Use of Plant-Growth Promoting Rhizobacteria and Mycorrhizal Fungi Consortium as a Strategy to Improve Chickpea (Cicer arietinum L.) Productivity under Different Irrigation Regimes

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Abstract: Climate change prediction indicates an increased likelihood of precipitation variability and droughts in the Mediterranean region. Previous studies demonstrated that microbial symbionts combined with supplementary irrigation could mitigate climate change effects and boost chickpea production in low-fertility soils. The aim of the study was to assess the effects of inoculation with a consortium of plant growth-promoting rhizobacteria (PGPR) (Pseudomonas sp., Burkholderia sp. and Mesorhizobium sp.) and arbuscular mycorrhizal fungi (AMF) (Rhizophagus irregularis, Funneliformis geosporum and Claroideoglomus claroideum) on growth, grain yield and crude protein content of chickpeas under the following irrigation regimes: I1-no water stress, I2-moderate water stress, I3-strong water-stress, I4-no water stress in critical growth stages of plant cycle development and I5-severe water stress. Plants irrigated only during the critical growth stages of flowering and pod filling showed higher grain yields compared to plants from other water deficit irrigation treatments. Additionally, chickpeas co-inoculated with PGPR and AMF, and irrigated only during critical growth stages, presented higher grain yield than non-inoculated plants without water stress (1.45- and 1.33-fold increase in 2018 and 2019, respectively). Inoculation with beneficial microorganisms and supplemental irrigation at critical stages benefits chickpea growth and should be considered for increasing crop productivity and promoting agricultural sustainability.

Keywords: chickpea; beneficial microorganisms; deficit irrigation; inoculants; low-input agriculture

1. Introduction

Agriculture in the 21st century faces multiple challenges in meeting growing global food demand. Despite the tremendous importance of grain legumes to sustainable agriculture, they lag far behind cereals in terms of area expansion and productivity gains [1]. Chickpeas (Cicer arietinum L.) are considered a highly nutritious and economically accessible food [2,3]. Chickpeas currently ranks second among cultivated grain legumes with an annual global production of around 17.2 million tons from 17.8 million cultivated ha [4].

In the Mediterranean region, it is traditionally grown as a spring-sown rainfed crop; thus, production depends on rainfall and the soil’s ability to store water [5]. In a changing environment, the availability of water resources is expected to become a major constraint for chickpea production. The crop often experiences prolonged periods of drought and consequently requires great care at sensitive physiological stages (i.e., flowering and pod filling) to avoid incurring yield penalties that can compromise up to 50% of its potential grain yield [6]. Thus, early phenology (flowering, podding and maturity) is a key trait
for adapting chickpea crops to spring sowing in Mediterranean-type environments [7–9]. Recent studies have shown positive effects on plant performance and grain yield in legumes with restricted irrigation at critical growth stages to overcome the detrimental impacts of terminal drought [10–12].

Moreover, inoculation of legume seeds with selected beneficial soil microorganisms is a promising biotechnological tool to improve plant growth either directly by facilitating nutrient availability and/or modulating plant hormone levels, or indirectly by attenuating the inhibitory effects of pathogens and/or alleviating abiotic stresses (e.g., heat, drought, salinity, heavy metals) [13–17].

Global trends restricting the use of agrochemicals that cause significant environmental and human health concerns promote the utilization of microbial formulations to ensure sustainable crop production. It has been reported that the symbiosis between legumes, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) may accelerate and increase plant growth through biological mechanisms including nitrogen fixation, nutrient channelization by solubilizing in absorbable forms, regulation of plant hormonal balance, and translocation of water from soil to plant via extraradical mycelium [18–20].

However, only a few field trials have investigated the effects of deficit irrigation combined with PGPR and AMF application on chickpea crop production to address water scarcity and problems related to low-fertility soils. Therefore, this study aimed to evaluate the effects of inoculation with a consortium of PGPR (*Pseudomonas* sp. DSM 33393, *Burkholderia* sp. DSM 33394 and *Mesorhizobium* sp. DSM 33395) and multiple AMF (*Rhizophagus irregularis*, *Funneliformis geosporum* and *Claroideoglomus claroideum*) on plant growth, grain yield and crude protein content of chickpeas under different irrigation regimes.

2. Materials and Methods

2.1. Microbial Inoculants and Seed Inoculation

The rhizobacteria used in this work were a mixture of *Pseudomonas* sp. DSM 33393, *Burkholderia* sp. DSM 33394 and *Mesorhizobium* sp. DSM 33395, obtained from the UTAD collection, Vila Real, Portugal. The isolates were identified by 16S rDNA sequencing (GenBank accession numbers MN880080, MN880078 and MN880079, respectively), characterized and selected according to their plant growth-promoting mechanisms.

Phosphate solubilization activity was determined using the National Botanical Research Institute’s phosphate growth medium (NBRIP) containing 5 g L\(^{-1}\) of tricalcium phosphate \(\text{TCP, } Ca_3(PO_4)_2\) or aluminum phosphate \(\text{AlPO}_4\) as the sole source of insoluble phosphorus [21]. The isolates were incubated at 28 °C for 3 days. The following formula \((\text{colony + colored zone diameter/colony diameter})\), was used to determine phosphate solubilization activity [22]. The same formula was used to evaluate siderophore production by the Chrome Azurol S assay [23] after incubation at 30 °C for 5 days. Indole acetic acid production (IAA) was assessed according to Brigido et al. [24] with some adaptations. Bacterial cultures were grown on Yeast Extract Mannitol (YEM) broth supplemented with tryptophan (250 µg mL\(^{-1}\)) at 28 °C for 72 h. Following incubation, the isolates were centrifuged at 8500 \(\times g\) for 5 min, and 2 mL of the supernatant was used to inoculate 4 mL of Salkowski’s reagent (1 mL 0.5 M FeCl\(_3\) solution in 50 mL of 35% HClO\(_4\)) and 100 µL of orthophosphoric acid. Absorbance was read after 25 min in the dark at 530 nm. The concentration of IAA was determined using a standard curve. Bacterial hydrogen cyanide (HCN) production was performed according to Bakker and Schippers [25].

For inoculant preparation, each bacterial isolate was grown on Yeast Mannitol Agar media (YMA) [26] at 28 °C for 3 days, suspended in sterilized saline solution (0.8% NaCl) and then transferred to sterilized peat. Bacterial concentration was adjusted to 10\(^8\) colony-forming units per gram. Chickpea seeds were surface sterilized with 0.5% \((v/v)\) sodium hypochlorite for 10 min, immersed in the bidding agent vegetable oil and dusted with the inoculum mixture in a rotating drum.
The AMF used for the inoculum contained isolates of *Rhizophagus irregularis* BEG140, *Funneliformis geosporum* BEG199 and *Claroideoglomus claroideum* BEG210 (1:1:1), and was provided by Symbiom, Lanškroun, Czech Republic. Each seed received 1 g of the inoculum, containing 60 viable spores of the final mycorrhizal blend.

### 2.2. Experimental Design

Chickpea seeds (cv. CHK 3357) were obtained from the University of Trás-os-Montes e Alto Douro (UTAD) collection. The field trial was conducted during the dry seasons of 2018 and 2019 at the UTAD campus, Vila Real, Portugal (41°17′08.9″ N, 7°44′28.6″ W). Composite samples of the tilled layer (0–20 cm) were collected for soil parameters analysis. The soil, a sandy loam, contained 189, 448, 220 and 143 g kg\(^{-1}\) of coarse sand, fine sand, silt and clay, respectively. The pH values of the water and KCl were 5.4 and 4.65, respectively, and electrical conductivity was 0.09 dS m\(^{-1}\). The organic matter content was 1.82%, total nitrogen 1.04 g kg\(^{-1}\), and Egner–Riehm’s extractable phosphorus (P) and potassium (K) concentrations were 174 and 237 mg kg\(^{-1}\), respectively. Total rainfall and mean air temperature were recorded at a nearby weather station (Table 1).

<table>
<thead>
<tr>
<th>Month</th>
<th>Rainfall (mm)</th>
<th>Mean Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2018</td>
<td>2019</td>
</tr>
<tr>
<td>May</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>June</td>
<td>121</td>
<td>38</td>
</tr>
<tr>
<td>July</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>August</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>September</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Season total</td>
<td>165</td>
<td>92</td>
</tr>
</tbody>
</table>

In the first year, conventional tillage with a moldboard plowing followed by disc harrowing was performed for seedbed preparation. In both years, sowing was manual in plots of 2.4 m\(^2\), and the seeding rate was 12.5 seed m\(^{-2}\) (20 cm × 40 cm spacing). Plots were organized in a randomized complete block design with three replicates. During the growing seasons, weeds were hand controlled.

The experimental design included the following microbial treatments: T1-non-inoculated plants, T2-plants inoculated with PGPR (*Pseudomonas* sp. DSM 33393, *Burkholderia* sp. DSM 33394 and *Mesorhizobium* sp. DSM 33395) and T3-plants inoculated with the previous mixture of PGPR and multiple AMF (*Rhizophagus irregularis* BEG140, *Funneliformis geosporum* BEG199 and *Claroideoglomus claroideum* BEG210). For each microbial treatment, five irrigation regimes were imposed: I1-no water stress [100% of plant water requirements (WRs) were fulfilled during the crop cycle], I2-moderate water stress (50% of WRs were fulfilled during the crop cycle), I3-strong water stress (25% of WRs were fulfilled during the crop cycle), I4-no water stress during critical growth stages (100% of WRs were fulfilled only during the flowering and pod filling stages) and I5-severe water stress (plants were grown under rainfed conditions).

Irrigation requirements were calculated as the difference between crop evapotranspiration (ET\(_c\)) and effective rainfall, considering the irrigation system’s efficiency (Ef). ET\(_c\) was estimated by multiplying referenced evapotranspiration (ET\(_o\)) by a crop coefficient (K\(_c\)). The K\(_c\) values were 0.54 and 0.97 during the vegetative and reproductive stages, respectively.

The volumetric water content through the soil profile was monitored using a multisensory capacitance probe based on a Frequency Domain Reflectometry (FDR) downhole sensor (Diviner 2000, Sentek Technologies, Stepney, Australia). Three access tubes were permanently installed within the active root system zone at 60 cm depth for each irrigation...
regime. A lateral line with in-line compensating emitter pressure was placed along each plant row. The emitters had a discharge rate of 1 L h\(^{-1}\) under an operating pressure of 1 atm. The emitter spacing was 0.33 m.

2.3. Agronomical Traits and Crude Protein Content

Chickpeas were harvested when 90% of the stems and pods were golden brown (harvest maturity). Plants were cut at soil level and allowed to dry at 60 °C to a constant weight. Subsequently, the quantitative traits shoot dry weight (SDW), number of pods per plant (NP), number of seeds per plant (NS), 100-seed weight (100SW) and grain yield were recorded in five plants per replicate. Grain dry samples were analyzed for total N as Kjeldahl N (no. 954.01) following the methods of the Association of Official Analytical Chemists [27]. Crude protein content was calculated as N \times 6.25.

2.4. Statistical Analysis

Data were submitted to normality and homogeneity of variance tests for each variable and further evaluated with analysis of variance (ANOVA). Differences between the means were separated by the Tukey’s multiple range test at the probability level of 0.05. Statistical analyses were performed using the SPSS 22.0 software package (IBM, Armonk, NY, USA).

3. Results

The rhizobacteria selected for the inoculant presented at least two plant growth-promoting activities (Table 2). All isolates were low inorganic solubilizers, except the *Mesorhizobium* sp., which exhibited better P-solubilizing activity with TCP. The isolate *Pseudomonas* sp. was unable to produce siderophores, but was the only isolate exhibiting the ability to produce HCN. The *Burkholderia* sp. and *Mesorhizobium* sp. produced siderophores, but only the *Mesorhizobium* sp. demonstrated the ability to synthesize IAA.

Table 2. Plant growth-promoting mechanisms of rhizobacteria.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>TCP Solubilization</th>
<th>AIPO(_4) Solubilization</th>
<th>Siderophore Production</th>
<th>HCN Production</th>
<th>IAA Production (µg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
<td>2</td>
<td>0.00</td>
</tr>
<tr>
<td>DSM 33393</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia</em> sp.</td>
<td>1.5</td>
<td>1.0</td>
<td>3</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>DSM 33394</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mesorhizobium</em> sp.</td>
<td>2.0</td>
<td>1.0</td>
<td>2</td>
<td>1</td>
<td>0.45</td>
</tr>
<tr>
<td>DSM 33395</td>
<td></td>
<td></td>
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</tbody>
</table>

Phosphate solubilization: negative (1), low (>1 and <2) and high (≥2); Siderophore production: negative (1), low (>1 and <2), medium (≥2 and <3) and high (≥3). HCN production: negative (1) and positive (2).

Agronomical traits of chickpea plants grown under different irrigation regimes and inoculation treatments are presented in Table 3. No differences were observed in the life cycle duration or the beginning of reproductive stages among treatments.

Yield parameters were significantly \((p < 0.05)\) affected by the growing season, except for shoot dry weight. On average, the number of pods and seeds per plant, number of seeds per plant and grain yield increased 50, 63 and 37%, respectively, in 2019 compared to 2018. However, the 100-seed weight was significantly lower in 2019 (34.2 g) compared to 2018 (41.1 g). Seed crude protein content was not affected by the year.

In both growing seasons, chickpea yield parameters were significantly affected by irrigation regimes and inoculations treatments \((p < 0.05)\) (Table 3). Plants without water stress throughout the growing season presented a significant increase in grain yield compared to plants under severe water stress (1.41- and 2.03-fold increase in 2018 and 2019, respectively). However, plants irrigated only during the critical growth stages of flowering and pod filling showed higher grain yields than plants from other water deficit irrigation treatments and even plants with no water stress throughout the growing season. Increased
water deficit was detrimental to plant performance since grain yield data followed the descending order of I4 > I2 > I3 > I5. Severe water stress significantly decreased all grain yield related parameters.

Table 3. Effect of different irrigation regimes and inoculation treatments and their interaction on shoot dry weight (g plant$^{-1}$), number of pods per plant, number of seeds per plant, 100-seed weight (g), grain yield (kg ha$^{-1}$) and crude protein content (%) of chickpea during 2018 and 2019 growing seasons.

<table>
<thead>
<tr>
<th>Irrigation Treatments</th>
<th>2018 Season</th>
<th>2019 Season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SDW NP NS 100SW GY PB</td>
<td>SDW NP NS 100SW GY PB</td>
</tr>
<tr>
<td>I1</td>
<td>105.2$^{ab}$ 95$^{a}$ 109$^{ab}$ 39.8$^{a}$</td>
<td>5347$^{b}$ 17.0$^{b}$ 126.2$^{ab}$ 135$^{ab}$ 190$^{ab}$ 34.6$^{a}$</td>
</tr>
<tr>
<td>I2</td>
<td>94.0$^{bc}$ 70$^{b}$ 90$^{d}$ 40.7$^{a}$ 4508$^{a}$ 18.7$^{a}$ 95.1$^{b}$ 110$^{b}$ 151$^{d}$ 33.4$^{a}$</td>
<td>6772$^{a}$ 18.0$^{a}$</td>
</tr>
<tr>
<td>I3</td>
<td>116.3$^{a}$ 96$^{b}$ 124$^{a}$ 41.0$^{a}$ 6276$^{a}$ 18.5$^{b}$ 138.8$^{a}$ 161$^{a}$ 213$^{a}$ 33.4$^{a}$</td>
<td>8982$^{a}$ 18.6$^{a}$</td>
</tr>
<tr>
<td>I4</td>
<td>76.1$^{c}$ 57$^{c}$ 73$^{d}$ 41.6$^{a}$ 3793$^{c}$ 19.1$^{a}$ 62.1$^{b}$ 71$^{c}$ 94$^{d}$ 34.6$^{a}$</td>
<td>4020$^{d}$ 18.0$^{a}$</td>
</tr>
<tr>
<td>I5</td>
<td>94.8$^{b}$ 67$^{b}$ 90$^{b}$ 39.4$^{a}$ 5185$^{a}$ 17.4$^{a}$ 110.0$^{ab}$ 124$^{ab}$ 164$^{d}$ 35.4$^{a}$</td>
<td>7555$^{a}$ 18.8$^{a}$</td>
</tr>
</tbody>
</table>

$p$-value | 0.011 | 0.002 | 0.003 | 0.007 | 0.946 | 0.003 | 0.041 | 0.009 | 0.258 | 0.006 | 0.002

SDW: shoot dry weight; NP: number of pods; NS: number of seeds; 100SW: 100-seed weight; GY: grain yield; PB: crude protein content; I1: no water stress (100% of WRs were fulfilled during crop cycle); I2: moderate water stress (50% of WRs were fulfilled during crop cycle); I3: strong water stress (25% of WRs were fulfilled during crop cycle); I4: no water stress in specific plant development stages (100% of WRs were fulfilled during flowering and pod fill stages); I5: severe water stress (rainfed conditions); T1: control plants (non-inoculated); T2: inoculation with a mixture of plant growth-promoting rhizobacteria (PGPR) (Pseudomonas sp. DSM 33393, Burkholderia sp. DSM 33394 and Mesorhizobium sp. DSM 33395); T3: inoculation with the previous mixture of PGPR together with multiple arbuscular mycorrhizal fungi (AMF) (Rhizopogon irregularis BEG140, Funneliformis mossorum BEG199 and Claroideoglosum clarioideum BEG210). Means in the same column followed by the same letter are not significantly different according to Tukey’s test at 0.05 level.

In the first growing season, seed crude protein content was significantly affected by the irrigation regime and plants experiencing severe water stress showed, on average, a higher seed crude protein content (19.1%). No effect for the irrigation regime was observed considering the 100-seed weight.

Regarding the microbial treatments, inoculation with PGPR had positive effects on plant growth and seed crude protein content compared to non-inoculated plants; however, significant increases were only observed for 100-seed weight in 2018 and grain yield in 2019 (Table 3).
In both growing seasons, the PGPR and AMF consortium significantly increased shoot dry weight, number of pods, number of seeds and grain yield compared to non-inoculated plants. In 2019, co-inoculation resulted in a significant increase in seed crude protein content of 4.4 and 3.9% compared to plants inoculated with PGPR and non-inoculated plants, respectively (Table 3).

In general, applying beneficial soil microorganisms within each irrigation regime positively affected plant growth parameters and resulted in grain yield increases. In the second growing season, chickpea inoculation with PGPR, or combined with AMF under severe water stress, resulted in the greatest production gains of 35.4 and 42.5%, respectively, compared to non-inoculated plants. Furthermore, microbial inoculation improved the seed crude protein content of plants under severe water stress (Table 3).

Overall, chickpeas inoculated with a consortium of PGPR and AMF and irrigated only during the critical growth stages of flowering and pod filling (I4T3) presented higher grain yield than non-inoculated plants without water stress (I1T1) (1.45- and 1.33-fold increase in 2018 and 2019, respectively).

The interaction effect between irrigation regimes and inoculation treatments significantly affected 100-seed weight, the number of pods per plant and seed crude protein content in the second growing season.

4. Discussion

Introducing beneficial microorganisms in agro-ecosystems is regarded as a biotechnological solution to improve the performance of plants, decrease synthetic fertilizer inputs and reduce the impacts of soil water deficit [28,29].

Inoculation with a single microbial species can benefit plant growth [30–32]. However, several studies reported better plant performance and superior yields in chickpeas inoculated with a consortium of PGPR [16,33–36] or together with AMF [37,38]. The authors state that the beneficial effects on plant growth could be attributed to induced plant nutrient acquisition with a particular emphasis on nitrogen and phosphorus nutrition, direct antagonism to pathogens, improved water uptake and synergistic interactions between microsymbionts.

In this study, inoculation with a mixture of PGPR improved plant growth parameters and grain yield; however, the consortium of PGPR and AMF had a synergistic effect that resulted in significant increases in grain yield. Indeed, combining different species in the same microbial formulation broadens the spectrum of action by targeting various biological mechanisms.

Notably, this study indicated that the inoculants employed have great potential for improving chickpea production and tackling problems arising from water deficit, since greater production gains over control plants were observed under severe water stress conditions.

Chickpeas are recognized as an important source of proteins, carbohydrates, fiber, minerals and vitamins in the human diet [39,40]. In this study, the effect of inoculation treatments and irrigation regimes on chickpea nutritional value is unclear. Irrigation treatments significantly affect seed crude protein content in the first year, while inoculation treatments had no significant effect (Table 3). In the second year, the opposite behavior was observed. Therefore, further insights into the effect of these agricultural practices on chickpea protein content and food quality are of great interest to benefit human health.

In grain legumes, drought stress during reproductive and grain filling stages is critical and usually results in significant yield losses [41]. Therefore, it is necessary to improve chickpea productivity in drought scenarios. In the present study, supplemental irrigation resulted in the development of plants with a larger vegetative structure capable of supporting more reproductive structures, thus leading to superior yields. These outcomes are consistent with other studies showing that a single irrigation [10,42], two irrigations [43] or three irrigations [11,44] throughout the growing season improved chickpea yield. The present study suggests that irrigation at critical growth stages (I4), instead of full irrigation throughout the growing season (I1), might be sufficient to achieve superior yields with
less water. These observations are in agreement with the findings of Kirnak et al. [11], who reported that single irrigation at 50% pod-set chickpeas had higher yields than fully irrigated chickpeas.

Plant growth was offset by microbial activity in all water regimes imposed. This observation is consistent with the findings of Laranjeira et al. [45], who reported increases in chickpea (‘Elixir’ cv) yield with the microbial formulations used in the present study under the same irrigation regimes, as well as by Erman et al. [46] who reported that both single and combined inoculation of chickpeas (‘Aziziye-94’ cv) with Mesorhizobium ciceri and Rhizophagus intraradices (former Glomus intraradices) improved seed yield under irrigated and rainfed conditions.

Overall, the application of microbial-based formulations coupled with limited irrigation at critical growth stages has great potential to mitigate the adverse effects of terminal drought stress and increase sustainable chickpea production.

5. Conclusions

In a changing environment, water availability is expected to be a major constraint in agricultural production. Thus, there is a need to improve chickpea productivity under drought scenarios. Inoculation with PGPR increased chickpea production in all irrigation treatments; however, the consortium of PGPR and AMF resulted in greater production gains. Under severe water stress conditions, the application of selected beneficial soil microorganisms enhanced grain quality by providing higher protein content than non-inoculated plants.

This study also indicated that irrigation only during the critical growth stages of flowering and pod filling combined with PGPR and AMF inoculation (I4T3) might be sufficient to obtain superior yields and enhance yield stability of chickpeas in drought-prone environments, instead of full irrigation throughout the growing season without the use of beneficial microorganisms (I1T1).

Overall, limited water input and the application of selected beneficial microorganisms can boost chickpea productivity under adverse environmental conditions and farmers should be encouraged to promote sustainable agriculture.


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References


