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Can arbuscular mycorrhizal fungi and rhizobacteria facilitate ^{33}P uptake in maize plants under water stress?

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) are able to provide key ecosystem services, protecting plants against biotic and abiotic stresses. Here, we hypothesized that a combination of AMF (*Rhizophagus clarus*) and PGPR (*Bacillus* sp.) could enhance ^{33}P uptake in maize plants under soil water stress. A microcosm experiment using mesh exclusion and a radiolabeled phosphorus tracer (^{33}P) was installed using three types of inoculation: i) only AMF, ii) only PGPR, and iii) a consortium of AMF and PGPR, alongside a control treatment without inoculation. For all treatments, a gradient of three water-holding capacities (WHC) was considered i) 30% (severe drought), ii) 50% (moderate drought), and iii) 80% (optimal condition, no water stress). In severe drought conditions, AMF root colonization of dual-inoculated plants was significantly lower compared to individual inoculation of the AMF, whilst ^{33}P uptake by dual-inoculated plants or plants inoculated with bacteria was 2.4-fold greater than the uninoculated treatment. Under moderate drought conditions the use of AMF promoted the highest ^{33}P uptake by plants, increasing it by 2.1-fold, when compared to the uninoculated treatment. Without drought stress, AMF showed the lowest ^{33}P uptake and, overall, plant P acquisition was lower for all inoculation types when compared to the severe and moderate drought treatments. The total shoot P content was modulated by the water-holding capacity and inoculation type, with the lowest values observed under severe drought and the highest values under moderate drought. The highest soil electrical conductivity (EC) values were found under severe drought in AMF-inoculated plants and the lowest EC for no drought in single or dual-inoculated plants. Furthermore, water-holding capacity influenced the total soil bacterial and mycorrhizal abundance over time, with the highest abundances being found under severe and moderate drought. This study demonstrates that the positive influence of microbial inoculation on ^{33}P uptake by plants varied with soil water gradient. Furthermore, under severe stress conditions, AMF invested more in the production of hyphae, vesicles and spore production, indicating a significant carbon drain from the host plant as evidenced by the lack of translation of increased ^{33}P uptake into biomass. Therefore, under severe drought the use of bacteria or dual-inoculation seems to be more effective than individual AMF inoculation in terms of ^{33}P uptake by plants, while under moderate drought, the use of AMF stood out.

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1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), drought is now recognised as the primary reason for agricultural production losses globally, costing the sector USD 37 billion overall from 2008 to 2018. However, other extreme events caused by climate change, such as floods and heatwaves, are also contributing to ongoing issues with food security (FAO, 2021). Therefore, improved management approaches are urgently required to improve agricultural sustainability. This is particularly relevant for the supply and exploitation of soil nutrients which have a finite supply, such as phosphorus (P). This is even more important when we consider the dramatic rise in fertilizer prices over the past year (Smith, 2022). To overcome the combined impact of drought stress and low nutrient use efficiency in cropping systems and the design of new management systems requires a greater fundamental understanding of plant-soil-microbial interactions. Mawarda et al. (2020) highlighted that arbuscular mycorrhizal fungi (AMF) and rhizobacteria may provide an environmentally friendly solution to this combined problem.

In highly weathered soils, where the exchange surfaces are dominated by aluminium and iron oxides/hydroxides, a large proportion of the applied phosphate fertiliser (ranging from 15% to 30%) becomes rapidly immobilized on the solid soil phase by adsorption and precipitation processes (Dhillon et al., 2017; Zavaschi et al., 2020). AMF may provide a tool to exploit native soil P reservoirs or residual fertiliser-derived P ("legacy P") that has accumulated over the past 50 years in these soils (Scrase et al., 2019; Pavinato et al., 2020a, 2020b). AMF is a key group of soil microorganisms that form symbiotic associations with more than 80% of all land plants and play an important role in the acquisition of nutrients (Smith and Read, 2008). For example, in maize (*Zea mays* L.), AMF is more important than root hairs for seedling growth under low P availability (Ma et al., 2021).

Concurrently, it is widely acknowledged that AMF and plant growth-promoting rhizobacteria (PGPR) can play an important role in the amelioration of a wide range of plant biotic and abiotic stresses such as drought, salinity, heavy metal exposure, and soil-borne pathogens (Pérez-De-Luque et al., 2017; Santoyo et al., 2021; Chen et al., 2022). There is also evidence that co-inoculation with AMF and PGPR can increase plant growth and health through additive and/or synergistic effects between them (Saia et al., 2015; Battini et al., 2017; Dutta and Neog, 2017; Nanjundappa et al., 2019). Understanding the plant-mycorrhizae-rhizobacteria interactions is crucial, as plants dedicate 5–30% of their photo-assimilate to supporting bacterial growth in soil (Carvalhais et al., 2011; Almeida et al., 2020). A further 20% of photo-assimilate is allocated to the maintenance of symbiotic AMF networks (Smith and Read, 2008).

Some studies have demonstrated the ability of AMF or PGPR alone to promote plant growth under water shortage events. It was postulated that AMF mechanisms, such as improvements in soil aggregation, photosynthetic efficiency, and nutrient uptake are primarily responsible for this response (Ji et al., 2019; Quiroga et al., 2019; Al-Arjani et al., 2020). In the case of PGPR, the main reported mechanisms include direct (e.g., changes in hormonal signalling, P solubilisation, biological nitrogen fixation) and indirect mechanisms (e.g., antibiotic production, cell wall degrading enzymes, induced systemic resistance, osmotic adjustment, quorum quenching, and siderophore production) (Glick, 2012; Olanrewaju et al., 2017; Naylor and Coleman-Derr, 2018; Araújo et al., 2020).

Overall, the majority of crop plant species are responsive to mycorrhizal symbiosis and rhizobacteria inoculation. This discovery has subsequently led to the search for novel microbes with the potential to increase crop yields, especially maize, as this represents one of the most important global crops (Zhao et al., 2017; Li et al., 2021). Recently, the use of maize has become more inviting due to the current scenario of bio-economy, which has incentives for production of biofuels to reduce CO_2 emissions. Thus, there is the possibility to intensify the biofuels market, making it more prosperous and stable (Eckert et al., 2018).

A myriad of earlier studies (Rhodes and Gerdemann, 1975; Jakobsen et al., 1992; Pearson and Jakobsen, 1993; Battini et al., 2017; Jongen et al., 2022) have demonstrated the ability of AMF hyphae to recover and translocate $^{32/33}$P located beyond the immediate root zone. Nevertheless, these studies do not consider how the efficiency of AMF-mediated P absorption process is affected under a gradient of soil water availability, especially when considering the presence of PGPR capable of tolerating low water activity. Here, we combine experiments that simultaneously addressed i) the P dynamics (sorption and diffusion) using radiolabeled phosphorus tracer (^{33}P), ii) the soil water gradient and iii) the presence or absence of specific AMF and PGPR strains. Our investigation was set up to test the hypothesis that the combined use of AMF and PGPR would enhance P uptake in maize plants under drought stress.

For this, we evaluated ^{33}P uptake in maize plants, the response of soil phosphatase activity, and key soil chemical attributes, as well as monitoring the abundance and dynamics of soil mycorrhizal and bacterial communities.

2. Material and methods

2.1. Experimental design

The microcosm experiment was set up in a completely randomized design, comprising a double factorial scheme (4 × 3) with three replicates. The first factor was the inoculation of microorganisms (either only AMF, only PGPR, or the consortium of AMF and PGPR, besides a control without any inoculation). The second factor was water stress (80%, 50%, and 30% of the water-holding capacity, simulating no drought, moderate drought, and severe drought, respectively). We established the water-holding capacity of 30%, 50% and 80% as severe, moderate and no drought, respectively, according to previous investigations (Kavamura et al., 2013; Araújo et al., 2020; Santos et al., 2020).

Each experimental unit comprised a plastic pot (8 cm internal diameter × 7 cm high), containing 200 g (dry weight) of sterilized soil (at 121 °C for 2 h) (Fig. 1a and b), ensuring that bacteria and fungi that were traced were only those from the inoculum. The soil was obtained from the Ah horizon (0–10 cm deep) of a field site located in Brazil (22°42' S, 47°38' W) and classified as an Arenosol (WRB-FAO, 2015), with low P content (Table 1). The A horizon has been used in the diagnosis of the main epipedons in soil taxonomy and here we use the Ah horizon as the most representative of the sampled area. Soil chemical characterization was evaluated according to van Raij et al. (2001).

Mesh exclusion (45 μm) was utilized to divide the pot into two compartments: a fertilized compartment and a planted/inoculated compartment, each of them receiving 100 g of sterilized soil. This mesh allowed fungal hyphae to pass through and absorb nutrients but prevented the ingrowth of roots from the plant compartment, inoculated or not. This approach has been used to investigate the role of mycorrhizae on plant growth and water supply (Cardoso et al., 2004; Neumann and Matzner, 2013; Scrase et al., 2019; Kakouridis et al., 2020). Nevertheless, we set up a preliminary experiment (Experiment 1) to confirm that roots were not able to pass through the mesh, while simultaneously also determining seed germination rate in the soil and the correction factor needed when calculating water-holding capacity over the course of subsequent experiments (Supplementary Note 1).

The mode of fertiliser amendment in the compartment was based on the application of 2.8 mL of 6 mM KH_2PO_4 (equivalent to 30 mg P kg^{-1} soil, as recommended for this type of weathered soil; van Raij et al., 1997) with a ^{33}P activity of 185 kBq. The fertiliser amendment was carried out after the soil acclimatization, sowing of seeds and inoculation of microbes in the planted/inoculated compartment (Fig. 1c). Furthermore, we set up an additional microcosm experiment to determine the distance that P can diffuse in the soil to confirm that the presence of ^{33}P in the planted/inoculated compartment could only occur

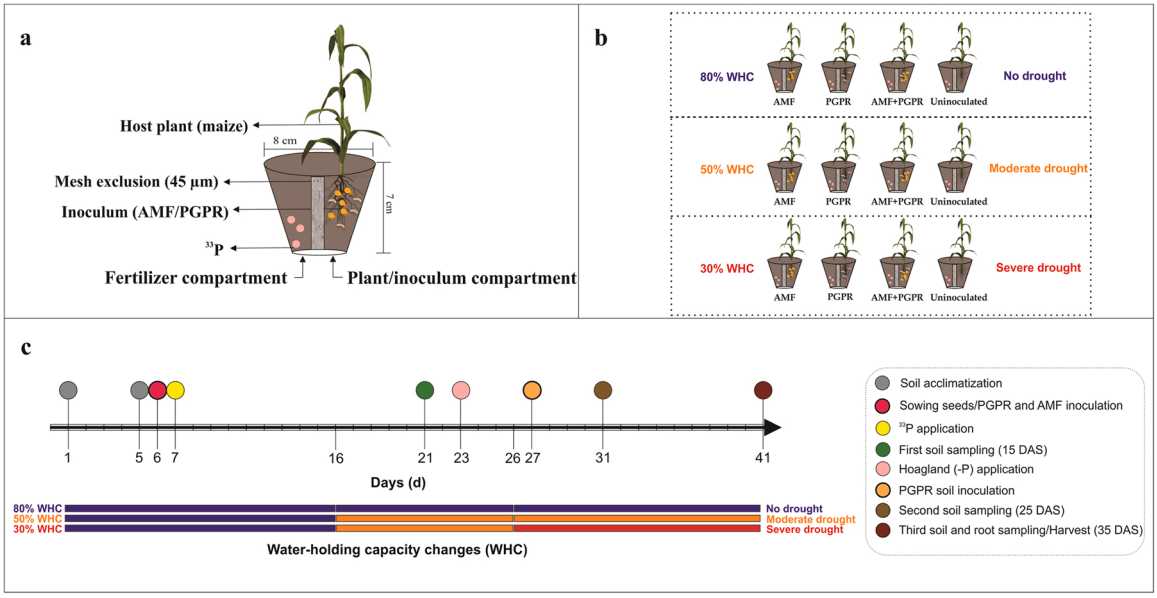

Fig. 1. Experimental representation, (a) pot using a root exclusion mesh to create two compartments, one of which was fertilized and the other planted and inoculated. (b) summary of the treatments evaluated in the experiment. (c) timeline of the experiment. WHC: water-holding capacity; DAS: days after sowing; PGPR: plant growth-promoting rhizobacteria; AMF: arbuscular mycorrhizal fungi.

Table 1
Physico-chemical characterization of the initial studied soil.

Material	pH*	O.M	P	S	K	Ca	Mg	Al	H+Al	SB	CEC	V*	Sand	Silt	Clay
		g dm^{-3}	mg dm^{-3}		mmol$_c$ dm^{-3}							%	g dm^{-3}		
Soil	4.1	15.0	< 6.0	18.0	0.8	4.0	3.0	7.0	28.0	7.8	35.8	22.0	751	23	226

pH: measured in $CaCl_2$, O.M: organic matter - colorimetric method, P: phosphorus with anion exchange resin, S: sulfur - 0.01 mol L^{-1} calcium phosphate, K, Ca and Mg: potassium, calcium and magnesium measured in anion exchange resin, Al: aluminum in 1 mol KCl L^{-1}, H + Al: potential acidity in SMP buffer, SB: sum of bases (K + Ca + Mg), CEC: cation exchange capacity, V: base saturation. mmol$_c$ dm^{-3}: millimoles of charge per kilogram of soil according to SI unit (International Standard of Units). *Soil pH was increased to 6.5 and base saturation to 70% after liming performed according to van Raij et al. (1997) using dolomitic lime (1.71 Mg ha^{-1}) with 100% relative power of total neutralization (80% Ca^{2+} and 20% Mg $^{2+}$).

via microbial transfer. In addition, we also measured P sorption to the soil to characterize the P-dynamics in this soil (Supplementary Note 2).

2.2. Fungal and bacterial inoculum

Fungal and bacterial strains were previously isolated from Serra do Ouricuri, Petrolina, Pernambuco, Brazil (39°3′ S, 8°28 W) in the Caatinga Biome, where a bio-prospecting program was developed to find microbes with the potential of helping crop plants to tolerate drought stress (Kavamura et al., 2013; Fernandes-Júnior et al., 2015). Initially, a pool of fungi and bacteria was isolated from the rhizosphere of *Tripogonella spicata* (Nees) plants, the so-called resurrection grass, due to its surprising rehydration capacity after a drought period (Fernandes-Júnior et al., 2015; Aidar et al., 2017). Then, those microrganisms were selected for their plant growth-promoting properties, such as the ability to grow under reduced water availability (Hallsworth et al., 1998), indole-3-acetic acid (IAA) production (Bric et al., 1991; Kuss et al., 2007) and calcium phosphate solubilization (Verma et al., 2001).

Bacillus sp. was cultivated in 10% (w/v) TSB (trypticase soy broth) culture medium at 30 °C and 150 rpm for 48 h. The inoculum was homogenized at OD_{550} = 0.2 and washed twice in a 0.85% (w/v) saline solution to obtain the bacterial suspensions (at 10^8 CFU mL^{-1}). A second bacterial inoculation (3 mL pot^{-1} at 10^8 CFU mL^{-1}) was done when the water-holding capacity was changed to 30% (i.e., twenty-one days after sowing). The second bacterial inoculation was performed due to the drastic reduction in soil water content, thus maintaining bacterial activity in the soils (Jeong et al., 2013; Armada et al., 2018; Mawarda et al., 2020). The same amount of sterile saline solution was provided to the AMF and control treatments (Fig. 1c).

Rhizophagus clarus spores were obtained from the pure trap culture, using maize as host plant, and transferred to the soil after surface-disinfection, directly under the seeds, in the form of 5 mL of sterile water containing 50 spores at the time of sowing. Spores of AMF were surface-sterilized by exposing them to 0.5% sodium hypochlorite in a filter unit allowing contact for 15 min and then repeatedly rinsing in sterile water (Johnson and Pfleger, 1992; Habte and Osorio, 2001; Maia and Yano-Melo, 2001). The germination rate of *R. clarus* spores in the soil was around 85% according to our third additional microcosm experiment, as reported in Supplementary Note 3.

2.3. Plant material, cultivation, and water-holding capacity management

Uniformly sized seeds of *Zea mays* L. (cv. BRS Gorotuba) obtained from the Brazilian Agricultural Research Corporation (EMBRAPA) were surface sterilized twice in 2% (v/v) sodium hypochlorite solution for 7 min, 70% (v/v) ethanol for 1 min, and rinsed thoroughly with sterile MilliQ water. The germination rate of seeds in soil and Petri dishes was around 90%. Seeds inoculated with PGPR or for doubled-inoculated plants with PGPR and AMF were soaked in the bacterial suspension for 2 h, whilst seeds for only AMF or with uninoculated plants were soaked in 0.85% (w/v) sterile saline solution for the same time (Kavamura et al., 2013). Two seeds were sown in the right compartment in each pot and thinning was done when one of the seedlings presented two true leaves. Soil bacterial inoculation was done 21 days after sowing, in

PGPR or consortium treatments, when pots were reduced to 30% of the water-holding capacity, according to the water content management described below. At the same time, 0.85% (w/v) sterile saline solution was applied to the AMF-inoculated or the uninoculated plants.

Plants were cultivated in a Conviron Adaptis® CMP 6010 growth chamber (Controlled Environments Ltd, Winnipeg, Manitoba, Canada) at the Environment Centre Wales, Bangor University, United Kingdom (53°13′ N, 4°7′ W). Plants were maintained under a day/night cycle of 16/8 h, 25/20 °C, 70% relative humidity, receiving artificial lighting at a photosynthetic photon flux density of 500 μmol m^{-2} sec^{-1}. Hoagland solution (without phosphorus) was applied 17 days after sowing, to keep nutritional balance of the plants. The final solution of pH 5.5 was composed of 4 mM Ca $(NO_3)_2$, 6 mM KNO_3, 2 mM $MgSO_4$, 1 mM Fe-EDTA, and 1 mM trace elements (Hoagland and Arnon, 1950).

The microcosms were randomized daily to ensure equal growth conditions and were weighed for 35 days, and the desired moisture was maintained with the addition of deionized and sterilized water when needed. At the beginning of the experiment, all microcosms ($n = 36$) were kept at 80% water-holding capacity to ensure seed germination. Ten days after sowing, $^2/_3$ of the microcosms ($n = 24$) were reduced to 50% water-holding capacity. Finally, twenty days after sowing, $^1/_3$ of the microcosms ($n = 12$) were reduced to 30% water-holding capacity (Fig. 1c). This approach was used to facilitate understanding the potential of inoculated microbes in the context of a decreasing gradient of soil water content (Ahmad et al., 2018; Czarnes et al., 2020; Lopes et al., 2021).

After 35 days of growth, the plants were harvested and separated into shoot and root material, whilst the soil was separated into fertilized and planted/inoculated compartments. In addition, soil was sampled in the planted/inoculated compartment five days after changing the water-holding capacity (i.e., on the 15th and 25th day after sowing) to monitor soil bacteria and mycorrhiza total abundance via quantitative polymerase chain reaction (qPCR). Sampling for assessing bacterial and fungal abundances were executed with a sterile polypropylene cylinder (8 mm inner diameter) to avoid disturbances during plant growth and not to affect the fungal hyphae.

2.4. Analytical procedures

At the end of the experiment (i.e., 35 days after sowing) plant height was measured using a metal ruler (Westcott Ltd., Chichester, England, United Kingdom) placed on the ground next to the tallest stem, while basal diameter was measured using a digital micrometer (Moore and Wright Ltd., Sheffield, England, United Kingdom). Shoot was cut off at the soil surface. Later, it was dried at 80 °C in paper bags, for 16 h before, quantifying the dry weight. The roots were separated from soil by gently shaking them and rinsing them with water. Then, 5 g of roots were placed in a 50 cm^3 polypropylene tube with 70% ethanol for analysis of mycorrhizal colonization percentage, and the remainder was dried to obtain the dry weight. For phosphorimager analysis to visualize the ^{33}P, dried plant tissue was placed in a 20 × 25 cm cassette for 1 h, and then analyzed in a Bio-Rad Molecular Imager® FX (Bio-Rad Laboratories Inc., Hercules, California, United States of America).

Soil available phosphorus was extracted using 0.5 M acetic acid (1:5 w/v) according to Fisher et al. (1998). Samples were extracted by shaking (200 rpm for 30 min at room temperature), centrifuging for 15 min (18,000 *g*), filtering and the supernatant was recovered for analysis. ^{33}P activity of the samples was determined in counts per minute (CPM) of ^{33}P using 1 mL of soil extract and 4 mL of HiSafe 3 Scintillation cocktail (PerkinElmer, Waltham, Massachusetts, United States of America) in a Wallac 1404 scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, United Kingdom). For plant tissue, the extract was obtained by placing 0.2 g in a muffle furnace and ashing at 500 °C overnight. Later, this was dissolved in 1 mL of 20% HCl and 9 mL of deionized water was added, according to Adrian (1973). The colorimetric P determination in soil and plant tissue was determined according to Murphy and Riley (1962).

The soil pH and electrical conductivity were determined according to Thomas (1996), whereby 10 g of 2 mm-mesh sieved soil was mixed with 25 mL of water and shaken (10 min at 200 rpm) and then allowing the sample to settle for 10 min before taking measurements with standard electrodes.

The acid and alkaline phosphatase activities (EC 3.1.3.2 and EC 3.1.3.21, respectively) were measured using the methodology described by Marx et al. (2001). For evaluation of AMF root colonization, the roots were prepared according to Vierheilig et al. (1998), with the roots dispersed in a Petri dish with a grid background and scored using a stereomicroscope according to Giovannetti and Mosse (1980).

2.5. Molecular analysis

Soil (0.25 g) was utilized for DNA extraction using DNeasy® PowerSoil® Pro Kit (QIAGEN Inc., Germany) according to the manufacturer's protocol. Extracted DNA was stored at − 80 °C before quantitative PCR analysis. DNA concentrations were determined using the Qubit quantification platform with Qubit 1X dsDNA HS Assay Kit (Invitrogen, Carlsbad, California, United States of America).

The quantitative PCR (qPCR) was used to determine gene copy number per gram of soil for bacteria (16 S rRNA) and AMF (LSU rDNA region), using the StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc., Carlsbad, California, United States of America) with the fluorescent marker GoTaq® qPCR Master Mix (Promega, Madison, Wisconsin, United States of America). All samples were analysed in triplicate.

16 S rRNA reactions were run in 10 μL comprising 5 μL of GoTaq® qPCR Master Mix, received 1 μL (5 μM) of each primer (Eub338 5′-CCTACGGGAGGCAGCAG-3′ and Eub518 5′-ATTACCGCGGCTGCTGG-3′), 0.1 μL de CXR Reference Dye, 2 μL of DNA template, and 0.9 μL nuclease free sterile water in the same conditions as described by Muyzer et al. (1993). Standard curves were obtained using 7-fold serial dilutions of purified PCR (10^2 to 10^8 copies) containing the targeted gene. The reliability of the standard curves was controlled by verifying reproducibility of the Ct values, the quality of the dilution series, and the efficiency (101.42%, $R^2 = 0.993$). The specificity of the primers was confirmed by melting curves analysis.

AMF reactions were run in 10 μL comprising 5 μL of GoTaq® qPCR Master Mix, received 1 μL (5 μM) of each primer (FLR3 5′-TTGAAAGGGAAACGATTGAAG T-3′ and FLR4 5′-TAC GTCAA-CATCCTTAACGAA-3′), 0.1 μL de CXR Reference Dye, 2 μL of DNA template, and 0.9 μL free sterile water. FLR3 is localized between the D1 and D2 domains of LSU rRNA, whilst FLR4 is in the D2 domain (Gollotte et al., 2004). Standard curves were obtained using 7-fold serial dilutions of purified PCR (10^2 to 10^8 copies) containing the targeted gene. The reliability of the standard curves was controlled by verifying reproducibility of the Ct values, the quality of the dilution series and the efficiency (101.46%, $R^2 = 0.975$). The specificity of the primers was confirmed by melting curves analysis.

2.6. Data analyses

Data were tested for normal distribution using the Shapiro-Wilk test, followed by the homogeneity of variances tests, using the Bartlett test. Having met the criteria (residuals normality and variance homoscedasticity), a two-way analysis of variance (ANOVA) was performed and, when appropriate, Tukey's posthoc pairwise comparison (cut-off significance at $p < 0.05$) was applied to determine individual differences between means.

Principal components analysis (PCA) was performed using the statistical packages FactoMineR and factoextra in the R® program (R Core Team, 2017). In PCA, to meet the premise of multivariate normality, the data were transformed into log ($x + 1$), and the attributes subject to collinearity were removed (Ramette, 2007). Additionally, using k-means

clustering algorithm, an unsupervised machine learning method of identifying and grouping similar data points, we classified our variables into groups (Jansson et al., 2022).

3. Results

In both soil compartments i.e., planted/inoculated and the fertilized compartment, we determined their ^{31}P and ^{33}P content alongside soil pH, electrical conductivity, and soil acid and alkaline phosphatase activity. In addition to plant P content, AMF root colonization and number of spores for the planted/inoculated compartment were determined. Here, we primarily concentrate on the results from the planted/inoculated compartment, while results from the fertilized compartment are present in the supplementary material. Data are reported on P uptake by arbuscular mycorrhizal hyphae, as revealed by the use of radioactive P (^{33}P), added the fertilized compartment. Overall, inoculation of AMF or PGPR positively influenced plant growth, radiolabelled P uptake, mycorrhization and soil characteristics, when compared to the uninoculated control. Nevertheless, the positive influence of microbial inoculation varied with soil water gradient.

3.1. ^{33}P uptake by plants, biomass, and P pool under different treatments

Soil water content greatly affected ^{33}P uptake by the plants, with the highest ^{33}P uptake (0.4 kBq.plant^{-1}, on average) observed in moderate drought (50% WHC) with the lowest uptake (0.2 kBq.plant^{-1}, on average) observed in the absence of drought (80% WHC) (Figs. 2a and 2b). Overall, for the planted/inoculated compartment, the highest levels of ^{33}P activity in soil were found in the presence of mycorrhizal inoculum under severe or moderate water stress, but this was not detected in soil from the fertilized compartment (Figs. S1a and S1b). In addition, for shoot biomass, the main difference among the inoculum types occurred in severe drought, with the highest biomass found in the presence of bacterial inoculum and the lowest biomass in the presence of mycorrhizal inoculum (Fig. S2a). This same pattern was detected for morphological traits, such as height and diameter (Fig. S2b and S2c).

Under severe drought (30% WHC), ^{33}P uptake in shoot was 2.4-fold greater ($p \leq 0.05$) in the PGPR and AMF+PGPR treatments than in uninoculated control. Whilst under moderate drought (50% WHC), ^{33}P uptake in shoot of the AMF treatment was 2.1-fold greater ($p \leq 0.05$) than in the uninoculated control, outperforming the other inoculation types. On the other hand, under optimal conditions (80% WHC), the highest ($p \leq 0.05$) ^{33}P uptake in shoot was found in AMF+PGPR and the lowest ($p \leq 0.05$) in AMF treatment (Fig. 2a, Fig. S3). The same pattern of ^{33}P uptake was seen in the root (Fig. 2b, Fig. S4).

The total shoot P content was modulated by the water-holding capacity and inoculation type, with the lowest values ($p \leq 0.05$) observed under severe drought (18.6 μg P plant^{-1}, on average) and the highest values ($p \leq 0.05$) under moderate drought (28.3 μg P plant^{-1}, on average), almost reflecting the results of those for ^{33}P uptake (Fig. 2c). However, this did not occur in the roots, in which there was an increase in P content with the increase of water content (Fig. 2d). Under severe drought, the highest shoot P content was observed in the PGPR treatment ($p \leq 0.05$) whilst under moderate drought the uninoculated control was, in general, superior to all other inoculation types. Under no water stress, shoot P content in AMF+PGPR and PGPR was 1.7 and 1.5-fold greater than in the AMF treatment, respectively (Fig. 2c). Regarding the P pool, it was observed that under severe drought in the AMF, PGPR, AMF+PGPR and uninoculated treatments the contents were 2.40, 2.74, 2.49, and 3.33 mg P 0.100 g soil^{-1}, respectively. Whist under moderate drought, following the same sequence of treatments, P contents were 2.07, 2.98, 3.45, and 3.06 mg P 0.100 g soil^{-1}. Finally, for no drought, the following P contents were observed 2.98, 3.22, 2.77, and 2.26 mg P 0.100 g soil^{-1} (Fig. S1c). Overall, soil P contents were higher in the planted/inoculated compartment than in the fertilized compartment (Figs. S1c and S1d).

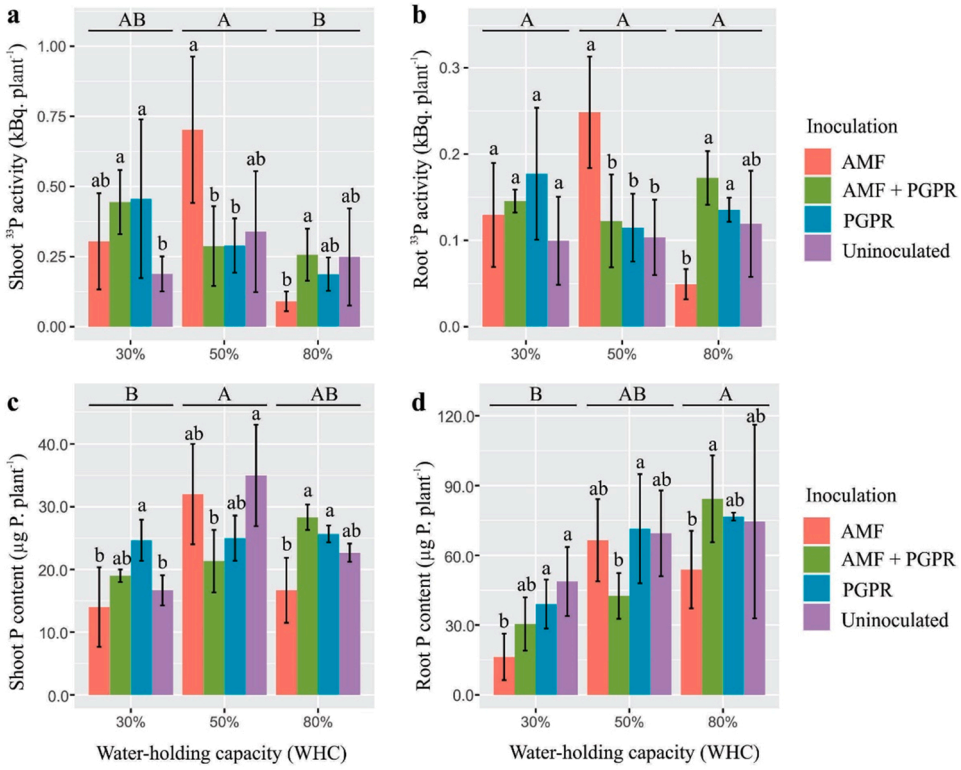

Fig. 2. Impact of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) inoculation on P acquisition under three different soil water-holding capacities (WHC). (a) ^{33}P activity in shoot and (b) root. (c) total P content (^{31}P + ^{33}P) in the shoot and (d) root. Uppercase letters compare differences in WHC, while lowercase letters compare differences according to inoculant types by Tukey's test at 5% ($p \leq 0.05$). Standard errors are shown ($n = 3$).

3.2. AMF root colonization and number of spores in soil

AMF root colonization was higher under severe (20.9%, on average) and moderate water stress (21.8%, on average) than in no drought (6.4%, on average). Under severe drought, the AMF treatment showed a higher ($p \leq 0.05$) AMF root colonization percentage (56.7 ± 7.4) than the AMF+PGPR treatment (26.7 ± 16.1). Whilst under moderate and no drought, there was no difference ($p > 0.05$) between them (Fig. 3a). The

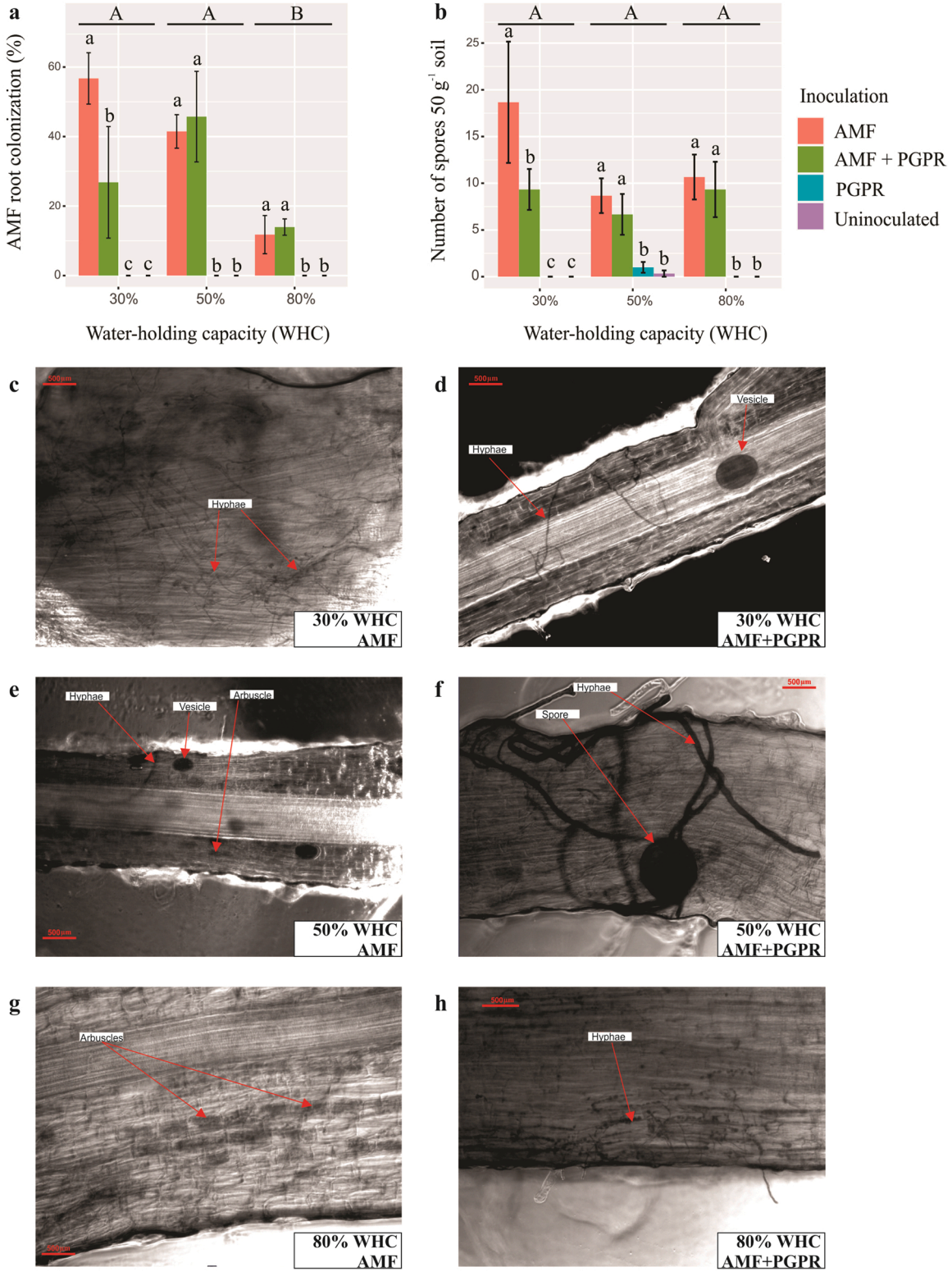

Fig. 3. Mycorrhization results, (a) Arbuscular mycorrhizal root colonization. (b) number of spores in soil. (c to h) microscopy results showing the arbuscular mycorrhizal structures (extraradical hyphae, vesicle, arbuscules, and spore) in the treatments inoculated with arbuscular mycorrhizal fungi (AMF) or consortium of AMF and plant growth-promoting rhizobacteria (AMF+PGPR) under three different water-holding capacities (WHC). Uppercase letters compare differences in WHC, while lowercase letters compare differences according to inoculant types by Tukey's test at 5% ($p \leq 0.05$). Standard errors are shown ($n = 3$).

number of spores differed only between the AMF and AMF+PGPR treatments under severe drought ($p \leq 0.05$), where the highest value was found in the AMF treatment (18 ± 6). Overall, the water-holding capacity did not influence ($p > 0.05$) the number of spores (Fig. 3b).

According to the microscopy results, a different pattern in WHC response occurred in the presence of AMF structures inside the roots. Considering a severe drought in the AMF treatment, there was a large presence of hyphae (60%), whilst in the AMF+PGPR treatment, we observed only about 40% of hyphae and 20% of vesicles (Figs. 3c and 3d, respectively). On the other hand, under a moderate drought, for AMF treatment, there was a higher presence of vesicles (50%), hyphae (35%), and arbuscules (10%) (Fig. 3e). Considering the AMF+PGPR treatment, there was the presence of spores inside the root (20%), as well as hyphae (20%) and vesicles (30%) (Fig. 3f). Without water stress, in the AMF treatment, there was a higher presence of arbuscules (25%), while in the AMF+PGPR we only noticed the presence of hyphae (20%) (Fig. 3g and h, respectively).

3.3. Soil pH and electrical conductivity, soil phosphatases activity, and microbial monitoring sampling time

The water-holding capacity did not influence the soil pH ($p > 0.05$), but it did influence ($p \leq 0.05$) the soil electrical conductivity (EC). The highest soil EC values were found under severe drought (86.1 $\mu S.cm^{-1}$, on average), and the lowest for moderate (64.7 $\mu S.cm^{-1}$, on average) and no drought (61.4 $\mu S.cm^{-1}$, on average). A subtle difference was found in soil pH between inoculum types, within the same soil water-holding capacity. Compared with the other treatments, the AMF treatment showed the lowest pH value under severe and moderate stress ($p \leq 0.05$), whilst non-water restriction showed the highest pH value ($p \leq 0.05$). The main difference in electrical conductivity (EC) was found under severe drought, where the AMF+PGPR treatment presented the lowest EC (Table 2).

The highest soil acid phosphatase activity was found in the uninoculated treatment without any water restriction, which was on average 3-fold higher than in the other treatments. There was no difference between treatments under severe drought, while under moderate drought, the uninoculated and PGPR treatments showed higher values compared to all other inoculation types. For soil alkaline phosphatase activity, the highest values were found, both, under moderate and non-stress conditions. An opposite behaviour was observed in the AMF+PGPR treatment, in which, under moderate stress, there was higher phosphatase activity, whereas in non-stress conditions, there was the lowest activity, when compared to the other treatments with the same water-holding capacity (Table 2). The results for pH, EC, acid, and alkaline phosphatase in the fertilized compartment are presented in Supplementary Table S1.

Overall, water-holding capacity influenced the total soil bacterial and mycorrhizal abundance over time (Table 3 and Table 4). Soil bacterial abundance increased from 15 days after sowing (DAS) to 25 DAS and decreased from 25 DAS to 35DAS (Table 3). Whilst mycorrhizal fungal abundance decreased from 15 DAS to 25 DAS (only in severe and moderate drought) and increased substantially from 25 DAS to 35DAS (Table 4) under absence of stress. For both, bacterial and mycorrhizal inoculum, the highest abundances were found under severe and moderate drought.

3.4. Principal components analysis (PCA)

Principal components analysis (PCA) was conducted to address relationships between water-holding capacity and inoculation type and to determine the major trait components that explain the variation in the original data.

The water-holding capacity and inoculum type influenced the attribute dynamics according to the visualization of residuals in the PCA, which explained about 48% in the 2 first components and 60%, when considering the three main components (Fig. 4a).

Overall, fungal parameters (AMF root colonization and number of spores), and ^{33}P uptake, besides soil EC and pH, were more correlated to the inoculum type under severe drought (square dots). Whilst plant parameters (biomass, diameter, and height), phosphatase dynamics, and phosphorus contents were more correlated with moderate stress and no-drought (triangular and circular dots, respectively). Despite the dispersion within the replicates, a subtle difference was evident in relation to the inoculum type, where the AMF+PGPR, only PGPR, and uninoculated treatments showed better clustering than the AMF treatment.

According to the results of the k-means clustering algorithm, three clusters of variables were identified, the first being composed of AMF root colonization, soil electrical conductivity, number of spores and ^{33}P activity (in soil and plant), which was more correlated to the AMF+PGPR treatment under severe drought. The second cluster was composed of plant parameters (diameter and height), and plant P content, while the third was composed of phosphatase activities and soil pH (Fig. 4b). We assessed the most important variables in explaining the variability in our data set according to the contribution level. The variables that contributed the most to the definition of the principal component 1 were AMF root colonization (12.81%), plant height (12.75%), root P content (10.65%), soil ^{33}P activity (10.31%), and number of spores (10.24%) (Fig. 4c). Whilst for the principal component 2, the most important variables were root and shoot ^{33}P activity (26.53% and 25.57%, respectively) (Fig. 4d).

Table 2
Soil pH, electrical conductivity (EC), acid phosphatase activity (Ac. Phosphatase) and alkaline phosphatase activity (Alk. Phosphatase) in the planted/inoculated compartment at three contrasting soil water-holding capacities (WHC) and with different types of inoculum (arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR), and consortium of AMF and PGPR)F.

WHC	Inoculum	pH	EC ($\mu S\ cm^{-1}$)	Ac. Phosphatase (nmol g^{-1} soil h^{-1})	Alk. Phosphatase (nmol g^{-1} soil h^{-1})
30% SD	AMF	6.5 ± 0.1 bA*	102 ± 10 aA	28.9 ± 10.7 aB	6.4 ± 0.7 bB
	AMF+PGPR	6.7 ± 0.1 abA	62.0 ± 6.0 bA	16.3 ± 7.9 aB	6.7 ± 0.7 bB
	PGPR	6.8 ± 0.0 aA	90.0 ± 4.0 abA	17.3 ± 4.6 aB	6.2 ± 0.2 bB
	Uninoculated	6.6 ± 0.1 abA	91.0 ± 22.0 abA	24.7 ± 8.1 aB	9.1 ± 1.0 aB
50% MD	AMF	6.8 ± 0.1 bA	65.0 ± 6.0 aB	18.5 ± 4.6 bB	7.9 ± 0.1 bA
	AMF+PGPR	7.1 ± 0.1 aA	69.0 ± 4.0 aB	26.3 ± 3.5 bB	12.0 ± 2.4 aA
	PGPR	6.5 ± 0.1 bcA	69.0 ± 17.0 aB	42.7 ± 5.5 aB	10.3 ± 0.3 abA
	Uninoculated	6.5 ± 0.0 cA	55.0 ± 2.0 bB	41.7 ± 4.7 aB	10.9 ± 0.5 aA
80% ND	AMF	7.0 ± 0.0 aA	63.0 ± 6.0 abB	38.0 ± 3.6 cA	11.1 ± 0.7 bA
	AMF+PGPR	6.7 ± 0.1 bA	60.0 ± 3.0 abB	64.1 ± 5.4 bA	9.3 ± 0.2 cA
	PGPR	6.7 ± 0.1 bA	64.0 ± 2.0 aB	39.4 ± 8.5 cA	12.3 ± 1.0 aA
	Uninoculated	6.8 ± 0.0 bA	58.0 ± 2.0 bB	114.2 ± 46.5 aA	10.7 ± 0.7 bA

*Uppercase letters compare differences in water-holding capacity, while lowercase letters compare differences according to inoculant types by Tukey's test at 5% ($p \leq 0.05$). Mean values are followed by standard errors ($n = 3$). SD: severe drought, MD: moderate drought, ND: no drought.

Table 3
Total soil bacterial abundance (gene copy number g soil^{-1}) over sampling time based on the qPCR of 16 S rRNA gene, considering the water-holding capacities (WHC), type of inoculum (arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR), and consortium of AMF and PGPR), and soil sampling taken 15, 25, and 35 days after sowing (DAS).

WHC	Inoculum	Sampling		
		15 DAS	25 DAS	35 DAS
30% SD	AMF	$3.20 \times 10^9 \pm 7.30 \times 10^8$ abA	$2.90 \times 10^9 \pm 1.40 \times 10^9$ aA	$2.00 \times 10^9 \pm 3.10 \times 10^8$ aA
	AMF+PGPR	$3.30 \times 10^9 \pm 7.80 \times 10^8$ abAB	$3.10 \times 10^9 \pm 1.60 \times 10^9$ aA	$1.40 \times 10^9 \pm 2.00 \times 10^8$ aB
	PGPR	$4.10 \times 10^9 \pm 1.20 \times 10^9$ aA	$4.50 \times 10^9 \pm 4.60 \times 10^7$ aB	$1.60 \times 10^9 \pm 5.10 \times 10^8$ aA
	Uninoculated	$1.90 \times 10^9 \pm 4.50 \times 10^7$ bA	$3.20 \times 10^9 \pm 4.40 \times 10^8$ aA	$2.00 \times 10^9 \pm 6.30 \times 10^8$ aAB
50% MD	AMF	$3.10 \times 10^9 \pm 9.60 \times 10^8$ abA	$4.10 \times 10^9 \pm 8.60 \times 10^7$ bA	$1.30 \times 10^9 \pm 2.30 \times 10^8$ aAB
	AMF+PGPR	$3.80 \times 10^9 \pm 1.70 \times 10^9$ aA	$2.90 \times 10^9 \pm 8.60 \times 10^8$ bA	$1.50 \times 10^9 \pm 3.10 \times 10^8$ aB
	PGPR	$2.00 \times 10^9 \pm 2.60 \times 10^7$ abB	$8.00 \times 10^9 \pm 5.00 \times 10^9$ aA	$1.50 \times 10^9 \pm 1.40 \times 10^8$ aA
	Uninoculated	$1.90 \times 10^9 \pm 2.20 \times 10^8$ bA	$2.10 \times 10^9 \pm 1.80 \times 10^8$ bA	$1.30 \times 10^9 \pm 1.80 \times 10^8$ aB
80% ND	AMF	$1.40 \times 10^5 \pm 6.30 \times 10^4$ bB	$2.90 \times 10^9 \pm 1.40 \times 10^8$ aA	$1.20 \times 10^9 \pm 2.10 \times 10^8$ cB
	AMF+PGPR	$2.20 \times 10^9 \pm 4.30 \times 10^8$ aB	$2.70 \times 10^9 \pm 3.30 \times 10^8$ aA	$2.90 \times 10^9 \pm 2.30 \times 10^8$ aA
	PGPR	$1.50 \times 10^9 \pm 7.40 \times 10^8$ abB	$2.20 \times 10^9 \pm 4.30 \times 10^8$ aB	$1.90 \times 10^9 \pm 6.70 \times 10^8$ bcA
	Uninoculated	$1.50 \times 10^9 \pm 6.00 \times 10^8$ abA	$4.80 \times 10^9 \pm 2.80 \times 10^9$ aA	$2.50 \times 10^9 \pm 7.50 \times 10^8$ abA

*Uppercase letters compare differences in water-holding capacity, while lowercase letters compare differences according to inoculant types by Tukey's test at 5% ($p \leq 0.05$). Mean values are followed by standard errors ($n = 3$). SD: severe drought, MD: moderate drought, ND: no drought

Table 4
Total soil mycorrhizal abundance (gene copy number g soil^{-1}) over sampling time based on the qPCR using FLR3 and FLR4 primers, considering the water-holding capacities (WHC), type of inoculum (arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR), and consortium of AMF and PGPR), and soil sampling taken 15, 25, and 35 days after sowing (DAS).

WHC	Inoculum	Sampling		
		15 DAS	25 DAS	35 DAS
30% SD	AMF	$3.78 \times 10^3 \pm 2.20 \times 10^3$ aA	$1.75 \times 10^3 \pm 1.04 \times 10^2$ aB	$1.04 \times 10^5 \pm 9.15 \times 10^4$ aB
	AMF+PGPR	$2.89 \times 10^3 \pm 9.07 \times 10^2$ abA	$2.52 \times 10^3 \pm 6.82 \times 10^2$ aB	$1.20 \times 10^5 \pm 5.30 \times 10^4$ aA
	PGPR	$1.84 \times 10^3 \pm 4.01 \times 10^2$ abA	$1.55 \times 10^3 \pm 5.57 \times 10^2$ aB	$2.15 \times 10^4 \pm 1.20 \times 10^4$ aA
	Uninoculated	$1.12 \times 10^3 \pm 1.65 \times 10^2$ bA	$1.49 \times 10^3 \pm 1.70 \times 10^2$ aA	$1.02 \times 10^4 \pm 2.98 \times 10^3$ aA
50% MD	AMF	$5.63 \times 10^3 \pm 4.14 \times 10^3$ aA	$2.59 \times 10^3 \pm 7.75 \times 10^2$ aB	$2.36 \times 10^5 \pm 1.82 \times 10^5$ aA
	AMF+PGPR	$2.22 \times 10^3 \pm 1.90 \times 10^2$ bAB	$1.86 \times 10^3 \pm 3.64 \times 10^2$ aB	$3.65 \times 10^4 \pm 1.52 \times 10^4$ bAB
	PGPR	$1.10 \times 10^3 \pm 1.35 \times 10^2$ bA	$1.42 \times 10^3 \pm 3.32 \times 10^2$ aB	$1.50 \times 10^4 \pm 2.65 \times 10^3$ bA
	Uninoculated	$1.35 \times 10^3 \pm 7.21 \times 10^1$ bA	$1.78 \times 10^3 \pm 2.51 \times 10^2$ aA	$6.83 \times 10^3 \pm 5.49 \times 10^2$ bA
80% ND	AMF	$8.49 \times 10^2 \pm 2.86 \times 10^2$ aB	$9.71 \times 10^3 \pm 1.83 \times 10^3$ bA	$4.77 \times 10^4 \pm 3.97 \times 10^4$ aB
	AMF+PGPR	$7.28 \times 10^2 \pm 4.33 \times 10^1$ aB	$1.87 \times 10^4 \pm 4.42 \times 10^3$ aA	$1.05 \times 10^4 \pm 6.01 \times 10^2$ aB
	PGPR	$1.48 \times 10^3 \pm 1.94 \times 10^2$ aA	$7.26 \times 10^3 \pm 1.75 \times 10^3$ bA	$6.83 \times 10^3 \pm 5.52 \times 10^2$ aA
	Uninoculated	$2.00 \times 10^3 \pm 5.64 \times 10^2$ aA	$3.46 \times 10^3 \pm 1.52 \times 10^3$ cA	$5.66 \times 10^3 \pm 2.48 \times 10^2$ aA

*Uppercase letters compare differences in water-holding capacity, while lowercase letters compare differences according to inoculant types by Tukey's test at 5% ($p \leq 0.05$). Mean values are followed by standard errors ($n = 3$). SD: severe drought, MD: moderate drought, ND: no drought

4. Discussion

Inoculated plants outperformed the uninoculated plants in terms of ^{33}P uptake, especially under drought conditions. Still, dual-inoculation or PGPR inoculation showed higher efficiency under severe drought compared to individual inoculation of AMF, which was more efficient under moderate drought. This was observed equally in instances where the measured plant growth variables of plants inoculated with either AMF or PGPR were higher when compared to co-inoculated plants. Therefore, considering our one-to-one model, the results indicate that the PGPR (*Bacillus* sp.) used here did not act as a mycorrhizal helper bacteria under moderate drought, at least not considering the time of evaluation. Thus, we partially rejected our initial hypothesis that the co-inoculation of AMF and PGPR enhances plant nutrient acquisition in treatments under any drought.

Nevertheless, we found that the co-inoculation (*Rhizophagus clarus* and *Bacillus* sp.), under severe drought, enhanced ^{33}P uptake 2.4-fold more intensely, than individual inoculation of *R. clarus*. In a similar approach, Battini et al. (2017) found that AMF inoculated plants showed higher ^{33}P uptake when also co-inoculated with bacteria. Karimi et al., (2017, 2018) also demonstrated the benefits of dual inoculation (AMF and PGPR) for phytoremediation of Pb-contaminated soils, showing that inoculated plants outperform non-inoculated ones in terms of growth and photosynthetic parameters. Furthermore, Hestrin et al. (2022) demonstrated that AMF inoculation has a protective effect on bacterial communities exposed to water limitation, thus ensuring plant growth and nutrition in droughted soils. Other studies have also demonstrated this strong synergistic effect between mycorrhizae and bacteria on plant biomass production (Larimer et al., 2014; Zhou et al., 2022). According to Jiang et al. (2021), mycorrhizae can control the interaction with the bacteria and actively recruit, transport and stimulate them to mineralize organic nutrients with benefit to the fungi, mainly in a region called mycorrhizosphere (a microhabitat in soil where plant roots are surrounded by fungal hyphae; Johansson et al., 2004). Conversely, the bacteria benefit from the release of carbon in hyphal exudates, representing cooperation between them, becoming allied with the symbiosis between the AMF and the plant root.

It is a well-known and proven response that the efficiency of this microbial synergism varies substantially with species identity of both fungi and plants, host phenology, soil nutrients or root exudation, which significantly impacts the rhizosphere and mycorrhizosphere microbial community (Pauwels et al., 2020; Jongen et al., 2022; Pérez-Castro, . et al., 2019; Ulrich et al., 2019). In our study, due to the lack of indigenous microorganisms in the soil system, only straightforward interactions between *Rhizophagus clarus* and *Bacillus* sp. were addressed, and therefore, most beneficial effects involving complex interactions harboured in the rhizosphere could not be exhibited, and different results can be obtained when these inoculants are challenged under

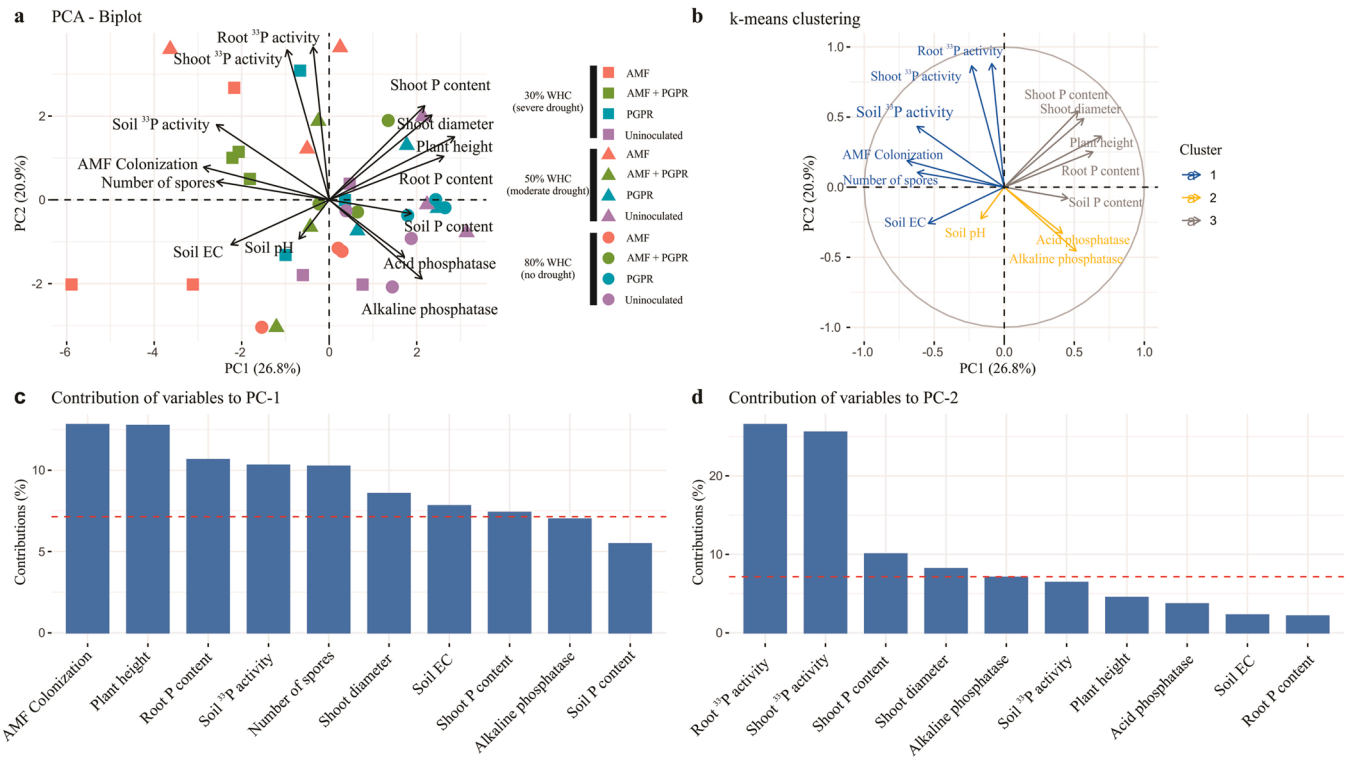

Fig. 4. Principal component analysis (PCA), (a) displaying relationships between the variables evaluated and treatments in the planted/inoculated compartment at three contrasting soil water-holding capacities (WHC). Equally, other factors, as different types of inoculum (arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR), and consortium of AMF and PGPR) were evaluated. (b) k-means clustering algorithm ordination, clustering similar variables based on an unsupervised machine learning method. (c and d) specific contribution of the variables to the main principal components (i.e., PC-1 and PC-2) of PCA. The red dashed line on the graph above indicates the expected average contribution. The third principal component explained 15% of the data variation.

realistic field conditions.

Although we have evidence of the ability of co-inoculation to increase ^{33}P uptake, this seems not to be related with the potential of drought mitigation effects on maize growth, as we did not observe increases in biomass production. Therefore, higher ^{33}P plant uptakes were not translated necessarily into higher growth. However, increase in biomass was present with individual inoculation of bacteria under severe drought. We argue that the absence of the beneficial effect of AMF on plant growth could be related to their rapid root colonization process, i.e., colonization of the plant root. There is a close association of the fungal life cycle with those of the plants, in which AMF sporulation happens at the end of the plant growth cycle, or when the plant growth slows down. Despite this, sporulation of the experimental AMF was observed at 35 days of plant growth when the plant was still in its vegetative phase, implying a significant carbon sink in the host plants (Smith and Read, 2008). This was also demonstrated by the results showing that AMF invested more in the production of hyphae and vesicles, in addition to the spore production, under severe and moderate stress conditions.

On the other hand, it is postulated, that the determination of C allocation to different fungal structures is driven by the severity of drought stress. Furthermore, the effectiveness of the plant–AMF interaction can lead to a plant physiological improvement and, consequently, to a higher C supply to the fungi even under drought stress, with severe drought increasing the C allocation to hyphae, and moderate drought to vesicles, and no stress investing in arbuscules (Kiers et al., 2011; Jongen et al., 2022). Likewise, in our study we observed the prevalence of arbuscules under no drought. Since, overall, arbuscules have a rapid turnover and are the exchange structures in mycorrhizal symbiosis (Smith and Read, 2008). Interestingly, although the highest presence of arbuscules found was under no drought stress, the highest ^{33}P uptake occurred under drought stress conditions (both severe and moderate). However, the arbuscules in the treatments under drought may have been formed and were active during earlier stages of the plant–AMF interaction, mainly due to the moment of water shortage experienced, explaining the observed AMF effects on the ^{33}P uptake.

In our study, we observed a higher ^{33}P uptake in shoots and roots of PGPR-inoculated maize plants than in AMF-inoculated plants under severe water-stress. Therefore, the use of bacteria as an agent mitigating the water stress seems to be more effective rather than using AMF, at least under the situation simulated in our investigation. Still, the second inoculation that took place 21 days after sowing (DAS) would potentiate these results, and, at this point, we are gathering information on the several types of inoculation of bacteria screened from a harsh environment to mitigate water shortage in soil, and the results brought about, will shed light on upon it (Mawarda et al., 2020).

The assessment of soil enzymes is crucial to understand the potential functioning response of the plant-microbe system since they are involved in the nutrient cycling. Thus, acid and alkaline phosphatase activities strongly control the biotic pathways of phosphorus (Margalef et al., 2017). Here, we observed an inverse relationship between enzyme activity and ^{33}P uptake by plants, which was somewhat expected, since the P source considered in our study (mono-potassium phosphate [KH_2PO_4]) is an inorganic compound, and the production of these enzymes in the soil is used to perform the acquisition of phosphate ions from organic molecules. Indeed, the high presence of inorganic P can repress the expression of *pho* genes, inhibiting soil phosphatase activities (Janes-Bassett et al., 2022).

In general, we observed that there was an increase in soil acid phosphatase activity with increasing soil water content, especially for the uninoculated treatment, which may be due to the high demand for P by plants since there was no microbial inoculation to facilitate the ^{33}P uptake. This result is interesting, considering that plants, although developing their adaptation to alleviate most biotic and abiotic stresses

in nature, also rely on their microbial partners when they are present to absorb nutrients such as P (Hassani et al., 2018). In general, for soil alkaline phosphatase, the lowest activity was found in the presence of individual inoculation with AMF, which may be related to the facilitation of phosphorus nutrition promoted by AMF via hyphal network, which reflects the ^{33}P uptake results obtained in our experiments.

Regarding changes in soil pH and electrical conductivity (EC), our results showed that the EC was dependent on water content, whereas pH was not affected. Soil EC increased with decreases in the soil water content, which may be due to other factors, since soil EC is also modulated by a combination of soluble salts, and soil temperature, for example (Bai et al., 2013). Pankaj et al. (2020), using PGPR to improve plant growth and crop yield of *Bacopa monnieri* (L.) Nash observed that soil EC and pH decreased in inoculated soils. Likewise, Al-Enazy et al. (2018) demonstrated that the inoculation of maize plants with *Azotobacter chroococcoccum*, *Bacillus megaterium* or *Pseudomonas fluorescens* also decreased soil EC and pH.

Over time, we observed an increase in the bacterial abundance from 15 DAS to 25 DAS, which may be attributed to the fact that soil bacterial inoculation occurred at 21 DAS, to assist the plants when the water-holding capacity decreased during a severe drought. After that, the bacteria abundance decreased from 25 DAS to 35 DAS, indicating a transient rather than persistent effect. At the same time, i.e., 25 DAS and 35 DAS, we observed an increase in AMF abundance under moderate drought, evidencing the complementary and synergistic effects provided by *R. clarus* and *Bacillus* sp. According to Mawarda et al. (2020), when inoculants are delivered into soil, they have low persistence due to a combination of high levels of competition from the native microbial community and a lack of available resources (e.g., C, water, nutrients). This transient effect, however, does not necessarily imply a lack of lasting legacy on plant growth. Therefore, the inoculant effects may not necessarily be due to the size of the inoculant populations, since various changes in soil community structure and functioning can be found, even though the number of inoculant cells declined following introduction into the soil. Florio et al. (2017) using rhizosphere soil from an experiment with maize seeds inoculated with *Azospirillum lipoferum* CRT1, under field conditions, could not detect the inoculated strain by qPCR at 37 DAS. Indeed, these authors maintain that, in general, bacterial inoculants stimulate root growth and modify plant metabolism at very early stages, and generate lasting effects on the root system, disappearing quickly, usually after a few weeks. Likewise, Silva et al. (2021), using bulk soil from an experiment with sugarcane, inoculated with a bacterial consortium, observed that bacterial abundance remains constant over time, while changes occur in its composition and functions.

In our study, a higher bacterial abundance was detected in the AMF treatments, even though the spores were previously disinfected, suggesting that the bacteria were located inside the spore walls. Indeed, bacteria belonging to the order *Bacillales*, which include the *Bacillus* strain used here, are intimately associated with AMF spores, increasing the AMF activity. Furthermore, they are often embedded in the outer or inner of the spore wall layers or the microniches formed by the peridial hyphae interwoven around the spores of various *Glomus* species, now assigned as *Rhizophagus* (Walley and Germida, 1995; Filippi et al., 1998; Rouphael et al., 2015; Selvakumar et al., 2016). In addition, it is important to consider that seeds have their microbiota, which comes from the flower microbiota (so-called anthosphere) and, therefore, bacteria can reside in and on seeds (Nelson, 2018; Johnston-Monje et al., 2021). In our investigation, as the seeds were sterilized, the bacteriome that lives inside the seeds may have contributed in some way to our results. However, there is still insufficient knowledge allowing us to determine which specific bacterial species would be helping AMF, as it will strongly depend on the associated soil and plant microbiome.

Our investigation is a breakthrough in the topic of dual microbe inoculation, shedding light on the beneficial use of *Rhizophagus clarus* and *Bacillus* sp. (potential new species) to increase the ^{33}P uptake by maize plants under severe drought stress. Furthermore, given that the obtained dataset was composed of three clusters of variables according to the k-means algorithm, we concluded that AMF root colonization, soil EC, and the number of spores (first cluster) were the main drivers to explain the ^{33}P uptake, especially using AMF+PGPR under severe drought. Therefore, we reinforce that the largest synergism between mycorrhizae and bacteria were more prevalent under severe drought rather than moderate drought.

5. Conclusions

We conclude that *Rhizophagus clarus* and *Bacillus* sp. inoculation offers a potential strategy to promote nutrient acquisition by plants in the context of the increasing frequency of drought gradient. This is supported by the enhanced uptake of ^{33}P in all inoculated plants at all moisture regimes, in comparison to the uninoculated plants. The major achievements of our investigation were i) demonstrating that the synergistic response of *Rhizophagus clarus* and *Bacillus* sp. increased under severe drought conditions and ii) that the use of mycorrhizae alone in conditions of moderate drought stood out in plant ^{33}P uptake. Therefore, the microbes tested in our investigation are potential candidates for a microbial inoculant in the near future. However, we emphasize that the straightforward interactions examined in the present study under controlled conditions may not represent complex systems in the rhizosphere, which harbours many microbes. Thus, further research is suggested to better understand how the application of these microbes can affect plant responses (physiological and biochemical) under the field in various edaphoclimatic conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability

Data will be made available on request.

Acknowledgments

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CRediT authorship contribution statement

AMMS and EJBNC conceived and designed the study. AMMS, DLJ, DRC, SRC, and APAP wrote the first draft of the manuscript. AMMS, XQ, and GVLJ collected the data. AMMS and FPM performed bioinformatic analyses. AMMS and VAVPA analysed the data. AMMS and all co-authors contributed to revisions. EJBNC, DLJ and DRC revised and edited the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127350.

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