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SHORT NOTE

Metabolite profiling of the hyphal exudates of *Rhizophagus clarus* and *Rhizophagus irregularis* under phosphorus deficiency

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Abstract

Arbuscular mycorrhizal (AM) fungal extraradical hyphae exude their metabolites into the soil. Root exudate metabolites are affected by plant species and P status. However, the effect of P status on AM hyphal exudate metabolites has been unknown. This study aimed to examine hyphal exudate metabolite composition of two AM fungal species and their response to P deficiency through metabolite profiling. *Rhizophagus clarus* and *R. irregularis* were grown in a two-compartment in vitro culture system of *Linum usitatissimum* roots on solid modified Strullu-Romand medium in combination with two P levels (3 µM (P3) and 30 µM (P30)). Hyphal exudates were collected from the hyphal compartment at 118 days after inoculation (DAI). The metabolite composition of the hyphal exudates was determined by capillary electrophoresis/time-of-flight mass spectrometry, resulting in the identification of a total of 141 metabolites at 118 DAI. In the hyphal exudates of *R. clarus*, the concentrations of 18 metabolites, including sugars, amino acids, and organic acids, were significantly higher ($p < 0.05$) under P3 than under P30 conditions. In contrast, the concentrations of 10 metabolites, including sugar and amino acids, in the hyphal exudates of *R. irregularis* were significantly lower ($p < 0.05$) under P3 than under P30 conditions. These findings suggest that the extraradical hyphae of AM fungi exude diverse metabolites of which concentrations are affected by P conditions and differ between AM fungal species.

Keywords Arbuscular mycorrhizal fungus · Hyphal exudate · Metabolite profiling · Phosphorus deficiency · Monoxenic culture

Introduction

Arbuscular mycorrhizal (AM) fungi symbiotically associate with approximately 80% of terrestrial plants and are known to support host plant growth by increasing the uptake of inorganic phosphorus (P_i) through their extraradical hyphal networks. The AM fungal extraradical hyphae grow into the soil beyond the root P_i depletion zone, thereby increasing the soil volume from which P_i is acquired (Li et al. 1991). The hyphal length of AM fungi ranged from 8 to 28 m g^{-1} soil, and extraradical hyphae spread at least 11 cm away from roots under pot culture conditions in 47 days after inoculation (DAI) (Jakobsen et al. 1992). The hyphal length of AM fungi was 111 m cm^{-3} soil in prairie and 81 m cm^{-3} in pasture communities in field conditions (Miller et al. 1995). P_i is taken up by the extraradical hyphae through H^+/P_i and Na^+/P_i symporters driven by H^+-ATPases and Na^+-ATPases, respectively. In addition to these mechanisms, the extraradical hyphae not only have the ability to take up nutrients but also can release some metabolites into the soil (Toljander et al. 2007; Bharadwaj et al. 2012; Sato et al. 2015). However, the hyphal exudate composition and concentration are poorly known.

The hyphal exudates of AM fungi and other soil fungi have been reported to play a role in the acquisition of unavailable nutrients. Koide and Kabir (2000) suggested that the hyphal exudates of AM fungi contained an acid phosphatase (ACP) that could hydrolyze organic P and increase P_i transfer into the host plant root. The hyphal exudates of AM, ectomycorrhizal, and phosphate-solubilizing fungi (PSF) by producing organic acids and

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lowering the soil pH also could solubilize unavailable inorganic P in the soil (Tawaraya et al. 2006; van Hees et al. 2006; Toljander et al. 2007; Li et al. 2019; Della Monica et al. 2020). Moreover, the hyphal exudates of ectomycorrhizal fungi and ericoid mycorrhizal fungi have been shown to mineralize not only P but also organic N, through their enzymatic activities (Hodge et al. 1995; Cairney and Burke 1998).

A hyphosphere is the zone where the interaction between AM or other soil fungal hyphae and soil takes place (Meier et al. 2015). The formation of a microbial community in this area is known to be affected by the chemical composition of the AM and other soil fungal hyphal exudates. Filion et al. (1999) confirmed that the hyphal exudate of *Glomus intraradices* plays an important role in the growth and composition of other soil microorganisms. This finding was later supported by Toljander et al. (2007) and Hooker et al. (2007), who explained that if a sugar compound is released as an AM hyphal exudate, it acts as a carbon source for the bacterial communities of the hyphosphere. Moreover, a recent study found that the fructose exuded by an AM fungus also plays a role as a signal molecule triggering the expression of the phosphatase gene in phosphate-solubilizing bacteria (Zhang et al. 2018).

Plant roots release a variety of metabolites into the soil through their exudates. The root exudate of *Arabidopsis thaliana* contains 64 different metabolites at 28 days after transplanting (DAT) (Witzel et al. 2017), whereas that of *Oryza sativa* contains 81 metabolites at 15 DAT (Tawaraya et al. 2018) and that of *Sorghum bicolor* contains 42 metabolites at 21 days after sowing (DAS) (Miller et al. 2019). Although we expect that AM extraradical hyphae also could release diverse metabolites through their exudates, the number of metabolites released through AM hyphal exudates has never been studied.

Root exudation in plants is affected by their P status. The concentrations of amino acids (glutamine, glutamic acid, valine, and methionine) and organic acids (citric acid, malic acid, and oxalic acid) in the root exudate of *Andropogon virginicus* were increased under P-deficient conditions (Edayilam et al. 2018). An increase in sugar compound exudation through *Zea mays* roots also was detected as a response to P deficiency because of carbohydrate accumulation in root tissues under P starvation conditions (Carvalhais et al. 2011). However, the response of metabolites released through AM hyphal exudates under low-P conditions still is poorly understood.

The metabolite response of root exudates to P-deficient conditions varied even among plant genotypes. The wild-type, P deficiency-insensitive *lpr1 lpr2* mutant, and P deficiency-hypersensitive *pdr2* mutant plants of *Arabidopsis thaliana* exhibit different root exudate metabolite responses to P deficiency. A total of 67, 49, and 21 metabolites of wild-type, *lpr1 lpr2*, and *pdr2* plants were changed under P-deficient conditions, respectively, with each genotype exhibiting different metabolite compositions (Ziegler et al. 2016). The differences in metabolite responses among plant genotypes might contribute to their different mechanisms to cope with P-deficient conditions. Therefore, we expect that different AM fungal species also will exhibit different hyphal exudate metabolic responses to P deficiency.

Knowledge of root exudate metabolite responses to P-deficient conditions is important to improve plant P acquisition in the face of the exhaustion of P fertilizer in the future. However, the metabolic responses of AM hyphal exudate metabolites other than acid phosphatase and organic acids, as a potential mechanism to acquire P_i under P-deficient conditions, have not yet been investigated.

The present study aimed to test the hypotheses that whether (1) similar to root exudate,AM fungi also can exude diverse metabolite compounds from their extraradical hyphae; (2) the exudation of those metabolites could be affected by the P concentration of media; and (3) the composition of the hyphal exudates and their responses to P concentration could be different between AM fungus species. To test our hypothesis, metabolite profiling of *R. clarus* and *R. irregularis* grown under two regimes of contrasting P availability was performed using capillary electrophoresis-time of flight-mass spectrometry (CE-TOF–MS).

Materials and methods

AM fungal spore preparation

AM fungal species, *R. clarus* CK001 (Sato et al. 2019) and *R. irregularis* DAOM197198 were used in the present study. *Agrobacterium rhizogenes*-transformed roots (hairy roots) of flax (*Linum usitatissimum* L.) were used for inoculum preparation. AM fungal spores were surface sterilized for 15 min in 2% chloramine T solution containing a few drops of Tween80 and 10 min in 0.02% streptomycin sulfate plus 0.01% gentamicin sulfate solution. After rinsing in sterilized deionized water, the spores were transferred onto 9 cm Petri dishes containing 20 mL of modified Strullu–Romand (MSR) medium (Declerck et al. 1998) with hairy roots, and were incubated at 27 °C in the dark for 60 days. The P concentration of the MSR medium was modified to 3 μM in order to increase AM colonization and spore production. New spores were collected by removing the hairy roots with tweezers, and the 60-day-old culture medium was transferred to 500 mL of 10 mM sodium citrate buffer (pH 6.0) to solubilize the gellan gum medium (Doner and Becard 1991). The spores were collected on a 30-μm nylon mesh, washed with sterilized water, and stored at 27 °C.

Two-compartment in vitro culture

An in vitro two-compartment culture was established according to St-Arnaud et al. (1996). One side of a two-compartment Petri dish (root compartment, RC) was filled with 20 mL of MSR medium with 3 (P3) or 30 (P30) µM of P in the form of potassium dihydrogen phosphate, on which a 1-cm piece of flax hairy root tip was placed. Twenty spores of each species were inoculated 2.5 cm from the root tip (2–10 replications per treatment) and grown at 27 °C in the dark. A hyphal compartment (HC) remained empty until the flax hairy root in the RC was colonized and extraradical hyphae crossed the central wall to the HC (Fig. 1a). The roots were trimmed weekly to prevent them from growing into the HC.

The extraradical hyphae of both AM fungi crossed the central wall at 50 days after inoculation (DAI). A slope was made from the top central wall to the bottom of petri dish of the HC at 1 cm from the central wall with 3 mL of MSR medium containing 3 or 30 µM P in the form of potassium dihydrogen phosphate. The petri dishes were placed at an angle in order to form a slope against the central wall until that medium was solidified. Then, 5 mL of liquid MSR medium containing 3 or 30 µM P was added to the HC (Fig. 1b).

Hyphal exudate collection and CE-TOF–MS

The liquid MSR medium in the HC was removed at 68 days after application, i.e., 118 DAI. The remaining liquid medium in the HC was removed additionally by washing the HC containing extraradical hyphae with sterilized deionized water three times carefully. To avoid hyphal damage, both the removal and wash processes were conducted without making any contact between hyphae and a pipette tip. To prevent detection of metabolite compounds other than AM hyphal exudates, the hyphal exudate was collected by adding 5 mL of sterilized deionized water to the HC which was incubated for 12 h at 27 °C (Fig. 1c). The 5 mL hyphal exudate solution was frozen immediately at − 30 °C. The frozen hyphal exudates were lyophilized. Sample preparation and CE-TOF-MS were performed as described by Tawaraya et al. (2013).

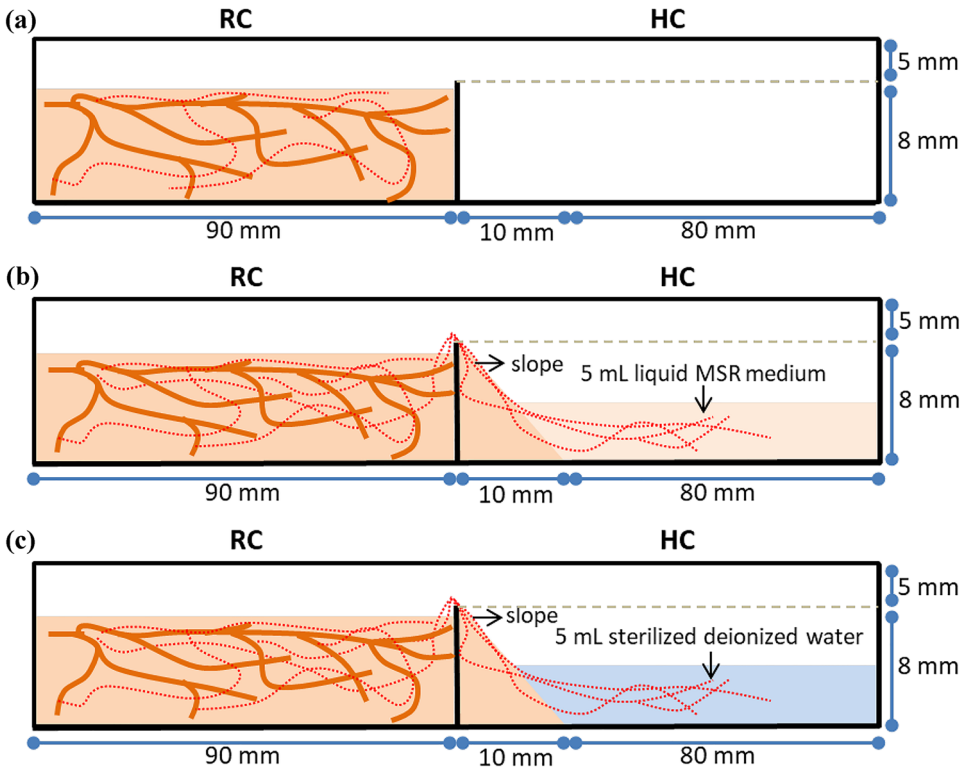

Fig. 1 In vitro two-compartment culture consisting of a root compartment (RC) and hyphal compartment (HC). *Linum usitatissimum* L. (flax) hairy roots inoculated with *R. clarus* CK001 and *R. irregularis* DAOM197198 spores were grown in the RC containing MSR medium with two different P regimes, i.e., 3 or 30 µM P. The HC remained empty until the flax hairy roots (solid lines) in the RC were colonized, and the extraradical hyphae (dotted lines) crossed the central wall to the HC (**a**). The slope connecting the RC and HC was made against the central wall by addition of 3 mL of MSR medium at 50 days after inoculation (DAI), the AM fungal extraradical hyphae in the HC were then cultivated in 5 mL liquid MSR medium until 118 DAI (**b**). At 118 DAI, the liquid MSR medium in the HC was removed and the HC was filled with 5 mL of sterilized deionized water for hyphal exudate collection (**c**)

The metabolites were quantified into 9 functional groups, i.e., amino acids and amines, nucleic acids, organic acids, carbohydrates and polyols, fatty acids, homologues, phosphate esters, phytohormones, and others, following Tawaraya et al. (2014b) and Yang et al. (2020).

Statistical analysis

The data of metabolite concentrations were statistically analyzed using the Student's *t* test along with the statistical software KaleidaGraph (Synergy Software, Reading, PA, USA) to identify significant differences ($P < 0.05$) between the P3 and P30 treatments, and between *R. clarus* and *R. irregularis*.

Results and discussion

Diverse metabolites detected in the hyphal exudates of *R. clarus* and *R. irregularis*

A total of 141 metabolites were detected in the hyphal exudates of *R. clarus* and *R. irregularis* grown under P3 and P30 conditions at 118 DAI (Tables S1 and S2). Of these 141 metabolites, 131 were detected in AM hyphal exudates for the first time. Previous studies by using ^{1}H-nuclear magnetic resonance (HNMR) have only detected 10 metabolite compounds in the hyphal exudates of *Glomus* sp. (Toljander et al. 2007). The sensitivity of the CE-TOF/MS analytical method used in this study was 10^{-23} mol and considerably higher than that of NMR (10^{-6} mol) (Sumner et al. 2003). The high sensitivity of CE-TOF/MS could detect many metabolites in hyphal exudate.

Most previous study of AM hyphal exudate metabolites was related to the interaction of AM fungi with other soil microbial community members and their populations (Toljander et al. 2006; Bharadwaj et al. 2012). Therefore, most information about AM hyphal exudate metabolites was restricted to the carbohydrate group. The present study shows that in addition to carbohydrates and polyols (9% of the total number of metabolites identified), AM hyphal exudate also contained other metabolite compounds, i.e., amino acids and amines (43%), nucleic acids (10%), organic acids (21%), fatty acids (1%), homologues (5%), phosphate esters (9%), phytohormones (1%), and others (1%).

Twelve *R. clarus* and *R. irregularis* hyphal exudates consisted of carbohydrate and polyols compounds. This number was higher than those of previous studies. Toljander et al. (2007) and Bharadwaj et al. (2012) reported that the extraradical hyphae of *Glomus* sp. MUCL 43205 and *Glomus irregularis* MUCL 41833 released only five different carbohydrate compounds, i.e., glucose, glycogen, raffinose, fructose, and trehalose under in vitro conditions.

Amino acids and amines were the most abundant metabolites (61/141) in both AM fungus species' exudates. This result was consistent with a previous study, which reported that extraradical hyphae of *Glomus irregularis* MUCL 41,833 exuded six essential amino acid compounds, including glutamine, tyrosine, asparagine, and arginine at 56 DAI (Bharadwaj et al. 2012). Mycelium of other fungi, such as ectomycorrhizal (ECM) fungal species, including *Hebeloma velutipes*, *Paxillus involutus*, *Piloderma byssinum*, *Rhizopogon roseolus*, *Suillus bovinus*, and *Suillus variegatus*, also exuded six amino acids, including asparagine, lysine, glutamine, arginine, threonine, and proline (Johansson et al. 2008).

The study of organic acid exudation through hyphal exudates commonly has been performed for the phosphate solubilizing fungi (PSF) species, such as *Aspergillus* sp., *Penicillium* sp., *Talaromyces* sp., and *Pirimorfosa* sp., because of their important roles in P cycling (Wu et al. 2018; Islam et al. 2019). Meanwhile, limited studies have been performed on organic acid exudation by mycorrhizal fungi. This study detected 30 metabolites in *R. clarus* and *R. irregularus* hyphal exudates that are organic acids. Tawaraya et al. (2006) conducted a study on organic acid exudation by AM fungal hyphae and found that *Gigaspora margarita* can release only citric acid in its hyphal exudate. *Glomus irregularis* MUCL 41833 also was found to release three different organic acids, i.e., citric acid, succinic acid, and uric acid in hyphal exudate (Bharadwaj et al. 2012). Meanwhile, a study on ECM fungi organic acid exudation mainly focused on the release of oxalic acid because of its high activity in mycelial exudates (Ahonen-Jonnarth et al. 2000; Van hees et al. 2006).

Phytohormones play a role as the molecular signal between AM fungi and host plants at the initiation stage of symbiosis (Oldroyd 2013). However, the exudation of phytohormones originated from AM hyphae was questioned in a previous study (Allen et al. 1980). Martin- Rodriguez et al. (2011) reported that AM fungi regulate abscisic acid (ABA) biosynthesis in roots, however biosynthesis of ABA in AM fungal hyphae was not known. Our present study found that both AM fungal species had ABA in their hyphal exudates.

P deficiency altered AM fungus hyphal exudation

No studies have been conducted thus far on the metabolite profiling of AM fungi hyphal exudates in response to P-deficient conditions. Among our results, the concentrations of 25 metabolites were significantly higher in *R. irregularis* hyphal exudate compared to *R. clarus* under the P30 condition (Tables S1 and S2). No metabolite showed a concentration significantly higher in *R. clarus* than in *R. irregularis* under the P30 condition. In contrast, under the P3 condition, the concentrations of 17 metabolites were significantly higher in *R. clarus* than in *R. irregularis* hyphal exudate (Tables S1 and S2), and no metabolite showed a

concentration significantly higher in *R. irregularis* than in *R. clarus* under the P3 condition. This indicates that the hyphal exudate metabolites of both AM fungus species responded differently to P availability.

An increase in hyphal exudation, especially of the acid phosphatase enzyme, by *R. clarus* CK001 under P-deficient conditions was reported in a previous study by Sato et al. (2019). Consistent with previous studies, of the 141 metabolites in the *R. clarus* hyphal exudates, the concentrations of 18 metabolites (5-oxoproline, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl 5′ monophosphate (AICAR), 2′-deoxycytidine-5′-monophosphate (dCMP), deoxyadenosine, deoxycytidine, deoxyguanosine, fructose-leucine, glutamine, homoserine, leucine, methionine sulfoxide, methylneuraminate, N-acetyl-b-alanine, prostaglandin E_2 (PGE2), raffinose, tartrate, theophylline, and tyrosine) were significantly higher under the P3 than under the P30 condition (Table 1). No metabolite showed a concentration significantly lower under P3 than under P30 conditions (Table S1). The concentrations of the remaining 123 metabolites did not statistically differ between the P3 and P30 conditions.

Of those 18 significantly increased metabolites in *R. clarus* hyphal exudates, 5-oxoproline, AICAR, glutamine, leucine, methionine sulfoxide, N-acetyl-b-Alanine, PGE2, theophylline and tyrosine belong to amino acids and amines (Table 1). There are no previous reports of the effect of P deficiency on amino acid concentrations in the AM or other soil fungi hyphal exudates. However, in plants, the concentrations of glutamine, glutamic acid, valine, and methionine were increased in *Andropogon virginicus* root exudates exposed to P-deficient conditions (Edayilam et al. 2018). Soybean (*Glycine max* cv. Suzuyutaka) plants also showed an increase in the concentration of leucine and glutamine in their shoot and root extracts in response to P-deficient conditions (Tawaraya et al. 2014b).

The concentrations of carbohydrates, such as raffinose and fructose-leucine, in the hyphal exudates of *R. clarus* also were significantly increased under P-deficient conditions (Table 1). That P deficiency alters the concentrations of carbohydrate compounds in AM hyphal exudates is in accord with many of the previous studies focused on carbohydrate exudation in relation to hyphosphere bacterial communities (Toljander et al. 2007; Zhang et al. 2020). At the plant level, the concentrations of inositol, erythritol, ribitol, fructose, glucose, and arabinose were increased in the root exudate of maize plants grown under P-deficient conditions (Carvalhais et al. 2011). The biosynthesis of raffinose in tomato roots also was shown to be increased under P-deficient conditions (Sung et al. 2015).

The concentrations of organic acids, especially of tartaric acid, in *R. clarus* hyphal exudate were significantly increased in response to P-deficient conditions (Table 1). The exudation of tartaric acid under P-deficient conditions also was confirmed in the PSF species *Aspergillus* sp., *Penicillium* sp., and *Talaromyces* sp. (Islam et al. 2019). Other organic acids, such as oxalic, malic, and citric acids, also were exuded by the PSF species *Piriformospora indica* colonizing *Brassica napus* in response to recalcitrant P addition (Wu et al. 2018). The increase in organic acid concentrations in response to P-deficient conditions also agrees with a previous study at the plant level (Tawaraya et al. 2014a). Furthermore, malic acid and citric acid were released in root exudates of *Lupinus albus* under P-deficient conditions (Zhou et al. 2020).

AM hyphal exudate metabolite response to P-deficient conditions is species-dependent

The response of *R. irregularis* to P-deficient conditions was different from that of *R. clarus*. In contrast to the metabolite response of *R. clarus*, of the 141 metabolites of the *R. irregularis* hyphal exudate, the concentrations of 10 metabolites (arginine, carnitine, deoxythymidine diphosphate (dTDP), N-acetyl-D-glucosamine 6-phosphate (GlcNAc-6-phosphate), glycerate, melilotate, N-acetylneuraminic acid (Neu5Ac), pipecolate, riboflavin, and theophylline) were significantly lower under P3 than under P30 conditions (Table 1). No metabolite showed a concentration significantly higher under P3 than under P30 conditions (Table S2). The concentrations of the other 131 metabolites were not different between the P3 and P30 conditions.

The concentrations of phosphate-containing metabolites, i.e., N-acetyl-D-glucosamine 6-phosphate (GlcNAc6P), thymidine diphosphate (dTDP), and pipecolate, were significantly decreased in the hyphal exudates of *R. irregularis* under P-deficient conditions (Table 1). This alteration could be a direct consequence of P deficiency in the growth medium. This result is similar to the results of Tawaraya et al. (2014a), who showed that the concentration of P ester metabolites decreased under P-deficient conditions in common bean root exudates.

Of the 10 significantly decreased metabolites in *R. irregularis*, 5 metabolites (i.e. arginine, carnitine, melilolate, pipecolate, and theophylline) belonged to amino acids and amines (Table 1). The decrease in amino acid concentrations under P-deficient conditions also was found at the plant level by Tawaraya et al. (2018) who reported that rice plants grown under P-deficient conditions showed a decrease in the concentration of amino acids in the shoot extract.

Theophylline was the only metabolite that differed significantly between P3 and P30 for both *R. clarus* and *R. irregularis* but the differences in theophylline concentrations between P3 and P30 were opposite for the two fungus species, consistent with the patterns of differences in all other metabolites.

Overall hyphal exudation by both fungus species responded oppositely to the two different P concentrations

Table 1 Concentration of significantly changed metabolites in the hyphal exudates of *Rhizophagus clarus* and *R. irregularis* grown under two P treatments (P3 and P30 representing 3.0 and 30.0 µM P, respectively) at 118 days after inoculation

Category of metabolites	Metabolite	*Rhizophagus clarus*						*Rhizophagus irregularis*					
		P3			P30		Log10(P3/P30)	P3			P30		Log10(P3/P30)
		(µM)						(µM)					
Amino acids and amines	Glutamine	7.62749 ± 2.22651	a	*	2.45573 ± 1.14961	b	0.49220	1.31684 ± 0.80421			2.00573 ± 0.27312		− 0.18274
	5-0xoProline	0.90341 ± 0.40384	a		0.24388 ± 0.07824	b	0.56871	0.17433 ± 0.10222			0.37622 ± 0.18242		− 0.33408
	Theophylline	0.61622 ± 0.05582	a	*	0.43248 ± 0.04646	b	0.15376	0.34974 ± 0.03587	b		0.60935 ± 0.02712	a	− 0.24112
	Leucine	0.33031 ± 0.09235	a		0.12940 ± 0.04388	b	0.40700	0.08694 ± 0.05004			0.33197 ± 0.22905		− 0.58189
	Tyrosine	0.07687 ± 0.02377	a		0.02970 ± 0.00727	b	0.41298	0.03336 ± 0.00619			0.02249 ± 0.00807		0.17126
	Methionine sulfoxide	0.02155 ± 0.00810	a	*	0.00370 ± 0.00249	b	0.76504	n.d. ± n.d.			0.02157 ± 0.02157		n.d
	PGE2	0.01346 ± 0.00523	a		0.00535 ± 0.00073	b	0.40088	0.00798 ± 0.00401			0.00871 ± 0.00259		− 0.03781
	N-Acetyl-b-Alanine	0.00781 ± 0.00138	a	*	0.00375 ± 0.00074	b	0.31853	0.00401 ± 0.00062			0.01641 ± 0.01338	*	− 0.61185
	AICAR	0.00040 ± 0.00012	a		0.00021 ± 0.00003	b	0.27481	0.00022 ± 0.00014			0.00026 ± 0.00001		− 0.07300
	Pipecolate	0.59636 ± 0.15360			0.39112 ± 0.17316		0.18319	0.18394 ± 0.10637	b		0.65503 ± 0.04580	a	− 0.55158
	Arginine	0.01541 ± 0.00453			0.02801 ± 0.00692		− 0.25962	0.01455 ± 0.00186	b		0.04046 ± 0.00450	a	− 0.44404
	Riboflavin	0.00453 ± 0.00044		*	0.00312 ± 0.00053		0.16159	0.00213 ± 0.00042	b		0.00487 ± 0.00071	a	− 0.35820
	Carnitine	0.00152 ± 0.00036			0.00213 ± 0.00058		− 0.14766	0.00202 ± 0.00034	b		0.00366 ± 0.00034	a	− 0.25729
	Melilotate	0.00231 ± 0.00043			0.00286 ± 0.00092		− 0.09223	0.00169 ± 0.00056	b		0.00635 ± 0.00170	a	− 0.57569
Nucleic acids	Deoxyadenosine	4.25393 ± 2.23400	a		0.81521 ± 0.27437	b	0.71752	2.01898 ± 1.07851			3.64960 ± 3.41879		− 0.25711
	Deoxycytidine	0.18927 ± 0.07897	a		0.05038 ± 0.01229	b	0.57479	0.08772 ± 0.04033			0.16181 ± 0.14958		− 0.26590
	Deoxyguanosine	0.10296 ± 0.03195	a		0.03001 ± 0.01110	b	0.53540	0.06473 ± 0.03136			0.10581 ± 0.10581		− 0.21344
Organic acids	Tartrate	0.18351 ± 0.06480	a		0.06308 ± 0.01554	b	0.46375	0.05499 ± 0.00650			0.07241 ± 0.01999		− 0.11955
	N-Acetylneuraminic acid	0.04307 ± 0.02935			0.00761 ± 0.00580		0.75269	0.00111 ± 0.00023	b		0.00272 ± 0.00002	a	− 0.38876
Carbohydrates and polyols	Raffinose	0.27750 ± 0.05043	a	*	0.13608 ± 0.02826	b	0.30945	0.07009 ± 0.03247			0.13335 ± 0.12056		− 0.27937
	Fructose-Leu	0.00989 ± 0.00094	a		0.00653 ± 0.00088	b	0.18015	0.00537 ± 0.00175			0.00425 ± 0.00303		0.10173
	Methylneuraminate	0.00890 ± 0.00469	a		0.00198 ± 0.00023	b	0.65344	0.00216 ± 0.00040			0.00236 ± 0.00011		− 0.03883
	Glycerate	0.12646 ± 0.00766		*	0.11187 ± 0.01090		0.05326	0.09759 ± 0.00537	b		0.20951 ± 0.06036	a	* − 0.33182
	GlcNAc6P	0.00037 ± 0.00011			0.00023 ± 0.00005		0.20561	0.00034 ± 0.00002	b		0.00046 ± 0.00004	a	− 0.12582
Homolog	HomoSerine	0.12789 ± 0.04180	a		0.04356 ± 0.01734	b	0.46777	0.03813 ± 0.02086			0.04058 ± 0.00490		− 0.02706

Table 1 (continued)

Category of metabolites	Metabolite	*Rhizophagus clarus*					*Rhizophagus irregularis*				
		P3		P30		Log10(P3/P30)	P3		P30		Log10(P3/P30)
		(μM)					(μM)				
Phosphate esters	dCMP	0.00285 ± 0.00103	a	0.00100 ± 0.00022	b	0.45506	0.00202 ± 0.00019	b	0.00265 ± 0.00209		− 0.11772
	dTDP	0.00192 ± 0.00018		0.00127 ± 0.00018		0.17939	0.00151 ± 0.00007	b	0.00242 ± 0.00041	a *	− 0.20446

Data presented are means ± standard error. The lack of letters indicates no significant difference ($P < 0.05$) between the P treatments for each AM fungus species; asterisks (*) indicate significantly different ($P < 0.05$) metabolite concentrations between the two AM fungi for the same P treatment by Student's *t* test

in the HC medium. Unfortunately, because of limited biomass of hyphae, we could not measure the P concentration within the extraradical hyphae, and it is possible that the P concentration of the medium was diminished by hyphal uptake and transport to the RC (Zhang et al. 2016). The effect of P concentration in the HC medium on the growth of extraradical hyphae should be further examined. Nevertheless, to our knowledge, our present study has shown for the first time that the hyphal exudates of *R. clarus* and *R. irregularis* have different metabolic responses to P deficiency. Our results suggest that *R. clarus* might tolerate P deficiency by increasing the concentrations of several hyphal exudate metabolites that could support an increase of P availability in the medium. In contrast, *R. irregularis* seems to respond to the low P condition by decreasing the concentration of some metabolites. How that might enable *R. irregularis* to cope with P deficiency remains unclear.

Functional roles of metabolites in AM hyphal exudates under P deficiency

The range of metabolite concentrations in *R. clarus* hyphal exudates was from 0.00007 to 7.62749 μM under P3 and from 0.00006 to 8.22426 μM under P30 (Table S1). The concentrations of the metabolites detected in the hyphal exudate of *R. irregularis* ranged from 0.00006 to 28.16739 μM under P3 and between 0.00004 and 25.95745 μM under P30 conditions (Table S2). The large ranges of metabolite concentrations in both *R. clarus* and *R. irregularis* under both P conditions, suggest generally different functional roles of the metabolites. The metabolites with high concentrations in the soil, both exuded by AM hyphae and plant roots, could clearly affect soil bacterial community structure and activities related to P availability in the soil. Zhang et al. (2020) reported that instead of 20 μmol kg^{-1}, addition 4 mmol kg^{-1} of fructose was effective in changing phosphate solubilizing bacteria in the maize hyphosphere.

Similarly, an increase of metabolite concentrations in hyphal exudates also could affect soil microbial growth and activity related to P availability. Raffinose, the carbohydrate compound with high concentrations in both *R. clarus* and *R. irregularis* (Tables S1 and S2) was previously reported to have ability to support the growth of Enterobacteriaceae bacterial taxa in the hyphosphere (Toljander et al. 2007 and Bharadwaj et al. 2012). These bacterial taxa are known to increase P uptake by AM extraradical hyphae because of the release of phosphate ions from rock phosphate and from a less-available indigenous P sources (Toro et al. 1997). Another carbohydrate compound, i.e., fructose-leucine, significantly increased in *R. clarus* hyphal exudates under the P3 condition (Table 1), is known to have ability to increase hyphosphere phosphatase activity by inducing phosphatase gene expression in the phosphate-solubilizing bacterium *Rhanella aquatilis* (Zhang et al. 2018, 2020).

Glutamine, the metabolite with highest concentration in both *R. clarus* and *R. irregularis* hyphal exudates (Tables S1 and S2), and tyrosine, the significantly increased amino acid in *R. clarus* hyphal exudates under the P3 condition (Table 1) are known to support the growth of *Pseudomonas* sp. In the *Glomus irregularis* MUCL 41833 hyphosphere and to increase the solubilization of sparingly soluble P (Villegas and Fortin, 2001). Amino acids play an important role in chelating metals and can enhance P mobility in the soil (Edaliyam et al. 2018). Therefore, the increase in the amino acid concentration in the hyphal exudates of *R. clarus* could be a strategy to cope with P-deficient conditions.

Citrate, the organic acid compound with high concentrations in both *R. clarus* and *R. irregularis* (Tables S1 and S2) has been reported to decrease the pH of growth medium and to increase P solubilizing activity (Chen et al. 2006). Following citrate, another organic acid with high concentrations in both *R. clarus* and *R. irregularis* exudates was gluconate (Tables S1 and S2). Rodriguez et al. (2004) reported that gluconate released by phosphate-solubilizing bacteria could reduce pH and increase solubilisation of calcium phosphate.

Tartate, the only organic acid that significantly increased in *R.clarus* hyphal exudate under the P3 condition, plays an important role in adaptation to P-deficient conditions. *Asperigillus aculeatus* produced 3 mM of tartaric acid and solubilized $Ca_3(PO_4)_2$ (Li et al. 2019). Hyphal exudate of *R. clarus* might solubilize some amount of "unavailable P" although the concentration of tartaric acid in the hyphal exudate (0.055–0.184 µM) was lower than that observed by Li et al. (2019).

Unlike *R. clarus*, the concentration of amino acids, especially of arginine, decreased in the hyphal exudates of *R. irregularis* in response to P-deficient conditions (Table 1). The arginine present in AM fungi hyphal exudates plays a role in N assimilation processes through the AM fungal pathway (Johansen et al. 1996). However, the role of arginine in N assimilation processes under P-deficient conditions is not known.

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Author contributions NL, NI, Tantriani, TS and KT designed the study and performed the experiments. NL, NI, Tantriani, TS AO, WC, and KT analyzed the data. NL and KT wrote the manuscript. All authors read and approved the final manuscript.

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Data availability Raw data of this study are available from the corresponding author Keitaro Tawaraya on request.

Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

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