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Effect of cyanobacterial exopolysaccharides on salt stress alleviation and seed germination

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Abstract: *Effect of exopolysaccharides (EPS) produced by a consortium of cyanobacteria on germination of three crops wheat, maize and rice was studied at different salt concentrations. Production of EPS was found to be stimulated by salts, which in turn had a significant Na^+ removal capability from aqueous solution. Seed germination, vigor index and mobilization efficiency in all the three crops remarkably improved when cyanobacterial EPS was applied. While germination improved significantly by 13 to 30%, mobilization efficiency increased marginally by 1.03 to 1.1 times and vigor index increased by 1.15 to 2.4 times in these crops in response to EPS under non-saline conditions. Salinity had an inhibitory effect on seed germination of all the species showing 18 to 54% reduction. However, in the presence of EPS, the salt induced inhibition diminished to 13 to 18%. Inhibitory effect of salt on chlorophyll concentration, vigor index and mobilization efficiency of the seedlings was much less in these crops in the presence of EPS, indicating the latter's role in salt stress alleviation.*

Key words: *Consortium, Exopolysaccharides, Mobilization efficiency, Vigor index*
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Introduction

Cyanobacterial production of extracellular polymers, mainly EPS is well documented (De Phillips *et al.*, 1998). Polysaccharides are characterised by an extreme structural diversity; as a result, they play very diverse roles in nature and may get modified under stress conditions (Ozturk and Aslim, 2009). Exopolysaccharides (EPS) have been reported to play a significant role in providing protection to the cell as a boundary layer (Caiola *et al.*, 1996), contributing to soil aggregation due to its gluing properties (Nisha *et al.*, 2007) and binding heavy metals due to the presence of several active functional groups onto it (Kaplan *et al.*, 1987; Sharma *et al.*, 2008).

Higher metal adsorptive capacity of EPS of *Lyngbya putealis* as compared to its immobilized biomass suggests it to be a better biosorbent for metal removal (Bala *et al.*, 2007). Extensive capacity for heavy metal removal by EPS has also been found to be a major trace metal transfer factor through water column to sediments in aquatic systems (Gonzales-Danila, 1995). Cyanobacterial polysaccharides have found applications in many industrial sectors because of their many interesting physical and chemical properties, such as stabilizing, suspending, thickening, gelling, and water-retention capability (Richert *et al.*, 2005). Cyanobacteria are also used in aquaculture, wastewater treatment, food, fertilizers, production of secondary metabolites including exopolysaccharides, vitamins, toxins, enzymes and pharmaceuticals (Abed *et al.*, 2009).Looking into the characteristics of EPS it was thought worthwhile to examine its possible application for binding sodium ions from saline medium, thereby alleviating salt stress for germinating seeds. Various applications among others such as improvement of water holding capacity of soil, detoxification of heavy metals and radionuclide-contaminated water and removal of solid matter from water reservoirs have been proposed for cyanobacterial EPS (Moreno *et al.*, 2000; Bender and Phillips, 2004; Freire-Nordi *et al.*, 2005).

Cyanobacterial biofertilizers have been reported to be very useful in ameliorating various physico-chemical properties of marginal soils and the EPS produced by the cyabobacteria seems to play an important role (Nisha *et al.*, 2007). Since salinity is a major constraint to crop growth and germination is the most syensitive and decisive stage for successful crop establishment (Soltani *et al.*, 2006), it would be worthwhile to study whether EPS application is useful in promoting seed germination in the presence of salts in the medium. The present study was therefore, undertaken to study the effects of application of cyanobacterial exopolysaccharides on seed germination and early seedling growth of three crop species at different salinity levels, with an objective to explore its applicability in improving germination of crops in saline soils.

Materials and Methods

Isolation and consortium formation of cyanobacterial strains: Various cyanobacterial strains were isolated from soil crusts of Hisar, Haryana, India (27°40'N, 30°56'and 74°28'E, 70° 35'W) using standard plating, isolation and culturing techniques in BG-11 culture medium (Stainer *et al.*, 1971). A consortium of four strains *Nostoc calcicola, Nostoc spongiaeformae, Nostoc linckia* and *Nostoc muscorum* was prepared by introducing equiproportional inocula (1ml each) in 400 ml nutrient broth (pH 7.5) contained in 500 ml flask. The cultures were maintained under control conditions of continuous light (3000 lux) using cool fluorescent tubes at 27±3°C for 15 days.

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EPS extraction and estimation: For extraction of the cyanobacterial EPS, 100 ml of the culture was subjected to centrifugation (3000 rpm) and after separating the settled biomass, cell-free culture containing the EPS was taken. It was concentrated half of its volume by using a rotary vacuum evaporator at a temperature of 35°C for further use in the experiment. In a similar way another set was used for quantitative estimation of EPS produced by the cyanobacteria at different NaCl concentration (S1= 55 mM, S2= 110 mM Na^+). After concentrating the EPS containing cell-free culture ten fold by evaporation at 40°C, it was precipitated with isopropanol. The precipitates were washed with isopropanol 2-3 times to remove any contaminants, dried at 37°C and hydrolysed with acid (2 M HCl) at 100°C for 2 hr. The hydrolysate was analyzed for glucose (Seifter, 1959) for all the treatments.

Removal of sodium by EPS: In order to see the Na removal efficiency of the EPS, to 100 ml of the cell free extract taken in Erlenmeyer flask 55 and 110 mM of Na^+ as NaCl was added, shaken on the illuminated orbital shaker with fluorescent light at 120 rpm at 25°C for 12 hr following Freire-Nordi *et al*. (2005) and Na^+ concentration in the aqueous medium was estimated on flame photometer (Allen *et al.*, 1986).

Seed germination studies: A total of 18 Petri dishes for each of the three crops (3 salt treatments X 2 EPS amendments X 3 replicates) were taken. Seeds of wheat, maize and rice taken for the study, were surface sterilized with 2% $HgCl_2$. Twenty five seeds of each crop per Petri plate were used for different salt concentrations (S_1= 55 mM Na^+, S_2= 110 mM Na^+, S_0= no salt). One set of the Petri dishes was amended with 5 ml of concentrated Cyanobacterial EPS (A) while the other set without amendment (A_0) served as control. Response of different crops under various salinity levels was assessed as % seed germination, mobilization efficiency, vigor index and total leaf chlorophyll in 7 days old seedlings. Mobilization efficiency was calculated as percent translocation of reserves from cotyledons to seedlings on dry weight basis (Singh *et al.*, 1990). Vigor index was calculated as the product of germination percent and axis (shoot length). Chlorophyll was estimated by hot extraction with methanol following Mckinney (1941). The data were statistically analyzed for testing the significance of differences due to treatment (Coolidge, 2000).

Results and Discusion

The cyanobacterial consortium produced significantly more ($p<0.05$) EPS in the sodium spiked culture medium, which was 2.4 to 3.2 times more than that in control, indicating that sodium stimulates EPS production by the cyanobacteria (Table1). Potential of the cyanobacterial EPS for Na^+ removal from the aqueous medium due to biosorption was also tested and the results are presented in Table 1. About 50% removal of Na^+ from the medium was observed, which was due to biosorption by the EPS. The biosorption of Na^+ by the EPS varies from 154 to 235 mg g^{-1}. Since cyanobacterial EPS has a rich array of ligands that can chelate metal ions (Aksu *et al.*, 2002), binding of Na^+ seems to take place on the functional groups.

Salinity had an inhibitory effect on seed germination of all the species. At S_1A_0, there was 9-20% suppression of germination in these species. But, on application of EPS, adverse effect of salinity was totally alleviated at this salt concentration. It is evident from the Table 2 that after amendment with EPS, wheat showed germination equivalent to that of control (S_0A_0), while the other two species performed still better, exhibiting percent germination even higher than control. At a high salt concentration of 110 mM Na^+ (S_2A_0), suppression in germination was more prominent for wheat (54%) and maize (32%), but on amendment with EPS, corresponding suppression was just 20 and 11% of control. In case of rice, there was 18% inhibition in germination due to S_2, which improved remarkably on amendment with EPS and the germination percent recovered to as high as 83.4%, which was even better than that in non-saline unamended (S_0A_0) as shown in Table 2. This indicates a distinct role of EPS in countering the adverse effect of salinity on seed germination. Statistically significant differences due to different treatments are indicated in the table.

Mobilization efficiency, indicating seedling dry weight per unit mass of cotyledon weight, also showed a similar significant decline ($p<0.05$), which was 28 to 33% in wheat and maize in response to increasing salt concentration. In case of rice, however, mobilization efficiency was not affected significantly ($p>0.05$) by high salt concentration. The cyanobacterial EPS helped in improving the mobilization of food reserves from the cotyledon for early seedling growth of the three species by about 5 to 25%, both in the presence and absence of salts (Table 3). EPS amendment improved mobilization of nutrient reserves in the three crops both in the presence and absence of salt.

Salinity significantly ($p<0.05$) decreased chlorophyll concentration in leaves of 7 day old seedlings, which was by 15% in wheat, by 76% in maize and by 36% in rice, but application of EPS had a distinct significant effect ($p<0.05$) on salinity alleviation as evident from recovery in the pigment concentration . There was 30-53% improvement in chlorophyll concentration in the leaves of the three crops at S_1 and 22-56% at S_2 in response to EPS amendment (Table 4).

Vigor index dramatically increased in rice and maize when EPS amendment was given in control, as percent germination as well as axis growth showed remarkable increase (30 to 70%). With increasing salt stress, vigor index in all the three species declined by 17 to 78%, but EPS amendment substantially reduced the adverse effect of salt and resulted in a marginal recovery of vigor index in maize (3-6%), in wheat (15%) at S_1 and very significantly in rice (52-85%). Statistically significant differences due to different treatments are indicated in the Table 5.

Salinity alleviation due to cyanobacterial EPS may be attributed to Na^+ removal by the latter from the aqueous medium

Table - 1: Production of exopolysaccharides (EPS) by cyanobacterial consortium and sodium adsorption/removal by EPS under different salt concentrations

Salt treatment	EPS production (g 100 ml^{-1})	Na^+ adsorption (mg g^{-1})	Na^+ removal (%)
S_0	0.172±0.04[a]	-	-
S_1	0.409±0.05[b]	154±2.2[a]	49.5±0.5[a]
S_2	0.555±0.06[c]	235±4.5[b]	51.5±0.08[b]

Values in each column followed by different superscripts (a, b, c) indicate statistically significant differences in the values (p<0.05); Values are mean ± SE (S_1 = 55 mM Na^+, S_2 = 110 mM Na^+, S_0 =No salt)

Table - 2: Effect of cyanobacterial EPS amendment on % seed germination of three crops under different salt concentrations

Treatment	% Seed germination		
	Wheat	Maize	Rice
S_0A_0	50.0±2.3[a]	63.3±3.0[a]	73.3±2.8[a]
S_0A	63.3±1.8[b]	93.3±2.6[b]	96.6±3.7[b]
S_1A_0	40.0±3.3[c]	53.4±3.0[ad]	66.6±4.8[c]
S_1A	50.0±1.7[a]	73.9±2.1[c]	86.6±3.4[b]
S_2A_0	23.3±1.9[d]	43.5±2.2[d]	60.0±2.5[c]
S_2A	40.0±1.1[c]	56.6±1.6[a]	83.4±2.7[ba]

Values in each column followed by different superscripts (a, b, c) indicate statistically significant differences in the values (p<0.05); Values are mean ± SE; (S_1 = 55 mM Na^+, S_2 = 110 mM Na^+, S_0 =No salt; A=Amended with Cyanobacterial EPS, A_0=Without EPS amendment)

Table - 3: Effect of cyanobacterial EPS amendment on mobilization efficiency of various crops under different salt concentrations

Treatment	Mobilization efficiency (%)		
	Wheat	Maize	Rice
S_0A_0	91.6±6.6[a]	97.4±11.6[a]	110.2±9.1[a]
S_0A	94.2±7.5[a]	107.7±9.04[a]	115.3±8.6[a]
S_1A_0	75.1±8.7[b]	64.2±10.3[b]	100.6±11.2[a]
S_1A	92.6±8.8[a]	95.7±14.9[a]	155.5±10.6[b]
S_2A_0	65.1±5.8[c]	64.7±7.02[b]	122.6±4.6[c]
S_2A	79.8±4.7[b]	75.7±7.6[b]	126.0±4.04[c]

Values in each column followed by different superscripts (a, b, c) indicate statistically significant differences in the values (p<0.05); Values are mean ± SE; (S_1 = 55 mM Na^+, S_2 = 110 mM Na^+, S_0 =No salt; A=Amended with Cyanobacterial EPS, A_0=Without EPS amendment)

Table - 4: Effect of cyanobacterial EPS amendment on total chlorophyll concentration in the seedling leaves of three crops under different salt concentrations

Treatment	Chlorophyll concentration (μg ml^{-1})		
	Wheat	Maize	Rice
S_0A_0	504±5.83[a]	868±7.64[a]	188±2.65[a]
S_0A	560±5.24[b]	1076±12.23[b]	204±3.47[b]
S_1A_0	428±3.90[c]	296±3.63[c]	128±1.80[c]
S_1A	556±3.86[b]	304±2.63[c]	196±2.42[a]
S_2A_0	424±3.55[c]	208±2.29[d]	120±1.37[d]
S_2A	520±3.35[d]	276±3.77[e]	188±1.96[a]

Values in each column followed by different superscripts (a, b, c, d) indicate statistically significant differences in the values (p<0.05); Values are mean ± SE; (S_1 = 55 mM Na^+, S_2 = 110 mM Na^+, S_0 =No salt; A=Amended with Cyanobacterial EPS, A_0=Without EPS amendment)

Table - 5: Effect of cyanobacterial EPS amendment on vigor index of various crops under different salinity levels

Treatment	Vigor index		
	Wheat	Maize	Rice
S_0A_0	208±3.93[a]	520±8.25[a]	398±5.24[a]
S_0A	240±3.34[b]	877±11.65[b]	960±14.45[b]
S_1A_0	172±1.47[c]	278±2.16[c]	278±2.22[c]
S_1A	199±0.95[d]	283±2.09[d]	422±4.12[d]
S_2A_0	124±1.25[e]	159±1.80[e]	222±3.76[e]
S_2A	103±2.70[f]	196±2.78[f]	412±6.51[d]

Values in each column followed by different superscripts (a,b,c,d,e,f) indicate statistically significant differences in the values (p<0.05); Values are mean ± SE ; (S_1 = 55 mM Na^+, S_2 = 110 mM Na^+, S_0 = No salt ; A=Amended with Cyanobacterial EPS, A_0=Without EPS amendment); Vigor index = % germination X axis length due to biosorption. Thus the osmotic as well as ionic effect of Na^+, which otherwise have an inhibitory effect on germination, seedling growth and chlorophyll concentration (Fernandes *et al.*, 1993) get substantially reduced due to the fact that after betting bound to the EPS, Na^+ ions are no more available as free ions in the medium. The cyanobacterial exudates also boost up germination and seedling growth of the crop species when no salts are present, indicating growth stimulating factors present in the EPS. Similar stimulation of crop growth has earlier been reported by Misra & Kaushik (1989). Thus application of cyanobacterial exopolysaccharides or extracts to seeds can significantly improve seed germination and seedling growth in different crops and can be particularly useful in salt-affected soils. The EPS seems to play a crucial role by binding the hazardous Na^+, thereby alleviating the adverse effects of salts on crops. While application of biofertilizer is known to be useful, the EPS produced by the cyanobacteria in the biofertilizer seem to play an important role. Based on the present study, further studies may be conducted to examine the possibility of further improving seed germination and seedling growth under saline conditions by direct pre-treatment of the seeds with EPS.

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