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Isolation, Characterization of Salt Tolerant *Azotobacter* and its Potential Role in Promoting Seed Germination of Indian Mustard Under Salt Stress

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ABSTRACT

Indian mustard (*Brassica juncea*) is one of the important oilseeds produced in India. It is used as an ingredient in the preparation of various cuisines and used as a green manure by vegetable growers. Overuse of chemical fertilizers, poor irrigation facilities, and other anthropogenic activities has led to increased salt concentrations in the soil. Salt stress has been found to decline the growth and yield of Indian mustard. Salinity negatively affects seed germination, which is the first stage of the plant's life cycle. Excessive salt has a huge impact on plant physiology. Mangrove soil provides shelter to various halotolerant plant growth-promoting bacteria like *Azotobacter,* which may provide a tool for sustainable crop improvement due to their multifarious features. Two fast-growing efficient isolates of halotolerant *Azotobacter* spp viz., AI, and AII were isolated from mangrove rhizospheric soil. Microscopic and biochemical characteristics of the isolates were studied. Both isolates showed PGP activities like phosphate solubilization, IAA, and ammonia production. A1 isolates tolerated salt concentration up to 0.5% and A2 till 2%. Seed germination parameters of uninoculated and isolate inoculated seeds under salt stress (0%, 0.1%, 0.2%, 0.3% and 0.4% of NaCl) were recorded. Salt stress significantly affected the germination traits. However, inoculation favored germination of mustard seeds in comparison to uninoculated ones under salt stress.

HIGHLIGHTS

- *Azotobacter* isolates A1 and A2 exhibited plant growth-promoting features like Ammonia production, Phosphate solubilization, and Indole acetic acid production.
- Isolates A1 and A2 tolerated salt concentrations up to 0.5% and 2%.
- Germination of seeds not inoculated with *Azotobacter* isolates reduced significantly with increasing concentrations of salt.
- Bioinoculation with isolates A1 and A2 boosted germination of seeds under salt stress.

Keywords: Indian mustard, salt stress, sustainable, halotolerant, *Azotobacter,* seed germination

In India, mustard seeds are used as one of the major ingredients in foods. Mustard seeds contribute 28.6% of the total oilseed production in India and rank second after groundnut in the Indian oilseed economy (Shekhawat *et al*. 2012). Oilseeds account for 14.1% of the total cropped area in India, out of which 3% are occupied by Mustard (Shekhawat *et al*. 2012). The areas of India where this commercially important crop is being cultivated are experiencing adverse soil conditions. Excessive use of fertilizers and other anthropogenic activities have turned arable lands saline. Salt stress profoundly affects the

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germination, growth, and productivity of mustard (Yousuf *et al*. 2017). High salt concentrations may cause inhibition of and delayed seed germination and also seed establishment (Cuneyt Ucarh 2020). Seed germination, seedling emergence, and early survival are particularly sensitive to substrate salinity (Sharma *et al*. 2013). Salt stress on seed germination may be attributed to either the osmotic effect and/or to specific ion toxicities to radicle emergence of seedling development (Sharma *et al*. 2013). Salt stress causes osmotic effects, decreased biosynthesis of chlorophyll and inefficiency of photosynthesis, nutrient deficiency, and toxicity by decreasing the water and mineral uptake and by excessive accumulation of Na^+ and Cl^- ions (Pillai 2011; Shah 2006).

Mangroves grow at the interface of land and sea in tropical and subtropical latitudes, where they exist in conditions of high salinity. The Rhizospheric region of mangroves harbors different plant growth-promoting bacteria, including *Azotobacter* spp (Sahoo and Dhal 2009). *Azotobacter* is an efficient biofertilizer enhancing seed germination, growth, and development by fixing atmospheric nitrogen and producing phytohormones, amino acids, vitamins, etc. (Pillai 2011). Further, the accumulation of compatible solutes by these species improves their salt tolerance (Klahn *et al*. 2009).

The present study deals with the isolation and characterization of halotolerant *Azotobacter* spp from salt-stressed mangrove rhizosphere and assessing its potential in promoting germination of mustard seeds under salt stress.

MATERIALS AND METHODS

Soil sampling

Soil sampling was carried out at the mangrove forest of Mansarovar, Navi Mumbai. Samples were collected from the rhizospheric region of mangrove plants at a depth of 10-15cms. Collected samples were refrigerated at 4°C.

Isolation of *Azotobacter* spp

0.5gm of soil sample was serially diluted up to 10^{-4} dilution. 0.1 ml of each dilution was spread plated on sterile N free Jensens agar medium containing per liter of distilled water: 20g sucrose, 1g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g NaCl, 0.001g Na_2MoO_4 , 0.01g $FeSO_4$,2g $CaCO_3$, 18g Agar, pH 7.0-7.2. After plating the samples, the medium was incubated at 30°C for 24-48 hours. Single, large and fast-growing colonies were selected and purified by repeated streaking on N free Jensen's media. The purified colonies were enriched in N-free Jensen's broth.

Characterization of isolates

Colony characteristics were studied on N-free Jensens agar medium. Microscopic characterization included Grams staining, Capsule staining (Manewal's method), and PHB staining (Sudan Black B staining). Biochemical characteristics of the isolates such as carbohydrate utilization, catalase test, and production of enzymes like amylase, urease, and cellulose were studied.

Screening for plant growth-promoting activities

Ammonia production

The isolates were grown in peptone water in tubes and incubated at 30°C for 48-72 hrs. To each tube, Nessler's reagent was added. The tubes were observed for the development of yellowish to brown color, indicating production of ammonia.

Phosphate solubilization

Phosphate solubilization test was done as per the method described by Aneja, (1996). The isolates grown on N free Jensen's agar were spotted on to sterile Pikovaskaya's agar medium containing per liter of distilled water: 10g Glucose, 0.5g Yeast extract, 0.5g $(NH_4)SO_4$, 0.2g KCl, 0.2g NaCl, 0.1 g $MgSO_4.7H_2O$, 0.002g $FeSO_4.7H_2O$, 5g $Ca_3(PO_4)2$, 18 g Agar, pH 7.0-7.2. The plates were incubated at 30°C for 48hrs and checked for the development of clear zones around the test colonies.

IAA production

IAA production was detected by the method described by Brick *et al*. (1991). A loopful of the culture was inoculated into 10ml sterile N free Jensen's broth amended with 100µg/ml of tryptophan and was incubated for 48 hrs at 30°C on a rotary shaker. After incubation, it was centrifuged at 10,000g for 15 minutes. 2 to 3 drops of O-phosphoric acid were added to 2ml of the

supernatant. Further, 4ml of Salkowski reagent (50ml of 35% perchloric acid, 1ml of 0.5M $FeCl_3$) was added to the aliquot, and the samples were incubated for 25 minutes at room temperature. The formation of pink color indicated IAA production.

Salt tolerance of the isolates

The bacterial isolates were spotted on sterile N free Jensen's Agar plates with various concentrations of NaCl (0% to 5% at intervals of 0.5%). The plates were checked for growth after incubation at 30ºC for 48-72 hrs.

Bioinoculation of mustard & seed germination assay

Mustard seeds were surface sterilized and inoculated with *Azotobacter* isolates by keeping the seeds immersed in culture suspensions of respective isolates for about 1 hour. Inoculated seeds were sown in different pots filled with sterilized soil with varying salt concentrations (0%, 0.1%, 0.2%, 0.3% and 0.4% of NaCl). Control was similarly set up using uninoculated seeds without the addition of salt in the soil.

Seed germination assay included seed germination percentage, germination index, germination energy, and relative germination rate determined by the following formula (Li, 2008).

$$\text{Germination percentage} = (a/b)*100;$$

$$\text{Germination index} = \Sigma\ Gt/Dt;$$

$$\text{Relative germination rate} = c/d$$

Where a = total number of germinated seeds; b = total number of seeds to germinate; Gt = germinated seeds int days; Dt = the number of germination days corresponding; c = germination percentage in salt treatment d = germination percentage in control experiments

RESULTS AND DISCUSSION

Microscopic and biochemical characteristics of the isolates

Various isolates of *Azotobacter* were obtained from rhizopsheric soil samples of mangrove forests. Two dominant, large and fast-growing colonies from N free Jensen's Agar medium were selected for the studies, which were designated as A1 and A2 isolates. Growth (colony), microscopic and biochemical characteristics of the isolates were recorded (Tab 1).

Table 1: Microscopic and Biochemical characteristics of *Azotobacter* isolates

Characteristics	A1 isolate	A2 isolate
Size	4 mm	2mm
Shape	Circular	Circular
Colour	Colourless	Light yellow
Margin	Entire	Entire
Opacity	Translucent	Translucent
Elevation	Convex	Convex
Consistency	Mucoid	Mucoid
Gram Nature	Gram –ve rods	Gram –ve rods
Capsule Staining	Capsulated	Capsulated
Polysaccharide production	+	+
Catalase test	+	+
PHB production	+	+
Amylase production (Starch hydrolysis)	+	+
Cellulase production	-	+

Key: *+ ve positive, - ve negative.*

Plant growth-promoting traits

The isolates A1 and A2 showed ammonia production, solubilization of phosphate, and synthesis of IAA (auxin) (Tab 2). Solubilized phosphates and ammonia serve as important nutrients for plant growth. Auxins like IAA may stimulate seed germination, control plant architecture and are involved in stress responses (Zhao *et al*. 2020). The exhibition of these traits indicates the plant growth-promoting potential of the *Azotobacter* isolates.

Table 2: Plant growth promoting Activities of *Azotobacter* isolates

Characteristics	A1 isolate	A2 isolate
Ammonia production	+	+
Phosphate Solubilization	+	+
IAA production	+	+

Key: *+ ve positive, - ve negative.*

Salt tolerance of the isolates

Tolerance of the isolates A1 and A2 to different concentrations of NaCl was studied (Tab 3). Isolate A1 tolerated up to 1% NaCl concentration and isolate A2 up to 2%. The tolerance feature of the isolates is an adaptation shown by the isolates which were thriving in the salty mangrove sediments. The PGP characteristics of the isolates and their resistance to salt concentrations show their capability to be used as a biofertilizer for plants exposed to saline stress.

Table 3: Salt tolerance of *Azotobacter* isolates

Salt Concentration (% NaCl)	A1 isolate	A2 isolate
0.5	+	+
1.0	+	+
1.5	-	+
2.0	-	+
2.5	-	-
3.0	-	-
3.5	-	-
4.0	-	-
4.5	-	-
5.0	-	-

Key: *+ ve positive, - ve negative.*

Seed germination traits under salt stress

Salt stress significantly affected the germination traits of Indian mustard. The germination percentage of all inoculated and uninoculated seeds, declined with increasing salt concentrations (Tab 4; Fig. 1). Similarly, germination index showed a reduction with increasing salinity (Tab 4; Fig. 2). Likewise, salinity influenced the relative germination rate of all seeds (Tab 4; Fig. 3). The reduction of these parameters, depicts the deleterious effects of salt stress on seed germination. Higher concentrations of NaCl retard the seed germination potential. However, in all the cases, it can be observed that the inoculated seeds performed very well in comparison to the uninoculated ones. These enhanced germination parameters of inoculated seeds is because of bioinoculation of *Azotobacter* isolates A1 and A2. The halotolerant isolates resisted the salt stress conditions and may have conferred some amount of resistance to the seeds as well. *Azotobacter* species are found to accumulate compatible solutes as a strategy for salt acclimation (Klahn *et al*. 2009). Also, the phytohormone auxin produced by the isolates and other PGP activities might have triggered the survival of seeds and their overall performance. The inoculated seeds showed

Table 4: Effect of *Azotobacter* inoculation on parameters of seed germination under salt stress conditions

Inoculation	Salt Concentration (%)	Germination %	Germination index	Relative germination rate
Uninoculated	0	90	3.91	1
	0.1	50	1.66	0.55
	0.2	40	1.41	0.44
	0.3	30	1.16	0.33
	0.4	20	0.83	0.22
Isolate A1 inoculated	0	90	4.5	1
	0.1	80	3.66	0.88
	0.2	70	3.16	0.77
	0.3	60	2.66	0.66
	0.4	60	2.41	0.66
Isolate A2 inoculated	0	90	4.5	1
	0.1	80	3.66	0.88
	0.2	80	3.41	0.88
	0.3	60	2.66	0.66
	0.4	60	2.0	0.66

40 percent more germination in comparison to uninoculated seeds.

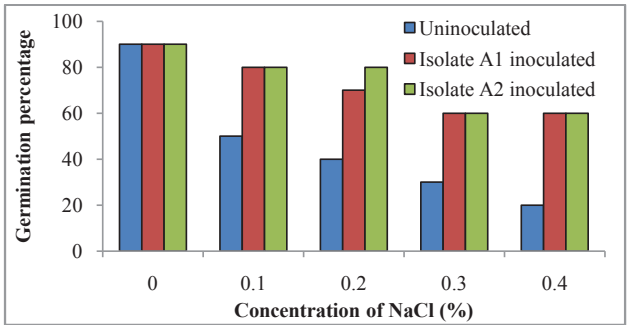

Fig. 1 Germination percentage of *Azotobacter* inoculated and unioculated mustard seeds under salt stress conditions

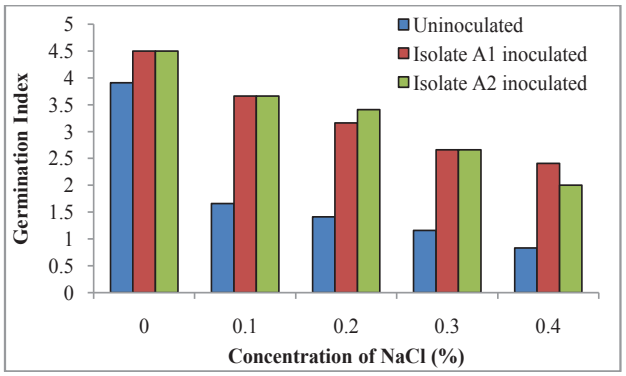

Fig. 2 Germination index of *Azotobacter* inoculated and unioculated mustard seeds under salt stress conditions

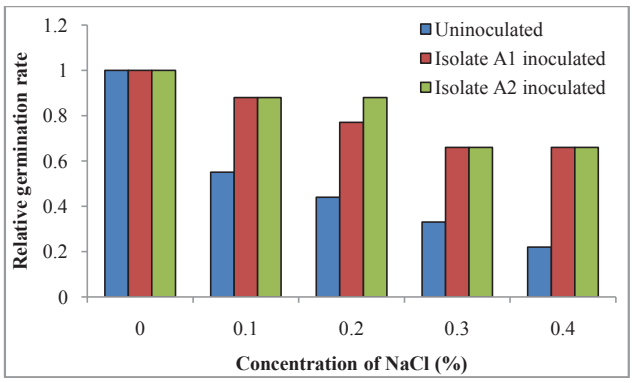

Fig. 3 Relative germination rate of *Azotobacter* inoculated and unioculated mustard seeds under salt stress conditions

CONCLUSION

The overall results indicate the suitability of the *Azotobacter* isolates A1 and A2 as a potential biofertilizer for enhancing seed germination in Indian mustard in different salt-affected areas. It would provide a cost-effective and eco-friendly tool for salt stress mitigation. The impact of inoculation on the growth and productivity of crops under salt stress needs to be evaluated.

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