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Development and Screening of Plant Growth Promoting Efficacy of Rhizobium-PGPR Based Formulation in *Vigna Unguiculata*

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Abstract: ***Cowpea is a food legume having significant importance worldwide as it is rich in proteins, vitamins and minerals. Rhizobium is a well-known nitrogen fixer which also produces some plant hormones. The present research work envisages the development of Rhizobium-PGPR consortia formulation involving enriched coir pith for growth enhancement in Vigna unguiculata. A total of four bacterial isolates were isolated from soil and root nodules and four isolates were confirmed through biochemical characterization as Rhizobium spp. Bioformulation involving Rhizobium and IAA producing Bacillus ascii using Coir pith as organic carrier was prepared. In a new attempt, the coir pith was enriched with nutritional solution and used as an organic carrier for development of said bioformulation. The pot culture study was undertaken with different treatments using the CRD design with three replicates to test plant growth efficacy of the bioformulation. Seed and soil treatment using enriched carrier material exhibited better shoot growth promotion (75.08±16.79) when compared with control (65.33 ±11.72) and commercially available biofertilizer (73.37±9.57) .This is a first attempt to develop a bioformulation using Rhizobium and IAA producing bacteria for growth enhancement in Cowpea. The results obtained clearly reveal a novel outcome wherein the enriched carrier material exhibited better growth enhancement than non enriched carrier materials. The bioformulation developed has been currently tested in field trials with farmers by means of a participatory approach.***
Keywords: Rhizobium, Bioformulation, Cowpea, Coirpith, PGPR.

I. INTRODUCTION

The use of chemical fertilizers for plant growth promotion is one of the major reason for environmental pollution. The replacement of chemical fertilizers with biofertilizer is currently widespread and it not only helps for plant growth promotion but also it improves soil health and protects environment from pollution. Soil contains many kinds of microorganisms that helps to maintain physical, chemical and biological properties of soil (Shahzad *et al*., 2012). The bacteria which helps for plant growth promotion is known as plant growth promoting rhizobacteria (PGPR). Rhizobia is one of the plant growth promoting rhizobacteria which is commonly seen in rhizosphere soil and in root nodules of leguminous plants. Nitrogen is one of the limiting nutrient for leguminous plant because that is present in the soil cannot support growth. It is essential for plants to synthesis enzymes, proteins and chlorophyll (Zephania et al., 2014). The useful effect of Rhizobium on legumes are because of biological nitrogen fixation.

Cowpea (*Vigna unguiculata*) is one of the leguminous crop having significant importance worldwide (Nandi *et a*l., 2013). Rhizobium is symbiotically associated with the root nodule of the cowpea and helps for biological nitrogen fixation. Some environmental factors suc as temperature, moisture, pH etc. affect the survival of cowpea *Rhizobium* (Nantakorn *et al* 1982). The present study was aimed to examine the plant growth promotion in cow pea by using enriched coir pith based bioformulation of Rhizobium and IAA producing PGPR as monoinoculant and co inoculants.

II. MATERIALS AND METHOD

A. Sample Collection, Isolation and Identification

The samples were collected from the cowpea fields in palakkad district, kerala, India. A total of 4 samples were collected, 2 from root nodules of cowpea and 2 soils samples from different region of the cowpea field. The soil sample was serially diluted and cultured in Yeast extract mannitol agar (Linu et al.,2009). Healthy root nodules were washed with tap water thrice to remove the soil particles and sterilized with 95% alcohol for 1-2min followed by washing with 1% sodium hypochlorite (Shahzad et al.,2012). The nodules were crushed with sterile distilled water and a loopfull of ground material was transferred to 5ml of sterile water, of which 0.1ml of sample was spread onto the surafe of yeast manitol agar.

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The plates were incubated at 28°C for 48 hours. Isolated colonies were restreaked and in YEMA plates for obtaining pure cultures. *Rhizobium* cultures were biochemically characterized as per the standard methods (Arora 2003).

B. Preparation of Carrier Material

For the preparation of bioformulation, coirpith was collected from Kerala Coir Board,India were selected as the carrier material. Treated coir pith with removing the excess chloride contents is used for developing the bioformulation.Coir pith was powdered using miller machine and sterilized at 121ºC at 15 lbs for 15 minutes using autoclavable polythene bags and stored at room temperature for further use. Coir pith was selected for making bioformulations with different combinations of microbes and enrichment material. Yeast extract mannitol broth and Nutrient broth were used as enrichment material. Enrichment material and carrier material was mixed in 2:1 proportion. The carrier material without any enrichment is taken as the contol. The culture of *Rhizobium* and *Bacillus ascii* was mixed with the bioformulation as mono-inoculant and co-inoculant.

C. Bioformulation Development

Bacterial inoculums were raised by growing Rhizobium sp. in YEM broth medium and Bacillus ascii in Nutrient broth medium for 48 hours at 28°C. After 48 hours of incubation, the broth containing 1×10^8 cfu/ml was used for the preparation of bioformulation. The formulation was developed as described by Prasanth et al., (2003). The CMC, Carrier and bacterial suspension in broth were used in the ratio 1:50:4. The bioformulation was prepared as YEM enriched coirpith with YEM broth (10gms of CMC+500gms of enriched coir pith+40ml of Rhizobial cells in YEM broth) ; Nutrient broth enriched coirpith ((10gms of CMC+500gms of enriched coir pith+40ml of *Bacillus ascii* cells in Nutrient broth) ; without enrichment mono-inoculant bioformulation (10gms of CMC+500gms of coir pith+40ml of Rhizobial cells in YEM broth) and without enrichment co-inoculant bioformulation (10gms of CMC+500gms of coir pith+20ml of Rhizobial cells in YEM broth and 20ml of *Bacillus ascii* cells in Nutrient broth). The materials were stored in sterile plastic bags at room temperature.

D. Pot Culture Studies

For the evaluation of growth promotional efficacy of the developed bioformulation, pot cultue study was undertaken with different treatments by using completely randomized block design (CRD) with three replications with five plants in each pot. Treatments involved in the experiment includes: Seed treatment , Seed + Soil treatment , Soil treatment .

Seeds of cowpea were surface sterilized with 1% sodium hypochlorite for 1-2 min and washed in sterile distilled water (Nandi *et al*., 2013). Coir pith bioformulation was mixed with sterile distilled water to form slurry in the ratio of 1:5 of the formulation and surface sterilized cowpea seeds were soaked in slurry of bioformulations enriched with several composition for 1 hour, then dried overnight in sterile conditions. Soil treatment is also done during sowing the seeds. 50 grams of different bioformulation was mixed with each pot. The experiment was carried out with 4 different treatments of coirpith bioformulation and untreated seeds served as control for the trials. Further the plant growth efficacy of formulations developed was compared with commercial Talc based fertilizer (*Pseudomonas fluorescens*) obtained from TNAU, Coimbatore.All experiments were done with 5 replicates. Each pot was mixed with sandy soil, red soil and farmyard manure in the ratio of 1:1:1 and another set up with Sandy soil and Red soil in the ratio of 1:1 without adding farmyard manure. Data on seed germination was recorded on the third day after sowing. Seedling length, root length, number of branches, leaves and nodules were recorded after 25 days of sowing.

E. Stastical Analysis

Data were statistically analyzed by ANOVA, Least Significant Difference (LSD) test at probability level of $P<0.05$ was used to check the means when ANOVA F test indicated a significant effect of the treatments.

III. RESULTS AND DISCUSSION

Four samples were characterized biochemically and were positive to the presence of *Rhizobium.* All isolates showed the same colony characteristics, after 48 hours of incubation. The colonies were white, translucent, circular in shape, shiny and raised with musky odour. All isolates were gram negative rods and all are catalase positive as confirmed by the liberation of effervescence of oxygen around the bacterial colonies. The samples were found to be methyl red, indole, citrate and voges prouskeur negative. The results of biochemical characteristics of isolated strains are summarized in Table 1.

The positive samples were also subjected to various sugar fermentation test and found positive to glucose, mannitol, sucrose, arabinose, maltose and lactose (Table 2).

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Table 1. Biochemical characteristics of Rhizobial isolates from cowpea

TEST	RN1	RN2	RN3	RN4
Gram staining	+	+	+	+
Catalase	+	+	+	+
Motility	+	+	+	+
Indole	-	-	-	-
Methyl red	-	-	-	-
Voges prouskeur	-	-	-	-
Citrate	-	-	-	-
Urea hydrolysis	+	+	+	+

+Positive -Negative

Table 2. Carbohydrate fermentation test

Carbohydrate	RN1	RN2	RN3	RN4
Glucose	A	A/G	A/G	A/G
Maltose	A/G	A/G	A/G	A/G
Lactose	A/G	A/G	A/G	A/G
Arabinose	A	A/G	A/G	-
Manitol	A/G	A/G	A/G	A/G
Sucrose	A/G	A/G	A/G	A/G

A- Acid production, A/G- Acid with gas production , - Negative

Table 3. Germination percentage of cowpea seeds after 3 days of sowing.

Experiments	% of germination Seeds germinated / Total number of seeds *100
ERB	89.42%
ERC	86.53%
NERB	88.46
NERC	82.69%
Biofertilizer	72%
Control	80%

Seed germination percentage was high for seeds treated with enriched *Rhizobium* (89.42%) when compared *Rhizobium* – Coinoculant (ERC) treatment (86.53%). The results confirmed that *Rhizobium* exhibits better seed germination rate than with Co-inoculant $FS_1$1.1**.** The results are summarized in table 3.

Table 4. Growth promotional studies in cowpea (Shoot).

PLANT GROWTH AFTER 25 DAYS WITH FYM				PLANT GROWTH AFTER 25 DAYS WITHOUT FYM		
Experiment	Shoot length	Number of leaves	Number of branches	Shoot length	Number of leaves	Num of branches
ERB						
1.Seed treatment	71.95 ±20.75	13.86±2.98	4.23±0.77	21.57± 2.52	7.2±1.13	1.9±0.31
2.Seed + Soil	73.56±13.39	16.2±2.33	4.33±0.72	24.56±5.52	8.1±1.44	2.2±0.42
3.Soil Treatment	69.81 ± 9.53	12.86± 2.26	3.13±0.35	21.79±3.31	7.8±1.54	1.7±0.48
ERC						
1.Seed treatment	72.61±13.34	14.20±1.93	3.53±0.91	20.87±1.44	7.8±0.63	
2.Seed + Soil	75.08±16.79	15.73±1.53	4.33±0.89	25.77±2.53	7.9±1.26	1.9±0.31
3.Soil Treatment	73.6±11.76	15.46±2.66	3.93±0.88	21.17±2.51	6.6±0.96	1.9±0.48
NERB						1.8±0.42
1.Seed treatment	67.8±15.58	12.06±1.63	2.42±0.35		6.6±1.07	
2.Seed + Soil	67.33±15.43	13.33±2.46	2.69±0.96	22.35±2.16	7.6±1.34	
3.Soil Treatment	62.86±18.09	12.08±2.14	2.23±0.88	23.4±1.34	6.8±1.92	1.9±0.31
NERC				23.55±2.94		2.2±0.42
1.Seed treatment	68.66±15.66	12.73±1.53	3.4±0.63		6.9±0.99	1.7±0.48
2.Seed + Soil	69.66±2.48	13.26±2.54	4.2±1.01	23.05±1.80	7.7±1.41	
3.Soil Treatment	67.4±23.65	12.8±2.14	3.4±0.91	24.00±0.81	7.00±1.76	2.00±0
Biofertilizer				23.65±2.86		2.4±0.51
1.Seed Treatment	71.45±11.77	13.1±2.72	3.9±0.73		7.2±1.13	1.7±0.48
2.Seed+Soil	73.37±9.57	15.7±1.70	4.3±0.48		7.2±0.83	
3.Soil	71.4±12.26	12.5±2.12	4.1±0.99	1.37±2.52	8±2.12	
Control	65.33 ±11.72	12.26±1.62	3.33±0.48	25.74±5.2	7.6±1.57	1.9±0.31
				25.8±1.9		2±0
				19.97±1.64		1.8±0.44
						2.3±0.67

The values are mean of 15 samples ± SD.

Shoot length, root length, nodule number, nodule weight, number of leaves and number of branches was calculated after 25 days of sowing. Highest shoot length was recorded in plants treated with Enriched *Rhizobium*-coinoculant (ERC) (75.08±16.79) grown along with Farm yard Manure (FYM) when compared to control (65.33 ± 11.72) and all other treatments. Among the three treatments studied using ERC with FYM, seed + soil treatment showed highest shoot length followed by soil treatment (73.06±11.76) than seed treatment. In addition the shoot length of ERC treated plants were found to be marginally better than talc based *Pseudomonas fluorescens* treated plants (73.37±9.57) used as positive control. This confirmed that seed + soil treatment along with FYM enhanced better shoot development in *Vigna unguiculata.* The results were tabulated in Table 4.

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Table 5. Growth promotional studies in cowpea (Root).

	ROOT LENGTH AFTER 25 DAYS WITH FYM			ROOT LENGTH AFTER 25 DAYS WITHOUT FYM		
Experiment	Root length	Nodule number	Nodule weight(Gms)	Root length	Nodule number	Nodule weight
ERB						
1.Seed treatment	12.89±2.34	9.00±3.67	0.28±08	8.33±1.26	8.2±2.58	0.028±001
2.Seed + Soil	12.66±2.98	13.2±1.92	0.37±0.04	8.13±1.2	7.6±1.81	7
3.Soil Treatment	10.12±1.80	7.8±1.78	0.12±0.02	9.28±2.3	11±2.64	0.036±0.01
ERC						3
1.Seed treatment						0.06±0.025
2.Seed + Soil	13.32±3.19	14.2±5.11	0.31±0.07	8.72±2.00	8.2±2.28	
3.Soil Treatment	14.46±0.89	12.4±3.70	0.4±0.035	9.53±1.83	8.2±1.78	
NERB	12.53±2.06	12.8±3.70	0.32±0.08	9.48±0.42	10.2±3.11	0.03±0.03
1.Seed treatment						0.188±0.01
2.Seed + Soil						0.11±0.13
3.Soil Treatment	8.99±1.41	7.6±1.14	0.10±0.03	9.43±1.32	8.2±.62	
NERC	9.63±1.44	8.4±2.0	0.10±0.01	9.33±1.44	5.2±3.56	
1.Seed treatment	8.96±1.15	7.0±2.44	0.06±0.04	8.96±1.15	6.0±1.58	0.06±0.04
2.Seed + Soil						0.005±0.00
3.Soil Treatment						6
Biofertilizer	8.59±0.89	6.8±2.38	0.06±0.05	8.96±1.71	4.2±0.83	0.007±0.00
1.Seed Treatment	9.53±1.40	9.0±2.0	0.2±0.1	8.53±1.61	6.4±1.81	4
2.Seed+Soil	9.89±0.86	8.6±2.3	0.09±0.05	8.66±1.71	6±3.80	
3.Soil						
Control	9.662±2.09	6.8±1.78	0.22±0.08	7.3±1.48	8.2±2.86	0.03±0.008
	10.06±2.48	9.6±1.78	0.22±1.10	9.2±1.64	10.0±2.0	0.06±0.04
	9.28±2.59	9.4±1.14	0.10±0.06	7.96±1.42	7.0±1.58	0.02±0.04
	9.46±1.07	2.2±2.04	0.006±0.005	7.33±2.65	2.6±2.60	0.05±0.06
						0.04±0.03
						0.04±0.03
						0.02±0.04

The values are mean values of 5 samples ± SD

Highest root length was recorded in plants treated with Enriched *Rhizobium*-coinoculant (ERC) (14.46±0.89) grown along with Farm yard Manure (FYM) when compared to control (9.46 ± 1.07) and all other treatments. Among the three treatments studied using ERC with FYM, seed + soil treatment showed highest root length followed by seed treatment (13.32±3.19) than soil treatment. This confirmed that seed + soil treatment along with FYM enhanced better root development in *Vigna unguiculata*. In addition the root length of ERC treated plants were found to be marginally better than talc based *Pseudomonas fluorescens* treated plants (10.06 ± 2.48) used as positive control. Application of Coinoculant along with *Rhizobium* was found to enhance better root development when compared to only *Rhizobium* treatment alone. The results were tabulated in Table 4.

IV. CONCLUSION

Farmers need high yield and quality for that they use fertilizers that are costly and it creates the environmental problems. The recent research moves towards the eco-friendly and sustainable agricultural practices. The use of chemical fertilizers will affect the soil health and the microorganism present in the soil. In the research findings shows that PGPR based bioformulation gave better yield and it provides plant growth promotion in Cowpea. The effect of microorganism with enriched coirpith, immensely helpful to boost the cowpea plantations by nutrient supply without using the chemical fertilizers. The current study provide the information on the use of Rhizobium and FS1.1 based bioformulation for the growth promotional activity in cowpea under ecofriendly manner without using the chemical fertilizers for sustainable yield and quality with better soil health environment.

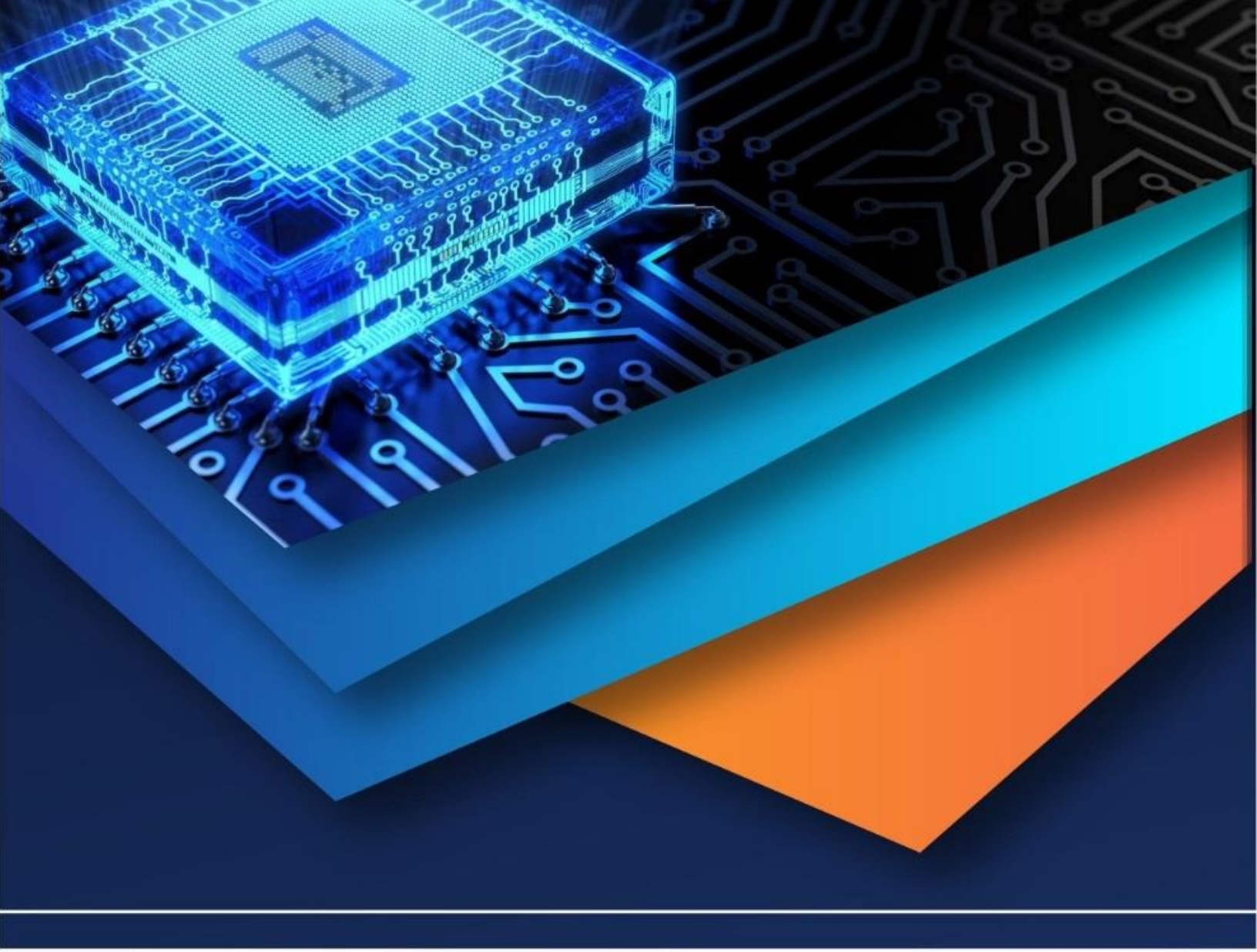
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