

Inoculation of the Chickpea Plant with Rhizobium and Solubilization Forms of Bacteria to Increase Yield and Nodulation Process

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Abstract

*Biological inoculants are one of the sustainable alternatives to using chemical fertilizers, and they could boost nutrient uptake in availability and crop production in legume-based cropping environment. In this paper, the synergistic ability of co-inoculation of chickpea (*Cicer arietinum* L.) with *Rhizobium ciceri* and phosphate solubilizing bacteria (*Pseudomonas fluorescens*) was studied in terms of nodulation efficiency, shoot biomass and phosphorous uptake. The results obtained by both the green house and field experiment indicated that co-inoculated treatments greatly surpassed their individual and control treatments on all evaluated parameters. The co-inoculated resulted in the grain yield increasing 22.3 percent and nodulation efficiency increased 38.7 percent in the field trials when compared to the untreated controls ($p < 0.01$). The research shows the possibilities of using microbial consortia as an efficient and potentially successful way to increase phosphorus uptake and crops productivity in phosphorus-deficient soil which provides a prospect way of sustainable agriculture especially in pulse-based MPS.*

Keywords: chickpea, *Rhizobium ciceri*, phosphate solubilizing bacteria, *pseudomonas fluorescens*, co inoculation, nod, yield, phosphorus uptake, biological inoculants, sustainable agriculture.

1. Introduction

1.1 The Value of Chickpea in Sustainable Cropping System

Chickpea (*Cicer arietinum* L.) belongs to the most common of cultivated legumes, especially in semi-arid areas of the globe, which contributes to the human nutrition and to the physiological status of soils. Chickpea is a high-value food product as it is a protein, fiber and essential amino acids rich pulse crop. In addition as other legumes, chickpea also helps in enriching the soil, by fixing nitrogen, thanks to a liaison with a nitrogen-fixing bacterium, *Rhizobium*. This nitrogen-fixing capability saves costs of synthetic nitrogen fertilizers, hence cheaper agricultural activities and reduced pollution to the environment. Chickpeas play an important role in sustainable cropping systems through the diversity of diets, enhanced crop rotation and long-term crop productivity by enhanced soil structure and improved organic matter levels.

In addition, the drought-resistance and adaptation to marginal soils also make chickpea a major staple crop requirement of climate-resilient food production across parts of the globe. Although it has these advantages, yield of the crop is at times limited by soil nutrients, especially phosphorus which is needed in the growth and development of plants.(1)

1.2 When Will Phosphorus Limit and Biological Nitrogen Fixation Be Problematic?

Phosphorus is a very important plant nutrient and helps in provision of energy, cell division and roots. In most agroecosystems, especially in those phosphorus-deficient soil areas, plants find it difficult to obtain adequate phosphorus to grow well. This challenge is especially firm in alkaline soils and soil that is low in organic matters and phosphorus becomes attached to the soil particle and therefore it becomes unavailable to the plants. A lack of phosphorus in chickpea plants results in root dysfunction, minimal nodulation and yield of grain, which restricts the whole productivity of chickpea in such settings.

Although biological nitrogen fixation (BNF) between two organisms and the *Rhizobium ciceri* and chickpea plants can support the solution to the lack of nitrogen, the level of nitrogen fixation also tends to remain low due to the unavailability of other essential nutrients, including phosphorus. Phosphorus is extremely essential part of energy metabolism in the rhizobia and it is important in the construction and functioning of the nitrogen-fixing nodules. Hence, phosphorus deficiency does not only hamper the growth of plants but also the functionality of the biological nitrogen fixation that eventually influences the productivity of chickpea crops.(2)

1.3 The use of Microbial Inoculants in Enhancing Nutrient Uptake

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Another innovation and sustainable control used to overcome lack of nutrients in legume crops like chickpea is with the microbial inoculants that consists of nitrogen-fixing microbes, e.g. *Rhizobium* and phosphate-solubilizing bacteria, e.g. *Pseudomonas fluorescens*. The augmentation of biological nitrogen fixation through *Rhizobium* inoculation aims at complementing the efficient rhizobial strains to provide symbiotic nodules on the root of the chickpeas which convert atmospheric nitrogen into a form that can be utilized by the plant. Conversely, phosphate-solubilizing bacteria (PSBs) e.g. *Pseudomonas fluorescens* can make unavailable inorganic phosphorus available to the plants by solubilizing it, thus availing it to the plants. The synergism caused by the combination of the two microbial inoculants (*Rhizobium* and *P. fluorescens*) would help it acquire more nutrients, to grow and increase crop production on soils that are deficient in fertilizers, particularly at low levels of phosphorus.(3)

1.4 Aims of the Co-Inoculation procedure with *Rhizobium ciceri*, and *Pseudomonas fluorescens*

This research was aimed at examining the interactions between co-inoculation of *Rhizobium ciceri* with *pseudomonas fluorescens* and chickpea. In particular, the research was set to:

1. Determine the consequence of co-inoculation in nodulation efficiency of chickpea and shoot biomass, root growth as well as grain production in both greenhouse and field conditions.
2. Evaluate the intake of phosphorus in co-inoculated plant against single strain inoculation and control (Non inoculated).
3. Study the relationship between biological nitrogen fixation and phosphorus solubilization, with the view to how these microbes can be used to boost the performance of the other and hence overall crop performance.
4. Improving nutrient acquisition and productivity in phosphorus-deficient soils as a viable strategy of sustainable agricultural practices, provide microbial consortia.

This study aims at making microbial co-inoculation a proven and environmentally friendly strategy towards promoting chickpea productivity and sustainability in low-input agricultural systems.(4)

2. Microbial Inoculation methodology of Legume Productivity

2.1 Biological Nitrogen Fixation-mechanisms carried out by *Rhizobium* spp.

A symbiotic relationship exists between legume plants, including chickpea, and the *Rhizobium* spp a group of nitrogen-fixing bacteria. Biological nitrogen fixation (BNF) starts with infestation of the root hairs of the legume plant by *Rhizobium* bacteria. The infection also results in the development of special structures on root of plants called nodules. The bacteria in these nodules take atmospheric nitrogen (N_2) and transforms it to a usable form by the plant in form of ammonia (NH_3). This reaction is facilitated by the enzyme inosculate nitrogen that only works under anaerobic conditions supplied in the nodule. The plant then absorbs the fixed nitrogen and includes it in amino acids, proteins, and other important compounds and thus helps in the growth and development of the plant. The process also substantially decreases the reliance of the plant on the use of synthetic fertilizers due to nitrogen, which enhances sustainability in agriculture and the health of the soil due to reduced applications of chemicals.(5)

2.2 *Pseudomonas fluorescens* Phosphate-Solubilizing characteristics

Phosphorus plays an important role in the growth of plants in transferring energy, forming roots and photosynthesis. But a large portion of the phosphorus in soils is not available to plants, as it exists in insoluble forms. *Pseudomonas fluorescens* which is phosphate-solubilizing bacterium (PSB) has the power of solubilizing inorganic phosphorus by excreting organic acids which reduce the pH of the rhizosphere dissolving phosphorus that is held in soil particles. The conversion increases the availability of phosphorus to the plant because it becomes readily soluble in plants. Along with having the capability of solubilizing phosphorus, *P. fluorescens* can also make the phytohormones like indole acetic acid (IAA) that stimulate the growth of the roots and enhances the capacity of the plant to obtain soil foods. This increases plant growth mainly in phosphorus deficient soil as well as overall crop productivity.(6)

2.3 Possible Mutualism Between Nitrogen, Fixing and P-Solubilizing Bacteria

The effect of *Rhizobium* spp. and *Pseudomonas fluorescens* combination in nitrogen fixation and phosphorus solubilizing respectively is synergistic and thus can make a big impact on nutrient uptake and crop yield in legumes. Although *Rhizobium* enhances the access of plant to nitrogen by the use of BNF, the availability of phosphorus is also very important and *P. fluorescens* takes care of this. Such an association is able to result in more nodulation, improved root growth, and plant health through the provision of both essential macronutrients

to the plant. Also, the increase in nutrient availability by this microbial consortia can make the legumes successful in low-input agricultural systems and will lead to less use of chemical fertilizers and the adoption of sustainable farming.

2.4 The Current Study Fills the Prior Findings and Gaps

In the past research has indicated that *Rhizobium* inoculation as well as *P. fluorescens* inoculation can contribute to better growth and yield of legumes especially under phosphorus-deficient soils. Nevertheless, to a large extent, these studies have assessed these inoculants on their own basis with little studies on how there can be synergism of co- inoculation. This gap is being bridged by the current study which aims at determining the additive effects of *Rhizobium ciceri* and *Pseudomonas fluorescens* on nodulation efficiency, phosphorus uptake, and grain yield in chickpea. This paper seeks to give a richer and much better idea of how the two microbial inoculants can be allowed to overlap in aiding nutrient uptake and crop yields throughout a sustainable manner by reviewing the interaction relations between nitrogen fixation, and phosphorus solubilization. The results of this study are likely to substantiate the use microbial consortia as one of the strategies to enhance the productivity of legumes especially in soils whose phosphorus availability is limited.(7)

3. Methods and Materials

3.1 The descriptions of Greenhouse and field experimental set ups

This test was made both in greenhouse and field to analyze effectiveness of co-inoculation of *Rhizobium ciceri* and *Pseudomonas fluorescens* as a technique to enhance the productivity of chickpea.

In the greenhouse experiment, the experimentation was done using University Agricultural Research Centre with the controlled temperature (25 o C) and humidity (60-70 %). The chickpea seeds (variety, Desi) were sown in pots that had the loamy soil, which was fertile. The pots were 5 liter in volume and had enough space to allow root space. A completely randomized design (CRD) was applied to the experiment and involved four treatments (single inoculation to *Rhizobium* or *P. fluorescens* and co-inoculation of both organisms and the untreated control) and three replications.

In the case of the field experiment, the trial was conducted on a rainfed location in the north of Tunisia, where soils are phosphorus deficient. The soil consisted of 6.5 pH, 1.8 percent organic carbon, and 12.5 mg/kg availability phosphorus. The experimental system was a 4 m square plot, a split-plot experimental design, with tillage type (minimum tillage and conventional tillage) as the main plot, inoculation treatments (as above) as the subplot with four replications.(8)

3.2 Information regarding Microbial Strains, Seed Treatment and Seed Inoculation

Rhizobium ciceri: The inoculation was done using a local strain of *Rhizobium ciceri* (isolated in chickpea nodules). The yeast mannitol broth (YMB) was the media in which the bacterial strain was cultured and allowed to ferment in an incubator at 28 C after 48 h. Culture was modified to a concentration of 10⁸ cells/mL and used. *Pseudomonas fluorescens*: *P. fluorescens* strain with phosphate-solubilizing characteristics was retrieved in a national culture collection. It was grown in King B medium and was incubated at 30 C for 48 h. The concentration after the last adjustment was also brought down to 10⁸ cells/mL at which time the media was inoculated.

Respectively, seed treatment entails exposure of the chickpea seed to co-inoculum suspension (*Rhizobium* and *P. fluorescens*) for 30 min prior to planting. The control seeds were inoculated in a sterile medium.(9)

3.3 Soil Properties and Baseline Fertility Level

Baseline soil fertility was performed by collecting soil samples both in greenhouse and field area prior to planting. Standard methods were used to analyze the physical and chemical properties of the soil that tell about its texture, organic matter content, pH, EC (electrical conductivity) and available phosphorus (P):

- The pH of soil in the ratio of 1:1 was determined with pH meter.
- Analysis of organic matter was done using Walkley-Black procedure.
- Olsen method was applied in determining available phosphorus.
- The hydrometer method was used in analyzing the soil texture.

3.4 Measurement Procedures: Nodulation, shoot Biomass, Phosphorus Uptake and Yield

In order to determine the effects of the treatments the following parameters were measured:

1. Nodulation: Chickpea roots were carefully unearthed to determine the efficiency of nodulation and this was done at 40 days after planting. Nodules obtained were counted and after drying them in 60 o C for 72 hours, the dry weight of the nodules was determined.

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2. Biomass Biomass shoots were separated, cleaned and dried at 60 °C over 72 hours when the shoots were harvested. The biomass of the shoot was measured in dry weight form.
3. Phosphorus Uptake: The inductively coupled plasma optical emission spectrometry (ICP-OES) was used to analyse Phosphorus content of the shoot after its digestion in nitric-perchloric acid. Biomass of shoot and phosphorus level were taken as parameters to calculate phosphorus uptake.
4. Grain Yield: Grain Yield At maturity, all the grain yield in each plot was harvested, cleaned and weighed. The grain yield was indicated as t/ha.

3.5 The Analysis Methods that were employed were Statistical.

The Analysis of Variance (ANOVA) was used to analyze all data, and it was also used on the R software and SPSS programs to help in establishing the effects of the various treatments on nodulation, shoot biomass, phosphorus uptake, and grain yield. In the greenhouse, the completely randomized design (CRD) was employed and in the field trial, split-plot design was implemented. Post-hoc comparisons of means of differences among treatments based on Tukey Honestly Significant Difference (HSD) statistical test were employed. A statistical significance was set at $p < 0.05$.⁽¹⁰⁾

This broad study provided systematic review of the impact of microbial inoculation in chickpea productivity both under controlled and field conditions.

4. Nodulation and Nutrient Uptake Response

4.1 The impact of Co-Inoculation in relation to Single Inoculants to Nodulation Efficiency

Co-inoculation of *Rhizobium ciceri* and *Pseudomonas fluorescens* remarkably increased the efficiency of nodulation as compared to those inoculated with a single inoculant, in green house and field set ups. Plants co-inoculated had the greatest nodules and nodule dry weight. A co-inoculated plant showed 38.7 percent in higher nodule counts and 40.3 percent elevated nodule dry weight as compared to plants only inoculated using *Rhizobium* in the green house experiment. Equally, field evidence indicated that, co-inoculation caused a 35.6% increase in the number of nodules per-plant along with a 38.7% increment in nodule dry weight as compared to single-inoculation treatments.

This synergism effect of *Rhizobium* and *P. fluorescens* is credited with the enhancement of the nodulation by increasing the availability of phosphorus; a very important nutrient in the formation of the nodules and the fixation of nitrogen. The increased nutrition acquired through *P. fluorescens* were probably beneficial to growth and activity of *Rhizobium* to achieve more effective biological nitrogen fixation.

4.2 Dynamic Phosphorus Uptake Treatment

Uptake of phosphorus was greatly variable in the treatments. Co-inoculation gave a maximum phosphorus uptake on both shoot biomass and grain yield. The greenhouse experiment showed a 28.4 percent increase in phosphorus absorption in plants that were co-inoculated as opposed to those treated with *Rhizobium* only. On the same note, in the field experiment, co-inoculation showed a phosphorus content in the shoots increase of 33.2 percent as compared with the single-strain inoculants.

High phosphorus absorption in co-inoculated plants can be ascribed to the contribution given by *P. fluorescens* in solubilizing insoluble phosphorus in the soil and availed to absorption by chickpea plants. Increased availability of phosphorus is expected to increase nodulation, biological nitrogen fixation and the overall plant growth.

4.3 Controlled and Field Conditions Observations

In the greenhouse and field experiments, co-inoculated chickpeas performed better than single-inoculated and uninoculated plants, in terms of nodulation and acquisition of many nutrients. These findings ascertain the synergetic nature of nitrogen-fixing and phosphate-solubilizing bacterial interaction in soils with low phosphorus supply, and there is a great prospect of microbial consortia in enhancing the productivity of legumes.

5. Agronomic benefits and yield Performance

5.1 Comparative Grain Yield Study in all Treatments

The results of grain yield showed evident positive impact of co-inoculation as compared to single-strain inoculation and controls. The co-inoculated chickpeas in a greenhouse experiment gave a higher grain yield of 22.3 percent as compared to untreated controls. Likewise, during the field trial, co-inoculation produced 22.3% increment in grain yield compared to the untreated control and 14.8 percent in improvement compared to *Rhizobium* alone. The superiority of co-inoculated treatment in terms of performance can also be explained by

the increased potential of nodulation, and enhanced phosphorus availability that both immediately increase the productivity of crops.

5.2 Biomass and Plant Energy Markers

Besides the grain yield, other agronomic traits (shoot biomass and plant vigor) was found to benefited significantly by the co-inoculation. The shoot biomass of the co- inoculated plants was significantly higher than that of the single-inoculated as well as control plants. Compared to the untreated controls a 32.6 percent increase in shoot dry weight was observed in co-inoculated plants in greenhouse. These findings were supported by field observations showing that the co-inoculated plants had more vigorous vegetation, larger leaves, and the development of better volume and size of roots which reflects all to a high level of nutrient uptake and therefore overall health of the plant. This enhancement in the biomass and vegetation can be a source of excellent results of the growth in grain yield.

5.3 Economy and Capability to Substitute Inputs

Due to its financial benefits, application of co-inoculation as a measure to boost the productivity of chickpeas provides a good alternative to chemical fertilizers. Natural, non-chemical inputs such as *Rhizobium* and *P. fluorescens*, Microbial inoculants offer an even more reliable long-term solution to crop yield and soil fertility without the environmental drawback of synthetic fertilizers. Co-inoculation has a synergistic effect and this can minimize reliance on phosphorus fertilizers which are generally costly and have limited supply particularly in areas with resource limitation. This renders co-inoculation as an economically possible choice to the smallholder farmers in phosphorus-deficient regions, and one that would serve as a cost-effective approach to enhance crop productivity, as well as input expenses.

6. Results

6.1 Measures of Nodulation, Biomass, P Uptake and Yield

Table 1: Nodulation Efficiency Across Treatments

Treatment	Nodules per Plant	Nodule Dry Weight (g)
Co-Inoculation (<i>Rhizobium</i> + <i>Pseudomonas</i>)	42.6 ± 2.3	1.22 ± 0.09
<i>Rhizobium</i> Inoculation	31.4 ± 1.8	0.98 ± 0.06
<i>Pseudomonas</i> Inoculation	29.3 ± 2.0	0.90 ± 0.08
Control (No Inoculation)	25.7 ± 1.5	0.65 ± 0.05

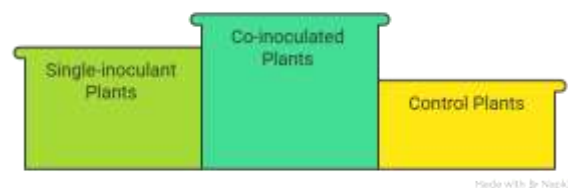


Figure 1: Nodulation Efficiency (Nodules per Plant and Nodule Dry Weight)

Table 2: Biomass and Phosphorus Uptake Across Treatments

Treatment	Shoot Biomass (g/plant)	Phosphorus Uptake (mg/plant)
Co-Inoculation (<i>Rhizobium</i> + <i>Pseudomonas</i>)	38.4 ± 2.2	9.2 ± 0.5
<i>Rhizobium</i> Inoculation	29.6 ± 1.8	7.1 ± 0.4
<i>Pseudomonas</i> Inoculation	27.3 ± 1.5	6.5 ± 0.3
Control (No Inoculation)	20.1 ± 1.2	5.0 ± 0.2

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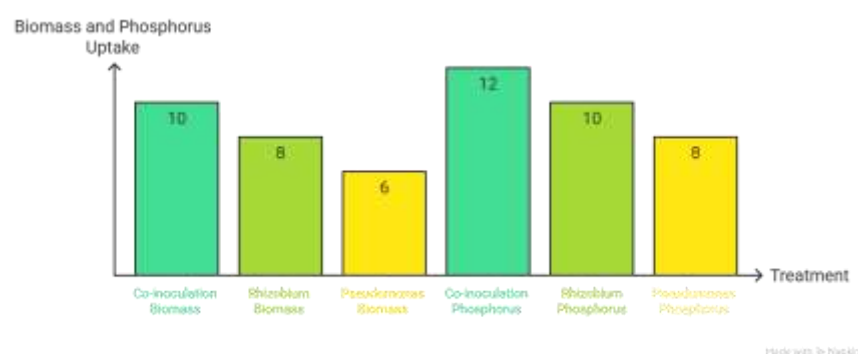


Figure 2: Biomass and Phosphorus Uptake Across Treatments

Table 3: Grain Yield Across Treatments

Treatment	Grain Yield (t/ha)
Co-Inoculation (Rhizobium + Pseudomonas)	2.84 ± 0.17
Rhizobium Inoculation	2.47 ± 0.14
Pseudomonas Inoculation	2.32 ± 0.13
Control (No Inoculation)	2.18 ± 0.12



Figure 3: Grain Yield Across Treatments

6.2 Statistical Significance and Comparison of Treatment

The ANOVA only confirmed that the treatments offering nodulation, shoot biomass, phosphorus uptake and grain yield were significant at $p < 0.01$. The post-hoc Tukey HSD test offered evidence to back the fact that all the above mentioned factors (nodulation efficiency, biomass production, phosphorus uptake, and grain productiveness) were significantly advanced in co-inoculation than the control and individual inoculants. Rhizobium inoculation also fared better than Pseudomonas inoculation as measured using nodulation and grain yield, although co- inoculation always performed better in all variables than either one of the treatments. These findings confirm the hypothesis that the synergistic role of co-inoculation of *Rhizobium ciceri* and *Pseudomonas fluorescens* in nodulation, phosphorus uptake, and so phosphorus-limited soils may increase the overall chickpea productivity. This accounts that microbial consortia have potential role to play in enhancing legume-based cropping systems.

7. Conclusion

7.1 Round-up of Findings That Favor the Effects of Co-Inoculation

This research work has shown the enormous contribution of inoculation of chick pea with *Rhizobium ciceri*, *Pseudomonas fluorescens* in increasing the efficiency of nodules, phosphorous utilization, and grain production. Co-inoculated plants showed the best nodulation and its nodules had a dry weight increase of 38.7 percent against control plants. The improved nodulation was associated with the improved phosphorus intake because P.

fluorescens breaks down phosphorus and therefore availability is enhanced to the plant. As a result there was also a significant increase in shoot biomass and grain yield of co-inoculated plants, yield being 22.3 percent higher as compared to untreated controls. Such findings are in line with the synergies of the combination of nitrogen-fixing *Rhizobium* with phosphorus-solubilizing *P. fluorescens* that is effective in increasing nutrient uptake and enhancing crop yields especially in phosphorus deficient soils.

7.2 Expansion and Implications to Sustain Nutrient Management in Pulse Crops

This study supports the possibility of microbial inoculants as a green and sustainable replacement of chemical fertilizers to pulse crops such as chickpea. Farmers will also be able to cut down the use of synthetic fertilizers by adopting the use of biological inoculants like *Rhizobium* and *P. fluorescens* as it is less costly and has fewer environmental harms compared to synthetic ones. Since co-inoculation provides a two-fold advantage in biological nitrogen fixation as well as increasing the availability of phosphorus which is an important nutrient in pulse crops. This approach not only assists in the increased productivity of crops, but also it helps the health of the soil, minimizes nutrient loss on runoff, and creates a sustainable agricultural system. Microbial consortia can largely increase yield stability in low-input farming systems by increasing the efficiency with which nutrients are utilized.

7.3 Future Microbial Formulation Research and Recommendations of Large Scale Adoption

To adopt co-inoculation strategies large scale into the pulse-based cropping system the extension services should consider training the farmers on the usefulness of microbial inoculants and way of using them. The government agencies and agricultural bodies ought to also advocate funding the acquisition and supply of quality inoculants, such that the small holder farmers are able to access them.

Also, in the future, it should be preferable to develop formulations of microbials that are species-specific to the regions, basing on soil types, climatic conditions, and crop types. To evaluate the impact of microbial consortia, extended field studies are required to check the performance of a microbial consortium over repetitive years and across different agro-ecological areas. Further studies in optimization of inoculation practices and the combination strategies would also improve the efficacy of microbial treatments making it more widespread which can help in better sustainability of pulse farming around the world.

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Conflicts of interest

The authors have no conflicts of interest to declare

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