Ectomycorrhiza: Phosphorus Source specific Economy and Potential in Resource Partitioning

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Das Leben ist absurd, aber du kannst es mit Ideen füllen.
..mit Enthusiasmus ..mit Freude.
(Nirmal Purja - Bergsteiger aus Nepal)

To my partner Paolo, my sister Viktoria,
my supervisors, colleagues, and friends,
my supper doggo Dora.

Abstract

Many natural and anthropogenic soils are phosphorus (P) limited, as P is largely immobilized in forms of low bioavailability affecting the plant productivity. One of the strategies to overcome this shortage lies in the symbiosis of plants with mycorrhizal fungi that increases the plant P uptake of these hardly accessible sources in exchange for photoassimilates. Nevertheless, the required investment to acquire the free phosphate from various P sources increases with decreasing P source bioavailability, indicating a different carbon (C) sink potential. Ectomycorrhizae were shown to be able to acquire various P sources, but experimental evidence for their P source dependent C sink potential as well as for their role in resource partitioning for P is missing.

The present study aimed to address following objectives: (i) to design a system to observe mycorrhizal mediated nutrient exchange (Study I); and to investigate (ii) whether the C investment from the host plant for the mycorrhizal mediated P derived from different P species is P source dependent (Study II); as well as the (iii) preferences of mycorrhizal plant to specific P sources from a mixed P pool (Study III). Following P sources were tested for the mycorrhizal mediated plant P uptake: PO_4^{3-} (oP), the primary mineral apatite (AP; Study II) or hydroxyapatite (HAP; Study III), the organic P species (P_{org}) phytic acid (Phy; Study II) or adenosine monophosphate (AMP; Study III), and goethite-bound PO_4^{3-} (gP) as secondary mineral-P adsorption complex.

The practical experience made in Study I revealed that, compared with the *in vitro* culture system, the construction and maintenance of the axenic rhizotrone and the mesocosm culture systems are less complicated and time consuming and at the same time more robust and very versatile systems that are also suitable for greenhouse conditions. In Study II a P source dependent trend in exchange of host C for ectomycorrhizal mediated P in roots was determined. The exchange of C for P in ectomycorrhizal roots of AP and gP treatments were of similar magnitude and could result from the applying the same mechanism, the exudation of LMWOAs and hyphal growth to acquire mineral P sources (mineral P effect). In contrast, the readily available P source oP, the soluble Phy, as well as the complete P limitation turn the ectomycorrhizal plant into a system of C retention in the mycorrhizal root interface. These results indicate a trend that the C allocation into the belowground is P source specific, which can have an impact on the C balance in soils of natural and anthropogenic ecosystems. The results of Study III show that an ectomycorrhizal plant is able to utilize all provided P sources via its mycorrhizal fungal associate. The acquisition timing was determined by the most bioavailable P sources, with oP and AMP over HAP and gP, and a mixed P pool over single P source. In contrast, the magnitude was defined by the amount of supplied P source and provision of additional nitrogen, hence AMP over oP and gP, as well as by P source complexity, with gP as the least favourable P form. Nevertheless, these results provide evidence that an ectomycorrhiza has the potential to occupy fundamental niches of various P sources differing in their bioavailability, indicating that being a generalist in P nutrition can facilitate adaptation to various nutritional settings in soil.

Keywords: ectomycorrhiza, mineral P, $^{15}N^{13}C$-urea labelling, nutrient exchange, organic P, P availability, P diversity, radioactive labelling, resource partitioning.

Zusammenfassung

Viele natürliche und anthropogene Böden sind Phosphor (P) limitiert, da P weitgehend in Formen geringer Bioverfügbarkeit immobilisiert ist und dadurch die Pflanzenproduktivität beeinflusst. Eine der Strategien zur Überwindung dieses Mangels liegt in der Symbiose zwischen Pflanze und Mycorrhizapilzen, welche die Bioverfügbarkeit von Nährstoffen und damit den Ernährungszustand von Pflanzen erhöhen können. Die zunehmende Mobilisierung von P durch die Mykorrhizapilze erfolgt durch Bodenexploration über extraradikale Hyphen und Anpassung der P-Erfassungsmechanismen an verschiedene chemische Formen von P, die unterschiedliche Ressourcennischen darstellen. Dabei steigt die erforderliche Investition, um das freie Phosphat aus verschiedenen P-Quellen zu beziehen, mit abnehmender Bioverfügbarkeit der P-Quelle, was auf ein unterschiedliches Senkenpotential von Kohlenstoff (C) hinweist. Ektomykorrhizen sollen in der Lage sein eine vielzahl von P-Ressourcennischen zu besetzen, aber experimentelle Demonstration für ihr P-Quellen-abhängiges C-Senkenpotential sowie für ihre Rolle bei der Ressourcenpartitionierung für P fehlen.

Folgende Ziele wurden aufgestellt: (i) Entwicklung eines Systems zur Beobachtung des Mykorrhiza-vermittelten Nährstoffaustauschs (Studie I); und zu untersuchen, (ii) ob die unterschiedlichen Bioverfügbarkeiten spezifischer P-Quellen, die von einem Ektomykorrhizapilz erworben wurden, die P-Aufnahmeeffizienz im Verhältnis zur C-Investition seiner Wirtspflanze beeinflussen; insbesondere, ob die C-Investition aus der Wirtspflanze für das Mykorrhiza-vermittelte P, das von verschiedenen P-Arten stammt, von der P-Quelle abhängt (Studie II); sowie die (iii) Präferenzen der Mykorrhizapflanze gegenüber spezifischen P-Quellen aus einem gemischten P-Pool (Studie III). Folgende P-Quellen wurden auf die Mykorrhiza-vermittelte Pflanzen-P-Aufnahme getestet: PO_4^{3-} (oP), das Primärmineral Apatit (AP; Studie II) oder Hydroxyapatit (HAP; Studie III), die organische P-Spezies (Porg) Phytinsäure (Phy; Studie II) oder Adenosinmonophosphat (AMP; Studie III) und Goethit-gebundenes PO_4^{3-} (gP) als sekundärer Mineral-P-Adsorptionskomplex.

Die in Studie I gemachten praktischen Erfahrungen haben gezeigt, dass im Vergleich zum in vitro-Kultursystem der Aufbau und die Aufrechterhaltung des axenischen Rhizotrons und der Mesokosmenkultursysteme weniger kompliziert und zeitaufwendig und gleichzeitig robuster und sehr vielseitiger sind. In Studie II wurde ein P-quellenabhängiger Trend im Austausch von Pflanzen-C gegen Ektomykorrhiza-vermitteltes P in Wurzeln bestimmt. Der Austausch von C gegen P in Ektomykorrhizawurzeln von AP- und gP-Varianten war von ähnlicher Größenordnung und könnte sich aus der Anwendung des gleichen Mechanismus, der Exsudation von LMWOAs und dem Hyphenwachstum zur Gewinnung von mineralischen P-Quellen ergeben. Im Gegensatz dazu verwandeln die leicht verfügbare P-Quelle oP, das lösliche Phy, sowie die vollständige P-Limitierung die Pflanze in ein System der C-Retention in der mykorrhizierten Wurzel. Diese Ergebnisse weisen auf einen Trend hin, dass die C-Zufuhr in den Untergrund P-Quellen-spezifisch ist, was sich auf die C-Bilanz in Böden natürlicher und anthropogener Ökosysteme auswirken kann. Die Ergebnisse von Studie III zeigen, dass eine Ektomykorrhiza in der Lage ist, alle bereitgestellten P-Quellen über ihren Pilz-Symbionten zu nutzen. Der Erfassungszeitpunkt wurde durch die Bioverfügbarkeit der P-Quellen (oP und AMP über HAP und gP) und P-Quellen-Vielfalt (gemischter P-Pool über einzelne P-Quelle) bestimmt. Im Gegensatz dazu wurde die Größenordnung durch die Menge der gelieferten P-Quelle und die Bereitstellung von zusätzlichem Stickstoff (AMP über oP und gP), sowie durch die P-Quellenkomplexität definiert, wobei gP die am

wenigsten verfügbare P-Form war. Dennoch belegen die Ergebnisse der Studie III, dass eine Ektomykorrhiza das Potenzial hat, grundlegende Nischen verschiedener P-Quellen mit unterschiedlicher Bioverfügbarkeit zu besetzen, was darauf hindeutet, dass ein Generalist in der P-Ernährung die Anpassung an verschiedene Ernährungssituationen im Boden erleichtern kann.

Schlüsselwörter: Ektomykorrhiza, Mineralisches P, $^{15}N^{13}C$-Harnstoff- Markierung, C-P-Austausch, organisches P, P-Verfügbarkeit, P-Vielfalt, radioaktive Markierung, Ressourcenaufteilung.

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Abbreviations

Al	aluminium
AM	arbuscular mycorrhizae
AMP	adenosine monophosphate
AP	apatite
C	carbon
Ca	calcium
CM	Cellophane membrane
ECM	ecto-mycorrhizae or ecto-mycorrhizal symbiosis
Fe	iron
gP	PO_4^{3-} adsorbed to goethite
HAP	hydroxyapatite
HC	hyphal compartments
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
IRMS	isotope ratio mass spectrometry
LMWOA	low molecular-weight organic anions
MMN medium	modified Melin-Norkrans medium
N	nitrogen
oP	*ortho*-phosphate
P	phosphorus
P_i	free phosphate ion
Phy	phytate/phytic acid
P_{org}	organic P
RC	root comparrtments
UDC	urea-derived ^{13}C
UDN	urea-derived ^{15}N
WPM medium	Woody plant medium

List of contributing Publications

The following publications contributed to this thesis:

1. Schreider K, Boy J, Sauheitl L, Figueiredo AF, Andrino A and Guggenberger G (2022). Designing a Robust and Versatile System to Investigate Nutrient Exchange in, and Partitioning by, Mycorrhiza (*Populus x canesces* x *Paxillus involutus*) Under Axenic or Greenhouse Conditions. Front. Fungal Bio. 3:907563.
   doi: 10.3389/ffunb.2022.907563

2. Schreider K, Hofmann D, Boy J, Andrino A, Fernandes Figueiredo A, Sauheitl L and Guggenberger G (2022). Mycorrhizal Mediated Partitioning of Phosphorus: Ectomycorrhizal (*Populus x canescens* x *Paxillus involutus*) Potential to Exploit Simultaneously Organic and Mineral Phosphorus Sources. Front. Soil Sci. 2:865517.
   doi: 10.3389/fsoil.2022.865517

3. Schreider, K., Boy, J., Andrino, A., Sauheitl, L., Fernandes Figueiredo, A., Guggenberger, G. (2022). P source dependent exchange of C for P in ectomycorrhiza (*Populus x canescens x Paxillus involutus*)

   Submitted in *Biogeochemistry* on 15.06.2022.

1 State of the Art and general Hypotheses

1.1 Phosphorus Limitations in Terrestrial Ecosystems

Phosphorus (P) is limited in many terrestrial ecosystems including natural systems such as forests (Holford, 1997; Clausing et al., 2020) and grasslands (Critchley et al., 2002; Ceulemans et al., 2017; Phoenix et al., 2020) but also 'anthropogenic' systems such as agricultural soils (Dissanayaka and Wasaki, 2020) and short rotation plantations with woody plants (Xu et al., 2000; Stanturf and van Oosten, 2014). Nevertheless, the total concentration of P in soils is often high (Dissanayaka and Wasaki, 2020), as P is abundant in the Earth's crust as inorganic P in primary minerals or it can accumulate in soils (Hinsinger et al., 2011; Plassard et al., 2011). Hence, the chemical nature of P makes it low available to plants. Plants are able to take up P in the form of orthophosphate (P_i) in soil solution (**Figure 1**). P_i but also soluble organic P (P_{org}) are poorly mobile in soil as they interact strongly with soil constituents (Hinsinger, 2001; Hinsinger et al., 2011) and can be adsorbed onto metal oxides and clay minerals in acidic soils, precipitate as (apatite-like) minerals in alkaline soils, or be immobilized in diverse organic forms (Holford, 1997; Hinsinger et al., 2011). So that significant amounts of 'available' P is prevented from circulation through sequestration forming accumulating organic and/or sorbed P pools (Walker and Syers, 1976).

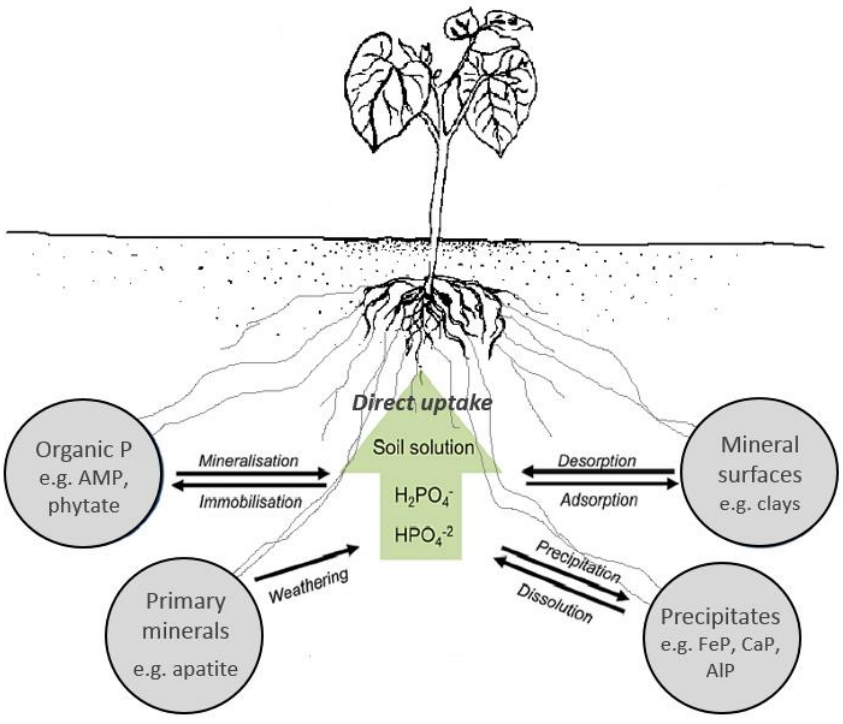

Figure 1 | Mycorrhizal fungal associated plant P uptake from a diverse pool of different P sources. Adopted from Owen et al. (2014).

That means e.g. for agricultural ecosystems that around 28 % of applied fertilizers and manures may stay unutilized and stored in the soils as 'legacy' P (Sattari et al., 2012), eventhought its accessibility to plants is higher compared with P originating from pedogenesis (Johnston et al., 2014). Furthermore, the productivity in forest ecosystems is declining not only due to the P limitation caused by the aging process of soils (Odum, 1969; Lang et al., 2017), but also by the anthropogenic effects such as N deposition, soil acidification (Peñuelas et al., 2013) as well as climate change causing lower rates in P mineralization and mobility in desiccated soils (Schachtman et al., 1998; Schimel et al., 2007).

Mycorrhizal fungi are a known strategy used by plants to increase crop production and to maintain terrestrial ecosystems (Gafur et al., 2003). What the mycorrhizae are and how they are able to help the plant to overcome the P limitation and sustain ecosystems limited in P is described in the following chapter 1.2.

1.2 Mycorrhizal Symbiosis as a Strategy to overcome Plant Phosphorus Limitations

To meet the P demand, plants cooperate in complex ecological associations, especially in mycorrhizal symbiosis (Lambers et al., 2018; Plassard and Dell, 2010, Becquer et al., 2014). These mycorrhizal partners associate with more than 90 % of all plant species including trees, wild grasses, and many crops (Carlile et al., 2001). Beside the benefits in plant nutrition, mycorrhizal fungi supply its host also with minerals (Smith and Read, 2008), water (Buscot et al., 2000) and protection from biotic and abiotic stresses (Buscot, 2015). The mycorrhizal fungi are ubiquitous and occur in diverse environments such as temperate (Smith and Read, 2008) and tropical forests, alpine and boreal zones, natural grasslands and agricultural systems (Bonfante and Genre, 2010).

1.2.1 Formation of Mycorrhizal Symbiosis and their Mechanisms to acquire Phosphorus

There exist different types of mycorrhizal associations. The main types of mycorrhizal symbiosis are the ectomycorrhizae (ECM; **Figure 2A**) and endomycorrhizae. Arbuscular mycorrhizae (AM; **Figure 2B**) are the widespread type of endomycorrhizae enclosing the fungi of the phylum Glomeromycota.

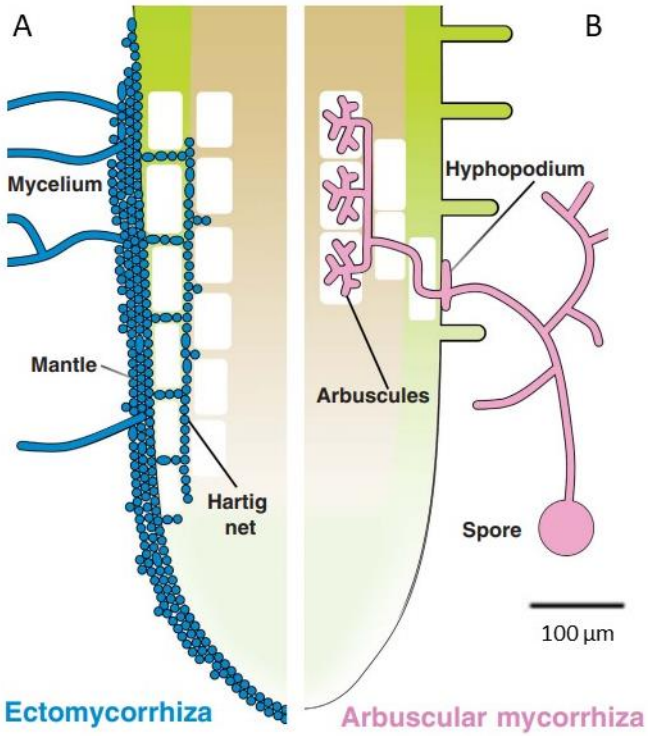

Figure 2 | The structure of root colonization by (**A**) ectomycorrhizal (ECM) and (**B**) arbuscular mycorrhizal (AM) symbios; adopted from Bonfante and Genre (2010). (**A**) The ECM (asco- or basidiomycotan) fungus covers a tip of a fine root with a hyphal mantel and penetrates into the root between the root cortical cells, forming a hartig net (Landeweert et al., 2001). (**B**) Whereas, the AM fungus develop from a spore, producing a hypopodium on the root surface and intraradically colonizing the root intra- and intercellularly by forming arbuscules inside the cortical cells (Bonfante and Genre, 2010). The hartig net and the arbuscules are considered to be the key element for the exchange of nutrients between fungus and plant.

The hyphae of both mycorrhizal types increase the volume of soil explored for nutrients and helping the plant roots to overcome depletion zones in soil resources. The ECM are found on roots of 95 % of trees and shape the present forests in temperate and boreal regions, but also colonize some tree taxa in tropical forests (Smith and Read, 2008). The formation of ECM is considered to be the most widespread strategy of trees to increase the P acquisition (Torres Aquino and Plassard, 2004; Smith and Read, 2008). In ECM (**Figure 2A**), the fungal partner covers the tips of fine roots with a hyphal mantle (Blasius et al., 1986) and extra-radical hyphae growing from this mantle (Becquer et al., 2014). At the root-hyphae interface occur the colonization of the apoplast between the root cortical cells forming the Hartig net, which serves for the exchange of nutrients between plant and mycorrhizal partner.

The contact between plant and AM fungus (**Figure 2B**) occurs by the grip of a hyphopodium to the root surface, once the AM fungus has developed from a spore (Bonfante and Genre, 2010). The intracellular AM fungal colonization follows from the epidermal and cortical cells. Intercellular hyphae develop between the cortical cells. In the inner cortical cells highly branched arbuscules are developed, where it is assumed the nutrient exchange between plant and the mycorrhizal partner does occur.

The mycorrhizal associates use different mechanisms for the acquisition of P: Specifically, to solubilise P from primary mineral such as apatite or P that is bound onto metal oxides and clay minerals, mycorrhizal fungi exude low-molecular-weight organic anions (LMWOA, e.g. oxalate, malate, citrate) (Plassard et al., 2011). Whereas, to hydrolyse P from organic monoesters, diesters, or phytate,

mycorrhizal fungi release substrate specific phosphatase enzymes, which can be surface-active, extracellular, or intracellular. However, the different mechanisms of P acquisition could represent an indicator for specialization of mycorrhizal fungi to acquire certain P sources or switch the mechanisms according to the available P source.

The ECM were shown to be more relevant in progressively developed ecosystems (Laliberté et al., 2012; Albornoz et al., 2016). With increasing pedogenesis the total P in soil decreases, accumulating as organic P fraction in soil, and plants shift their mycorrhizal associates from AM to ECM in order to adjust their P acquisition strategy. Due to the suggested capabilities of ECM fungi in acquisition of differently available P forms, the following dissertation is focusing mainly on ECM.

1.2.2 The Exchange of Carbon-Phosphorus in Mycorrhizal Symbiosis

In exchange for nutrients, the plant supplies the fungus with carbon (C) such as sucrose (Buscot et al., 2015), which the plant synthesizes by the process of photosynthesis. C is transferred within the plant to the organs and tissues that are in need and a share of up to 30% of daily fixed C is allocated by plant to their ectomycorrhizal fungal partner (Finlay and Söderström, 1992; Hobbie, 2006). Therefore, ECM are an essential sink for C (Finlay and Söderström, 1992). Nevertheless, the plant C allocation to the ECM is governed by soil accessibility and plant needs in nutrients (van der Heijden, 2001; Lambers et al., 2018). These facts lead to the assumption, that the plant adjusts its C allocation to the mycorrhizal partner to increase the acquisition of the limited nutrient (Albornoz et al., 2016). The study of Kiers et al. (2011) has shown, that to achieve stability in plant-mycorrhizal mutualism, the exchange of photoassimilates for nutrients is considered to be reciprocally regulated. Eventhough, their study was performed with AM, it demonstrated that both symbionts can sense variations in the resource allocation by the other partner and consequently adjust their own resource share. This could indicate that when a highly specialized fungus invests more to acquire free phosphate from complex P sources, it would be able to provide the host plant with less P but also rewarded with less carbon (C), in comparison to fungi, which provide their host with more P from simpler or many different chemical P forms, as these P forms do not require higher C investment into the acquisition of the P source.

1.3 Resource Partitioning for Soil Phosphorus and Mycorrhizal Contribution

The diversity in P acquiring mechanisms can ensure more efficient use of different biologically available P forms by plants through resource partitioning (Turner, 2008). As proposed by Turner (2008), resource partitioning for soil P is an additional framework (**Figure 3**) to explain the co-existence of plant species, which adapt on, and specialize to acquire, different chemical forms of P to reduce competition. The investment to acquire the free phosphate from various P sources increases with decreasing biological availability of the P source. This model comprises only the organic P pool and organic P secondary mineral complexes, but it is also applicable to the inorganic P pool in the soil, which includes mineral P, co-precipitates and sorbed P.

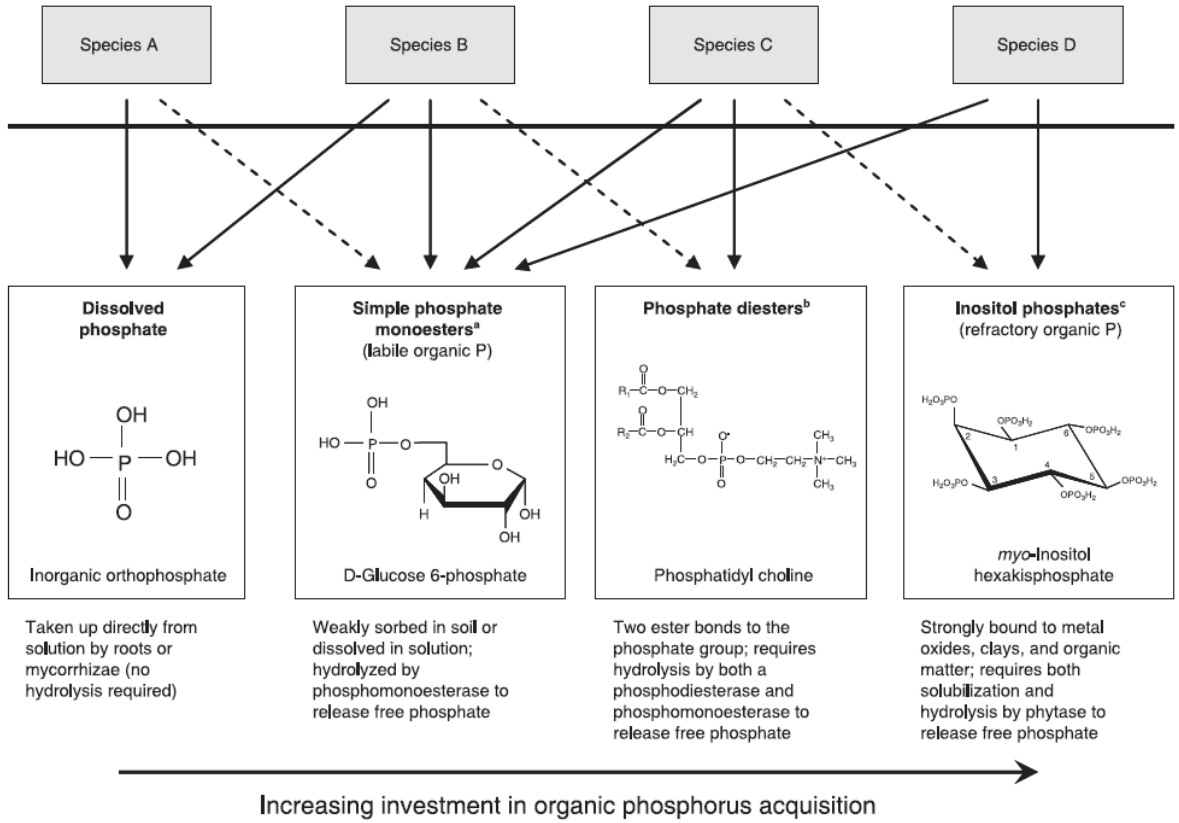

Figure 3 | The conceptual framework of resource partitioning for soil P introduced by Turner (2008) (the figure is from the same refenrece). It represents different different organic P sources in the soil with increasing investment for acquisition: the dissolved phosphate need to be just taken up from the soil solution; simple monoesters have to be hydrolysed by phosphomonoesterase; phosphodiesters are due to 2 ester bounds more complex and have to be hydrolysed by phosphomono- and phosphodiesterase enzymes; and the phytate which is strongly bound to any soil constituents, has to be solubilised and hydrolysed by phytase.

Studies on resource partitioning for soil P (e.g. Ceulemans et al., 2017; Phoenix et al., 2020) were performed in several mesocosm experiments using grasses and forbs as co-ocurring plant species in European grasslands. These grasses and forbs exhibit contrasting adaptations to P limitation in soils such as formation of mycorrhizae or specific root traits. Mixed communities or monocultures of 7-14 of these plants were exposed to organic or inorganic P sources or to a mixture of both. Their main findings were that the abundance of specific plants was related to the acquisition and uptake of specific P source. Hence, these finding support the hypothesis that each plant species finds its niche of an appropriate P source, increasing the biodiversity under P limitation (Olde Venterink et al., 2011; Ceulemans et al., 2014). Hence, resource partitioning for soil P could help to explain the loss of biodiversity due to the enrichment of readily available P in terrestrial ecosystems, resulting in competitive disadvantage for species specializing on accessing complex P sources (Wassen et al., 2005; Ceulemans et al., 2014).

But, also the framework of resource partitioning for soil P highlights the fact that plant P uptake under P limiting or co-limiting conditions is, most likely, mycorrhizae associated (Turner, 2008). For instance, the study of Liu et al. (2018) demonstrated that co-occurring plant species of tropical and subtropical forests partition P through mycorrhizal association. They worked with plant species from a less divers overstorey dominated by ECM tree species and a divers understorey dominated by AM tree species. Their results suggest a variation in the capacity to acquire different organic P sources and free phosphate within and between the ECM and AM species. A possible trade-off was suggested could occur through the increased investment of C from host plants to acquire complex organic P through ECM, which could put a boundary to the ECM plants from competitive dominance (Jiang et al., 2017) in tropical and subtropical forests (Liu et al., 2018), which would allow the co-occurring of different plant species with different mycorrhizal types and species.

1.4 Designing a Test System using compatible Plant and Mycorrhizal Fungal Material

In order to enable the understanding of (i) C investment from ECM plant in relation to the mining of different chemical forms of P as well as (ii) partitioning of different chemical forms of P available in the same pool, adequate experimental systems consisting of compatible plants and mycorrhizal fungi have to be developed. To ensure a robust test of nutrient acquisition and exchange models, the plant and mycorrhizal fungal material have to be functional to each other (Gafur et al., 2004; Müller et al., 2013), as it was shown that only functional associates can increase plant P uptake under natural,

nutrient limited field-conditions (Rousseau et al., 1994; Smith and Read, 2008). Only in functional symbiosis the mycorrhizal associates can penetrate into the plant root, forming a Hartig net between the cortical cells (Rousseau et al., 1994; Garfur et al., 2004). This Hartig net is assumed to serve for the exchange of nutrients between the symbionts (Landeweert et al., 2001).

Poplar plant species *Populus x canescens* as well as the ECM fungus *Paxillus involutus* strain MAJ were reported to be such organisms that are functional to each other (Gafur et al., 2004; Müller et al., 2013). They were shown to form ECM under axenic conditions (Cripps and Miller, 1995; Hampp et al., 1996; Loewe et al., 2000). Under field conditions, poplars can form symbiotic associations with different mycorrhizal types such as ECM and AM fungi (Gherghel et al., 2014). It was also shown that *P. involutus* and *Rhizophagus irregularis* were the first two mycorrhizal species colonizing poplar clones under various field conditions (Gherghel et al., 2014). Furthermore, poplars are considered as beneficial model organisms due to the ease of *in vitro* micropropagation and rooting (Confalonieri et al., 2003), having identical clones, stochastic variation would be reduced compared to the seedlings. Also *P. involutus* was severally reported to propagate in culture that are easy to maintain (Cripps and Miller, 1995). Moreover, *P. involutus* is able to exude oxalic acid to acquire P from mineral sources (Lapeyrie et al., 1991) and release surface-bound phosphatases to mineralise organic P sources (McElhinney and Mitchel, 1993).

1.5 Motivation and General Hypotheses

Mycorrhizal fungi are a beneficial opportunity not only to safe chemical P fertilizer but also to enhance soil biological processes, increasing aboveground diversity (Bender et al., 2016) and therefore ensure a resilient ecosystem functioning (Oliver et al., 2015). Due to the importance of finding ways to enhance a more efficient use and to recycle the soil P, it is important to understand the capabilities of ECM fungi to acquire differently available P sources and related C costs required from the host plant to support the mycorrhizal mediated P acquisition. Studies by Jones et al. (1991, 1998) or Tinker et al. (1994) using an ectomycorrhizal willow calculated higher benefits (P uptake) in relation to C costs compared to non-mycorrhizal plants. The studies of Andrino et al. (2019, 2921) indicated that the AM plant invests more C to sustain the symbiotic relationship with access to more complex P sources. To our knowledge, data on P source dependent C exchange in ECM are missing, representing a research gap. But ECM associates represent an essential sink for C (Finlay and Söderström, 1992) in forest ecosystems worldwide.

The ECM fungi are known to dominate in temperate (Moser, 1967; Meyer, 1973) and boreal (Singer and Morello, 1960; Meyer, 1973) forest ecosystems. The bioavailability of P in temperate forests is assumed to be limited mainly due to the advanced aging of soils (Walker and Syers, 1976; Turner et al., 2007). The P limitation in boreal forests is caused by including nitrogen (N) deposition (Hedwall et al., 2017) as well as groundwater discharge redistributing Al and Fe that sorbs P and increasing N turnover through keeping the pH in the humus high (Giesler et al., 2002). Nevertheless, the tropical and subtropical forests consisting of a less diverse overstorey dominated by ECM tree species and a diverse understorey dominated by AM tree species (Liu et al., 2018) could serve as a scenario to increase the stake of agroforestry. So that ECM plants could access complex P sources and return it e.g. through litter fall, senescence of hyphae, and decomposition/mineralisation of both back to the P cycle in soil and so, supply the crops (which are usually AM plants) with P in a more environmentally sustainable manner.

Nevertheless, none of the studies researching about phosphorus partitioning addressed the sole mycorrhizal contribution and the choices and preferences of mycorrhizal fungus to specific P sources, when the mycorrhizal plant is exposed to a pool consisting of different P sources of different availabilities. To test the exclusively mycorrhizal mediated plant P uptake, an adequate experimental setup has to exclude the plant roots from the supplied P sources. Many attempts have been made to build culture systems with separate compartments for the P sources, where only the fungal hyphae had access (e.g. Andrino et al., 2019, 2021). In order to test (i) the P source specific C allocation from the host to the fungal associate or (ii) the resource partitioning of P that are exclusively accountable to the P acquisition by the mycorrhizal fungus a compartmental culture system has to be developed. The traits of *P. x canesces* and *P. involutus* described above are promising associates to test a mycorrhizal mediated plant P uptake from differently available P forms.

The review of current literature relevant for the research of phosphorus economy in ECM reveals some open questions, which are formulated as main aims of this study. The purpose of the present study was

(i) to design a system to observe mycorrhizal mediated nutrient exchange; and

to investigate

(ii) whether the different bioavailabilities of specific P sources (including PO_4^{3-} (oP), the primary mineral apatite (AP), the organic P species (P_{org}) phytic acid (Phy) or adenosine monophosphate (AMP), and goethite-bound PO_4^{3-} (gP) as secondary mineral-P adsorption complex) acquired by an ectomycorrhizal fungus influence the P uptake efficiency in relation to the C investment of its host plant; specifically, whether the carbon (C) investment from

the host plant for the mycorrhizal mediated P derived from different P species is P source dependent; and

(iii) the preferences of mycorrhizal plant to specific P sources from a mixed P pool.

Based on the theoretical background above, the following general research hypotheses have been addressed:

H1 When the exchange of C for P is reciprocally regulated (Kiers et al., 2011) and the plant C allocation to the ectomycorrhizal fungal associate is governed by soil P accessibility and plant needs in nutrients (van der Hejden, 2001; Lambers et al., 2018), plant P uptake mediated by ectomycorrhizal fungus from easily available source oP will cause less C costs compared to the more complex P sources (Phy and mineral P sources AP and gP).

H2 Due to the differences in the required P acquiring mechanisms, the ectomycorrhizal C drain from the host plant for the acquisition of the recalcitrant, organic P source Phy will differ compared to the C drain for the mineral P sources AP and gP, as both P source types require different acquiring mechanisms (Plassard et al., 2011; Hinsinger et al., 2011).

H3 As the mycorrhizal fungi are important in plant P acquisition and are known to be able to access organic and sorbed P sources (Hodge et al., 2001; Plassard and Dell, 2010), all P sources within the diverse P pool are available to mycorrhizal fungi, and therefore to plant, but the order/magnitude of acquisition will depend on P source complexity/amount.
In order to differentiate the P uptake between the different P sources, application of radioisotope labelling with 33P would be necessary, which would enable the direct visualisation of mycorrhizal fungal preference to specific P sources (**Figure 4**): whether the mycorrhizal plant is able to utilise (H3-I) only the free phosphate, (H3-II) all affordable P sources, depending on biological availability of the P source, or (H3-III) specialise to acquire P from (a) organic or (b) mineral sources, but still also utilising inorganic orthophosphate.

H4 Furthermore, in order to support the general assumption that a minor diversity in belowground resources, which can occur as a side effect of fertilisation (Rajaniemi, 2002; Rieger et al., 2019), result in less efficient uptake of P (Zavičić et al., 2018), we hypothesise that mineral source (HAP) as a single P source is less available than within a pool of diverse P sources.

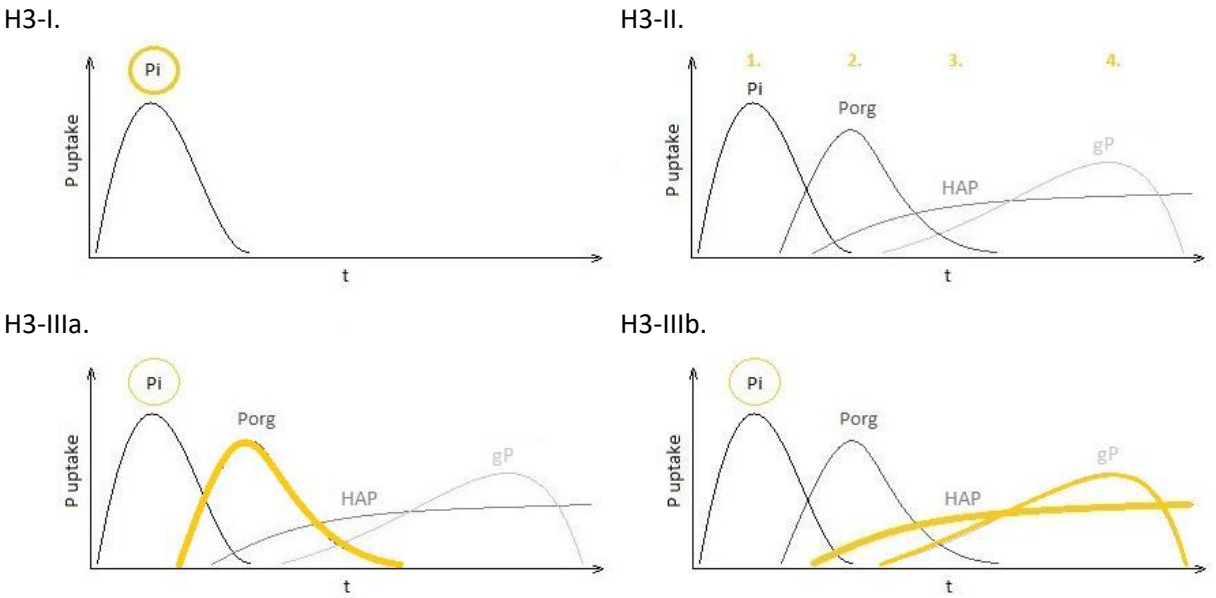

Figure 4 | Uptake of various P sources by mycorrhizal plant in a time course of the experiment in dependence of biological availability of P (Pi..inorganic P; Porg..AMP (organic P); HAP..hydroxyapatite (primary mineral); and gP..inorganic P bound to goethite; t..time): Ability of mycorrhizal fungus to utilise (H3-I) only the free phosphate, (H3-II) all affordable P sources, or (H3-III) specialisation of mycorrhizal fungus to acquire P from (a) organic or (b) mineral P sources.

1.6 Scientific Approach

In the following dissertation three studies were performed: Study I aimed to design different culture systems for test of hypotheses of Study II and III. Study II focused on P source dependent plant development and translocation of C (and N) from plants aboveground to the belowground organs that are exclusively governed by mycorrhizal fungal associate. In Study III, mycorrhizal mediated resource partitioning for P and transfer to the host plant was investigated. A short report (and an illustration in *Fig. 5*) of the materials and methods used for each study are described as following:

Study I – Culture systems for Study II (mesocosms) and Study III (rhizotrone systems) were developed using the compatible poplar plants *P. x canescens* and ectomycorrhizal fungus *P. involutus* as model organisms as well as separate compartments for the exclusive access of the P sources by mycorrhizal fungus. Materials and methods included development of *in vitro* multiplication and rooting of poplar plants and axenic propagation of mycorrhizal fungal material in liquid culture and on substrate carrier to produce the inoculum.

Study II – A greenhouse experiment using mesocosms developed in Study I was performed. In separate compartments different P sources were supplied: ortho-phosphate (oP), phytic acid (Phy), apatite (AP), oP bound to goethite (gP), and H_2O as a no P control. To trace the P source dependent C allocation to the belowground parts of the plant, the plant was leaf-fertilised using $^{13}C^{15}N$-labelled urea. The analyses included the urea-derived ^{13}C and ^{15}N, the absolute amounts of P, C, N, mycorrhization of plant roots of harvested plant material.

Study III – Resource partitioning for P by mycorrhizal was investigated using rhizotrone culture systems developed in Stidy I. Again in separate compartments and simultaneously following P sources were supplied: oP, adenosine monophosphate (AMP), hydroxyapatite (HAP), and gP were supplied. To differentiate the P uptake between the different P sources, radio-isotope labelling with ^{33}P was applied. The mycorrhizal mediated P uptake of HAP supplied as single P source was compared with the partitioning of the different P sources from a pool. Imaging of culture systems in a time course of the experiment and of harvested plants was performed. Further analysis included mycorrhization grade of plant roots, P content and ^{33}P activity in plant material.

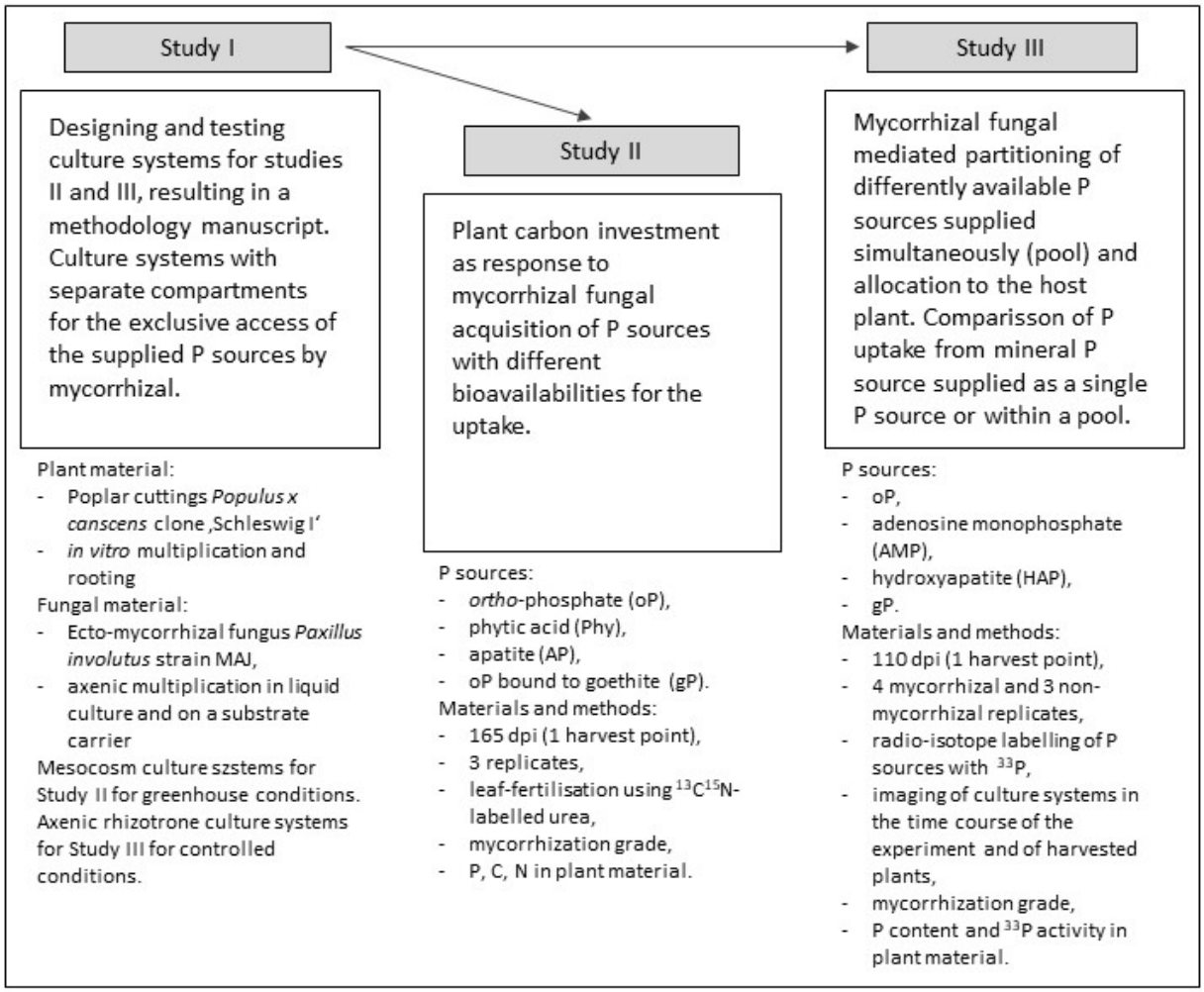

Figure 5 | Overview of the materials and methods used in each study.

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2 Study I

Designing a Robust and Versatile System to Investigate Nutrient Exchange in, and Partitioning by, Mycorrhiza (*Populus x canesces* x *Paxillus involutus*) Under Axenic or Greenhouse Conditions

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Author contribution:

The idea of the experiment came from JB with contributions from KS, AA, GG, AF, and LS. KS prepared the plant and fungal material, prepared the different culture systems, and conducted the experiments. KS performed data analysis and wrote the manuscript. JB and GG supervised the research. All authors contributed to the article and approved the submitted version.

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Phosphorus (P) bioavailability affects plant nutrition. P can be present in soils in different chemical forms that are not available for direct plant uptake and have to be acquired by different mechanisms, representing different resource niches. These mechanisms, of which many seem to be attributed to mycorrhiza, likely influence the diversity and stability of plant communities in natural ecosystems, as they also might help to overcome a future shortage of P supply in agro-ecosystems. In order to understand the mechanisms of P acquisition, the associated carbon costs, and the resource partitioning by mycorrhizal fungi, the ecosystem situation has to be mimicked in smaller scaled experiments. Here, different experimental setups are evaluated using plantlets of *Populus x canescens* and its functional ectomycorrhizal (ECM) fungus *Paxillus involututs* strain MAJ. To investigate resource partitioning involving mycorrhizae, the protocols of this study describe preparation of an *in vitro* and a rhizotrone culture systems for studies under axenic conditions as well as a mesocosm culture system for greenhouse conditions. We also describe the construction of separate compartments containing nutrients and excluding plant roots as well as the progress that has been made in *in vitro* propagation of plant and ECM fungal material. The practical experience made in our

study shows that the *in vitro* culture system is prone to desiccation and its construction and maintenance are more time consuming and complicated. In contrast, with the axenic rhizotrone culture system and the mesocosms we have created more robust and very versatile systems that are also suitable for greenhouse conditions.

Keywords: ectomycorrhizal, *in vitro*, laboratory protocols, mesocosm, *Paxillus involutus*, *Populus x canscens*, rhizotrone, phosphorus availability

HIGHLIGHT

Compartmental culture systems have been developed to study the mycorrhizal associations to investigate the mycorrhizal fungal mediated P resource partitioning and plant P uptake, required host carbon investment and mycorrhizal fungal acquisition mechanisms in dependence of P form bioavailability.

INTRODUCTION

More than 90% of phosphorus (P) in the soil is present in different chemical forms that are unavailable to plants (Mengel et al., 2001). The association of plants with mycorrhizal fungi can increase the bioavailability of P (Hinsinger, 2001; Hinsinger et al., 2011; Plassard et al., 2011) and so meliorate their nutritional state (Gafur et al., 2004). Moreover, resource partitioning of P associated with mycorrhizal fungi could contribute to more efficient use of different P forms by plants, reducing competition for soil P (Turner, 2008). It is hypothesised that mycorrhizal fungi are the key component in resource partitioning under P impoverished conditions in soil.

Ectomycorrhizae (ECM) were shown to mine different chemical forms of P (reviewed by Plassard et al., 2011) in exchange for energy derived from hosts' photosynthesis (Buscot, 2015) and increase cost-efficiently the soil volume explored for nutrients *via* its extra-radical hyphae (Jones et al., 1998). To understand if (i) ECM mobilizes P from differently accessible sources and (ii) consequences of higher energy costs for the plant development due to the acquisition of more complex P forms exist, adequate and innovative experimental systems consisting of compatible plants and mycorrhizal fungi have to be developed. The use of compatible plant and ectomycorrhizal (ECM) fungal species provide valuable model systems for a more robust test of nutrient acquisition and exchange models (Gafur et al., 2004). In functional symbiosis, the mycorrhizal associates can penetrate the plant root, forming a Hartig net between the cortical cells (Rousseau et al., 1994; Hampp et al., 1996; Gafur et al., 2004), where the exchange of nutrients between the symbionts is supposed to happen (Landeweert et al., 2001). In contrast, incompetent ECM fail to penetrate the host roots, causing a defence reaction by thickening the cell wall of the epidermis (Lei et al., 1990; Gafur et al., 2004). It was shown that only functional associates could increase plant P uptake in nature under impoverished nutrient conditions (Rousseau et al., 1994; Smith and Read, 2008; Hoeksema et al., 2010). Poplar plant species *Populus x canescens* and the ECM fungus *Paxillus involutus* strain MAJ are the reassuring organisms on this matter (Gafur et al., 2004; Müller et al., 2013).

Under field conditions, poplars can form symbiotic associations with different mycorrhizal types such as arbuscular mycorrhizal (AM) and ECM fungi (Khasa et al., 2002; Gherghel et al., 2014). The study of Gherghel et al. (2014) could show that *P. involutus* subsequent to *Rhizophagus irregularis* colonized poplar clones under various field conditions. Among poplars, *P. involutus* (Basidiomycetes) has a wide variety of hosts able to form ECM with many forest tree species belonging to gymnosperms and angiosperms (Duddridge, 1987; Baum et al., 2000; Gafur et al., 2004) and be an appropriate help for trees in 'bare-root' conditions (Jarosch and Bresinsky, 1999; Hönig et al., 2000). *P. involutus* was also shown to be able to exude oxalic acid to acquire P from mineral sources (Lapeyrie et al., 1991) and to release surface-bound phosphatases that can mineralize organic P forms (McElhinney and Mitchell, 1993; Alvarez et al., 2004). Moreover, *P. involutus* is easy to maintain and propagate in culture and is therefore increasingly used in ECM studies (Wallander and Soderstrom, 1999; Gafur et al., 2004; Müller et al., 2013).

The natural hybrid *P. x canescens* (grey poplar) result through pollination of *P. alba* (white poplar) by *P. tremula* (European aspen) (Lexer et al., 2005). *P. x canescens* and *P. alba* occur sympatrically in European river valleys (Rajora and Dancik, 1992; Fossati et al., 2004; Lexer et al., 2005), whereas *P. tremula* is an important pioneer tree species covering forests in the upland (Adler et al., 1994; van Loo et al., 2008). Furthermore, the economic and scientific importance of *Populus* trees increased. Due to their natural distribution and genetic variability, they can be cultivated under polluted and degraded soil conditions (Chen and Polle, 2010) and contribute to a site's positive carbon balance. Thereby, the poplar supplies the industry with wood biomass, fibre, bioenergy and chemicals (Klass, 1998). Furthermore, poplars are considered as beneficial model organisms due to the ease of micropropagation (Confalonieri et al., 2003), which reduces stochastic variation by the use of clones instead of seedlings. Because of all these benefits, poplars gained importance in scientific fields, including biotechnology, molecular biology, and other areas related to nutrition, abiotic pressures, or the plant-soil interface (reviewed by Müller et al., 2013).

The latest and first study aiming to investigate the ECM mediated resource partitioning for P was performed by Schreider et al. (2022), revealing that the ECM *P. involutus* has the potential to occupy fundamental niches of various P sources, whereby the readily available phosphate was not as expected the

most favourable P source for uptake within a mixed P pool. Previously, progress in studying partitioning for soil P has been achieved by examining plant responses to single P sources (Steidinger et al., 2014) or a mixed pool of different P sources (Liu et al., 2018), but the sole contribution of mycorrhizal fungi in resource partitioning was neglected in these studies. The studies of Andrino et al. (2019; Andrino et al., 2021) were the first to investigate the mycorrhizal mediated acquisition of differently available P sources using arbuscular mycorrhiza. Nevertheless, Andrino et al. (2019; Andrino et al., 2021) have determined that higher amounts of C were invested by the plant into the association with a mycorrhizal fungus that had access to more complex P sources, whereby the P sources were supplied as a single P source. By using separate compartments for the nutrients, it is possible to mimic the ecosystem situation with an mycorrhizal plant having access to widely distributed nutrient patches with different bioavailabilities through mycorrhizal fungus, excluding the direct competition of plant roots and mycorrhizal hyphae for P and depending on the experimental conditions revealing the true capabilities of mycorrhizal fungal associate in nutrient acquisition.

The main aim of this study is to provide practical experience and to describe the learning curve in developing a culture system that is robust and versatile to investigate mycorrhizal mediated resource partitioning. Three different experimental setups using poplar plantlets of *P. x canescens* and its functional ECM fungus *P. involututs* strain MAJ for axenic and greenhouse conditions are presented. The present study covers also modified protocols (Müller et al., 2013) of the *in vitro* multiplication and rooting of plant material and production of ECM fungal inoculum under axenic conditions that suited our experiments the most. Furthermore, the co-cultivation of poplar and ECM fungi and maintenance steps in different culture systems under axenic and greenhouse conditions are reported. Also, the construction of separate compartments for the nutrient supply that are adaptable into different experimental set-ups is described.

AXENIC MULTIPLICATION AND ROOTING OF PLANT MATERIAL

All steps regarding multiplication, maintenance, and preparation of plant and fungal material were performed under sterile conditions under laminar airflow. As a model plant, poplar species are beneficial because of their ease for *in vitro* propagation (Müller et al., 2013). For the propagation of poplar plantlets *P. x canescens* clone 'Schleswig I,' Woody Plant Medium [WPM, (Lloyd and McCown, 1980) composed as published by Müller et al. (2013)] without hormones (ready to use purchased from dephyte e.K., Langenberg, Germany) was used. After pH was adjusted to 5.2, 8 g/L plant agar (Duchefa Biochemie B.V, Haarlem, Netherlands) was added as a solidifying agent. Explants were established from lateral meristems with one leaf and bud. Always eight cuttings were placed in one vessel (sterile, PP, 10 cm high, 7 to 9 cm in diameter; Plastikbecher.de GmbH, Giengen an der Brenz, Germany) containing around 70 ml WPM multiplication medium (autoclaved at 121°C for 20 min) and these vessels kept under controlled environmental conditions (24°C; 60% humidity; photosynthetic active radiation (PAR) of 150 µmol m^{-2} s^{-1} at plant height for a light period of 16 h). The multiplication step was repeated every eight weeks to maintain the culture homogeneous and fresh. This period of time has to be considered if high quantities of plantlets for the experiments have to be produced starting with a few vessels only.

For rooting, the poplar plantlets' apical buds with 1.5 cm height were used. The WPM medium used for rooting contained one-third of sucrose and vitamins only. The medium was poured (around 90 ml) into Microboxes (autoclavable, 13 cm high, 9 to 10 cm in diameter; O118/80, by Sac O_2, Deinze, Belgium). The leads of these Microboxes had an installed membrane for sterile gas exchange (81.35 GE day^{-1}) so that CO_2 could diffuse into and O_2 outside the vessel. The last two steps were necessary to shorten the acclimatization time of the plants in the greenhouse so that the plants already start to perform photosynthesis, not only taking C from the medium. After six weeks, the plantlets developed roots of up to 8 cm in length and were 6 to 8 cm in height. In this developmental stage, the plantlets are ready to be transferred into the culture systems and the direct co-culture with the mycorrhizal fungus.

AXENIC PRODUCTION OF ECTOMYCORRHIZAL INOCULUM

Before used as inoculum, the ectomycorrhizal fungus *P. involutus* strain MAJ was propagated and maintained in pure culture on modified Melin-Norkrans medium (MMN, ready to use salts purchased from dephyte e.K., Langenberg, Germany). The composition of MMN medium was published by Müller et al. (2013). After the pH of the medium was adjusted to 5.5, 10 g/L plant agar was added as a solidifying agent. Petri dishes (ø 9 cm) were filled with 25 ml MMN medium (autoclaved at 121°C for 20 min) and inoculated by a fungal plug from the previous culture. Petri dishes were sealed with Parafilm, wrapped in aluminium foil to simulate darkness, and kept with the lid down at room temperature at around 20 to 23°C.

For different culture systems, there exist various ways to produce the inoculum of *P. involutus*. The cultivation techniques of ECM fungi were reviewed in detail, e.g. by Harvey (1991). For our experimental purposes following techniques worked well: For *in vitro* systems, it is suggested to pre-grow the ECM fungus on a cellophane membrane (CM; Gel drying frames, Sigma, Z377600-1PAK, LOT# 3110) in axenic culture (Felten et al., 2009; Navarro-Rodenas et al., 2012; Müller et al., 2013). The CM would stop the fungus from growing into the medium and, due to its fine porosity, it would supply the fungus with the necessary nutrients from the medium (Müller et al., 2013). The CM would ease the transfer of the fungus into the culture system that is maintained on a different medium that is different from the propagation medium and should not contain specific elements (e.g. P, C, or N) and vitamins. Therefore, to prevent this, the fungal plug should be cut out before using the pre-inoculated CM for the experiments. Further benefits of using CM is to shorten the time for the mycorrhization of the plant and to foster the proliferation of mycelium in the system.

The CM (1.6x6cm) was boiled in 1 mM EDTA (Merck, 1.08417.1000) solution for one hour and washed in deionized water (dH_2O) (Navarro-Rodenas et al., 2012). After autoclaving, the CM was plated on MMN medium, inoculated with two fungal plugs of MAJ, and sealed and incubated as described above.

In experiments with mesocosms or rhizotrone systems which use growth substrates like Perlite, it is necessary to produce higher quantities of inoculum, as it is usually applied proportions of around 10% compared to the substrate [reviewed by Harvey (1991)]. This is possible by propagating *P. involutus* in liquid culture. For this purpose, one culture plate of *P. involutus* has to be vortexed and transferred in 0.2 L MMN liquid medium in 0.5 L glass bottles. Under horizontal shaking for around eight weeks, the fungus grows in more or less concentric spheres, which can be perfectly used to proliferate further on 0.8 L Perlite carrier and 0.2 L MMN. The later step is required to obtain a more homogeneously inoculated substrate for the actual experiments.

CO-CULTIVATION IN COMPARTMENTAL CULTURE SYSTEMS

In the following study, the presented culture systems consisted of a root compartment (RC) and physically separated hyphal compartment (HC) to exclude the plant roots from the contents of the hyphal compartments (**Figure 1**). The design of the systems allows the insertion of more than one HC. For our aims, up to four HCs with different chemical forms of P, representing different bio-availabilities, were introduced into the RC.

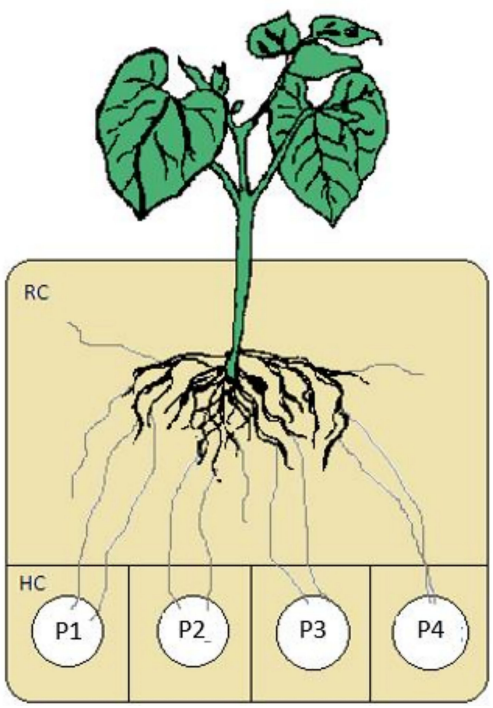

FIGURE 1 | Experimental setup for mycorrhizal mediated uptake of P of different bioavailabilities and translocation to the plant.

Before deciding about the experimental setup, the designer has to consider the key boundary conditions: (i) the aim/research question, (ii) the variables and parameters that has to be measured or visualised, (iii) the experimental necessities to test the hypothesis (e.g. axenic or outdoor conditions), or (iv) the resources to establish the system. The following presents three different experimental approaches with their benefits and disadvantages.

Indication of the Hartig Net in Mycorrhizal Roots of *P. x canscens* Associated With *P. involutus*

Characteristically for successful mycorrhization of poplar roots by MAJ is the change in root morphology such as specific branching of root tips and absent development of root hairs (Brundrett et al., 1996). From our pre-experiment of synthesizing *P. x canescens* with *P. involutus*, we could observe the Hartig net hyphae between the cortical cells inside the roots (**Figure 2**) and a dense hyphal mantel around the root tips (**Figure 2B**). Such observations of *P. x canescens* with *P. involutus* (MAJ) were also previously detected by Gafur et al. (2004).

Phosphorus Sources and Their Sterilisation

In our studies, we have tested resource partitioning for soil P using the following P chemical forms to mimic the conditions and cover the most abundant P pools available in soil: *ortho*-phosphate (oP), organic P source [P_{org}; e.g. Phytate (Phy) or adenosine monophosphate (AMP)], mineral P source (e.g. apatite (AP; Krantz Company, Bonn, Germany) or synthesised hydroxyapatite [HAP; according to Wolff et al. (2018)]; < 0.2 mm), oP secondary mineral adsorption complex (oP bound to goethite [gP; according to Andrino et al. (2019)]; < 0.63 mm). The sources oP and P_{org} were supplied in liquid form and filter-sterilised using a sterile syringe filter (Filtropur S, PES, 0.2 µm pore size, Sarstedt AG & Co. KG, Nümbrecht, Germany) prior filling into the HCs. In contrast, the mineral sources underwent a fractionated sterilisation (three times sintered at 105°C for 30 min over three days), which is called tyndallization and is used when the mineral structure should not be changed through the sterilization process. The consistency of gP with a high volume to mass ratio compared to other P sources has to be taken into account when considering the amounts of each P source that have to be supplied to the system at the HC.

In vitro Culture System With Controlled Conditions

Successful *in vitro* synthesis of plants of *Populus* sp. and ectomycorrhizal fungi, including *P. involutus*, were reported in previously conducted studies (e.g. Hampp et al., 1996; Gafur et al., 2004; Felten et al., 2009; Müller et al., 2013). First attempts were to

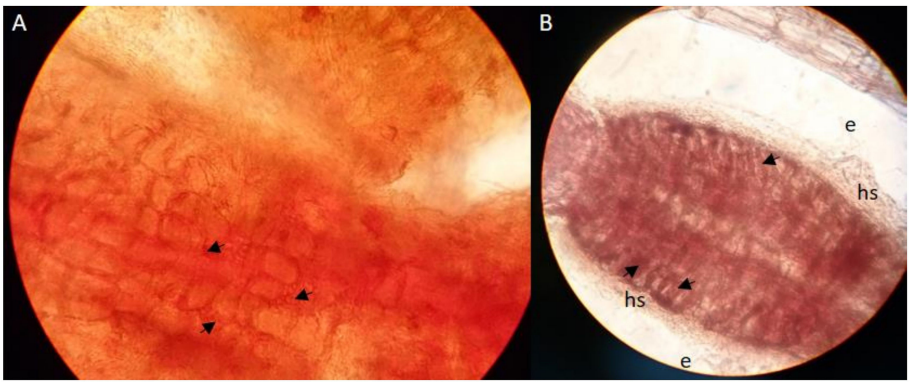

FIGURE 2 | Cleared and stained (acid fuchsin) root tips [**(A)** x100 magnification and **(B)** x40 magnification] of *P. x canescens* (Schleswig I) colonised by ECM fungus *P. involutus* (MAJ). The arrows indicate the Hartig net hyphae between the root cortical; hs: hyphal mantle around the root tips (mycorrhizal root tips).

use divided Petri dishes in order to create a compartmental *in vitro* system for studying AM uptake of P (Joner and Johansen, 2000; Koide and Kabir, 2000; Nielsen et al., 2002) and other elements (Dupre de Boulois et al., 2006; Dupre de Boulois et al., 2009). We have combined these practices to create a compartmental *in vitro* system to study resource partitioning and elemental exchanges between the mycorrhizal fungus and its host plant.

All steps were conducted under sterile conditions under laminar airflow. The culture system (**Figure 3**) consisted of a square Petri dish (120 x 120 x 10 mm, sterile; Greiner Bio-One GmbH, Frickenhausen, Germany), serving as RC. The MMN media formulation was shown to be too poor for successful co-cultivation of *P. tremula* L. and *P. involutus* (Langer et al., 2008). After macronutrients and vitamins were introduced into the formulation, the plant survival rate and mycorrhization increased. The WPM medium formulation contains all necessary macro- and micronutrients for plant development and, from our own experience, also for a successful mycorrhization. Therefore, Petri dishes were poured with around 70 ml WPM medium lacking P, C, and vitamins. As long as the medium was warm, four lids of Petri dishes (ø 35 x 100 mm, sterile; Sarstedt AG & Co. KG, Nümbrecht, Germany), serving as HC, were pressed slightly into the medium so that the medium extended bellied above the lid wall (Dupre de Boulois et al., 2006). Due to the gravitational attraction, the plant roots could grow into the medium underneath the HCs. The HCs were filled with four different, sterile P sources. The CM pre-inoculated with *P. involutus* was placed on the top of the medium between the HCs and the plant with the roots on the top of the CM to obtain mycorrhizal treatments or directly on the top of the medium to obtain non-mycorrhizal controls. The RC was closed with the lid of the Petri dish, where on one side or on the top, a hole was provided for the plant shoot and sealed with a sterile (autoclaved at 121°C for 20 min) silicon grease (Voets et al., 2005) to avoid contamination. The RC of the system was sealed with Parafilm and wrapped in aluminium foil to maintain the roots in the dark. A successful mycorrhization (**Figure 3**) of *P. x canescens* and *P. involutus* was clearly detectable after 17 days post-inoculation (dpi).

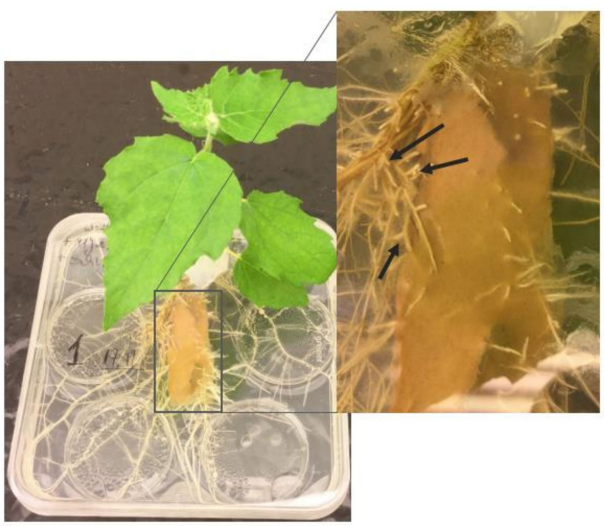

FIGURE 3 | Compartmental *in vitro* culture system synthesising *P. x canescens* (Schleswig I) and the ectomycorrhizal fungus *P. involutus* (MAJ, pregrown on CM) on WPM medium lacking P, C, and vitamins; at 17 dpi and maintained at 24°C and >80% humidity. The arrows in the zoom-in point to specific branching of fine root tips that indicate successful mycorrhization of plant roots.

To shorten the time for mycorrhization of plant roots, the plant can be pre-inoculated in a closed culture system (**Figure 4A**). The same square Petri dishes were used. Only a half of the petri dish was filled with WPM media, containing only one-third of P and C as required from the formulation and no vitamins. Two plants were placed per system with roots on top of CM pre-inoculated with *P. involutus* and the shoots inside the dish. These systems were sealed with Parafilm, and the bottom part was covered in an opaque plastic bag to keep the roots in the dark. After 22 dpi, the plants can be transferred into the compartmental *in vitro* systems (**Figure 4B, C**). We could see a proliferation of up to 2 cm of fungal mycelium from the mycorrhizal root tips in the medium after only 33 dpi (**Figure 4B**).

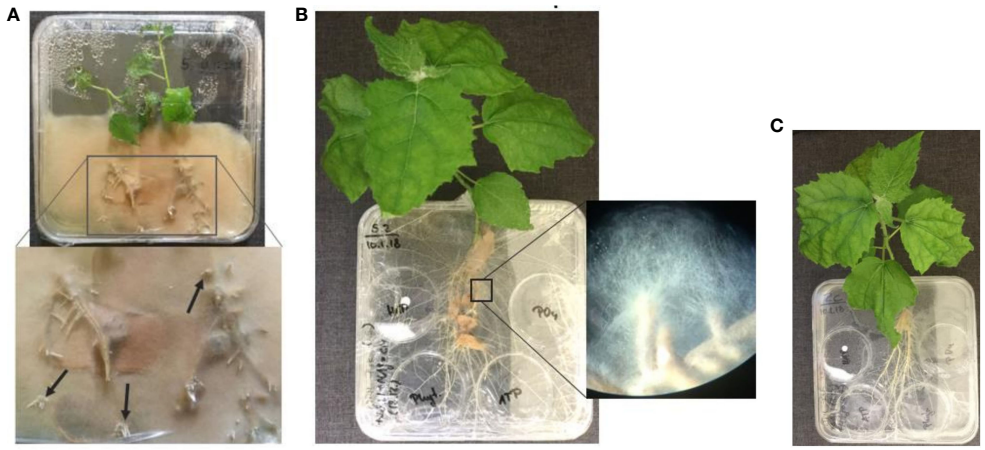

FIGURE 4 | **(A)** Pre-inoculated *P. x canescens* (Schleswig I) by the ectomycorrhizal fungus *P. involutus* (MAJ, pre-grown on CM) in a closed culture system on WPM medium containing one-third P, C and no vitamins (22 dpi); **(B)** Compartmental *in vitro* culture system prepared from pre-inoculated Schleswig I with MAJ (pregrown on CM) and **(C)** the non-mycorrhizal control poplar plant on WPM medium lacking P, C, and vitamins (55 dpi). All systems were maintained at 24°C and >80% humidity. The arrows in the zoom-in **(A)** point to specific branching of fine roots tips that indicate successful mycorrhization of plant roots; and **(B)** show the proliferation of fungal mycelium from the mycorrhizal root tips in the culture medium.

These systems are vulnerable to desiccation and thus have to be maintained under high humidity > 80%, a temperature of around 22°C, and PAR of 200 µmol m^{-2} s^{-1}. In the first two weeks, the freshly transferred plantlets in the system were covered with translucent vessels (e.g. sterile PP vessels used for plant material propagation). Especially, the non-mycorrhizal control plants were sensitive to desiccation and started to wilt after around 80 dpi, indicating that the ECM fungus *P. involutus* makes the plant more resilient to abiotic stress. If the high humidity in the growth chamber cannot be preserved, it is possible to keep the systems in a translucent container that can be closed. But they need to be opened regularly to ensure an exchange of CO_2 and O_2. Further, the *in vitro* systems are prone to contamination. With each additional preparation step, the contamination rate of the prepared systems increases. To maintain the system, new medium has to be added frequently to the system after around 80 dpi. For that the system has to be opened, increasing the contamination rate.

Besides the complicated maintenance, the *in vitro* culture system has also benefits: By using this type of culture system with agar or gellan gum as a solidifying agent for the medium in RC, the roots and fungal structures can be extracted from the gel using sodium citrate buffer [e.g. 10 mM, pH 6.6, at temperatures above 30°C (Doner and Becard, 1991)]. This makes the mycorrhizal fungal mycelium and plant roots well accessible for various analyses. The plant roots can easily be separated, and the hyphae filtered from the medium in the RC. This culture system also enables the collection of hyphae from the HCs separately by cutting them at the RC-HC interface.

Nevertheless, the proliferation of fungal mycelium in substrates like Perlite is faster. Therefore, one should consider whether it is necessary to make all this effort to establish an *in vitro* culture system or focus on other culture systems, which are more robust and much easier to maintain.

Preparing Separate Hyphal Compartments for Axenic Rhizotrone and Mesocosm Culture Systems

The separate hyphal compartments (HCs) for the P sources can be easily prepared from different SafeSeal reaction tubes to Falcon tubes to plastic bottles, depending on the experimental scale. For our culture systems, the HCs were prepared of (a.) 5 ml SafeSeal reaction tubes or (b.) 15 ml Falcon tubes (**Supplementary Figure 1**) (both from Sarstedt AG & Co. KG, Nümbrecht, Germany). For construction of both, at around 2 ml height a hole of 1 cm in diameter was burned into the tube and sealed with a combination of two types of membranes. A hydrophobic PTFE membrane (5 µm, Pieper Filter GmbH, Bad Zwischenahn, Germany) was installed at the P source side, preventing the liquids from mass flow and ions from diffusion into the root compartment (Mäder et al., 1993; Vierheilig et al., 1998; Andrino et al., 2019). A nylon mesh (20 µm, Franz Eckert GmbH, Waldkirch, Germany) was attached on the top of the PTFE membrane at the plant root side (Watkins et al., 1996), protecting the hydrophobic PTFE membrane additionally from a higher force applied by a growing root. The HCs were autoclaved at 121°C for 20 min and filled with the different P sources under sterile conditions. All containers were filled up to (a.) 3 ml or (b.) 4 ml with dH_2O. The HCs filled with P sources were always prepared at least one day before using them in the culture systems. If the HCs were prepared for axenic culture systems, they were stored in sterile containers until use.

Axenic Rhizotrone Culture System With Controlled Conditions

With the axenic rhizotrone culture systems (**Supplementary Figure 2**), we have excluded the weaker points of *in vitro*

culture system to establish a more robust compartmental culture system with an additional feature to supply the plants in the culture systems with the nutrient solution without the need to open it. This system also offers controlled conditions in the belowground in relation to rhizo- and mycosphere.

All preparing steps were conducted under sterile conditions under laminar airflow. The rhizotrone culture systems (**Supplementary Figure 2**) were made of square Petri dishes (100 x 100 x 20 mm, sterile; Sarstedt AG & Co. KG, Nümbrecht, Germany). The HCs used for this culture system were made of 5 ml SafeSeal reaction tubes, and up to four of these HCs can fit into the system. Four HCs were filled with different chemical forms of P such as oP, AMP, HAP, and gP. In a randomized order, these HCs were inserted one centimetre from the bottom into the rizotrones. Then, the rhizotrones were filled with Perlite (Perligran® classic, Knauf Aquapanel GmbH, Dortmund, Germany) as a nutrient free substrate. The Perlite was two times washed with dH_2O and autoclaved (at 121°C for 20 min). To obtain treatments with mycorrhized plants, the substrate was inoculated with 10% of *P. involutus* that was proliferated on Perlite as a carrier. For the non-mycorrhizal plant treatments, the inoculated substrate was autoclaved one more time before use. The rooted poplar plantlets were placed with the roots on the top of the HCs and covered with an additional thin layer of Perlite. The rhizotrone can be covered with the lid of the Petri dish. But for our experiments, we have used a thin sterile PVC foil (100 x 100 x 0.2 mm, Modulor GmbH, Germany) as a cover glued with a hot glue. Compared with the original lid from the Petri dish, the foil reduces the shielding of radio-isotopes (e.g. ^{33}P). The skip for the plant stem at the top and the opening to release the excess of nutrient solution at 2 cm height from the bottom of the rhizotrone were closed with a sterile cotton plug.

To maintain axenic conditions of the system during watering, the rhizotrones were equipped with a sterile syringe filter (Filtropur S, PES, 0.2 µm pore size, Sarstedt AG & Co. KG, Nümbrecht, Germany), which was connected to the rhizotrone through an autoclaved PVC pipe. From 0 dpi, the mesocosms were supplied with a WPM nutrient solution containing macro- and micro-elements without P and vitamins (WPM –P), which was balanced with KNO_3 and $(NH_4)_2SO_4$ to adjust the desired concentration of K. This is a modified formulation described by Müller et al. (2013). WPM nutrient solution were purchased as ready-to-use salts (dephyte e.K., Langenberg, Germany) that had to be dissolved in dH_2O only.

To obtain no P controls, the rhizotrones were prepared with HCs containing dH_2O only. The plantlets were kept under a plastic cover and included moisturized protecting paper plugs for the first two weeks for acclimatization and, thus, for protection from lower ambient air humidity and higher UV radiation. The rhizotrones were kept in a climate chamber at 80% ambient humidity and 20/18°C at an 18 h/6 h day/night cycle.

The results and the discussion of this experiment are published in Schreider et al. (2022).

Mesocosm Culture System With Greenhouse Conditions

If the controlled (axenic) conditions are not a key boundary for the research aim, the compartmental system can be up-scaled to the greenhouse conditions. In this paragraph we describe a mesocosm culture system (**Figure 5**) to investigate mycorrhizal mediated resource partitioning for P under greenhouse conditions.

The mesocosm culture systems (**Figure 5**) were prepared from pots and HCs (**Supplementary Figure 1**) to supply the different P sources. The rooted poplar plantlets of *P. x canscens* were transferred directly in pots (Ø 13 cm, 0.83 L) with Perlite as substrate (washed with dH_2O and autoclaved at 121°C for 20 min two times). The substrate was inoculated with 10% of *P. involutus* (proliferated on Perlite as a carrier) to obtain mycorrhizal plant treatments. For the non-mycorrhizal plant treatments, the inoculated substrate was autoclaved one more time before use. The mycorrhizal and non-mycorrhizal plantlets were kept under a plastic cover and a protecting cloth for four weeks for acclimatization, as the temperature in

FIGURE 5 | Mesocosm culture systems with four different chemical forms of P (4P) supplied in four separate HCs under greenhouse conditions: the poplar plants *P. x canescens* (Schleswig I) mycorrhized by ectomycorrhizal fungus *P. involutus* (MAJ) and non-mycorrhizal control plants at 58 dpi. The HCs for the different P sources were made of 15 ml Falcon tubes and hydrophobic and nylon meshes (see **Supplementary Figure 1** and **Figure 7**).

December in the greenhouse could drop to 14°C. In other seasons, the acclimatization period can be shortened to two weeks. The mesocosms were supplied with a WPM nutrient solution (without vitamins) containing a low concentration in P of 1.1 mg l^{-1} (WPM LP) as described by Müller et al. (2013) to support the P nutrition before the mycorrhizal fungus starts to acquire P from the HCs. At 58 dpi, four separate HCs containing each a different chemical P form such as oP, Phy, AP, or gP were inserted into the mesocosms (**Figure 5**). From 72 dpi, the mesocosms were watered with a WPM solution without any P (WPM –P). Both nutrient solutions WPM LP and WPM –P were balanced with KNO_3 and $(NH_4)_2SO_4$ to adjust the concentration of K. All WPM nutrient solution variants were purchased as ready-to-use salts (dephyte e.K., Langenberg, Germany). In the experiment's time course, the mesocosms were watered with dH_2O on demand.

The poplar plantlets and *P. involutus* were co-cultured in the mesocosm culture system for up to eight months. In case the experimental period has to be longer than ten months, we suggest using bigger pots, as the non-mycorrhizal plants respond to P deficiency with higher root biomass (**Figure 6**) in foraging for P. Also, in this culture system, we could observe abundant branching of mycorrhizal fine root tips specific for *P. involutus* (**Supplementary Figures 3, 4**), confirming high mycorrhization of poplar roots. The substrate and, especially, the entrance points to the P sources at HCs (**Supplementary Figure 3** and **Figures 7A, B**) were highly colonised by the mycelium of *P. involutus*. We could also observe the mycorrhizal mycelium between the membranes (**Figures 7C**) and inside the HC (**Figure 7D**) of all provided P sources in mycorrhizal treatments.

Compared to the axenic culture systems, the mesocosms are more robust after a short acclimatization period, easy to maintain and construct, and very versatile. Depending on the number and amount of supplied resources with the HCs and further additional tests, the system can be up- or down-scaled by using a different size of pots and containers used to prepare the HCs. This system can be easily used for labelling experiments, such as labelling the plant with ^{13}C-Urea *via* leaf-fertilisation to trace the C transfer from plant to the mycorrhizal fungus growing towards the different P sources (**Figure 8**). Different transfer rates of labelled C can indicate different investment costs required from the mycorrhizal partner to acquire the different chemical forms of P (Andrino et al., 2019). Further tests could include studying the P acquiring mechanisms of the mycorrhizal fungus by measuring the enzymes or low molecular organic anions in the HCs containing the different P sources.

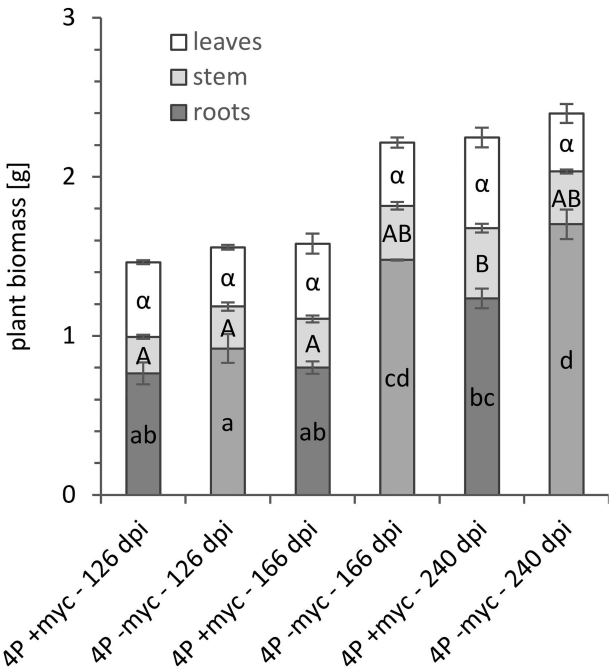

FIGURE 6 | Plant biomass [mg] in *P. involutus* ectomycorrhizal (+myc) and non-mycorrhizal (-myc) poplar plants of *P. x canescens* from 4P treatments harvested at 126, 166, and 240 dpi. The error bars show the standard deviation (n=3). The letters in the bars indicate significant differences in plant biomass [g] between mycorrhizal treatments and harvesting time points: $P < 0.05$.

CONCLUSION

The present study revealed that the construction and maintenance of the axenic rhizotrone and the mesocosm culture systems are less complicated and time consuming compared with the *in vitro* culture

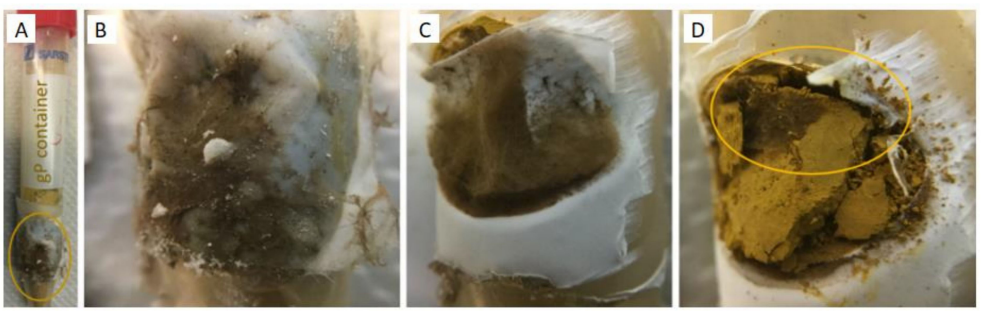

FIGURE 7 | **(A)** HC containing gP as a P source at 166 dpi; **(B)** nylon mesh, **(C)** recumbent hydrophobic PTFE membrane, and **(D)** gP colonised by *P. involutus* (MAJ).

FIGURE 8 | ^{13}C-labelling of *P. involutus* ectomycorrhizal poplar plant *P. x canescens* using leaf-fertilisation via ^{13}C-labelled urea or non-labelled urea as controls at 167 dpi.

system. But also, the *in vitro* culture system can be equipped with the external apparatus with sterile syringe filters (as reported for the axenic rhizotrone culture system) to supply the plants with a nutrient solution without the need to open the system in the time course of the experiment. Nevertheless, especially the mesocosms are robust and very versatile. But all presented culture systems enable the user to comprise additional tests, including labelling of the plant with ^{13}C or determination of P acquisition mechanisms.

Also the separate compartments for nutrient supply are well adaptable to different experimental set-ups and enable the simulation of an ecosystem situation with an ECM plant having access to widely distributed P source patches with different bioavailabilities (**Figure 1**) through mycorrhizal fungus, excluding the direct strife of plant roots and mycorrhizal hyphae for P. *P. involutus* is a long distance exploration type ECM fungus (Gronbach, 1988) with few but highly differentiated rhizomorphs (review by Agerer, 2001). These type of ECM fungi were shown to transport efficiently water and higher rates of P. Moreover, the ectomycorrhizal fungus *P. involutus* (strain MAJ) is compatible with the poplar plant species *P. x canescens* (clone 'Schleswig I') and both are easy to maintain and propagate *in vitro*. Hence, these organisms provide valuable model systems for a more robust test of nutrient acquisition and exchange models (Gafur et al., 2004; Müller et al., 2013).

Therefore, the design of a compartmental culture system using these compatible ectomycorrhizal associates was a solid choice to down-scale the ecosystem situation of P source dependent host C exchange for mycorrhizal P as well as of mycorrhizal mediated P resource partitioning. Since the protocol described by Rygiewicz et al., 1988, the present study was the first providing details on practical experience and evaluated protocols for the design and maintenance of the experimental set-ups to investigate such ecosystem situations. Moreover, these culture systems were designed not only for outdoor but also for controlled conditions excluding interferences with other micro-organisms, revealing the true capabilities of the mycorrhizal fungi in nutrient acquisition.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

The idea of the experiment came from JB with contributions from KS, AA, GG, AF, and LS. KS prepared the plant and fungal material, prepared the different culture systems, and conducted the experiments. KS performed data analysis and wrote the manuscript. JB and GG supervised the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffunb.2022.907563/full#supplementary-material

Supplementary Figure 1 | The separate hyphal compartments (HCs) for the different P sources made of 15 ml Falcon tubes and hydrophobic and nylon meshes.

Supplementary Figure 2 | Axenic rhizotrone culture system with four different chemical forms of P (4P) supplied in four separate hyphal compartments (HCs): the poplar plants *Populus x canescence* (Schleswig I) **(A)** mycorrhized (+Myc) by ectomycorrhizal fungus *Paxillus involutus* (MAJ) and **(B)** non-mycorrhizal (-Myc) control plants at 7 dpi. The HCs for the different P sources were made of 5 ml SafeSeal tubes and hydrophobic and nylon meshes.

Supplementary Figure 3 | Root tips of *Populus x canescence* (Schleswig I) colonised by the ectomycorrhizal fungus *Paxillus involutus* strain (MAJ) and its mycelium traversing the substrate, holding it as a block (at 126 dpi; viewed from **(A)** the side and **(B)** from above); hs: hyphal mantle around the root tips (mycorrhizal root tips); e: extraradical hyphae.

Supplementary Figure 4 | Mycorrhization grade of plant roots expressed as mycorrhizal root tips per cm root length of *Paxillus involutus ectomycorrhizal* (+myc) roots of poplar plants *Populus x canescens* from different harvesting time points (126, 166, 250, and 243 dpi). The root length was determined using the grid-line intersection method (Giovannetti and Mosse, 1980; Brundrett et al. 1996). The error bars show the standard deviation (n=4). There was no significant ($P < 0.05$) difference in the mycorrhization grade of root tips between the different harvesting time points.

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Supplement of

Designing a Robust and Versatile System to Investigate Nutrient Exchange in, and Partitioning by, Mycorrhiza (*Populus x canesces* x *Paxillus involutus*) Under Axenic or Greenhouse Conditions

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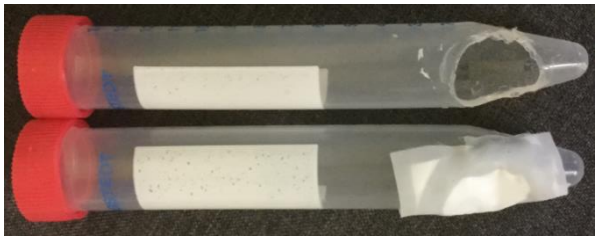
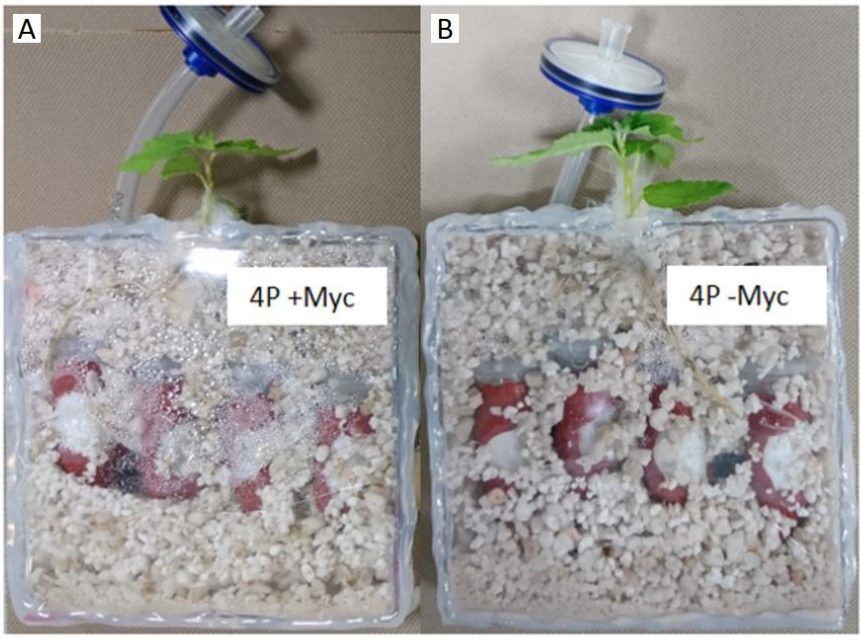
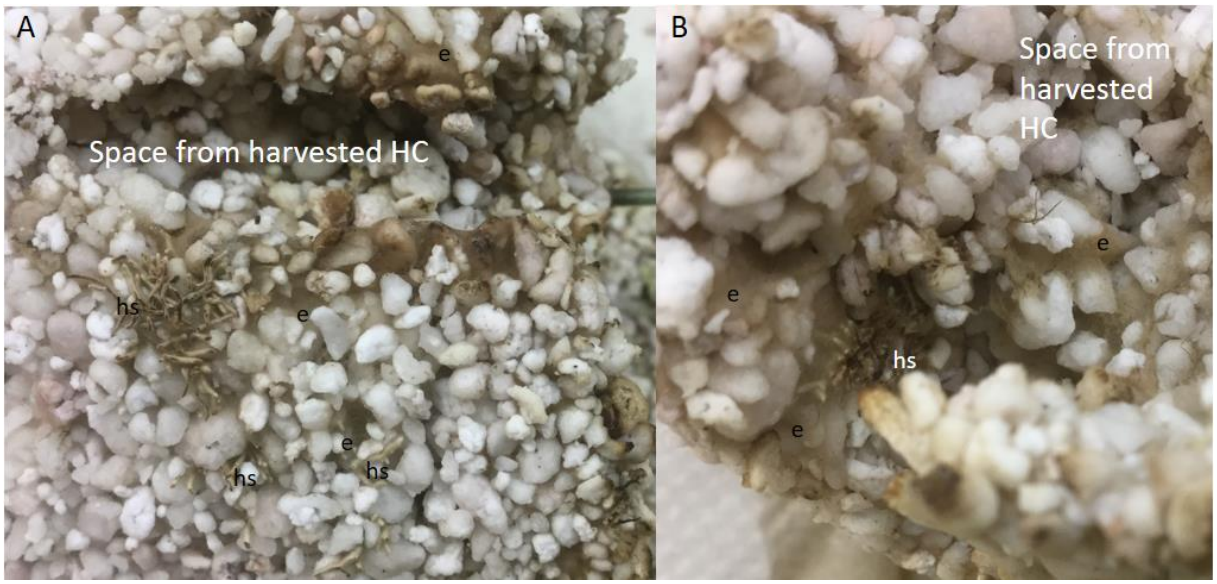


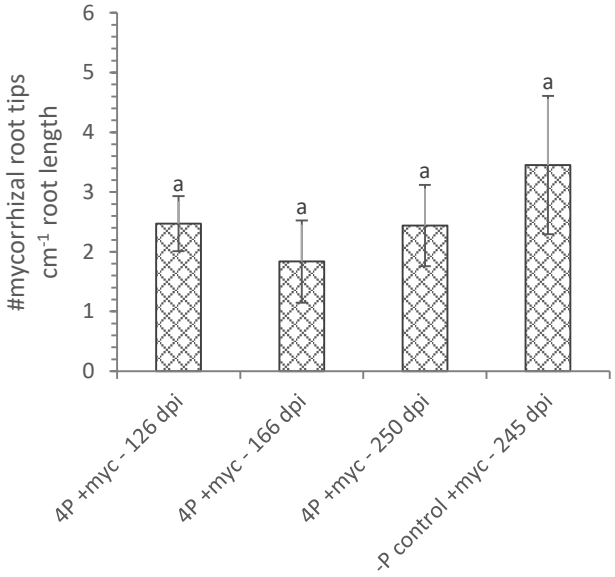
Supplementary Figure 1 | The separate hyphal compartments (HCs) for the different P sources made of 15 ml Falcon tubes and hydrophobic and nylon meshes.



Supplementary Figure 2 | Axenic rhizotrone culture system with four different chemical forms of P (4P) supplied in four separate hyphal compartments (HCs): the poplar plants *Populus x canescence* (Schleswig I) (A) mycorrhized (+Myc) by ectomycorrhizal fungus *Paxillus involutus* (MAJ) and (B) non-mycorrhizal (-Myc) control plants at 7 dpi. The HCs for the different P sources were made of 5 ml SafeSeal tubes and hydrophobic and nylon meshes.



Supplementary Figure 3 | Root tips of *Populus x canescence* (Schleswig I) colonised by the ectomycorrhizal fungus *Paxillus involutus* strain (MAJ) and its mycelium traversing the substrate, holding it as a block (at 126 dpi; viewed from (A) the side and (B) from above); hs: hyphal mantle around the root tips (mycorrhizal root tips); e: extraradical hyphae.



Supplementary Figure 4 | Mycorrhization grade of plant roots expressed as mycorrhizal root tips per cm root length of *Paxillus involutus* ectomycorrhizal (+myc) roots of poplar plants *Populus x canescens* from different harvesting time points (126, 166, 250, and 243 dpi). The root length was determined using the grid-line intersection method (Giovannetti and Mosse, 1980; Brundrett et al. 1996). The error bars show the standard deviation (n=4). There was no significant ($P < 0.05$) difference in the mycorrhization grade of root tips between the different harvesting time points.

3 Study II

Phosphorus source dependent exchange of carbon for phosphorus in ectomycorrhiza (*Populus x canescens* x *Paxillus involutus*)

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Author contribution:

The idea of the experiment came from JB with contributions of AA, KS, AFF, TW, and GG. KS prepared the plant and fungal material, prepared the mesocosms, and conducted the experiment. KS performed all the necessary sample and data analysis. LS supervised data analysis. JB, GG, and TW supervised the research. KS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Biogeochemistry.

Abstract Soils of many terrestrial ecosystems are phosphorus (P) limited, as P is largely immobilized in forms of low bioavailability. Ectomycorrhizae were shown to mine different chemical forms of P in exchange for energy derived from hosts' photosynthesis. Nevertheless, experimental evidence for P source dependent C sink potential of ectomycorrhizae is missing. We conducted a study using mesocosms that were supplied with four different P sources of different bioavailability. To trace the C allocation from the plant to its fungal partner, leaf fertilisation of plants with $^{13}C^{15}N$-urea was performed and the urea-derived ^{13}C (UDC) was determined in plant shoots and roots. The latter represents an interface for the exchange of host UDC for mycorrhizal P. Our results indicate that the mycorrhizal plants were significantly higher in P and had significantly higher shoot biomass. However, the non-mycorrhizal control plant had significantly higher root biomass, indicating that the control plants were investing their photoassimilates into extended root growth in quest for P. In contrast, the mycorrhizal plants invested their C into mycorrhizal association and therefore could develop higher aboveground biomass. Furthermore, our results could show a P source dependent trend in the exchange of host C for mycorrhizal mediated P in roots. The retention of C in the mycorrhizal root interface of the apatite and phosphate bound to goethite treatments was of the lowest magnitude, indicating a high C drain potential of the P acquiring mechanisms (such as exudation of LMWOAs and hyphal growth) to access P from mineral P sources (mineral P effect). In contrast, the readily available phosphate, the soluble phytate, and the complete P limitation turn the ectomycorrhizal plant into a system of C retention in the mycorrhizal root interface. Our results indicate an ectomycorrhizal associated, P source specific C allocation into the belowground, which has an impact on the C balance in soils of natural and anthropogenic ecosystems.

Keywords apatite · ectomycorrhiza · goethite P · P availability · phytic acid · $^{15}N^{13}C$-urea labelling

Introduction

Many terrestrial ecosystems are limited or co-limited in phosphorus (P) (Elser et al. 2007), as its nature makes it low available to plants (Hinsinger et al. 2011; Plassard et al. 2011). Plants are able to take up P in the form of orthophosphate (P_i) from the soil solution, but P_i is poorly mobile in the soil as it interacts strongly with soil constituents (Hinsinger 2001; Hinsinger et al. 2011). P_i can be adsorbed onto metal oxides and clay minerals, precipitate as (apatite-like) minerals or be immobilized in diverse organic forms (Hinsinger et al. 2011). Therefore, P is present in soil with up to 90% in chemical forms that are not readily available to plants (Mengel and Kirby, 2001). Additionally, anthropogenic leverages such as N deposition and increasingly prevalent summer droughts caused by global change accelerate the P undersupply in many natural ecosystems including forests (Talkner et al. 2015; Sardans et al. 2016; Dirks et al. 2019). The decline in P availability is apparent in diminishing P concentrations in plants and reduced growth in numerous regions of Europe (Elser et al. 2007; Peñuelas et al. 2012).

To increase the uptake of P by plants, mycorrhizal fungi received essential attention (Smith and Smith 2011). Especially in P deficient soils, mycorrhizal fungal extraradical hyphae proliferate beyond the nutrient

depletion zone near the root and expand into mineral micropores (3–10 µm) foraging for nutrients and water that are unreachable to plant roots (Hoffland et al. 2003). In exchange for nutrients, the plant supplies the fungus with carbon (C) from photosynthesis (review of Buscot (2015)). One of the first studies providing evidence for host plant C translocation to the fungal structures in endomycorrhizae was shown by using ^{14}C (Ho and Trappe 1973). A ground-breaking study by Pearson and Jakobsen (1993) examined the efficiency in the exchange of ^{14}C for ^{32}P in arbuscular mycorrhizae. Another studies by Jones et al. (1991; 1998) or Tinker et al. (1994) using an ectomycorrhizal willow calculated higher benefits (P uptake) in relation to C costs compared to non-mycorrhizal plants. Due to the lower C costs per unit of mycorrhizal fungal P required to construct hyphae than plant roots, the P uptake via the mycorrhizal fungal pathway is more efficient (Jones et al. 1998), as the hyphae offer greater absorptive surface area compared to roots (Harley 1989).

However, the required investment to acquire the free phosphate from various P sources increases with decreasing bioavailability of the P source (Turner 2008). Specifically, to weather primary minerals such as apatite or solubilise P that is bound onto metal oxides and clay minerals, mycorrhizal fungi exude low molecular-weight organic anions (LMWOA) (Plassard et al. 2011). To recycle organic P sources, mycorrhizal fungi release substrate specific phosphatase enzymes (Hinsinger et al. 2011; Smith et al. 2015). Besides other organic P forms existent in soil, phytic acid, a six-fold dihydrogenphosphate ester, is the most resistant to acquisition and P uptake and usually accumulates in soils, representing up to 50% of the total soil organic P pool (Ognalaga et al. 1994). Previously, the study of Kiers et al. (2011) has shown that the exchange of host C for arbuscular mycorrhizal fungal P is reciprocally regulated. Recently, the studies of Andrino et al. (2019; 2021) indicated that the arbuscular mycorrhizal plant invests more C to sustain the symbiotic relationship with access to more complex P sources.

Nevertheless, it was assumed that the arbuscular mycorrhiza are able to acquire inorganic and more readily available P sources, while the ectomycorrhizal fungi were shown to be capable of enhancing the uptake of organic and sorbed P (Hodge et al. 2001; Plassard and Dell 2010). Hence, the ectomycorrhizal fungi are more relevant in progressively developed ecosystems (Laliberté and Tylianakis 2012; Albornoz et al. 2016). With increasing pedogenesis, the total P in soil decreases, accumulating as an organic P fraction (20–80% (Mengel and Kirby 2001)) in soil, and P uptake becomes more efficient, shifting to the P recycling strategy (Odum 1969). Still, a proportion of P leaches into mineral soil layers and the subsoil and is strongly adsorbed onto pedogenic Fe and Al oxides at low soil pH (Walker and Syers 1976; Hinsinger 2001). In ecosystems with P recycling strategies, plants shift their mycorrhizal associates from arbuscular to ectomycorrhiza to adjust their P acquisition strategy (Albornoz et al. 2016). ectomycorrhizal fungi are essential for the nutrition of forest trees in temperate regions (Becquer et al. 2014; Nehls and Plassard 2018) and are found on the roots of 95% of trees (Hobbie 2006). Up to 30% of daily fixed C is allocated by the plant to their ectomycorrhizal associates, representing an essential sink for C (Finlay and Söderström 1992).

In order to compare the plant C investment into the ectomycorrhizal fungal acquisition of differently available P sources, the C fluxes and P uptake efficiencies in the plant have to be determined. The purpose of the following study was to investigate whether the different bioavailabilities of specific P sources (including PO_4^{3-} (oP), the primary mineral apatite (AP), the organic P species (P_{org}) phytic acid (Phy), and goethite-bound PO_4^{3-} (gP) as secondary mineral-P adsorption complex) acquired by an ectomycorrhizal fungus influence the P uptake efficiency in relation to the C investment of its host plant. With this, we aimed to extend the studies of Andrino

et al. (2019; 2021) by using an ectomycorrhizal fungus as a symbiotic partner. For this, a greenhouse experiment was conducted using a mesocosm culture system and the mycorrhizal associates of the poplar plant *P. x canescens* and the ectomycorrhizal fungus *P. involutus*. After 58 dpi, the mesocosms were supplied with one of four different chemical forms of P or H_2O in separate compartments so that only the mycorrhizal fungus had access to the P sources. To investigate the required P dependent investment of a plant to support the symbiosis, the plants were leaf-fertilised with $^{13}C^{15}N$-labeled and non-labelled urea. Since the C compound in urea is accompanied by nitrogen (N), the double labelling enabled us to resolve the fate of fertilised, complemented N in a system that is not limited in N. We addressed the following hypotheses: When the exchange of C for P is reciprocally regulated (Kiers et al. 2011) and the plant C allocation to the ectomycorrhizal fungal associate is governed by soil P accessibility and plant needs in nutrients (van der Hejden 2001; Lambers et al. 2018), we hypothesise (H1) that plant P uptake mediated by ectomycorrhizal fungus from easily available source oP will cause fewer C costs compared to the more complex P sources (Phy and mineral P sources AP and gP). Furthermore, due to the differences in the required P acquiring mechanisms, we hypothesise (H2) that the ectomycorrhizal C drain from the host plant for the acquisition of the recalcitrant, organic P source Phy will differ compared to the C drain for the mineral P sources AP and gP, as both P source types vary in their acquiring mechanisms (Plassard et al. 2011; Hinsinger et al. 2011).

Material and Methods

Plant and mycorrhizal material

Poplar species *Populus x canescens* clone 'Schleswig I' was used as a model plant *P. x canescens* and its functional ectomycorrhizal fungal strain MAJ of *Paxillus involutus* (Gafur et al. 2004) was used as a symbiotic partner. The reasons to use these model organisms for our experiments were described in detail by Schreider et al. (2022a). Also, the procedures for *in vitro* multiplication and rooting of poplar plantlets and the production of inoculum of *P. involutus* were described by Schreider et al. (2022a). Briefly: The poplar plantlets were propagated on Woody Plant Medium (WPM (Müller et al. 2013)) without hormones (dephyte e.K., Langenberg, Germany) by establishing explants from the first two lateral meristems with one leaf and bud. For rooting, only the apical buds with 1.5 cm height were used for rooting on WPM medium but with one-third amounts of sucrose and vitamins in Microboxes (O118/80, by Sac O_2, Deinze, Belgium) with a membrane for sterile gas exchange (81.35 GE day^{-1}). *P. involutus* was propagated in liquid culture of 0.2 L of modified Melin-Norkrans (MMN, ready to use purchased from dephyte e.K., Langenberg, Germany) medium (Gafur et al. 2004; Müller et al. 2013) in 0.5 L glass bottles under shaking and proliferated on 0.8 L perlite carrier and 0.2 L MMN.

Preparing phosphorus sources

Four different P sources and deionized H_2O (dH_2O) as no P control were supplied in separate P compartments. We choose *ortho*-phosphate (oP) as an easily available P source, phytic acid (Phy) as a complex organic P source, apatite (AP) as a primary mineral, and oP bound to goethite (gP) as a secondary mineral adsorption complex.

To obtain the P sources oP and Phy, the salts of $KH_2PO_4 \cdot H_2O$ (for analysis, EMSURE®, Merck KGaA, Darmstadt, Germany) and phytic acid sodium salt hydrate (from rice, SigmaAldrich, Merck KGaA, Darmstadt, Germany) were dissolved in dH_2O to obtain P solutions with concentrations of 20 mg P ml^{-1}. The proportion of P_i in Phy was measured spectrophotometrically according to the ammonium vanadate-molybdate method (Gericke and Kurmies 1952) and accounted for less than 0.8%. The pH of the oP and Phy solutions was adjusted to 5.2 using $Na_2HPO_4 \cdot 2H_2O$ solution (20 mg P ml^{-1}) and 1 M HCl, respectively. These P solutions were filtered using sterile syringe filter (Filtropur S, PES, 0.2 µm pore size, Sarstedt AG & Co. KG, Nümbrecht, Germany).

To prepare the adsorption complexes of oP to goethite (gP), 90 ml of 1 M $NaH_2PO_4 \cdot 2H_2O$ solutions and 18 g of synthetic goethite (α-FeOOH < 0.045 mm, Bayferrox® 920 Z, Lanxess Deutschland GmbH, Cologne, Germany) were mixed and filled up to 900 ml with ultrapure water (dH_2O) centrifuge bottle (Nalgene™, Thermo Scientific™, Germany). After adjusting the pH to 4, the slurry in the bottles was placed for 3 h into the ultrasonic bath and equilibrated for 24 h on an overhead shaker. Then, the slurry was repeatedly centrifuged (4500 *g;* for 10 min) and washed with dH_2O until electric conductivity was <40 µS cm^{-1} (at least four wash cycles). The gP pellet was dried at 50 °C and sieved through a 0.63 mm sieve. To determine the P content of gP, 106 mg were solubilized in 20 ml 32% HCl (AnalaR NORMAPUR® for analysis, VWR International GmbH, Darmstatd, Germany), shaken on an overhead shaker for 72 h, and filtered using folded filters (Grade: 3 hw; ø 185 mm; 65 g/m^2; Lot # 18-004, Sartorius). P analysis was performed via Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Varian 725-ES). The P content of gP accounted for 1.1 ± 0.1 mg g^{-1} goethite (n = 3).

The apatite (AP; Krantz Company, Bonn, Germany) was ground with mortar and pestle and passed through a 0.20 mm sieve. The P content of AP accounted for 187.2 mg g^{-1} AP (Andrino et al. 2019; 2021). To determine the easy desorbable P from gP and AP, 0.32 g and 0.52 g (respectively) were suspended in 10 ml dH_2O, shaken on an overhead shaker for 24 h, centrifuged (3000 × g, 15 min), and the supernatant was filtered through a 0.45-mm syringe filter (C.A. 25 mm, 0.45 µm; Berrytec® GmbH, Grünwald; 1171006, Lot# 1770313). The analysis of PO_4^{3-} was performed via Ion Chromatography (IC, 930 Compact IC Flex, Metrohm). The easy resorbable P accounted for 0.137 ± 0.001 mg g^{-1} goethite and 0.0021 ± 0.0002 mg g^{-1} AP, so a maximum of 12% P from P bound to goethite and 0.39% P from P in AP could be mobilized without any action of the ectomycorrhizal fungus.

The P sources gP and AP were sintered three times at 105 °C for 30 min, including the subsequent incubation period at 30 °C for 2 h the first two times. The last step is called tyndallisation and is used for fractionated sterilization without changing the mineral structure.

Preparing mesocosms and the P containers

The experiment was conducted in the greenhouse of ZVA from December 2018 until May 2019. The mesocosm culture systems used in the following study were described in detail by Schreider et al. (2022a). Briefly: the rooted poplar plantlets were planted in pots (Ø 12 cm, 0.65 L) on Perlite as substrate. To obtain ectomycorrhizal plant treatments, the substrate was inoculated with 10 vol.% of *P. involutus*-Perlite carrier. The theoretical P content of the carrier accounted for less than 8 µg P per pot. Half of the inoculated substrate was autoclaved one more time to obtain non-mycorrhizal control treatments. The ectomycorrhizal and non-

mycorrhizal plantlets were kept under a plastic cover and a protecting cloth for four weeks for acclimatisation, as the temperature in December in the greenhouse could drop to 14 °C.

The mesocosms were supplied with a WPM nutrient solution containing macro- and micro-elements (without vitamins) as described by Müller et al. (2013), but with a low concentration in P of 1.1 mg l^{-1} (WPM LP) and receiving 489 µg P in total until two weeks after the time point where the P containers were inserted into the mesocosms. This was done to avoid the mortality of plant material and complete loss of leaves due to the fulsome P limitation, as it was observed in pre-experiments. Thereafter, the mesocosms were supplied with a WPM solution without any P (WPM --P). The mesocosms were watered with deionised H_2O (dH_2O) on-demand in the experiment's time course.

At 58 dpi (after the next four weeks), the ectomycorrhizal poplar plantlets were exposed to one P source such as oP, Phy, AP, gP, and dH_2O (as a no P control) in the separate compartments in five replicates each. Eight non-mycorrhizal (control) replicates were equipped with one P container enclosing one of the five different P sources. The P sources were supplied in separate containers made of 15 ml Falcon tubes as described by Schreider et al. (2022a); briefly: At around 2 ml height, a hole of 1 cm in diameter was burned into the tube and sealed with a hydrophobic PTFE membrane (5 µm, Pieper Filter GmbH, Bad Zwischenahn, Germany) and nylon mesh (20 µm, Franz Eckert GmbH, Waldkirch, Germany), so that only the ectomycorrhizal partner could have access to the P sources. The containers were autoclaved (121 °C, 20 min) and filled with the different P sources under sterile conditions in a laminar flow cabinet. The containers were filled with the following P amounts: Always 1 ml of oP and Phy solutions (20 mg P ml^{-1}) were pipetted into the P container and around 19.9 ± 0.3 mg P in the form of AP and 5.53 ± 0.01 mg P in the form of gP were weight into the P containers. All containers were filled up to 4 ml with sterile dH_2O. Nevertheless, based on the P amount of 20 mg per pot used to sustain beech saplings for 14 months in the study of Klotzbücher et al. (2020), the P amount of 20 mg supplied per system in our study was considered as sufficient to maintain ectomycorrhizal poplar plantlets for up to 6 months. The gP was supplied to the systems in a 3.6-fold smaller P amount, as due to its voluminous consistency only up to 6 mg gP could fit into the P container. But since the amount of easy desorbable P from gP (689 µg P in total) was clearly higher compared to AP (42 µg P in total), the smaller gP amount allowed a closer comparison of these two mineral P sources.

Plant labelling with $^{13}C^{15}N$-Urea

In order to determine the P source dependent C and N fluxes through the plant to the roots, stable isotope labelling with $^{13}C^{15}N$-urea was applied. For this purpose, 0.5% urea ml^{-1} dH_2O ($^{13}C^{15}N$-Urea or non-labelled urea) was prepared by dissolving 5 mg of dual labelled-urea (99 atom% ^{13}C and 98 atom% ^{15}N, SigmaAldrich, Merck KGaA, Darmstadt, Germany) in 995 mg dH_2O. At 159 dpi, the plants were leaf-fertilised in triplicates with $^{13}C^{15}N$-labelled urea and in duplicates with non-labelled urea to obtain the control treatments. For the labelling via leaf-fertilisation, the edges of always the third plant leaf from the top were cut, and the leaf was inserted into a 2 ml Eppendorf SafeLock Tube containing 1ml of 5 mg urea, resulting in a concentration of 0.5% urea ml^{-1} and an additional supply of maximum 1.0 mg C and 2.4 mg N per plant. The 2 ml tube with the leaf inside was sealed with Parafilm®M to avoid evaporation. The mesocosms were harvested 5 to 7 days after

the labelling, starting with the Phy treatment (on day 5), followed by oP, AP (on day 6), gP and dH_2O (on day 7), which is described in more detail in the next paragraph.

Harvest of the mesocosms and processing of plant material and substrates

For the harvest of the mesocosms (at 164–166 dpi), the plant material was separated into leaves, stem, and roots. The roots were separated from the substrate and washed. The fresh biomass of the different plant parts was recorded. A root aliquot of up to 0.3 g of each plant was conserved in 70% Ethanol to assess mycorrhization grade. The plant material and substrate were frozen at -20 °C, freeze-dried, the dry weight (dw) recorded, and for further analysis milled in a ball-mill (Retsch® GmbH, Haan, Germany) at the vibrational frequency of 20 Hz for 40 secs for leaves and substrate or 120 sec for stem and roots.

Analysis of P and other macro- and micronutrients

The analysis of total P was performed via ICP-OES (Varian 725-ES, Agilent Technologies, Santa Clara, United States). For this analysis, ~ 50 mg of milled plant material was digested through dry oxidation at 480 °C overnight. The nutrients were extracted using 4 ml of 2 M HCl. After a minimum 10 min incubation time, a further 8 ml dH_2O were added to the extract, mixed, and filtered using a filter paper (thickness of 0.14 mm, the pore size of 2-3 µm; LABSOLUTE®, Th. Geyer GmbH & Co. KG, Renningen, Germany). The standards for the calibration were prepared using the same matrix. The element content in plant shoots and roots was calculated by multiplying the concentration of the relevant element by shoot or root biomass, respectively.

Analysis of C, N, ^{13}C and ^{15}N

For the analysis of C, N, ^{13}C, and ^{15}N via a stable Isotope Ratio Mass Spectrometer (IRMS; IsoPrime PrecisION, Elementar UK Limited, IsoPrime House, Cheadle, UK) that was coupled to an elemental analyser (EA; vario ISOTOPE cube, Elementar Analysensysteme GmbH, Hanau, Germany), 5–10 mg of milled plant material or 16 mg of milled substrate were weighed into tin capsules (4x4x6 mm or 6x6x12 mm, respectively; IVA Analysentechnik GmbH & Co. KG, Meerbusch, Germany) as duplicates. Isotope values were measured in the delta notation and recalculated to atom% heavy isotope for further mass balance calculations. The $\delta^{13}C$ values are expressed relative to the Vienna Pee Dee Belemnite (VPDB) standard (Hut 1987) and $\delta^{15}N$ values relative to atmospheric air-N_2. Recalculation of measured raw values to the mentioned international units was based on a calibration by isotope standards of the international atomic energy agency (IEAE): Three Ammonium Sulphates with differing isotopic compositions (USGS-25, IAEA-N-1 and IAEA-N-2; International Atomic Agency, Terrestrial Environment Laboratory, Vienna, Austria), Cellulose (IAEA-CH-3), and Caffeine (IAEA-600). The reproducibility and precision of ^{13}C and ^{15}N measurements was determined based on the repeated measurements of an in-house standard (High Organic Sediment; 7.5% C, 0.52% N; IVA33802150, IVA Analysentechnik GmbH & Co. KG, Meerbusch, Germany). The element content of C and N was determined via a long-term stable calibration function and corrected with a daily factor calculated based on measured values of the standard chemical Acetanilide (pro analysi, Merck KGaA, Darmstadt, Germany).

Enrichment of ^{13}C and ^{15}N in harvested plants was calculated by subtracting the mean atom% value of the control plants (leaf-fertilised with non-labelled urea) from the atom% of the labelled plants (leaf-fertilised with $^{13}C^{15}N$-urea), yielding excess ^{13}C and excess ^{15}N [atom%].

Calculation of urea-derived ^{13}C and ^{15}N in plant

To determine the P source dependent allocation of C and N from plants' aboveground to belowground parts, the recovery of urea-derived ^{13}C (R_{udc}) and ^{15}N (R_{udn}) [µg mg^{-1}] from leaf-fertilisation in plant material were determined (Eq. 1) according to the method proposed by Balesdent and Mariotti (1996) and modified by Langenbruch et al. (2014) using ^{13}C as an example:

$$R_{udc}\ [\mu g\ mg^{-1}] = \frac{atom\%^{13}C_{p,l} - atom\%^{13}C_{p,nl}}{atom\%^{13}C_{u,l} - atom\%^{13}C_{u,nl}} \cdot 10^{3} \cdot \frac{C_p}{C_{u,l}} \quad (1)$$

with $atom\%^{13}C_p$ values of the plant (p) material, $atom\%^{13}C_u$ values of urea (u), $atom\%^{13}C_l$ values of labelled (l) compound, $atom\%^{13}C_{nl}$ values of non-labelled (nl) compound, c_p, the absolute C amount [mg] in plant material, and $c_{u,l}$, the absolute C amount [mg] of applied urea. Finally, the R_{udc} [µg mg^{-1}] has to be normalised by the exposure time of the plant to urea (5 to 7 d), resulting in UDC [µg mg^{-1} h^{-1}] (Eq. 2):

$$UDC\ [\mu g\ mg^{-1}\ h^{-1}] = \frac{R_{udc}\ [\mu g\ mg^{-1}]}{t_l} \quad (2)$$

with t_l, exposure duration of plant leaf to urea [h].

Assessment of mycorrhization grade of plant roots

Characteristically for successful mycorrhization of poplar roots by MAJ is the change in root morphology, e.g. specific branching of root tips and no development of root hairs. The mycorrhization grade of plant roots was expressed as 'number of mycorrhizal root tips per centimetre analyzed root length.' For this purpose, 66 ± 10 cm of ectomycorrhizal and 37 ± 7 cm of non-mycorrhizal fine roots were inspected for the mycorrhizal root tips using a stereo zoom microscope (45x magnification; KERN OZM-542, Kern & Sohn GmbH, Balingen, Germany). After each counting, the mycorrhizal root tips were counted in triplicates by rearranging the same root sample in the Petri dish. The exact root length was determined from root photographs via the open-source Fiji plugin rhizoTreck (Möller et al. 2019). The mycorrhization grade of the analyzed root aliquot was calculated as follows:

$$mycorrhization\ grade\ [\#mycorrhizal\ root\ tips\ cm^{-1}\ root\ length] = \frac{\#\ \text{mycorrhizal root tips} \cdot \text{scale (pixels cm}^{-1})}{\text{root length (pixels)}} \quad (3)$$

Statistical analysis

Three of five replicates of non-mycorrhizal, leaf-fertilised ($^{13}C^{15}N$-urea) plants were chosen for the data analysis using the stratified sampling without replacement (Excel Analysis ToolPak, Microsoft Office Standard 2013). The correlation analysis was performed via Microsoft® Excel® 2016 MSO (16.0.4266.1001, © 2016

Microsoft Corporation, USA). All further statical tests were performed via the Software IBM® SPSS® Statistics 26.0 (IBM Corporation, US) at the probability level of 0.05.

All data were tested for normal distribution using the Shapiro-Wilk test; and homogeneity of variances using Levene's test, where the p-value was calculated based on the mean. To test for significant differences in data (normally distributed) with only two independent groups, the independent samples t-test was performed. To test for significant differences in data with more than three independent groups, one-factorial analysis of the variances (ANOVA) with replicates was performed on normally distributed data. In case of significant differences, Scheffé procedure (for data sets with no equal variances, as it is insensitive to violation of homogeneity of variances) or the Tukey-HSD test (for data sets with equal variances) as a post hoc test was used. In case the data was not normally distributed Kruskal-Walis-H test was used. It is a non-parametric equivalent of the One-Way ANOVA to test for significant differences between more than two groups using medians. In case of significant differences between the groups, the Mann-Witney-U test was performed.

Results

Plant growth parameters and P uptake and use efficiencies

At the end of the experiment, at around 166 dpi, we determined the biomass (Fig. 1A and Tab. S1) and P uptake efficiency (Fig. 1A) of different parts of leaf-fertilised plants.

In sum, the plant biomass [g dry weight] (Fig. 1A) did not differ significantly between the P treatments. However, the root biomass of mycorrhizal AP (AP +myc) and no P control (dH_2O +myc) treatment was significantly ($P < 0.05$) lower compared with the non-mycorrhizal P (P -myc) treatment. Whereas the shoot biomass as well as the shoot:root ratio (Tab. S1) of non-mycorrhizal plant treatment were significantly ($P < 0.05$) lower compared to the mycorrhizal P treatments (except the stem biomass of AP +myc treatment).

The absolute content of P [mg] (Fig. 1B), reflecting the P uptake efficiency, did not differ in roots independent of P or mycorrhizal treatment, but the shoots of mycorrhizal gP (gP +myc) and H_2O +myc treatments had significantly ($P < 0.05$) higher P contents compared with the non-mycorrhizal treatment.

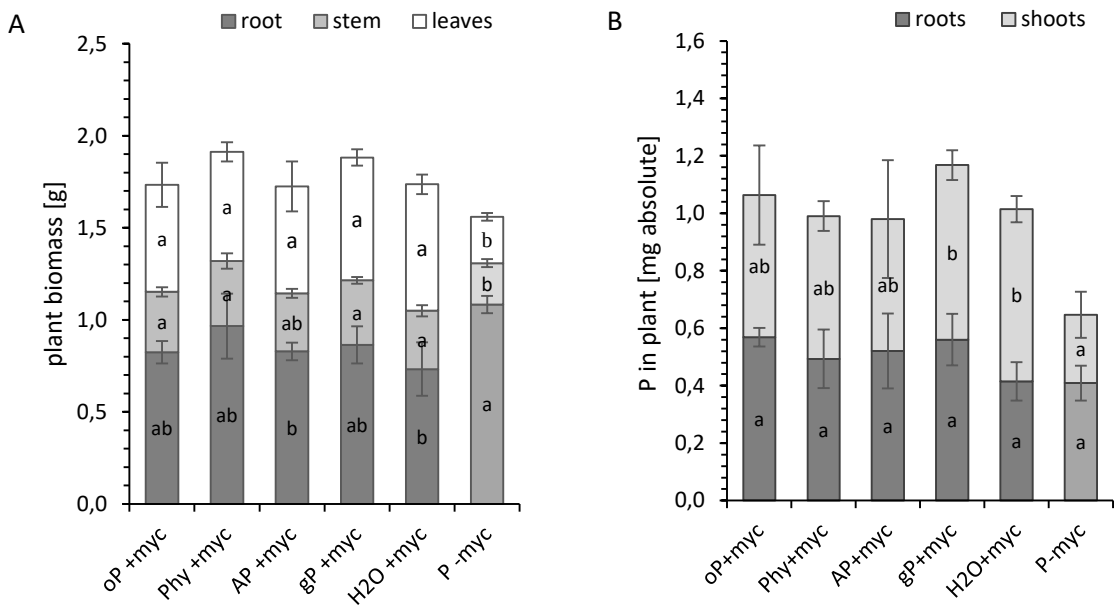

Fig. 1 Plant (**A**) biomass [mg] (leaves, stem, roots) and (**B**) P content [mg absolute] of ectomycorrhizal (+myc, *P. involutus*) and non-mycorrhizal (-myc) poplar plants *P. x canescens* at 166 dpi. These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. Plants underwent a leaf-fertilisation with $^{13}C^{15}N$- and non-labelled urea. The error bars show the standard deviation (n = 3). The letters indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

Mycorrhization of plant roots

In order to measure the response of the plant to ectomycorrhizal inoculation and the P source dependency, we determined the mycorrhization grade in ectomycorrhizal plant roots (Fig. S1) expressed as the number of mycorrhizal root tips per cm root length.

The ectomycorrhizal plants always showed a pronounced mycorrhization of their roots by *P. involutus*: A dense hyphal mantel around the root tips (Fig. S1A) could be observed. The proof of the compatibility of the used poplar plant *P. x canescens* and ectomycorrhizal fungus *P. involutus* strain MAJ was shown in Schreider et al. (2022a) but was also published by Gafur et al. (2004). Nevertheless, the 'non-mycorrhizal' plants showed a significantly ($P < 0.008$) lower mycorrhization grade compared with that of the ectomycorrhizal treatments (Fig. S1B).

^{13}C and ^{15}N enrichment and urea-derived ^{13}C and ^{15}N in plant

In order to investigate the C and N fluxes from plant aboveground to belowground parts, representing the costs required from the plant to support the ectomycorrhizal fungal acquisition of differently available P sources, we determined the enrichment in ^{13}C and ^{15}N [atom% excess] (**Figure 3**) in harvested plant material from the leaf-fertilisation experiment. Proportionally higher ^{15}N enrichment [atom% excess] compared to the ^{13}C enrichment [atom% excess] was incorporated in plants (Fig. 2). Thereby, plant shoots of mycorrhizal gP

treatment compared to the mycorrhizal AP and non-mycorrhizal treatments (Fig. 2A) were significantly ($P < 0.05$) lower enriched in ^{13}C, although there was no significant difference in ^{13}C in roots between the different P treatments (Fig. 2B). There was no ^{13}C enrichment in non-mycorrhizal plant roots. In contrast, the ^{15}N enrichment in non-mycorrhizal plant roots (Fig. 2E) was significantly ($P < 0.05$) lower compared to the mycorrhizal P and no P control treatments but indifferent compared to the mycorrhizal Phy treatment. The ^{15}N enrichment in plant shoots (Fig. 2D) of mycorrhizal gP treatment was significantly ($P < 0.05$) lower compared to the mycorrhizal oP, Phy, and AP treatments. The enrichment of ^{13}C and ^{15}N in the one (labelled) leaf that was exposed to $^{13}C^{15}N$-labelled urea was significantly ($P < 0.05$) higher in non-mycorrhizal treatment compared to mycorrhizal oP treatment (Fig. 2C,F) as well as compared to the mycorrhizal no P control for ^{13}C enrichment (Fig. 2C).

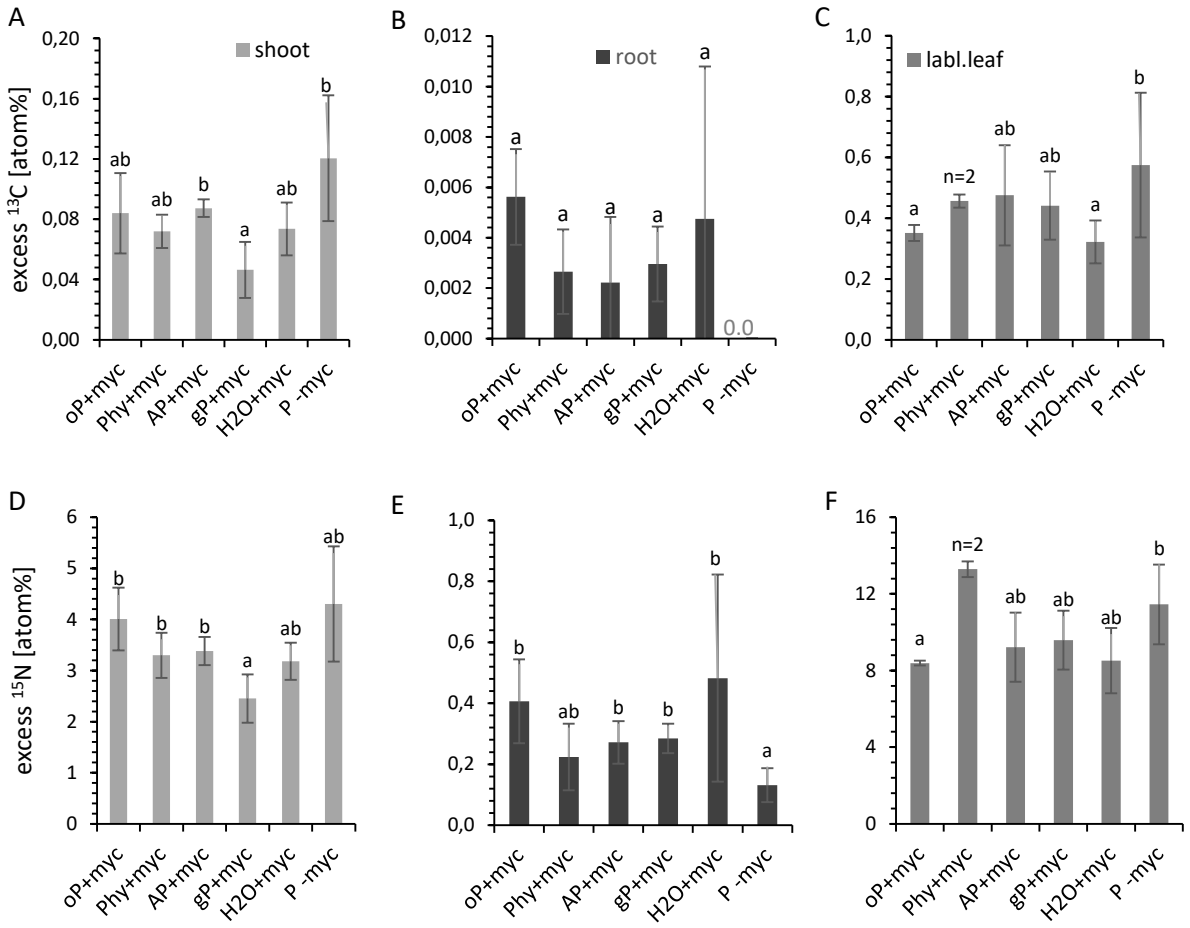

Fig. 2 Excess (**A-C**) ^{13}C and (**D-F**) ^{15}N [atom%] incorporated in plants' (**A**,**D**) shoots, (**B**,**E**) roots, (**C**,**F**) labelled leaf (labl.leaf, that was in contact with urea) from leaf-fertilisation of ectomycorrhizal (+myc) and non-mycorrhizal (-myc) plants with $^{13}C^{15}N$-labelled and non-labelled urea for 5–7 d (165 dpi, n = 30). These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. The error bars show the standard deviation (n = 3). The letters indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

Furthermore, to normalise the data of C and N fluxes for the C and N contents (Fig. S2) in the plant as well as for the 5–7 d exposure time of the plant to urea, we calculated the urea-derived ^{13}C (UDC) and ^{15}N (UDN) [µg mg^{-1} h^{-1}] (Eq. 1–2) in harvested plant material (Fig. 3). The UDC and UDN (Fig. S3) were found at the same

proportions in the one (labelled) leaf exposed to $^{13}C^{15}N$-labelled urea. Thereby, the UDC and UDN found in shoots were significantly ($P < 0.05$) and up to 50-fold higher compared to roots and to some extent higher proportion of UDN compared with UDC was found in the shoots and roots. Nevertheless, there was no significant difference between the mycorrhizal P treatments in UDC and UDN found in plant shoots or roots (Fig. 3) but also the ratios of UDC and UDN [µg mg^{-1} h^{-1}] per [mg] P incorporated in plant shoots or roots (Fig. 4). Instead, there is a trend of lower UDC and UDN in ectomycorrhizal roots of the mineral P source treatments, AP and gP.

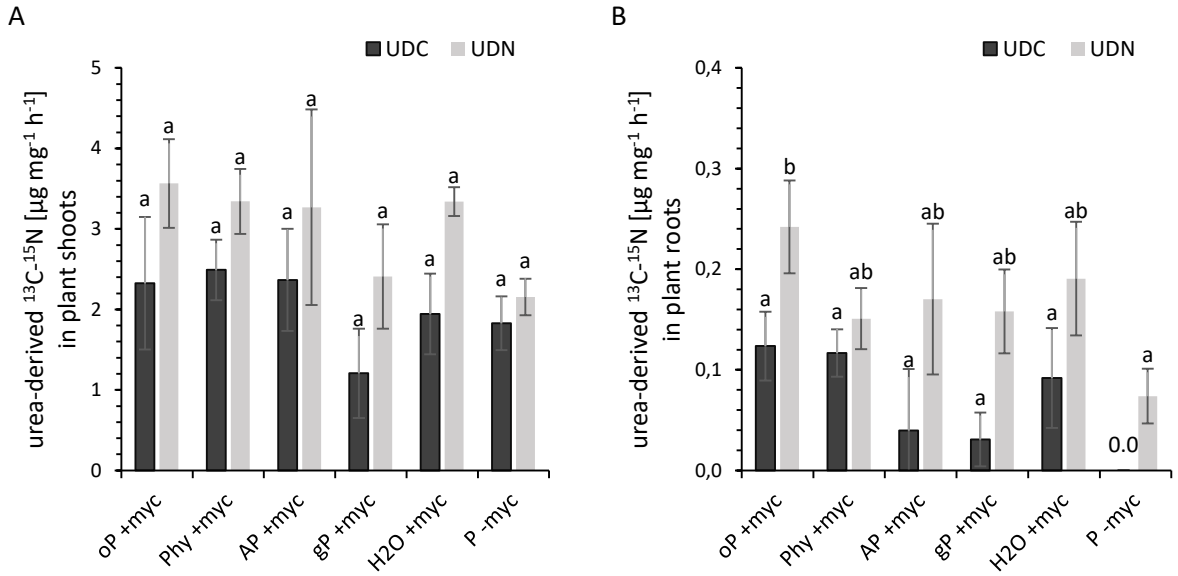

Fig. 3 Urea-derived ^{13}C (UDC) and ^{15}N (UDN) [µmol h^{-1}] in ectomycorrhizal (+myc) and non-mycorrhizal (-myc) plant (**A**) shoots and (**B**) roots from leaf-fertilisation of plant with $^{13}C^{15}N$-labelled urea and an exposure time of 5–7 d. The harvest of these mesocosm systems occurred at 165 dpi. These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. The error bars show standard deviation (n=3). The letters (a, b) indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

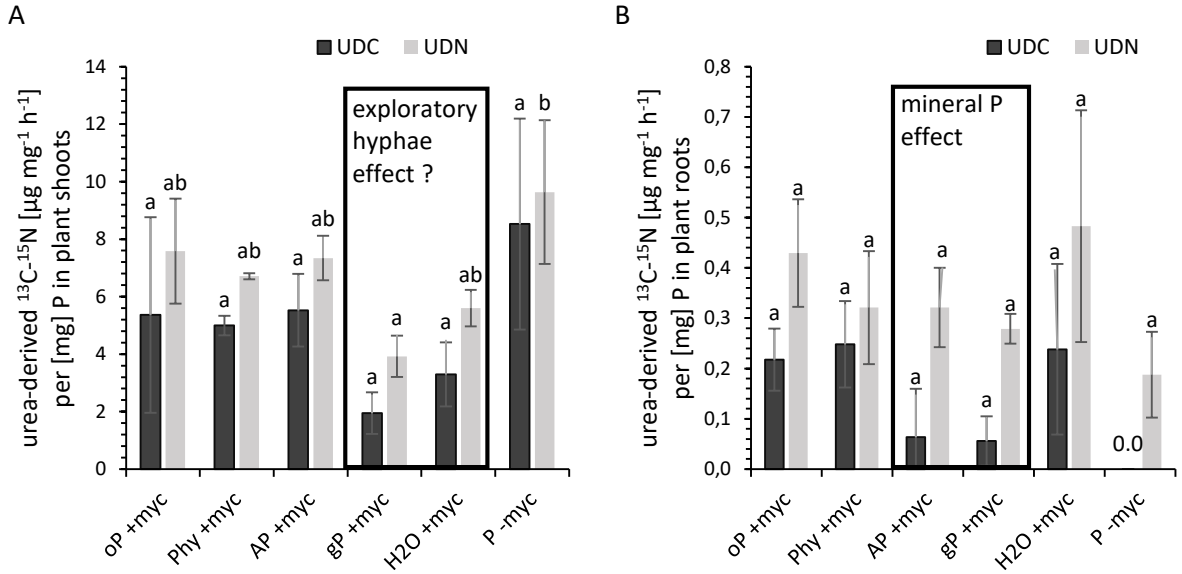

Fig. 4 Urea-derived ^{13}C/P (UDC/P) [µmol h^{-1} mg^{-1}] and urea-derived ^{15}N/P (UDN/P) [µmol h^{-1} mg^{-1}] ratios in plant (**A**) shoots and (**B**) roots of ectomycorrhizal (+myc) and non-mycorrhizal (-myc) plant treatments from leaf-fertilisation of plant (at 159 dpi) with $^{13}C^{15}N$-labelled urea and an exposure time of 5–7 d. These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. The error bars show the standard deviation (n = 3). The letters (a, b) indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

Discussion

P is present in soil with up to 90% in chemical forms that are not readily available to plants (Elser et al. 2007), resulting in P limitation in many terrestrial ecosystems (Mengel and Kirby 2001). In these ecosystems, mycorrhizal fungi are known to increase plant P uptake (Smith and Smith 2011) in exchange for plant's photoassimilates (review of Buscot (2015)). Thereby, the required investment to acquire the free phosphate from various P sources increases with decreasing bioavailability of the P source (Turner 2008). Our previous studies (Andrino et al. 2019; 2021) investigated the exchange of photosynthetically fixed C per unit arbuscular mycorrhizal fungal P and could confirm that the plant C investment in the mycorrhizal fungal provision of P is dependent on the P source bioavailability. In the present study, to investigate the required P source dependent investment of a plant to support the ectomycorrhizal symbiosis, the plants were leaf-fertilized with $^{13}C^{15}N$-labeled and non-labelled urea for 5–7 d before harvesting the mesocosms at 165 dpi. The labelling of plants with $^{13}C^{15}N$-urea allowed us to look at the partitioning of urea-derived ^{13}C (UDC; Eq. 1–2) between plant shoots and roots. The present study looked closer into, and found a trend for, dependencies between the P source bioavailability and the related exchange of plant C for ectomycorrhizal fungal P.

Evaluation of leaf-fertilization of plants with ^{13}C-^{15}N-urea and of urea-derived ^{13}C and ^{15}N determination in plant

The leaf-fertilized ^{13}C-^{15}N-urea was efficiently incorporated into the plant tissue and transferred into the root system and substrate. The double labelling enabled us to determine also the N fluxes, as urea-derived ^{15}N (UDN) is a composite of urea. Proportionally higher excess ^{15}N [atom%] than excess ^{13}C was integrated into the plant tissue (Fig. 3). This result is consistent with previously performed studies using double labelling with $^{13}C^{15}N$-urea mainly for grasses, forbs, legumes (Putz et al. 2011) or maize (Schmidt and Scrimgeour 2001), and it was attributed to the atomic consistency of urea ($CO(NH_2)_2$) with two N and one C atoms (Lakkineni et al. 1995). Once incorporated, one molecule of urea is rapidly hydrolyzed in the cytosol of the plant leaf to two molecules of NH_3 and one molecule of carbonic acid (Gooding and Davis 1992; Sirko and Brodzik 2000; Witte et al. 2002), and it is suggested that the urea-derived and the atmospheric C in the plant are assimilated and analogously partitioned in the plant (Allison et al. 1954; Clifford et al. 1973). However, a loss of ^{13}C through respiration was discussed as a possible reason for the higher enrichment of ^{15}N in the plant compared to ^{13}C (Lakkineni et al. 1995). This assumption could be supported by our observations of a higher proportion of UDN compared to the UDC transferred to shoots and roots (Fig. S3) normalized for the plant's C and N contents and for the plant's exposure time to urea. In contrast, the UDC and UDN found in the one (labelled) leaf exposed to $^{13}C^{15}N$-labelled urea were still at the same proportions (Fig. S3A). Nevertheless, our results instead indicate that the lower ^{13}C enrichment

is caused to a greater extent by fractionation due to the 10–20-fold higher absolute C content than the plant's absolute N content (Fig. S2), resulting in a higher dilution of ^{13}C in plant material. This could also be the reason for the observed significant differences in excess ^{13}C but also excess ^{15}N incorporated in plant shoots and roots between the different P and mycorrhizal treatments, which are not so pronounced when looking at the UDC and UDN (Fig. 3).

Partitioning of urea-derived ^{13}C and ^{15}N in plant

The partitioning and allocation of C and inorganic nutrients in the plant were shown to be a composite of different requirements, including symbionts and fluxes into the belowground, storage, or growth (review by Friend et al. (1994)) that are regulated by competing sources and sinks (Reynolds and Chen 1996). Thereby, leaf-fertilisation of plants with urea in our study induced a sudden event of N addition, turning the plant beside C also into N source and shifting our system more into P limitation and a higher demand for P, as it was severely shown that N deposition goes hand in hand with enhanced plant development and induction of P limitation (Jonard et al. 2015; Talkner et al. 2015). Even though the UDC and UDN (Fig. 3) incorporation in plant shoots and roots were independent of the P source availability, the enrichment of UDC and UDN found in shoots was significantly ($P < 0.05$) and up to 50-fold higher than in roots, which could indicate that the UDC and UDN are used mainly for the shoot development but not for root growth. The effect of further shoot development and stagnancy of root growth was documented previously e.g. for *Hebeloma cylindrosporum* ectomycorrhizal maritime pine seedlings, whereas the non-mycorrhizal seedlings developed lower shoot:root ratios compared to their ectomycorrhizal counterparts (Torres Aquino and Plassard 2004). Also, in our study, the non-mycorrhizal poplar plantlets developed significantly higher root biomass with higher contents of P, C, and N compared to the shoots, which indicates that without a symbiotic partner, poplar plants invest in the root growth with the need to explore the belowground for P, which was limited in these systems. Nevertheless, due to the higher absorbing surface of the external hyphae (Rousseau et al. 1994) for the lower construction costs of fungal hyphae compared with the roots (Jones et al. 1998), the host plant can afford to stop the root growth and to invest its resources into aboveground biomass and their functions.

Ectomycorrhizal roots – the exchange interface for elements between host and fungal partner

In our study, the ectomycorrhizal plants always showed high mycorrhization of their roots with a dense hyphal mantel around the root tips (Fig. S1A). We can assume that the lower enrichment of UDC and UDN in roots compared to shoots and the fact that we could observe an enrichment only in ectomycorrhizal plant roots make the ectomycorrhizal roots to the interface for the exchange of C but also N for P between plant and ectomycorrhizal fungus in our study. Hence, when looking at the UDC [$\mu g\ mg^{-1}\ h^{-1}$] per [mg] P (UDC/P) in ectomycorrhizal plant roots, we can assume this ratio reflect closely the P source dependent exchange of C for P between the host plant and its ectomycorrhizal fungal partner. Supporting evidence for our assumption was provided by the study of Schreider et al. (2022b), which could show with images of harvested plants that the highest ^{33}P label was incorporated in roots with high intensity in the branching of the root tips. This observation

indicates that a high proportion of incorporated ^{33}P label in the plant was detectable at the mycorrhizal fungus-root interface. Furthermore, the study of Bücking and Heyser (2001) could localize a microautoradiographic distribution of P in exchange for C in the median zone of ectomycorrhizal roots of poplar seedlings. Their study could show that photosynthetically fixed ^{14}C was translocated to the hyphae of the Hartig net and homogeneously spread and accumulated in the hyphal mantel around the root tips. Previously, Bauer et al. (1991) could also observe an accumulation of host ^{14}C in the hyphal mantel and presumed that the hyphal mantel serves as storage or is an interface for C metabolism, where the host C is transformed to fungal sugars (trehalose, mannitol, glycogen (Lewis and Harley 1965)). Bücking and Heyser (2001) also observed that P_i taken up by the ectomycorrhizal fungus was quickly accumulated in the hyphal mantel and was slowly allocated through the Hartig net into the cortical cells of the host root and further transferred to young leaves of plant shoots. Thereby, the molecular study of Becquer et al. (2018) under controlled conditions revealed that the host plant induces the P transfer through the ectomycorrhizal fungal Hartig net to plant roots. The labelling was performed at 165 dpi, when the system including the ectomycorrhizal fungus in the belowground were already increasingly established (Fig. S1A), indicating another possible reason for the low transfer rates of UDC into the mycorrhizal root interface. In contrast, at this stage, the UDC found in the mycorrhizal roots could reflect the real demand of the ectomyccorhizal fungus to acquire the respective P sources.

Exchange of urea-derived ^{13}C for the different P sources

Our data show a trend for P source dependent exchange of UDC for P in ectomycorrhizal roots of mineral P source treatments, gP and AP (Fig. 4B), compared to the other mycorrhizal P treatments, indicating a trend of an P source dependent effect of how the ectomycorrhizal plant is managing its resources. Evolving strategies of a mycorrhizal plant to deal with nutrient scarcities are the production and exudation of LMWOA to increase the release and availability of mineral P_i (Plassard et al. 2011). The P acquiring strategies require the investment of C by the host plant. This context was observed e.g. by the study of Leake et al. (2008) where an *P. involutus* ectomycorrhizal *Pinus sylvestris* allocated preferentially ^{14}C to apatite patches, while the weathering of apatite increased three-fold. Also goethite associated P_i release is increased via the ligand exchange with LMWOAs, which otherwise is caused mainly by the equilibrium of sorption and desorption processes and the diffusion transport (Yan et al., 2015). Hence, the similarities in UDC and UDC/P for ectomycorrhizal roots of AP and gP treatments could result from applying the same mechanism and the demand of the ectomycorrhizal fungus *P. involutus* to produce and exude LMWOAs to release P_i from the mineral P sources (mineral P effect; Fig. 4B). In contrast, to take up P_i from the solution, the ectomycorrhizal fungus has to take up the free phosphate (in our case, the oP) from the solution via the plasma membrane phosphate transporters at fungal hyphae (Becquer et al., 2014).

The extended growth of extraradical hyphae is another strategy to explore the soil matrix for nutrients beyond the depletion zones and is an essential constituent for the fungus and the host plant (Smith and Read, 2008), which application we could expect from all mycorrhizal P treatments but especially from the mycorrhizal no P treatment, as all treatments received a starting supply of 0.5 mg P. Furthermore, the tendentially lower UDC/P ratio in plant shoots of mycorrhizal gP treatment (followed by the mycorrhizal no P control) compared to

other P treatments could be caused either by the later harvesting time point (at 7th day after leaf-fertilisation) compared to other P treatments (at 5th or 6th day) or instead by the need of the ectomycorrhizal fungus to extend the mycelial infrastructure in foraging for P (exploratory hyphae effect; Fig. 4A). To support the latter assumption, compared to AP, the P source gP has a far more voluminous consistency. Hence, to reach the P from gP, the ectomycorrhizal fungus has to spread its mycelium more intensively, requiring proportionally more C for the construction of the mycelial infrastructure than AP. We could assume that the higher proportion of easy desorbable P from gP (0.69 mg P) compared to AP (0.04 mg P) made it affordable to the host plant to retain less UDC in the shoots of mycorrhizal gP treatments compared to the mycorrhizal no P and AP treatments. Moreover, our study detected a more extensive mycelial growth inside the P compartments containing gP as P sources compared to other P treatments.

Furthermore, it is assumed that ectomycorrhizae invest considerable C amounts into the production and release of enzymes to mobilise nutrients (Antibus et al. 1997). Hence, the phosphatase activity is considered to be induced by the substrate or a low concentration of P_i (Lang et al. 2017). The phosphoester bonds of phytate, including its hydrolysis products, are resistant to abiotic decomposition and have to be cleaved by phytase (Cosgrove and Irving 1980; Mullaney and Ullah 2003). These cleaving enzyme proteins belong to the class of phosphatase enzymes (Herrmann et al. 2019) specialised in progressive cleaving of five of six P groups of phytate (Konietzny and Greiner 2002). Phosphatases and their activity are an arising adjustment of the physiological system of the ectomycorrhizal plant to a nutrient shortage (Antibus et al. 1997). The activity of phytase was documented for the ectomycorrhizal fungi, demonstrating their ability to acquire P from this relatively recalcitrant organic P source (Antibus et al. 1992). Also, the ectomycorrhizal building Basidiomycete *P. involutus* in axenic culture was previously shown to exhibit wall-bound phytase activity [mg P released g^{-1} dry mass h^{-1}] by utilising sodium phytate as a P source in the incubation medium (McElhinney and Mitchell 1993). Thereby, phytate hydrolysis appeared to be not as efficient as other less complex organic P substrates. Another study by Hilger and Krause (1988) observed that *P. involutus* grown aseptically accumulated P_i during the cell lysis phase, indicating that hydrolysis of phytate happened through the released, intracellular phosphatases. Enzymes are considered to be a costly resource to be released into the surrounded environment under nutrient limiting and with other microorganisms competing conditions (Pritsch and Garbaye 2011). However, our results do not indicate a considerable investment of UDC for ectomycorrhizal fungal production of enzymes, as the UDC (Fig. 3) and UDC/P (Fig. 4) in plant roots of mycorrhizal Phy, oP, and no P control (H_2O) treatments were of similar magnitude. Rennenberg and Herschbach (2013) proposed that ectomycorrhizal fungi can take up organic P sources as a whole molecule via glycerophosphoinositol transporters in the hyphae, which would need to be taken up from the solution in the P compartment. But the activity of these transporters was not yet proved experimentally (Becquer et al. 2014; 2018). Due to the absence of axenic conditions in the mesocosm culture system, the ectomycorrhizal fungal hyphae entering the P compartment could introduce also other microorganisms into the Phy solution. The experiment under greenhouse conditions in the present study does not represent natural soil conditions, and we did not determine the presence of other microorganisms in the P compartments, but numerous microorganisms were identified as being able to release phytase, comprising bacteria (Greaves et al. 1967; Shimizu 1992; Cosgrove et al. 1970; Richardson and Hadobas 1997) or fungi (Howson and Davis 1983; Ullah and Gibson 1987). Some bacteria are not as efficient as ectomycorrhizal fungi

(Irshad et al. 2012) but some soil isolates of fluorescent *Pseudomona* spp. were shown to be efficient in utilising Phy as a P source (Richardson and Hadobas 1997; Li et al. 2013) but also as a C source (Richardson and Hadobas 1997). Nevertheless, we could rather assume that some of the mechanisms discussed above in this paragraph could influence the hydrolysis and/or uptake of Phy and could explain the absence of the expected C drain in ectomycorrhizal roots of the Phy treatment compared to the readily available P source oP.

Instead, we can observe that the P uptake, the C utilisation in aboveground biomass and C exchange for P occur with similar efficiencies in oP treatment and the more recalcitrant Phy treatment. The study of Schreider et al. (2022b) could observe higher incorporation of the ^{33}P label and especially significantly higher plant P uptake from an organic P source, adenosine monophosphate (AMP), compared to oP, which is a surprising result, as also the AMP is assumed to require a phosphomonoesterase to release the P_i for uptake (Turner 2008). These results indicate that organic P sources are (AMP) or can be (Phy) more readily available for the mycorrhizal mediated plant P uptake. Dissolved AMP was favoured over oP and Phy required no more C investment compared to oP and, therefore, it could be the case that the adsorption to Fe, Al (in acidic soils), or Ca (in alkaline soils) (Hinsinger 2001) would make the organic P sources but also the oP unavailable for uptake. Also under natural conditions the organic P sources and P_i are usually not present in dissolved forms and are rapidly sorbed to secondary minerals and metal oxides (Hinsinger et al. 2011). Hence, the results of the present study could indicate that not the organic P source exhibit an enormous C sink potential by *P. involutus* but rather the production and the release of LMWOAs as well as the extended hyphal growth.

Nevertheless, we had only one harvesting time point. Because, the P uptake efficiency and the related C investment are considered to be dependent on the demand for P and the affordable supply of C at the plant level (Yanai et al., 1995), it is possible that we could see more pronounced differences in P incorporation efficiencies and the P source dependent C investment between the mycorrhizal P treatments, when the exposure of ectomycorrhizal plants to the different P sources would have been for a more extended time period (e.g. at least one vegetation period) with more than one harvesting time point, indicating the need for further investigations.

Conclusion

In the present study, we determined a P source dependent trend in the exchange of host UDC for ectomycorrhizal mediated P in roots. Based on the discussion and the citing literature above, we can be sure of *P. involutus*'s ability to acquire the differently available P sources in our study. Hence, the emergence of these different mechanisms required to access the various chemical forms of P in our study could be responsible for the differences in UDC allocation and partitioning within the different plant parts that represent the different C costs for the acquisition of different P sources. Henceforth, the *P. involutus* ectomycorrhizal poplar plantlets can adjust the internal C investment according to the external nutrient availability by modulating the acquisition mechanisms through trade-off in nutrient acquisition and C investment. Thereby, a functional trait of a plant can mirror its adaptive strategy but also its influence on ecosystem functioning (Liu et al. 2021). The C investment in the nutrient acquisition of an ectomycorrhizal plant represents such a functional trait. This would imply that an ectomycorrhizal plant that acquires P from (primary and secondary) mineral P sources could be seen as a system

that invests a considerable amount of C into belowground mycelial infrastructure and the acquiring mechanism of exudation of LMWOAs. These findings are in accordance with the findings made by the studies of Andrino et al. (2019; 2021) using a different mycorrhizal type, the arbuscular mycorrhizae. Eventhough, a direct comparison of P source dependent C investment in the present study and the studies performed by Andrino et al. (2019; 2021) is not possible, as both studies have used different parameters to express the C investment, but these individual observations imply that both mycorrhizal types could respond similarly to more complex mineral P sources. In contrast, the readily available P source oP, the soluble Phy, but also the complete limitation in P (no P control) turn the ectomycorrhizal plant into a system of C retention in the ectomycorrhizal root interface.

We can further conclude that the used double labelling with ^{13}C-^{15}N-urea, including the determination of UDC and UDN according to Langenbruch et al. (2014), is a versatile technique to study not only the P source dependent C but also N dynamics in ectomycorrhiza.

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Author contributions The idea of the experiment came from JB with contributions of AA, KS, AFF, TW, and GG. KS prepared the plant and fungal material, prepared the mesocosms, and conducted the experiment. KS performed all the necessary sample and data analysis. LS supervised data analysis. JB, GG, and TW supervised the research. KS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability statement The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflict of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Supplementary Material The supplementary material for this article can be found at the end of this article.

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Supplement of

Phosphorus source dependent exchange of carbon for phosphorus in ectomycorrhiza (*Populus x canescens* x *Paxillus involutus*)

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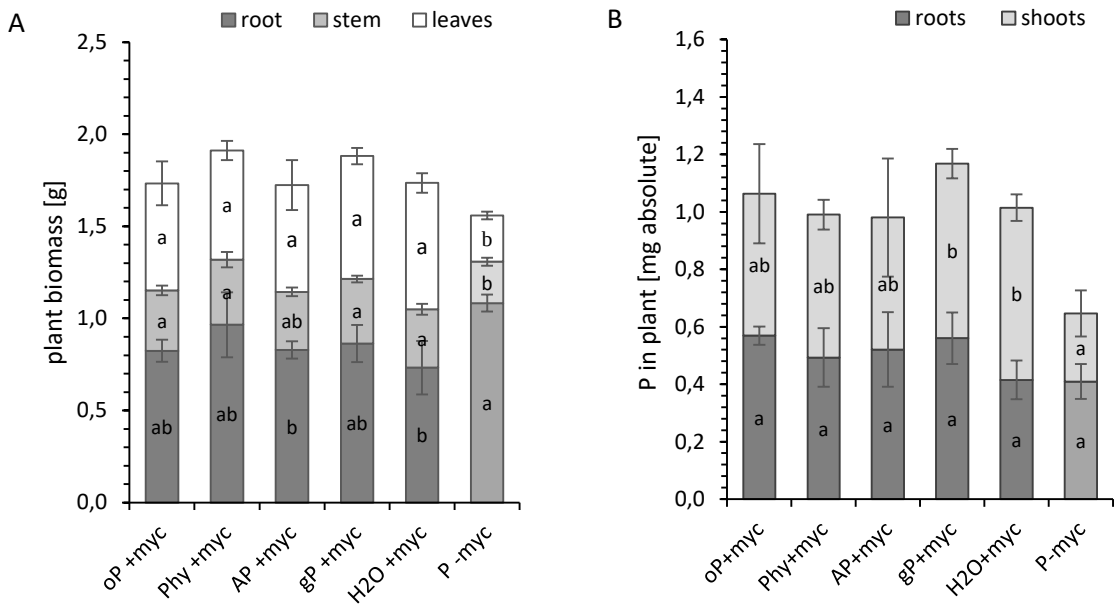

Fig. 1 Plant (**A**) biomass [mg] (leaves, stem, roots) and (**B**) P content [mg absolute] of ectomycorrhizal (+myc, *P. involutus*) and non-mycorrhizal (-myc) poplar plants *P. x canescens* at 166 dpi. These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. Plants underwent a leaf-fertilisation with $^{13}C^{15}N$- and non-labelled urea. The error bars show the standard deviation (n = 3). The letters indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

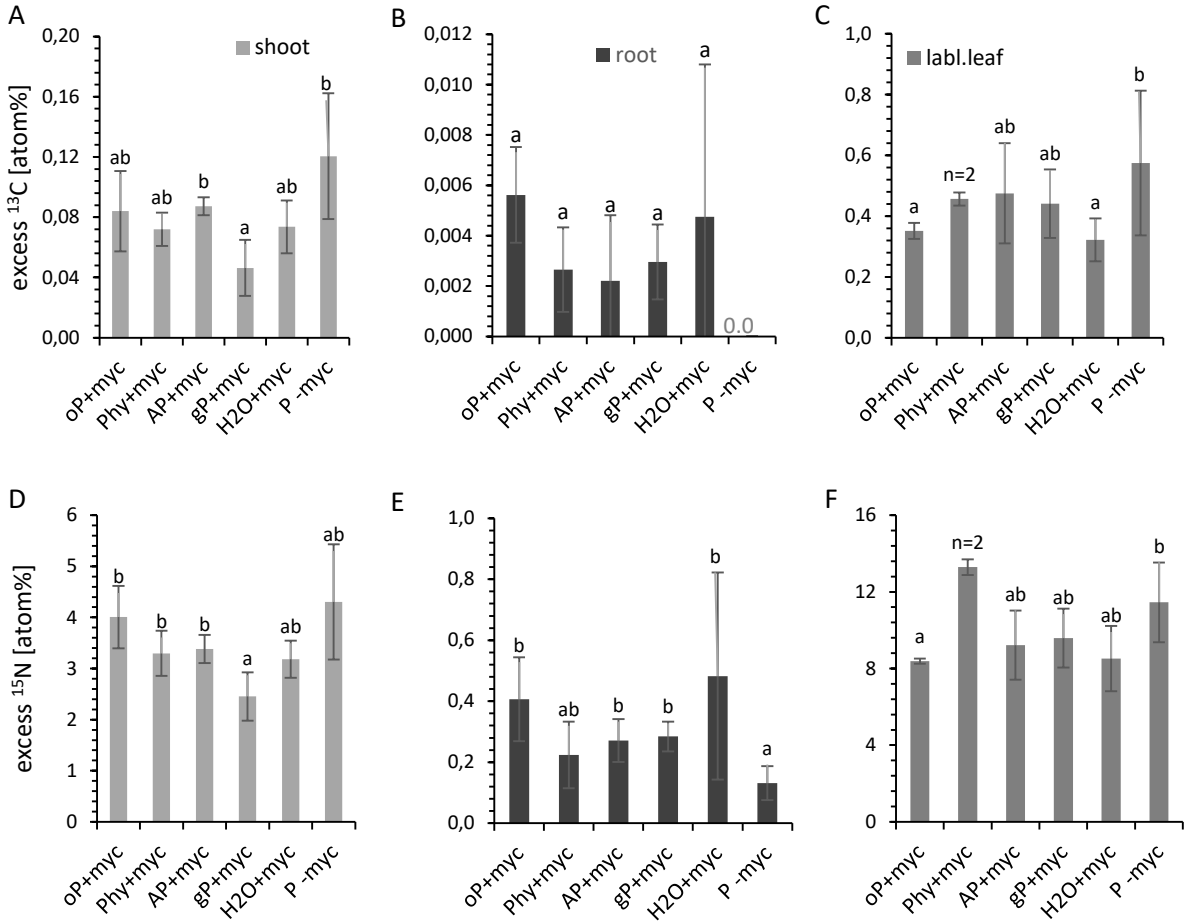

Fig. 2 Excess (**A-C**) ^{13}C and (**D-F**) ^{15}N [atom%] incorporated in plants' (**A**,**D**) shoots, (**B**,**E**) roots, (**C**,**F**) labelled leaf (labl.leaf, that was in contact with urea) from leaf-fertilisation of ectomycorrhizal (+myc) and non-mycorrhizal (-myc) plants with $^{13}C^{15}N$-labelled and non-labelled urea for 5–7 d (165 dpi, n = 30). These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. The error bars show the standard deviation (n = 3). The letters indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

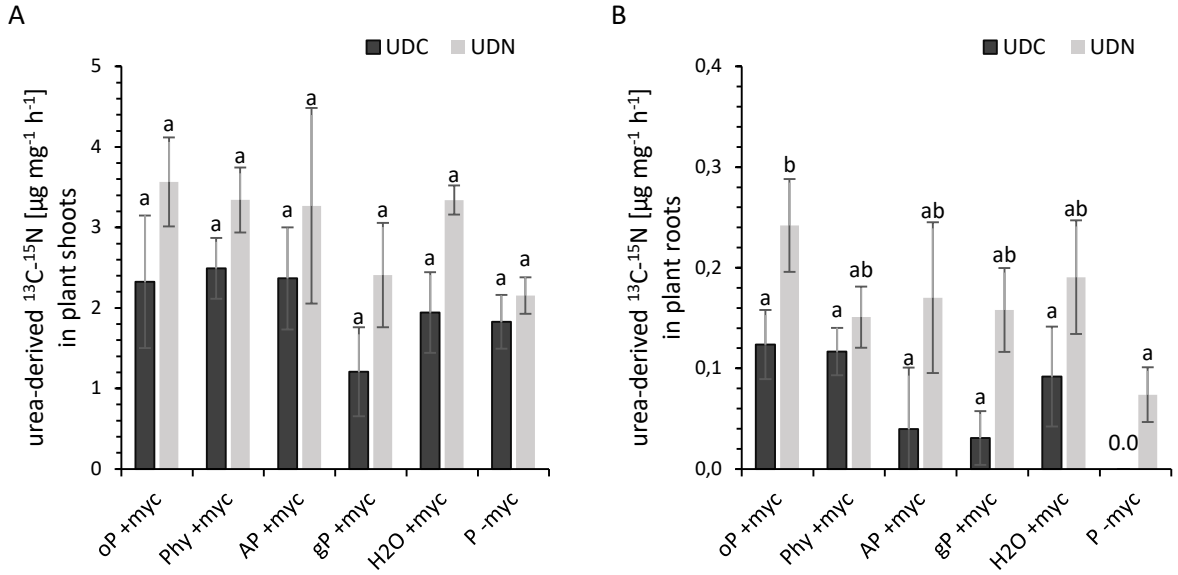

Fig. 3 Urea-derived ^{13}C (UDC) and ^{15}N (UDN) [µmol h^{-1}] in ectomycorrhizal (+myc) and non-mycorrhizal (-myc) plant (**A**) shoots and (**B**) roots from leaf-fertilisation of plant with $^{13}C^{15}N$-labelled urea and an exposure time of 5–7 d. The harvest of these mesocosm systems occurred at 165 dpi. These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. The error bars show standard deviation (n=3). The letters (a, b) indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

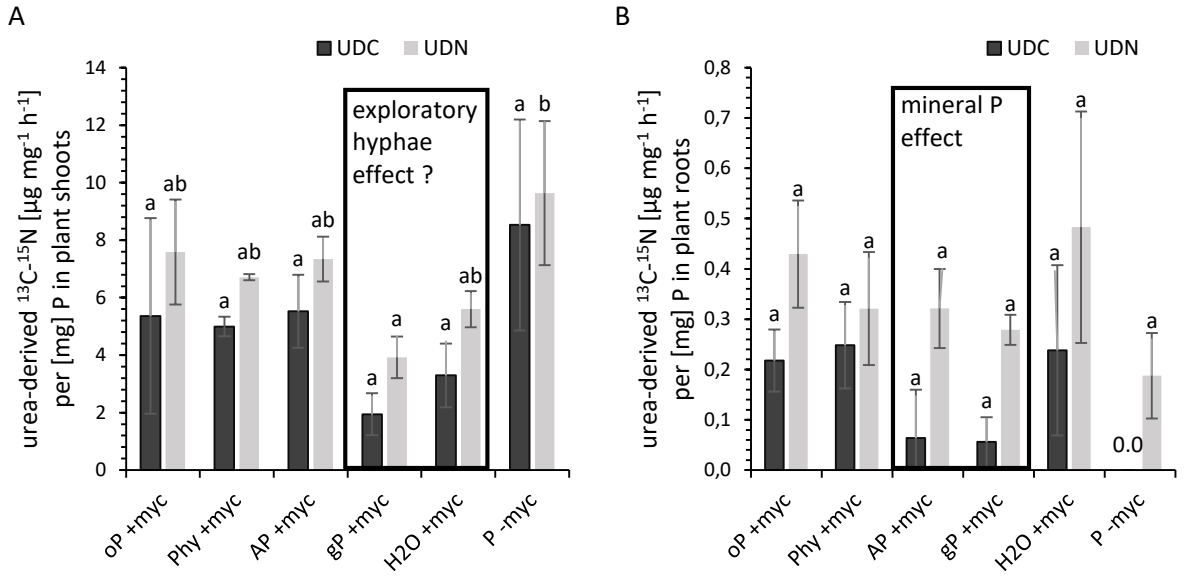

Fig. 4 Urea-derived ^{13}C/P (UDC/P) [µmol h^{-1} mg^{-1}] and urea-derived ^{15}N/P (UDN/P) [µmol h^{-1} mg^{-1}] ratios in plant (**A**) shoots and (**B**) roots of ectomycorrhizal (+myc) and non-mycorrhizal (-myc) plant treatments from leaf-fertilisation of plant (at 159 dpi) with $^{13}C^{15}N$-labelled urea and an exposure time of 5–7 d. These mesocosms were supplied with one of four different chemical forms of P or no P (H_2O) in separate compartments, so that only the ectomycorrhizal fungus had access to the P sources. The error bars show the standard deviation (n = 3). The letters (a, b) indicate significant differences between the P and mycorrhizal treatments: $P < 0.05$.

4 Study III

Mycorrhizal mediated partitioning of phosphorus: ectomycorrhizal (*Populus x canescens* x *Paxillus involutus*) potential to exploit simultaneously organic and mineral phosphorus sources

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JB, KS, AA, GG, AF, and LS designed the experiment. KS prepared the plant, fungal material, everything for the rhizotrone culture systems, performed the experiment, sample, data analysis, and wrote the paper. DH synthesized the hydroxyapatite and irrigated and imaged the systems from day 10 to 88 as well as performed LSC analysis of plant extracts. JB and GG supervised the research. All authors contributed to the article and approved the submitted version.

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Mycorrhizal Mediated Partitioning of Phosphorus: Ectomycorrhizal (*Populus x canescens x Paxillus involutus*) Potential to Exploit Simultaneously Organic and Mineral Phosphorus Sources

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Many natural and anthropogenic soils are phosphorus (P) limited often due to larger P stocks sequestered in forms of low bioavailability. One of the strategies to overcome this shortage lies in the symbiosis of plants with mycorrhizal fungi, increasing the plant P uptake of these hardly accessible sources. However, little is known about mycorrhizal fungal mediated partitioning of differently available P forms, which could contribute to more efficient use of P by plants and, thereby, reduce competition for soil P. This study aimed to investigate the uptake of P from differently bioavailable P sources by ectomycorrhiza. For that, we conducted a rhizotrone study using *Populus x canescens* and its compatible ectomycorrhizal fungus *Paxillus involutus*. Four different P sources [*ortho*-phosphate (oP), adenosine monophosphate (AMP), hydroxyapatite (HAP), and oP bound to goethite (gP)] or only HAP as 1P control were supplied in separate compartments, where only the fungal partner had access to the P sources. The amount of the specific P sources was increased according to their decreasing bioavailability. In order to distinguish between the P sources, we applied ^{33}P to track its incorporation in plants by a non-destructive analysis *via* digital autoradiography. Our results show that an ectomycorrhizal plant is able to utilize all provided P sources *via* its mycorrhizal fungal associate. The acquisition timing was determined by the most bioavailable P sources, with oP and AMP over HAP and gP, and a mixed P pool over a single P source. In contrast, the magnitude was defined by the by the amount of supplied P source provision of additional nitrogen, hence AMP over oP and gP, as well as by P source complexity, with gP as the least favorable P form. Nevertheless, the results of the present study provide evidence that an ectomycorrhiza has the potential to occupy fundamental niches of various P sources differing in their bioavailability, indicating that being a generalist in P nutrition can facilitate adaptation to various nutritional settings in soil.

Keywords: adenosine monophosphate, ectomycorrhizal fungi, goethite P complex, hydroxyapatite, P availability, P diversity, radioactive labeling, resource partitioning

INTRODUCTION

Phosphorus is an essential element for the plant net primary productivity (1), but it is also limited in soils in many ecosystems [e.g., (2)], as more than 90% of P in the soil is present in different chemical forms that are not readily available to plants (3). Plants are able to take up P in the form of free phosphate (P_i) in soil solution (4). However, P_i is poorly mobile, as it interacts strongly with soil constituents and can be adsorbed onto metal oxides and clay minerals, and precipitate as (apatite-like) minerals (5, 6). In addition, P is immobilized in diverse organic forms. In young soils with parent material high in P, plants and microorganisms acquire P from primary minerals (7). However, with increasing soil development, primary minerals are getting depleted, and the plants and soil organisms recycle P from organic matter in the upper soil horizons (7, 8) or pedogenic Fe and Al oxides mainly in the subsoil (9, 10). Therefore, with increasing soil succession and associated depletion in readily available P sources, more efficient plant P uptake and recycling (8) are necessary.

The association of plants with mycorrhizal fungi defines a strategy to cope with P limitation (1). With their extraradical hyphae, the mycorrhizal symbionts are able to increase the volume of soil explored for nutrients beyond the rhizosphere (11, 12). Furthermore, mycorrhizal fungi actively contribute to P mobilization through exudation of low-molecular-weight organic anions (LMWOAs) to release P from primary and secondary minerals and release phosphatases to cleave P from organic P sources [reviewed by (1)]. The mycorrhiza associated P uptake is thereby more cost-effective, as less carbon (C) units are needed for the uptake of one unit P, while the hyphae provide a higher adsorbing length for the same infrastructural investment as compared to roots (13). Furthermore, depending on the inherent P acquiring mechanisms, mycorrhizal fungi are able to mobilize P from mineral and/or organic P sources (14, 15) and could, therefore, contribute to the necessary efficient use of different forms of P in soil. Also, ectomycorrhizal fungi showed an adaptation to a high variety of P sources such as inorganic phosphate (P_i), as well as different mineral (16) and organic P sources (17). Furthermore, the study of Zavičić et al. (18) has shown that fertilization of soils with triple phosphate negatively affected the P uptake efficiency of mycorrhizal beech compared to P uptake efficiency in P deficient soils.

According to the framework of Turner (19), the different chemical forms of P are partitioned by plants with different P acquiring adaptations to P limitation. This framework highlights the fact that mycorrhizal fungi are the key players to reduce competition for P and facilitate the co-existence of plants. Plant communities developing on soils with limited bioavailabilities of P are globally some of the most biodiverse compared with ecosystems limited in other nutrients (20, 21). Therefore, besides affecting the net primary productivity of terrestrial ecosystems, P limitation influences also the diversity and persistence of different plant species (20–22).

Progress in studying P partitioning for soil P has been achieved by examining plant responses to single P sources (23, 24) or a mixed pool of different P sources (25). The study of Andrino et al. (26, 27) investigated the mycorrhizal fungal acquisition of differently available P sources, which was determined by different amounts of C invested by the plant into the association with a mycorrhizal fungus that had access to differently available P sources. Nevertheless, the P sources were supplied as a single P source. The demonstration of the uptake of P with different bioavailabilities is challenging due to the need to differentiate one particular P source from a mixture of various P sources. Starting with Pearson and Jacobsen's (28) groundbreaking study on P uptake *via* mycorrhiza, recently, a few studies have used radioisotope labeling with ^{33}P to demonstrate differences in plant P uptake from different chemical forms of P offered as a sole P source (21) and as a mixture of different P sources (29). However, the sole contribution of ectomycorrhizal fungi in resource partitioning for P remains unclear. Hence, the aim of our study was to investigate whether an ectomycorrhizal fungus shows preferences to specific P sources of different bioavailability when offered in a mixed pool. This should help understand how the belowground nutrient P diversity shapes the mycorrhizal mediated aboveground plant biomass and P uptake. The present study addressed the following hypotheses: as the mycorrhizal fungi are essential in plant P acquisition and are known to be able to access organic and sorbed P (1, 30), we hypothesized (H1) that all P sources within the diverse P pool are available to mycorrhizal fungi, and therefore to plants, but the order and magnitude of acquisition depend on the P source complexity. We expected that the mycorrhizae would favor the readily available P_i over organic or mineral P sources, whereby the amount of each P source applied into the system was increased according to its complexity. Furthermore, in order to support the general assumption that a minor diversity in belowground resources, which can occur as a side effect of fertilization (31, 32), result in less efficient uptake of P (18), we hypothesize (H2) that mineral source (HAP) as a single P source is less available than within a pool of diverse P sources.

MATERIALS AND METHODS

Plant and Mycorrhizal Material

Poplar species *Populus x canescens* clone "Schleswig I" was used as a model plant and its functional ectomycorrhizal fungal strain MAJ of *Paxillus involutus* (33) was used as a symbiotic partner. Both were identified as functional associates which provide valuable model systems for a more robust test of nutrient acquisition and exchange models (33). The poplar plantlets were propagated on Woody Plant Medium [WPM (34)] without hormones (dephyte e.K., Langenberg, Germany) by establishing explants from the first two lateral meristems with one leaf and bud. The apical buds with 1.5 cm height were used for rooting on WPM medium but with one-third amount of sucrose and vitamins in Microboxes (O118/80, by Sac O_2, Deinze, Belgium) with a membrane for sterile gas exchange (81.35 GE day^{-1}). *P. involutus* was propagated in liquid culture of 0.2 L of modified Melin-Norkrans (MMN; ready to use salts, dephyte e.K., Langenberg, Germany) medium (33) in 0.5 L glass bottles under shaking and proliferated on 0.8 L Perlite as a carrier with 0.2 L MMN.

Preparation of the Phosphorus Sources and the Containers

To investigate mycorrhizal mediated plant P uptake from a pool of different P sources, the following chemical forms of P were tested: free *ortho*-phosphate (oP), adenosine monophosphate (AMP), hydroxyapatite (HAP), beside phosphate bound to goethite (gP) as a P adsorption complex with the Fe oxide, which is one of the most profuse and naturally occurring in soils (35). To differentiate the uptake between the different P sources, the use of radio-labeling with ^{33}P in combination with the non-destructive digital autoradiography was made. As ^{33}P labeled compounds [P33] phosphoric acid (FF-1) and [alpha-P33] AMP (FF-217) were purchased from Hartmann Analytic GmbH (Braunschweig, Germany).

To obtain the P sources oP and AMP in concentrations of 0.9 and 3.8 mg P ml^{-1}, respectively, $NaH_2PO_4 \cdot 2H_2O$ (for analysis, EMSURE®, Merck KGaA, Darmstadt, Germany) and adenosine 5′-monophosphate sodium salt (from yeast, >99%, SigmaAldrich, Merck KGaA, Darmstadt, Germany) were dissolved in deionized H_2O (dH_2O) and the pH adjusted to 5.2 using 1 M Na_2HPO_4 for oP and 1 M HCl for AMP. For labeling with ^{33}P, an aliquot of the oP and AMP solutions was spiked with 0.20 ml 74 MBq $H_3^{33}PO_4$ and 0.20 ml 74 MBq AM^{33}P, respectively. The oP and AMP were filtered using sterile syringe filter (Filtropur S, PES, 0.2 μm pore size, Sarstedt AG & Co. KG, Nümbrecht, Germany).

The HAP was produced as multistep synthesis at 25°C according to the protocol described by Wolf et al. (36). The only modification of this protocol was performed in the second step for the application of ^{33}P label, in which the 2 M H_3PO_4 solution was spiked with 0.5 ml 185 MBq $H_3^{33}PO_4$. The HAP pellet was ground with mortar and pestle and passed through a 0.20 mm sieve. The resulting ^{33}P activity of HAP accounted for 117.8 MBq g^{-1}.

To prepare the adsorption complexes of oP to goethite (gP), the procedure described by Andrino et al. (26, 27) was used with following modifications: for the loading of goethite with oP, 1 M NaH_2PO_4 solution was used. To prepare the ^{33}P labeled gP (gP-^{33}P), 24.5 ml of 1 M NaH_2PO_4 solution were spiked with 0.5 ml 185 MBq $H_3^{33}PO_4$. Always 6.2 ml 1 M NaH_2PO_4 (non-labeled or ^{33}P-labeled) and 5 g of synthetic goethite (α-FeOOH < 0.045 mm, Bayferrox® 920 Z, Lanxess Deutschland GmbH, Cologne, Germany) were mixed and filled up to 220 ml with dH_2O in centrifuge bottles (250 ml, Nalgene™, Thermo Scientific™, Waltham, Massachusetts, USA). Further steps are described in detail by Andrino et al. (26, 27) up to the step of drying the gP-^{33}P pellet. Due to the labeling with ^{33}P, gP had to be dried at 50°C in a drying oven without ventilation. After cooling down, the gP pellet was sieved through a 0.63 mm sieve. The resulting ^{33}P activity of gP accounted for 245.6 kBq g^{-1}.

Both gP and HAP (labeled and non-labeled) were sintered three times at 105°C for 30 min in a drying oven, including a subsequent incubation period at 30°C for 2 h the first two times. This step is called tyndallization and is used for fractionated sterilization without changing the mineral structure. The Raman spectrum of HAP with its phosphate typical bands (**Supplementary Figure 1**) is aligned with the spectra obtained by Wolf et al. (36), confirming the main characteristics of HAP.

To determine the P content of gP and HAP, 102 mg of each were solubilized in 10 ml 32% HCl (AnalaR NORMAPUR® for analysis, VWR International GmbH, Darmstadt, Germany), shaken on an overhead shaker for 24 h, and filtered using filter papers (thickness of 0.14 mm, a pore size of 2–3 μm; LABSOLUTE®, Th. Geyer GmbH & Co. KG, Renningen, Germany). P analysis was performed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Varian 725-ES, City, State). In order to determine the easy desorbable P from gP and HAP, 55 mg gP or 101 mg HAP were suspended in 10 ml dH_2O, shaken on an overhead shaker for 24 h, and filtered through a filter paper (as described above). The analysis of P in the filtered solution was performed *via* ICP-OES. The P content of gP accounted to 1.04 ± 0.06 mg g^{-1} goethite ($n = 7$), of which 0.17 ± 0.01 mg P g^{-1} gP were easy desorbable, so that maximum of 16% P from P bound to goethite could be mobilized without any action of the mycorrhizal fungus. The P content of HAP accounted for 197.5 ± 5.3 mg g^{-1} HAP ($n = 3$), of which 7.1 ± 0.1 mg P g^{-1} HAP (3.6% P in HAP) were easy desorbable. According to the dissolution studies of HAP conducted by Wolf et al. (36), HAP is (independent of the synthesis temperature) otherwise stable in aqueous systems at pH values above 3.8.

All P sources were supplied in separate containers made of 5 ml SafeSeal reaction tubes. At around 2 ml height, a hole of 1 cm in diameter was burned into the tube and sealed with a combination of two types of membranes, allowing only the mycorrhizal partner to access the P sources in the P containers as described in more detail by Andrino et al. (26, 27). Briefly, a hydrophobic PTFE membrane (5 μm, Pieper Filter GmbH, Bad Zwischenahn, Germany) was installed at the P source side and a nylon mesh (20 μm, Franz Eckert GmbH, Waldkirch, Germany) at the plant root side. The containers were autoclaved (121°C, 20 min) and filled with the different labeled and non-labeled P sources under sterile conditions in a laminar flow cabinet. All containers were filled up to 3 ml with dH_2O.

Preparation of the Rhizotrone Culture Systems

A small-scaled, continuously closed rhizotrone culture system with four separate containers for the simultaneous supply of four different P sources (oP, AMP, HAP, and gP) was established to test hypothesis H1. In this 4P-^{33}P experiment, only one P source was labeled with ^{33}P before installing into a rhizotrone culture system. The overall P amount per system accounted for 13.2 mg P and was considered sufficient to sustain the system for 3 months. The specific P sources were supplied as 0.9 mg P in the form of oP, 3.8 mg P in the form of AMP, 6.2 ± 0.1 mg P in the form of HAP, and 2.3 ± 0.1 mg P as gP, whereby 223 and 368 μg P from mineral P sources HAP and gP (respectively) were easy desorbable (**Table 1**). The supplied P amounts increased with the increasing complexity of the P sources. The activity of ^{33}P (**Table 1**) accounted for 7,452 kBq per P container for oP and

TABLE 1 | Amount of P [mg], ^{33}P activity [kBq], and the calculated specific ^{33}P activity [Bq mg^{-1} P] of the different P sources applied in the P compartments in the rhizotrone systems.

P source in P compartment	4P +oP-^{33}P	4P +AMP-^{33}P	4P +HAP-^{33}P	4P +gP-^{33}P	1P +HAP-^{33}P
P [mg]	0.88	3.77	6.26 ± 0.12	2.32 ± 0.07	19.9 ± 0.4
^{33}P activity [kBq]	7,452	7,452	2,547 ± 47	638 ± 20	8,090 ± 158
specific ^{33}P activity [kBq mg^{-1} P]	8,498	1,975	407	275	407

Data indicate means ± standard deviation (n = 7). 4P experiment with simultaneous supply of four different P sources: oP, ortho-phosphate; AMP, adenosine monophosphate; gP, oP bound to goethite; HAP, synthesized hydroxyapatite; and 1P control experiment supplied with HAP.

AMP and 2,538 and 410 kBq per P container for HAP and gP (respectively) at 7.5 days post inoculation (dpi).

To test hypothesis H2, a 1HAP-^{33}P experiment was established to test the synthesized, ^{33}P labeled hydroxyapatite (1HAP-^{33}P) as a sole P source as a control to the 4P-^{33}P experiment. The P containers contained 19.9 ± 0.4 mg P in the form of HAP to provide a complex P source in surplus to ensure uptake. The activity of ^{33}P in HAP accounted for 8,080 kBq per P container at 3 dpi.

The rhizotrone culture systems used in the present study were made of square Petri dishes (10 × 10 × 2 cm, Sarstedt AG & Co. KG, Nümbrecht, Germany). The P containers were inserted 1 cm from the bottom into the rizotrones in a randomized order. Then, the rhizotrones were filled with Perlite (washed with dH_2O; autoclaved at 121°C for 20 min two times; Perligran® classic, Knauf Aquapanel GmbH, Dortmund, Germany) as nutrient-free substrate. To obtain mycorrhizal plant treatments, the substrate was inoculated with 10 vol.% of *P. involutus*-Perlite carrier. For the non-mycorrhizal plant treatments, the inoculated substrate was autoclaved one more time before use. The rooted poplar plantlets were placed on the top of the P containers and covered with an additional thin layer of Perlite. Next, the rhizotrone was covered with a thin, sterile PVC foil (10 × 10 × 0.02 cm, Modulor GmbH, Germany) to minimize radio-active shielding and afterwards sealed with hot glue. The skip for the plant stem at the top and the opening to release the excess of nutrient solution at 2 cm height from the bottom of the rhizotrone were closed with sterile cotton wool.

To keep the system sterile during the watering, the rhizotrones were equipped with a sterile syringe filter (Filtropur S, PES, 0.2 µm pore size, Sarstedt AG & Co. KG, Nümbrecht, Germany), which was connected to the rhizotrone through an autoclaved PVC pipe. From 0 dpi, the mesocosms were supplied with a Woody Plant (WPM) nutrient solution containing macro- and micro-elements without P and vitamins (WPM –P; dephyte e.K., Langenberg, Germany), which is a modification from a recipe described by Müller et al. (34) and which was balanced with KNO_3 and $(NH_4)_2SO_4$ to adjust the desired concentration of K.

For each ^{33}P labeled P source, four replicates were prepared as mycorrhizal treatments and three replicates as non-mycorrhizal treatments. To obtain no P controls with P compartments containing H_2O only, two mycorrhizal and two non-mycorrhizal rhizotrone culture systems were prepared. The plantlets were kept under a plastic cover that included moisturized protecting paper plugs for the first 2 weeks for acclimatization and, thus, for protection from lower ambient air humidity and higher UV radiation. The rhizotrones were kept in a climate chamber set at an 18/6 h day/night cycle with 20/18°C and 80% ambient humidity.

Autoradiography and Harvest of Rhizotrones and Plant Material

In the time course of the experiment, the rhizotrones were regularly exposed to phospho-imaging screens for 4 h (at 7, 21, 34, 52, 70, and 80 dpi) and 72 h (at 42 and 94 dpi) in order to monitor the transfer of ^{33}P through the ectomycorrhizal fungal partner from the P containers into the plant shoots. To do this, the whole rhizotrone surfaces, including the above-ground plants, were overlaid by phospho-imaging screens (20 × 40 cm and 35 × 43 cm; Dürr NDT GmbH & Co. KG, Germany). They were wrapped into an extra thin cover foil to protect the screens from contamination with the ^{33}P label and damage by an outflow from the rhizotrone. To ensure possibly tight contact between the plant leaves and stems and the phospho-imaging screen, a foam of a suitable thickness was placed under the plant shoots. Another foam and a heavier plate were placed from the phospho-imaging screens' backside to ensure tight contact between the rhizotrone and screen from the top side. As reference to the ^{33}P label, a set of ^{14}C polymer standards (IPcal test source array; Elysia-raytest, Straubenhardt, Germany) were used with different activities (in dpm cm^{-2}): 1,107,000, 424,000, 112,000, 41500, 13,350, and 3,950, supplemented by an activity free background. Thereby, each activity had a surface area of 1 cm^2. The exposition of rhizotrones to the phosphor-imaging screens must be in the dark. The phospho-image screens were read out immediately using an image plate scanner (CR-35 BIO, Elysia-raytest, Liège, Belgium) in sensitive mode with 100 µm resolution. Each scan is saved as a set of two raw data files which can be converted subsequently into one (e.g., JPG) data file using the Aida Image Analyser v5.0 (Elysia-raytest, Straubenhardt, Germany).

The mesocosms from 1HAP-^{33}P and 4P-^{33}P experiments were harvested at 108 dpi and between 110 and 112 dpi, respectively. The plant roots were separated from the substrate, washed, and dried with paper clothes. Fine root aliquots of up to 0.15 g of each plant were conserved in 70% ethanol to assess mycorrhization grade. The freshly harvested plant material was immediately imaged first for 4 h and afterwards for 72 h (as described above). The same imaging procedure was performed with the plant material after drying at 40°C overnight. After the last imaging, the plant material was separated into leaves, stem, and roots, and the biomass of the different plant parts was recorded.

The substrate was dried at 40°C for 96 h. The liquids in the P containers were collected in 2 ml SafeSeal reaction tubes,

weighed, and controlled for the pH using pH-indicator strips (Neutralit® pH5-10, Merck KGaA, Darmstadt, Germany; pH-Fix 0-6, Macherey-Nagel GmbH & Co. KG, Düren, Germany; and pH-Fix 0-14, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The pH-indicator stripes may not provide the most accurate result, but it offers reasonable control of changes in pH attributable to P uptake.

Quantification of ^{33}P and P and Calculation of Related Parameters

In order to quantitatively analyse the activity of ^{33}P in plant material, all parts of plant material (leaves, stem, roots) and substrates underwent thermal digestion at 480°C for a minimum of 6 h. ^{33}P, P, and nutrients were extracted using 3.6 M HNO_3. After 10 min incubation, the extracts were filtered using a folded filter (Sartorius™ FT-4-303-185, Grade 3hw, ø150 mm, 65 g m^{-2}, Sartorius AG, Goettingen, Germany). Thereafter, an aliquot of plant/substrate extracts (diluted to obtain a 2.3 M HNO_3 solution and prevent any phase separation) was mixed with 10 ml scintillation cocktail (Ultima Gold™, PerkinElmer Inc., USA) and analyzed for ^{33}P activity (dpm single program under blank correction) by liquid scintillation counting (LSC, Tri-Carb 3110TR, PerkinElmer Inc., Waltham, USA). The obtained ^{33}P activity was corrected by subtracting the background value, obtained as a blank undergoing the same extraction and preparation procedures as the plant material. The calibration/normalization of the LSC was performed daily with instrument inherent external standards. The reliability and accuracy of the ^{33}P activity data were ensured by a serial dilution of a sample extract with a high ^{33}P activity at 260 Bq at the day of measurement (**Supplementary Figure 2**). The reproducibility of measured values down to 1.5 Bq did not deviate significantly from the theoretical values. The measured values starting from 1.0 to 0.5 Bq were with 8–5% (respectively) less reproducible. ^{33}P activity values below 0.2 Bq were with 16% standard deviation less trustful, and as a consequence, excluded from data analysis.

The ^{33}P activity incorporated in different plant parts (leaves, stem, and roots) was determined as follows:

$$^{33}P\ activity\ \left[Bq\right] = \frac{\left(^{33}P\ signal\ \left[Bq\right]\ sample\ -\ ^{33}P\ signal\ \left[Bq\right]\ blanc\right) \bullet\ extract\ volume\ \left[ml\right] \bullet\ plant\ part\ biomass\ \left[g\right]}{extract\ aliqoute\ \left[ml\right] \bullet\ plant\ part\ aliqoute\ \left[g\right]} \quad (1)$$

The correction of ^{33}P activity in plant material from LSC measuring date and experiment starting date was calculated as follows:

$$^{33}P\ activity\ \left[\mathrm{Bq}\right] = \frac{^{33}P\ activity\ \left[Bq\right]}{e^{\left(\left(\frac{-\ln(2)}{25.34}\right) \bullet\ (t_0 - t_{LSC})\right)}} \quad (2)$$

Since not only the activity but also the P amounts applied with different P sources varied, we had to determine the specific activity in each P compartments containing ^{33}P labeled P source (**Table 1**) by using the following equation:

$$specific\ ^{33}P\ in\ P\ compartment\ \left[Bq\ mg^{-1}\right] = \frac{^{33}P\ \left[kBq\right]\ in\ P\ compartment\ \bullet\ 10^3}{P\ in\ P\ compartment\ \left[mg\ absolute\right]} \quad (3)$$

The recovery of ^{33}P (5) in plant material and substrate as well as the total P uptake in plant material (6) was calculated as follows:

$$^{33}P\ recovery\ [\%] = \frac{^{33}P\ \left[Bq\right]\ in\ plant\ or\ substrate}{^{33}P\ in\ P\ compartment\ \left[Bq\right]} \bullet 100\% \quad (4)$$

$$P\ uptake\ in\ plant\ \left[ug\right] = \frac{^{33}\ P\ \left[Bq\right]\ in\ plant\ \bullet\ 10^3}{specific\ ^{33}P\ \left[kBq\ mg^{-1}\right]\ in\ P\ compartment\ \bullet\ 10^3} \quad (5)$$

The analyses of total P in plant material and substrate were performed *via* ICP-OES (Varian 725-ES, Agilent Technologies, Santa Clara, United States). The standards for the calibration were prepared using the same matrix.

Assessment of Mycorrhization Grade of Mycorrhizal Plant Roots

In order to record the plants' response to mycorrhizal inoculation and the P source dependency and/or diversity, we determined the mycorrhization grade in mycorrhizal plant roots. Characteristically for successful mycorrhization of poplar roots by *P. involutus* (MAJ) is the change in root morphology, e.g., specific branching of root tips and no development of root hairs. The grade of mycorrhization of plant roots was determined using the gridline intersection method (37) as modified and described in detail by Brundrett et al. (38). Thereby, aliquots of 82 ± 27 cm of mycorrhizal and 28 ± 7 cm of non-mycorrhizal fine roots were inspected for the mycorrhizal root tips and the intersections of roots with the gridlines in triplicates (by rearranging the same root sample after each counting) using a stereo zoom microscope (45x magnification; KERN OZM-5, KERN & SOHN GmbH, Balingen, Deutschland). Mycorrhization grade was expressed as number of mycorrhizal root tips per cm^{-1} analyzed root length:

$$mycorrhization\ grade\quad [\#mycorrhizal\ root\ tips\ cm^{-1}\ root\ length] = \frac{number\ mycorrhizal\ root\ tips}{cm\ root\ length} \quad (6)$$

As expected, the fine roots of mycorrhizal plants were abundantly mycorrhized at 108 dpi (**Supplementary Figure 3**; **Supplementary Table 1**), while the non-mycorrhizal controls were not mycorrhized.

Statistical Analysis

All statical tests were performed with SPSS® Statistics 26.0 [IBM® Corporation, USA (39)] at the probability level of 0.05. All data were tested for normal distribution using the Shapiro-Wilk test and homogeneity of variances using Levene's test, where the *P*-value was calculated based on the mean. To test for significant differences in data of plant biomass, P content, and pH of P liquids (normally distributed) between mycorrhizal treatments (with two independent groups), the independent samples *t*-test was performed. To test for significant differences in data of plant biomass, plant P content, ^{33}P activity, ^{33}P recovery, and plant P uptake between the different P and mycorrhizal treatments (with more than three independent groups), the following statistical procedure was applied: a one-factorial analysis of the variances (ANOVA) with replicates was performed on normally distributed data. In case of significant differences, the Scheffé procedure (for data sets with no equal variances, as it is insensitive to violation of homogeneity of variances) or the Tukey-HSD test (for data sets with equal variances) as a *post-hoc* test were used. In case the data were not normally distributed, the Kruskal-Walis-*H*-test was used. It is a non-parametric equivalent of the One-Way ANOVA to test for significant differences between more than two groups using medians. In case of significant differences between the groups, the Mann-Witney-*U*-test was performed.

RESULTS

Distribution of Biomass and Absolute P Content in Plant Parts Differ Between Mycorrhizal and P Treatments

At the end of the experiment at 108 dpi, we determined in both experiments (4P-^{33}P and HAP-^{33}P) the biomass (**Figure 1A**) of, and the absolute P content (**Figure 1B**) in, different plant parts and substrate in order to investigate, whether these two parameters differ between the different plant parts and P pools applied into the system.

In both experiments, mycorrhizal plants developed significantly higher biomass at 108 dpi (**Figure 1A**) compared with the non-mycorrhizal plants ($P < 0.0005$). In addition, both treatments (mycorrhizal and non-mycorrhizal) had significantly ($P < 0.00001$) higher root biomass compared with the shoot biomass. Unfortunately, only two non-mycorrhizal 1HAP-^{33}P and mycorrhizal no P (H_2O) controls and only one non-mycorrhizal no P control could be harvested at the end of the experiment so that no statics could be done on these treatments.

The absolute P content (**Figure 1B**) follows almost the same trend as the plant biomass, as independent of the P species and mycorrhizal treatment, the absolute P content in plant correlated positively with the plant biomass ($R^2 = 0.64$, $P < 0.001$; **Supplementary Figure 4**). The absolute P content in mycorrhizal plants from 4P-^{33}P systems was significantly higher ($P < 0.001$) than in their non-mycorrhized counterparts. Mycorrhizal and non-mycorrhizal plants incorporated significantly more P ($P < 0.005$) in roots than in their stems and leaves. Furthermore, the P content in mycorrhizal 4P-^{33}P plants was significantly higher ($P < 0.04$) than in mycorrhizal 1HAP-^{33}P plants.

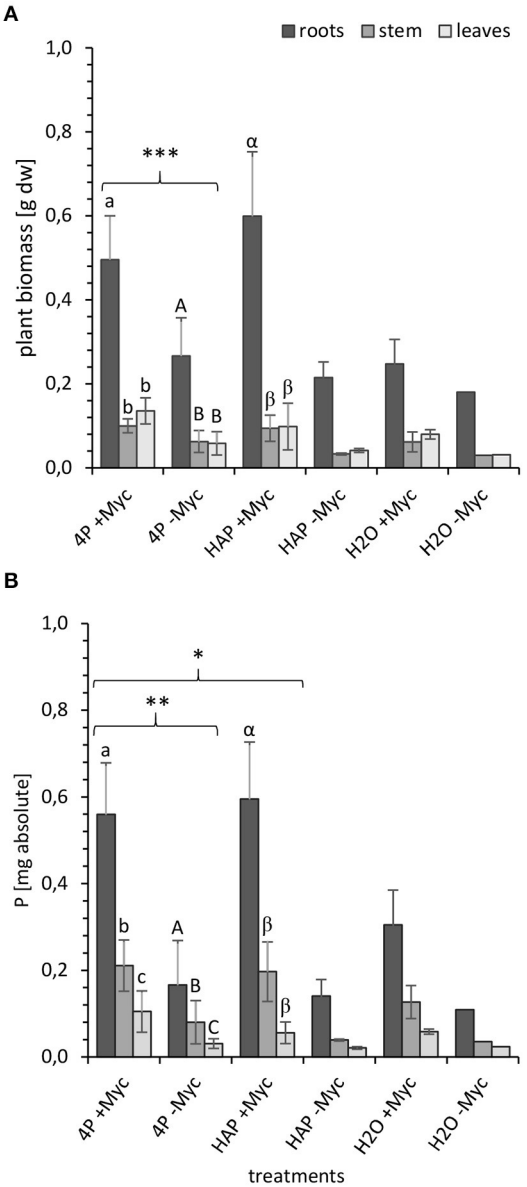

FIGURE 1 | (A) Biomass [g dry weight] and **(B)** P content [mg absolute] in different parts of mycorrhizal (+Myc) and non-mycorrhizal (-Myc) poplar plant at 108 dpi. 4P experiment with simultaneous supply of four different P sources: oP, *ortho*-phosphate; AMP, adenosine monophosphate; gP, oP bound to goethite; HAP, synthesized hydroxyapatite; and 1P control experiment supplied with HAP. The error bars show the standard deviation (4P: +Myc: $n = 16$; -Myc: $n = 12$; HAP: +Myc: $n = 4$; -Myc: $n = 2$; H_2O: +Myc: $n = 2$; -Myc: $n = 1$). The letters (a, A, α) indicate significant differences between the plant parts: **(A)** a-b and A-B: $P < 0.001$; α-β: $P < 0.003$; **(B)** a-c: $P < 0.001$; A-C and α-β: $P < 0.005$. Marking with (*) indicates significant differences between mycorrhizal and non-mycorrhizal treatments: **(A)** ***$P <$ 0.001 for whole plant and for leaves, stem, and roots); **(B)** **$P < 0.02$ for the whole plant; $P < 0.001$ for leaves, stem, and roots; *$P < 0.04$ for the whole plant; $P < 0.001$ for leaves and stem; $P < 0.01$ for roots.

The plant biomass and the P content in plants from mycorrhizal no P control systems are almost 2-fold lower than the mycorrhizal P treatments. This gain in biomass and P content of mycorrhizal P treatments accounted for 0.35 ± 0.15 g biomass and 0.38 ± 0.17 mg P. This is the gain alone from the P uptake from the P compartments.

^{33}P Incorporation in Plants and Its Distribution Between Plant Parts Differ Between P Treatments

In order to differentiate the plant P uptake from the different P sources and its partitioning in the plant, we have exposed the rhizotrone culture systems in the time course of the experiment (**Supplementary Figure 5**) as well as the harvested plants (**Figure 2**) to phospho-imaging screens for 4 and 72 h. Further, for the same reason, we determined the ^{33}P activity in harvested plants (leaves, stem, and roots) and substrate (**Figure 3**; **Supplementary Table 3**).

No incorporation of ^{33}P label in plant material was detected at 21 dpi. In contrast, the images of rhizotrone culture systems at 34 dpi revealed that the mycorrhizal fungus started to acquire the P sources oP and AMP (**Supplementary Figures 5A,B**, respectively) almost simultaneously. From HAP supplied in a pool of four different P sources (**Supplementary Figure 5C**), incorporation of ^{33}P label in plant shoots was visible for the first time at 42 dpi, while ^{33}P from HAP supplied as a single P source (**Supplementary Figure 5D**) was detectable for the first time in the plant at 94 dpi, when the rhizotrone culture systems were imaged for 72 h. No incorporation of ^{33}P label could be detected in the plant from HAP supplied as a single P source, nor from gP supplied in a pool of four different P sources at that time point.

The images of harvested plants of 4P systems with ^{33}P-labeled oP, AMP, and HAP (**Figure 3**) showed a trend in ^{33}P activity incorporated in plant: After 4 h exposing time of harvested plant material to the phospho-imaging screens, the highest ^{33}P activity was detected in 4P systems with ^{33}P-labeled AMP (**Figure 2B**), followed by oP (**Figure 2A**), and HAP (**Figure 2C**). The blue color indicates areas with high ^{33}P activity—these are the areas of roots with high intensity in the branching of the root tips (**Supplementary Figure 2**). As the main proportion of ^{33}P label in the plant is incorporated in roots, the ^{33}P incorporated in the plant from gP (**Figure 2D**) could be detected the first time in harvested plant material by exposing the dried plant to the phosphor-imaging plate for 72 h.

The quantitative analysis of ^{33}P activity (**Figure 3**) in plants showed the same trend as the images of harvested plant material. Moreover, the ^{33}P activity was significantly lower in plants from mycorrhizal gP treatments compared to the other mycorrhizal P treatments. The non-mycorrhizal P treatments were evidently lower enriched in ^{33}P than the mycorrhized counterparts, and the ^{33}P activity for the non-mycorrhizal 4P+gP-^{33}P and 1P+HAP-^{33}P treatments was even under the detection limit. When comparing different plant parts, the leaves of 4P+AMP-^{33}P treatment incorporated significantly ($P < 0.03$) more ^{33}P compared with the 4P+oP-^{33}P treatment and significantly higher amounts of ^{33}P in leaves, stem, and substrate compared with 1P+HAP-^{33}P treatment. The main proportion of ^{33}P was found in the plant roots (>57%).

^{33}P Recovery in Plant and Plant P Uptake Differ Between the Specific P Sources From a Mixed P Pool

In order to relate the ^{33}P activity incorporated in plant material to the ^{33}P activity of the initially applied P sources, we investigated the ^{33}P recovery [%] in the harvested plant (**Figure 4A**; **Supplementary Table 4**). Furthermore, in order to standardize the different ^{33}P activity added with different P amounts applied with each P source, we used the ^{33}P activity [Bq] incorporated in plant material, and the specific ^{33}P activity [kBq mg^{-1}] of the initially applied P sources to determine the plant P uptake [μg] (**Figure 4B**; **Supplementary Table 4**) from the specific ^{33}P labeled P source.

The recovery of initially applied ^{33}P in plant (**Figure 4A**) from the ^{33}P-labeled P sources accounted to a maximum of 5%. Significantly ($P < 0.03$) higher ^{33}P recovery could be observed in mycorrhizal plants grown in 4P rhizotrones with labeled AMP-^{33}P (followed by HAP-^{33}P and oP-^{33}P) compared with gP-^{33}P and single HAP-^{33}P and non-mycorrhizal treatments. Although the initial P content of 1P rhizotrone system containing labeled HAP-^{33}P as a single P source was 32% higher compared with the 4P system, the recovery of ^{33}P in plant shoots (leaves and stems) was significantly lower ($P < 0.03$) from the former as compared to the latter.

Looking at the plant ^{33}P uptake [μg] (**Figure 4B**) standardized by the initially applied P content, the uptake from HAP-^{33}P as a single P source (44 ± 66 μg P) tended to be lower than from HAP-^{33}P with the 4P system (119 ± 59 μg P), which is possibly due to the higher standard deviation in data from 1P+HAP-^{33}P treatment. Furthermore, P taken up from AMP-^{33}P (115 ± 73 μg P) and HAP-^{33}P from a mixed P pool were similar. However, the P uptake from the readily available oP-^{33}P (7.1 ± 1.6 μg P) and the more complex gP-^{33}P (0.61 ± 0.76 μg P) was significantly lower ($P < 0.03$) compared with other P treatments. Furthermore, the calculated plant P uptake from the mineral P sources HAP and gP (max. 168 μg P and 1.6 μg P, respectively) was lower compared to the easy desorbable P (max. 224 μg P from HAP and 369 μg P from gP).

DISCUSSION

The present study tested the suggestion highlighted in the framework of Turner (19) that the partitioning of different chemical P forms in the soil by plants is mediated by mycorrhizal fungi. For this purpose, we performed an experiment using an axenic rhizotrone culture system and the mycorrhizal associates of the poplar plant *P. x canescens* and the ectomycorrhizal fungus *P. involutus*. The system was supplied simultaneously with four different chemical forms of P in separate compartments so that only the mycorrhizal fungus had access to the P sources. To differentiate the mycorrhizal mediated plant P uptake between the different P sources, labeling with the radio-isotope ^{33}P was applied. Finally, the obtained results from this experiment were compared with the mycorrhizal mediated plant P uptake from

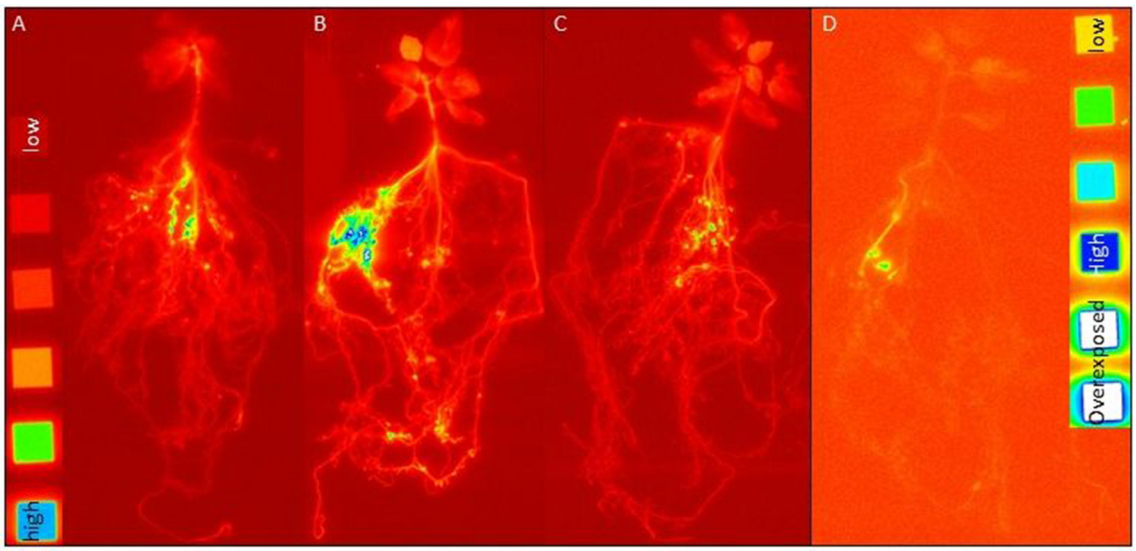

FIGURE 2 | Images of harvested plants from rhizotrone systems labeled with **(A)** ^{33}P-oP, **(B)** -AMP, and **(C)** -HAP (4 h exposing time to imaging plates; images developed at factor 2.3) as well as **(D)** ^{33}P-gP (72 h exposing time to imaging plates; images developed at factor 3.6) after 108 dpi. As reference, C-14 polymer sources were used with following activities [dpm cm^{-2}]: 1,107,000, 424,000, 112,000, 41,500, 13,350, and 3,950, supplemented by an activity free background (each activity had a surface area of 1 cm²). The blue color indicates areas with high ^{33}P activity.

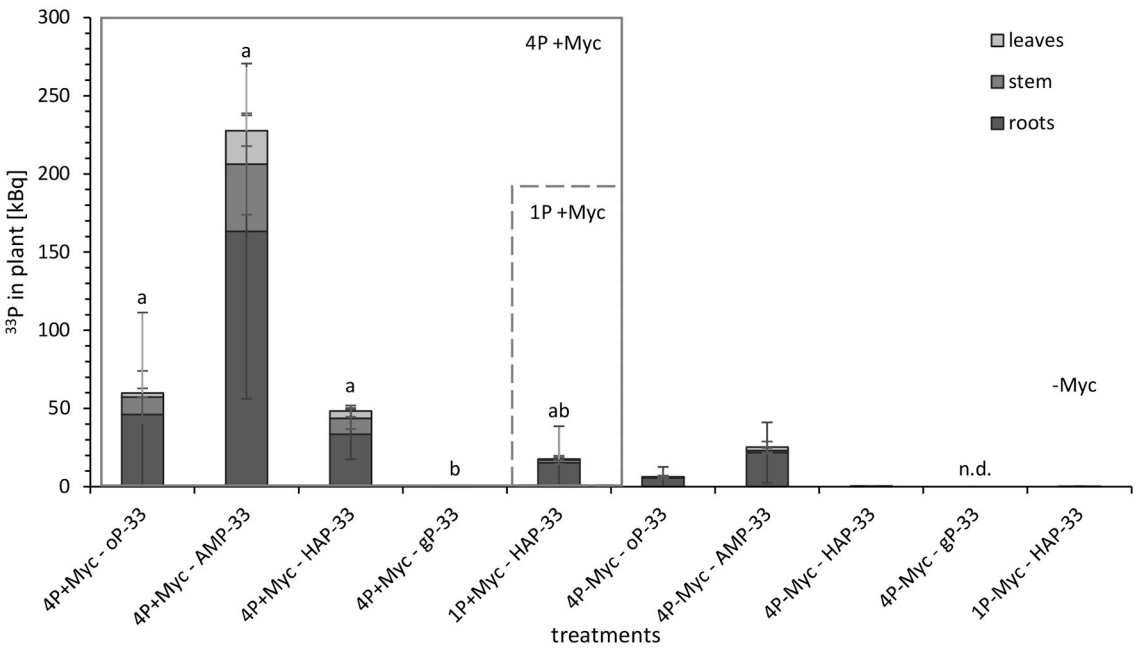

FIGURE 3 | ^{33}P activity [kBq] in different parts of mycorrhizal (+Myc) and non-mycorrhizal (-Myc) poplar plants at 108 dpi. 4P experiment with simultaneous supply of four different P sources: oP, *ortho*-phosphate; AMP, adenosine monophosphate; gP, oP bound to goethite; HAP, synthesized hydroxyapatite; and 1P control experiment supplied with HAP. The error bars show the standard deviation (4P+Myc: $n = 4$; 4P-Myc: $n = 3$; 1P+Myc: $n = 4$; 1P-Myc: $n = 2$). Data replaced by n.d. was below detection limit. The letters (a, b, c) indicate significant differences in ^{33}P activity in plant between 4p-^{33}P and 1HAP-^{33}P treatments: $P < 0.03$ (Kruskal-Wallis-*H*-test and subsequent Mann-Witney-*U*-test; see **Supplementary Table 3** for detailed information on statistical analysis).

a mineral P form supplied as a single P source in the system to support the general assumption that a minor diversity in belowground resources, e.g., due to fertilization, resulting in a reduced uptake efficiency (18). We can confirm the previous findings of Andrino et al. (26, 27), as our compartmental system using nylon and hydrophobic membranes to separate the P

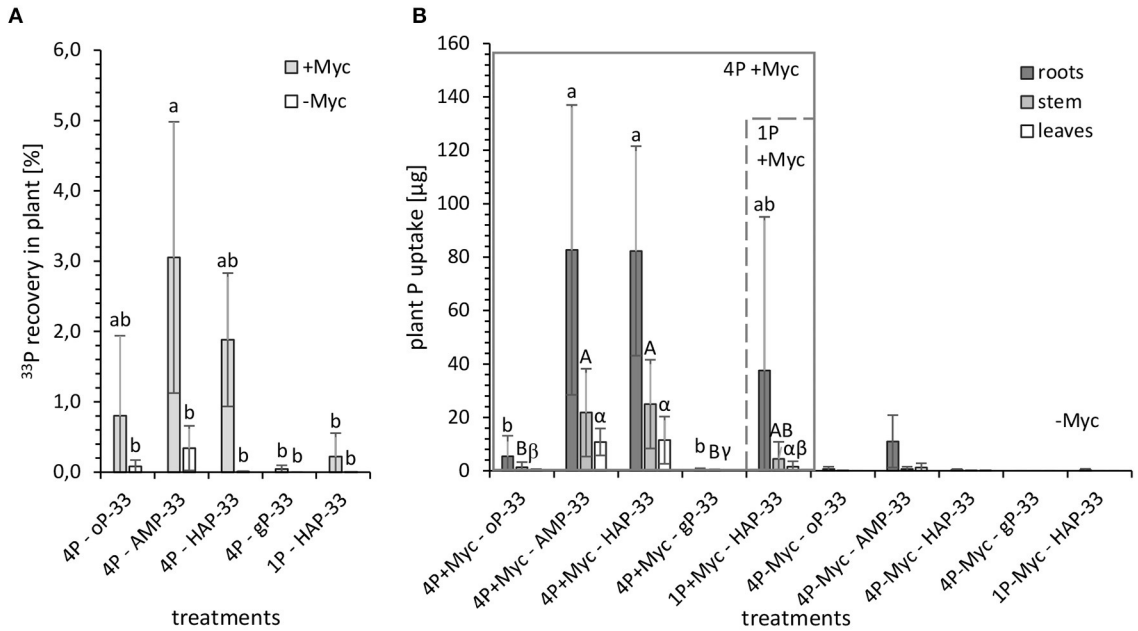

FIGURE 4 | (A) ^{33}P recovery [%] in plant and **(B)** plant P uptake [µg] from ^{33}P labeled P sources from mycorrhizal (+Myc, $n = 4$) and non-mycorrhizal (-Myc, $n = 3$ for 4P-^{33}P; and $n = 2$ for 1HAP-^{33}P) rhizotrones equipped with P containers with different P sources: 4P experiment with simultaneous supply of four different P sources: oP, *ortho*-phosphate; AMP, adenosine monophosphate; gP, oP bound to goethite; HAP, synthesized hydroxyapatite; and 1P control experiment supplied with HAP. The error bars show the standard deviation. The letters (a, A, α) indicate significant differences between the P treatments of 4P-^{33}P and 1HAP-^{33}P experiments: $P < 0.003$ (see **Supplementary Table 4** for more details).

sources from plant roots was a good choice to test mycorrhizal mediated plant P uptake. The small and negligible quantities of ^{33}P activity detected in non-mycorrhizal treatments possibly result from "leakiness" due to the process of autoclaving of the P compartments to sterilize them and create axenic conditions inside the system. After the autoclaving, the membranes could not adhere so tight anymore at some weaker spots of the P compartment, which we did not examine in this study. The autoclaving of the P compartments was chosen, as it was possible to sterilize a vast amount of P compartments simultaneously and thereby avoid the use of any chemicals and safe time of the preparation of the culture systems.

Our results confirm a successful mycorrhizal mediated plant P uptake from the P compartments, independent of the P form taken up from a pool of differently available P sources or a single P source. The mycorrhizal mediated plant P uptake from the P compartments of the P treatments resulted in the gain in plant biomass of a similar magnitude as absolute plant P content compared to the mycorrhizal no P control treatment. Also, the incorporation of the ^{33}P label (**Figures 2–4**) in mycorrhizal plants indicates a P uptake from all supplied P sources. Due to the axenic conditions prevalent in our system, we have excluded any competition with other microorganisms inhabiting the plant rhizosphere (40) and the fungal hyphosphere (41), which consume nutrients released by hyphae and root or also by feeding on hyphae (40, 42, 43), and which could reduce the control on the mycorrhizal fungal supply of P to plant. The images of harvested plants (**Figure 2**) showed areas with the highest 33P label incorporated in roots with high intensity in the branching of the root tips (**Supplementary Figure 3**), indicating that a high proportion of incorporated 33P label in the plant was detectable at the mycorrhizal fungus-root interface. This observation could result from a higher shielding of 33P by roots and shoots compared to the hyphal mantel around the root tips. In contrast to this assumption, the microradiographic localization of 33P in ectomycorrhizal poplar roots performed by Bücking and Heyser (44) has revealed that P taken up by hyphae accumulates rapidly in the hyphal mantel around the root tip and is slowly allocated through the Hartig net to the root cortical cells. Also, previous studies using excised roots detected an accumulation of P in the fungal sheet around the root tips and linked it to mycorrhizal fungal control on the amounts of P translocated to its host plant (11, 30). Nevertheless, results from experiments using excised plant roots should be handled with care, as they do not provide the same extent of a whole plant controlling its P demand and the P translocation by ECM fungus. The first successful attempt to describe the molecular processes of P transfer through the ectomycorrhizal Hartig net to plant roots under controlled conditions were made by the study of Becquer et al. (45). The H^+:P_i transporter, HcPT2, of *Hebeloma cylindrosporum* was determined not only in the extra-radical hyphae, serving for the P_i entry into the mycelium, but also in the Hartig net, aiding for the transfer of P_i to the host plant *Pinus pinaster*. Thereby, the host plant was also found to induce the expression of HcPT2, revealing that the host plant is regulating its P supply by the mycorrhizal fungus.

Nevertheless, mycorrhizal fungal P uptake preferences in an axenic system should more closely reflect the potential

capabilities (niches) (46, 47) in acquiring differently available P sources. The higher incorporation of the ^{33}P label and especially the significantly higher plant P uptake from AMP compared to oP was surprising. We have also expected that the fungus would start the P acquisition from oP, but within a week (27–34 dpi), we have detected a simultaneous uptake of oP and AMP. Nevertheless, our results cannot approve the assumption that the mycorrhizae prefer or would start to acquire the readily available oP. Free P_i has to be just taken up from the solution *via* the plasma membrane phosphate transporters at fungal hyphae (40), whereby the mycorrhizal fungus would not need to apply any acquiring mechanisms, causing no costs for the P mining. In contrast, the AMP is assumed to require a phosphomonoesterase to release the phosphate ion (P_i) (19). Previous studies provide evidence that the ectomycorrhizal fungal species *P. involutus* can produce phosphatases, which are largely surface-bound (48, 49). The phosphatase activity is considered to be induced by the substrate or a low concentration of P_i (7). Furthermore, the results of Scheerer et al. (50) have proposed that AMP (and/or ADP) or at least the nucleoside adenosine consisting of ribose and adenine could be taken up by excised roots of poplar cuttings and beech seedlings *via* some nucleotide transporters of the root, which were not indicated to date. Their assumption was built due to the uptake rates of ^{13}C and ^{15}N but also of ^{33}P of ATP tested as a P source for plant P uptake. We could not find experimental evidence for the uptake of the whole molecule of AMP or adenosine by mycorrhizal mycelium in the present literature. Still, it was already suggested by Rennenberg and Herschbach (51) that ectomycorrhizal fungi could take up organic P sources as a whole molecule. Many mycorrhizal species, including *P. involutus*, possess the ortholog of the yeast P_{org} transporter, ScGit1p (52). This transporter of *Saccharomyces cerevisiae* is upregulated under P limiting conditions and can import phospho-diesters (53). The activity of these transporters in ectomycorrhizal fungi/symbiosis was not identified to date and present a knowledge gap in the biochemistry of mycorrhizal plant P uptake. Nevertheless, this hypothesized mechanism could probably explain the mycorrhizal favoritism of AMP over oP in our study. The adenine could serve as an additional C and N source to the mycorrhizal fungal associate, reducing the dependency on C supply from the host plant.

However, the plasma membrane P_i transporters of ectomycorrhizal fungi of Basidiomecete are coupled with H^+ symporter (54). Also, the Git1p was shown to function as an H^+ symporter (55). Hence, with each imported P_i/P_{org} molecule, the P solution would lose one H^+, by which the pH in the P solution would increase. Also, the performance of Git1p was shown to drop with increasing pH (56). Likewise, in our study, the pH (**Supplementary Figure 6B**) increased significantly in harvested AMP solution of mycorrhizal treatments compared to the non-mycorrhizal treatments but also compared to the pH of 5.2 set initially. Considering the higher incorporation of the ^{33}P label from AMP, followed by oP and HAP, the pH increase in these P solutions in mycorrhizal treatments should be a consequent outcome.

On the one hand, if the activity of the Pi or Git1p transporters decreases with increasing pH, the similar plant P uptake from AMP and HAP could be explained by the pH increase in the AMP solution. We could detect the first incorporation of the 33P label in the plant from HAP only 1 week later compared to AMP and oP in the 4P system. On the other hand, HAP was supplied with a 2-fold higher P amount initially, which could indicate that besides the P source availability also the P amount of a P source is additionally essential for the choice of P uptake from a pool with different P sources by mycorrhizal fungus and its delivery to the plant. To underpin this assumption, shifts in nutritional strategies from acquisition (weathering of P rich primary minerals) to recycling (of accumulated P stocks in organic or secondary mineral P forms) were already observed from P-rich to P-poor temperate beech forests (7), suggesting that the most profuse P sources in an ecosystem are (of course depending on their bioavailability) the most favorable for the acquisition.

Inorganic P bound to metal oxides is considered not or only hardly accessible for P uptake by mycorrhizal plants and accumulating in the Lüss subsoil of forests limited in P (10). This was indebted to the high abundance of Fe and Al oxides in the Bw horizon and comprehensive adsorption of P to the mineral surface. Thus, it might not be surprising that the mycorrhizal mediated plant P uptake from gP was of the lowest magnitude compared to the other P sources supplied in the mixed pool. The desorption of P_i from goethite in H_2O is affected by sorption-desorption equilibrium and diffusion transport (35). Further, low molecular organic anions (LMWOAs) increase the release and availability of P_i sorbed to goethite *via* ligand exchange. The ectomycorrhizal fungal genera Paxillus were described to release extensive amounts of LMWOAs (57, 58). *P. involutus* was also shown to produce and exude oxalate (59–61), which is suggested to be also a key component in apatite dissolution for the P uptake by ectomycorrhizal fungi and immobilization of Ca into Ca-oxalate (62). Furthermore, it was shown for arbuscular mycorrhizae that if the P source occurs in a not dissolved form, the P is more uniformly shared between the mycorrhizal associates (26). The higher retention of P in hyphae was considered to be a response to P bioavailability (63) and enabled the arbuscular mycorrhizal fungus to invest in the growth of its hyphae to reach the not soluble P sources (64, 65). However, the plant P uptake from gP (**Figure 4B**; **Supplementary Table 4)** was lower compared to the easy desorbable P from the same P source. Nevertheless, this parameter represents the maximal desorption rate of P bound to goethite supplied in dH_2O suspended form, which was obtained by shaking the gP suspension for 24 h. In our study, the filled P compartments and the rhizotrone systems were handled with care and did not experience extensive shaking. We could instead attribute the lower P uptake from gP to its voluminous but at the same time compact consistency, which indirectly categorizes gP as a more complex P source within the mixed pool in our study. It has been suggested that ectomycorrhizal fungi can function as "biosensors" searching for nutrients from mineral sources and differentiate the mineralogy and grain sizes (61, 62). This could have lead *P. involutus* to favor HAP over gP in our study. Nevertheless, for all these reasons, we can conclude that the later and lower P incorporation in the *P. involutus* ectomycorrhizal plant could be due to the higher complexity of gP as a P source and the lower ^{33}P activity applied initially compared with other P sources.

The absolute P content in the plant and leaves of 4P treatments was significantly higher compared to the 1P treatment. This indicates that the fungus can supply the plant with more P either due to the presence of specific P forms in a mixed P pool or due to a general effect of P source diversity. To explain the latter effect of P source diversity with more favorable and readily available P sources besides the more complex P sources, we could assume that a mixed P pool might work as a "spiking" instrument to ensure an easier and faster familiarization of ectomycorrhiza to P sources with different bioavailabilities. Nevertheless, both statements are strongly supported by the significantly higher incorporation of the ^{33}P label [kBq] from AMP and its significantly higher ^{33}P recovery [%] in plant leaves compared to the 1P-HAP treatment. Higher P content in leaves of 4P plant treatments presumably allowed the earlier acquisition of HAP in 4P systems compared to the 1P system, as a higher C translocation to the HAP compartments is required to release Pi through exudation of organic anions (62). In contrast, photosynthesis is restricted under P limiting conditions (66), reducing at the same time the translocation of C to the below-ground infrastructure in order to mine the HAP patches. The lower P content in leaves of 1P treatment could result in lower photosynthetic fixation of CO_2 that consequently could result in a lower C allocation to the mycorrhizal partner for the acquisition of HAP supplied as a single P source and could explain its later uptake. Nevertheless, there was no difference in plant P uptake from HAP supplied as a single P source or with different P sources, even though the amount of P supplied with HAP as a single P source was 1/3-fold higher compared with the sum of P amounts supplied with the four different P sources in the 4P system.

CONCLUSION

In the conducted rhizotrone experiment under axenic conditions, our results indicate that the representative ectomycorrhizal fungus *P. x involutus* was able to access all P sources (oP, AMP, HAP, and gP) we have supplied with the P pool, suggesting that this ectomycorrhizal fungus can act as a generalist in P acquisition. We assume a different weighting between the ectomycorrhizal species offering a playground for further research. Nevertheless, we confirm that specialization to the acquisition of one or a few specific P sources while having access to many in order to find a niche of an appropriate resource occur as an option or need due to competition for limited soil resources (67). Ectomycorrhizal fungi, compared with other P acquiring strategies of plants, could be unequally capable of occupying a broad variety of niches (68), which could explain the dominating occurrence of ectomycorrhizal fungi in many boreal (69–71) and temperate (70, 72, 73) forest ecosystems limited in P, controlling the occurrence of other species. Our results indicate that nutrient niching is possible in one species of ectomycorrhiza and that P resource diversity improves plant nutrition, providing supporting evidence for higher functional diversity by nutrient source partitioning.

In our study, from the mixed P pool, the sources AMP and HAP (over oP) were the most favorable to ectomycorrhizal mediated plant P uptake. We conclude that the magnitude of plant P uptake from a mixed pool containing P sources of different availabilities depends on the P species itself, additional nutrients coming with the specific P sources, and the complexity of the P source. In contrast, the order of uptake of different P forms depended on their complexity (oP and AMP over HAP and gP) and supplied diversity (HAP in a pool over HAP alone).

Even though we have used one mycorrhizal fungus and one plant only, to our knowledge, we are the first in showing that the ectomycorrhizal *P. x canescens* can occupy simultaneously fundamental niches of various P sources through the mediation of its mycorrhizal fungal partner *P. involutus*, indicating that the acquisition of differently available P sources by an ectomycorrhiza can facilitate niche plasticity and adaptation to specific nutrient limiting conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JB, KS, AA, GG, AF, and LS designed the experiment. KS prepared the plant, fungal material, everything for the rhizotrone culture systems, performed the experiment, sample, data analysis, and wrote the paper. DH synthesized the hydroxyapatite and irrigated and imaged the systems from day 10 to 88 as well as performed LSC analysis of plant extracts. JB and GG supervised the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.865517/full#supplementary-material

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Supplement of

Mycorrhizal mediated partitioning of phosphorus: ectomycorrhizal (*Populus x canescens* x *Paxillus involutus*) potential to exploit simultaneously organic and mineral phosphorus sources

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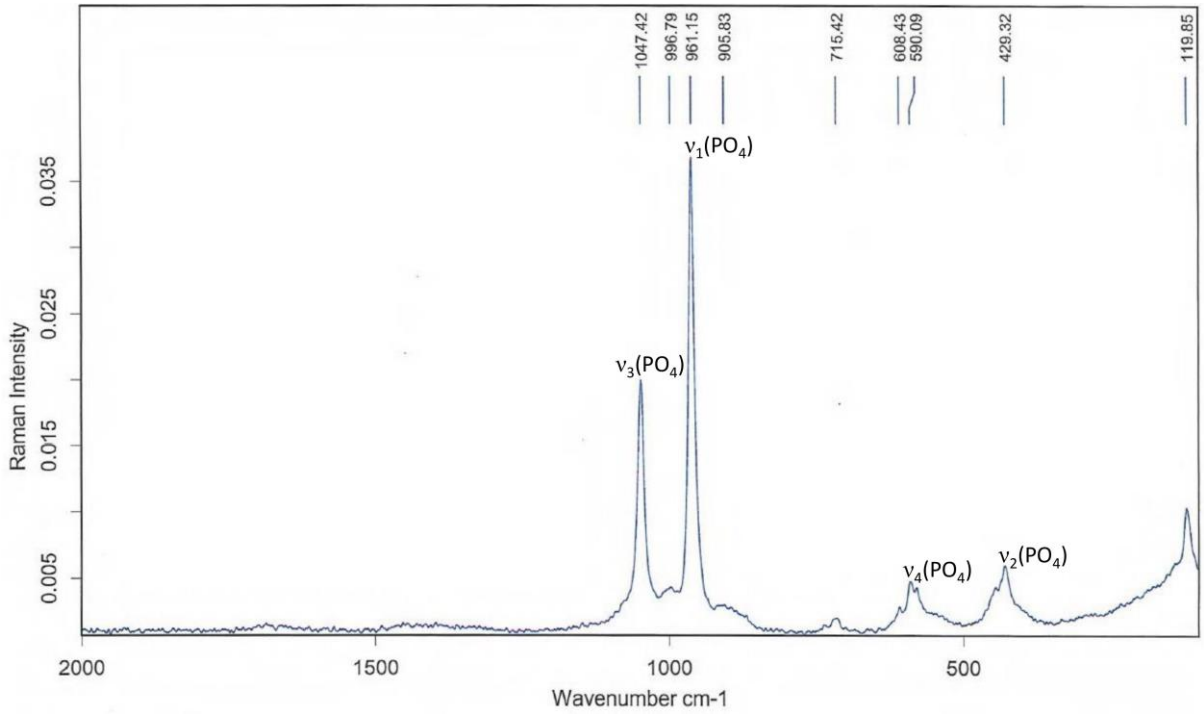
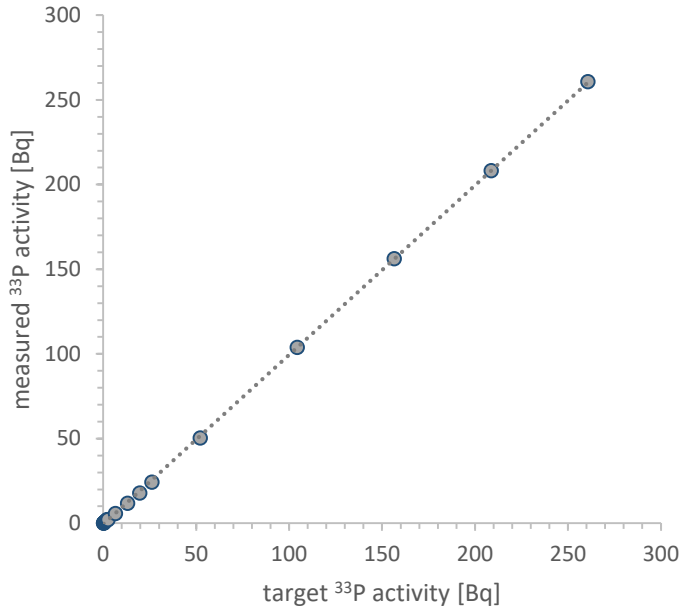
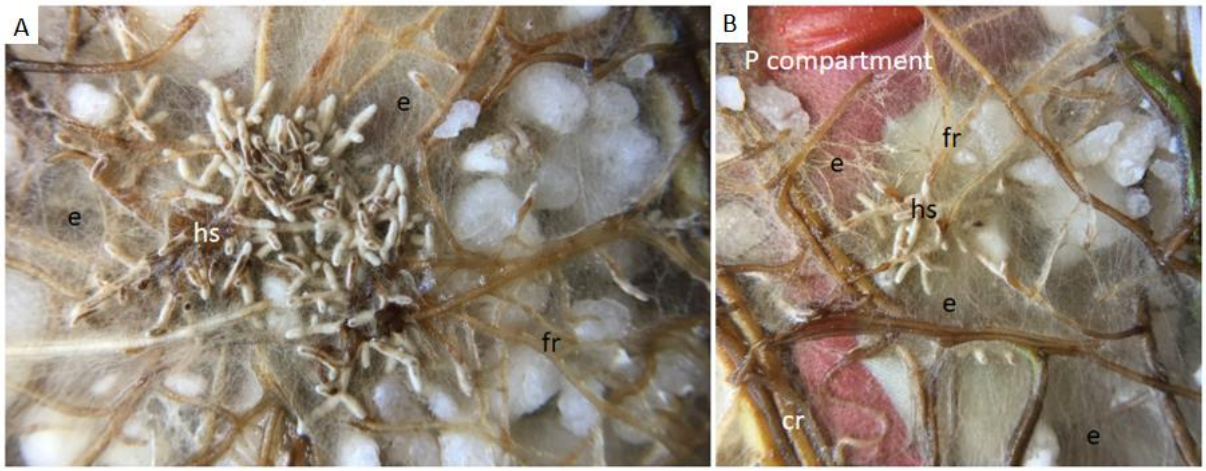


Supplementary Figure 1 | Raman spectrum (2° cm^{-1}) of hydroxyapatite (HAP), synthesized **at 25 °C**. The typical phosphate bands are highlighted as ν_1, ν_2, ν_3, and ν_4. The Raman analysis was conducted using a Bruker RFS 100/S FT-Raman spectrometer (including a Nd:YAG laser (1064 mm, 500 mW) as an excitation source and the OPUS software).



Supplementary Figure 2 | Linear regression between the measured and the targeted ^{33}P activity [Bq] (n=7 per measuring point) with a coefficient of determination (R^2) of 0.9999 ($P < 0.001$) and a slope (m) of nearly 1 (f(x)=1.00x-0.58). The linear regression was investigated using SPSS® Statistics 26.0 (IBM® Corporation, USA). The reliability of the ^{33}P measurements via the LSC was tested by a serial dilution of a sample extract with a high ^{33}P activity at 260 Bq at the day of measurement. The reproducibility of measured values down to 1.5 Bq didn't deviate significantly from the theoretical values. The measured values starting from 1.0 to 0.5 Bq were with 8 to 5% (respectively) less reproducible. ^{33}P activity values below 0.2 Bq were with 16% standard deviation less trustful, and as a consequence, excluded from data analysis.



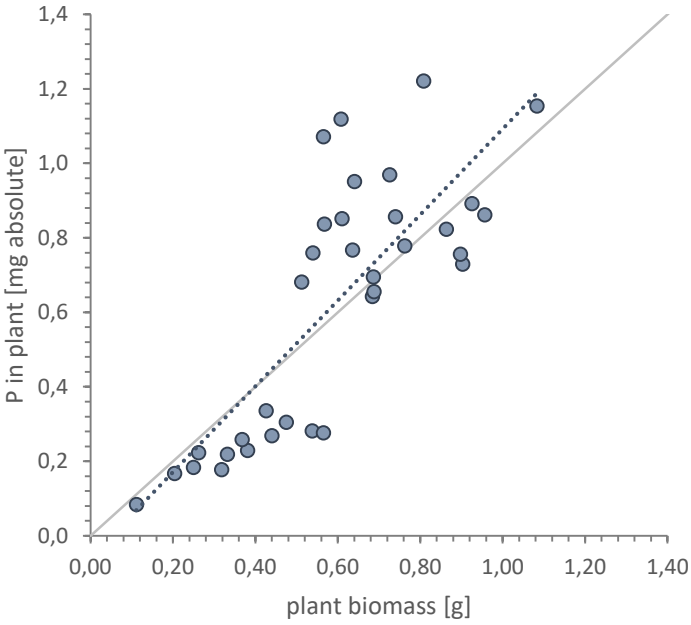
Supplementary Figure 3 | Root tips of *Populus x canescence* clone ‚Schleswig I' colonised by *Paxillus involutus* strain MAJ (108 dpi) and grown on (**A**) Perlite and (**B**) around a P container enclosing gP as a P source. hs: mantle, hyphal sheath around root tips forming the mycorrhizal root tips; e: extraradical hyphae; fr: fine roots; and cr: coarse roots.

Supplementary Table 1 | Mycorrhization grade (Eq. 6) expressed as number of mycorrhizal root tips cm^{-1} root length determined for *Paxillus involutus* ectomycorrhizal plant roots of poplar plantlets *Populus x canscens* from 4P-^{33}P (n=15), 1HAP-^{33}P (n=4), and H_2O control (n=2) experiments. Data indicate means ± standard deviation.

System	# mycorrhizal root tips cm^{-1} root length
4P-^{33}P	1.88 ± 0.74
1HAP-^{33}P	2.70 ± 1.22
H_2O control	2.88 ± 0.35

Supplementary Table 2 | The data of plant biomass and the absolute P content (n=37) in different parts of mycorrhizal (+Myc) and non-mycorrhizal (-Myc) plants (see **Figure 1** for details on significant differences). Abbreviations are: 4P experiment with simultaneous supply of four different P sources: oP (*ortho*-phosphate), AMP (adenosine monophosphate), gP (oP bound to goethite), and HAP (synthesised hydroxyapatite); and 1P control experiment supplied with HAP.

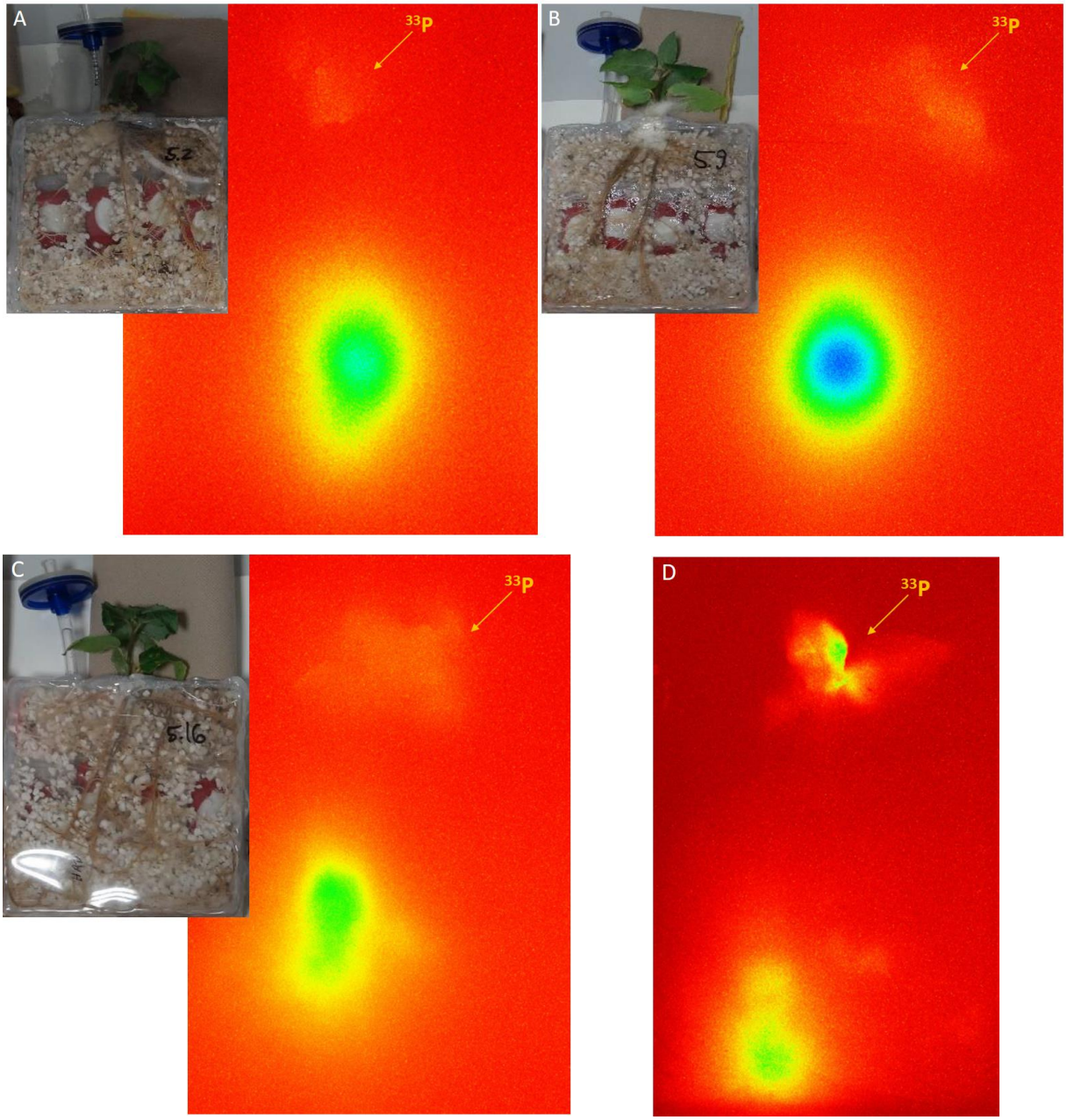
		4P-^{33}P		1HAP-^{33}P		H_2O control	
		+Myc	-Myc	+Myc	-Myc	+Myc	-Myc
biomass	plant	0.73 ± 0.13	0.38 ± 0.14	0.79 ± 0.23	0.29 ± 0.04	0.39 ± 0.05	0.24
[g]	leaves	0.14 ± 0.03	0.06 ± 0.03	0.10 ± 0.06	0.04 ± 0.01	0.08 ± 0.01	0.03
	stem	0.10 ± 0.02	0.04 ± 0.03	0.10 ± 0.03	0.03 ± 0.00	0.06 ± 0.02	0.03
	root	0.50 ± 0.10	0.27 ± 0.09	0.60 ± 0.15	0.22 ± 0.04	0.25 ± 0.06	0.18
	substrate	10.4 ± 1.2	9.2 ± 1.3	12.2 ± 0.9	11.0 ± 1.6	14.6 ± 1.5	10.5
P [mg]	plant	0.88 ± 0.16	0.27 ± 0.15	0.85 ± 0.22	0.20 ± 0.03	0.49 ± 0.11	0.17
	leaves	0.11 ± 0.05	0.03 ± 0.01	0.06 ± 0.03	0.02 ± 0.00	0.06 ± 0.01	0.02
	stem	0.21 ± 0.06	0.08 ± 0.05	0.20 ± 0.07	0.04 ± 0.00	0.13 ± 0.04	0.04
	root	0.56 ± 0.12	0.17 ± 0.10	0.60 ± 0.13	0.14 ± 0.04	0.31 ± 0.08	0.11
	substrate	0.26 ± 0.09	0.19 ± 0.10	0.21 ± 0.01	0.32 ± 0.12	0.40 ± 0.09	0.20



Supplementary Figure 4 | Linear regression between the absolute P content in plant and plant biomass (n=37) with a coefficient of determination (R^2) of 0.638 ($P < 0.001$) and a slope (m) of nearly 1 (f(x)=1.15x+0.06). The linear regression was investigated using SPSS® Statistics 26.0 (IBM® Corporation, USA).

Supplementary Table 3 | The data of ^{33}P activity in plant parts and substrate of mycorrhizal (+Myc) and non-mycorrhizal (-Myc) treatments. Abbreviations are: 4P experiment with simultaneous supply of four different P sources: oP (*ortho*-phosphate), AMP (adenosine monophosphate), gP (oP bound to goethite), and HAP (synthesised hydroxyapatite); and 1P control experiment supplied with HAP. Data indicate means ± standard deviation. Following number of replicates were existent: 4P-^{33}P: +Myc, n=4, and -Myc, n=3; 1HAP-^{33}P: +Myc, n=4, and -Myc, n=2. Data replaced by n.d. was below detection limit. Data cursively highlighted is not normally distributed. All data had not homogeneous variances. The letters (a-b) indicate significant differences in ^{33}P between the P treatments of 4p-^{33}P and 1HAP-^{33}P experiments: $P < 0.03$ (Kruskal-Wallis-H test and subsequent Mann-Witney-U test; there were no significant differences between the non-mycorrhizal treatments).

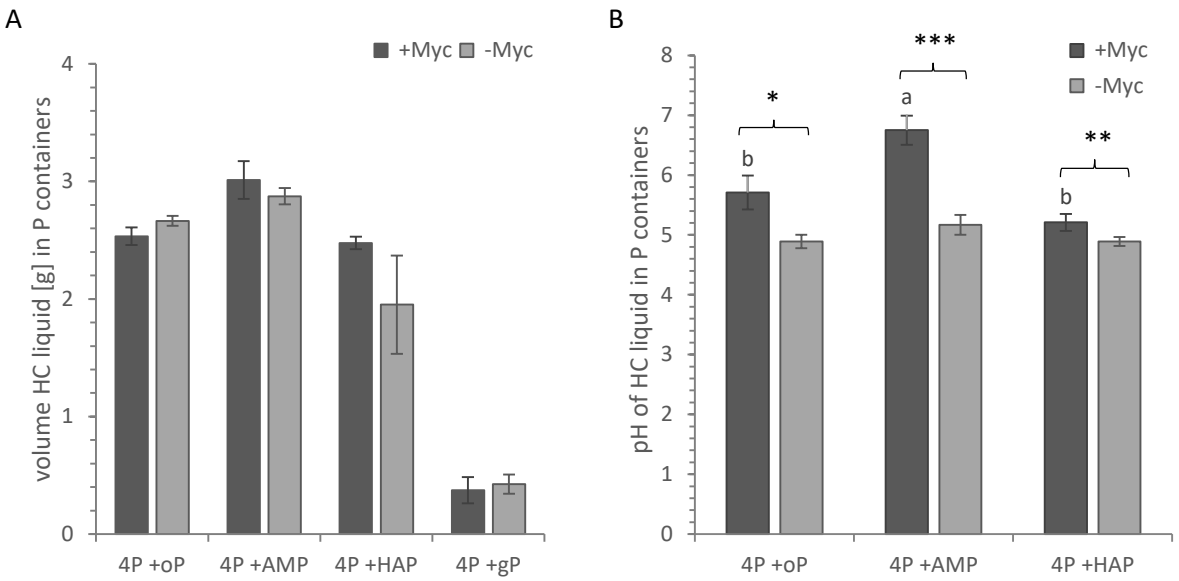
		^{33}P activity [kBq]	
		+Myc	-Myc
4P +oP-^{33}P	whole plant	*59.9 ± 84.6* [a]	*6.1 ± 6.8*
	leaves	2.62 ± 2.92 [b]	0.52 ± 0.74
	stem	*11.2 ± 16.7* [a]	0.24 ± 0.32
	root	*46.1 ± 65.2* [a]	5.56 ± 7.10
	substrate	5.20 ± 4.43 [a]	*0.002 ± 0.004*
4P +AMP-^{33}P	whole plant	227 ± 144 [a]	25.4 ± 23.7
	leaves	21.3 ± 9.9 [a]	2.29 ± 3.42
	stem	43.0 ± 32.5 [a]	1.34 ± 1.61
	root	163 ± 107 [a]	21.7 ± 19.3
	substrate	32.2 ± 32.0 [a]	4.32 ± 3.89
4P +HAP-^{33}P	whole plant	48.3 ± 24.1 [a]	0.21 ± 0.19
	leaves	4.67 ± 3.60 [b]	*0.02 ± 0.04*
	stem	10.2 ± 6.8 [a]	*0.03 ± 0.06*
	root	*33.5 ± 16.0* [a]	0.15 ± 0.13
	substrate	9.2 ± 10.5 [ab]	n.d.
4P +gP-^{33}P	whole plant	0.17 ± 0.21 [b]	n.d.
	leaves	n.d.	n.d.
	stem	*0.05 ± 0.09* [b]	n.d.
	root	*0.12 ± 0.14* [b]	n.d.
	substrate	n.d.	n.d.
1P +HAP-^{33}P	whole plant	*17,7 ± 26.8* [a]	0.17 ± 0.24
	leaves	0.64 ± 0.83 [b]	n.d.
	stem	1.80 ± 2.61 [ab]	n.d.
	root	*15.3 ± 23.4* [a]	0.11 ± 0.20
	substrate	0.16 ± 0.20 [b]	n.d.



Supplementary Figure 5 | Images of 4P rhizotrone culture systems ^{33}P labelled (**A**) oP, (**B**) AMP (4h exposing time to imaging plates; images developed at factor 2) after 34 dpi, and (**C**) HAP (72 h exposing time to imaging plates; images developed at factor 2) after 42 dpi, as well as (**D**) 1P control system with ^{33}P labelled HAP (72 h exposing time to imaging plates; images developed at factor 1.3) after 94 dpi. The blue color indicates areas with highest ^{33}P activity. The yellow arrows point the ^{33}P label incorporated in plant leaves.

Supplementary Table 4 | The calculated ^{33}P recovery [%]in plant parts and substrate as well as P uptake by plant and P retention in substrate [µg] of mycorrhizal (+Myc) and non-mycorrhizal (-Myc) treatments. Abbreviations are: 4P experiment with simultaneous supply of four different P sources: oP (*ortho*-phosphate), AMP (adenosine monophosphate), gP (oP bound to goethite), and HAP (synthesised hydroxyapatite); and 1P control experiment supplied with HAP. Data indicate means ± standard deviation. Following number of replicates were existant: 4P-^{33}P: +Myc, n=4, and -Myc, n=3; 1HAP-^{33}P: +Myc, n=4, and -Myc, n=2. Data cursively highlighted is not normally distributed. All data had not homogeneous variances. Marking with (a, b, c, d) indicate significant differences in ^{33}P recovery between 4p-^{33}P and 1HAP-^{33}P treatments: $P < 0.03$ (Kruskal-Wallis-H test and subsequent Mann-Witney-U test).

		^{33}P recovery [%]		P uptake/retention [µg]	
		+Myc	-Myc	+Myc	-Myc
4P +oP-^{33}P	whole plant	*0.80 ± 1.13* [a]	0.082 ± 0.091	*7.05 ± 9.95* [b]	0.72 ± 0.80
	leaves	0.035 ± 0.039 [a,c]	*0.007 ± 0.010*	0.31 ± 0.34 [b]	*0.061 ± 0.087*
	stem	*0.15 ± 0.22*	0.0032 ± 0.0043	*1.31 ± 1.97* [b]	0.028 ± 0.038
	root	*0.62 ± 0.87* [a]	*0.074 ± 0.095*	*5.43 ± 7.67* [b]	0.65 ± 0.83
	substrate	0.070 ± 0.059 [a,b]	0.00003 ± 0.00005	0.61 ± 0.52 [b]	*0.0003 ± 0.0004*
4P +AMP-^{33}P	whole plant	3.05 ± 1.93 [a,b]	0.34 ± 0.32	115.28 ± 72.83 [a,b]	12.84 ± 12.01
	leaves	0.29 ± 0.13 [a,b,c]	0.031 ± 0.46	10.79 ± 5.03 [b]	1.16 ± 1.73
	stem	0.58 ± 0.44 [a,b]	0.018 ± 0.022	21.79 ± 16.44 [a,b]	0.68 ± 0.82
	root	2.19 ± 1.44 [a]	0.29 ± 0.26	82.70 ± 54.30 [a,b]	11.01 ± 9.80
	substrate	0.43 ± 0.43 [a,b]	0.058 ± 0.052	16.30 ± 16.22 [b,c]	2.19 ± 1.97
4P +HAP-^{33}P	whole plant	1.88 ± 0.95 [a]	0.0081 ± 0.0075	118.75 ± 59.28 [a,b]	0.51 ± 0.47
	leaves	0.18 ± 0.14 [a,b]	*0.0009 ± 0.0015*	11.47 ± 8.84 [b]	*0.053 ± 0.093*
	stem	0.40 ± 0.27 [a,b]	*0.0014 ± 0.0023*	24.98 ± 16.64 [a,b]	*0.085 ± 0.147*
	root	*1.30 ± 0.62* [a]	0.0059 ± 0.0051	*82.3 ± 0.052* [a,b]	0.37 ± 0.32
	substrate	0.36 ± 0.41	-	82.31 ± 39.23	-
4P +gP-^{33}P	whole plant	0.027 ± 0.035 [a,c]	-	0.61 ± 0.76 [a]	-
	leaves	-	-	-	-
	stem	*0.008 ± 0.015* [a,c]	-	*0.17 ± 0.34* [a]	-
	root	0.019 ± 0.022 [a,c]	-	*0.43 ± 0.51* [a]	-
	substrate	-	-	-	-
1P +HAP-^{33}P	whole plant	*0.22 ± 0.33* [b]	0.0014 ± 0.0024	*43.59 ± 65.82*	0.28 ± 0.48
	leaves	0.008 ± 0.010 [b]	-	1.57 ± 2.05	-
	stem	0.022 ± 0.033 [b]	-	4.42 ± 6.40	-
	root	*0.19 ± 0.29* [b]	0.0014 ± 0.0024	*37.59 ± 57.50*	0.28 ± 0.48
	substrate	0.002 ± 0.002 [b]	-	0.39 ± 0.49 [c]	-



Supplementary Figure 6 | (**A**) The liquid content [g] and (**B**) its pH of P containers enclosing different P sources (oP: ortho-phosphate; AMP: adenosine-mono-phosphate; HAP: synthesized hydroxyapatite; and gP: oP bound to goethite) of mycorrhizal (+Myc) and non-mycorrhizal (-Myc) poplar plants of the 4P-33 experiment. The error bars show the standard deviation ((**A**) +Myc oP and AMP, n=16; +Myc HAP, n=13; +Myc gP, n=12; -Myc oP and AMP, n=11; -Myc HAP and gP, n=9; (**B**) +Myc oP and AMP, n=15; +Myc HAP, n=12; -Myc oP and AMP, n=12; -Myc HAP, n=9). Different letters indicate significant differences between the pH of mycorrhizal treatments ($P < 0.0006$); and *** $P < 0.00002$, ** $P < 0.04$, * $P < 0.05$).

5 General Discussion

P is an essential element for the plant net primary productivity (Plassard and Dell, 2010), but it is also limited in soils in many terrestrial ecosystems (e.g. Elser et al., 2007), as more than 90% of P in the soil is present in different chemical forms that are not available to plants (Mengel et al., 2001). The association of plants with mycorrhizal fungi can increase the bioavailability of P (Hinsinger, 2001; Plassard et al., 2011). Moreover, resource partitioning of P associated with mycorrhizal fungi could contribute to more efficient use of different P forms by plants, reducing competition for soil P (Turner et al., 2008). Ectomycorrhizae were shown to mine different chemical forms of P (reviewed by Plassard et al., 2011) in exchange for energy derived from hosts' photosynthesis (Buscot et al., 2015), indicating a high potential as C sink (Finlay and Söderström, 1992). Nevertheless, experimental evidence for P source dependent C sink potential of ectomycorrhizae as well as for their sole contribution in resource partitioning for P is missing. In order to understand such ecosystem situations, (i) in Study I, I have developed compartmental mesocosm and rhizotrone culture systems for the Studies II and III, respectively. Further, I aimed to investigate, (ii) the P source dependent C investment from the host plant for the mycorrhizal mediated P derived from different P species (oP, Phy, AP, gP; Study II); as well as (iii) the ectomycorrhizal preferences to specific P sources from a mixed P pool (oP, AMP, HAP, gP; Study III) and the comparison of the P uptake from the mineral P source, HAP, from a pool or as a single P source.

In Study II, I have leaf-fertilized the plants with $^{13}C^{15}N$-labeled and non-labelled urea, which allowed me to look at the partitioning of urea-derived ^{13}C (UDC [$\mu g\ mg^{-1}\ h^{-1}$]; Eq. 1-2) between plant shoots and roots. I could find a trend for dependencies between the P source bioavailability and the related exchange of plant C for ectomycorrhizal fungal P. Whereas, in Study III, in order to distinguish the uptake between the different P sources from a mixed pool, I have applied the labelling with ^{33}P. The results showed that an ectomycorrhizal plant has the potential to utilize all provided P sources via its mycorrhizal fungal associate.

5.1 Culture System to mimic Ecosystem Situations

To investigate the research objectives (ii) and (iii), in Study I, I have developed compartmental mesocosm and rhizotrone culture systems for greenhouse or controlled conditions, respectively. By

using separate compartments for the different P sources, I aimed to mimic the ecosystem situation with an ectomycorrhizal plant having access to widely distributed P source patches with different bioavailabilities (**Figure 1**) through mycorrhizal fungus, excluding the direct strife of plant roots and mycorrhizal hyphae for P. *P. involutus* is a long distance exploration type ectomycorrhizal fungus (Gronbach, 1988) with few but highly differentiated rhizomorphs (review by Agerer, 2001). These type of ectomycorrhizal fungi were shown to transport efficiently water and higher rates of P. Moreover, the ectomycorrhizal fungus *P. involutus* is compatible with the poplar plant species *P. x canescens*. Hence, these organisms provide valuable model systems for a more robust test of nutrient acquisition and exchange models (Gafur et al., 2004; Müller et al., 2013). In functional symbiosis, the mycorrhizal associates can penetrate the plant root, forming a Hartig net between the cortical cells (Rousseau et al., 1994; Hampp et al., 1996; Gafur et al., 2004), where the exchange of nutrients between the symbionts is supposed to happen (Landeweert et al., 2001). In contrast, incompetent ECM fail to penetrate the host roots, causing a defence reaction by thickening the cell wall of the epidermis (Lei et al., 1990; Gafur et al., 2004). Nevertheless, it was shown that only functional associates could increase plant P uptake in nature under impoverished nutrient conditions (Rousseau et al., 1994; Smith and Read, 2008; Hoeksema et al., 2010).

In each study, I have observed that the plant roots of *P. x canescens* were heavily mycorrhized by *P. involutus*, including changes in root morphology such as specific branching of root tips and absent development of root hairs (Brundrett et al., 1996). I could also detect the Hartig net hyphae between the cortical cells inside the roots (**Figure 2**, Study I) and a dense hyphal mantel around the root tips (**Figure 2B**, Study I). These observations of mycorrhization of *P. x canescens* with *P. involutus* (MAJ) are in line with findings made in the study of Gafur et al. (2004). The substrate and, especially, the entrance points to the P sources at the separate compartments (**Figure 9B** and **Figure 11A,B**, Study I) but also from inside (**Figure 11D**, Study I) of all provided P sources in mycorrhizal treatments were colonised by the mycelium of *P. involutus*.

Furthermore, the study of Gherghel et al. (2014) could show that *P. involutus* subsequent to *Rhizophagus irregularis* colonized poplar clones under various field conditions. Among poplars, *P. involutus* has a wide variety of hosts able to form ectomycorrhiza with many forest tree species belonging to gymnosperms and angiosperms (Duddridge, 1987; Baum et al., 2000; Gafur et al., 2004) and be an appropriate help for trees in 'bare-root' conditions (Jarosch and Bresinsky, 1999; Hönig et al., 2000). *P. involutus* was also shown to be able to exude oxalic acid to acquire P from mineral sources (Lapeyrie et al., 1991) and to release surface-bound phosphatases that can mineralize organic P forms (McElhinney and Mitchel, 1993; Alvarez et al., 2004). Furthermore, due to the natural distribution and genetic variability of *Populus* trees, they can be cultivated under polluted and degraded soil conditions (Chen and Polle, 2010) and contribute to a site's positive carbon balance.

For all these reasons, the design of a compartmental culture system using these compatible ectomycorrhizal associates was a solid choice to down-scale the ecosystem situation of P source dependent host C exchange for mycorrhizal P as well as of mycorrhizal mediated P resource partitioning. Since the protocol described by Rygiewicz et al. in 1988, the present study was the first providing details on practical experience and evaluated protocols for the design and maintenance of the experimental setups to investigate such ecosystem situations. Moreover, these culture systems were designed not only for outdoor (mesocosms; Study II) but also for controlled conditions (rhizotrones; Study III) excluding interferences with other micro-organisms, revealing the true capabilities of the mycorrhizal fungi in P acquisition.

5.2 Mycorrhizal Roots as Exchange Interface of Carbon for Phosphorus

In Study II, I have determined the UDC [µg mg^{-1} h^{-1}] as well as the UDC per [mg] P (UDC/P) in ectomycorrhizal plant roots. I have proposed that the UDC/P ratio reflect closely the P source dependent exchange of C for P between the host plant and its ectomycorrhizal fungal partner for the following reasons: The ectomycorrhizal plants in the present study showed always high mycorrhization of their roots with a dense hyphal mantel around the root tips (**Figure 3A**, Study II). Nevertheless, the images of harvested plants (**Figure 2**) in Study III showed areas with the highest ^{33}P label incorporated in roots with high intensity in the branching of the root tips (**Supplementary Figure 3**), indicating that a high proportion of incorporated ^{33}P label in the plant was detectable at the mycorrhizal fungus-root interface. But also the significantly ($P < 0.05$) and up to 50-fold lower incorporation of UDC in mycorrhizal plant roots as well as no translocation of UDC to the roots of non-mycorrhizal treatments indicate that the ectomycorrhizal fungus is the sink for the UDC, which make the ectomycorrhizal roots to the interface for the exchange of C for P between plant and ectomycorrhizal fungus in the present study. Supporting evidence for this assumption was provided by the study of Bücking and Heyser (2001), which localized a microautoradiographic distribution of P in exchange for C in the median zone of ectomycorrhizal roots of poplar seedlings. Their study could show that photosynthetically fixed ^{14}C was translocated to the hyphae of the Hartig net and homogeneously spread and accumulated in the hyphal mantel around the root tips. They also observed that P_i taken up by the ectomycorrhizal fungus was quickly accumulated in the hyphal mantel and was slowly allocated through the Hartig net into the cortical cells of the host root. Thereby, the molecular study of Becquer et al. (2018) under controlled conditions revealed that the host plant induces the P transfer through the ectomycorrhizal fungal Hartig net to plant roots.

Furthermore, the enrichment of UDC in plant was accompanied by urea-derived ^{15}N (UDN). Nevertheless, since the UDC and UDN were with significantly larger proportion incorporated in plant shoot, I assume that the plant utilizes these elements for shoot development but not for the root growth. The effect of further shoot development and stagnancy of root growth was documented previously e.g. for *Hebeloma cylindrosporum* ectomycorrhizal maritime pine seedlings, whereas the non-mycorrhizal seedlings developed lower shoot:root ratios compared to their ectomycorrhizal counterparts (Torres Aquino and Plassard, 2004).

I have performed the labelling at 165 dpi, when the system including the ectomycorrhizal fungus in the belowground were already progressively developed (**Figure 2**, Study II; **Figure 9**, Study I), indicating another possible reason for the low transfer rates of UDC into the mycorrhizal root interface. In contrast, at this stage, the UDC found in the mycorrhizal roots could reflect the real demand of the ectomyccorhizal fungal partner to acquire the respective P sources.

5.3 Phosphorus Source Specificity and their Impact on associated Carbon Costs and Phosphorus Resource Partitioning

Eventhought, the first or second hypotheses (H1 and H2) could not be approved, the data obtained from Study II showed a trend for a P source dependent exchange of UDC for P (UDC/P) in ectomycorrhizal roots. I could observe that the UDC but also the UDC/P ratio in ectomycorrhizal roots of mineral P treatments, AP and gP (mineral P effect; **Figure 5B**, Study II), were of similar magnitude and lower compared to the other P treatments. I assumed that this might result from the ectomycorrhizal fungal application of the same P acquiring mechanisms such as the exudation of low-molecular organic anions (LMWOAs) to release the free P_i (Plassard et al., 2011), requiring C investment from the host plant. To confirm this assumption, the study of Leake et al. (2008) observed an *P. involutus* ectomycorrhizal *Pinus sylvestris* allocating preferentially photosynthetically fixed ^{14}C to apatite patches and at the same time increasing its weathering. Goethite associated P_i release is increased via the ligand exchange with LMWOAs, which otherwise is caused mainly by the equilibrium of sorption and desorption processes and the diffusion transport (Yan et al., 2015). *P. involutus* was shown to produce and exude the LMWOA oxalate (Lapeyrie et al., 1987, 1991; Schmalenberger et al., 2015), which is suggested to be also a key component in apatite dissolution for the P uptake by ectomycorrhizal fungi and immobilisation of Ca into Ca-oxalate (Smith et al., 2008). Furthermore, I have observed a tendentially lower UDC/P ratio in plant shoots of mycorrhizal gP treatment (followed by the mycorrhizal no P control) compared to other P treatments. I assumed that this effect could be caused by the need of the ectomycorrhizal fungus to extend the mycelial infrastructure in foraging for

P (exploratory hyphae effect; **Figure 5A**), as compared to AP, the P source gP has a far more voluminous consistency. Hence, to reach the P from gP, the ectomycorrhizal fungus has to spread its mycelium more intensively, requiring proportionally more C for the construction of the mycelial infrastructure than AP and I could detect an extensive mycelial growth inside the P compartments containing gP (e.g. **Figure 11**, Study I) as a P source. But I cannot postulate this effect with high certainty, as the mycorrhizal treatments gP and the no P control were harvested the least (at 7th day after leaf fertilisation) compared to other mycorrhizal P treatments (at 5th or 6th day). Nevertheless, the results from Study II could imply, that the mineral P sources (and especially gP due to its consistency) are more complex and, thus, require higher C investment compared to the other mycorrhizal P treatments tested in the present study.

Also in Study III, I could identify the mineral P source gP as the least favourable P source for the mycorrhizal mediated plant P uptake from a mixed P pool that included also oP, AMP, and HAP (each P source supplied in separate compartments). The ectomycorrhizal mediated plant P uptake from the mineral P source HAP was not different to the P uptake from the most favourable P source AMP within the mixed P pool in Study III. In contrast to AP and gP, HAP was synthesised at 25 °C to represent a mineral P source that is still well accessible for P uptake (Wolf et al. 2018, 2020). Hence, as opposed to HAP, the calculated plant P uptake from the secondary mineral adsorption complex gP (max. 1.6 µg P) was clearly lower compared to the easy desorbable P (max. 369 µg P from gP), which could result from its consistency (as described above) but also from the lower ^{33}P activity applied initially with this P source. To support the former assumption, the findings from the study of Andrino et al. (2019) have to be mentioned, where it was shown for arbuscular mycorrhizae that if the P source occurs in a not dissolved form, the P is more uniformly shared between the mycorrhizal associates. The higher retention of P in hyphae was considered to be a response to P bioavailability (Ezawa et al., 2002) and enabled the arbuscular mycorrhizal fungus to invest in the growth of its hyphae to reach the not soluble P sources (Olsson et al., 2008; Hammer et al., 2011). These observations from arbuscular mycorrhizal systems could be translated to the ectomycorrhizal systems and, therefore, could explain the lowest incorporation of P from gP in Study III.

Nevertheless, I can conclude that an ectomycorrhizal plant that acquires P from more complex mineral P sources (AP and gP) could be explained as a system that invests a considerable amount of C into belowground mycelial infrastructure and the acquiring mechanism of exudation of LMWOAs. These findings are in line with the findings made previously by the studies of Andrino et al. (2019, 2021) using a different mycorrhizal type, the arbuscular mycorrhizae. Eventhough, a direct comparison of P source dependent C investment in the present study and the studies performed by Andrino et al. (2019, 2021) is not possible, as both studies have used different mycorrhizal types and different parameters

to express the C investment, but these individual observations imply that both mycorrhizal types could respond similarly to more complex mineral P sources.

In Study II beside the mineral P sources, I have tested the ectomycorrhizal fungal C sink potential for the acquisition of the organic P source Phy in dissolved form. I have expected to observe a high C drain potential in the Phy treatment, as it is considered that Phy is a more recalcitrant organic P source that requires the activity of specialised phosphatase such as phytase (Turner, 2008) and it is assumed that ectomycorrhizae invest considerable C amounts into the production and release of enzymes to mobilise nutrients (Antibus et al., 1997). Also, the ectomycorrhizal fungus *P. involutus* in axenic culture was previously shown to exhibit wall-bound phosphatase activity, eventhough, phytate hydrolysis appeared to be not as efficient as other less complex organic P substrates (McElhinney and Mitchell, 1993). Another study by Hilger and Krause (1988) observed that *P. involutus* hydrolysed phytate during the cell lysis phase through the released, intracellular phosphatases. The latter mentioned effect but also some other possible reasons discussed in detail in Study II could be responsible for the absence of the expected considerable investment of UDC for ectomycorrhizal fungal production of enzymes or uptake of P_i from Phy, as the UDC and UDC/P ratio (**Figures 4** and **5**, respectively; Study II) in plant roots of mycorrhizal Phy, oP, and no P control (H_2O) treatments were of similar magnitude. To consume the P_i, the ectomycorrhizal fungus has to take up the free phosphate (oP) from the solution via the plasma membrane phosphate transporters at fungal hyphae (Becquer et al., 2014), representing lower C sink potential compared to specialising P acquiring mechanisms such as production and release of LMWOAs or phosphatases but also extended hyphal growth. Nevertheless, I could rather observe that the P uptake, the C utilisation in aboveground biomass and the C exchange for P occur with similar efficiencies in oP treatment and the more recalcitrant Phy treatment. Therefore, I can only assume and postulate that the readily available P source oP, the soluble Phy, but also the complete limitation in P (no P control) could turn the ectomycorrhizal plant into a system of C retention in the ectomycorrhizal root interface.

Furthermore, the higher incorporation of the ^{33}P label and especially the significantly higher plant P uptake from AMP compared to oP (**Figure 4B**; Study III) was also surprising. I have expected that the fungus would start the P acquisition from oP, but within a week (27-34 dpi), I could observe a simultaneous uptake of oP and AMP. Also the AMP is assumed to require a phosphomonoesterase to release the P_i for uptake (Turner, 2008). Nevertheless, these results could not approve the assumption that the ectomycorrhizae prefer or would start to acquire the readily available oP. Instead, these results indicate that organic P sources are (AMP) or can be (Phy) more readily available for the mycorrhizal mediated plant P uptake. Dissolved AMP was favoured over oP (Study III) and Phy required no more C investment compared to oP (Study II) and, therefore, it could be the case that the adsorption to Fe, Al (in acidic soils), or Ca (in alkaline soils) (Hinsinger, 2001) would make the organic P sources

but also the oP unavailable for uptake. Also under natural conditions the organic P sources and P_i are usually not present in dissolved forms and are rapidly sorbed to secondary minerals and metal oxides (Hinsinger et al., 2011). Hence, the results of the present dissertation could indicate that not the organic P source exhibit an enormous C sink potential by *P. involutus* but rather the production and release of LMWOAs as well as the extended hyphal growth. Henceforth, the results indicate that the mineral P sources required higher C investment compared to oP and Phy (Study II), but also were the latest to be acquired from a mixed P pool (partially with lower proportions for gP; Study III).

Nevertheless, as hypothesised (H3) all supplied P sources within the mixed P pool were available for the ectomycorrhizal fungal mediated uptake by the host plant (Study III). To support this result, the study of Clausing and Polle (2020) found that the constellation of ectomycorrhizal fungi was indifferent between the soil layers, but mycorrhizal roots showed higher P uptake efficiencies in soil layers with higher P provision to plant roots (Clausing and Polle, 2020), indicating an adaptation of mycorrhizal fungi to specific environmental settings (Zavišić and Polle, 2018). Hence, it could not be surprising that from the mixed P pool (Study III), the sources AMP and HAP (over oP) were the most favourable to ectomycorrhizal mediated plant P uptake, as AMP provide additional nutrients and both AMP and HAP higher P amount. Nevertheless, there was no difference in plant P uptake from HAP supplied as a single P source or with different P sources (rejecting the last hypothesis (H4)). However, the results of Study III suggest that an ectomycorrhiza has the potential to occupy simultaneously fundamental niches of various P sources differing in their bioavailability, indicating that the acquisition of differently available P sources by *P. involutus* and provision of P to its host plant could facilitate niche plasticity.

6 Conclusion

The present dissertation provides new insights in P source dependent economy in, and resource partitioning for P by an ectomycorrhiza (*P. x canescens x P. involutus*). To my knowledge, this is the first study that has investigated C investment into acquisition of the most representative P sources differing in their bioavailability (oP, Phy, AP, gP). Also, for the first time, this study examined ectomycorrhizal mediated resource partitioning of specific P sources with different bioavailabilities from a mixed pool (oP, AMP, HAP, gP).

The tested hypotheses are presented and explained here in detail:

H1 Plant P uptake mediated by ectomycorrhizal fungus from easily available source oP will cause less C costs compared to the more complex P sources (Phy and mineral P sources AP and gP) – This hypothesis could not be completely confirmed, as only a P source dependent trend in C allocation into the ectomycorrhizal roots could be determined that represent an interface for the exchange of host C for mycorrhizal P. Thereby, the exchange of C for P was of similar magnitude for the readily available oP source, the soluble refractory organic P source Phy, or the complete P limitation in system, concluding that these P source/limiting conditions turn the mycorrhizal plant into a system of C retention in the mycorrhizal root interface. In contrast, the mineral P sources showed a different trend, as expected in hypothesis H2:

H2 Due to the differences in the required P acquiring mechanisms, the ectomycorrhizal C drain from the host plant for the acquisition of the recalcitrant, organic P source Phy will differ compared to the C drain for the mineral P sources AP and gP, as both P source types vary in their acquiring mechanisms – The retention of C in the mycorrhizal root interface of the mineral P treatments was of lowest magnitude, indicating a high sink potential for host C for these P acquiring mechanisms.

H3 All P sources within the diverse P pool are available to mycorrhizal fungi, and therefore to plant, but the order/magnitude of acquisition will depend on P source complexity/amount – The results show that an ectomycorrhizal plant is able to utilize all provided P sources simultaneously via its ectomycorrhizal fungal associate. In contrast, the magnitude was defined by the amount of supplied P source and provision of additional nutrients (AMP over oP and gP) as well as by P source complexity (gP as the least favourable P form). The

acquisition timing was determined by the most bioavailable P sources (oP and AMP over HAP and gP) and P source diversity (mixed P pool over single P source).

H4 Mineral source (HAP) as a single P source is less available than within a pool of diverse P sources – There was no difference in mycorrhizal mediated P uptake from HAP. But the mining of HAP in a mixed pool occurred faster compared to HAP supplied as single P source.

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Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der Naturwissenschaftliche Fakultät der Gottfried Wilhelm Leibniz Universität Hannover zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Ectomycorrhiza: Phosphorus Source specific Economy and Potential in Resource Partitioning

am Lehrstuhl für Bodenkunde unter der Anleitung und Betreuung von Prof. Dr. Georg Guggenberger gemäß §6(1) der Promotionsordnung eigenständig verfasst habe. Ich versichere, dass ich keine anderen, außer den genannten Literaturquellen und Hilfsmitteln, verwendet habe. Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Hannover, 05.07.2022, K. Schneider

Ort, Datum, Unterschrift

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Lebenslauf

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10/2016 – 03/2020	Wissenschaftliche Mitarbeiterin Gottfried Wilhelm Leibniz Universität Hannover Thema: ‚Phosphor-Ökonomie innerhalb der Mykorrhiza' Finanzierung: DFG-Förderungsprogram GRK1798 ‚Signaling at the Plant-Soil Interface' (10/2016-09/2019), Institutsmittel (10/2019-03/2020 und 8/2021-11/2021) sowie Promotionsabschlussförderung (02/2021-08/2021).
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Scientific Communication

Veröffentlichungen

Schreider K, Hofmann D, Boy J, Andrino A, Fernandes Figueiredo A, Sauheitl L, Guggenberger G (2022). Mycorrhizal mediated partitioning of phosphorus: ectomycorrhizal (*Populus x canescens* x *Paxillus involutus*) potential to exploit simultaneously organic and mineral phosphorus sources. *Front. Soil Sci.* 2:865517. doi: 10.3389/fsoil.2022.865517

Schreider K, Boy J, Sauheitl L, Fernandes Figueiredo A, Andrino A, Guggenberger G (*2022*). Designing a robust and versatile system to investigate nutrient exchange in, and partitioning by, mycorrhiza (*Populus x canesces* x *Paxillus involutus*) under axenic or greenhouse conditions. *Front. Fungal Biol*. 2:865517. doi: 10.3389/fsoil.2022.865517

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Präsentationen

Schreider K (2021). Mycorrhizal mediated resource partitioning for phosphorus. Projektdarstellungen IBG-3, Forschungszentrum Jülich, Institut für Bio- und Geowissenschaften, held online on 26th October, 2021.

Schreider K (2019). Carbon-Phosphorus Economy of mycorrhizal symbiosis. 5th Doctoral Researcher's Conference 2019 of the Research Training Group GRK1798, Hannover, 25th - 27th September, 2019.

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Poster

Schreider K, Figueiredo A, Andrino A, Boy J, Winkelmann T, Guggenberger G (2017). Developing an In-vitro Culture System for investigation into Phosphorus Economy of mycorrhizal symbiosis. 4th Doctoral Researcher's Conference 2017 of the Research Training Group GRK1798, Bad Salzdetfurth, 27th - 29th September, 2017.

Hilke I, Schreider-Goidenko K, Froehlich B, Henkel K (2015). How to quantify organic carbon in soils containing carbonates reliably, precisely, fast? A comparison of three different analytical approaches. Jahrestagung der Deutschen Bodenkundlichen Gesellschaft, München, 5th - 10th September 2015.

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