RESEARCH ARTICLE

# Characterization effective of *Rhizobium* and *Agrobacterium* species used as a bio fertilizers on growth of chick pea (*Cicer arietinum*)

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The present study was conducted to evaluate combine effect by using *Rhizobium sp* and *Agrobacterium sp* as a biofertilizer on nodule formation and growth of chick-pea plant and to study the efficiency of seed inoculation for nitrogen fixation. *Rhizobium sp* and *Agrobacterium sp* effect was studied using *Cicer arietinum* and controlled condition. *Rhizobium sp* and *Agrobacterium sp* were isolated from root nodules of *Cicer arietinum*, sterilized, prepared serial dilutions, incubated then applied on *Cicer arietinum* seeds and also on controlled seeds, pot experiments were conducted to evaluate the effect of bacterized seeds and controlled seeds. Therefore, it can be concluded that combined biofertilizer (*Rhizophium sp* and *Agrobacterium sp*) application was the most effective in terms of yield in *Cicer arietinum* cropping pattern and can be used to reduce the use of chemical fertilizers for sustainable crop production in terms of yield and soil fertility as well as environmental safety. However, more and intense systematic studies are required to provide better understanding of biofertilizer use in making crop production more profitable income generating.

**Key Words:** *Rhizobium sp; Agrobacterium sp; Cicer arietinum; Seeds; Biofertilizer*

## INTRODUCTION

Rhizobia are famous for their potency to embed a symbiosis with legumes. They inhabit root nodules, where they reduce atmospheric nitrogen and make it available to the plant. The Rhizobium bacterial strains in the starter cultures were needed to be grown on a large scale for which their mass production was urgently needed. In order to produce large quantities for a microorganism, the inoculums must be purified and liquid form would be cultured. Next, have to select the culture vessel used for liquid culture. In this case the size of the culture vessel is adjusted as required and productivity depends on vessel size [1].

Environmental requirements for microbial culture vessels have to be ensured. Mass propagation at a higher rate after isolation of micirobial biofertilizer is a challenging issue. This involves the culturing of large volumes of bacteria to obtain enough material to spectrally characterize an isolated compound. Currently, it is estimated that upwards of 90% of all culturing experiments in biotechnology are performed through shaken cultures [2]. The benefits of using shaken cultures are numerous and include the ability to culture in high density, a relatively low-cost barrier, and the use of easily accessible equipment. There are, however, a number of drawbacks to using shake flasks [3]. Primarily, shake flasks rely on surface aeration to transfer oxygen into the culture, resulting in low oxygen transfer compared to stirred bioreactors [4].

For natural product researchers, the application of shake flasks for the large-scale cultivation of microorganisms for natural product isolation has additional challenges. Although high-capacity shakers capable of handling numerous flasks at one time are available, they are expensive. This necessitates the use of smaller, cheaper orbital shakers that requires multiple rounds of culturing to generate enough material for compound isolation and characterization [5]. Conventional funnel or balloon shaped glass containers are taken for the purpose of cultivating more bacteria in a short period of time. It is not cost effective as low cost parts are assembled. Agitation of these types of bioreactors is accomplished by the flow of sterile air instead of mechanical stirring. Sometimes fermenters also used; the equipment, which provides the proper environment for the growth of a desired organism. It is generally a large vessel in which, the organism is kept at the required temperature, pH, dissolved oxygen concentration and substrate concentration. A sophisticated fermenter contains pH regulator, oxygen level regulator, anti-foam device, temperature controller, etc [6].

## MATERIALS AND METHODS

### Mass cultivation of *Rhizobium sp* and *Agrobacterium sp*

*Rhizobium sp* and *Agrobacterium sp* mass culture was prepared by using broth culture. Selective medium broth was prepared on Erlenmeyer flask and was sterilizes at 121°C for 15 minutes. Then the liquid medium was kept for cooling. After cooling down, a large amount of *Rhizobium sp* and *Agrobacterium sp* were transferred aseptically from the agar medium to liquid medium with the aid of a sterile inoculating needle. The flask containing broth and isolates was then placed on the shaker at 280°C under 120 rpm for three days to accelerate the growth of *Rhizobium sp* and *Agrobacterium sp*. After three days, growth was observed on the flask and it was taken out from the shaker for further process.

### Antibiotics resistance tests

PGPR strains were grown overnight in Luria-Bertani broth (for 1 liter: peptone 10 g; yeast extract 5 g; NaCl 5 g; $H_2O$ ad 1 liter ) and washed with 0.9% saline solution. They were then streaked on LB agar containing the antibiotics and on a LB agar control without antibiotics. They were incubated for one week at room 37°C.

### Rhizosphere colonization assay

Selected isolates were assessed for their ability to colonise the root system of *Cicer arietinum* in agnotobiotic screening method [7]. Bacteria were grown in 5 mL NB for 48 hours at 28°C on a rotary shaker at 200 rpm, then washed twice in sterile 0.03 M $MgSO_4$, to ensure that the pellet was free of the bacterial growth medium, and resuspended at an OD 600 of 0.15 in fresh 0.03 M $MgSO_4$. $MgSO_4$ was chosen in place of saline to avoid deleterious effects the saline may have had on seed germination. *Cicer arietinum* seed was surface sterilized as above and uniform seeds were placed into sterile Petri dishes and each dish was incubated at room temperature for 1 hour with 10 ml of either sterile 0.03 M $MgSO_4$ (used as a negative control) or bacterial suspensions in sterile 0.03 M $MgSO_4$. The seeds were then transferred to sterile test tubes containing 0.6% water-agar with 4 replicates per bacterial isolate. Growth of the seedlings and bacteria in the tubes was monitored for 12 days and the presence of bacterial colonies on the roots recorded at 12 days.

### *In vitro* assay for *Rhizobium sp* and *Agrobacterium sp*

**Auxin production:** Auxin production by the rhizobacterial strains obtained

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from the initial isolation procedure was determined in the presence or absence of L-Tryptophan (L-TRP) by colorimetric analysis using the Salkowski reagent method [8]. Pure bacterial colonies were looped into sterile 5 ml sterile Nutrient Broth (NB) (Becton Dickinson) in McCartney bottles and shaken on a rotary shaker at 200 rpm and 28°C for four days.

Cultures at 4 days old were adjusted to an OD 600 of 0.5 and 1 ml of culture solution was added to 10 ml of Glucose Peptone Broth (GPB) (glucose 10.0 g, peptone 5.0 g, 1000 ml $H_20$, pH 7.0) in McCartney bottles giving a final volume of 11.0 ml. To test for auxin activity in the presence of L-tryptophan (L-TRP), 1 ml of the OD adjusted culture solution was also added to GPB amended with 1 ml of filter sterilized (0.2 µm membrane filter, Schleicher and Schuell) 0.2% L-TRP solution giving a final concentration of 0.167 g/l L-TRP and volume of 12.0 ml. Cultures were incubated at 28°C for 48 hours in the dark at 200 rpm.

Non-inoculated controls with and without L-TRP were included for comparison. After incubation, 1.5 ml of the culture medium was centrifuged (5000 g, 10 min) to remove the bacterial cells. A 1 ml aliquot of the supernatant was mixed with 4 ml Salkowski reagent and allowed to stand for 30 min for colour to develop before absorbance at 535nm was measured. Auxin compounds expressed as IAA (µg $ml^{-1}$) equivalents were determined by comparison with an IAA (Sigma) standard curve over the range 0 to 50 µg $ml^{-1}$. To 4 ml Salkowski reagent, 1 ml of each IAA standard was added, allowed to stand for 30 min then the absorbance measured at 535 nm.

**ACC deaminase production:** To confirm the production of ACC deaminase by the isolates the method of Li et al., was used. A single colony of selected isolates was looped into 5 ml sterile tryptone- yeast broth (5 g tryptone, 3 g yeast extract, 0.89 g $CaCl_2.2H_2O$, 1000 ml $H_2O$; TY) in triplicate and incubated overnight at 28°C on a rotary shaker at 200 rpm. The bacterial isolate *Sinorhizobium meliloti* SM1021 does not have ACC deaminase activity and was included as a negative control. Two ml of the overnight cultures were centrifuged at 8000 g for 5 min, and then the cell pellet washed twice with 1 ml sterile DF salts solution and finally resuspened in 1 ml fresh DF salts. The 1 ml culture suspension was added to a sterile 30 ml tube containing 1 ml DF salts and to this 12 µl of a 0.5 mm ACC solution was added to give a final concentration of 3.0 mm. Two ml of sterile DF salts amended with 3.0 mm ACC was prepared as a blank. All the tubes were incubated for 24 hours on a shaker at 200 rpm and 28°C. Just prior to conducting the ACC consumption assay, the ninhydrin reagent was prepared. The ninhydrin reagent consisted of reagent A: (1 m citrate buffer; 1.53 g citric acid, 15.42 g sodium citrate, 60 ml $H_2O$) combined with reagent B: (500 mg ninhydrin, 15 mg l-ascorbic acid, 60 ml ethylene glycol) to make up a final volume of 120 ml. A standard curve was prepared using DF salts as a diluent for concentrations of ACC ranging from 0.05-0.50 mm. A 1 ml aliquot of each culture and blank was centrifuged in a 1.5 ml centrifuge tube at 8000 g for 5 min and the supernatant was diluted 1/10 with fresh DF salts. Diluted cultures (60 µl), blanks and standards were added to separate wells in a 96 well chimney top PCR microplate (Axygen) and to each well 120 µl of the ninhydrin reagent was added. The PCR plate was covered with a silicon mat (Axygen) and placed in a thermocycler (Bio-Rad MyCycler Version 1.065) at 100°C for 30 min. The plate was then allowed to cool and the colour to develop for 10-15 min at room temperature. The absorbance was then read at 570 nm on a microplate reader (Beckman and Coulter DTX 880 Multimode Detector).

**Cellulase production:** Cellulase production was assessed using the methods of Matthysse et al., [9] and Hameed et al., [10]. Yeast extract (1.2 $gL^{-1}$), cellulose (10 $gL^{-1}$) 0.2 $gl^{-1}$ Congo red and agar (8 $gL^{-1}$, Grade A) were added to 800 ml $H_2O$ and autoclaved at 121°C for 20 min. After autoclaving, 200 ml sterile M9 salts, 2 ml of sterile 1 m $MgSO_4$ and 100 µl of sterile 1 M $CaCl_2$, were added. Twenty µl of a 0.5 OD 600 nm culture solution of each PGPB in 0.89 % (w/v) NaCl solution was placed in the centre of a cellulose plate in triplicate. After 7 days of incubation at 28°C, isolates surrounded by clear halos were considered positive for cellulase production.

**Pectinase production:** Pectinase production was also determined in M9 medium except the cellulose was replaced with pectin (4.8 g/liter) and the Congo red omitted. Twenty µl of a 0.5 OD600 culture solution in 0.89% (w/v) NaCl solution was placed in the centre a pectin plate in triplicate. After 7 days of incubation at 28°C, the plates were flooded with 2 M HCl and isolates surrounded by clear halos were considered positive for pectinase production [9].

**Phosphorus solubilisation:** The ability of selected isolates to solubilise phosphorus in vitro was assessed on several different media. In a method adapted from Oresnik et al., [11], sterile calcium phosphate precipitate produced from combining 50 ml 10% (w/v) $K_2HPO_4$ and 100 ml 10% (w/v) $CaCl_2$, was added to 850 ml autoclaved Potato Dextrose Agar (PDA) while stirring to produce a final volume of 1000 ml. The PGPB isolates were patch inoculated on to the $CaHPO_4$ plates using sterile orange sticks from cultures grown on TY agar at 28°C for 2 days. The inoculated $CaHPO_4$ plates were incubated for 7 days at 28°C and the assay was repeated twice.

**Polyamine production:** Polyamine production by the bacteria was determined using a modified Moeller's Decarboxylase Agar Medium (MDAM) [12]. This media contains 5.0 gm peptone, 3.0 gm yeast, 0.02 gm phenol red 0.03 gm $MnSO_4$ 15.0 gm agar in 1000 ml deionized water. L-arginine-hydrochloride (1$gL^{-1}$) was added to the MDAM prior to autoclaving. The control MDAM did not contain L-arginine. Bacteria were patch plated onto the MDAM plates and incubated in the dark for 24 hours at 28°C. The ability to produce arginine decarboxylase and hence putrescine from the arginine contained in these agar plates is indicated by a dark red halo beneath the bacterial colony [12].

**Indole acetic acid production:** YEMA Culture media add tryptophan (0.1%) then all isolates inoculated with this media. Then it kept shaking incubator (32°C, 100 rpm) for 48 hours.

**Siderophore production:** To detect siderophore production by the isolates, Chrome Azurol S (CAS) agar plates [13] were prepared using the modified method of Denarie et al., [14]. The PGPB were patched from TY agar onto the CAS agar plates using a sterile orange stick. There were 4 PGPB per CAS agar plate and 3 replicate plates per PGPB. The plates were incubated at 28°C for up to 48 hours and an orange halo produced around the bacterial colonies was considered positive for siderophore production. As Hexadecyl Trimethyl Ammonium Bromide (HDTMA), which is used to complex the CAS and the ferric solution, has antibacterial properties [14], the effect of this cationic detergent was evaluated by preparing 0.1x TSA with 73 mg $L^{-1}$ HDTMA and bacterial growth was compared with bacteria patched onto unamended 0.1x TSA. To detect siderophore production by the isolates, Chrome Azurol S (CAS) agar plates [13] were prepared using the modified method of Denarie et al., [14]. The PGPB were patched from TY agar onto the CAS agar plates using a sterile orange stick. There were 4 PGPB per CAS agar plate and 3 replicate plates per PGPB. The plates were incubated at 28°C for up to 48 hours and an orange halo produced around the bacterial colonies was considered positive for siderophore production. As Hexadecyl Trimethyl Ammonium Bromide (HDTMA), which is used to complex the CAS and the ferric solution, has antibacterial properties [14], the effect of this cationic detergent was evaluated by preparing 0.1 × TSA with 73 mg $L^{-1}$ HDTMA and bacterial growth was compared with bacteria patched onto unamended 0.1 × TSA. Each test was carried three times and statistically, they were analyzed.

## RESULTS AND DISCUSSION

The present study, a total of 18 out of 166 Rhizosphere isolates were obtained by the preliminary isolation technique. However, this does not represent the total culturable proportion of Rhizosphere organisms in these soils. The similar results reported already, the culturable fraction of soil microorganisms is generally found to be less than 5% of the total microscopically countable cells [15] which is attributed to the intrinsic selectivity of any medium utilized as well as the incubation conditions [16]. Furthermore, in the current study, the true population of bacteria in the soils may be underrepresented due to loss of viability of some bacteria during drying of the rhizosphere soils before plating onto the agar media as well as the selection of only fast growing colonies on the ½ NA agar.

This rich diversity is due to existence of microbes in all niches where life is possible. The microbial processes control the transformation of nutritionally agri product to a protein- rich food substrate which represents a significant contribution to energy and nutrient budgets of the terrestrial ecosystems [17,18]. A lot of physico-chemical parameters are significantly influencing the microbial activity in the mangrove environment [19].

Microbial diversity in Indian soil has been studied especially for nitrogen fixers (azotobacter and azospirillum), phosphate solublizers, cyanobacteria, actinomycetes and fungi [20]. The current study influence of bio-fertilizers on

seed germination of *Cicer arietinum* at various stages of its growth was reported. The previous study demonstrated in enhancement of seed germination might be attributed to the role of Rhizobacteria, Azospirillum and phosphobacteria in enhancing the availability of nitrogen and phosphorus in the soil thus making these nutrients available to the germinating seed with consequent enhancement in the metabolic activity resulting in higher germination [21].

The current study influence of bio-fertilizers on total leaf area of *Cicer arietinum* at various stages of its growth was reported in Table 1. The similar result were updated already for this research, increase of leaf area is attributed to the nitrogen that was made available in the soil due to the organism present in the bio-fertilizers. Nitrogen application increases the metabolism rate and transport of growth promoters in the plants which results in promoting length of the leaf and leaf area [22].

Previous studies have shown that around 80% of rhizosphere organisms are able to produce auxin [23] and in the current study 97% of the organisms tested produced detectable levels of auxin. A greater number of high auxin producing (>10.0 µg $mL^{-1}$ in presence of L-TRP) isolates were obtained from the rhizosphere of crop species than from native species. For instance, 16 high auxin producing isolates were obtained from the rhizosphere soils of peas and wheat at Meckering compared with four from the rhizosphere soil of native species at Chittering. The two highest auxin producing isolates were obtained from peas at Meckering (*P. granadensis* PMK4) and from native species at Chittering (NCH7) (Table 1).

Auxin production by the rhizobacterial isolates was substantially increased with the addition of L-TRP to the culture medium. The number of isolates capable of producing >1.0 µg $mL^{-1}$ IAA equivalents increased by 50% with the addition of L-TRP to the medium. These results complement those of other workers on the increases in microbial production of IAA upon the addition of L-TRP to culture media and soils [24], and indicate that L-TRP serves as a physiological precursor for the biosynthesis of auxins in microbes.

Root exudates are a natural source of the amino acid tryptophan for rhizosphere microflora in soil [25]. Sheldrake [26] found detectable amounts of tryptophan in the root exudates of some, but not all varietes of wheat, and it is suggested, therefore, that not all plants release tryptophan in quantities adequate for the microbial production of auxin. However, Steel et al., [27] showed that *Azospirillum brasilense* has a tryptophan independent auxin biosynthesis pathway that accounts for 90% of the auxin produced by this bacterium in the absence of exogenously applied L-TRP.

It has frequently been demonstrated that formulations of the Salkowski reagent show varying specificity for auxin and indolic compounds. For instance, Sarwar et al., [28] examined the specificity of three versions of the Salkowski reagent and the three formulations also reacted with Indolepyruvic Acid (IPyA) and Indoleacetamide (IAM), which are intermediates in the IAA biosynthesis pathway. As a consequence, Glickman and Dessaux suggest the production of IPyA and IAM by bacterial isolates may overestimate IAA production using these techniques. In support of this, using Reverse Phase High Performance Liquid Chromatography (RP-HPLC) Sheldrake [26] detected high levels of indole-3-lactic acid, which is detected by some forms of the Salkowski reagent, as well as IAA and IAM in the culture supernatant of a *P. putida* strain. Furthermore, Stewart and Anderson determined IAA production by *Pseudomonas aeruginosa* strain OG by the spectrophotometric (Salkowski) method as 28.83 µg $mL^{-1}$ whereas using High Performance Thin Layer Chromatography (HPTLC), production of IAA was 1.60 µg $mL^{-1}$. This indicates that IAA intermediate compounds.

Two of the PGPB were able to improve plant growth and yields in the field in the current study. This includes the isolate VBSR 8 and VBRS 5 which increased nodulation of the peas at the *Cicer arietinum* field site compared with the rhizobia only controls. Shoot Dry Weights (SDW) and grain yields were not significantly increased upon co-inoculation with VBSR 8 and VBRS 5 at the Site 1, possibly due to the black spot infection which may have reduced the yields at this site, although they were higher than the average for India. Slatter et al., repoted early research in the field pea yields in Western Australia can exceed 2.0 tonne $ha^{-1}$ under favourable growing conditions particularly with the newer varieties [29]. Therefore, Rhizobium needs to be investigated in the field with other varieties of pea to determine if nodulation and yields are improved in these varieties with this PGPB. This isolate demonstrates several of the common traits associated with beneficial plant-microbe interactions, including production of the auxin IAA, production of ACC deaminase, siderophore production, as well as the solubilization of sparingly soluble phosphates in both solid and liquid media (Table 2) [30-34].

**TABLE 1**

**Root, Shoot length and Leaf area measured at time interval of 15 days upto 60 days**

| Parameters | Days | *Cicer arietinum* | | |
|---|---|---|---|---|
| | | Control | Bio-fertilizers | Chemical fertilizers |
| Root length (cm) | 1 | 0.08 ± 0.015 | 7.5 ± 0.013 | 3 ± 0.010 |
| | 15 | 1.2 ± 0.015 | 12.7 ±0 .013 | 4.5 ± 0.021 |
| | 30 | 1.5 ± 0.015 | 20.5 ± 0.013 | 6 ± 0.011 |
| | 45 | 2 ± 0.015 | 27.5 ± 0.013 | 7.2 ± 0.013 |
| | 60 | 2.5 ± 0.015 | 42.7 ± 0.013 | 13 ± 0.023 |
| Shoot length (cm) | 1 | 0.07 ± 0.015 | 8.3 ± 0.015 | 3.1 ± 0.012 |
| | 15 | 7.14 ± 0.015 | 22.1 ± 0.023 | 20.3 ± 0.011 |
| | 30 | 11.9 ± 0.015 | 29.8 ± 0.020 | 23.2 ± 0.014 |
| | 45 | 15.6 ± 0.015 | 48.3 ± 0.015 | 33.1 ± 0.012 |
| | 60 | 2.05 ± 0.015 | 52.1 ± 0.023 | 35.3 ± 0.011 |
| Leaf area ($cm^2$) | 1 | 0.07 ± 0.015 | 0.14 ± 0.011 | 0.09 ± 0.013 |
| | 15 | 0.12 ± 0.015 | 0.32 ± 0.013 | 0.13 ± 0.01 |
| | 30 | 0.26 ± 0.015 | 0.56 ± 0.011 | 0.26 ± 0.012 |
| | 60 | 0.32 ± 0.015 | 0.73 ± 0.011 | 0.33 ± 0.013 |

**TABLE 2**

***In vitro* assay for *Rhizobacterium sp* and *Agrobacterium sp***

| Origin | PGP properties | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Strain | Auxin | IAA | ACC | Siderophore | Pectinase | Phosphorus | Polyamine | Cellulose |
| 1 | + | + | - | + | - | + | + | - |
| 2 | + | + | + | + | + | + | + | + |
| 3 | + | + | + | + | - | + | + | - |
| 4 | - | - | + | + | - | - | + | - |
| 5 | + | - | - | + | + | + | - | + |
| 6 | + | + | + | - | + | + | + | + |
| 7 | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + |
| 9 | + | + | - | + | - | + | + | - |
| 10 | + | + | + | - | + | + | + | + |

**Note:** '+'=presence; '-'=absence.

## CONCLUSION

The present study was conducted to investigate the effect of *Rhizobium sp* and *Agrobacterium sp* different growth and yield parameters of *Cicer arietinum* intercropping system. PGPR inoculation was found significant on pod length in pea monoculture and in pea intercropped with wheat. Inoculation with combined biofertlizers was found to be significant on days to maturity, number of branches per plant, number of seeds per pod and seed yield in pea monoculture and it showed a significant influence on days to maturity, number of branches per plant and on pod length in pea intercropped with wheat. It gave the highest yield in pea monoculture. Therefore, it can be concluded that combined biofertilizer (*Rhizophium sp* and *Agrobacterium sp*) application was the most effective in terms of yield in *Cicer arietinum* cropping pattern and can be used to reduce the use of chemical fertilizers for sustainable crop production in terms of yield and soil fertility as well as environmental safety. However, more and intense systematic studies are required to provide better understanding of biofertilizer use in making crop production more profitable income generating.

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