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ORIGINAL ARTICLE

Isolation and Quantification of EPS Produced by Rhizobacteria of Cluster Bean under Abiotic Stress Conditions

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ABSTRACT
Abiotic stresses such as drought and salinity have an adverse impact on agricultural productivity limiting crop yield. Rhizobacteria are known to possess multifarious plant growth promoting traits which help plants in tolerating such abiotic stresses. Among these various plant growth promoting traits, one of the ways in which microbes help plant to tolerate abiotic stresses is through the production of exopolysaccharide (EPS). The present study focuses on isolation of EPS producing bacteria from the Rhizospheric soil of cluster bean under abiotic stress conditions i.e Drought and Salinity. In total 10 isolates showed high tolerance to drought (up to -0.73 MPa water potential) and salinity (2.0 M NaCl). These isolates were further analyzed for their EPS producing capability under stress condition and screened using gravimetric analysis of EPS dry weight and quantification assay for total carbohydrate content by phenol sulphuric acid method. Four isolates coded as KM1, KM6, AK17 and MN40 produced significantly higher EPS compared to other bacterial isolates and the EPS production increased with increase in stress levels. These 4 isolates were identified as Bacillus and Pseudomonas species on the basis on their morphological and biochemical behavior. Quantification of EPS by phenol sulphuric acid method shows that isolate AK17 was highest EPS producer with 2.7g/100ml of EPS at -0.73 MPa water potential and at 1.6M NaCl concentration isolate KM6 showed highest EPS production i.e 2.0 g/100ml. The results indicate that isolates are having potential for producing high level of EPS and further field study could establish their positive role in alleviating abiotic stresses in plants.
***Keywords**: EPS, Drought, Salinity, Bacillus spp, Pseudomonas spp*

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INTRODUCTION

Agricultural production and productivity are highly vulnerable to the effects of abiotic stresses. Major abiotic stresses comprises of drought, salinity, temperature extremes, floods, low pH/acid sulfate conditions and nutrient deficiencies [1, 2, 3]. Abiotic stresses can reduce the yield of crop by up to 50% [4]. With the global climate change, the intensity as well as the adverse impact of these abiotic stresses has increased manifold, which in turn further limit the agricultural productivity. Water stress adversely affects various physiological characteristics of plants including the photosynthetic capability [5]. Prolonged exposure to water stresses results in reduction of leaf water potential and stomatal opening, suppresses root growth, reduces size of leaf, reduces size, number and viability of seed, delay in fruiting and flowering thus limiting overall plant growth and productivity [6, 7] . Salinity also adversely affects plant growth and productivity [8]. Salinity causes ion toxicity within plant cells and prolongs exposure to salinity caused disruption of osmotic balance. Combined effect of these ionic as well as osmotic shocks has an adverse impact on plant growth and development [9].

Microorganisms colonizing the rhizosphere/endo-rhizosphere area play a significant role in alleviation of environmental stresses in plants. One of the mechanism through which microorganisms help plants tolerate environmental stresses is by secretion of exopolysaccharides(EPS). Although bacteria produce EPS to protect itself against extreme environmental condition (i.e drought, high temperature, salinity and UV radiations), plants also benefits from produced EPS [10]. EPS help to mitigate salinity stress by reducing the content of Na+ available for plant uptake [11]. Furthermore, EPS producing microbes helps

in increasing soil aggregation resulting in retention of moisture in the rhizosphere, thereby increasing nutrient uptake by plants and protect from drought stress [12]. Association of EPS producing bacteria with the roots of the plant result in biofilm formation around the roots and the soil. This sheath or biofilm formation helps to stabilize soil aggregates, increases water flow across plant roots and nutrient uptake by plants thus promoting overall plant growth (13, 14). In this study we aim at isolating the most efficient EPS producing bacterial colony under Abiotic stresses i.e drought and salinity from the Rhizospheric region of cluster bean.

MATERIAL AND METHODS

Sample Collection: Rhizospheric soil sample of cluster bean were collected from three different regions of Gujarat. Soil samples were collected in sterile plastic bags and transferred to laboratory for further study.

Isolation and Purification: Serial dilution method was used for isolation of bacteria from soil sample. Dilution up to 10^7 fold was done and aliquots (100 ml) of the diluted sample were spread on the Nutrient agar media. The best growth culture were selected and purified. The purified cultures were stored at 4 ^{0}C for further use.

Screening for tolerance to Abiotic stresses: Purified bacteria were screened out on the basis of their drought and salt tolerant ability. Isolated bacteria were tested for their drought tolerance by growing them under different water potential (-0.05 MPa, -0.15 MPa, -0.30 MPa, -0.49 MPa, -0.73 MPa) using polyethylene glycol (PEG 6000) and for salt tolerance by using different molar concentration of NaCl (0.2 M to 2.0 M). Bacterial colonies showing best result under both the stress were selected and further screened out for EPS production.

Selection of EPS producing bacteria under Abiotic stress: Bacterial colonies showing ability to tolerate drought and salt stress were further analyzed for EPS production under normal and stress condition. For this the selected colonies were first cultivated on the nutrient agar media supplement with 5% sucrose and incubated for 3 days at 30 ^{0}C. Colonies showing thick mucoid like appearance on plates were selected (15). Then qualitative and quantitative analysis of EPS production under stress and unstressed condition were carried out.

Qualitative Assay: Qualitative Assay was done using dry weight of EPS produced by selected bacteria cultures in liquid medium. An inoculum of each culture was prepared by growing bacteria for overnight in liquid medium. When bacterial suspension reached 1.5 x 10^8 bacteria/ml the inoculum were inoculated in already autoclaved 50 ml nutrient medium supplemented with 5% sucrose and different concentration of PEG 6000 and NaCl. These flasks were kept in rotary shaker at 30 ^{0}C for 3 days at 120 rpm. Then the culture were centrifuged at 10000 rpm for 15 minutes to obtain cell free supernatant by adding three volume of pre chilled acetone and precipitated after refrigerating at 4 ^{0}C overnight. The precipitates were collected on a pre dried filter paper and dried at 60 ^{0}C to constant weight. Now the EPS were estimated by measuring the total dry weight of the dry precipitate (16 ,17)

Quantitative Assay: Quantification of EPS was done by estimating the total carbohydrate content in the precipitated EPS by using phenol sulphuric acid method (18). The precipitated EPS obtain after adding three volume of ethanol were washed and dissolved in distilled water. From this dissolved EPS sample 1ml was taken. To this sample, 0.5 ml of 5% phenol and 3.5 ml of concentrated sulphuric acid were added and incubated at 30-40 ^{0}C for 10-20 minutes in hot water bath. Absorbance was taken at 490 nms and the amount of carbohydrate was determined using glucose as standard.

Physiological and Biochemical Identification: Selected bacterial cultures were characterized morphologically and biochemically using standard methodologies as described by Gerhardt et.at(19). Morphological characterization was based on size, shape, colony morphology, capsule formation and gram staining. Different biochemical test involves Indole test, Voges Proskauer test, methyl red test, catalase test, oxidase test, production of H_2S, sugar fermentation etc.

Statistical analysis: All the experiments were performed in three replicates and analyzed statistically. Mean, standard deviations and standard errors of the means were calculated. The spread of the values shown in the figure as error bars represents standard errors of the means.

RESULT

Isolation of Rhizobacteria: Soil sample were collected from three different region of Gujarat and then serially diluted and placed on nutrient agar medium. The best grown thirty cultures were selected and purified for further study.

Screening for Abiotic stress tolerance: Isolates were tested for drought and salinity tolerance by growing them under different concentration of PEG(6000) and NaCl in liquid media. Tolerance for

drought (-0.49 MPa) was recorded for 85% of test isolates while 50% of the isolates withstood up to -0.73 MPa of water potential. With regard to salt stress 70% of the isolates were able to tolerate upto 1.2 M NaCl concentration while only 25% were able to grow at 2.0 M salt concentration. On the basis of above result 10 isolates (MN5, MN7, MN21, MN36, MN40, AK3, AK12, AK17, KM1, KM6) were selected for their EPS production capability.

EPS production under stress condition: Out of 10 isolates, only 7 showed a ropy like or mucoid forming appearances on the EPS media plates. These isolates were further investigated for Qualitative and Quantitative estimation of EPS production under non-stressed and stressed condition. Gravimetric measurement of EPS dry weight showed that in some isolates EPS production increased with increase in stress level while in other isolates there was a decrease in EPS production with increase in stress levels. Isolates KM6, AK17, MN40, KM1 showed >1.2gm/100ml of EPS production under stress condition while other isolates showed decrease in EPS production as stress increased.

Quantification was done by estimating the total carbohydrate content using phenol sulphuric acid method. A significant increase in EPS production was observed in salt stress (0.4 to 1.6M NaCl) and drought stress (-0.05 to -0.73 MPa) as compared to non stressed condition. Strains AK17 and KM6 produced highest amount of EPS under drought stress condition (-0.73 MPa) i.e 2.7 g/100ml and 2.3 g/100ml respectively followed by KM1 and MN40 i.e 2.0 g/l and 1.8 g/l respectively(Fig 1). Under salt stress (1.6 M) higher EPS production was noticed in KM6 (2.0g/100ml) followed by AK17 but at 2.0M salt stress EPS production was decreased in all four strains.(Fig 2).

Physiological and Biochemical identification: Four strains were selected on the basis of their drought and salt tolerance ability and EPS production under stressed and non stressed condition. These strains were KM1, KM6, AK17 and MN40. These strains were further identified by Bergey's Manual of Determinative Bacteriology on morphological, microscopic and biochemical characteristics. On the basis of their morphological and biochemical traits these strains were identified as belonging to *Bacillius* and *Pseudomonas* species (Table 1).

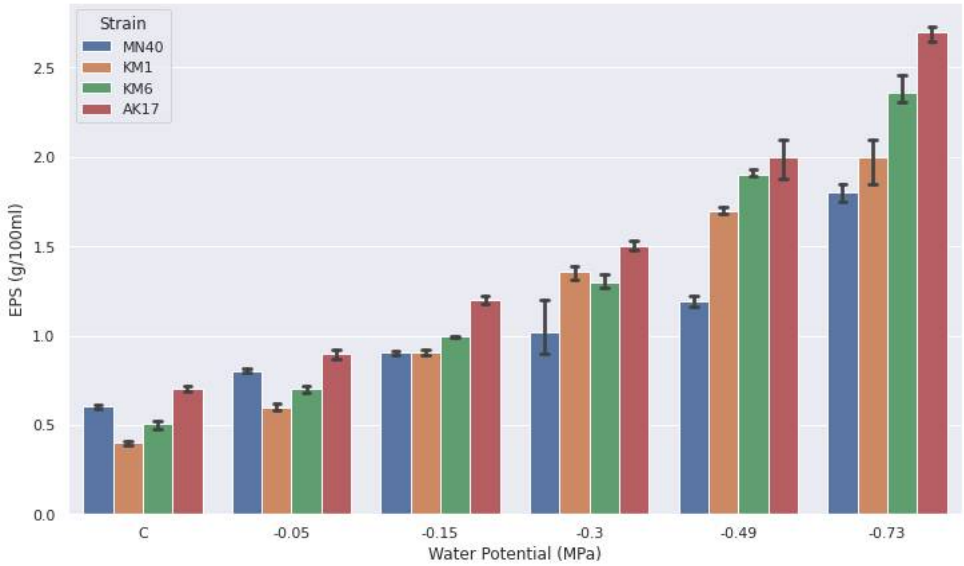

Fig. 1. EPS production by isolates under control (c) and different levels of Drought stress.

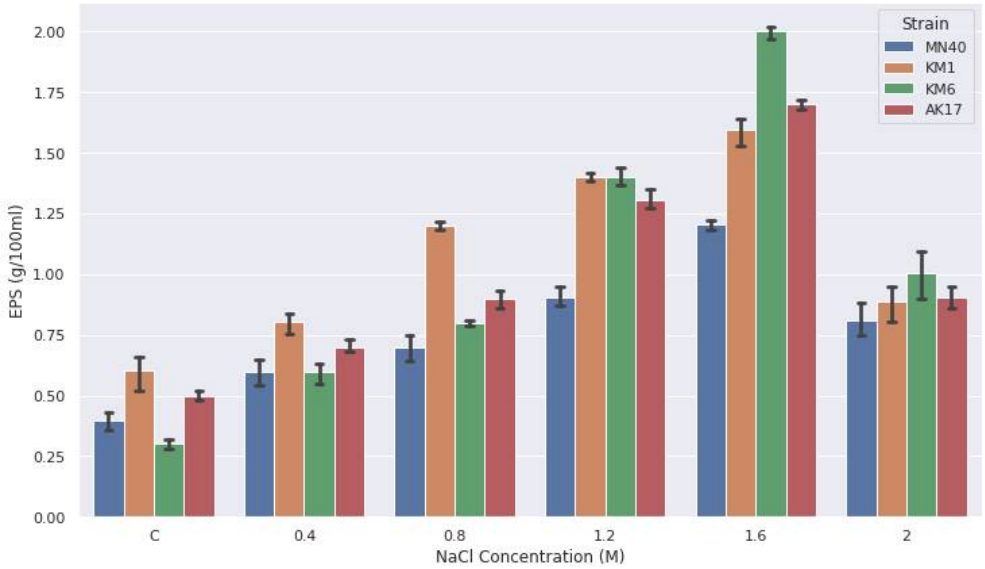

Fig. 2. EPS Production by isolates under control (c) and different levels of salt stress.

Table 1: Morphological and Biochemical characterization of EPS producing bacteria

Test	**MN40**	**KM1**	**KM6**	**AK17**
Morphological Characteristic				
Margin	Irregular	Irregular	Irregular	Even
Elevation	Convex	Flat	Convex	Convex
Consistency	Moist	Dry	Moist, Mucoid, Shiny	**Dry**
Motility	+	+	+	+
Pigmentation	-	-	-	-
Endospore Formation	+	-	-	+
Gram Nature	**+**	**+**	**+**	**-**
Opacity	Opaque	Opaque	Translucent	Opaque
Biochemical Test				
Voges-Praoskauer	+	+	+	+
Indole	-	-	-	-
Methyl Red	+	+	-	-
Catalase	+	+	+	+
Oxidase	-	+	-	-
Citrate	+	+	+	+
Starch hydorlysis	+	+	-	+
Gelatin hydrolysis	+	-	+	+
N_2S production	-	-	-	-
Nitrate reduction	-	+	+	+
Glucose	+	+	+	+
Galactose	+	-	+	-
Mannitol	+	+	+	-
Rhamnose	-	-	-	-
Arabinose	-	+	+	-
Sorbitol	+	+	-	-
Raffinose	+	+	+	+
Xylose	-	-	-	-
Fructose	-	+	-	+
Maltose	-	+	+	-
Sucrose	+	+	+	+

DISCUSSION

Abiotic stresses such as drought, salinity, high temperature etc result in a decrease in agricultural productivity (20, 21). Microbial population inhabiting naturally in soil have capability to overcome these extreme environmental condition (22, 23). The role of these Rhizospheric bacteria such as *Bacillus* (24,25, 26, 27), *Psuedomonas* (28, 29) has been reported for mitigating multiple types of abiotic stresses and thus help in plant growth. So rhizobacteria gain importance in overcoming these abiotic stresses. It has been reported that the production of exopolysaccharide (EPS) act as stress response and survival mechanism for many bacteria (30). Similarly other workers (31,32) found that the production of EPS and Bioflim development result in tolerance against various stresses. So in the present work, we aimed to isolate bacteria which were able to tolerate abiotic stresses and produce EPS under such harsh condition, as the EPS produced by microorganism help them to survive under low water potential and other stresses.

Bacteria were isolated from the Rhizospheric soil of cluster beans and screened out on the basis of their drought and salt tolerance ability. Some isolates screened out were able to tolerate drought stress up to -0.73 Mpa water potential and salinity up to 2.0M NaCl concentration. Other workers like Zhang et al. (2018) reported that their bacterial isolates were able to tolerate a salt concentration up to 2.5 M (33) while Sharma et.al (2015) found that their bacterial isolates have salt tolerance to more than 0.6 M NaCl (34). A.Susilowati et.al (2018) reported that their isolates *Bacillus licheniformis, Bacillus megaterium* and *Bacillus pumilus* were able to tolerate water potential up to -2.0 Mpa and consider as highly drought tolerant species (35). Similarly Ansari et.al find that their isolate FAP3 (*Pseudomonas spp*.) shows higher CFU under all stresses i.e water, temperature and salt stress and enhanced activity help in plant growth promotion (36).

Selected bacterial isolates having capacity to tolerate high drought and salt stress were further tested for EPS production capability under stress condition as it has been reported that the exopolysaccharide(EPS) production by Rhizosphere bacteria is one of the important mechanisms in striving drought tolerance. EPS produce by microorganism under stress condition get accumulated on the cell surface, cause cells to aggregate and thus protect the membrane by stabilizing it under harsh environmental conditions. It also help in soil aggregation and thus improves soil water holding capacity and fertility (37, 38). Isolates showing ropy or mucoid like appearance on plates were tested for EPS production on liquid medium. Gravimetric measurement of EPS dry weight was done and it showed that 4 isolates KM1, KM6, AK17 and MN40 produced >1.5gm/lt EPS under stress condition. Sandhya et al reported an increase in the production of EPS by *Pseudomonas putida* GAP-P45 with increase in abiotic stresses i.e drought, temperature and salt stresses (39). Similarly *Rhodopseudomonas acidophila* showed an increase in EPS production under high salt concentrations as reported by Sheng et al. (40). Quantification of EPS was done by calculating total carbohydrate using phenol sulphuric acid method. Our result reveals that there is increase in EPS production with increase in stress level. It has been reported earlier that when Bacterial sand cultures are exposed to desiccation there is an increase in EPS production, signifying that EPS confers some competitive advantage on microorganism during desiccation.(41).Isolate AK17showed a significant increase in EPS production under drought(-0.73MPa) and isolate KM6 at salt stress(1.6 M). Physiological and Biochemical data shows that isolates KM1, KM6 and MN 40 are *Bacillus spp*. while AK17 is *Pseudomanas spp.* Sen *et. al* (2014) also found that their isolate *Pseudomonas spp*. PMDzncd2003 showed better EPS producing capability and root colonizing capacity thus helping in tolerance to salinity [42]]. Similarly Khan *et al* [43] found that inoculation of *Bacillus pumilus* improved rice growth even in salinity stress. Vardharajula *et al*. [26] also found an increase in EPS production under water stressed condition compared to non-stressed condition by *Bacillus amyloliquefacaciens*. Increase in EPS result in microaggregates formation, thus improve plant growth and RAS/RT (Root-adhering soil/ root tissue) ratio under drought stress.

CONCLUSION

This study aimed at isolating the bacteria having highest EPS producing ability under stressed conditions of drought and salinity. Four strains having highest EPS production ability under such stresses were screened and identified as *Bacillus* and *Pseudomonas spp* based on their morphological and biochemical traits. EPS molecules play an important role in providing energy and nutrients in polysaccharide production and protecting *Bacillus* and *Pseudomonas spp*. cells against salt and water limited environments and thus help in improving soil structure. As such, these isolates may serve as effective bioinoculant for mitigating abiotic stresses in agriculture field.

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