses. A greater understanding of PGPEB tolerance to individual and combined effects of salinity and drought stress will improve the storage, persistence and application of plant growth promoting endophytic bacteria in stress agriculture. Although microbe-mediated individual stress tolerance in plants [5,302,303] has been extensively elucidated, however, our understanding of combined

stress tolerance bacteria remains lacking. Our findings collectively suggest that 32A and D7G regulate cellular and metabolic pathways to adapt to individual and combined salinity and drought stress. This behavior shows that 32A and D7G can sustain under extreme salinity and drought conditions, even under the combination of two stresses (salinity and drought). Given our results on tolerance of these strains, 32A and D7G reflects the dynamics of complex salt-microbe, drought-microbe, and salt-drought-microbe interactions.

3.5. Conclusions

We evaluated the individual and combined effects of salinity and drought stress on the growth of *Enterobacter ludwigii* 32A and *Pantoea agglomerans* D7G using a data set generated through de novo assembly of next generation sequencing data. Salinity, drought, and combined stress treatments caused significant changes in genes, 642 in 32A and 1243 in D7G. Our results suggest, 32A survives under individual and combination of stresses through various pathways, such as, membrane transport, cell motility, RNA transcription and degradation and amino acid metabolism. In addition to these pathways, D7G survive through carbohydrate metabolism, and amino acid metabolism. Under combined stress, more DEGs in 32A were involved in metabolic, ABC transporter and sugar biosynthesis pathways as compared to the sum of DEGs involved in these pathways under individual salinity and drought stress. So, the bacteria strategy to survive under a combination of two stresses is not an additive survival strategy of the individual stresses. A potential candidate gene in 32A and D7G can also be extracted from our data for functional analysis of their survival under extreme conditions. Exploration of stress responsive gene for tolerance to salinity, drought, and combined stress can be cloned or stimulated before using the bacterial strains for further experiments.

Chapter 4-
Gluconacetobacter diazotrophicus Pal5 enhances plant robustness status under the combination of moderate drought and low nitrogen stress in *Zea mays* L.

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Graphical Abstract

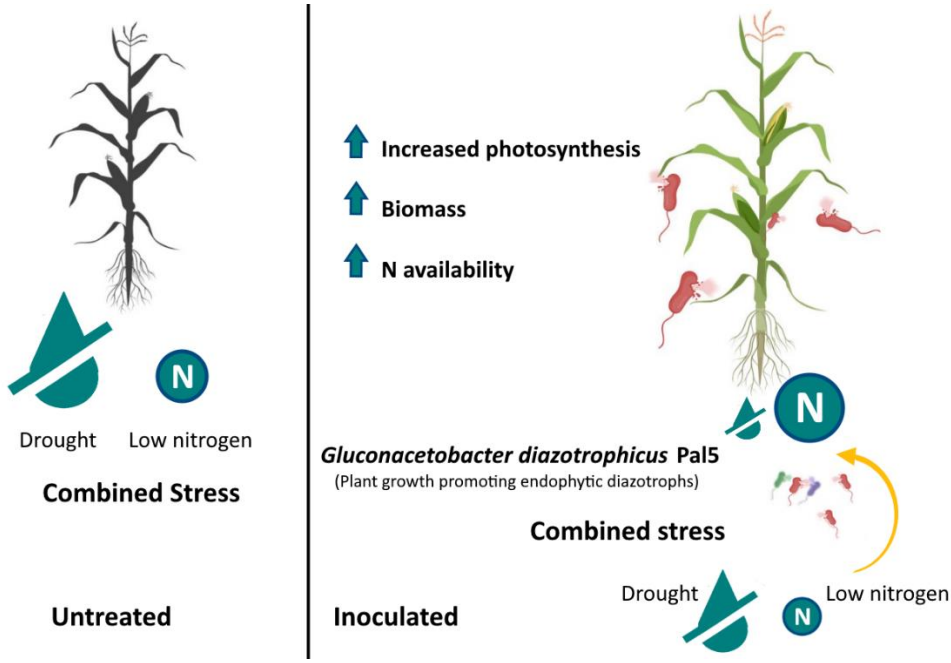

Figure 4a 1: Graphical Abstract

Abstract

Plant growth promoting endophytic bacteria, which can fix nitrogen, plays a vital role in plant growth promotion. Previous authors have evaluated the effect of *Gluconacetobacter diazotrophicus* Pal5 inoculation on plants subjected to different sources of abiotic stress on an individual basis. The present study aimed to appraise the effect of *G. diazotrophicus* inoculation on the amelioration of the individual and combined effects of drought and nitrogen stress in maize plants (*Zea mays* L.). A pot experiment was conducted whereby treatments consisted of maize plants cultivated under drought stress, in soil with a low nitrogen concentration and these two stress sources combined, with and without *G. diazotrophicus* seed inoculation. The inoculated plants showed increased plant biomass, chlorophyll content, plant nitrogen uptake, and water use efficiency. A general increase in copy numbers of *G. diazotrophicus*, based on 16S rRNA gene quantification, was detected under combined moderate stress, in addition to an increase in the abundance of genes involved in N fixation (*nifH*). Endophytic colonization of bacteria was negatively affected by severe stress treatments. Overall, *G. diazotrophicus* Pal5 can be considered as an effective tool to increase maize crop production under drought conditions with low application of nitrogen fertilizer.

4.1. Introduction

The main limiting environmental factors influencing maize production worldwide are drought and low N stress [304-306]. Drought stress cause up to a 15% annual yield loss in maize, which is gradually increasing due to climate change [307,308]. In the current situation, agriculture is significantly impacted by climate change, causing a global threat to food security [309]. This is mostly derived from the loss of arable land due to drought stress, land degradation, and environmental restrictions on agricultural production as reported by the UN General Assembly [310]. In the 20th century, chemical synthesis of nitrogen fertilizer through the Haber–Bosch process improved agriculture production and food security [311]. Owing to the instability of synthetic nitrogenous fertilizers, over half of the world's nitrogen fertilizer is lost to leaching in groundwater and volatilization in the atmosphere in the form of nitrous oxide, a potent greenhouse gas [312,313]. Another leading agricultural challenge is to supply adequate and sufficient nitrogen to cereal crop plants. Cereals contain 75% carbohydrates and up to 15% protein, contributing 50% in global terms of energy supply [314].

Plants face diverse biotic and abiotic stresses in hostile environments. Drought stress has been counted as a critical issue that negatively affects plant growth in different developmental stages, and, more importantly, crop yield [315]. Several approaches are employed to enhance drought tolerance and nitrogen use efficiency in plants with higher yields. Current agricultural production approaches are costly and non-renewable, e.g.,

improper use of chemical fertilizers can contribute to greenhouse gas emissions and cause various environmental problems [316,317].

Plants have natural mechanisms for defending against multiple stresses and one of them is the synergic interaction with microorganisms [318]. Such plant-beneficial microorganisms, specifically bacteria, offer several advantages to their host plants and allow them to withstand various biotic and abiotic stresses that can have detrimental effects on their growth and development [63,64,319]. Endophytic bacteria that, directly or indirectly, support plant growth, development, and health status are usually known as endophytic plant growth promoting bacteria (PGPEB). PGPEBs can increase productivity and confer plant immunity and systemic resistance to abiotic stresses that can induce physiological, molecular, and biochemical changes in plants. These PGPEBs improve osmotic adjustment, phytohormone regulation, nutrient (N, P, K etc.) acquisition, enzymatic and non-enzymatic antioxidants' activation mechanisms, and osmo-protectants' production [73,74,320]. Particularly, endophytic diazotrophic bacteria are of special interest since they are capable of fixing atmospheric nitrogen, entrapping N_2 and converting it in NH_3, a form that is readily utilized by plants [321]. This process is catalyzed by the oxygen-sensitive enzyme nitrogenase, formed by various subunits encoded by the *nifH*, *nifD*, and *nifK* genes. Among these three genes, *nifH* has become the most used reference marker in studies of diversity and abundance of nitrogen-fixing microorganisms [322]. Furthermore, association with nitrogen-fixing PGPEBs may increase the leaf nitrogen concentration of plants which is essential to synthesize chlorophylls, nucleic acids, and proteins [323]. Some nitrogen-fixing endophytes are being currently tested as biofertilizers, and these bacteria include members of the genera Azoarcus, Achromobacter, Burkholderia, Gluconoacetobacter, Herbaspirillum, Klebsiella and Serratia [324]. *Gluconacetobacter diazotrophicus* Pal5 is an endophytic diazotrophic bacteria, which has previously been reported in ameliorating the effects of drought stress in rice and sugarcane plants and promoting plant growth in low nitrogen environments [325-327].

Maize (*Zea mays* L.) is a multipurpose crop with wide adaptability to different agro-climatic conditions. It is grown in most parts of the world and is preferred by farmers because it is a C4 cash crop with a high photosynthetic rate and grain production potential, with a dual-purpose use as food source (grain and fodder) and raw material for industry [328-331].

Therefore, the aim of the present study was to investigate the potential of the diazotrophic bacteria endophyte, *Gluconacetobacter diazotrophicus* Pal5, for improving the growth of maize plant under individual and combined effects of drought and low nitrogen stress. Our findings suggests that use of endophytic diazotrophic bacteria can be a promising solution to improve plant tolerance to a combination of multiple stresses.

4.2. Materials and Methods

4.2.1. Experimental Design

4.2.1.1. Inoculum Preparation

The bacterial strain *Gluconacetobacter diazotrophicus* Pal5 (Gd) type strain (DSM 5601) was ordered from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Leibniz Institute, Germany (Retrieved on March 06, 2019, from https://www.dsmz.de/). The bacterium was cultured in 250 mL Sabouraud 2% Glucose (SG) broth at 28 °C for 48 h at 180 rpm in an orbital shaker. The culture optical density was measured at $\lambda = 600$ nm using a spectrophotometer and adjusted to 0.1 to obtain a uniform population of bacteria, 10^8 colony forming units (CFU) mL^{-1}, for inoculation.

4.2.1.2. Pot Experiment Setup

A pot experiment was conducted in the growth room to evaluate the effectiveness of the nitrogen fixing bacterial strain Gd for promoting growth and yield of maize under nitrogen and water deficit conditions. Maize seeds (variety: KXB 5146, batch number: 16V-4053, KWS, Einbeck, Germany) were surface sterilized before inoculation, according to Naveed et al. [332]. Sterile maize seeds were incubated in a 10^8 CFU mL^{-1} of SG broth overnight culture of Gd for 2 h. Untreated seeds were maintained for 2 h in sterile 2% SG broth. Five seeds, either inoculated or not, were sown in plastic pots containing 550 g of sterile commercial soil with fewer amount of available nutrients (50–200 mg L^{-1} nitrogen, 80-150 mg L^{-1} P_2O_5 and 150–300 mg L^{-1} K_2O; Gardol). Two days after germination, the plants were trimmed to one. Plastic pots were sterilized using 70% ethanol and soil was sterilized two times at 121 °C for 40 min. Prior to seed sowing, an equal amount of sterile distilled water was applied to the pots to maintain optimal soil moisture. Temperature was set to 25 ± 2 °C, the photoperiod to a 16 h light and 8 h dark with 36% humidity.

In total, 6 treatments were set up (Table 1). The experiment comprised three levels of nitrogen and three levels of drought stress. The soil moisture regimes were: 35% (Dr.35), 50% (Dr.50), and 100% (Dr.100) of soil water holding capacity (WHC), representing severe, moderate, and no water stress conditions, respectively. Soil water content regimes were controlled by weighing the pots and irrigating the plants during the experimental period starting from 12 days after sowing. The nitrogen treatments consist of no-N (N-Free, 0 mg N pot^{-1}), 50% N (N-50, 150 mg N pot^{-1}), and 100% N (N-100, 300 mg N pot-1) of recommended Nitrogen dose. Nitrogen doses were applied in the form of modified Hoagland solution, 7 days after sowing. A modified Hoagland solution was prepared using calcium nitrate, as a source of nitrogen fertilizer [333]. An

N-100 dose was calculated based on nitrogen, phosphorous, and potassium (N-P-K:160-100-60 kg ha^{-1}) fertilization recommended by Naveed et al. [332]. Five replicates per treatment were set up, using either untreated or Gd inoculated seeds, making a total of 60 experimental units.

Table 4. 1: Treatment plan

Treatments	Description
T1	Soil moisture regime 35% of WHC with 100% nitrogen application
T2	Soil moisture regime 50% of WHC with 100% nitrogen application
T3	No nitrogen application with 100% WHC
T4	50% nitrogen application of recommended dose with 100% WHC
T5	Soil moisture regime 35% of WHC with 50% of nitrogen application
T6	Soil moisture regime 50% of WHC with 50% of nitrogen application

4.2.2 Plant Analysis

Maize plants were harvested 26 days after sowing and taken from all five pots in each treatment. Bulk soil attached to the roots was removed by gently shaking and followed by a water rinse. Roots, stems, and leaves of each plant were separated for further analysis. Samples needed for molecular analysis were immediately stored at −80 °C.

The following parameters related with the plant were measured: plant biomass, chlorophyll content and relative water content, plant water consumption and efficiency, and leaf rolling score. Shoot and root weight were measured with a weighing balance. Plant images were captured with a centimetre scale and analysed with the open access software platform FIJI (ImageJ) [334]. Plant growth parameters such us shoot length, root length, stem diameter, and leaf width were measured. Leaf relative water contents (RWC) were calculated according to [335]. To evaluate the photosynthetic efficiency, *Chl a*, *b* and carotenoids were measured. Therefore, 0.5 g of fresh leaf cut from the middle part of the older leaves was ground in 4.5 mL acetone (80%) using a porcelain mortar and then centrifuged at 3000 rpm for 5 min. The mixture was brought to the volume of 20 mL by adding distilled water. The final solution was exposed to a wavelength of 646 and 663 nm to determine the concentration of *Chl a* and *b*, respectively, and 470 nm for carotenoids using a spectrophotometer. Chlorophyll concentration per mg of fresh weight was determined based on the method described by Lichtenthaler and Wellburn (1983). Plant shoot samples, three replicates per treatment, were sent to LUFA® (https://www.lufa-nord-west.com/ on August 31, 2020) for nitrogen analysis. Then, shoot nitrogen uptake and nitrogen use efficiency were

calculated as described by [336]. Plant water consumption (PWC), i.e., the total evapotranspiration from maize plant and soil, was calculated from the water balance in each experimental pot according to Wang et al. [337]. For the whole plant, water use efficiency (WUE) was calculated as the ratio between shoot dry matter (DM) and PWC during the experimental period. The leaf rolling score included five levels: 1, leaf is unrolled and turgid; 2, leaf rim starting to roll; 3, leaf has a shape of a 'V'; 4, rolled leaf rim covers part of leaf blade; and 5: leaf is rolled like an onion [306].

4.2.3. DNA Isolation

Root, stem, and leaf samples from three plants per treatment were used for DNA isolation. Namely, 0.5 g of tissue were cut and sterilized with 70% ethanol for 30 sec, treated with 2% NaClO for 10 sec, and followed by 3 times washing with sterile distilled water for 1 min each. Surface sterilized samples were grounded in liquid nitrogen using autoclaved pistil and mortar. Finally, DNA was isolated from the grounded plant samples using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific GmbH, Dreieich, Germany), according to the manufacturer's instructions. Pure DNA was stored at −20 °C until further needed.

4.2.4. G. diazotrophicus Pal5 Detection

In order to detect *G. diazotrophicus* in roots, stems, and leaves of the inoculated maize plants, a nested PCR approach was implemented. In the first PCR round, a primer pair targeting the whole 16S rRNA gene was used, the 16S-27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and16S-1492R (5′-TACGGYTACCTTGTTA CGACTT-3′) [338]. In the second PCR run of the nested approach, the bacterial primer pair PAl5F2 (5'-GGCTTAGCCCCTCAGTGTCG-3´) and PAl5R2 (5´-GAAACAGCCATCTCTGACTG-3´) was used to amplify 16S rRNA gene fragments of *G. diazotrophicus* [339]. For the first PCR round, the reaction mixture (50 mL) contained: 1 µL DNA template, 1.25 Units DreamTaq DNA polymerase (Thermo Fisher Scientific, Germany), 1X DreamTaq Buffer, 0.2 mM of each dNTP, 0.125 µM of each primer. In the second PCR round, for 50 mL reactions, the following reactive concentrations were used: 1 µL PCR product, 1.25 Units DreamTaq DNA polymerase (Thermo Fisher Scientific, Germany), 1X DreamTaq Buffer, 0.2 mM of each dNTP, 0.125 µM of each primer. The gene fragments were amplified with a Mastercycler® gradient thermocycler (Eppendorf, Hamburg, Germany). Thermal cycling conditions in both PCR rounds were: initial denaturation step of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min and a final extension step for 7 min at 72 °C. The presence and correct

size of PCR product was checked in a 1.5% agarose gel. The 16s rRNA gene fragments derived from the second PCR run were purified and sent to sequenced, in Starseq® (Mainz, Germany), to confirm that DNA from *G. diazotrophicus* was amplified.

4.2.5. Design of Novel nifH Primers and Validation

In order to detect the *nifH* gene in the inoculated plants tissue, the *nifH* universal primer pair designed by Ueda et al. [340] was used. After PCR run, no amplification was detected. Therefore, a new primer pair was designed to detect *nifH* gene in *G. diazotrophicus* Pal5 specifically. Moreover, *G. diazotrophicus* Pal5 genome was compared with the universal *nifH* primers proposed by Ueda et al. [340], using the QIAGEN CLC Genomics workbench (QIAGEN, Hilden, Germany). The Gd genome regions, where the *nifH* universal primers were attached in silico, were the sequences used to design the new primer pair for this study. The novel primer pair Gd-nifH-F (5´-GCCTTTTATGGAAAGGGAGG-3´) and Gd-nifH-R (5´-AAGCCGCCGCAGACCACGTC-3´) were used to amplify *nifH* gene in the inoculated plants' root, stem, and leaf tissues. For 50 mL PCR reactions, the following concentrations were used: 1 µL DNA template, 1.25 Units DreamTaq DNA polymerase (Thermo Fisher Scientific, Germany), 1X DreamTaq Buffer, 0.2 mM of each dNTP, 0.45 µM of each primer. The gene fragments were amplified with a Mastercycler® gradient thermocycler (Eppendorf, Hamburg, Germany). PCR conditions consisted of an initial denaturation at 94 °C for 5 min, which was followed by 40 cycles of 94 °C for 50 sec, annealing at 62 °C for 45 sec, extension at 72 °C for 1 min, and the final extension for 7 min at 72 °C. The presence and correct size of PCR product was checked in a 1.5% agarose gel and verified by sequencing.

4.2.6. Quantification of nifH and G. diazotrophicus Pal5 16S rRNA Genes in Plant Tissues

The abundance of the *nifH* and the *G. diazotrophicus* 16S rRNA genes was assessed by quantitative PCR (qPCR). The qPCR was carried out with 2x GoTaq® qPCR Master Mix containing a low level of carboxy-X-rhodamine (CXR) reference dsDNA-binding dye (Promega, Germany) on an Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems (ThermoFisher Scientific, Bremen, Germany). The oligonucleotide primer pairs used were PAl5F2/PAl5R2 and Gd-*nifH*-F/Gd-*nifH*-R (see above) at a concentration of 333 nM. The thermal cycling conditions for *G. diazotrophicus* 16S rRNA genes were one DNA-denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min., 58 °C for 1 min, and 72 °C 1 min. For *nifH* genes, the qPCR conditions were one cycle at 94 °C for and then continued with 40

cycles of 94 °C for 50 sec., 62 °C for 45 sec., 72 °C for 1 min. The 10-log-fold standard curves were produced as follows: *G. diazotrophicus* Pal5 DNA was used as a template for conventional PCR amplification of the *nifH* and 16S rRNA genes (see above, Section 2.5 and 2.4, respectively). The PCR products, with the expected size, were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), quantified with NanoDrop 2000c (Thermo Scientific, Wilmington, DE, USA), and the gene copy numbers were calculated with scienceprimer (Retrieved on August 06, 2020 from http://scienceprimer.com/copy-number-calculator-forrealtime-pcr). Ten-fold serial dilutions of *nifH* and *Gd 16S rRNA* genes PCR products were prepared and used to generate the qPCR standard curve.

The quantification of these genes in plants' roots, stems, and leaves was carried out with 1 mL of DNA template added to the PCR master mix in 96-well plates. Negative controls without DNA template and standards were included in all plates, and the melting curves were evaluated to confirm the purity of the amplified products.

4.2.7. Statistical Analysis

The statistical analysis was performed using the R (version 4.0.4) package agricolae (version 1.3-3). After corroborating the normality and homogeneity assumptions, a one-way ANOVA was performed followed by a Tukey's HSD test ($\alpha = 0.05$). Data of qPCR were analyzed using StepOne™ software v. 2.3. A regression analysis was used to determine the relationships between the measured parameters. The graphs were designed using a ggplot2 package in the R environment and Microsoft Excel.

4.3. Results

4.3.1. Effect of G. diazotrophicus Inoculation on Maize Plant Growth

In this study, significant differences were observed in fresh and dry weights of root and shoot parts of the inoculated plants as compared to non-inoculated plants (Figure 4.1a–d). Under severe drought (T1) and N deficiency (T3), no significant differences were observed in shoot and root weights (fresh and dry) between untreated and inoculated plants. However, when water holding capacity was at 50% (T2), i.e., moderate drought stress, Gd inoculation significantly increased the shoot fresh weight by 65%, shoot dry weight by 67%, root fresh weight by 30%, and root dry weight by 80% of maize plants (Figure 4.1a–d). Shoot fresh weight of maize plants increased by 66% with Gd inoculation when grown under medium N deficiency (T4) (Figure 4.1a), but no

differences were seen in root fresh weight and shoot and root dry weights (Figure 4.1b–d). On the other hand, a significant increase of 28% in root fresh weight was observed in Gd inoculated plants under severe combined stress (T5) (Figure 4.1c). The highest increase in plant shoot fresh weight was observed in the moderate combined drought and nitrogen stress (T6) that modulated from 9 ± 2 g to 24 ± 3 g (Figure 4.1a). The shoot dry weight was also increased from 560 ± 99 mg to 1490 ± 234 mg in T6 treatment) (Figure 4.1b). However, no significant differences were observed in root weights in moderate drought and nitrogen stress treatment combined (T6; Figure 4.1c,d).

Gd inoculated plants showed an increase in shoot length when grown under moderate drought stress (50% WHC, T2), severe nitrogen stress (T3), and moderate combined stress (T6) (Figure 4.2a). The greatest increase in shoot length occurred when the plants were subjected to moderate drought and nitrogen stress (T6) and raised from 254 ± 68 cm in untreated plant to 385 ± 43 cm in Gd-inoculated plants (Figure 4.2a). In the same treatment, T6, the largest root sizes were also found, with a length of 170 ± 14 cm in untreated plants and 416 ± 95 cm for inoculated plants, meaning a 145% increase (Figure 4.2b). In addition, Gd inoculation caused an increase of 46% in root length when maize plants were grown under moderate nitrogen stress (150 mg N pot^{-1}, T4) (Figure 4.2b). Whereas no differences were observed in other treatments in shoot and root lengths of inoculated maize plants as compared to untreated controls (Figure 4.2a,b).

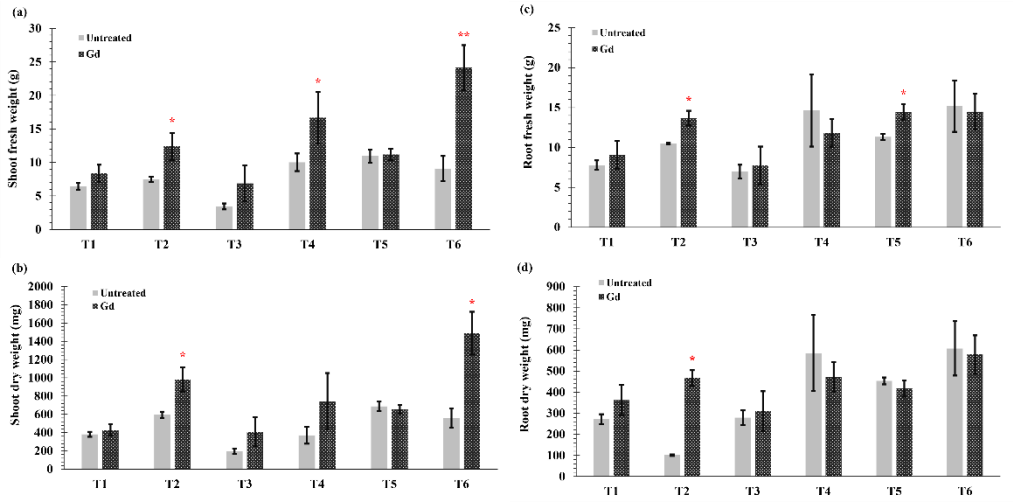

Figure 4. 1: Evaluating the effects of severe water stress (35%-WHC; T1), moderate water stress (50%-WHC; T2), severe nitrogen stress (0 mg N pot^{-1}; T3), moderate nitrogen stress (150 mg N pot^{-1}; T4), combination of severe water stress and moderate nitrogen stress (35% WHC and 0 mg N pot^{-1}; T5), and combination of moderate water stress and moderate nitrogen stress (50% WHC and 150 mg N pot^{-1}; T6) on (a)- Shoot fresh weight, (b) shoot dry weight, (c) root fresh weight, and (d) root dry weight of maize plants inoculated with *Gluconacetobacter*

diazotrophicus Pal5 (Gd) in comparison with untreated plants. Bars represented means of three (n = 3) replicates with standard errors (SEs). * and ** above bars indicate significance at $p < 0.05$ and $p < 0.01$ and bars without any * are non-significant ($p > 0.05$)

In plants inoculated with *G. diazotrophicus*, an increase was shown in the leaf relative water content (RWC) of maize plants by 8%, 10%, and 6% under moderate water stress (50% WHC; T2), moderate nitrogen stress (150 mg N pot^{-1}; T4), and moderate combined stress (50% WHC and150 mg N pot^{-1}, T6) treatments (Figure 4.3). In other treatments, no significant differences were observed in Gd inoculated plants as compared to untreated control (Figure 4.3).

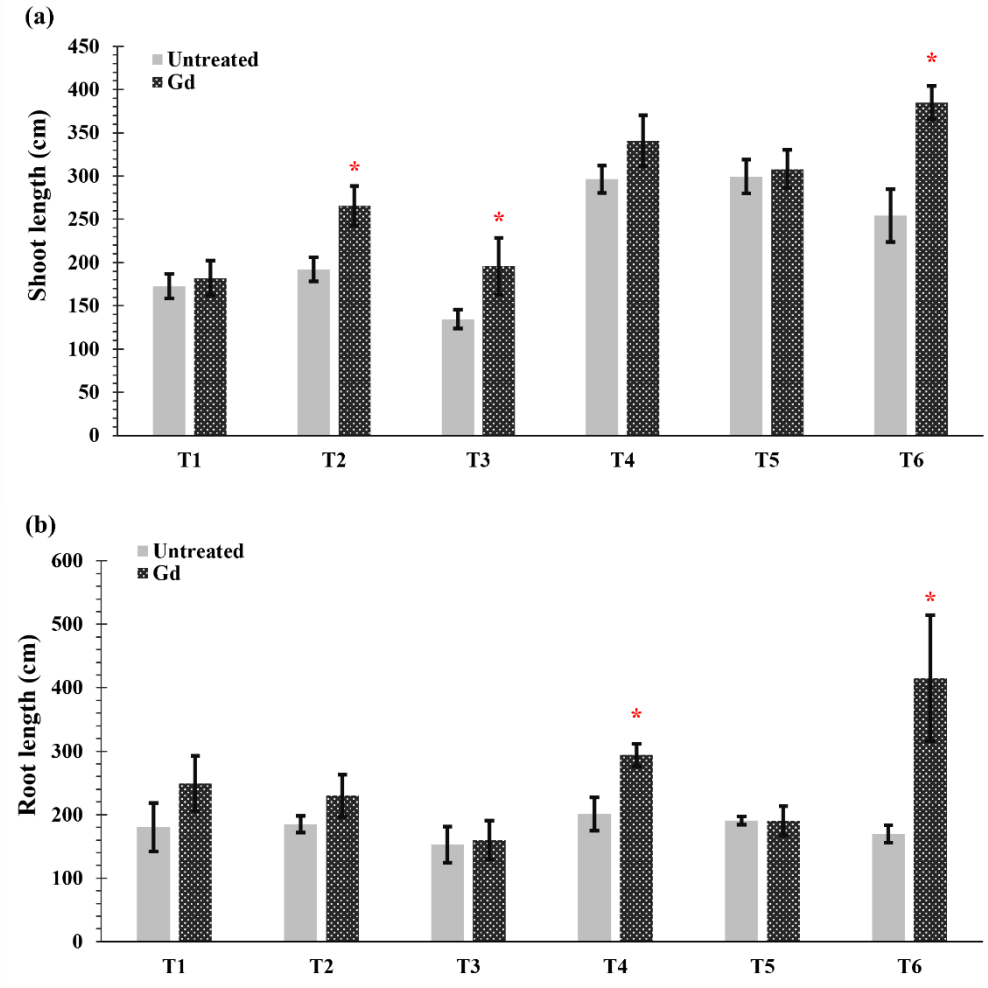

Figure 4. 2: Evaluating the effects of severe water stress (35%-WHC; T1), moderate water stress (50%-WHC; T2), severe nitrogen stress (0 mg N pot^{-1}; T3), moderate nitrogen stress (150 mg N pot^{-1}; T4), combination of severe water stress and moderate nitrogen stress (35% WHC and 0

mg N pot^{-1}; T5), and combination of moderate water stress and moderate nitrogen stress (50% WHC and 150 mg N pot^{-1}; T6) on (a) shoot length and (b) root length of maize plants inoculated with *Gluconacetobacter diazotrophicus* Pal5 (Gd) in comparison with untreated plants. Bars represented means of three (n = 3) replicates with standard errors (SEs). * above bars indicate significance at $p < 0.05$ and bars without any * are non-significant ($p > 0.05$)

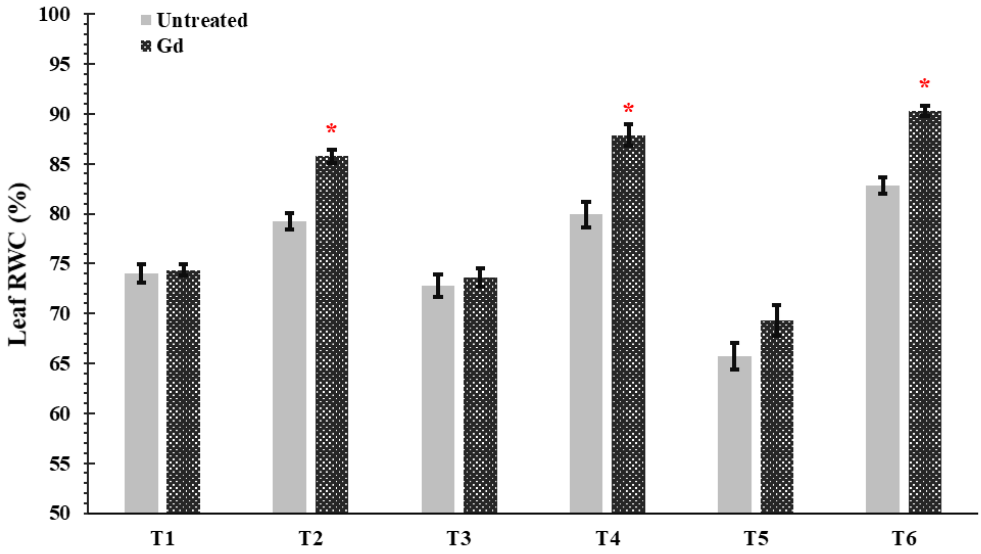

Figure 4. 3: Evaluating the effects of severe water stress (35%-WHC; T1), moderate water stress (50%-WHC; T2), severe nitrogen stress (0 mg N pot^{-1}; T3), moderate nitrogen stress (150 mg N pot^{-1}; T4), combination of severe water stress and moderate nitrogen stress (35% WHC and 0 mg N pot^{-1}; T5), and combination of moderate water stress and moderate nitrogen stress (50% WHC and 150 mg N pot^{-1}; T6) on Leaf relative water contents (RWC) of maize plants inoculated with *Gluconacetobacter diazotrophicus* Pal5 (Gd) in comparison with untreated plants. Bars represented means of three (n = 3) replicates with standard errors (SEs). * above bars indicate significance at $p < 0.05$ and bars without any * are non-significant ($p > 0.05$)

4.3.2. Plant Photosynthetic Efficiency

Differences in chlorophyll (a and b) contents between inoculated and untreated plants were found when the maize plants were grown under moderate drought stress (T2), moderate nitrogen stress (T4), and moderate combined stress (T6) (Figure 4.4a–c). The largest increase in chlorophyll content occurred under individual moderate nitrogen stress (T4), going from 2.8 ± 0.11 to 3.5 ± 0.12 mg g^{-1} fresh weight (FW) in chlorophyll a and from 1.4 ± 0.04 to 1.7 ± 0.06 mg g^{-1} FW in chlorophyll b (Figure 4.4a,b). Carotenoid contents, under moderate nitrogen stress (T4) and moderate combined stress (T6), were increased by 22% and 28%, respectively, when maize plants were inoculated with Gd (Figure 4.4d).

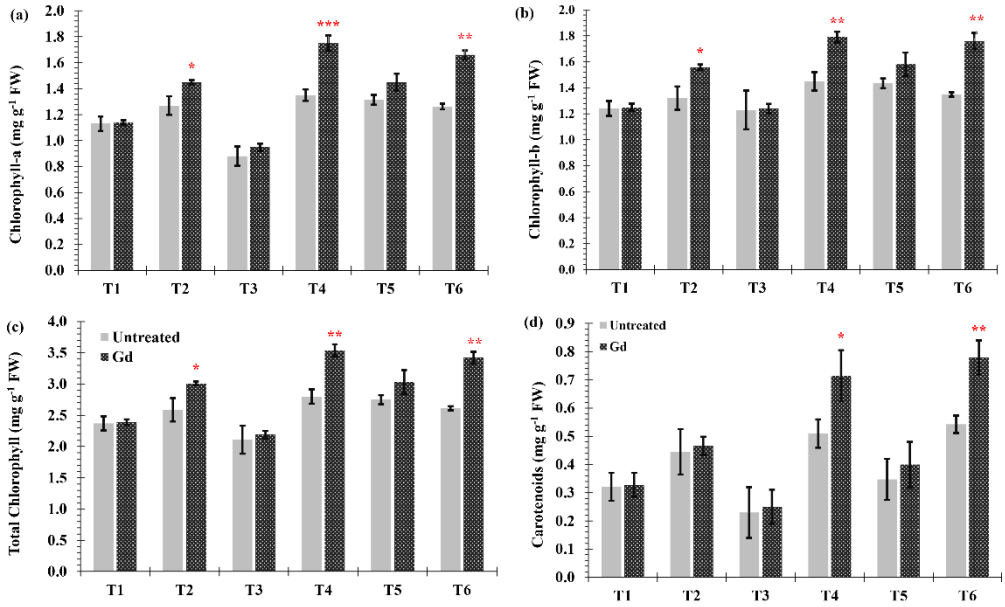

Figure 4. 4: Evaluating the effects of severe water stress (35%-WHC; T1), moderate water stress (50%-WHC; T2), severe nitrogen stress (0 mg N pot^{-1}; T3), moderate nitrogen stress (150 mg N pot^{-1}; T4), combination of severe water stress and moderate nitrogen stress (35% WHC and 0 mg N pot^{-1}; T5), and combination of moderate water stress and moderate nitrogen stress (50% WHC and 150 mg N pot^{-1}; T6) on (a) Chlorophyll-a and (b) Chlorophyll-b, (c) total Chlorophyll and (d) carotenoids of maize plants inoculated with *Gluconacetobacter diazotrophicus* Pal5 (Gd) in comparison with untreated plants. Chlorophyll is shown in milligram per gram of fresh weight (mg g^{-1} FW) of plants. Bars represented means of three (n = 3) replicates with standard errors (SEs). *, ** and *** above bars indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Bars without any * are non-significant ($p > 0.05$)

4.3.3. Nitrogen Contents in Plants and NUE

Gluconacetobacter diazotrophicus inoculated maize plants showed a significant increase in nitrogen content in shoots, as compared to untreated control, when growing under moderate drought stress (T2), severe N deficiency (T3), severe combined stress (T5), and moderate combined stress (T6). The highest increase in shoot nitrogen uptake was observed in T6 treatment, combined stress with 150 mg N pot^{-1} and 50 % WHC. This increment was 1.73 times higher in the inoculated plants than in the untreated control (Figure 4.5).

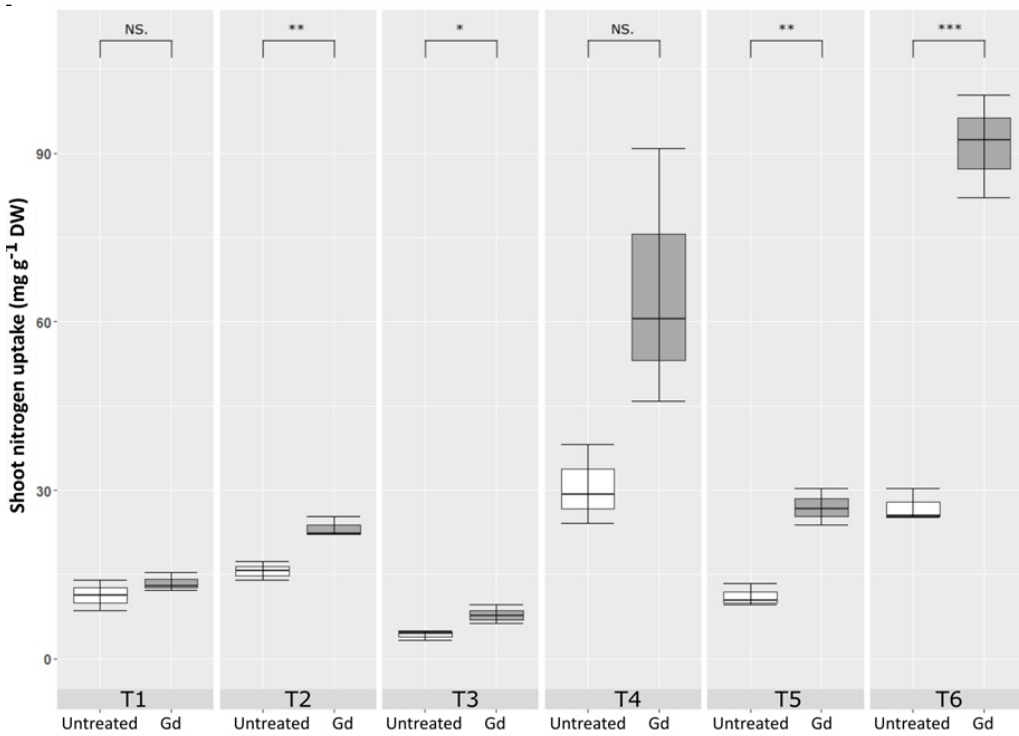

Figure 4. 5: Evaluating the effects of severe water stress (35%-WHC; T1), moderate water stress (50%-WHC; T2), severe nitrogen stress (0 mg N pot^{-1}; T3), moderate nitrogen stress (150 mg N pot^{-1}; T4), combination of severe water stress and moderate nitrogen stress (35% WHC and 0 mg N pot^{-1}; T5), and combination of moderate water stress and moderate nitrogen stress (50% WHC and 150 mg N pot^{-1}; T6) on carotenoids of maize plants inoculated with *Gluconacetobacter diazotrophicus* Pal5 (Gd) in comparison with untreated plants. Shoot nitrogen uptake is given in milligram per gram of dry weight (mg g^{-1} DW) of plants. Boxplots show the third quartile and first quartile (box edges), median (middle line) and range of the data (whiskers). Each boxplot represents the average of three samples. *, ** and *** above boxes indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Boxes without any * are non-significant ($p > 0.05$)

Nitrogen use efficiency (NUE) was significantly increased in plants inoculated with Gd when growing under moderate drought stress (T2), moderate N deficiency (T4), severe combined stress (T5), and moderate combined stress (T6), as compared to untreated control plants. The increments in NUE were 50%, 163%, 204.05%, and 274%, respectively. No significant differences were observed under severe drought and N stress treatments (T1 and T3) (Figure 4.6).

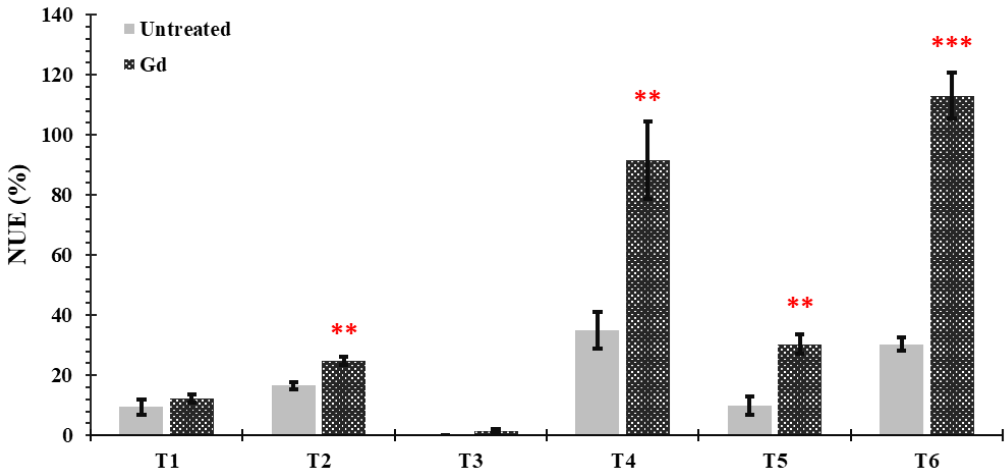

Figure 4. 6: Evaluating the effects of severe water stress (35%-WHC; T1), moderate water stress (50%-WHC; T2), severe nitrogen stress (0 mg N pot^{-1}; T3), moderate nitrogen stress (150 mg N pot^{-1}; T4), combination of severe water stress and moderate nitrogen stress (35% WHC and 0 mg N pot^{-1}; T5), and combination of moderate water stress and moderate nitrogen stress (50% WHC and 150 mg N pot^{-1}; T6) on nitrogen use efficiency of maize plants inoculated with *Gluconacetobacter diazotrophicus* Pal5 (Gd) in comparison with untreated plants. Bars represented means of three (n = 3) replicates with standard errors (SEs). ** and *** above bars indicate significance at $p < 0.01$ and $p < 0.001$, respectively. Bars without any * are non-significant ($p > 0.05$)

4.3.4. Plant Water Consumption, Water Use Efficiency, and Leaf Rolling Scores

In terms of plant water consumption (PWC), the highest values were obtained in maize plants growing under severe and moderate N stress (T3 and T4), in both untreated and Gd inoculated (Table 4.2). Plants were well irrigated in these two treatments. However, the PWC of maize plants significantly increased under severe and moderate drought stress (T1 and T2) and under moderate combined stress (T6) when inoculated with Gd as compared to untreated controls (Table 4.2). Additionally, the highest increase in PWC was observed in T6, by 3.6% (Table 4.2). Plant water use efficiency (WUE) generally increased when maize plants were inoculated with Gd as compared to the untreated ones (Table 4.2). The highest WUE levels were observed in the T6 treatment, when the plants were inoculated and growing under moderate combined stress (Table 4.2). Leaf rolling is one of the main plant reactions against drought stress in maize crops. There was a clear reduction in the leaf rolling scores in plants growing under moderate drought stress (T2) and severe and medium combined stress (T5 and T6) when Gd was inoculated as compared to the untreated controls (Table 4.2).

Table 4. 2: The effects of water stress levels and N rates on plant water consumption (PWC), water use efficiency (WUE), and leaf rolling score of maize plants at the time of harvest. *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

Treatments	PWC (mL)			WUE (mg/mL)			Leaf Rolling Score		
	Unt.	Gd	AOV	Unt.	Gd	AOV	Unt.	Gd	AOV
T1	719.7	747.1	*	0.35	0.39	ns	4.8	4.5	ns
T2	824.9	838.0	*	0.41	0.72	**	2.1	1.6	**
T3	1069.4	1100.6	ns	0.16	0.37	*	1.3	1.2	ns
T4	1120.2	1141.4	*	0.33	0.83	***	1	1	ns
T5	738.4	745.0	ns	0.41	0.49	ns	4.1	3.5	*
T6	809.0	840.5	**	0.69	1.99	***	2.2	1.5	**

4.3.5. nifH and G. diazotrophicus 16S rRNA Genes Abundance in Plant Tissues

The presence of *G. diazotrophicus* in roots, stems, and roots was confirmed by nested PCR targeting the *16S rRNA* gene. Regarding the qPCR results, the assays were highly reproduceable, and the standard errors were very low in the case of leaf and root tissues; however, stem tissues showed high standard errors making all the samples significantly similar to each other. The Gd 16s rRNA gene abundance ranged from 3.57 ± 0.11 (copy n° in T1) to 5.35 ± 0.14 gene copy number (log10) g^{-1} of fresh plant tissue (copy n° in T4). *G. diazotrophicus* 16s rRNA gene copy numbers were higher in inoculated plants growing under moderate N fertilization (T4) ranging from 4.31 ± 0.22 to 5.35 ± 0.11 gene copy number (log10) g^{-1} and moderate combined stress going from 4.59 ± 0.35 to 5.17 ± 0.15 gene copy number (log10) g^{-1} of fresh plant tissue (T6) (Figure 4.7). Moreover, in leaf tissues, the abundance of this gene was significantly higher in treatments T2 (moderate drought stress), T4, and T6 (Figure 4.7). No significant differences were observed in stem tissues; however, T5 showed least *Gd 16S rRNA* gene copy numbers as compared to other treatments. On the other hand, in *G. diazotrophicus* inoculated samples from T6 treatment, moderate combined stress sources, significantly higher *nifH* gene copy numbers were found in root and leaf tissues, as compared to the other treatments (Figure 4.7). No significant differences were observed in *nifH* gene abundance among treatments in stem tissues (Figure 4.7).

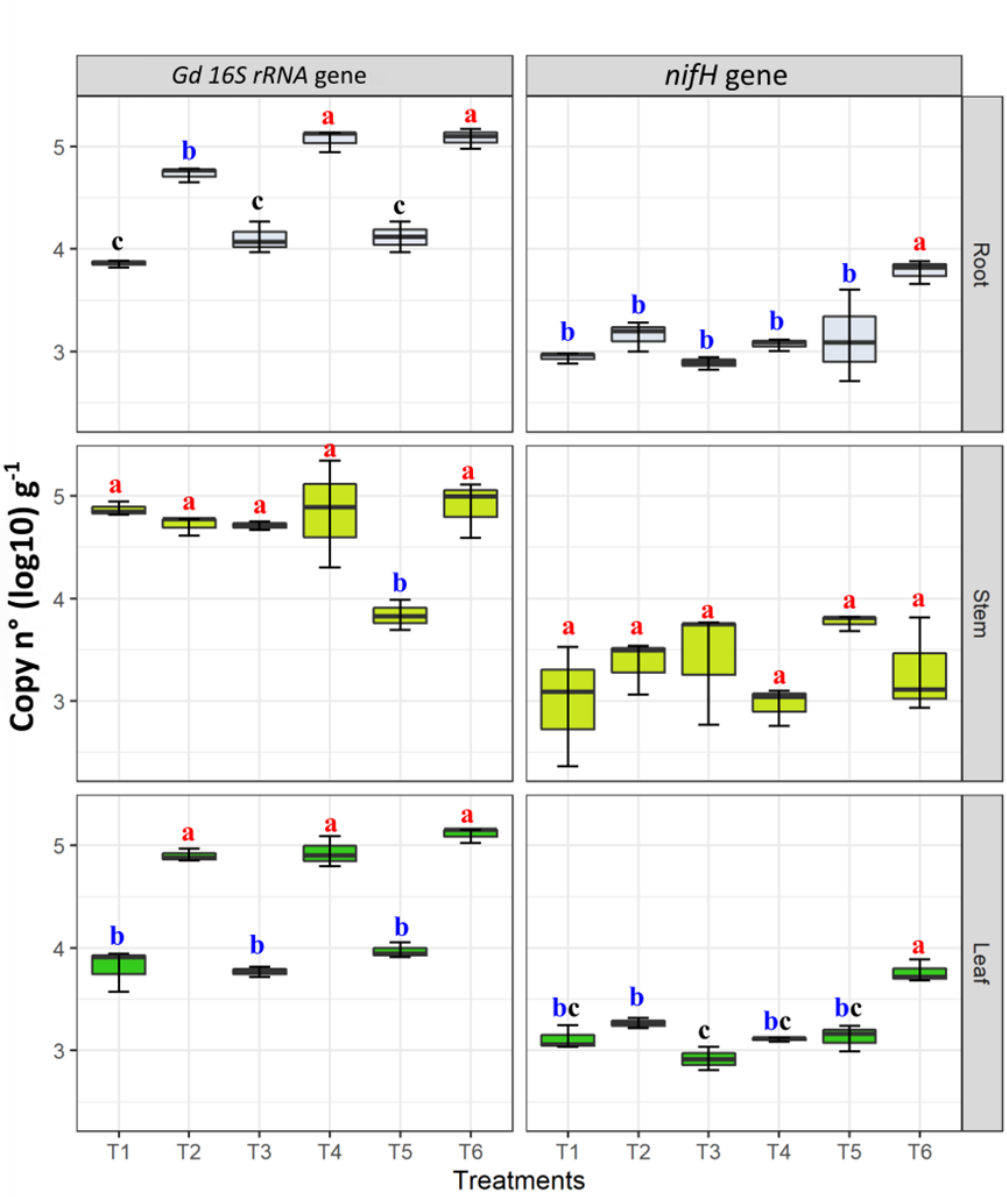

Figure 4. 7: Quantification of *G. diazotrophicus* (Gd) *16S rRNA* and *nifH* gene copies in samples of maize plant tissues (leaves, stems, and roots) grown plants under water and nitrogen stress treatments, applied individually and in combination (T1-T6). Gene abundance was copy numbers log 10 per gram of fresh plant tissue (copy n° (Log 10) g^{-1}). Boxplots show the third quartile and first quartile (box edges), median (middle line) and range of the data (whiskers). Each boxplot represents the average of three samples. Boxplots having the same letters are significantly similar according to the Tukey HSD test at $p < 0.05$

4.4. Discussion

The main limiting environmental influences in maize production worldwide is drought and low nitrogen stress, which have received considerable attention in recent years [304,306]. Plants growing under drought and low nitrogen stress display a series of physiological, biochemical, and genetic changes which have an adverse effect on plant growth and production [306,341]. Evolutionary plants have evolved mechanisms to cope with environmental unfavorable conditions, a process widely known as stress resilience [342,343]. Many studies found that plant growth promoting endophytic bacteria (PGPEB) can improve plant resilience to water deficit conditions [320]. Moreover, nitrogen fixing PGPEB can give dual benefits to plants, improving drought tolerance and supplying fixed nitrogen, for their better growth [344]. Few studies are currently available in the literature that investigate the effect that PGPEB inoculation has on the plant under two sources of combined stress [345]. However, the role of diazotrophic endophytic bacteria to relieve the combined effects of both stresses, drought, and low nitrogen has not been studied. Results obtained in this study showed that *Gluconacetobacter diazotrophicus* Pal5 strain may better colonize maize plants under moderate drought, low nitrogen, and combined stress based on the *nifH* and Gd 16S rRNA genes analysis. This might be due to the tolerance mechanism of *G. diazotrophicus* decreasing the level of drought stress [346].

In general, the fresh and dry weight of the root and shoot is greater in *G. diazotrophicus* inoculated plants. Under severe drought and nitrogen stress, the differences are not significant, while, at intermediate stress levels, 50% water holding capacity and 50% nitrogen addition, either in combination or individually, the inoculated bacteria have an effect on plant weight. *G. diazotrophicus* Pal5 is known to produce auxin phytohormones, activate plant defense mechanisms against abiotic stresses, and fix atmospheric nitrogen while living inside the plants [344,347]. A *Gluconacetobacter diazotrophicus* strain was reported to produce indoleacetic acid, a molecule which is active in tricarboxylic acid cycle expression, glyoxylate shunt and amino acid biosynthesis, contributing to the induction of plant growth [348]. The inhibitory effects of moderate drought and low nitrogen stress on Gd inoculated plants may have been ameliorated via hormonal action and/or nitrogen availability to the plants as suggested by Egamberdieva et al. [349]. Nitrogen is an essential plant nutrient that affects plant growth and metabolic pathways including photosynthesis [344]. Increase in shoot and root growth can possibly be explained by the increment on N availability in shoot when the plants were inoculated with *G. diazotrophicus*. Shirinbayan et al. [350] reported that nitrogen fixing plant growth promoting bacteria *Azotobacter* strains increased the

nitrogen concentrations and shoot dry weight, shoot length, chlorophyll content, and water use efficiency in maize plant under drought stress at 40% field capacity. Similarly, Gd inoculation increased the shoot N uptake by 1.3 to 2.1 times, nitrogen use efficiency by 1.1 times to 3.8 times, water use efficiency by 2.31 to 2.88 times, and plant water consumption by 1.03 to 1.05 times. This might be due to an increase in N-fixation efficiency and phytohormone production ability of the endophyte. The *nifH* gene in *G. diazotrophicus* is involved in the nitrogen fixing process [351,352]. Therefore, a higher number of *nifH* gene copy numbers in leaves and stems might be responsible for more N-fixation in moderately stressed treatments. Furthermore, it was shown that auxins promote cell elongation and formation of lateral roots and root hairs. Hence, the stimulation of maize plant growth might be due to the endogenous auxins and the Gd produced auxins inside the plants [353]—thus resulting in increased length and biomass to absorb more water and nutrients from the soil. This is in accordance with our results: Gd-treated plants show higher shoot and root length at moderate levels of drought stress, either individually or in combination with N deficiency. This is similar to the findings of Sandhya et al. in [354], where *G. diazotrophicus* inoculation improved shoot length of maize plants. Ref. [355] have shown that, when root biomass and length increased conjointly, the water uptake of plants increased, and, therefore, the hydration status of leaves was higher when the intensity of stress conditions was lower. A larger and denser root system will not only influence the nutrient uptake, as described above, but also the water uptake [356]. In our study, the leaf relative water content was higher when the inoculated plants were grown under moderate drought and N stress conditions, but there was no effect of inoculation under severe water stress and N-free treatments. Several studies have found that drought and low nitrogen stress can negatively affect root length, morphology, and biomass. Peng et al. [357] demonstrated that N deficiency suppressed the lateral root growth and increased root death causing a decrease in root length but increased N supply increased the toot length and biomass. In accordance with this, Gd inoculated plants in moderate N stress treatments (T4 and T6) showed increased root length as compared to untreated plants. In addition, increased nitrogen supply by bacteria might be the reason for increased root length. Our results further indicate the importance of interactive effects of drought and low nitrogen stress on root length, biomass, and morphology with and without Gd inoculation. In the cases of plants under severe drought stress, plants close their stomata to avoid water loss by transpiration and preserve more water inside plants, in order to sustain the water deficit condition [358]. Interestingly, growth of maize plants and N-uptake is much lower in the N-free treatment than in the 50% nitrogen dose treatment, as the plants clearly showed lower

leaf rolling in the former. This was mainly due to the weaker growth and longer root systems [359]. The main function of abscisic acid is to control the stomatal closure under drought stress conditions preventing plant water loss [360]. Cytokinins are involved in cell division and improve photosynthesis of the plant [361]. In this sense, maize plants inoculated with *G. diazotrophicus* Pal5 may have downregulated the abscisic acid concentration in stomatal cells or upregulated the levels of cytokinin, thereby regulating the stomal closure and photosynthesis [362]. In accordance with Gururani et al., we found that the net photosynthesis activity was reduced due to drought stress, and inoculation with PGPEB increased the photosynthetic rate of plants. Similarly, our findings indicate that photosynthetic efficiency is lower in plants growing under severe drought and nitrogen stress conditions. Therefore, Gd presence in the shoot might be involved in increasing the chlorophyll contents in moderately stressed plants, leading to increased pumping of photosynthesis-generated glucose, which is needed for plant growth processes [205]. Chlorophyll and carotenoids are products of the photosynthesis that are directly involved in sugar synthesis in plants [363]. Sugars and carbohydrates play important roles in signaling and defending stressed plants as they are the primary building framework and energy supply for the processing and maintenance of biomass [206]. These observations of increasing chlorophyll and carotenoids contents are in accordance with the previous reports on the use of PGPEB for improving plant tolerance to drought stress [332,354].

4.5. Conclusions

In conclusion, *G. diazotrophicus* Pal5 was shown to ameliorate the individual and combined effects of drought and low nitrogen stress from maize plants, by regulating plant defense mechanisms. Furthermore, it has the potential to promote maize plant growth under water deficit conditions and with low nitrogen application, thus it could be used effectively in sustainable agriculture. Therefore, seed inoculation with *G. diazotrophicus* can be a very successful tool for inducing individual and combined stress tolerance in maize plants.

Chapter 5-
Conclusions

During this PhD thesis, the individual and combined effects of abiotic stresses on plant growth promoting endophytic bacteria (PGPEB) and plant growth was evaluated using a multi-technique approach. The meta-analysis showed that PGPEB could promote plant growth under non-stressed and salinity stress conditions in similar manner. Data extracted from 42 articles published in the last ten years (2011-2020) containing 614 observations, confirmed that PGPEB inoculated plants perform better under salinity-stress conditions than the non-stressd inoculated plants. Traits that play a crucial role in plant biomass production such as photosynthetic rate, chlorophyll contents, shoot and root lengths, and root biomass were increased in plants when inoculated with PGPEB under salinity-stressed conditions, confirming the ability to increase overall biomass production of plants. However, PGPEB's tolerance to abiotic stress also plays a significant role to increase plant biomass under extreme conditions. If a bacterium is tolerant to a single stress that might also be tolerant to varied multitude of multiple abiotic stresses. The study of two PGPEB strains *Enterobacter ludwigii* 32a and *Pantoea agglomerans* D7G tolerance to the individual and combined effect of salinity and drought stress, brought the complexity of co-occurrence of multiple abiotic stresses. The plant growth promotion related characteristics such as indole 3-acetic acid production by 32a and D7G were significantly similar or increased under individual and combined stresses compared to non-stressed condition. Endophytic colonization related features such as motility, and biofilm formation were differentially modulated exhibiting the complexity of PGPEB tolerance mechanisms under combined stress conditions. Endophytic colonization of tomato plants by 32a and D7G increased under combined stress conditions showing the complexity of concurrent abiotic stress.

However, the transcriptome of individual and combined abiotic stress tolerant strains *Enterobacter ludwigii* 32a and *Pantoea agglomerans* D7G uncovered several tolerance mechanisms of two different bacterial genera to salinity, drought, and their combination. In general, differentially expressed genes (DEGs) in D7G was higher than the 32a, showing the complexity of tolerance strategy of D7G. Indeed, the genes related to cell motility and membrane transport was largely impacted after exposure to salinity and drought stresses. However, under combined stress treatment, some pathways showed an additive effect of both individual stresses, and others showed antagonistic effects. For instance, D7G motility, a fundamental trait for endophytic colonization had an additive effect under combined stress. Growth related genes in 32a exhibited an additive effect related to the survival of bacteria under extreme conditions. In KEGG pathways, more

DEGs in 32a were involved in flagellar assembly than D7G, showing a greater impact on motility. Interestingly, a number of up-regulated genes in 32a under combined stress is 30% less than the addition of up-regulated genes under both salinity and drought stress, showing an antagonistic effect. On the other hand, up-regulated genes in D7G after combined stress exposure are 32% more as compared to the sum of both salinity and drought stress treatments, showing an additive effect. These findings suggest that combined effects of abiotic stresses may have an additive or antagonistic effect on PGPEB tolerance mechanisms that varies among bacterial genera. However, the combined effect of abiotic stresses may be different in case of PGPEB-plant interaction. Interestingly, the study of maize plant growth under individual and combined effects of drought and low nitrogen stresses by a well know PGPEB diazotrophic bacteria *Gluconacetobacter diazotrophicus* Pal5 (Gd) showed that the impact of combined abiotic stress on PGPEB-plant interactions largely depends on the intensity of each individual stresses, when combined. Molecular analysis showed that Gd can endophytically colonize the maize plants under a combination of severe drought and low nitrogen stress, however, Gd had no significant effect on plant growth promotion. Conversely, Gd significantly improved plant growth promotion and tolerance under moderate combination of drought and low nitrogen stresses.

Chapter 6-
Future perspectives

The use of stress-tolerant plant growth promoting endophytic (PGPEB) is just the beginning to draw the attention of the farming community, researchers, and bio-stimulant industry. One of the main reasons for such interest is the lifestyle of endophytic bacteria, who spend whole or a part of their life cycle inside plant tissues, providing direct benefits to plant with significantly greater efficacy than microbes living outside the plants, even under extreme conditions [68,75]. Understanding the importance of PGPEB in crop plants and, more specifically, determining how they positively affect the plants will lead to endophyte-based biotechnologies for increasing crop production under stress conditions. Therefore, in our study, we aimed at designing a delivery strategy to increase the efficiency of PGPEB-based biostimulants. Firstly, a meta-analysis of studies spanning one decade (2011-2020) was conducted, which suggests that:

- Stress tolerant PGPEB can perform better under salinity-stress as compared to no-stress
- Seed inoculation is most widely used inoculation method (Figure 2.4a)
- Most of the experiments were conducted in either in *In-vitro* or greenhouse and very few trials were conducted in fields (Figure 2.4b)
- *Bacillus* and *Pseudomonas* were the main PGPEB genera used in the last decade to improve salinity stress tolerance in plants (Figure 2.4c)
- Salinity tolerant plants showed less significant effect sizes than salinity sensitive plants

With our meta-data analysis, we can not conclude with precision that all stress tolerant PGPEB can perform better under salinity with seed inoculation under greenhouse trials to improve the tolerance of salinity sensitive plants, mainly since the number of experiments with salinity tolerant plants was limited. Therefore, more research is needed with stress tolerant PGPEB genera other than the Bacillus and Pseudomonas with different inoculation methods under In-vitro, greenhouse and field conditions as well, which might have significantly better efficacy than the current dominant methods of PGPEB assisted salinity tolerance of crop plants.

Secondly, we hypothesized based on the introduction (Chapter 1) and meta-analysis (Chapter 2), that if a bacterium is tolerant to a single stress that might also be tolerant to varied multitude of multiple abiotic stresses. The study of two PGPEB strains *Enterobacter ludwigii* 32a and *Pantoea agglomerans* D7G tolerance to the individual and combined effect of salinity and drought stress, brought the complexity of co-

occurrence of multiple abiotic stresses (Chapter 3). Our data suggested that bacterial characteristics under no-stress conditions might be as similar as under individual and combination of salinity and drought stresses, based on their tolerance mechanisms. Transcriptome analysis of PGPEB reveled the importance evaluating bacterial tolerance to a multitude of abiotic stresses.

Thirdly, we tested the efficacy of diazotrophic PGPEB *Gluconacetobacter diazotrophicus* Pal5 to promote maize plant growth under individual and combination of drought and low nitrogen stresses. Our findings suggested that *G. diazotrophicus* Pal5 can improve the maize plant tolerance to moderate drought and low nitrogen stresses [364] (Chapter 4).

However, salinity, drought, and low nitrogen stresses are not the only stress combination but PGPEB and plant face a combination of several biotic and abiotic stresses under field conditions including but not limited to salinity, drought, temperature (heat and/or cold) and nutrient stress, pathogenic, insect attack etc. The effects of these abiotic stresses may vary according to the intensity, type, and number of abiotic stresses combinations. Therefore, we suggest evaluating the effects of the individual as well as combinations of abiotic and biotic stresses on PGPEB and host-plants to uncover the underlying mechanisms of PGPEB and host-plant tolerance to these stresses, which will provide an insight to use PGPEB based biostimulants specific to the crop type, stress type, stress intensity, and geographical location with particular stresses. However, the use of stress-tolerant plant growth promoting endophytic bacteria can be an efficient tool to increase crop production under a multitude of abiotic stresses, if used precisely, based on PGPEB characteristics and mechanisms of tolerance and plant-microbe interactions.

Chapter 7-
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