



# Carbon Investment Required for the Mobilization of Inorganic and Organic Phosphorus Bound to Goethite by an Arbuscular Mycorrhiza (*Solanum lycopersicum* x *Rhizophagus irregularis*)

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Nutrient supply in phosphorus (P)-limited ecosystems, with most P being associated with secondary minerals, has to rely on efficient nutrient allocation strategies, such as those involving mycorrhizal symbioses. Yet, little is known about the extent of photo-assimilate transfer to the fungal partner, who in turn mobilizes mineral-bound P sources required by the plant. This study aims to explore the carbon (C)–P trade between an arbuscular mycorrhizal (AM) plant and its ability to incorporate P from differently accessible P sources. We compared P uptake rates of AM plants for orthophosphate (OP) and phytic acid (PA), applied to mesocosms in either dissolved form or bound to goethite ( $\alpha$ -FeOOH). The design of the mesocosms allowed the plant to only access the P in the fungal compartment via the AM hyphae. We hypothesized the AM plant to invest more C into the symbiosis, if P is present in the less accessible form. To estimate the C budget of the symbiosis, we determined total organic carbon (OC), 16:1 $\omega$ 5c phospholipid fatty acid (PLFA; AM fungi extraradical mycelium), 16:1 $\omega$ 5c neutral lipid fatty acid (NLFA; AM fungi energy storage), and CO<sub>2</sub> cumulative respiration in the fungal compartment. A ratio to the total C translocated into the fungal compartment (OC+CO<sub>2</sub>-C cumulative respiration) and the P incorporated into the AM plant (Total C/P) was calculated to estimate the C investment made by the AM plant into its symbiotic partner. AM plants incorporated P derived from all four P sources exclusively via the mycorrhizal pathway in different amounts and kinetics. The Total C/P ratio was significantly larger for those AM plants accessing the goethite-bound P compounds. They also transferred significantly larger amounts of PLFA and NLFA to their fungal partner, both indicating a larger plant C investment per P taken up. Our data provide first evidence about the ability of an AM plant to incorporate P from an organic source bound to a secondary mineral. The different C investments of AM plants into P allocation from variably available sources suggests a broad nexus between P mining strategies, resource partitioning in soil, and the amounts of C accumulated in terrestrial soils.

**Keywords:** goethite, organic phosphorus, inorganic phosphorus, arbuscular mycorrhiza, carbon-phosphorus trading, PLFA 16:1 $\omega$ 5c, NLFA 16:1 $\omega$ 5c

## INTRODUCTION

Phosphorus (P) is an essential element for plant growth and productivity. While excessive utilization of P as fertilizer has led to widespread eutrophication of inland and coastal waters (Rowe et al., 2016), its deficiency is still a major constraint to agricultural productivity, affecting an estimated area of >20 million km<sup>2</sup> worldwide (Oberson et al., 2001). This is especially true for tropical soils where secondary minerals immobilize P to a large extent. In 2014, phosphate rocks were included in the list of the 20 critical raw materials of the European Commission (George et al., 2016), suggesting that this finite resource could be exhausted within this century (Gilbert, 2009). Consequently, all plant strategies which increase P uptake and improve its use-efficiency will be increasingly valuable to prevent P loss to adjacent ecosystems and lower the need of P fertilizers (Smith and Smith, 2011).

Plants can only take up P as free phosphate anions, either as H<sub>2</sub>PO<sub>4</sub><sup>-</sup> or as HPO<sub>4</sub><sup>2-</sup> (Becquer et al., 2014). Phosphate concentrations in soil solutions span from 1 to 10 μM, being about 1,000-fold smaller than cellular contents in plants, thus are often insufficient for optimal growth (Smith and Smith, 2012). Soil P occurs in forms of varying accessibility to plants with >90% of it being bound in plant-inaccessible forms (Mengel and Kirkby, 2001). Phosphorus occurs either as inorganic phosphates, primarily in associations with Ca, Fe, and Al, or in organic forms. Organic P constitutes 20–80% of total soil P and includes phosphomonoesters such as inositol phosphates (IP), phosphodiester as ribonucleic acid, deoxyribonucleic acid, lipoteichoic acid, phospholipid fatty acids, and organic polyphosphates, e.g., adenosine triphosphates. The most prevalent phosphomonoester is myo-IP6, which contains six phosphate groups bound to cyclohexane and has nine stereoisomers. The myo-IP6 stereoisomer or phytic acid (PA) is the most common form of inositol phosphates and represents >50% of the organic P in soils (Ognalaga et al., 1994; Nash et al., 2014).

Phosphorus deficiency of plants is caused by strong adsorption of inorganic and organic P to Al and Fe hydroxides and oxides (summarized as “oxides”), making large proportions of total P unavailable to plants (Osorio and Habte, 2001; Javaid, 2009). The specific adsorption and high affinity to soil oxides is greater for the organic P forms (He and Zhu, 1998). As compared to inorganic P forms, the dynamics of organic P species in soils are much less investigated. This is surprising since this fraction is highly relevant to the supply of P to crops in deeply weathered soils like Oxisols (Rodrigues et al., 2016). To overcome soil P limitation, plants developed different strategies to acquire P from soil solution. One strategy is to increase the exchange-surface of the root–soil interface; another strategy lies in solubilizing complexed P by root exudates, such as low-molecular-weight organic acids and phosphatases. The most widespread strategy nevertheless is to rely on symbiotic associations with mycorrhizal fungi and P-solubilizing or organic P-mineralizing bacteria (Richardson et al., 2009; Giles et al., 2012).

In P-deficient environments, e.g., Spodosols, Ultisols, or Oxisols (Yang et al., 2013), selection pressure commonly results

in the proliferation of free-living microorganisms and symbiotic associations with mycorrhizal fungi which have the potential to mobilize and mineralize unavailable organic P (Nash et al., 2014). Most terrestrial plants form symbioses with one or more kinds of mycorrhizal fungi. About 80% of plant species are associated with arbuscular mycorrhizal fungi (AMF) (Cairney, 2000; Smith et al., 2008; Johnson, 2010), which enhance the plant's access to limiting belowground resources (Read, 1991).

Plants that establish symbiosis with an AMF can acquire part or almost all of the phosphorus for their metabolic activities, over this pathway (Javot et al., 2007; Willis et al., 2013; Lambers et al., 2015). As they are able to mine soils for P, research on P release rates from secondary minerals is important to improve models on plant nutrient cycling by AMF (Cardoso et al., 2006). Though some studies have shown that mycorrhizal and non-mycorrhizal plants seem to use the same labile P sources (Bolan et al., 1984; García, 2000; Frossard et al., 2011), others demonstrated that mycorrhizal plants obtain P from usually unavailable sources of organic and inorganic P (Rychter et al., 2016). In addition, Bolan et al. (1987) proposed that AMF may cleave the bond between Fe and P and thus release P, but they did not depict the underlying mechanisms.

Turner (2008) suggested that the different soil P species constitute a gradient of biological availability based on the plant's investment to access the phosphate. For example, phytate, being most resistant to hydrolysis, is hypothesized as the metabolically most expensive P source. This makes necessary to understand if there exists a C-P trading in arbuscular mycorrhizal symbiosis.

In return for providing almost all P needed by the AM plant, the fungus receives up to 20% of net plant photosynthates under ambient atmospheric CO<sub>2</sub> (Pfeffer et al., 2004). The C assimilates are transported mainly as lipid droplets and glycogen to the AM fungal hyphae (Bago et al., 2002). Recently, three publications have shown that AMF do not have the capacity to produce fatty acids completely on their own (Bravo et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017). Instead, AMF were found completely depending on plant-derived fatty acids. These studies suggest a model in which a chain length of 16 carbons (C16) b-monoacylglycerol molecules are transported from the root cell through the periarbuscular membrane to the fungus. Fatty acids of chain lengths up to C16 are needed, because the fungus apparently lacks genes encoding multi-domain cytosolic fatty acid synthase subunits. This makes fatty acids a major good of trade between plant and fungus in AM.

To compare the true costs of incorporating different P forms into the plant by the AMF pathway requires the quantification of the C fluxes. The development of AM itself already presents a complex series of trade-offs between the C cost of the fungus and the benefits of enhanced nutrient supply to the plant (Cavagnaro et al., 2008). Quantification of the fluxes in mycorrhiza is one of the most important, yet little explored tasks of mycorrhizal physiology and ecology. In order to identify the behavior of AM in mobilizing P from differently accessible P forms, we carried out a mesocosm experiment under controlled conditions. Our work aimed at (i) clarifying whether the AM plant can take up P from less accessible sources and (ii) to determine whether there exists a trading between C and P over the AM. We hypothesize that

less accessible P sources cause larger photoassimilate investments by the plant partner, resulting in differing kinetics of the AMF providing P to the host plant.

## MATERIALS AND METHODS

### Plant Growth and Mycorrhization

We selected the AM mycorrhizal association between *Solanum lycopersicum* L. (var. MoneyMaker) x *Rhizophagus irregularis* (DAOM 197198) for our experiment. The association has been previously investigated to elucidate processes related with the nutritional benefits offered by the AMF to the tomato plant (Nagy et al., 2005; Schaarschmidt et al., 2006; Giovannetti et al., 2012). The reason for using the AMF *R. irregularis* is due to its global distribution and adaptation to intensive agricultural practices. This ubiquitous occurrence indicates that *R. irregularis* is compatible with a wide range of soil conditions like pH (5.6–8.0), P availability (0.3–18.8 mg/kg), sand content (17.5–57.0%), and C content (1.0–10.5%) (Köhl et al., 2016). *Rhizophagus irregularis* DAOM 197198 (syn: *Glomus irregulare*) recently reassigned from *Glomus intraradices* Schenck and Smith (Krüger et al., 2012) was used as the mycorrhizal inoculum. It consisted of 0.4 g of spores, hyphae, and root fragments from a *Sorghum bicolor* trap plant culture (Brundrett, 1996). Seeds of *Solanum lycopersicum* L. var. MoneyMaker (Volmary GmbH) were surface sterilized with 5% H<sub>2</sub>O<sub>2</sub> for 10 min and washed three times with sterile distilled water. Seeds were germinated on petri dishes for 3 days at 27°C. MoneyMaker germinated seeds were sowed on 75 ml pots QP96 (HerkuPlast Kubern GmbH) containing the AM inoculum and 70 ml autoclaved acid washed quartz sand, which has been successfully used as plant and fungal growth substrate before (Johansen et al., 1996; Olsson and Johansen, 2000). Allowing the AM mycelium to grow from a colonized root into purified quartz sand, a relatively pure mycelium could be extracted and used for our studies. Mycorrhized and non-mycorrhized control tomato plants were grown in a greenhouse (16/8 light/dark, 24/20°C light/dark, 50–60% relative humidity, photon flux density of 175–230 μmol/m<sup>2</sup>/s). Seedlings were watered every day with 10 ml deionized water and were fertilized with 5 ml low P (0.32 mM) Long Ashton nutrient solution pH 6.5 (Hewitt, 1966) on alternate days. The complete root system of 10 individuals was processed to test for the presence of arbuscular mycorrhiza in the roots of 4 week old plants before transplanting. A root subsample was digested with 10% KOH (35 min, 95°C) and stained using the ink and vinegar staining technique for AMF (Vierheilig et al., 1998b). Stained root fragments were mounted on glass slides and observed at 400× magnification using an Olympus BH2 microscope (Olympus Optical Company Ltd, Tokyo, Japan), to determine the mycorrhizal status using the methodology from McGonigle et al. (1990), before the plants were transplanted to the mesocosms.

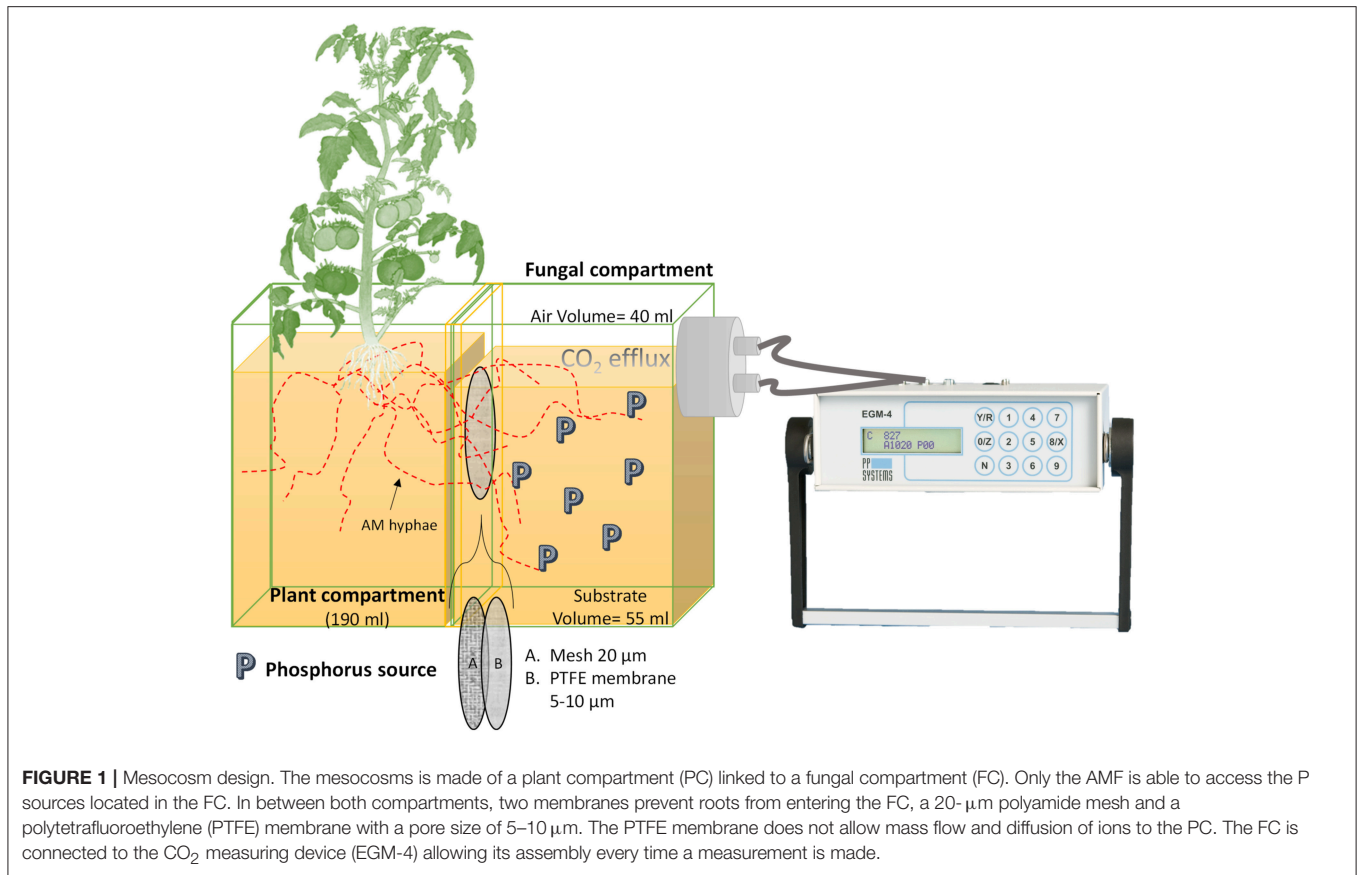
### Mesocosm Experiment

Each mesocosms consisted of a plant compartment connected to a fungal compartment in which only the mycelium was able to develop and access the different P sources due to two barriers (Figure 1). Each mesocosms was fabricated from a

Nalgene® 250 ml square polypropylene bottle cut at 45° angle. The threaded section of the bottle was glued with Pattex® hot glue on the side with the largest surface of the bottle base. A second Nalgene® 100 ml square polypropylene bottle was cut into two sections, one as irrigation compartment (30 ml) and the second as support for the mesocosms. Through a 3 mm diameter perforation at the base of the plant compartment, a 3 mm diameter fiberglass wick (Ortmann Kapillarbewässerung, Vlotho, Germany) was passed through, leaving 5 cm of the wick inside the irrigation compartment, where twice a week 10 ml deionized water were applied (Supplementary Figure 1). The wick irrigation system operates in a closed cycle, without runoff and covers maximum water requirements of the plant (Son et al., 2006; Kuntz, 2013). At the plant compartment side a polyamide mesh with a pore size of 20 μm and 30 mm side (Franz Eckert GmbH) separated mycorrhizal roots and mycelium (Watkins et al., 1996; Fitter et al., 1998). The second barrier was a 25 mm diameter polytetrafluoroethylene membrane with a pore size of 5–10 μm (Pieper Filter GmbH). The second membrane allowed the AM hyphae to cross but prevented mass flow and diffusion of ions into the plant compartment (Mäder et al., 1993, 2000; Vierheilig et al., 1998a). Hence, P sources were exclusively accessible to the plant by the hyphae. There were no other P sources present in the system as the cultivation substrate in the plant compartment consisted of acid washed quartz sand (1–2 mm) free of nutrients (data not shown). Four-week-old mycorrhized and non-mycorrhized seedlings were transplanted into each mesocosm and placed in a glasshouse under controlled climatic conditions (24/20°C light/dark; photoperiod 16/8 h light/dark; 50–60% relative humidity; photon flux density of 175–230 μmol/m<sup>2</sup>/s).

Once a week all mesocosms were rotated to achieve homogeneous growth conditions and were fertilized twice a week on top of the plant substrate with 5 ml no-P Long Ashton nutrient solution at pH 6.5 (Hewitt, 1966). The P sources offered in the fungal compartment, including the control treatments, are summarized in Table 1. The 55 ml volume of the fungal compartment was amended by a total P amount of 30 mg and water volume was adjusted to field capacity. The water volume at the fungal compartment was determined with a time-domain reflectometry probe Trime Pico connected to a Trime-FM version P2 (Imko Micromodultechnik GmbH, Ettlingen, Germany). The treatments containing quartz sand as a substrate (60 g) in the fungal compartment exhibited 19% water volume while the ones containing goethite (24.3 g) exhibited 41% water volume. The water volume was checked once a week and amended with autoclaved MilliQ water to field capacity. A nylon black cloth with a 1 mm pore was placed on the mouth of the fungal compartment to prevent algae growth, water evaporation and to facilitate gas diffusion to the outside of the fungal compartment (Supplementary Figure 1).

The P uptake and C investment was tracked for 91 days. The first harvest point was on the day of transplanting (day 0) to determine the P stocks of the plants ( $n = 5$ ), followed by another six sampling points at days 7, 21, 35, 49, 77, and 91. In each of the six harvest points, three biological replicates of each treatment were processed.



**TABLE 1 |** Description of the treatments tested by the time course experiment.

Treatment	Treatment code	Fungal compartment (Total volume: 55 ml)	P content (mg)
Mycorrhizal plant without P	M+	Acid washed quartz sand (Awqs) (60 g) + MilliQ water (16 ml)	0
Non mycorrhizal plant without P	M-	Awqs (60 g) + MilliQ water (16 ml)	
Mycorrhizal plant with goethite	GOE	Goethite (24.3 g) + MilliQ water (28 ml)	
Mycorrhizal plant with orthophosphate bound to goethite (1.24 mg P/g)	GOE-OP	GOE-OP (24.3 g) + MilliQ water (28 ml)	30
Mycorrhizal plant with phytic acid bound to goethite (1.79 mg P/g)	GOE-PA	GOE-PA (16.7 g) + Goethite (7.6 g) + MilliQ water (28 ml)	
Mycorrhizal plant with orthophosphate ( $\text{KH}_2\text{PO}_4$ )	OP	Awqs (60 g) + MilliQ water (16 ml P solution)	
Mycorrhizal plant with phytic acid ( $\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$ )	PA	Awqs (60 g) + MilliQ water (16 ml P solution)	

## Preparation of Phosphorus Sources and Phosphorus Desorption

Each P source was placed inside the fungal compartment as explained in **Table 1**. The orthophosphate (OP) source was added as  $\text{KH}_2\text{PO}_4$  (Sigma Aldrich, Taufkirchen, Germany) and the organic P source was added as phytic acid (PA) sodium salt hydrate (Sigma Aldrich). Two adsorption complexes were produced using the OP and the PA bound to goethite. The adsorption complexes were prepared equilibrating the P compounds with goethite (Bayferrox 920 Z). First, 50 g of goethite were equilibrated for 16 h in 250 ml MilliQ  $\text{H}_2\text{O}$  adjusted to pH 4. Then, 250 ml MilliQ  $\text{H}_2\text{O}$  containing either 17 g  $\text{KH}_2\text{PO}_4$  or 0.7191 g  $\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$  were added to the goethite

suspension and equilibrated for 48 h on an overhead shaker. The goethite-P suspensions were centrifuged ( $3,000 \times g$ , 15 min) and the pellets were subsequently washed with MilliQ  $\text{H}_2\text{O}$  until the electric conductivity was  $<40 \mu\text{S}/\text{cm}$ . The resulting goethite-P associations were shock-frozen in liquid  $\text{N}_2$ , and freeze-dried. The OP and PA bound to the goethite adsorption complexes was determined in triplicate by hydrolysing 5 mg of the goethite-P associations in concentrated  $\text{HNO}_3$  and subsequent measurement of P contents by ICP-MS Agilent 7500C (Agilent Technologies Ireland Ltd., Cork, Ireland). The adsorption complexes contained 1.24 mg P/g in case of GOE-OP and 1.79 mg P/g for GOE-PA, respectively. A second analysis on the adsorption complexes was carried out to determine the

amount of desorbable P, by shaking 1.0 g of each goethite-P association ( $n = 3$ ) in 30 ml of MilliQ water for 24 h on an overhead shaker. The centrifuged supernatant ( $3,000 \times g$ , 15 min) was filtered through a  $0.45 \mu\text{m}$  syringe filter (PVDF) and 1 ml was mixed with 1 ml 30%  $\text{HNO}_3$  and the mixture was filled up to 10 ml with MilliQ water and P content was then measured by ICP-MS.

## Plant and Fungal Phosphorus Contents

At each harvest point, shoots and roots were air dried ( $70^\circ\text{C}$ , 48 h), weighed and ball milled. Plant samples were incinerated at  $480^\circ\text{C}$  for 8 h, digested with 1 ml 30%  $\text{HNO}_3$ , filtered and analyzed by ICP-MS. The P stocks were determined for the whole plant. The amount of total P incorporated by the AM plant over time was calculated as the difference of the P stock at each harvest point and the P stock initially present in the transplanted plant on day 0 (determined from five subsamples). The separation of hyphae/spores took place on day 91 following a modified method of Brundrett (1996). First, 4 g of goethite-P associations (GOE-OP, GOE-PA) or 10 g of sand (OP, PA) were sampled from each fungal compartment in 50 ml Falcon test tubes. Tubes were then filled with a fixative solution (0.9 g/l NaCl plus 3% glutaraldehyde), equilibrated for 2 h on an overhead shaker, and then centrifuged ( $3,000 \times g$ , 15 min). The supernatant was filtered through a polyethersulfone  $0.45 \mu\text{m}$  filter (Supor<sup>®</sup> PES membrane disc filters, Pall Life Sciences, Hampshire, UK), using a vacuum pump system. Pellets were re-suspended in a 50% sucrose solution by vigorously shaking plus an equilibration step (1 h) on an overhead shaker. The samples were then centrifuged for 15 min at  $3,000 \times g$  to facilitate the separation of hyphae and spores in the glucose density gradient. Immediately after centrifugation, hyphae and spores in the sucrose supernatant were poured onto the same polyethersulfone  $0.45 \mu\text{m}$  filter and carefully washed with MilliQ  $\text{H}_2\text{O}$  to remove the sucrose and the fixative solution. This step was repeated three times per sample. After rinsing the hyphae and spores, the filters were placed in petri dishes. The hyphae/spores were collected under a stereomicroscope with a glass pipette and deposited in 2 ml Eppendorf tubes. The fungal material was air-dried at  $50^\circ\text{C}$  for 96 h. The P content (P mg /hyphae mg) was determined for an aliquot of fungal material. Samples were incinerated at  $480^\circ\text{C}$  for 8 h, digested with 1 ml 30%  $\text{HNO}_3$ , filtered, and analyzed by ICP-MS. Additionally, the P stock incorporated into the plant tissues at day 91 was calculated as the percentage of the initial total 30 mg P offered at the fungal compartment, for each P source.

## Organic Carbon and Carbon Dioxide Production at the Fungal Compartment

At each harvest point, 2 g from each fungal compartment were air-dried ( $70^\circ\text{C}$ , 48 h), weighed, and ball milled. The C content of these samples was measured using an Elementar vario MICRO cube C/N analyzer (Elementar GmbH, Hanau, Germany). For the calculation of the C stock in the fungal compartment, it was multiplied the C content (mg/g), determined at the analyzer, by the total weight of substrate in the fungal compartment reported in **Table 1**. As there was no inorganic C in the fungal

compartment, all C measured in the fungal compartment was considered as organic carbon (OC).

The  $\text{CO}_2$  efflux ( $\text{mmol CO}_2 \text{ m}^{-2} \text{ h}^{-1}$ ) was measured in the fungal compartment twice a week using an EGM-4 infrared gas analyzer (PP-systems, Hitchin, UK), a close dynamic system (Vermue et al., 2008). At each measuring point, the black cloth was removed, the airtight lid was placed in the mouth of the fungal compartment and the inlet and outlet tubes were connected to the EGM-4 (**Supplementary Figure 1**).  $\text{CO}_2$  efflux was automatically calculated for a headspace volume of 40 ml and an exposed area of  $0.0012 \text{ m}^2$  of the fungal compartment. The  $\text{CO}_2$  content was measured every 4.8 s and the  $\text{CO}_2$  flux was measured for at least 3 min or until a good quadratic fit was obtained. Cumulative  $\text{CO}_2$  production was calculated in mg by interpolation using a cubic spline function (Gentsch et al., 2018) for each fungal compartment.

At each time point, the Total C/P ratio was used to evaluate and estimate the investment made by the AM plant into its symbiotic partner at the fungal compartment per plant P incorporated (Equation 1), since all the C measured at the fungal compartment, in the form of total OC and cumulative  $\text{CO}_2$ -C will be exclusively carried by the AMF.

$$\text{Total C/P} = \frac{\text{OC (mg)} + \text{cumulative CO}_2 - \text{C (mg)}}{\text{P incorporated (total mg per plant)}} \quad (1)$$

Where OC is the total C (mg) per fungal compartment; cumulative  $\text{CO}_2$ -C is the total C present in the cumulative respired  $\text{CO}_2$  (mg) at the fungal compartment.

## Fatty Acid Analysis and *R. irregularis* Biomass Estimation

Using a chloroform-methanol-citrate buffer (1:2:0.8 v/v/v), lipids were extracted twice from 16 g of fungal compartments containing quartz sand or 8 g for the ones with goethite. Thereafter, extracts were fractionated by solid phase extraction with activated Silica gel (Sigma Aldrich, pore size  $60 \text{ \AA}$ , 70–230 mesh) into neutral lipid fatty acids (NLFA), glycolipids, and phospholipid fatty acids (PLFA) by elution with 5 ml of chloroform, 20 ml of acetone, and 20 ml of methanol, respectively. The PLFA and NLFA samples were subjected to mild alkaline methanolysis, which transformed the neutral lipids and the phospholipids into free fatty acid methyl esters, as outlined in Frostegård et al. (1991) with modifications by Bischoff et al. (2016). The methyl esters were then separated by gas chromatography using an Agilent 7890A GC system (Agilent Technologies Ireland Ltd., Cork, Ireland) equipped with a 60 m Zebron capillary GC column (0.25 mm diameter and  $0.25 \mu\text{m}$  film thickness; Phenomenex, Torrance, California, USA) and quantified with a flame ionization detector, using He as carrier gas. Glycerol tridodecanoate (25  $\mu\text{g}$ ) and nonadecanoic acid (25  $\mu\text{g}$ ) were used as internal standards during the extraction and tridecanoic acid methyl ester (15  $\mu\text{g}$ ) was added to each sample and standard before analysis as a recovery standard. Identification of the fatty acids was achieved by use of the relative retention times, in comparison to that of the internal

standard using the GC ChemStation (B.03.02.341) software (Agilent Technologies Ireland Ltd., Cork, Ireland).

The AMF *R. irregularis* DAOM 197198 model organism (Daubois et al., 2016) has a fatty acid composition ranging from C16:0 to C22:2 with 16:1 $\omega$ 5 as major fatty acid (Aarle and Olsson, 2003; Calonne et al., 2010; Wewer et al., 2014). The PLFA 16:1 $\omega$ 5c can be used for evaluating the amount of extraradical mycelia of AMF, making it a good predictor for the amount of C allocated to AMF (Olsson and Johansen, 2000; Aarle and Olsson, 2003; Marschner, 2007). In addition, the NLFA 16:1 $\omega$ 5c is considered a good predictor on energy storage by the fungus (Bååth, 2003). NLFA are stored in intraradical vesicles, spores, extraradical mycelium and make up a large proportion of the AM fungal biomass. They are metabolized in the mycelium through the glyoxalate cycle, and might provide the major fungal energy source as respiratory substrate (Olsson and Wilhelmsson, 2000; Aarle and Olsson, 2003; Olsson and Johnson, 2005). Therefore, PLFA and NLFA 16:1 $\omega$ 5c were analyzed to assess the biomass and energy storage of AMF in the fungal compartment, respectively (Johansen et al., 1996; Olsson et al., 1997, 2002; Larsen et al., 1998; Stumpe et al., 2005). Ratios of 16:1 $\omega$ 5c PLFA to plant P uptake (Equation 3) and NLFA to plant P uptake (Equation 4), were used to estimate the investment made by the tomato plants into their fungal partner in either biomass or energy storage to obtain P from each source, respectively. The NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c ratio (Equation 5) was calculated as an index for the growth strategy of *R. irregularis* (Green et al., 1999; Rinnan and Bååth, 2009). For each P source, the mean NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c ratio was calculated for sampling points belonging to the periods where no P incorporation was detected in the AM plant tissue as well as for those where we detected P in the AM plant.

$$\frac{\text{PLFA } 16:1\omega 5c}{P} = \frac{\text{PLFA } 16:1\omega 5c \text{ (total } \mu\text{g at the fungal compartment)}}{P \text{ incorporated (total mg per plant)}} \quad (2)$$

$$\frac{\text{NLFA } 16:1\omega 5c}{P} = \frac{\text{NLFA } 16:1\omega 5c \text{ (total } \mu\text{g at the fungal compartment)}}{P \text{ incorporated (total mg per plant)}} \quad (3)$$

$$\frac{\text{NLFA}}{\text{PLFA}} = \frac{\text{NLFA } 16:1\omega 5c \text{ (total } \mu\text{g at the fungal compartment)}}{\text{PLFA } 16:1\omega 5c \text{ (total } \mu\text{g at the fungal compartment)}} \quad (4)$$

A high NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c ratio indicates preferential C allocation to storage products in form of neutral lipids. Moreover, the ratio identifies the origin of the 16:1 $\omega$ 5c fatty acid. At NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c > 1, the majority of this fatty acid is considered to originate from AM fungi and not from bacteria (Olsson, 1999; Hammer et al., 2011a; Vestberg et al., 2012; Cozzolino et al., 2016). To estimate the amount of AMF grown in the fungal compartment, we used a conversion factor of 1.2 nmol PLFA 16:1 $\omega$ 5c per mg dry hyphae (Olsson and Johansen, 2000; Olsson and Wilhelmsson, 2000; van Diepen et al., 2010). The estimated total AM biomass was used together with the fungal P content to infer the P stock incorporated by the AMF. Furthermore, we calculated the percentage of the initial total 30 mg P offered at the fungal compartment incorporated into the AMF biomass at day 91, for each P source.

## Data Analysis

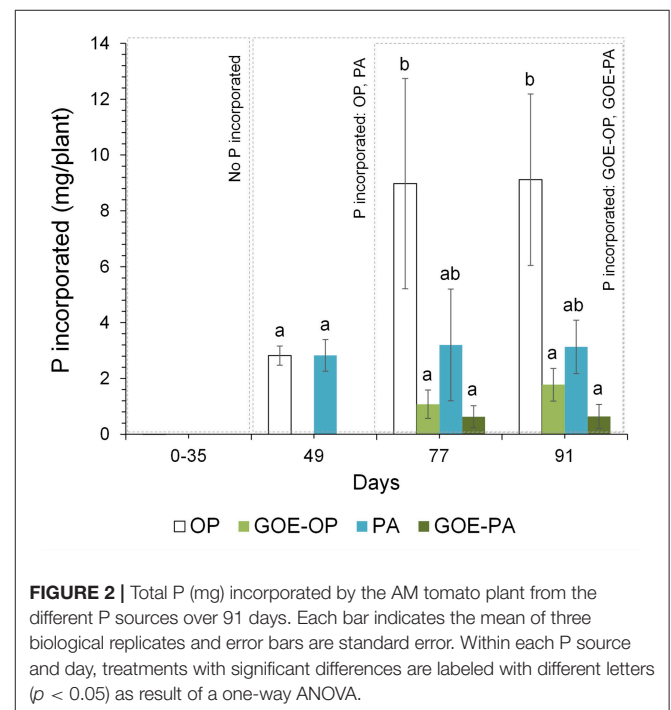
The normality of the data was verified with the Shapiro-Wilk's test and homogeneity of variances using the Levene's

test. One-way ANOVA analysis of the variance and the Tukey *post-hoc* test (Supplementary Tables 1–6) were employed to test for differences of mean values ( $P < 0.05$ ) of measured variables between the treatment groups, presented by the different P sources offered in the fungal compartment. Data analysis was performed using SPSS v.24 for Windows (IBM Corporation, 2016).

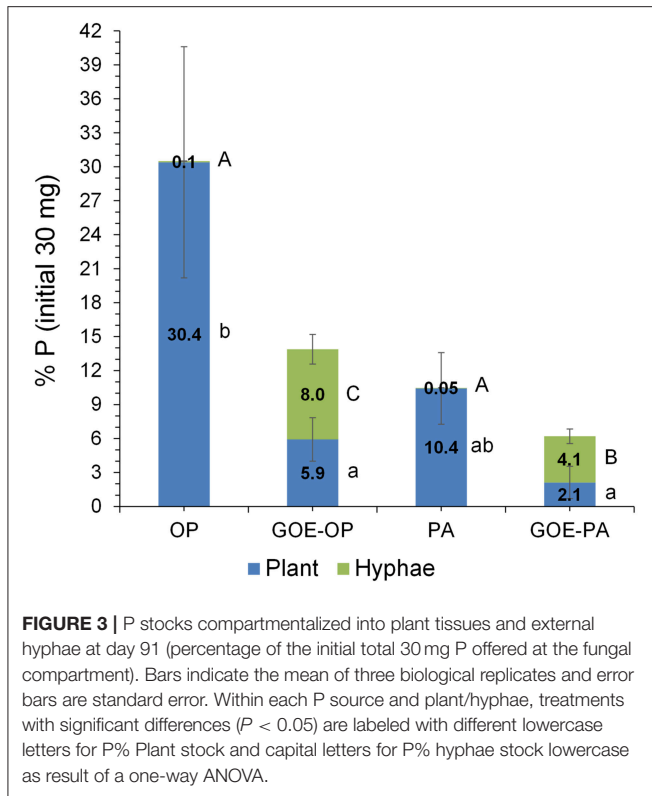
## RESULTS

Phosphorus derived from each offered source was incorporated by the AM tomato plants, while none of the controls (GOE, M+, and M–; Table 1) showed P incorporation (Figure 2). Those AM plants which had access to OP incorporated the highest amount of P, followed by PA, GOE-OP, and GOE-PA. The first P incorporation was detected at day 49 in case of the treatments offering OP and PA and at day 77 in case of GOE-OP and GOE-PA as P sources. At day 91, AM plants reached the maximum P incorporation, with P taken up in the following order: OP (30.4% of the initially added P) > PA (10.4%) > GOE-OP (5.9%) > GOE-PA (2.1%) (Figure 3).

AM plants with access to OP had significantly larger P contents in their hyphae and spores, followed by plants supplied with GOE-OP and PA. The GOE-PA and M+ (day 0) treatments exhibited significantly smaller P contents in the AMF (Figure 4). Although the hyphal content of P was significantly larger for the fungal compartment containing OP, the total amount of P accumulated in the hyphae was smaller, as there was significantly less AMF biomass. On average, the extraradical hyphae accumulated less P in the fungal compartment containing OP (0.03 mg P; 0.1%) and PA (0.015 mg P; 0.05%) as compared to the goethite-bound forms GOE-OP (2.39 mg P; 8%), and P for



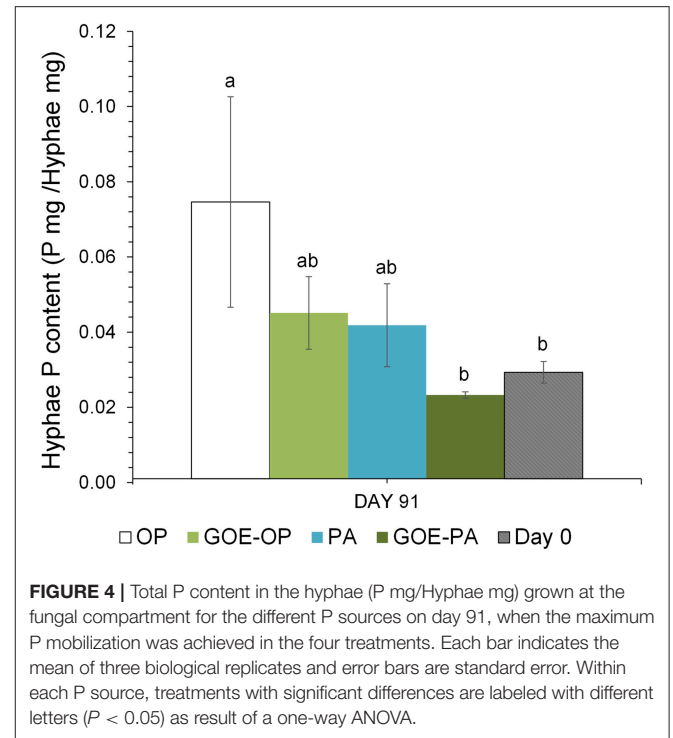
**FIGURE 2** | Total P (mg) incorporated by the AM tomato plant from the different P sources over 91 days. Each bar indicates the mean of three biological replicates and error bars are standard error. Within each P source and day, treatments with significant differences are labeled with different letters ( $p < 0.05$ ) as result of a one-way ANOVA.



GOE-PA (1.22 mg P; 4.1%) (**Figure 3**). When these P amounts are summed up to the P incorporated by the plant, *R. irregularis* mobilized 9.15 mg P (30.5% of the initially added P) from the OP, followed by GOE-OP (4.17 mg P; 13.9%), PA (3.14 mg P; 10.5%), and GOE-PA (1.86 mg P; 6.2%) (**Figure 3**). Compared to that, P desorption from the goethite in water was 7.4% for GOE-OP and 1.2% for GOE-PA. These proportions corresponded to P amounts of  $2.2 \pm 0.25$  mg in the GOE-OP treatment and  $0.35 \pm 0.008$  mg P in the GOE-PA treatment, which likely were mobilized by desorption processes. Hence, the difference between the P incorporated in the plant tissues/AM mycelium and the P desorbed in water of the goethite-bound P sources, resulted in a net P mobilization from the goethite complexes by the AMF between 5% (GOE-PA) and 6.5% (GOE-OP) of the initially added P.

Organic C contents (mg/g fungal compartment) show that the M- control treatment did not accumulate any C throughout the experiment. From day 7 to day 91, fungal compartments containing OP, PA and M+ control showed significantly smaller accumulation of OC compared to GOE-OP, GOE-PA, and GOE control. From day 7 to 77, the fungal compartment containing PA showed significantly larger OC contents than those with OP and control without P (M+) (**Figure 5A**). The same occurred for fungal compartments containing GOE-PA as compared to those with GOE-OP and GOE control (**Figure 5A**).

In case of cumulative  $\text{CO}_2$  production (mg/g fungal compartment), the M- treatment, without mycorrhiza and without P, showed significantly smaller production than any other treatment (**Figure 5B**). Fungal compartments containing OP, PA and the M+ control showed significantly smaller



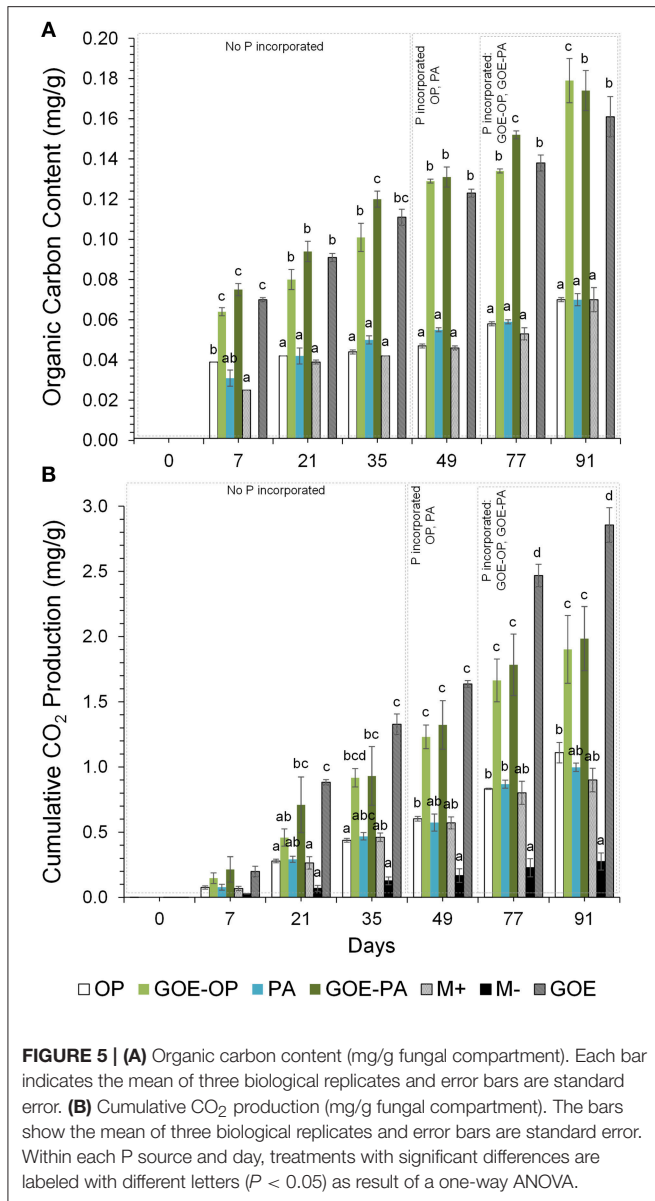
cumulative  $\text{CO}_2$  production from day 7 to 91 as compared to the production of GOE-OP, GOE-PA, and the GOE control. The cumulative  $\text{CO}_2$  production showed no significant difference between those containing OP, PA and the M+ control. Throughout the experiment, the GOE control showed significantly larger cumulative  $\text{CO}_2$  production values than any other treatment.

At days 77 and 91, when AM plants reached their maximum P incorporation, the Total C/P was significantly higher for those AM plants that had access to GOE-OP, GOE-PA, and PA, as compared to OP (**Figure 6**). The P incorporated from GOE-OP showed larger PLFA 16:1 $\omega$ 5c/P ratios than the OP and PA treatments at days 77 and 91, while for GOE-PA this ratio was larger than for OP and PA at day 91 only (**Figure 7A**). The NLFA 16:1 $\omega$ 5c/P was significantly larger for both P sources bound to goethite in comparison to OP and PA (**Figure 7B**). For all P sources, the NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c ratio was above one, meaning that the PLFA and NLFA originated largely from AM fungi (**Figure 7C**). The NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c ratios increased for all AM plants accessing a P source in the fungal compartment during the period of P incorporation. The NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c ratio showed always significant larger values for the treatment offering GOE-OP as P source.

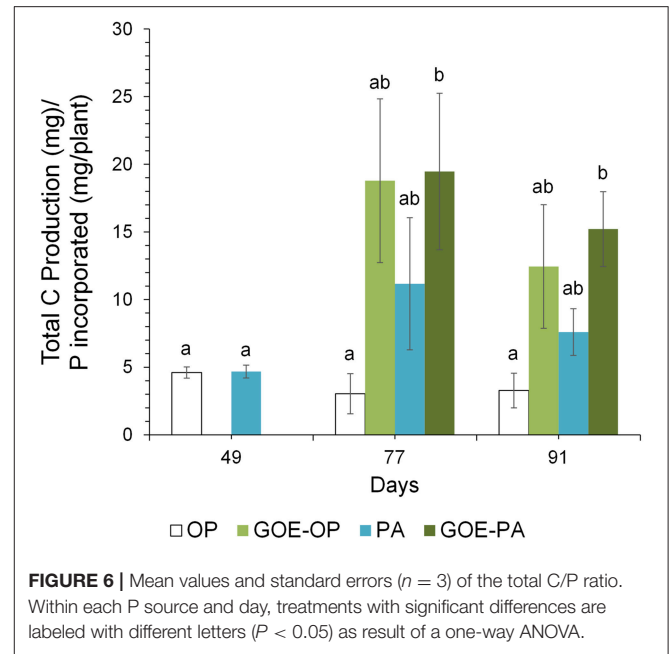
## DISCUSSION

### Phosphorus Plant Uptake From Different Sources

We tested the role of AMF in taking up P from four different sources having a different accessibility. For this, a mesocosm was designed where exclusively the AM hyphae were involved in plant



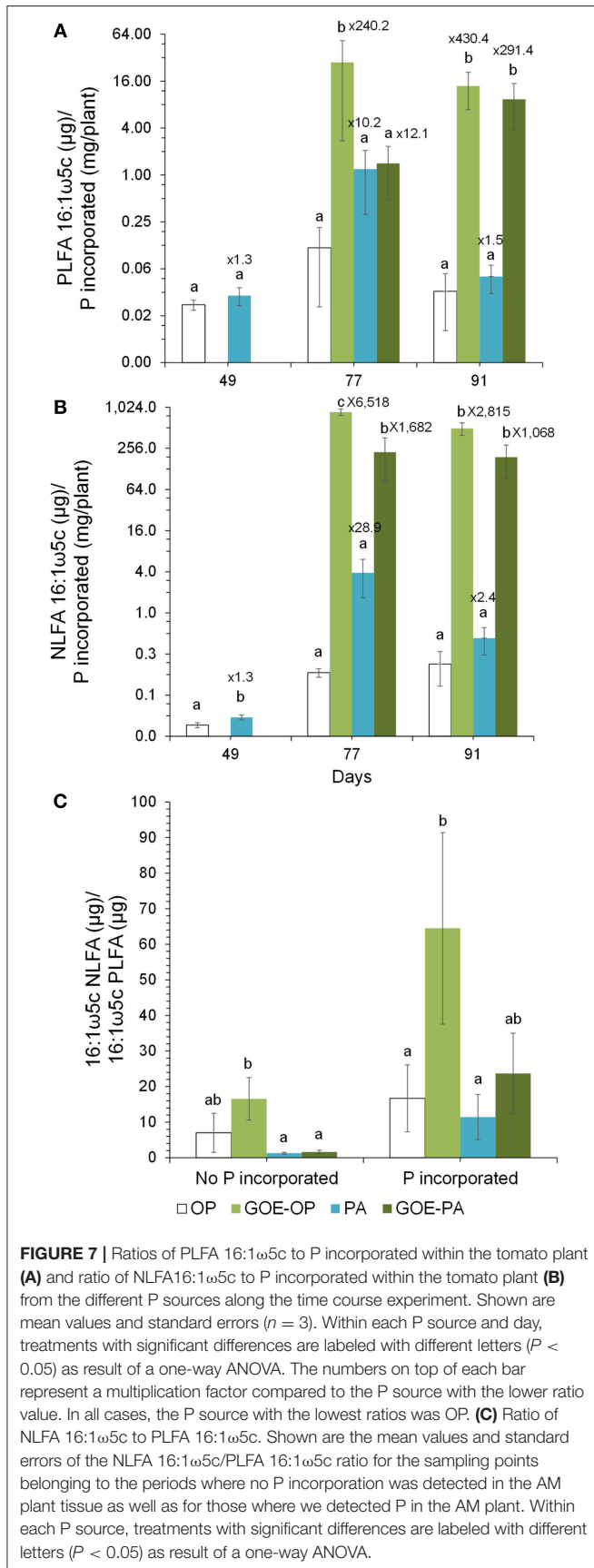
P acquisition. Our results indicate that all AM plants colonized with *R. irregularis* incorporated P derived from inorganic (OP) or organic (PA) sources, no matter if added either in their free form or bound to goethite. Phosphorus from the different P forms was taken up in different amounts and kinetics via the mycorrhizal pathway. In contrast to other studies where roots and hyphae are only separated by a 20 μm nylon mesh (Argüello et al., 2016; Jakobsen et al., 2016; Zhang et al., 2016; Sawers et al., 2017), the hydrophobic polytetrafluoroethylene membrane used in our mesocosms avoided not only the direct P uptake by roots, but also the influence of root exudates and the diffusive transport from the fungal compartment to plant compartment, as well as the P absorption by the roots. This membrane type has been successfully used in other studies investigating on nitrogen transfer by AMF (Mäder et al., 1993, 2000; Frey et al., 1998), but not for P nutrition via the mycorrhizal pathway. The



hydrophobic and selective nature of this barrier, however, is of paramount importance for separating the effects of the root and the fungus and quantifying the nutrient fluxes. These first results do thus not only give evidence of the importance for mycorrhiza in the uptake of P but also proof the advantage of hydrophobic polytetrafluoroethylene membranes (Figure 1).

Plant P incorporation appeared quickest in OP and PA treatments, followed 1 month later by the P forms bound to goethite. In addition, P from the freely accessible forms of OP and PA was incorporated to a greater extent (Figure 2). The faster plant P uptake in case of OP can be explained by the fact that an AM plant can absorb OP directly via the mycorrhizal pathway (Smith et al., 2003). Usually most of the studies on plant nutrition only consider inorganic phosphate—but not the organic P fraction—as biologically available, even though organic P represents the major part of the total soil P (Turner, 2008). To be available for the plant, PA has to be hydrolyzed first by phytases, a group of phosphatases, which are either of plant or microbial origin (Li et al., 1997; Baldwin et al., 2001, 2008; Javaid, 2009). In our study we did not measure enzymatic activities, but it has been demonstrated that *R. irregularis* DAOM 197198 is able to secrete acid phosphatase, which contributes to the mineralization of P-bearing organic compounds in soil and thus increases the concentration of available inorganic P (Tisserant et al., 2012). Tarafdar and Marschner (1994a,b) reported the mobilization of organic P sources for plant P uptake by exudation of fungal acid phosphatase in an experiment using *Glomus mosseae*. Accumulating evidence suggests that *Rhizophagus* species can hydrolyze organic P forms and the resultant inorganic P can be taken up and transported to host roots (Joner et al., 2000; Koide and Kabir, 2000; Sato et al., 2015). Utilization of organic P is thus assumed to contribute in a comparable manner to AM plant P nutrition as inorganic P (Feng





et al., 2003), as we did not find significant differences among the amount of P mobilized from OP and PA.

We also did not find differences in the amount of P incorporated into the plant between the treatments offering GOE-OP and GOE-PA as P sources. Phosphorus from both P-goethite associations was incorporated by the mycorrhizal plant on average less and also later than free OP. Concerning OP, our results support earlier conclusions by Parfitt (1979), who provided the only available study addressing the mobilization of OP adsorbed to goethite. They showed that *Lolium perenne* mycorrhized with *Glomus tenuis* desorbed phosphate from goethite after 55 days. However, in this experiment there was no physical separation between the plant and hyphae. For that reason, Parfitt's results should be interpreted with caution, as desorption may be partially caused by the acidification of the rhizosphere mediated by root exudates containing an excess of protons, and not exclusively by the AMF (Hinsinger and Gilkes, 1996). To our knowledge, this is the first experiment proving that an AM plant incorporated P from PA bound to a secondary mineral. Noteworthy, the amounts of mobilized P were larger than the portion of most weakly sorbed PA, as determined in the separate desorption experiment. This finding suggests that AMF play an active role in the mobilization of mineral-bound P. A possible mechanism used by AMF to desorb P from the surface of the goethite is through the release of exudates containing organic acids, as observed by Tawarayama et al. (2006). The delay to incorporate P from GOE-OP and GOE-PA associations may be due to two reasons: firstly, both P forms had to be desorbed from the goethite by means of organic acids; with the P diffusion being likely impaired in the goethite substrate. Secondly, in contrast to the desorbed OP, the released PA had still to be hydrolyzed through the action of phosphatases.

AMF not only supplied P to the plant, but also accumulated it into the hyphae grown in the fungal compartment. On the one hand, the P contents in the AM hyphae were larger for the treatments offering free P forms OP and PA, followed by those treatments offering GOE-OP and GOE-PA as P sources (Figure 4). On the other hand, the P stocks in the fungal biomass for OP and PA were significantly smaller than those determined in GOE-OP and GOE-PA (Figure 3). Thus, the P from the goethite-bound forms was evenly distributed between the fungus and the plant, while the non-bound forms were preferably incorporated into the plant. Our results point to a smaller P content inside the hyphae growing in the fungal compartments with the lees P accessible sources, GOE-OP and GOE-PA. This result would be in accordance with the observations made by Ezawa et al. (2002), who found that the P content in the hyphae appears to respond flexibly to P availability in the environment, varying its internal content proportionally.

*Rhizophagus irregularis* did not transfer all of the mobilized P from the goethite sources to the plant but stored it within its hyphae, as a potential strategy of the fungus to accumulate P under deficient conditions to keep a well-balanced homeostasis (Solaiman et al., 1999). Another possible interpretation of the P accumulation within the hyphae/spores in a broader sense, could be significant from the perspective of the AM plant in the common mycorrhizal network, where accumulated P indicates a

likely support for the growth of a new mycelium and maybe even act as an “entrance fee” for a new colonization (Olsson et al., 2008; Hammer et al., 2011b). Likewise, this accumulation of P would not be a futile waste of energy, due to the fact that P storage within microbial biomass may account for 1–10% (10–100 kg of P/ha) of the total soil P (Richardson, 2001) and may become available to plants once the microbes die (Deubel and Merbach, 2005).

## Carbon and Phosphorus Trading

No OC was detected in the fungal compartment for M-control treatment, while treatments containing PA and goethite-P associations showed larger OC contents as compared to those fungal compartments for OP and control M+ (**Figure 5A**). AMF tends to develop more readily under nutrient-poor conditions than under nutrient-rich or heavily P-fertilized conditions (Bryla and Eissenstat, 2005; Olsson et al., 2010), as would be the case for those fungal compartments where there was GOE, PA and sources of goethite-P. Before any P was incorporated between days 0 and 35, the GOE-OP, GOE-PA and GOE treatments exhibited significantly higher OC contents. The data suggests that OC increased more rapidly in those fungal compartments containing goethite compared to fungal compartments with OP or PA. Supposedly, this is due to a greater investment of photoassimilates via the AMF to mobilize less accessible forms before and during the P acquisition. The organic substances exuded by the AMF may be also well stabilized against fast microbial decomposition, as goethite effectively binds organic molecules via ligand exchange below its point of zero charge (Kaiser and Guggenberger, 2007).

Along with the larger OC contents in the fungal compartment of the AM plants, we detected a larger CO<sub>2</sub> production at all sampling points in any of the AM plant treatments, especially those where goethite was present, as for the M- treatment (**Figure 5B**). The total CO<sub>2</sub> production might have been a bit overestimated as we also measured a small CO<sub>2</sub> production in the M- treatment, which was likely caused by gas permeability of the two membranes between the plant and the fungal compartment. We also cannot exclude the presence of root CO<sub>2</sub> production in treatments with AM plants. However, similar mesocosm systems were used to indirectly estimate CO<sub>2</sub> production of extraradical mycelium (Heinemeyer et al., 2006; Nottingham et al., 2010; Karasawa et al., 2012), and different calculations were applied to estimate the contribution of AM extraradical mycelium, such as the difference in total CO<sub>2</sub> emission between AM and non-AM fungal compartments (Karasawa et al., 2012). We did not indirectly estimate the CO<sub>2</sub> production of the extraradical mycelium as a variable soil nutrient availability is known to demand different amounts of root and respiratory energy (Atkin et al., 2009). In line with Atkin et al. (2009), we could show that AM tomato plants were associated with larger CO<sub>2</sub> rates in the fungal compartment compared with M-plants and increased soil CO<sub>2</sub> release. The nature of the respired CO<sub>2</sub> at the fungal compartment, is the sum of the autotrophic and the heterotrophic respirations. The autotrophic respiration comprises the fraction derived from current photosynthates (Olsson et al., 2005). In our case includes the respiration by mycorrhizal fungi, and likely other microorganisms using C

that has been recently fixed by plant photosynthesis, since we inoculated the plants with *R. irregularis* carrying the microorganisms naturally associated with its hyphosphere. The heterotrophic respiration is due to the decomposition of OC (Kuzuyakov and Gavrichkova, 2010) made up mostly of dead hyphae and bacteria, mediated by the microbes present at the fungal compartment. Taking together the results of OC content and CO<sub>2</sub> production in the fungal compartment, we can assume that treatments containing less accessible phosphorus sources showed higher C investments into autotrophic and heterotrophic metabolic processes, before and during the incorporation of P in the AM plant. Consequently, the Total C/P ratio was significantly larger in those fungal compartments containing free PA and both P sources bound to goethite (**Figure 6**). The total C as the sum of total OC and cumulative CO<sub>2</sub> accounted for most of the C exclusively carried by the AMF into the fungal compartment. The results point to a greater investment of plant photoassimilates compared to the free OP treatment and controls. Similarly, a more active metabolism for the less accessible P sources was indicated by a larger CO<sub>2</sub> production for the same amount of P mobilized from PA, GOE-OP and GOE-PA compared to OP. Respiratory energy is required by the microbiont for constructing new intraradical and extraradical fungal tissue (including reproductive structures), for maintenance and repair of existing fungal tissue and for cellular processes in the fungal tissue associated with the absorption and transfer of nutrients to the host (Bryla and Eissenstat, 2005). The photoassimilate investment into respiration (fast) or structural (slow) C pools in soil has been reported frequently before (Zhu and Miller, 2003), but was not related to a direct nutrient allocation mechanism as in our case. Staddon et al. (2003) suggested AM hyphae do not contribute substantially to C sequestration in soil. In contrast, our results point toward larger C sequestration rates and metabolic activity in the less accessible sources, being consistent with the general line of argumentation of Turner (2008). This energy investment into fungal compartments with less accessible P sources, taken together with more structural C contents found in the respective compartments clearly show that AM fungi are able to adjust to the requirements imposed by a given nutrient source.

## Growth of *R. irregularis* Under Variable Phosphorus Availability

The PLFA 16:1 $\omega$ 5c /P and NLFA 16:1 $\omega$ 5c/P showed a significant larger amount of both fatty acids accumulated into the fungal compartment relative to P uptake, in those fungal compartments containing GOE-OP and GOE-PA compared to OP and PA (**Figures 7A,B**). The fungal lipids extracted from the fungal compartment indicate a different growth strategy to mobilize P from the goethite-bound compounds compared to the free forms. The AMF grown in those fungal compartment containing GOE-OP and PA accumulated two and three orders of magnitude more PLFA and NLFA, respectively, compared to OP and PA. This observation confirms a larger C investment in energy storage and mycelium per unit of P incorporated into the AM plant. *Rhizophagus irregularis* grown in the fungal compartment

containing GOE-OP accumulated significant more NLFA, as indicated by the ratio NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c, meaning that the AMF stored energy in the form of neutral lipids before and during the P mobilization, compared to GOE-PA and the free P forms (Figure 7C). One potential explanation for the more pronounced development of fungal infrastructure in the presence of P forms bound to goethite is the need to establish a sufficient extraradical hyphae of *R. irregularis* to mobilize and transport P more efficiently to the plant (Abbott and Robson, 1982; Joner et al., 2000; Smith et al., 2003). According to Bryla and Eissenstat (2005), mycorrhizas tend to be favored under nutrient-poor conditions, as in case of the goethite-P treatments. The reduced hyphal growth in both fungal compartment containing OP and PA with a high P content indicate that the roots may reduce the C flux to the fungus under larger P availability (Olsson et al., 1997, 2002). It has been suggested that a reduced carbohydrate allocation would be a regulatory saving mechanism by the plant host where there is an excess of phosphate ion (Torres de los Santos et al., 2016; Konvalinková et al., 2017). Bååth (2003) showed the NLFA 16:1 $\omega$ 5c/PLFA 16:1 $\omega$ 5c is a good indicator to express preferential C allocation to storage products, when comparing different nutritional scenarios. Our results corroborate this observation and suggest that the preferential energy storage in the form of neutral lipid could be used to support the extensive growth of *R. irregularis* mycelium in the goethite systems.

The plant is often considered as being in control of the symbiosis, and this “phytogenic” view is supported by the idea that AM symbiosis is a strategy that could be suppressed by the plants at sufficient P supply. But in natural ecosystems, instead of luxurious P availability, suppression of AM colonization by the plant is not the normal situation (Smith and Smith, 2012), as plants have evolved diverse adaptations to acquire different forms of soil P (Ceulemans et al., 2017). Hammer et al. (2011b) suggest a fungal control mechanism of the P transfer to the plant, and that the C-P exchange between the symbionts is closely linked. Our results, showing a different content and partitioning of C into NLFA and PLFA 16:1 $\omega$ 5c for those *R. irregularis* grown in treatments with mineral-bound P sources, corroborate that point. This more “mycogenic” view of symbiosis explains the evolutionarily stable mutualistic relationship between these symbionts. If AMF are able to reduce the delivery of P to plants with a limited supply of C (Kiers et al., 2011), they possess an important function to actively increase their fitness by choosing the best plant partner, which in turn could increase selection pressure on the plant species within a community (Hammer et al., 2011b). Under our experimental conditions, where the tomato plants could only acquire the P through the AMF, we observed different trading costs between C for P, distinct P acquisition strategies resulting in differing C costs for the AM plant. Our results show that the mobilization of a certain P source by a plant, comes at differing photoassimilatory costs and, by this, necessarily changes the functional trait of this plant. Thus, we propose this mechanism as a potential driver

of nutritional partitioning in plant communities, as described by e.g., Turner (2008).

## CONCLUDING REMARKS

Our study provided evidence that AM plants are able to mobilize P sources differing in their accessibility. This mobilization happened exclusively via the mycorrhizal pathway at contrasting kinetics and accumulation rates, and was driven by the amount of photoassimilates needed for the mobilization of the P sources. The mobilized P was redistributed between fungus and plant in differing amounts, which again was influenced by the P species. Both P sources adsorbed to goethite (GOE-OP, GOE-PA) facilitated a greater investment of the AM plant into the production of fungal vegetative structures than in case of the free OP and PA. Larger contents of PLFA 16:1 $\omega$ 5c and NLFA 16:1 $\omega$ 5c were observed especially in systems with goethite-associated P, suggesting the accumulation of significantly larger amounts of extraradical hyphae. Further, the lipid accumulation in form of PLFA 16:1 $\omega$ 5c and NLFA 16:1 $\omega$ 5c suggests different growth strategies to mobilize P from the goethite-bound compounds. This is also mirrored by the larger C investment per P incorporated, as compared to OP and PA. Our data also suggest that the C investment by mycorrhized plants into P acquisition from differently available P sources has a direct effect on the amount of C accumulating in soils.

## AUTHOR CONTRIBUTIONS

JB, GG, AA, and RM designed the experiment. AA prepared the plant and fungal material. AA conducted the experiment and analyzed the data. JB, LS, GG, and RM supervised the research. AA wrote the paper with contributions from JB, GG, LS, and RM.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2019.00026/full#supplementary-material>

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