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

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HIGHLIGHTS

- Mycorrhizae and rhizobacteria led to a higher plant ^{33}P uptake under drought
- ^{33}P facilitation was modulated by the soil water content
- Consortium or only rhizobacteria were more efficient under severe drought
- Under moderate drought conditions, mycorrhizae alone stood out in plant ^{33}P uptake

31 **ABSTRACT**

32 The role of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting
33 rhizobacteria (PGPR) in delivering important ecosystem services and protecting plants
34 against biotic and abiotic stress is well recognized. Here, we hypothesized that a
35 combination of AMF and PGPR could enhance P uptake in maize plants under drought
36 stress. A microcosm experiment using mesh exclusion and a radiolabeled phosphorus
37 tracer (^{33}P) was established using three types of inoculation: i) only AMF, ii) only PGPR,
38 and iii) a consortium of AMF and PGPR, alongside a control treatment without
39 inoculation. For all treatments, a gradient of three water-holding capacities (WHC) was
40 considered i) 30 % (severe drought), ii) 50 % (moderate drought), and iii) 80 % (optimal
41 condition, no water stress). Under severe drought conditions, the use of the consortium
42 (AMF+PGPR) or PGPR alone both increased ^{33}P uptake by 2.4-fold compared to the
43 uninoculated treatment. In contrast, under moderate drought the use of AMF promoted
44 the highest ^{33}P uptake by plants, increasing it by 2.1-fold compared to the uninoculated
45 treatment. Without drought stress, AMF showed the lowest ^{33}P uptake and, overall, plant
46 P acquisition was lower for all inoculation types when compared to the severe and
47 moderate drought treatments. Considering the water gradient, the plant physiological
48 response, such as altering signalling networks and root exudation, may have been decisive
49 in the success of the plant-microbial interaction investigated here. We found that AMF
50 colonization, soil electrical conductivity, and the number of spores were the main drivers
51 which explained plant ^{33}P uptake. In conclusion, this study demonstrates that a one-size-
52 fits-all solution for plant bio-inoculants is an unexpected outcome, where microbial
53 inoculants, whether constituted of purified strains or in consortia, are apt to vary in their
54 ^{33}P uptake efficiency according to the soil water gradient.

55 **Keywords:** Phosphate nutrition, isotope tracer, water shortage, plant symbiosis, soil-
56 dwelling microbes.

57 **INTRODUCTION**

58 According to the Food and Agriculture Organization of the United Nations (FAO),
59 drought is now recognised as the primary reason for agricultural production losses
60 globally, costing the sector USD 37 billion overall from 2008-2018. However, other
61 extreme events caused by climate change, such as floods and heatwaves, are also
62 contributing to ongoing issues with food security (FAO 2021). Therefore, improved
63 management approaches are urgently required to improve agricultural sustainability. This

64 is particularly relevant to the supply and exploitation of soil nutrients which have a finite
65 supply, such as phosphorus (P), especially given the dramatic rise in fertilizer prices over
66 the past year (Smith 2022). To overcome the combined impact of drought stress and low
67 nutrient use efficiency in cropping systems and the design of new management systems
68 requires a greater fundamental understanding of plant-soil-microbial interactions. In this
69 context, it has been highlighted that arbuscular mycorrhizal fungi (AMF) and
70 rhizobacteria may provide an environmentally friendly solution to this combined problem
71 (Mawarda et al. 2020).

72 In highly weathered soils where the exchange surfaces are dominated by aluminium
73 and iron oxides/hydroxides, a large proportion of the applied phosphate fertilizer (ranging
74 from 15-30 %) becomes rapidly immobilized on the solid phase by adsorption and
75 precipitation processes (Dhillon et al. 2017; Zavaschi et al. 2020). AMF may provide a
76 tool to exploit native soil P reservoirs or residual fertiliser-derived P ("legacy P"), that
77 has accumulated over the past 50 years in these soils (Scrase et al. 2019; Pavinato et al.
78 2020a; Pavinato et al. 2020b). AMF is a key group of soil microorganisms that form
79 symbiotic associations with more than 80 % of all land plants and play an important role
80 in the acquisition of nutrients (Smith and Read 2010). For example, in maize (*Zea mays*
81 L.), AMF is more important than root hairs for seedling growth under low P availability
82 (Ma et al. 2021).

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

95 Some studies have demonstrated the ability of AMF or PGPR alone to promote plant
96 growth under water shortage events. It has been postulated that AMF mechanisms, such
97 as improvements in soil aggregation, photosynthetic efficiency, and nutrient uptake are

98 primarily responsible for this response (Ji et al. 2019; Quiroga et al. 2019; Al-Arjani et
99 al. 2020). In the case of PGPR, the main purported mechanisms include direct (e.g.,
100 changes in hormonal signalling, P solubilisation, biological nitrogen fixation) and indirect
101 mechanisms (e.g., antibiotic production, cell wall degrading enzymes, induced systemic
102 resistance, osmotic adjustment, quorum quenching, and siderophores production) (Glick
103 2012; Olanrewaju et al. 2017; Naylor and Coleman-Derr 2018; Araújo et al. 2020).
104 Overall, the majority of crop plant species are responsive to mycorrhizal symbiosis
105 and rhizobacteria inoculation. This discovery has subsequently led to the search for novel
106 microbes with the potential to increase crop yields, especially maize, as this represents
107 one of the most important global crops (Zhao et al. 2017; Li et al. 2021). Recently, the
108 use of maize has become more inviting due to the current scenario of bio-economy, which
109 has incentives for production of biofuels to reduce CO_2 emissions. Thus, there is the
110 possibility to intensify the biofuels market, making it more prosperous and stable (Eckert
111 et al. 2018).
112 A myriad of early studies (Rhodes and Gerdemann 1975; Jakobsen et al. 1992; Pearson
113 and Jakobsen 1993; Battini et al. 2017; Jongen et al. 2022) have demonstrated the ability
114 of AMF hyphae to recover and translocate $^{32/33}P$ located beyond the immediate root zone.
115 Nevertheless, these studies do not consider how the efficiency of AMF-mediated P
116 absorption is affected under a gradient of soil water availability, especially when
117 considering the presence of PGPR capable of tolerating low water activity. Here, our
118 investigation was set up to test the hypothesis that the combined use of AMF and PGPR
119 could enhance P uptake in maize plants under drought stress. For this, we evaluated ^{33}P
120 uptake in maize plants, the response of soil phosphatase activity, and key soil chemical
121 attributes, as well as monitoring the abundance and dynamics of soil mycorrhizal and
122 bacterial communities.

123 **MATERIAL AND METHODS**

124 **Experimental design**

125 The microcosm experiment was set up in a completely randomized design, comprising
126 a double factorial scheme (4 × 3) with three replicates. The first factor was the inoculation
127 of microorganisms (either only AMF, only PGPR, or the consortium of AMF and PGPR,
128 besides a control without any inoculation). The second factor was water stress (80, 50,
129 and 30 % of the water-holding capacity, simulating no drought, moderate drought, and
130 severe drought, respectively). Each experimental unit comprised a plastic pot (8 cm

131 internal diameter × 7 cm high), containing 200 g (dry weight) of sterilized soil (at 121 ºC
132 for 2 h) (Figure 1A and 1B). The soil was obtained from the Ah horizon (0-10 cm deep)
133 of a field site located in Brazil (22°42' S, 47°38' W) and classified as an Arenosol (WRB-
134 FAO 2015), with low P content (Supplementary Table 1).

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

145 The fertilized compartment received 2.8 mL of 6 mM KH_2PO_4 (equivalent to 30 mg P
146 kg^{-1} soil, as recommended for this type of weathered soil; van Raij et al. 1997) with a ^{33}P
147 activity of 185 kBq after the soil acclimatization, sowing of seeds and inoculation of
148 microbes in the planted/inoculated compartment (Figure 1C). Furthermore, we set up an
149 additional microcosm experiment to determine the distance that phosphorus can diffuse
150 in the soil to confirm that the presence of ^{33}P in the planted/inoculated compartment could
151 only occur via microbial transfer. In addition, we also measured P sorption to the soil to
152 characterize the P-dynamics in this soil (Supplementary Note 2).

153 **Plant material, cultivation, and water-holding capacity management**

154 Uniformly sized seeds of *Zea mays* L. (cv. BRS Gorotuba) were surface sterilized
155 twice in 2 % (v/v) sodium hypochlorite solution for 7 min, 70 % (v/v) ethanol for 1 min,
156 and rinsed thoroughly with sterile MilliQ water. The germination rate of seeds in soil and
157 Petri dishes was around 90 %. Seeds inoculated with PGPR or for consortium treatments
158 were soaked in the bacterial suspension for 2 h, whilst seeds for AMF and uninoculated
159 treatments were soaked in 0.85 % (w/v) saline solution for the same time (Kavamura et
160 al. 2013). Two seeds were sown in each pot and thinning was done when one of the
161 seedlings presented two true leaves. Soil bacterial inoculation was performed 21 days
162 after sowing, in PGPR or consortium treatments, when pots were reduced to 30 % of the
163 water-holding capacity according to the water content management described below. At

164 the same time, 0.85 % (w/v) saline solution was applied to the AMF, and uninoculated
165 treatments

166 Plants were cultivated in a controlled environment chamber (Conviron Adaptis® CPM
167 6010) at the Environment Centre Wales, Bangor University, United Kingdom (53º13' N,
168 4º7' W), under a day/night cycle of 16/8 h, 25/20 °C, 70 % relative humidity, receiving
169 artificial lighting at a photosynthetic photon flux density of 500 µmol m^{-2} sec^{-1}. Hoagland
170 solution (without phosphorus) was applied 17 days after sowing, to keep nutritional
171 balance of the plants. The final solution of pH 5.5 was composed of 4 mM $Ca(NO_3)_2$, 6
172 mM KNO_3, 2 mM $MgSO_4$, 1 mM Fe-EDTA, and 1 mM trace elements (Hoagland and
173 Arnon 1950).

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

183 After 35 days of growth, the plants were harvested and separated into above and
184 belowground material, whilst the soil was separated into fertilized and planted/inoculated
185 compartments. In addition, soil was sampled in the planted/inoculated compartment five
186 days after changing the water holding capacity (i.e., on the 15th and 25th day after sowing)
187 to monitor soil bacteria and mycorrhiza total abundance via quantitative polymerase chain
188 reaction (qPCR).

189 **Fungal and bacterial inoculum**

190 Fungal and bacterial strains were previously isolated from Serra do Ouricuri, Petrolina,
191 Pernambuco, Brazil (39°3' S, 8°28 W) in the Caatinga Biome, where a bio-prospecting
192 program was developed to find microbes with the potential of helping crop plants to
193 tolerate drought stress (Kavamura et al. 2013). Initially, a pool of fungi and bacteria was
194 isolated from the rhizosphere of *Tripogonella spicata* (Nees) plants, the so-called
195 resurrection grass, due to its surprising rehydration capacity (Fernandes-Júnior et al.
196 2015; Aidar et al. 2017). Then, they were selected for their plant growth-promoting

197 properties, such as the ability to grow under reduced water availability (ca. 0.9 Aw)
198 (Hallsworth et al. 1998), indole-3-acetic acid (IAA) production (Bric et al. 1991; Kuss et
199 al. 2007) and calcium phosphate solubilization (Verma et al. 2001).

200 *Bacillus* sp. was grown in 10 % (w/v) TSB (trypticase soy broth) culture medium at
201 30 °C and 150 rpm for 48 h. The inoculum was homogenized at OD_{550} = 0.2 and washed
202 twice in a 0.85 % (w/v) saline solution to obtain the bacterial suspensions (at 10^8 CFU
203 mL^{-1}). A second bacterial inoculation (3 mL pot^{-1} at 10^8 CFU mL^{-1}) was done when the
204 water-holding capacity was changed to 30 % (i.e., twenty days after sowing). The same
205 amount of saline solution was provided to the AMF and control treatments (Figure 1C).

206 *Rhizophagus clarus* spores were obtained from the pure trap culture, using maize as
207 host plant, and transferred to the soil after disinfection, directly under the seeds, in the
208 form of 5 mL of sterile water containing 50 spores at the time of sowing. The germination
209 rate of *R. clarus* spores in the soil was around 85 % according to our third additional
210 microcosm experiment, as reported in Supplementary Note 3.

211 **Analytical procedures**

212 At the end of the experiment (i.e., 35 days after sowing), the shoot was cut off at the
213 soil surface and dried at 80 ºC in paper bags for 16 h to quantify dry weight. The roots
214 were separated from soil by gently shaking them and rinsing them with water. Then, 5 g
215 of roots were placed in a Falcon tube with 70 % ethanol for analysis of mycorrhizal
216 colonization percentage, and the remainder was dried to obtain the dry weight. For
217 phosphorimager analysis to visualize the ^{33}P, dried plant tissue was placed in a 20 × 25
218 cm cassette for 1 h, and then analyzed in a Bio-Rad Molecular Imager® FX.

219 Soil available phosphorous was extracted using 0.5 M acetic acid (1:5 w/v) according
220 to Fisher et al. (1998). Samples were extracted by shaking (200 rpm for 30 min at room
221 temperature), centrifuging for 15 min (18,000 *g*), filtering and the supernatant was
222 recovered for analysis. ^{33}P activity of the samples was determined in counts per minute
223 (CPM) of ^{33}P using 1 mL of soil extract and 4 mL of HiSafe 3 Scintillation cocktail
224 (PerkinElmer Inc., Waltham, MA, USA) and analyzing in a liquid scintillation counter
225 with an automated quench correction (Wallac 1404 liquid scintillation counter,
226 PerkinElmer Inc). For plant tissue, the extract was obtained by placing 0.2 g in a muffle
227 furnace and ashing at 500°C overnight. Later, this was dissolved in 1 mL of 20 % HCl
228 and 9 mL of deionized water was added, according to Adrian (1973). The colorimetric P

229 determination in soil and plant tissue was determined according to Murphy and Riley
230 (1962).
231 The soil pH and electrical conductivity were determined according to Thomas (1996),
232 whereby 10 g of 2 mm-mesh sieved soil was mixed with 25 ml of water, placing it on an
233 orbital shaker for 10 min at 200 rpm, and then allowing the sample to settle for 10 min
234 before taking measurements with standard electrodes.
235 The acid and alkaline phosphatase activities (EC 3.1.3.2 and EC 3.1.3.21, respectively)
236 were measured using the methodology described by Marx et al. (2001). For evaluation of
237 AMF root colonization, the roots were prepared according to Vierheilig et al. (1998), with
238 the roots dispersed in a Petri dish with a grid background and scored using a
239 stereomicroscope according to Giovannetti and Mosse (1980).

240 **Molecular analysis**

241 Soil (0.25 g) was utilized for DNA extraction using DNeasy® PowerSoil® Pro Kit
242 (QIAGEN Inc., Germany) according to the manufacturer's protocol. Extracted DNA was
243 stored at -80 °C before quantitative PCR analysis. DNA concentrations were determined
244 using the Qubit quantification platform with Qubit 1X dsDNA HS Assay Kit (Invitrogen,
245 Carlsbad, CA, USA).
246 The quantitative PCR (qPCR) was used to determine gene copy number per gram of
247 soil for bacteria (16S rRNA) and AMF (LSU rDNA region), using the StepOnePlus™
248 Real-Time PCR System (Applied Biosystems Inc., Carlsbad, CA, USA) with the
249 fluorescent marker GoTaq® qPCR Master Mix (Promega, Madison, WI, USA). All
250 samples were analysed in triplicate.
251 16S rRNA reactions were run in 10 µL comprising 5 µL of GoTaq® qPCR Master Mix,
252 received 1 µL (5 µM) of each primer (Eub338 5'-CCTACGGGAGGCAGCAG-3' and
253 Eub518 5'-ATTACCGCGGCTGCTGG-3'), 0.1 µL de CXR Reference Dye, 2 µL of
254 DNA template, and 0.9 µL nuclease free sterile water in the same conditions as described
255 by Muyzer et al. (1993). Standard curves were obtained using 7-fold serial dilutions of
256 purified PCR (10^2 to 10^8 copies) containing the targeted gene. The reliability of the
257 standard curves was controlled by verifying reproducibility of the Ct values, the quality
258 of the dilution series, and the efficiency (101.42 %, R^2 = 0.993). The specificity of the
259 primers was confirmed by melting curves analysis.
260 AMF reactions were run in 10 µL comprising 5 µL of GoTaq® qPCR Master Mix,
261 received 1 µL (5 µM) of each primer (FLR3 5'-TTGAAAGGGAAACGATTGAAG T-

262 3’ and FLR4 5’-TAC GTCAACATCCTTAACGAA-3’), 0.1 µL de CXR Reference Dye,
263 2 µL of DNA template, and 0.9 µL free sterile water. FLR3 is localized between the D1
264 and D2 domains of LSU rRNA, whilst FLR4 is in the D2 domain (Gollotte et al. 2004).
265 Standard curves were obtained using 7-fold serial dilutions of purified PCR (10^2 to 10^8
266 copies) containing the targeted gene. The reliability of the standard curves was controlled
267 by verifying reproducibility of the Ct values, the quality of the dilution series and the
268 efficiency (101.46 %, R^2 = 0.975). The specificity of the primers was confirmed by
269 melting curves analysis.

270 **Data analyses**

271 Data were tested for normal distribution using the Shapiro-Wilk test, followed by the
272 homogeneity of variances tests, using the Bartlett test. Having met the criteria (residuals
273 normality and variance homoscedasticity), a two-way analysis of variance (ANOVA) was
274 performed and, when appropriate, Tukey’s posthoc pairwise comparison (cut-off
275 significance at $p < 0.05$) was applied to determine individual differences between means.
276 Principal components analysis (PCA) was performed using the statistical packages
277 *FactoMineR* and *factoextra* in the R® program (R Core Team 2017). In PCA, to meet the
278 premise of multivariate normality, the data were transformed into log (x+1) and the
279 attributes subject to collinearity were removed (Ramette 2007). Additionally, using k-
280 means clustering algorithm, an unsupervised machine learning method of identifying and
281 grouping similar data points, we classified our variables into groups (Jansson et al. 2022).

282 **RESULTS**

283 In both soil compartments (i.e., planted/inoculated and fertilized compartments) we
284 determined their ^{31}P and ^{33}P content alongside soil pH, electrical conductivity, and soil
285 acid and alkaline phosphatase activity. In addition to plant P content, AMF root
286 colonization and number of spores for the planted/inoculated compartment were
287 determined. Here, we primarily concentrate on the results from the planted/inoculated
288 compartment, while results from the fertilized compartment are presented in the
289 supplementary material. Data is reported on P uptake by arbuscular mycorrhizal hyphae,
290 as revealed by the use of radioactive P (^{33}P), added to fertilized compartment.

291 **^{33}P uptake by plants, biomass, and soil P content**

292 Soil water content greatly affected ^{33}P uptake by the plants, with the highest ^{33}P uptake
293 (0.4 $kBq.plant^{-1}$, on average) observed in moderate drought (50 % WHC) with the lowest
294 uptake (0.2 $kBq.plant^{-1}$, on average) observed in the absence of drought (80 % WHC)
295 (Figure 2A and 2B). Overall, for the planted/inoculated compartment, the highest levels
296 of ^{33}P activity in soil were observed in the presence of mycorrhizal inoculum under severe
297 or moderate water stress, but this was not observed in the fertilized compartment
298 (Supplementary Figures 1A and 1B). In addition, for shoot biomass, the main difference
299 among the inoculum types occurred in severe drought, with the highest biomass found in
300 the presence of bacterial inoculum. This same pattern could be detected for morphological
301 traits, such as height and diameter (Supplementary Figure 2).

302 Under severe drought (30 % WHC), ^{33}P uptake in shoot was 2.4-fold greater ($p \leq 0.05$)
303 in the PGPR and AMF+PGPR treatments than in uninoculated control. Whilst under
304 moderate drought (50 % WHC), ^{33}P uptake in shoot of AMF treatment was 2.1-fold
305 greater ($p \leq 0.05$) than uninoculated control, outperforming the other inoculation types.
306 On the other hand, under optimal conditions (80 % WHC), the highest ($p \leq 0.05$) ^{33}P
307 uptake in shoot was found in AMF+PGPR and the lowest ($p \leq 0.05$) in AMF treatment
308 (Figure 2A, Supplementary Figure 3). The same pattern of ^{33}P uptake was observed in the
309 root (Figure 2B, Supplementary Figure 4).

310 The total shoot P content was modulated by the water-holding capacity and inoculation
311 type, with the lowest values ($p \leq 0.05$) observed under severe drought (18.6 µg P $plant^{-1}$,
312 on average) and the highest values ($p \leq 0.05$) under moderate drought (28.3 µg P $plant^{-1}$,
313 on average), almost reflecting the results of those for ^{33}P uptake (Figure 2C). However,
314 this did not occur in the roots, in which there was an increase in P content with the increase
315 of water content (Figure 2D). Under severe drought, the highest shoot P content was
316 observed in the PGPR treatment ($p \leq 0.05$), whilst under moderate drought the
317 uninoculated control was, in general, superior to the other inoculation types. Under no
318 water stress, shoot P content in AMF+PGPR and PGPR was 1.7 and 1.5-fold greater than
319 in the AMF treatment (Figure 2C). Overall, soil P contents were higher in the
320 planted/inoculated compartment than in the fertilized compartment (Supplementary
321 Figures 1C and 1D).

322 **Mycorrhizal root colonization and number of spores in soil**

323 AMF root colonization was higher under severe (20.9 %, on average) and moderate
324 water stress (21.8 %, on average) than in no drought (6.4 %, on average). Under severe

325 drought, the AMF treatment showed a higher ($p \leq 0.05$) AMF root colonization
326 percentage (56.7 ± 7.4) than the AMF+PGPR treatment (26.7 ± 16.1). Whilst under
327 moderate and no drought, there was no difference ($p > 0.05$) between them (Figure 3A).
328 The number of spores differed only between the AMF and AMF+PGPR treatments under
329 severe drought ($p \leq 0.05$), where the highest value was found in the AMF treatment (18
330 ± 6). Overall, the water-holding capacity did not influence ($p > 0.05$) the number of spores
331 (Figure 3B).

332 According to the microscopy results, a different pattern in WHC response occurred in
333 the presence of AMF structures inside the roots. Considering a severe drought in the AMF
334 treatment, there was a large presence of hyphae (60 %), whilst in the AMF+PGPR
335 treatment, we observed only about 40 % of hyphae and 20 % of vesicles (Figure 3C and
336 3D, respectively). On the other hand, under a moderate drought, for AMF treatment, there
337 was a higher presence of vesicles (50 %), hyphae (35 %), and arbuscules (10 %) (Figure
338 3E). Considering the AMF+PGPR treatment, there was the presence of spores inside the
339 root (20 %), as well as hyphae (20 %) and vesicles (30 %) (Figure 3F). Without water
340 stress, in the AMF treatment, there was a higher presence of arbuscules (25 %), while in
341 the AMF+PGPR we only noticed the presence of hyphae (20 %) (Figure 3G and 3H,
342 respectively).

343 **Soil pH and electrical conductivity, soil phosphatases activity, and microbial**
344 **monitoring over sampling time**

345 The water-holding capacity did not influence the soil pH ($p > 0.05$), but it did influence
346 ($p \leq 0.05$) the soil electrical conductivity (EC). The highest soil EC values were found
347 under severe drought (86.1 $\mu S.m^{-1}$, on average), and the lowest for moderate (64.7 $\mu S.m^{-}$
348 ^{1}, on average) and no drought (61.4 $\mu S.m^{-1}$, on average). A subtle difference was found
349 in soil pH between inoculum types, within the same soil water-holding capacity.
350 Compared with the other treatments, the AMF treatment showed the lowest pH value
351 under severe and moderate stress ($p \leq 0.05$), whilst non-water restriction showed the
352 highest pH value ($p \leq 0.05$). The main difference in electrical conductivity (EC) was
353 found under severe drought, where the AMF+PGPR treatment presented the lowest EC
354 (Table 1).

355 The highest soil acid phosphatase activity was found in the uninoculated treatment
356 without any water restriction, which was on average 3-fold higher than the other
357 treatments. There was no difference between treatments under severe drought, whilst

358 under moderate drought, the uninoculated and PGPR treatments showed higher values
359 compared to other inoculation types. For soil alkaline phosphatase activity, the highest
360 values were found both under moderate and non-stress conditions. An opposite behaviour
361 was observed in the AMF+PGPR treatment, in which, under moderate stress, there was
362 higher phosphatase activity, whereas in non-stress was the lowest compared to the others
363 treatment with the same water-holding capacity (Table 1). The results for pH, EC, acid,
364 and alkaline phosphatase in the fertilized compartment are presented in Supplementary
365 Table S2.

366 Overall, water-holding capacity influenced the total soil bacterial and mycorrhizal
367 abundance over time (Supplementary Table S3 and Table S4). Soil bacterial abundance
368 increased from 15 days after sowing (DAS) to 25 DAS and decreased from 25 DAS to
369 35DAS (Supplementary Table S3). Whilst mycorrhizal fungal abundance decreased from
370 15 DAS to 25 DAS (only in severe and moderate drought) and increased substantially
371 from 25 DAS to 35DAS (Supplementary Table S4). For both, bacterial and mycorrhizal
372 inoculum, the highest abundances were found under severe and moderate drought.

373 **Principal components analysis (PCA)**

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

377 The water-holding capacity and inoculum type influenced the attribute dynamics
378 according to the visualization of residuals in the PCA, which explained about 48 % in the
379 first two components (Figure 4A). Overall, fungal parameters (AMF root colonisation
380 and number of spores), and ^{33}P uptake, besides soil EC and pH, were more correlated to
381 the inoculum type under severe drought (square dots). Whilst plant parameters (biomass,
382 diameter, and height), phosphatase dynamics, and phosphorus contents were more
383 correlated with moderate stress and non-drought (triangular and circular dots,
384 respectively). Despite the dispersion within the replicates, a subtle difference was evident
385 in relation to the inoculum type, where the AMF+PGPR, only PGPR, and uninoculated
386 treatments showed better clustering than AMF treatment.

387 According to the results of the k-means clustering algorithm, three clusters of variables
388 were identified, the first being composed of AMF root colonization, soil electrical
389 conductivity, number of spores and ^{33}P activity (in soil and plant), which was more
390 correlated to AMF+PGPR treatment under severe drought. The second was composed of

391 plant parameters (diameter and height) and plant P content, while the third was composed
392 of phosphatases activity and soil pH (Figure 4B). We assessed the most important
393 variables in explaining the variability in our data set according to the contribution level.
394 The variables that contributed the most to the definition of the principal component 1
395 were AMF root colonisation (12.81 %), plant height (12.75 %), root P content (10.65 %),
396 soil ^{33}P activity (10.31 %), and number of spores (10.24 %) (Figure 4C). Whilst for the
397 principal component 2, the most important variables were root and shoot ^{33}P activity
398 (26.53 % and 25.57 %, respectively) (Figure 4D).

399 **DISCUSSION**

400 Inoculated plants outperformed the uninoculated plants in terms of ^{33}P uptake,
401 especially under drought stress. The enhanced drought tolerance promoted by AMF
402 inoculated plants can be ascribed to several mechanisms. For instance, Quiroga et al.
403 (2019), when utilizing maize inoculated with *Rhizophagus irregularis*, under drought
404 conditions, demonstrated an increase in stomatal conductance in the shoots and, thereby,
405 enhanced photosynthesis. Other mechanisms were extensively detailed by Li et al. (2019),
406 Ji et al. (2019), Al-Arjani et al. (2020), Chen et al. (2021), and Zhang et al. (2021). Also,
407 we showed a synergistic effect of the consortium (AMF+PGPR), which significantly
408 increased ^{33}P uptake under severe drought. More importantly, we provide evidence that
409 AMF responsiveness in a hostile environment may be due to the presence of PGPR.
410 Therefore, our results demonstrated that the presence of AMF-helper bacteria (i.e.,
411 *Bacillus* sp. screened from an extreme environment), contributes to ^{33}P uptake and maize
412 growth. Thus, we confirmed our initial hypothesis that inoculation of AMF and PGPR
413 enhances plant nutrient acquisition in treatments with increased frequency of drought.

414 We found that either bacterial (*Bacillus* sp.) or microbial consortia (*Rhizophagus*
415 *clarus* and *Bacillus* sp.), under severe drought, enhanced ^{33}P uptake 2.4-fold more
416 intensely, than *R. clarus* alone. In a similar approach, Battini et al. (2017) found that AMF
417 inoculated plants showed higher ^{33}P uptake when also co-inoculated with bacteria.
418 According to Jiang et al. (2021), mycorrhizas can control the interaction with the bacteria
419 and actively recruit, transport and stimulate it to mineralize organic nutrients with benefit
420 to the fungi, mainly in a region called mycorrhizosphere (a microhabitat in soil where
421 plant roots are surrounded by fungal hyphae (Johansson et al. 2004)). Conversely, the
422 bacteria benefit from the released of carbon in hyphal exudates, representing cooperation
423 between them, which is allied with the symbiosis between the AMF and plant. However,

424 this cooperation seems to be closely dependent on the soil water level since, under
425 moderate drought, *R. clarus* alone showed higher ^{33}P uptake as compared to co-inoculated
426 treatment. Thus, this efficiency may vary substantially with species identity of both fungi
427 and plants, host phenology, soil nutrients or root exudation, which significantly impacts
428 rhizosphere/mycorrhizosphere microbial community (Pauwels et al. 2020; Jongen et al.
429 2022; Pérez Castro et al. 2019; Ulrich et al. 2019).

430 Although we have evidence of the ability of inoculation to increase ^{33}P uptake, this
431 may not be related to the potential of mitigation of drought effects on maize growth, since
432 we did not observe great differences in biomass production. Therefore, higher ^{33}P plant
433 uptake did not necessarily translate into higher growth. Perhaps, under highly stress
434 conditions more P is utilized by the plant to support the given conditions, such as
435 supporting the mycorrhizae-rhizobacteria interaction, whilst under less stress conditions
436 the plant can utilize the more absorbed P for growth.

437 We observed higher AMF colonization under severe and moderate water stress.
438 However, the prevalence of arbuscules was observed under no drought. Our results may
439 indicate a close association between the *R. clarus* and the maize genotype (*Zea mays* L.
440 cv. BRS Gorotuba), with the determination of C allocation to the different fungal
441 structures driven by the severity of drought stress. The effectiveness of plant–AMF
442 interaction can lead to a plant physiologic improvement and, consequently, to a higher C
443 supply to the fungi even under drought stress, with severe drought increasing the C
444 allocation to hyphae, and moderate drought to vesicles, and no stress investing in
445 arbuscules (Kiers et al. 2011; Jongen et al. 2022). Overall, arbuscules have a rapid
446 turnover and are the exchange structure in mycorrhizal symbiosis (Smith and Read 2008).
447 Interestingly, although the highest presence of arbuscules was found under no drought
448 stress, the highest ^{33}P uptake occurred under drought stress conditions (both severe and
449 moderate). However, the arbuscules in the treatments under drought would probably be
450 formed and functional during earlier stages of the plant–AMF interaction, mainly due to
451 the moment of water shortage experienced, explaining the observed AMF effects on the
452 ^{33}P uptake.

453 The assessment of soil enzymes is crucial to understand the potential functioning
454 response of the plant-microbe system since they are involved in nutrient cycling. Thus,
455 soil phosphatase activities (e.g., acid and alkaline) strongly control phosphorus biotic
456 pathways (Margalef et al. 2017). Here, we observed an inverse relationship between

enzyme activity and ^{33}P uptake by plants, which was somehow 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 (Margalef et al. 2021). Indeed, the high presence of inorganic P can repress the expression of *pho* genes, inhibiting soil phosphatase activities (Nannipieri et al. 2011).

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). Overall, for soil alkaline phosphatase, the lowest activity was found in the presence of microbial inoculation with AMF alone, 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.), observed that soil EC and pH decreased in inoculated soils. Likewise, Al-Enazy et al. (2018) demonstrated the inoculation of maize plants with *Azotobacter chroocococcum*, *Bacillus megaterium* and *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 soil bacterial inoculation that 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, 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

490 levels of competition from the native microbial community and a lack of available
491 resources (e.g., C, water, nutrients). This transient effect, however, does not necessarily
492 imply a lack of lasting legacy on plant growth. Therefore, the inoculant effects may not
493 necessarily be due to the size of the inoculant populations, since various changes in soil
494 community structure and functioning can be found, even though the number of inoculant
495 cells declined following introduction into the soil. Florio et al. (2017) using rhizosphere
496 soil from an experiment with maize seeds inoculated with *Azospirillum lipoferum* CRT1,
497 under field conditions, could not detect the inoculated strain by qPCR at 37 DAS. Indeed,
498 these authors maintain that, in general, bacterial inoculants stimulate root growth and
499 modify plant metabolism at very early stages, and generate lasting effects on the root
500 system, disappearing quickly, usually after a few weeks. Likewise, Silva et al. (2021),
501 using bulk soil from an experiment with sugarcane, inoculated with a bacteria consortium,
502 observed that bacterial abundance remains constant over time, whilst changes occur in its
503 composition and functions.

504 In our study, a higher bacteria abundance was detected in the AMF treatments,
505 even though the spores were previously disinfected, suggesting that the bacteria were
506 located inside the spore walls. Indeed, bacteria belonging to the order *Bacillales*, which
507 include the *Bacillus* strain used here, are found to be intimately associated with AMF
508 spores, increasing the AMF activity. Furthermore, they are often embedded in the outer
509 or inner of the spore wall layers or the microniches formed by the peridial hyphae
510 interwoven around the spores of various *Glomus* species, now assigned as *Rhizophagus*
511 (Walley and Germida, 1995; Filippi et al. 1998; Rouphael et al. 2015; Selvakumar et al.
512 2016). On the other hand, other authors maintain that bacteria belonging to the order
513 *Acidobacteriales* can suppress AMF activity (Svenningsen et al. 2018). In addition, it is
514 important to take into account that seeds have their microbiota, which comes from the
515 flower microbiota (so-called anthosphere) and, therefore, bacteria can reside in and on
516 seeds (Nelson, 2018; Johnston-Monje et al. 2021). In our investigation, as the seeds were
517 sterilized, the bacteriome that lives inside the seeds may have contributed in some way to
518 our results. However, there is still insufficient knowledge allowing us to determine which
519 specific bacterial species would be helping AMF, as it will strongly depend on the
520 associated soil and plant microbiome.

521 Our investigation is a breakthrough in the topic of dual microbe inoculation,
522 shedding light on the beneficial use of *Rhizophagus clarus* and *Bacillus* sp. (potential new

523 species) to increase the ^{33}P uptake by maize plants under drought stress. Furthermore,
524 given that our obtained dataset was composed of three clusters of variables according to
525 the k-means algorithm, we concluded that AMF root colonization, soil EC, and the
526 number of spores (first cluster) were the main drivers to explain the ^{33}P uptake, especially
527 using AMF+PGPR under severe drought and, therefore, reinforcing the synergism
528 between mycorrhizae and bacteria.

529 **CONCLUSIONS**

530 We conclude that *Rhizophagus clarus* and *Bacillus* sp. inoculation offers one potential
531 strategy to promote nutrient acquisition by plants in the context of the increasing
532 frequency of drought seen in most cropping regions of the world. This is supported by the
533 enhanced uptake of ^{33}P in all inoculated plants at all moisture regimes in comparison to
534 the uninoculated plants.

535 The synergistic response of *Rhizophagus clarus* and *Bacillus* sp. increased under drought
536 conditions, representing greater stress tolerance. This supports the future use of microbial
537 consortia as an inoculation technology to alleviate drought stress under future climate
538 change scenarios, as expected in the coming future, resulting in longer dry periods and a
539 decrease in rains in certain areas of the world.

540 Thus, future investigations should be carried out to test whether the microbes that have
541 emerged here under water scarcity events can respond in the same way considering the
542 presence of indigenous microbial communities. In addition, recent research has revealed
543 the potential of microbes, especially bacteria, associated with Brazilian Caatinga biome
544 plants as an inoculant in promoting plant growth under drought conditions. Therefore, the
545 microbes tested in our investigation are potential candidates for a microbial inoculant in
546 the near future, combining both bacteria and mycorrhizae.

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