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Exogenously supplied osmoprotectants confer enhanced salinity tolerance in rhizobacteria

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Abstract
The response of eight rhizobacterial isolates obtained from the rhizosphere of *Salicornia brachiata* to osmotic stress (salinity) in minimal medium M9 to evaluate their osmotolerance properties. These rhizobacteria could tolerate NaCl upto 0.714 M in M9 minimal medium. It was observed that all isolates demonstrated different response to salt stress in the presence of glycine, proline, betaine, glycerol and yeast extract in the growth medium. The maximum osmoprotective effect under salinity stress was registered by yeast extract followed by glycerol, proline, glycine and betaine. The present findings suggested that proline, glycine and betaine played a critical role in osmotic adaptation at high osmolarity. Among the rhizobacterial isolates, *Zhihengliuella* sp. and *Brachybacterium* sp. synthesized highest proline as osmoprotective substance under salinity stress.

Keywords: Rhizobacteria, proline, glycine, betaine, salinity tolerance

INTRODUCTION

Bacteria have to cope with a range of abiotic stresses caused by fluctuations in their surroundings. High salinity constitutes an environmental stress also for rhizospheric bacteria. The exposure of bacteria to high osmolarity conditions decreases water activity in their cytoplasm (Epstein, 1986) and most of the cellular proteins and other biological macromolecules as well as essential functions are impaired (Bakker et al., 1987). In addition to general effects outlined above, there is marked alteration of proteins involved in the initial attachment steps (adsorption and anchoring) of bacteria to plant roots in symbiotic interaction occur as well as inhibition of bacterial nodulation and nitrogen fixation activity, alteration of exopolysaccharide (EPS) and lipopolysaccharide (LPS) composition of the bacterial cell surface and inhibition of bacterial mobility and chemotaxis toward plant roots. High salinity decreases bacterial numbers colonizing root cells endophytically because plants utilize proline, glycine betaine and glutamate under osmotic stress conditions and thus deprive rhizobacteria of these substances as energy and carbon sources. Bacterial cells have developed powerful strategies to proliferate and survive under stressful conditions. These rhizobacteria protect them against high external osmolarity by accumulating osmoprotective organic compounds in their cytoplasm (Bremer and Krämer, 2000). Osmoprotectants are highly soluble compounds that carry no net charge at physiological pH and are nontoxic at high concentrations. They raise osmotic pressure in the cytoplasm and also stabilize proteins and membranes when salinity levels are unfavorable. They play an important role in the adaptation of cells to various adverse environmental conditions. There are number of compounds including sugars (trehalose), free amino acids (e.g., glutamate and proline), and quaternary ammonium compounds (e.g., glycine betaine, proline betaine, butyrobetaine, and carnitine) that bacteria accumulate from *de novo* synthesis or imported into the cell from external environment (Ko et al., 1994; Glaasker et al.,1998; Le Rudulier et al., 2002). Bacteria also have the capacity to use compatible solutes such as glycine betaine and proline as carbon and nitrogen sources in addition to osmoprotection (Goldmann et al., 1994; Alloing et al., 2006). In particular, proline and glycine betaine (GB) display a variety of beneficial physiological effects for salt-stressed cells (Csonka and Epstein, 1996). Proline and glycine betaine accumulation in response to stress is well documented in plants and microbes. There are many reports of these osmolytes being involved in ameliorating salinity stress (Beumer et al., 1994; Delamare et al., 2003; Ameur et al., 2011).

Since rhizobacteria play a prominent role in maintenance of soil fertility and productivity, any loss in rhizobacterial diversity may result into perceptible consequences. Therefore, it is becoming imperative to study and investigate the effect of stress in naturally occurring moderately halotolerant rhizobacteria. This work investigates the osmoprotective effect of proline (amino acid), trimethylglycine (glycine betaine), glycine, polyalcohol glycerol and a complex substrate yeast extract on the growth of rhizobacteria under high salt concentrations. The study also emphasizes the accumulation of proline in these rhizobacteria when exposed to saline conditions.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were isolated from the

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rhizosphere of a halotolerant plant *Salicornia brachiata*. These rhizobacteria were identified as *Pseudomonas putida*, *Cronobacter sakazaki*, *Rhizobium radiobacter*, *Zhihengliuella* sp. *Mesorhizobium* sp. *Brachybacterium saurashtrense*, *Vibrio alginolyticus, Brevibacterium* sp.

Effect of different concentration of NaCl on the bacterial growth and Osmoprotective effect of proline, glycine, betaine, glycerol and yeast extract on the bacterial growth under salinity

The bacteria were maintained on nutrient agar medium. Before each experiment the bacteria were cultured for three times on M9 medium to avoid the interference of nutrient broth components. Experiments for salinity tolerance were carried out in M9 medium amended with 0.178, 0.357, 0.535, 0.714 and 0.89 M sodium chloride and M9 medium was used as control. Effect of various osmoprotectants (1 mM proline, 2 mM glycine, 1 mM betaine, 0.05% glycerol and 0.5% yeast extract) under 0.714 M NaCl (4%) was also studied. Ten milliliter of M9 medium supplemented with 4% NaCl and different concentrations of proline, glycine, betaine, glycerol and yeast extract, were inoculated with 100 µl of exponentially growing cultures of each rhizobacteria. The tubes were incubated at 30ºC in orbital shaker (175 rpm) for 24 h. Cell growth was evaluated by measurements of optical density at 600 nm on a UV-VIS spectrophotometer UV-3101 (Shimadzu, Japan). Cell viability was determinate by plating and colony counting on nutrient broth medium.

Proline content

For determination of proline content of bacteria, they were grown in M9 medium supplemented with 4% of NaCl at 30ºC for 24 h. Bacterial cultures were harvested by centrifugation. Proline extraction and determination was performed according to Bates et al. (1973) with minor modifications. Bacterial cultures (~100 mg) were vertexed in 1.2 ml 3% aqueous sulphosalicylic acid and centrifuged at 13,000 rpm for 10 min. After centrifugation, 500 ml of supernatant was made up to 1 ml with distilled water and reacted with 1 ml of glacial acetic acid and 1 ml of ninhydrin (2% in acetone). The mixture was incubated at 90°C for 1 h. The samples were cooled in ice bath and 2 ml of toluene was added and vortexed for 2 min. Upper phase was aliquoted to read the absorbance at 520 nm. The proline content was calculated by comparing with a standard curve drawn with the known concentrations of proline and expressed as mg/100 mg fresh weight of bacterial cells.

RESULTS AND DISCUSSION

To analyze the response of rhizobacteria to increased osmolarity and bacteria were cultivated in M9 medium supplemented with 0.178-0.89 M NaCl. All the bacteria showed comparable growth with control and M9 medium amended up to 0.357M NaCl. Bacterial strains *Zhihengliuella* sp. *Brachybacterium saurashtrense*, *Vibrio alginolyticus, Brevibacterium* sp. could tolerate NaCl upto 0.714 M. The halotolerance characteristics of these rhizobacteria are summarized in Fig 1. Growth gradually and significantly decreased as NaCl concentrations increased. Effect of three amino acids (proline, glycine, and betaine), a polyalcohol (glycerol), and a complex substrate (yeast extract), on the growth of rhizobacteria in the presence of 0.89 M NaCl was evaluated. The results presented in Fig. 2 showed that all the osmoprotectants used in the experiment conferred, the rhizobacterial strains, the ability to grow under high salt concentration. The maximum osmoprotective effect was registered by yeast extract followed by glycerol, proline, glycine and betaine.

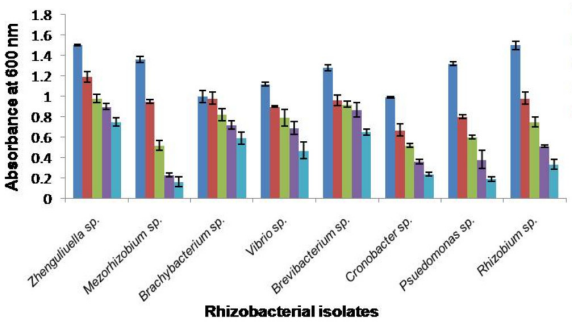

Fig 1. Effect of different concentrations of NaCl on growth of rhizobacterial isolates

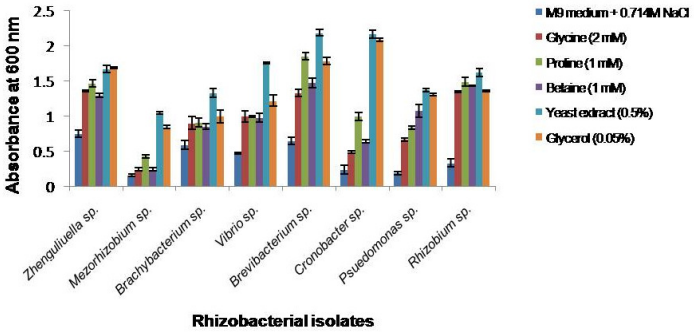

Fig 2. Effect of exogenously supplied glycine (2 mM), proline (1 mM), betaine (1 mM), yeast extract (0.5%) and glycerol (0.05%) on growth of rhizobacterial isolates growing in M9 medium amended with 0.714 M NaCl

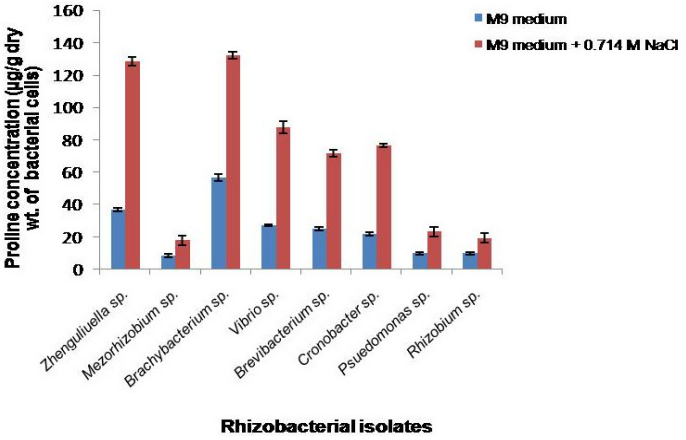

Fig 3. Proline synthesis/accumulation in rhizobacterial isolates growing in M9 medium and M9 medium amended with 0.714 M NaCl

The protective effect of yeast extract is probably due to the synergistic effect of several components like potassium salts, glutamic acid, proline, trehalose, and glycerol (Delamare et al., 2003). Glycerol is widely known for its osmo and cryoprotectant properties. Glycine is a substrate for glycine betaine synthesis, thus the decrease of glycine is probably related to the salinity response of

cells by increasing betaine content. Exogenously supplied proline and betaine are osmoprotective for bacteria, facilitating growth in highly saline environments (Csonka and Hanson, 1991; Yancey, 1994; Delamare et al., 2003; Ameur et al., 2011). The physicochemical basis for osmoprotective effect is not fully understood, but there is good evidence that it lies partly in the exclusion of osmoprotectant molecules from the water layer in contact with protein surfaces. This creates a situation in which native (*i.e.* folded) protein structures are thermodynamically favored because they present the least possible surface area to the water. Most other solutes such as NaCl or $MgSO_4$ interact directly with protein surfaces and favor unfolding, which leads to denaturation (McNeil et al., 1999).

The accumulation of proline in the rhizobacteria as a response to the exposure of saline conditions was also studied. Rhizobacteria grown under unstressed condition, registered lower levels of proline accumulated. At 0.714 M NaCl, accumulation of proline was significantly higher as compared to unstressed condition. As the NaCl concentration increased to 0.714 M the proline levels also increased by 2-3-folds for the rhizobacteria as shown in Fig 3. Accumulation of osmoprotectant amino acid like proline to combat salt stress is a well known phenomenon for many bacteria (Kempf and Bremer, 1998). Proline has been shown to function as molecular chaperone with the ability to protect protein integrity and enhance the activities of different enzymes (Szabados and Savourè, 2009).

CONCLUSION

Understanding the mechanism of osmoadaptation in plant growth promoting rhizobacteria is expected to contribute to the long-term goal of improving plant-microbe interactions for the exploitation of salinity affected fields for crop productivity. In view of the bacteria-specific differences in osmoregulatory mechanisms information on a large number of osmotolerant rhizobacterial strains and their ability to utilize different osmoprotectant compounds is desirable. These selected rhizobacteria which are resistant and /or tolerant to fluctuations in the environmental conditions could be ideal to be used as biofertilizers for reclaiming saline lands for agricultural usage. The rhizobacterial strains from the present work are halotolerant and have high proline accumulation/synthesizing potential so they may prove to be ideal candidates for use as biofertilizers.

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