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Direct and indirect influences of arbuscular mycorrhizal fungi on phosphorus uptake by two root hemiparasitic *Pedicularis* species: do the fungal partners matter at low colonization levels?

Ai-Rong Li[1,2,*], Kai-Yun Guan[1], Rebecca Stonor[2], Sally E. Smith[2] and F. Andrew Smith[2]

[1]*Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences, PR China and* [2]*Soils Group, School of Agriculture, Food and Wine, The University of Adelaide, Australia*
** For correspondence. E-mail airongli@mail.kib.ac.cn*

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• *Background and Aims* Because most parasitic plants do not form mycorrhizal associations, the nutritional roles of arbuscular mycorrhizal (AM) fungi in them have hardly been tested. Some facultative root hemiparasitic *Pedicularis* species form AM associations and hence are ideal for testing both direct and indirect effects of AM fungi on their nutrient acquisition. The aim of this study was to test the influence of AM inoculation on phosphorus (P) uptake by *Pedicularis rex* and *P. tricolor*.
• *Methods* ^{32}P labelling was used in compartmented pots to assess the contribution of the AM pathway and the influence of AM inoculation on P uptake from a host plant into the root hemiparasites. Laboratory isolates of fungal species (*Glomus mosseae* and *G. intraradices*) and the host species (*Hordeum vulgare* 'Fleet') to which the two *Pedicularis* species showed obvious responses in haustorium formation and growth in previous studies were used.
• *Key Results* The AM colonization of both *Pedicularis* spp. was low (< 15 % root length) and only a very small proportion of total plant P (< 1 %) was delivered from the soil via the AM fungus. In a separate experiment, inoculation with AM fungi strongly interfered with P acquisition by both *Pedicularis* species from their host barley, almost certainly because the numbers of haustoria formed by the parasite were significantly reduced in AM plants.
• *Conclusions* Roles of AM fungi in nutrient acquisition by root parasitic plants were quantitatively demonstrated for the first time. Evidence was obtained for a novel mechanism of preventing root parasitic plants from overexploiting host resources through AM fungal-induced suppression of the absorptive structures in the parasites.

Key words: Arbuscular mycorrhizal fungi, *Glomus*, *Hordeum vulgare*, barley, Orobanchaceae, *Pedicularis rex*, *P. tricolor*, phosphorus uptake, root hemiparasitic plant.

INTRODUCTION

Parasitic plants are common in many natural and semi-natural ecosystems, comprising > 1 % of terrestrial plant species and having various life forms (Phoenix and Press, 2005). Through specialized organs known as haustoria, parasitic plants derive nutrients and water from their hosts (Press and Graves, 1995; Cameron *et al.*, 2008; Tesitel *et al.*, 2011), directly affecting performance of the hosts. Furthermore, parasitic plants often differentially affect other plant species and consequently alter plant community structure and other properties of the ecosystems in which they live (Press and Phoenix, 2005; Bardgett *et al.*, 2006; Westwood *et al.*, 2010).

With regard to studies of parasitic plants, the emphasis has been on interactions between hosts and parasites (Li and Guan, 2008). Investigations of multispecies interactions and interactions between parasitic plants and other organisms have been few in comparison. In their natural habitats, root parasitic plants and their hosts do not occur in isolation, but live in close association with other organisms (Sanon *et al.*, 2009; Johnson *et al.*, 2011; Van Hoveln *et al.*, 2011), including extensive root systems and abundant soil micro-organisms. As a consequence, sub-terranean interactions are typically more complicated and difficult to manipulate than aerial interactions, but are essential to gain a better understanding of the interactions in the wild.

As almost ubiquitous components of the soil microflora, arbuscular mycorrhizal (AM) fungi colonize a majority of terrestrial plants and play significant ecological roles including nutrient absorption and cycling (Smith and Read, 2008). Numerous and extensive studies have been carried out on AM fungal associations with many plant species. A small but growing number of investigations observed an indirect influence of AM fungi on the performance of parasitic plants during co-infection of host plants (Davies and Graves, 1998; Salonen *et al.*, 2001; Gworgwor and Weber, 2003; Fernandez-Aparicio *et al.*, 2010). However, because most parasitic plants do not form mycorrhizal associations (Atsatt, 1973; Brundrett, 2002), studies concerning direct interactions between parasitic plants and AM fungi are largely lacking (Li *et al.*, 2012*b*).

Pedicularis (Orobanchaceae) is a large lineage of photosynthetic root hemiparasitic plants (approx. 600 species), widely distributed in the northern temperate hemisphere and best represented in south-west China (Yang *et al.*, 1998). It forms interspecific haustoria with xylem connections to host roots as well as intraspecific haustoria parasitizing rootlets of its own species/individual (Piehl, 1963; Li *et al.*, 2012*b*). Although the genus was thought to be non-mycorrhizal (NM) (Harley and Harley,

1987), a majority of *Pedicularis* plants examined from Yunnan Province (south-west China) were found to harbour AM fungi in the wild (Li and Guan, 2007; Li and Guan, 2008). Based on high AM colonization and well-developed fungal structures, it was suggested that AM fungi may play significant roles in nutrient acquisition by the hemiparasites (Li and Guan, 2008). Recently, we observed AM fungal-induced suppression of haustorium formation and some obvious growth responses in *P. tricolor*, with or without the presence of a host plant of the hemiparasite (Li *et al.*, 2012*b*), indicating direct interactions between AM fungi and the root hemiparasite. Since haustoria are exclusive organs responsible for nutrient transfer from a host to a parasite, a reduction in numbers of haustoria after AM inoculation may have a marked influence on acquisition of host-derived nutrients. Therefore, the fungal partners may have a significant influence on both direct nutrient uptake from soil and host-derived nutrient acquisition by *Pedicularis*. As far as we are aware, however, no effort has been made to investigate direct nutrient contributions by AM fungi to root parasitic plants. Furthermore, quantitative analysis and physiological evidence are still lacking with regard to the influence of AM inoculation on nutritional interactions between a root parasitic plant and its host plant.

In previous studies, we observed significant differences in the capacity for haustorium formation by two *Pedicularis* species (*P. rex* C. B. Clarke and *P. tricolor* Hand.-Mazz.). Haustoria appeared much later and occurred in far fewer numbers in *P. rex* than in *P. tricolor* (Li *et al.*, 2012*a*), suggesting different parasitic capacity between the two species. It is intriguing to test whether the observed suppression of haustorium formation by AM fungi found in *P. tricolor* (Li *et al.*, 2012*b*) also occurs in *P. rex*. In addition, it is relevant to test whether AM fungi play similar nutritional roles in the two species.

In this study, we attempted to shed some light on the roles of AM fungi in phosphorus (P) acquisition by *P. rex* and *P. tricolor*. In two pot experiments we quantified the influence of AM inoculation on P acquisition by *Pedicularis*, with or without the presence of a host plant, using ^{32}P-labelled growth medium. Specifically, we tested: (1) the contribution of the AM pathway to P uptake from soil by the *Pedicularis* species; (2) the influence of AM inoculation on acquisition of P from the host plant by the *Pedicularis* species; and (3) the interspecific variation in haustorium formation and growth responses to AM inoculation between the two *Pedicularis* species. The knowledge obtained will enable us to better characterize the nutritional interactions between parasitic plants and their symbiotic fungi, and will help broaden our understanding of the function of AM fungi in root hemiparasitic plants.

MATERIALS AND METHODS

Experimental design

The study consisted of two experiments that were set up as described by Li *et al.* (2012*b*), but slightly modified for the radioisotopic tracer study. Based on molecular determination of AM fungal species associated with the two *Pedicularis* species in their natural habitats and determination of host preference of the hemiparasites in cultivation experiments, we used laboratory isolates of fungal species and host species to which the two *Pedicularis* species showed obvious responses in haustorium formation and growth (Li *et al.*, 2012*a*, *b*).

Experiment 1 investigated the contribution of two different AM fungal species, *Glomus intraradices* Schenck and Smith [DAOM 181602, now renamed *Glomus irregulare* (Stockinger *et al.*, 2009)] and *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (WFVAM45/BEG161) to P uptake from soil by *P. rex* and *P. tricolor* in the absence of a host plant for the hemiparasites. The experiment had three AM fungal treatments, NM or inoculated with *G. intraradices* or *G. mosseae*. There were six replicates per treatment. Plants were grown in a compartmented pot system (closely similar to that used by Smith *et al.*, 2003) in which ^{32}P-labelled soil was accessible only to external AM hyphae. The main pot constituted the root hyphal compartment (RHC) filled with 1·5 kg of soil:sand mix, incorporating NM mock inoculum or AM inoculum (see below). Each pot contained a hyphal compartment (HC) consisting of a small plastic tube filled with 42 g of sterile soil, labelled with carrier-free $H_3^{32}PO_4$ to provide 72 kBq g^{-1} dry soil. A buffer zone (about 2 mm in depth) of 10 g of unlabelled sterile soil mix was put on top of the labelled soil to prevent ^{32}P uptake by root hairs penetrating the mesh and diffusion of ^{32}P out of the HC. The open end of the tube was covered with 30 μm nylon mesh that allowed fungal hyphae to pass through but prevented passage of roots. The tube was buried in the centre of the pot with the open end facing up. As *Pedicularis* grows very slowly at the early seedling stage, to obtain sufficient plant material for ^{32}P activity determination, five individuals of *P. rex* or *P. tricolor* were grown in each pot.

In expt 2, we examined the influence of AM fungi on removal of P by *P. rex* and *P. tricolor* from a host (*Hordeum vulgare* 'Fleet'; barley), using compartmented pots and a split-root system (Fig. 1). We supplied ^{32}P-labelled soil to half of the root system of the host plant. *Pedicularis* plants were grown with the other half of the host root system, inoculated with either the AM fungus *G. intraradices* or with NM mock inoculum. *Glomus intraradices* was chosen for this experiment based on the more obvious appearance of ^{32}P in the shoots of *Pedicularis* inoculated with this fungal species than with *G. mosseae* in expt 1, which was monitored using a hand-held monitor. Experiment 2 had a factorial design for each *Pedicularis* species, using barley as host plant and *G. intraradices* as test AM fungus. Barley was chosen for two reasons: (1) to determine if the different growth of the two *Pedicularis* species observed with this plant in previous pot cultivation experiments (Li *et al.*, 2012*a*) would influence the effects of AM inoculation; and (2) because it does not show marked increases in growth or total P uptake when mycorrhizal (Christophersen *et al.*, 2009), so its competition with *Pedicularis* would not be different between NM and AM treatments. The parasite–host–AM fungal combinations were as follows: (1) two *Pedicularis* plants of the same species, no AM fungus or host plant, NM H–; (2) two *Pedicularis* plants of the same species, inoculated with AM fungus but no host plant, AM H–; (3) two *Pedicularis* plants of the same species, no AM fungus and with one host plant, NM H + ; and (4) two *Pedicularis* plants of the same species, with AM fungus and one host plant, AM H + . The first two plant combinations were set up as controls for the last two to determine any transfer of ^{32}P via the soil when *Pedicularis* grew alone. There were six replicate pots per treatment. In this experiment, we used a three-compartment pot method and split-root system in which ^{32}P-labelled soil was accessible only

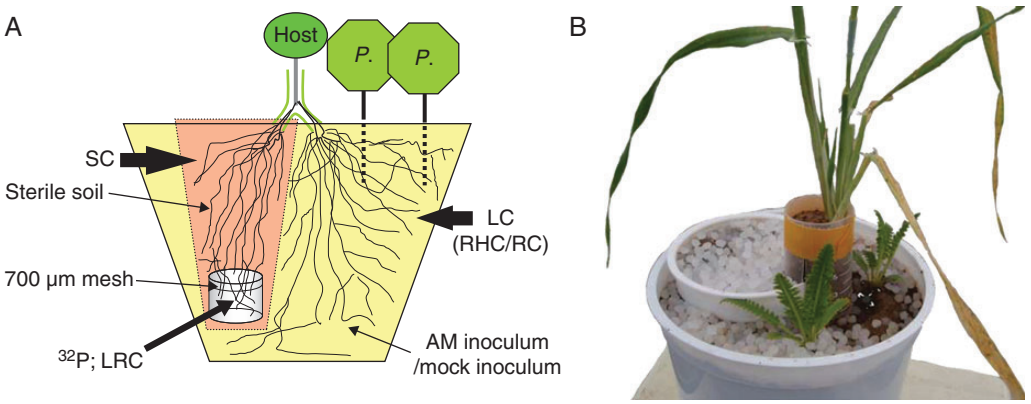

FIG. 1. Experimental set-up for quantitative determination of influence of arbuscular mycorrhizal (AM) fungi on phosphorus (P) acquisition by *Pedicularis* (*P.*) from its host plant. (A) Schematic diagram of the three-compartment pot design with a split-root system. Note that the drawing is not to scale. (B) An outside view of the study system. SC, small compartment; LC, large compartment [as root and hyphae compartment (RHC) when inoculated with AM fungi, or root compartment (RC) when not inoculated with AM fungi]; LRC, labelled root compartment.

to one half of the barley roots (Fig. 1). A small plastic pot was put in a second large plastic pot (about three times larger than the small pot) to make a solid barrier between the two compartments, thus forming a two-chamber split-root device. The large compartment (LC) was filled with 1 kg of soil mix incorporating either NM mock inoculum or *G. intraradices* inoculum. The small compartment (SC) was a root compartment filled with 0·4 kg of sterile soil in which only barley roots grew. Each SC contained a small plastic tube (hereafter referred to as the labelled root compartment, LRC) filled with 42 g of sterile soil, labelled with carrier-free $H_3^{32}PO_4$ providing 56 kBq g^{-1} dry soil. A buffer zone of 10 g of unlabelled sterile soil was put on the top of the labelled soil to minimize diffusion of ^{32}P. The open end of the tube was covered with 700 μm nylon mesh that kept the labelled soil separated from unlabelled soil but allowed passage of barley roots. The LRC was buried in the centre of the SC with the open end facing up.

Growth medium

The growth medium was the same as used by Li *et al.* (2012*b*). Briefly, the medium was a mix of 10 % autoclaved soil (collected from the Waite Arboretum, University of Adelaide) and 90 % autoclaved fine sand, with 2·6 mg of plant-available P per kg of dry soil and a pH value of approx. 6·0.

Plant materials

Seeds of *P. rex* and *P. tricolor* were collected from Shangri-la, Yunnan Province of China, in September 2008 and were stored in paper bags at 4 °C until used, except during transit to Australia. Seed P content was on average 18 μg per seed for *P. rex* and 13 μg for *P. tricolor* (AR Li, Kunming Institute of Botany, CAS, China, unpubl. res.). To promote germination, the seeds were surface sterilized in 4·5 % commercial sodium hypochlorite for 10 min, rinsed thoroughly with running reverse osmosis (RO) water and soaked in 1000 mg L^{-1} gibberellic acid for 2 h, and then stratified at 4 °C for 1 week (Li *et al.*, 2012*b*). Germination was carried out on filter papers at 20 °C in total darkness for 6 d.

Barley seeds were surface sterilized in 4·5 % commercial sodium hypochlorite for 10 min, rinsed with RO water, and germinated on filter papers at 25 °C in total darkness for 3 d.

AM fungal inoculum

Inoculum of *G. mosseae* and *G. intraradices* consisted of colonized root fragments, soil and spores, and was derived from the same batch of pot cultures as those used by Li *et al.* (2012*b*). *Glomus mosseae* pot cultures were prepared with *Medicago truncatula* Gaertn. grown in 1:9 Waite Arboretum soil:fine sand mix (pH approx. 6·0), and *G. intraradices* with *Trifolium subterraneum* L. grown in 1:9 Mallala soil:sand mix (pH approx. 7·4). Non-inoculated pot cultures from the same pot culture batch as *G. mosseae* were used as mock inoculum. Ten per cent (w/w) of each type of inoculum was used in soil of the appropriate compartments for both experiments.

To introduce microbes other than AM fungi in all treatments, 20 mL of soil filtrate was added to each pot in both experiments. In expt 2, 6 mL of filtrate was added to the SC and 14 mL to the LC. The filtrate was made from a mix of all the types of inocula (approx. 50 g of each) used in the corresponding experiment, suspended in 1 L of RO water and then filtered through Whatman filter papers #1 and #42 (Li *et al.*, 2012*b*).

Planting and growth conditions

In expt 1, uniform newly germinated seeds of *Pedicularis* were planted directly into experimental pots. In expt 2, *Pedicularis* seedlings were pre-grown in nurse pots inoculated with *G. intraradices* or mock inoculum for 3 weeks and then transplanted into corresponding experimental pots at the same time as well germinated barley seedlings, which had an average root length of 6–8 cm to facilitate the root splitting process. Root systems of single barley seedlings were divided into two roughly equal parts which were planted into the LC and SC (one on each side of the solid barrier formed by the wall of the small pot). The exposed roots were protected from light and desiccation by a partially split 50 mL centrifuge tube spread across the junction between SC and HC, and through which the shoot of

the barley seedling protruded vertically. The tube was filled with 25 g of sterile soil and fixed in place with waterproof tape (Fig. 1).

The surface of the soil was covered with autoclaved polyethylene beads (Qenos Pty Ltd, Australia) to retain moisture. Pots were watered to weight with RO water whenever necessary to maintain water content at approx. 10 % oven-dry soil. Long Ashton nutrient solution minus P but with increased N [2 mM K_2SO_4, 1·5 mM $MgSO_4{\cdot}7H_2O$, 4 mM $CaCl{\cdot}2H_2O$, 0·1 mM FeEDTA, 4 mM $(NH_4)_2SO_4$, 16 mM $NaNO_4$, 2·86 mg L^{-1} H_3BO_3, 1·81 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0·5 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 0·08 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 0·025 mg L^{-1} $NaMoO_4{\cdot}2H_2O$] was applied weekly (15 mL per pot) after transplanting. Pots were fully randomized on a single bench for each experiment and re-randomized at each watering to reduce position effects.

The experiments were conducted in an environmentally controlled glasshouse at the Waite Campus, University of Adelaide, Australia. The light intensity ranged from 450 to 1000 μmol m^{-2} s^{-1} and temperature ranged from 16 to 31·3 °C in the glasshouse during the study.

Harvest and sampling

Plants were harvested at 8 weeks after planting (expt 1) or transplanting (expt 2). At harvest, shoots were cut at the soil surface and separated from roots for subsequent analysis. In expt 2, split barley root halves were harvested separately. To obtain sufficient plant tissues for ^{32}P analysis, shoots and roots of *Pedicularis* from the same pot were separately pooled together as one sample. Shoot dry weight (DW) per pot was determined after oven drying at 85 °C for 48 h. Roots were washed thoroughly and fresh weights (FWs) were determined after blotting with paper towels. *Pedicularis* roots were separated from barley roots under a stereomicroscope. Haustoria tightly attached to barley roots were carefully cut off with as little barley tissue as possible and pooled with *Pedicularis* roots for subsequent analysis. A weighed sub-sample was taken randomly from each root sample and stored in 50 % ethanol for later assessment of AM colonization and haustorium formation in different plant combinations. The remainder of each root sample was oven-dried at 85 °C for 48 h and DW determined. Total DW of the roots was determined from the FW:DW ratio of this sample and the total FW of the roots.

Soil adjacent to (2–3 mm above) the HC mesh in expt 1 was sampled separately to test the effectiveness of the buffer zone (i.e. diffusion of any ^{32}P). Soil in each compartment was well mixed and sub-samples were taken for determination of hyphal length density (HLD) and water content. External hyphae were collected on Millipore filters (8 μm pore size, 25 mm diameter) as described by Jakobsen *et al.* (1992) and their length (m g^{-1} oven-dry soil) determined using a grid intersect method (Tennant, 1975).

Assessment of AM colonization and examination of internal structures of haustoria (Ha) were conducted as described by Li *et al.* (2012*b*). Because the total number of Ha and presumably functional haustoria (showing vascular connections, PFHa) can be directly linked to nutrient uptake quantity via the host plant, these data were presented herein instead of standardized numbers of Ha or PFHa per unit root DW. Total numbers of Ha and PFHa per pot were calculated from the number per g of FW of the root sub-sample and the total root FW of *Pedicularis.* Since haustorial connections were easily broken during the excavation and washing processes, it was impossible to discriminate PFHa that were attached to barley roots from the self-parasitized ones. As a consequence, numbers of PFHa in treatments with barley include both PFHa formed on barley roots and those formed between *Pedicularis* rootlets.

Plant tissue P concentrations were determined following digestion of dried material in concentrated HNO_3 (69·8 wt%) and analysis using the phosphovanado-molybdate method (Hanson, 1950). Plant P content was calculated from the P concentration and DW of the plant material. Plant-available P concentrations in soil from different compartments were determined using a resin P extraction method (McLaughlin *et al.*, 1994) from oven-dried samples (approx. 2 g). The same samples were used to measure ^{32}P activity and hence specific activity (SA) of available P in soil of the corresponding compartments. The rest of the ^{32}P-labelled soil was stored at 5 °C until ^{32}P had largely decayed. The ^{32}P activity in plant tissue digests or soil extracts (the same as used for P concentration determination; 2 mL) was measured by Cerenkov ^{32}P counting in an LKB-1215 Rackbeta II liquid scintillation counter (Wallac, Finland) and corrected for isotopic decay.

Calculation of ^{32}P SA and AM pathway contribution to shoot P

The ^{32}P SA in shoot digests or soil extracts was calculated by dividing the total ^{32}P activity by the total P in shoot or soil samples. We used shoot SA rather than the whole-plant SA to avoid overestimation of hyphal P transfer by inclusion of ^{32}P retained in the intraradical hyphae (Grace *et al.*, 2009). Because seed P reserves of both *P. rex* and *P. tricolor* were small and it was impossible to determine what proportion became shoot P, we did not include seed P content to the calculation of the plant P budget. The percentage contribution of the AM P pathway to shoot P uptake by *Pedicularis* in expt 1 was calculated according to the following equation (Grace *et al.*, 2009):

$$\%\text{AM pathway contribution to shoot P} = (SA^{32}P \text{ shoot}/SA^{32}P \text{ in HC soil}) \times (\text{P in RHC}/\text{P in HC}) \times 100$$

Data analysis

All data were analysed using a non-parametric method for analysis of variance (ANOVA), PERMANOVA, as most of the data did not fulfil the assumptions of either normality or homogeneity of variances required for a parametric ANOVA. The analyses were based on Bray–Curtis dissimilarities using unrestricted permutation of raw data. A random sub-set of 9999 permutations was used. When any main effect was statistically significant, pair-wise *a posteriori* comparisons of the corresponding means among levels of the factor were done according to the User's Guide of this program (Anderson, 2005). When a significant interaction between the factors was found, pair-wise *a posteriori* comparisons of the corresponding means among levels of one factor within each level of the other factor were done to determine exactly which parts of the interaction are significant. AM inoculation was used as the only factor (fixed; three levels) in the analysis of data from expt 1. For expt 2, AM inoculation and host presence were used as factors (fixed; two levels for

each) in the analyses of shoot P concentration (SPConc), shoot P content (SPC) per pot, shoot DW (SDW) per pot, root DW (RDW) per pot, whole-plant DW (WDW) per pot, SA of ^{32}P in shoot (SAP), total number of haustoria (THa) and number of PFHa per pot of *Pedicularis*. The interaction effect of AM fungi and host was included in the partition of variation. Independent samples *t*-tests were conducted to compare AM colonization, biomass and P content of plant materials between two appropriate treatments.

RESULTS

Experiment 1

AM colonization and external hyphal length densities (HLDs). No colonization by AM fungi occurred in non-inoculated plants. Colonization levels were generally very low in both *Pedicularis* species, with values significantly lower for *G. mosseae* than for *G. intraradices* (Table 1). Few arbuscules and vesicles were observed, so that hyphae were virtually the only fungal structures present in the roots. External HLDs were extremely low in all plant–fungus combinations, being $<0{\cdot}3$ m g^{-1} oven-dry soil and negligibly different from values in NM soil (results not shown). No significant differences were observed in HLDs between HCs and RHCs.

Biomass and P response. Inoculation with *G. intraradices* did not result in any significant change in shoot DW, shoot P concentration or shoot P content of either *Pedicularis* species (Fig. 2A–C). *Glomus mosseae* significantly increased shoot DW and shoot P content in *P. rex*, but not shoot P concentration. Inoculation with *G. mosseae* significantly increased shoot P concentration in *P. tricolor*, but the increases in shoot DW and shoot P content were not significant. Neither fungal species showed any influence on root DW or whole-plant DW (Table 2, Fig. 2A).

^{32}P uptake and contribution of the AM pathway. No ^{32}P was detectable in soil adjacent to the HC mesh or in NM *P. rex* shoots. Negligible levels of ^{32}P were detected in NM *P. tricolor* shoots. Levels of ^{32}P detected in shoots of the *Pedicularis* species inoculated with AM fungi were very low, as indicated by the shoot ^{32}P SA (Fig. 2D). However, the SA in *Pedicularis* inoculated with *G. intraradices* was higher than with *G. mosseae*. The percentage contribution of the AM pathway to P uptake by both *Pedicularis* species was generally <1 % of the total P uptake.

Haustorium formation. Pedicularis tricolor produced many more intraspecific haustoria than *P. rex* in the absence of a host plant (Table 1). Some haustoria had long haustorial hairs (up to 5 mm long in a few cases) that could have penetrated the HC mesh. Inoculation with AM fungi had no obvious effect on either the number of total haustoria or the number of PFHa in *P. rex*, but significantly reduced both the number of total haustoria and PFHa in *P. tricolor*. Inoculation with *G. mosseae* reduced the total number of haustoria (but not the number of PFHa) to a greater extent than *G. intraradices* in *P. tricolor*.

Experiment 2

AM colonization and external HLDs. The mean colonization values by *G. intraradices* in *P. rex* and *P. tricolor* were generally less than 15 % root length (Table 3). Hyphae were the only fungal structures observed in roots of the hemiparasites. Barley roots were highly colonized (approx. 90 % root length; Table 3) with well developed arbuscules and vesicles. In the absence of barley, HLDs in LC soil inoculated with *G. intraradices* were similar to those of NM treatments ($<0{\cdot}4$ m g^{-1} oven-dry soil). In the presence of barley, HLDs in AM-inoculated LC soil were $3{\cdot}1 \pm 0{\cdot}8$ and $2{\cdot}8 \pm 0{\cdot}3$ m g^{-1} oven-dry soil, in *P. rex* and *P. tricolor* pots, respectively. Although more mycelium was available in pots with barley (as shown by increased external hyphal length densities) to spread the infection in *Pedicularis*, no significant difference was observed for root colonization by *G. intraradices* in either *Pedicularis* species between treatments with barley and those without barley (Table 3).

Biomass and P response. Pedicularis rex and *P. tricolor* showed significant biomass responses to both AM inoculation and host attachment, but with different patterns (Table 4, Fig. 3A). In *P. rex*, interaction effects between AM inoculation and host presence were observed on shoot DW, root DW and whole-plant DW, with greater reduction in DWs in the presence of both *G. intraradices* and barley than with either fungus or plant alone. Inoculation with *G. intraradices* significantly reduced shoot DW and whole-plant DW of the hemiparasite (attached and unattached alike), but had no effect on root DW. Shoot DW, root DW and whole-plant DW of *P. rex* were all reduced when

TABLE 1. *Arbuscular mycorrhizal (AM) colonization level and haustorium formation in* Pedicularis rex *and* P. tricolor *inoculated with* Glomus intraradices, G. mosseae *or non-mycorrhizal mock inoculum and grown for 8 weeks at a low P level (approx. 2·6 mg kg^{-1}) in the absence of host plants of the root hemiparasites*

Pedicularis species	AM treatment	% Colonization	No. of Ha per pot (5 plants)	No. of PFHa per pot (5 plants)
P. rex	Non-mycorrhizal	–	16 ± 5^{a}	2 ± 1^{a}
	G. intraradices intraradices	14 ± 2^{b}	$23 \pm 10_{a}$	1 ± 1^{a}
	G. mosseae	5 ± 2^{a}	13 ± 5^{a}	2 ± 1^{a}
P. tricolor	Non-mycorrhizal	–	690 ± 326^{z}	93 ± 47^{y}
	G. intraradices intraradices	14 ± 2^{y}	75 ± 8^{y}	8 ± 3^{x}
	G. mosseae	3 ± 1^{x}	31 ± 11^{x}	6 ± 2^{x}

Colonization data are presented as total percentage root length colonized.
Ha, haustoria; PFHa, presumable functional haustoria.
Values are the means ± s.e. of six replicates. Values with the same letter for each *Pedicularis* species in the same column are not significantly different at the $P<0{\cdot}05$ level.

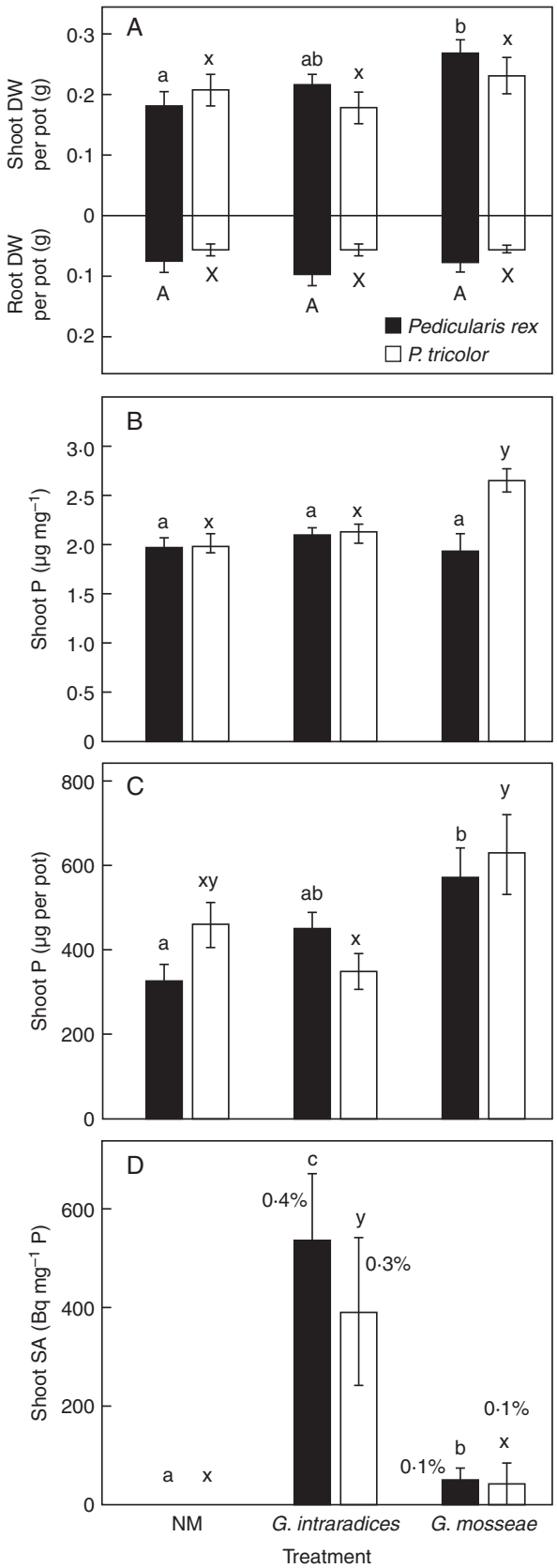

FIG. 2. Plant dry weight (DW; g per pot), shoot phosphorus (P) concentration, shoot P content per pot and specific activity (SA) of ^{32}P in shoots of *Pedicularis rex* and *P. tricolor* (as indicated in the key) inoculated with either *Glomus mosseae* or *G. intraradices*, or without inoculation (NM), grown in P-deficient soil for 8 weeks in expt 1. (A) Shoot (above the zero line) and root (below the zero line) DW (g) per pot. (B) Shoot P concentration (μg mg^{-1}) in *Pedicularis*. (C) Shoot P content (μg per pot) in *Pedicularis*. (D) Specific activity (SA) of ^{32}P (Bq mg^{-1} P) in *Pedicularis* shoot; percentage values near the bars are the average percentage contribution of P uptake via the hyphal pathway. NM, non-mycorrhizal treatment inoculated with mock inoculum. Data are presented as the mean ± s.e. of six replicate pots. Statistics are done separately for each *Pedicularis* species. Bars with different letters within the same *Pedicularis* species indicate a statistically significant difference at the $P<0{\cdot}05$ level.

attached to barley with or without AM inoculation. In *P. tricolor*, interaction effects between AM inoculation and presence of barley were observed only for root DW, with root biomass further reduced by a combination of the two factors. Shoot DW, root DW and whole-plant DW were all reduced when inoculated with *G. intraradices*. The presence of (and presumably attachment to) barley affected neither shoot DW nor whole-plant DW, but significantly reduced root DW regardless of the AM status.

In *P. rex*, interaction effects between AM inoculation and host presence were observed on shoot P content. The hemiparasite had the least shoot P when inoculated with *G. intraradices* in the presence of barley. Both inoculation with *G. intraradices* and the presence of barley separately and significantly reduced shoot P concentration (Fig. 3B) and shoot P content (Fig. 3C). In *P. tricolor*, no significant interactions between AM inoculation and host attachment were observed in terms of shoot P content (Table 4). Inoculation with *G. intraradices* significantly reduced shoot P concentration and shoot P content. The presence of (and again presumably attachment to) barley significantly increased shoot P concentration in NM plants of *P. tricolor* (Fig. 3B), but the increase in total shoot P content was not significant (Fig. 3C).

Inoculation with *G. intraradices* had no significant effect on barley growth or P content in either *Pedicularis*–barley pair (Table 3).

Effects of AM inoculation on P uptake by Pedicularis *from barley.* No correlation was observed between SA values in shoots of *Pedicularis* and root weights of barley in LCs (data not shown). Both *P. rex* and *P. tricolor* removed substantial amounts of P from barley roots, as shown by the SA values in shoots of both root hemiparasites (Fig. 3D). Strong interaction effects between AM inoculation and host were observed in *P. tricolor* but not in *P. rex* in terms of host-derived P uptake (Table 4). Acquisition of P from the barley hosts by *P. rex* inoculated with *G. intraradices* was apparently reduced (by an average factor of 3–4) when compared with NM plants, but the reduction was not statistically significant due to large variability in NM H + . Removal of P from barley was strongly suppressed (by a factor of 7) in AM-inoculated *P. tricolor* compared with NM individuals.

Haustorium formation. Pedicularis tricolor produced far more haustoria than *P. rex* in NM treatments (Table 3). Barley promoted haustorium formation per unit DW of roots in both *Pedicularis* species (data not shown), but not the total number of haustoria per pot. Inoculation with *G. intraradices* strongly repressed initiation and differentiation of haustoria in both *P. rex* and *P. tricolor*, with or without the presence of barley. In consequence, the numbers of Ha and PFHa were very low in these treatments.

TABLE 2. *PERMANOVA results (*P*-values) for shoot dry weight (SDW), root dry weight (RDW), whole-plant dry weight (WDW), shoot phosphorus concentration (SPConc), shoot P content (SPC), specific activity of ^{32}P in shoot (SAP), total number of haustoria (THa) and number of presumably functional haustoria (PFHa) per pot of* Pedicularis rex *and* P. tricolor *from expt 1*

Pedicularis species	Source of variation	SDW (g per pot)	RDW (g per pot)	WDW (g per pot)	SPConc (μg mg^{-1})	SPC (μg per pot)	SAP (Bq mg P^{-1})	THa	PFHa
P. rex	AM inoculation	**0·0370**	0·6589	0·1686	0·6143	**0·0132**	**0·0144**	0·8452	0·3388
	d.f. residuals	15	15	15	15	15	15	15	15
P. tricolor	AM inoculation	0·4640	0·9218	0·5510	**0·0057**	0·0786	0·0777	**0·0001**	**0·0029**
	df residuals	15	15	15	15	15	15	15	15

Values in bold indicate significant effects and thus were taken into consideration for pairwise comparisons of the corresponding means.

TABLE 3. *Arbuscular mycorrhizal (AM) colonization and haustorium formation in* Pedicularis rex *and* P. tricolor *inoculated with* Glomus intraradices *or non-mycorrhizal mock inoculum and grown for 8 weeks (after transplanting of 3-week-old seedlings) at a low phosphorus (P) level (approx. 2·6 mg kg^{-1}) in the presence or absence of a heavily mycorrhizal host (barley) in expt 2*

Pedicularis species	Treatment	% AM colonization in *Pedicularis* root	No. of Ha per pot (2 plants)	No. of PFHa per pot (2 plants)	% AM colonization in barley root	Root DW (g) of barley in LCs	Root P content (mg) of barley in LCs	Shoot DW (g) of barley	Shoot P content (mg) of barley
P. rex	NM H−	–	69 ± 17^{b}	6 ± 2^{b}	–	–	–	–	–
	NM H+	–	25 ± 10^{b}	6 ± 2^{b}	–	0·61 ± 0·22^{a}	0·88 ± 0·30^{a}	2·67 ± 0·28 a	7·30 ± 0·84^{a}
	AM H−	8 ± 2^{a}	3 ± 2^{a}	1 ± 1^{a}	–	–	–	–	–
	AM H+	13 ± 3^{ab}	4 ± 2^{a}	2 ± 1^{a}	89 ± 3	0·28 ± 0·08^{a}	0·57 ± 0·17^{a}	2·46 ± 0·16 a	6·34 ± 0·23^{a}
P. tricolor	NM H−	–	379 ± 125^{z}	75 ± 31^{y}	–	–	–	–	–
	NM H+	–	98 ± 45^{y}	40 ± 17^{y}	–	0·42 ± 0·08^{x}	0·72 ± 0·15^{x}	2·29 ± 0·22 x	6·87 ± 0·56^{x}
	AM H−	15 ± 7^{xy}	7 ± 5^{x}	1 ± 1^{x}	–	–	–	–	–
	AM H+	5 ± 5^{x}	8 ± 7^{x}	1 ± 1^{x}	91 ± 1	0·50 ± 0·11^{x}	1·02 ± 0·22^{x}	2·41 ± 0·11 x	6·18 ± 0·43^{x}

NM, non-mycorrhizal treatment inoculated with mock inoculum; AM, inoculated with *Glomus intraradices*; H−, absence of a host plant; H +, presence of a host (barley); Ha, haustoria; PFHa, presumably functional haustoria; LC, large compartments that constitutes *Pedicularis* roots, barley root half and AM fungi or NM mock inoculum.

Colonization data are presented as the total percentage root length colonized.

Values are the means ± s.e. of six replicates. Values with the same letter for each *Pedicularis* species in the same column are not significantly different at the $P<0·05$ level. Note that DWs and P contents of barley between AM and NM treatments are not significantly different for either *Pedicularis* species.

TABLE 4. *PERMANOVA results (*P*-values) for shoot dry weight (SDW), root dry weight (RDW), whole-plant dry weight (WDW), shoot phosphorus concentration (SPConc), shoot P content (SPC), specific activity of ^{32}P in shoot (SAP), total number of haustoria (THa) and number of presumably functional haustoria (PFHa) per pot of* Pedicularis rex *and* P. tricolor *from expt 2*

Pedicularis species	Source of variation	SDW (g per pot)	RDW (g per pot)	WDW (g per pot)	SPConc (μg mg^{-1})	SPC (μg per pot)	SAP (kBq mg P^{-1})	THa	PFHa
P. rex	Inoculation (I)	**0·0021**	0·2444	**0·0055**	**0·0035**	**0·0024**	0·3491	**0·0001**	**0·0067**
	Host (H)	**0·0001**	**0·0001**	**0·0001**	**0·0001**	**0·0001**	**0·0001**	0·1296	0·3918
	I × H	**0·0472**	**0·0256**	**0·0102**	0·0683	**0·0046**	0·4884	0·2860	0·4168
	d.f. residuals	20	20	20	20	20	20	20	20
P. tricolor	Inoculation (I)	**0·0001**	**0·0001**	**0·0001**	**0·0001**	**0·0001**	**0·0045**	**0·0001**	**0·0001**
	Host (H)	0·3580	**0·0005**	0·1617	0·2873	0·3450	**0·0001**	0·2659	0·3364
	I × H	0·5641	**0·0003**	0·1750	0·2988	0·2900	**0·0020**	0·0683	0·3320
	d.f. residuals	20	20	20	20	20	20	20	20

Values in bold indicate factors or interactions that had significant effects and thus were taken into consideration for pairwise comparisons of the corresponding means.

DISCUSSION

Direct influence of AM inoculation on P uptake from soil by the hemiparasites

Radioisotopic evidence suggests that the AM fungal pathway made little contribution to P uptake from soil by either *Pedicularis* species (Fig. 2D). This can be explained by low colonization levels, absence of arbuscules and poor development of AM hyphae in the soil observed here. In view of the high colonization by *G. intraradices* in barley roots, the inoculum potential of the AM inocula used and the growth conditions were not the causes of the low AM colonization observed in the root

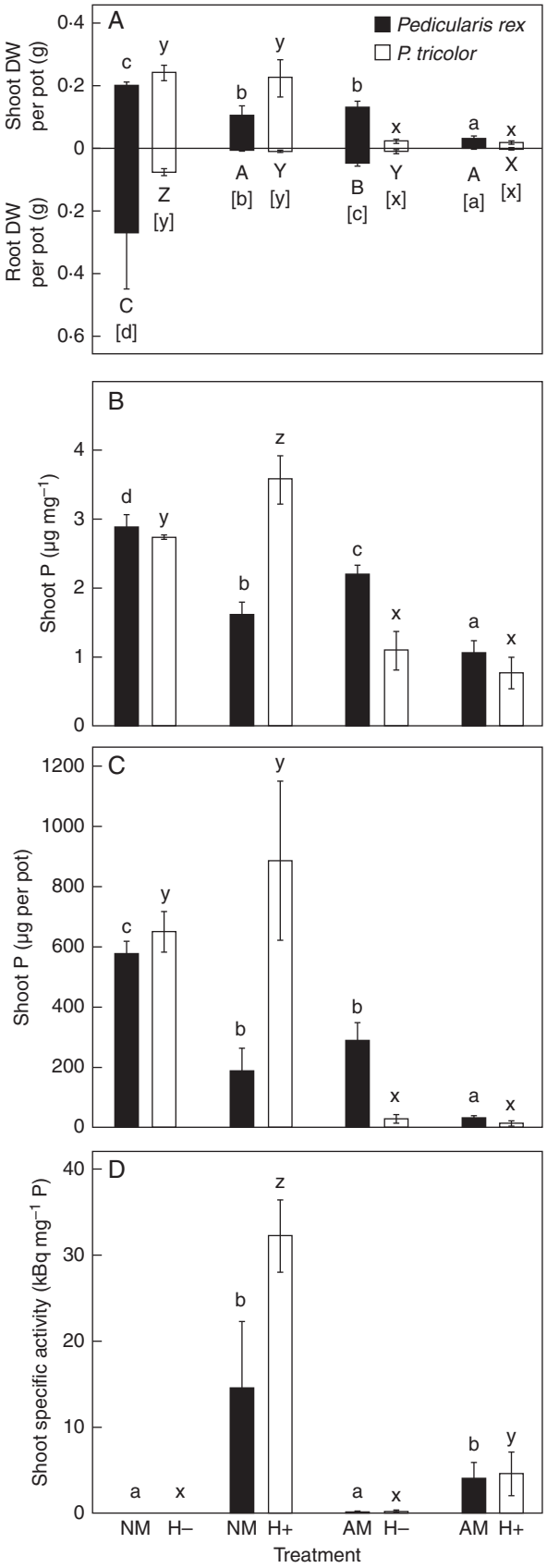

FIG. 3. Influence of *Glomus intraradices* and host plant (barley) on plant dry weight (DW), shoot P concentration, shoot P content and specific activity (SA) of shoot ^{32}P in *Pedicularis rex* and *P. tricolor* (as indicated in the key) grown in P-deficient soil for 8 weeks after transplanting (pre-grown in nurse pots for 3 weeks). (A) Shoot (above the zero line; lower case letters), root (below the zero line; upper case letters) and whole plant (in square brackets) DW (g) per pot. (B) Shoot P concentration (μg mg^{-1}) in *Pedicularis*. (C) Shoot P content (μg per pot) in *Pedicularis*. (D) Specific activity (SA) of ^{32}P (kBq mg^{-1} P) in *Pedicularis* shoot. Statistics are done separately for each *Pedicularis* species. Bars with different letters within the same *Pedicularis* species indicate a statistically significant difference at the $P<0{\cdot}05$ level. NM H−, non-mycorrhizal mock inoculation and no host plant; NM H +, non-mycorrhizal mock inoculation and in the presence of a host plant; AM H−, with AM inoculation and no host plant; AM H +, with AM inoculation and in the presence of a host plant.

hemiparasites (Tables 1 and 3). Rather, poorly compatible fungus–plant pairs may be an explanation, as indicated by obvious variations between colonization levels by different AM fungal species used here and in other studies (Li and Guan, 2008; Li *et al.*, 2012*b*). It remains to be tested whether AM fungal isolates from natural habitats of *Pedicularis* can colonize the hemiparasites more effectively than those used here and hence contribute more to P uptake via the AM pathway.

Despite the apparently negligible contribution of the AM pathway to P acquisition by *Pedicularis* from soil, inoculation with *G. mosseae* significantly increased shoot P content and shoot DW of *P. rex* and shoot P concentration of *P. tricolor* (expt 1; Fig. 2). Previously we suggested that reduction in the number of intraspecific haustoria, that are theoretically of little benefit, could have saved energy of the root hemiparasites (Li *et al.*, 2012*b*), leading to increased nutrient and biomass accumulation. However, this interpretation cannot explain the increases in *P. rex* after AM inoculation, as the number of intraspecific haustoria in *P. rex* inoculated with *G. mosseae* was similar to that of NM individuals. Additionally, *P. tricolor* inoculated with *G. intraradices* did not grow any better than NM individuals, though the former produced far fewer intraspecific haustoria than the latter. Enhanced nutrient uptake and increased plant growth at low AM colonization levels have been found in other plant species (Asghari *et al.*, 2005). It was suggested that apart from a direct nutrient contribution via AM hyphae, AM fungus-induced alterations to root system architecture or microbial communities may also contribute to growth and nutrient acquisition (van der Heijden, 2001; Asghari *et al.*, 2005). Specific mechanisms for the enhanced growth of *P. rex* when poorly colonized by *G. mosseae* require further investigation.

Effects of AM inoculation on P acquisition from barley into the hemiparasites

The influence of AM inoculation on removal of a nutrient (in this case P) by a root hemiparasitic plant from its host was quantified for the first time (expt 2). As expected, inoculation with AM fungi strongly interfered with P acquisition from barley by *Pedicularis*. Barley root P content in LCs and total shoot P were both similar between AM and NM treatments for each *Pedicularis* species, suggesting that the quantity of host-derived P resource was not significantly different between AM and NM treatments. Decreased host-derived P acquisition after AM inoculation (Fig. 3D) can be attributed to a significant reduction in numbers of haustoria (particularly PFHa; Table 3), which

serve as the only functional connection for nutrient transfer between *Pedicularis* and its host. Suppression of haustorium formation and depression in growth of *Pedicularis* after AM inoculation in *Pedicularis*–barley pairs does not agree with results observed in *Rhinanthus minor*–*Lolium perenne* association (Davies and Graves, 1998), where the root hemiparasite produced more haustoria and grew better when grown with a mycorrhizal host. Davies and Graves (1998) suggested that enhanced haustorium formation and growth of *R. minor* attached to a mycorrhizal host was the product of a positive feedback loop. It was envisaged that an AM-induced increase in organic carbon (C) sink in the root may have facilitated formation of secondary haustoria and hence host-derived nutrient acquisition. This was obviously not the case in *Pedicularis*–barley associations shown here. Growth depression in *Pedicularis* attached to mycorrhizal barley does agree with indirect (i.e. via the host plant) inhibitory effects of AM fungal inoculation on growth of a few non-mycorrhizal parasitic plants (Gehring and Whitham, 1992; Gworgwor and Weber, 2003; Lendzemo *et al.*, 2005). However, as *Pedicularis* forms a direct connection with AM fungi and is subject to a direct influence of the fungal partner, which has not been reported in other parasite–host plant pairs, the underlying mechanisms of the growth depression are likely to be very different.

The differential influence of barley on the two *Pedicularis* species was obvious in this study. Attachment to barley greatly benefited *P. tricolor* in terms of a large amount of host-derived P. While *P. rex* did derive P from barley, the DW, shoot P concentration and P content of this root hemiparasite were significantly reduced in the presence of this host (Fig. 3). In view of the late response and small number of haustoria produced by *P. rex*, we presume that parasitic benefit by attachment to barley was an insufficient trade-off with competition for this species pair (Li *et al.*, 2012*a*). In a strongly P-deficient soil, as used in this study, competition for soil P between slow growing *Pedicularis* and fast growing barley may be very strong. The presence of dense mycorrhizal fungal hyphae in the soil and a large difference in root colonization of the paired plants may further exaggerate the contrast. However, despite the different growth and P responses of *P. rex* and *P. tricolor* to the presence of barley, inoculation with *G. intraradices* caused growth depression and reduction in P uptake in both *Pedicularis* species. This suggests that interference with host-derived P in *Pedicularis* by AM fungi may be independent of outcomes of parasitic plant–host plant interaction, at least in the *Pedicularis*–barley association.

Inconsistent effects of AM fungi on growth of the hemiparasites

Although we observed growth depression in *Pedicularis* after AM inoculation with *G. intraradices* in expt 2 (comparing the treatments without barley plants; Fig. 3A), there was no such depression in expt 1 (Fig. 2A). We have no definite explanation for the discrepancy at present, but we suggest that different seedling ages (8 weeks in expt 1 and 11 weeks in expt 2) and the effects of transplanting in expt 2 may have affected the interactions to some extent. For example, in the absence of barley, *G. intraradices* did not reduce the number of haustoria in *P. rex* as occurred in *P. tricolor* in expt 1, but the haustorium number of both *Pedicularis* species was reduced in expt 2. This can be explained by the different responses of haustorium formation between the two root hemiparasites, with *P. tricolor* producing haustoria much earlier and in larger number than *P. rex* (Li *et al.*, 2012*a*). According to our previous observations, the numbers of haustoria and biomass produced by the same *Pedicularis* species increased up to ten times from 6 weeks to 14 weeks for *P. rex* and *P. tricolor* (Li *et al.*, 2012*a*). The pre-growing of *Pedicularis* plants in nurse pots for 3 weeks allowed more time for the repressive effects of *G. intraradices* on haustorium formation and growth to become fully manifest in expt 2.

Based on the existing literature, it is increasingly acknowledged that the performance of a parasitic plant can be affected by the mycorrhizal status of its host plant. However, controversial results (promotion vs. suppression) have been reported regarding the influence of AM fungi on performance of parasitic plants during co-infection of a host plant (Gehring and Whitham, 1992; Davies and Graves, 1998; Salonen *et al.*, 2001; Gworgwor and Weber, 2003; Lendzemo *et al.*, 2005; Li *et al.*, 2012*b*). We believe that the differences in plant identity (hence different potential to be colonized by AM fungi and responses to the fungal partners) of parasitic plant–host plant pairs and differences in effectiveness of different AM fungi (as shown in expt 1) may account for the considerable variation and even controversy. In view of interspecific variation in effects of AM fungi on growth of *Pedicularis* in the absence of a host, it will be intriguing to test if inoculation with a growth-promoting AM fungus (e.g. *G. mosseae* for *P. rex*) interferes with nutrient acquisition from its host by the root hemiparasites to some extent. In addition, it will also be relevant to test the influence of AM colonization on *Pedicularis*–host interactions using an AM-responsive host (e.g. a legume), as a positive growth response to AM colonization in a host plant has been suggested to affect outcomes of the tripartite interactions (Salonen *et al.*, 2001).

Conclusions

Our findings provide the first quantitative evidence that AM fungi play important roles in nutrient acquisition by root parasitic plants. Due to the low AM colonization levels, a direct contribution by the AM pathway to P uptake in the two *Pedicularis* species tested was negligible. However, a dramatic reduction in the number of haustoria and hence P acquisition from barley by AM fungal-inoculated *Pedicularis* plants was observed, despite their different responses to barley attachment. This study provides the physiological evidence for a novel mechanism that appears to prevent parasitic plants from overexploiting host resources, via suppression of absorptive structures in the parasites. Since the transfer of nutrients from host to a xylem parasite (i.e. forming only a xylem connection with its host) such as *Pedicularis* is mostly non-selective (Jiang *et al.*, 2004, 2010), P is not the only nutrient that will be decreased after inoculation with AM fungi. The AM fungi may therefore play a role in regulating *Pedicularis*–host nutritional interactions that may minimize potential overexploitation of host resources. Further investigations are required to determine any ecological significance of such negative effects of AM fungi on haustorium formation in the root hemiparasites.

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