ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 6, Issue, 08, pp.7741-7745, August, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

SCREENING OF SALINITY RESISTANT RHIZOBACTERIA FROM RHIZOSPHERIC SOIL OF *CAJANUS CAJAN* L VAR. MANAK

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ARTICLE INFO

Article History:
Received 07th May, 2014
Received in revised form
15th June, 2014
Accepted 20th July, 2014
Published online 06th August, 2014

Key words:
Cajanus cajan,
Phosphate,
Antibiotic, NaCl

ABSTRACT

In the present study, rhizospheric soil samples were collected from *Cajanus cajan* cultivated field from Hailakandi district of Assam, India. A total of thirteen (13) bacterial strains were isolated and identified on the basis of their morphological and biochemical characteristics and classified as *Pseudomonas* sp., *Klebsiella* sp., *Bacillus* sp. and *Staphyllococcus* sp.. All the strains were unable to solubilize phosphate and showed sensitivity against different antibiotics such as amikacin, tetracycline, streptomycin and gentamycin. Salt tolerance was observed upto 5% NaCl concentration for all the isolates and only two isolates showed moderate growth at 10% NaCl. Since GN1 and GN7 were able to survive at high salt concentration, they may be useful in crop fields or agricultural farms where the level of salt concentration is extremely high. In this way, it can also help in the process of bioremediation.

INTRODUCTION

Pigeon pea (*Cajanus cajan* L var. Manak) a member of the family Fabaceaeis an economically important Kharif green legume crops of the tropics and subtropics. Pigeon pea is a very popular food grain in India and is a major source of protein for most of the vegetarian population worldwide. India is a principal pigeon pea-growing country contributing nearly 90% of total world's production (Dubey *et al*., 2010). The benefits of incorporating pigeon pea into cropping systems include its role as a soil ameliorant, ability to fix nitrogen and extract phosphorous, and high drought tolerance. The uses of pigeon pea are widely varied and include being a protein source for humans and livestock, windbreaks or shade for smaller crops, a fuel source, a food for commercial insects, and a versatile intercropping and rotational plant (Nene and Sheila, 1990). The term rhizobacteria is used to describe a subset of rhizosphere bacteria capable of colonizing the root environment. Microorganisms that colonize the rhizosphere can be classified according to their effects on plants and the way they interact with roots, some being pathogens whereas other trigger beneficial effects. To be an affective rhizobacteria, the bacteria must be able to colonize roots because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects (Gangwar *et al*., 2013). A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology to a greater extent, especially considering their competitiveness in root colonization (Antoun and Kloepper, 2001; Barriuso *et al.,* 2008).The rhizobacteria influence the plant physiology by uptake of nutrient, phytohormone production, nitrogen fixation and phosphate solubilizing.

Salinity is one of the major anthropogenic as well as environmental stresses that reduce growth and yield of plants. Although many new physical and chemical methods have been employed along with traditional methods to bring salt affected landmass under under irrigation, not much success is viewed anywhere. However the use of soil rhizosphere bacteria possessing the traits of plant growth promotion under saline stress is becoming prevalent worldwide to achieve sustainable agriculture along with soil reclamation through phytoremediation as well as bioremediation (Tank and Saraf, 2010).With increased salinity, ACC levels increase, resulting in high ethylene concentration that ultimately increases plant damage (Nadeem *et al*., 2009). Our results indicate that it is very likely that these PGPR strains promoted root growth by lowering the endogenous inhibitory levels of ethylene in roots because of their ACC deaminase activity along with other associated characteristics. The present investigation was undertaken to isolate and screen rhizobacteria based on their salinity tolerance, isolated from rhizospheric soil of pigeon pea plants.

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MATERIALS AND METHODS

Collection of soil sample

Soil samples were collected from the rhizospheric soil of pigeon pea (*Cajanus cajan* L var. Manak) from Hailakandi district of Assam, India. The actively growing *Cajanus cajan* plant were selected and rhizosphere soil were dug out at a depth of 10 to 20 inch. Soil samples were then collected in polythene bags with proper labelling and immediately brought to the laboratory for isolation of bacteria.

Isolation of rhizospheric bacteria

1 gram of rhizospheric soil was taken in a test tube, and 9 ml of distilled water was added to it. The test tube was shaken for 10 min. A series of dilution of the suspension was made by pipetting 1 ml aliquot into 9 ml sterilized distilled water. The serial dilution technique was performed upto 10^{-5} dilution. Samples (dilution factor 10^{-3}, 10^{-4} and 10^{-5}) were then inoculated onto petridish containing 18-20 ml Nutrient Agar (NA) media and kept in an incubator at 37^0C for 48 hrs. To enumerate the bacterial cultures, standard plate count method was used. The number of viable bacterial cells per unit volume of a sample in NA media was enumerated using a colony counter and expressed as cfu (colony forming units) per gram of soil.

Identification of bacteria

Fine isolated and distinct colonies were picked up and streaked freshly on NA plates and incubated at 37^0C. After the recovery isolates in pure form, the rhizobacteria were identified on the basis of the standard protocol given by Cappuccino and Sherman (2005) and Dubey and Maheshwari (2011). Morphological characteristics of all isolates viz., Colony morphology (colour, shape, margin, elevation and surface) and cell morphology (size, shape and arrangement) were studied followed by biochemical tests viz, catalase test, indole production test, methyl red test, voges proskauer test, citrate utilization test, starch hydrolysis test, urease test, nitrate reduction test etc. as described by (Cappuccino and Sherman (2005).

Effect of salinity on bacterial growth

Isolates were tested for osmotic stress. The growth of any bacteria can be affected by the amount of water entering or leaving the cell. If the medium has low amount of solute, it is hypotonic and has high osmotic pressure on the cell. On the other hand, if the solute is high, medium is hypertonic and growth is considerably inhibited. In such cases, cytoplasm dehydrates and shrinks away from the cell wall. Nutrient Agar (NA) medium was prepared in five different conical flasks supplemented with NaCl at concentrations 0.5%, 2%, 5%, 10% and 20% and one kept as a control. The NA media were poured in petriplates, bacterial strains were streaked on each plate and incubated at 37°C for 24 hrs (Dubey and Maheshwari, 2011).The influence of NaCl concentrations on degree of inhibition of bacterial growth was recorded.

Antibiotic sensitivity and resistance pattern of the bacterial isolates

Susceptibility of microorganisms against antibiotics was determined by Kirby-Bauer disc diffusion method (Bauer *et al.,* 1966). The antibiotic discs that were procured from 'HIMEDIA' are Amikacin (AK, 30 mcg), Gentamycin (HLG, 120 mcg), Streptomycin (S, 10 mcg), Rifampicin (RIF, 5 mcg), Vancomycin (VA, 5 mcg), Tetracycline (TE, 30 mcg), Polymyxin – B (PB, 300 mcg) and Penicillin (P, 10 mcg). Antibiotic discs of different concentrations were placed over the bacterial lawns on Mueller Hinton Agar (MHA) medium and by measuring the zones of inhibition, the sensitivity of antibiotics against the isolate was determined (Cappuccino *et al*., 2005). The diameter of the inhibition zones was measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method.

Phosphate solubilizing test

Phosphate solubilization of isolates was evaluated from the ability to solubilize inorganic phosphate. Pikovskaya's agar medium containing calcium phosphate as the inorganic form of phosphate was used in assay.Bacterial culture were streaked on Pikovskaya's agar plates and kept for incubation at 28°C for 4-5 days. The appearanceof transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

RESULTS

Isolation and enumeration of bacteria

Total viable counts ranges from 17 X 10^4 (CFU/g) to 43 X 10^4 (CFU/g). A total of thirteen rhizospheric bacteria from *Cajanus cajan* L var. Manak were isolated. Colony morphology of all the isolates was examined. The surface characteristics of bacterial isolates were found to be smooth, rough, dry, glistening, shiny and colonies had a diameter of 2-3 mm. Most of the colonies, which were selected visually based on differences with naked eye, were whitish and cream colour whereas very few colonies were found to be yellow or greenish colour. Individual colonies of bacteria which varied in shape and color were picked up and purified by streaking on nutrient agar followed by their biochemical characterization. Out of the 13 strains, 69% were Gram negative rod shaped and 23% were Gram-positive rods.

Biochemical characteristics of recovered isolates

The biochemical characteristics of bacterial isolates are shown in table 1. It has been observed that all the isolates were indole negative, MR positive, VP negative and catalase positive. Bacterial culture grown on starch agar when flooded with iodine solution, a clear zone (yellowish in appearance) was observed around the colonies indicates that the isolates have the potential to hydrolyze starch present in the medium. Out of 13 recovered isolates, 5 strains (GN1, GN2, GN7, GN12 and GN13) were found to be positive for starch hydrolysis assay, whereas appearance of blue colour in rest of the test plates

signifies negative result. Isolates GN5, GN7, GN10 and GN13 have the ability to hydrolyze urea. The appearance of pink colour in urease test by these isolates infers that the organism splits urea into alkaline end products of ammonia, carbon dioxide, and water. Positive result for citrate test was detected by five isolates (GN1, GN2, GN3, GN4, GN5, GN9, GN10, GN12 and GN13) infers the ability of these organisms to utilize citrate as the sole source of carbon and energy. All the isolates were also found to produce an enzyme "nitrate reductase" resulting in the reduction of nitrate (NO_3). According to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) the strains were identified as: GN6, GN7 and GN8were identified as *Bacillus* sp., GN3, GN4, GN9, GN10, GN12 and GN13as *Pseudomonas* sp., GN1, GN2 and GN5 as *Klebsiella* sp., and GN11 was identified as *Staphylococcus* sp.

Antibiotic sensitivity and resistance pattern

All the recovered isolates were checked for antibiotic resistance and sensitivity pattern (table 2). It has been found that, most of the isolated strains were sensitive to amikacin, tetracycline, streptomycin and gentamycin. Around 46% of the isolate (GN5, GN6, GN8, GN9, GN11 and GN12) were found to sensitive towards polymyxin – B, whereas 62% of the isolated strains were sensitive to vancomycin. Isolates GN3 and GN4 does not show any inhibition zone for vancomycin, polymyxin – B and penicillin. Resistance pattern against penicillin and rifampicin was observed by all recovered isolates.

Effect of salinity on bacterial growth

The salt concentration in an environment is the major contributor to the osmotic effect of ions on growth. Bacteria require ions that are provided by salts and typically moderate salt concentrations. High salt or high sugar in the environment leads to loss of water from cells and, ultimately, to the death. Some bacteria require high level of salt surprisingly to begin growth, whereas other bacteria would be immediately killed in high levels of salt. In the present study, all isolates showed 5% NaCl tolerance, while only five strains (GN1, GN2, GN7, GN8 and GN12) showed growth at 10% NaCl concentration (table 3). It has been found that, no isolates were able to withstand higher concentration (20%) of NaCl concentration.

Solubilization of phosphate by isolated strains

All isolated bacterial strains were screened for phosphate solubilisation. It was found that no hallow zones appeared around the bacteria grownin Pikovskaya's agar media signifies their incompetency to solubilize phosphate.

DISCUSSION

In the present study, 13 rhizobacteria were isolated from the rhizosphere of pigeon pea and majority of those were rod shaped. All the isolates are positive to catalase and MR test and all the isolates are negative to the indole and VP test. Five isolates shows positive to starch hydrolysis test, citrate test and nitrogen reduction tests. The result showed some close similarity with the work of Dubey *et al*. (2010), who isolated 8

Table 1. Biochemical characteristics of the isolated strains

Bacterial Isolates	Gram staining	Shapes	Indole	MR	VP	Catalase	Starch	Urease	Citrate	NO_3 production	Suspect Organism
GN1	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	*Klebsiella* sp.
GN2	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	*Klebsiella* sp.
GN3	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	*Pseudomonas* sp.
GN4	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	*Pseudomonas* sp.
GN5	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	*Klebsiella* sp.
GN6	Gram +ve	Rod	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	*Bacillus* sp.
GN7	Gram +ve	Rod	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	*Bacillus* sp.
GN8	Gram +ve	Rod	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	*Bacillus* sp.
GN9	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	*Pseudomonas* sp.
GN10	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	*Pseudomonas* sp.
GN11	Gram +ve	Coccus	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	*Staphylococcus* sp.
GN12	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	*Pseudomonas* sp.
GN13	Gram –ve	Rod	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	*Pseudomonas* sp.

Table 2. Antibiotic sensitivity and resistance pattern of isolated bacteria

	Amikacin	Polymyxin-B	Tetracycline	Penicillin	Streptomycin	Rifampicin	Gentamycin	Vancomycin
Isolates	AK 30	PB 300	TE 30	P 10	S 10	RIF 5	HLG 120	VA 5
GN1	25(S)	11(R)	18(S)	NZ	26(S)	13(R)	29(S)	19(S)
GN2	25(S)	8(R)	24(S)	NZ	25(S)	11(R)	27(S)	17(S)
GN3	28(S)	NZ	19(S)	NZ	22(S)	7(R)	30(S)	NZ
GN4	26(S)	NZ	17(S)	NZ	20(S)	7(R)	32(S)	NZ
GN5	10(R)	14(S)	13(I)	NZ	16(S)	7(R)	24(S)	NZ
GN6	24(S)	13(S)	23(S)	10(R)	24(S)	15(R)	29(S)	18(S)
GN7	20(S)	8(R)	18(S)	NZ	23(S)	NZ	27(S)	18(S)
GN8	24(S)	14(S)	24(S)	NZ	25(S)	12(R)	29(S)	20(S)
GN9	30(S)	12(S)	22(S)	NZ	25(S)	10(R)	23(S)	19(S)
GN10	24(S)	11(R)	22(S)	NZ	25(S)	11(R)	26(S)	17(S)
GN11	23(S)	12(S)	19(S)	NZ	22(S)	NZ	24(S)	14(R)
GN12	30(S)	14(S)	22(S)	10(R)	24(S)	15(R)	26(S)	17(S)
GN13	23(S)	11(R)	22(S)	NZ	6(R)	9(R)	15(S)	10(R)

Letters in parenthesis indicates: NZ=No Zone, R=Resistance; I=Intermediate; S=Susceptible

Table 3. Effect of salinity on bacterial growth

Isolates	Control	0.5% NaCl	2% NaCl	5% NaCl	10% NaCl	20% NaCl
GN1	+++	+++	+++	+++	++	-
GN2	+++	+++	+++	+++	+	-
GN3	+++	+++	+++	+++	-	-
GN4	+++	+++	+++	+++	-	-
GN5	+++	+++	+++	+++	-	-
GN6	+++	+++	+++	+++	-	-
GN7	+++	+++	+++	+++	++	-
GN8	+++	+++	+++	+++	+	-
GN9	+++	+++	+++	+++	-	-
GN10	+++	+++	++	++	-	-
GN11	+++	+++	+++	+++	-	-
GN12	+++	+++	+++	+++	+	-
GN13	+++	+++	+++	+++	-	-

* +++ = Excellent growth; ++ = Moderate growth; + = Less growth; - = No growth

bacterial strains from root nodules of pigeon pea. The improvement of soil fertility is one of the most common strategies to increase agricultural production. Phosphorus is major essential macronutrients for biological growth and development. Microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic phosphorus of soil and make it available to the plants. The ability of some microorganisms to convert insoluble phosphorus to an accessible form, like orthophosphate, is an important trait in a PGPB for increasing plant yields (Rodriguez *et al*., 2006; Chen *et al*., 2006). Bacterial plant growth promotion is achieved by the activities of more than one plant growth-promoting traits exhibited by the associated bacterium (Lifshitz el al., 1987). In the present study, no bacterial isolates were able to solubilize phosphate in Pikovskaya's agar; thus showed incompetency when compared with the work of Dastager *et al*., (2010), who isolated bacterial strain from root-free soil of rhizosphere of cowpea, all of which were phosphate-solubilizing.

Damodaran *et al*., (2013) isolated 16 bacteria from sodic rhizospheres, from which five showed tolerance to high salt concentration (7.5 % NaCl) and among them two isolates, *B. pumilus* and *B. subtilis* had higher uptake of sodium when cultured under in-vitro conditions in 1 M NaCl solution. It has also been reported previously that bacteria isolated from saline soil are more likely to withstand saline conditions (Upadhyay *et al*., 2009). In the present study, all bacterial isolates were able to grow upto 5% NaCl, five isolates shows the in vitro efficiency to grow in 10% NaCl concentration. However, at 20% NaCl concentration, no growth was observed by any isolated strains even after 48 hrs of incubation. *Bacillus* sp. (GN6, GN7 and GN8), *Pseudomonas* sp. (GN3, GN4, GN9, GN10, GN12 and GN13), *Klebseilla* sp. (GN1, GN2 and GN5), and *Staphylococcus*sp. (GN11) were found to be resistant to penicillin and rifampicin. Vancomycin proved resistance against *Pseudomonas* sp. (GN3, GN4 and GN13), *Klebseilla* sp. (GN5) and *Staphylococcus* sp. (GN11), whereas they were susceptible to other antibiotics (amikacin, tetracycline, streptomycin and gentamycin) upto some extent. Nath *et al.* (2013) also isolated *Pseudomonas* sp., *Klebseilla* sp., and *Staphylococcus* sp. from the crop field of Barak Valley region of Assam, India and found that these strains were resistant to amoxycillin, ampicillin, cefalexin, cefixime, kanamycin, methicillin and tetracycline.

Conclusion

Cajanus cajan L var. Manak is found to be a rich source of protein and a most important forage crop. Isolation and characterization of bacteria from the rhizospheric soil of *Cajanus cajan* helps in better understanding of the basic composition of the rhizosphere ecology, including the function and diversity of inhabiting microorganisms. Further knowledge to optimize soil microbial technology to the benefit of plant-growth and health in the natural environment is also necessary for selective isolation of valuable microorganisms. In the present study isolate GN1 (*Klebsiella* sp.) and GN7 (*Bacillus* sp.) was found to be most efficient in tolerating salt concentration at 10%. Their plant growth promotion activities ought to be determined, as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers. Further research are still required to isolate some valuable microbes from *Cajanus cajan* L var. Manak rhizosphere that can produce Indole Acetic Acid (IAA) and other siderophores. Study of their tolerance capacity in extremely saline conditions may bring new insight and application in environmental stress areas. Proper efficacy testing by pot/field trials is also necessary for sustainable use of these microorganisms in agricultural purpose.

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