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RESEARCH ARTICLE

SCREENING OF ROOT NODULE BACTERIA FROM WILD LEGUMES FOR THEIR POSSIBLE ROLE IN PLANT GROWTH PROMOTION.

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Abstract

In the present study, root nodule bacteria (RNB) were studied for their plant growth promoting (PGP) properties. For recovery of RNB wild leguminous plants viz., *Abrus precatorious* (*A. precatorious*), *Crotolaria retusa* (*C. retusa*), *Crotolaria turnesia* (*C. turnesia*), *Desmodium sp. Gliricidia sp* and *Mimosa pudica* (*M. pudica*) were collected from different areas of Bhopal. A total of 31 isolates (7 from *A. precatorious*, 3 from *C. retusa*, 4 from *C. turnesia*, 15 from *Desmodium sp*., 1 from *Gliricidia sp*. and 1 from *M. pudica*) were recovered and were studied for PGP activities viz., siderophore production, phosphate solubilization (P.solubilization), Indole acetic acid (IAA) production and ACC deaminase activity. 45.1% of the recovered isolates were positive for siderophore production with isolate DM1e showing maximum production i.e. 72.6 µg ml^{-1}. About 22.5% of the isolates produced IAA with isolate DM1f showing the maximum production of 10 µg ml^{-1}. P. solubilization and ACC deaminase activity was recorded in 54.8 and 87% isolates respectively. Further study on nitrogen fixation is underway. RNB such as rhizobia are known to promote plant growth by fixing nitrogen within root nodules and provide it to the plant for nutrition. Multifaceted beneficial effects can be obtained on plant growth and health by such RNB if they possess additional PGP properties as worked out in this study. Moreover, benefits can be obtained even if they fail to nodulate the plant (a constraint for rhizobia) but colonize the rhizosphere and compete with indigenous microbial population. Such microbes with multiple PGP properties could be developed as bioinoculants and have the potential to fetch the profit in growing biotechnology sector of India.

Introduction:-

Legumes are important in different agriculture and natural environment. Symbiosis between legumes and rhizobia are of considerable environmental and agricultural importance since they are responsible for most of the atmospheric nitrogen fixed on land.

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RNB are soil borne micro-symbionts capable of forming nodules in plants of family *Leguminosae*. Within the nodules, the rhizobia fix atmospheric nitrogen and transport the fixed nitrogen to their hosts in the form of amino acid; this symbiosis is considered to be the most economic and ecofriendly resources for mankind and received an extensive study. The majority of bacteria that form root legume symbiosis belong to α proteobacteria (e.g. *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium*) (Sprent, 2001). Recently some β proteobacteria, close to *Burkholderia, Cupriavidus, Pseudomonas* and *Ralstonia* and even some γ proteobacteria able to form nitrogen fixing nodules with legumes, have been discovered (Sprent, 2007). Legumes also interact with a variety of plant growth promoting bacteria (PGPB) which are representatives of the genera *Agrobacterium, Azospirullum, Bacillus, Burkholderia, Erwinia, Flavobacterium, Paenibacillus and Pseudomonas*.

Rhizobia are important members of plant growth promoting rhizobacteria (PGPR) showing various PGP activities (Glick, 1994). Direct PGP activities include production of IAA and siderophore, P. solubilization, etc. (Deshwal*et al*., 2003). Possession of these PGP activities along with nitrogen fixation by rhizobia may account for better growth promotion in plants. Rhizobia are the first group of bacteria which have the ability to release IAA that can help to promote growth in plants (Mandal*et al*., 2007). Similarly, P solubilization activity has also been reported in *Rhizobium* (Mikanova and Kubat, 1999). Siderophore production may be of particular significance in rhizobia-legume symbiosis, since iron is required in nodule formation as well as synthesis of components required for nitrogen fixation, such as nitrogenase complex and leghaemoglobin

Material and Method:-

Study site and sample Collection:-

Samples (plants bearing root nodules) were collected from leguminous plants growing wildly in different areas of Bhopal such as Barkatullah University (B.U) campus, Ekant park and Swarna Jayanti park. The leguminous plant species collected for isolation of root nodule bacteria were *Abrus precatorious*,*Crotolaria* sp, *Desmodium* sp.,*Gliricidia* sp. and *Mimosa* pudica (Table 1).

Isolation of root nodulating bacteria from root nodules and their morphological Characterization:-

Isolation of bacteria from nodules (Fig 1B) was done following the standard procedure given by (Somasegaran and Hoben 1985). To ensure that the nodules were devoid of any microbial contamination, the surface sterilized nodules were placed on Yeast Extract Mannitol Agar (YEMA) plates (g/l; mannitol-10.0; K_2HPO_4-0.5; $MgSO_4.2H_2O$-0.2; NaCl-0.1; Agar-agar-20; Congo red dye-25 ppm; distilled water-1000 ml; pH-6.8) and incubated at 28° C overnight (Fig 1A). The nodules showing any microbial growth were discarded and only the clean nodules were selected for isolation of the microsymbiont. Individual nodules were crushed in 200µl of sterile distilled water in a test tube with the help of blunt ended forceps; the resultant turbid bacteroid suspension was streaked on YEMA plates and incubated at 28° C for growth of bacteria. From these cultures, gummy white transparent single cell colonies were picked up and re-streaked on YEMA plates to recover pure cultures. Colony morphology (shape, margin, elevation, colour) of the isolates were observed.

PGPR Attributes:-

Siderophore Production:-

For qualitative test, all the cultures were spot inoculated on Chrome Azurol Medium (Chrome Azurol Test); the plates were then incubated at 28±2° C for 3 days. The formation of orange halos around the bacterial cultures indicated positive test for siderophore production.

Quantification of siderophore produced in culture broth was carried in Standard succinate medium using extinction coefficient of siderophore (E= 16500, pH 7.2) (Meyer and Abdallah, 1978). Pure cultures of selected isolates were grown in standard succinate broth for 24 h. 1 ml active culture was inoculated in 100 ml standard succinate broth and flasks were incubated at 28±2° C (120) rpm. After 72 h of incubation, 10 ml of culture was withdrawn and centrifuged at 10,000 rpm for 5 min. The O.D of the supernatant was read at 400 nm and siderophore was quantified based on the extinction coefficient of pyoverdine as under:

$$\text{Siderophore (mg/L)} = A_{400} \times \frac{\text{Mol. weight of compound}}{E\lambda \times 10^{-3}}$$

Mol weight of Pyoverdine = 1,500 Da
Extinction coefficient (Eλ) = 16,500

Test for phosphate solubilization was done on Pikovyskya agar medium. Test cultures were spot inoculated on plates containing Pikovyskya agar medium, the plates were then incubated at 28±2 °C for 3 days. The formation of clear zones around the bacterial cultures indicated positive test for phosphate solubilization. For the quantitative measurement of P, 100 ml of Pikovyskaya broth containing tricalcium phosphate (TCP) were inoculated with 1 ml of fresh culture (10^8 cells ml^{-1}) and incubated for 5 d on a shaker (120 rpm) at 28±2 °C. The culture broth was centrifuged (12000 rpm for 30 min) and the amount of water soluble phosphate released into the supernatant was estimated by the chlorostannous-reduced molybdophosphoric acid blue method.

IAA Production:-
Indole acetic acid when react with $FeHClO_4$ (Salkowasky reagent) gives stable pink colour. Salkowasky reagent (1 ml 0.5M $FeCl_3$; 50ml 35% $HClO_4$).

M_9 minimal broth was prepared. 12.5mg/250 ml Tryptophan was added to media. 10 ml broth was taken in the tubes and inoculated with test cultures, the tubes were incubated at 28±2°C for 3 days (120 rpm). Culture broth was centrifuged at 10,000 rpm for 5 min to remove cells. To 1 ml cell free extract 2 ml salkowasky reagent was added and tubes were incubated at room temperature for 30 min. Absorbance of pink coloured product was read at 530 nm.

ACC deaminaseActivity:-
ACC (1-aminocyclopropane-1-carboxylate) deaminase producing bacterial isolates were detected qualitatively using Dworkin and Foster minimal medium simply known as DF medium (Dworkin and foster, 1958). DF medium was supplemented with 3mM 1-aminocyclopropane-1-carboxylate and bacterial isolates were streaked on the plates and incubated at 28° C for 72 hours. Simultaneously control was prepared by supplementing DF medium with ammonium sulfate (2g/l) to detect whether they utilized nitrogen source or not. Bacterial isolates were streaked upon it. Growth of bacterial isolates in medium supplemented with ACC indicated ACC deaminase production .

Antagonistic Activity:-
The isolates were screened against fungal pathogen ie. *Fusarium oxysporum* (*F.oxysporum*), *Macrophomina phaseolina* (*M.phaseolina*), *Sclerotenia sclerotiorum* (S.*sclerotiorum*) by *in vitro* dual plate assay (Huang and Hoes, 1976).

Result:-

Isolation of root nodulatingBacteria:-
A total of 31 isolates (7 from *A. precatorius* , 3 from *C. retusa* , 4 from *C. turnesia* , 15 from *Desmodium sp.*,1 from *Glricidia sp.* , 1 from *M. pudica*) were isolated from 6 leguminous plant species.

Colony morphology and Gram Reaction:-
Colonies of all the isolates were round, mucoid, transparent/off white/white on YEMA amended with congo red dye (25 ppm) dye (Fig.1C). All the isolates were Gram negative long and short rods (Fig 1D). Morphological characteristics of all the isolates are presented in (Table .1).

Table 1:- Colony morphology (colour, size, shape) and Gram reaction of recovered RNB from different wild legumes

S.no	Source Plant	Isolates	Colony colour	Colony size	Gram reaction	Cell shape
1	*A. precatorious*	AP1a	Off white	2mm	Gram negative	Short rods
2		AP1b	Off white	1mm	Gram negative	Short rods
3		AP1c	White	1mm	Gram negative	Long rods
4		AP1d	Off white	1mm	Gram negative	Long rods
5		AP2a	Off white	<1mm	Gram negative	Long rods
6		AP2b	Off white	<1mm	Gram negative	Short rods
7		AP2c	White	1mm	Gram negative	Short rods
8	*C. retusa*	CR1a	White	<1mm	Gram negative	Long rods
9		CR1b	White	2mm	Gram negative	Long rods

10		CR1c	Trans parent	2mm	Gram negative	Short rods
11	*C. turnesia*	CT1a	White	1mm	Gram negative	Long rods
12		CT1b	White	1mm	Gram negative	Short rods
13		CT1c	White	1mm	Gram negative	Long rods
14		CT1d	Off white	1mm	Gram negative	Long rods
15	*Desmodium sp.*	DM1a	White	2mm	Gram negative	Short rods
16		DM1b	White	2mm	Gram negative	Long rods
17		DM1c	Off white	1mm	Gram negative	Short rods
18		DM1d	Off white	1mm	Gram negative	Short rods
19		DM1e	White	1mm	Gram negative	Long rods
20		DM1f	Off white	1mm	Gram negative	Long rods
21		DM1g	Off white	1mm	Gram negative	Short rods
22		DM2a	White	1mm	Gram negative	Long rods
23		DM2b	White	2mm	Gram negative	Short rods
24		DM2c	White	2mm	Gram negative	Short rods
25		DM2d	White	1mm	Gram negative	Long rods
26		DM2e	White	1mm	Gram negative	Long rods
27		DM2f	White	1mm	Gram negative	Short rods
28		DM2g	Off white	1mm	Gram negative	Long rods
29		DS1a	White	1mm	Gram negative	Long rods
30	*Gliricidia sp.*	GG1a	Off white	<1mm	Gram negative	Short rods
31	*M. pudica*	MP1a	White	<1mm	Gram negative	Long rods

Table 2:- PGPR attributes by RNB isolated from different wild leguminous plants

S.No.	Isolates	Siderophore production	P.Sol.*	IAA	ACC Deaminase	Antagonism against
1	AP1a	-	0.37 ±0.03	-	+	*F. oxysporum*
2	AP1b	-	0.23 ±0.02	-	+	
3	AP1c	-	0.16 ±0.03	-	+	*F. oxysporum*
4	AP1d	-	0.20 ±0.05	-	+	
5	AP2a	-	0.28 ±0.04	-	+	
6	AP2b	-	0.07 ±0.02	-	+	
7	AP2c	8.75± 0.12	0.31 ±0.03	-	+	*M.phaseolina, S.sclerotorium*
8	CR1a	6.21± 0.08	0.39 ±0.05	-	+	
9	CR1b	-	-	-	+	
10	CR1c	11.03± 0.11	0.22 ±0.07	-	+	*M.phaseolina, S.sclerotorium*
11	CT1a	-	0.32 ±0.06	-	+	
12	CT1b	4.48± 0.09	-	-	+	
13	CT1c	13.15± 0.19	-	-	+	
14	CT1d	-	-	-	+	
15	DM1a	1.15± 0.05	-	-	-	*M.phaseolina, S.sclerotorium*
16	DM1b	-	-	5.4 ± 0.05	+	
17	DM1c	36.57± 0.19	-	-	+	*S. sclerotorium*
18	DM1d	-	0.15 ±0.03	-	+	
19	DM1e	1.36± 0.06	-	-	+	
20	DM1f	72.60± 0.20	0.47 ±0.03	2.3 ± 0.04	-	
21	DM1g	-	0.24 ±0.04	10 ± 0.18	+	*S. sclerotorium*
22	DM2a	6.81± 0.11	0.14 ±0.03	-	+	
23	DM2b	41.06 ±0.25	-	4.9± 0.05	+	
24	DM2c	-	-	-	-	

25	DM2d	-	0.48 ±0.03	4.85±0.08	+	
26	DM2e	24.51± 0.10	-	-	+	
27	DM2f	10.72± 0.20	-	4.82 ± 0.07	+	*M.phaseolina*, *S.sclerotorium*
28	DM2g	-	-	-	-	
29	DS1a	-	0.17 ±0.02	-	+	M.phaseolina, *S.sclerotorium*
30	GG1a	-	-	2.5 ± 0.07	+	
31	MP1a	12.15± 0.20	0.25 ±0.03		+	

A '+' denotes positive test and '-' denotes negative test; Values represent mean±SE (n=3) P.Sol-Phosphate solubilization, *F.oxysporum-Fusarium oxysporum*, *M. phaseolina- Macrophomina phaseolina*, *S.sclerotorium-Sclerotinia sclerotorium.*

PGPR Attributes:-

In a plate assay for siderophore production 45% isolates showed positive results. Siderophore production ranged from 4.9-72.60 µg/ml, wherein, maximum amount of siderophore was produced by isolate DM1f (72.60µg/ml) (Table 2, Fig.1E)

Only 22.5% isolates were found positive for IAA production. IAA production ranged between 2.3-10µg/ml of IAA. Highest IAA production was recorded by isolate DM1g (10µg/ml) (Table 2, Fig 1F)

On Pikovaskya agar, 54.8% isolates showed positive results for P solubilization by formation of clear zone around the bacterial colony. Based on solubilization efficiency, isolate DM2d was found to be most efficient P solubilizer under *in vitro* conditions. Results of P solubilization by RNB are presented in (Table 2, Fig 1G).

All the isolates were tested for ACC deaminase activity. 87% isolates showed positive results for the test.

Antagonistic activity of all the isolates were performed against three fungal pathogens viz., *Macrophomina phaseolina, Fusarium oxysporum* and *Sclerotenia sclerotorium*. Only 2 isolates AP1a & AP1c showed zone of inhibition against *F. oxysporum* ,5 isolates AP2c,CR1c, DM1a, DM2f, DS1a showed antagonistic activity against both *M. phaseolina* and *Sclerotenia sclerotorium.* Two isolates DM1c and DM1g showed inhibition against *S.sclerotorium* (Table 2, Fig 1H)

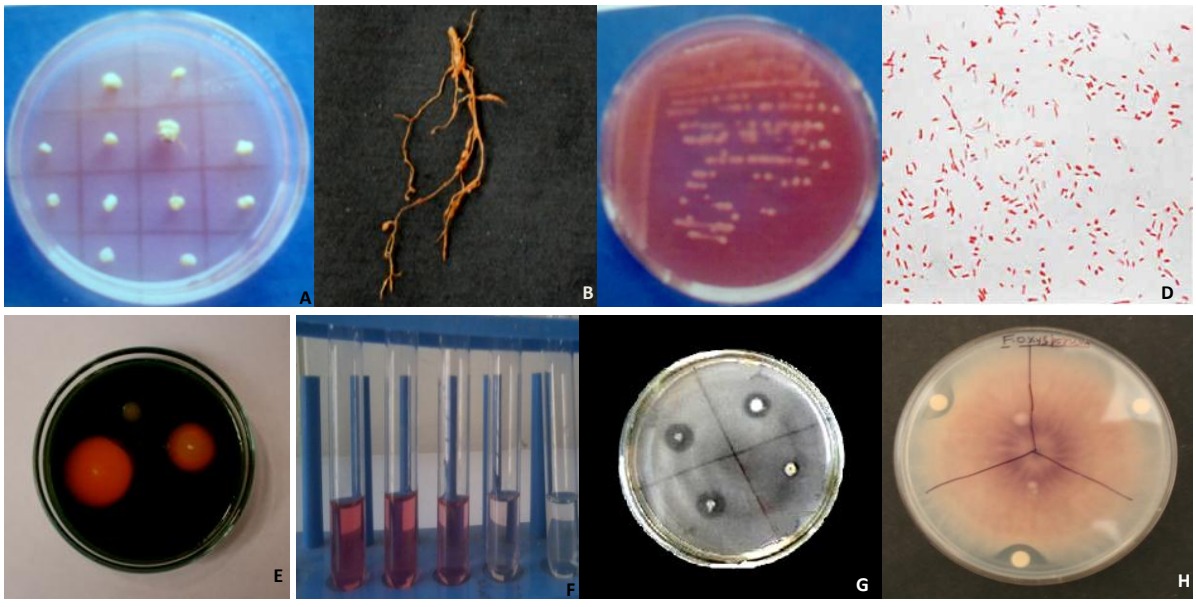

Fig.1:-Test for surface sterilization on YEMA (A); Root nodules of *Mimosa sp*.(B); Pure culture of RNB on YEMA(C); Gram staining of RNB (D); Siderophore production on CAS Medium (E); IAA production by RNB (F); P. solubilization on Pikovyaskya medium(G); Antagonistic activity of RNB against *F. oxysporum* (H)

Discussion:-

It has been described frequently that a plant obtains almost everything directly from the soil to support growth. Therefore the soil structure must be physically capable of supporting the above-ground half of the plant through its developing root system as it grows. The soil needs to be maintained at an appropriate pH, provide protection from toxic substances and pathogens and contain suitable water level. In addition to this all the essential mineral elements that plant requires are obtained from soil. Most of these elements are taken from the soil solution in their ionic form (White, 2003). Plant associated bacteria is probably one of the most important cause for improving growth and yields of various crops (Ali et al.2009).

Microorganisms having the mechanisms that facilitate nutrient uptake or increase nutrient availability or stimulate plant growth are commonly referred to as biofertilizers. The most well studied PGPR considered biofertilizers correspond to nitrogen fixation and utilization of insoluble forms of phosphate (Martinez et al. 2010).

P is an essential plant nutrient with low availability in many agricultural soils. Most of the insoluble P forms are present as aluminium and iron phosphate in acidic soils (Mulen, 2005) and calcium phosphates in alkaline soils (Goldstein and Krishnaraj ,2007).

The ability of rhizosphere bacteria to solubilize insoluble P minerals has been attributed to their capacity to reduce pH by excretion of organic acids (eg. Giuconate, citrate, lactate, and succinate and protons (during the assimilation of ammonium ions (Mullen, 2005).

Many important plant- microbial interactions center on the production of Auxin which is responsible for division, expansion and differentiation of plant cells and tissues and stimulates root elongation. The ability to synthesize IAA has been detected in many rhizobacteria as well as in pathogenic, symbiotic and free living bacterial isolates. (Tsavkelova et al., 2006)

Various studies have demonstrated that plants treated with PGPR bacteria that produce ACC deaminase have increased their resistance to environmental stress (Grinchko and Glick, 2001).

In recent years, rhizobacteria containing ACC deaminase activity has been used to alleviate deleterious effects of ethylene under stressed conditions of high salt content, heavy metals , flooding and drought (Nadeem et al.,2010)
Treatment of *V. radiata* seeds with rhizobacteria exhibiting ACC deaminase activity significantly enhanced root length (upto 50%) and number of nodules (upto47%) (Akhtar and Ali 2011).

Indirect plant growth promotion includes the prevention of deleterious effect of phytopathogenic organisms (Glick and Pasternak 2003). This can be achieved by the production of siderophores, i.e. small iron binding molecules. In soil iron is found predominately as ferric ions, a form that cannot be directly assimilated by microorganisms. Siderophore production enables bacteria to compete with pathogen by removing iron from the environment (Hayat et al. 2010).

Conclusion:-

Assays on plant growth promoting properties, viz., P-solubilization, siderophore production, IAA production and ACC deaminase activity, revealed that many of isolated RNB have potential for the development of bioinoculants. These properties may promote growth, even if, these bacteria fail to nodulate the plant root. These RNB are of economic importance in low input sustainable agriculture, agroforestry and land reclamation.

Since these bacteria possess the plant growth promoting properties, these may be helpful in enhancing the yield and active principles of the selected medicinal plants, and thus may provide economic gains to the nurserymen and farmers of the state.

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Conflict Of Interest:-

The authors have no conflict of interest to declare.

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