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Screening of rhizobial isolates for the production of plant growth promoting bioformulations by using different carriers

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ABSTRACT

The irrational use of chemical fertilizer and pesticide increase the production but they are deleterious to environment indirectly directly. So, there is utmost need of alternate method or fertilizer to combat the fertility problem of soil and crops. In environment there is presence of microorganism which can increase the soil fertility as well as production of some inhibitory substance which inhibit the growth of harmful phytopathogens. In the present investigation five carriers- sawdust, charcoal, rice bran, wheat bran and sugarcane bagasse were evaluated for the production of bioinoculants. The bacterial population was determined up to three month storage, other parameter such as shoot length, root length, number of nodule, fresh weight and dry weight was also evaluated. Out of tested carrier sawdust was proved to be the best carrier in maintaining the bacterial population and also useful in enhancement of the growth of *Cicer arientinum* compared to control after the addition of inoculants. The finding of this study suggests that sawdust based carrier was much better than other carrier based inoculants taken in the study.

Keywords : Bioformulation, *C. arientinum,* carriers

There is growing interest in the use of biological approaches to replace chemicals in fertilizing soil. In this regard plant growth promoting rhizobacteria (PGPR) have a potential role. Such PGPRs also fix nitrogen. Legume crops like red-gram, black-gram, groundnut, cowpea, and soyabean which help in saving 20-40 kg chemical nitrogen. 50-300 kg nitrogen fixation from soil and helps in saving about 25-100 kg chemical nitrogen i.e. 55-220 kg urea per hectare. Rhizobium is a soil habitat Gram-negative bacterium, which can able to colonize the legume roots and fixes atmospheric nitrogen symbiotically (Gomere *et al*. 2013). Although N_2 is abundant (around 80%) in the atmosphere. Atmospheric N_2 is not readily available for plant uptake and some bacteria are capable of N_2 fixation from the atmospheric N_2 pool. Many free living N_2 fixing bacteria occur in soil. The amount of N_2 fixed by these organisms is considerable because of the close proximity they have with their host plant (Kumar 2014).

Rhizobia are unique in that they are the only nitrogen fixing bacteria living in a symbiotic relationship with legume (Singh *et al*. 2014). Biological nitrogen fixation by rhizobia is one of the effective methods to improve the plant growth and productivity (Deshwal *et al*. 2013).

Formulations generally composed of the active material which must be preserved or maintained in viable condition to produce its biological effect; the carrier material may or may not include the incorporation of enrichment materials or additives. The active material is mixed with carrier materials such as water, clay, talc, oil or others to make the formulation safer to handle, easier to apply and better suited for storage (Omer *et al*. 2010). A good quality formulation promotes survival of bacteria, maintaining a viable

population sufficient to exude growth promoting effects on plants (Aeron *et al*. 2011). For ideal bioformulation or biofertiliser it should or must contain good quality carrier, which make bioformulation highly effective, and also maintain the viability of inoculants for longer duration. Various authors or researcher used different carrier based formulation for seed or soil inoculation such as alginate-perlite dry granules, celite, kaoline, diatoms, coal, wheat bran, composed sawdust, Agriperlite, cheese whey, sugarcane baggase, nutrient supplement pumice, waste water sludge etc.

So, present study was aimed to examine the potential of different carrier based bioformulations of root nodulating *Rhizobium spp.* on growth promotion of chickpea (*Cicer arientum*).

MATERIALS & METHODS

Isolation of Rhizobium species—The fresh root nodules were collected from *Vigna unguiculata* (Lobiya bean, Lb)*, Vigna mungo* (Urad bean, Ub)*, Vigna radiate* (Mung bean)*, Cajanus cajan* (Arhar bean, Ar) plants from farmer's field at different locations of Kanpur of Uttar Pradesh, India. Root nodules of plants were sterilized in 95% (v/v) ethanol for 10 s and then washed 7 times with sterile distilled water. Individual nodules were crushed with sterile glass rods and streaked onto Yeast Extract Mannitol (YEM) agar containing 0.0025% (w/v) Congo red. After incubation for 48-72hr at 30°C, single colonies were selected. After 48 hr days of incubation, Rhizobium colonies were obtained. Pure isolates were maintained on YEM slant in refrigerator at 4°C till further analysis and tests were performed.

Identification of Rhizobium species—The colony morphology of isolates was examined on both YEM agar plates. After an incubation of 2-3 days at 30°C 30°C, individual colonies were characterized based on their color, shape, Gram staining (Holt *et al.* 1994)

Indole acetic acid (IAA) production by Rhizobium spp.—IAA production by Rhizobium isolates was determined following the methods of Gordon and Weber (1951). The bacterial isolates were grown on Luria Bertani (LB) Broth and incubated at 28° C for 24 h at 129 rpm. Exponentially grown culture (10^8 cells per cell) was centrifuged at 10000 rpm for 15 min at 4° C to collect supernatant. Two drops of orthophosphoric acid was added to 2 ml of supernatant. Appearance of pink color confirmed the production of IAA.

Production of HCN by Rhizobium isolates—Hydrogen cyanide production by Rhizobium isolates was determined using the method given by Kloepper *et al*. (1991). According to the method isolates of Rhizobium are spread plated on King's B medium supplemented with 4.4 g/L of glycine. Filter paper strips soaked in picric acid solution (2.5 g picric acid + 12.5 g Na_2CO_3 in 1L of water) were placed on the lid of plate. The Petriplate were sealed with parafilm and incubated for 72 hours. Production of HCN was indicated by the change in color of the filter paper strips from yellow to brown.

Siderophores production by *Rhizobium* isolates—500 ml King's B agar medium was prepared and autoclaved. Solution A (CAS indicator solution) was prepared by dissolving 60.5 mg of chrome azurol in 50 ml of double distilled water. Solution B (acidic ferric chloride solution) was prepared by dissolving 1.62 mg $FeCl_3$ and 8.5 µl concentrated HCL in total 10 of double distilled water. Solution C (CTAB solution) was prepared by dissolve 40 mg of CTAB in 40 ml of double distilled water. The solution C was first mixed with solution B thoroughly and then this mixture was mixed with sterilized and molten King's B agar and poured in Petri plate. Active cultures of *Rhizobium spp.* either spot inoculated or inoculated in previously prepared wells on agar surface. The yellow zone indicates the positive results.

Phosphate solubilisation by Rhizobium spp.—Mineral phosphate solubilization activity was checked on Pikovaskaya Agar Media (Hi Media). The plates were spot inoculated with log phase culture of bacterial isolates and halo zone is observed around colony after 4 days of incubation at 28°C. Phosphate Solubilization Index (SI) was measured using following formula :

$$PSI = \frac{\text{colony diameter} + \text{halo zone diameter}}{\text{Colony diameter}}$$

The colony forming clear halo were consider as phosphate solubiliser (Pikovskaya 1948)

Bio-formulation and shelf-life studies of Bacterial isolates—The selected strains were used for the preparation of bio-formulations using different carrier materials such as charcoal, wheat bran, rice bran, saw dust and sugarcane bagasse and evaluated for their viable cell count during storage period of 180 days in

laboratory conditions. Population of Rhizobium in all the carriers was determined up to six months (180 days). The population density was measured by mixing 1 g of the sample in 10 ml of distilled water aseptically and serially diluted upto 10^{-7}. Average cell number per sample was calculated by estimating cfu on YEM agar. Finally, the effect of bioformulations on plant productivity was determined. Twenty-five grams of carrier based (six months stored) bioformulation was mixed with 100 ml of 2% carboxymethylcellulose solution and the resultant slurry used for pelleting seeds of *C. arientum.* The earthen pots (60 × 60 × 60 mm) were filled with unsterilized soil and the pelleted seeds were sown in these pots. All experiments were done in five replicates. After 40 days of growth, plants were uprooted to measure plant biomass and number of nodules / plant.

RESULTS & DISCUSSION

Isolation, identification and biochemical characteristics of Rhizobium isolates -The results of morphological and bio-chemical characteristics of isolated strains of bio-fertilizers are summarized in Table 1. The isolates were evaluated for morphological, cultural and biochemical characteristics as per Bergeys manual of systematic bacteriology. On the basis of biochemical characteristic and nodule formation in test plant, there are 14 isolates of *Rhizobium spp* has been identified. These isolates are tested for possibility of having plant growth promotion activity and certain test such as phosphate solubilisation, HCN production, Indole acetic acid test, siderophore production test and salt tolerance test were performed. These tests are generally prerequisite for good plant growth promotion bioinoculant. All the tested strain for HCN test, gelatinase, MR-VP and amylase are negative, isolate Lb3, Ur2, Ur3, Ar2 and Ar3 shown excellent IAA production followed by Lb4 and Mg2 rest of the isolates are producing IAA in low concentration, Mg1, Ar2, Ar3 isolates showed good siderophore production, however Lb1, Ur1, Mg3 are not able to produce siderophore, rest of the isolates produce siderophore in feeble quantity. Phosphate solubilisation test was shown by Lb2, Lb3, Ar1, Ar2, Ur3, Mg2 and Mg3. The most phosphate solubilising bacterial isolates were Lb3 and Ar2.

Shelf life study of carrier based formulation— Shelf life studies of bioformulation were done by determining the viable count at 30 day of interval upto 180 days. On the basis of determination of population density by spread plate method. It was found that population density in case of carriers such as wheat bran, rice bran and baggase decrease drastically i.e. no growth was obtained after 180 day of storage in all three bioformulation containing Ar2, Mg1 and Lb1 Rhizobium isolates.

However, in all three tests, saw dust and charcoal supported colony count of for *Rhizobium spp.* after 180 days. (Fig.1, Fig.2 and Fig.3). Similar finding was also reported by Arora *et al*. (2008) in which they reported that saw dust supported the colony count of 10.01 log cfu/gm of *Rhizobium spp*. As per the guideline laid down by the Bureau of Indian standard all the bacterial inoculants should have minimum CFU of 5×10^7 per g of carrier. Our saw dust carrier based formulations are within the permissible limit after six month of storage.

Plant Growth Promotion Activity of isolates-On the basis of biochemical characteristic of Rhizobium isolates, only three isolates viz. Lb1, Mg1 and Ar2 was selected for plant growth promotion test. Table 2 showed that five carrier based bioformulation such as charcoal, wheat bran, rice bran, saw dust, and baggase was used. The results of a greenhouse experiment 60 days after sowing indicated that isolate were show different growth rate with different carrier , the saw dust based carrier with Lb3 , the wheat bran based carrier with Mg1 , the sugarcane bagasse based carrier with Ar2, show good result of plant growth promotion effect on gram (*C. arientinum)* seed and in between time the viability of microbial cell was observed by cfu count and it was found that cell viability decreased with time. The plants inoculated with Rhizobium isolates significantly increase the shoot length, root length, number of nodules, fresh weight and dry weight of tested plant (Table 2). The chlorophyll content of inoculated plant was increased as compared to control plant. The increase may be due to an increase in photosynthesis, transpiration, stomatal conductance and increased plant growth. The other reason for enhanced chlorophyll may be due to the presence of large number of bundle sheath chloroplast (Arumugam *et al*. 2010).

For preparation of bioinoculant choice of carrier is important and to become a good carrier, the material should be non toxic to bacterial inoculants, and plant, good moisture absorbance capacity, easy to process, free of

Table1—Biochemical characteristic of Rhizobium isolates.

S . No.	Isolate names	Biochemical Characteristics of isolate									
		Colony characte-ristics	**Amylase Test**	**Catalase Test**	**Gelati-nase Test**	**MR-VP Test**	**Salt toler-ance**	**Phosphate solubili-sation**	**HCN produc-tion tion**	**Sidero-phore produc-tion1**	**IAA produc-**
1.	Lb1	Shape-Round, Margin-Gummy	-	+	-	-	++	-	-	-	++
2	Lb2	Shape-Round Margin-Gummy	-	+	-	-	++	++	-	++	++
3	Lb3	Shape-Round Margin-Gummy	-	+	-	-	+++	+++	-	+++	++++
4	Lb4	Shape-Round Margin-Gummy	-	+	-	-	++	+	-	++	+++
5	Ur1	Shape-Round Margin-Gummy	-	+	-	-	++	-	-	-	+
6	Ur2	Shape-Round Margin-Gummy	-	+	-	-	+	-	-	+++	+++
7	Ur3	Shape-Round Margin-Gummy	-	+	-	-	++	+	-	+++	++++
8	Mg1	Shape-Round Margin-Gummy	-	+	-	-	+	++	-	++++	+++
9	Mg2	Shape-Round Margin-Gummy	-	+	-	-	++	+	-	++	++
10	Mg3	Shape-Round Margin-Gummy	-	+	-	-	+	++	-	-	++
11	Mg4	Shape-Round Margin-Gummy	-	+	-	-	+	-	-	+	++
12	Ar1	Shape-Round Margin-Gummy	-	+	-	-	+	+	+	-	++
13	Ar2	Shape-Round Margin-Gummy	-	+	-	-	+++	+++	-	++++	++++
14	Ar3	Shape-Round Margin-Gummy	-	+	-	-	++	–	-	+++	++++

(-) No production, (+) poor, (++) - fair, (+++) - good, (++++) - Excellent

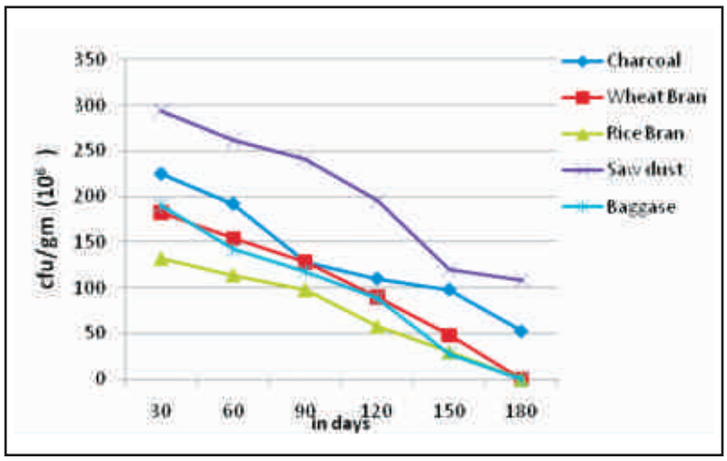

Fig. 1— Cfu count of Ar2 with different types of carriers

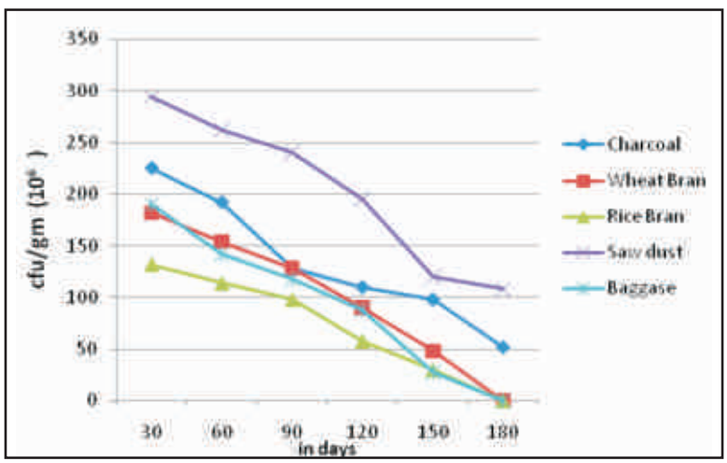

Fig. 2— Cfu count of Mg1 with different types of carriers

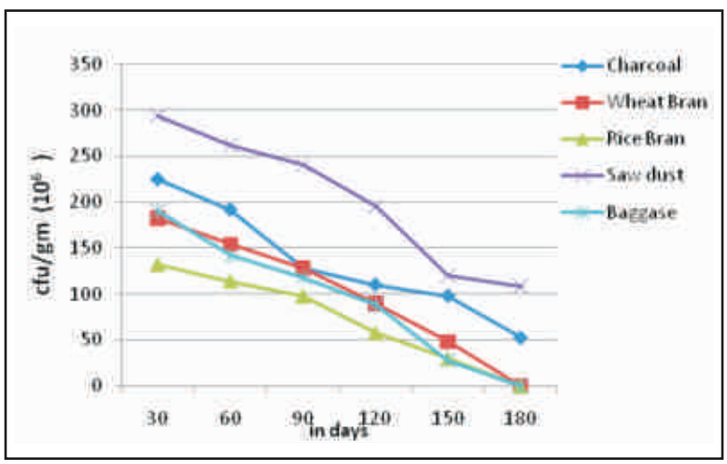

Fig. 3— Cfu count of Lb1 with different types of carriers

lump forming material, easy to sterilize by autoclaving, inexpensive, good adhesion to seed and good pH buffering capacity. In search of efficient PGPR strains with multiple activities, we prepared fifteen bioformulations using three isolates of Rhizobium. For the development the formulations mineral carriers (wheat bran, charcoal, rice bran, sugarcane bagasse, saw dust) has been used for increasing stability in interaction between associated PGPR and *C. arientinum* plants, preparation of bacterial suspension and development of bioformulations included a binder (Carboxymethylcellulose). Out of five tested carriers, the saw dust based carrier with Lb3 , the wheat bran based carrier with Mg1 , the sugarcane bagasse based carrier with Ar2, show good result of plant growth promotion effect on gram (*C. arientinum*) seed and in between time the viability of microbial cell was observed by cfu count and it was found that cell viability decreased with time. Similar finding was also reported by Arora *et al*. (2008) and Singh *et al*. (2014) in which they also found that out of tested carrier, saw dust bioformulation was the best resulting good population density of tested inoculants.

It was well understood that *Rhizobium spp.* have the ability to produces various enzymes like nitrogenase (Baoling *et al*. 2007). Some bacterial and fungal species have the ability to synthesize plant growth regulators such as indole -3 – acetic acid and other indole related compound (Furukawa *et.al*. 1996), siderophore production which is responsible to chelate iron from soil and make available to plant is known to directly promote plant growth by supplying iron and contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitat (Loper & Henkels 1997). Phosphorus is one of the most important plant nutrients and a large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and becomes unavailable to plants (Nautiyal 1999). Mechanisms of growth promotion by PGPR include production of plant growth activators such as IAA, release of volatile growth-stimulating compounds, and inhibition of deleterious Rhizobacteria via competition for iron (Ramazan *et al*. 2005, Zaidi & Mohammad 2006, Turan *et al*. 2006). The formularize materials were not particularly

Table 2—Effect of different bioformulation on growth promotion of *C. arientum*

S . No.	Treatment	Shoot length (cm)	Root length (cm)	No of nodule	Fresh weight (gm)	Dry weight (gm)	Chla (mg/g)	Chlb (mg/g)	Total chlorophyll (mg/g)
1.	Control	5.0±0.1	7.0±0.2	-	0.560±0.1	0.110±0.05	1.078±0.2	0.803±0.03	1.859±0.02
2.	Lb3+seed	7.5±0.3	11.0±0.1	-	0.588±0.2	0.156±0.03	1.797±0.8	1.442±0.05	3.200±1.02
3.	Mg1+seed	6.9±0.2	16.0±0.8	-	0.550±0.05	0.167±0.12	1.206±0.4	1.422±0.05	2.600±1.05
4.	Ar2+seed	6.2±0.1	12.0±0.6	-	0.998±0.10	0.226±0.05	1.195±0.6	1.529±0.09	2.696±0.60
5.	Lb3+seed+charcoal	8.0±0.1	11.5±0.1	-	1.910±0.50	0.175±0.03	1.654±0.6	1.615±0.08	3.233±1.15
6.	Mg1+seed+charcoal	7.5±0.2	23.0±0.9	-	0.741±0.02	0.254±0.20	1.068±0.4	0.811±0.02	1.857±0.80
7.	Ar2+seed+charcoal	9.0±0.5	22.0±0.1	-	0.924±0.60	0.271±0.04	0.996±0.2	0.353±0.06	1.330±0.90
8.	Lb3+seed+wheat bran	9.0±0.5	15.7±0.5	2	1.890±0.80	0.157±0.05	2.126±0.9	2.835±0.03	4.910±1.52
9.	Mg1+seed+wheat bran	9.5±0.2	19.0±0.3	-	0.872±0.08	0.194±0.03	1.808±0.2	1.561±0.07	3.330±1.04
10.	Ar2+seed+wheat bran	8.0±0.1	22.5±0.4	-	0.701±0.90	0.167±0.06	1.417±0.1	1.675±0.05	3.059±1.52
11.	Lb3+seed+rice bran	9.6±0.6	13.0±0.8	-	1.810±0.30	0.263±0.03	1.511±0.1	0.631±0.01	2.334±0.90
12.	Mg1+seed+rice bran	8.2±0.4	16.0±1.02	-	0.980±0.02	0.145±0.04	1.224±0.8	1.132±0.01	2.330±0.50
13.	Ar2+seed+rice bran	8.5±0.2	13.5±0.5	-	0.774±0.02	0.319±0.05	1.149±0.5	0.603±0.05	1.729±0.70
14.	Lb3+seed+saw dust	11.2±0.2	25.0±0.5	-	1.970±0.02	0.410±0.02	1.141±0.9	0.999±0.02	2.115±0.10
15.	Mg1+seed+saw dust	9.5±0.1	25.0±0.7	-	1.398±0.02	0.371±0.01	1.124±0.8	0.469±0.0	11.571±0.90
16.	Ar2+seed+saw dust	7.9±0.7	18.0±0.3	1	1.421±0.02	0.353±0.01	1.084±0.6	1.504±0.0	82.562±0.02
17.	Lb3+seed+ bagasse	8.0±0.5	11.5±0.6	-	1.760±0.50	0.261±0.05	1.439±0.1	1.471±0.0	22.878±0.35
18.	Mg1+seed+ bagasse	8.5±0.4	15.0±0.5	-	0.727±0.70	0.230±0.15	1.430±0.5	0.979±0.0	12.095±0.40
19.	Ar2+seed+ bagasse	10.5±0.3	22.0±0.4	-	0.878±0.10	0.228±0.03	1.100±0.7	1.231±0.0	12.306±1.12

beneficial when used to bioformulate cells of Rhizobium for storage purpose, but rendered more benefits to viable cell when they are meant for field application. Nakkeeran *et al*. (2005) mentioned that the performance of bioformulation can be increased by the incorporation of water soluble adjuvant, oils, stickers, and emulsion. The use of plant growth promoting rhizobacteria (PGPR) has a potential role in developing sustainable systems for crop production.

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