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Solubilization of calcium phosphate as a consequence of carbon translocation by *Rhizoctonia solani*

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Abstract

A model system based on arrays of three concentric rings of discrete agar droplets is described which allowed study of fungal growth in vitro in nutritionally-heterogeneous conditions. Droplets containing different combinations of glucose and calcium phosphate were used to study the consequences of spatially separating these components in relation to metal phosphate solubilization by *Rhizoctonia solani*. A pH indicator, bromocresol purple, was added to the agar to visualise the localised production of acidity by the fungus. In the presence of the fungus, solubilization of calcium phosphate on homogeneous agar plates only occurred when glucose was present in the underlying medium. However, solubilization occurred in droplets containing calcium phosphate, but no glucose, when glucose was present in other droplets within the tessellation and where fungal hyphae spanned the droplets. This demonstrates that substrate was transported via mycelia from glucose-containing domains, with the functional consequence of metal phosphate solubilization. In another design, where the inner ring of droplets contained glucose and the outer ring contained only calcium phosphate, acidification of all droplets in the outer ring was observed when the inner droplets contained glucose. However, solubilization of calcium phosphate only occurred when the concentration of glucose in the inner droplets was greater than 2% (w/v). This indicated that a threshold concentration of carbon source may be required before such mechanisms of solubilization are invoked. There was also evidence for reverse translocation of substrate from newly colonised glucose-containing droplets in the outer ring to the central droplets, where fungal growth had originated.

Keywords: Translocation; Nutritional heterogeneity; Metal phosphate solubilization; Calcium phosphate; *Rhizoctonia solani*

1. Introduction

The majority of environments in which fungi grow are spatially heterogeneous and this is particularly true for soils due to their complex physical structure. Soil particles, pores, nutrients, moisture, and temperature can create considerable spatio-temporal variation. Pollutants, such as organic (e.g. phenols) and toxic metal-contaminated wastes, and soil amendments, such as fertilisers, increase the heterogeneity of the edaphic environment. Fungi are able to grow in a variety of conditions, are very efficient at exploring space in the soil, and have an important role in the transformation of metals in soils [1,2]. Various metabolites, such as organic acids, enable fungi to solubilize insoluble metal compounds and in some instances immobilize them in the form of oxalate crystals [1]. The release of phosphates and sulfates from insoluble sources and the redistribution of nutrients through mycelial networks is an important process for both plant and microbial nutrition. The phosphate-solubilising ability of *Penicillium bilaii* and other free-living and symbiotic fungi has been documented, as well as potential roles in increasing plant growth [1,3].

Rhizoctonia solani is an economically important fungus and occurs in a wide range of agricultural soils [4]. The role of *R. solani* as a plant pathogen and saprophyte is well documented, although the extent of its saprophytic abilities is still being investigated [5,6]. The redistribution of certain nutrients by *R. solani* in heterogeneous environ-

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ments has previously been reported [7–9]. In these experiments, microcosms consisting of heterogeneous arrays of discrete agar domains [7,8] and gradients [9] of differing nutritional status were used to study the interaction between the fungus and its environment. *R. solani* was observed to grow in nutrient-sparse regions by translocating material from parts of the mycelium growing in nutrient-rich conditions. However, experimental information in relation to the functional consequences of metal phosphate solubilization under nutritionally-heterogeneous conditions is lacking. Furthermore, the significance of carbon translocation, which may allow fungi to affect changes in metal mobility in nutritionally impoverished areas, is unknown. In this paper these issues are addressed using microcosm systems comprising agar droplets of different nutritional status and observing the growth responses and metal phosphate solubilizing activity of *R. solani*.

2. Materials and methods

2.1. Organisms, media and culture conditions

R. solani Kühn anastomosis group 4 (R3) was maintained on potato dextrose agar (PDA, Oxoid) at 20°C. Before being used for inoculation, the fungus was grown on tap water agar (TWA) containing 10 g l^{-1} Noble agar (Difco). Experiments were carried out on a glucose–mineral salt medium (MSM), comprising (all per litre distilled water): 5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.05 g $CaCl_2{\cdot}6H_2O$, 0.1 g NaCl, 0.0025 g $FeCl_3{\cdot}6H_2O$, 0.004 g $ZnSO_4{\cdot}7H_2O$, 0.004 g $MnSO_4{\cdot}4H_2O$, 0.0004 g $CuSO_4{\cdot}5H_2O$ 10 g Noble agar, 20 g D-glucose, unless otherwise stated. Salts were autoclaved individually before being added to the molten agar and glucose at approximately 50°C. Media were sterilised by autoclaving at 121°C for 20 min.

2.2. Experimental design

2.2.1. Experiment 1: Effect of pH indicators on growth of R. solani

The effect of the pH indicators bromocresol purple (BDH) and alizarin S (Riedel-de Haën) on the growth rate of *R. solani* was tested using MSM. The medium was amended with either 2% (w/v) glucose, 5 mM $Ca_3(PO_4)_2$, 2% glucose and 5 mM $Ca_3(PO_4)_2$ or left unamended. The control did not contain a pH indicator. The pH indicators were diluted to 1% (w/v) with distilled water and 1% (v/v) was added to the agar. The pH of MSM in the absence of $Ca_3(PO_4)_2$ was adjusted to pH 7 using 2% (v/v) pH 7 phosphate buffer (BDH) to ensure the agar was of the same pH as the calcium phosphate amended medium. Five replicate agar plates were inoculated with 4 mm diameter plugs of *R. solani* and incubated at 25°C. Radial growth was measured as the mean of two perpendicular radii of growing colonies, and recorded daily. Colony radial growth rate was calculated by linear regression of colony radius versus time.

2.2.2. Experiment 2: Growth of R. solani on tessellated agar droplets

The four combinations of MSM used in the growth rate experiment were also used in a tessellated agar droplet system. $Ca_3(PO_4)_2$ and bromocresol purple were added to the molten medium (50°C) before the agar was dispensed. Agar droplets were produced by pipetting 175 μl molten agar into a sterile 90 mm diameter Petri dish in a hexagonal array so that the droplets were 10 mm in diameter and 2 mm apart at the closest point, to prevent diffusion of nutrients between adjacent droplets. The basic design of the arrays was of 19 droplets arranged as three concentric domains, i.e. a central droplet, an inner ring and an outer ring comprising 1, 6 and 12 droplets respectively.

The tessellations of the four media adopted in the second experiment are shown in Fig. 1. The central and inner ring of agar droplets comprised different amended media to that of the outer ring, resulting in 12 different tessellations. Four 'control' tessellations, where all agar droplets comprised the same medium, were also established. The central droplet in each tessellation was inoculated with a 4 mm diameter plug of *R. solani* hyphae previously grown on TWA, with excess agar being cut away from the hyphae: each tessellation combination was replicated five times. Plates were sealed with Parafilm M, covered with moist tissue paper to prevent the agar droplets from drying out and incubated at 25°C in the dark. Uninoculated controls were also incubated.

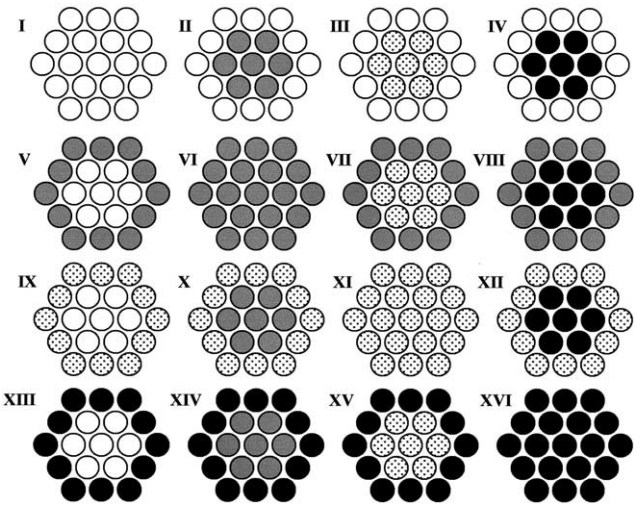

Fig. 1. Experimental design to determine the growth and solubilization by *R. solani* in nutritionally heterogeneous conditions. Agar droplets were amended with both 2% (w/v) glucose and 5 mM $Ca_3(PO_4)_2$ (black circles), only 2% (w/v) glucose (stippled circles), only 5 mM $Ca_3(PO_4)_2$ (grey circles) or left unamended (open circles). Droplets were 10 mm in diameter and 2 mm apart at the closest point.

Table 1
Effect of the pH indicators bromocresol purple and alizarin S on the growth rate of *R. solani* on media amended with different combinations of 5 mM $Ca_3(PO_4)_2$ and 2% (w/v) glucose

Amendment	Growth rate (mm day^{-1})		
	Control	Bromocresol purple	Alizarin S
+glucose+$Ca_3(PO_4)_2$	10.7 ± 0.4	10.2 ± 0.4	6.7 ± 0.5*
+glucose−$Ca_3(PO_4)_2$	8.2 ± 0.5	7.0 ± 0.6	3.8 ± 0.5*
−glucose+$Ca_3(PO_4)_2$	10.5 ± 0.3	8.1 ± 0.3	7.3 ± 0.4*
−glucose−$Ca_3(PO_4)_2$	9.8 ± 0.5	7.4 ± 0.3	4.6 ± 0.6*

Results are means of five replicates ± S.E.M.
*Significantly different from the control (ANOVA $P < 0.05$).

2.2.3. *Experiment 3: Effect of glucose concentration on mobility of the substrate*

In a third experiment the concentration of glucose added to the medium was varied in the central and inner droplets, the concentrations used were 0.1, 1, 2, 5 or 10% glucose (w/v); the outer ring of droplets consisted of MSM amended with 5 mM $Ca_3(PO_4)_2$ (Fig. 1; Tessellation VII). The two controls comprised the central and inner droplets being unamended MSM, and MSM amended with 5 mM $Ca_3(PO_4)_2$. The central agar droplet was inoculated with *R. solani* as before. Uninoculated controls were also incubated.

2.3. *Assessment*

The agar tessellations were visually assessed daily for 8 successive days. Fungal density, colonisation of agar droplets, acidification of agar droplets and solubilization of calcium phosphate were observed and scored on relative qualitative scales. Images of the tessellations were also captured daily by scanning the underside of the Petri dish using a Hewlett-Packard ScanJet ADF scanner.

3. Results and discussion

The addition of the pH indicators alizarin S and bromocresol purple demonstrated a decrease in pH of the agar, when *R. solani* was present, probably a result of the production of protons and organic acids by *R. solani*. Alizarin S was found to significantly reduce the growth rate of *R. solani* when grown on mineral salt medium amended with combinations of calcium phosphate and glucose (Table 1). Cunningham and Kuiack [10] also observed a reduction in growth of *P. bilaii* in the presence of alizarin S. It was noted in the present study that *R. solani* hyphae turned pink in the presence of alizarin S, which can also be used as a stain for calcium [11]. Bromocresol

Table 2
Growth, acidification of media and solubilization of calcium phosphate by *R. solani* grown on heterogeneous systems after 168 h

Tessellation	Location of droplets								
	Fungal density[a]			Droplets acidified			Droplets solubilised[b]		
	inner	central	outer	inner	central	outer	inner	central	outer
I	*	*	*	−	−	−	na	na	na
II	*	*	*	−	−	−	−	−	na
III	***	***	**	*	*	*	na	na	na
IV	**	**	***	*	*	*	++	++	na
V	*	*	*	−	−	−	na	na	−
VI	*	*	*	−	−	−	−	−	−
VII	**	**	**	*	*	*	na	na	+
VIII	**	**	***	*	*	*	++	++	+
IX	*	*	**	*	*	*	na	na	na
X	*	**	***	*	*	*	+	+	na
XI	**	**	**	*	*	*	na	na	na
XII	***	***	***	*	*	*	++	++	na
XIII	**	***	***	*	*	*	na	na	++
XIV	***	***	***	*	*	*	+	+	++
XV	**	**	***	*	*	*	na	na	++
XVI	***	***	***	*	*	*	++	++	++

See Fig. 1 for key to tessellations. Typical results are shown from one of five replicate treatments.
[a]*, sparse; **, moderate; ***, dense.
[b]na, not applicable; −, none; +, partial solubilization; ++, full solubilization.

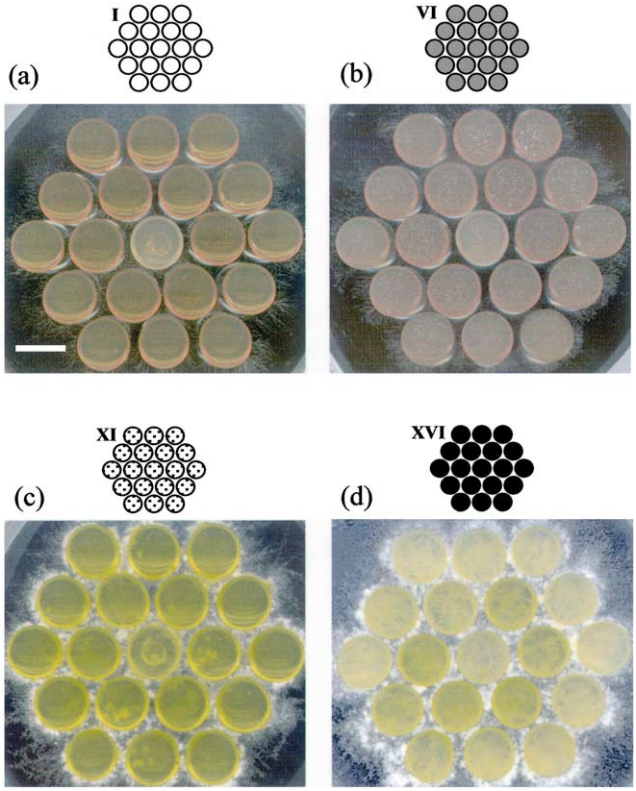

Fig. 2. Growth of *R. solani* after 168 h on agar droplets containing (a) no glucose or $Ca_3(PO_4)_2$ (Tessellation I), (b) only 5 mM $Ca_3(PO_4)_2$ (Tessellation VI), (c) only 2% (w/v) glucose (Tessellation XI) and (d) both 5 mM $Ca_3(PO_4)_2$ and 2% (w/v) glucose (Tessellation XVI). Scale bar = 1 cm. See Fig. 1 for key to tessellations. Typical results are shown from one of five replicate treatments.

purple did not significantly affect the growth rate of *R. solani* ($P < 0.05$), and so was used in subsequent experiments.

When *R. solani* was grown on conventional agar plates such as those used in Experiment 1, colony growth was characteristically symmetrical independent of the nutrient status. The colony density of *R. solani* reflected the nutrient status of the underlying agar when grown on uniform agar plates, with the mycelium being considerably more dense when grown on high nutrients. This was also apparent when *R. solani* was grown on the nutritionally uniform tessellations (Tessellations I, VI, XI and XVI; Fig. 2), but not the case when grown on nutritionally heterogeneous tessellations (Table 2). Hyphal density did not correlate with the nutrient status of the agar droplet immediately underneath the colony when the fungus had previously grown on a higher concentration nutrient source. For example, the hyphal density in the outer ring of Tessellations III and IV was greater than that of Tessellations I and II even though nutrients were not present in the outer droplets for any of the tessellations. This was also observed in Tessellations X and XIII where the hyphae on the central and inner droplets, devoid of glucose, became denser over time after the fungus had colonised the outer glucose-containing droplets (Table 2). The growth responses of *R. solani* on the tessellations of agar droplets were generally asymmetrical except for the glucose-free droplets (Tessellations I and VI, Fig. 2; Tessellations II and V, Table 2). In the tessellations devoid of glucose (Tessellations I, II, V and VI), fungal growth was recorded as sparse, even in the presence of calcium phosphate (Tessellations V and VI; Table 2).

Table 3
Growth and production of organic acids by *R. solani* on agar droplets containing different amounts of glucose in the central and inner ring and 5 mM $Ca_3(PO_4)_2$ in the outer ring (Tessellation VII; Fig. 1)

Assessment	Time after inoculation (h)	Amount of glucose (%) in inner and central droplets						
		0	0.1	1	2	5	10	0+metal
Fungal density	24	–	*	*	*	*	*	*
	48	*	*	*	**	**	**	*
	72	*	*	**	**	**	***	*
	96	*	*	**	**	***	***	*
	120	*	*	**	**	***	***	*
	144	*	*	**	***	***	***	*
Number of droplets colonised	24	0	1	1	1	1	1	1
	48	1	7	7	7	7	7	7
	72	7	19	19	19	15	7	19
	96	19	19	19	19	19	19	19
	120	19	19	19	19	19	19	19
	144	19	19	19	19	19	19	19
Number of droplets acidified	24	0	0	0	0	0	1	0
	48	0	3	3	3	1	1	0
	72	0	11	8	14	7	7	0
	96	0	16	19	19	18[a]	12	0
	120	0	18	19	19	19[a]	19[a]	0
	144	0	19	19	19[a]	19[a]	19[a]	0

Density of fungal growth: –, no growth; *, sparse; **, moderate; ***, dense.
[a] = $Ca_3(PO_4)_2$ solubilized.

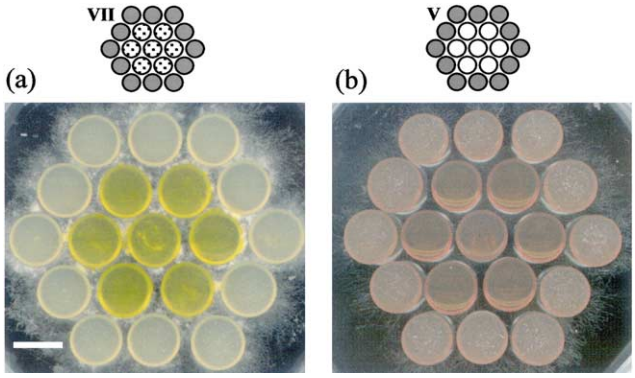

Fig. 3. Growth of *R. solani* after 168 h on agar droplets containing (a) 2% (w/v) glucose in the central and inner droplets and only 5 mM $Ca_3(PO_4)_2$ in the outer droplets (Tessellation VII), and (b) no glucose or $Ca_3(PO_4)_2$ in the central and inner droplets and only 5 mM $Ca_3(PO_4)_2$ in the outer droplets (Tessellation V). Scale bar = 1 cm. See Fig. 1 for key to tessellations. Typical results are shown from one of five replicate treatments.

Droplets in the uninoculated control tessellations did not become acidified and there was no solubilization in these treatments. Therefore growth and activity of the fungus were responsible for such actions when they occurred. In tessellations containing calcium phosphate but devoid of glucose (Tessellations II, V and VI), solubilization of calcium phosphate did not occur. This confirms that insoluble metal phosphates are not solubilized by *R. solani* unless a sufficient carbon source is present [12]. This was evident in Tessellation VII, where glucose was present in the central and inner droplets and calcium phosphate was present in the outer ring. The production of organic acids and protons by fungi is an important process in biogeochemical cycles, making ions such as phosphate and metal cations available for intracellular uptake [2]. It was evident in the above experiments that the presence of a carbon source was essential for acidification of the agar and solubilization of calcium phosphate (Fig. 2). *R. solani* has previously been reported to produce oxalic acid, and calcium oxalate crystals in vitro and in vivo in plants [13]. Biosynthesis of oxalic acid from glucose occurs by hydrolysis of oxaloacetate to oxalate and acetate catalysed by cytosolic oxaloacetase (oxaloacetate (acetyl)hydrolase) [1]. The production of acidity by *R. solani* was not apparently dependent on the presence of metal phosphates in this experiment (Fig. 2c). However, in certain other fungi, organic acid production can be markedly influenced by the addition of metal phosphates [14,15]. Furthermore, it has also been shown that carbon levels can affect metal toxicity and biomass distribution in *Trichoderma viride* and *Rhizopus arrhizus* [16].

In Experiment 3, solubilization of calcium phosphate in the outer ring only occurred when the concentration of glucose in the central and inner droplets was 2% (w/v) or above, whereas acidification of the agar occurred in all droplets for the concentrations of glucose used in this experiment (Table 3). No acidification or solubilization occurred when glucose was absent from the central and inner droplets (Tessellation V and Fig. 3b). Fungal expansion was fastest at lower concentrations of glucose and acidification occurred approximately 24 h after the agar droplet had been colonised. Therefore, it appears that a certain concentration of organic acid or protons is needed for the solubilization of calcium phosphate. In laboratory studies, it has been found that oxalic acid concentrations of < 0.1 mM are able to solubilize gypsum [17].

It seems likely that substrate was transported by the fungus from the central and inner droplets to the outer droplets by translocation within the mycelium. However, the transfer of nutrients by 'wicking', i.e. the external movement of a substance by capillary action and diffusion along the hyphae, may also be a potential mechanism by which substances could be transported from one domain to another [18]. Although this process is not considered to be a form of biological translocation, it nevertheless may facilitate the movement of nutrients via the mycelial network.

When the central and inner droplets contained calcium phosphate and the outer droplets contained glucose (Tes-

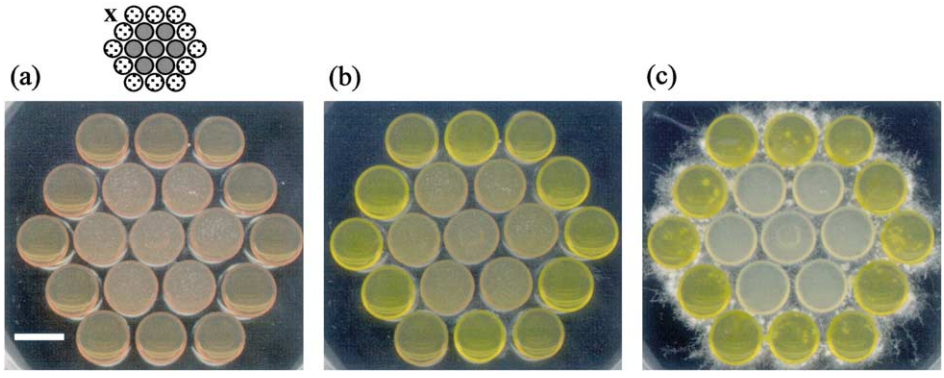

Fig. 4. Growth of *R. solani* after (a) 24 h, (b) 72 h and (c) 168 h on agar droplets containing only 5 mM $Ca_3(PO_4)_2$ in the inner droplets and only 2% (w/v) glucose in the outer droplets (Tessellation X). Scale bar = 1 cm. See Fig. 1 for key to tessellation. Typical results are shown from one of five replicate treatments.

sellation X), the fungus grew sparsely over the inner droplets for the first 72 h (Fig. 4a,b) and did not affect the pH of the agar until the outer droplets were colonised (Fig. 4c). Subsequently, after 96 h, the inner agar droplets became acidified and the calcium phosphate was solubilized, indicating that substrate was redistributed to a site the fungus had already colonised. This exploitation of previously unavailable resources may be facilitated by either reverse translocation, or by growth of the fungus back to the inoculated domain. In the absence of glucose, solubilization of calcium phosphate is apparently untenable. However, when substrate becomes available to the mycelium via the colonisation of a glucose-containing domain, the calcium phosphate is then solubilized and utilized by the fungus with a concomitant increase in hyphal density (Fig. 4, Table 2). Bi-directional translocation of nutrients, carbohydrates and phosphate has been reported in a number of fungi [19–21]. Lindahl et al. [21] postulated that nutrient transport can occur in all directions independently of polarities within the mycelium; however, concentration gradients cause fluxes in different directions that result in net translocation from source to sink.

R. solani acidified the medium in the presence of glucose regardless of a metal phosphate being present. The transfer of carbon substrate to a remote zone occurred in both the presence and absence of calcium phosphate. Therefore, the substrate was probably distributed, in part, by diffusion (passive translocation within the hyphae), and the functional consequence of this was the solubilization of the calcium phosphate. However, there were certain combinations of tessellations that lacked the fungal growth symmetry associated with simple diffusive translocation. For example, the biomass densities of Tessellations III and IX, and VII and X (Table 2) suggest the fungus was better at translocating carbon towards the colony periphery than towards the colony centre. Comparison of biomass in Tessellations III and VII also suggests that fungal growth on the inner agar droplets is dependent upon the status of the outer agar droplets, a result that cannot be explained by standard diffusion models. In a mathematical model produced by Boswell et al. [22], it was hypothesised that *R. solani* may possess a mechanism by which different translocatory mechanisms may be induced in certain circumstances, and where gradients in internal and external nutrients act as a switch between mechanisms. Our results here provide further experimental support for such a hypothesis.

In conclusion, *R. solani* was able to redistribute carbon, either glucose or derived products, to a remote location lacking a carbon source. The consequence of this phenomenon was the solubilization of calcium phosphate. Such solubilization and nutrient translocation by fungi may be environmentally significant since it may influence the location of fungal species and relocation of otherwise immobile elements such as metals and phosphate in the soil. Furthermore, given interactions may influence the location and extent of functional processes such as solubilization, this has implications for management of biological processes in soils.

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