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Is a specialist root-feeding insect affected by arbuscular mycorrhizal fungi?

Amanda F. Currie[a], Philip J. Murray[b], Alan C. Gange[a,*]

[a] School of Biological Sciences, Royal Holloway University of London, Egham Hill, Egham, Surrey TW20 0EX, UK
[b] Department of Sustainable Soils and Grassland Systems, Rothamsted Research, North Wyke, Okehampton, Devon EX20 2SB, UK

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are known to reduce the growth of generalist root-feeding insects, but whether the same is true for a specialist insect is unknown.

White clover (*Trifolium repens*) was inoculated with the AM fungi *Glomus fasciculatum* and *Glomus mosseae* individually and in combination, and larvae of the clover root weevil (*Sitona lepidus*) reared on mycorrhizal and non-mycorrhizal plants. On emergence, adult weevils were weighed and the percentage of larvae surviving to adulthood was calculated for each treatment.

Larval survival to adulthood was increased by both species of fungi, but weight was unaffected. Larval feeding reduced foliar biomass, but had no effect when two fungi colonized the root system. Although larval survival was greatest in the dual fungal treatment, the proportion of grazed root nodules was lower, suggesting that AMF may improve root quality for the herbivore. Root feeding caused an increase in arbuscular colonization in the dual fungal treatment, and this may have enabled plants to tolerate herbivory, through enhanced mycorrhizal benefit.

We conclude that a specialist root feeder is less affected by the presence of AMF than are generalist species. However, AMF enable a plant to tolerate the effects of root loss, and this is dependent on the number of mycorrhizal species in the root system.

1. Introduction

Studies on the effects of arbuscular mycorrhizal fungi (AMF) on insect herbivores have focussed primarily on foliar feeding insects (Gange, 2007; Koricheva et al., 2009). The latter paper reports the results of a meta-analysis, in which it was found that generalist insects seem to respond negatively to the presence of mycorrhizas, while specialists tend to respond positively. AM fungi are known to alter both constitutive and induced defences in foliar tissues (Bennett et al., 2009; Kempel et al., 2010) and it is likely that insect responses are due to such chemical changes (Bowers and Puttick, 1988). As AMF cause large changes in secondary metabolites in roots (Schliemann et al., 2008), it might be expected that root-feeding insects would be influenced more by mycorrhizal presence, than foliar feeding species. However, to date, relatively few studies have involved insect root herbivores and all of these have used generalist feeding species in the genus *Otiorhynchus*, feeding on roots of *Taraxacum officinale* F.H. Wigg, *Fragaria* L. × *ananassa* (strawberry) or various trees. These studies do conform to the general pattern, in that AMF reduce the growth of root-feeding larvae (Gange et al., 1994; Gange, 1996, 2001) as do ectomycorrhizal fungi (Halldorsson et al., 2000; Oddsdottir et al., 2010). However, a feature of these studies is that while any one species of AMF reduces larval growth and survival, combinations of fungi seem to have much less effect. For example, Gange (2001) used *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe and *G. fasciculatum* (Thaxter Sensu. Gerd.) Gerd. & Trappe, as individual and combined inocula on strawberry plants infested with *Otiorhynchus sulcatus* (Fab.) larvae. When either fungus was present alone, larval survival and weight was halved, but when both fungi were present, growth and survival was no different to that observed in the non-mycorrhizal treatment. A similar pattern was also found by Gange (1996), where *G. mosseae* and *Glomus intraradices* (Schenck & Smith) were used as individual and combined sources of inoculum. Thus, while the responses of specialist and generalist insects to mycorrhizas are known to differ, it is unknown whether this also occurs in the soil, as no published study has yet involved a root-feeding insect with a narrow diet (Koricheva et al., 2009).

To an extent, this lack of study may reflect the fact that the majority of root-feeding insects have a wide host range (Brown and Gange, 1990). However, many root-feeding insects are notorious pest species and can have dramatic effects on plants in crop situations and natural communities (Blossey and Hunt-Joshi, 2003). Indeed, several of the worst pest species have relatively restricted diets and thus it is important to understand whether AMF can be used as any form of plant protectant against these species.

* Corresponding author. Tel.: +44 01784 443 188; fax: +44 01784 414 224.
E-mail address: a.gange@rhul.ac.uk (A.C. Gange).

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The clover root weevil *Sitona lepidus* Gyllenhal (Coleoptera: Curculionidae) exhibits a preference for *Trifolium* spp. (Murray and Clements, 1994) and in the field feeds on white clover (*Trifolium repens* L.) which is the dominant legume in grazed pastures in the UK. The adults feed on the foliage, first instar larvae on the nodules, and later instars on progressively larger roots (Gerard, 2001). Consequently root (Murray and Clements, 1992) and foliar biomass (Murray et al., 1996) are reduced, as well as the functioning of the nitrogen-fixing nodules (Murray et al., 2002), and there is increased risk of pathogenic fungal attack at feeding sites (Kilpatrick, 1961). As a result, species of *Sitona* have long been regarded as pests of legumes in the UK and elsewhere (e.g. Jackson, 1920; Morrison et al., 1974; Goldson et al., 1988). Clements and Murray (1993) found that up to 30% of the photosynthetic area of a clover plant could be removed by adult weevils especially in late winter when a temporary rise in ambient temperature allowed the weevils to feed on the clover plants when they were either growing slowly or not at all. Consequently, the competitive ability of clover in a mixed grass/clover sward may be weakened leading to reduced clover content in the sward. It is desirable to have *T. repens* in the sward due to its nitrogen-fixing capabilities (Newbould, 1982) and its feeding value for livestock (Bax and Schills, 1993). AMF are known to increase nodulation of *T. repens* (Crush, 1974; Barea et al., 1989) and thus the question addressed in this paper is whether these fungi also affect the growth and survival of *S. lepidus*, thus presenting the first study of the responses of a specialist root-feeding insect to these fungi. We hypothesised that extra availability of root nodules, mediated by AMF, might increase larval survival and growth, as the larvae are highly dependent on this crucial resource (Johnson et al., 2004).

2. Materials and methods

Seeds of *T. repens* (cv. Kent Wild White clover) were germinated on moistened filter paper in Petri dishes before two seedlings were transplanted into each of 120 pots (100 mm diameter) filled with a 1:1 volumetric mix of horticultural sand and TerraGreen® (an attapulgite clay soil conditioner, Turfpro Ltd., Staines, UK) (Staddon et al., 1998). A supply of P in the form of bonemeal (Gem Horticulture, Accrington, UK) was added at a rate of 0.25 g l^{-1} (Staddon et al., 1998). Four treatments (each with 30 replicates) were established, consisting of a non-mycorrhizal control inoculum (*G. mosseae* and *G. fasciculatum*) steam sterilised at 121 °C for 20 min, applied at a rate of 1.5 g per pot of each AM species, single treatments of *G. mosseae* and *G. fasciculatum*, each applied as an individual inoculum at a rate of 3 g per pot, and a fourth treatment consisting of a mix of the two AM species (dual inoculation), each of which was added at a rate of 1.5 g per pot. These corresponded to the inoculum levels in Gange (2001). The inoculum consisted of clay granules, containing a mixture of spores, hyphal fragments and roots, taken from individual cultures of each fungal species maintained on *Plantago lanceolata* (L.). The inoculum was added as a horizontal layer, 30 mm below the rim of the pot.

Each pot was watered with 50 ml of distilled water and inoculated with 4 ml of a mixture of 5 strains of *Rhizobium leguminosarum* (Frank) biovar *trifolii* (WPBS 501, WPBS 502, WPBS 505, WPBS 509, WPBS 511) culture to establish nodules on the clover roots. Each pot was maintained in a Sunbag® (Sigma–Aldrich Company Gillingham, UK) in a constant environment room with a light/dark cycle of 16/8 h and a temperature of 20 °C. Light levels were 620 μmol m^{-2} at pot level.

After 14 days the weaker seedling was removed and the remaining seedlings were watered with 20 ml of Rorison's nutrient solution, amended to contain no phosphorus, and diluted to a fifth of its normal strength (Koide and Li, 1990). Cobalt was added to the nutrient solution, at a rate of 0.2 ml l^{-1}, to ensure the development of nodules on the clover roots. The plants were watered once a week with this nutrient solution.

To check on the success of mycorrhizas and *Rhizobium*, 10 pots from each treatment were harvested after 12 weeks, roots were washed thoroughly and the number of active (pink) nodules on the roots was counted (Murray et al., 1996). AM fungal colonization was recorded using a root sample of 0.5 g.

The root sample was cut into 10 mm long sections and the method of AM visualisation used was that of Staddon et al. (1998). The amount of time that roots were cleared for was amended to 4.5 min. Clearing occurred in 10% potassium hydroxide in a waterbath at a temperature of 80 °C, after which roots were rinsed with tap water and acidified at room temperature in 1% hydrochloric acid for 1 min. After acidification, roots were immediately immersed in 0.01% acid fuchsin, dissolved in destaining solution (14:1:1 lactic acid:glycerol:water), and stained for 20 min in the waterbath at 80 °C. Stained root samples were stored in destaining solution for 24 h before being mounted in the same solution on a microscope slide. AM quantification was conducted with the cross-hair eyepiece method (McGonigle et al., 1990) at 200× and at 400× magnification for further clarification of the structures.

AM colonization was found in all inoculated plants, and all roots were well nodulated (mean of over 100 per plant). The remaining 20 plants in each treatment were randomly separated into two groups of 10, one group of which was infested with eight *S. lepidus* eggs per pot, delivered in 1 ml of sterile water around the main stem of the clover plant. The remaining plants not receiving eggs, were given 1 ml of sterile water. The eggs were collected from adult *S. lepidus* weevils which had been caught in the field and kept in pots designed to collect eggs (Murray et al., 1996). Previous viability tests indicated an 84% eclosion rate after approximately 10 days. All plants continued to receive the nutrient solution described above. Nine weeks after eggs were added to the pots, adult weevils started to emerge. These were weighed, and all pots were checked daily over a period of 3 weeks, after which no more adults were found.

At this point the plants were harvested as described above. Active (pink) nodules on roots were counted, as well as the number of nodules showing herbivore damage on infested plants. Roots were assessed for AM colonization levels as described above. The percentage of eggs developing into adult weevils was calculated for each treatment, as well as mean adult weevil fresh weight (mg) per pot in each treatment.

The dried plant materials (leaves and petioles, stolons and roots) from the final harvest were ground in an analytical mill (IKA-A10, Janke and Kunkel, Staufen, Germany), and the total (mg) carbon (C) and nitrogen (N) as well as percentage C and N in each plant part was analysed using an elemental analyser (Carlo Erba NA 2000, Milan, Italy).

All data were tested for normality and homogeneity of variances. Percentage data was subject to the angular transformation prior to analysis (Zar, 1999). Foliar (leaves and stolons) and root dry biomass, root/shoot ratio, the number of active and grazed nodules; and the percentage of C and N of the plant parts were analysed by three factor ANOVA, employing *G. fasciculatum*, *G. mosseae* and herbivory as main effects. No AM colonization was detected in any of the plants receiving sterilised inoculum. Percent root length colonized (%RLC) was therefore analysed by a two factor ANOVA, employing fungi and larvae as main effects. Adult weevil weight and the percentage of larvae developing into adults were analysed by two factor ANOVA with AM fungal species as factors. Differences between means were separated using the Tukey HSD test. All analyses were conducted with the UNISTAT® statistical package.

3. Results

3.1. Plant and microbial attributes

After 12 weeks, colonization of roots by arbuscules averaged between 4 and 10% and there was no difference between the treatments (data not shown). Colonization by *G. mosseae* increased nodulation ($F_{1,36} = 2.93$, $p < 0.05$) with the greatest nodule number occurring in the dual fungal plants (mean of 114.0 ± 12.0 per plant). Small amounts of colonization were found in a few control plants, but in no case did this exceed 1%.

All plants had good levels of AMF colonization at the end of the experiment (Fig. 1). Root herbivory had no overall effect on arbuscule number, but there was a significant interaction between herbivory and fungi ($F_{2,54} = 4.9$, $p < 0.05$). When larvae attacked plants colonized by either fungus alone, arbuscule number was reduced. However, when roots of the dual colonization treatment were attacked, arbuscule number increased (Fig. 1a). Herbivory reduced colonization by vesicles ($F_{1,54} = 15.53$, $p < 0.001$), but not in the *G. mosseae* treatment, leading to a significant interaction term ($F_{2,54} = 3.79$, $p < 0.05$). Larvae had no effect on hyphal density, but *G. fasciculatum* treatments produced considerably more intra radical hyphae than did *G. mosseae* ($F_{2,54} = 11.9$, $p < 0.001$; Fig. 1c).

Larvae were clearly active on the roots, reducing total live nodule number in all treatments ($F_{1,72} = 37.79$, $p < 0.001$; Fig. 2a). Neither fungus affected total nodule number. However, there was a significant interaction term between fungal species in the proportion of nodules that were grazed ($F_{1,36} = 3.99$, $p < 0.05$; Fig. 2b). This was because the dual colonization treatment resulted in a significant reduction in grazing, while colonization by either fungus alone had no effect.

Overall, root herbivory reduced total dry foliar biomass ($F_{1,72} = 13.55$, $p < 0.001$; Fig. 3a). Neither fungus affected foliar production, but there was an interaction term between *G. mosseae* and larvae ($F_{1,72} = 4.47$, $p < 0.05$). In the treatments where this fungus was present, herbivory had no effect on foliar biomass. This was not due to a lack of feeding on roots, as herbivory reduced dry root biomass in all treatments ($F_{1,72} = 32.19$, $p < 0.001$; Fig. 3b) and no interaction terms were found. Overall, herbivory had no effect on the root/shoot ratio, but because of the differential effects on foliar and root biomass, the ratio was reduced by herbivory only when *G. mosseae* was present ($F_{1,72} = 4.04$, $p < 0.05$; Fig. 3c).

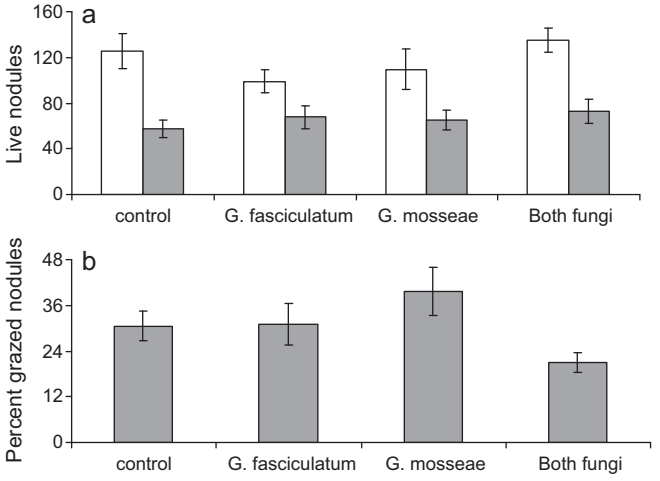

Fig. 2. The total number of live root nodules (a) and the percentage that were grazed (b) by larvae of *S. lepidus*. Shading as in Fig. 1. $N = 10$ in all treatments.

3.2. Plant chemistry

Mycorrhizal colonization had no effect on the percentage of carbon in shoots (Fig. 4a). There was a weak effect of herbivory ($F_{1,72} = 5.08$, $p < 0.05$), as feeding reduced C content in shoots of plants that were uncolonized or colonized by *G. mosseae*. Mean-

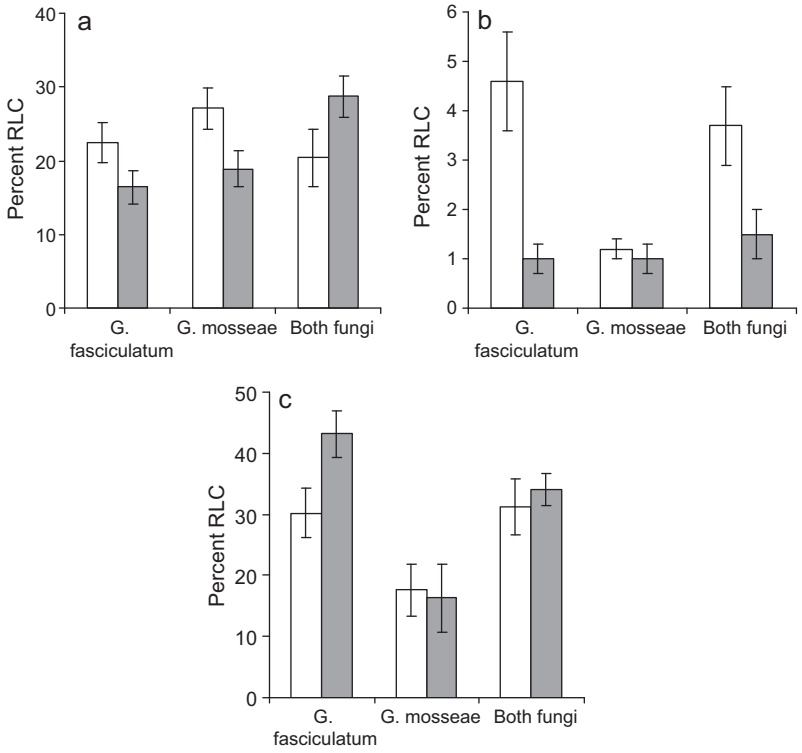

Fig. 1. Colonization, expressed as percent root length colonized (Percent RLC), of *Trifolium repens* roots by arbuscules (a), vesicles (b) and hyphae (c) when inoculated with either *Glomus fasciculatum*, *Glomus mosseae* or both fungi. Open bars: no herbivory, shaded bars: root herbivory by larvae of *Sitona lepidus*. Values are means ± one standard error.

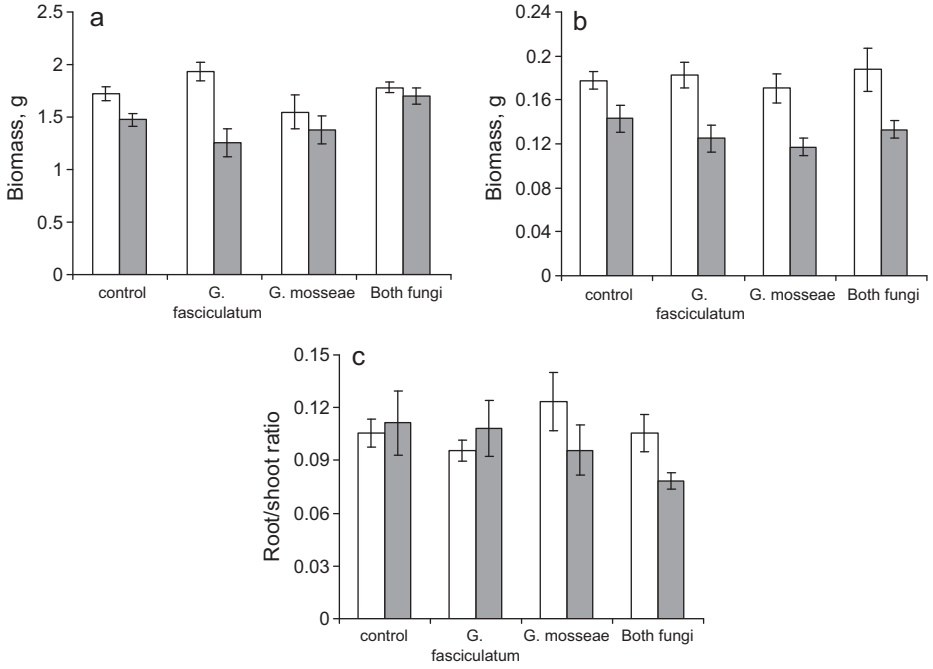

Fig. 3. Dry shoot biomass (a), root biomass (b) and the root/shoot ratio of plants with and without mycorrhizal colonization and root herbivory. Shading as in Fig. 1. $N = 10$ in all treatments.

while, colonization by *G. mosseae* increased the percentage of N in shoots ($F_{1,72} = 5.51$, $p < 0.05$; Fig. 4b), leading to a reduction in the foliar C/N ratio when this fungus was present in the root system (Fig. 4c).

Neither larvae nor mycorrhizas had any effect on the percentage of C in the roots, but both fungi increased the percentage of N in the roots (Fig. 5b). There was also a significant interaction between the fungal species ($F_{1,72} = 13.87$, $p < 0.001$), because the fungal effect did not seem to be additive, with N in the dual treatment being similar to or less than that of either fungus alone (Fig. 5b). The effects of fungi on root N were also seen in the C/N ratio in roots, in which this parameter was reduced by mycorrhizas, but the effect was not additive (Fig. 5c).

3.3. Insect attributes

The development time (data not shown) and adult weevil weight were unaffected by either fungal treatment (Fig. 6a). However, weevil survival was increased by the presence of both *G. fasciculatum* ($F_{1,36} = 4.91$, $p < 0.05$) and *G. mosseae* ($F_{1,36} = 6.23$, $p < 0.05$) and there was no interaction between the treatments, with survival highest in the dual colonization treatment (Fig. 6b).

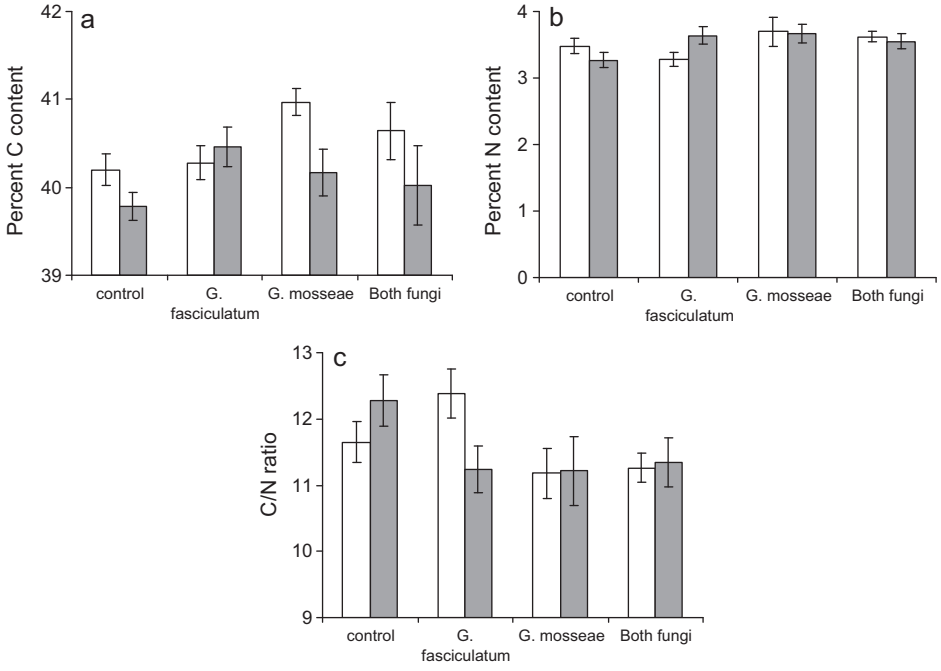

Fig. 4. Shoot percentage C content (a), N content (b) and the C/N ratio of plants with and without mycorrhizal colonization and root herbivory. Shading as in Fig. 1. $N = 10$ in all treatments.

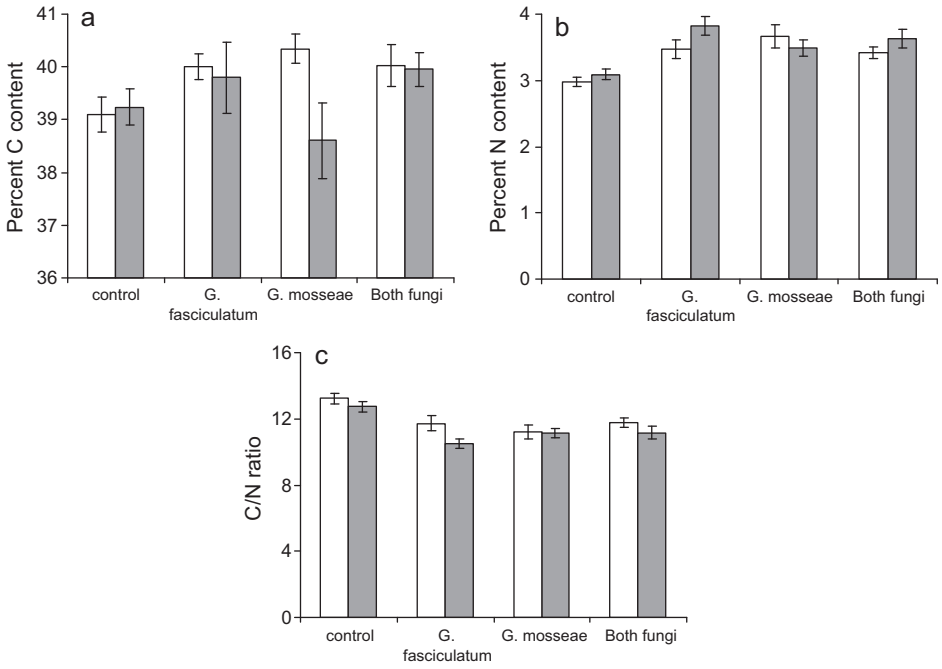

Fig. 5. Root percentage C content (a), N content (b) and the C/N ratio of plants with and without mycorrhizal colonization and root herbivory. Shading as in Fig. 1. $N = 10$ in all treatments.

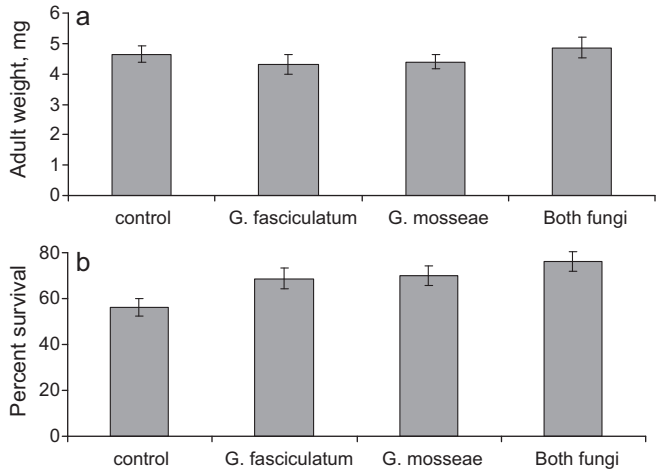

Fig. 6. Adult weight (a) and percentage survival of larvae to adult (b) of *S. lepidus* when reared on plants of different mycorrhizal status. Shading as in Fig. 1. $N = 10$ in all treatments.

4. Discussion

In this study, there were clear interactions between weevil larvae and the mycorrhizal fungi. Herbivory increased the colonization of roots by arbuscules but decreased vesicles in the dual fungal treatment, and increased hyphal density of *G. fasciculatum*. Currie et al. (2006) also found that root feeding by the larvae of *Tipula paludosa* Meigen increased mycorrhizal colonization in the grass, *Agrostis capillaris* L. It is thought that the effect is due to changes in root exudates, to which AMF respond, caused by root herbivory (Dawson et al., 2004; Narula et al., 2009). The extent to which this effect benefits or disadvantages the plant host are unknown. The plant may benefit from an enhanced nutrient uptake via elevated mycorrhizal colonization, but, if the amount of fungus increases greatly relative to the root (which is being lost to herbivory), the association could become detrimental to the plant. This is because the cost of C outflow to the herbivore and the fungus outweighs the benefit of nutrient input from the mycorrhiza (Gange and Ayres, 1999). Furthermore, confounding this interaction is the direct effect of the fungi on the insect herbivore.

We found that AMF colonization had no adverse affect on the growth of *S. lepidus* larvae, with adults being of a similar size in all treatments. However, weevil survival was greatest on plants colonized by both fungi. In this treatment, the proportion of root nodules that were grazed was lower. One might expect that a higher density of larvae would have produced higher levels of attack, or that competition within the pots would have forced larvae to consume more root, rather than the N-rich nodules. However, this did not seem to occur, as root biomass was not lower in the dual fungal treatment with larvae. *S. lepidus* larval survival is highly dependent on nodule number (Johnson and McNicol, 2010) and at the time when weevils were added to the plants in our study, nodule number was higher in dual fungal plants. Therefore we conclude that weevil establishment was better on plants colonized by both fungi, due to the enhanced availability of nodules.

The null or positive effect of AMF on *S. lepidus* survival is in contrast to that seen with polyphagous root-feeding insects (Halldorsson et al., 2000; Gange, 2001; Oddsdottir et al., 2010). In particular, Gange (2001) in a similar experimental design, found that *G. mosseae* and *G. fasciculatum* reduced *O. sulcatus* growth and survival. However, our results do tend to fit with the general pattern of AMF effects on insects, in which negative effects are seen on generalist species, while null or positive effects are found with specialist species (Koricheva et al., 2009). It has been suggested that these effects are mediated via changes in plant secondary metabolites, caused by the mycorrhiza (Gange, 2007). The chemistry of *T. repens* has recently been reviewed and there is no clear effect of AMF on secondary metabolites in roots (Carlsen and Fomsgaard, 2008). This species contains cyanogenic glycosides, but typical of many specialist insects, *S. lepidus* seems unresponsive to changes in these chemicals (Mowat and Clawson, 1996; Murray, 1996). Thus, even if cyanogenic compounds were increased in mycorrhizal clover roots in our study, it seems unlikely that this will have been the cause of enhanced larval survival. We found no effects of

AMF on root C carbon contents, but N concentration was increased, providing further evidence to support the suggestion that mycorrhizal clover roots provided a higher quality of food for larvae than non-mycorrhizal roots. The improved food quality via N content may also explain why the proportion of grazed nodules was lower when larval survival was highest, as insects can regulate the amount of dietary intake, depending on the nitrogen content of tissues (Simpson and Simpson, 1990).

Feeding by *S. lepidus* larvae considerably reduces both foliar and root biomass of *T. repens* (Murray and Clements, 1992; Murray et al., 1996). In the present study, a reduction in foliar biomass was seen in all treatments except for that in which both fungi were inoculated, while root biomass was reduced in every treatment. Therefore, the mitigation of effects in the dual fungal treatment cannot be due to a lessening of herbivory on the root system. It was noticed that larvae increased colonization of roots by arbuscules in the dual treatment, which one would expect to translate into increased P uptake and thus plant growth (Gange and Ayres, 1999). Furthermore, the proportion of nodules that were grazed was also reduced in plants inoculated with both fungi. As weevil size was unaffected by treatment, we suggest that the effect seen in foliar biomass is one in which plants are able to tolerate herbivory. When AMF provide the plant with a surplus of resources, an enhanced ability to tolerate above-ground herbivory is often seen (Borowicz, 1997; Bennett et al., 2006). The increases seen in both foliar and root N in mycorrhizal plants suggest that either nodulation was more efficient or that plants were able to uptake a greater amount of N from soil. Either way, it suggests that mycorrhizas improved plant resource supply, enabling them to tolerate herbivory. While such effects have been seen with above-ground insects (e.g. Borowicz, 1997), this is the first report of mycorrhizas enabling a plant to tolerate root herbivory.

Many previous studies with mycorrhizas and foliar- and root-feeding insects have shown that combinations of fungi have different effects on insect growth and survival, compared to single species inoculations (Gange, 2007). Molecular techniques have shown that plants in natural communities harbour a much greater diversity of AMF species than was previously thought (Öpik et al., 2008). In the latter study, a mean of about three fungal species per plant was found for several herbaceous species. Perhaps of more importance is the fact that mycorrhizal fungal species do not all provide the same amount of benefit to every plant, resulting in a degree of specificity with their hosts (Hoeksema et al., 2010). Indeed, within a legume root system, it is known that different mycorrhizal species inhabit the roots and the root nodules (Scheublin et al., 2004). Thus, in any plant community, but especially in crop systems such as pasture grassland, a diverse mycorrhizal community is beneficial, in terms of enhancing ecosystem services and production (Barrios, 2007). Our results show that mycorrhizal diversity is important in protecting plants against specialist insects, many of which are pest species. Indeed, it would be interesting to expand the experiments reported here to three or more AMF and determine the effects on insect performance. Highly managed grasslands often show reduced microbial abundance (Jangid et al., 2008), but our results emphasise the importance of maintaining soil biodiversity in such systems.

5. Conclusions

Maintaining the diversity of mycorrhizal fungi in a ryegrass/clover pasture is important, because these fungi help clover plants to tolerate root herbivory by larvae of *S. lepidus*. When two mycorrhizal fungi were present in the root system, weevil survival was higher than when no fungi were present, but the increase in root quality meant that fewer of the N-fixing root nodules were grazed. The reduction in foliar biomass caused by herbivory when no fungi were present, did not occur in plants colonized by two AMF species. Overall, AMF had no detrimental effects on the performance of a specialist root-feeding insect, in contrast to the effects seen with generalist species. It would be useful to investigate the mycorrhizal diversity of pastures and to determine whether other AMF species have similar effects on the growth of this insect.

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