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Bacillus pumilus enhances tolerance in rice (*Oryza sativa* L.) to combined stresses of NaCl and high boron due to limited uptake of Na^+

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) confer plant tolerance to abiotic stresses like salinity and high boron (B) due to limited uptake of toxic ions as well as increased production of antioxidants. The current study was aimed to investigate whether particular PGPR strain is responsible either for the decreased uptake of B together with salt toxic ions or to promote rice growth through an efficient antioxidative system under combined stresses of salinity and high B. Rice seedlings were maintained in pots according to completely randomized design (CRD) and stressed with high B (0.92 mmol L^{-1} or 10 ppm) and NaCl (150 mmol L^{-1} or ECw of 14.7 dS m^{-1}) for 8 weeks. Half of the pots received *Bacillus pumilus*-inoculated rice seedlings, whereas the other half received un-inoculated ones. Subsequently, plants were harvested and analyzed for mineral composition and antioxidation activity either using atomic absorption spectrometer (AAS) or spectrophotometer. In the absence of PGPR, NaCl salinity significantly enhanced the leaf B and salt toxic ions concentrations, thereby resulting in the shoot growth reduction when compared with the control. Similarly, combined treatment increased the leaf and xylem sap B as compared to NaCl alone, however, remained insignificant for salt toxic ions. Contrary, NaCl + high B decreased the leaf B concentrations as compared to high B alone. Application of PGPR enhanced the plant growth under individual stresses due to enhanced activity of certain of antioxidative enzymes. In combined treatment, *B. pumilus* showed a positive potential for limiting the Na^+ accumulation in rice leaves, but not for leaf B. Moreover, limited uptake of Na^+ resulted in the decreased plant antioxidation activity irrespective of increasing leaf B concentrations which in turn enhanced the rice tolerance.

1. Introduction

Rice (*Oryza sativa* L.) is an important staple crop worldwide, facing problems due to cultivation under abiotic stresses like soil salinity and high B. Soil salinity is considered a major limiting factor for the decreased crop productivity (Munns and Tester, 2008) in arid and semi-arid regions like Pakistan. In these regions, higher B concentrations are often associated with salinity (Grieve and Poss, 2000). Although, B is an essential micronutrient for normal plant growth, but higher soil B concentrations become toxic to plants including rice (Ochiai et al., 2008). High soil B concentrations, usually in the range of 1–5 mM inhibit the plant growth (Reid et al., 2004). Plants experience high B and salt concentrations resulting from high temperatures, limited availability of good quality water, and the use of marginal land and waste water carrying high concentrations of toxic ions (Diaz and Grattan, 2009).

In recent years, interactive effects of salinity and B toxicity on the plants have gained enormous recognition. Several studies reported the combined effects of salinity and high B on growth response of different crops (Bastias et al., 2010; Grieve et al., 2010; Masood et al., 2012a; Javid et al., 2014), however, lacking the consensus, as different plant species exhibited different growth responses. Some reports showed an additive effect of salinity and high B (Alpaslan and Gunes, 2001; Ismail, 2003), while the other

Abbreviations: B, boron; Ca, calcium; Cl, chloride; Mg, magnesium; K, potassium; Na, sodium; POD, peroxidase; SOD, superoxide dismutase; PGPR, plant growth promoting rhizobacteria; CAT, catalase.

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indicated no interaction for shoot B concentrations (Wimmer et al., 2003). In some studies, salinity alleviated the B toxicity by inhibiting the shoot B concentrations of different plant species (Ismail, 2003; Diaz and Grattan, 2009), whereas synergistic effects of combined stresses have also been observed (Grieve and Poss, 2000), however, some of these results were inconsistent (Masood et al., 2012a; Smith et al., 2010b, 2013; Yermiyahu et al., 2008). Despite of an antagonism between salt stress and high B on B uptake, both the stresses have been shown to affect the plant physiological processes such as membrane damage (Eraslan et al., 2007a), nutrient disorders (Masood et al., 2012a; Smith et al., 2010b) and oxidative stress in wheat plants (Masood et al., 2012b). According to Smith et al. (2010b), high B in the presence of sulphate salinity significantly affected the Mg^{2+}, Cl^{-} and B accumulation in broccoli leaves as compared to the high B or salinity treatments alone. Similarly, NaCl salinity and high B significantly affected the Ca^{2+}, Cl^{-} and B concentrations in wheat leaves (Masood et al., 2012a) as compared to the individual stresses of high B and NaCl. High B supply increases the B accumulation in older leaves via transpiration stream (Tanaka and Fujiwara, 2008) that may affect the Mg^{2+} concentrations and result in the loss of leaf chlorophyll contents (Reinbott et al., 1997). Furthermore, it may impose an ionic imbalance by affecting the ionic ratios in plants.

Salinity produces oxidative stress in plants through the production of reactive oxygen species (ROS) (Zhu et al., 2007), which act as damaging molecules to plant membranes **(**Miller et al., 2010**).** Hence, ROS are considered as the key inducers of the programmed cell death in plants (De Pinto et al., 2012). In order to avoid the osmotic stress caused by abiotic stresses, plants evolve an efficient antioxidative system. The antioxidative system is comprised of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) or ascorbate peroxidase (APX) and non-enzymatic antioxidants like ascorbate, glutathione, α-tocopherol (Miller et al., 2008) and proline (Cervilla et al., 2012). There is increasing evidence that alleviation of oxidative damage through the enhanced production of antioxidants increases the resistance to environmental stresses (Cakmak et al., 1993). Antioxidant enzymes have been reported to minimize the B toxicity effects in some plant species (Gunes et al., 2006). Recently, plant growth promoting rhizobacteria (PGPR) are known to increase the abiotic stress tolerance in plants through the enhanced production of antioxidants (Shao et al., 2009; Islam et al., 2014**).** Certain microbes like *Pseudomonas*,*Bacillus*, *Pantoea*, *Burkholderia* and *Rhizobium* were effective to provide tolerance against various abiotic stresses like drought, temperature stress and salinity in pea, maize, wheat, grapevine and common bean (Arshad et al., 2008; Marulanda et al., 2010; Egamberdiyeva and Hoflich, 2003; Barka et al., 2006; Figueiredo et al., 2010). *Bacillus* species like *B. subtilis* produced some metabolites in tomato plants, which stimulated the plant growth and alleviated the salt-ion toxicity (Stavropoulou, 2011). Accordingly, Jha and Subramanian (2013) reported that the inoculation of plants with *B. pumilus* provides resistance in rice plants against salt stress. To our knowledge, this is first report to assess whether (1) particular PGPR strain, *Bacillus pumilus* either responsible for higher or lower uptake of B along with salt toxic ions and also (2) provides resistance in rice plants against combined stresses of salinity and high B through the enhanced antioxidants synthesis.

2. Materials and methods

2.1. Pre-soil analysis and rice cultivation

The soil (top soil: 0–15 cm depth) used for the experiment was collected from a fallow agricultural field and analyzed for EC 3.29 dS m^{-1}, pH 7.7, soil organic matter (SOM) 0.83%, total N 52.14 mmol kg^{-1}, available P 0.24 mmol kg^{-1}, available K 4.87 mmol kg^{-1} and available B 0.041 mmol kg^{-1}. The soil textural class was determined by hydrometer method using the USDA textural triangle (Moodie et al., 1959). Soil was clayey in nature and contained 25, 30, 45% sand, silt and clay, respectively. A pot experiment was carried out in the greenhouse facility of Quaid-i-Azam University, Islamabad, Pakistan located between latitude and longitude of 33.14° N and 73.13° E. Each pot was filled with 6 kg of sterilized soil and sand (3:1). The soil was well mixed with recommended doses of NPK fertilisers (120 kg N + 90 kg P_2O_5 + 60 kg K_2O ha^{-1}) viz urea, di-ammonium phosphate and sulphate of potash, respectively. Rice [*O. sativa* L. cultivar KSK-133; salt-sensitive (Khan et al., 2013)] nursery was initially raised from seeds treated with 1 mM $CaSO_4 \cdot 2H_2O$ solution. After two weeks, sixteen uniform rice seedlings were transplanted after soaking the roots in bacterial culture for 3–4 h. Bacterial strain, *B. pumilus* with the accession number KF875447 was used for rice seedlings inoculation which was isolated from the field area of Quaid-i-Azam University, Islamabad, Pakistan and purified (Mufti et al., 2015). For this purpose, fresh inoculum of *B. pumilus* was prepared in 250 mL LB culture media, placed in shaker-incubator (ECELLA E23, California, USA) for 24 h and centrifuged at 3000 rpm for 20 min. Supernatant was discarded and the pellet was diluted using sterile water, whereas optical density (OD) of the supernatant was maintained to 1 using spectrophotometer (HITACHI, U-1500) at wave length of 660 nm. After one week of transplantation, high B was supplied once along with irrigation water, whereas 25 mM NaCl increments were applied daily to the plants till the final concentrations of 150 mM NaCl to avoid osmotic shock. Following treatments; control, high B (0.92 mmol L^{-1} or 10 ppm), NaCl (150 mmol L^{-1} or ECw of 14.7 dS m^{-1}), and NaCl + high B either received the inoculated rice seedlings or non-inoculated ones. High B and NaCl stresses were applied as irrigation treatments. The experiment was consisted of 8 treatments with 3 replications and repeated twice. Plants were grown in flooded conditions for about 8 weeks.

2.2. Leaf chlorophyll content determination and xylem sap collection

After the appearance of visible B toxicity symptoms, leaf chlorophyll contents were monitored daily using Spad meter (SPAD-502, Minolta Camera Co., Ltd., Japan) until the plants were harvested after 8 weeks of exposure to combined stresses. For the determination of leaf chlorophyll contents, middle leaves were chosen randomly. Xylem sap was collected early in the morning by cutting the stem about 10 cm above the ground. Initial exudates were cleaned with tissue paper and later bleeding sap was collected in 2 mL *Eppendorf* tubes with the help of pipette. Thereafter, fresh leaf material and xylem fluids were stored at −80 °C for further analysis. In parallel, rice leaf samples were also oven dried at 65 °C for mineral composition analysis.

2.3. Mineral analysis including whole leaf and xylem sap B

For the determination of minerals (Na, Ca, Mg and K), a digestion mixture; HNO_3:$HClO_4$ (3:1 v/v) was prepared. The dried ground leaf material (1 g) was digested with 8 mL of digestion mixture and kept overnight. Afterwards, the digestion flasks containing leaf samples were placed on hot plate and heated until the brown fumes turned to white. On cooling, the digested mixtures were diluted with 40 ml of distilled water and filtered through Whatman No.42 filter paper. The collected filtrates were used for mineral analysis with the help of atomic absorption spectrophotometer (AAS) (Spectra AA240 FS, Varian, New Jersey, USA).

Whole leaf B analysis was carried out by dry ashing at 550 °C after taking 1 g air-dried leaf material in crucible. Later, the ash was