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Inoculation of plant growth promoting bacteria and arbuscular mycorrhizal fungi improve chickpea performance under water deficit conditions

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ABSTRACT

Chickpea (*Cicer arietinum* L.) currently ranks second in the world's production of grain legumes and it is considered a cheap source of plant-based protein. In Mediterranean regions, predicted changes in climate are likely to further worsen drought stress and increase the economic vulnerability of chickpea production. Plant growth promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) have the potential to improve plant growth and ameliorate the adverse effects of drought stress. The aim of this study was to assess the effects of inoculation with PGPB (*Mesorhizobium* sp., *Burkholderia* sp. and *Pseudomonas* sp.) and AMF (*Rhizophagus irregularis*, *Funneliformis geosporum* and *Claroideoglomus claroideum*) on the growth, grain yield and protein content of chickpea in a field experiment under different irrigation regimes throughout the growing season (100% water requirements (WR), 50% WR, 25% WR, 100% WR only during reproductive stages, and rainfed).

Based on two years of results, the beneficial effects of co-inoculation (PGPB+AMF) on plant growth parameters of chickpea allow a cumulative grain yield of about 13,838 kg ha^{-1}, resulting in an increase of 6% when as compared to a single inoculation and 24% over the non-inoculated plants. Plants inoculated with PGPB+AMF, and irrigated only during the reproductive stages, had the highest cumulative grain yield (18,157 kg ha^{-1}), resulting in an increase of 16% and 237% over fully irrigated plants inoculated with PGPB+AMF and non-inoculated plants under rainfed conditions, respectively.

In water-scarce environments, deficit irrigation only during the reproductive stage allows farmers to achieve higher yields with less water consumption, which, when combined with microbial inoculation, has the potential to benefit agricultural production of chickpea.

1. Introduction

Chickpea (*Cicer arietinum* L.) currently ranks second in the world's production of grain legumes. Total production is about 17.2 million tons, of which 77% are produced in Asia (FAOSTAT, 2018). Europe has a production deficit of plant proteins, including chickpea that is valued as a source of carbohydrates and proteins (which together constitute about 80% of the total dry seed weight), fat, fiber, vitamins and minerals (Bar-El Dadon et al., 2017; FAOSTAT, 2018; Jukanti et al., 2012).

Chickpea is often cultivated in areas where climate conditions, poor soil fertility and limited access to synthetic fertilizers reduce yield (Merga and Haji, 2019; Oliveira et al., 2017). However, it has the ability to fix atmospheric nitrogen in symbiosis with rhizobia, thus reducing the need for nitrogen fertilizer applications (Wolde-meskel et al., 2018).

Chickpea plants also establish mutualistic relationships with arbuscular mycorrhizal fungi (AMF) that benefit plant nutrient uptake, phytohormonal balance and water relationships (Desai et al., 2016; Hashem et al., 2019).

In rhizobia-AMF-legume tripartite symbiosis, microbial partners can act synergistically to promote plant nutrition, particularly nitrogen and phosphorus, resulting in overall yield benefits and tolerance against biotic and abiotic stresses (Abd-Alla et al., 2019; Erman et al., 2011; Foyer et al., 2019).

Furthermore, plant growth promoting bacteria (PGPB), commonly referred to as a heterogeneous group of bacteria living close to or on the surface of roots, can either directly or indirectly facilitate plant growth

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(Ahkami et al., 2017). Mechanisms of action employed by these bacteria include the production of auxin, indole acetic acid, ACC deaminase, cytokinin, gibberellin, hydrogen cyanide, siderophores, nitrogen fixation, phosphorous solubilization, induced systemic resistance and biocontrol against phytopathogens (Glick, 2015). Exploitation of bacteria with a wide range of functional traits can be advantageous for the formulation of biofertilizers with multiple agricultural purposes.

In order to achieve global food security, the resilience of agricultural systems has been considered to be as important as their productivity. Chickpea is mostly cultivated in rainfed areas, where terminal drought stress is a significant factor limiting productivity (Oweis et al., 2004; Sinha et al., 2019; Waqas et al., 2019). Therefore, strategies to optimize soil biological interactions and to maximize water use efficiency are important ways of increasing agricultural productivity (Sindhu et al., 2019; Soltani et al., 2016).

Microbial inoculation of seeds is considered a promising tool for enhancing plant growth and resilience under adverse conditions (O'Callaghan, 2016). However, the outcomes in field conditions have often been inefficient due to the failure to establish symbiotic relationships with the host or unfavorable environmental conditions, thus reducing their acceptance and widespread application by farmers. Therefore, it is desirable to improve the agronomic efficiency of microbial inoculants adapted to different challenging edaphoclimatic conditions, including water deficit conditions, with reproducible plant responses (Brígido et al., 2017; Schütz et al., 2018).

This study aims to assess the effects of seed inoculation with PGPB (*Mesorhizobium* sp., *Burkholderia* sp. and *Pseudomonas* sp.) and AMF (*Rhizophagus irregularis*, *Funneliformis geosporum* and *Claroideoglomus claroideum*) on the growth, grain yield and protein content of chickpea under five different water regimes, particularly under water deficit conditions.

2. Material and methods

2.1. Isolation of bacteria endophytes from chickpea roots

The isolation of root nodule bacteria was performed in chickpea plants from agricultural fields as described by Callow (1971). Surface-sterilized nodules (3% sodium hypochlorite for 2 min, 70% ethanol for 1 min and serial washes in sterile distilled H_2O) were crushed and streaked on yeast mannitol agar medium (YMA) supplemented with 0.0025% Congo red.

2.2. Screening of plant growth promoting characteristics in vitro

The isolates were evaluated for their ability to induce root nodules by inoculating seedlings in tubes containing N-free nutrient solution (Somasegaran and Hoben, 1994).

Phosphate solubilization activity was performed in the National Botanical Research Institute's phosphate growth medium (NBRIP) containing 5 g L^{-1} of tricalcium phosphate (TCP, $Ca_3(PO_4)_2$) or aluminum phosphate ($AlPO_4$) as a single phosphorus source (Mehta and Nautiyal, 2001). After incubation at 28 °C for 3 days, solubilizing activity was detected by the formation of a distinguishable clear halo around the colonies (Brígido et al., 2017; Gupta et al., 1994).

Indole acetic acid (IAA) production was assessed by the method described by Patten and Glick (2002) with some modification. Bacterial cultures were grown for 3 days at 28 °C in YMA supplemented with tryptophan (250 μg mL^{-1}). After incubation, bacterial cells were removed by centrifugation and 2 mL of the supernatant were mixed with 4 mL of Salkowski's reagent (1 mL 0.5 M $FeCl_3$ solution in 50 mL of 35% of perchloric acid) and 100 μL of orthophosphoric acid. Following incubation at room temperature for 25 min, in the dark, the absorbance was measured at 530 nm.

Siderophore production was determined on Chrome Azurol S medium (Alexander and Zuberer, 1991). A color change from blue to orange indicated siderophore production (Brígido et al., 2017).

Hydrogen cyanide (HCN) production was detected by the qualitative method of Bakker and Schippers (1987). Bacterial cultures were grown on Luria-Bertani (LB) agar supplemented with glycine (4.4 g L^{-1}). A Whatman No.1 filter paper soaked in 0.5% picric acid and 2% of sodium carbonate was placed on the upper lid of the petri plate. Changes in filter color indicated HCN production.

2.3. Molecular identification and phylogenetic analysis

DNA extraction was performed as described by Laguerre et al. (1994) with some modifications. Briefly, bacterial cells were lysed with a CTAB extraction buffer and sterile glass beads. A phenol, chloroform and isoamyl alcohol (25:24:1) solution was used to denature proteins. DNA was precipitated by adding 0.6 volume isopropanol. The pellet was washed with 70% ethanol, dried and suspended in sterilized ultra-pure water.

Amplification of 16S rDNA was performed with the set of primers fD1 and rD1 or 27F and 1492R (Heuer et al., 1997; Weisburg et al., 1991). PCR reaction mixtures contained 7.5 μL DNA extract, 10 μL 2× My Taq HS Mix (Bioline) and 1 μL of each primer (10 μM). PCR cycling conditions were as follows: preheating for 3 min at 95 °C, 34 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 54 °C, extension for 2 min at 72 °C, and a final 10 min at 72 °C. The PCR products were sequenced by StabVida (Portugal) and compared with the GenBank nucleotide data bank from the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). Sequences were deposited in the GeneBank with Accession Numbers: MN880078, MN880079 and MN880080.

2.4. Experimental design

The experiment was carried out over two consecutive dry seasons (2018 and 2019) in an agricultural field located at the University of Trás-os-Montes and Alto Douro (UTAD) campus (41°17′08.9″N; 7°44′28.6″W), Vila Real, Portugal. At the outset of experimentation,

Table 1
Soil characteristics in the 0–20 cm depth layer.

Parameters	
Particle-size distribution (g kg^{-1})	
Coarse sand (200–2000 μm)	189
Fine sand (20–200 μm)	448
Slit (2–20 μm)	220
Clay (<2 μm)	143
Water pH	5.4
KCl pH	4.65
Soil reaction	Moderately acid
Organic matter content (%)	1.82
Phosphorus (mg P_2O_5 kg^{-1})	174
Potassium (mg K_2O kg^{-1})	237
Boron (mg B kg^{-1})	0.255
Copper (mg Cu kg^{-1}) method EDTA	13.51
Zinc (mg Zn kg^{-1}) method EDTA	3.45
Iron (mg Fe kg^{-1}) method EDTA	47.45
Manganese (mg Mn kg^{-1}) method EDTA	47.1
Exchangeable bases ($cmol_c$ kg^{-1})	
Ca	5.53
Mg	0.645
K	0.525
Na	0.135
Al	0.155
Potencial CTC (pH = 7.0) ($cmol_c$ kg^{-1})	9.145
Degree of base saturation (%)	97.7
Degree of aluminum saturation (%)	2.3
Electric conductivity (dS m^{-1})	0.09
Total nitrogen (g N kg^{-1})	1.04
C:N ratio	10.15
Texture class	Sandy loam

topsoil subsamples (0–20 cm) were collected to evaluate soil chemical properties and soil granulometry (Table 1). Monthly rainfall and mean air temperature were collected from a local weather station (Table 2).

The field trial was conducted with chickpea cv 'Elixir' harvested for grain. Three microbial treatments to seeds were imposed: non-inoculated (C); single inoculation with a mix of plant growth promoting bacteria (*Mesorhizobium* sp. UTADM31, *Burkholderia* sp. UTADB34, *Pseudomonas* sp. UTAD11.3, PGPB), and inoculation with the previous bacterial mix with AMF inoculant (PGPB+AMF) provided by Symbiom Ltd. (Czech Republic). The AMF fungi used were a mix of *Rhizophagus irregularis* BEG140, *Funneliformis geosporum* BEG199 and *Claroideoglomus claroideum* BEG210 (1:1:1) grown for 8 months in multispore pot cultures containing a 1:2 (v/v) mixture of clinoptilolite and expanded clay with *Trifolium pratense* L. and *Zea mays* L. as host plants (Pereira et al., 2019).

Conventional tillage with a 20 cm deep moldboard plow followed by disc harrowing was performed in the first year for weeding control and seedbed preparation. In the second year, seedbed preparation was manually performed. The experiment was set up in a randomized complete block design with three replicates being sown manually in both years. Each plot area was 2.4 m^2 with 1.2 m paths separating the plots to prevent contamination by superficial run-off, and the seeding rate was 12.5 seeds m^{-2} (20 cm × 40 cm spacing).

For inocula preparation, bacterial isolates were grown on Yeast Mannitol Agar (YMA) medium for 3 days at 28 °C. The cultures were then suspended in sterilized NaCl 0.8% to reach the optical density of 0.5 at 600 nm (OD600) corresponding to a total colony-forming unit (CFU) of about 1×10^8 mL^{-1}.

Before sowing, seeds were surface-sterilized with 0.5% (v/v) sodium hypochlorite for 10 min and washed twice with sterile water. Then, seeds were submerged in vegetable oil as a binder and coated by gradually adding the inoculant preparation with the coating mixture (sterilized peat) according to the pan coating method (Pedrini et al., 2017). Non-inoculated control seeds were coated only with sterilized peat. Mycorrhizal fungi were manually added on sowing. Each seed received 1 g of the AMF mix, containing 60 viable spores per g of final mycorrhizal blend.

Weeds were manually controlled. In the second year, the insecticide TUREX (*Bacillus thuringiensis* subsp. *aizawai*), authorized for organic production, was applied at a rate of 1 kg ha^{-1} at R5 and R7 growth stages against *Helicoverpa armigera*.

Water requirements (WR) were quantified based on the difference between crop evapotranspiration (ETc) and effective rainfall taking into account the efficiency of the irrigation system (Ef). Crop evapotranspiration (*ETc*) was estimated by multiplying referenced evapotranspiration (*ETo*) by a crop coefficient (*Kc*), [$ETc = ETo \times Kc$]. The crop coefficient was 0.54 during the vegetative stage and 0.97 during the reproductive stage.

Irrigation levels throughout the growing season were as follows: 100% of water requirements (WR), assumed to be the irrigation control treatment (100WR); 50% of WR (50WR); 25% of WR (25WR); 100% of WR only during the reproductive stages (RGS); and rainfed (R).

The irrigation system was installed with one drip lateral line to each plant row. Drip lines had emitters with 1 L h^{-1} flow rate and a 0.33 m emitter spacing. The irrigation controller allowed for the variable rate irrigation for different plots according to the pre-defined set-point.

The soil water content was monitored through the soil profile based on a time-domain reflectometry (TDR Delta-T Devices PR1/4d-02 connected to a handheld TRIME-FM). The permanently installed access tubes were located within the active root system zone at 60 cm measured from the furrow. Three replicates for each irrigation regime were used.

2.5. Agronomic characterization and crude protein content

Yield by plant fraction was measured by randomly collecting five plants per plot at physiological maturity for evaluation of plant growth (shoot dry weight, SDW; number of pods, NP; pod weight, PW; number of seeds, NS; seed weight, SW; 100 seed weight, 100SW; and harvest index, HI). Grain yield (GY) was determined by harvesting all the aboveground biomass in each plot.

Relative grain yield (RGY) of each treatment to the control treatment irrigated with 100% water requirements (RGY:C100%WR) was determined based on the data of GY. Calculations were performed according to the following equation:

$$RGY = \frac{GYTx - GYC100\%WR}{GYC100\%WR} \times 100$$

where, GYTx corresponds to the GY of treatments to be compared with the GY of the control treatment irrigated with 100% water requirements (GYC100%WR).

According to the Association of Official Analytical Chemists guidelines, dried grain samples were analyzed for total N as Kjeldahl N (no. 954.01) (AOAC, 1990). Crude protein content was determined as N × 6.25.

2.6. Statistical analysis

Statistical analysis was performed using Software SPSS V.25 (SPSS-IBM, New York, USA). Statistical differences were evaluated by one-way and two-way analysis of variance (ANOVA), followed by the *post hoc* Tukey's multiple range test at the probability level of 0.05, establishing irrigation regimes and inoculation effects. One-way ANOVA, establishing year effects on chickpea plant growth parameters, was also performed.

3. Results

3.1. Molecular identification and biological mechanisms of bacteria

In search of effective PGPB strains with multiple plant growth promoting traits, three isolates belonging to *Pseudomonas*, *Burkholderia* and *Mesorhizobium* genera were selected to be applied as inoculants in the field trial. All the isolates exhibited at least two plant growth promoting mechanisms (Table 3).

Considering the ability to solubilize inorganic phosphorus, all the isolates were able to solubilize TCP. However, in the medium containing insoluble mineral phosphate $AlPO_4$, the only bacterial strain capable of solubilizing phosphorus was *Pseudomonas* sp. UTAD11.3, which also

Table 2
Average mean temperature (MTT), average minimum temperature (MTN), lowest minimum temperature (MTX), average maximum temperature (TNN), highest maximum temperature (TXX) and rainfall during the experiment (2018–2019) and the 30-year mean (1981–2010).

Month	MTT (°C)			MTN (°C)		MTX (°C)		TNN (°C)		TXX (°C)		Rainfall (mm)		
	2018	2019	30-yr mean	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	30-yr mean
May	14.9	16.4	14.9	9.6	9.7	3.7	4.5	20.7	22.8	26	31.7	24	6	71
Jun	18.0	16.1	19.2	13.0	10.6	7.6	5.5	23.8	21.8	32.6	31.7	121	38	34
Jul	19.9	21.5	21.3	14.3	15.0	10.6	9.1	26.4	28.9	32.0	36.2	10	15	15
Aug	24.0	20.9	21.7	16.4	14.8	11.8	9.3	31.8	28.3	39.0	34	6	20	27
Sep	22.2	19.0	18.5	16.2	13.2	12.6	9.4	29.7	26.3	35.0	32.5	4	13	55

Table 3
Origin, biological mechanisms and GenBank accession number of the isolates. Classes of P solubilization: no solubilization (1), low solubilization (>1 and ˂2) and high solubilization (≥2); Classes of siderophore production: no production (1), low production (>1 and ˂2), medium production (≥2 and ˂3) and high production (≥3). Classes of HCN production: no production (1) and production (2).

Isolate	Origin site	TCP solubilization	$AlPO_4$ solubilization	Siderophore production	HCN production	IAA production (μg mL^{-1})	Genbank accession number
Pseudomonas sp. UTAD11.3	Aveiro	1.5	1.5	1	2	0.00	MN880080
Burkholderia sp. UTADB34	Portalegre	1.5	1.0	3	1	0.00	MN880078
Mesorhizobium sp. UTADM31	Portalegre	2.0	1.0	2	1	0.45	MN880079

exhibited the ability to produce HCN. Both *Burkholderia* sp. UTADB34 and *Mesorhizobium* sp. UTADM31 were able to produce siderophores, the latter also being able to synthesize IAA (Table 3).

3.2. Chickpea growth and seed crude protein content

All chickpea plant growth parameters were significantly affected by the year, except shoot dry weight (SDW) and seed crude protein content (PB). In general, higher numbers of reproductive structures were recorded in 2019 compared to 2018.

Overall, chickpea plant growth parameters were significantly affected by the inoculation treatments and irrigation regimes imposed (Table 4). Irrigation applied only during the reproductive stages (RGS) significantly increased shoot dry weight (SDW), number of pods (NP), pod weight (PW), number of seeds (NS) and seed weight (SW). In general, within each irrigation regime, co-inoculated plants presented the highest values, followed by single inoculation and non-inoculated plants for plant growth and yield parameters (Table 4). Plants under rainfed conditions without microbial inoculation significantly decreased chickpea plant growth parameters (Table 4).

Based on the results over two years, the lowest values for the weight of 100 seeds (100SW) and the harvest index (HI) were observed in the treatment PGPB+AMF and PGPB respectively, both in water deficit treatment 25WR (Table 4).

Regarding crude protein content, no significant differences between inoculation treatments were observed in the first year. However, in the

Table 4
Effects of different inoculation treatments (non-inoculated, C; single inoculation with a mix of plant growth promoting bacteria, PGPB; and dual inoculation with a mix of plant growth promoting bacteria and multiple AMF, PGPB+AMF) under different irrigation levels throughout the growing seasons (100% of water requirements (WR), 100WR; 50% of WR, 50WR; 25% of WR, 25WR; 100% of WR only during reproductive growth stages, RGS; and rainfed, R) on plant growth (shoot dry weight, SDW; number of pods, NP; pod weight, PW; number of seeds, NS; seed weight, SW; 100-seed weight,100SW and harvest index, HI), grain yield, GY; relative grain yield to control treatment under 100WR conditions, RGY:C100WR, and crude protein content, PB. Values are means, $n = 15$ plants per treatment group.

Year	Irrigation (I)	Inoculation (T)	SDW (g)	NP	PW (g)	NS	SW (g)	100SW (g)	HI (%)	GY (kg ha^{-1})	RGY:C100WR%	PB (%)
2018	R	C	62.6	56	33.2	62	23.5	38.1^{ab}	38.9	2933.0	-52.3	19.7
		PGPB	82.2	89	47.9	91	32.0	35.3^{ab}	41.2	3996.7	-35.1	19.0
		PBPB + AMF	109.2	92	60.4	98	35.3	37.2^{ab}	37.2	4414.6	-28.3	18.6
	25WR	C	94.0	93	51.5	113	38.4	34.5^{b}	43.2	4800.5	-22.0	16.3
		PGPB	121.1	108	61.3	111	38.9	34.9^{ab}	34.6	4864.4	-21.0	18.6
		PBPB + AMF	115.5	101	59.7	127	41.3	33.5^{b}	40.3	5161.8	-16.1	17.7
	50WR	C	88.7	83	44.8	99	36.7	37.4^{ab}	46.2	4582.4	-25.0	18.8
		PGPB	110.7	112	59.1	122	43.0	35.5^{ab}	43.6	5375.9	-12.7	18.5
		PBPB + AMF	97.5	110	60.1	119	45.2	38.4^{ab}	47.2	5655.0	-8.1	19.8
	RGS	C	119.7	132	75.6	158	59.5	38.4^{ab}	54.0	7432.6	20.8	17.9
		PGPB	164.3	171	95.1	191	70.5	37.8^{ab}	46.6	8814.2	43.2	16.7
		PBPB + AMF	155.4	159	84.9	199	70.0	35.2^{ab}	45.6	8744.8	42.1	19.5
	100WR	C	118.9	128	72.1	140	49.2	35.0^{ab}	42.8	6153.9	0.0	17.4
		PGPB	111.3	111	64.1	143	53.4	37.8^{ab}	50.3	6680.3	8.6	18.3
		PBPB + AMF	113.7	115	69.3	134	54.0	40.8^{a}	48.5	6756.3	9.8	18.4
P (I)			***	***	***	***	***	**	**	***	***	*
P (T)			**	*	*	*	**	ns	ns	**	**	ns
P (I*T)			ns	ns	ns	ns	ns	*	ns	ns	ns	ns
2019	R	C	36.7	58	26.5	61	19.6	32.1	53.5	2452.3	−64.8	18.2^{abc}
		PGPB	71.7	104	48.7	113	36.4	31.9	50.0	4549.1	−34.6	17.4^{bcde}
		PBPB + AMF	79.3	112	55.9	132	42.5	32.5	53.9	5309.8	−23.7	18.4^{ab}
	25WR	C	88.0	136	59.6	145	44.9	31.3	50.8	5610.7	−19.4	16.8^{de}
		PGPB	102.0	125	66.0	145	49.3	33.6	47.5	6167.9	−11.4	18.2^{abc}
		PBPB + AMF	106.3	147	71.4	174	53.8	31.2	48.2	6729.9	-3.3	18.3^{abc}
	50WR	C	93.0	123	66.9	144	49.2	34.5	52.5	6154.3	−11.6	17.3^{cde}
		PGPB	104.3	134	69.8	152	53.1	34.8	51.0	6633.3	-4.7	18.4^{ab}
		PBPB + AMF	117.7	182	85.9	205	65.3	31.8	56.1	8165.0	17.3	17.7^{abcde}
	RGS	C	135.3	189	95.5	208	71.2	36.8	52.0	8903.1	28.0	17.8^{abcd}
		PGPB	149.0	189	100.0	226	74.7	33.1	49.8	9336.8	34.2	18.4^{ab}
		PBPB + AMF	155.7	215	101.5	238	75.6	31.9	49.1	9412.8	35.3	18.7^{a}
	100WR	C	104.3	147	73.9	171	55.7	32.4	52.9	6958.6	0.0	17.9^{abc}
		PGPB	143.7	192	96.0	210	69.8	33.2	48.7	8726.6	25.4	16.7^{e}
		PBPB + AMF	124.7	174	90.1	210	70.7	33.8	57.1	8841.9	27.1	18.7^{a}
P (I)			***	***	***	***	***	ns	*	***	***	**
P (T)			***	**	**	***	***	ns	**	***	***	***
P (I*T)			ns	ns	ns	ns	ns	ns	ns	ns	ns	***

Within each column and year, values followed by a different letter are significantly different at $P < 0.05$. Asterisks indicate a significant effect at the level of $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$; ns, non-significant effect.

second year, and similar to other plant growth parameters, significant differences were observed, with the highest value (18.7%) for the treatments PGPB+AMF RGS and PGPB+AMF 100WR (Table 4).

3.3. Grain yield and relative grain yield

Yield performance of chickpea was evaluated based on grain yield (GY) and relative grain yield (RGY) of the treatments as compared to the control (non-inoculated plants under 100% of water requirements; C 100WR). As previously shown for plant growth parameters, yield parameters were also significantly affected by inoculation treatments, irrigation levels and year. On average, chickpea grain yield increased in 2019 by 20% compared to 2018.

In 2019, under rainfed conditions, chickpea inoculation with PGPB resulted in significant grain yield increases compared to the respective non-inoculated control plants. In both years, under rainfed conditions, plants inoculated with PGPB+AMF significantly increased grain yield compared to the control plants. In 2019, in plants irrigated with 50% water requirements, inoculation with PGPB+AMF significantly increased grain yield compared to the respective control treatment. Overall, within the same irrigation regime, inoculation treatments resulted in an increase in chickpea grain yield when compared to the respective non-inoculated controls (Table 4).

Regarding the irrigation levels, chickpea irrigated only during the reproductive stage (RGS) had the highest grain yield (Table 4).

Considering the cumulative grain yield of the two-year experiments (2018 and 2019), higher values were observed in the treatments PGPB+AMF RGS (18,158 kg ha^{-1}) and PGPB RGS (18,151 kg ha^{-1}), while the C R treatment resulted in the lowest value (5385 kg ha^{-1}) (Fig. 1A). In all irrigation scenarios, co-inoculation with PGPB+AMF resulted in higher grain yield when compared to the single inoculation (PGPB) (Fig. 1A). Regarding relative grain yield, the results varied from −58.9% to 38.5% for RGY:C 100WR with greater values for the treatments PGPB+AMF RGS and PGPB RGS and the lowest for C R (Fig. 1B).

4. Discussion

Biofertilizers based on plant growth promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) are a promising tool to achieve higher yields and plant resilience in a climate-changing environment. However, inoculants are still poorly used in legume production (Abd-Alla et al., 2019; Romdhane et al., 2007) since competition from native microbiota, adaptation to unfavorable environmental conditions and management practices affect inoculation efficiency (McConnell et al., 2002; O'Callaghan, 2016).

In the present study, the combination of *Mesorhizobium* sp. UTADM31, *Burkholderia* sp. UTADB34 and *Pseudomonas* sp. UTAD11.3 strains comprise several microbial activities, such as phosphate solubilization, siderophore, IAA and HCN production, to support chickpea growth.

The average of the two-year field experiments showed that inoculation of chickpea with PGPB resulted in an increase of 23%, 17%, 18%, 16% and 16% in shoot dry weight (SDW), number of pods (NP), pod weight (PW), number of seeds (NS) and seed weight (SW), respectively, as compared to non-inoculated plants. Positive effects on plant growth parameters resulted in an increase of 16% in grain yield when compared to non-inoculated plants.

These findings are corroborated by other studies in chickpea plants that have revealed beneficial effects of dual and triple inoculation with PGPB on the nodulation, root dry weight, shoot dry weight, seed protein content and yield (Elkoca et al., 2007; Fabbri et al., 1995; Goel et al., 2002; Romdhane et al., 2007). In other crops, such as common bean, maize, tomato and soybean, increase in growth and yield parameters have also been reported with PGPB inoculation (de Souza and de Souza and Ferreira, 2017; Di Salvo et al., 2018; El-Nahrawy and Omara, 2017; He et al., 2019; Ulzen et al., 2016). These studies observed that PGPB improved the symbiotic performance of rhizobia, as well as crop productivity through direct and indirect mechanisms that enhanced the availability of nutrients, mineralized organic compounds, produced

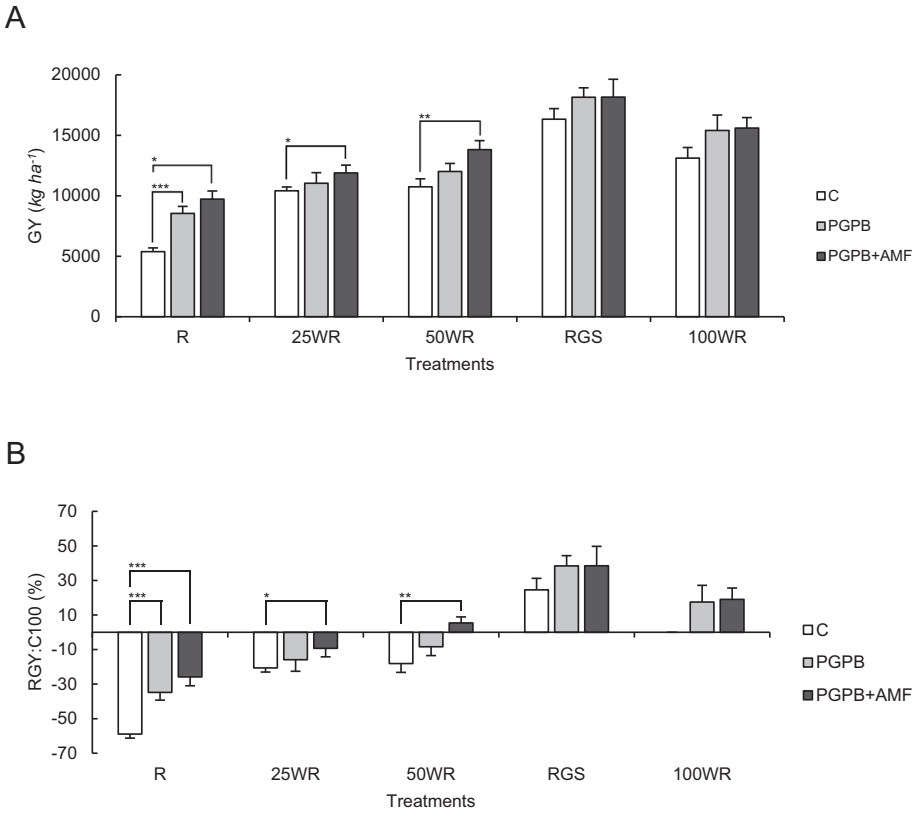

Fig. 1. Cumulative grain yield (2018 and 2019) (A); relative grain yield to non-inoculated plants under 100WR, RGY:C100WR (2018 and 2019) (B). Inoculation treatments (I): non-inoculated, C; single inoculation with a mix of plant growth promoting bacteria, PGPB; and dual inoculation with a mix of plant growth promoting bacteria and multiple AMF, PGPB+AMF. Irrigation levels throughout the growing season: 100% of water requirements (WR), 100WR; 50% of WR, 50WR; 25% of WR, 25WR; 100% of WR only during reproductive growth stages, RGS; and rainfed, R. Within the same irrigation level, statistical differences between inoculation treatments were set at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***), while absence of superscript indicates no significant differences. Data represent the mean ± standard error.

phytohormones and mediated the extent and quality of plant growth (Orozco-Mosqueda et al., 2018; Rocha et al., 2019; Vejan et al., 2016). Such interactions are crucial, mainly under eco-friendly legume production, that relies on multiple biological mechanisms instead of chemical inputs to maintain soil fertility and sustainable crop production (Ma, 2019; Vessey, 2003).

More insight into the data shows that co-inoculation of chickpea with PGPB+AMF promoted an increase of about 25%, 23%, 23%, 26% and 24% in SDW, NP, PW, NS and SW, respectively, as compared to non-inoculated plants (C). Grain yield increased 24% when compared to non-inoculated plants (C).

These observations agree with the findings of Rocha et al. (2019) and Singh et al. (2010), who reported the consortium of rhizobacteria and mycorrhizae as a viable way of improving chickpea production. Inoculation with PGPB+AMF also leads to increases in growth and productivity in bean (Singh, 2011), faba bean (Abd-Alla et al., 2014; Pereira et al., 2019), pea (Xavier and Germida, 2003), cowpea (Omirou et al., 2016), soybean (Meghvansi et al., 2008) and wheat (Raklami et al., 2019).

A proper given set of PGPB+AMF species depends on the particular combination between microsymbionts, since their interactions may, or may not, be beneficial for plant growth under different environmental conditions (Herrmann and Lesueur, 2013). Therefore, the functional compatibility of the symbionts and the assessment of their performance is desirable when used as biofertilizers (Meena et al., 2018).

The results of this study suggest that, regardless of the irrigation level, PGPB inoculation has beneficial effects on plant performance but co-inoculation (PGPB+AMF) shows a far greater potential to improve plant productivity. The synergistic effects of the consortium PGPB+AMF leads to a cumulative grain yield of 13,838 kg ha^{-1}, an increase of 6% compared to single inoculation and 24% over the non-inoculated plants. In plants under rainfed conditions, single and dual inoculations result in higher grain yield increases (59% and 81%, respectively) when compared to the control treatment (Fig. 1A). These findings suggest that the inoculum developed is adapted to drought stress conditions.

Although water deficit affects plant growth at any stage, its occurrence during late vegetative and reproductive stages is critical and usually results in variable and low yields (Nadeem et al., 2019; Sinha et al., 2019).

Our results show that grain yield was 19% higher when 100% water requirements were fulfilled during the reproductive stage (RGS), from flowering to grain filling, as compared with 100% water requirements over the whole crop cycle. This might be due to the increase in dry matter accumulation and the high tolerance of chickpea to harsh environmental conditions, including water stress. These outcomes emphasize the global guidelines regarding the selection of the optimum period for irrigation to obtain higher grain yields and to reduce water use where it is in short supply.

In other irrigation studies, Singh et al. (2016) showed that a single irrigation during pod formation resulted in higher chickpea yields, when compared to irrigation at flowering (2626 kg ha^{-1} and 2202 kg ha^{-1}, respectively), while Kemal et al. (2018) observed that irrigation once at the vegetative stage (2917 kg ha^{-1}) showed higher seed yield over irrigation at flowering (2533 kg ha^{-1}) or pod formation (2456 kg ha^{-1}).

Overall, this work highlights the positive effects on chickpea plant growth parameters caused by the treatment PGPB+AMF in plants irrigated only during the reproductive growth stages (RGS) (Table 4). Based on the two-year results, the cumulative grain yield for this treatment was 18,158 kg ha^{-1}, resulting in an increase of 12,772 kg ha^{-1} and 2559 kg ha^{-1} compared to non-inoculated plants under rainfed conditions (C R) and fully irrigated co-inoculated plants (PGPB+AMF 100WR), respectively (Fig. 1A). The effect of the PGPB+AMF RGS treatment resulted in a relative grain yield of 38.5% as compared to C 100WR (Fig. 1B).

Furthermore, considering the average of the two-year experiment, we found that the PGPB+AMF RGS treatment had the highest crude protein content (19.1%), which can contribute to improved food quality and lead to benefits in human health. This observation agrees with the findings of Oliveira et al. (2017) who reported an increase in grain protein content of chickpea inoculated with *Mesorhizobium mediterraneum* + *Rhizophagus irregularis* under conditions of moderate water deficit.

From an environmental viewpoint, enhancing legume production with beneficial microorganisms and greater water use efficiency is in compliance with the world's concerns about climate change. In this study, single and dual inoculations increased chickpea yield regardless of the irrigation level. Moreover, irrigation only during the reproductive stage of chickpea provides a new insight into how to achieve the millennium development goals – to produce more with fewer resources and tackle problems arising from water scarcity.

5. Conclusion

Introducing plant growth promoting bacteria and arbuscular mycorrhizal fungi in legume production is regarded as a means to increase the growth and resilience of plants against biotic and abiotic stresses. Investigation into the effects of inoculation on chickpea productivity with beneficial microorganisms under different irrigation regimes is scarce, especially in Mediterranean regions.

This study highlights the beneficial effects of PGPB+AMF inoculation on chickpea growth parameters, which increased grain yield of 6% compared to a single inoculation, and 24% over the non-inoculated plants. Additionally, grain yield increased 19% in plants subjected to irrigation only during the reproductive stages compared to fully irrigated plants.

Overall, inoculation with PGPB+AMF combined with irrigation only during the reproductive stage resulted in the highest grain yield and can be recommended as a biotechnological tool and agronomic strategy of great relevance regarding the future of sustainable chickpea production.

These findings provide scientific support that encourages the further use of biofertilizers and deficit irrigation to increase chickpea production, particularly under adverse environmental conditions, where chickpea has great economic and agronomic value as a source of protein.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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