Research

Oikos 118: 634–640, 2009
doi: 10.1111/j.1600-0706.2009.17418.x,

Subject Editor: Heikki Setälä. Accepted 11 November 2008

Symbiotic soil microorganisms as players in aboveground plant–herbivore interactions – the role of rhizobia

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Rhizobia play a key role for performance of leguminous plants and ecosystem productivity. However, no studies to date have investigated the importance of the rhizobial symbiosis for legume–herbivore interactions. The additional nitrogen provided by the rhizobia improves the nutritional quality of plants, but may also be used for the synthesis of defence compounds. We performed greenhouse experiments with nodulating and non-nodulating, as well as cyanogenic and acyanogenic strains of *Trifolium repens* to study the effects of rhizobia *Rhizobium leguminosarum* on plant growth and the performance of the chewing herbivore *Spodoptera littoralis* and the phloem-sucking aphid *Myzus persicae*. We demonstrate that for nodulating strains of *T. repens* rhizobia increased plant growth and the performance of *Spodoptera littoralis*. However, this positive effect of rhizobia on the caterpillars did not occur in a cyanogenic clover strain. Reproduction of the phloem-sucking aphid *Myzus persicae* was inconsistently affected by rhizobia. Our study provides evidence that the additional nitrogen provided by the rhizobia may be used for the production of nitrogen-based defence compounds, thereby counteracting positive effects on the performance of chewing herbivores. The symbiosis with rhizobia is therefore an important driver of legume–herbivore interactions.

In almost all terrestrial ecosystems mutualistic interactions between plants and microorganisms in the soil play a major role for the structure and dynamics of plant communities (van der Heijden et al. 2008). An important group of mutualists are the nitrogen-fixing bacteria associated with legumes (Turkington et al. 1988, Sprent 2001). It is well known time that legumes are of key importance in many ecosystems (van der Heijden et al. 2006, Temperton et al. 2007) and their growth and establishment in natural ecosystems is sometimes limited by the presence of rhizobia (Larson and Siemann 1998). However, despite their impact on the global and local nitrogen cycles (Sprent and Sprent 1990), the effect of rhizobia within food webs is largely unexplored, especially the effect of rhizobia on herbivores feeding on aboveground parts of plants.

Depending on the feeding mode of the herbivores, the mutualism between plants and microorganisms may affect associated herbivores in contrasting ways. For mycorrhizal fungi it has been shown that generalists and leaf-chewers tend to be negatively affected by the association between the host plant and the fungi, whereas specialists and sap feeding herbivores tend to be positively affected (reviewed by Gehring and Whitham 2002). This difference may result from changes in the chemical composition of plants and the additional production of secondary compounds due to the presence of the mycorrhiza. Similarly, rhizobia may have contrasting effects on herbivores. The additional nitrogen provided by the rhizobia to the host plant increases the nitrogen content of plants (Sprent and Sprent 1990). Nitrogen is crucial for the performance of insect herbivores (Mattson 1980, Schädler et al. 2005, 2007). Therefore, herbivores may benefit from feeding on plants associated with rhizobia. However, the mutualism with rhizobia may also affect the plant's defence system. Many defence substances contain nitrogen (e.g. cyanogenic compounds in many legumes) and plants may use the additional nitrogen provided by the rhizobia to produce N-rich substances for herbivore control (Herms and Mattson 1992, Koricheva et al. 2004). Cyanogenic gas (HCN) is known to be released after the reaction of two precursors of cyanide when tissue damage allows the mixing of substrate and enzyme (Hayden and Parker 2002). Hydrogen cyanide gas is known to repel mainly leaf-chewing herbivores (Nahrstedt 1985, Jones 1998) and appears to be an effective inhibitor of feeding by generalist herbivores rather than by specialists (Compton and Jones 1985, Nahrstedt 1985, Schappert and Shore 1999). Schwarz et al. (1996) were able to show feeding avoidance of *S. littoralis* on leaves of cyanogenic plants. The production of HCN is a constitutive defence and has been shown to be not inducible by herbivory (Hayden and Parker 2002). Cyanogenesis in plants is strongly demanding for leaf N (Miller and Woodrow 2008) and it can therefore be expected that symbiotic nitrogen fixation is an integral part of the defence system in legumes. According to the carbon/nutrient balance hypothesis the allocation of resources to plant

defences is controlled by the carbon-nutrient status of the plant (Bazzaz et al. 1987, Bryant et al. 1983, but see Hamilton et al. 2001). Following this hypothesis, investment in nitrogen-based defences should increase with increasing nitrogen availability.

Although rhizobial bacteria are ubiquitous keystone species (Wardle 2002), their effects on plant–herbivore interactions have been largely neglected by ecologists. Recently, Dutta et al. (2007) demonstrated that rhizobia in combination with free-living plant growth-promoting bacteria may be involved in the production of defence-related enzymes in pigeon pea. Johnson et al. (2006) found that clover root weevils laid more eggs on white clover plants with nodules than on those without. Johnson and Bentley (1991) demonstrated that symbiotic nitrogen fixation increases the alkaloid content of leaves of lupines. In turn, reduction of photosynthesis by herbivory can reduce nitrogen fixation rates and therefore attenuate the positive effects of rhizobia on plant growth (Johnson and Bentley 1991). However, to our knowledge no studies have explored the influence of rhizobia on the performance of herbivores associated with aboveground parts of legumes. In two greenhouse experiments with strains of *Trifolium repens* which differ in their ability to form an association with rhizobia or produce N-based secondary compounds we investigated the role of rhizobia on interactions between legumes and herbivores feeding above ground. In the first experiment we compared the effects of rhizobia on the performance of a chewing and a sucking herbivorous insect on a nodulating and a non-nodulating strain of white clover *Trifolium repens*. In the second experiment we used a cyanogenic and an acyanogenic strain of white clover to evaluate the performance of the two insect species on these strains in the presence and absence of rhizobia. Insects with sucking mouthparts, however, cause minimal tissue disruption and may thereby avoid the defence effect of this compound (Schreiner et al. 1984). In particular, we aimed to test the hypothesis that the symbiosis with rhizobia positively affects the performance of herbivores, but this effect may be overridden by the plant's ability to use additional nitrogen for the production of defence compounds. Furthermore, the interacting effects of rhizobia and the ability to produce defence compounds should be more pronounced for a leaf-chewing herbivore than for a sap-sucking herbivore. Since rhizobia may not only increase the plant's ability to produce secondary compounds but also the ability to compensate tissue loss due to herbivory, we further hypothesize that negative effects of herbivores on plant growth are attenuated by rhizobia.

Material and methods

Study organisms

White clover *Trifolium repens* is a perennial herb belonging to the Fabaceae. It forms a close association with N_2-fixing soil bacteria of the family Rhizobiaceae. This symbiosis results in visible, ball-like structures formed on the roots (nodules). *Trifolium repens* shows polymorphism with regard to the production of HCN. In white clover many herbivore species, especially snails and slugs, prefer acyanogenic plants to cyanogenic plants (Crawford-Sidebotham 1972, Angseesing 1974, Dirzo and Harper 1982a, 1982b, Horrill and Richards 1986, Mowat and Shakeel 1988). As a representative of polyphagous herbivorous insect species (generalists), we used the larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the green peach aphid *Myzus persicae* (Hemiptera: Aphididae). Larvae of *S. littoralis* are known to be a serious pest of many crops in North Africa, the Mediterranean as well as the Middle East. They feed on plants belonging to at least 40 plant families (Brown and Dewhurst 1975) and are therefore often used in experiments that compare leaf palatability across very different plant species (Hendriks et al. 1999, Schädler et al. 2005, 2007). *Myzus persicae* is a heteroecious, holocyclic aphid with a worldwide distribution. It shows host plant alternation with *Prunus persica* (Rosaceae) as its primary host and more than 400 plant species from different families as secondary hosts (Blackman and Eastop 2000).

Experiment 1

Here we compared the performance of the two insect species on white clover in the presence and absence of rhizobia. Bacteria disperse easily through air and soil. Therefore, a major difficulty in experiments with rhizobia is to avoid contamination of the rhizobia-free treatments. Thus, in addition to a clover strain (CON) that produces active nodules, we used an inbred line of *T. repens* (NIL) which is not able to do so. The strains belong to a number of inbred lines originating from a small number of plants and were cultivated at the Inst. of Grassland and Environmental Research (Abberton et al. 1998). Using these strains allowed us to compare the effects of the plant's ability to form an association with rhizobia with the effects of the presence of rhizobia per se.

The experiment was conducted in a greenhouse with temperature maintained at 15 to 25°C and a constant day length of 14 h, with additional light supplied by high-pressure sodium lamps. Photosynthetic photon flux density varied depending on weather conditions between 80 and 350 μmol s^{-1} m^{-2}. At the end of December 2006, seeds of the NIL and CON strain were surface-sterilized with 1% H_2O_2 for 10 min, washed with sterilized water and placed on sterile moist filter paper in petri dishes. Half of the seeds of each strain were inoculated with a suspension of a strain of the N-fixing bacterium *Rhizobium leguminosarum* bv. *trifolii* (Radicin no. 1, minimum 10^7 bacteria ml^{-1}). Seeds of the non-rhizobia treatment received an autoclaved inoculum. Sterilized experimental pots (ø 9 cm, height 7 cm) were filled with steam-sterilized (100 C, 4 h) soil and sand (ratio 1:1 v/v). The soil was taken from an old fallow grassland site (Lahnberge near Marburg; Hesse, Germany). In this area sandstone is the major rock type and as a consequence the soil is rather nutrient-poor. Nevertheless, to leach nutrients from the soil which became available during the steaming process pots were irrigated each day with 40 ml of deionised water for 3 days prior to the start of the experiment. This resulted in an initial nitrogen concentration of 2.88 mg NH_4^+ kg^{-1} and 1.16 mg NO_3^- kg^{-1}. After germination, seedlings were transplanted

into the experimental pots. All pots were watered every two days with distilled water. After a further eight days, the inoculation process was repeated with 2 ml of the rhizobia suspension to ensure nodulation.

After eight weeks of plant establishment, one second to third-instar larva of *S. littoralis* was added to one third of the pots and left for five days. To the second third of all pots four reproductive females of *M. persicae* were added and left for four weeks to reproduce. The remaining pots were left without herbivores. During the experimental period with insects, all pots were enclosed with nylon gauze (200 μm mesh, height 60 cm). The experiment was set up in a full-factorial design, with two white clover strains (NIL/CON), with and without rhizobia treatments and with three levels of leaf herbivory (aphids, caterpillar or without). The full complement of 12 combinations was established with 10 replicates each, resulting in 120 pots. To buffer possible effects of environmental heterogeneity, pots were randomly assigned into 10 blocks in the greenhouse and randomised within the blocks every other week.

Larval mass of *S. littoralis* before and after the feeding period of five days was recorded. Three non-rhizobia treated plants of the CON strain (two with *S. littoralis* and one with *M. persicae*) were excluded from the analysis, as plants were highly contaminated by rhizobia. Aphids were removed from the plants by brushing and counted. Aboveground plant parts were harvested, dried at 70°C to a constant weight, and weighed. Roots were washed, checked for rhizobial colonisation, and nodulation was quantified by counting the number of nodules per plant. Roots were dried at 70°C to a constant weight and also weighed.

Experiment 2

In the second experiment we used a cyanogenic white clover strain (Huia) which is able to produce hydrogen cyanide gas (HCN) and an acyanogenic strain (Milkanova) (Caradus and Woodfield 1997). Both strains produce active nodules.

The experimental layout and procedure was basically the same as in experiment 1, but with the white clover strains being the cyanogenic and the acyanogenic variant. The soil and the inoculation procedure was similar to experiment 1. After eight weeks of plant establishment, one third to fourth-instar larva of *S. littoralis* was added to one third of the pots and left for five days. To the second third of all pots four reproductive females of *M. persicae* were added and left for four weeks to reproduce. In total, there were two clover strains (HCN −/HCN +), three herbivory treatments (without, caterpillar, aphids), two rhizobia treatments (without/with) in a full factorial design with 10 replicates per treatment combination in 10 randomized blocks (120 replicates). Two pots of the larvae (2 × Milkanova) and three pots of the aphid treatment (2 × Huia and 1 × Milkanova) were excluded from the analysis because herbivores showed no feeding activity or plants failed to establish. Assessment of caterpillar mass, aphid reproduction, nodulation and plant growth was similar to experiment 1.

Statistical analyses

Data were visually checked for normality of residuals and, if necessary, log-transformed prior analyses. Count data (number of aphids), if necessary, were subjected to square root transformation. The effects of block, strain, rhizobia and herbivory (none, aphids, caterpillars) on aboveground and belowground biomass and the effects of block, strain and rhizobia on number of aphids were analysed using ANOVAs (Statistica 6). The effects of block, strain and rhizobia on final larval mass were analysed using an ANCOVA (Statistica 6) with initial larval mass as the covariate. By using type I sums of squares, the confounding effects of initial larval mass were removed and we obtained an estimate of the relative growth rate of the larvae (Raubenheimer and Simpson 1992, Horton and Redak 1993).

In all analyses, interactions with the factor block were pooled into the error term (Newman et al. 1997). Every ANOVA yielding significant effects was followed by a post-hoc test (Tukey's honest significant difference test; HSD; Statistica 6). For the post-hoc analyses and presentation of larval mass of caterpillars and the correlation with nodule numbers we used the values of final biomass adjusted for the effect of initial biomass (ANCOVA). The relationship between number of nodules and final larval mass controlled for initial mass was assessed using partial correlation (Statistica 6).

Results

Experiment 1

Plant performance

Although we could not avoid contamination of the rhizobia-free treatments, number of nodules on rhizobia-treated *Trifolium repens* plants of the CON strain were much higher (357 ± 20.7 nodules plant^{-1}; means ± SE) than on non-rhizobia treated plants (27.9 ± 21.8 nodules plant^{-1}; $F_{1,46} = 120.8$, $p < 0.001$). Therefore, our results can be interpreted as a conservative examination of the results of inoculation. Roots of the NIL strain also showed small nodule-like structures which were much smaller in size and white or green in colour. In contrast, active nodules of the CON strain tend to be pinkish. Those non-active nodules develop due to the infection of the roots by *Rhizobium*, but are not able to fix N_2 because of an abnormality in the genome of the NIL strain (Abberton et al. 1998, see also Blauenfeldt et al. 1994).

Overall, the presence of rhizobia led to an increase in aboveground biomass of *T. repens* (ANOVA, $F_{1,96} = 29.8$, $p < 0.001$; Fig. 1a). However, this effect was highly dependent on the identity of the clover strain (strain: $F_{1,96} = 42.1$, $p < 0.001$; strain × rhizobia interaction: $F_{1,96} = 31.4$, $p < 0.001$). While the NIL strain did not show a change in biomass, rhizobia increased aboveground biomass of the CON strain by 103%. Similarly, belowground biomass was also significantly increased due to rhizobial association only in the CON strain (+50%, Fig. 1b, rhizobia: $F_{1,96} = 9.7$, $p = 0.002$; strain: $F_{1,96} = 8.8$, $p = 0.004$; rhizobia × strain interaction: $F_{1,96} = 21.1$,

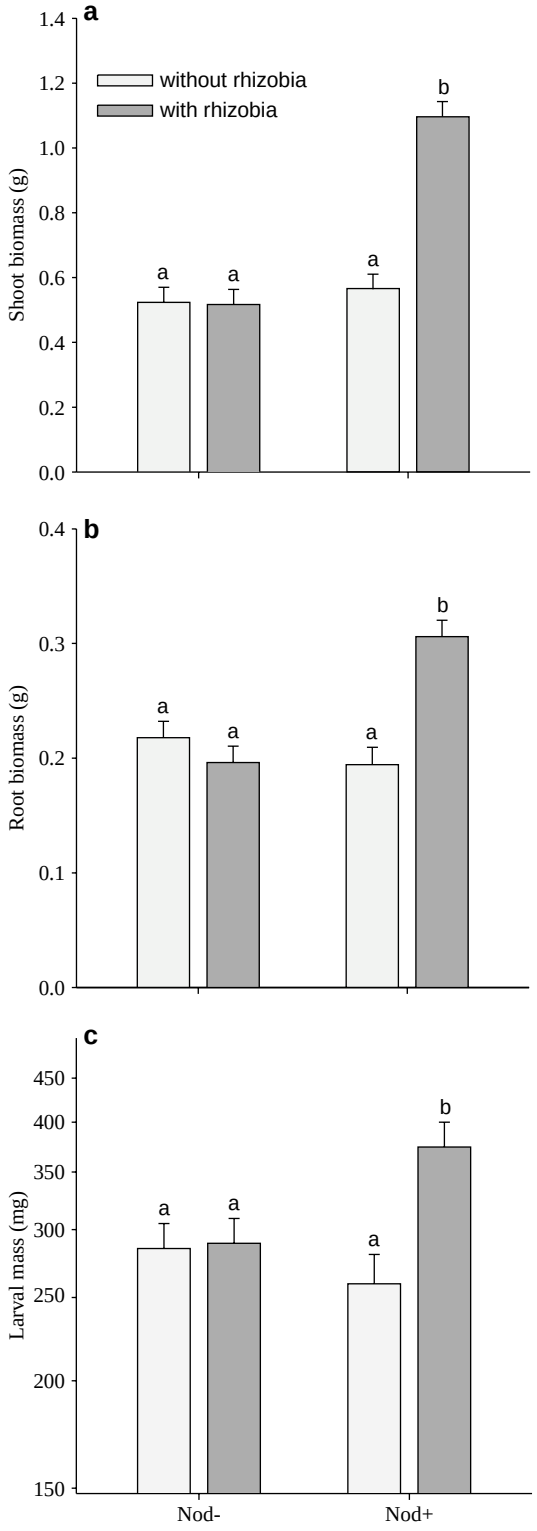

Figure 1. Results of experiment 1: the effects of the identity of the white clover strain and rhizobia on (a) shoot biomass and (b) root biomass of *Trifolium repens* as well as (c) final larval mass (adjusted for initial larval mass) of *Spodoptera littoralis*. Values are means with standard error. Nod– = non-nodulating strain (NIL), Nod+ = nodulating strain (CON). Note that the y-axis in (c) is log-transformed. Different letters indicate significant differences between means following post hoc tests (Tukey HSD, $p < 0.05$).

$p < 0.001$; Fig. 1b). Herbivory by larvae of *S. littoralis* had a negative effect on aboveground and belowground plant biomass (means $\pm$ SE; aboveground: $F_{2,96} = 12.7$, $p < 0.001$, without herbivory 0.78 ± 0.04 g; with *M. persicae* 0.75 ± 0.04 g, with *S. littoralis* 0.50 ± 0.04 g; belowground: $F_{2,96} = 5.8$, $p = 0.004$, without herbivory 0.24 ± 0.01 g, with *M. persicae* 0.25 ± 0.01 g, with *S. littoralis* 0.19 ± 0.01 g). However, this effect of herbivory on plant growth was not affected by the symbiosis with rhizobia or the identity of the *T. repens* strain (no significant interactions). The number of nodules on the rhizobia-treated CON strain did not differ significantly between plants with and without herbivores (without herbivory 394 ± 46.4 nodules plant^{-1}, with *M. persicae* 368 ± 46.4 nodules, with *S. littoralis* 310 ± 46.4 nodules; $F_{2,18} = 0.83$, $p = 0.45$).

Herbivore performance

The symbiosis with rhizobia led to increased larval mass of *S. littoralis* on the CON strain only (rhizobia: $F_{1,24} = 6.45$, $p = 0.018$; strain $\times$ rhizobia interaction: $F_{1,24} = 6.2$, $p < 0.02$; Fig. 1c). Furthermore, the number of nodules per plant was positively related to larval mass adjusted for initial mass (partial correlation, $R = 0.57$, $p = 0.045$). Including number of nodules as a covariate into the analysis therefore removed the effect of rhizobia on larval mass ($F_{1,95} = 1.2$, $p = 0.32$).

Symbiosis with rhizobia had only a marginally significant effect on the total number of aphids that developed during the experiment (mean $\pm$ SE: without rhizobia 102.5 ± 19.3, with rhizobia 145.8 ± 19.3; $F_{1,26} = 3.85$, $p = 0.06$). There was no interaction between the factors strain and rhizobia on aphid reproduction.

Experiment 2

Plant performance

Rhizobia-free treatments were again slightly contaminated with rhizobia but number of nodules was still much higher in the rhizobia treatment (rhizobia treatment 219 ± 11.0 nodules plant^{-1}; non-rhizobia treatment 73 ± 11.1 nodules plant^{-1}; $F_{1,102} = 86.5$, $p < 0.001$). The addition of rhizobia increased aboveground ($F_{1,94} = 80.98$, $p < 0.001$) and belowground biomass ($F_{1,94} = 11.91$, $p < 0.001$) for both the acyanogenic and the cyanogenic strain of *T. repens* (Fig. 2a–b). Furthermore, strains differed in aboveground ($F_{1,94} = 25.48$, $p < 0.001$) as well as belowground biomass ($F_{1,94} = 11.18$, $p = 0.001$) independently from rhizobial colonisation whereas the acyanogenic outweigh the cyanogenic strain by 33% above ground and 20% below ground. There was no interacting effect between strain and rhizobia on plant biomass.

The negative effect of herbivory (aboveground biomass: $F_{2,94} = 45.25$, $p < 0.001$, without herbivory 1.25 ± 0.05 g, *M. persicae* 1.21 ± 0.05 g, *S. littoralis* 0.64 ± 0.05 g; belowground biomass: $F_{2,94} = 18.49$, $p < 0.001$, without herbivory 0.27 ± 0.01 g, *M. persicae* 0.26 ± 0.01, *S. littoralis* 0.18 ± 0.01 g; means $\pm$ SE) on plant growth was not mediated by the symbiosis with rhizobia or the identity of the *T. repens* strain (no significant interactions). The number of nodules on rhizobia-treated plants of both strains was significantly reduced due to herbivory ($F_{2,43} = 3.89$,

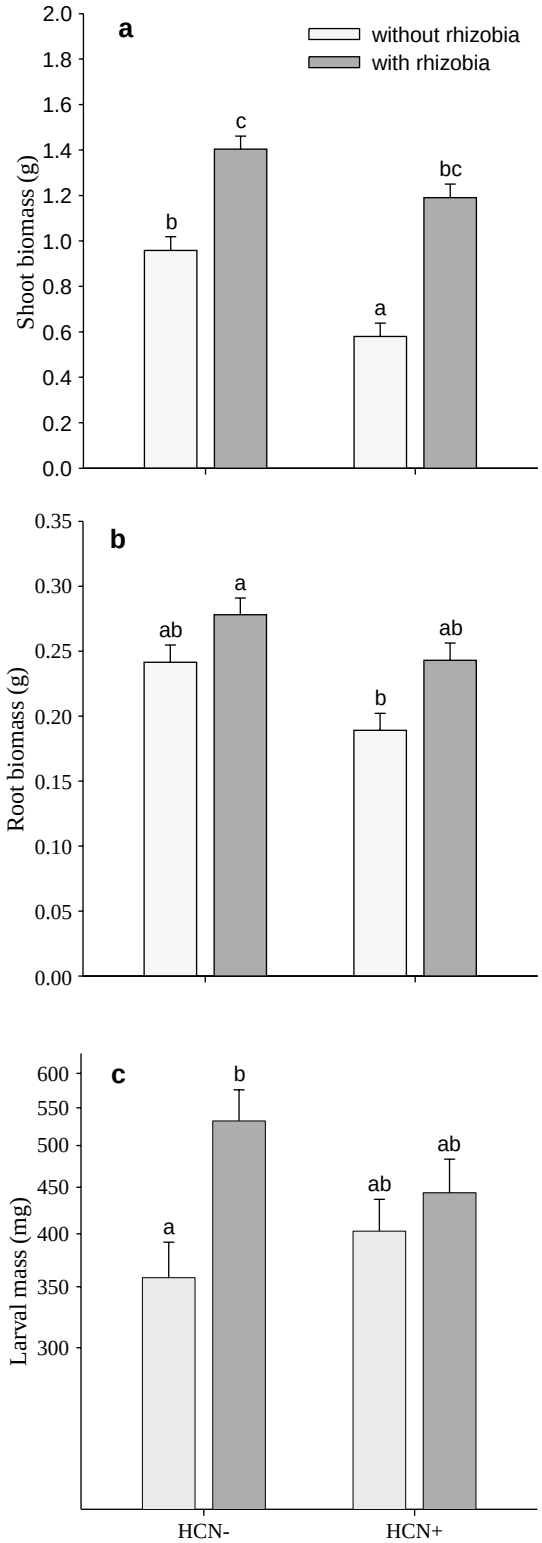

Figure 2. Results of experiment 2: the effects of the identity of the white clover strain and rhizobia on (a) shoot biomass and (b) root biomass of *Trifolium repens* as well as (c) final larval mass (adjusted for initial larval mass) of *Spodoptera littoralis*. Values are means with standard error. HCN− = acyanogenic strain (Milkanova), HCN+ = cyanogenic strain (Huia). Note that the y-axis in (c) is log-transformed. Different letters indicate significant differences between means following post hoc tests (Tukey HSD, $p < 0.05$).

$p = 0.028$; without herbivory 262 ± 22.7 nodules, *M. persicae* 220 ± 23.4 nodules, *S. littoralis* 171 ± 23.4 nodules; means $\pm$ SE). However, including belowground biomass, which also declined due to herbivore feeding, as a covariate into the analysis removed the negative effect of herbivory on number of nodules ($F_{2,42} = 0.69$, $p = 0.51$). Thus, the effect of herbivores on the number of nodules was attributable to their effects on root biomass.

Herbivore performance

Overall, larval mass of *S. littoralis* increased with presence of rhizobia (ANCOVA, $F_{1,24} = 9.23$, $p = 0.006$), but differed not between strains. This positive effect of rhizobia was only evident for the acyanogenic strain of *T. repens* (marginally significant interaction, $F_{1,24} = 2.91$, $p = 0.1$, Fig. 2c) whereas on the cyanogenic strain larval mass did not increase with rhizobia. Numbers of nodules were again positively related to larval mass adjusted for initial mass (partial correlation, $R = 0.44$, $p < 0.001$). Including number of nodules as a covariate into the analysis therefore removed the effect of rhizobia on larval mass ($F_{1,93} = 0.28$, $p = 0.60$). Aphid reproduction was not affected by rhizobia or the identity of the *T. repens* strain.

Discussion

Our study demonstrates an influence of rhizobia on insect herbivores feeding on aboveground parts of plants and that this effect is modulated by the plant's ability to produce nitrogen-based defence compounds. The effects of rhizobia on plant growth and herbivore performance was not observed in a plant strain which is not able to produce active root nodules. Therefore, the inoculum did not per se affect plant growth and herbivore performance but successfully initiated the development of active nodules within the range of nodule numbers reported by Abberton et al. (1998). As expected, for nodulating plants, the presence of rhizobia resulted in a significant increase in aboveground as well as belowground biomass. This positive effect of rhizobia on plant biomass has been well studied in several economically important crop species (Marschner 1995) as well as in grassland ecosystems (van der Heijden et al. 2006).

Our study demonstrates convincingly that rhizobia do not only increase the growth of legumes but also that rhizobia are an overlooked but important factor in plant–herbivore interactions. The symbiosis of *T. repens* with rhizobia resulted in a substantial increase in larval growth of *S. littoralis* in acyanogenic strains of white clover. Aphid reproduction also benefited from rhizobia in the first experiment. Furthermore, we could show that larval growth as well as number of aphid offspring was related to the number of nodules formed on the roots of white clover. It is well known that the N-content of a plant affects herbivore performance (Mattson 1980, Schädler et al. 2007). Therefore, the additional nitrogen supplied by rhizobia may have improved food quality and therefore increased herbivore performance.

It has been shown for mycorrhizal fungi that the positive effects of mutualistic microorganisms on plant growth do not necessarily translate into positive effects for the associated herbivores (Gehring and Whitham 2002). One explanation is that plants may not only use the resources supplied by the mutualistic partner for plant growth but also for the production of defence substances. Accordingly, in experiment 2 we found no positive effect of rhizobia on the growth of the caterpillars on a cyanogenic strain of white clover which suggest that the additional nitrogen gained from rhizobial symbiosis was used for the production of nitrogen-based secondary compounds such as hydrogen cyanide in the acyanogenic strain. Such substances may override the positive effects of the increased nutritional N due to rhizobia, which presumably caused the positive effects on herbivore performance on the acyanogenic strain.

Cyanogenic plants are known to store nitrogen in the toxic form for anti-herbivore defense (Gleadow and Woodrow 2002). Hence, nitrogen provided by rhizobia may have been in part stored in the form of a cyanoglycoside and a beta-glucosidase enzyme. Both compounds are necessary for the plant's ability to release HCN. However, cyanogenesis only occurs when the cyanoglycoside is mixed with the enzyme, for example, when the tissue is chewed. Insects with sucking mouthparts cause minimal tissue damage and therefore avoid the mixing of the cyanoglycoside with the enzyme (McMahon et al. 1995, Gleadow and Woodrow 2002). This would explain why the performance of *M. persicae* on cyanogenic and acyanogenic strains of white clover was not significantly affected during our experiment. Other studies report similar observations concerning the importance of feeding strategy for plant–insect interactions. For example, aphids failed to distinguish between cyanogenic and acyanogenic strains of *Pteridium aquilinum* whereas sawflies did so (Schreiner et al. 1984). However, studies where aphids preferred acyanogenic to cyanogenic *T. repens* plants have also been reported (Dritschilo et al. 1979). Furthermore, aphids have been shown to react sensitively to the composition and quality of nitrogen-based compounds (e.g. amino acids) in plants (Van Emden and Bashford 1969, Dixon 1998). These traits may have also been affected by the activity of rhizobia and the amount of available N in plant tissue.

Herbivory by *S. littoralis* reduced aboveground as well as belowground plant biomass. In our experiment this top–down effect on plant growth was not affected by the presence of rhizobia. One explanation might be the higher compensatory growth of plants with rhizobial symbiosis. Gómez et al. (2007) demonstrated a shift of biomass allocation towards the leaves following herbivory by *Spodoptera exigua* in *T. repens.* Johnson and Bentley (1991) suggested that the lowered production of photosynthates with herbivory reduces nitrogen fixation by rhizobia and offsets the positive effect on plant growth. We could not show a significant effect of herbivory on nodulation intensity of white clover plants. Studies with mycorrhizal plants showed that removing photosynthetically active leaf tissue by herbivores decreased allocation of carbon to the mutualistic fungi and led to a weaker mycorrhization (reviewed by Gehring and Whitham 2002). Further studies are needed to show whether N-translocation to the plant from the rhizobia and plant C-allocation to rhizobia are dependent on aboveground herbivory.

We are aware that working with an artificial system and sterilized soil may not reflect the net effect of rhizobia in natural systems. Very few is known on which soil organisms may antagonize or synergize the effects of these symbionts (de Varennes and Goss 2007, Dutta et al. 2007) and this point should be addressed in future studies. To our knowledge, this is the first study demonstrating the effect of rhizobia on aboveground plant–herbivore interactions. This effect depends on the plant's ability to produce nitrogen-based secondary compounds. We suggest that the surplus of nitrogen which became available through the mutualism with rhizobia may be used for both plant growth and production of defence compounds. These direct and indirect interactions in the rhizobia–legume–herbivore system may be of crucial ecological importance, because the functioning of ecosystems is often influenced by the presence and abundance of leguminous herbs. Our study further shows that the use of genotypes and inbred lines of plants is an important tool for analyzing the complex interactions within food webs.

Acknowledgements – We specially thank Terry Michaelson-Yeates for providing the white clover seeds and Elisabeth Engels (JOST GmbH, Iserlohn/Germany) for providing the rhizobial suspension and useful comments. Marcel van der Heijden provided valuable comments on a former version of the manuscript.

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