New Phytologist

Research

A neglected alliance in battles against parasitic plants: arbuscular mycorrhizal and rhizobial symbioses alleviate damage to a legume host by root hemiparasitic *Pedicularis* species

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Received: *22 May 2018*
Accepted: *7 July 2018*

New Phytologist (2018)
doi: 10.1111/nph.15379

Key words: arbuscular mycorrhizal (AM) fungi, dual inoculation, legume host, multi-species interaction, nutritional symbioses, *Pedicularis*, rhizobia, root hemiparasitic plant..

Summary

• Despite their ubiquitous distribution and significant ecological roles, soil microorganisms have long been neglected in investigations addressing parasitic plant–host interactions. Because nutrient deprivation is a primary cause of host damage by parasitic plants, we hypothesized that beneficial soil microorganisms conferring nutrient benefits to parasitized hosts may play important roles in alleviating damage.

• We conducted a pot cultivation experiment to test the inoculation effect of an arbuscular mycorrhizal fungus (*Glomus mosseae*), a rhizobium (*Rhizobium leguminosarum*) and their interactive effects, on alleviation of damage to a legume host (*Trifolium repens*) by two root hemiparasitic plants with different nutrient requirements (N-demanding *Pedicularis rex* and P-demanding *P. tricolor*).

• Strong interactive effects between inoculation regimes and hemiparasite identity were observed. The relative benefits of microbial inoculation were related to hemiparasite nutrient requirements. Dual inoculation with the rhizobium strongly enhanced promotional arbuscular mycorrhizal effects on hosts parasitized by *P. rex*, but reduced the arbuscular mycorrhizal promotion on hosts parasitized by *P. tricolor*.

• Our results demonstrate substantial contribution of arbuscular mycorrhizal and rhizobial symbioses to alleviating damage to the legume host by root hemiparasites, and suggest that soil microorganisms are critical factors regulating host–parasite interactions and should be taken into account in future studies.

Introduction

Interspecific interactions have been shown to have strong effects on productivity and composition of plant communities and eventually ecosystems as a whole (Callaway *et al.*, 2002; Larimer *et al.*, 2014). Due to the multitrophic nature of species interactions, the direction and magnitude of these effects on plant communities are often context-specific, depending on which organisms are involved (Callaway *et al.*, 2002; Klabi *et al.*, 2014; Weremijewicz *et al.*, 2018), the nature of the interactions (Larimer *et al.*, 2014), and the abiotic environments in which the interactions occur (Pugnaire *et al.*, 2004; Larimer *et al.*, 2014; Clark *et al.*, 2017). Among the interactions, those involving nutrient acquisition are fundamental for plant communities in most ecosystems (Rodríguez-Echeverría *et al.*, 2013; Clark *et al.*, 2017). Land plants have developed different strategies to increase their capability for nutrient acquisition by associating with other species, including forming mutualistic symbioses with soil microorganisms, such as arbuscular mycorrhizal (AM) fungi for more efficient phosphorus (P) acquisition, and nitrogen-fixing bacteria for enhanced nitrogen (N) uptake (de Varennes & Goss, 2007; Smith & Smith, 2011; Castagno *et al.*, 2014). On the other hand, the 1% of angiosperms that are parasitic plants have evolved a strategy to directly extract nutrients from other plants via parasitic organs called haustoria, resulting in host growth depression, productivity reduction and probably changes in plant community structure (Press *et al.*, 1999; Press & Phoenix, 2005; Irving & Cameron, 2009; Joel *et al.*, 2013). Because beneficial soil microorganisms and parasitic plants have marked but contrasting effects on their host plants, the microorganisms may have profound impacts on the outcomes of parasite–host interactions. This may particularly be the case for root hemiparasitic plants whose interactions with host plants occur in soil where the beneficial microorganisms occur. As far as we know, very few investigations addressing parasitic plant–host interactions have taken soil microorganisms into account (Davies & Graves, 1998; Salonen *et al.*, 2001; Stein *et al.*, 2009; Li *et al.*, 2013a; Sui *et al.*, 2014). This deficiency greatly hinders our understanding of underlying mechanisms for variations in outcomes of parasite–host interactions.

In our investigations of tripartite interactions among root hemiparasitic *Pedicularis* species, AM fungi and their grass hosts, we found that AM fungi significantly alleviated parasitic effects of *Pedicularis* on their hosts by suppressing haustorial connections and growth of the hemiparasites (Li *et al.*, 2013a; Sui *et al.*, 2014). However, because the grass hosts showed little response to AM colonization in terms of nutrient acquisition and plant growth, any influence of the effects of mutualistic soil microorganisms and hemiparasitic plants was not fully tested. To better understand the roles of mutualistic soil microorganisms in regulating root hemiparasite–host interactions, it is relevant to test how a host plant with positive growth response to the microorganisms interacts with root hemiparasitic plants.

In contrast to grasses, leguminous plants commonly associate with both AM fungi and rhizobia, which confer complementary resources (AM fungi primarily for P and rhizobia for N) to their host plants (Larimer *et al.*, 2014). It has been well documented that inoculation with suitable rhizobia or AM fungi can enhance plant N and P acquisition and improve growth of a legume host (El-Ghandour *et al.*, 1996; Mortimer *et al.*, 2008; Smith *et al.*, 2009; Wang *et al.*, 2011). Because nutrient deprivation is a primary cause of host damage by root hemiparasites (Irving & Cameron, 2009), associations that improve host nutrient acquisition could be helpful in enhancing host tolerance to the hemiparasites. Therefore, we hypothesized that AM and rhizobial symbioses may significantly improve tolerance of a legume host to root hemiparasites. High tolerance in legume hosts has already been reported for some root hemiparasite–host pairs (Davies *et al.*, 1997; Cameron *et al.*, 2006; Decleer *et al.*, 2013). However, the previous observations were based either on field studies (Davies *et al.*, 1997; Decleer *et al.*, 2013) or on pot cultivation in non-sterilized growth substrate (Cameron *et al.*, 2006). In those cases, the effects of AM fungi and rhizobia could not be separately tested, as the ubiquitous microbial symbionts may have already existed in the soil. Manipulated inoculation experiments with comparable non-inoculated controls are therefore needed to test the impact of these symbionts on tolerance of legume hosts to root hemiparasites. Because the two microbial partners confer different nutrient benefits to a legume host, it is of interest to test if they have differential alleviating effects on damage caused by root hemiparasitic plants with different nutrient requirements.

In previous studies with root hemiparasitic *Pedicularis* species, we found that *P. rex* C. B. Clarke and *P. tricolor* Hand.-Mazz. had different nutrient requirements. While growth of *P. rex* is more sensitive to N deficiency, *P. tricolor* is more sensitive to P deficiency (Li *et al.*, 2013b). We also showed that *P. tricolor* acquired much more P from its hosts than *P. rex* (Li *et al.*, 2012a, 2013a). Although we have not quantitatively tested whether the two *Pedicularis* species differ in host N acquisition, N supply levels have been found to have a stronger impact on *P. rex* than on *P. tricolor* when attached to a host whose growth was sensitive to N deficiency (A.R. Li, R.J. Xue & Y. Chen unpublished data). These findings suggest that *P. rex* and *P. tricolor* may cause differential nutrient deprivation when attached to a host, with *P. rex* causing more stress on host N and *P. tricolor* causing more stress on host P acquisition. We therefore hypothesized that a legume host benefits more from N_2-fixing rhizobial association when parasitized by N-demanding *P. rex*, but benefits more from AM association when parasitized by P-demanding *P. tricolor*. Considering functional complementarity between AM fungi and rhizobia, dual inoculation was expected to have a synergistic effect, as on non-parasitized legume plants (Barea *et al.*, 1996; Jia *et al.*, 2004; Larimer *et al.*, 2014). However, because N and P deficiency have strong but different impacts on proper development and function of the symbiotic associations, often with N being a limiting factor for AM associations (Johnson *et al.*, 2015; Püschel *et al.*, 2016) and P a limiting factor for rhizobial associations (Mortimer *et al.*, 2008; Larimer *et al.*, 2014), the relative benefits of the microbial symbioses depend on N and P supply (Larimer *et al.*, 2014). We hence hypothesized that the interactive effect of these symbionts on the legume host would differ between the plants parasitized by *P. rex* and *P. tricolor*.

The objective of this study was therefore to investigate the effects of AM and rhizobial symbioses on alleviation of damage to a legume host by *P. rex* and *P. tricolor* in a manipulated pot cultivation experiment. The following questions were addressed. (1) Does inoculation with an AM fungus and rhizobium alleviate damage to the legume host by the root hemiparasites? (2) Is dual inoculation more beneficial than single inoculation of either symbiont? (3) Do alleviating effects of the soil microorganisms differ when the host is parasitized by different *Pedicularis* species with differential nutrient requirements? The knowledge obtained will not only enable better understanding of the function of soil microorganisms in alleviating host damage induced by root hemiparasites, but also help broaden understanding of how multiple contrasting associations with an individual host affect each other.

Materials and Methods

Experimental design

Four inoculation regimes and three plant combinations were included. The inoculation regimes were: (1) non-inoculated control (CK), (2) rhizobium inoculation with *Rhizobium leguminosarum* (+RH), (3) AM inoculation with *Glomus mosseae* (+AMF), and (4) dual inoculation with *R. leguminosarum* and *G. mosseae* (+RH+AMF). *Trifolium repens* L., a legume commonly found in natural habitats of the two *Pedicularis* species, was used as a host. In order to test effects of *P. rex* and *P. tricolor* on growth of the legume under different inoculation regimes, three plant combinations were included for each of the four inoculation regimes: one *T. repens* plant per pot (TR), one *T. repens* and one *P. rex* per pot (TR+PR), and one *T. repens* and one *P. tricolor* per pot (TR+PT). In total, there were 12 treatments, each with five replicates.

Plant materials

Seeds of *P. rex*, *P. tricolor* and *T. repens* were collected from Shangri-la Alpine Botanical Garden (99°38′E, 27°54′N, 3328 m), Yunnan, China. All seeds were stored in paper bags at 4°C

until used. Seeds were surface-sterilized in 4.5% commercial sodium hypochlorite for 8 min (for *Pedicularis* seeds) or 5 min (for *T. repens* seeds), and rinsed thoroughly with reverse osmosis (RO) water. Germination was carried out on moist filter papers at 18°C : 25°C (night : day) in an incubator. The photoperiod was 12 h light with 22.2 μmol photons m^{-2} s^{-1} illumination and 12 h dark.

Inocula

The rhizobium inoculum (*R. leguminosarum* Frank) was isolated from *T. repens* nodules collected from Kunming Botanical Garden (102°41′E, 25°01′N, 1990 m) using standard procedures on yeast extract mannitol agar (YMA; Vincent, 1970). Before use, the bacteria were streaked onto fresh YMA plates and incubated at 28°C in darkness for 4 d, then suspended in sterile RO water as rhizobial inoculant. The liquid inoculant had an ocular density of *c.* 0.5 at 600 nm. The suspension was added weekly to pots (10 ml per pot after thorough shaking) designated +RH and +RH+AMF for the first 3 wk. Non-inoculated pots were watered with the same volume of sterile RO water.

Inoculum of *G. mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (BGC YN05) consisted of colonized root fragments, soil and spores, derived from pot cultures prepared with *Sorghum bicolor* (L.) Moench grown in coarse sand. The inoculation rate was 20 g for each pot, designated +AMF or +RH+AMF, containing > 100 spores. To keep microbes other than AM fungi identical in all treatments, 20 ml of inoculum soil filtrate (prepared as described by Li *et al.*, 2012b) was added to all pots.

Planting and growth conditions

Uniform newly germinated seeds of *P. rex, P. tricolor* and *T. repens* were planted directly into pots. One host seedling was planted into the center of each 1.4-l pot with three drain holes at the bottom, containing 2.1 kg of a soil mixture of 10% soil collected from Kunming Botanical Garden, and 90% fine sand as described by Sui *et al.* (2014). The soil mixture was autoclaved at 121°C for 2 h before use. Plant available N (by the Kjeldahl method), available P (by the phosphovanado-molybdate method) and available K (by the flame photometry method) concentrations of the soil mixture were 14.3, 2.7 and 62.4 mg kg^{-1} dry soil, respectively. Ten days after planting the host, five germinated seeds of *P. rex* or *P. tricolor* were planted into pots designated as host–parasite pairs, each at a distance of 1.5–2 cm from the host. Seedlings of *Pedicularis* were thinned to one per pot 7 wk after planting, when establishment of functional connections between the hemiparasites and the host was confirmed by enhanced growth of the hemiparasites.

All plants were grown under outdoor conditions in Kunming Botanical Garden, protected with a glass roof and fly net to reduce the influence of rainwater and insects. The experiments were conducted for 16 wk (9 wk after successful establishment of parasitic associations) from late-April (spring) to late-August (summer) in 2016, with temperatures usually ranging from 10°C to 24°C, but occasionally up to 30°C. In order to facilitate the formation of root nodules, 20 ml nutrient solution with low nitrogen content (Chen & Wang, 2011; 0.10 mM Ca$(NO_3)_2{\cdot}4H_2O$, 3.38 mM $CaSO_4$, 0.24 mM $MgSO_4{\cdot}7H_2O$, 0.78 mM $K_2HPO_4{\cdot}3H_2O$, 0.31 mM $FeC_6H_5O_7$, 1.01 mM KCl, 46.26 nM H_3BO_3, 11.99 nM $MnSO_4$, 1.37 nM $ZnSO_4$, 0.12 nM H_2MoO_4, 5.01 nM $CuSO_4$) was added to each pot during the first 4 wk. Subsequently, 20 ml full-strength Long Ashton nutrient solution (Li *et al.*, 2013b) was supplied weekly. The pots were watered daily to field capacity with 350 ml of tap water. All pots were fully randomized and re-randomized weekly to reduce position effects.

Harvest and sampling

Plants were harvested 16 wk after planting (9 wk after successful establishment of parasitic associations). Replicate pots in which plants died before the end of this experiment were not included in data collection and analysis, leaving three–five intact replicates of each treatment. Shoots were cut at the soil surface and separated from roots for each pot. Shoot dry weights (DWs) of host and *Pedicularis* species were determined separately after oven-drying at 75°C for 48 h. Root samples were washed carefully to remove soil debris and kept in 50% ethanol until further analysis.

Pedicularis roots were separated from those of their host plants under a stereomicroscope. Haustoria on host roots were carefully cut off with minimized host tissue and pooled with *Pedicularis* roots. Fresh root weights (FWs) of host and *Pedicularis* species were determined separately after blotting with paper towels. The number of haustoria formed by each *Pedicularis* plant was counted under a stereomicroscope. The numbers of haustoria per gram dry root were used for further analysis. Nodules were cut from the legume roots using a razor blade. Nodule DW was measured after oven-drying. For assessment of AM colonization in *T. repens* and the root hemiparasites, a weighed subsample of root material less than 1 mm in diameter was randomly taken. AM colonization levels in root samples were determined by the magnified intersections method (McGonigle *et al.*, 1990), after clearing in 10% KOH and staining in a 5% ink-vinegar solution (Vierheilig *et al.*, 1998). The remaining root samples were oven-dried and weighed. The DW of a subsample used for checking AM colonization was obtained from the ratio between FW and DW of the remainder and FW of the subsample. Total root DW is presented as the sum of nodule DW (for the hosts), DW of roots used for assessment of AM colonization and DW of the remaining root samples.

Measurement of shoot N and P status

Three replicate pots were randomly chosen from each treatment to determine shoot N and P content. Dried shoot tissue of host and *Pedicularis* species from each pot was separately ground in a pestle and mortar, and digested in a 5 ml sulfuric–salicylic acid mix using the Kjeldahl method (Allen *et al.*, 1974). Shoot N concentrations were determined by distillation in a Kjeldahl apparatus (BUCHI K360, Switzerland) followed by titration with 0.01 N H_2SO_4. Shoot P concentrations were determined using the

phosphovanado-molybdate method (Hanson, 1950) and a spectrophotometer (UV1601 Shimadzu, Japan). Total shoot element content (mg per plant) was calculated by multiplying the element concentration by corresponding shoot DW.

Statistical analyses

Two-way ANOVAs were used to analyze AM colonization data, with the three-level factor 'parasitism by *Pedicularis* plants (PP)' (non-parasitized, parasitized by *P. rex*, parasitized by *P. tricolor*) and two-level factor 'rhizobium inoculation (RH)' (−, +) as factors for AM colonization in host roots, two-level factors 'parasite identity (PI)' (*P. rex*, *P. tricolor*) and RH (−, +) as factors for AM colonization in the hemiparasites. All other data on plant responses were analyzed by factorial three-way ANOVAs. For the host, the three-way ANOVAs were conducted with PP (non-parasitized, parasitized by *P. rex*, parasitized by *P. tricolor*), RH (−, +) and 'AM inoculation (AMF)' (−, +) as factors. For the hemiparasites, the three-way ANOVAs were conducted with PI (*P. rex*, *P. tricolor*), RH (−, +) and AMF (−, +) as factors. Assumptions of normality and equality of variance were assessed based on standardized residual analysis. Data were transformed if necessary using the appropriate transformations, specifically arcsine transformation for AM colonization levels, natural logarithm ($\log_e$) transformation for DWs, R : S ratios and host shoot nutrient contents, and square-root transformation for number of haustoria and hemiparasite shoot nutrient contents. The significance level for all tests was 0.05. Partial eta squared (η_p^2) was used to indicate effect size. All the analyses were performed using the Statistical Product and Service Solution (SPSS) software (version 16.0; SPSS China, Shanghai, China).

Results

Arbuscular mycorrhizal colonization in host plants and root hemiparasites

Arbuscular mycorrhizal colonization levels in *G. mosseae*-inoculaed *T. repens* were above 65% of root length on average (Fig. 1a). Dual inoculation with *R. leguminosarum* and *G. mosseae* greatly increased the percentage of root length colonized in *T. repens* ($P < 0.01$, $\eta_p^2 = 0.515$), particularly in the non-parasitized plants (Table 1; Fig. 1a). Parasitism by the hemiparasites caused a non-significant increase in AM colonization of non-rhizobium-inoculated *T. repens*. In contrast to high AM colonization levels in the host, colonization was low (< 10% of root length) in both *Pedicularis* species regardless of inoculation regimes (Fig. 1b). Neither dual inoculation nor parasite identity had any significant effects on AM colonization of the root hemiparasites (Table 1). No AM colonization was observed in non-inoculated roots.

Nodulation in *T. repens* roots

Spontaneous nodules were observed on *T. repens* roots from non-rhizobium-inoculated pots (Fig. 1c). The spontaneous nodules were elongated and slightly ovoid, morphologically resembling normal nodules produced by rhizobium-inoculated plants, except that they were white while those on inoculated plants were pinkish. Parasitism and AM inoculation had significant interactive effects on nodule DWs ($P < 0.05$, $\eta_p^2 = 0.164$; Table 2). Arbuscular mycorrhizal inoculation markedly increased (up to 10-fold) nodule DWs in both parasitized and non-parasitized *T. repens* (Fig. 1c; Table 2). Parasitism by *P. rex* or *P. tricolor* halved nodule DWs in non-mycorrhizal *T. repens*, but had little influence on those of AM hosts (Fig. 1c). Spontaneous nodules were observed at a similar level in non-rhizobium-inoculated *T. repens* (CK and +AMF) as the inoculated ones (+RH and RH+AMF; Fig. 1c).

Microbial-mediated alleviation of damage to *T. repens* by the hemiparasites

Significant three-way interactions were detected in terms of impact on shoot biomass of the host ($P < 0.05$, $\eta_p^2 = 0.193$; Table 2). When compared with non-parasitized counterparts, parasitism by either *P. rex* or *P. tricolor* reduced shoot DWs of *T. repens* to some extent in most cases (Fig. 2a). The only exception was that parasitism by *P. tricolor* increased shoot DWs of the host by an average of 42.4% after single AM inoculation (+AMF; Fig. 2a). The promoting effect of the AM fungus on *T. repens* parasitized by *P. tricolor* was greater than on those parasitized by *P. rex* (Fig. 2a). Dual inoculation (+RH+AMF) had different effects on the outcome of single inoculation (+AMF) on hosts parasitized by *P. rex* or *P. tricolor* (Fig. 2a). While rhizobium increased the beneficial effect of +AMF on shoot DWs of non-parasitized *T. repens* (an average increase of 57.1%) and those parasitized by *P. rex* (an average increase of 93.3%), it reduced the AM effect when the host was parasitized by *P. tricolor* (average decrease of 33.1%; Fig. 2a).

Parasitism and AM inoculation had similar effects on root biomass as on host nodule DWs, with significant interaction effects ($P < 0.01$, $\eta_p^2 = 0.268$; Table 2). Parasitism by either *P. rex* or *P. tricolor* significantly reduced root DWs of non-mycorrhizal hosts, by an average of 66.7% and 77.8%, respectively, but had negligible impact on AM hosts (Fig. 2a). AM inoculation greatly increased root DWs of both non-parasitized and parasitized *T. repens*. Rhizobium inoculation had little influence on root DWs of *T. repens*. Both parasitism ($P < 0.05$, $\eta_p^2 = 0.151$) and AM inoculation ($P < 0.001$, $\eta_p^2 = 0.257$) reduced R : S ratios in *T. repens* (Fig. 2b; Table 2). Inoculation with rhizobium had no effect on the R : S ratios.

Significant three-way interactive effects on host shoot N ($P < 0.05$, $\eta_p^2 = 0.298$) and P contents ($P < 0.05$, $\eta_p^2 = 0.214$) were observed (Table 2). The overall patterns were similar to those for host shoot DWs (Fig. 2a,c,d). When compared with plants inoculated with AM fungus alone (+AMF), dual inoculation with the AMF fungus and rhizobium had different effects on plant acquisition of N and P in the presence of different *Pedicularis* species. Shoot N and P acquisition was strongly increased in *T. repens* parasitized by *P. rex* after dual inoculation, but reduced in those parasitized by *P. tricolor* (Fig. 2c,d).

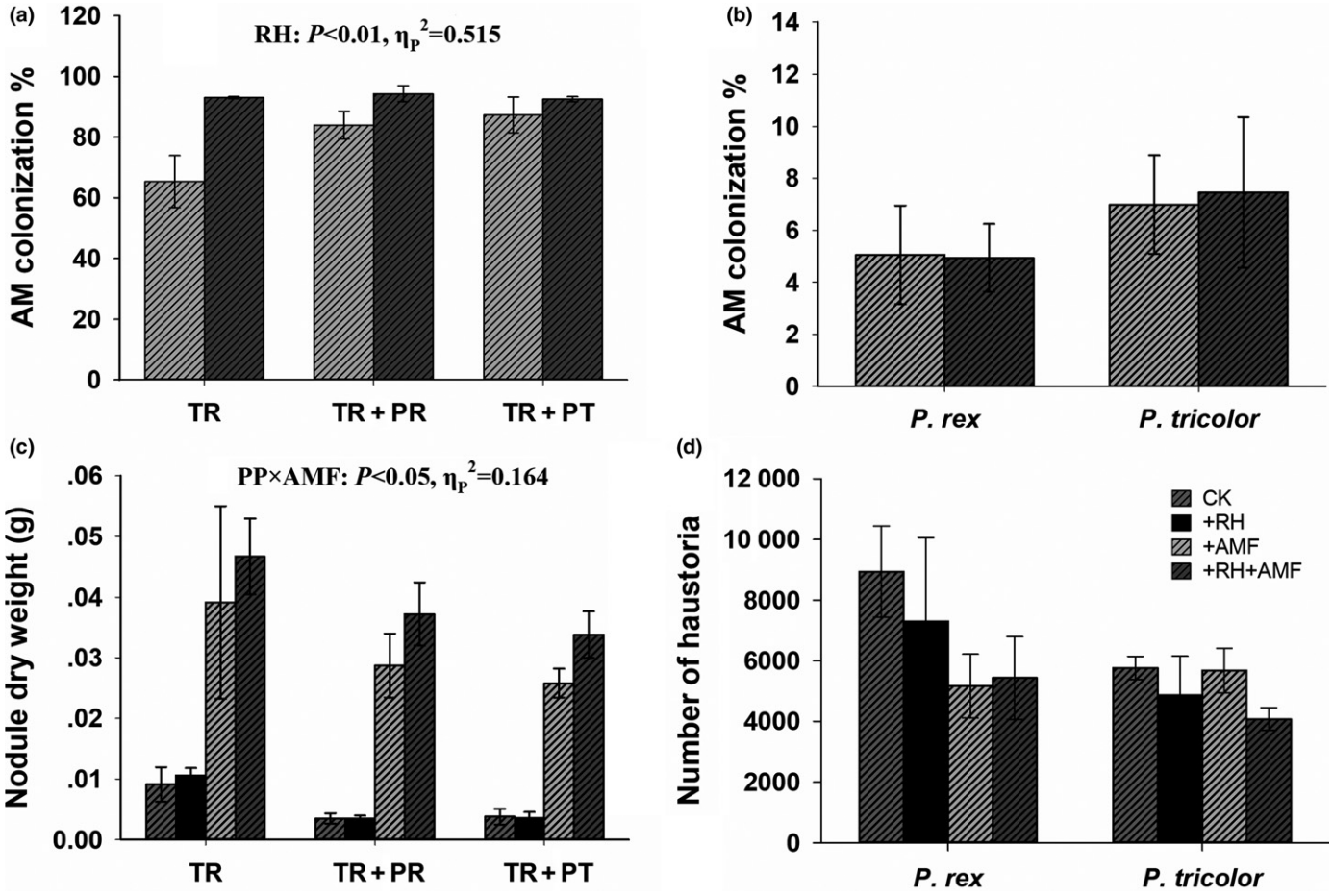

Fig. 1 Arbuscular mycorrhizal (AM) colonization (% root length colonized), nodulation and haustorium formation in *Trifolium repens* or *Pedicularis* spp. (a) AM colonization in *Trifolium repens*, (b) AM colonization in *Pedicularis rex* and *P. tricolor*, (c) nodule dry weights (DW) of *T. repens* per plant, and (d) number of haustoria per gram dry root of *P. rex* and *P. tricolor*. Data are presented as mean ± SE (n = 3–5). The significant higher-order interaction or the main factor effects and partial eta squared (η_p^2) indicating effect size are shown. For other values, see Tables 1 or 2. Plant combinations: TR, a single plant of *T. repens*; TR+PR, one *T. repens* plant parasitized by one *P. rex* plant; TR+PT, one *T. repens* plant parasitized by one *P. tricolor* plant. Inoculation regimes: CK, non-inoculated control; +RH, inoculation with *Rhizobium leguminosarum*; +AMF, AM inoculation with *Glomus mosseae*; +RH+AMF, dual inoculation with rhizobium and AM fungus.

Table 1 Summary of two-way factorial ANOVA testing effects of *Rhizobium leguminosarum* inoculation (RH) and parasitism by *Pedicularis* plant (PP) on arbuscular mycorrhizal (AM) colonization levels (% root length) in roots of *Trifolium repens* (TR) and *Pedicularis* plants inoculated with AM fungus *Glomus mosseae*

		% AM col. in host			% AM col. in *Pedicularis*	
Factors	df	F	η_p^2	df	F	η_p^2
RH	1,12	**12.749****	**0.515**	1,8	0.007	0.001
PP	2,12	2.527	0.296	1,8	1.141	0.125
PP × RH	2,12	2.3	0.277	1,8	0.02	0.002

Data were arcsine transformed before analysis. Partial eta squared (η_p^2) is presented to indicate effect size. df, degrees of freedom. Values suggesting significant effects are given in bold. Significance level: **P < 0.01.

Overall, AM hosts acquired markedly more N and P than non-mycorrhizal ones.

Performance of the root hemiparasites

One–two attached *Pedicularis* plants died in a few treatments between attachment and the harvest of the experiment, with no clear pattern in terms of treatment effect. Significant three-way interactions were detected for shoot biomass of the root hemiparasites (P < 0.05, η_p^2 = 0.196; Table 3). *P. rex* had lower shoot DWs than *P. tricolor* when not inoculated with any symbionts, but had similar shoot DWs to *P. tricolor* when inoculated with rhizobia, due to a significant reduction in shoot DWs of *P. tricolor* (Fig. 3a). AM inoculation (+AMF) greatly increased shoot DWs of *P. rex* (by 31-fold relative to non-mycorrhizal control), but showed little promoting effect on *P. tricolor*. As a result, *P. rex* had much higher shoot DWs than *P. tricolor* in +AMF pots. Dual inoculation (+RH+AMF) slightly (though not significantly) decreased shoot DW of *P. rex* while it greatly increased shoot DW of *P. tricolor* when compared with their counterparts inoculated with the AM fungus alone (+AMF), resulting in similar shoot DWs of both the parasites.

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Table 2 Summary of three-way ANOVA testing the effects of parasitism by *Pedicularis* plants (PP), rhizobia (RH) and arbuscular mycorrhizal fungi (AMF) on shoot dry weights (DWs), root DWs, root : shoot (R : S) ratio, nodule DWs, shoot nitrogen (N) content and shoot phosphorus (P) content of *Trifolium repens*

		Shoot DWs		Root DWs		R : S ratio		Nodule DWs			Shoot N content		Shoot P content	
Factors	df	*F*	η_p^2	*F*	η_p^2	*F*	η_p^2	*F*	η_p^2	df	*F*	η_p^2	*F*	η_p^2
PP	2,37	**27.763*****	**0.600**	**22.114*****	**0.544**	**3.284***	**0.151**	**9.033*****	**0.328**	2,24	**41.689*****	**0.776**	**35.018*****	**0.745**
RH	1,37	3.406	0.084	0.864	0.023	0.157	0.004	2.664	0.067	1,24	4.136	0.147	**12.472****	**0.342**
AMF	1,37	**613.298*****	**0.943**	**198.763*****	**0.843**	**12.801*****	**0.257**	**202.085*****	**0.845**	1,24	**775.02*****	**0.970**	**1247*****	**0.981**
PP × RH	2,37	**7.577****	**0.291**	1.714	0.085	0.727	0.038	0.187	0.01	2,24	**10.301*****	**0.462**	**14.18*****	**0.542**
PP × AMF	2,37	**10.658*****	**0.366**	**6.768****	**0.268**	0.151	0.008	**3.617***	**0.164**	2,24	**27.032*****	**0.693**	**30.345*****	**0.717**
RH × AMF	1,37	0.735	0.019	0.530	0.014	2.046	0.052	0.384	0.01	1,24	0.561	0.023	0.016	0.001
PP × RH × AMF	2,37	**4.418***	**0.193**	0.614	0.032	2.092	0.102	0.067	0.004	2,24	**5.089***	**0.298**	**3.27***	**0.214**

Data were natural logarithm ($\log_e$) transformed before analysis. Partial eta squared (η_p^2) is presented to indicate effect size. df, degrees of freedom. Values suggesting significant effects are given in bold. Significance levels: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

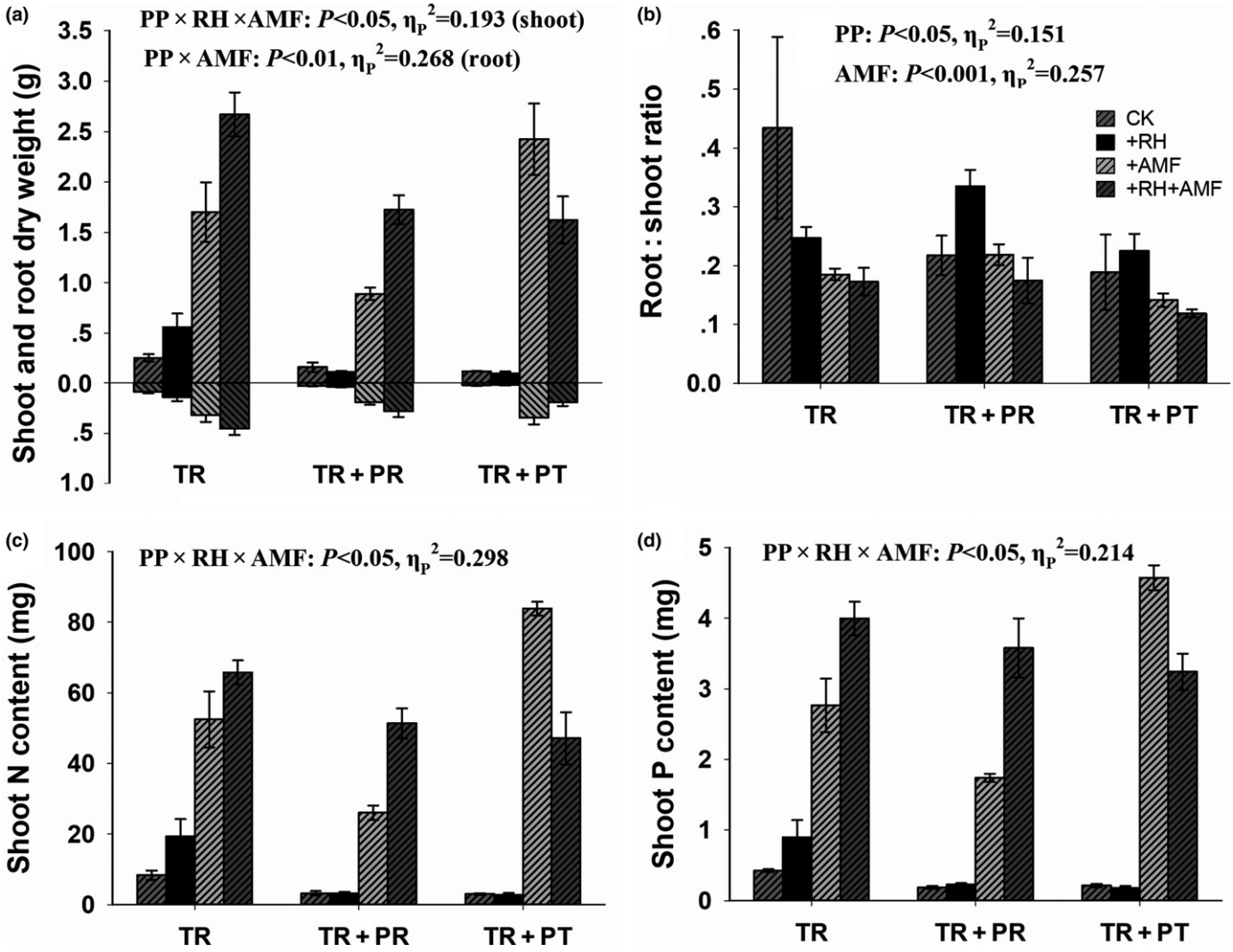

Fig. 2 Growth and nutrient contents of *Ttifolium repens.* (a) Shoot and root dry weight (DW), (b) root : shoot DW ratio, (c) shoot nitrogen (N) content, and (d) phosphorus (P) content with or without parasitism by a *Pedicularis* plant under different inoculation regimes. Data are presented as mean ± SE of three–five replicates. The significant higher-order interaction or the main factor effects and partial eta squared (η_p^2) indicating effect size are shown. For other values, see Table 2. Plant combinations: TR, a single plant of *T. repens*; TR+PR, one *T. repens* plant parasitized by one *P. rex* plant; TR+PT, one *T. repens* plant parasitized by one *P. tricolor* plant. Inoculation regimes: CK, non-inoculated control; +RH, inoculation with *Rhizobium leguminosarum*; +AMF, arbuscular mycorrhizal (AM) inoculation with *Glomus mosseae*; +RH+AMF, dual inoculation with rhizobium and AM fungus.

Table 3 Summary of three-way ANOVA testing the effects of parasite identity (PI), rhizobium (RH) and arbuscular mycorrhizal fungi (AMF) on shoot dry weights (DWs), root DWs, root : shoot (R : S) ratio, number of haustoria per gram dry root, shoot nitrogen (N) content and shoot phosphorus (P) content of *Pedicularis rex* and *P. tricolor*

		Shoot DWs		Root DWs		R : S ratio		Num. haustoria per gram dry root			Shoot N content		Shoot P content	
Factors	df	*F*	η_p^2	*F*	η_p^2	*F*	η_p^2	*F*	η_p^2	df	*F*	η_p^2	*F*	η_p^2
PI	1,23	0.000	0.000	**17.215*** **	**0.428**	**21.777*** **	**0.486**	1.711	0.069	1,16	**6.528***	**0.290**	**9.374****	**0.369**
RH	1,23	1.266	0.052	**9.302****	**0.288**	3.510	0.132	1.344	0.055	1,16	0.073	0.005	0.058	0.004
AMF	1,23	**88.494*** **	**0.794**	**34.997*** **	**0.603**	**42.229*** **	**0.647**	2.168	0.086	1,16	**156.262*** **	**0.907**	**264.848*** **	**0.943**
PI × RH	1,23	0.483	0.021	**5.073***	**0.181**	2.479	0.097	0.083	0.004	1,16	0.490	0.030	3.833	0.193
PI × AMF	1,23	2.621	0.102	**8.560****	**0.271**	1.082	0.045	1.107	0.046	1,16	1.783	0.100	4.47	0.218
RH × AMF	1,23	**4.817***	**0.173**	**5.678***	**0.198**	0.146	0.006	0.152	0.007	1,16	2.647	0.142	3.067	0.161
PI × RH × AMF	1,23	**5.596***	**0.196**	**9.06****	**0.283**	0.008	0.000	0.441	0.019	1,16	**5.510***	**0.256**	**12.737****	**0.443**

Data were natural logarithm ($\log_e$) transformed for DWs and R : S ratios, and square-root transformed for number of haustoria per gram dry root and nutrient contents before analysis. Partial eta squared (η_p^2) is presented to indicate effect size. df, degrees of freedom. Values suggesting significant effects are given in bold. Significance levels: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Parasite identity, rhizobium and AM inoculation showed significant three-way interactive effects on root DWs of the hemiparasites ($P < 0.01$, $\eta_p^2 = 0.283$; Table 3). Rhizobium inoculation had no effect on root DWs of *P. rex* in non-mycorrhizal pots, but slightly reduced the promotional AM effect in +RH+AMF pots. On the contrary, rhizobium inoculation reduced root DWs of *P. tricolor* in non-mycorrhizal pots, but had no significant effect in +RH+AMF pots (Fig. 3a). Overall, R : S ratios were higher in *P. rex* than in *P. tricolor* ($P < 0.001$, $\eta_p^2 = 0.486$; Fig. 3b). Arbuscular mycorrhizal inoculation significantly reduced R : S ratios of both hemiparasites ($P < 0.001$, $\eta_p^2 = 0.647$). Neither parasite identity nor inoculation regimes showed any statistically significant effect on number of haustoria produced per gram dry roots (Fig. 1d; Table 3).

Significant three-way interactive effects on shoot N ($P < 0.05$, $\eta_p^2 = 0.256$) and P contents ($P < 0.01$, $\eta_p^2 = 0.443$) of the hemiparasites were detected, in similar patterns as observed for their shoot and root biomass (Fig. 3c,d; Table 3). Single inoculation with rhizobium (+RH) had little effect on *P. rex*, and slightly reduced shoot N and P contents in *P. tricolor* in non-mycorrihzal pots. By contrast, dual inoculation (+RH+AMF) greatly increased shoot N and more so P contents in *P. tricolor*, but decreased those in *P. rex* when compared with plants inoculated with AM fungus alone (+AMF). Overall, the hemiparasites acquired markedly more N and P in AM treatments than the non-mycorrhizal ones (Fig. 3c,d).

Discussion

Arbuscular mycorrhizal fungi and rhizobia play important roles in alleviating host damage

As expected, the AM fungus and rhizobium had robust alleviating effects on host damage by the hemiparasites (Fig. 2; Table 2). Complex and significant interactive effects between inoculation regimes and hemiparasite identity on host performance were observed (Table 2). This confirmed our hypothesis that beneficial soil microorganisms may impact the direction and magnitude of parasite–host interaction, whereas the interaction outcomes may be context dependent.

Arbuscular mycorrhizal inoculation accounted for a great proportion of treatment effects on both the host and hemiparasites for most parameters tested, as shown by the large η_p^2 (Tables 2, 3). A strong influence of AM fungi on interactions between a root hemiparasitic plant and its host plant has been observed in a few other parasite–host pairs. Both positive (Davies & Graves, 1998; Salonen *et al.*, 2001; Stein *et al.*, 2009) and negative (Lendzemo & Kuyper, 2001; Gworgwor & Weber, 2003; Li *et al.*, 2013b; Sui *et al.*, 2014) effects have been reported. Based on the limited number of studies, direction and magnitude of host response to AM inoculation seems to play a key role in determining the direction and magnitude of the AM effect on the hemiparasites. Taking *Pedicularis* species as examples, while AM fungal inoculation had promoting effects on *Pedicularis* plants attached to a strongly responsive legume host in this experiment (Fig. 3), AM effects on *Pedicularis* species attached to non-responsive grass hosts were suppressive (Li *et al.*, 2013b; Sui *et al.*, 2014). In both responsive and non-responsive cases, however, AM alleviated host damage induced by parasitism of the *Pedicularis* plants, despite contrasting effects on the root hemiparasites and probably different mechanisms involved.

Although the nodule DWs between non-inoculated plants and those of *R. leguminosarum*-inoculated legume hosts were similar (Fig. 1c), significant growth responses were detected only for *R. leguminosarum*-inoculated plant pairs (Fig. 2a). This suggested that the spontaneous nodules observed in non-inoculated plants were not fixing N_2 (Blauenfeldt *et al.*, 1994; Tirichine *et al.*, 2006). Inoculation with *R. leguminosarum* alone had negligible effects on most parameters measured for both parasitized *T. repens* and the hemiparasites (Tables 2, 3). However, the marked effects of dual inoculation (+RH+AMF) on growth of both the host and hemiparasites suggested significant roles of this bacterium in regulating legume–parasite interactions (Figs 2, 3;

Tables 2, 3). Considering the fact that legumes are preferred hosts for many parasitic plants and are well known to associate with N_2-fixing rhizobia, it is surprising that very few efforts have been made to address the influence of rhizobia on host–parasite interactions (Mabrouk *et al.*, 2007; Jiang *et al.*, 2008; Lu *et al.*, 2013; Cirocco *et al.*, 2017). Unfortunately, most of these studies did not include a strict control (i.e. with other factors identical between rhizobium-inoculated and non-inoculated treatments). As a consequence, the exclusive effect of the rhizobia on the interactions between a parasitic plant and its legume host remain largely unexplored. In their controlled investigation into effects of several rhizobium strains on interactions between the root holoparasite *Orobanche crenata* and peas, Mabrouk *et al.* (2007) found that *R. leguminosarum* decreased parasitism of the host. However, effects of the N_2-fixing rhizobia varied among strains depending on their compatibility with the legume host. This may partially explain the observed variations in rhizobia effects on different parasite–host plant pairs.

Relative benefits of microbial inoculation depended on nutrient requirements of the hemiparasites

Distinct patterns in response to +AMF and dual inoculation (+RH+AMF) by different host–parasite pairs were observed. While dual inoculation strongly enhanced the promotional effect of +AMF inoculation on growth and nutrient acquisition by non-parasitized *T. repens* plants and those parasitized by *P. rex*, it significantly reduced the AM promotion when the host was parasitized by *P. tricolor* (Fig. 2). The different response patterns may be explained by differential nutrient requirements of the hemiparasites and hence differential nutrient stress on the host (Li *et al.*, 2012a, 2013b). Although differential nutrient stress caused by parasitism between *P. rex* and *P. tricolor* were not detected on the non-mycorrhizal *T. repens* plants, contrasting patterns in shoot N and P contents of the AM hosts parasitized by the two *Pedicularis* species were observed (Fig. 2c,d). The results indicated that the two *Pedicularis* species may have differentially affected

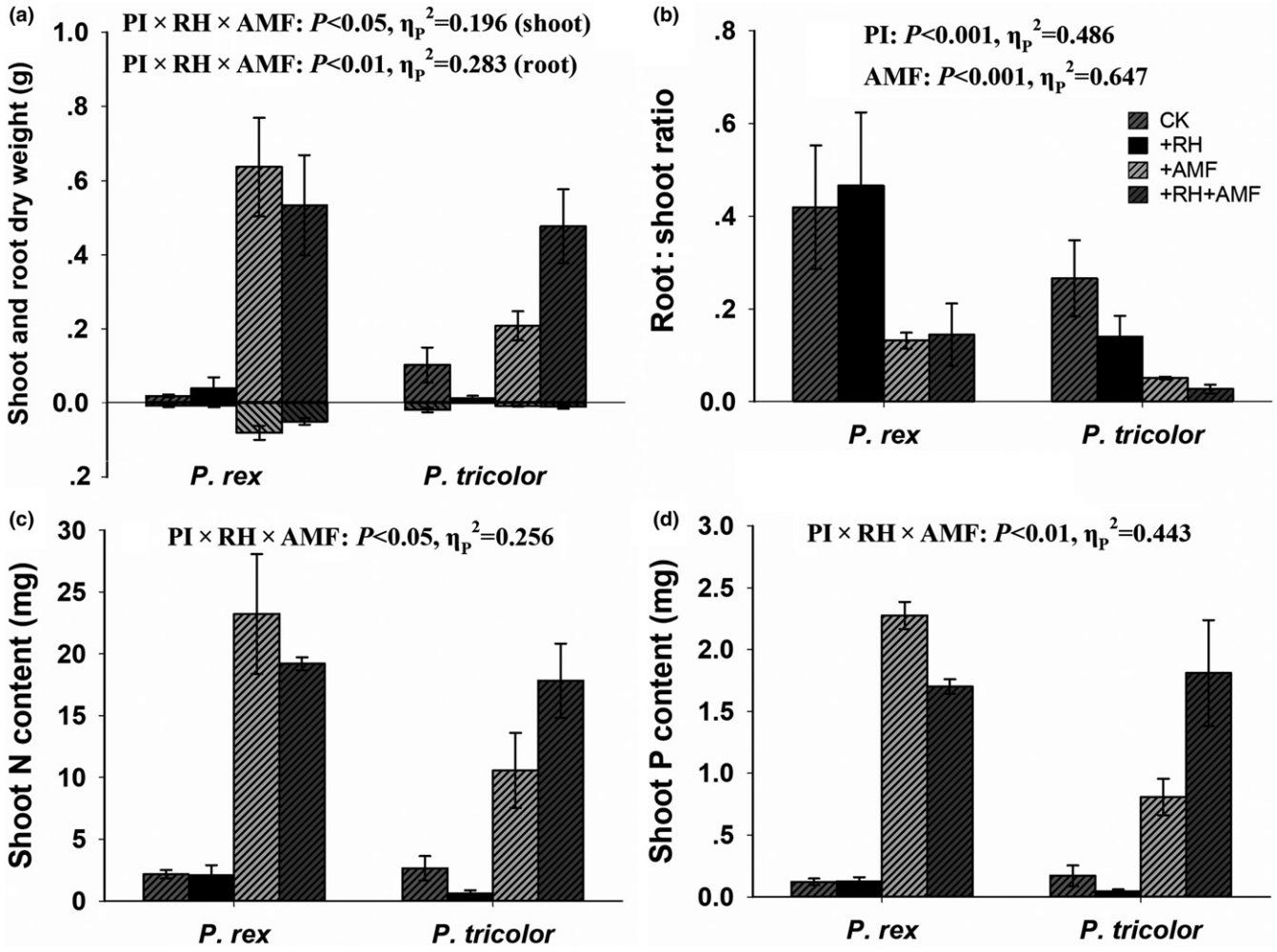

Fig. 3 (a) Shoot and root dry weight (DW), (b) root : shoot weight ratio, (c) shoot nitrogen (N) content, and (d) phosphorus (P) content of *Pedicularis rex* and *P. tricolor* attached to a *Trifolium repens* plant. Data are presented as mean ± SE of three–five replicates. The significant higher-order interaction or the main factor effects and partial eta squared (η_p^2) indicating effect size are shown. For other values, see Table 3. Inoculation regimes: CK, non-inoculated control; +RH, inoculation with *Rhizobium leguminosarum*; +AMF, arbuscular mycorrhizal (AM) inoculation with *Glomus mosseae*; +RH+AMF, dual inoculation with rhizobium and AM fungus.

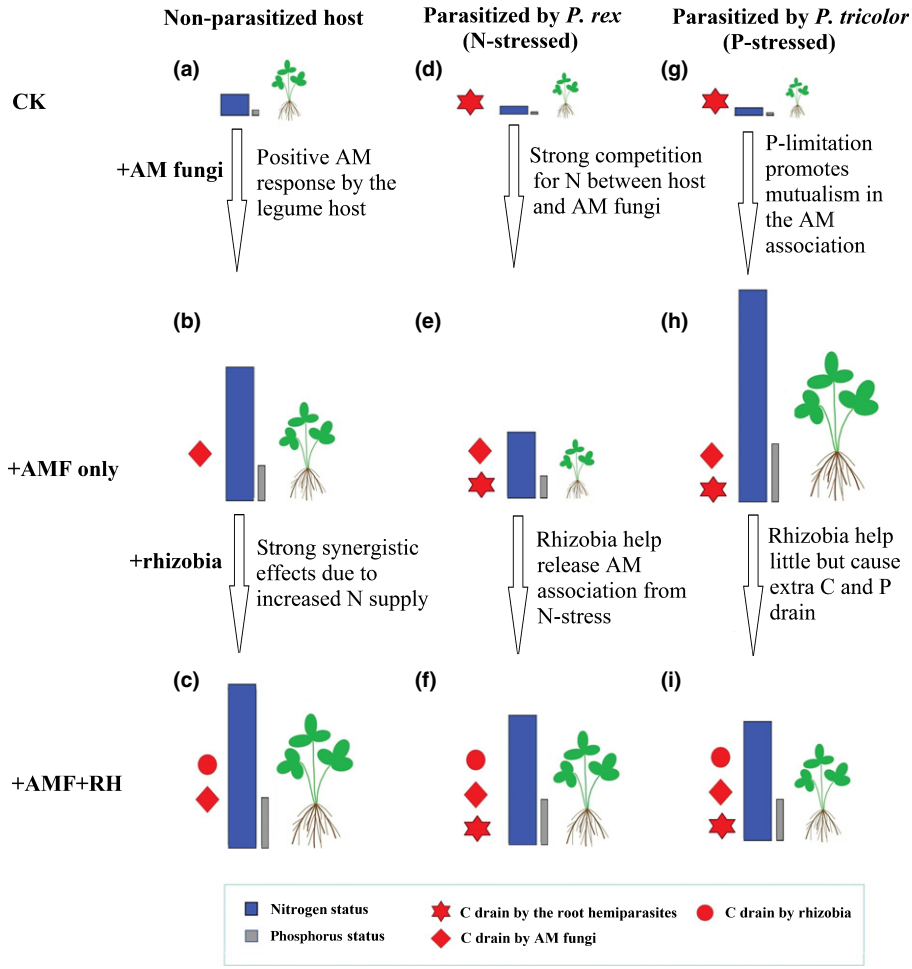

Fig. 4 Proposed model for differential effects of dual inoculation (+AMF+RH) on *Trifolium repens* parasitized by *Pedicularis rex* and *P. tricolor*, as shown by schematic representation of nitrogen (N), phosphorus (P) and carbon (C) dynamics within the 'host–parasite–arbuscular mycorrhizal (AM) fungus–rhizobium' system. Inoculation regimes: CK, non-inoculated control; +AMF, inoculation with AM fungus *Glomus mosseae*; +AMF+RH, dual inoculation with the AM fungus (+AMF) and *Rhizobium leguminosarum* (+RH). Based on our previous studies, *P. rex* may cause stronger N-stress and *P. tricolor* may cause stronger P-stress in their hosts. The sizes of the components are not proportional to the real values. (a) Non-parasitized *T. repens* without microbial inoculation, (b) *T. repens* plants showing positive AM response when inoculated with AMF alone (+AMF), (c) synergistic effects of dual inoculation on *T. repens* plants due to increased N supply by the N_2-fixing rhizobia, (d) *T. repens* plants parasitized by N-demanding *P. rex* showing C, N and P drain and reduced growth, (e) less beneficial AM association with presumably N-stressed *T. repens* plants when parasitized by *P. rex*, (f) the N_2-fixing rhizobia release the AM association from N-stress and improve host nutrient status and growth, (g) *T. repens* plants parasitized by P-demanding *P. tricolor* showing C, N and P drain and reduced growth, (h) more beneficial AM association with presumably P-stressed *T. repens* plants when parasitized by *P. tricolor*, (i) the N_2-fixing rhizobia help little in P-limitation, but cause extra C and P drain to maintain nodules and thus reduce *T. repens* growth when compared with +AMF.

interactions between their hosts and the symbiotic soil microorganisms. A schematic diagram showing N, P and C dynamics within the 'host–parasite–AM fungi–rhizobia' system of different plant combinations is presented as a framework to facilitate the interpretation of the differential inoculation effects (Fig. 4).

For the non-parasitized *T. repens* plants (Fig. 4a), AM inoculation promoted nutrient acquisition and plant growth (Fig. 4b), and dual inoculation (+AMF+RH) had strong synergistic effects (Fig. 4c) as expected (Barea *et al.*, 1996; Jia *et al.*, 2004; Larimer *et al.*, 2014). Because AM fungi and their hosts are more likely to form mutualistic associations under P-limited conditions, but commensal or even parasitic associations under N-limited conditions (Johnson *et al.*, 2015; Püschel *et al.*, 2016), parasitism by the two *Pedicularis* species that cause differential nutrient stress on the host may have shifted the direction and magnitude of the symbiotic interactions. Parasitism by N-demanding *P. rex* may have caused N-stress in the host, and hence strong competition for N between the host and the AM fungus (Fig. 4d). This may have shifted the mutualistic AM association toward commensalism, resulting in reduced plant biomass of the host (Fig. 4e) when compared with non-parasitized AM plants (Fig. 4b). In this case, dual inoculation with rhizobium may have released the N-stress caused by *P. rex* and hence restored the mutualism between AM fungi and the host, as shown by significantly higher legume DW in dual-inoculated plants (Fig. 4f) compared with single AM-inoculated plants (Fig. 4e). On the other hand, parasitism by P-demanding *P. tricolor* may have caused P-stress to the host (Fig. 4g) and hence shifted the AM association towards mutualism, as shown by markedly increased host biomass (Fig. 4h) when compared with the non-parasitized plants (Fig. 4b). In this case, the

N_2-fixing rhizobium was of little help. As the rhizobia within nodules require both carbohydrates and P to support growth and propagation (Mortimer *et al.*, 2008; Larimer *et al.*, 2014), maintenance of the symbiosis was merely a carbon and P drain to the host, resulting in decreased plant biomass following dual inoculation (Fig. 4i) when compared with single AM inoculation (Fig. 4h).

The dynamics of changes in plant nutrient status throughout symbiosis development and quantitative analysis of nutrient exchange between the organisms were outside the scope of this study. Nevertheless, knowledge of these aspects is essential for a better understanding of the regulation processes as well as underlying mechanisms. Quantitative tracer studies with multiple harvests and measurements of nutrient transfer during symbiosis development will further our understanding of the complex multi-species interactions.

Possible mechanisms of alleviation of hemiparasite-induced host damage by arbuscular mycorrhizal and rhizobial symbioses

Association with AM fungi and rhizobia directly improved nutrient status and growth of the host (Fig. 2). The marked increase in shoot DW of the host suggested greatly increased competition for light with the tiny *Pedicularis* plants, which offset benefits of improved host nutritional status and suppressed growth of the hemiparasites (Matthies, 1995; Těšitel *et al.*, 2011; Li *et al.*, 2012a). The suppressed growth of hemiparasites could further reduce relative biomass loss in parasitized hosts.

Apart from improved host nutrient status and strong light competition from vigorously growing host plants, direct competition for host resources from the below-ground hyphae of AM fungi and rhizobia in nodules may also account for the alleviation effects. Rhizobia and AM fungi have been well documented to facilitate N and P absorption in legume plants in exchange of host carbohydrates (Kistner & Parniske, 2002; Fitter, 2006; Smith *et al.*, 2009), with rhizobia receiving 6–30% host carbohydrates and AM fungi 10–23% (Mortimer *et al.*, 2008). In addition, nodulation requires a large amount of P (Mortimer *et al.*, 2008), and development of AM fungal structures requires substantial N (Johnson *et al.*, 2015; Püschel *et al.*, 2016). Direct competition between the microbial symbionts and the hemiparasite for host-derived resources may have limited growth of the hemiparasites, as shown by the significant reduction in shoot and root DW of *P. tricolor* when attached to rhizobium-inoculated *T. repens* (Fig. 3a). However, due to the complex multitrophic nature of multi-species interactions, outcomes of the interactions did not always appear to be a simple sum of component interactions. More work is required for a better understanding of the shape and relative contribution of each interaction to observed outcomes under various conditions.

Conclusions

Interactive effects of AM fungi and rhizobia on parasitic plant–host interactions were tested for the first time. Our results demonstrated strong effects of beneficial microorganisms on alleviating host damage by root hemiparasites. By using two root hemiparasites with contrasting nutrient requirements, we showed that the relative benefits of microbial inoculation were related to different nutritional interactions between the host and hemiparasites. The results also showed, though indirectly, that different hemiparasites may impose differential nutrient deprivation on their hosts. Future work needs to take soil microorganisms into account when addressing host–parasite interactions involving root hemiparasitic plants. Investigations of the multi-species interactions along a gradient of nutrient supply will increase understanding of regulation mechanisms influencing the interactions between such nutrient-driving symbioses.

Acknowledgements

The authors thank Ms Ying Qi and Mr Zhanghao Yang from Kunming Institute of Botany, Chinese Academy of Sciences, for their excellent help with harvest. The authors thank Professor Naomi S. Altman from Pennsylvania State University for her valuable comments on the statistical analysis. The authors are grateful to Professor Sally Smith and Professor Andrew Smith from the University of Adelaide, and three anonymous reviewers for their instructive comments and valuable suggestions that greatly improved the manuscript. The research was financially supported by National Natural Science Foundation of China (no. 31400440, no. 31370512 and U1303201), Natural Science Foundation of Yunnan Province (2016FB059), and funding for Airong Li from Youth Innovation Promotion Association of Chinese Academy of Sciences and Young Academic and Technical Leader Raising Foundation of Yunnan Province (2014HB047).

Author contributions

X-L.S. and A-R.L. conceived and designed the experiment. X-L.S., T.Z., Y-Q.T. and R-J.X. performed the experiment and collected the data. X-L.S. analyzed the data and wrote the first draft of the paper. A-R.L. assisted in data analysis, interpreted the analysis and led the revision of the paper.

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