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BRIEF COMMUNICATION

Spore associated bacteria of arbuscular mycorrhizal fungi improve maize tolerance to salinity by reducing ethylene stress level

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Abstract The role of spore associated bacteria of arbuscular mycorrhizal fungi (AMF) in improving plant growth and alleviating salt stress is a potential area to explore. In the present study, 22 bacteria isolated from the spore walls of AMF were identified to contain 1-aminocyclopropane-1-carboxylate deaminase. These were tested for their ability to improve seed germination and alleviate salt stress in the early growth of maize. Among the isolates, 19 bacteria that were able to grow at 4% NaCl were used for germination assay. Two bacteria and seven bacteria significantly improved maize seed germination at 100 mM NaCl and 200 mM NaCl, respectively. Based on the presence of plant growth promoting (PGP) characters and the ability to improve seed germination, five strains were chosen for further experiments. At 0 mM NaCl, all the strains were able to increase maize shoot and root growth significantly. At 25 mM NaCl, except for *Bacillus aryabhattai* S210B15, all the strains were able to increase shoot and root growth significantly. At 50 mM NaCl, *Bacillus aryabhattai* S110B3 and *B. aryabhattai* S210B15 significantly improved shoot length, whereas, *Pseudomonas koreensis* S2CB35 and *B. aryabhattai* S210B15 significantly increased root length. Although salinity increased ethylene production in maize, bacterial inoculation significantly reduced the ethylene level at 0, 25 and 50 mM NaCl. Among the five strains, only *P. koreensis* S2CB35 showed the presence of PGP functional traits of *nif*H, *acd*S and *nod*A genes.

Keywords Spore associated bacteria · ACC deaminase · Salt stress · Ethylene emission · PGP functional traits

Introduction

Many of the bacteria present in the soil provide benefits to plants by forming symbiotic relationship with roots. The symbiosis involves formation of specialized structures or nodules on the host plant. In addition, free living soil bacteria especially present in the rhizosphere were also shown to promote plant growth either indirectly or directly. Beneficial free living bacteria also known as plant growth promoting (PGP) bacteria promotes plant growth directly by synthesizing the compound such as auxins, phytohormones and facilitating the increase of nutrient uptake from the environment (Glick et al. 1999; Yim et al. 2013). The indirect plant growth promotion by bacteria involves the alleviation of the detrimental effects of biotic and abiotic stress on plants. PGP bacteria have different ways to facilitate plant growth such as fixing atmospheric nitrogen, production of various phytohormones, solubilization of minerals, production of siderophores and synthesis of enzymes.

The seedling stage is the most important stage of plant ontogeny as it is very responsive to various environmental conditions (Bewley and Black 1978; Bishnoi et al. 1993; Kuriakose and Prasad 2007). Salinity has been reported to negatively affect seed germination of various crops

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including tomato (Cuartero and Fernandez-Munoz 1999), canola (Jalilia et al. 2009) and wheat (Egamberdieva 2009). Like any other stress factor, salinity also elevates the level of stress ethylene (Mayak et al. 2004). Although little amount of ethylene is needed to break seed dormancy (Esashi 1991), following seed germination, high amounts of ethylene production inhibit root elongation. The production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase by bacteria has been well studied due to their significance in alleviating stress ethylene level in seedlings. Siddikee et al. (2015) reported that ACC deaminase containing halotolerant bacteria ameliorated response of canola seedlings under salt stress by reducing the stress ethylene level.

Physiologically evolved stress ethylene has been reported to be more detrimental and can cause more damage in plants compared to the direct effects of the abiotic stress (Grichko and Glick 2001; Ali et al. 2014; Subramanian et al. 2015). Bacteria containing ACC deaminase activity cleaves ACC, a precursor of ethylene into ammonia and α-ketobutyrate, thus reducing ethylene production in seedlings (Mayak et al. 2004; Ali et al. 2014). Plant growth promotion through ACC deaminase activity of soil bacteria and plant associated endophytic bacteria have been studied previously. However, the role of ACC deaminase containing spore associated bacteria (SAB) of arbuscular mycorrhizal fungi (AMF) in reducing ethylene level is still uncertain. Spore associated bacteria of AMF were shown to improve plant growth and protect plants from phytopathogenic attack (Li et al. 2007; Bharadwaj et al. 2008). However, the exact mechanism on which these spore associated bacteria improve plant growth and help plants resist various detrimental environmental conditions is still not clear. In our previous study, we isolated spore associated bacteria from the surfaces of *Glomus caledonium, Racocetra alborosea* and *Funneliformis mosseae* (Selvakumar 2016) and characterized for their association with spore walls. Thus, this study aimed to evaluate the plant growth promoting characters of spore associated bacteria and their ability to improve resistance of maize plants to salt stress.

Materials and method

Plant growth promoting characters of spore associated bacteria

ACC deaminase containing 22 spore associated bacteria were selected for this study (Table S1). Salt tolerance of the isolates was determined in nutrient agar media amended with different concentrations of NaCl (2, 4, 6, 8, 10, 12 and 14%). ACC deaminase activity was determined by growing the bacterial strains in nitrogen-free medium (JNFb—Jensen's Nitrogen Free media) amended with 3 mM ACC as nitrogen source (Penrose and Glick 2003) and the amount of α-ketobutyrate produced by the enzymatic hydrolysis of ACC was estimated. Production of Indole-3-acetic acid (IAA) by the isolates was quantified in the presence and absence of tryptophan (Bano and Musarrat 2003). Filter sterilized tryptophan was supplied in the medium at a concentration of 500 µg ml^{-1}. The ability to solubilize insoluble phosphate was carried out in NBRIP media (Mehta and Nautiyal 2001). Atmospheric nitrogen fixing ability was determined by allowing the isolates to grow in nitrogen free media. Siderophore production was studied on CAS agar plates prepared according to Alexander and Zuberer (1991).

Maize seed germination under salt stress

For seed germination test, maize (*Zea mays* L.) seeds were surface sterilized by immersing in 70% ethanol for 1 min and 6% NaOCl for 5 min, followed by thorough rinsing with sterile distilled water seven to ten times. The sterilization was confirmed by incubating in nutrient agar media for 24 h. Ten surface sterilized maize seeds per treatment in triplicate were used to check germination on sterilized filter paper (Whatman No. 2) soaked in solutions of 0, 50, 100, 150 and 200 mM NaCl in Petri dishes. Germination test was carried out for 96 h at 28 ± 1 °C with a cycle of 12 h of dark followed by 12 h of light (18 µmol m^{-2} s^{-1}) in a plant growth chamber (DS 54 GLP, DASOL Scientific Co., Ltd., Republic of Korea). The number of germinated seeds was recorded every after 24 h.

To determine the bacterial efficiency of the isolated spore associated bacteria in improving seed germination, the bacteria were grown in nutrient broth supplemented with 4% NaCl and cells were collected, washed and re-suspended in sterile 0.03 M $MgSO_4$ (10^8 cells ml^{-1}). Bacterial treatment of the surface sterilized seeds was done by immersing them in bacterial cells suspended in $MgSO_4$ solution for 2–4 h in shaker at 60 rpm. Ten seeds per treatment were placed on sterilized filter paper (Whatman No. 2) in Petri dishes with 0, 100 and 200 mM NaCl and incubated for 4 days. Seeds without any treatment served as negative control and seeds treated only with saline solution were used as positive control. The petri dish covers were removed every 12 h at an open air circulation area to record the germination and to moisten the filter paper.

Gnotobiotic assay

Five bacteria were chosen for further gnotobiotic assay based on their PGP characters and their ability to improve seed germination under saline condition. Maize seeds were surface sterilized as mentioned above and allowed to

germinate in Petri dishes containing moistened filter paper for 2 days. Evenly germinated seedlings were then transferred to sterilized growth pouches containing 0, 25 and 50 mM NaCl solutions. Four replicates were maintained for each treatment with three maize seedlings per pouch. Sterile Hoagland's nutrient solution with the desired concentration of NaCl was used to moisten the pouch every day. After 7 days, shoot and root lengths of the maize seedlings were measured.

Determination of stress ethylene production

Ethylene emission from the maize seedlings were measured following the protocol of Mayak et al. (2004) with slight modification. Maize seeds were surface sterilized, then treated with SAB as described previously. One piece of filter paper was placed inside a 120 ml narrow neck bottle and 2 ml of de-ionized water was added to each bottle then autoclaved at 121 °C for 15 min. After cooling to room temperature, 10 seeds were placed in each bottle then incubated in a plant growth chamber. Three days after germination, treated seeds were additionally supplied with 2 ml of bacterial suspension in 30 mM $MgSO_4$. Six days after germination, the excess liquid was drained and 2 ml of 0, 25, and 50 mM NaCl solution was added. Four hours after the addition of saline solution, the bottles were closed for 20 h with a rubber septum and the ethylene from the headspaces were collected and analyzed. The gas samples were injected in a Gas Chromatograph (dsCHROM 6200, Donam Instruments Inc., Republic of Korea) packed with a Poropak-Q column and equipped with a flame ionization detector. The gas chromatograph was adjusted to 40, 150 and 250 °C for oven, injection and detection temperatures, respectively. The flow rates of N_2, H_2 and air were 35, 30 and 300 ml min^{-1}, respectively. The amount of ethylene produced was expressed as nmol of ethylene (g seed)$^{-1}$ h^{-1} by comparing to a standard curve generated with pure ethylene.

Presence of functional genes

The presence of *nif*H gene was determined by amplifying the 390 bp fragment using a pair of specific primers, 19F (5′-GCIWTYTAYGGIAARGGIGG-3′) and 407R (5′-AAICCRCCRCAIACIACRTC-3′) as described in Ueda et al. (1995). The conditions of the polymerase chain reaction (PCR) were: initial denaturation at 94 °C for 4 min then 30 cycles containing 30 s at 94 °C, 1 min at 50 °C, and 30 s at 72 °C and the final extension at 72 °C for 7 min. The presence of *acd*S gene was determined by amplifying the 558 bp fragment using primers F1936f (5′-GH GAM GAC TGC AAY WSY GGC-3′) and F1939r (5′-GA RGC RTC GAY VCC RAT CAC-3′) as described in Blaha et al. (2006). The PCR conditions were as follows: initial denaturation at 94 °C for 4 min then 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C and the final extension at 72 °C for 7 min. The presence of *nod*A gene was determined by amplifying the genomic DNA using a pair of specific primers, nodA-1 (5′-TGC RGT GGA ARN TRN NCT GGG AAA-3′) and nodA-2 (5′-GGN CCG TCR TCR AAW GTC ARG TA-3′) as described by Palaniappan et al. (2010). The PCR reaction was performed using the following conditions: an initial denaturation step (94 °C, 5 min), followed by 20 cycles of denaturation (94 °C, 30 s), annealing (from 60 °C to 50 °C, 30 s) and extension (72 °C, 42 s), followed by 22 cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30 s), extension (72 °C, 42 s) and final extension (72 °C, 7 min). The amplified products were resolved on a 1 % agarose gel in 1× TAE buffer and visualized under UV light (Bio-Rad Laboratories, CA, USA).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and the mean significant differences were compared by *t* test and Duncan's multiple range test (DMRT) at $P \leq 0.05$. All data was analyzed using SAS package, Version 9.4.

Results and discussion

Plant growth promoting potential of spore associated bacteria

This study demonstrates the effectiveness of spore associated bacteria containing ACC deaminase activity in improving seedling germination and inducing salt tolerance during the early stages of maize growth. Of the 22 isolates characterized to be associated with AMF spores, several PGP traits have been identified in all isolates (Table 1). Twelve bacteria were able to grow in over 10 % NaCl, four bacteria in 6 % NaCl and three bacteria in 4 % NaCl. All of the 22 spore associated bacteria were able to produce varying amounts of IAA in the presence of L-tryptophan, a precursor of IAA biosynthesis. IAA plays an important role in growth promotion by increasing the total root surface area which leads to increased mineral uptake by plants (Spaepen and Vanderleyden 2010). Strain *Bacillus aryabhattai* S210B8 showed the highest IAA production in the presence of tryptophan followed by *P. koreensis* S2CB35. All of the 22 bacteria showed ACC deaminase activity, in particular, *Variovorax paradoxus* S2CB41 showed the highest activity among the strains followed by *B. aryabhattai* S1CB4. ACC deaminase activity is an important PGP trait as this is involved in reduction of stress ethylene (Glick et al. 2007). Nineteen bacteria which were able to grow in 4 % NaCl or greater concentration were tested for phosphate solubilizing ability.

Table 1 Plant growth promoting characters of spore associated bacteria

Strain	Salt tolerance (%)	IAA production[a]		ACC deaminase activity[b]	P solubilization[c]	N fixation	Siderophore production
		Without tryptophan	With tryptophan				
Bacillus aryabhattai S1CB3	>10	1.31±0.01	3.46±0.66	3.12±0.28	15.03±4.94	+	–
Bacillus aryabhattai S1CB4	>10	1.34±0.01	5.64±1.74	14.1±7.09	2.13±0.04	+	–
Bacillus aryabhattai S110B2	>10	1.34±0.01	3.24±0.58	10.52±2.43	29.68±8.61	+	–
Bacillus aryabhattai S110B3	6	1.31±0.07	6.32±1.52	2.37±1.48	22.92±6.15	+	–
Bacillus aryabhattai S110B4	6	1.36±0.02	3.77±0.41	1.35±0.65	0.53±0.04	+	–
Bacillus aryabhattai S120B2	>10	2.12±0.08	2.52±0.24	2.68±1.57	50.9±16.28	+	–
Bacillus aryabhattai S120B3	>10	1.29±0.00	2.11±0.19	4.14±3.49	22.04±4.03	+	–
Sphingomonas aquatilis S3CB10	2	2.81±0.09	2.63±0.21	8.15±1.56	ND	ND	ND
Bacillus aryabhattai S310B3	>10	2.71±0.30	6.98±3.63	1.80±1.22	26.25±1.75	+	–
Bacillus aryabhattai S2CB31	>10	1.33±0.03	2.38±0.41	4.06±1.17	76.43±27.73	+	–
Pseudomonas koreensis S2CB32	4	2.00±0.04	6.43±0.04	0.65±0.34	105.64±2.33	+	+
Pseudomonas koreensis S2CB35	4	2.01±0.03	8.47±0.73	0.78±0.48	108.54±2.88	+	+
Pseudomonas koreensis S2CB37	4	2.05±0.07	6.34±0.14	2.38±1.39	113.1±1.31	+	+
Variovorax paradoxus S2CB41	2	1.93±0.11	1.91±0.07	14.5±0.16	ND	ND	ND
Pseudomonas koreensis S2CB45	6	2.01±0.04	6.16±0.14	1.65±0.53	108.18±1.52	+	+
Sphingomonas aquatilis S2CB54	2	0.83±0.42	2.87±0.06	2.13±0.47	ND	ND	ND
Bacillus aryabhattai S210B8	>10	1.63±0.03	10.95±2.08	2.46±2.46	79.85±2.45	+	–
Bacillus aryabhattai S210B11	>10	1.88±0.19	6.37±1.30	1.95±1.95	108.27±1.47	+	–
Bacillus aryabhattai S210B15	>10	1.85±0.05	5.21±0.52	2.59±2.14	34.94±5.96	+	–
Paenibacillus xylanexedens S210B16	6	0.78±0.39	1.67±0.04	4.48±4.48	109.32±1.21	–	–
Bacillus aryabhattai S220B2	>10	1.27±0.04	3.21±0.68	1.12±1.12	94.32±1.21	+	–
Bacillus aryabhattai S220B4	>10	1.54±0.05	3.17±0.69	2.11±1.39	95.90±0.70	+	–

Each value represents the mean of three replications±SE

+, positive; –, negative; *ND* not determined

[a] µg ml^{-1}

[b] µmol α-ketobutyrate (mg protein)$^{-1}$ h^{-1}

[c] g l^{-1}

All the 19 bacteria were able to solubilize tricalcium phosphate. P solubilization ability of bacteria can help plants to uptake more P by solubilizing the insoluble P into soluble P (Liu et al. 2014). The highest P solubilization ability was observed in *Paenibacillus xylanexedens* S210B16 followed by *P. koreensis* S2CB35. Eighteen bacteria from different genera were able to fix atmospheric nitrogen. Four *Pseudomonas* bacteria were able to produce siderophores, which can facilitate iron nutrition for the plants and are involved in soil borne disease suppression.

Seed germination

To confirm the PGP activities of the isolates in association with plants, seed germination assays were conducted. Salinity is shown to decrease seed germination of most crop plants mainly by causing osmotic stress to the seeds (Siddikee et al. 2015). A greater reduction in maize seed germination was observed as salt concentration increased. After 96 h, maize seed germination was reduced to 73, 63 and 30% in 100, 150 and 200 mM NaCl, respectively (Fig. S1). Egamberdieva (2009) reported that a much lower percentage of wheat seed germination was observed at 100 mM NaCl compared to non-saline controls. In our study, maize seed germination was high under optimal condition. However, as salinity increased, germination percentage decreased. Bacteria which were able to grow over 4% of NaCl were tested for their ability to improve seed germination under salt stress. Under 0 mM NaCl, no significant difference was observed between bacterial treatment and control (Fig. 1a, *t* test, $P \leq 0.05$). Under 100 mM NaCl, two bacteria treatments showed significantly higher germination percentage compared to control (Fig. 1b, *t* test, $P \leq 0.05$). Under 200 mM NaCl, seven bacterial treatments showed significantly higher germination percentage than control (Fig. 1c, *t* test, $P \leq 0.05$). Similar results were obtained by Jalilia et al. (2009) with PGP bacterial inoculation significantly enhancing seed germination of canola at 11 and 14 dS m^{-1} salinity.

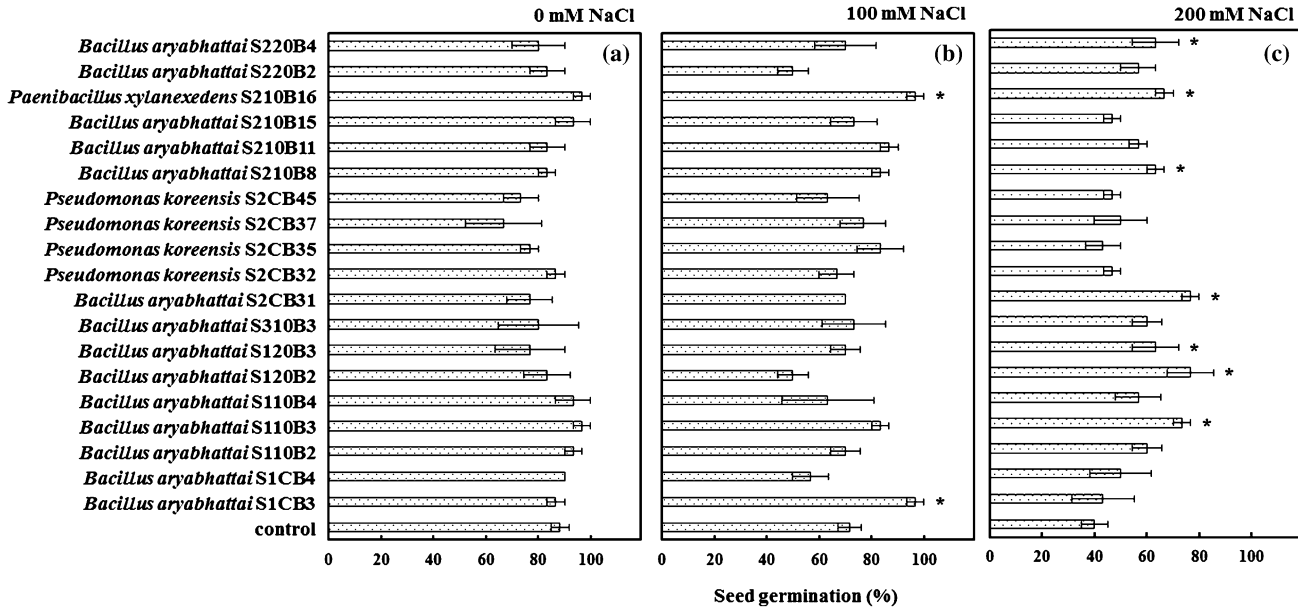

Fig. 1 SAB inoculation effect on maize seed germination. **a** 0 mM NaCl concentration, **b** 100 mM NaCl concentration, **c** 200 mM NaCl concentration. Each value represents the mean of three replications ± SE. *Denotes significant difference compared to control at $P \leq 0.05$ (*t* test, SAS v9.4)

In another study, Siddikee et al. (2015) reported that under 120 mM NaCl, canola seed germination was increased by halotolerant PGP bacterial inoculation compared to control.

Early growth of maize

Based on the PGP characters and the ability to improve maize seed germination, five bacteria namely, *B. aryabhattai* S110B3, *P. koreensis* S2CB35, *B. aryabhattai* S210B8, *B. aryabhattai* S210B15 and *P. xylanexedens* S210B16 were selected and studied for their ability to improve plant growth in gnotobiotic condition. As the salinity level increased, maize shoot and root growth were reduced. At 25 mM NaCl, salinity reduced the shoot and root growth by 13 and 4%, respectively compare to 0 mM NaCl (Fig. 2a, DMRT, $P \leq 0.05$), whereas at 50 mM NaCl, the growth further reduced to 43 and 11%, respectively compared to 0 mM NaCl (Fig. 2b, DMRT, $P \leq 0.05$). At 0 mM NaCl, all the bacterial treatments showed significantly higher shoot and root growth. At 25 mM NaCl, all the treatments significantly improved maize growth over the control except for treatment with *B. aryabhattai* S210B15, whereas, at 50 mM NaCl, *P. koreensis* S2CB35 and *B. aryabhattai* S210B15 showed significantly higher root growth compared to control. ACC deaminase plays an important role in functional interactions of various plant associated bacteria and fungi. Under salt stress, ACC deaminase activity has shown to improve plant growth (Nadeem et al. 2007; Siddikee et al. 2010) and root elongation (Wang et al. 2000; Belimov et al. 2002).

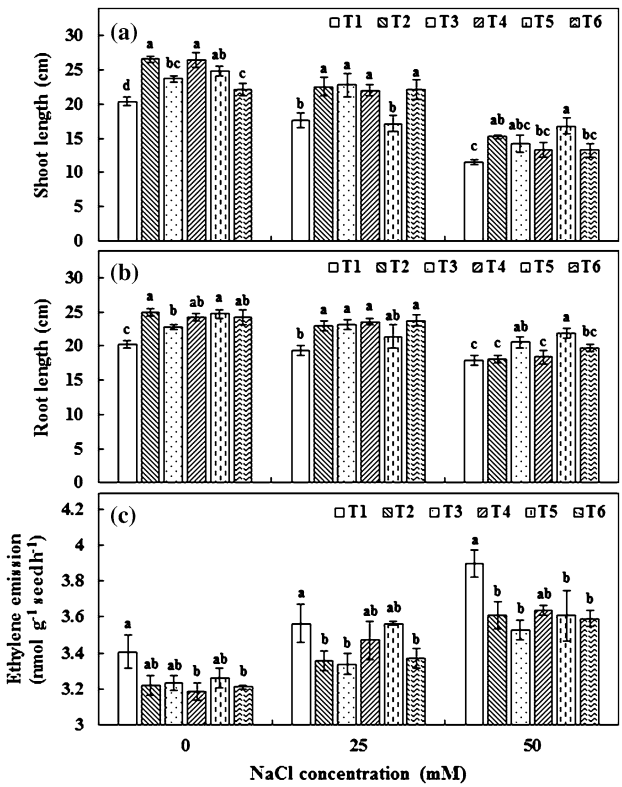

Fig. 2 Early growth and ethylene emission of maize seedlings inoculated with spore associated bacteria under different salt levels. **a** Shoot length, **b** root length and **c** ethylene emission. T1, control, T2, *B. aryabhattai* S110B3, T3, *P. koreensis* S2CB35, T4, *Bacillus aryabhattai* S210B8, T5, *Bacillus aryabhattai* S210B15, T6, *Paenibacillus xylanexedens* S210B16. *Alphabets on top of columns* indicate statistical grouping based on DMRT ($P \leq 0.5$)

Ethylene emission

A biphasic pattern of ethylene emission proposed by Renata and Gniazdowska (2012) indicated that extremely low ethylene emission is crucial for the activation of embryo axis during the very early stage of germination and the amount of ethylene emission increased during embryo expansion and radical protrusion in variety of seeds. Balancing the ethylene level during seed germination promotes successful physiological performance of the seed and plant establishment (Patricia et al. 2010). Mayak et al. (2004) reported that ethylene is involved in a plant's response to abiotic stress. The present study shows that when salinity increases, ethylene emission from maize seedlings also increased, however, inoculation of ACC deaminase containing bacteria reduced the ethylene emission from the seedlings (Fig. 2c, DMRT, $P \leq 0.05$). Under 0 mM NaCl, *B. aryabhattai* S210B15 and *P. xylanexedens* S210B16 significantly decreased ethylene emission by 4 and 6%, respectively. Under 25 mM NaCl, *B. aryabhattai* S110B3, *P. koreensis* S2CB35 and *P. xylanexedens* S210B16 significantly reduced ethylene emission by 6, 6 and 5%, respectively. Under 50 mM NaCl, except for *B. aryabhattai* S210B8, all the SAB were able to reduce ethylene emission of maize significantly. A previous study by Madhaiyan et al. (2007) reported that ACC deaminase producing plant endophytic *Methylobacterium* spp. strains reduced ethylene stress level and improved canola seedlings growth. Another study by Siddikee et al. (2015) reported that ACC deaminase producing halotolerant bacteria reduced ethylene stress levels of canola seedlings under salt stress.

Presence of PGP functional traits

All the five bacteria were targeted for presence of PGP functional traits *nif*H, *acd*S and *nod*A genes by PCR amplification. Only *P. koreensis* S2CB35 showed the presence of all the three genes and *P. xylanexedens* S210B16 was positive for *nif*H gene (Fig. S2). Although *Bacillus* and *Paenibacillus* strains showed quantitative ACC deaminase activity, *acd*S gene was not found in their genome. The phylogeny and evolutionary study of ACC deaminase by Nascimento et al. (2014) reported that despite the known ACC deaminase activity shown by bacteria belonging to Bacteroidetes/Firmicutes, the presence of *acd*S gene in their genome was not identified. This explains that although known amount of ACC deaminase activity was present in the bacterial strains, *acd*S gene was not amplified in their genome.

Conclusion

Spore associated bacteria of AMF exhibited multiple plant growth promoting characters. Bacteria showing ACC deaminase activity improved maize seed germination and early growth under salt stress. Significant reduction in ethylene emission in maize seedlings by inoculation of *Bacillus, Paenibacillus* and *Pseudomonas* strains demonstrate the effect of bacterial ACC deaminase activity with respect to salinity stress. In addition, the presence of PGP functional traits in these strains infers that they might have helped the maize plants to overcome the detrimental effects of salinity. Further studies with PGP functional gene mutants of these bacteria can help identify the vital PGP characters to improve plant resistance to salt stress.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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