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Improved soil structure and citrus growth after inoculation with three arbuscular mycorrhizal fungi under drought stress

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ABSTRACT

In a controlled potted experiment, citrus (*Poncirus trifoliata*) seedlings were inoculated with three species of arbuscular mycorrhizal (AM) fungi, *Glomus mosseae*, *G. versiforme* or *G. diaphanum*. Two soil-water levels (ample water, −0.10 MPa; drought stress, −0.44 MPa) were applied to the pots 4 months after transplantation. Eighty days after water treatments, the soils and the citrus seedlings were well colonized by the three AM fungi. Mycorrhizal fungus inoculation improved plant biomass regardless of soil-water status but decreased the concentrations of hot water-extractable and hydrolyzable carbohydrates of soils. Mycorrhizal soils exhibited higher Bradford-reactive soil protein concentrations than non-mycorrhizal soils. Mycorrhizas enhanced >2 mm, 1–2 mm and >0.25 mm water-stable aggregate fractions but reduced 0.25–0.5 mm water-stable aggregates. Peroxidase activity was higher in AM than in non-AM soils whether drought stressed or not, whereas catalase activity was lower in AM than non-AM soils. Drought stress and AM fungus inoculation did not affect polyphenol oxidase activity of soils. A positive correlation between the Bradford-reactive soil protein concentrations, soil hyphal length densities, and water-stable aggregates (only >2 mm, 1–2 mm and >0.25 mm) suggests beneficial effects of the AM symbiosis on soil structure. It concluded that AM fungus colonization enhanced plant growth under drought stress indirectly through affecting the soil moisture retention via glomalin's effect on soil water-stable aggregates, although direct mineral nutritional effects could not be excluded.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are widely beneficial fungi, symbiotically associated with higher plant roots. An increasing number of experiments has shown that AM alters plant water relations and prevents drought stress (DS) under certain conditions [3]. An improved drought tolerance may result not only from direct water supply by extraradical mycorrhizal fungal hyphae [14,24], but also from indirect mycorrhizal effects, such as an improved nutrient status [19], hormonal regulation of stomata [15], a better osmotic adjustment in AM plants [6,29,38], and increased antioxidant levels in AM

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plants [1,40,41]. However, previous studies on mycorrhizal water relations mainly focused on plant performance [4].The effects of AM fungi exhibit on soil structure under conditions of DS is known just a little.

Soil structure refers to pore space as well as to aggregates. Soil aggregate stability is a crucial soil property affecting soil sustainability, crop production, biological activity, soil carbon storage, and the movement and storage of water [2]. AM fungi and roots interacted as factors that affect soil aggregate stability, although the mechanism is not known [9,22]. Because soil aggregates regulate soil water flow [23], it seems logical to suspect that AM fungus colonization may improve the water relations of plants. Glomalin, a glycoprotein produced by AM fungi, and first reported by Wright and Upadhyaya [35], is long lived in soils [27] and tightly correlated with soil aggregate stability [25,36]. Glomalin could influence soil carbon storage indirectly by stabilizing soil aggregates [45]. The operationally defined fractions of glomalin obtained from soils are termed glomalin-related soil protein (GRSP) [25]. To better understand drought tolerance of AM plants, it appears important to include GRSP in studies on mycorrhizal effects on soil structure.

Soil enzyme activity has been proposed as potential indicator of soil quality because of their sensitivities to various environmental stresses such as DS [17]. Wang et al. [32] observed that *G. caledonium* or mixed inocula increased the activities of phosphatase and urease in the multi-metal-contaminated soils of *Elsholtzia splendens* and *Zea mays*. Little is known about soil enzymes, especially antioxidant enzyme activity responses of mycorrhizal plants in pots cultures under DS conditions.

In this paper, we analyzed the soil properties in drought-stressed citrus. Our objectives were to determine (1) whether AM symbiosis altered soil structure to improve water relations of plants according to GRSP, soil water-stable aggregates and soil carbohydrates, and (2) whether soil antioxidant enzymes were affected by AM fungi.

2. Materials and methods

2.1. Experimental design

The present experiment consisted of a randomized block design with two factors: (1) mycorrhizal treatments (*Glomus versiforme* (Karsten) Berch, *G. mosseae* (Nicol.& Gerd.) Gerdemann & Trappe, *G. diaphanum* Morton & Walker, and non-AM fungal control) and (2) soil-water status (ample water and drought stress). Each of the eight treatments was replicated six times, leading to a total of 48 pots.

2.2. Experimental set-up

The experiment was conducted over summer in a plastic greenhouse, lacking light and temperature control. The average day/night temperature was 30/24 °C during entire water treatments, and the average relative air humidity was 80%.

Seeds of trifoliate orange (*Poncirus trifoliata* (L.) Raf.), provided by the Institute of Fruit and Tea, Hubei Academy of Agricultural Science, China, were surface-sterilized with 70% alcohol for 15 min and placed on sterilized moist filter paper for germination in darkness at 28 °C. Seven days after germination, six seedlings were transplanted to a plastic pot (15 × 20 cm) containing 3.4 kg of autoclaved (121 °C, 0.11 MPa, 2 h) soils (pH 5.4, total phosphorus 0.56 g kg^{-1}, available phosphorus 18.45 mg kg^{-1}, organic matter 8.7 g kg^{-1}) collected from the Fruit Sample Garden, Huazhong Agricultural University, and inoculated with 1400 spores per pot separately with *G. versiforme*, *G. mosseae* or *G. diaphanum*. Non-AM fungal controls received the sterilized (121 °C, 0.11 MPa, 2 h) mixed inocula. The spores were placed 5 cm below citrus seedlings. The inocula were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences. The seedlings were thinned 30 days after transplanting to three seedlings per pot. These seedlings were not gotten additional nutrients during the entire experiment.

To allow for AM fungus colonization, the DS treatment began 4 months after transplantation/inoculation. Due to the drought-sensitivity of trifoliate orange, half of the pots were kept at ample water (AW) conditions at field capacity (−0.10 MPa), and the other half were subjected to DS at 73% of field capacity (−0.44 MPa). The soil-water contents were maintained at the target levels by weighting pots daily and adding appropriate volumes of distilled water. The soil water potential was measured using Psypro Dew Point Water Potential System (Wescor Inc., USA).

2.3. Plant and soil measurements

Plants were harvested 80 days after commencement of the water treatment/200 days after inoculation. The following parameters were measured: AM fungus colonization, soil hyphal density, GRSP concentration, root and shoot dry weights, fractions of >2 mm, 1–2 mm, 0.5–1 mm, 0.25–0.5 mm and >0.25 mm water-stable aggregates, hot water-extractable and hydrolysable carbohydrate concentrations, and the activities of peroxide (POD), catalase (CAT), polyphenol oxidase (PPO) in soils.

AM fungal colonization of 1 cm root pieces was assessed in 10% KOH (m/v) at 90 °C and staining with 0.05% (w/v) trypan blue in lactophenol according to Phillips and Hayman [21]. Quantification was performed using the following formula:

$$\text{AM fungus infected percentage (\%)} = \frac{\text{root length infected}}{\text{root length observed}} \times 100$$

Mycorrhizal dependency was defined as the ratio of the dry weight (dry wt.) of the AM seedlings and non-AM seedlings [16].

At harvest, a homogenized soil sub-sample was refrigerated for subsequent analyses. The rest of the soils was air-dried and passed through a 4 mm sieve before the measurements of water-stable aggregates.

Soil hyphal density was determined by the method of Bethlenfalvay and Ames [8].

There are currently two detecting methods utilized to quantify GRSP: Bradford protein assay, yielding Bradford-reactive soil protein (BRSP), and an ELISA, yielding immunoreactive soil protein (IRSP) [28]. BRSP was measured based on the protocols of Wright and Upadhyaya [36] and Wright and

Jawson [34]. Briefly, a 1.0-g sample of well-mixed soils was suspended in 8 ml of 50 mM trisodium salt of citric acid (pH 8.0, adjusted with HCl). Samples were vortexed, autoclaved for 60 min at 121 °C and 0.11 MPa, and centrifuged at 10,000 × *g* for 3 min to remove soil particles. After three cycles of extraction and centrifugation, the supernatant showed clear/light yellow. The BRSP concentration in the extracts was determined with a Bradford [11] assay using bovine serum albumin as a standard.

Water-stable aggregation of air-dried soils was determined as described by Yan [42]. A 40-g sample of soils was spread evenly over the top of a nest of sieves (2 mm, 1 mm, 0.5 mm, and 0.25 mm) and wet-sieved for 10 min by hand. The reserved soils of each sieve were oven-dried at 70 °C for 72 h and weighed. The percentage of water-stable aggregates was calculated by dividing the mass of the oven-dried water stable fraction by the original sample mass.

Extraction of hot water-extractable and hydrolyzable carbohydrates was done following the method of Li et al. [18] with slight modification. A 0.75-g air-dried soil sample was placed in a cuvette, added into 10 ml distilled water, incubated in 100 °C for 2 h, and filtered using filter paper. The process of anthrone method described by Wu and Xia [38] using sucrose as the standard was used to determined hot water-extractable carbohydrate contents of the filtered liquid. A 0.50-g air-dried soil sample was placed in a cuvette, added into 10 ml H_2SO_4 (2.5 M), incubated in 100 °C for 20 min, filtered by two filter papers and diluted properly. Hydrolysable carbohydrate of extracts was determined by the procedure of anthrone method described by Wu and Xia [38] using sucrose as the standard.

Activities of POD, CAT and PPO in soils were performed as described by Yan [42]. CAT activity was defined as the consumption of $KMnO_4$ (0.1 M) for 1 min and 1 g air-dried soil. Activities of POD and PPO were expressed in the amount of hydroxybenzene during 3 h for 100 g air-dried soil.

2.4. Statistical analysis

Experimental data were subjected to a two-way ANOVA with AM fungus treatments and water treatments [30]. The variance was related to the main treatments and to the interaction between them. Probabilities of significant difference were used to test the significance among treatments and interactions, and Fisher's protected least significant differences multiple comparison test at 5% level was used to compare means among treatments.

3. Results

AM seedlings were infected by AM fungi and the average root colonization varied from 13.64% to 67.21% (Table 1). *G. mosseae*-inoculation showed the highest AM fungus colonization and hyphal density regardless of soil-water status and *G. diaphanum*-inoculation the lowest AM fungus colonization and fungal density. Drought stress notably reduced *G. versiforme*- and *G. mosseae*- infection to roots but did not affect the colonization by *G. diaphanum*. Drought stress markedly decreased the hyphal density of only *G. mosseae* among the three fungi. Soils from AM seedlings showed a higher amount of BRSP than that from non-AM seedlings regardless of soil-water status (Table 1).

Mycorrhizal seedlings showed a shoot, root and plant biomass enhancement compared to uninoculated seedlings (Table 2). Under AW conditions, only *G. mosseae*- and *G. versiforme*-inoculation among these fungi significantly increased shoot, root and plant dry weights. Under DS conditions, only *G. mosseae*- and *G. diaphanum*-inoculation among these fungi significantly increased shoot and root biomass, but all of three fungi notably increased total plant biomass. *G. mosseae* stimulated plant growth most among the three studied AM fungal species. Mycorrhizal dependency of trifoliate orange was the highest for *G. mosseae* under AW and DS conditions, respectively. Drought stress notably depressed the shoot, root and plant dry weights of *G. versiforme*- or *G. mosseae*-inoculated seedlings but did not affect those of *G. diaphanum*-colonized seedlings and control.

Hydrolyzable carbohydrate content of soils was more than 10-times higher than hot water-extractable carbohydrate contents of soils subjected to the same treatment (Table 3). Drought stress decreased hot water-extractable and hydrolyzable carbohydrates to a certain extent. Mycorrhizal soils remained lower soil carbohydrates when compared to non-mycorrhizal soils. Mycorrhizal fungus colonization by *G. versiforme* or *G. diaphanum* markedly reduced hot water-extractable carbohydrate regardless of soil-water status. All mycorrhizal fungus inoculation notably reduced hydrolyzable carbohydrate under AW conditions, and mycorrhizal fungus colonization by *G. versiforme* or *G. mosseae* significantly reduced hydrolyzable carbohydrate under DS conditions.

Drought stress increased >0.25 mm water-stable aggregates to a certain extent (Table 4). AM fungus colonization altered water-stable aggregates, too. When seedlings were subjected to AW, *G. mosseae*-inoculation notably increased

Table 1 – AM fungus colonization, hyphal density and Bradford-reactive soil protein (BRSP) of AM or non-AM *Poncirus trifoliata* seedlings grown under ample water (AW) or drought stress (DS) conditions

Water status	AMF	AM fungus colonizatioin (%)	Hyphal density (m g^{-1})	BRSP (mg g^{-1})
AW	*G. versiforme*	46.91b	0.78b	1.64b
	G. mosseae	67.21a	0.98b	1.89a
	G. diaphanum	23.10cd	0.64b	1.81ab
	Non-AMF	0.00e	0.00c	1.10c
DS	*G. versiforme*	22.23cd	1.03b	1.86ab
	G. mosseae	24.76c	1.67a	1.93a
	G. diaphanum	13.64d	0.88b	1.81ab
	Non-AMF	0.00e	0.00c	1.34c
ANOVA				
DS		**	*	*
AMF		**	**	**
DS × AMF		**	NS	NS

Means of three observations followed by the same letter within a column are not significantly different among treatments at $P < 0.05$. Data were analyzed with ANOVA. *$P < 0.05$, **$P < 0.01$. NS, not significant.

Table 2 – Shoot, root and plant dry weights and mycorrhizal dependency of AM or non-AM *Poncirus trifoliata* seedlings grown under ample water (AW) or drought stress (DS) conditions

Water status	AMF	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Plant dry weight (g plant^{-1})	Mycorrhizal dependency (%)
AW	*G. versiforme*	1.05b	0.48b	1.53b	213
	G. mosseae	1.51a	0.69a	2.20a	306
	G. diaphanum	0.51d	0.31c	0.82c	114
	Non-AMF	0.44de	0.28cd	0.72cd	
DS	*G. versiforme*	0.46de	0.29cd	0.75c	127
	G. mosseae	0.92c	0.45b	1.37b	232
	G. diaphanum	0.50d	0.30c	0.80c	136
	Non-AMF	0.34e	0.25d	0.59d	
ANOVA					
DS		**	**	**	
AMF		**	**	**	
DS × AMF		**	**	**	

Means of six observations followed by the same letter within a column are not significantly different among treatments at $P < 0.05$. Data were analyzed with ANOVA. *$P < 0.05$, **$P < 0.01$.

>2 mm and 0.5–1 mm water-stable aggregates, and *G. mosseae* and *G. versiforme* treatments 1–2 mm and >0.25 mm water-stable aggregates. When seedlings were exposed to DS, all fungal inoculation increased >2 mm water-stable aggregate, and inoculation with *G. versiforme* >0.25 mm water-stable aggregate. However, mycorrhizal soils remained lower 0.5–1 mm and 0.25–0.5 mm water-stable aggregates.

G. mosseae- and *G. diaphanum*-inoculation notably enhanced POD activity of soils under AW and DS conditions when compared with the uninoculated treatment, and *G. versiforme*- and *G. diaphanum*-inoculation markedly reduced CAT activity of soils (Table 5). Drought stress and mycorrhizal fungus colonization did not affect the PPO activity of soils.

Table 3 – Soil hot water-extractable and hydrolyzable carbohydrate contents of AM or non-AM *Poncirus trifoliata* seedlings grown under ample water (AW) or drought stress (DS) conditions

Water status	AMF	Hot water-extractable carbohydrate (mg g^{-1})	Hydrolyzable carbohydrate (mg g^{-1})
AW	*G. versiforme*	0.61c	8.51cd
	G. mosseae	0.76ab	9.86b
	G. diaphanum	0.69bc	8.43cd
	Non-AMF	0.86a	11.01a
DS	*G. versiforme*	0.45d	6.98e
	G. mosseae	0.70bc	7.66de
	G. diaphanum	0.61c	7.86cde
	Non-AMF	0.74ab	8.88bc
ANOVA			
DS		**	**
AMF		**	**
DS × AMF		NS	NS

Means of three observations followed by the same letter within a column are not significantly different among treatments at $P < 0.05$. Data were analyzed with ANOVA. *$P < 0.05$, **$P < 0.01$. NS, not significant.

4. Discussion

In our experiment, AM seedlings were infected by AM fungi and hyphal density varied from 0.64 to 1.67 m g^{-1}. Augé et al. [5] inoculated bean plants (*Phaseolus vulgaris*) with *G. intraradices* and indicated that hyphal density ranged from 0.9 to 2.3 m g^{-1} during a continuous soil drying episode. This value is slightly higher than ours, probably due to the differences in fungal species or AM fungal colonization level. Ample internal and external hyphae would provide a direct transported pathway for water and mineral nutrition, especially phosphorus along and through the mycelium [14], thus improving water relations of host plants. The improvement of mycorrhizal plant water relations in turn affected plant growth, such as shoot dry weight, root dry weight and plant dry weight. The result agrees with those obtained by Wu and Xia [38]. Therefore, mycorrhizal plants present an adaptive effect in arid climates. Bolandnazar et al. [10] also reported that mycorrhizal onions (*Allium cepa*) became less responsive to water deficit (longer irrigation interval). In addition, *G. mosseae*-inoculation showed higher plant biomass and mycorrhizal development than other two fungal treatments, implying that *G. mosseae* is more efficient fungus in *P. trifoliata*. The result is in agreement with the finding of Wu et al. [39].

The concentration of BRSP in mycorrhizal soils of trifoliate orange seedlings varied from 1.64 to 1.93 mg g^{-1}. This result is in coincidence with that of Wright and Upadhyaya [36], who observed that the BRSP concentration was less than 2 mg g^{-1} in Texas soils. Drought stress enhanced slightly BRSP concentration, thought the differences were not significant. The trend of BRSP subjected to DS was similar to hyphal density, which elucidates the connection between BRSP and hyphal density ($r = 0.7169$, $P < 0.0001$). When the ANOVA for BRSP was redone without the data for the uninoculated seedlings, DS did not show a significant effect of BRSP concentration, and the BRSP concentration was similar among these fungi whether drought stressed or not.

Extraradical hyphae development and soil aggregation of pepper (*Capsicum annuum*) plants inoculated with *G. deserticola*

Table 4 – Water-stable aggregates of AM or non-AM *Poncirus trifoliata* seedlings grown under ample water (AW) or drought stress (DS) conditions

Water status	AMF	>2 mm water-stable aggregate (%)	1–2 mm water-stable aggregate (%)	0.5–1 mm water-stable aggregate (%)	0.25–0.5 mm water-stable aggregate (%)	>0.25 mm water-stable aggregate (%)
AW	*G. versiforme*	6.00abc	4.61a	9.75bc	10.31b	30.67abc
	G. mosseae	6.41ab	4.69a	10.71ab	10.30b	32.10ab
	G. diaphanum	4.79bcd	4.09ab	10.01bc	10.42b	29.31cd
	Non-AMF	4.30cd	3.41b	9.07c	10.51b	27.29d
DS	*G. versiforme*	7.17a	4.53a	10.15bc	11.16ab	33.01a
	G. mosseae	6.51ab	4.59a	10.92ab	10.73b	32.75ab
	G. diaphanum	6.00abc	4.61a	10.86ab	10.42b	31.90ab
	Non-AMF	2.92d	4.20a	11.62a	11.87a	30.62bc
ANOVA						
DS		NS	NS	**	*	**
AMF		**	**	NS	NS	**
DS × AMF		NS	NS	NS	NS	NS

Means of three observations followed by the same letter within a column are not significantly different among treatments at $P < 0.05$. Data were analyzed with ANOVA. *$P < 0.05$, **$P < 0.01$. NS, not significant.

were enhanced by drought acclimation [12]. Our results also confirmed that DS increased the hyphal density of *G. mosseae*-inoculated seedlings only in these fungi used. In addition, our results obtained indicated that mycorrhizal soils remained high in >2 mm, 1–2 mm and >0.25 mm water-stable aggregates and low in 0.25–0.5 mm water-stable aggregates, when compared to non-mycorrhizal soils. The correlation coefficient between hyphal density and >2 mm, 1–2 mm or >0.25 mm water-stable aggregates was 0.6331 ($P < 0.001$), 0.5344 ($P < 0.01$) and 0.5467 ($P < 0.001$), respectively. Fungal hyphae may initiate aggregate formation and affect drying and wetting actions, shrinking and swelling of clays, which stabilize soil aggregates [13,31]. Therefore, when plants are inoculated with AM fungi, formed extraradical hyphae will enhance soil aggregate status. Fungal hyphae, especially those of AM fungi, grow into the soil matrix to create the skeletal structure that holds primary soil particles together via physical entanglement [4]. The improved conditions of soils are propitious to formation of microaggregates and smaller microaggregates into macroaggregate structures [4]. The changed process might be connected with glomalin. The glomalin can glue smaller macroaggregates (e.g. 0.25–0.5 mm water-stable aggregates) into macroaggregates (e.g. >2 mm and 1–2 mm water-stable aggregates). BRSP was positively correlated with water-stable aggregates (>2 mm, $r = 0.6217$, $P < 0.01$; 1–2 mm, $r = 0.6153$, $P < 0.01$; >0.25 mm, $r = 0.6481$, $P < 0.001$). This agrees with previous reports [26,33,36,37]. Mycorrhizal soils maintained better soil structure, especially soil water-stable aggregates and BRSP, which are important for: (1) maintaining soil porosity, (2) increasing stability against wind and water erosion, and (3) storing C by protecting organic matter from microbial decomposition [20]. The correlation has linked soil aggregates with increased drought resistance [4]. Augé et al. [7] reported that mycorrhizal soils had more water-stable aggregates and consequently a higher soil moisture. Thus, the increased water-stable aggregate and

Table 5 – Soil peroxide (POD), catalase (CAT), polyphenol oxidase (PPO) activities of AM or non-AM *Poncirus trifoliata* seedlings grown under ample water (AW) or drought stress (DS) conditions

Water status	AMF	POD [mg (pyrogallic acid) $(3\ h)^{-1}$]	CAT [0.1 M $KMnO_4$ ml $(g\ h)^{-1}$]	PPO [mg (pyrogallic acid) $(3\ h)^{-1}$]
AW	*G. versiforme*	21.84cd	0.87c	0.79a
	G. mosseae	27.23ab	0.98abc	0.91a
	G. diaphanum	27.04ab	0.79c	0.43a
	Non-AMF	19.91d	1.11ab	1.57a
DS	*G. versiforme*	26.19abc	0.89bc	0.79a
	G. mosseae	30.18a	1.13a	0.73a
	G. diaphanum	28.43a	0.83c	1.51a
	Non-AMF	22.45bcd	1.15a	1.27a
ANOVA				
DS		*	NS	NS
AMF		**	**	NS
DS × AMF		NS	NS	NS

Means of three observations followed by the same letter within a column are not significantly different among treatments at $P < 0.05$. Data were analyzed with ANOVA. *$P < 0.05$, **$P < 0.01$. NS, not significant.

BRSP due to mycorrhizal symbiosis lead to better soil structure and might alter soil moisture retention properties that, in turn, lead to better plant drought resistance and plant production, although direct mineral nutritional effects cannot be excluded.

In our study, AM fungus inoculation reduced hot water-extractable and hydrolyzable carbohydrate contents of soils. The BRSP concentration was negative correlated with hot water-extractable carbohydrate ($r = -0.4328$, $P < 0.05$) or hydrolyzable carbohydrate content ($r = -0.6268$, $P < 0.01$), whereas correlations were not very strong. The contribution of AM fungi hyphae to C cycling lies not only with extraradical hyphae themselves but also with exudates from hyphae [45]. Glomalin, a exudate produced by AM fungi, is tightly bound in the fungal mycelium. The amount of C in glomalin represented a sizeable amount (ca. 4–5%) of total soil C in tropical soils [27]. Thus, a lower C content would be observed in mycorrhizal soils owing to C storage of glomalin.

Soil enzymes play an essential role in catalyzing reactions necessary for organic matter decomposition and nutrient cycling in ecosystems [44], and have been used as indices of microbial activity, soil fertility and land quality [43]. Findings from this study showed that AM fungus inoculation could increase POD, decrease CAT, and not affect PPO activities in soils, suggesting that the productions of reactive oxygen species in soils are dubious when colonized by AM fungi. POD activity were positive correlated with hyphal density ($r = 0.6861$, $P < 0.001$) or with BRSP ($r = 0.7342$, $P < 0.0001$), implying that POD only in these enzymes is relatively sensitive to changes in mycorrhizal soils. Relationships between AM fungus inoculation and alterations of soil enzyme obviously need further research.

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