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Enhanced Plant Performance in *Cicer arietinum* L. due to the Addition of Combination of Plant Growth Promoting Bacteria

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Abstract

Six bacterial strains with differing abilities to produce varying concentrations of Indole Acetic Acid were tested individually and in consortia for plant growth promoting and fitness related traits of *Cicer arietinum*. In all experiments the presence of the nitrogen fixer *Mesorhizobium ciceri* resulted in increased biomass production. In the absence of this strain, IAA *Psedomonas putida* and *Bacillus megaterium* hinder plant growth and fitness related traits. The application of mixes of the three strains always resulted in better plant performance when *M. ciceri* was present. Whereas *P. putida* has a noticeable plant growth-promoting effect *B. megaterium* resulted less effective. The low levels of IAA produced by the selected strains had a significantly greater positive effect on plant biomass accumulation, flower, pods and seed production as well as on total plant nitrogen and nitrogen concentration in seed than high IAA producer strains.

Keywords: *Cicer arietimum*; Indole Acetic Acid; *Bacillus megaterium; Pseudomonas putida; Mesorhizobium ciceri*

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1. Introduction

Cicer arietinum L., commonly known as chickpea, is one of the most important legume crop all over the world [1]. It represents an important source of protein for humans and fodder for livestock in temperate and semiarid climates [2]. It is in these semiarid areas where chickpea's production can be jeopardized due to impoverished soils and hindered biogeochemical cycles, due to high temperatures and low rainfall. The addition of fertilizers has been the traditional way of improving soil fertility and the only way to obtain reasonable crops. However, chemical fertilizers are costly and have shown negative effects on the soil fertility in long and intermediate terms [3]. Traditional nutrient management has been mainly based on external fertilizer inputs for the maintainance of high crop productivity [4]; however, in the last decades, crop yield has not increased proportionally with increasing fertilizer inputs, leading to low nutrient use efficiency and strong environmental imbalances [4]. To overcome the lack of fertilization at no expenses of soil pollution implies to explore alternative routs of nutrient use and input, possible through biological rhizosphere processes.

Soil microbes are paramount for soil health and they are responsible for maintaining nutrients cycling [5]. They have the ability to convert fixed forms of nitrogen and phosphorus in soil into soluble forms that can be easily taken up by plants [6]. In addition, rhizobial strains are known to establish positive symbiosis with plant in the legume family that result in biological nitrogen fixation and enhanced plant growth [7]. Not only rhizobia, but many other microorganisms, concentrated in the rhizospere of plants are described as plant growth promoting bacteria (PGPB) [8]. Several studies have reported many of the beneficial aspects induced by PGPB to the growth of plants [9] and to the entire community [10]; such benefits include the increase in growth, N and P-uptake by plants, increased photosynthesis and decreased carbon construction costs, through the inoculation of elite strains in pot experiments [11] and under field conditions [12, 13, 7]. PGPB can also increase the growth of plants thorugh the production of phyto-hormones such as Indole acetic acid (IAA) [14]which are well known to be plant growth promoters. Therefore, inoculation of plants by selected microorganisms at different concentration is likely to have beneficial effect in plant performance. Moreover, as different microbial strains are able to produce different levels of the same phyto-hormone, results might vary

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from strain to strain. In addition and as previously proposed by [15], only one type of microorganism may not be effective for sufficient plant growth enhancement. Single strains might not be able to compete with native microorganisms or could not colonize properly the rhizosphere in new soil environment. In consequence, we propose that the answer of plants to inoculation would vary according to the strain used and that it is likely that a consortium of PGPB is preferred for soil inoculations to enhance plant crop.

The present study investigates the effects of six strains of *Pseudomonas putida*, *Bacillus megaterium* and *Mesorhizobium ciceri* alone and in combination, on plant performance of *Cicer arietinum*. We hypothesize that chickpea plants inoculated with indole acetic producing bacteria and Rhizobium are likely to produce more biomass, flowers and viable seed than those inoculated with only one of the bacterial strains. We used low and high IAA producing strains for the three microorganisms. The study was conducted in a growing chamber and in a glass house setting during the spring season (March to June, 2015).

2. Material and Methods

2.1. Biological material

The strains of *P. putida*, *B. megaterium* and *M. ciceri* were from our own collection obtained from rhizosphere of wild plants collected from a cropland located in a farm next of the University Pablo de Olavide, Seville (Spain). The site is located at latitude 37° 36'12.72″N, longitude 5° 93′40.19″E, and an elevation of 8 m above the sea level. The area is characterized by a continental climate in the range of Mediterranean Type Climate with annual average rainfall ranging from about 600-700 mm, most of which is irregular and falls in autumn and spring. The monthly mean temperature ranges from a minimum of 0°C to a maximum of 42°C. The soil from where plants were collected is loamy and its chemical properties are described below. Bacteria were extracted by the dilution methods, isolated in selective media and single colonies were amplified for further sequencing. Sequences were submitted to the Basic Local Alignment Search Tool (BLAST on line), and gave a likelihood of 99% of similarity of with strains of *P.putida*, *B. megaterium* and *M. ciceri*, for what they were selected for the present study. Indole Acetic Acid (IAA) production was identified and measured using the Salkowski reagent [30].

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The strains were maintained on yeast extract Mannitol (YEM) agar and Louria Bertani media [16] at 4 ºC. For inoculation of seedlings, cultures were grown for 6 days in the appropriate broth (LB or YMA) at 23 ºC in an orbital shaker at 100 rpm before dilution to the required concentration of cells.

Seeds of *C. arietinum* were surface-sterilized in 70 % ethanol for 5 min and 1 % sodium hypochlorite for 3 min and then washed six times in sterile distilled water. Once germinated, 15 seedlings were transplanted to the appropriate treatment.

2.2. Experimental setting

Experiments were conducted in glasshouse in bleached plastic pots (20 x 18 cm), using sterile loamy soil (pH 6.2, organic matter 1.77%, available-N 12 mg kg^{-1}, available-P 2mg kg^{-1}, K 112 mg kg^{-1}, Ca 0.417 mEq 100 g^{-1} soil, Mg 0.084 mEq 100 g^{-1} soil, 1.5 kg soil per pot). Soil was thoroughly mixed and passed through a 2 mm sieve to remove large particulate matter. The surface of the filled pots was covered with sterile polyurethane beds to prevent airborne contamination, and watering was conducted weekly through a capped watering pipe. All pots were randomly arranged in the greenhouse at the University Pablo de Olavide (Seville, Spain) and rotated each week to avoid environmental positional effects within the greenhouse. The pots of each inoculation treatment were maintained on independent benches 2 m apart from each other.

The seeds were inoculated in 15 different sets of pots with fifteen replications each. These sets included: (1) sterile soil; (2) soil + *B. mgaterium* Low IAA producer (BmL); (3) soil + *P. putida* Low IAA (PpL); (4) soil + *M. ciceri* Low IAA (McL); (5) soil + *B. mgaterium* High IAA producer (BmH)); (6) soil + *P. putida* High IAA (PpH); (7) soil + *M. ciceri* High IAA (McH); (8) soil + BmL + PpL; (9) soil + BmL + McL; (10) soil + PpL+ McL; (11) soil + BmH + PpH; (12) soil + BmH + McH; (13) soil + PpH+ McH; (14) soil + BmL + PpL+ McL; (15) soil + BmH + PpH+ McH. The control treatment consisted of sterile un-inoculated soil watered with 50% strength of a Hoaglands solution [17]. Inoculation treatments consisted of growth phase broth cultured inoculant at 1x 10^6 cells ml^{-1}. The three strains, *P. putida* (Pp), *B. megaterium* (Bm) and *M. ciceri* (Mc) were first individually applied to pots. For each bacteria we had a high and a low IAA producer (Table 1).

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Table 1. Effect of plant growht promoting bacterias, *B. megaterium*, *P. putida* and *M. ciceri* (L-Low and H-High Indole Acetic Acid producers) on plant performance of chickpea plants grown in sterile soil. Different letters next to the numbers indicate significant differences amongst treatments after significant ANOVA.

Treatment	Nº Flowers	Nº Pods	Nº Seed	N in Seed (mg N. g plant^{-1})
sterile soil	36^{a}	24.6^{a}	26.0268^{a}	1.4923^{a}
BmL	132^{b}	90.2^{c}	90.39^{b}	2.4621^{a}
PpL	176^{b}	147.6^{d}	161.86^{c}	2.7240^{a}
McL	121^{b}	86.1^{c}	99.659^{b}	5.0547$^{a\,b}$
BmH	62^{b}	49.2^{b}	45.701^{b}	2.5665^{a}
PpH	99^{b}	77.9^{c}	82.4182^{b}	4.2804$^{a\,b}$
McH	86^{b}	65.6^{b}	61.502^{b}	3.7236^{a}
BmL + PpL	49^{a}	36.9^{a}	39.0402^{b}	2.6187^{a}
BmL + McL	98^{b}	73.8^{c}	78.0804^{b}	4.3326$^{a\,b}$
PpL+ McL	252^{c}	213.2^{d}	266.789^{c}	12.6672^{c}
BmH + PpH	139^{b}	108.9^{d}	104.029^{c}	4.1412$^{a\,b}$
BmH + McH	77^{b}	69.7^{c}	73.7426^{b}	4.4283$^{a\,b}$
PpH+ McH	163^{b}	135.3^{d}	143.1474^{c}	8.3172^{c}
BmL + PpL+ McL	149^{c}	118.9^{d}	115.041	10.9272^{c}
BmH + PpH+ McH	73^{b}	53.3^{c}	56.3914	6.3945$^{a\,b}$

One germinated seed was transferred to each pot in all treatments. At the emergence of the cotyledons 100 ml of the appropriate inoculum was added according to treatment and covered with soil. After four hours each pot was watered with distilled sterile water. Water was added as necessary to maintain soil moisture through the length of the experiment. The average number of flowers and pods were calculated in each treatment.

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Plants were harvested after maturity, 17 weeks after planting. Their shoot and root dry weights, seed number and weight was measured and recorded. The nitrogen accumulated in shoots was calculated by multiplying the weight of dry shoots by the nitrogen content as measured by the semi micro-Kjedahl method [18].

2.3. Statistical analysis

Pot experiments were arranged in completely randomized block design. Statistical analysis was conducted using one or two-way ANOVA statistical package for social sciences (SPSS) software, version 11.5. Comparisons of means were performed by the LSD test at pp0.05.

3. Results

Three bacterial strains, *B. megaterium*, *P. putida* and *M. ciceri*, with differential ability to produce Indole Acetic Acid (IAA), chosen for their capacity to induce growth promotion in plants, were tested for their ability to promote growth of *C. arietinum* in sterile soils. Simultaneously, we tested the ability of the same strains to exert a positive in other plant traits, like flower, pod and seed production and the amount of nitrogen accumulated by both, shoots and seeds. The individual and combined effect positive of the strains on the growth and seed production of chickpea plants was observed in pots containing only sterile soil with low nutrients soil available supplemented with a 50% strength of a Hoaglans solution.

The inoculation of McL and McH IAA producer strains had the maximum significant (p = 0.0021) stimulatory effect, in terms of shoot production (Figure 1). Shoot biomass production ranked between 1.085 mg in the sterile soil and 3.562mg in the PpL+McL treatment. Nodules were only produced in plants inoculated with *M. ciceri* (McL and McH) indicating that the sterile conditions were observed all through the length of the experiment. The higher number of nodules was consistently and significantly ($p > 0.0001$) obtained in the presence of the Low IAA producer strain (Figure 1). Similarly, plants in all treatments with McL alone or in combination with PpL attained the greatest significant ($p = 0.0403$) total N content per gram of dry biomass (Figure 1). Nitrogen concentrations

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were in the range of 2.7445 mg of N/g of biomass, in plants grown in sterile soil and 20.096 mg of N/g of biomass, in plants under the BmL+PpL+McL treatment.

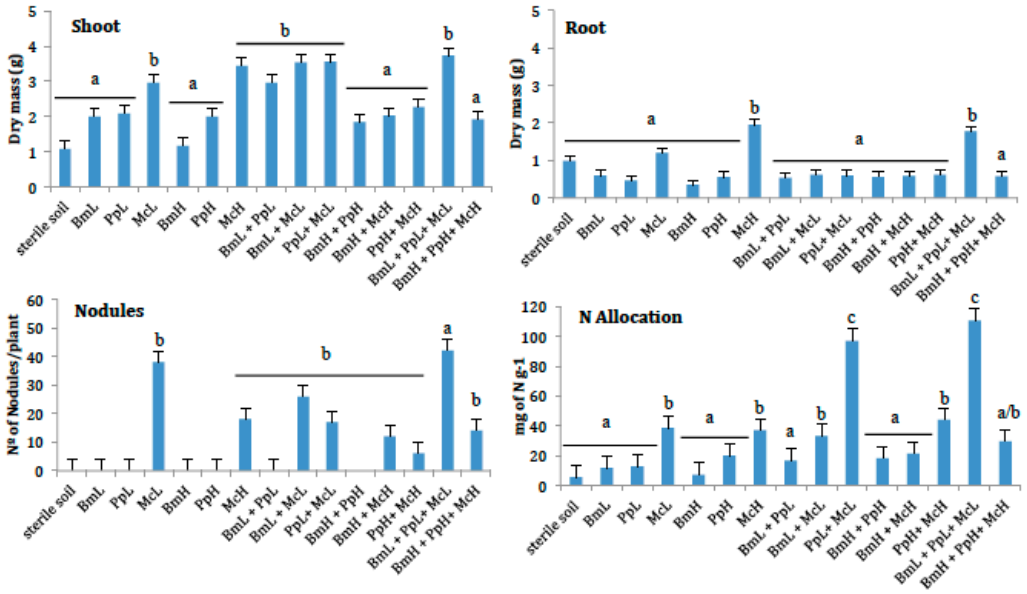

Figure 1. Plant biomass (Shoot and Root), number of nodules and nitrogen per gram of plant biomass, produced by plants of *Cicer arietinum* inoculated with 15 combinations of low and high IAA producer bacteria Different letters on top of the columns indicate significant differences amongst treatments after significant ANOVA.

In pots containing only soil, the number of seed, pod and seeds per pot per plant attained values of 36, 24.6 and 26.03 respectively (Table 1). These values significantly (p = 0.0334) differed from those obtained in plants under the PpL+McL treatment which values for the same variables were 252, 213.2 and 266.79. Hig values of flower, pod and seed production were also attained in plants treated only with PpL, followed by those treated with PpH+McH and Bml+PpL+McL. Also significantly high values were achieved by treatments BmH+PpH , MmL and McL (Table 1).

Significant differences were observed in seed nitrogen accumulation with the greatest value of 12.6672 mg of N per g of dry biomass in plants treated with PpL+McL, BmL+PpL+McL, PpH+McH, BmH+PpH+McH and McL (Table 1). The lowest value of 1.4923 mg of N per g of dry biomass, was reached again in plants grown in the sterile soil. Significantly low values (p >0.0001) were observed in plants grown inoculated with

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BmL (2.4621 mg N. g biomass), BmH (2.5665 mg N. g biomass), BmL+PpL (2.6187 mh N. g biomass) and PpL (2.7240 mh N. g biomass) (Table 1).

4. Discussion

Symbiotic relationships between plants and microorganisms in natural soils are responsible for plant performance. Agriculture could take advantage of these interactions to enhance plant productivity and crops, by manipulating the composition of soils microbial communities [19, 20]. It has been reported that different bacterial strains have the ability to induce growth promotion on diverse crop production [21, 22, 20, 23] through either the induction of nitrogen fixation or the production of varying plant hormones. Results from the present study reinforce this and puts the stress on the fact that the individual effect of one single strain is modified by the presence of one or more other strains, both in the same species or different.

We had hypothesized that chickpea plants inoculated with indole acetic producing bacteria and *Rhizobium* are likely to produce more plant biomass, flowers and viable seed than those inoculated with only one of the bacterial strains and that the level of IAA produced by the selected strains might have an effect on the total plant performance. The results of our study support this hypothesis. The seedlings treated with *M. ciceri* attained high values of shoot biomass. Any time that this strain was present in the treatments, both biomass and total nitrogen concentration in plants was enhanced. Nevertheless, when the strain tested was the one producing high levels of IAA, the enhancing effect was hindered. Simultaneously, plant growth-promoting effect was observed in plants inoculated with the low IAA producing *B. megaterium* and *P. putida* bacteria. Interestingly, any of the consortia that included McL, BmL and PpL resulted in great increase of all studied plant parameters, while the use of any of the H strains resulted in the reverse. This is not the first time we describe the need for relatively low levels of IAA to enhance plant performance. In a previous experiment [24] we describe how intermediate production of IAA resulted in both enhanced root cluster production and plant biomass crop in *Leucadendron salicifolium*, *Viminaria juncea* and *Lupinus albus*. This experiment supports the previous ones in terms of low IAA needed for the increase in plant performance in *C. arietinum.*

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It was also interesting to observe that flowers, pods and seed production were not enhanced at their maximum by McL or McH, but by PpL alone or in combination with Mc. One would have expected to see how the nitrogen fixing *M. ciceri* would have had the best possible effect on plant performance. Nevertheless it was the presence of PpL in all Mc treatments (H and L) that had the greatest effect on plant reproduction traits. This could be due to the IAA added to the plant, that has a synergistic effect in combination with *M. ciceri*. *B. megaterium* also produces IAA and its presence at both H or L values did not give the same results as the observed in the presence of *P. putida*. This fact led us to think that (i) not only IAA but other compounds produced by the bacteria enter into the plant growth-promoting equation [3, 6]; (ii) there is competition amongst the strains that result in better expression of one or another depending on the better competitor, a fat that has been described in several soil types [25], under differing levels of nutrients [26] or under changing plant community composition [27]; (iii) there is plant sanctioning against particular strains as it has been observed in the case of legumes inoculated with particular rhizobial strains that result in the infection of only one strain [28, 29, 7]. These are complex systems that for sure occur in nature, and that are still to be identified in order to better understand and use the plant enhancing effects by soil microorganisms.

5. Conclusion

Our study has proven that *C. arietinun* growth is enhanced by both individually applied plant growth-promoting bacteria and by elite consortiums of such bacteria. The plant hormone IAA is crucial in determining both, plant biomass production and N accumulation in shoot and seed. Low amounts of IAA induce greater biomass and N accumulation than high concentrations (according to the values used in this study).

Author Contributions: Both authors have equally contributed to the experimental design, glasshouse experiment performance, writing and revising the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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