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Isolation and Screening of Beneficial Endophytic Bacteria from Rice Grown under Coastal Saline Soils

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A B S T R A C T

Salinity causes disturbance in symbiotic performance of plant and increases susceptibility of plants to soil-borne pathogens. Endophytic bacteria which are associated with their host plants have a beneficial effect for many different plant species and are determinant of cross-tolerance to biotic and abiotic stresses in plants. To maintain the growth and development of crops in saline condition, plant growth promoting rhizobacteria (PGPR) were isolated, and detected their plant growth promoting (PGP) potential under salt stress was investigated. In this study, fifteen endophytic bacteria were isolated from aerial parts of the rice which were grown in different regions of coastal soils. It was concluded that isolates Ri 4 and Ri 12 exhibited higher IAA production, isolates Ri 4 and Ri 5 showed better solubilization in phosphorous, Ri 3 and Ri 4 showed better solubilization of potassium. The result of this study can be used for further investigation in enhancing crop production and maintaining soil health in coastal saline soil environment.

Introduction

Rice (*Oryza sativa*) is one of the major staple food crops consumed globally and its production is highly affected by salinity. In India, nearly 9.38 million ha area is occupied by salt -affected soils out of which 5.5 million ha are saline (including coastal regions). Better management practices are needed to improve the productivity and quality of such low productive salty soils. Under the saline conditions the growth and development of rice are reduced because of the salinity-induced changes in metabolism and acidification of apoplast which affects the turgor pressure of cell (Munns and Tester, 2008). In plants, the effect of salinity leading to inhibition of germination, difficulties in crop area establishment, leaf area development, decrease in dry matter production, delay in seed set and also even sterility can occur **(**Khatun and Flowers., 1995).

Endophytes are the group of microorganisms that colonize the internal tissues of plants

either by symbiotically or in a mutualistic relationship (Dudeja *et al.,* 2012). However some of the endophytic bacteria exert several beneficial effect on host plants, such as stimulation of plant growth, nitrogen fixation, secretion of plant growth regulators (eg: IAA, phosphate solubilization activity)and induction of resistance to plant pathogens (Hung *et al.,* 2004).Osmotic adjustment, stomatal regulation, modification of root morphology, enhance uptake of minerals and alteration of nitrogen accumulation and metabolism are some of the other effects of endophytes infection on the host plants (Zinniel *et al.,* 2008, Stoltzfus *et al.,*1997). Various researchers reported that bacteria isolated from saline environment are more likely to withstand salt stress. The present investigation was carried out to isolate and screen the beneficial endophytic bacteria from rice (*Oryza sativa*) which are cultivated in coastal saline soil regions of Tamil Nadu.

Materials and Methods

Sample collection

The samples (rice crop) were collected from different coastal regions of Tamil Nadu(Cuddalore, Puducherry, Karaikal, Nagapattinam, Ramanathapuram). The samples were asymptomatic and healthy. The sterile plastic bags were used to collect all the samples and transported to the laboratory aseptically. The pre-treatment/ surface sterilization and isolation of endophytic bacteria from the collected samples were carried out according to the method described bySun*et al.,*(2006) with some modification.

Surface sterilization

Fresh leaf sample were washed in running tap water and surface disinfection were carried out in stepwise washing in 70% ethanol for 5min, 2% sodium hypochlorite solution for 1min, 70% ethanol for 30sec and followed by two rinses of sterile water and samples were dried Araujo *et al.,* (2002). To conform whether surface sterilization was done properly, the sterilized leaf samples were imprinted in LB media or the aliquot of sterile water used in final rinse were inoculated into sterile LB broth to examine growth for overnight at 37ºC, ifno growth were observed then the disinfection process is successful.

Isolation of endophytic bacteria

After pretreatment, the samples were cut into small pieces and crushed in sterile water using pestle and mortar. About 1ml of crushed leaf sample was serially diluted up to 10^{-6} dilution and 0.1ml of aliquot from 10^{-5} and 10^{-6}were spread on sterile petri-plate containing LB media using sterilized glass rod. All the plates were incubated at 37ºC for 5 days and observations were recorded regularly in order to recover maximum amount of colonies. After incubation, morphologically different isolates were streaked on petri-plate containing LB media and incubated it for 3-5 days at 37ºC.

Morphological and biochemical characterization of endophytic bacterial isolates

Classical gram staining method was used to determined cell morphology (Bathlomew, 1962). Biochemical test for citrate utilization, starch hydrolysis, indole test, methyl red and voges-proskauer were carried out according to the procedure described by Cappuccino and Sherman (2002).

Screening for indole acetic acid producing activity

Indole acetic acid production (Glickmann and Dessaux 1995) was examined colorimetrically using Salkowski'sreagent(1 ml of 0.5M $FeCl_3$

in 49 ml of 35% perchloric acid).To measure IAA qualitatively, isolated strains were aseptically cultured in respective broth containing 100µg L-tryptophan per ml and incubated at 37ºC for 7 days.

After incubation period, the grown isolates were centrifuged at 10,000 rpm for 20 minutes, 1ml of supernatant were mixed with two ml of Salkowski's reagent and one drop of ortho-phosphoric acid and incubated at room temperature for 25-30 min. Development of pink colour showed IAA production. For quantitative analysis, the absorbance of developed pink colour was measured at 530nm and IAA concentrations were calculated by using IAA standard curve.

Quantitative determination of phosphate solubilization activity

Bacterial isolates were screened for their potential to solubilize insoluble calcium phosphate on Pikovskaya agar medium as described by Pikovskaya (1948).Fifteen endophytic bacterial strains were further evaluated quantitatively (Murphy and Riley, 1962), for their P-solubilizing ability in Pikovskaya's liquid medium in which 0.5% of tri-calcium were added as substrate. The strains were inoculated into 10ml of respective broth and incubated at 30ºC for 7 days. Un-inoculated liquid broth was taken as control. After incubation period, the samples were centrifuged at 10,000 rpm for 10min. One ml of supernatant from each sample were taken and 2ml of freshly prepared colour reagent (A and B) was added immediately and final volume were makeup to 6mlusing distilled water and incubated for 15 min to develop colour. The blue colour intensity was measured at 830nm by UV-spectrophotometer. KH_2PO_4 was used as standard and total amount of phosphate solubilization were expressed as total P release mg/L.

Quantitative determination of K release activity

Plates of modified Alexandrov´s medium were supplemented withinsoluble form of potassium (mica) Aleksandrov *et al.,* (1967). For quantitative analysis, the isolated strains were inoculated into 10ml of Aleksandrov's broth supplemented with insoluble form of K (Stanford *et al.,* 1949) and culture flask were incubated at 30ºC for 10 days. After incubation period, the samples were centrifuged at 6,000 rpm for 10 min to remove insoluble potassium. Five ml of culture filtrate was added with 25ml of 1N Ammonium acetate and the mixture samples were kept in mechanical shaker for 10 min. After incubation the samples were filtered through Whatman No.1 filter paper and the collected filtrate were fed into flame photometer to determine the K content. The amount of potassium released were calculated using standard curve in which KCl used as standard. Un-inoculated broth was used as control.

Statistical analysis

The experiments were conducted in a completely randomized block design. The mean of three replicates were used to present the results. Standard deviation used to estimate the sample variability. Analysis of variance on the data at CD(0.05%)

Results and Discussion

Endophytic bacterial isolates were isolated from rice crop that are grown in coastal saline regions of Tamil Nadu (Cuddalore, Karaikal, Nagapattinam, Puducherry, Ramanathapuram) by using LB media. Some of the endophytic strains have been isolated from aerial tissues of four agronomic crops and from prairie plant species and also from other parts of the plants such as roots(Asraful

et al.,2010), stems and seeds (Magnani *et al.*, 2010), petioles, tuber tissues and flowers (Reiter and Sesssitsch, 2004) can also be used in isolation of endophytes.

Fifteen isolates were selected for further investigation based on their growth and morphology was shown in Table 1.Gayathri*et al.*, 2010 isolated 36 bacterial strains based on different morphology from mangrove and salt marsh plants. In morphological characterization the endophytic bacterial isolates exhibit the diverse colony shape, colour, margins, elevation including round, circular to irregular colonies with regular and wavy margins.

Out of 15 isolates 6 were pigmented and 9 were non pigmented. Regarding the cell shape and gram staining, 7 gram negative bacilli, 1 gram negative cocci, 4 gram positive bacilli and 3 gram positive cocci were observed. Zinniel *et al.*, (2002) reported an equal presence of both gram positive and negative bacteria in their study about endophytic bacteria isolation. Biochemical tests were carried out in which Ri 3, Ri 4, Ri 6, Ri 7, Ri 10, Ri 11 and Ri 12 were positive in citrate utilization test, they produced blue colour in Simmon's citrate agar (Table 2).

Phosphate solubilization potential of the isolates was evaluated and the results indicated a significant variation among the isolates to solubilize different quantities of PO_4 and release of P into the broth.

Maximum P release was noticed in Ri 5 (0.747±0.003 mg l^{-1}) followed by Ri 4 (0.590±0.004 mg l^{-1}). Minimum was reported with the isolate Ri 15 (0.034±0.004 mg l^{-1}).

Table.1 Morphological characterization of endophytic bacteria isolated from aerial parts of rice crop grown in coastal saline soils

ISOLATES	GRAM REACTION	SHAPE	COLONY MORPHOLOGY			
			Form	Elevation	Margin	Colony
Ri1	gram negative	Bacilli	Circular	Raised	Entire	Translucent, slimy
Ri2	gram positive	Bacilli	Circular	Raised	Entire	Opaque
Ri3	gram negative	Bacilli	Circular	Flat	Entire	Colourless
Ri4	gram positive	Cocci	Irregular	Raised	Wavy	Dark brown
Ri5	gram negative	Bacilli	Irregular	Raised	Wavy	Light yellow
Ri6	gram positive	Cocci	Circular	Raised	Entire	Rough yellow
Ri7	gram negative	Cocci	Irregular	Umbonate	Entire	Opaque
Ri8	gram negative	Bacilli	Round	Flat	Entire	Transparent,slimy,glistering
Ri9	gram positive	Bacilli	Regular	Convex	Entire	Translucent
Ri10	gram positive	Cocci	Round	Convex	Entire	Yellow
Ri11	gram positive	Bacilli	Round	Raised	Entire	Opaque
Ri12	gram negative	Bacilli	Round	Flat	Entire	Opaque
Ri13	gram positive	Bacilli	Irregular	Undulate	Wavy	Colourless
Ri14	gram negative	Bacilli	Circular	Flat	Entire	Orange
Ri15	gram negative	Bacilli	Circular	Raised	Entire	Light brown

Table.2 Results of biochemical tests for selected endophytic bacteria from rice

ISOLATES	MR	VP	CATALASE	SIMMON CITRATE UTILIZATION	STARCH HYDROLYSIS	INDOLE
Control	-	-	-	-	-	-
Ri1	+	+	+	-	-	-
Ri2	-	+	+	+	+	+
Ri3	+	-	-	+	-	+
Ri4	+	-	+	-	+	++
Ri5	-	+	-	+	-	++
Ri6	+	-	+	-	+	-
Ri7	+	-	+	+	+	+
Ri8	-	+	+	+	-	-
Ri9	+	+	+	-	-	-
Ri10	+	-	+	-	+	-
Ri11	+	+	-	-	+	++
Ri12	+	-	+	-	+	-
Ri13	-	-	-	+	-	-
Ri14	-	-	-	-	-	-
Ri15	+	-	-	+	-	++

++ highly positive, + positive, - negative

Table.3 IAA production, P solubilization and K solubilization activity of endophytic bacterial isolates

Endophytic bacterial isolates	Phosphorous solubilization (P release mg l^{-1})	Potassium solubilization (K release mg l^{-1})	Indole Acetic Acid (µg ml^{-1})
Control	0.013 ± 0.002^{o}	12.14 ± 0.093^{k}	0.177 ± 0.001^{j}
Ri1	0.312 ± 0.004^{g}	14.18 ± 0.220^{g}	0.922 ± 0.008^{e}
Ri2	0.402 ± 0.001^{f}	15.56 ± 0.252^{c}	1.110 ± 0.009^{d}
Ri3	0.590 ± 0.004^{b}	20.14 ± 0.407^{a}	1.112 ± 0.002^{c}
Ri4	0.747 ± 0.003^{a}	19.45 ± 0.165^{b}	1.382 ± 0.009^{a}
Ri5	0.489 ± 0.005^{d}	18.56 ± 0.212^{c}	1.229 ± 0.003^{b}
Ri6	0.236 ± 0.003^{j}	14.77 ± 0.257^{f}	0.541 ± 0.006^{i}
Ri7	0.520 ± 0.006^{c}	19.74 ± 0.125^{a}	1.362 ± 0.016^{a}
Ri8	0.215 ± 0.003^{k}	13.19 ± 0.218^{i}	0.726 ± 0.011^{g}
Ri9	0.155 ± 0.004^{l}	15.15 ± 0.128^{f}	0.654 ± 0.007^{h}
Ri10	0.448 ± 0.004^{e}	12.89 ± 0.016^{i}	1.091 ± 0.003^{d}
Ri11	0.114 ± 0.004^{m}	15.7±0.220e	1.247 ± 0.008^{b}
Ri12	0.247 ± 0.001^{i}	16.05 ± 0.352^{d}	1.355 ± 0.027^{a}
Ri13	0.401 ± 0.002^{f}	16.88 ± 0.172^{h}	0.937 ± 0.013^{e}
Ri14	0.034 ± 0.001^{n}	13.57 ± 0.247^{f}	0.564 ± 0.004^{i}
Ri15	0.28 ± 0.005^{h}	14.94 ± 0.127^{j}	0.219 ± 0.001^{f}
Grand Mean	**0.324**	**15.791**	**1.001**
SE.D	**0.006**	**0.384**	**0.019**
CD(0.05)	**0.013**	**0.782**	**0.039**

The results were given in Table 3.Endophytic bacteria residing within plant tissues have been reported to promote the plant growth either directly or indirectly through the production of phytohormones and the improvement of nutritional status (Pandey *et al.,* 2008). Huang *et al.,* (2005) reported that most of the phosphate solubilizing endophytic bacteria belonging to *Bacillus, Pseudomonas, Klebsiella* and *Acinetobacter* were isolated from maize and rape plants. Thamizh Vendan *et al.,* (2010) studied the endophytes of Gingseng plants and the endophytic isolates belonging to *Bacillus cereus* and *Bacillus megaterium* showed notable P-solubilization activity by detecting extracellular solubilization of precipitated tri-calcium phosphate with glucose as sole carbon. Phosphate solubilization by *Bacillus* sp from salt stressed environment had been observed (Sun *et al.,* 2006) earlier. The amount of potash solubilized was assessed by using Flame photometer. Among the fifteen isolates, significantly higher release of K was observed in Ri3 (20.14 ± 0.407 mg l^{-1}) followed by Ri4 (19.45 ± 0.165 mg l^{-1}). Minimum was reported in Ri15 (12.89 ± 0.016 mg l^{-1}) and the results were tabulated in Table 3.Padma and Sukumar, (2015) reported that a considerably higher concentration of potassium solubilizing bacteria (KSB) are commonly found in the rhizosphere in comparison with non-rhizosphere soil.KSB are usually present in all soils and have been isolated from rhizosphere soil, non-rhizosphere soil, paddy soil (Bakhshandeh *et al.,* 2017) and saline soil (Bhattacharya *et al.,* 2016). IAA production was observed in 15 isolates of endophytic bacteria which were grown in LB broth supplemented with 0.1% tryptophan and the results were presented in Table 3. Most of the isolates exhibited significant variation in IAA production.In this study the isolate Ri 4 produced higher quantity of IAA (1.382±0.009 µg ml^{-1}) which was significantly higher than other isolates. The minimum production of IAA was recorded in Ri 6 which produced about 0.541±0.006µg ml^{-1}. Long *et al.,*(2008) reported the production of IAA by the endophytic isolates of *Solanumnigrum*. Endophytes have also been shown to promote plant growth by producing IAA (Mendes *et al.,* 2007), increases root size and distribution, resulting in greater absorption of nutrient from the soil(Li *et al.,* 2008).

In this study, fifteen endophytic bacteria were isolated from aerial parts of the rice which were grown in different regions of coastal saline soils. It was concluded that isolate Ri 4 and Ri 12 exhibited higher IAA production, isolate Ri 4 and Ri 5 showed better solubilization in phosphorous, Ri 3 and Ri 4 showed better solubilization of potassium. These endophytic bacteria have the potential for phosphate and potassium solubilization and sufficient amount of IAA production. In future studies, these isolates could possibly be utilized for bio-remediation of salt affected soils for agricultural crop production.

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