# Growth and nutrition of cowpea (*Vigna unguiculata*) under water deficit as influenced by microbial inoculation via seed coating

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## Abstract

Drought can drastically reduce cowpea [*Vigna unguiculata* (L.) Walp.] biomass and grain yield. The application of plant growth- promoting rhizobacteria and arbuscular mycorrhizal fungi can confer resistance to plants and reduce the effects of environmental stresses, including drought. Seed coating is a technique which allows the application of minor amounts of microbial inocula. Main effects of the factors inoculation and water regime showed that: severe or moderate water deficit had a general negative impact on cowpea plants; total biomass production, seed weight and seed yield were enhanced in plants inoculated with *P. putida*; inoculation of *R. irregularis* significantly increased nitrogen (N) and phosphorus (P) shoot concentrations; and *R. irregularis* enhanced both chlorophyll b and carotenoids contents, particularly under severe water deficit. Plants inoculated with *P. putida* + *R. irregularis* had an increase in shoot P concentration of 85% and 57%, under moderate and severe water deficit, respec-tively. Singly inoculated *P. putida* improved potassium shoot concentration by 25% under moderate water deficit. Overall, in terms of agricultural productivity the inoculation of *P. putida* under water deficit might be promising. Seed coating has the poten-tial to be used as a large-scale delivery system of beneficial microbial inoculants.

**KEYWORDS**

arbuscular mycorrhizal fungi, plant growth-promoting bacteria, seed inoculation

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## 1 | INTRODUCTION

The agriculture sector is facing a real challenge against climate change (Vurukonda, Vardharajula, Shrivastava, & Skz, 2016). With the increase in heat waves, storms, droughts, floods or heavy pre-cipitation, crop productivity and food security are being endangered (Hansen, Sato, & Ruedy, 2012; Sundström et al., 2014). Among these climate change threats, drought is expected to dramatically ham-per plant growth and development for more than 50% of the arable lands by 2050, decreasing crop productivity worldwide (Kasim et al., 2013; Li et al., 2014). From moderate and short to extremely severe and prolonged periods, drought can disturb plant water potential and turgor and thus modify physiological and morphological traits of plants (Rahdari & Hoseini, 2012).

Some beneficial soil microorganisms can help plants overcome problems caused by abiotic stress (Bardi & Malusà, 2012; Bhardwaj, Ansari, Sahoo, & Tuteja, 2014; Egamberdieva & Adesemoye, 2016; Vassilev et al., 2015). The exploitation of plant beneficial microbes, such as plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi for drought stress mitigation in plants, is gaining importance (Li et al., 2014; Nadeem, Ahmad, Zahir, Javaid, & Ashraf, 2014; Vurukonda et al., 2016). Besides their contribution to nutrient acquisition and biocontrol, PGPR can also confer drought tolerance in plants by osmotic adjustment, antioxidant metabolisms and phytohormone modulation (Rubin, van Groenigen, & Hungate, 2017; Vurukonda et al., 2016). AM fungal symbiosis can improve plant antioxidant activity, osmotic regulation, photosynthetic rates and pigments, root water absorption and transport and uptake of nutrients, especially phosphorus (P) (Li et al., 2014; Oliveira, Rocha, Ma, Vosátka, & Freitas, 2016; Oliveira, Ma et al., 2016; Quiroga, Erice, Aroca, Chaumont, & Ruiz-Lozano, 2017).

Grain legumes are important for a variety of reasons, since they are a significant and cheap source of protein, are able to fix N in agricultural ecosystems and can be used for industrial and medicinal purposes (Farooq et al., 2017). Cowpea [*Vigna unguiculata* (L.) Walp.] is an important seed crop legume for human consumption (seeds and pods) and for soil amendment and fertilisation (e.g. green manure and organic material) (Vurukonda et al., 2016). Plant biomass and grain yield of legumes can be seriously hampered by moderate to severe drought stress (Farooq et al., 2017). Inoculation with AM fungi and PGPR has been considered to be a promising strategy to increase plant drought tolerance (Bhardwaj et al., 2014; Dodd & Ruiz-Lozano, 2012). Some studies presented the effects of beneficial microbes on plant under water stress, such as improved grain yield and protein content (Oliveira et al., 2017a,b) increment on nutrient (Ngakou et al., 2007) and water uptake and increased transpiration and photosynthesis rates (Virakornphanich, Masuhara, & Adachi, 1994). Therefore, it is imperative to develop feasible strategies for application of these beneficial microbes in open agricultural fields using minor amounts of inoculum for precision agriculture. Seed coating is a process where exogenous materials are applied to the surface of the seed and can be used for delivering active ingredients, including beneficial microbes (Pedrini, Merritt, Stevens, & Dixon, 2017). This technique intends to use minor amounts of inocula in a more precise application that should be as efficiently as conventional soil inoculation. Seed coating could serve as a powerful tool for large-scale inoculation of beneficial microorganisms (Oliveira, Rocha et al., 2016).

The main goal of the present study was to assess the impact of the application of PGPR and AM fungi via seed coating in cowpea production under drought stress.

## 2 | MATERIALS AND METHODS

### 2.1 | Seeds and soil material

Seeds of cowpea [*Vigna unguiculata* (L.) Walp.] cv. Fradel were used in this study. The soil used in the experiment presented a loam texture with pH (1:2.5 w/v water) 7.1, electrical conductivity 0.045 dS/m, 0.16% organic matter, 0.11 g/kg total N, 3,542 mg/kg extractable (Egner-Riehm) P and 13 mg/kg potassium (K). Previous to use the soil was sieved through a 4-mm mesh and autoclaved twice (121°C for 25 min) on consecutive days.

### 2.2 | Microbial inocula and seed coating

The AM fungus used was *Rhizophagus irregularis* PH5 grown for 8 months in a multispore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Zea mays* L. as host plant. Regarding the seed coating procedure, the *R. irregularis* inoculum was sieved through a 500-μm mesh and mixed with starch/silicon dioxide mixture (coating material) in the proportion of 1:1 (w/w) (the inoculum-coating material mixture was provided by Symbiom Ltd., Czech Republic). *Pseudomonas putida* strain GP was isolated from an agricultural soil in central Portugal used to grow *Lupinus albus* L. and tested positively for indoleacetic acid (IAA) (Brick, Bostock, & Silverstone, 1991), ammonia (Cappuccino & Sherman, 1992) and siderophores production (Schwyn & Neilands, 1987), phosphate solubilisation (Gaur, 1990), N fixation (Dobereiner, Marriel, & Nery, 1976), biofilm formation in the presence of different salt concentrations, 0.5 to 2.5 M (Christensen et al., 1985) and water stress tolerance (Ma, Rajkumar, Zhang, & Freitas, 2016). For the seed coating with bacteria, *P. putida* was grown in LB media for 17 hr at 28–30°C and 150 rpm, centrifuged at 3,500 rpm for 15 min and re-suspended in ringer solution with 1% carboxymethylcellulose (as an adhesive agent). The bacterial suspension at a concentration of $10^8$ colony-forming unit (CFU)/ml was mixed with the coating material (1:1 v/w). Both AM fungus and bacterium were also coated together using the same procedure and proportions (1:1:1 w/v/w) as aforesaid. For seeds coated with *R. irregularis*, the AM fungal propagules per seed estimated by most probable number were 21 (Porter, 1979). Cowpea seeds were coated by the pan coating method (Scott, Hill, & Jessop, 1991) as described by Oliveira, Rocha et al. (2016). Non-inoculated control seeds were coated only with the starch/silicon dioxide mixture.

### 2.3 | Experimental design

This study was conducted in a heated greenhouse (temperature ranging from 18 to 30°C) with an average photoperiod of 12 hr using pots of 2 L disposed in a fully randomised scheme. Each pot received 1 seed. The positions of the pots were periodically swapped to minimise differences caused by their location in the greenhouse. All pots received 50 ml of microbial populations filtrate (Whatman No. 1 filter) from the original non-sterile soil as described by Oliveira, Castro, Dodd, and Vosátka (2006), in order to provide a common soil microbiota for all the treatments. The experimental design involved twelve treatments, resulting from the combination of four inoculation treatments via seed coating [non-inoculated controls (Control); plants inoculated with

*Rhizophagus irregularis* PH5 (RIcoat); *Pseudomonas putida* (PPcoat) and a mix of *R. irregularis* + *P. putida* (MIXcoat)] and three water regimes [no water deficit, 80–75% of water holding capacity (D0); moderate water deficit, 60–55% of water holding capacity (D1); and severe water deficit, 30–25% of water holding capacity (D2)]. Each treatment had six replicates. During the first 3 weeks of plant growth, water was supplied daily to reach 80% of water holding capacity in all treatments. Volumetric soil moisture was measured with a ML2x ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), where changes in the apparent dielectric constant of moist soil allowed measuring the volumetric soil moisture content (Roth, Malicki, & Plagge, 1992; White, Knight, Zegelin, & Topp, 1994). Before starting the experiment, measures were performed to match the water holding capacity of the soil with the volumetric soil moisture. The 100%, 85–80%, 60–55% and 30–25% of soil water holding capacity corresponded to 22, 16, 10–9 and 6–5% volumetric soil moisture, respectively. In order to control water deficit and maintain it at the desire level, the soil water content was measured daily with the ThetaProbe ML2x at the end of the afternoon (5:00–6:00 p.m.) and the amount of water lost was added to each pot. For fertilisation, each plant received 20 ml of modified white mineral solution P2N3 (Gryndler, Vejsadová, & Vančura, 1992) twice a week.

## 2.4 | Gas exchange parameters

The steady-state net photosynthesis A ($P_n$), stomatal conductance (gs), intercellular $CO_2$ concentration (Ci) and transpiration rate (Tr) were determined using a Li-6400 IRGA (LI-COR, Lincoln, NE, USA). A 300 µmol/s flow of non-contaminated air was provided to the leaves using a leaf chamber and mass flow controllers. The analysed leaves were exposed to a saturating photosynthetic photon flux density of 1000 µmol m$^{-2}$ s$^{-1}$, block leaf temperature of 25°C and with the relative humidity of the air within the apparatus ranging between 45 and 55%. In all cases, only mature, fully expanded leaves were selected for measurements from four different plants of each experimental condition. The measurements for gas exchange were recorded between the late morning (9:00–11:00 a.m.) and early afternoon (1:00–3:00 p.m.). The instantaneous water use efficiency (WUE) (µmol $CO_2$ per mmol $H_2O$) was calculated by dividing the values of steady-state net photosynthesis by the transpiration rate ($P_n$/Tr).

## 2.5 | Chlorophylls and carotenoids content

Fresh cowpea leaves (about 0.2 g) were homogenised in chilled N, N-dimethylformamide and stored overnight in the dark at 4°C (Moran & Porath, 1980). The absorptions were measured at 664, 647 and 461 nm using a HACH DR/4000U spectrophotometer (HACH Company, Loveland, CO, USA). Chlorophyll a and chlorophyll b were estimated using the equations of Inskeep and Bloom (1985) and carotenoids using the equation of Chamovitz, Sandmann, and Hirschberg (1993).

## 2.6 | Biomass production, seed yield and nutrients acquisition

At harvest, pods were separated and weighted to determine fresh weights. After recording the weight of pods, seeds were collected and weighted. Shoots and roots were dried for 2 days at 75°C to obtain dry weights. Seed yield was calculated by multiplying the number of pod per plant by the number of seeds per pod and the seed weight mean (Sinha, 1977). After drying, shoots were grinded and digested according to the European Standard EN 13805 (2014). A segmented flow analyser was used for total N evaluation (Skalar Inc. SanPlus, The Netherlands) and inductively coupled plasma optical emission spectrometry (ICP-OES; GBC Quantima, Australia) for total P and K. The ICP-OES operating conditions were as follows: 1,000 W RF power—1,000 W, 15.0 L/min plasma gas flow rate, 1.2 L/min auxiliary gas flow rate, 1.0 L/min carrier gas flow rate, 50 scan/reading, 3 measurement replicates and dual detector.

## 2.7 | Mycorrhizal development

Mycorrhizal colonisation in the roots of cowpea was assessed by microscopic methods. The roots were carefully washed and stained as described in a modified Phillips and Hayman (1970) protocol (Oliveira, Vosátka, Dodd, & Castro, 2005). The percentage of root length colonised (RLC) was evaluated by the grid-line intersect method (Giovannetti & Mosse, 1980) under a stereomicroscope (Leica EZ4 HD, Germany).

## 2.8 | Statistical analysis

Normality and homogeneity of variances were confirmed and data analysed by one-way and two-way analysis of variance (ANOVA) for each dependent variable versus the independent variables (inoculation and water regime). In some cases, transformation was performed before analysis, to normalise skewed distributions before ANOVA. This was the case of data of mycorrhizal colonisation ($x^2$), N shoot concentration (1/x), stomatal conductance ($x^{1/3}$), transpiration rate ($\sqrt{x}$), water use efficiency (1/x) and carotenoids leaf content ($\sqrt{x}$). The main effects of the factors inoculation (Control, PPcoat, RIcoat and MIXcoat), water regime (D0, D1 and D2) and their interaction were analysed. When a significant *F*-value was obtained ($p < 0.05$), treatment means were compared using Duncan's multiple range test. All statistical analyses were performed with the SPSS 25.0.0 software package (IBM SPSS Statistics, USA).

## 3 | RESULTS

## 3.1 | Plant growth, yield and nutrients concentration

Seeds coated with *R. irregularis* inoculum (singly or mix) took approximately 7 days to final emergence from the soil, while those inoculated with bacteria and control took 4 days. Shoots, roots and total dry weights of cowpea were negatively affected by

**TABLE 1** Biomass production and seed yield of *Vigna unguiculata* (L.) Walp. under different inoculation treatments [non-inoculated (Control) *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix *R. irregularis* + *P. putida* (MIXcoat)] and no water deficit (D0), moderate water deficit (D1) and severe water deficit (D2)

| Inoculation | Water regime | Shoot dry weight (g) | Root dry Weight (g) | Total plant dry weight (g) | Root/Shoot ratio | Seed weight (g) | Seed yield (g) |
|---|---|---|---|---|---|---|---|
| Control | D0 | 1.2 ± 0.1 cd | 0.9 ± 0.1 e | 2.0 ± 0.2 g | 0.8 ± 0.0 abc | 0.07 ± 0.0 ab | 0.4 ± 0.1 a |
| | D1 | 0.7 ± 0.0 b | 0.5 ± 0.1 cd | 1.2 ± 0.1 def | 0.7 ± 0.1 ab | 0.10 ± 0.0 ab | 0.5 ± 0.1 a |
| | D2 | 0.4 ± 0.0 a | 0.3 ± 0.1 abc | 0.8 ± 0.1 bc | 0.8 ± 0.1 bc | 0.08 ± 0.0 ab | 0.2 ± 0.1 a |
| PPcoat | D0 | 1.3 ± 0.1 d | 0.9 ± 0.1 e | 2.1 ± 0.1 g | 0.7 ± 0.0 ab | 0.22 ± 0.0 c | 1.0 ± 0.2 c |
| | D1 | 0.8 ± 0.0 b | 0.6 ± 0.1 d | 1.4 ± 0.1 ef | 0.8 ± 0.1 bc | 0.15 ± 0.0 bc | 0.9 ± 0.0 bc |
| | D2 | 0.4 ± 0.0 a | 0.5 ± 0.1 cd | 0.9 ± 0.1 bcd | 1.1 ± 0.2 c | 0.12 ± 0.0 abc | 0.4 ± 0.1 a |
| RIcoat | D0 | 1.1 ± 0.1 c | 0.4 ± 0.0 bcd | 1.5 ± 0.1 f | 0.4 ± 0.0 a | 0.06 ± 0.0 a | 0.3 ± 0.2 a |
| | D1 | 0.6 ± 0.1 b | 0.2 ± 0.0 ab | 0.8 ± 0.1 bc | 0.4 ± 0.1 ab | 0.08 ± 0.0 ab | 0.4 ± 0.1 a |
| | D2 | 0.3 ± 0.1 a | 0.1 ± 0.0 a | 0.4 ± 0.1 a | 0.6 ± 0.2 ab | 0.07 ± 0.0 ab | 0.2 ± 0.1 a |
| MIXcoat | D0 | 1.0 ± 0.0 c | 0.4 ± 0.1 bcd | 1.4 ± 0.1 ef | 0.4 ± 0.1 a | 0.05 ± 0.0 a | 0.4 ± 0.1 a |
| | D1 | 0.8 ± 0.1 b | 0.3 ± 0.1 abc | 1.1 ± 0.1 cde | 0.5 ± 0.1 ab | 0.11 ± 0.0 ab | 0.5 ± 0.1 ab |
| | D2 | 0.4 ± 0.1 a | 0.3 ± 0.1 abc | 0.7 ± 0.2 ab | 0.6 ± 0.2 ab | 0.05 ± 0.0 a | 0.2 ± 0.1 a |

*Note*. Means (±1 SE) followed by letters that indicate significant differences between treatments according to Duncan's multiple range test at *p* < 0.05.

water regime, especially by severe water deficit (Tables 1 and 2). In general, the roots and total biomass were significantly affected by the inoculation treatments, positively by *P. putida* and negatively by *R. irregularis* (Table 2). There was no significant effect of inoculation on shoot dry weight under the different water regimes when compared with control (Table 1). Overall, PPcoat treatment had a significant enhancement effect in total plant dry weight, seed weight and seed yield of cowpea (Table 2). Under moderate water deficit, plants inoculated with *P. putida* presented a significant increase in seed yield (Table 1). RIcoat treatments presented lower root biomass when compared with the PPcoat and control

treatments and consequently inferior values of root biomass over shoot (Table 2). The seed yield was significantly impaired by the severe water deficit (Table 2). Inoculation and water regime had significant main effects on cowpea shoot nutrients concentration (Table 2). In general, the presence of *R. irregularis* increased N and P shoot concentrations when compared with control (Table 2). Yet, the interaction between water regime and inoculation showed only significant increase of N in plants under no water deficit (Figure 1), with an increase of 38% in shoot concentration. Comparing with the corresponding control, P shoot concentration was significantly increased in the treatments of RIcoat D0, Mix D1

**TABLE 2** Main effects of the factors inoculation and water regime and two-way ANOVA *F*-values and significances for biomass production, seed yield and nutrient shoot concentration of *Vigna unguiculata* (L.) Walp

| Main Effects | | Shoot dry weight (g) | Root dry Weight (g) | Total plant dry weight (g) | Root/Shoot ratio | Seed weight (g) | Seed yield (g) | N (g kg$^{-1}$) | P (g kg$^{-1}$) | K (g kg$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| Inoculation (I) | Control | 0.7 ab | 0.6 b | 1.3 b | 0.8 b | 0.1 a | 0.4 a | 12.3 b | 1.5 a | 25.3 a |
| | PPcoat | 0.8 b | 0.6 b | 1.5 c | 0.9 b | 0.2 b | 0.7 b | 9.3 a | 1.4 a | 27.3 a |
| | RIcoat | 0.7 a | 0.3 a | 0.9 a | 0.5 a | 0.1 a | 0.3 a | 14.7 c | 2.0 b | 26.1 a |
| | MIXcoat | 0.7 a | 0.3 a | 1.0 a | 0.5 a | 0.1 a | 0.4 a | 15.4 c | 2.3 c | 26.4 a |
| Water regime (WR) | D0 | 1.1 z | 0.6 z | 1.7 z | 0.6 x | 0.1 x | 0.5 y | 12.2 x | 1.8 x | 22.6 x |
| | D1 | 0.7 y | 0.4 y | 1.2 y | 0.6 x | 0.1 x | 0.6 y | 12.5 x | 1.6 x | 27.0 y |
| | D2 | 0.4 x | 0.3 x | 0.7 x | 0.8 y | 0.1 x | 0.2 x | 13.1 x | 1.8 x | 30.6 z |
| **Two-way ANOVA *F*-values and significances** | | | | | | | | | | |
| Inoculation (I) | | 3.8* | 19.9*** | 16.0*** | 8.3*** | 8.0*** | 9.2*** | 17.9*** | 15.0*** | 3.3* |
| Water regime (WR) | | 140.5*** | 24.0*** | 85.5*** | 3.8* | 1.4 ns | 8.9*** | 0.9 ns | 2.9 ns | 41.0*** |
| I × WR | | 1.2 ns | 1.5 ns | 1.5 ns | 0.5 ns | 1.1 ns | 1.1 ns | 0.9 ns | 3.8** | 1.0 ns |

*Notes*. Letters indicate significant differences according to Duncan's Multiple Range test. *, **, ***significant effect at the level of *p* < 0.05, *p* < 0.01 and *p* < 0.001, respectively; ns, non-significant effect. Control, non-inoculated; PPcoat, *Pseudomonas putida*; RIcoat, *Rhizophagus irregularis*; MIXcoat, mix of *R. irregularis* and *P. putida*; D0, no water deficit; D1, moderate water deficit; D2, severe water deficit.
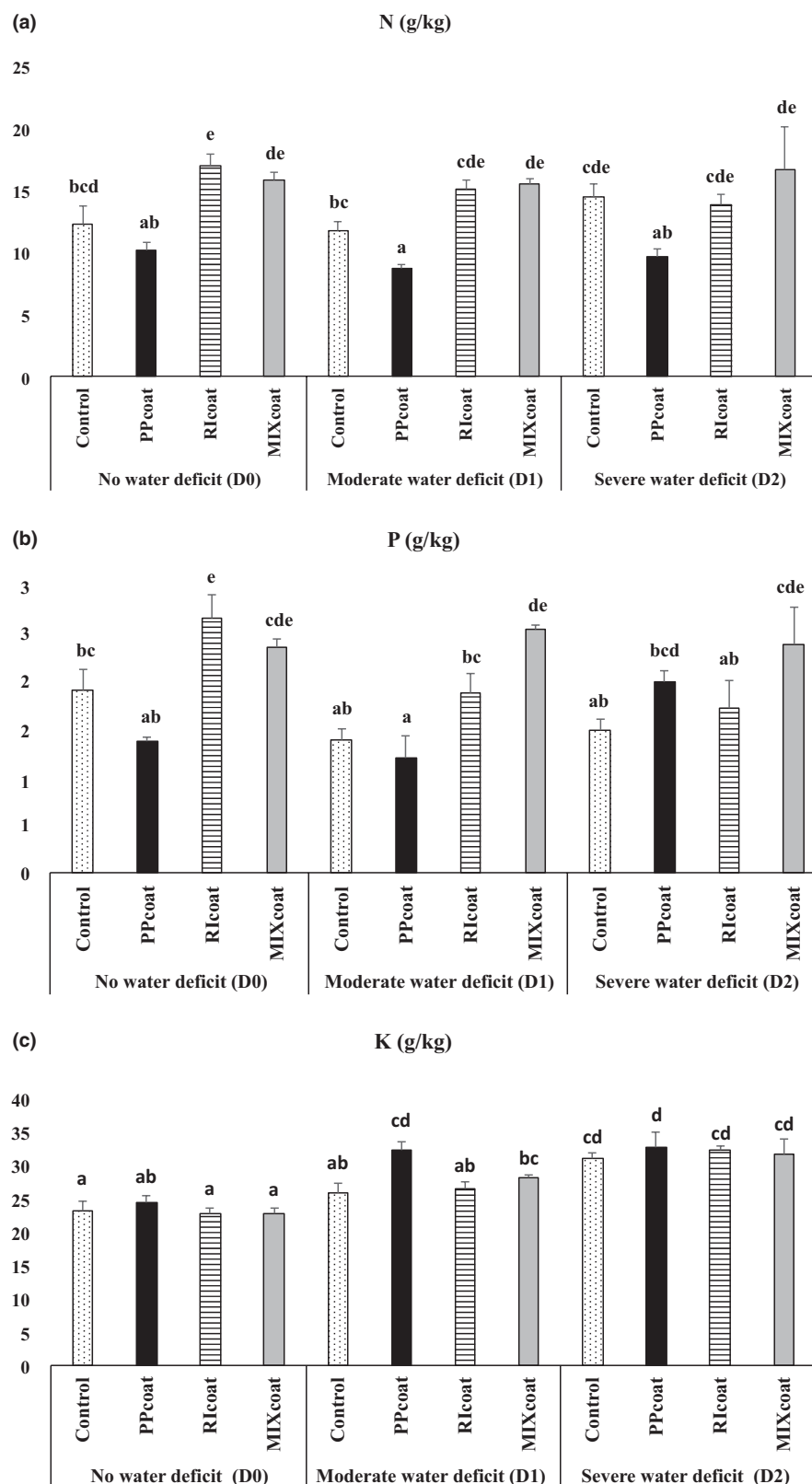
**(a)** N (g/kg)

**(b)** P (g/kg)

**(c)** K (g/kg)

**FIGURE 1** Effects of different inoculation treatments [non-inoculated (Control), with *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix *R. irregularis* + *P. putida* (MIXcoat)] and water regimes on N (a), P (b) and K (c) shoot concentration in *Vigna unguiculata* (L.) Walp. Values are means ± 1 *SE* and letters indicate significant differences (*p* < 0.05) according to Duncan's multiple range test

and D2 by 39%, 85% and 57%, respectively. The accumulation of K in cowpea shoots was mainly affected by the water regime, being increased by moderate and severe water deficits (Table 2). Singly inoculated *P. putida* improved K shoot concentration by 25% under moderate water deficit (Figure 1).

## 3.2 | Mycorrhizal root colonisation

Plants without *R. irregularis* inoculation (control and *P. putida* inoculation) had no root mycorrhizal colonisation. Treatments where *R. irregularis* was inoculated had root colonisation that varied with water
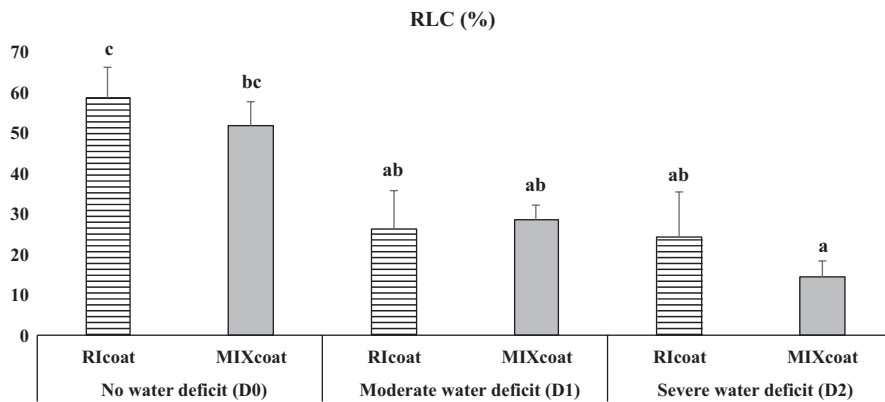
**RLC (%)**

**FIGURE 2** Percentage of root length colonisation (% RLC) in the roots of *Vigna unguiculata* (L.) Walp. inoculated with *Rhizophagus irregularis* (RIcoat) or the mix *R. irregularis* + *Pseudomonas Putida* (MIXcoat) via seed coating under different water regimes. Values are means ± 1 *SE* and letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test

regime (Figure 2). Both moderated and severe water restrictions negatively affected the presence of *R. irregularis* in the roots. When no water deficit was imposed, the percentage of RLC was higher than 50%. Inoculation with *P. putida* did not have a significant impact on root colonisation by *R. irregularis*.

## 3.3 | Leaf parameters

Both water regime and microbial inoculation influenced cowpea leaf gas exchange parameters (Figure 3a–e and Table 3). Severe water deficit negatively affected the gas exchange parameters in both non-inoculated and inoculated treatments (Figure 3 and Table 3). The presence of mycorrhiza singly and in combination with *P. putida* significantly enhanced $P_n$ when no water deficit was imposed (Figure 3a). Also, under no water deficit, the treatment MIXcoat presented higher values of gs and Tr (Figure 3b,d). Intercellular $CO_2$ concentration was adversely impacted by severe water deficit (Table 3). Plants singly inoculated with *P. putida* showed the lower values of $P_n$, gs and Tr in all water regimes. WUE (Figure 3e) was significantly higher in plants under severe water deficit and in the presence of microbial inoculants.

Chlorophyll and carotenoids varied according to microbial inoculation and water regime (Figure 4 and Table 3). Plants under moderate and severe water deficit had significantly lower concentrations the leaf pigments, irrespective of microbial inoculation (Table 3). In general, plants inoculated with *R. irregularis* enhanced both chlorophylls and carotenoids contents, even under severe water deficit, when compared with PPcoat and control treatments (Table 3).

## 4 | DISCUSSION

The frequency and intensity of drought can dramatically decrease plant biomass and grain yield (Farooq et al., 2017). Ahmed and Suliman (2010) showed cowpea yield reductions of 34–66% under water stress during the reproductive stage of crop development, and Akyeampong (1985) revealed 29% of declination during pod filling. Our results showed that both moderate and severe water deficit decreased shoots, roots and total biomass and that severe water deficit significantly reduced seed yield (Table 2). The negative

variation on gas exchange parameters such as photosynthesis, stomatal conductance or transpiration imposed by water stress can hamper plant growth (Farooq et al., 2008; Li et al., 2014), which was shown in our results (Table 1 and Figure 3). Equally, water deficit significantly decreased the content of chlorophyll a, chlorophyll b and carotenoids in cowpea leaves (Table 3). Photosynthetic pigments are important for plants to harvest light and produce reducing powers. Carotenoids play a key role in plant antioxidant defence system by quenching singlet oxygen and peroxyl radicals, protecting the photosynthetic tissue from oxidative damage (Jaleel et al., 2009).

Legume crops are able to establish symbiotic interactions with microbes (e.g. PGPR and AM fungi), which help them cope with unfavourable environmental conditions such as drought (Oliveira et al., 2017a,b; Zahran, 2010).

Cowpea is considered to be highly mycotrophic (Molla & Solaiman, 2009) which leads to enhancement of below and above ground biomass, nutrients accumulation, protein content and grain yield under different water regimes (Kwapata & Hall, 1985; Oliveira et al., 2017a; Oruru, Njeru, Pasquet, & Runo, 2018; Rabie, Aboul-Nasr, & Al-Humiany, 2005). However, our results showed that association between AM fungi and cowpea did not result in increased plant growth or seed yield (Tables 1 and 2). Moreover, for root weight and root/shoot ratio the values of plants inoculated with *R. irregularis* were lower than control. This can be related 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 (Table 2). Consequently, plants adjust belowground C allocation contributing to the formation of a shorter mycorrhizal root system (Jacobsen, Smith, & Smith, 2002), relying on the fungal mycelium for nutrient uptake (Smith, 2000). In fact, there was a significant enhancement in shoot nutrient content (Table 2), particularly N and P, which has also been described in other studies with inoculated cowpea (Boby, Balakrishna, & Bagyaraj, 2008; Oruru et al., 2018; Sanginga, Lyasse, & Singh, 2000; Yaseen, Burni, & Hussain, 2011). Still, this enhancement in nutrient content was not enough to result in greater yields, fact perhaps associated with the sink of carbohydrates of the fungal mycelium that the plant could not allocate to seed development and filling. Also, the observed delay on seedling emergence of plants inoculated with AM fungi might have a negative influence on cowpea yield or even adaptation to the water deficit. Faster germination
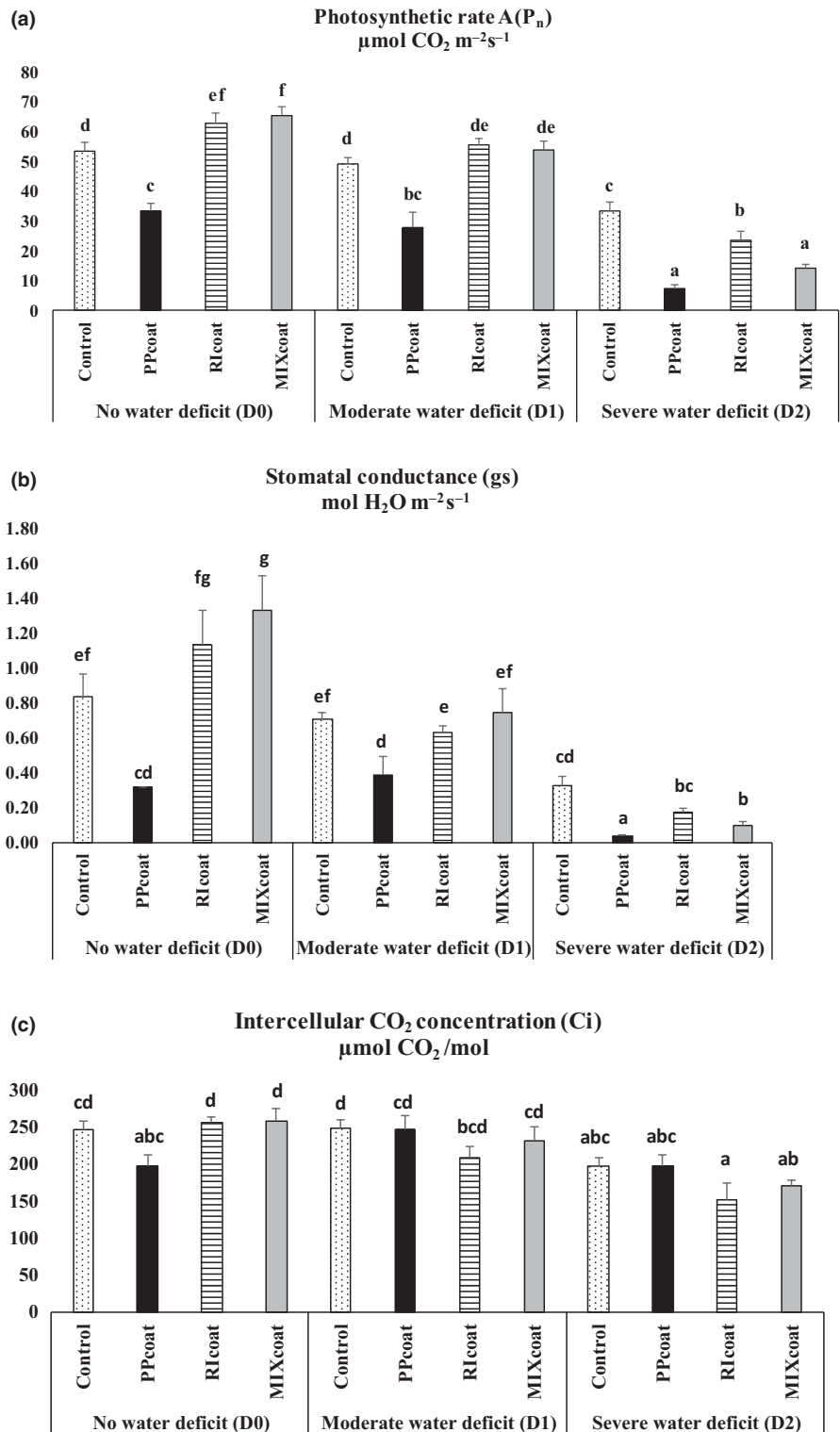
**(a)**

### Photosynthetic rate A($P_n$) $\mu$mol $CO_2$ m$^{-2}$s$^{-1}$



**(b)**

### Stomatal conductance (gs) mol $H_2O$ m$^{-2}$s$^{-1}$



**(c)**

### Intercellular $CO_2$ concentration (Ci) $\mu$mol $CO_2$ /mol



**FIGURE 3** Effects of microbial inoculation [non-inoculated (Control), *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix of *R. irregularis* + *P. putida* (MIXcoat)] and water regime on $P_n$ (a), gs (b), Ci (c), Tr, (d) and WUE (e) of *Vigna unguiculata* (L.) Walp. Letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test

and establishment increases the opportunity of seedlings to achieve a positive C and nutrient balance, which is crucial, especially under stress conditions (de Albuquerque & de Carvalho, 2003). Further studies are, therefore, needed to improve this limitation on the germination of cowpea seeds coated with AM fungi.

On the other hand, when compared with control, there was an overall enhancement on chlorophyll and carotenoids contents

in *R. irregularis*-inoculated plants (Table 3), particularly under severe water deficit for chlorophyll a and b (Figure 4). WUE, one of the mechanisms of plants to increase drought resistance (Vivas, Marulanda, Ruiz-Lozano, Barea, & Azcón, 2003), was increased in plants inoculated with *R. intraradices* and *P. putida* under severe water deficit (Figure 3). The presence of mycorrhiza significantly enhanced photosynthetic rate, stomatal conductance and transpiration
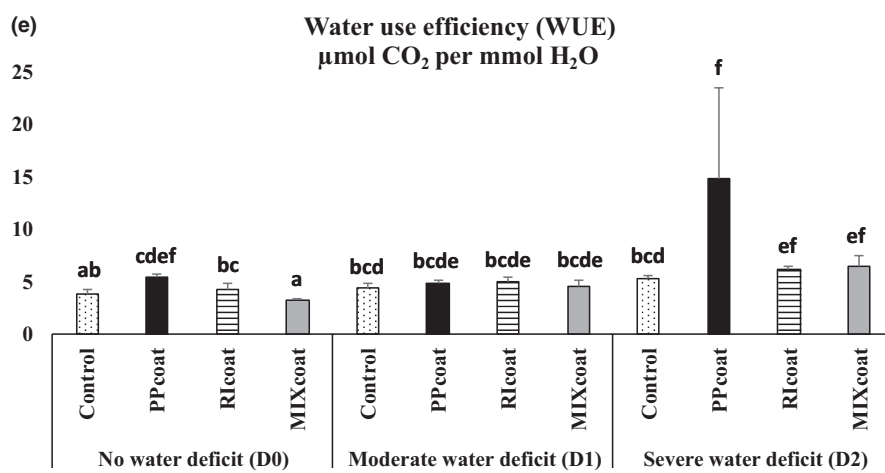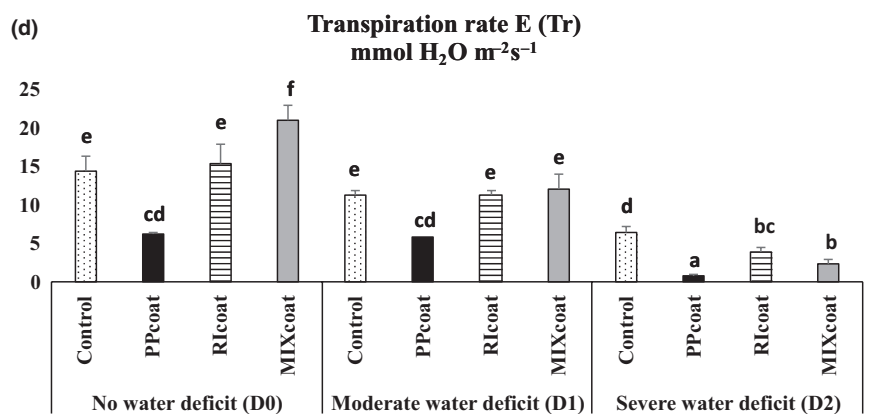
**(d)**

### Transpiration rate E (Tr) mmol $H_2O$ m$^{-2}$s$^{-1}$



**(e)**

### Water use efficiency (WUE) µmol $CO_2$ per mmol $H_2O$



**FIGURE 3** Continued

rate (Figure 3) under no water deficit, corresponding to the water regime where the colonisation was higher (Abdel-Salam, Alatar, & El-Sheikh, 2017). The increased rate of photosynthesis was probably a result of the increased use of fixed C (Fitter, 1991) and/or higher chlorophyll content (Gusain, Singh, & Sharma, 2015), under no water deficit (Figures 3 and 4). Under severe water deficit, this relationship between photosynthesis and chlorophyll content was not so obvious. Water deficit affects various physiological and biochemical processes of plants, limiting stomata and transpiration and resulting in reduced photosynthesis (Farooq, Wahid, Kobayashi, Fujita, & Basra, 2009). These physiological limitations and decreased photosynthetic rate under water deficit possibly eliminated the compensatory effect of mycorrhiza shown in plants without water deficit. In fact, under water deficit the decrease in photosynthetic activity was also greater in mycorrhizal plants, as shown by Birhane, Sterck, Fetene, Bongers, and Kuyper (2012). Thus, this photosynthetic depression could have been responsible for the lower percentage of AM root colonisation. AM fungal colonisation is negatively influenced by water deficit (Kaya, Higgs, Kirnak, & Tas, 2003; Oliveira et al., 2017a; Wu & Xia, 2006), which, in the present study, might have been related to the observed reduction of cowpea fitness and to the lower production of photosynthates, meaningless C for the fungal symbiont.

PGPR singly or in combination with AM fungi play a significant role in alleviating drought stress in plants (Vurukonda

et al., 2016). In our results, the co-inoculation (PGPR + AM fungi) apparently did not present any extra benefit to the plants. On the other hand, plants singly inoculated with *P. putida* showed a significant increase in seed yield (Table 2), including under moderate water deficit (Table 1). Overall, *P. putida* significantly enhanced total plant biomass (Table 2). The accumulation of K in cowpea shoots was enhanced by 25% in plants singly inoculated with *P. putida* under moderate water deficit (Figure 1). K is an essential nutrient for plants and plays an important role in drought conditions, cell membrane stability, root growth and leaf area increase, water uptake and water conservation improvement (Wang, Zheng, Shen, & Guo, 2013). The enhancement of K under moderate water deficit might be one of the factors responsible for improving cowpea tolerance to the stress and positively influencing seed yield, when comparing to the reaming treatments under the same water regime. The ability of PGPR to increase plant biomass, yield and protein content both under greenhouse and field conditions was shown before in legumes (Oliveira et al., 2017a,b; Sindhu, Dua, Verma, & Khandelwal, 2010). Many studies with various crops showed a positive relationship between PGPR inoculation and drought tolerance (Figueiredo, Burity, Martínez, & Chanway, 2008; Gusain et al., 2015; Kohler, Hernández, Caravaca, & Roldán, 2008; Naseem & Bano, 2014). In these studies, the production of phytohormones and the production of exopolysaccharides helped with drought stress alleviation and/or

**TABLE 3** Main effects of the factors inoculation and water regime and two-way ANOVA F-values and significances for leaf parameters and chlorophyll and carotenoids contents of *Vigna unguiculata* (L.) Walp

| Main effects | | Photosynthetic rate A ($P_n$) µmol $CO_2$ m$^{-2}$s$^{-1}$ | Stomatal conductance ($g_s$) mol $H_2O$ m$^{-2}$s$^{-1}$ | Intercellular $CO_2$ concentration (Ci) µmol $CO_2$/mol | Transpiration rate E (Tr) mmol $H_2O$ m$^{-2}$s$^{-1}$ | Water use efficiency (WUE) µmol $CO_2$ per mmol $H_2O$ | Chlorophyll a (µg/mg fresh leaf) | Chlorophyll b (µg/mg fresh leaf) | Carotenoids (µg/mg fresh leaf) |
|---|---|---|---|---|---|---|---|---|---|
| Inoculation (I) | Control | 46.40 b | 0.67 b | 233.20 a | 11.11 b | 4.40 a | 0.18 ab | 0.04 a | 0.07 a |
| | PPcoat | 22.87 a | 0.25 a | 215.00 a | 4.26 a | 8.34 c | 0.11 a | 0.02 a | 0.10 a |
| | RIcoat | 47.27 b | 0.65 b | 206.78 a | 10.21 b | 5.09 bc | 0.28 b | 0.07 b | 0.14 b |
| | MIXcoat | 46.60 b | 0.78 b | 224.80 a | 12.83 b | 4.54 ab | 0.31 b | 0.07 b | 0.15 b |
| Water regime (WR) | D0 | 54.62 z | 0.94 z | 241.92 y | 14.83 z | 4.05 x | 0.34 y | 0.08 y | 0.16 y |
| | D1 | 47.37 y | 0.65 y | 235.85 y | 10.49 y | 4.62 y | 0.18 x | 0.04 x | 0.10 x |
| | D2 | 19.58 x | 0.16 x | 180.42 x | 3.36 x | 8.12 z | 0.14 x | 0.03 x | 0.14 x |
| Two-way ANOVA F-values and significances | | | | | | | | | |
| Inoculation (I) | | 43.87*** | 22.13*** | 1.42 ns | 32.70*** | 5.65** | 4.62* | 5.36** | 6.91** |
| Water regime (WR) | | 145.14*** | 94.78*** | 18.92*** | 112.87*** | 18.34*** | 9.37*** | 10.50*** | 8.54*** |
| I × WR | | 5.90*** | 3.75** | 2.82* | 4.61** | 1.77 ns | 1.36 ns | 1.88 ns | 1.15 ns |

*Notes.* Letters indicate significant differences according to Duncan's multiple range test. *, **, ***, significant effect at the level of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; ns, non-significant effect. Control, non-inoculated; PPcoat, *Pseudomonas putida*; RIcoat, *Rhizophagus irregularis*; MIXcoat, mix of *R. irregularis* and *P. putida*; D0, no water deficit; D1, moderate water deficit; D2, severe water deficit.

increased seed yield and protein content. The increase of drought stress tolerance by PGPR can be related to several mechanisms, such as production of phytohormones (abscisic acid, gibberellic acid, cytokinins and IAA); ACC deaminase; induced systemic tolerance; and production of exopolysaccharides (Vurukonda et al., 2016). The *P. putida* strain used in the present study is a strong IAA producer, which is physiologically the most active auxin in plant growth and development. Various plant species inoculated with IAA producing bacteria increased root growth thus increasing water and nutrient uptake and tolerance to water deficit (Vurukonda et al., 2016). More studies on the microbial mechanisms behind the increase in drought stress tolerance and yield are essential.

# 5 | CONCLUSIONS

It is imperative to improve agricultural productivity, in a sustainable way, against unfavourable environmental conditions. Understanding plant responses to drought is of great importance, since this is one of the main constraints to crop yield. Microbial inoculation is known to confer drought resistance to plants. In this study, results showed a general positive effect of bacterial inoculation via seed coating on crop productivity under moderated water deficit, which might be relevant for agricultural applications. AM fungal inoculation via seed coating had an overall positive influence on cowpea regarding the uptake of nutrients, leaf pigments content and gas exchange parameters, nonetheless mostly obtained under no water deficit. The application of PGPR and AM fungi represents a key approach for agricultural systems and should be integrated with or without drought stress, yet more studies concerning the microbe–plant interaction and the mechanisms that confer the stress alleviating abilities are necessary. Selecting the microbe that better potentiates plant tolerance is critical for the efficiency of microbial inoculation. On the other hand, seed coating can be a promising tool for efficiently delivering microbial inocula. Nonetheless, additional studies are needed to address the cowpea seed germination reduction and improve the technique. Moreover, field studies under real agricultural context are indispensable to prove the possible application of seed coating with PGPR and AM fungi in a large-scale approach.
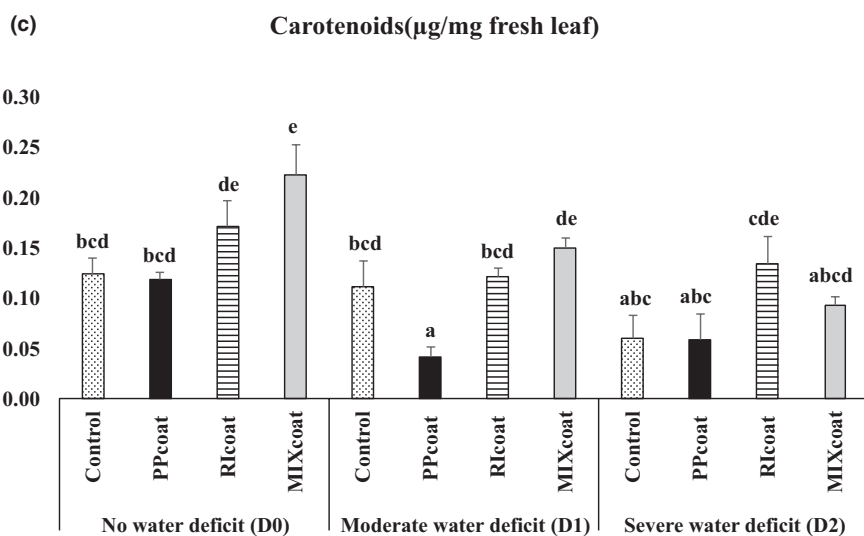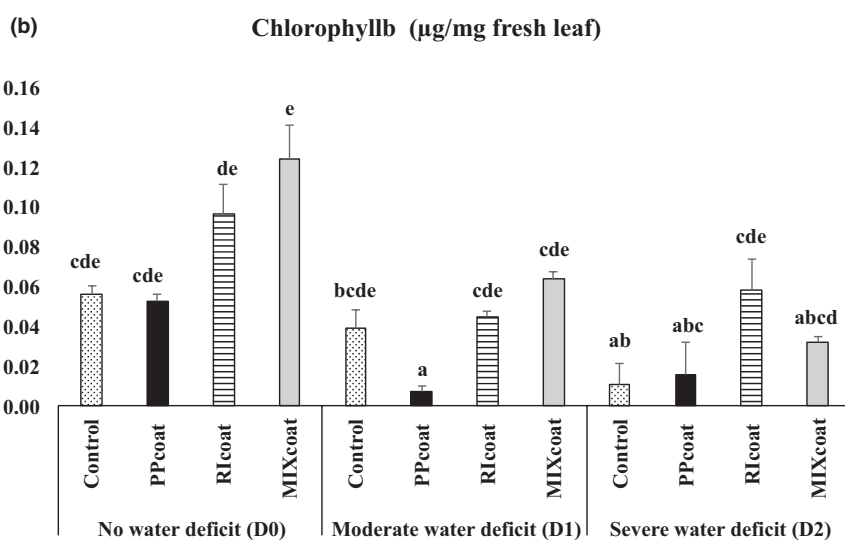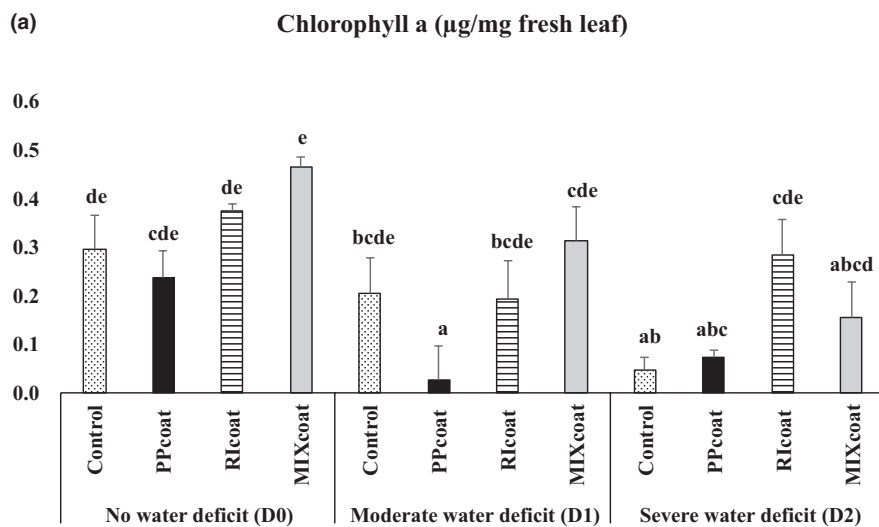
**(a)**

**Chlorophyll a (µg/mg fresh leaf)**



**(b)**

**Chlorophyllb  (µg/mg fresh leaf)**



**(c)**

**Carotenoids(µg/mg fresh leaf)**



**F I G U R E 4**   Chlorophyll a (a), chlorophyll b, (b) and carotenoids (c) leaf concentrations of *Vigna unguiculata* (L.) Walp. under different inoculation treatments [non-inoculated controls (Control) and inoculated with *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix of *R. irregularis* +  *P. putida* (MIXcoat)] and water regimes. Letters indicate significant differences  ($p < 0.05$) according to Duncan's multiple range test

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