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Influence of salinity variations on exocellular polysaccharide production, biofilm formation and flocculation in halotolerant bacteria

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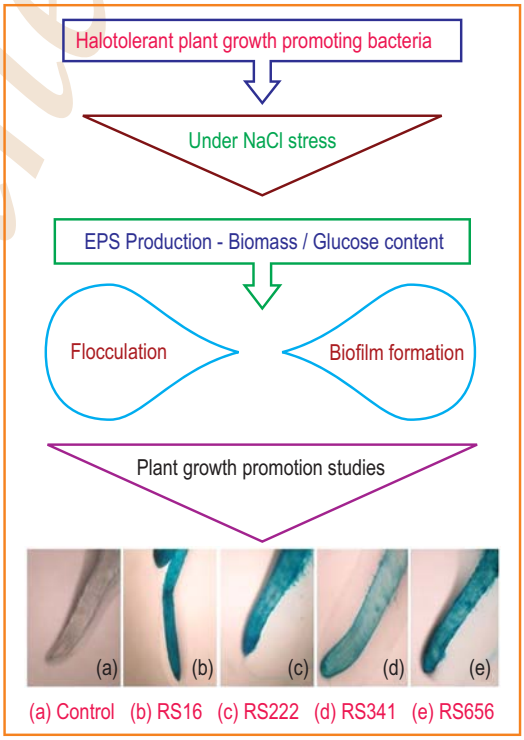
Abstract

Aim: The present study was carried out to evaluate the exopolysaccharide (EPS) production, biofilm formation and flocculation in four halotolerant plant growth promoting (PGP) bacterial strains i.e., *Brevibacterium iodinum* RS16, *Micrococcus yunnanensis* RS222, *Bacillus aryabhattai* RS341, *B. licheniformis* RS656 under different salt levels.

Methodology: Four halotolerant plant growth promoting bacterial strains namely *B. iodinum* RS16, *M. yunnanensis* RS222, *B. aryabhattai* RS341 and *B. licheniformis* RS656 were studied for their exopolysaccharide (EPS) production, biofilm formation and flocculation efficiency under different NaCl levels. EPS production of halotolerant plant growth promoting strains was quantified in terms of dry weight and glucose content. Biofilm formation of the plant growth promoting halotolerant strains was quantified based on a micro titer plate assay. Plant growth-promoting efficiency of halotolerant strains was quantified in red pepper plants in terms of vigor index and fresh weight.

Results: Among the strains, strain B. aryabhattai RS341 recorded the highest EPS dry weight (5.80 g l^{-1}), while the highest floc yield (0.60 g l^{-1} dry weight) was recorded by *B. licheniformis* RS656 at 2 mM NaCl concentration. Regression analysis revealed that EPS content, expressed in terms of dry weight, and flocculation in terms of floc yield, correlated positively with NaCl levels. The highest biofilm formation in Canola root surfaces was observed in the strain *B. iodinum* RS16 in all NaCl concentrations. All the halotolerant strains promoted plant growth as evidenced by the increased vigor index and fresh weight irrespective of the level of NaCl.

Interpretation: It was observed that halotolerant bacterial strains were able to promote plant growth in the presence of NaCl through the mechanisms of EPS production, flocculation and biofilm formations.



Introduction

Soil salinity is one of the major factors affecting agricultural productivity in the world and especially in arid and semiarid regions (Ranjbar and Jalali, 2016).Salinity refers to the presence of excessive sodium ions at the root surface leading to the inhibition of plant growth and the excess of Na^+ ions disrupts plant potassium uptake, which is considered to be a critical factor in plant metabolism (Tester and Davenport, 2003). Some plant growth promoting halotolerant bacteria such as *Anabaena torulosa, Serratia proteamaculans and Rhizobium leguminosarum* are able to thrive under salt stress conditions and are able to promote plant growth (Upadhyay *et al.,* 2011; Nanjani and Soni, 2012). The reason for this is that the growth of halotolerant bacteria is often accompanied by the production of exocellular polysaccharides, which possess numerous ecological and physiological functions (Ashraf *et al.,* 2005).

EPS helps to protect the bacteria from inhospitable conditions, thereby enabling their survival (Vurukonda *et al.,* 2016). EPS is essential for flocculation or aggregation formation in bacteria, which can be explained as specific adsorption of polymeric segment and polymer bridging between cells (Tenney and Stumm, 1965).Flocculation in bacteria, as a response to salinity, helps the bacteria to survive in stressed environments, and this further helps the host plant against various stresses (Qurashi and Sabri, 2012a). Moreover, EPS play a major role in the formation of bacterial biofilm, which plays a critical role in bacterial colonization on plant root surfaces (Chen *et al.,* 2013).

Taking all these factors into account, the present study was carried out to assess the effect of salinity on EPS production in terms of dry weight and glucose content. The biofilm formation, flocculation and plant growth promoting efficiency of halotolerant bacterial strains (*Brevibacterium iodinum* RS16, *Micrococcus yunnanensis* RS222, *Bacillus aryabhattai* RS341, *B. licheniformis* RS656) were also evaluated at different salinity levels.

Materials and Methods

Plant growth promoting halotolerant bacteria and their growth condition : Four plant growth promoting halotolerant bacterial strains viz., *B. iodinum* RS16, *M. yunnanensis* RS222, *B. aryabhattai* RS341 and *B. licheniformis* RS656, used in the present study were previously isolated from the coastal saline region of the Yellow Sea, Incheon, South Korea (Siddikee *et al.,* 2011; Siddikee *et al.,* 2012) and these isolates were able to grow in the presence of 1.75 M (~10%) NaCl concentration.

Bacterial EPS production : For quantitative analysis of bacterial EPS production, tryptic soy broth (TSB) with different concentrations of NaCl (0, 0.5, 1, 1.5, 2 M) were inoculated with 24 h bacterial culture (>7 Log CFU ml^{-1}) and incubated in a shaking incubator (DS-310RL, Dasol, Korea) at 30 ºC, 150 rpm for 72 h To extract EPS, the above-mentioned cultures were centrifuged at 10000 rpm for 15 min at 4 ºC. To this supernatant, three volumes of pre-chilled ethanol (95%) were used to precipitate the EPS. This precipitated EPS was separated by centrifugation at 15000 rpm for 20 min. For determining dry weight, the EPS was dried at 58 ºC for 24 h in the same centrifuge tube to minimize the EPS loss. The amount of EPS was calculated in terms of dry weight following to the methods of Verhoef *et al*. (2003) and De Vuyst *et al.* (1998) and quantified in terms of total carbohydrate content (DuBois *et al.,* 1956).

Biofilm formation experiments : A microtiter plate based protocol was used for the estimation of biofilm formation, with minor modifications (Christensen, 1985). The halotolerant bacterial strains were initially grown at 30 ºC, for 24 h in TSB medium with different concentrations of NaCl (0, 0.5, 1, 1.5, 2 M). The cells were harvested and re-suspended in the same medium and adjusted to OD_{600} of 0.3 in a spectrophotometer (UV-1601, Shimadzu, Japan). Two hundred microlitre of the re-suspended bacterial suspension was added to each well on a flat-bottomed 96-well microtiter plate and incubated at 30 ºC for 72 h, without shaking. After 72 h, the medium was then removed from the wells and the biofilms formed on the wall of microtiter plates were stained with 0.01% crystal violet for 20 min. The dye that had stained the bacteria present in the biofilm was then dissolved in 200µl ethanol (95%), and the amount of dye was quantified by measuring the absorbance at OD_{590}.

Estimation of flocculation : Flocculation expressed as floc yield was carried out following the protocols of Sadasivan and Neyra (1985). Bacteria were grown at 30 ºC for 72 h in TSB media and flocs were harvested by filtering through Whatman filter paper (No. 1). To measure dry weight, the filter paper with the flocs was placed in a desiccator oven at 60 ºC. After 2 h, the dried filter paper was weighed and floc yield was expressed as mg per liter of medium (mg l^{-1}).

Plant growth promotion efficiency and biofilm formation under in vitro conditions: Halotolerant bacterial strains were grown in Luria-Bertani broth in a shaking incubator at 150 rpm, at 28±2 °C for 48 h. Bacterial cells were harvested and adjusted to OD 0.5, which corresponds to a bacterial load of > 10^8 cfu ml^{-1}. Plant growth promotion ability of halotolerant strains was studied by roll towel method (ISTA, 1993). Germination percentage was calculated on the 7th day and vigor index (VI) was estimated on the 14th day, following the method of Abdul-Baki and Anderson (1983) by the following formula: *VI=GP* (RL+SL)* where, *RL* is the root length, *SL* is the Shoot length and *GP* is the Germination percentage. The plant fresh weight was also measured on the 14th day and expressed as mg $plant^{-1}$. The biofilm formation on Canola root surfaces was estimated according to the methods of Fujishige *et al.* (2006) with required modifications. In brief, to evaluate the biofilm formation, the roots were detached and washed in sterile distilled water in an orbital shaker to remove the loosely associated cells. To evaluate the number of bacterial cells,

about 4-5 cm of root piece was vortexed in 0.1 mM PBS and plated on nutrient agar plates. The plant roots were stained with 0.1% aqueousalcian blue dye and observed microscopically as described by Gerhardt *et al.* (1981).

Statistical analysis: For statistical analysis, data were subjected to one or two way ANOVA, followed by DMRT with critical differences (C.D) calculated at *P* level of 0.05%. Regression analysis was performed with Microsoft Excel 2013 and *P* level were calculated at 0.05, 0.01 and 0.001%.

Results and Discussion

EPS production of the four halotolerant bacterial strains (*B. iodinum* RS16, *M. yunnanensis* RS222, *B. aryabhattai* RS341, *B. lichenifomis* RS656) was evaluated in terms of dry mass and the results are presented in Fig 1a. It was observed that the EPS content of the studied strains increased as NaCl concentration increased. Except for *B. iodinum* RS16, the highest EPS dry weight was observed in strain *B.aryabhattai* RS341at 2M NaCl concentration. Regression analysis between salt concentrations and EPS content of all strains were also found to be significant (Table 1). Several researchers (Qurashi and Saabri, 2012a; Zhang *et al.,* 2013;Wang *et al.,* 2016) reported that EPS production is a survival strategy adopted by these bacteria to tolerate high salt levels by maintaining a mini assembly to retain water level around the cells. Zaki *et al.* (2013) reported carbohydrate as a major component in thermo-stable bio-flocculant obtained from strain *Bacillus velezensis* 40B.

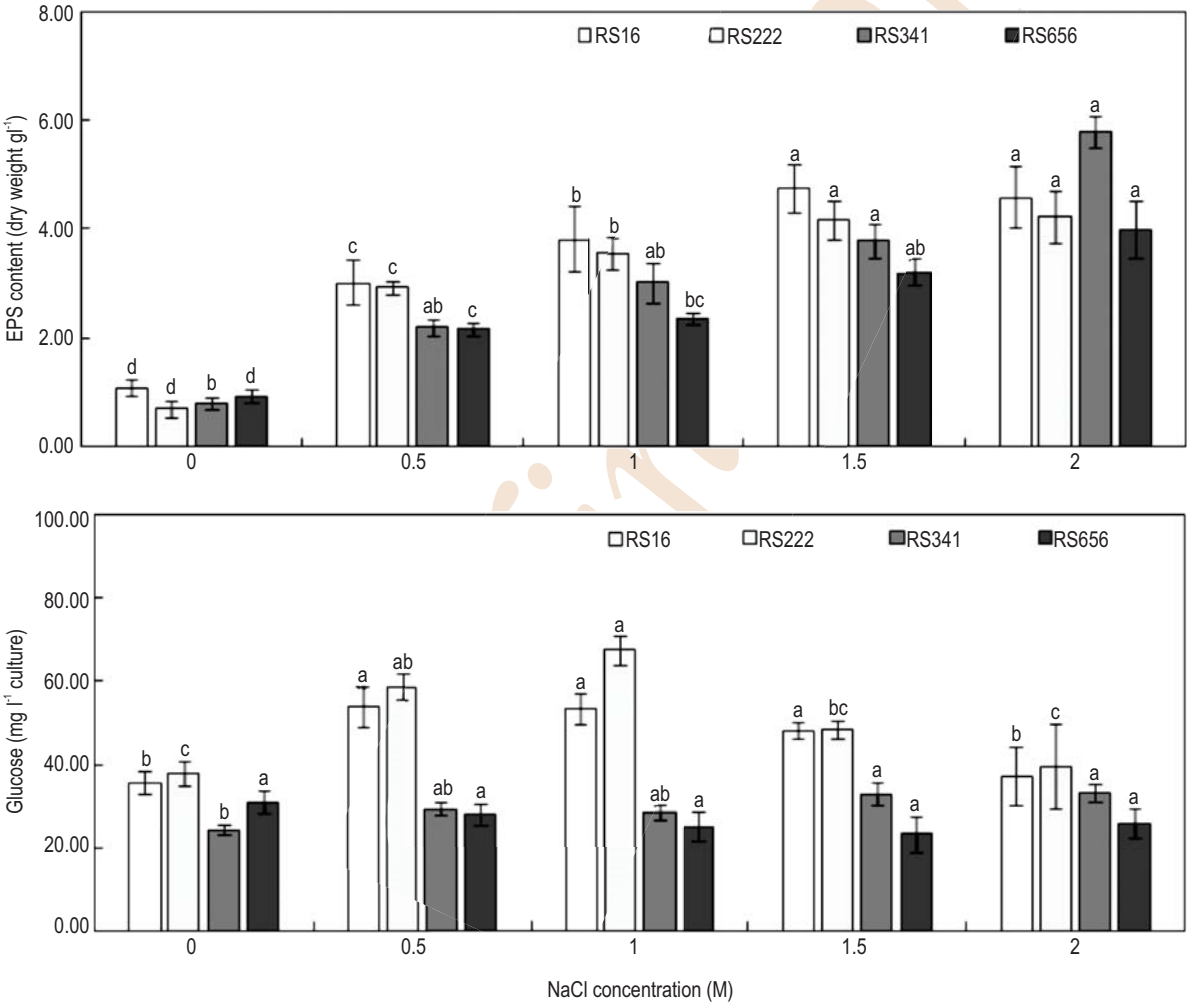


Fig. 1 : Effect of NaCl concentrations on (A) EPS-dry mass and (B) EPS glucose content (as glucose mg l^{-1} culture)of halotolerant bacterial strains. Each value represents the mean of three replicates ± SE. Same letters in the graph are not significantly different according to DMRT at $P \leq 0.05$

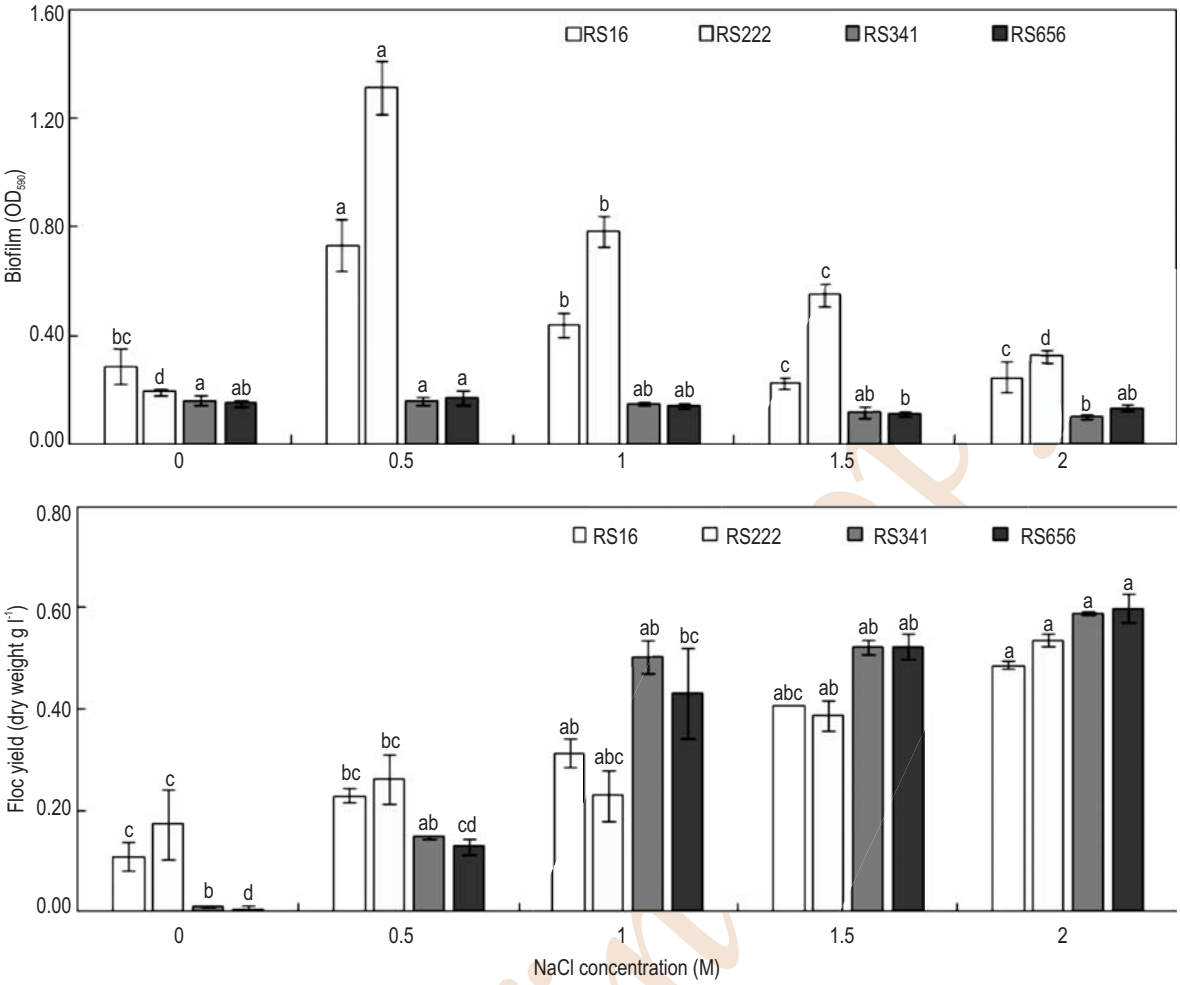


Fig. 2 : Effect of NaCl concentration on (A)biofilm formation of halotolerant bacterial strains; (B) flocculation, expressed in terms of floc yield, among halotolerant bacterial strains in TSB medium. Each value represents the mean of three replicates ± SE. Same letters in the graph are not significantly different according to DMRT at $P \leq 0.05$

Table 1 : Regression analysis between salt concentration and different EPS content, glucose content, biofilm formation and floc yield

Parameters	Significance F				
	RS16	**RS222**	**RS341**	**RS656**	**Overall**
EPS content	*	*	**	**	**
Glucose content	ns	ns	*	ns	ns
Biofilm formation	ns	ns	ns	ns	ns
Floc. yield	***	*	*	**	***

* - significant at $P \leq 0.05$; ** - significant at $P \leq 0.01$; *** - significant at $P \leq 0.001$; ns – not significant

On analyzing EPS content of the studied strains in terms of glucose content, it was observed that strain *M. yunnanensis* RS222 showed highest EPS-glucose content at all the studied NaCl concentrations (Fig. 1b). Strains *B. iodinum* RS16 and *M. yunnanensis* RS222 showed maximum EPS-glucose level at 1 M NaCl concentration, while *B. aryabhattai* RS341 and *B. licheniformis* RS656 exhibited maximum EPS-glucose at 0 and 1.5 M NaCl concentration, respectively. Strain *M. yunnanensis* RS222

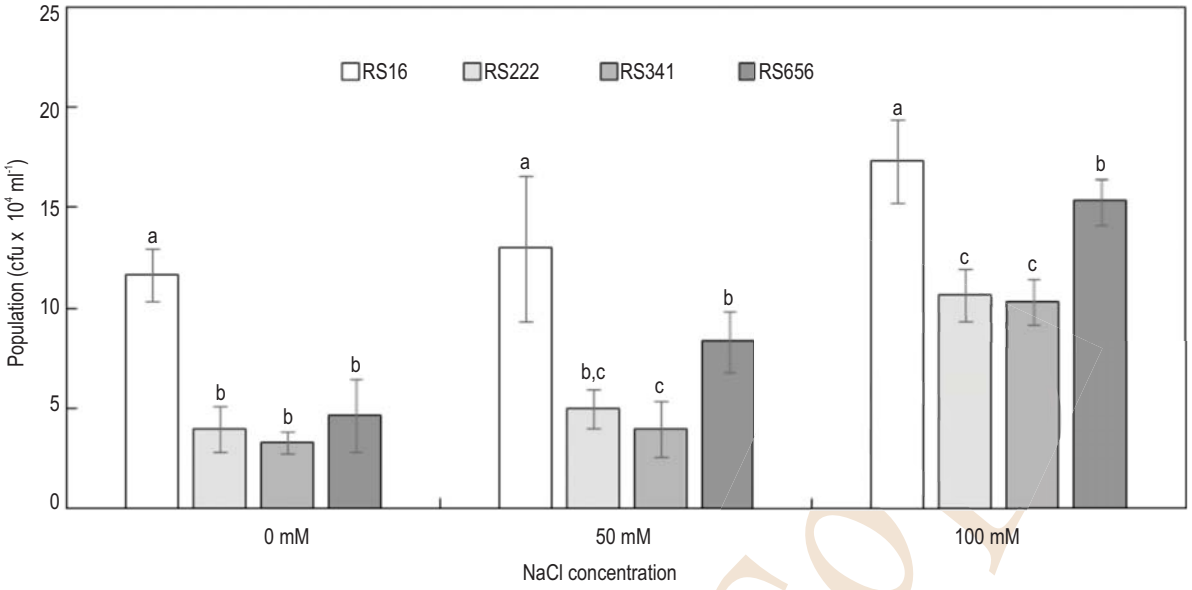


Fig. 3 : Effect of NaCl concentrations on bacterial biofilm, expressed in terms of CFUs, in canola (*Brassica campestris* L.) seedling on 15th day. Each value represents the mean of three replicates ± SE. Same letters in the graph are not significantly different according to DMRT at $P \leq 0.05$

showed highest EPS-glucose level of 67.32 mg l^{-1} at 1 M NaCl concentration among the studied strains (Fig. 1b) and the overall response of strain *B. aryabhattai* RS341 to EPS-glucose levels under salt stress was also found to be significant (Table 1).The high EPS-glucose content of these strains under different NaCl levels in this study is in confirmation with the earlier report of Bahat-Samet *et al*. (2004) where glucose was the main sugar in the EPS of *A.brasilense* AP7 strain during the exponential growth phase. Failure to observe overall significant differences in glucose levels under salt stress in other strains may be due to the possible involvement of other sugars in the EPS formation (Table 1). Qurashi and Sabri (2012b) and Satpute and Banat (2010) reported that the composition of EPS molecules produced by bacteria is variable with the presence of a number of sugar molecules such as glucose, galactose, mannose, xylose, glucuronic and galacturonic acids.

Biofilm formation of the bacterial strains varied under different NaCl concentrations based on microtiter plate assay (Fig. 2a). *B. iodinum* RS16, *M. yunnanensis* RS222, and *B. lichenifomis* RS656 strains showed higher biofilm formation at 0.5 M NaCl concentration. In general, biofilm formation of the strains decreased at concentrations above 0.5 M NaCl concentration, but biofilm formation in *B. lichenifomis* RS656 increased above 1.5 M NaCl concentration. The biofilm formation of strain *M. yunnanensis* RS222 was found to be highest among all the halotolerant strains (1.31, OD_{590}) at 1.5 M NaCl. These results are in line with the earlier finding of Rode *et al.*(2007), wherein NaCl in combination with glucose increased the biofilm formation in *Staphylococcus aureus* strains. This result could be explained based on the fact that biofilm formation is a strategy used by the bacteria to protect themselves from various environmental stresses (Flemming and Wingender, 2001). The reduction of biofilm formation in *B. iodinum* RS16 with respect to NaCl concentration go well with the previous studies of Martinez *et al.*(2011) and Havasi *et al.* (2008), where a decrease in growth and biofilm formation with an increase in NaCl concentration in the cultures of *S. maltophilia* X26332 and *P. aeruginosa* was reported. These authors explained that reduction of biofilm formation was due to the inhibitory role of saline in motility and growth of these bacterial strains.

A concomitant increase in floc yield was observed with increase in NaCl concentration in all the studied strains. The highest floc yield was observed in most of the strains at 2M NaCl concentration (Fig. 2b), however, *B. iodinum* RS16 showed maximum floc yield at 1.5 M NaCl concentration. The highest floc yield of 0.60 g l^{-1} was observed in strain *B. licheniformis* RS656 at this concentration. The overall increase in the floc yield was found to be related to the increase in NaCl concentration (Table 1). These results in the increase in floc yield at various NaCl concentration confirms with the previous report of Watanabe *et al.* (1998), who observed that increasing NaCl concentration up to 6%, increased the flocculation ability in marine photosynthetic bacterium *Rhodovulum* sp. by 80%. Jensen *et al.* (2007) reported the reason for this increase in flocculation to the change in adhesion pattern in *Listeria monocytogenes* strains in the presence of 5% NaCl in TSB.

Biofilm formation of four halotolerant bacteria in the root region of canola plants was compared at 0, 50 and 100 mM NaCl

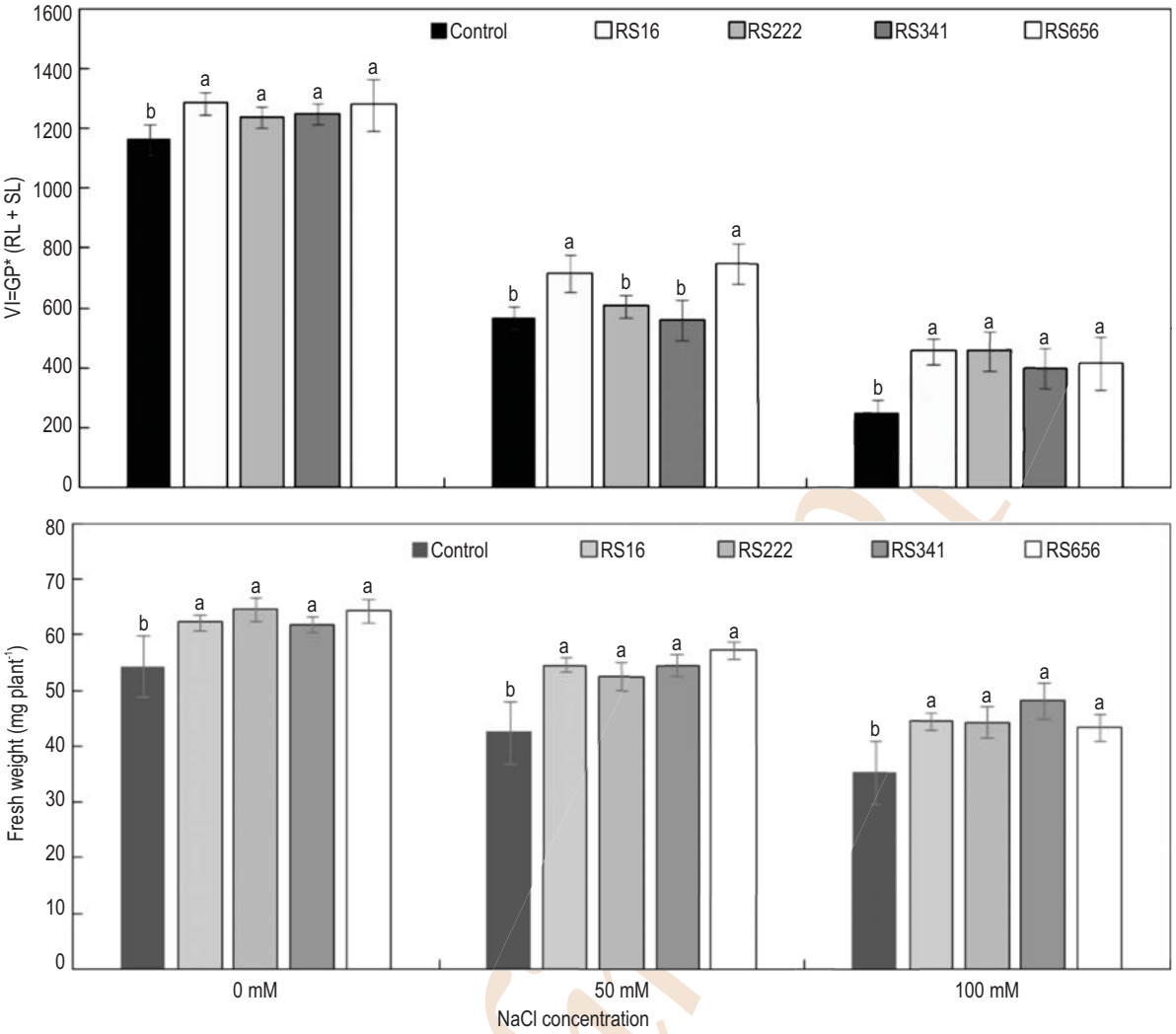


Fig. 4 : Effect of NaCl concentrations on (A) Vigor index of canola (*Brassica campestris* L.) seedlings at 0 mM, 50mM and 100 mM NaCl concentrations on 15th day, (B) Plant fresh weight at different NaCl concentration. Each value represents the mean of three replicates ± SE. Same letters in the graph are not significantly different according to DMRT at $P \leq 0.05$

concentrations. It was observed that the biofilm formation efficiency expressed as cfu ml^{-1} in all the strains increased as NaCl concentration increased (Fig. 3a). Strain *B.iodinum* RS16 showed the highest biofilm formation in all the studied NaCl concentrations. At highest concentration (100mM NaCl), the highest biofilm formation of 17.3 × 10^4Cfu ml^{-1} was observed with *B. iodinum* RS16, and this was followed by *B. licheniformis* RS656 (17.3 × 10^4Cfu ml^{-1}). Alcian blue staining showed the presence of biofilms in all the bacterial treatments, and its absence in the control treatment (data not shown). Microscopic analysis also revealed the presence of more biofilm formation in the root tips, as compared to other root regions.

The effect of halotolerant bacteria in salt stress alleviation in canola seedlings was compared at 0, 50 and 100 mM NaCl concentration for a period of 15 days. The vigor index of plants increased by10.2, 6.1, 7.2, and 9.7 % in *B. iodinum* RS16, *M. yunnanensis* RS222, *B. aryabhattai* RS341 and *B. licheniformis* RS656 strains, respectively, compared to non-inoculated treatment at 0 mM NaCl concentration (Fig. 4a). At 50 mM NaCl concentration, only *B.iodinum* RS16 and *B. licheniformis* RS656 treated plants showed significant improvement in vigor index as compared to control. At 100 mM NaCl stress, vigor index increased by 126.6, 115.2, 175.4 and 158.8 % over control with inoculation of *B. iodinum* RS16, *M. yunnanensis* RS222, *B.*

aryabhattai RS341 and *B. licheniformis* RS656, respectively. Interestingly all the halotolerant bacteria treated plants showed higher plant fresh weight as compared to control, irrespective of NaCl concentrations (Fig 4b).

In the plant tests, seedlings inoculated with *B. iodinum* RS16 and *B. aryabhattai* RS341 showed an increase in plant root lengths compared to control at 100 mM NaCl concentration. Previous studies (Siddikee *et al.,* 2011; Siddikee *et al.,* 2012; Siddikee *et al.,* 2015) demonstrated that the plant growth promoting potential of these four halotolerant strains, improved plant growth by producing various plant hormones and stress alleviating enzymes under salinity stress.

In the present study, it is understood that the halotolerant strains exhibited high EPS production and can efficiently form biofilms, which in turn can reduce the effect of stress in plants grown under in vitro conditions.

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