RESEARCH ARTICLE

Co-inoculation with rhizobia and mycorrhizal fungi increases yield and crude protein content of cowpea (*Vigna unguiculata* (L.) Walp.) under drought stress

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HIGHLIGHTS

- **Cowpea is one of the most consumed legumes worldwide due to its high seed protein content.**
- **Rhizobial bacteria and arbuscular mycorrhizal fungi can improve growth and yield of leguminous plants.**
- **The selection of appropriate microorganisms is essential to the success of symbiosis.**
- **Co-inoculation with selected beneficial microorganisms increased crude protein content in the grain of plants under drought stress.**
- **This eco-friendly strategy can be a useful tool in more sustainable agriculture to mitigate climate changes.**

KEYWORDS **AMF, drought, rhizobia, tripartite symbiosis, *Vigna unguiculata* (L.) Walp.**

Abstract

Recent trends in sustainable agricultural production seek improved bioinoculants that can improve crop adaptation and production and reduce external inputs of pesticides and synthetic fertilisers, particularly under abiotic and biotic stress conditions. Drought is one of the critical and more frequent conditions that can drastically reduce plant biomass and yield. In this sense, the use of bioinoculants is a biological strategy to mitigate climate change and reduce the water needs of plants. Leguminous plants are very important in improving sustainable cropping systems because they can form effective symbiotic associations with both nitrogen-fixing bacteria and arbuscular mycorrhizal fungi. These microorganisms can act as an alternative source of nitrogen and can increase phosphorus utilisation from soils and fertilisers. Cowpea is a multipurpose crop that has caused a great interest due to its resistance to abiotic stress. This pot experiment in a greenhouse with non-sterilised soil aimed to test the effect of three previously selected rhizobial bacteria (*Rhizobium* sp. (B1), *Bradyrhizobium elkanii* (B2) and *Bradyrhizobium* sp. (B3)) and arbuscular mycorrhizal fungi (*Claroideoglomus claroideum* BEG210) on the yield and crude protein content of cowpea under drought conditions and also to compare the competitiveness of the inoculated bacteria with native rhizobial bacteria naturally present in the soil. The combined inoculation with each bacteria and arbuscular mycorrhizal fungi *Claroideoglomus claroideum* BEG210 was shown to increase the crude protein content of cowpea seeds in plants under drought stress (25 % of field capacity) by 13 %, 17 %, and 30 %, respectively. This study shows that these microorganisms are potentially resistant to drought and can be used as a biotechnological tool for sustainable agriculture under drought conditions.

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1 Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an annual legume crop native of Africa and is the most widely cultivated seed-legume in arid and semi-arid areas (Alkama et al., 2009; Johnson et al., 2013; Lazaridi et al., 2017). It is adapted to high temperatures (20 to 35 °C) and can grow well in a wide range of soil textures and with only 188 mm of annual rainfall. Its growth period can range between 90 to 240 days, depending on the climatic conditions and the maturity period of the cultivar (Ngalamu et al., 2014; Carvalho et al., 2017).

It has been estimated that the total cultivated area has increased in the last years from approximately 2.4 million hectares in 1961 to around 12.5 million hectares in 2017 (FAOSTAT, 2017). Despite the wide distribution of cowpea, around 98 % of the world production is located in Africa (12.3 million hectares) (Alkama et al., 2009; Oliveira et al., 2017).

Cowpea seeds provide a rich source of proteins (23 %), carbohydrates (56 %), fibre (4 %) and calories, as well as minerals and vitamins, and for this reason are sometimes called "poor man's meat" (Iqbal et al., 2006). Additionally, cowpea can also provide an alternative protein source for people that suffer from allergies to soybean protein (Ravelombola et al., 2016).

Nowadays, the increasing food demand, the rising global temperatures, and global water scarcity have led to a need to produce more food with less water (Oliveira et al., 2017). Water scarcity is one of the main reasons for the reduction in agricultural productivity because it can lead to anatomical, morphological, physiological, and biochemical modifications that affect plant growth and development (Bezerra et al., 2003). In fact, according to Bastos et al. (2011), well-watered cowpea plants can produce more than 1,000 kg grain ha^{-1}, but water scarcity can reduce this potential to approximately 360 kg ha^{-1}. In this sense, the understanding of the physiological, biochemical, and agromorphological mechanisms that can explain the resistance of cowpea varieties to drought is of extreme importance (Cruz de Carvalho, 2000). The physiological mechanisms include the closing of the stomata when the water in the soil is not sufficient and the decrease in the transpiration and photosynthetic rates. The biochemical mechanisms involve the osmotic adjustment which is characterised by the accumulation of organic solutes to maintain the cell turgor, and the agromorphological processes include the turning of the leaves upwards to protect them from excessive temperatures and the reduction in the root volume (Krouma, 2010; Hall, 2012; Halilou et al., 2015). Despite the inherent resistance of cowpea plants to the drought, the inoculation of cowpea and other legumes with beneficial and drought-resistant microorganisms, such as rhizobial bacteria and arbuscular mycorrhizal fungi (AMF), also has a great potential to reduce the negative effects of water scarcity and global warming on cowpea plants. A heterogeneous group of slow-growing rhizobial bacteria belonging to the genus *Bradyrhizobium* and known as "cowpea-miscellany" has the ability to nodulate cowpea roots (Allen and Allen, 1981; Appunu et al., 2009), increasing plant resistance to high temperatures and water deficit and reducing the need for chemical fertiliser inputs. *Bradyrhizobium elkanii*, *B. yuanmingense*, and *B. japonicum* are among the main rhizobial species associated with cowpea (Zhang et al., 2008).

The association with AMF is a non-specific, highly compatible, and long-lasting mutualism, whereby both partners have advantages (Abdel-Fattah et al., 2011; Harrison, 1998). AMF can be applied to increase the growth potential and reduce water and fertiliser inputs. Indeed, in this symbiosis, the fungal hyphae (thread-like structures) spread through the soil, taking up nutrients such as phosphorus and absorbing water and transporting them to the plant root, while receiving sugars from the plant in return. This association between AMF and plants can increase drought tolerance (Augé et al., 2001; Oliveira et al., 2017) and consequently improve cowpea yield under adverse environmental conditions.

Co-inoculation with both rhizobia and AMF in legumes results in a mutualistic tripartite symbiosis (Antunes and Goss, 2005) that usually leads to a higher increase of growth and yield than that resulting from single inoculation with one microorganism (Chalk et al., 2006; Marulanda et al., 2006). In fact, in this kind of symbiosis, the presence of one microorganism can affect the activity of the other and, consequently, the interaction of both has normally a positive effect on the host plant (Vejsadova et al., 1993; Xie et al., 1995).

The objective of the present work was to evaluate the effect of single and co-inoculation with several rhizobial bacteria (*Rhizobium* sp., *Bradyrhizobium elkanii* and *Bradyrhizobium* sp.) and AMF (*Claroideoglomus claroideum* BEG210) on the growth, yield, and crude protein content of cowpea seeds under drought conditions and compare the competitiveness of the inoculated bacteria with those naturally present in the soil.

2 Materials and methods

2.1 Bacterial inoculant and arbuscular mycorrhizal fungi inoculant

The bacterial strains used in this work were isolated from fresh surface sterilised root nodules of cowpea plants and previously selected among others according to their performance in in vitro experiments. Bacteria B1 and B2 were collected in Elvas, Portugal (39'23'59.72''N, 7'53'25.99''W), in July 2014, and bacteria B3 were collected in Vila Real, Portugal (41'28.54''N, 7'74.14''W), in September 2014. The bacteria identification was performed by amplification of 16S rDNA using the universal primers fD1 and rD1 (Weisburg et al., 1991). Furthermore, for multilocus sequence analysis (MLSA) and in order to identify the isolates at the species level, this analysis was complemented with six housekeeping genes: *recA* (DNA recombination protein), *gyrB* (DNA gyrase B), *SMc00019* (conserved hypothetical protein), *thrA* (homoserine dehydrogenase), *atpD* (atpD synthase β-subunit), and *truA* (RNA pseudouridine synthase A) (Haukka et al., 1998; Gaunt et al., 2001; Zhang et al., 2012). Taxonomic position at the symbiovar level was determined by the inferred phylogenies based on the symbiotic genes of nodulation: *nodA* (N-acyltransferase nodulation protein A) and *nodC* (N-acetylglucosaminyltransferase) (*Table 1*). PCR mixtures were performed with 7.5 µl of

TABLE 1
List of primers used in this work for the molecular identification of collected rhizobial bacteria

Primers	Sequence (5'–3')	Reference
fD1	AGA GTT TGA TCC TGG CTC AG	Weisburg et al., 1991
rD1	AAG GAG GTG ATC CAG CC	
thrAB-F	TGC TTC GTC GAR YTG ATG G	Zhang et al., 2012
thrAB-R	ACR CCC ATC ACC TGY GCR ATC	
thrAMRS-F	TAA TAC GAC TCA CTA TAG GGG CNG GBG GYA TYC CSG TBA TCA AG	modified by Tampakaki from Zhang et al., 2012
thrAMRS-R	GAT TTA GGT GAC ACT ATA GCG YTC GAT NCG RAT SAC YTG SGG	
SMc00019B-F	CAT TCV KCS GAR GGV GCS ATG GGY ATC	Zhang et al., 2012
SMc00019B-R	GCG TGB CCB GCS KCG TTS GAV AGC AT	
SMc00019MRS-F	TAA TAC GAC TCA CTA TAG GGC ADT TCC TBA THG CCA TGC C	modified by Tampakaki from Zhang et al., 2012
SMc00019MRS-R	GCV GGR CAN KTS AGC CAD CCR TT	Zhang et al., 2012
truAB-F	TAA TAC GAC TCA CTA TAG GGC GCT ACA AGC TCA YYA TCG A	modified by Tampakaki from Zhang et al., 2012
truAB-R	CCS ACC ATS GAG CGB ACC TG	Zhang et al., 2012
truAR-F	TGA CCG TSG AAT ATG ACG G	
truAR-R	ACA TCS AGY CGG TCV AGS GT	
truAMS-F	TAA TAC GAC TCA CTA TAG GGC AGG TSG CDC ATS TCG AYC T	modified by Tampakaki from Zhang et al., 2012
truAMS-R	GAD CGB AYC TGG TTR TGM AG	Zhang et al., 2012
gyrB340F-T7	TAA TAC GAC TCA CTA TAG GGT TCG ACC ARA AYT CYT ACA AGG	modified by Tampakaki from Zhang et al., 2012
gyrB1057R-SP6	GAT TTA GGT GAC ACT ATA GCC AAY TTR TCC TTG GTC TGC G	
gyrB-F	ACC GGT CTG CAY CAC CTC GT	Spilker et al., 2009
gyrB-R	YTC GTT GWA RCT GTC GTT CCA CTG C	
recA6F	CGK CTS GTA GAG GAY AAA TCG GTG GA	Gaunt et al., 2001
recA555R	CGR ATC TGG TTG ATG AAG ATC ACC AT	
atpD273F	SCT GGG SCG YAT CMT GAA CGT	Gaunt et al., 2001
atpD-294F	TAA TAC GAC TCA CTA TAG GGA TCG GCG AGC CGG TCG ACG A	modified from Gaunt et al., 2001
atpD771R	GCC GAC ACT TCC GAA CCN GCC TG	Gaunt et al., 2001
nodA-1	TGC RGT GGA ARN TRN NCT GGG AAA	Haukka et al., 1998
nodA-2	GGN CCG TCR TCR AAW GTC ARG TA	
nodCF	AYG THG TYG AYG ACG GTT C	Laguerre et al., 2001
nodCFu	AYG THG TYG AYG ACG GIT C	
nodCI	CGY GAC AGC CAN TCK CTA TTG	

master mix (MyTaq HS Mix, 2 x of Bioline), 1 µl of each forward and reverse primer, and 5.5 µl of DNA template, with the final volume of 15 µl. Amplified samples were sequenced in Stabvida, Portugal. Nucleotide sequences were corrected using BioEdit software, and homology searches were performed on the National Center for Biotechnology Information (NCBI) server using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

Bacteria B1, B2, and B3 were identified as *Rhizobium* sp., *Bradyrhizobium elkanii*, and *Bradyrhizobium* sp., respectively, and the obtained sequences for 16S ribosomal RNA region were deposited in Genbank database with the accession numbers MH938299-MH938301.

For the inoculum preparation, each type of bacteria was grown on six plates of Yeast Mannitol Agar media (1 g L^{-1} of yeast extract, 10 g L^{-1} of mannitol, 0.5 g L^{-1} K_2HPO_4,0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} NaCl, and 15 g L^{-1} agar) supplemented with 0.1 g L^{-1} bromothymol blue. After 3 to 5 days of growing, bacterial inoculant was suspended in sterilised 0.8 % NaCl and then transferred to a sterilised mix of peat and vermiculite (1:1).

The AMF isolate *Claroideoglomus claroideum* BEG210 was grown for eight months in a multi-spore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Zea mays* L. as the host plant.

2.2 Plant culture and experimental design

Cowpea seeds were surface-sterilised with 0.5 % (v/v) sodium hypochlorite (NaClO) for 20 minutes, followed by serial washes with sterilised distilled water. Seeds from cultivar *Fradel*, the only cowpea cultivar registered at the Portuguese National Catalog for commercial use (CNV, 2019), were used. After germination, three seedlings of similar size were kept in each plastic pot (6 litres), containing a mixture of soil, vermiculite, sand and, peat (1:1:1:1, w/w). Non-sterilised soil was used in this work. Chemical analyses of soil mixture revealed the

following values: 8.10 % organic matter, pH (1:2.5 w/v water) 5.0, 51 mg kg^{-1} P, and 132 mg kg^{-1} P (method of Égner-Riehm). Each pot was inoculated with approximately 1 g of mix with the selected bacteria or AMF inoculant, according to the different treatments. All pots from the non-bacterial treatments received the same amount of autoclaved peat and vermiculite and sterilised 0.8 % NaCl, and every pot from non-mycorrhizal treatments received the same amount of AMF inoculum autoclaved twice (121 °C, for 30 minutes) on two consecutive days.

The study was conducted in a greenhouse at the University of Trás-os-Montes e Alto Douro, Vila Real, Portugal, during the growing season of cowpea (May to September 2015) under natural conditions of light, temperature, and humidity. Pots were occasionally rotated to different places to minimise the effect of the location in the greenhouse.

For each treatment, twelve pots were prepared and distributed equally for the two water regimes used in the experiment (25 % and 75 % of field water capacity (FC)), in a total of six pots (biological replicates) per treatment and water regime. The FC of the soil in the pots was determined according to Grewal et al. (1990). The water regime of 25 % FC was used to simulate the drought stress, and 75 % FC was used to simulate well-watered plants. After inoculation and during four weeks, all pots were kept at 75 % FC by weighting and watering the pots every two days. The drought stress was initiated four weeks after plant emergence, and it lasted two months until the flowering stage. During this period, the plants were weighed and watered accordingly in order to ensure the amount of required water.

2.3 Nodule number and biomass and assessment of AMF colonisation

After a growth period of three months, plants were harvested at full maturation stage, and the number and weight of root nodules were determined.

After counting and weighing the nodules, root systems were used for the estimation of the extent of root colonisation by AMF. For this purpose, roots were cleared in potassium hydroxide (KOH) 2.5 %, at 80 °C for 40 minutes, followed by rinsing with water. Roots were immersed in a staining solution containing 5 % blue ink in vinegar and kept at 80 °C for 5 minutes (Vierheilig et al., 1998). After washing away the staining solution, roots were de-stained with tap water containing some drops of vinegar and examined under a compound microscope for quantitative colonisation assessment by the magnified-intersection method according to McGonigle et al. (1990).

2.4 Biomass production, seed yield, and crude protein determination

At harvest, shoots and roots were separated for the evaluation of dry weight. The number of seeds and the weight of 100 seeds were also determined.

Dry samples were analysed for ash (942.05) and for total N (954.01) as Kjeldahl N following the methods of the Association of Official Analytical Chemists (AOAC). Total nitrogen was converted to crude protein using the formula N x 6.25.

2.5 Statistical analysis

Statistical analysis was performed using Software SPSS V.25 (SPSS-IBM, Orchard Road-Armonk, New York, NY). Statistical differences were evaluated by one-way and two-way analysis of variance (ANOVA), followed by the post-hoc Duncan's multiple range test ($P<0.05$), to establish treatments and water regime effects. One-way ANOVA was also performed to establish treatment effect within each water regime.

3 Results

3.1 Cowpea growth

Taking into account the single application of beneficial microorganisms, a significant increase was observed in the shoot weight (*Figure 1A*) of plants under drought stress (25 % of FC) and inoculated with *B. elkanii* (B2), *Bradyrhizobium* sp. (B3), and AMF comparing to the control (1.77, 1.96, and 2.06-fold increase, respectively). Under this water regime, plants single-inoculated with the bacteria B2 and B3 also presented significantly higher shoot weight than plants co-inoculated with the respective bacteria and fungi (B2+AMF and B3+AMF).

No effect was observed in the shoot weight after co-inoculation with rhizobial bacteria and AMF. On the other hand, comparisons between water regimes showed that, with the exception of a single inoculation with B2 that presented similar shoot weight in both water regimes, all of the other treatments resulted in higher shoot weight in well-watered plants (75 % of FC) than in plants under drought stress (25 % of FC). In fact, shoot weight was affected by the water regime ($P<0.001$) and the interaction between the treatment and the water regime ($P<0.001$).

Similarly, root weight was also affected by the water regime ($P<0.001$) and the interaction between the treatment and the water regime ($P<0.05$). Root weight (*Figure 1B*) of well-watered plants (75 % of FC) was not affected by microbial inoculation (either with single or in combination). However, under drought stress (25 % of FC), simple inoculation with fungi led to a 1.69-fold increase in root weight when compared with control cowpea plants. In general, this parameter was higher in well-watered plants (75 % of FC) than in plants under drought stress (25 % of FC), with the exception of plants inoculated with AMF, which presented similar root weight in both water regimes.

3.2 Cowpea seed yield

The number of seeds was affected by the water regime ($P<0.001$) and the interaction between the treatment and the water regime ($P<0.05$). The number of seeds (*Figure 2A*) of well-watered plants (75 % of FC) was positively affected by a single inoculation with AMF in comparison to the control group, with 1.53-fold increase. There was no effect of co-inoculations in both water regimes. In general, this parameter was higher in well-watered plants (75 % of FC) than in plants under drought stress (25 % of FC), with the exception of plants co-inoculated with B2 and AMF. The weight of 100 seeds was affected by the treatment ($P<0.001$) and the water regime ($P<0.05$). Although no significant differences were

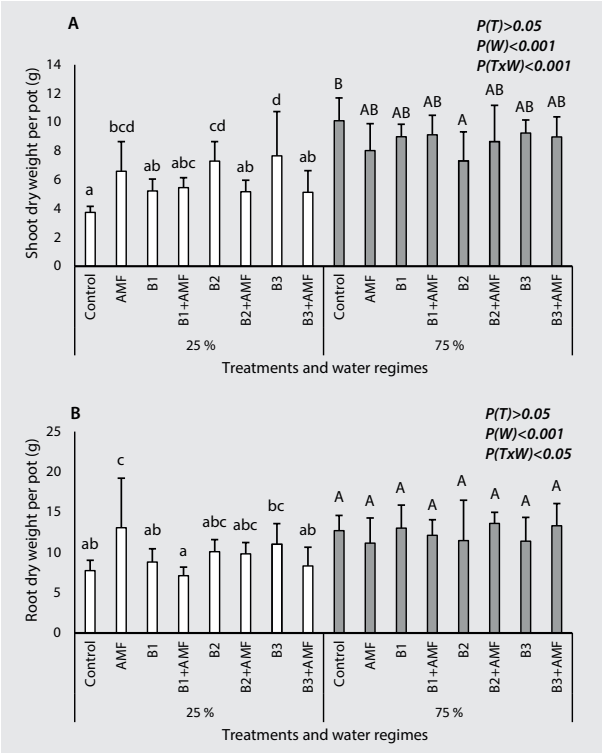

FIGURE 1
Shoot dry weight (A) and root dry weight (B) of cowpea plants uninoculated (control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. (B1), *Bradyrhizobium elkanii* (B2), and *Bradyrhizobium* sp. (B3)), a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF, and B3+AMF) subjected to two different water regimes (25 and 75 % of field water capacity). Capped lines indicate standard deviations. Different lowercase letters indicate significant differences (P<0.05) among treatments of plants under drought stress (25% of field capacity), and uppercase letters indicate significant differences (P<0.05) among treatments of well-watered plants (75 % of field capacity), according to Duncan's test.

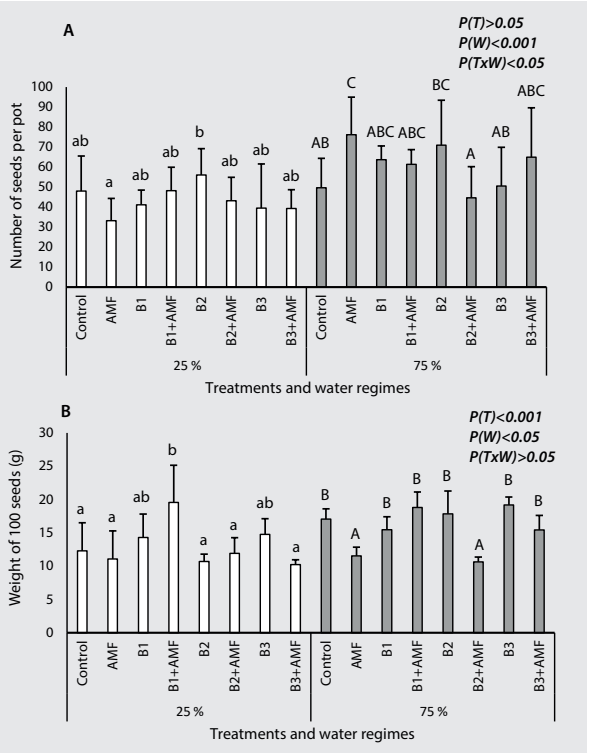

FIGURE 2
The number of seeds (A) and the weight of 100 seeds (B) of cowpea plants uninoculated (control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. (B1), *Bradyrhizobium elkanii* (B2), and *Bradyrhizobium* sp. (B3)), a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF, and B3+AMF) subjected to two different water regimes (25 and 75 % of field water capacity). Capped lines indicate standard deviations. Different lowercase letters indicate significant differences (P<0.05) among treatments of plants under drought stress (25 % of field capacity), and uppercase letters indicate significant differences (P<0.05) among treatments of well-watered plants (75 % of field capacity), according to Duncan's test.

observed by single inoculations in the weight of 100 seeds (*Figure 2B*), the co-inoculation of plants under drought stress (25 % of FC) with B1 and AMF presented significantly heavier seeds than control (1.59-fold increase). In well-watered plants (75 % of FC), single inoculation with fungi and co-inoculation with B2 and fungi significantly decreased the weight of seeds comparing with all the other treatments. In general, seeds were slightly heavier in well-watered plants (75 % of FC) than in plants under drought (25 % of FC).

3.3 Cowpea seed crude protein

Crude protein content was affected by the treatment (P<0.001), the water regime (P<0.001), and the interaction between the treatment and the water regime (P<0.001).

All plants under drought stress (25 % of FC) and co-inoculated with one bacteria and fungi presented significantly higher (P<0.05) crude protein content in the seeds (*Figure 3*), with a 1.2, 1.3 and, 1.3-fold increase following the co-inoculation with B1 and AMF, B2 and AMF, and B3 and AMF, respectively, when compared to the control. A positive effect was observed by the addition of AMF to B2 and B3 since plants co-inoculated with one of these bacteria and fungi presented significantly higher crude protein in the seeds than plants single-inoculated with either each bacteria or with each fungi. In well-watered plants (75 % of FC), crude protein content in the seeds was significantly higher in plants single-inoculated with fungi and with B2 than in plants co-inoculated with both microorganisms together, with a 1.29-fold increase for each. Comparing single inoculation with all the bacteria, B1 and B2 presented significantly higher crude protein in the seeds than single inoculation with B3 (1.22-fold increase for each).

Taking in account the crude protein yield per pot (*Figure 4*), calculated by taking into account the number of seeds and their weight and the crude protein percentage per treatment

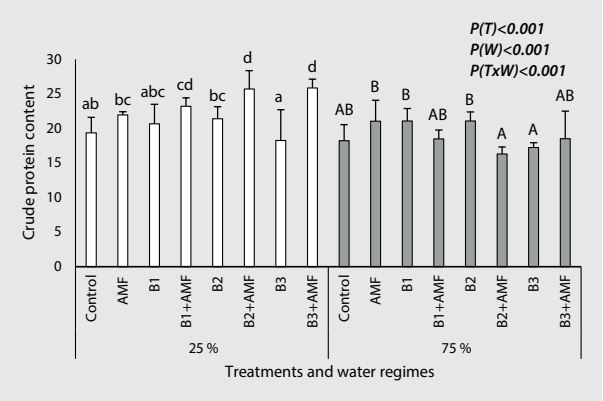

FIGURE 3
Crude protein content in the grains of cowpea plants uninoculated (ontrol) and inoculated with three rhizobial bacteria (*Rhizobium* sp. (B1), *Bradyrhizobium elkanii* (B2), and *Bradyrhizobium* sp. (B3)), a mixture of arbuscular mycorrhizal fungi (AMF), and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF, and B3+AMF) subjected to two different water regimes (25 and 75 % of field water capacity). Capped lines indicate standard deviations. Different lowercase letters indicate significant differences (P<0.05) among treatments of plants under drought stress (25 % of field capacity), and uppercase letters indicate significant differences (P<0.05) among treatments of well-watered plants (75 % of field capacity), according to Duncan's test.

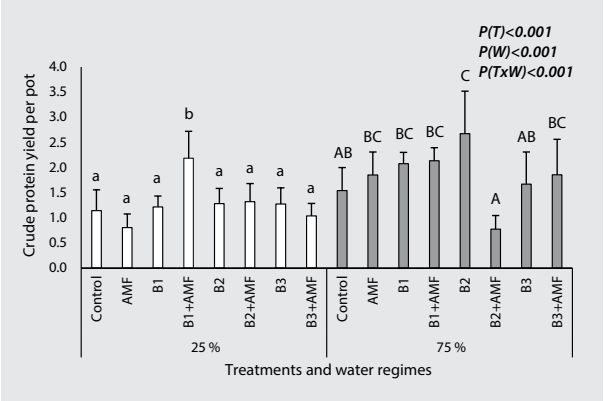

FIGURE 4
Crude protein yield per pot of cowpea plants uninoculated (control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. 32–B1, *Bradyrhizobium elkanii* 57–B2 and *Bradyrhizobium* sp. 63–B3), a mixture of arbuscular mycorrhizal fungi (AMF) and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF and B3+AMF) subjected to two different water regimes (25 and 75 % of field water capacity). Capped lines are standard deviations. Different lowercase letters indicate significant differences (P<0.05) among treatments, within plants under drought stress (25 % of field capacity) and uppercase letters indicate significant differences (P<0.05) among treatments, within well-watered plants (75 % of field capacity), according to Duncan´s test.

under water stress, only plants co-inoculated with B1 and AMF showed significantly higher crude protein yield than the control plants. On the other hand, the well-watered plants inoculated with B2 showed a significantly higher crude protein yield than control plants, plants co-inoculated with the same bacteria and AMF, and plants single-inoculated with the bacteria B3. Similarly, to crude protein content in the grain, crude protein yield per pot was also affected by the treatment (P<0.001), the water regime (P<0.001), and the interaction between the treatment and the water regime (P<0.001).

3.4 Microbial performance

The number of nodules was only affected by the treatment (P<0.05). Although a higher number of nodules (*Figure 5A*) was observed in all inoculated plants under drought stress (25 % of FC), a significant increase was only observed in plants inoculated with B3 when compared to control plants. On the other hand, in well-watered plants (75 % of FC), the number of nodules was positively affected by single inoculation with B2 and B3 and co-inoculation with B1 or B3 and fungi in comparison to the control and plants inoculated only with fungi. A positive correlation was observed between the number and weight of nodules (r=0.444).

The weight of nodules was affected by the treatment (P<0.05), the water regime (P<0.001), and the interaction between both (P<0.05). Well-watered plants (75 % of FC) single- and co-inoculated with each bacteria and AMF presented significantly heavier nodules (*Figure 5B*) than control and plants single inoculated with AMF. Despite the similar number of nodules observed in both water regimes, they were heavier in well-watered plants (75 % owf FC) in all the performed treatments.

Under drought stress (25 % of FC), mycorrhizal colonisation rate (*Figure 5C*) was positively affected by single inoculation with fungi and co-inoculation with *Bradyrhizobium* sp. B3 and AMF, with a 1,41 and 1,44-fold increase compared to control, respectively. Although no significant differences were observed, co-inoculation with bacteria *Rhizobium* sp. B1 or B. elkanii B2 and AMF also increased the mycorrhizal colonisation of plants under drought stress (25 % of FC). In well-watered plants (75 % of FC), co-inoculation with *B. elkanii* B2 and AMF was the unique treatment that increased significantly mycorrhizal colonisation rate comparing with control, with a 1.47-fold increase. Mycorrhization rate followed the same profile within each water regime. Indeed, this parameter was only affected by the treatment (P<0.05).

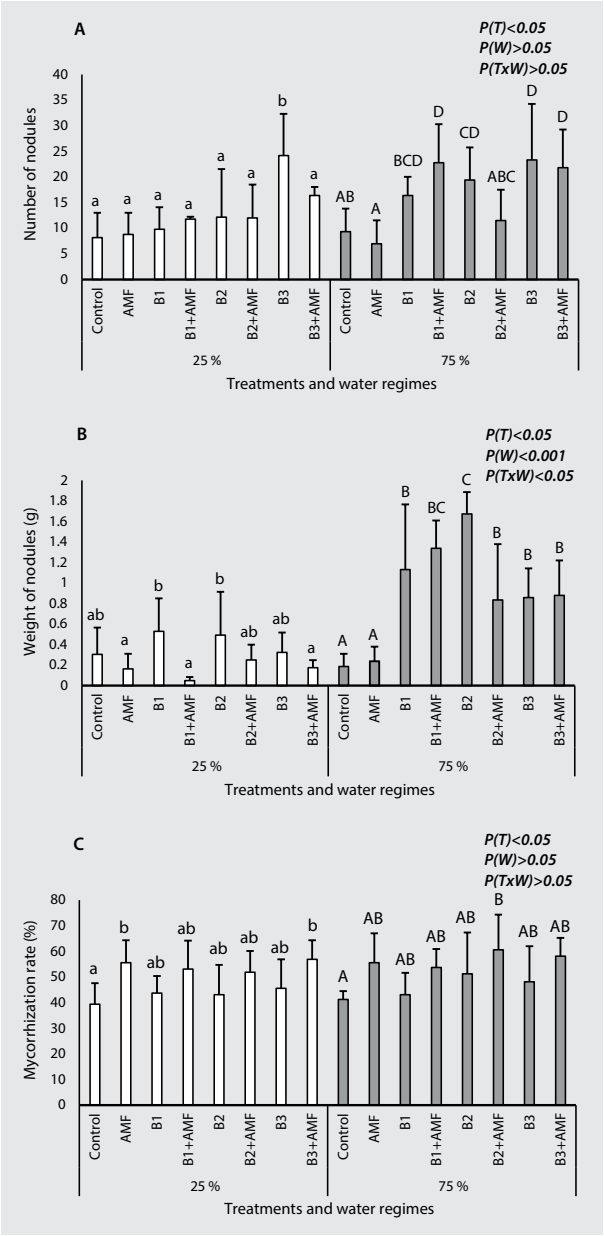

FIGURE 5
Number of nodules (A), weight of nodules (B) and mycorrhization rate (C) of cowpea plants uninoculated (control) and inoculated with three rhizobial bacteria (*Rhizobium* sp. 32–B1, *Bradyrhizobium elkanii* 57–B2 and *Bradyrhizobium* sp. 63–B3), a mixture of arbuscular mycorrhizal fungi (AMF) and co-inoculated with each bacteria and AMF (B1+AMF, B2+AMF and B3+AMF) subjected to two different water regimes (25 and 75 % of field water capacity). Capped lines are standard deviations. Different lowercase letters indicate significant differences (P<0.05) among treatments, within plants under drought stress (25 % of field capacity) and uppercase letters indicate significant differences (P <0.05) among treatments, within well-watered plants (75 % of field capacity), according to Duncan´s test

4 Discussion

Although cowpea has been referred to as a well-adapted plant to abiotic stress, drought is one of the main concerns in its production. Thus, inoculation with selected rhizobial bacteria and AMF has great potential to reduce the impact of water scarcity (Oliveira et al., 2017). Though, the selection of appropriate combinations of specific AMF and rhizobia is very important to improve the yield of cowpea since the response of a legume host to a given set of AMF-*Rhizobium* partners may or may not be favourable for plant growth depending on the interaction of symbionts (Xavier and Germida, 2003). In fact, Ahmad (1995) demonstrated that symbiotic effectiveness depends on a combination of AMF species, *Rhizobium strain*, and also the host plant.

In our work, the inoculation and co-inoculation with the studied microorganisms influenced the plant performance mainly under drought stress. In well-watered plants, the beneficial effects of the inoculation were less evident. This could be due to the presence of other native bacteria and fungi in the soil that also interact with plants, giving them the advantages of symbiosis, even in control plants. However, some differences between control and inoculated plants under drought stress could be observed, suggesting that the native microorganisms present in the soil were not so resistant to drought as the inoculated strains. As shown in other studies, drought, among other stresses, affects the ability to grow and even the basic survival of native microorganisms (Haruta and Kanno, 2015; Goufo et al., 2017).

In general, in plants under drought stress, single inoculation with the studied microorganisms did not improve their responses; however, when both microorganisms were inoculated together, an improvement in the general plant performance was observed. This can be due to the simultaneous improvement in the nitrogen fixation ensured by the bacteria (Hardarson and Atkins, 2003) and the improvement in the uptake of water and other minerals ensured by the fungi (Nadeem et al., 2014). According to previous studies, in general, co-inoculation with rhizobial bacteria and AMF (tripartite symbiosis) improves the water and nutritional status of plants on a larger scale than single inoculation with one microorganism. This can be explained by the fact that nodulation process by rhizobia requires a high amount of P and therefore, the association with AMF helps in the development and function of symbiotic nodules (Ribet and Drevon, 1996). As described in some studies, this symbiosis ameliorates plant photosynthetic efficiency (Jia et al., 2004; Kaschuk et al., 2009) and consequently increases photoassimilate production, which can be used by the plants to improve their growth, productivity, and/or quality. Indeed, the impact that the microbial symbionts have on photosynthetic rates appears to be mediated by their effects on the plant N:P ratio (Jia et al., 2004).

In the present study, co-inoculation did not affect the growth of plants, taking in account the absence of significant differences in the shoot and root weight between control and co-inoculated plants. In line with this, Diallo et al. (2001) found no benefits in plant root and shoot biomass with AMF

inoculation. The authors attributed this lack of effect to the fact that the production of fungal mycelium is much more cost-effective in terms of organic carbon (C) than the production of equivalent root length. Consequently, plants adjust belowground C allocation contributing to the formation of a shorter mycorrhizal root system, relying on the fungal mycelium for nutrient uptake (Smith et al., 2000).

Moreover, in the present study, co-inoculations also did not influence the productivity parameters since the number and weight of seeds were not affected, except for the mix of B1 and AMF that resulted in heavier seeds than the control.

We observed a significant increase in the crude protein content (derived from the nitrogen level by the Kjeldahl method) in the seeds of plants under drought stress (25 % of FC) and co-inoculated with one bacteria and AMF in comparison to the control plants, which suggests that these plants have the ability to mobilise photoassimilates to the seed, which is a sink of protein production, in detriment of growth and yield. Despite the increase in nitrogen observed in co-inoculated plants under water stress, it is not possible to distinguish between protein nitrogen and non-protein nitrogen with this method; therefore, it cannot be ruled out that this increase occurred in the non-protein fraction of nitrogen.

In a meta-analysis with 12 legume species performed in a previous study, it was also observed that inoculation with rhizobia in the field and with AMF in pots increased seed protein content (Kaschuk et al., 2010). In fact, according to Dubova et al. (2015), protein accumulation in the seeds depends not only on plant biosynthetic activity but can also be affected by microbial symbionts. From the results of this study, it can be concluded that the microorganisms used in this study were efficient and competitive under drought stress (25 % of FC), benefiting the plants to a greater extent than the native microbiota present in the soil (control plants). In previous studies, it was also shown that these beneficial microorganisms can increase plant resistance to high temperatures and water deficit and that their application can reduce the needs of chemical fertiliser inputs in agriculture (Peoples et al., 1995; Oliveira et al., 2017), as soil microbes are critical for a sustainable functioning of natural and managed ecosystems (Sharma et al., 2018). Additionally to the treatment influence, the crude protein content was also affected by the water regime, being higher in plants under drought stress. This can be explained by the increase in nitrogenous compounds, such as the amino acid proline usually synthesised in large amounts in plants under stress, previously described by da Costa et al. (2011). In fact, proline demonstrates high sensitivity to stress conditions (Ashraf et al., 2011), increasing its concentration by up to 100 times compared to that observed in plants grown under normal conditions (Verbruggen and Hermans, 2008). This increase can occur through de novo synthesis or by inhibiting the oxidation process of proline. The accumulation of proline and other compatible solutes (glycine betaine, trehalose, sucrose, polyamines, mannitol, pinitol and others) in vacuole or cytosol contributes to the maintenance of water balance and the preservation of the integrity of proteins, enzymes, and cell membranes (Marijuan and Bosch, 2013). These solutes also have an osmoprotective function against toxic by-products of metabolism, resulting from water stress. This accumulation is not harmful to cell metabolism and, by increasing the osmotic pressure inside the cells, maintains the water absorption and the turgor pressure of the cells, which allows the continuity of physiological processes (Marijuan and Bosch, 2013). Considerable accumulation of proline is a feature in the response of plants under water stress (Fukutoku and Yamada, 1981; Levy, 1983). Furthermore, water stress induces a net loss of leaf protein since its synthesis is inhibited and its degradation is stimulated, leading to an accumulation of free amino acids (Cooke et al., 1979; Dungey and Davies, 1982). Thus, a relationship between proline accumulation and protein metabolism has been described, since protein may be a source of nitrogen for proline synthesis during water stress. In these conditions, as reported by Fukutoku and Yamada (1984), a loss of leaf protein-^{15}N occurs, which is balanced by a gain in ^{15}N in the free amino acids, namely proline and asparagine.

The use of non-sterilised soil makes this work very useful because we can extrapolate the results obtained in pots to the real conditions in the field. However, it is important to note that the potential of the microorganisms used in this work, especially the fungi, could be underestimated due to the confined space of the pot, which does not allow the maximum development of the root. According to the results obtained in this work, it is possible to extrapolate that the studied bacteria should have the same strategies to cope with stressful conditions, which can be, among others, the formation of cysts and spores, changes in cellular membranes, expression of repair enzymes for damage, synthesis of molecules for relieving stresses (Storz and Hengge, 2011). These strategies make them potentially resistant to drought, which can be used as an improved biotechnological tool for sustainable agriculture in drought situations. Indeed, climate change will seriously impact food security and nutrition, making it crucial to support a transition toward smart and sustainable food systems that take climate into account (FAO, 2008). With this eco-friendly approach, it is possible to increase the nutritional and commercial value of leguminous plants, a cheap and alternative source of protein for human consumption, by increasing their crude protein content without chemical fertiliser applications and genetic improvements.

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