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Plant growth-promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare* L.) under salt stress

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ABSTRACT

Soil salination is a major concern of modern agriculture, specifically regarding irrigation and arid regions. However, plant growth promoting rhizobacteria (PGPR) can increase plant resistance to abiotic stress and represent an environmental friendly approach to alleviate salt stress in crops. The plant growth promoting (PGP) activities of *Hartmannibacter diazotrophicus* E19^T, a new genus recently isolated from the rhizosphere of *Plantago winteri* from a natural salt meadow, were assessed in pure culture experiments and *in vivo* assays. ACC-deaminase activity for strain E19^T at 1, 2 and 3% NaCl were 0.56 ± 0.20, 1.29 ± 0.82 and 2.60 ± 1.2 μmol α-ketobutyrate mg protein^{-1} h^{-1} respectively, and production of IAA was not detected. *H. diazotrophicus* E19^T inoculated summer barley seedlings exposed for 2 h to 200 mM and 400 mM NaCl stress showed reduced ethylene emission in comparison to uninoculated plantlets exposed to same conditions. Inoculation of barley plants (*Hordeum vulgare* L.) with strain E19^T in non-sterile soil under salt stress conditions significantly increased root (308%) and shoot (189%) dry weight. The relative increase of water content in the root system was 378% than the control treatment, and the root-to-shoot ratio was increased more than double compared to control. *H. diazotrophicus* inoculation showed no effect on both Na^+ and K^+ concentration in leaf blades or sheaths, but decreased root surface sodium uptake. The capability of strain E19^T to colonize barley roots under salt stress conditions was revealed with a specific designed fluorescence *in situ* hybridization (FISH) probe. *H. diazotrophicus* strain E19^T positively promotes barley growth under salt stress conditions, and indicates that the mode of action is based on ACC-deaminase production.

1. Introduction

Salinity affects about 20% of all irrigated agricultural fields and over 7% of the world land surface (Szabolcs, 1994), by reducing the ability of crops to take up water and by ion toxicity. The physiological consequences of this is the loss of cropyield by inhibition of seed germination, seedling growth, flowering and fruit set (Sairam and Tyagi, 2004). Economical global annual costs by loss in crop production caused by salt-induced land degradation were estimated to be US$ 27.3 billion (Qadir et al., 2014). Moreover future climate change-predicted scenarios show the increasing risk of salinization at different latitudes, and therefore a special effort will be required for maintaining crop production under salt stress (Turral et al., 2011).

It is widely reported that the application of plant growth promoting rhizobacteria (PGPR) influences plant growth by different mechanisms such as fixation of atmospheric nitrogen, solubilization and mobilization of phosphorus, sequestration of iron by siderophores, production of phytohormones, 1-amino-cyclo-propane-1-carboxylate (ACC) deaminase, antibiotics, hydrogen cyanide, organic molecules such as vitamins, amino acids and volatile compounds and synthesis of hydrolytic enzymes (Babalola, 2010). Moreover, PGPR can also help plants to tolerate abiotic stresses such as salinity, drought, waterlogging and heavy metals *e.g.* by inducing systemic tolerance (Yang et al., 2009). Bacterial-mediated plant tolerance to salt stress has been reviewed and includes diverse functional and taxonomical groups of bacteria (Dimkpa et al., 2009).

One of the mechanisms of PGPR to alleviate salt stress is the synthesis of ACC deaminase in the rhizosphere, which lowers the level of ethylene accumulation in stressed plants (Glick et al., 1998; Mayak et al., 2004). Ethylene is a modulator of growth and

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development in plants and is involved in the response of plants to stress. A sustained high level of ethylene, due to stress, would inhibit root elongation leading to an abnormal root growth, which would affect plant growth and development (Babalola, 2010). Bacteria producing ACC deaminase are able to promote root elongation and plant growth by lowering ethylene levels in the roots of developing plants (Dey et al., 2004). Diversity of rhizobacteria-mediated plant tolerance to salinity stress involving ACC deaminase activity in different plant species have been reviewed by Nadeem et al. (2010) and Paul and Lade (2014). ACC deaminase production has been reported in strains belonging to *Proteobacteria*,*Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Glick, 2014; Nadeem et al., 2010). Nevertheless, no particular bacterial genus and species have the same genetic makeup and metabolic capabilities to consider a specific bacterial group as PGPR (Glick, 2014).

The use of PGPR is a promising agricultural method to help salt affected crops to maintain an acceptable level of productivity under higher salt concentrations (Nadeem et al., 2012; Singh et al., 2011). Amelioration of adverse effect of salt stress by different rhizobacteria on several crops is reviewed by Paul and Lade (2014). Barley (*Hordeum vulgare* L.) is one of the world's most extensively cultivated crops and the most salt tolerant cereal, reported to become seriously damaged only after extended periods at salt concentrations higher than 250 mM NaCl (equivalent to 50% seawater) (Munns et al., 2006). As far in our knowledge not many studies have been done in plant growth promotion by PGPR on barley (*H. vulgare* L.) (Omar et al., 2009; Chang et al., 2014; Hmaeid et al., 2014; Nabti et al., 2014) probably due to its natural salt tolerance compared to other agricultural crops such as corn, soybean, wheat, tomato and rice. Therefore, increasing research on PGPR strains to ameliorate salt plant stress on barley will help to improve its natural tolerance on fields and also its potential use in salt remediation of salt impacted soils (Chang et al., 2014).

Salt impacted environments are sources of potential PGPR able to ameliorate salt stress in agriculture. Meta-analysis of publicly available 16S rRNA gene sequences suggested that in saline soil habitats less than 25% of bacterial diversity has been recovered using molecular techniques (Ma and Gong, 2013) and only a small part has been isolated. Recent studies on PGPR from salt environments reported the presence of a possible new species ('*Haererehalobacter*', Jha et al., 2012) and new species, with plant-growth promotion potential (Suarez et al., 2014a,b,c; Gontia et al., 2011)

We hypothesized that a bacterium naturally occurring in a naturally salinated soil and coevolved over long time with salt resistant plants could be a suitable alleviator of salt stress on crops such as barley. Therefore, we tested the *in vitro* and *in vivo* PGP activities of a new genus recently isolated in our laboratory from a natural salt meadow (Suarez et al., 2014a). We investigated the effect of *H. diazotrophicus* on growth of summer barley (*H. vulgare* L) in a greenhouse experiment with non-sterile soil and under high salinity.

2. Material and methods

2.1. Bacterial strain and cultivar

H. diazotrophicus E19^T (Suarez et al., 2014a) was grown routinely in half concentrated marine broth (Carl Roth GmbH, Germany) at 28 °C. Liquid cultures were incubated 48 h in an orbital shaker, at 28 °C and 150 rpm. The strain does not grow on NaCl < 1%. Optimal growth occurs at NaCl concentration of 1–3%. The strain is available by the LMG and KACC culture collection under the code LMG 27460^T and KACC 17263^T. For all plant assays, the cultivar Propino of summer barley (*H. vulgare* L.) was used.

2.2. Plant growth promotion activities in vitro

ACC-deaminase activity was determined by the amount of α-ketobutyric acid generated from the cleavage of ACC following the protocol proposed by Penrose and Glick (2003) with following modifications: Strain E19^T was grown in 20 ml half marine broth (Roth) for 48 h at 28 °C and in order to evaluate enzyme activity under different salt concentration DF minimal salt medium supplemented with ACC (Penrose and Glick, 2003) was adjusted to 1, 2 and 3% NaCl final concentration. Reaction was determined at 540 nm absorbance comparing the values of the samples to a standard curve of α-ketobutyrate ranging between 0.1 and 1 μM. Protein concentration was determinated by Bradford assay (Bradford, 1976) with Bovine Serum Albumin (BSA) as standard protein for ACC-deaminase activity. Indole acetic acid (IAA) production was previously reported as negative for this strain by growth on Luria Bertani (LB) agar supplemented with L-tryptophan and it was qualitatively confirmed in this study by inoculating 200 μl of liquid E19^T culture grown for 48 h at 28 °C in 10 ml (LB) broth supplemented with L-tryptophan (100 μg ml^{-1}) and adjusted to 1, 2 and 3% NaCl final concentration. Grown culture (1.5 ml) was centrifuged at 16,200×*g* for 5 min and 50 μl supernatant were added to 100 μl Salkowsky reagent (Gordon and Weber, 1951). After 30 min incubation at room temperature IAA concentration was measured at 530 nm absorbance comparing the values of the samples to a standard curve of IAA (5, 15, 25, 35 and 45 μg ml^{-1}). A *Pseudomonas* species (isolate E8, isolated in our laboratory and producing a high amount of ACC deaminase and IAA; unpublished) was used as positive control.

2.3. ACC deaminase activity in vivo

Barley plants ethylene emission was measured following the protocol of Siddikee et al. (2011) with modification. Strain E19^T cells were grown in half marine broth, centrifuged at 3345×*g* for 10 min, re-suspended in ACC supplemented DF minimal salt medium (1, 2 and 3% NaCl final concentration) and incubated for 48 h at 28 °C in agitation (120 rpm). Cells were harvested, washed and re-suspended in sterile 30 mM $MgSO_4$ solution to reach a concentration of 10^7–10^8 CFU ml^{-1}. Sterilized barley seeds (*H. vulgare* L, cv. Propino) were incubated for 1 h with the bacterial suspension, seeded into rectangular plastic pots (45.5 cm × 27.5 cm × 5 cm depth, 40 seeds per pot) filled with ~4500 ml (~1620 g dry weight) non-sterile classic clay substrate ED 73 (Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co. KG, Sinntal–Altengronau, Germany). Three replicate pots per treatment were prepared. Physico-chemical properties of the substrate were: pH ($CaCl_2$) 5.8, KCl 2.5 g l^{-1}, EC 0.3–0.9 dS m^{-1}, nitrogen ($CaCl_2$) 250 mg l^{-1}, phosphate 300 mg l^{-1}, potassium 400 mg l^{-1}, sulphur 200 mg l^{-1}, magnesium 700 mg l^{-1}. The maximum water holding capacity (WHC) was estimated to be 2000 ml, and each pot was irrigated with 1500 ml rain water (~75% m. WHC). Growth conditions were daylight from 06:00 to 22:00 (artificial light switched on when natural light was less than 10 Klx), and temperature of 20 °C and 16 °C (day and night, respectively). Eight days after seeding, the soils were irrigated with 0, 200 and 400 mM NaCl solved in deionized water, to impose different salt stress levels. Not inoculated seeds, with and without salt stress, were used as control treatments. From each pot, 30 seedlings were uprooted, washed in order to remove soil from roots using respective NaCl solution and placed in 120 ml flasks (Schott, Mainz, Germany). After 30 min, the flasks were sealed using butyl rubber stoppers. One milliliter air samples from headspace were sampled after 4 h and 24 h of incubation at room temperature. Ethylene concentrations were measured using a PerkinElmer Auto system XL chromatograph equipped with a