

Increased protein content of chickpea (*Cicer arietinum* L.) inoculated with arbuscular mycorrhizal fungi and nitrogen-fixing bacteria under water deficit conditions

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Abstract

BACKGROUND: Chickpea (*Cicer arietinum* L.) is a widely cropped pulse and an important source of proteins for humans. In Mediterranean regions it is predicted that drought will reduce soil moisture and become a major issue in agricultural practice. Nitrogen (N)-fixing bacteria and arbuscular mycorrhizal (AM) fungi have the potential to improve plant growth and drought tolerance. The aim of the study was to assess the effects of N-fixing bacteria and AM fungi on the growth, grain yield and protein content of chickpea under water deficit.

RESULTS: Plants inoculated with *Mesorhizobium mediterraneum* or *Rhizophagus irregularis* without water deficit and inoculated with *M. mediterraneum* under moderate water deficit had significant increases in biomass. Inoculation with microbial symbionts brought no benefits to chickpea under severe water deficit. However, under moderate water deficit grain crude protein was increased by 13%, 17% and 22% in plants inoculated with *M. mediterraneum*, *R. irregularis* and *M. mediterraneum* + *R. irregularis*, respectively.

CONCLUSION: Inoculation with N-fixing bacteria and AM fungi has the potential to benefit agricultural production of chickpea under water deficit conditions and to contribute to increased grain protein content.

Keywords: drought; grain legume; grain protein content; *Mesorhizobium mediterraneum*; *Rhizophagus irregularis*

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second most important legume crop consumed worldwide, especially in North Africa, South-East Asia, the Middle East, southern Europe, America and Australia.¹ Globally, it is one of the most cultivated pulses in terms of world production, with a total production of 14.2 million t and an average yield of 0.96 t ha⁻¹.² Chickpea has been considered an important source of proteins, carbohydrates, minerals, vitamins and health-promoting fatty acids in the human diet.^{3,4} As a cheaper source of protein it is particularly important for low-income consumers around the world and in developing countries, where large segments of populations have limited access to food of animal origin.⁵ Chickpea is usually cultivated in regions where climate variability, drought and limited use of fertilizers significantly reduce productivity. However, being a leguminous crop, it exhibits the important characteristic of fixing atmospheric nitrogen (N) through its symbiosis with rhizobia, enabling cultivation in many N-poor soils with acceptable yields. Chickpea rhizobia are included in the *Mesorhizobium* genus⁴ and can fix up to 140 kg N ha⁻¹ per annum, depending on soil and climatic conditions.⁶

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Legume plants also establish mutualistic relationships with arbuscular mycorrhizal (AM) fungi, which together with rhizobia are often designated by tripartite symbioses.^{7,8} The environmental benefits of legumes are the reduced use of mineral N fertilizer,^{9,10} the improvement of soil conditions and the yield increase of succeeding crops in the rotation.^{11,12} Relatively few studies have addressed the influence of AM fungi in the performance of legume–rhizobia symbiosis. Nevertheless, benefits of AM fungi have been demonstrated on legume growth, N fixation, grain yield,^{13,14} plant uptake of nutrients,^{15–17} hormonal balance and water relations.^{18,19}

In Mediterranean regions drought stress is known to hamper plant production.²⁰ In legumes, drought has a considerable negative impact on nodule functioning, due to premature senescence and consequent reduction of N fixation.²¹ It has been demonstrated that AM fungi can efficiently alleviate drought stress by several mechanisms, including regulation of plant hormonal balance,²² increased photosynthetic rate and leaf gas exchange,²³ and translocation of water from soil to plant via extraradical mycelium (ERM).^{24,25} Since prolonged periods of drought are responsible for yield losses, optimization of the relationships of legumes with drought-tolerant microbial symbionts is required to develop effective methods for minimizing the effects of exposure to drought.

The aims of the present study were (i) to determine the effects of an N-fixing bacterium and an AM fungus on the growth and grain yield of chickpea under three different water regimes, and (ii) to assess whether microbial inoculation could improve grain protein content, particularly under water deficit.

MATERIALS AND METHODS

Plant material and soil

The seeds used in this study were from chickpea (*Cicer arietinum* L. cv. Chk 4198) obtained from the collection of UTAD. Soil was collected from the uppermost 10 cm layer of an organic farm in northern Portugal, sieved through a 4 mm mesh and autoclaved twice (121 °C for 25 min) on consecutive days. This was a sandy soil with pH (1:2.5 w/v water) 6.5, electrical conductivity 0.1 dS m⁻¹, 1.2% organic matter, 3.8 g kg⁻¹ total N, 48.8 mg kg⁻¹ extractable (Egner–Riehm) P, 4.3 g kg⁻¹ K, 1.6 g kg⁻¹ Ca, 66 mg kg⁻¹ Mg and 147 mg kg⁻¹ Na.

Microbial inocula

The bacterial isolate *Mesorhizobium mediterraneum* UPM-Ca36 was grown in Tryptone Yeast (TY) medium²⁶ for 3 days at 28 °C and 0.5 × g. The culture was then centrifuged at 4930 × g for 10 min and the pellet was washed with saline solution (0.85% NaCl). The pellet was resuspended in saline solution and the colony-forming unit (CFU) adjusted to 10⁹ mL⁻¹.

The AM fungal isolate *Rhizophagus irregularis* BEG140 was grown for 8 months in a multi-spore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Zea mays* L. as a host plant. The fungal inoculum was provided by Symbiom Ltd, Czech Republic.

Experimental design and setup

Experimental units (1 dm³ pots filled with soil) were arranged in a fully randomized manner using a 2 × 2 × 3 factorial design, where the first factor was bacterial inoculation (non-inoculated plants and plants inoculated with *M. mediterraneum*), the second factor

was fungal inoculation (non-inoculated plants and plants inoculated with *R. irregularis*) and the third factor was water deficit (no water deficit (N), moderate water deficit (M) and severe water deficit (S)). Thus for each water regime there were four treatments: non-inoculated plants; plants inoculated with *M. mediterraneum*; plants inoculated with *R. irregularis*; and dually inoculated plants. Each treatment combination was replicated 10 times. Seeds of chickpea were surface sterilized with 0.5% (v/v) sodium hypochlorite for 20 min, placed on moist paper towels and germinated at 20 °C in the dark. After germination, seedlings of similar size were transplanted: one plant into each pot. A nitrocellulose membrane filter (24 mm diameter and 0.4 µm pore size) (Pragopore, Pragochema Ltd, Czech Republic) was inserted vertically into each pot for future measurements of ERM length.²⁷ At transplanting, each pot from the bacterial treatments received 4 mL bacterial suspension (described above). Every pot from the non-bacterial treatments received 4 mL autoclaved bacterial suspension. Each pot from the mycorrhizal treatments received 10 g inoculum consisting of colonized root fragments, hyphae and spores in the mixture of zeolite and expanded clay, placed 2 cm below the root system. Every pot from the non-mycorrhizal treatments received 10 g inoculum autoclaved twice (121 °C for 25 min) on consecutive days. In order to eliminate differences in bacterial populations introduced with the AM fungal inoculum, 5 mL of a filtrate of AM fungal inoculum was added to all pots from the non-mycorrhizal treatments.²⁸ The filtrate was prepared as described in Oliveira *et al.*²⁹ Field capacity of the soil in the pots was determined³⁰ and during the first 4 weeks soil moisture in all pots was kept at 75% of field capacity by weighing the pots every 2 days and watering accordingly with deionized water. Then soil moisture was kept at 75%, 50% and 25% of field capacity by weighing the pots for the treatments with no water deficit, moderate water deficit and severe water deficit, respectively. Plants were grown in a greenhouse under natural light with an average photoperiod of 12 h. Temperature and relative humidity ranges were 12–42 °C and 55–85%, respectively. Pots of different treatments were periodically rotated to different bench positions to minimize differences due to their location in the greenhouse.

Plant and microbial analyses

After a growth period of 3 months, grains were harvested and the number of grains per plant, fresh weight of grains per plant and fresh weight per grain were determined. Grain samples were dried at 80 °C for 48 h and analysed for total Kjeldahl N following the methods of the Association of Official Analytical Chemists.³¹ Crude protein was calculated as N × 6.25.³² Plants were removed from the pots, and the root system was separated from the shoot and gently washed to remove adhered soil. Shoot height was measured and the number of root nodules was counted. A fresh subsample (0.2 g) of roots was collected to assess AM colonization (described below). The remaining root system was weighed and dried at 80 °C for 48 h together with the shoot. The dried root system and shoot were then reweighed. The dry root mass of the subsample was calculated by multiplying its fresh mass by the dry-to-fresh mass ratio of the root system. The sum of the dry mass of the root subsample with the dry mass of the root system and the dry mass of the shoot gave the total dry weight per plant. The subsample of fresh roots was cut into 1 cm pieces and stained with trypan blue using a modified Phillips and Hayman³³ protocol.³⁴ Percentage root length colonized (RLC) by AM fungi was assessed for each plant species using the gridline intersect method³⁵ under a stereo microscope (Olympus SZ61, Japan). The ERM length was determined by the

Table 1. *F*-values and three-way ANOVA significance of plant growth parameters according to bacterial inoculation, fungal inoculation and water deficit

	Shoot height	Root dry weight	Shoot dry weight	Total plant dry weight
Bacterial inoculation (B)	2.1 ns	1.4 ns	3.6 ns	2.9 ns
Fungal inoculation (F)	36.4***	0.01 ns	10.3**	1.1 ns
Water deficit (W)	12.8***	2.0 ns	14.8***	5.6**
B × F	0.1 ns	7.6**	2.7 ns	6.8*
B × W	1.4 ns	0.9 ns	1.3 ns	0.7 ns
F × W	0.2 ns	1.3 ns	0.01 ns	0.6 ns
B × F × W	0.4 ns	0.3 ns	3.1 ns	0.8 ns

Asterisks indicate a significant effect at the level of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; ns, non-significant effect.

inserted membrane technique,²⁷ followed by the gridline intersect method under a compound microscope (Leica DM 750, Germany), using an ocular grid at × 200 magnification.³⁶ Background lengths of mycelium found in non-mycorrhizal treatments were subtracted from the values obtained in the corresponding mycorrhizal treatments and the ERM length expressed in cm of hyphae per 1 cm² of the inserted membrane filter.

Statistical analysis

Normality and homogeneity of variances were confirmed and data analysed using three-way analysis of variance (ANOVA) for each dependent variable (plant parameters) *versus* independent variables (bacterial inoculation, fungal inoculation and water deficit). When a significant *F*-value was obtained ($P < 0.05$), treatment means were compared using Duncan's multiple range test. Microbial parameters data were analysed by two-way ANOVA without including the respective non-inoculated control treatments. All statistical analyses were performed with the SPSS 23.0.0.0 software package (IBM SPSS Statistics, USA).

RESULTS AND DISCUSSION

Plant growth

Single inoculation with the AM fungus *R. irregularis* significantly increased shoot height of chickpea in all water regimes when compared with the respective non-inoculated controls (Fig. 1A). Tufenkci *et al.*³⁷ also showed that inoculation with an AM fungal isolate resulted in higher shoot height of chickpea. However, in their study water deficit was not imposed to the plants. Dually inoculated (*M. mediterraneum* + *R. irregularis*) plants had significantly higher shoot height than non-inoculated controls under moderate water deficit (M). There was no effect on shoot height of single inoculation with the N-fixing bacterium *M. mediterraneum* (Table 1). Water deficit significantly influenced shoot height of chickpea, particularly in non-inoculated control plants where increased water deficit significantly decreased shoot height; in plants inoculated with *R. irregularis* where moderate water deficit significantly reduced shoot height in comparison with plants without water deficit; and in dually inoculated plants where severe water deficit (S) resulted in a reduction of shoot height of chickpea when compared with plants without water deficit. Reduced irrigation in order to maintain severe water stress was shown to be detrimental to the growth of chickpea, since a reduction in plant height under such conditions has been reported.³⁸

Overall there was no significant effect of bacterial inoculation, fungal inoculation and water deficit on the dry weight of roots of chickpea (Table 1). The exception was in plants inoculated

with *M. mediterraneum* and subjected to moderate water deficit, where root dry weight was significantly greater than the respective non-inoculated control (Fig. 1B).

Under severe water deficit there were no significant differences in shoot dry weight between non-inoculated controls and all inoculation treatments, whereas without water deficit all inoculations (single and dual) resulted in significantly improved shoot dry weight of chickpea (Fig. 1C). Additionally, dually inoculated plants subjected to moderate water deficit also had significantly improved shoot dry weight compared with non-inoculated control. Significant reductions in shoot dry weight of singly and dually inoculated chickpea were observed in plants under severe water deficit in comparison with those without water deficit.

Inoculation with *R. irregularis* was shown to significantly improve total biomass of chickpea without water deficit. Plants inoculated with *M. mediterraneum* without water deficit and under moderate water deficit had higher biomass than the corresponding non-inoculated controls (Fig. 1D). Under severe water deficit there were no significant differences in total plant dry weight between non-inoculated control plants and those of any inoculation treatment.

N-fixing bacteria and AM fungi have the potential to improve plant growth and alleviate drought stress under water-limiting conditions.^{39–46} Considering such potential, we aimed at assessing whether inoculation with *M. mediterraneum* and *R. irregularis* could improve plant growth, grain yield and protein content under water deficit.

Our results showed that shoot height was increased by inoculation with *R. irregularis* under moderate and severe water deficit and by inoculation with *M. mediterraneum* + *R. irregularis* under moderate water deficit, whereas plant biomass was only improved in plants inoculated with *M. mediterraneum* under moderate water deficit.

Erman *et al.*⁴⁷ reported improved plant biomass of chickpea grown without irrigation and inoculated singly and dually with AM fungi and N-fixing bacteria. The authors suggested that both bipartite and tripartite symbioses contributed to better plant nutrition, particularly regarding N and P, which resulted in improved plant growth. Enhanced nutrient uptake in inoculated chickpea has also been demonstrated by Farzaneh and colleagues.¹⁵

There was no AM fungal colonization or nodules of *M. mediterraneum* in the roots of non-inoculated control plants. All plants inoculated with *R. irregularis* had root mycorrhizal colonization and all plants inoculated with *M. mediterraneum* developed root nodules (Table 2). Inoculation with *R. irregularis* resulted in a %RLC that, among treatments, varied between 21% and 52%, which is within the range reported by Tavasolee *et al.*⁴⁸ for chickpea.

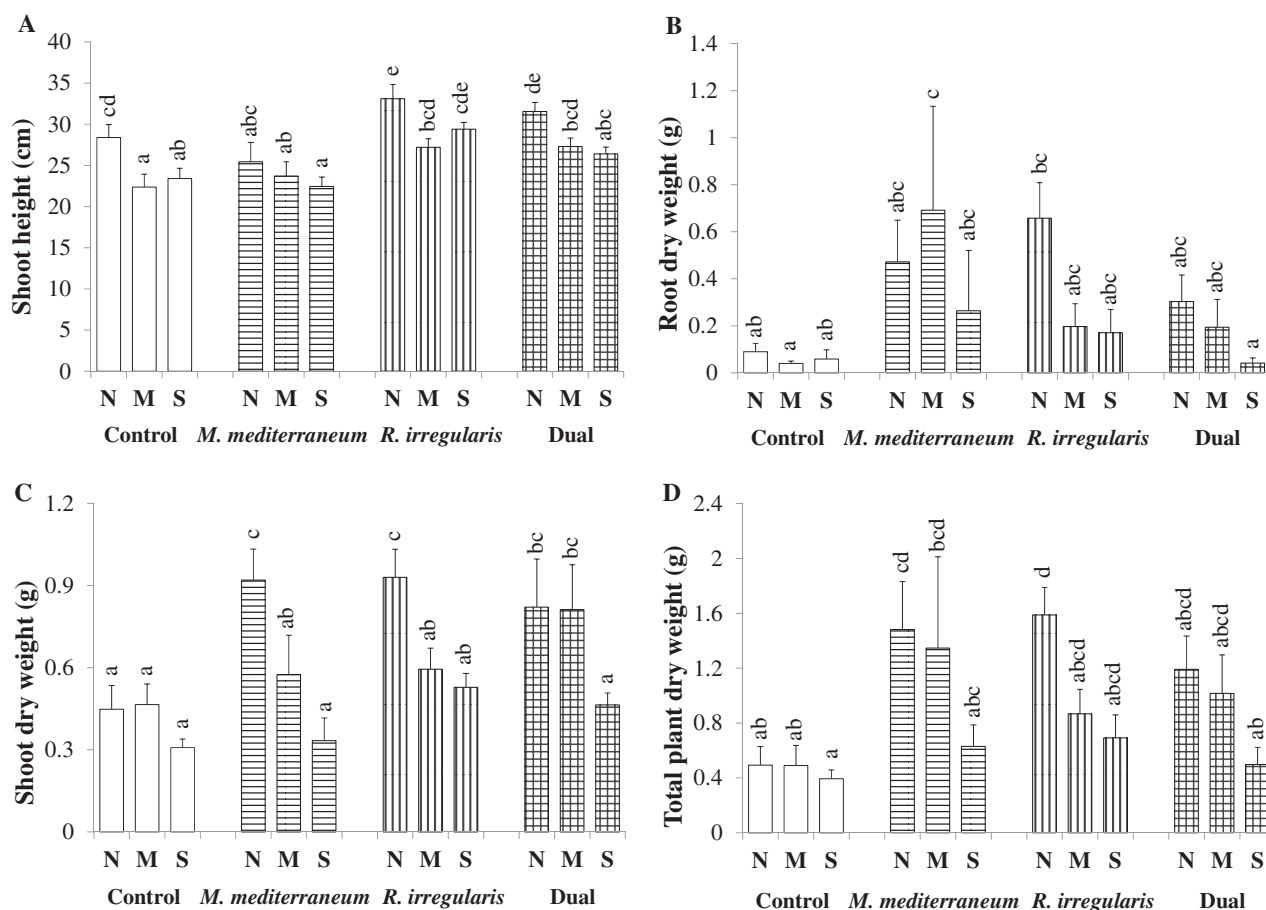


Figure 1. Shoot height (A), root (B), shoot (C) and total plant (D) dry weight of *Cicer arietinum* inoculated singly or dually with *Mesorhizobium mediterraneum* and *Rhizopagus irregularis* under different water deficits. Values are means \pm 1 SE. Columns marked with different letters are significantly different according to Duncan's multiple range test at $P < 0.05$. N, no water deficit; M, moderate water deficit; S, severe water deficit.

The number of root nodules was significantly reduced in plants under severe water deficit when compared with those without water deficit. Erman *et al.*⁴⁷ showed that without irrigation the number of nodules was significantly reduced in the roots of chickpea. Drought may reduce the number of nodules, which can negatively affect symbiosis. It seems, therefore, that severe water deficit was detrimental to the microbial symbionts, which may have contributed to the less evident plant growth benefits observed under severe than moderate water deficit. This is in agreement with the findings of Ruiz-Lozano *et al.*,²¹ who demonstrated that drought has a considerable negative impact on nodule functioning, resulting in decreased ability of N fixation. Inoculation with *R. irregularis* did not influence the number of root nodules. There was no effect of bacterial inoculation or water deficit on the ERM length.

Grain yield and protein content

There was a significant increase in the number of grains produced per plant of chickpea with inoculation of *R. irregularis* and *M. mediterraneum* + *R. irregularis* without water deficit (Fig. 2A). However, under moderate and severe water deficit there was no increase in number of grains in any inoculation treatment when compared with the respective non-inoculated controls. Water deficit significantly influenced the number of grains per plant (Table 3). In all inoculation treatments, the number of grains per plant was significantly reduced in plants under moderate and severe water deficit when compared with those without water deficit (Fig. 2A).

Similarly, the weight of grains per plant was significantly reduced in plants under moderate and severe water deficit when compared with those without water deficit in all inoculation treatments (Fig. 2B). Additionally, a significant reduction in weight of grains per plant was observed in non-inoculated control plants under severe water deficit when compared with those without water deficit. A significant increase in weight of grains per plant of singly and dually inoculated chickpea was only observed in plants without water deficit. These observations are in agreement with the findings of Erman *et al.*,⁴⁷ who reported that both single and dual inoculation of chickpea with *Mesorhizobium ciceri* and *Glomus intraradices* resulted in increased grain yield. Zaidi *et al.*⁴⁹ also showed that dual inoculation with an N-fixing bacterium and an AM fungus led to increased grain yield of chickpea.

There was no influence of any inoculation treatment in the weight per grain of chickpea (Table 3). However, in plants inoculated with *M. mediterraneum* there was a significant reduction in the weight per grain of plants under severe water deficit when compared with those without water deficit (Fig. 2C).

Under moderate and severe water deficit there were no improvements of single or dual inoculation on grain yield. However, in plants without water deficit and in those under moderate water deficit, dual and single inoculation resulted in significant increases of the content of crude protein of chickpea grains in comparison with the respective non-inoculated controls (Fig. 2D). There were no significant differences in the content of crude protein

Table 2. Effect of inoculation and water deficit on the number of root nodules, mycorrhizal colonization and length of the extraradical mycelium

Inoculation	Water deficit	No. of nodules	AMF colonization (%RLC)	ERM length (cm cm ⁻²)
<i>Mesorhizobium mediterraneum</i>	N	27.6 ± 3 cd	0	0
	M	20.6 ± 3abc	0	0
	S	14.8 ± 2a	0	0
<i>Rhizophagus irregularis</i>	N	0	48 ± 5bc	15 ± 6
	M	0	33 ± 5ab	15 ± 5
	S	0	52 ± 8c	5 ± 2
<i>Mesorhizobium mediterraneum</i> + <i>Rhizophagus irregularis</i>	N	32.2 ± 3d	35 ± 7abc	17 ± 9
	M	25.2 ± 2bcd	31 ± 5ab	16 ± 5
	S	19.8 ± 1ab	21 ± 2a	13 ± 4
<i>Two-way ANOVA F-values and significance</i>				
Bacterial inoculation (B)			10.9**	0.5 ns
Fungal inoculation (F)		6.1*		
Water deficit (W)		14.5***	1.4 ns	0.9 ns
B × W			3.2 ns	0.2 ns
F × W		0.005 ns		

Means (±1 SE) followed by the same letters within each column are not significantly different according to Duncan's multiple range test; asterisks indicate a significant effect at the level of **P* < 0.05, ***P* < 0.01 and ****P* < 0.001, respectively; ns, non-significant effect. AMF, arbuscular mycorrhizal fungal; RLC, root length colonized; ERM, extraradical mycelium; N, no water deficit; M, moderate water deficit; S, severe water deficit.

Table 3. F-values and three-way ANOVA significance of grain parameters according to bacterial inoculation, fungal inoculation and water deficit

	Number of grains per plant	Fresh weight of grains per plant	Fresh weight per grain	Crude protein of grains
Bacterial inoculation (B)	0.5 ns	1.2 ns	1.1 ns	5.8*
Fungal inoculation (F)	3.3*	1.0 ns	0.7 ns	24.2***
Water deficit (W)	14.4***	55.1***	8.5***	8.4**
B × F	1.0 ns	1.5 ns	0.004 ns	4.5*
B × W	0.6 ns	3.0 ns	0.7 ns	3.7*
F × W	0.5 ns	0.5 ns	1.4 ns	0.4 ns
B × F × W	1.1 ns	3.4*	0.9 ns	2.7 ns

Asterisks indicate a significant effect at the level of **P* < 0.05, ***P* < 0.01 and ****P* < 0.001; ns, non-significant effect.

of grains between inoculated (singly and dually) plants and non-inoculated controls under severe water deficit. Under moderate water deficit inoculation with *M. mediterraneum*, *R. irregularis* and *M. mediterraneum* + *R. irregularis* led to increase in the content of crude protein of chickpea grains of 13%, 17% and 22%, respectively. An increase in grain protein content of chickpea inoculated with AM fungi has also been reported by Pellegrino and Bedini.⁵⁰ The authors speculated that the increase could have been related to the larger ERM length of AM fungi, which can improve inorganic and organic soil N mobilization. However, this was not clear in our study, since we not only obtained grain protein increases in plants inoculated with *R. irregularis*, where abundant ERM was observed, but also in plants inoculated with *M. mediterraneum*, where no AM fungal ERM was present (Table 2).

Grains of chickpea are highly valued for human consumption owing to their high level of protein and adequate proportions of carbohydrates and oil.⁵¹ Therefore, increases in grain protein content, as shown in our study, can be of importance for agricultural production of chickpea. Overall, grain yield was higher without water deficit. Microbial inoculation further improved grain yield. With the rise of global temperatures, extreme summer heat and prolonged drought will reduce soil moisture and become a major

issue in agricultural practice.^{20,52} Our results showed that inoculation with an N-fixing bacterium and an AM fungus can contribute to improve the quality of chickpea grains under moderate water deficit.

CONCLUSIONS

Inoculation with *R. irregularis* and *M. mediterraneum* + *R. irregularis* increased grain productivity of chickpea without water deficit. However, under moderate and severe water deficit there was no improvement of grain yield in inoculated plants. Water availability is predicted to become a major constraint for agriculture under the warmer, drier climate of the future. Thus there is a need to improve the productivity of chickpea under drought conditions. Inoculation with *M. mediterraneum*, *R. irregularis* and *M. mediterraneum* + *R. irregularis* was shown to increase the content of crude protein of chickpea grains under moderate water deficit conditions. Additionally, there was an increase in biomass of plants inoculated with *R. irregularis* or *M. mediterraneum* without water deficit and inoculated with *M. mediterraneum* under moderate water deficit. N-fixing bacteria and AM fungi alone and in combination have great potential to benefit the agricultural production of chickpea under adverse environmental conditions, contributing to improve food quality leading, ultimately, to benefits in human health.

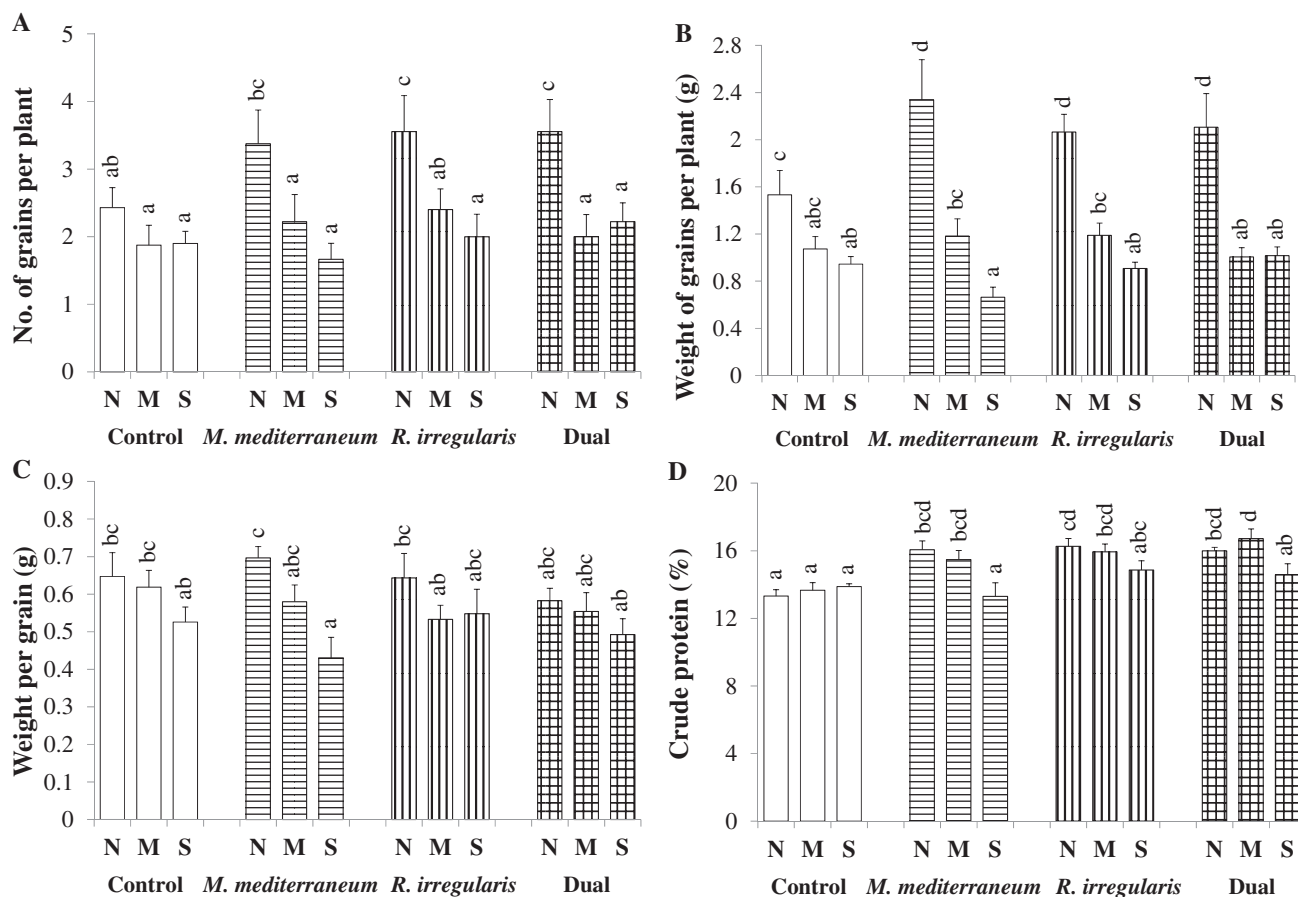


Figure 2. Number of grains per plant (A), fresh weight of grains per plant (B), fresh weight per grain (C) and percentage of crude protein of grains (D) at harvest of *Cicer arietinum* inoculated singly or dually with *Mesorhizobium mediterraneum* and *Rhizophagus irregularis* under different water deficits. Values are means \pm 1 SE. Columns marked with different letters are significantly different according to Duncan's multiple range test at $P < 0.05$. N, no water deficit; M, moderate water deficit; S, severe water deficit.

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