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Original Research Paper

Microbiology

ISOLATION AND SCREENING OF SIDEROPHORE PRODUCING PGPR FROM COMMERCIAL CROP.

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KEYWORDS :

INTRODUCTION:

India is an agricultural country, there is increasing demand of food every day. The use of important microbes in agricultural production systems enhance plant tolerance to adverse environmental stresses, which include salt and drought stress, nutrient deficiency, heavy metal contaminations and weed infestation (Compant et al., 2005and Egamberdieva, 2010).

PGPR bacteria were isolated from rhizosphere of different commercial crops of wheat, sorghum, cotton, maize, groundnut, pigeon pea, and sugarcane from Jalna District of Maharashtra State, India. For isolation of siderophore producing bacteria deferrated succinate medium was used.

Siderophores are the most important secondary metabolite produced by bacteria in the rhizosphere soil. Siderophores are defined as relatively low molecular weight ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress. Siderophores form high-spin, kinetically labile chelates with ferric ion which are characterized by exceptional thermodynamic stability (Schwarzenbach *et al.*, 1963 and Raymond and Carrano., 1979).

Iron is the third most limiting nutrients for plant growth and metabolism. Primarily due to low solubility of the oxidized iron form in aerobic environment it unavailable to plants. (Zhang et al., 2010 and Rungin et al, 2012). Micronutrient iron in plant is required for cholorophyll biosynthesis, redox reactions and some important physiological activities (Briat et al., 1995). Plant require 10^{-17} mol/litre of iron at neutral PH while the level of of available iron required by microorganism is 10^{-6} mol/litre under neutral PH (Omiduari et al., 2010).

Pyoverdine siderophores produced by *Pseudomonas* species can enhance plant growth (Kloepper et al., 1980). Microbial siderophore is an essential component for plant growth (Masalha et al., 2000). Microbial siderophores are major source of irons in plants (Crowley, 2006). *E.coli* from rhizosphere of sugarcane and rye grass (*Lolium perenne*) is associated with siderophore production and thus enhances the plant growth (Gangwar and Kaur, 2009).

Siderophores produced by an endophytic *Streptomyces* sp. isolated from the roots of thai jasmine , rice plant induce plant growth and elevated root and shoot biomass and length(Rungin et al, 2012). Siderophores produced by *Aspergillus niger*, *Penicillium citrinum* and *Trichoderma harzianum* increases the shoot and root length of chickpeas (*Cicer arcelinum*) (Janardan et al., 2011).

Siderophores are effective in solubilizing and increasing the mobility of wide range of metals like Cd, Cu, Ni, Zn, Pb and Th(IV), Pu(IV) (Schalk et al., 2011). Siderophores ability depends on ligand functionalities. Siderophores may have a strong affinity or selectivity for a particular metal other than iron with regards to the stability constants of the metal siderophore complex (Hernlem et al., 1999).

MATERIALS AND METHODS

Soil Sampling

The soil sample was collected from different region of the Jalna district of Maharashtra. The rhizospheric soil of wheat, sorghum, maize, cotton, groundnut, pigeon pea and sugarcane was collected aseptically in polythene bags and stored at 4^0c temperature in laboratory.

Use Of Selective Media For Isolation Of PGPR Isolates:

Preparation of soil suspension was done by taking 1 gram of soil in 10 ml of distilled water in small tubes and were shaken well. The tube were allowed to stand still for 30 minutes. Later serial dilutions were prepared by taking the clear supernatant from the tube containing 1 gm soil.

These dilutions were spread on nutrient agar plate and were incubated at 30° C for 48 hours. The colonies isolated were allowed to grow on Succinate medium i.e. minimal media for isolation of potential isolates for 48 hours at 30°C. 28 different colonies were isolated by repeated culturing and were maintained at -4°C.

Ammonia Production

Cappuccino and Sherman method (1992) was used for determining production of ammonia. 10ml peptone broth was inoculated with isolate and incubated at 30°C for 48 hours on orbital shaker at 120 rpm. 0.5ml Nessler's reagent was added to each tube after development of faint yellow to brown color indicating the production of ammonia.

HCN Production

Isolate was spread on nutrient agar medium containing 4.4g per liter of glycine. Whatman filter paper No. 1 soaked in 0.5 % picric acid solution containing 2% sodium carbonate was placed inside the lid of plate. Plates were sealed with parafilm and incubated at 30°C for 4 days. Light brown to dark brown color spots indicates HCN production (Castric, 1975).

IAA Production

Indole-3-acetic acid (IAA) production was tested by using nutrient broth containing 0.1% dl-tryptophan (Gordon and Weber. 1951). The isolate were inoculated and incubated for 48 hr at 30°C at 120rpm on rotary shaker. After incubation, cultures were centrifuged at 3000 rpm for 30 minutes. 2 ml supernatant was mixed with 2 drops of O-phosphoric acid and 4 ml of Salkowaski reagent (50ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) was used to determine IAA production. Formation of pink color indicates IAA production.

Isolation of Phosphate Solubilizing Bacteria (PSB)

For isolation of PSB, the isolates were spot inoculated on sterile Pikovaskaya's agar plate of $_{P}$H 7. Plates were kept at room temperature for 7 days until highest number of colony developed. Amphotericin B 200mg/L added in PKA to avoid fungal growth (Afzal and Bano, 2008).

Other Test: Starch degradation, Gelatin degradation, Casein degradation test of PGPR isolates was performed.

Nitrogen Free Medium:
Jensen's medium is recommended for detection and cultivation of nitrogen fixing bacteria. Jensen's medium was prepared in agar plates and the PGPR strains were streaked on Jensens agar (plate $_{P}$H 7) and kept at room temperature for 24 hours.

Antibiotic Sensitivity Test:
Himedia Antibiotic octadiscs were used to test the antibiotic sensitivity of the isolated phosphate solubilizing bacteria.

Siderophore Production:
Quanitative estimation of siderophore was performed by assay suggested by payne (1994).quantitative assay was performed by using CAS assay medium (Barbhaiya and Rao, 1985).For siderophore production iron free succinic acid medium was used. All the glassware were deferrated. Cultures were grown in a minimal medium at room temperature for 24 hours under shaker conditions (100 rpm) at 48 hours. The cells were removed by centrifugation at 3000 rpm for 15 mins. 0.5 ml of the culture supernatant was then mixed with 0.5 ml CAS solution and 10μl shuttling solution (sulfosalicylic acid). The color obtained was determined using the spectrophotometer at 630 nm after 20 mins of incubation. Necessary blank (minimal medium) & reference solution (minimal medium + CAS dye + shuttle solution) were used during the determination. percent siderophore units in the aliquot were calculated by the formula

$$\frac{Ar\text{-}AS \times 100}{Ar} = \%\ \text{siderophore}$$

Where, Ar- absorbance of reference,
As – absorbance of sample.

Germination Test:
Seed Germination Test (Plate Assay)
The influence of BN7 isolate which produce maximum siderophore units as compared to other isolate was checked on the germination of Wheat seed by plate assay method. Seed germination test was done wheat seed which was of Ajeet (111) variety. Seeds were collected from local market of Jalna district. Wheat (*Triticum aestivum)* were selected which were free from visible damage and surface sterilized by soaking in 95% ethanol for 30 seconds. Then 1.2% sodium hypochlorite solution was used and seeds were kept for 5 min, followed by 5 rinses with sterile water. (Singh et al., 2008). Surface sterilized seeds were inoculated with the suspension of isolate (OD_{600} 1.0) kept for 4 hours at 30°C at 120rpm. (khalifa et al., 2016). Germination test were carried out by paper towel assay. Seeds treated with isolates were placed in between paper towels. Petri dish was kept in a well ventilated room at room temperature. Paper towels were regularly watered and daily analyzed for germination of seeds for 4 days.

RESULT AND DISCUSSION:
8 Culture were isolated from soil rhizospheric soil of wheat, sorghum, maize, cotton, groundnut, pigeon pea and sugarcane plant from different region of the Jalna district of Maharashtra. The isolate culture were named BN followed by number. Out of 8 culture BN7 showed all the PGPR traits which was isolated from maize rhizosphere soil was selected for further research. Results of material and methods 1.1 to 1.11 are

Table1. Screening of Soil Bacteria for PGPR traits.

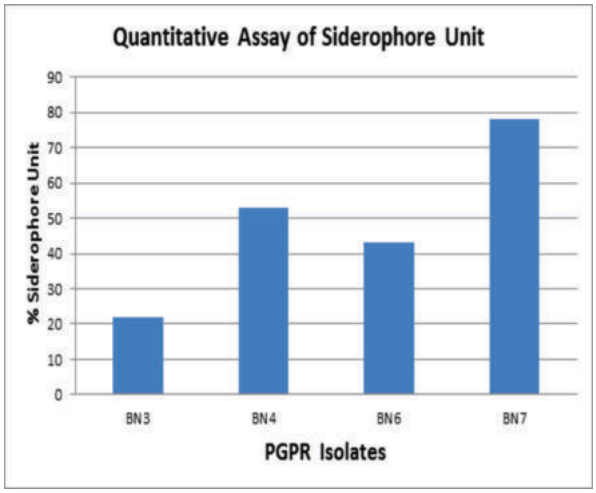
Isolates	Siderophore Producion	IAA Production	Ammonia Production	HCN	PSB
BN1 (wheat)	-	+	+	+	+
BN2 (Maize)	-	+	+	+	+
BN3 (wheat)	+	+	+	+	+
Bn4 (cotton)	+	-	+	+	+
BN5 (Sugarcane)	-	+	+	-	+
Bn6 (Jawor)	+	+	+	-	+
BN7 (Maize)	+	+	+	+	+
BN8 (pigeon pea)	-	-	+	-	+

(IAA= indole-3-acetic acid, HCN= hydrogen cyanide, Key + = Positive, - = Negative)

Siderophore Production:
The bacteria isolates BN3, BN4, BN6 and BN7 showed positive CAS test which shows the ability for the production of the Siderophore. Further results are shown in table 1.

Siderophore produce by bacteria is measured by spectrophotometric method. CAS reagent is mixed with supernatant and optical density of each sample was taken to estimate quantity of siderophore. The culture filterate filtrate of different isolate , which showed change in colors (Brown-yellow), further subjected to quantitative estimation of siderophore production and percentage siderophore was calculated.

Photo1: CAS test of the PGPR Isolates

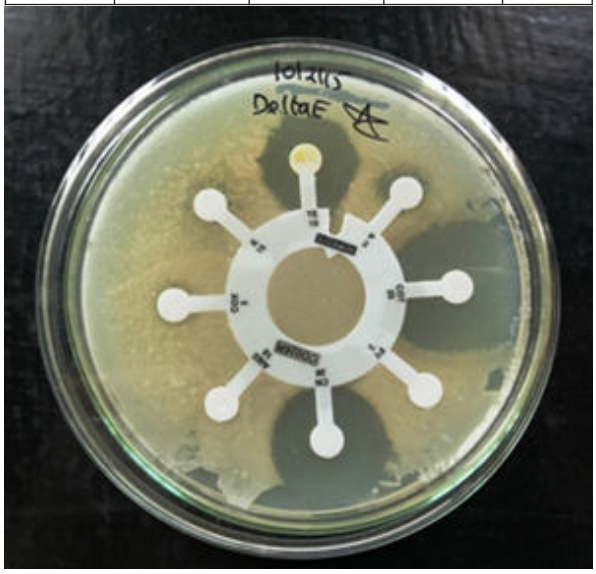


Graph 1. Isolate BN7 produced maximum siderophore unit.

Other Test:
Results of Starch degradation, Gelatin degradation, Casein degradation test and growth of PGPR isolates on Nitrogen free Jensen's media are expressed in table 2.

Table 2 : Different Test Performed On PGPR Isolates.

PGPR ISOLATES	DIFFERENT TEST PERFORMED			
	Starch Degradation	Gelatin Degradation	Casein Degradation	Growth Jensen's medium
BN1	+	---	+	+
BN2	+	---	---	-
BN3	+	---	---	-
BN4	--	+	---	+
BN5	+	---	+	-
BN6	+	---	---	+
BN7	+	---	---	+
BN8	+	---	---	-



Photos 2 : Antibiotic sensitivity checking of the PGPR isolate Bn7.

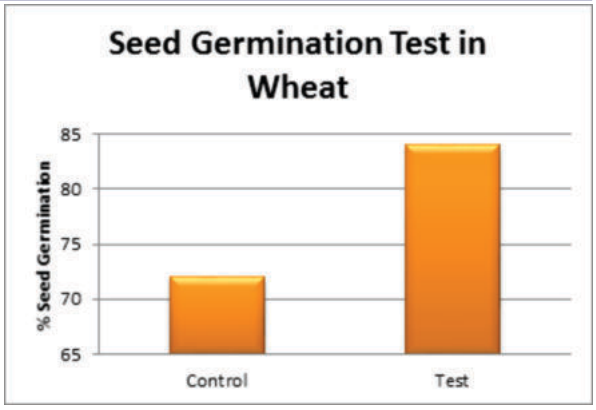
Wheat Germination Test

Wheat seeds were treated with BN7 isolate. The number of bacterial cells per seed, determined via serial dilutions, was approximately 108 CFU/seed. Treated seed germinate fast, compared to untreated seed. It was observed that treated seed germinate fast (30-36 hrs) than the control seed (36-48hr).nearly 88% seed germination was recorded in bacterized seed , whereas 75 % in un-bacterized seed. 13% increased in seed germination was observed in bacterized seeds.

(Photo.3)

(Photo.4)

(Photo. 5)



CONCLUSION:

From the present study it is revealed that rhizospheric soil of the commercial crops contains various siderophore producing plant growth promoting rhizobacteria (PGPR). Siderophore are iron chelating secondary metabolite, play an important role in plant growth promotion activity. The phosphate solubilizing bacteria are very much important for conversion of insoluble phosphate to soluble form phosphates to the plants. The PGPR also have the multiple role like production of siderophores, exopolysaccharide and ability to grow in nitrogen free medium. The germination of wheat seeds was increased as compared to the untreated wheat seeds, this indicates that the rhizospheric bacteria have important role in seed germination. The antibiotic sensitivity test is also very much important for the preliminary detection of the antibiotic sensitive bacteria. Plant growth promoting bacteria is beneficial for plant growth and sustainable agriculture.

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