# BIODIVERSITY AND FUNCTION OF ACC-DEAMINASE PRODUCING BACTERIA ASSOCIATED WITH GRASS ROOTS

By

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ABSTRACT OF THE THESIS

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Poaceae grass root associated ACC-deaminase producing bacteria were collected from four locations in New Jersey pine barrens. A total of 186 bacterial isolates were obtained, belonging to 6 orders, 9 families and 10 genera based on 16S rDNA sequencing. The most prevalent genus was *Burkholderia* (71.5%), followed by *Herbaspirillum* (9.1%), *Pseudomonas* (8.6%) and *Paenibacillus* (5.4%). A high throughput method was employed to quantify the ACC deaminase activity, which ranged from 0 to as high as 40024 nmol ketobutyrate/mg/h among all the isolates. A root elongation assay showed three isolates promoted root elongation significantly under well-watered condition and one isolate was able to promote root elongation under salinity condition. ACC-deaminase producing bacteria may play an important role in assisting the establishment of Poaceace grasses in the dry and infertile pine barrens ecosystem.

The second part of this study tested the potential of ACC-deaminase bacteria

inoculation in promoting plant growth and salinity stress tolerance and examined growth traits, nutrition status, and physiological changes associated with the ACC-deaminase bacteria inoculation. Perennial ryegrass (*Lolium perenne* L.) were inoculated with two ACC-deaminase bacteria, *Burkholderia phytofirmans* and *Burkholderia gladioli* under well-watered or salinity condition. The inoculums suppressed ACC production in both shoots and roots. Shoot and root growth was inhibited by salinity stress whereas the bacteria inoculation increased turf quality, tiller number, and promoted root development and biomass accumulation of both roots and shoots under well-watered and salinity stress. The inoculation also promoted physiological tolerance of plant to salinity stress, as manifested by higher leaf relative water content and photochemical efficiency, as well as lower electrolyte leakage of plants exposed to salinity stress. The inoculated plants had significantly lower Na content and higher $K^+/Na^+$ ratio in shoots under both well-watered and salinity conditions. The inoculation increased nitrogen content in shoot and root and potassium content in shoot under both well-watered and salinity conditions, but caused reduction in Ca, Mg, Fe, and Al content in shoots and roots under salinity conditions. The results demonstrated ACC-deaminase bacteria had beneficial roles in promoting perennial ryegrass growth and salinity tolerance, which could be potentially used in turfgrass establishment and maintenance.

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# GLOSSARY

| | |
|---|---|
| 1-MCP | 1-methylcyclopropene |
| 2, 5-NBD | 2, 5-norbornadiene |
| ACC | 1-aminocyclopropane-1-carboxylate |
| acdS | ACC deaminase structural gene |
| ATP | adenosine triphosphate |
| AVG | L-α-aminoethoxyvinylglycine |
| BSA | Bovine serum albumin |
| DAT | day after treatment |
| DF agar | Dworkin and Foster Agar |
| DW | dry weight |
| EL | electrolyte leakage |
| FW | fresh weight |
| GACC | 1-(-L-glutamylamino) cyclopropane-1-carboxylic acid |
| HGT | horizontal gene transfer |
| IAA | indole-3-acetic acid |
| ISR | induced systematic resistance |
| LB | lysogeny broth |
| Met | L-methionine |
| MTA | 5'- methylthioadenosine |
| PGPB | Plant Growth Promoting Bacteria |

| | |
|---|---|
| PGPR | Plant Growth Promoting Rhizobacteria |
| RWC | relative water content |
| SAM | *S*-adenosyl methionine |
| SAM | S-adenosyl-L-methionine |
| STS | silver thiosulphate |
| TQ | turf quality |
| TW | turgid weight |
| WUE | water use efficiency |

# LITERATURE REVIEW

## Introduction

Almost all plants in nature have bacteria on their surfaces (epiphytes) or in their tissues (endophytes). The ubiquitous plant-bacterial interactions range from pathogenic, neutral to beneficial (Lynch, 1990). The beneficial bacteria that can promote the growth of the colonized plants are defined as the Plant Growth Promoting Bacteria (PGPB) (Bashan and Holguin, 1998). PGPB promote the plant growth through various mechanisms, one of which is lowering ethylene level in plant by the bacterium produced 1-aminocyclopropane-1-carboxylate (ACC) deaminase. An increasing number of PGPB with ACC deaminase activity have been isolated from natural environments. However, to my knowledge, no study has been published on ACC deaminase producing PGPB of turfgrass species and their physiological responses to PGPB inoculation.

## Plant Growth Promoting Bacteria

PGPB can be divided into two groups according to the different ecological niches they colonize. Those colonize the rhizosphere were first defined as Plant Growth Promoting Rhizobacteria (PGPR) by Kloepper and Schroth (1978), which were the most widely studied PGPB. The other group is the endophytic PGPB that reside within living plant tissues. It has been assumed that endophytic bacterial community might initiate from root zone colonization (van Peer *et al.*, 1990; Sturz, Christie & Nowak, 2000). In fact some PGPB can be both rhizosperic and endophytic, for example, the

*Burkholderia phytofirmans* PsJN (Weilharter *et al.*, 2011).

The plant growth promotion mechanisms employed by PGPB include direct mechanisms such as nitrogen fixation, phosphate solubilization, siderophore production, phytohormone level modulation, and indirect mechanisms such as induced systematic resistance (ISR) (Glick *et al.*, 1999; Compant, Clément, & Sessitsch, 2010).

Nitrogen fixation might be the best-studied mechanism of beneficial relationship between bacteria and plants. The nitrogen fixing PGPB can convert atmospheric nitrogen to ammonium, which is absorbable to plants (Compant *et al.*, 2005). Most of these PGPB belong to the genus *Rhizobium*, while species from *Azospirillum, Azotobacter, Clostridium, Klebsiella* (Bashan and Holguin, 2002), *Burkholderia* (Reis *et al.*, 2004), *Azoarcus* (Krause *et al.*, 2006) and *Enterobacter* (Peng *et al.*, 2009) were also reported to be able to fix nitrogen.

Beside nitrogen, phosphorus is another essential mineral nutrient limiting plant growth. However, soil phosphorus is normally in an insoluble state that cannot be utilized by plants. Many PGPB are capable of producing organic acids of small molecular weight as well as phosphortase, which help convert the insoluble phosphorus to soluble forms (Kim *et al.*, 1997). Through solubilization of phosphorus nutrient availability is enhanced and thus the growth of host plants promoted (Richardson and Simpson, 2011).

Bioavailable iron usually is also limited in soil. There are PGPB that promote plant growth by producing siderophores, which are compounds with high chelating affinity with $Fe^{3+}$ (Hilder and Kong, 2010). Siderophore producing PGPB can increase the bioavailability of iron by either sequestering $Fe^{3+}$ from the surrounding soils or from neighboring microorganisms with low chelating affinity (Scavino and Pedraza, 2013).

They can also act as bio-control agents by out competing plant pathogens with siderophores of higher iron affinity (Compant *et al.*, 2005).

PGPB can also promote plant growth through producing or regulating plant hormones, which play important roles in plant growth and development. It has been reported that certain PGPB produce gibberellins (Gutiérrez-Mañero *et al.*, 2001; Bottini, Cassán and Piccoli, 2004), cytokinin (Timmusk *et al.*, 1999; García de Salamone *et al.*, 2001) or auxin (Mayak, Tirosh and Glick, 1999; Asghar *et al.*, 2002) and their growth promotion effect has been shown on various plant species. Indole-3-acetic acid (IAA) as the most well-known auxin involves in various plant developmental process include cell division, extension and differentiation, root formation and vegetative growth. Bacterial IAA enhances plant growth mainly by increasing root length and surface area, which results in a better access to soil nutrients (Glick, 2012). However, detailed mechanism of plant growth promoting effects by gibberellins and cytokinin producing PGPB are still not well understood. Ethylene generally is considered as a plant growth inhibitory phytohormone and can be regulated by a group of ACC deaminase containing PGPB. Plant ethylene level can be decreased through the cleavage of ACC, the intermediate precursor of ethylene in higher plants, by bacterial ACC deaminase (Glick, 2005). As a result, plant root elongation inhibition by high level of ethylene can be prevented under stressed conditions (Jackson, 1991).

## Ethylene

Ethylene as a gaseous phytohormone involves actively in the regulation of plant growth and development including seed germination, root elongation, tissue

differentiation, flowering and fruiting, leaf senescence and response to various environmental stimuli (Abeles *et al.*, 1992).

Ethylene synthesis in higher plants starts from L-methionine (Met) and proceed through the intermediates, S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984). SAM as an intermediate in the ethylene biosynthetic pathway is synthesized from methionine and adenosine triphosphate (ATP). ACC as the immediate precursor of ethylene is usually the biosynthetic step that limits ethylene production in plant tissues (Taiz and Zeiger, 2006). In this pathway SAM synthetase catalyzes the conversion of methionine to SAM (Giovanelli *et al.* 1980), ACC synthase is responsible for the hydrolysis of SAM to ACC and 5'- methylthioadenosine (MTA) (Kende, 1989), and ethylene along with carbon dioxide and cyanide are metabolized by ACC oxidase from ACC (John, 1991). Beside ethylene, ACC can also be converted to N-malonyl ACC, which is a conjugated form (Amrhein *et al.*, 1981; Martin and Saftner, 1995) by ACC N-malonyltransferase or to 1-(-L-glutamylamino) cyclopropane-1-carboxylic acid (GACC) (Martin *et al.*, 1995).

Ethylene has long been recognized as a growth inhibitor since the early report on seedling's triple response to ethylene, which are shortening and thickening of hypocotyl with apical hook (Abeles *et al.*, 1992). However, there are increasing reports on the stimulation effect of ethylene on plant growth under relatively low concentrations or different nutrient conditions (Smalle *et al.*, 1997) and light quality (Pierik *et al.*, 2004). Therefore it was proposed in recent years that ethylene might act biphasically. It can either inhibit or stimulate plant growth depending on its concentration, interaction with other hormone pathways and the plant species and

tissues (Pierik *et al.*, 2006; Dugardeyn and Van Der Straeten, 2008).

An increase of ethylene synthesis typically takes place when plants are exposed to various abiotic and biotic stresses including high temperature, flooding, drought, salinity, toxic metals and organic pollutants, radiation, wounding, insect predation, and microbial pathogens (Morgan and Drew, 1997). The ethylene synthesized by plant in response to the environmental stresses is called "stress ethylene" (Abeles *et al.*, 1992), which is suggested to be produced in two peaks (Stearns and Glick, 2003; Pierik *et al.*, 2006). The first peak, which occurs a few hours after the stress, is typically a small fraction of the magnitude of the second peak and is thought to function as a signal to turn on the transcription of protection related genes (van Loon and Glick, 2004). The second peak, which occurs one to three days after the stress, is much larger and might reach the concentration that can exacerbate the stress symptoms such as senescence, chlorosis and leaf abscission (Glick 2007). These symptoms due to environmental stresses can be alleviated by inhibitors of ethylene synthesis or action (Glick 2007).

## ACC deaminase Producing Bacteria

Bacterial ACC deaminase, which is capable of hydrolyzing ACC, was first isolated in 1978 from *Pseudomonas* sp. strain ACP (Honma and Shimomura, 1978). ACC deaminase (EC: 4.1.99.4) has also been detected in the fungus, *Penicillium citrinum* (Honma, 1993) and the yeast, *Hansenula saturnus* (Minami *et al.*, 1998). Other bacterial strains that were reported to have ACC deaminase activity include: *Pseudomonas* sp. strains 6G5, 3F2, ACP and 17 (Klee et al., 1991; Sheehy et al. 1991; Campbell and Thomson 1996), *Pseudomonas putida* GR12-2 (Jacobson *et al.*, 1994), *Enterobacter cloacae* CAL2 and CAL3 (Glick *et al.*, 1995; Mayak *et al.*, 2001),

*Pseudomonas putida* UW4 (Shah et al., 1998), *Kluyvera ascorbata* SUD165 (Burd et al 1998), *Pseudomonas brassicacearum* Am3 (Belimov et al. 2007), *Rhizobium leguminosarum* bv. *viciae*, *Rhizobium hedysari* (Ma *et al.*, 2003), *Bacillus circulans* DUC1, *Bacillus firmus* DUC2, *Bacillus globisporus* DUC3 (Ghosh *et al.*, 2003), and *Methylobacterium fujisawaense* (Madhaiyan *et al.*, 2006).

The ACC-deaminase producing bacteriais mainly identified by the method of inoculating on DF-ACC, which is a minimal medium with ACC as the sole nitrogen source (Honma and Shimomura, 1978). ACC deaminase activity is quantified by measuring the hydrolysis product of ACC, α-ketobutyrate, and the protocol developed by Penrose and Glick (2003) is most widely used by researchers. It is reported that the ACC deaminase activity varies among different organisms, which can be up to 100 fold (Glick, 2005).

ACC deaminase activity is suggested to be a relatively common trait in soil microorganisms (Glick, 2014). Jacobson *et al.* (1994) argued that ACC as a unique and novel nitrogen source may provide ACC-deaminase producing bacteriawith a competitive advantage over other rhizosphere microorganisms. While a detailed understanding of this competitive advantage is lacking, there are several researches that are consistent with this argument. Five out of thirteen rhizobial strains were shown to have ACC deaminase activity from the work of Ma *et al.* (2003). Among a collection of 233 rhizobial strains from Southern Saskatchewan, Canada, 27 (12%) strains showed positive result in the ACC deaminase activity assay (Duan *et al.*, 2009). In an examination of ACC deaminase genes from *Mesorhizobium* strains, 10 out of 12 *Mesorhizobium* type strains and all 18 chickpea-nodulating *Mesorhizobium* isolates contain ACC deaminase genes (Nascimento *et al.*, 2012 a).

## ACC deaminase gene

Functional ACC deaminase genes have been cloned from a wide range of soil bacteria (Klee *et al.*, 1991; Sheehy *et al.*, 1991; Glick *et al.*, 1995; Campbell and Thomson 1996; Shah *et al.*, 1998; Ma *et al.*, 2003; Belimov *et al.*, 2007). A large number of genes were annotated as ACC deaminase structural gene (acdS) by searching through sequenced bacterial and fungal genomes. However it is suggested the precise identity of these genes should be further tested as the majority of them might not be able to encode active gene (Glick, 2014). For example, none of the acdS genes that have been identified from the DNA sequences of *Escherichia coli*, various Archaebacteria and *Arabidopsis thaliana* displayed ACC deaminase activity but these genes cluster separately from genes encoding active ACC deaminase in rhizobacteria and fungi (Duan, 2007).

It was proposed that some ACC deaminase genes might evolve through horizontal gene transfer (HGT) based on the comparison between a phylogenetic analysis of a few acdS genes and the phylogeny of the 16S rDNA sequences from the same bacteria (Hontzeas *et al.*, 2005). Similarly, it was suggested that horizontal gene transfers was very common in Proteobacteria by Blaha *et al.* (2006). In agreement with this suggestion, it was suggested acdS genes is subjected to horizontal gene transfer among the strains of *Mesorhizobium* species (Nascimento *et al.*, 2012 a). Recently a more comprehensive study on ACC deaminase phylogeny and evolution showed that ACC deaminase genes are predominantly vertically inherited in various bacterial and fungal species, while a considerable degree of horizontal gene transfer events exist (Nascimento *et al.*, 2014).

**The Plant Growth Promoting Model By ACC-deaminase Producing Bacteria**

The possession of ACC deaminase is suggested by Glick (2014) to be a key bacterial trait in facilitating plant growth among the various plant growth promoting mechanisms. Stress ethylene, which is detrimental to plant, can be decreased by bacterial ACC deaminase by breaking down ACC in plant (Glick, 1995).

In a model described by Glick *et al.* (1998), ACC deaminase–producing PGPB first bind to the surface of plant root (rhizosphere) or seed, attracted by the large amounts of sugars, amino acids and organic acids which can serve as bacterial food source. The associated bacteria are able to synthesis and secrete indole-3-acetic acid (IAA) in response to the small molecules like tryptophan in the plant root exudates. The secreted IAA, when taken up by the plant, can trigger various responses along with the endogenous IAA. The IAA either facilitates plant cell proliferation and/or elongation by loosening plant cell walls, or induces ACC and ethylene production through the stimulated transcription of plant ACC synthase. Some of the synthesized ACC are secreted to the rhizosphere along with other small molecules in the root exudates (Penrose *et al.*, 2001). Then the exuded ACC might be taken up by the associated ACC-deaminase producing PGPB as nitrogen source (Penrose and Glick, 2003).

In this described model the ACC-deaminase producing PGPB exist as a sink for ACC. The elevated ethylene production in response to environmental stresses is lowered through the hydrolysis of ACC. Therefore the plant growth inhibition by excessive ethylene under a wide range of abiotic and biotic stresses is alleviated in plants associated with ACC-deaminase producing PGPB.

As reviewed previously, stress induced ethylene comes in two peaks: the first

one which is small and thought to be a signal to turn on plant defense responses, and the second one which is much larger and deleterious. It was suggested that the ACC-deaminase producing PGPB can selectively lower the second ethylene peak, which is detrimental to the plant growth, without affecting the first one (Glick, 2014). It was argued that ACC deaminase activity is relatively low in bacteria before it is induced. At the first ethylene peak which is immediately after plants are exposed to stresses, the level of plant ACC is low as is the ACC deaminase level in the associated PGPB (Glick, 2014). The second peak of ethylene is resulted from the increasing level of plant ACC, which in turn induces the ACC deaminase in the associated PGPB. Therefore the induced ACC deaminase can significantly reduce the second ethylene peak and ameliorate the deleterious effects. Since ACC oxidase has a higher affinity for ACC than ACC deaminase does (Glick *et al.*, 1998), it is also suggested ACC deaminase must function before the induction of ACC oxidase by stress in order to reduce ACC effectively (Glick, 2014).

Though IAA stimulates plant cell proliferation and/or elongation, it also stimulates the transcription of ACC synthesis, thus might results in an elevated level of ACC and ethylene, which might in turn inhibit the plant growth. However it was argued by Glick (2014) that the elevated ethylene will act as a negative feedback which inhibit IAA signal transduction, therefore limiting the amount of elevated ACC by IAA. With the bacterial ACC deaminase, plant ethylene level is lowered and so is the negative feedback on IAA signal transduction. Therefore more IAA can promote plant growth in PGPB with both IAA and ACC deaminase compared with those with no ACC deaminase. The plant growth promoting effect of ACC-deaminase producing bacteria turned out to be a crosstalk effect of between ACC-deaminase and IAA.

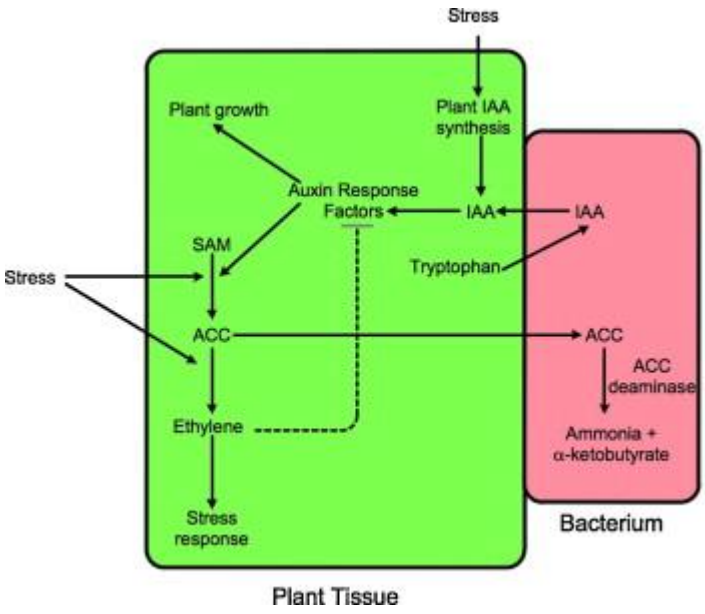


**Figure 1.** A schematic model of how plant growth-promoting bacteria that both produce ACC deaminase and synthesize IAA may facilitate plant growth (Glick, 2014).

## ACC-deaminase Producing PGPB and Plant Stresses

As reviewed in previously, ethylene inhibitors should be able to alleviate the adverse effects of stress ethylene on plant and help facilitate plant growth under a variety of environmental stresses. Ethylene action inhibitor such as 2, 5-norbornadiene (2, 5-NBD), silver thiosulphate (STS), and 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1999), and ethylene synthesis inhibitor such as L-α-aminoethoxyvinylglycine (AVG) (Yang and Hoffman, 1984) have been reported to control fruit ripening, flower and leaf senescence, and other ethylene responses. However the application of these chemicals has drawbacks such as the high cost of AVG and STS and the potential pollutions posed to the environment (Abeles *et al.*, 1992). The use of PGPB with ACC-deaminase activity in ameliorating stress damage to plant has received increased attention in recent years (Saleem et al., 2007; Yang, Kloepper & Ryu, 2009).

Salinity is a worldwide agriculture problem as it is estimated that 20% of the

world's cultivated land are affected by salinity and this number continues growing as a result of agricultural practices such as irrigation (Zhu, 2001). Like many other stresses, the plant growth inhibition by salinity stress is partially attributed to the production of stress ethylene. Mayak *et al.* (2004 a) first reported the stress alleviation effect of *Achromobacter piechaudii* ARV8, a known ACC-deaminase producing PGPB, on tomato (*Solanum lycopersicum* L.) seedlings under salt stress (up to 172 mM NaCl). Ethylene production, which was stimulated under the salinity treatment, was reduced by the bacteria inoculation. An increase in water use efficiency (WUE) was also observed in the bacteria inoculated plants under salinity conditions. Thereafter, plant growth promoting effects of ACC-deaminase producing PGPB have been reported on various plant species. The growth of canola (*Brassica napus* L.) under salinity was reported to be improved significantly by the ACC-deaminase activity of *Pseudomonas putida* UW4 (Cheng et al., 2007). The same bacterial strain also conferred salt tolerance improvement on cucumber (*Cucumis sativus* L.) by increasing biomass accumulation, root and shoot development, and photosynthetic efficiency (Gamalero *et al.*, 2010), Inoculation of three ACC-deaminase producing bacteria significantly increased the shoot and root growth of red pepper (*Capsicum annuum* L.) under salinity treatment, and reduced the ethylene emission (Siddikee *et al.*, 2012). The contents of N, P, K, Ca, Mg were increased by the bacteria inoculation while the Na content was decreased. ACC-deaminase producing bacteria strains isolated from saline soil were shown to promote shoot and root growth, increase chlorophyll content and reduce ethylene production in rice (*Oryza sativa* L.) under salinity stress (Bal *et al.*, 2013). Inoculation of ACC-deaminase producing *Pseudomonas* on mungbean (*Vigna radiata* L.) reduced the adverse effects of salinity by increasing relative water content, water use efficiency,

chlorophyll content, N, P and K content while reducing Na content (Ahmad *et al.*, 2013). Growth promoting effects including increased plant height, root length, total biomass, cob mass, and grain yield, and increased salt tolerance through higher K+/Na+ ratio, relative water content and chlorophyll content were observed on maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) inoculated with ACC-deaminase producing bacteria under salinity stresses (Nadeem *et al.*, 2007, 2010).

Plant growth promoting and stress alleviation effects by ACC-deaminase producing bacteria were also reported in other stresses. Increased tolerance to flooding stress by ACC-deaminase producing bacteria has been confirmed in tomato (*Solanum lycopersicum* L.), *Ocimum sanctum* and cucumber (*Cucumis sativus* L.) (Grichko and Glick, 2001; Barnawal *et al.*, 2012; Li *et al.*, 2013). It was reported by Mayak *et al.* (2004 b) that the ACC-deaminase producing *Achromobacter piechaudii* ARV8 conferred drought resistance in tomato (*Lycopersicum esculentum* Mill *cv*. F144) and pepper (*Capsicum annuum* L. *cv.* Maor) plants. Increased drought stress tolerance was reported on various plant species (Arshad *et al.*, 2008; Zahir *et al.*, 2008; Belimov *et al.*, 2009; Shakir *et al.*, 2012). ACC-deaminase producing bacteria were also explored to be used in metal phytoremediation after the first report of decreased nickel toxicity to canola plants by ACC-deaminase producing PGPB (Burd *et al.*, 1998). The mechanism typically involves a cooperation of the stress ethylene suppression by ACC-deaminase, the plant growth promotion by IAA and the ion maintenance by siderophores in the presence of overwhelming metals (Glick, 2014). Biotic stresses, which are the infections by various pathogens, can be exacerbated by the increased levels of stress ethylene. It has been shown by an increasing number of studies that inoculation with ACC-deaminase producing PGPB can ameliorate the damage caused by infection of

bacteria (Wang *et al.*, 2000; Hao *et al.*, 2007; Toklikishvili *et al.*, 2010; Hao *et al.*, 2011), fungi (Wang *et al.*, 2000; Husen *et al.*, 2011; Nascimento *et al.*, 2011 b), and nematodes (Nascimento *et al.*, 2013).

While ACC-deaminase producing PGPB assist plants to overcome various environmental stresses, it was suggested that ACC-deaminase producing PGPB should occur more frequently in stressed environment due to the selective pressure (Glick, 2014). This is supported by the result of a recent study on the prevalence of ACC-deaminase producing bacteria among bacterial isolations from the rhizosphere of wild barley (*Hordeum spontaneum*) growing in Northern Israel (Timmusk *et al.*, 2011). Bacteria were isolated from the North Facing Slope which is free of drought and has better vegetation, and the South Facing Slope which suffers from frequent drought and has sparse vegetation. The result showed 4% of the North Facing Slope isolates possess ACC-daminase activity while half of the South Facing isolates do. Therefore there might be a higher chance to obtain ACC-deaminase producing PGPB in stressed environment than in unstressed environment.

## New Jersey Pine Barren

Pine barrens is a general name for a unique type of ecosystem that has acidic, sandy and oligotrophic soils (Forman 1998). Pines and oaks are the dominant trees in pine barrens, whereas the grasses (Poaceae), sedges (Cyperaceae) and the heath family (Ericaceae) are dominant on disturbed open and dry areas. The largest and most uniform area of pine barrens in the United States is the 1.4 million acre (57,000 $km^2$) pine barrens of New Jersey. The podzolic soil in this region is sandy, dry (low moisture holding capacity) acidic (pH ~4.0 with very low cation exchange capacity), and nutrient

poor (low in P, N, K, etc) (Joffe and Watson, 1933; Forman 1998; Tedrow 1952; Turner *et al.*, 1985). The New Jersey Pine Barrens represents one of a series of barrens ecosystems along the eastern seaboard of the United States and one of a series of similar ecosystems around the world. There are over 50 native Poaceae grass species documented in the New Jersey Pine Barrens (McCormick 1979; Boyd 1991). However, the soils of in this area would not support the growth of domesticated grain crops. While success in the pine barrens may be owing to the characteristics of the plants themselves, microorganisms associated with them also may play a role in helping the plants survive in the stressed environment. Nevertheless, little is known about the bacteria communities in the pine barrens ecosystem. Shah et al. (2011) reported that in the Long Island Pine Barrens soils, ammonia oxidizing bacteria occupy a major fraction of microbial community, indicating that they may play a role in the nitrogen cycle in the ecosystem.

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# ACC-DEAMINASE PRODUCING BACTERIA ASSOCIATED WITH GRASS ROOTS IN THE OLIGOTROPHIC PINE BARRENS ECOSYSTEM

## Introduction

Pine barrens is a general name for a unique type of ecosystem that has acidic, sandy and oligotrophic soils (Forman 1998). Pines and oaks are the dominant trees in pine barrens, whereas the grasses (Poaceae), sedges (Cyperaceae) and the heath family (Ericaceae) are dominant on disturbed open and dry areas. The largest and most uniform area of pine barrens in the United States is the 1.4 million acre (57,000 $km^2$) pine barrens of New Jersey. The podzolic soil in this region is sandy, dry (low moisture holding capacity) acidic (pH ~4.0 with very low cation exchange capacity), and nutrient poor (low in P, N, K, etc) (Joffe and Watson, 1933; Forman 1998; Tedrow 1952; Turner et al., 1985). The New Jersey Pine Barrens represents one of a series of barrens ecosystems along the eastern seaboard of the United States and one of a series of similar ecosystems around the world. There are over 50 native Poaceae grass species documented in the New Jersey Pine Barrens (McCormick 1979; Boyd 1991). However, the soils of in this area would not support the growth of domesticated grain crops. This raised our interest on how the native grass species thrive in the dry and infertile soils of the pine barrens. While success in the pine barrens may be owing to the characteristics of the plants themselves, microorganisms associated with them also may play a role in helping the plants survive in the stressed environment.

Plant-bacterial interactions are ubiquitous in nature, and some bacteria are beneficial to the growth of the associated plants that are called Plant Growth Promoting

Bacteria (PGPB) (Bashan and Holguin, 1998). Stress alleviation by applying PGPB to plants is drawing increasing attention in recent years (Saleem et al. 2007; Yang et al. 2009). PGPB may promote plant growth through direct stimulation such as nitrogen fixation, secretion of phytohormones and siderophores, or indirect mechanisms such as biocontrol (Glick and Bashan, 1997). One of the well-studied mechanisms is the breakdown of the plant ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) by microbial ACC deaminase, a trait that has been reported in various bacterial species and a few fungi (Saleem et al. 2007). It is believed that besides promoting plant growth under normal condition, ACC-deaminase producing bacteria can alleviate the damaging effects of overproduction of ethylene in plants under various abiotic stresses (Glick 2004). Mayak et al. (2004) first confirmed that ACC-deaminase producing bacteriapromoted drought and salinity stress tolerance in tomato and pepper plants. Their results showed an increased fresh weight and dry biomass and water use efficiency, and reduced ethylene production of the inoculated tomato seedlings under drought and salinity stresses. A reduced depression effect by ACC-deaminase activity was also reported in maize, the inoculated plants of which showed higher relative water contents, chlorophyll concentration and production under salinity stress (Nadeem et al. 2007). Similar results were reported by this research group for wheat (Nadeem et al. 2010). ACC-deaminase producing bacteriaalso have been shown to promote growth in other crop species, including canola (*Brassica napus* L.) (Cheng et al. 2007), cucumber (*Cucumis sativus* L.) (Gamalero et al. 2010), red pepper (*Capsicum annuum* L.) (Siddikee et al. 2012), rice (*Oryza sativa* L.) (Bal et al. 2013), grapevine (*Vitis vinifera* L.) (Ait et. al 2006), and soybean (*Glycine max* L.) (Kasotia et al. 2012).

Plants in the pine barrens are under natural drought and low nutrient stresses.

However, little is known about the diversity and function of microbes associated with plants in the pine barrens ecosystem (Forman 1998; Tuininga and Dighton, 2004). Shah et al. (2011) reported that in the Long Island Pine Barrens soils, ammonia oxidizing bacteria occupy a major fraction of microbial community, indicating that they may play a role in the nitrogen cycle in the ecosystem. The objectives of this study were to 1) identify ACC-deaminase producing bacteriaassociated with Poaceae grass roots in the New Jersey Pine Barrens; 2) assess the correlation between the ACC deaminase activity level and the phylogenetic relationship of the ACC deaminase producing bacteria; and 3) screen the ACC-deaminase producing bacteriafrom the New Jersey Pine Barrens for potential PGPB through a modified high-throughput pipeline.

# Materials and Methods

## Sampling

Grass root samples were taken from four locations in the New Jersey Pine Barrens. Colliers Mills and Assumpink Wildlife Management area were sampled in June, 2012. Wharton State Forest and Pygmy Pine Plains were sampled in June of 2013. Twenty apparently healthy Poaceae grass samples were collected from each location and kept on ice. In order to maximize the biodiversity coverage, grass samples were collected randomly, with a distance of at least 10 meters between each pair of sampled plants. Bacterial isolation was performed within 24 hours of collection.

## Screening of Endophytic ACC Deaminase Containing Bacteria

The grass root samples from the same location were pooled and surface sterilized. The roots were first rinsed in tap water to remove soil particles on the surface, then treated with 1% hypochloride solution for 1 min, followed by 70% (v/v) ethanol for 1 min, and finally rinsed in sterile distilled water for at least five times. After surface sterilization two grams of roots per location were cut into 2 mm segments and homogenized using mortar and pestle. For samples collected in 2012, homogenized root tissues were diluted and spread on Nutrient Agar and incubated at 28°C. After two days, 90 bacterial colonies per location were randomly picked and purified twice by streaking for single colonies. To screen for strains with ACC deaminase activity, the purified isolates were streaked on the DF-ACC medium, a modified Dworkin and Foster (DF) Minimum Agar (Dworkin and Foster, 1958) with 3 mM ACC as the sole nitrogen source. Bacteria that were able to form colonies on the modified DF-ACC agar

were considered to be potential ACC deaminase producing bacteria. For samples collected in 2013, the dilutions of root homogenate were directly spread on the DF-ACC Agar with the Nutrient Agar step omitted. For each location, 30 colonies were picked randomly and further purified. The purified bacterial isolates were preserved in 15 % glycerol (v/v) at -80°C.

**DNA Extraction, PCR and Sequencing**

Bacterial DNA was extracted by a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instruction manual. The 16S rRNA gene was amplified using the primers F27 (5'-AGAGTTTATCMTGGCTCAG-3') and R1492 (5'-GRTACCTTGTTACGACTT-3') (Weisburg et al., 1991) under the following conditions: 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and primer extension at 72 °C for 60 s. A final extension of 10 min at 72 °C was followed. The amplified 16S rRNA was treated with ExoSap-IT (USB Corporation, OH, USA) and the sequencing was conducted by GenScript (Piscataway, NJ, USA) using primers F27 and R1492.

**Phylogenetic Analysis**

The bacterial 16S rRNA gene sequences were edited and aligned using Bioedit (Hall 1999). Maximum Likelihood (ML) tree was generated with MEGA 6 (Tamura et al., 2013) using 500 bootstrap replications. K2+G was selected as the best model by the model test in MEGA 6, which was used in the ML analysis. Bacterial identification was

based on the BLAST search in GenBank (www.ncbi.nlm.nih.gov/BLAST) and the phylogenetic analysis.

**ACC Deaminase Activity Measurement**

ACC deaminase activity quantification was based on the measurement of α-ketobutyrate produced by deamination of ACC. We modified the method of Penrose and Glick (2003) to a smaller reaction volume but higher throughput using 96-well plates. Bacterial isolates were first revived by adding 10 µL -80°C stored suspension into 1 mL LB broth and incubated at 28 °C and 150 rpm for 48 h. Bacterial cells were then collected by centrifuging at 8000 *g* for 5 min. The supernatants were discarded and the pellet was washed twice in 1 mL 0.1 M Tris-HCl (pH=7.5) buffer. The cells were re-suspended in 1 mL DF minimum medium with 3 mM ACC as the sole nitrogen source, and incubated at 28 °C and 150 rpm for 24 h to induce ACC deaminase activity. Bacterial cells were then harvested and washed twice following the same protocol as described above. Cell pellet was re-suspended in 200 µL 0.1 M Tris-HCl (pH=8.5) with 5% (v/v) toluene to labialize - the cells. 50 µL of the labialized cells were mixed with 5 µL 500 mM ACC in a 96 well PCR plate and incubated at 30 °C for 30 min. After adding 100 µL 2.8 N HCl into each well, the 96- well plate was centrifuged at 20000 *g* for 5 min. 50 µL supernatant (or standard solution of α-ketobutyrate for standard curve) was transferred into a 96-well microplate, and mixed with 40 µL 0.56 M HCl and 15 µL DNF solution in each well. The microplate was incubated at 30 °C for 30 min. 100 µL 2M NaOH was added into each well. Absorbance at 540 nm was measured using a microplate reader.

Protein assay was based on the method of Bradford (1976). 20 µL of the toluene

labialized cell of each sample was pipetted into a 96-well PCR plate. 80 µL 0.25 M NaOH was added into each well and the plate was heated at 100 °C for 10 min. The samples were then centrifuged at 8000 *g* for 5 min. 40 µL supernatant of each sample was transferred to a 96-well microplate and 160 µL 4× diluted Braford protein reagent (Bio-Rad Lab., USA) was added and mixed thoroughly with each sample. Protein concentration was determined by measuring the absorbance at 595 nm. Bovine serum albumin (BSA) was used to construct a standard curve.

**Root Elongation Assay**

Perennial ryegrass (*Lolium perenne*) cultivar "Nightshade" was used in the root elongation assay. Seeds were surface sterilized following the previous described protocol of root surface sterilization and placed on moist filter paper in a Petri dish to germinate at room temperature for 3 days. The bacteria inoculum was prepared by first culturing the bacteria in LB broth for 2 days, then collecting the cells by centrifuge at 8000 *g* for 5 min. and washed the cells in $ddH_2O$. Germinated Perennial ryegrass seeds of similar size were soaked in the bacterial suspension (OD=1) overnight. Sterile $ddH_2O$ was used as control. After inoculation, plant seeds were placed in seed germination pouches (Cyg seed germination pouch, Mega International, Minneapolis, MN) with 5 seeds per pouch and 3 pouches for each treatment. Seedlings were either supplied with sterile $ddH_2O$ for watered condition or first $ddH_2O$ for 2 weeks followed by 240 mM NaCl for salinity stress treatment. After 3 weeks growth in a growth chamber set at 23/18 $^oC$ (day/night temperature), 12-h photoperiod, and 610 µmol photons $m^{-2}$ $s^{-1}$, the roots in each pouch were strained with 1% crystal violet and scanned. Root morphometric analyses were done for the scanned pictures with

Win-RHIZO Basic V.2002 software (Regent Instruments, Quebec, Canada) .

# Results

## ACC-Deaminase Producing Bacteria

A total of 186 bacterial isolates were obtained with the DF-ACC agar method from Poaceae grasses in New Jersey Pine Barrens. Among them, 65 were from Colliers Mills, 68 from Assumpink Wildlife Management area, 28 from Warton State Forest and 25 from the Pygmy Pine Plains.

Based on the 16S rRNA sequences, the 186 bacterial isolates belong to 6 orders, 9 families and 10 genera (Fig. 1). Isolates from the same genus were all grouped together in the phylogenetic tree (Fig. 2). There were 2, 2, 6 and 5 genera found in Colliers Mills, Assumpink, Wharton State Forest and Pygmy Pine Plains, respectively. The most frequently isolated genus was *Burkholderia*, which were uncovered from all four sampling locations although most were from Colliers Mills and Assumpink Wildlife Management Area. Within the *Burkholderia* clade, isolates from Colliers' Mills and Assumpink formed a subclade while those from Wharton State Forest and Pygmy Pine plains were grouped together in the phylogenetic tree (Fig. 2). The 133 *Burkholderia* isolates took 71.5% of the total collection, followed by *Herbaspirillum*, *Pseudomonas* and *Paenibacillus*, which took 9.1%, 8.6% and 5.4% respectively. The 17 *Herbaspirillum* isolates including 5 *Herbaspirillum huttiense* and 12 *Herbaspirillum rhizosphaerae* were all from Pygmy Pine Plains. The 13 *Pseudomonas* isolates were from Colliers Mills and Pygmy Pine Plains. *Leifsonia* (2.7%) and *Microbacterium* (1.6%) were all from Wharton State Forest. *Paenibacillus* included 7 *Paenibacillus pabuli* isolates and 3 *Paenibacillus barcinonensis* isolates, most of which were isolated from Wharton State Forest, with one *Paenibacillus barcinonensis* isolate

from Pygmy Pine Plains. For the genus *Bacillus* (from Assumpink Wildlife Management Area), *Bosea* (from Pygmy Pine Plains) *Chryseobacterium* and *Variovorax* (both from Wharton State Forest), only one isolate were obtained in the collection. The top BLAST matches for them were *Bacillus megaterium*, *Bosea* sp., and *Chryseobacterium taichungense* respectively.

**ACC deaminase Activity Assay**

The ACC deaminase activity assay included 187 bacterial isolates, of which 185 (65 from Colliers Mills Wildlife Management Area, 68 from Assumpink Wildlife Management Area, 28 from Wharton State Forest, 24 from Pygmy Pine Plains) were our collections from New Jersey Pine Barrens. *Burkholderia phytofirmans* PsJN, a well-studied plant growth promoting strain and *Burkholderia gladioli* RU1, an ACC deaminase producing strain from Kentucky bluegrass (*Poa pratensis* L.) were also included in the assay as positive controls.

The ACC deaminase activity assay was conducted three times for each bacteria strain. Mean values were calculated as showed in Table 1. The ACC deaminase activity among different isolates ranged from 0 to as high as 40024 nmol ketobutyrate/mg/h. There were 12 strains considered with no or little ACC deaminase activity based on the assay result. Eleven of them were from the Wharton State Forest collections, the other one was from Assumpink Lake Wildlife Management Area. The isolates PP4, PP28 and CM1-29 failed to be revived for the assay. Five Pygmy Pine Plains isolates and 8 Wharton State Forest isolates had ACC deaminase activities less than 1000 nmol ketobutyrate/mg/h. A total of 75 isolates exhibited an ACC deaminase activity between 1000 and 2500 nmol ketobutyrate/mg/h, 65 of which were from Assumpink Wildlife

Management Area. From 2500 to 5000 nmol ketobutyrate/mg/h, there were 39 isolates, 29 of which were from Colliers Mills Wildlife Management Area. There were 28 isolates with ACC deaminase activities between 5000 and 10000 nmol ketobutyrate/mg/h, most of which were from Colliers Mills Wildlife Management Area. From 10000 to 20000 nmol ketobutyrate/mg/h there are 6 isolates. 7 isolates had the activity above 20000 nmol ketobutyrate/mg/h. 6 of them were from the Pygmy Pine Plains (Fig. 3).

The ACC deaminase activity was also labeled on the phylogentic tree (Fig. 2). It is shown that strains with high ACC deaminase activity were mainly from *Burkholderia* and *Herbaspirillum*. Six out of seven highest (~ 20000 nmol ketobutyrate/mg/h) ACC deaminase activity isolates were from the *Herbaspirillum rhizosphaerae* clade, while those in the *Variovorax*, *Leifsonia*, *Bacillus* and *Paenibacillus* clades had low ACC deaminase activities. Large variation in ACC deaminase activity was observed in the *Burkholderia* and *Pseudomonas* clades.

**Root Elongation Assay**

Four bacterial isolates from our collections with high ACC deaminase activity, *Herbaspirillum huttiense* PP19, *Burkholderia* sp. WSF26, *Burkholderia* sp. CM2-8 and *Herbaspirillum rhizosphaerae* PP06, along with PsJN and RU1 were chosen to test their potential growth promotion effect to plant. Root analyze results were shown in Table 2A for well-watered condition and Table 2B for salinity condition. Under well-watered condition, PP19, WSF26 and CM2-8 treatment had significant higher total root length than the control. No significant differences were observed among the average root diameters. Under salinity treatment only the PP19 treatment had

significantly higher total root length than control. RU1 significantly decreased the total root length while increased the average root diameter (Fig. 4 A, B).

## Discussion

In this study, we collected 186 endophytic ACC-deaminase producing bacteria strains from the roots of grass species in the pine barrens ecosystem. Those strains were identified to a total of 10 genera, of which *Burkholderia* was revealed to be the most dominant one. Based on their study of 45 strains in 20 *Burkholderia* species, Onofre-Lemus and Janette et al. (2009) suggested that ACC deaminase activity is a widespread feature in *Burkholderia* species. This might explain why they formed the dominant part of our endophytic ACC-deaminase producing bacteriacollection and were discovered in all four sample sites. *Pseudomonas putida* GR12-2 (Jacobson et al., 1994), *Pseudomonas putida* UW4 (Shah et al., 1998) and other *Pseudomonas* strains also have shown ACC deaminase activity and plant growth promoting effects. Nadeem et al. (2007) reported that inoculation of ACC deaminase producing *Pseudomonas syringae* S5 and *Pseudomonas chlororaphis* S9 improved salt tolerance of maize. Several isolates of these two species from our pine barrens grass root collection also have ACC deaminase activities. *Variovorax paradoxus* 5C-2 is a widely studied PGPR (Jiang et al., 2012; Chen et al., 2013), and this species was also isolated in this study but with relatively low ACC deaminase activity. *Leifsonia shinshuensis* and *Bacillus megaterium* isolated from this study also have also been reported to have ACC deaminase activity in previous studies (Anandham et al., 2008; Zhang et al., 2011).

ACC deaminase activity has already been reported in *Microbacterium azadirachtae* AI-S262 (Madhaiyan et al., 2010), *Microbacterium arborescens*, *Microbacterium testaceum* (Rasche et al., 2006), *Microbacterium* sp. F10a (Sheng et al., 2009), and other *Microbacterium* species. This study first reports the ACC deaminase activity of *Microbacterium trichothecenolyticum*. *Herbaspirillum* species are well

known as plant-associated bacteria which can increase plant growth and productivity (Monteiro et al., 2012). There has been increasing reports on the ACC deaminase activity of *Herbaspirillum* species (Li et al., 2001). Our study first reports the ACC deaminase activity of *Herbaspirillum huttiense* and *Herbaspirillum rhizosphaerae*, which composed 9.1% of our total collection and presented relatively high ACC deaminase activities. *Paenibacillus pabuli, Paenibacillus barcinonensis, Chryseobacterium taichungense* and *Bosea* sp. are also first reported in our study presenting ACC deaminase activity.

We have conducted the ACC deaminase activity assay in 96-well plates modified from the protocol of Penrose and Glick (2003). The smaller volume reaction system might generate higher variation but provides a higher throughput, which facilitates the screening of large numbers of uninvestigated natural biodiversity (Nelson 2004). The ACC deaminase activity of our positive control strain PsJN was measured as 17143 nM α-ketobutyrate $mg^{-1}$ $h^{-1}$, which is close to 18480 nM α-ketobutyrate $mg^{-1}$ $h^{-1}$ reported by Sessitsch et al. (2005). In our study, variation in ACC deaminase activity was observed among different isolates of the same bacterial species, which may be explained by the horizontal gene transfer in bacteria (Hontzeas et al., 2005) rather than the errors in measurements.

It has been reported that an ACC deaminase activity of 1200 nM α-ketobutyrate $mg^{-1}$ $h^{-1}$ is sufficient to provide plant growth promoting effects (Penrose and Glick, 2003). A total of 154 isolates from our collection meet this criterion, with the ACC deaminase activity ranging from 1259 to 40024 nM α-ketobutyrate $mg^{-1}$. Though the presence of a plant growth promoting trait *in vitro* cannot guarantee a certain isolate the plant growth promoting effects (Dey et al., 2004), our plant inoculation assay showed

that 3 out of 4 strains with high ACC deaminase activities from our pine barrens collection significantly increased the total root length of Perennial ryegrass under normal watered condition, and one strain also significantly increased the total root length under salinity stress.

It was suggested that endophytic PGPB may play a more important role to the plant host than the rhizospheric bacteria because they have more intimate contact with plant tissues and less competition with rhizospheric microorganisms (Glick, 2004; Naveed et al., 2014). Results from this study indicate that the endophytic ACC-deaminase producing bacteriamay play an important role in assisting the establishment of Poaceace grasses in the dry and infertile pine barrens ecosystem. The newly reported ACC deaminase producing bacterial species along with the diversity of ACC deaminase activity among different isolates of the same species might suggest a high potential of discovering new PGPB strains from natural environments.

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**Table 1.** BLAST result and ACC-deaminase activity of collected isolates. ACC-deaminase activities are averages from three measurements. Isolates labeled with asterisks are not included in the phylogenetic analysis. "." in BLAST or ACC-deaminase activity column indicates missing data.

| Strain | Best Match from BLAST | ACC-deaminase activity (nmol ketobutyrate/mg/h) |
|---|---|---|
| WSF1 | *Chryseobacterium taichungense* | 3687 |
| WSF2 | *Paenibacillus pabuli* | 359 |
| WSF3 | *Leifsonia shinshuensis* | 709 |
| WSF4 | *Leifsonia shinshuensis* | 888 |
| WSF5 | *Microbacterium trichothecenolyticum* | 3467 |
| WSF6 | *Paenibacillus barcinonensis* | 0 |
| WSF7 | *Burkholderia sp.* | 0 |
| WSF8 | *Paenibacillus barcinonensis* | 350 |
| WSF9 | *Leifsonia shinshuensis* | 1259 |
| WSF10 | *Paenibacillus pabuli* | 208 |
| WSF11 | *Microbacterium trichothecenolyticum* | 4490 |
| WSF12 | *Paenibacillus pabuli* | 0 |
| WSF13 | *Burkholderia sp.* | 0 |
| WSF14 | *Burkholderia sp.* | 19027 |
| WSF15 | *Burkholderia sp.* | 0 |
| WSF16 | *Burkholderia sp.* | 0 |
| WSF17 | *Paenibacillus pabuli* | 0 |
| WSF18 | *Microbacterium trichothecenolyticum* | 3125 |
| WSF19 | *Paenibacillus pabuli* | 0 |
| WSF20 | *Paenibacillus pabuli* | 1045 |
| WSF21 | *Burkholderia sp.* | 0 |
| WSF22 | *Leifsonia shinshuensis* | 975 |
| WSF23 | *Burkholderia sp.* | 17690 |
| WSF24 | *Variovorax paradoxus* | 0 |

| | | |
|---|---|---|
| WSF25 | *Paenibacillus sp.* | 0 |
| WSF26* | *Burkholderia sp.* | 21504 |
| WSF27* | . | 1150 |
| WSF28 | *Burkholderia sp.* | 0 |
| WSF29 | *Leifsonia shinshuensis* | 895 |
| WSF30 | *Paenibacillus sp.* | 260 |
| PP2 | *Pseudomonas chlororaphis* | 504 |
| PP3 | *Herbaspirillum autotrophicum* | 422 |
| PP4 | *Herbaspirillum autotrophicum* | . |
| PP6 | *Herbaspirillum rhizosphaerae* | 39876 |
| PP7 | . | 311 |
| PP11* | *Paenibacillus barcinonensis* | . |
| PP12 | *Herbaspirillum rhizosphaerae* | 25962 |
| PP13 | *Herbaspirillum rhizosphaerae* | 3225 |
| PP14 | *Herbaspirillum rhizosphaerae* | 39623 |
| PP15 | *Pseudomonas chlororaphis* | 755 |
| PP16 | *Herbaspirillum rhizosphaerae* | 40024 |
| PP17 | *Herbaspirillum rhizosphaerae* | 33978 |
| PP18 | *Herbaspirillum rhizosphaerae* | 33134 |
| PP19 | *Herbaspirillum huttiense* | 5266 |
| PP20 | *Herbaspirillum huttiense* | 1629 |
| PP21 | *Burkholderia sp.* | 1998 |
| PP22 | *Bosea sp.* | 3580 |
| PP23 | *Herbaspirillum sp.* | 4133 |
| PP24 | *Burkholderia sp.* | 4468 |
| PP25 | *Pseudomonas chlororaphis* | 692 |
| PP26 | *Herbaspirillum sp.* | 4180 |
| PP27 | *Herbaspirillum rhizosphaerae* | 5025 |
| PP28 | *Herbaspirillum rhizosphaerae* | . |
| PP29 | *Herbaspirillum sp.* | 2109 |
| PP30 | *Burkholderia glathei* | 11955 |
| CM1_4 | *Burkholderia sp.* | 9919 |

| | | |
|---|---|---|
| CM1_5 | *Burkholderia sp.* | 9848 |
| CM1_7 | *Burkholderia sp.* | 8178 |
| CM1_8 | *Pseudomonas syringae* | 4332 |
| CM1_9 | *Burkholderia sp.* | 5917 |
| CM1_11 | *Burkholderia sp.* | 10227 |
| CM1_13 | *Burkholderia sp.* | 8356 |
| CM1_14* | . | 5205 |
| CM1_15 | *Burkholderia sp.* | 8657 |
| CM1_16 | *Pseudomonas syringae* | 4630 |
| CM1_17 | *Burkholderia sp.* | 8742 |
| CM1_18 | *Burkholderia sp.* | 8966 |
| CM1_19 | *Burkholderia sp.* | 9506 |
| CM1_20 | *Burkholderia sp.* | 9681 |
| CM1_21 | *Burkholderia sp.* | 9316 |
| CM1_22 | *Burkholderia sp.* | 3129 |
| CM1_23 | *Pseudomonas syringae* | 5683 |
| CM1_24 | *Burkholderia sp.* | 7483 |
| CM1_25 | *Burkholderia sp.* | 9526 |
| CM1_26 | *Pseudomonas syringae* | 4563 |
| CM1_27 | *Burkholderia sp.* | 8000 |
| CM1_28 | *Burkholderia sp.* | 8636 |
| CM1_29 | *Burkholderia sp.* | . |
| CM1_30 | *Burkholderia sp.* | 8584 |
| CM2_1 | *Burkholderia sp.* | 9884 |
| CM2_4 | *Pseudomonas syringae* | 3999 |
| CM2_5 | *Burkholderia sp.* | 8641 |
| CM2_6 | *Burkholderia sp.* | 9038 |
| CM2_8 | *Burkholderia sp.* | 11119 |
| CM2_9 | *Pseudomonas syringae* | 4376 |
| CM2_11 | *Burkholderia sp.* | 9308 |
| CM2_12 | *Burkholderia sp.* | 10189 |
| CM2_13 | *Burkholderia sp.* | 8695 |

| | | |
|---|---|---|
| CM2_14 | *Burkholderia sp.* | 9029 |
| CM2_15 | *Burkholderia sp.* | 8994 |
| CM2_16 | *Burkholderia sp.* | 7957 |
| CM2_18 | *Burkholderia sp.* | 9103 |
| CM2_19 | *Pseudomonas syringae* | 3295 |
| CM2_20 | *Pseudomonas syringae* | 4221 |
| CM2_24 | *Burkholderia sp.* | 3336 |
| CM2_25 | *Pseudomonas syringae* | 1901 |
| CM2_26 | *Burkholderia sp.* | 3255 |
| CM2_27 | *Burkholderia sp.* | 3376 |
| CM2_29 | *Burkholderia sp.* | 3321 |
| CM2_30 | *Burkholderia sp.* | 3155 |
| CM3_2 | *Burkholderia sp.* | 3000 |
| CM3_4 | *Burkholderia sp.* | 3358 |
| CM3_6 | *Burkholderia cenocepacia* | 1007 |
| CM3_7 | *Burkholderia sp.* | 3240 |
| CM3_9 | *Pseudomonas syringae* | 1855 |
| CM3_10 | *Pseudomonas syringae* | 2679 |
| CM3_12 | *Burkholderia sp.* | 3455 |
| CM3_13 | *Burkholderia sp.* | 3306 |
| CM3_14 | *Pseudomonas syringae* | 1975 |
| CM3_15 | *Burkholderia sp.* | 3175 |
| CM3_16 | *Burkholderia sp.* | 3457 |
| CM3_17 | *Burkholderia sp.* | 3184 |
| CM3_18 | *Pseudomonas syringae* | 1935 |
| CM3_19 | *Burkholderia sp.* | 3447 |
| CM3_22 | *Burkholderia sp.* | 3180 |
| CM3_24 | *Burkholderia sp.* | 3319 |
| CM3_26 | *Burkholderia sp.* | 3339 |
| CM3_28 | *Burkholderia sp.* | 3114 |
| CM3_29 | *Burkholderia sp.* | 2667 |
| CM3_30 | *Burkholderia sp.* | 3166 |

| | | |
|---|---|---|
| AL1_2 | *Burkholderia ambifaria* | 2353 |
| AL1_3 | *Burkholderia ambifaria* | 2325 |
| AL1_4 | *Burkholderia ambifaria* | 1988 |
| AL1_5 | *Burkholderia ambifaria* | 2347 |
| AL1_6 | *Burkholderia ambifaria* | 2142 |
| AL1_7 | *Burkholderia ambifaria* | 1944 |
| AL1_8 | *Burkholderia ambifaria* | 2034 |
| AL1_9 | *Burkholderia ambifaria* | 1838 |
| AL1_12 | *Burkholderia ambifaria* | 2249 |
| AL1_13 | *Burkholderia ambifaria* | 2072 |
| AL1_14 | *Burkholderia ambifaria* | 2788 |
| AL1_17 | *Burkholderia ambifaria* | 2110 |
| AL1_18 | *Burkholderia ambifaria* | 2133 |
| AL1_19 | *Burkholderia ambifaria* | 2379 |
| AL1_20 | *Burkholderia ambifaria* | 2137 |
| AL1_21 | *Burkholderia ambifaria* | 1786 |
| AL1_22 | *Burkholderia ambifaria* | 2082 |
| AL1_23 | *Burkholderia ambifaria* | 2053 |
| AL1_24 | *Bacillus megaterium* | 0 |
| AL1_25 | *Burkholderia ambifaria* | 2133 |
| AL1_26 | *Burkholderia ambifaria* | 2080 |
| AL1_27 | *Burkholderia ambifaria* | 2242 |
| AL1_28 | *Burkholderia ambifaria* | 1884 |
| AL1_29 | *Burkholderia sp.* | 2165 |
| AL1_30 | *Burkholderia ambifaria* | 2307 |
| AL2_3 | *Burkholderia ambifaria* | 2280 |
| AL2_4 | *Burkholderia ambifaria* | 1866 |
| AL2_5 | *Burkholderia ambifaria* | 1143 |
| AL2_8 | *Burkholderia ambifaria* | 2108 |
| AL2_11 | *Burkholderia ambifaria* | 2130 |
| AL2_14 | *Burkholderia ambifaria* | 1909 |
| AL2_18 | *Burkholderia ambifaria* | 1966 |

| | | |
|---|---|---|
| AL2_19 | *Burkholderia ambifaria* | 2018 |
| AL2_20 | *Burkholderia ambifaria* | 2439 |
| AL2_21 | *Burkholderia ambifaria* | 1966 |
| AL2_22 | *Burkholderia ambifaria* | 2046 |
| AL2_23 | *Burkholderia ambifaria* | 1646 |
| AL2_24 | *Burkholderia ambifaria* | 2081 |
| AL2_26 | *Burkholderia ambifaria* | 2314 |
| AL2_27 | *Burkholderia ambifaria* | 1905 |
| AL2_28 | *Burkholderia ambifaria* | 1730 |
| AL2_29 | *Burkholderia ambifaria* | 1327 |
| AL3_2 | *Burkholderia ambifaria* | 1689 |
| AL3_3 | *Burkholderia ambifaria* | 2345 |
| AL3_5 | *Burkholderia ambifaria* | 2182 |
| AL3_6 | *Burkholderia ambifaria* | 2540 |
| AL3_7 | *Burkholderia ambifaria* | 1712 |
| AL3_8 | *Burkholderia ambifaria* | 1867 |
| AL3_10 | *Burkholderia ambifaria* | 2029 |
| AL3_11 | *Burkholderia cenocepacia* | 2022 |
| AL3_12 | *Burkholderia ambifaria* | 2294 |
| AL3_13 | *Burkholderia ambifaria* | 2029 |
| AL3_14 | *Burkholderia ambifaria* | 2100 |
| AL3_15 | *Burkholderia ambifaria* | 1828 |
| AL3_16 | *Burkholderia ambifaria* | 1974 |
| AL3_17 | *Burkholderia ambifaria* | 1868 |
| AL3_18 | *Burkholderia ambifaria* | 1914 |
| AL3_19 | *Burkholderia cenocepacia* | 2081 |
| AL3_20 | *Burkholderia ambifaria* | 1812 |
| AL3_21 | *Burkholderia ambifaria* | 2040 |
| AL3_22 | *Burkholderia ambifaria* | 2026 |
| AL3_23 | *Burkholderia ambifaria* | 1975 |
| AL3_24 | *Burkholderia ambifaria* | 1317 |
| AL3_25 | *Burkholderia ambifaria* | 1935 |

| | | |
|---|---|---|
| AL3_26 | *Burkholderia cenocepacia* | 1970 |
| AL3_28 | *Burkholderia ambifaria* | 1578 |
| AL3_29 | *Burkholderia ambifaria* | 1506 |
| AL3_30 | *Burkholderia ambifaria* | 1893 |

**Table 2.** Root analysis of 2 weeks old perennial ryegrass seedlings of different treatments. A, well-watered condition; B, salinity condition.

A.

| | total length (cm) | average diameter (mm) |
|---|---|---|
| Control | 125.71[b] | 0.35[a] |
| PsJN | 191.79[ab] | 0.33[a] |
| RU1 | 122.46[b] | 0.36[a] |
| PP19 | 193.47[a] | 0.33[a] |
| WSF26 | 237.14[a] | 0.28[a] |
| CM2-8 | 219.41[a] | 0.32[a] |
| PP6 | 181.10[ab] | 0.30[a] |

B.

| | total length (cm) | average diameter (mm) |
|---|---|---|
| Control | 74.00[bc] | 0.36[b] |
| PsJN | 94.01[abc] | 0.39[ab] |
| RU1 | 56.72[c] | 0.54[a] |
| PP19 | 125.74[a] | 0.37[b] |
| WSF26 | 111.59[ab] | 0.37[b] |
| CM2-8 | 94.38[ab] | 0.41[ab] |
| PP6 | 70.31[ab] | 0.51[ab] |

**Figure 1.** Composition of the collected ACC-deaminase producing bacteria from New Jersey pine barrens at genus (outer circle) and order (inner circle) level.

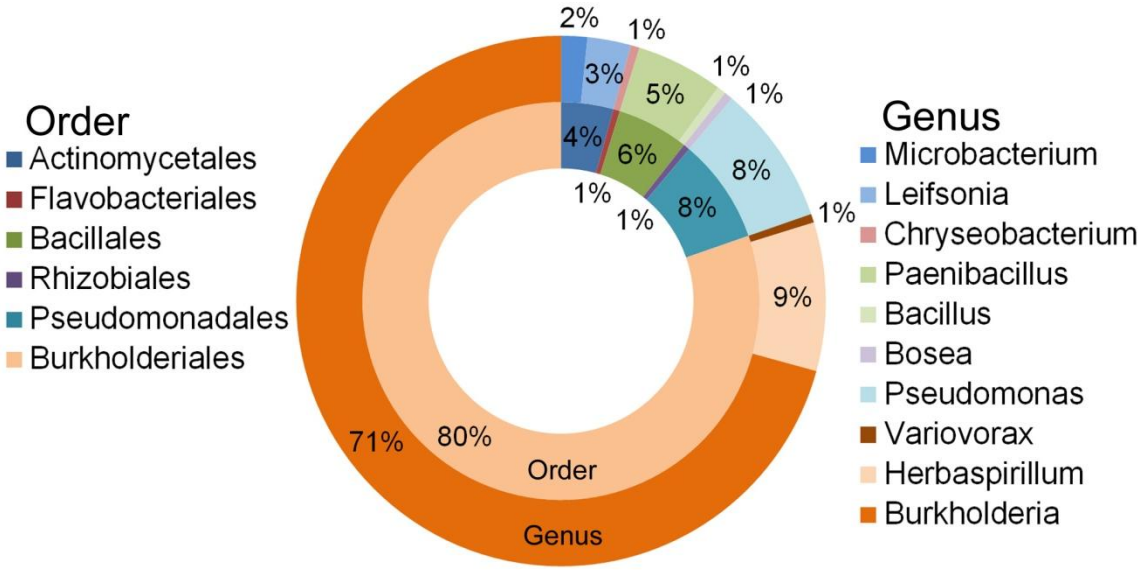


**Figure 2.** The maximum likelihood tree inferred from 16S rDNA sequences. MP boot strap value ≥ 50% are noted above internodes. Quantified ACC-deaminase activities (nmol ketobutyrate/mg/h) are represented by blocks in a grey scale. Sample sites are mapped on the tree by lines of 4 different colors.

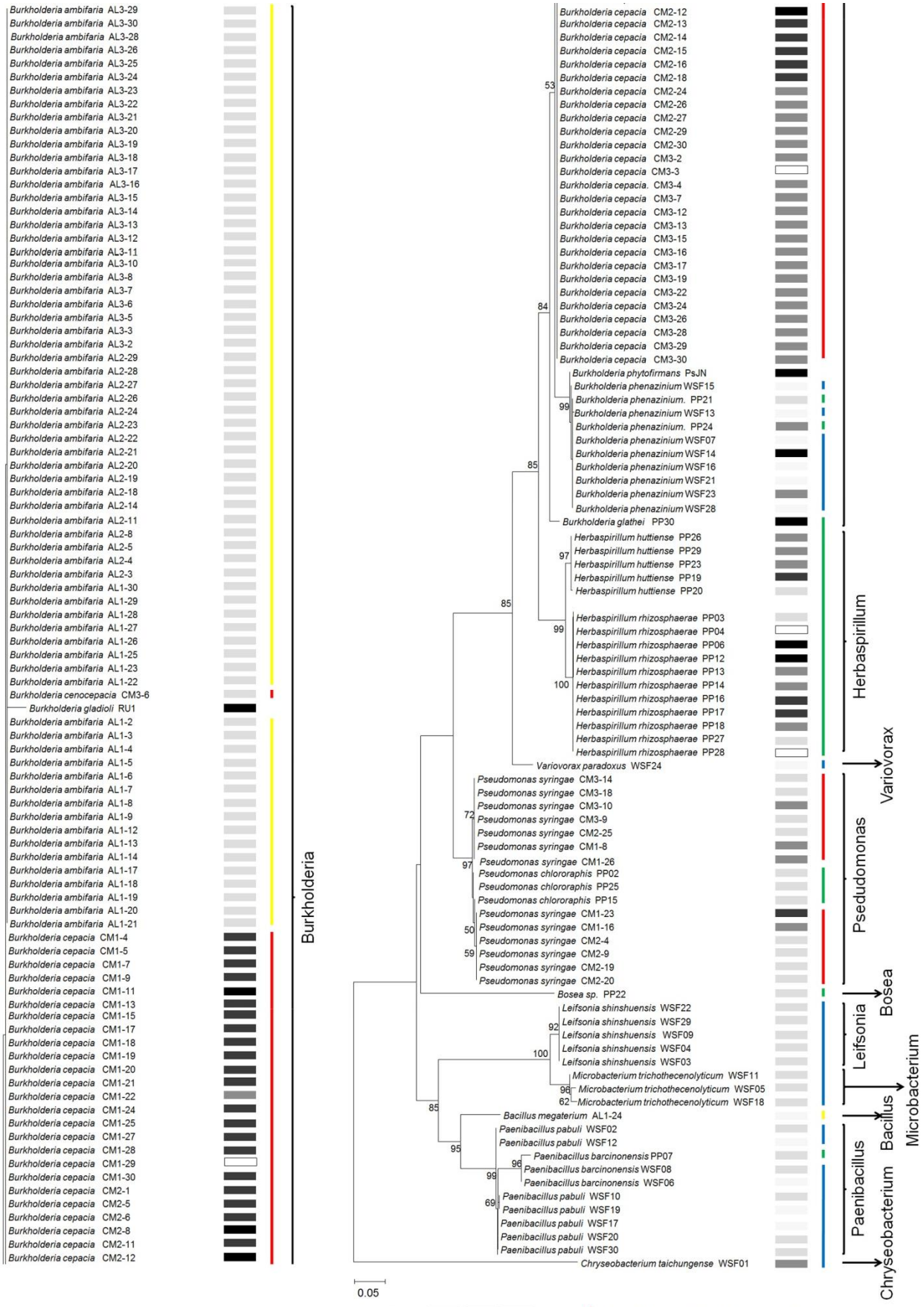

Burkholderia ambifaria AL3-29
Burkholderia ambifaria AL3-30
Burkholderia ambifaria AL3-28
Burkholderia ambifaria AL3-26
Burkholderia ambifaria AL3-25
Burkholderia ambifaria AL3-24
Burkholderia ambifaria AL3-23
Burkholderia ambifaria AL3-22
Burkholderia ambifaria AL3-21
Burkholderia ambifaria AL3-20
Burkholderia ambifaria AL3-19
Burkholderia ambifaria AL3-18
Burkholderia ambifaria AL3-17
Burkholderia ambifaria AL3-16
Burkholderia ambifaria AL3-15
Burkholderia ambifaria AL3-14
Burkholderia ambifaria AL3-13
Burkholderia ambifaria AL3-12
Burkholderia ambifaria AL3-11
Burkholderia ambifaria AL3-10
Burkholderia ambifaria AL3-8
Burkholderia ambifaria AL3-7
Burkholderia ambifaria AL3-6
Burkholderia ambifaria AL3-5
Burkholderia ambifaria AL3-3
Burkholderia ambifaria AL3-2
Burkholderia ambifaria AL2-29
Burkholderia ambifaria AL2-28
Burkholderia ambifaria AL2-27
Burkholderia ambifaria AL2-26
Burkholderia ambifaria AL2-24
Burkholderia ambifaria AL2-23
Burkholderia ambifaria AL2-22
Burkholderia ambifaria AL2-21
Burkholderia ambifaria AL2-20
Burkholderia ambifaria AL2-19
Burkholderia ambifaria AL2-18
Burkholderia ambifaria AL2-14
Burkholderia ambifaria AL2-11
Burkholderia ambifaria AL2-8
Burkholderia ambifaria AL2-5
Burkholderia ambifaria AL2-4
Burkholderia ambifaria AL2-3
Burkholderia ambifaria AL1-30
Burkholderia ambifaria AL1-29
Burkholderia ambifaria AL1-28
Burkholderia ambifaria AL1-27
Burkholderia ambifaria AL1-26
Burkholderia ambifaria AL1-25
Burkholderia ambifaria AL1-23
Burkholderia ambifaria AL1-22
Burkholderia cenocepacia CM3-6
Burkholderia gladioli RU1
Burkholderia ambifaria AL1-2
Burkholderia ambifaria AL1-3
Burkholderia ambifaria AL1-4
Burkholderia ambifaria AL1-5
Burkholderia ambifaria AL1-6
Burkholderia ambifaria AL1-7
Burkholderia ambifaria AL1-8
Burkholderia ambifaria AL1-9
Burkholderia ambifaria AL1-12
Burkholderia ambifaria AL1-13
Burkholderia ambifaria AL1-14
Burkholderia ambifaria AL1-17
Burkholderia ambifaria AL1-18
Burkholderia ambifaria AL1-19
Burkholderia ambifaria AL1-20
Burkholderia ambifaria AL1-21
Burkholderia cepacia CM1-4
Burkholderia cepacia CM1-5
Burkholderia cepacia CM1-7
Burkholderia cepacia CM1-9
Burkholderia cepacia CM1-11
Burkholderia cepacia CM1-13
Burkholderia cepacia CM1-15
Burkholderia cepacia CM1-17
Burkholderia cepacia CM1-18
Burkholderia cepacia CM1-19
Burkholderia cepacia CM1-20
Burkholderia cepacia CM1-21
Burkholderia cepacia CM1-22
Burkholderia cepacia CM1-24
Burkholderia cepacia CM1-25
Burkholderia cepacia CM1-27
Burkholderia cepacia CM1-28
Burkholderia cepacia CM1-29
Burkholderia cepacia CM1-30
Burkholderia cepacia CM2-1
Burkholderia cepacia CM2-5
Burkholderia cepacia CM2-6
Burkholderia cepacia CM2-8
Burkholderia cepacia CM2-11
Burkholderia cepacia CM2-12
Burkholderia
Burkholderia cepacia CM2-12
Burkholderia cepacia CM2-13
Burkholderia cepacia CM2-14
Burkholderia cepacia CM2-15
Burkholderia cepacia CM2-16
Burkholderia cepacia CM2-18
Burkholderia cepacia CM2-24
Burkholderia cepacia CM2-26
Burkholderia cepacia CM2-27
Burkholderia cepacia CM2-29
Burkholderia cepacia CM2-30
Burkholderia cepacia CM3-2
Burkholderia cepacia CM3-3
Burkholderia cepacia. CM3-4
Burkholderia cepacia CM3-7
Burkholderia cepacia CM3-12
Burkholderia cepacia CM3-13
Burkholderia cepacia CM3-15
Burkholderia cepacia CM3-16
Burkholderia cepacia CM3-17
Burkholderia cepacia CM3-19
Burkholderia cepacia CM3-22
Burkholderia cepacia CM3-24
Burkholderia cepacia CM3-26
Burkholderia cepacia CM3-28
Burkholderia cepacia CM3-29
Burkholderia cepacia CM3-30
Burkholderia phytofirmans PsJN
Burkholderia phenazinium WSF15
Burkholderia phenazinium. PP21
Burkholderia phenazinium WSF13
Burkholderia phenazinium. PP24
Burkholderia phenazinium WSF07
Burkholderia phenazinium WSF14
Burkholderia phenazinium WSF16
Burkholderia phenazinium WSF21
Burkholderia phenazinium WSF23
Burkholderia phenazinium WSF28
Burkholderia glathei PP30
Herbaspirillum huttiense PP26
Herbaspirillum huttiense PP29
Herbaspirillum huttiense PP23
Herbaspirillum huttiense PP19
Herbaspirillum huttiense PP20
Herbaspirillum rhizosphaerae PP03
Herbaspirillum rhizosphaerae PP04
Herbaspirillum rhizosphaerae PP06
Herbaspirillum rhizosphaerae PP12
Herbaspirillum rhizosphaerae PP13
Herbaspirillum rhizosphaerae PP14
Herbaspirillum rhizosphaerae PP16
Herbaspirillum rhizosphaerae PP17
Herbaspirillum rhizosphaerae PP18
Herbaspirillum rhizosphaerae PP27
Herbaspirillum rhizosphaerae PP28
Variovorax paradoxus WSF24
Pseudomonas syringae CM3-14
Pseudomonas syringae CM3-18
Pseudomonas syringae CM3-10
Pseudomonas syringae CM3-9
Pseudomonas syringae CM2-25
Pseudomonas syringae CM1-8
Pseudomonas syringae CM1-26
Pseudomonas chlororaphis PP02
Pseudomonas chlororaphis PP25
Pseudomonas chlororaphis PP15
Pseudomonas syringae CM1-23
Pseudomonas syringae CM1-16
Pseudomonas syringae CM2-4
Pseudomonas syringae CM2-9
Pseudomonas syringae CM2-19
Pseudomonas syringae CM2-20
Bosea sp. PP22
Leifsonia shinshuensis WSF22
Leifsonia shinshuensis WSF29
Leifsonia shinshuensis WSF09
Leifsonia shinshuensis WSF04
Leifsonia shinshuensis WSF03
Microbacterium trichothecenolyticum WSF11
Microbacterium trichothecenolyticum WSF05
Microbacterium trichothecenolyticum WSF18
Bacillus megaterium AL1-24
Paenibacillus pabuli WSF02
Paenibacillus pabuli WSF12
Paenibacillus barcinonensis PP07
Paenibacillus barcinonensis WSF08
Paenibacillus barcinonensis WSF06
Paenibacillus pabuli WSF10
Paenibacillus pabuli WSF19
Paenibacillus pabuli WSF17
Paenibacillus pabuli WSF20
Paenibacillus pabuli WSF30
Chryseobacterium taichungense WSF01
Herbaspirillum
Variovorax
Pseudomonas
Bosea
Leifsonia
Microbacterium
Bacillus
Paenibacillus
Chryseobacterium
0.05
0
2500
5000
10000+
Colliers Mills Wildlife Management Area
Assumpink Wildlife Management Area
Wharton State Forest
Pygmy Pine Plains

**Figure 3.** ACC-deaminase activities (nmol ketobutyrate/mg/h) by sampling locations. CM, Colliers Mills Wildlife Management Area. AL, Assumpink Wildlife Management Area. WSF, Wharton State Forest. PP, Pygmy Pine Plains.

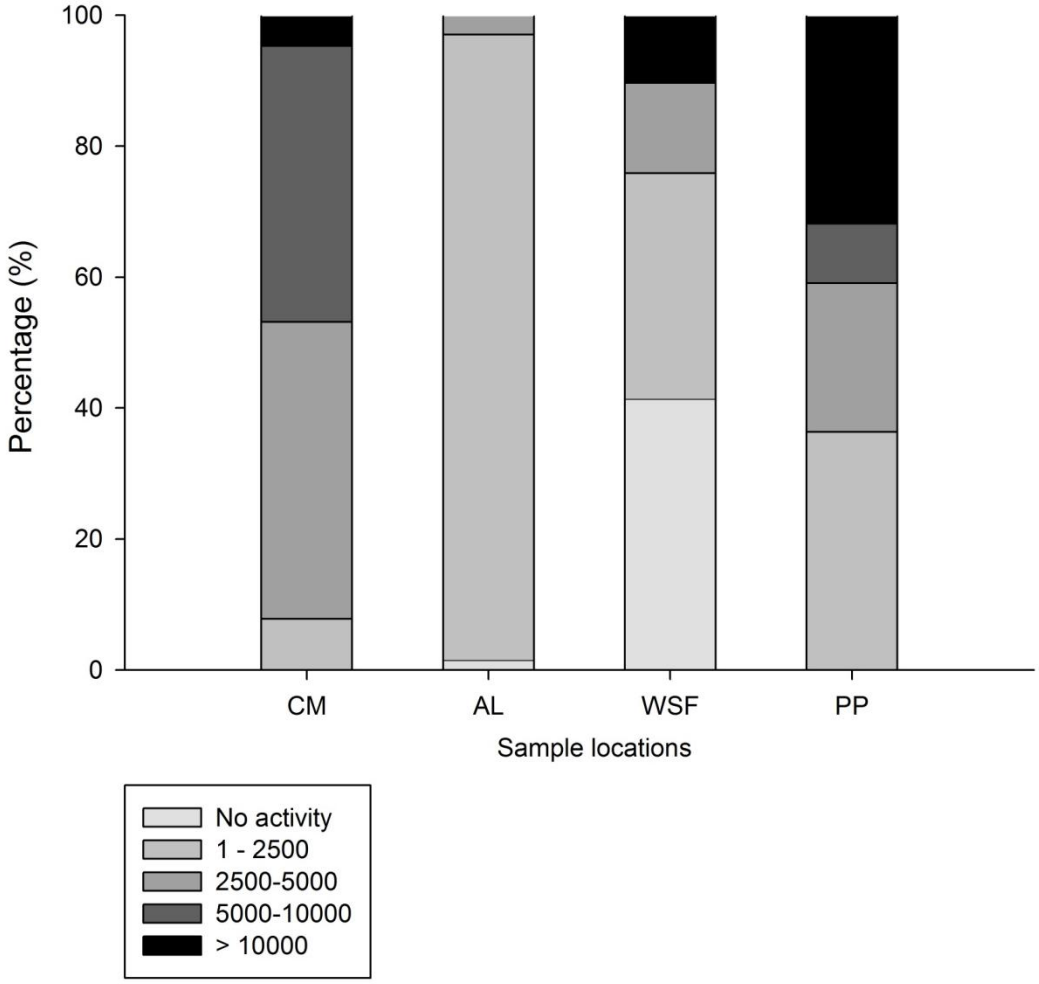
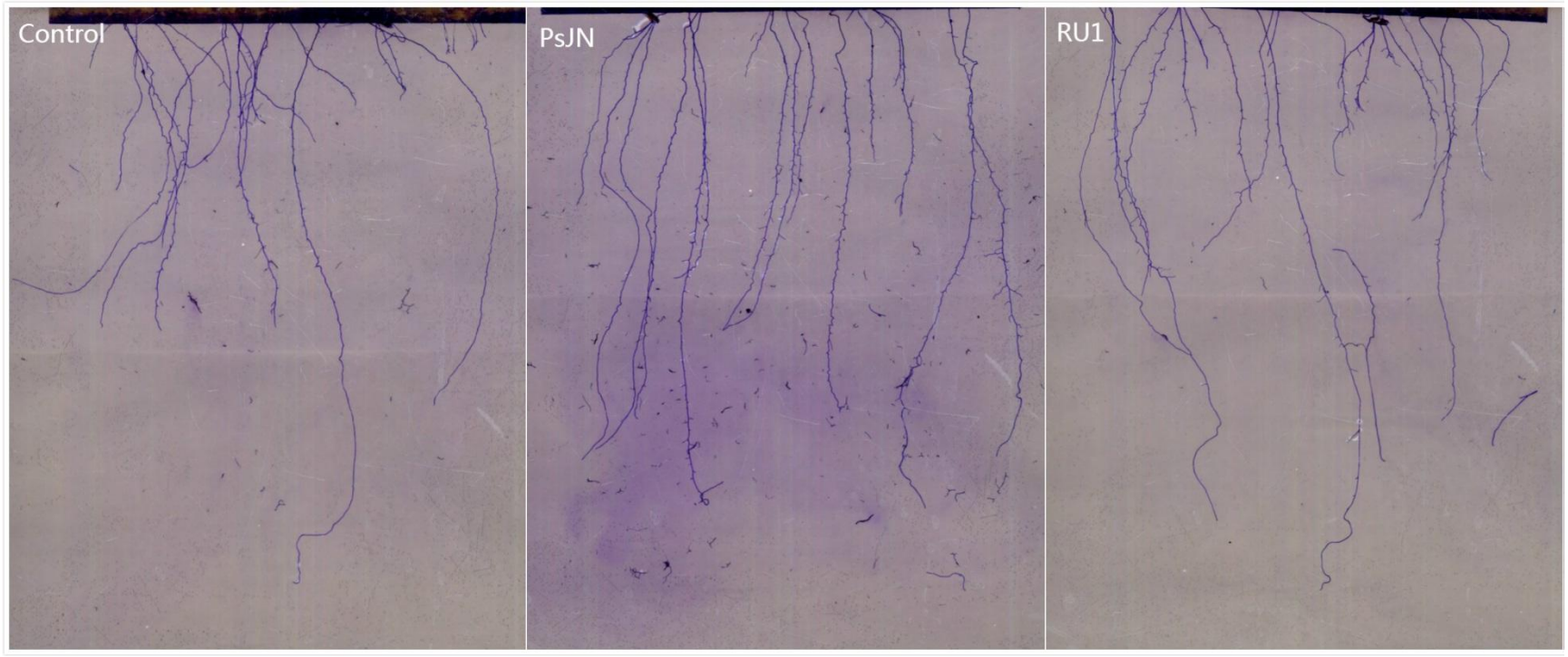
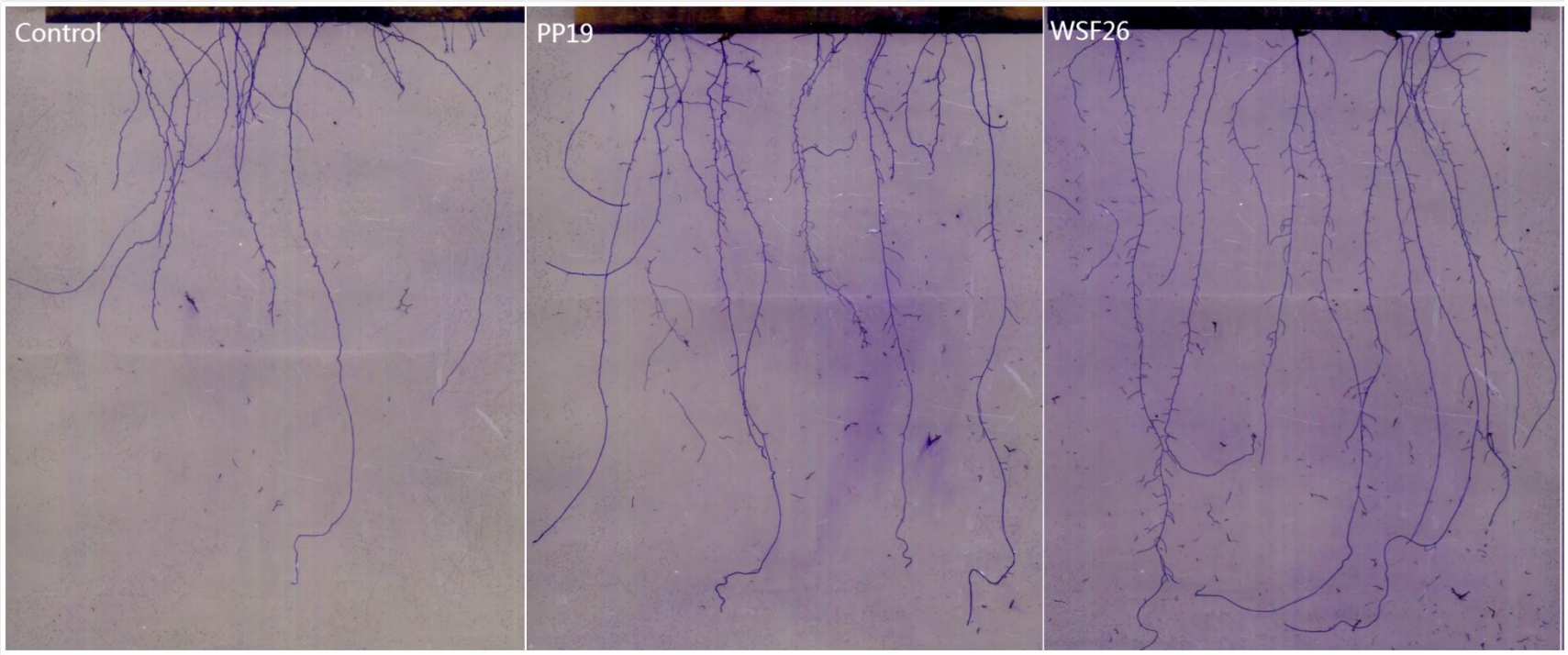
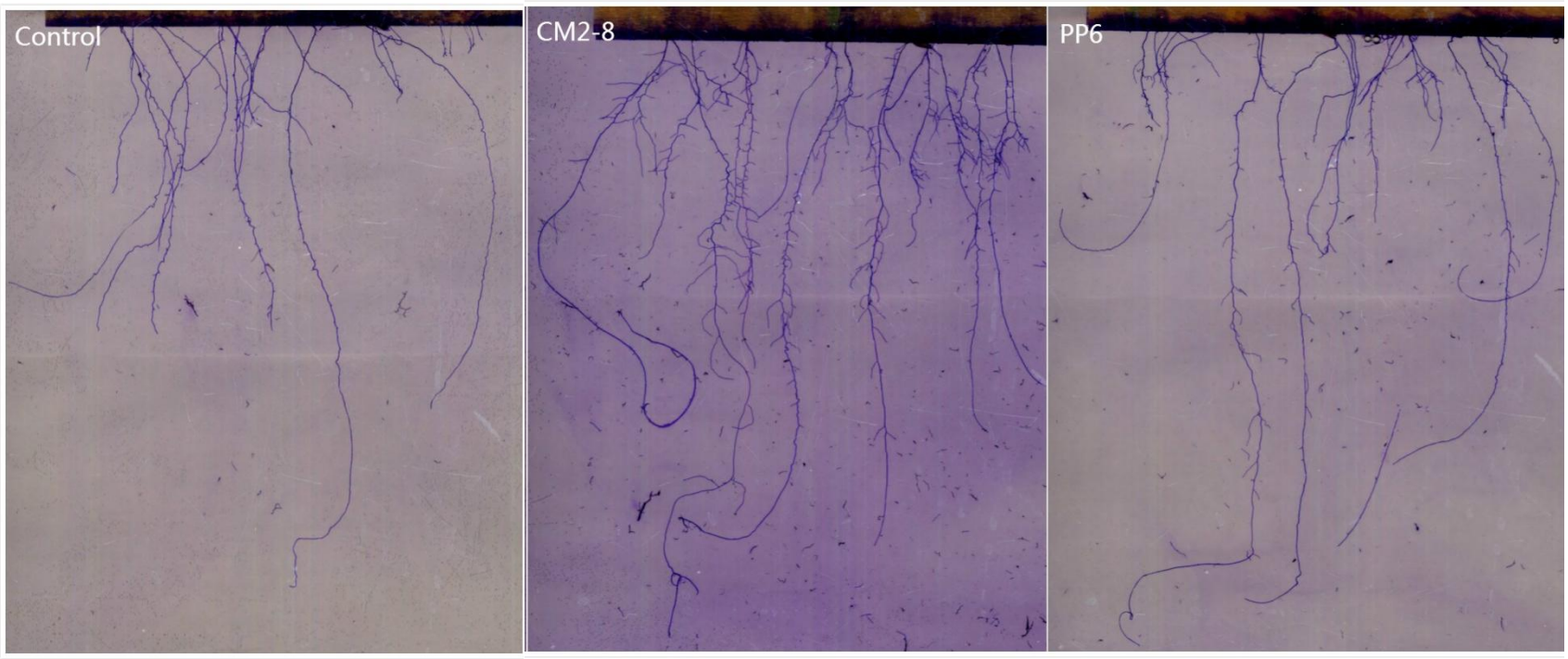


**Figure 4.** Root scanning of 2 weeks old perennial ryegrass seedlings of different treatments. A, well-watered condition; B, salinity condition. Bacterial treatments are labeled on the figures.

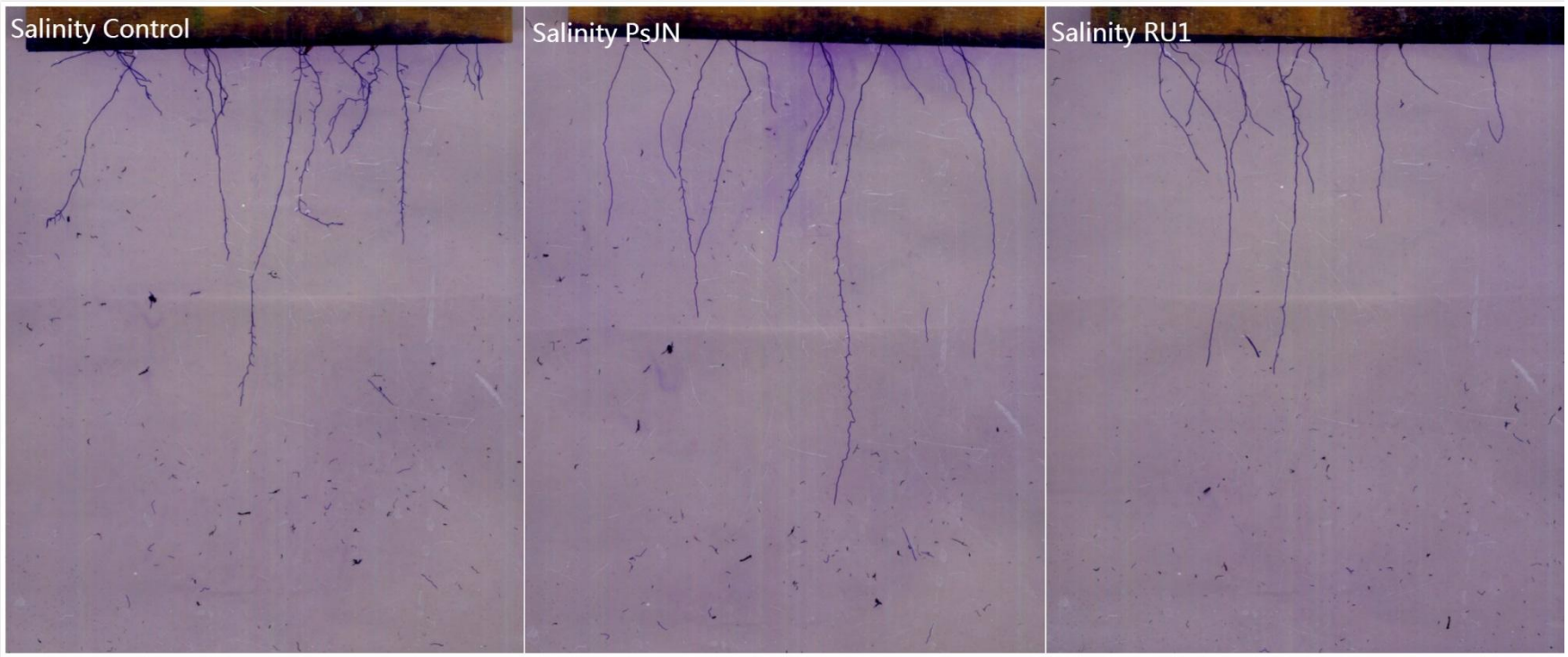
A.

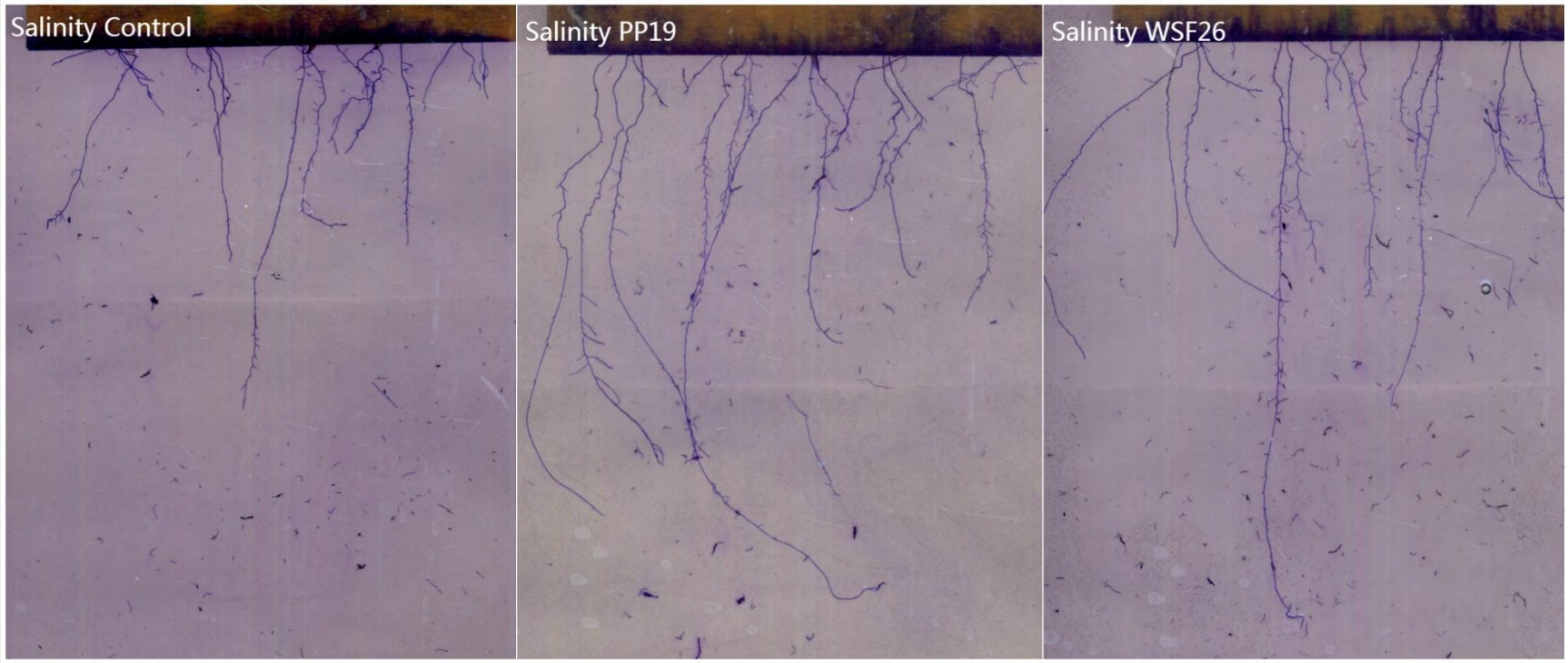


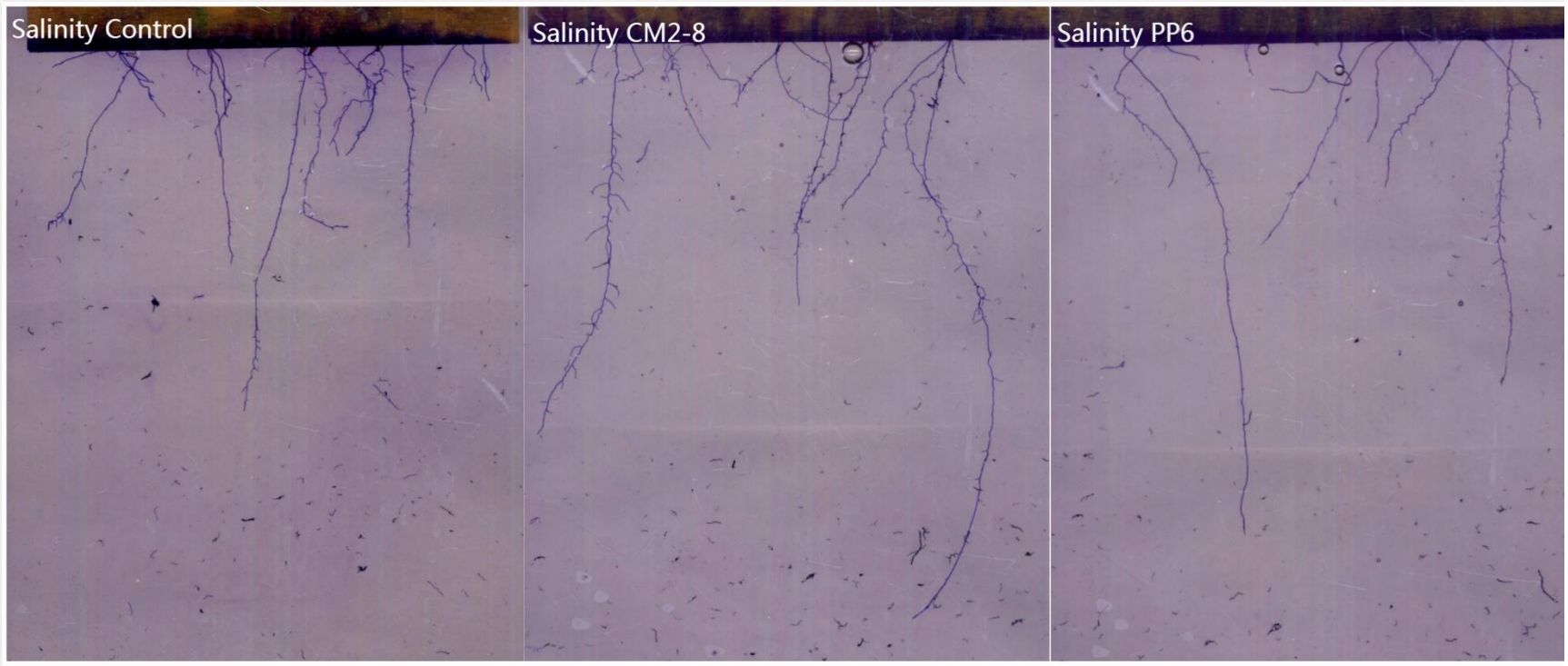

Control
PP19
WSF26


Control
CM2-8
PP6

B.




Salinity Control
Salinity PP19
Salinity WSF26


Salinity Control
Salinity CM2-8
Salinity PP6

# EFFECTS OF ACC-DEAMINASE PRODUCING BACTERIA ON PERENNIAL RYEGRASS GROWTH AND PHYSIOLOGICAL RESPONSES TO SALINITY STRESS

## Introduction

Plant growth promoting bacteria (PGPB) are beneficial bacteria that form symbiosis with plants, which have been reported to promote plant growth and stress tolerance (Glick & Bashan, 1997; Saleem et al., 2007; Yang, Kloepper & Ryu, 2009). The growth promoting and stress alleviating effects of PGPB may be achieved through different mechanisms with different bacteria species. Most studied PGPB include those for nitrogen fixation improving plant nutrition and changing hormone status within the rhizosphere or plants that indirectly regulate plant growth and responses to environmental stresses (Glick & Bashan, 1997).

Ethylene has been considered as a stress hormone, which typically accumulates in plants exposed to stresses, including salinity stress, restricting shoot and root growth (Abeles, Morgan and Saltveit, 2012). Some PGPB contains enzymes, such as *Burkholderia* phytofirmans and *Burkholderia gladioli* breakdown an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC deaminase, and use ACC produced from plants as the nitrogen source (Saleem et al., 2007).
ACC-deaminase producing bacteria can promote plant growth and alleviate the adverse effects of ethylene accumulation in plants under various stresses, include salinity, as mostly found in agronomic and horticultural crop species, such as tomato (*Solanum lycopersicum* L.) (Mayak et al. 2004), canola (*Brassica napus* L.) (Cheng et al., 2007),

cucumber (*Cucumis sativus* L.) (Gamalero et al., 2010), red pepper (*Capsicum annuum* L.) (Siddikee et al., 2012), rice (*Oryza sativa* L.) (Bal et al., 2013), mungbean (*Vigna radiata* L.) (Ahmad et al., 2013), maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) (Nadeem et al., 2007, 2010). The alleviated salinity stress by ACC-deaminase producing bacteria was characterized as increased fresh and dry biomass (Mayak et al. 2004; Gamalero et al., 2010; Siddikee et al., 2011; Siddikee et al., 2012; Bal et al., 2013; Nadeem et al., 2007, 2010), affected root architecture such as increased total root length and surface area (Gamalero et al., 2010; Siddikee et al., 2011; Siddikee et al., 2012; Bal et al., 2013), increased water use efficiency (WUE) (Mayak et al. 2004), higher relative water content (RWC) (Nadeem et al., 2007, 2010; Ahmad et al., 2013) and chlorophyll content (Mayak et al. 2004; Nadeem et a., 2007; Bal et al., 2013), and nutrient accumulation (Siddikee et al., 2011; Nadeem et al., 2007, 2010).

With the increasing shortage of freshwater, saline water is becoming a substitution of freshwater irrigation in large unban landscaping, such as parks and golf courses (Miyamoto & Chacon, 2006). Salinity can impose severe damages in turfgrass growth in salt-affected soils. However, the use of ACC-deaminase bacteria in turfgrass growth and culture and the mechanism of ACC-deaminase associated with salinity tolerance for turfgrasses are not yet reported. Studying ACC-deaminase producing bacteria on turfgrass can help explore the application of PGPB in turfgrass improvement under stress conditions. Therefore, the objectives of this study was to determine whether ACC-deaminase producing bacteria could promote growth and salinity tolerance for a widely-used turfgrass species, perennial ryegrass (*Lolium perenne* L.) and to investigate physiological effects of ACC-deaminase producing bacteria inoculation on perennial ryegrass responses to salinity stress.

## Materials and Methods

### Plant materials and growth condition

Perennial ryegrass (cv. Pangea) was established from tillers collected from the turfgrass research farm at Rutgers University. Tillers of similar sizes were briefly surface sterilized by soaking in 1% sodium hypochlorite for 30 s and then rinsed twice in autoclaved water. Ten tillers were transplanted into each pot filled with autoclaved fritted clay as growth medium. Plants in each pot were watered daily and supplied with 50 mL of sterilized half-strength Hoagland's solution (Hoagland and Arnon, 1950) every week. Plants were established in a greenhouse for 4 weeks (September-October, 2013) with natural sunlight and average day/night temperature of 24/15 $^{o}$C, and then moved to growth chambers for bacterial inoculation and salinity treatment. The growth chamber was set at 23/18 $^{o}$C (day/night temperature), 12-h photoperiod, and 610 µmol photons $m^{-2}$ $s^{-1}$.

### Bacteria inoculation and salinity treatment

Two ACC-deaminase producing bacteria species, *Burkholderia phytofirmans* (PsJN) and *Burkholderia gladioli* (RU1) were used to inoculate perennial ryegrass plants. Bacteria species were revived from frozen stock vials stored at −80 °C by culturing on nutrient agar plates. Single colonies were picked and inoculated in LB (lysogeny broth) broth and incubated at room temperature on a shaker set at 200 rpm for 48 h. Bacteria suspension was centrifuged at 8000 g for 10 min and then re-suspended in deionized and distilled water (dd$H_2O$). The centrifuge and re-suspension process was repeated twice to remove the LB medium. The prepared bacteria suspension were

adjusted to OD=1.

Plants were inoculated by soil drenching with 30 mL prepared bacterial inoculum into each pot twice at an interval of 6 h. The control group for the bacterial inoculation treatment was watered with 30 mL of dionized distilled water ($ddH_2O$). Salinity treatment was started the day after the inoculation. A 50 mL NaCl solution was watered into each pot every day with an increasing concentration of 20, 40, 80, 160, and 250 mM. Thereafter, each pot was watered daily with 50 mL 250 mM NaCl . The control group for salinity treatment was watered daily with 50 mL $ddH_2O$.

**Physiological analysis**

Electrolyte leakage (EL) of leaves was measured as an indicator of cell membrane stability (Blum and Ebercon, 1981). Approximately 0.2 g fresh leaf tissues was collected, rinsed with deionized water to remove exogenous solutes, and placed in a test tube containing 30 mL deionized water. Tubes were placed on a conical flask shaker for 12 h and the initial conductance ($C_i$) measured using a YSI Model 32 Conductivity Meter (Yellow Spring, OH). Leaf samples were killed by autoclaving at 120 °C for 20 min and again shaken for 12 h. The maximal conductance of killed tissue ($C_{max}$) was then measured. EL was calculated using the formula (%) = ($C_i/C_{max}$) ×100.

Relative water content (RWC) was measured as an indicator for leaf hydration status (Barrs and Weatherley, 1962). Leaf RWC was calculated based on leaf fresh weight (FW), turgid weight (TW), and dry weight (DW) using the formula (%) = [(FW – DW)/ (TW – DW)] ×100. FW of leaves was determined with a mass balance immediately after leaves detached from the plant. Samples were then wrapped in

tissue paper and submerged in deionized water for 12 h at 4 °C. Leaf tissue was removed from the water, blotted dry, and again weighed for TW. Following a drying period of three days at 80 °C, samples were weighed a final time for DW.

Leaf photochemical efficiency was estimated by measuring chlorophyll fluorescence expressed as the ratio of variable to maximum fluorescence ($F_v/F_m$) with a fluorescence induction monitor (OS 1FL, Opti-Sciences, Hudson, NH). Leaves were exposed to darkness for 30 min before $F_v/F_m$ was measured.

The protocol of ACC concentration measurement was adopted from the method of Lizada and Yang (1979). About 0.1 g of fresh leave tissue was ground by liquid nitrogen and dissolved in 1.5 mL ethanol. The sample was then centrifuged at 10,000 g for 15 min and the supernatant was evaporated to dryness under reduced pressure at 50 °C. The centrifuge tube was added with 0.75 mL $H_2O$ and 0.75 mL chloroform, vortexed and centrifuged at 10,000 g for 15 min. 0.5 mL extract (water phase) was transferred to glass tube (about 10 mL) with rubber cap, 10 μL 0.1M $HgCl_2$ was added and the volume was brought up to 0.8 mL with water. A 0.2 mL ice cold mixture (v/v=2:1) of commercial bleach and saturated NaOH was injected by a syringe and the glass tube was vortexed. After 3 min incubation on ice, 1 mL air sample in the glass tube was withdrawn with a syringe and then injected into gas chromatography for ethylene measurement.

**Analysis of shoot and root growth**

Visual evaluation of turf quality (TQ) was performed biweekly during the salinity treatment. TQ was rated on a scale of 1 to 9, with 1 being brown and desiccated

turf, 6 being the minimal acceptable level, and 9 being green and dense turf. Ratings were based on parameters such as uniformity, visual attractiveness, leaf color, and canopy density (Beard, 1973). Tiller density was determined by manually counting the numbers of tillers in each pot every 5 d.

Shoot and root dry weights were measured at 10 d and 20 d of salinity treatment. Whole plant was harvested and separated by shoot and root. Root materials were washed by flowing tap water to remove the fritted clay. Both shoots and roots were dried in an 80°C oven for three days. Then dry weight was measured using a mass balance.

Root morphological parameters (root length, root volume, surface area, and average diameter) were analyzed upon harvest. Root samples were analyzed for morphological parameters with WinRhizo software (Regent Instruments, Loretteville, Canada) after dying with 1% crystal violet.

**Nutrient analysis**

All plant samples were divided into shoot and root parts at 20 day DAT. They were carefully washed with deionized water and dried at 80 °C for 3 days. The dry plant samples were ground with liquid nitrogen and passed through a 2 mm mesh sieve. Around 0.2 g sample were analyzed for nutrient content in shoots and roots. Nitrogen content was determined using the combustion method of Horneck and Miller (1998). The content of P, K, Ca, Mg, Mn, Fe, Cu, B, Al, Zn and Na was measured by the dry ash method (Miller, 1998).

**Statistical analysis**

Analysis of variance (ANOVA) was performed using the general linear model of SAS program (SAS 8.1. SAS Institute Inc., Cary, NC) for the determination of bacteria inoculation and salinity effects, and their interactions. Treatment means were compared using Fisher's protected least significance difference (LSD) test at the 0.05 P level.

## Results

### Shoot and root growth as affected by the inoculation with ACC-deaminase producing bacteria

No significant differences in turf quality were observed between bacteria-inoculated plants and the non-inoculated control plants at either 10 or 20 d of well-watered conditions (Table 1). Salinity caused significant reduction of turf quality in non-inoculated plants at 10 and 20 d of salinity treatment, and inoculated plants only at 20 d. Under salinity treatment, a significant improvement in TQ was detected in the inoculated plants with both bacteria species at 20 d, compared to TQ of the non-inoculated plants. No significant difference in TQ was observed between the two bacteria treatments under salinity stress. Bacteria inoculation significantly increased the number of tillers under both well-watered and salinity conditions, but to a greater extent under well-watered conditions, particularly with PsJN inoculation (Fig.1 A, B). PsJN inoculation showed a stronger promoting effect on tiller formation than RU1 treatment.

Both bacteria inoculation showed significant positive effects on shoot and root biomass accumulation at 10 d and 20 d of salinity stress, compared to the non-inoculated plants. Shoot biomass of PsJN-inoculated plants was significantly higher than that of RU1-inoculated plants at 10 d of salinity conditions and at 20 d of both well-watered and salinity conditions (Fig. 2 A). For root biomass, the difference between PsJN and RU1 inoculation was not significant at 10 d of both well-watered and salinity conditions and at 20 d of salinity conditions. At 20 d, root biomass of PsJN-inoculated plants was significantly lower than that of RU1-inoculated plants under well-watered conditions (Fig. 2 B).

Both bacteria-treated plants have significantly higher root length and root volume under well-watered conditions, but under salinity condition the difference was significant only between RU1-inoculated and the non-inoculated control, but not between PsJN and the control (Fig. 3 A, C). Both bacteria species increased root surface area under well-watered conditions, and only RU1 inoculation significantly increased root surface area under salinity conditions (Fig. 3 B). No difference in root diameter was observed among bacterial treatments and the control under both well-watered and salinity conditions (Fig. 3 D).

**Physiological responses**

Leaf photochemical efficiency declined under salinity conditions (Fig. 4 B). All bacteria inoculated plants had higher $F_v/F_m$ under both well-watered and salinity conditions. No significant differences existed between the two bacteria inoculations under either well-watered or salinity conditions (Fig. 4 A, B).

Leaf EL was lower in bacteria-inoculated plants than the non-inoculated plants under either well-watered or salinity conditions (Fig. 5 A, B). There was no difference in leaf EL between plants inoculated with the two bacteria species under well-watered conditions (Fig. 5 A). After 15 d of salinity treatment, EL of PsJN-inoculated plants was significantly higher than RU1-inoculated plants (Fig. 5 B).

Under well-watered conditions, RWC remained around 90% and no significant differences existed among bacteria-inoculated plants and non-inoculated control plants (Fig. 6 A). Under salinity conditions, RWC of the non-inoculated plants was significantly lower than that of plants inoculated with either bacteria strain (Fig. 6 B).

There was no significant difference in RWC between the two bacteria speciesinoculations during most of the treatment period under salinity conditions.

Both shoot and root ACC concentration under salinity treatment was higher than that of well-watered plants (Fig. 7 A, B). Shoot ACC concentration was significantly lower in plants inoculated with PsJN at 20 d of well-watered conditions (Fig. 7 A). Under salinity conditions, shoot ACC concentrations were significantly lower in the two bacteria-treated plants than in the non-inoculated plants (Fig. 7 A). No significant difference in shoot ACC concentration was detected between plants inoculated with the two bacteria species under salinity conditions (Fig. 7 A). In root tissues (Fig. 7 B), ACC concentration was not significantly affected by bacteria inoculation under well-watered conditions. Under salinity conditions, root ACC concentration of PsJN- or RU1-inoculated plants was significantly lower than that in the non-inoculated plants. Plants inoculated with the two bacteria species had no significance differences in root ACC concentration under either well-watered or salinity conditions (Fig. 7 B).

**Shoot and root nutrient status**

Na content was increased dramatically by salinity treatment in both shoots and roots (Table 2). In two bacteria-inoculated plants, a significant lower Na concentration was observed in shoot tissues under both well-watered and salinity conditions, compared to that in the non-inoculated plants. No significant difference in root Na content was detected between bacteria-inoculated and non-inoculated plants under salinity conditions. For K content, shoots of both PsJN-treated and RU1-treated plants

had higher content than the non-inoculated plants under both well-watered and salinity conditions (Table 2). Roots of PsJN treated plants had higher K content than the non-inoculated plants under salinity conditions (Table 2). Bacteria-inoculated plants had greater K/Na ratio in both shoots under both well-watered and salinity conditions.

The inoculation of plants with two bacteria species had differential effects on macronutrients and micronutrients under well-watered and salinity conditions. For N content, there was a significant increase in both shoots and roots in the inoculated plants compared to the non-inoculated plants under either well-watered or salinity conditions (Table 3). No effects of bacteria inoculation were observed on shoot and root P content under either well-watered or salinity conditions (Table 3). For Ca and Mg content, the effect of bacteria inoculation was only showed under salinity condition, with significant lower content in bacteria-inoculated plants than non-inoculated plants in both shoots and roots.

Shoot Fe contents were significantly lower in both bacteria-inoculated plants than non-inoculated control under both well-watered and salinity conditions (Table 3). PsJN-inoculated plants had significantly lower root Fe content than the non-inoculated plants under both well-watered and salinity conditions, while the difference in root Fe content between RU1 inoculated plants and the non-inoculated control was not significant. Al content of both shoots and roots were significantly lower in both bacteria-inoculated plants than those in the non-inoculated control under salinity condition. Under well-watered condition, shoot Al content of RU1 inoculated plants was significant lower than that of the non-inoculated control; root Al content of PsJN-inoculated plants was significant lower than that of the non-inoculated control (Table 3). Bacteria inoculation had no significant effects on Mn and Zn content in roots.

Shoot Mn content was significantly lower in PsJN-inoculated plants compared to the non-inoculated plants under well-watered condition. Shoot Zn content was significantly lower in both bacteria inoculation under well-watered condition and in RU1-inoculated plants under salinity condition compared to the non-inoculated plants (Table 3). .

## Discussion

Increases in ACC levels with salinity stress have been reported in leaves (Arbona et al., 2003; Zapata et al., 2004; Ghanem et al., 2008) and roots (Gómez-Cadenas et al., 1998; Kukreja et al., 2005) of various plant species. Stress-induced ethylene accumulation is known for its inhibitory effects on plant growth under various stress environments, including salinity (Morgan & Drew, 1997). In this study, leaves of perennial ryegrass have also produced higher ACC under salinity stress than those under well-watered conditions. The inoculation of perennial ryegrass plants by *Burkholderia phytophirmans* (PsJN) or *Burkholderia gladioli* (RU1) significantly lowered ACC concentration in both root tissues and shoot tissues, suggesting that ACC-deaminase producing bacteria suppressed ACC accumulation in plant tissues, which could contribute to their positive growth and physiological effects on improving salinity tolerance in perennial ryegrass in this study. Siddikee et al. (2012) showed similar results where red pepper (*Capsicum annuum* L.) seedlings inoculated by ACC-deaminase producing bacteria showed significant lower levels of ACC under salinity condition. Other related studies measured ethylene production rate instead of ACC concentration and found the same results (Grichko & Glick, 2001; Mayak et al., 2004; Siddikee et al., 2011). The improved growth of transgenic canola with ACC deaminase activity under salinity stress suggested that ethylene is responsible for the inhibited growth of salinized plants (Sergeeva et al., 2006). In our study, plants inoculated with *Burkholderia phytophirmans* (PsJN) or *Burkholderia gladioli* (RU1) also showed an increase in turf quality, tiller formation and shoot biomass, indicating both ACC-deaminase bacterial species improved shoot growth of perennial ryegrass through the bacterial hydrolysis of ACC. In addition, a more extensive root system was observed for plants inoculated with either bacteria species under well-watered

conditions, with higher total root length and root volume although root diameter was not affected. Root growth promotion effects were also observed under salinity conditions in plants inoculated with *Burkholderia gladioli* (RU1). This better developed root system could attribute to the increased salinity tolerance of the inoculated plants.

Physiological analysis, measured as RWC, EL, and $F_v/F_m$, indicated that the inoculation of perennial ryegrass with *Burkholderia phytophirmans* (PsJN) or *Burkholderia gladioli* (RU1) also helped to maintain better cellular hydration and membrane stability, and greater photochemical efficiency under salinity stress. The increased RWC by PGPB under salinity stress has also been reported in maize (Nadeem et al., 2007), wheat (Nadeem et al., 2010) and mung bean (Ahmad et al., 2013). Mayak et al. (2004) reported increased water use efficiency of PGPB-inoculated plants. The enhanced RWC could be related to root growth promotion for enhanced water uptake capacity. Naveed et al. (2014) also reported an increase of $F_v/F_m$ in PGPB inoculated maize under normal growth conditions. Lowering EL has also been reported in PGPB inoculated peanut (*Arachis hypogaea* L.) under salinity by Shukla et al. (2012). Our results suggested that the reduction in ethylene accumulation under salinity stress through ACC-deaminase bacteria promoted physiological tolerance of perennial ryegrass to salinity stress.

Salt tolerance of a plant can be indicated by the $K^+/Na^+$ ratio (Hamdia et al., 2004). Mayak et al. (2004) reported that the main effect of the ACC-deaminase producing PGPR inoculation was an increase in the uptake of K, which plays an important role in balancing osmotic potential of the vacuole (Hu T. et al., 2011). Nadeem et al. (2007, 2010) also reported a higher $K^+/Na^+$ ratio in ACC

deaminase-containing PGPR inoculated maize and wheat under salinity stress. In our study, inoculated perennial ryegrass with either bacterial species had a significant higher $K^+/Na^+$ ratio in shoot tissues under both normal watered and salinity conditions. In addition, salinity caused increases in shoot and root Na content, but shoot Na content was lower in bacteria inoculated plants, although root Na content did not differ between the inoculated and the non-inoculated plants. These results indicated that ACC-deaminase bacteria could affect shoot exclusion or extrusion of Na in shoots, and helped to maintain K and Na balance to minimize the toxic effects of Na. However, mechanisms of how lowered ACC production in plant tissues by ACC-deaminase bacteria may affect Na accumulation and K balance are not clear.

The ACC-deaminase bacteria inoculation also increased N content in shoots and roots, in addition to K under well-watered or salinity conditions. The increased K and N content could be due to increased root growth for nutrient uptake and also was reflected in the increased turf quality. In contrast to K and N, the content of Ca and Mg decreased with bacterial inoculation. The mechanisms of how ACC-deaminase bacteria may decrease Ca and Mg accumulation are not clear, despite of their positive effects on improving salinity tolerance, which deserves further investigation. For micronutrients, accumulation of Fe, Al, Mn, and Zn can be detrimental to plant growth (Foy et al., 1978). In this study, plants inoculated with ACC-deaminase bacteria had accumulate lower amount of Fe, Al, Mn, and Zn, suggesting that they could have beneficial roles for plant growth under salinity stress by lowering the potential toxic effects of those micronutrients.

In summary, this study first reported positive effects of ACC-deaminase producing bacteria inoculation on turfgrass species for promoting plant growth and

salinity tolerance. The hydrolysis of ACC in plant roots by the two ACC-deaminase producing PGPB could reduce ethylene production, which may be responsible for alleviation of salinity stress on perennial ryegrass. The ACC-deaminase producing PGPB could be useful for turfgrass establishment and maintenance in salt-affected areas. Further effort should be taken to examine metabolic mechanisms or pathways of ACC-deaminase bacteria involving ethylene regulation of growth and stress tolerance.

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**Table 1.** Effects of bacteria inoculation on turf quality of perennial ryegrass at 10 and 20 d of well-watered or salinity treatment. Values are means of six replicates. Values with the same letter within each column indicated no significant difference based on LSD test at $p = 0.05$.

| | Turf Quality (10 d) | Turf Quality (20 d) |
|---|---|---|
| Watered | $9.0^{a}$ | $7.8^{b}$ |
| Watered + PsJN | $8.8^{ab}$ | $8.0^{ab}$ |
| Watered + RU1 | $8.9^{ab}$ | $8.3^{a}$ |
| Salinity | $8.5^{c}$ | $6.2^{d}$ |
| Salinity + PsJN | $8.8^{abc}$ | $6.8^{c}$ |
| Salinity + RU1 | $8.5^{bc}$ | $6.8^{c}$ |

**Table 2.** K, Na content and K/Na ratio in shoots and roots of perennial rye plants under well-watered or salinity treatment. Values are means of four replicates. Values with the same letter within each column indicated no significant difference based on LSD test at $p = 0.05$.

| | | | K % | Na % | K / Na |
|---|---|---|---|---|---|
| Shoot | Water | Control | 1.36[b] | 0.08[a] | 16.87[b] |
| | | PsJN | 1.73[a] | 0.05[b] | 37.39[a] |
| | | RU1 | 1.91[a] | 0.04[b] | 45.33[a] |
| | Salinity | Control | 1.07[b] | 3.98[a] | 0.27[b] |
| | | PsJN | 1.57[a] | 1.81[b] | 0.88[a] |
| | | RU1 | 1.65[a] | 0.93[c] | 1.81[a] |
| Root | Water | Control | 1.76[a] | 0.14[b] | 12.68[a] |
| | | PsJN | 1.88[a] | 0.17[a] | 11.63[a] |
| | | RU1 | 1.3[b] | 0.15[ab] | 8.78[b] |
| | Salinity | Control | 1.38[b] | 0.38 | 3.68[ab] |
| | | PsJN | 2[a] | 0.48 | 4.22[a] |
| | | RU1 | 1.34[b] | 0.39 | 3.4[b] |

**Table 3.** Mineral nutrient content in shoots and roots of perennial rye plants under well-watered or salinity treatment. Values are means of four replicates. Values with the same letter within each column indicated no significant difference based on LSD test at $p = 0.05$.

| | | | N % | P % | Ca % | Mg % | Mn ppm | Fe ppm | Al ppm | Zn ppm |
|---|---|---|---|---|---|---|---|---|---|---|
| Shoot | Water | Control | 1.39$^{b}$ | 0.24 | 0.49 | 0.23 | 967.09$^{a}$ | 1088.07$^{a}$ | 873.44$^{a}$ | 31.15$^{a}$ |
| | | PsJN | 1.6$^{a}$ | 0.25 | 0.48 | 0.23 | 685.76$^{b}$ | 726.93$^{b}$ | 703.19$^{ab}$ | 20.85$^{b}$ |
| | | RU1 | 1.58$^{a}$ | 0.29 | 0.45 | 0.23 | 809.68$^{ab}$ | 743.68$^{b}$ | 654.86$^{b}$ | 20.67$^{b}$ |
| | Salinity | Control | 1.35$^{b}$ | 0.28 | 0.71$^{a}$ | 0.28$^{a}$ | 801.54 | 1428.26$^{a}$ | 1162.96$^{a}$ | 30.06$^{a}$ |
| | | PsJN | 1.62$^{a}$ | 0.26 | 0.55$^{b}$ | 0.24$^{b}$ | 638.53 | 935.33$^{b}$ | 808.75$^{b}$ | 24.08$^{ab}$ |
| | | RU1 | 1.63$^{a}$ | 0.28 | 0.49$^{b}$ | 0.21$^{b}$ | 609.63 | 381.06$^{c}$ | 368.29$^{c}$ | 22.37$^{b}$ |
| Root | Water | Control | 0.74$^{c}$ | 0.24 | 0.22 | 0.12$^{a}$ | 569.2 | 3550.47$^{a}$ | 3480.78$^{a}$ | 42.16 |
| | | PsJN | 0.9$^{a}$ | 0.25 | 0.25 | 0.11$^{b}$ | 514.53 | 2296.84$^{b}$ | 2690.81$^{b}$ | 44.36 |
| | | RU1 | 0.83$^{b}$ | 0.25 | 0.26 | 0.12$^{a}$ | 547.99 | 3009.21$^{a}$ | 3527.91$^{a}$ | 46.24 |
| | Salinity | Control | 0.71$^{b}$ | 0.24 | 0.34$^{a}$ | 0.16$^{a}$ | 709.96 | 4053.75$^{a}$ | 4454.38$^{a}$ | 44.68 |
| | | PsJN | 0.87$^{a}$ | 0.17 | 0.26$^{b}$ | 0.12$^{b}$ | 485.61 | 2485.11$^{b}$ | 2837.24$^{b}$ | 32.71 |
| | | RU1 | 0.83$^{a}$ | 0.19 | 0.26$^{b}$ | 0.12$^{b}$ | 504.33 | 2785.37$^{ab}$ | 3238.48$^{b}$ | 38.08 |

**Figure 1.** Tiller number of RU1, PsJN inoculated and non-inoculated perennial ryegrass under well-watered condition (A) and salinity condition (B). Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.

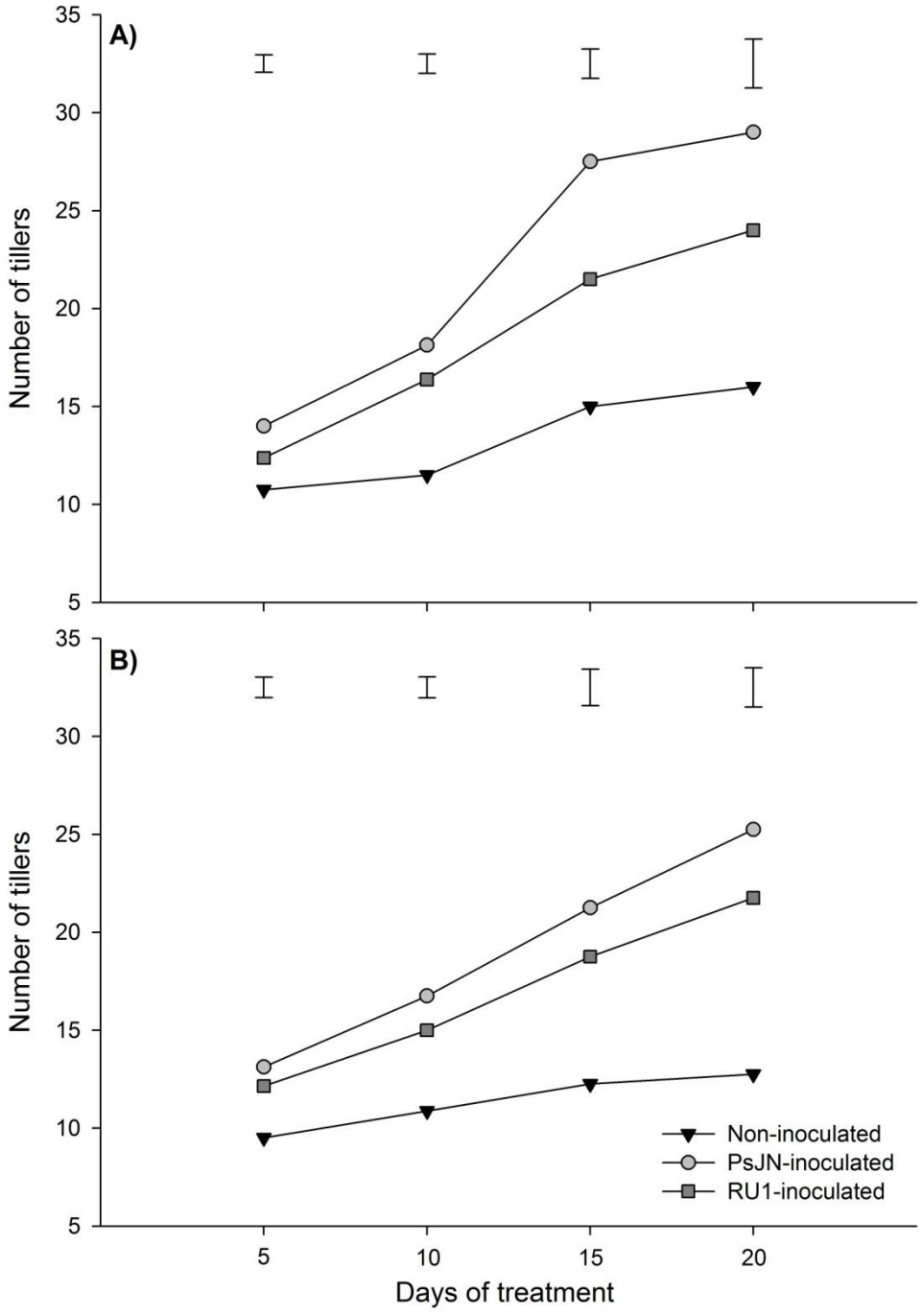


**Figure 2.** Shoot biomass (A) and root biomass (B) of RU1, PsJN inoculated and non-inoculated perennial ryegrass at 10 and 20d of well-watered and salinity conditions (DAT). LSD bars and different letters atop bars indicate significant differences exist at $P \leq 0.05$ within each group (non-inoculated, PsJN inoculated, RU1 inoculated).

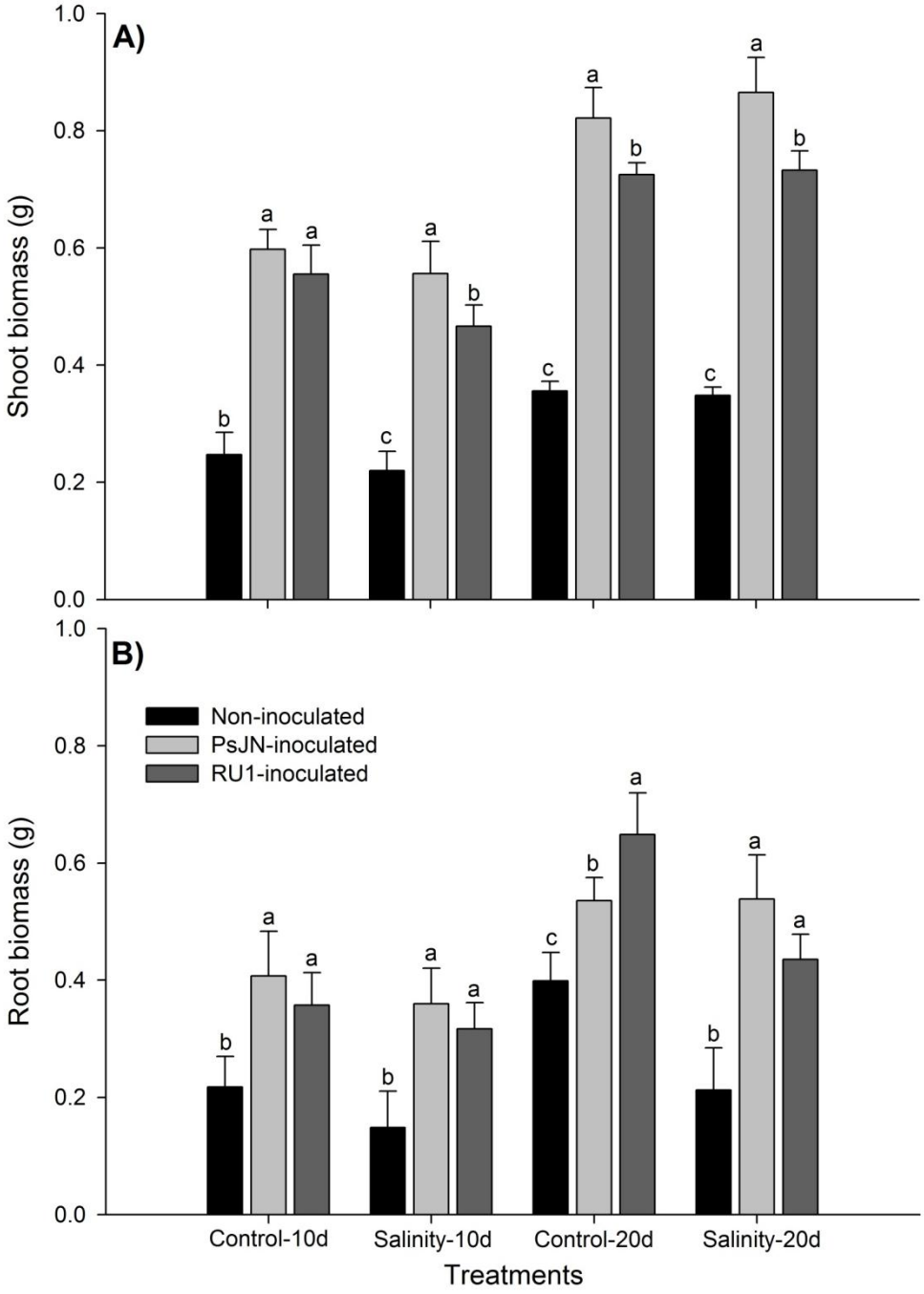


**Figure 3.** Root total length, root surface area (B), root volume (C), root average diameter (D) of RU1, PsJN inoculated and non-inoculated perennial ryegrass under well-watered and salinity conditions. LSD bars and different letters atop bars indicate significant differences exist at $P \leq 0.05$ within each group (non-inoculated, PsJN inoculated, RU1 inoculated).

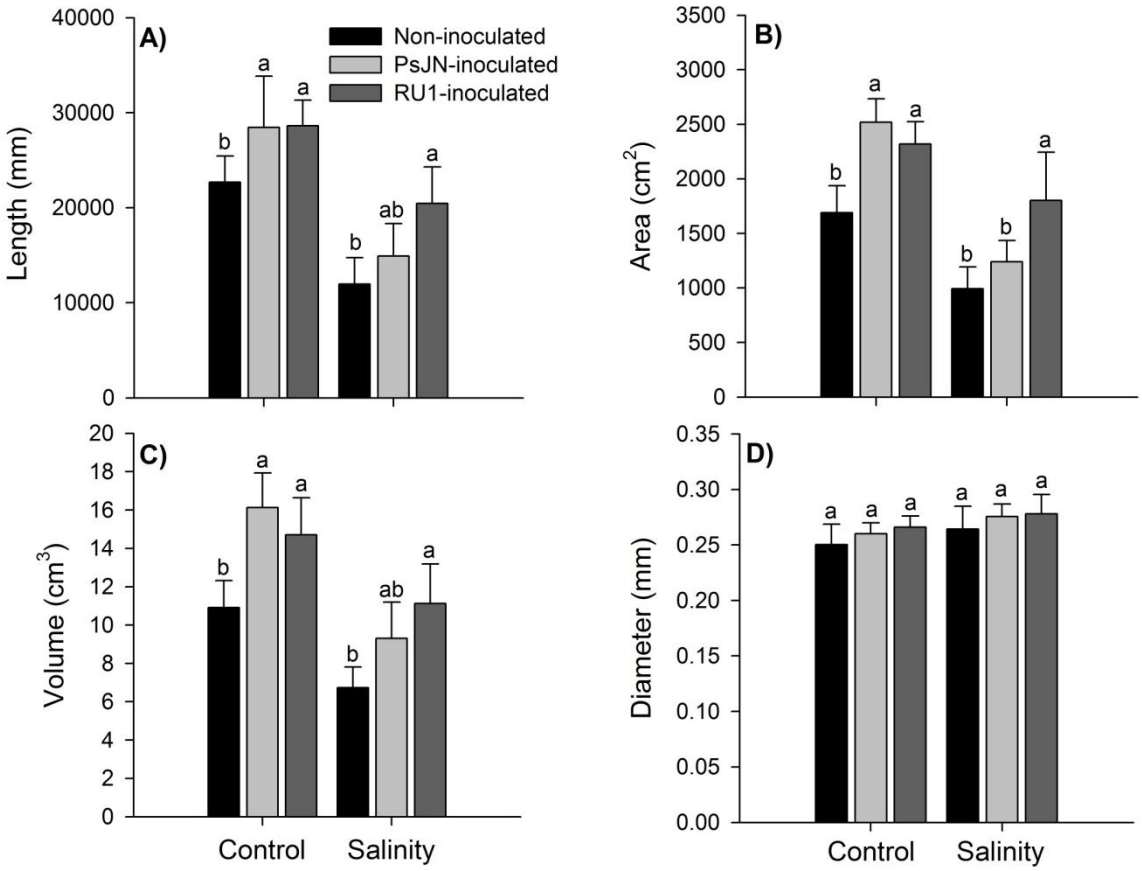


**Figure 4.** Leaf photochemical efficiency ($F_v/F_m$) of RU1, PsJN inoculated and non-inoculated perennial ryegrass under well-watered condition (A) and salinity condition (B). Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.

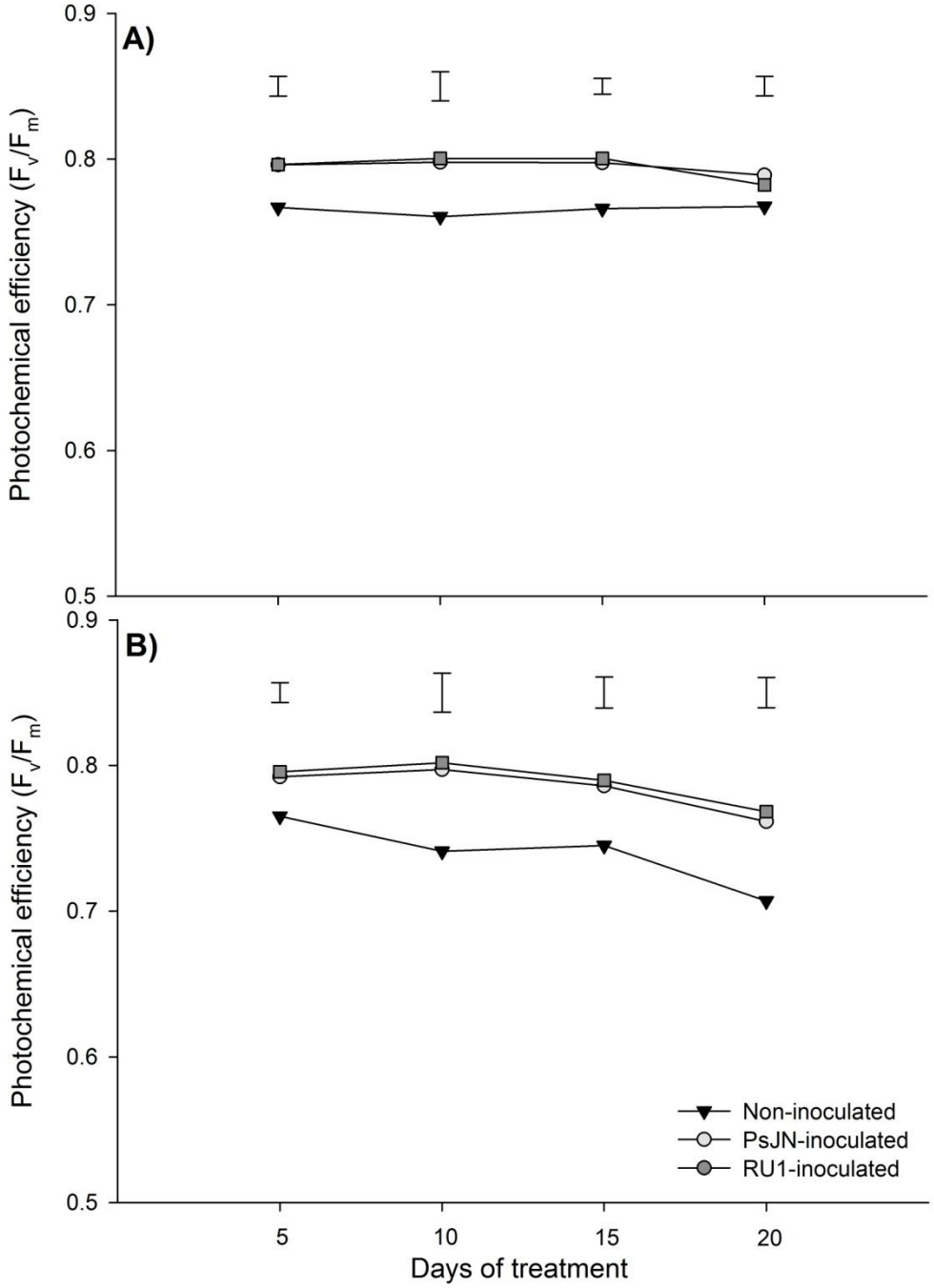


**Figure 5.** Electrolyte leakage (EL) of RU1, PsJN inoculated and non-inoculated perennial ryegrass under well-watered condition (A) and salinity condition (B). Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.

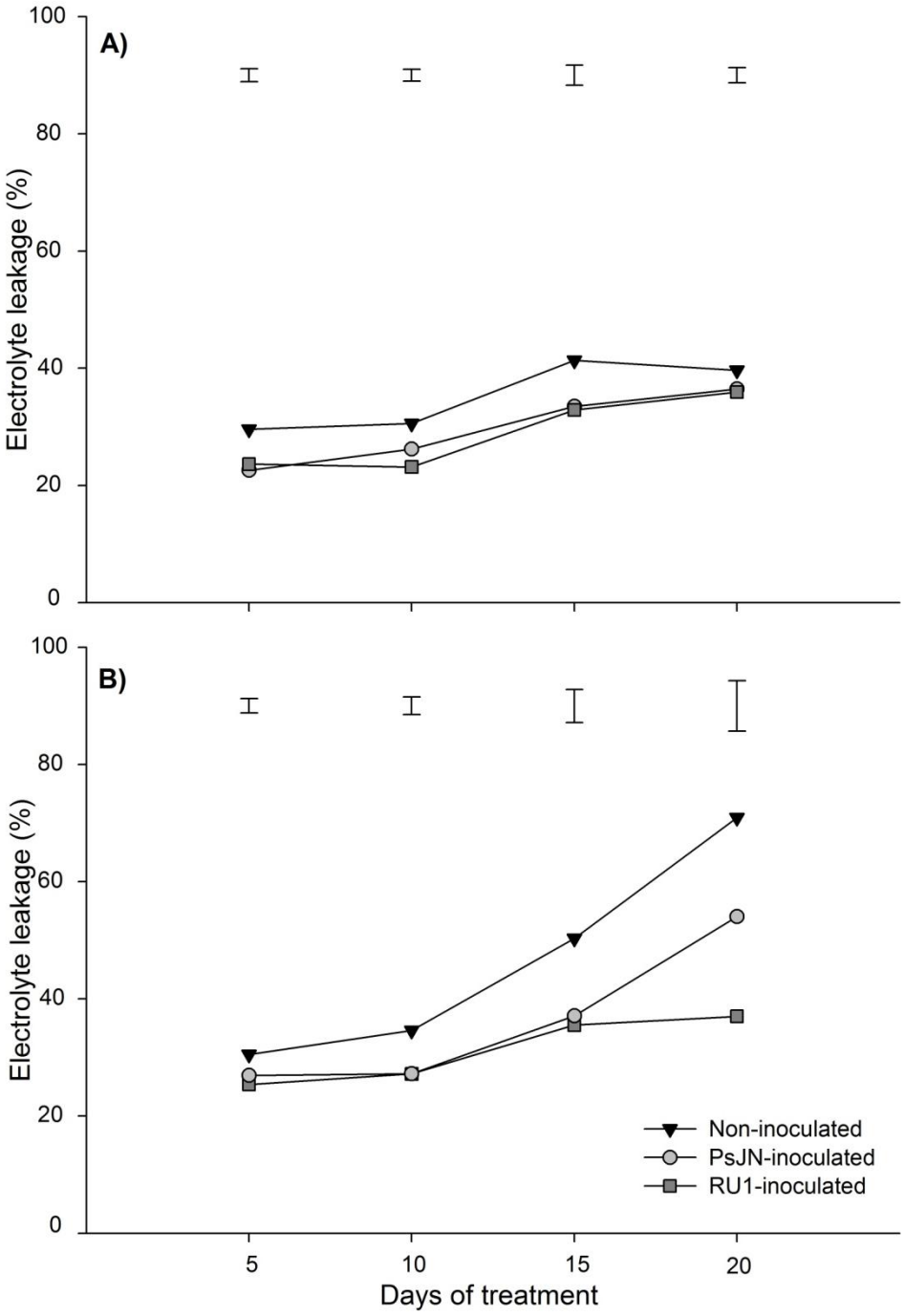


**Figure 6.** Relative water content (RWC) of RU1, PsJN inoculated and non-inoculated perennial ryegrass under well-watered condition (A) and salinity condition (B). Vertical bars indicate LSD values ($P \leq 0.05$) for comparison between treatments at a given day of treatment where significant differences were detected.

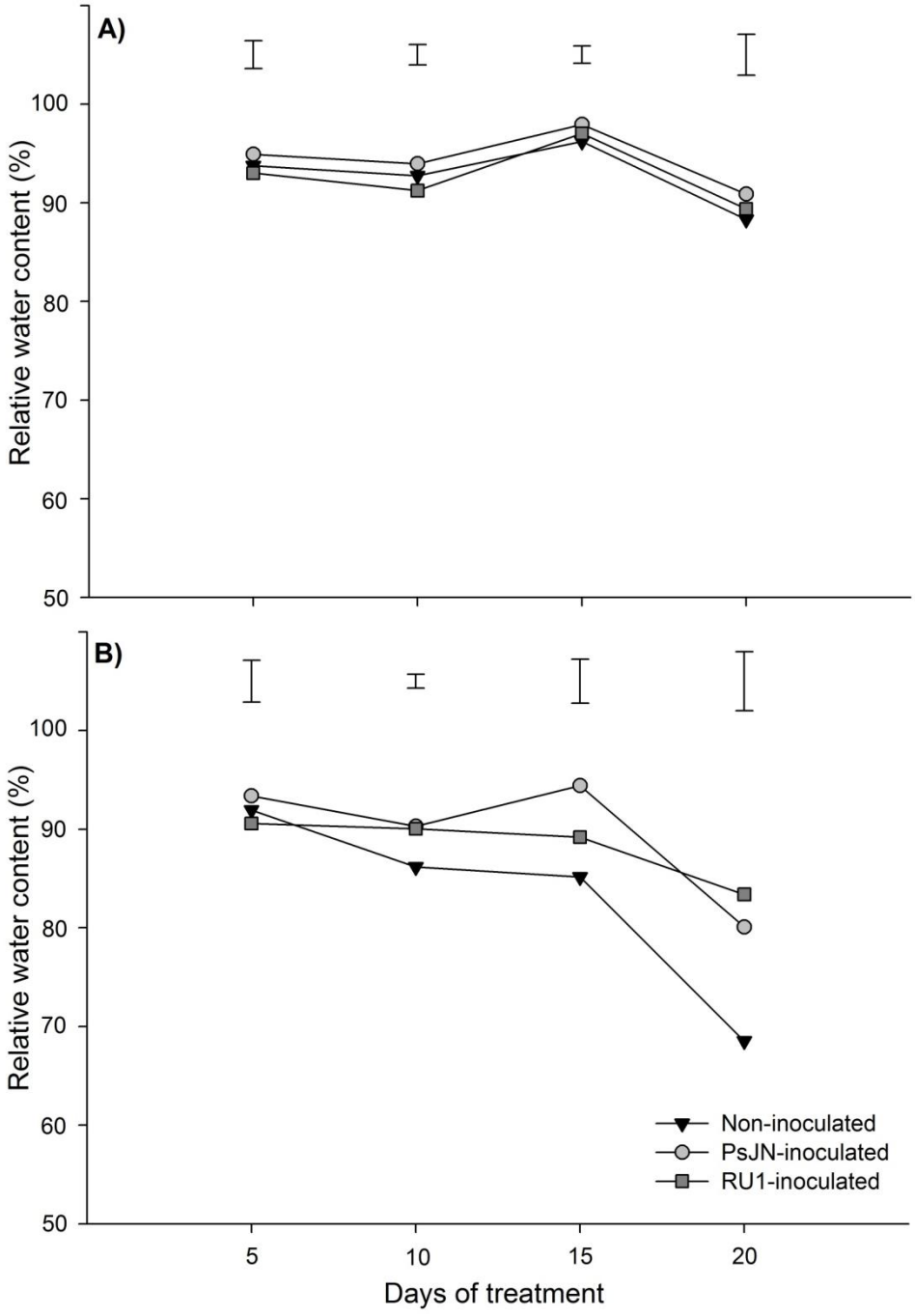


**Figure 7.** Shoot ACC concentration (A) and root ACC concentration (B) of RU1, PsJN inoculated and non-inoculated perennial ryegrass under well-watered and salinity conditions. LSD bars and different letters atop bars indicate significant differences exist at $P \leq 0.05$ within each group (non-inoculated, PsJN inoculated, RU1 inoculated).

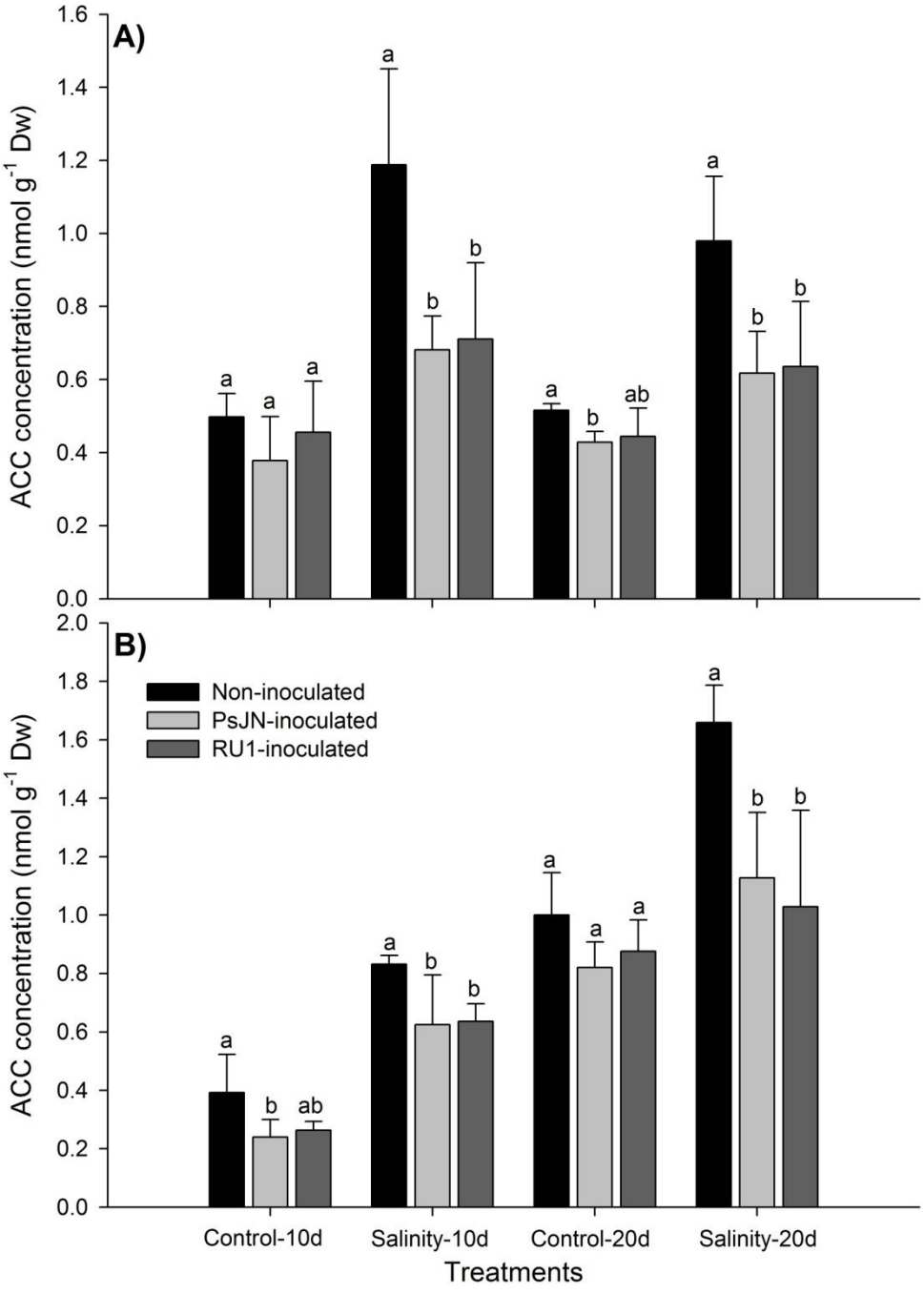
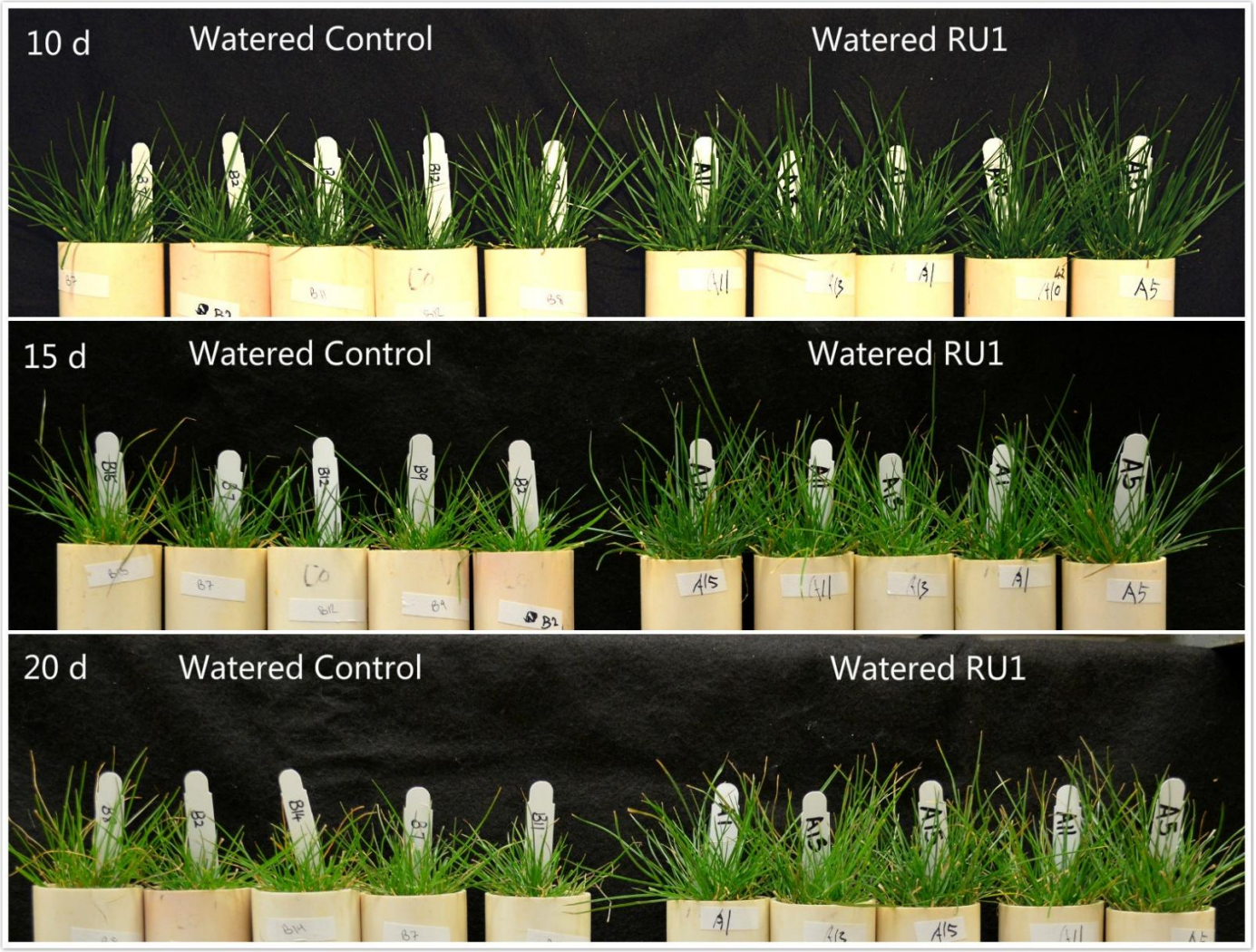
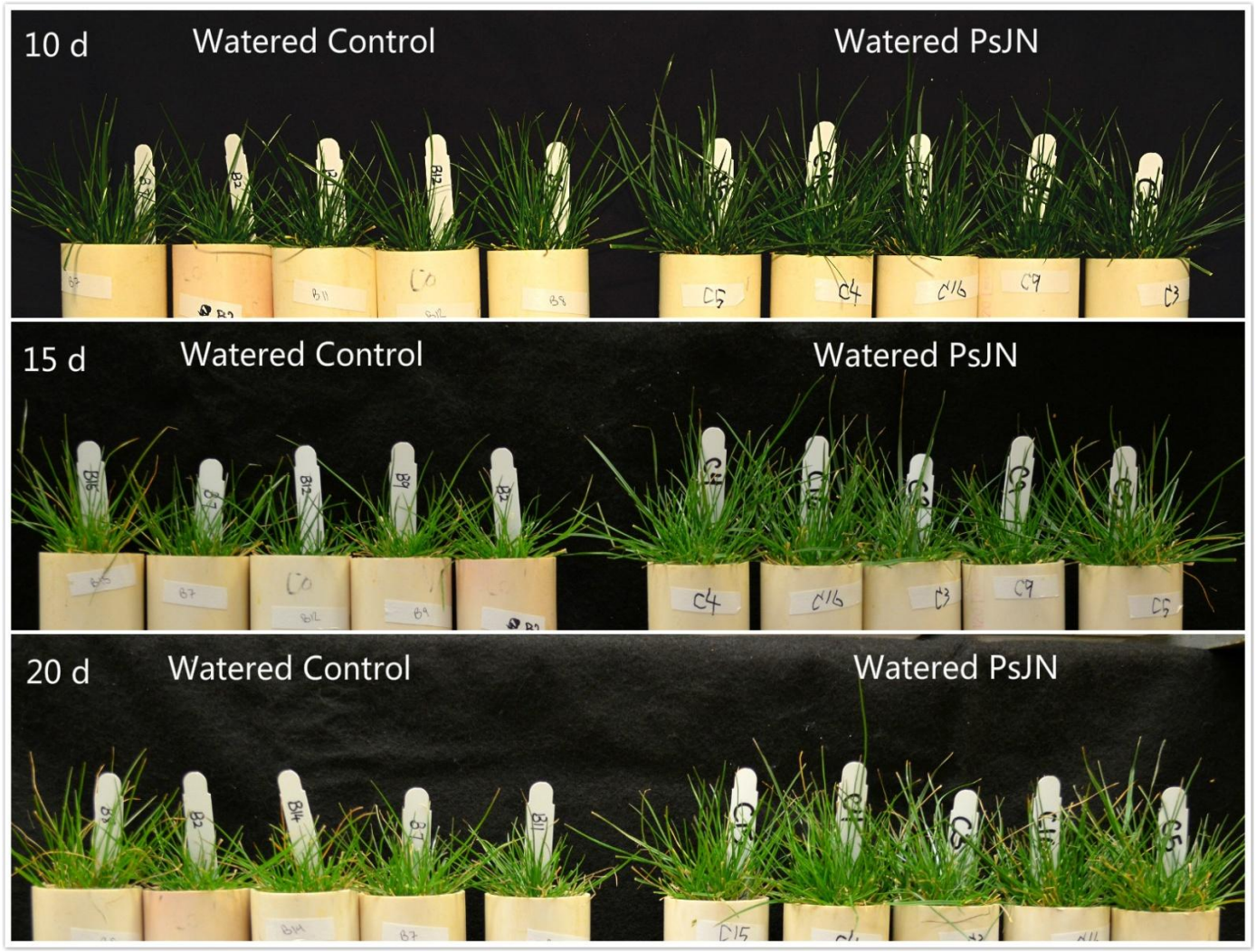


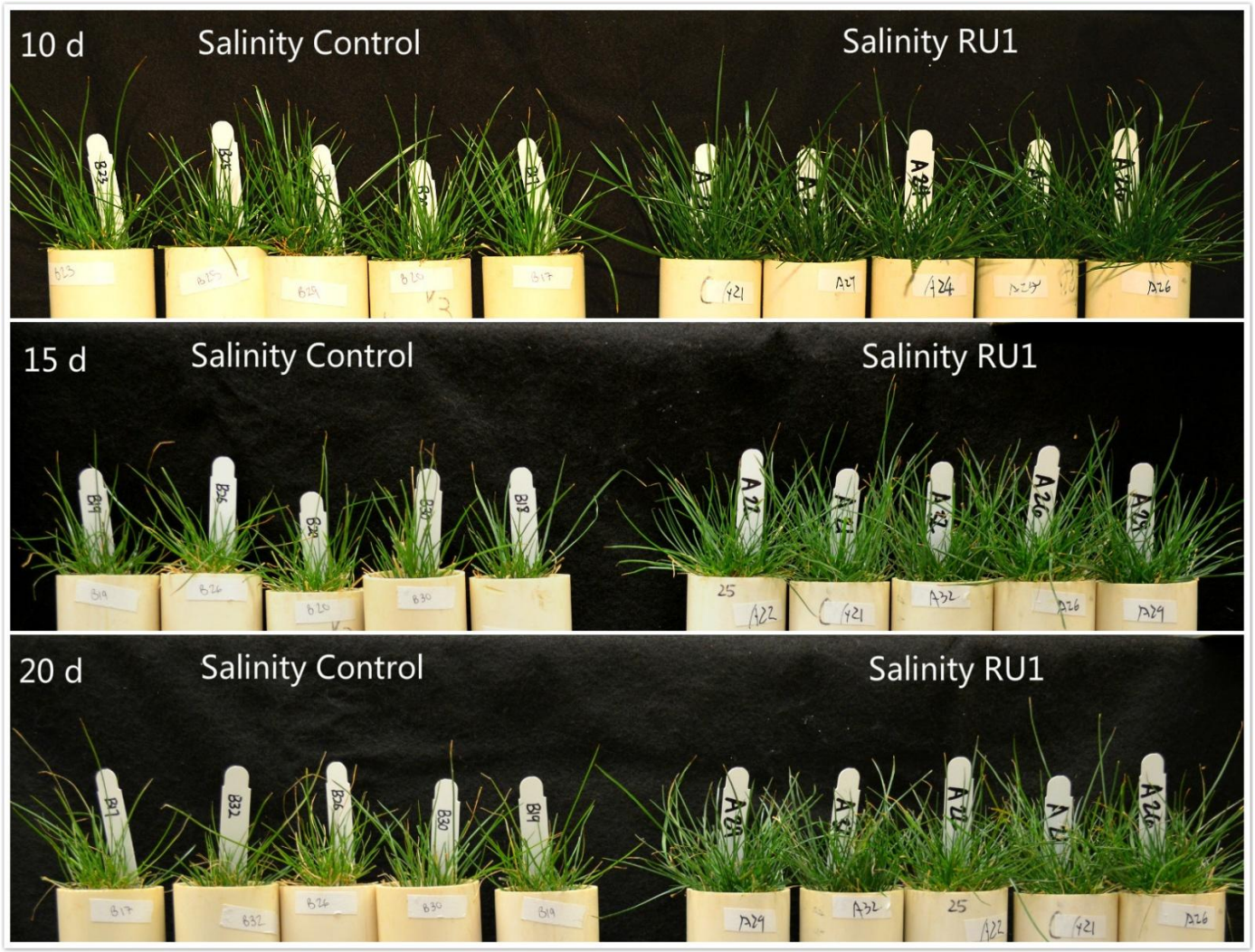
**Figure 8.** Perennial ryegrass with different inoculations under A, well-watered condition; B, salinity condition.

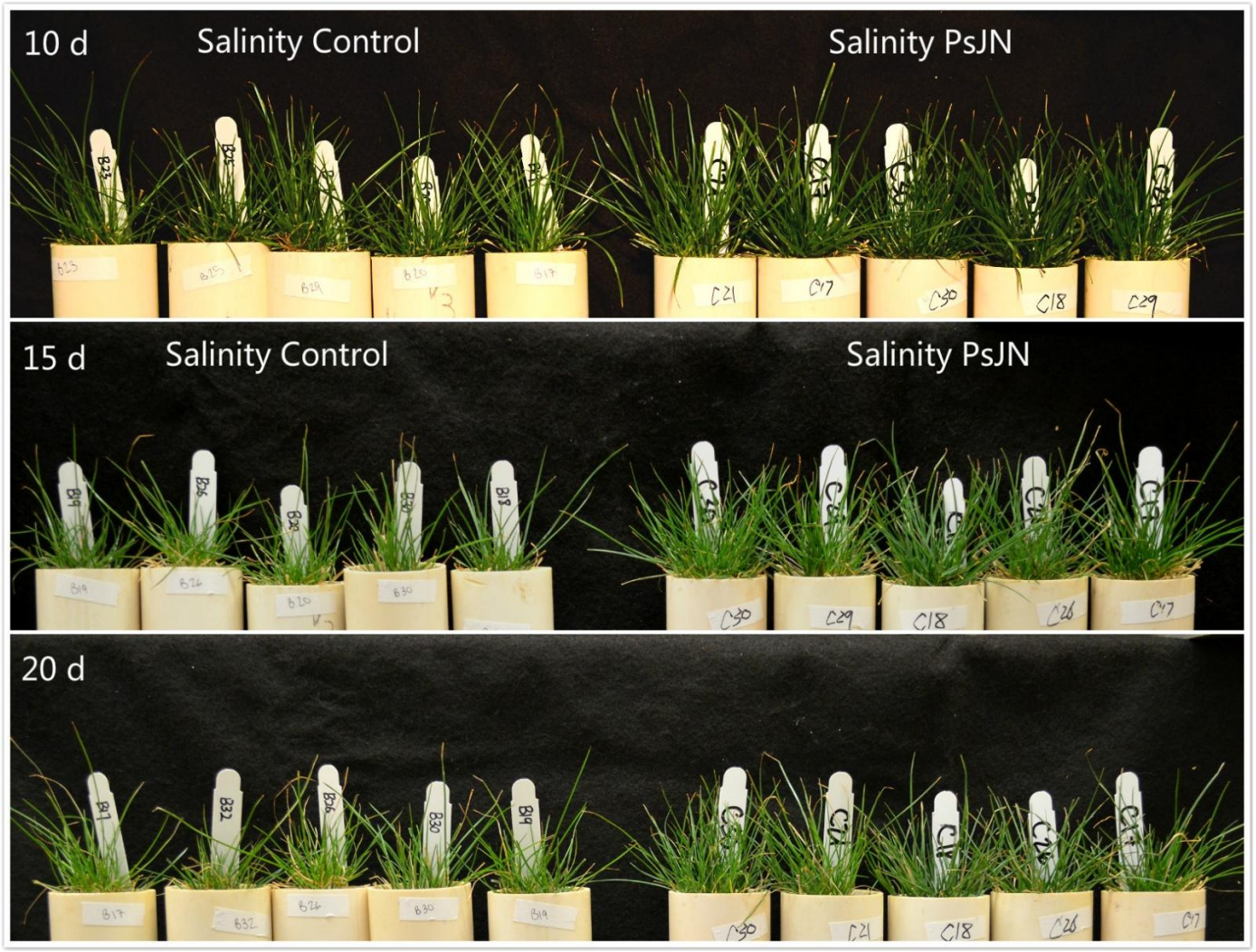
A.


10 d
Watered Control
Watered RU1
15 d
Watered Control
Watered RU1
20 d
Watered Control
Watered RU1


10 d
Watered Control
Watered PsJN
15 d
Watered Control
Watered PsJN
20 d
Watered Control
Watered PsJN

B.


10 d
Salinity Control
Salinity RU1
15 d
Salinity Control
Salinity RU1
20 d
Salinity Control
Salinity RU1


10 d
Salinity Control
Salinity PsJN
15 d
Salinity Control
Salinity PsJN
20 d