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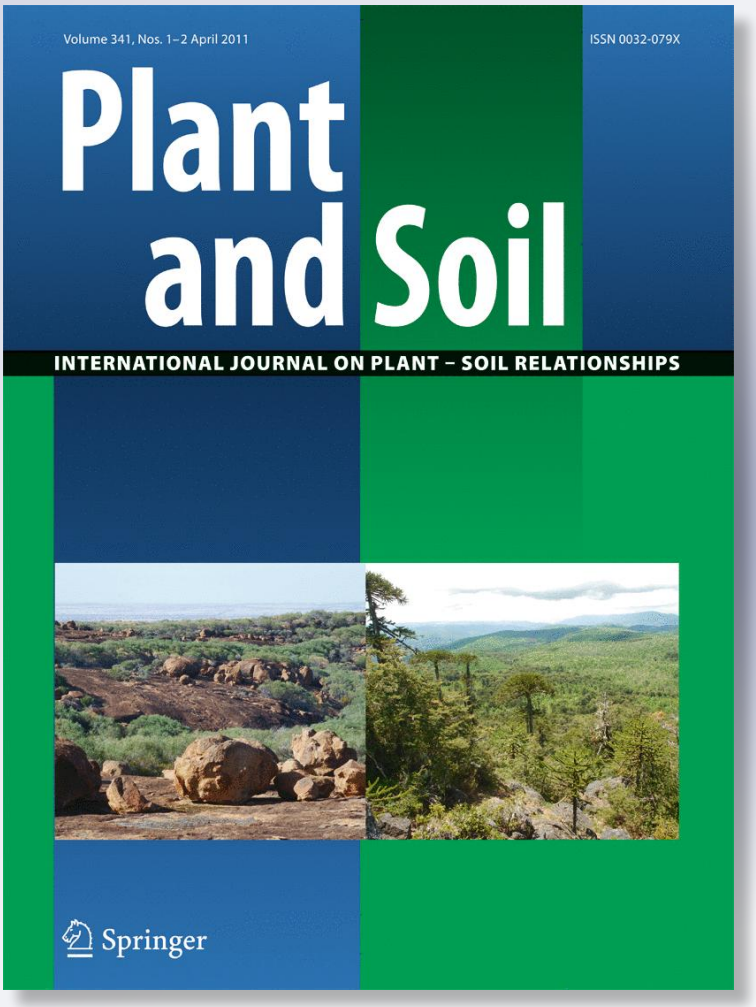

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REGULAR ARTICLE

Dual benefit from a belowground symbiosis: nitrogen fixing rhizobia promote growth and defense against a specialist herbivore in a cyanogenic plant

Sylvia Thamer · Martin Schädler · Dries Bonte · Daniel J. Ballhorn

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Abstract Legume-associated nitrogen-fixing bacteria play a key role for plant performance and productivity in natural and agricultural ecosystems. Although this plant-microbe mutualism has been known for decades, studies on effects of rhizobia colonisation on legume-herbivore interactions are scarce. We hypothesized that additional nitrogen provided by rhizobia may increase plant resistance by nitrogen-based defense mechanisms. We studied this below-aboveground interaction using a system consisting of lima bean (*Phaseolus lunatus* L.), rhizobia, and the Mexican bean beetle (*Epilachna varivestis* Muls.) as an insect herbivore. We showed that the rhizobial symbiosis not only promotes plant growth but also improves plant defense and resistance against herbivores. Results of our study lead to the suggestion that nitrogen provided by rhizobia is allocated to the production of nitrogen-containing cyanogenic defense compounds, and thereby crucially determines the outcome of plant-herbivore interactions. Our study supports the view that the fitness benefit of root symbioses includes defence mechanisms and thus extends beyond the promotion of plant growth. Since the associations between legumes and nitrogen-fixing rhizobia are ubiquitous in terrestrial ecosystems, improved knowledge on rhizobia-mediated effects on plant traits—and the resulting effects on higher trophic levels—is important for better understanding of the role of these microbes for ecosystem functioning.

Keywords Above-belowground interactions · Cyanogenesis · Ecosystem functions · Legumes · Nitrogen fixation · Symbiosis

Abbreviations

HCNp	cyanogenic potential; amount of cyanogenic precursors in a given plant tissue
L:D	light:dark period

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Introduction

Mutualistic interactions between plants and soil microbiota are ubiquitous in terrestrial ecosystems and crucially determine plant diversity and ecosystem productivity (Carney and Matson 2005; van der Heijden et al. 2008). Despite focus on plant-microbe interactions, little is known about the three-way interaction of plant-associated soil microorganisms, plants, and higher trophic levels (but see Goverde et al. 2000; Gehring and Whitham 2002; Gange et al. 2003; Bezemer et al. 2005; Hempel et al. 2009; Pozo and Azcon-Aguilar 2007). While most studies on the ecological function of mutualistic plant-microbe interactions focus on effects of mycorrhizal fungi on plants and plant-associated organisms, specific bottom-up effects of nitrogen-fixing, root colonizing bacteria remain elusive (Sprent 2001; van der Putten et al. 2001; van der Heijden et al. 2006, 2008). This is surprising since nitrogen-fixing bacteria play a key role for global and local nitrogen cycles (Sprent and Sprent 1990). Over 15,000 plant species from more than 12 families are able to form associations with these bacteria and therefore have access to the atmospheric nitrogen pool (Sprent 2001). Amongst them, legumes (Fabaceae) associated with nitrogen-fixing rhizobia are of enormous economic and ecological importance (Wardle 2002).

Depending on the mode of feeding and the degree of specialisation, colonization of plants by symbionts like mycorrhizal fungi can lead to negative (generalists and leaf-chewing herbivores) or positive effects (specialists and sap-feeders) for plant consumers (Koricheva et al. 2009). In this line, in one of the few available studies on effects of rhizobia-colonization on herbivores, Kempel et al. (2009) observed increased performance of the generalist leaf-chewing herbivore *Spodoptera littoralis* only on an acyanogenic clover strain, whereas no such effects were found on a cyanogenic cultivar. In additional experiments using the same plant genotypes, the phloem-feeding aphid *Myzus persicae* was inconsistently affected by rhizobia-colonization of hosts. Thus, belowground symbionts affect both growth and defense in plants and may therefore be important mediators of the trade-off between both processes (Herms and Mattson 1992).

The observed differences in effects of mycorrhizal fungi or rhizobia on specific herbivores may result from changes in the chemical composition of plants' nutritive and defensive compounds. Besides alkaloids, cyanogenic glycosides belong to the most widely distributed nitrogen-containing compounds in higher plants including many legume species (Møller and Seigler 1999). Cyanogenesis is known to mainly repel leaf-chewing herbivores (Jones 1998) and appears to be an effective defense against generalist rather than specialist herbivores (Schappert and Shore 1999). However, more recent studies on cyanogenic lima bean (*Phaseolus lunatus*) showed detrimental effects of cyanogenic glycosides on performance and food choice also of specialist insect herbivores, both in laboratory (Ballhorn et al. 2007, 2008, 2010a) and field studies (Ballhorn et al. 2009a).

Cyanogenesis in plants strongly demands leaf nitrogen (Miller and Woodrow 2008). For example, in *Eucalyptus cladocalyx* as much as 15% of leaf N can be allocated to the cyanogenic glycoside prunasin (Gleadow et al. 1998). Therefore, considering the large demand that cyanogenesis makes on plant resources, we expect symbiotic nitrogen fixation to be an integral part of chemical defenses in legumes (Kempel et al. 2009). However, until now no quantitative data on the impact of nitrogen fixation by rhizobia on cyanogenesis and resulting effects on herbivores have been available. To better understand effects of nitrogen-fixation by plant symbionts on higher trophic levels, we conducted climatic chamber experiments with cyanogenic wildtype lima bean from a natural population in southern Mexico, a rhizobia strain isolated from lima bean at the same natural site, and the Mexican bean beetle as a natural insect herbivore of lima bean. In comparative feeding trials using colonized and uncolonized plants we tested the hypothesis that plant resistance to herbivores can be mediated via rhizobia. To our knowledge, this is the first study in which quantitative effects of rhizobia-colonization on defensive and nutritive leaf traits and arising effects on herbivores were demonstrated. This study further contributes to our knowledge how root symbionts simultaneously affect plant growth, tissue quality and anti-herbivore defense.

Material and methods

Study organisms

Lima bean (Fabaceae: *Phaseolus lunatus* L.) forms a close association with N_2-fixing soil bacteria of the

family Rhizobiaceae in natural systems. Screenings of wild lima bean plants in nature revealed nodulation of all plants investigated. However, there was quantitative variability in number of nodules (DJB; unpublished data). The nodules are visible (0.5–5 mm in diameter), ball-like structures formed on the roots containing the rhizobia in a structurally modified form (bacteroids) (Van Brussel et al. 1977). Lima bean plants used in this study were grown from seeds collected in a natural population in southern Mexico (15°55′N; 097°09′W, elevation 15 m). Plants were cultivated in a climatic chamber (Thermotec-Weilburg GmbH & Co.KG., Weilburg, Germany) adjusted to resemble conditions at natural sites in Mexico as recorded for September to October 2007. Light in the chamber was provided by a combination (1:1) of HQI-BT 400W (Osram) and RNP-T/LR 400W (Radium) lamps with a light regime of 13:11L:D under a photon flux density of 450–500 μmol photons $m^{-2}s^{-1}$ at table height. Temperature was 30°C in the light period and 23°C in the dark period and relative air humidity was adjusted to 70–80%. Plants were cultivated in plant-containers of 10×10×11 cm (width, length, height; one plant per pot) in a 1:1 ratio of standard substrate (TKS®-1-Instant, Floragard®, Oldenburg, Germany) and sand (grain size 0.5–2.0 mm). The substrate was dried in an oven at 75°C for 72 h to remove any insects and reduce fungal contaminations. Autoclaving the substrate was not necessary since European strains of rhizobia form no association with wildtype lima bean (DJB; personal observation). All plants were fertilized with 50 mL of a 0.1% aqueous solution of Flory-3® [NPK+Mg (%); 15, 10, 15 +2-Fertilizer, EUFLOR GmbH, Munich, Germany] once a week and watered daily. We applied this low amount of fertilizer to avoid a strongly retarded growth of control plants, which might affect other parameters than tested with potential effects on leaf palatability to herbivores (leaf toughness, leaf tissue hydration). To avoid contamination, control plants were placed in plastic trays (50×70 cm). Position of trays in the climatic chamber was changed every 3 days to exclude any position effects. Experiments and chemical analyses of leaf material were conducted after a plant cultivation period of 5 weeks.

Rhizobia—Cultivation and inoculation of plants

The rhizobia strain used in our study was isolated from lima bean roots derived from natural sites in Mexico according to Eilmus (2009). Based on 16S rDNA sequence data (GenBank accession no EU842041) bacteria were classified as *Rhizobium* (Eilmus 2009). Rhizobia were cultivated in liquid medium (pH 7.0) containing 1 g yeast extract (AppliChem, Darmstadt, Germany), 10 g mannite (Roth, Karlsruhe, Germany), 800 ml deionized water, and 200 ml soil extract. The soil extract was prepared from 160 g dry, non-fertilized loamy soil (taken from a grass-covered area the Botanical Garden of the University of Duisburg-Essen) that was suspended in 400 ml deionized water under addition of 0.4 g sodium carbonate (Roth, Karlsruhe, Germany) and autoclaved at 121°C for 30 min at a pressure of 1260 mbar. Three days prior to plant inoculation, rhizobia were cultivated at 28°C and 180 rpm on a laboratory shaker (Eppendorf, Wesseling-Berzdorf, Germany). The bacteria solution was then diluted with tap water in a ratio of 1:10 and plants were watered with 100 ml of this solution. Media solutions applied to the control plants contained no bacteria while all other parameters remained unchanged.

Establishment of rhizobia (i.e. nodulation; forming of visible root nodules) as well as the rhizobia-free status of controls were evaluated at regular time intervals by carefully removing plant containers from the root system and recording occurrence of nodules at its periphery.

Mexican bean beetles

The Mexican bean beetle (Coccinellidae: *Epilachna varivestis* Mulsant) is an oligophagous insect that feeds on a range of legumes but with a distinct preference for *Phaseolus* species and especially lima bean (Flanders 1984). The Mexican bean beetle is native to southern Mexico, as are lima beans of the Mesoamerican gene pool. Beetles were obtained from Prof. CPW Zebitz (Dept. Applied Entomology, University of Hohenheim, Germany). Beetles were maintened on acyanogenic snap bean leaves to prevent them from developing any preferences for cyanogenic food. Conditions for the cultivation of beetles (light period and intensity, temperature, humidity) were identical to conditions adjusted for plant cultivation.

Leaf material

For analyzing the quantitative impact of rhizobia-colonization on chemical leaf traits and resistance to herbivores, we selected certain defined leaf develop-

mental stages to reduce uncontrolled variation due to leaf ontogeny (Ballhorn et al. 2008, 2010a). According to their insertion position at the stem, leaves were classified as 'young,' 'intermediate,' or 'mature'. By definition, leaves at the apex of the shoot or a side shoot that were fully unfolded for at least 4 days, but no longer than 6 days were considered 'young'. Leaves located on the stem two positions below the category 'young' were defined as 'intermediate'. Leaves assigned to this category still showed a thin and delicate leaf tissue. 'Mature' leaves were located on the stem two positions below 'intermediate' leaves. These leaves were characterized by a dark green color and a hardened midrib; they were always completely expanded.

Of each individual trifoliate lima bean leaf, one randomly selected leaflet was used for chemical analyses of cyanide and soluble protein concentration, while the other two leaflets were used in feeding trials. Chemical and physical leaf traits were assumed to be similar among the three leaflets of individual leaves, as previous studies have shown distinct homogeneity of traits within individual trifoliate leaves (Ballhorn et al. 2009a).

Cyanogenic potential of leaves

Leaves of lima bean plants were analyzed for their cyanogenic potential (HCNp; concentration of cyanogenic precursors) following the procedure described in Ballhorn et al. (2005). In short, this method is based on complete enzymatic degradation of cyanogenic glycosides in closed Thunberg vessels and subsequent spectrophotometric measurement (585 nm) of HCN released from the cyanide-containing compounds using the Spectroquant® cyanide test (Merck KGaA, Darmstadt, Germany). For enzymatic degradation, we used specific β-glucosidase isolated from rubber tree (Euphorbiaceae: *Hevea brasiliensis*). This plant species possesses the same cyanogenic glycosides as lima bean, i.e. linamarin and lotaustralin. We added external β-glucosidase in excess to leaf extracts to guarantee for total conversion of cyanogenic glycosides into free cyanide and to accelerate the enzymatic reaction (Ballhorn et al. 2006).

Soluble protein concentration

Co-variation of cyanogenic and nutritive plant traits may strongly determine the overall attractiveness or resistance of plants to herbivores. Thus, in addition to cyanogenic traits, we considered leaf soluble protein as an important nutritive trait (Ganzhorn 1992). Soluble protein concentration was quantified according to Bradford (1976) and following the modifications described in Ballhorn et al. (2007). Leaf material was homogenized in ice-cold sodium acetate buffer (pH 5.0). Leaf extracts were centrifuged at 13,000g (4°C), and the supernatant was filtered over NAP™ columns containing Sephadex™ G-25 DNA-Grade (GE Healthcare, München, Germany). Subsequently, 5 µl of the eluate were pipetted on microplates (96-well Microplates, F-bottom; Greiner Bio-One, Frickenhausen, Germany), and 250 µl Bradford reagent (diluted with deionized water in the ratio 1:4) were added. Protein concentration of samples was spectrophotometrically quantified at 595 nm. Bovine serum albumin solutions (Merck) in the range between 10 and 600 µg ml^{-1} served as standard.

Morphological plant traits

Shoot length of plants was measured and secondary leaves were counted at the end of the 5 week growth period. Leaf area was determined using digital photographs of leaves on a scale (Canon, EOS 40D; 10,000 pixels) and the analySIS software (Olympus, Hamburg, Germany). Leaf mass per area (LMA) was calculated on fresh weight basis of leaves. Substrate was carefully washed off from roots with water and above and below ground biomass was evaluated by drying plant material in an oven at 50°C for 5 days until constancy of weight. From rhizobia-inoculated plants all visible root nodules were collected and number and dry weight of nodules was determined on an analytic scale (Kern 770, Kern & Sohn GmbH, Balingen-Fromern, Germany).

Feeding trials

For feeding trials, adult Mexican bean beetles were used that had moulted at least 24 h but no longer than 5 days prior to the experiment. Beetles were food deprived for 2 h before the experiment. Feeding trials were conducted in Petri dishes (9.5 cm diameter) lined with slightly moist filter paper to avoid wilting of leaves. In binary choice feeding experiments comparing rhizobia-colonized vs. rhizobia-free plants, leaf material (leaf discs of 2.73 mm diameter) of a

given leaf stage (young, medium, and mature) was offered to individual beetles for 2 h. After the experimental period leaf disc were digitally photographed on a scale (Canon, EOS 40D; 10,000 pixels) and consumed leaf area was computer-based quantified with the analySIS software (Olympus, Hamburg, Germany). Leaf discs in the Petri dish were placed with a distance of 2 cm to each other. Positions on the lamina at which the discs were removed were the same for both leaflets. We used leaf discs to exclude potential effects on preference of herbivores resulting from different leaf size or shape. In addition, to exclude effects of leaf color (leaves of rhizobia-inoculated leaves were considerably darker green than leaves of control plants) we carried out two experimental series (n=18 feeding trials per series) under controlled conditions, one in the light (photon flux density of 200 $\mu mol\,m^{-2}s^{-1}$) and one in the dark. Temperature and ambient air humidity were identical in both series (22°C, 70–80% relative air humidity).

Statistical analysis

The effect of rhizobia on above- and belowground biomass, number of leaves and plant height was tested using a one-way ANOVA. The effects of rhizobia and leaf stage on leaf area and chemical traits (HCNp, soluble protein) were tested by means of a split-plot analysis since leaves of different age were analysed from every plant (subject). Here, rhizobia were used as between-subject term whereas the within-subject term consisted of leaf stage and the leaf stage x rhizobia-interaction. In feeding trials, leaf stage was considered as between-subject term, which was assigned to the specific plant pairings (blocking factor). Since we used one leaf each of a control plant and an inoculated plant per trial, rhizobia were considered as within-subject term and feeding trials were analysed as a blocked split-plot design. The feeding trials in the light and in the dark ran as separate experiments and were analyzed, separately. We used linear contrasts to compare means of different leaf stages. In case of a significant leaf stage × rhizobia-interaction linear contrasts were calculated separately for plants with and without rhizobia. Data were checked visually for normal distribution of residuals and homogeneity of variances. Biomass data were log transformed to meet the assumptions of ANOVA. Statistical analyses were carried out using SAS 9.2 (SAS institute Inc., Cary, NC, USA).

Results

Plant performance

Rhizobia-inoculation of beans resulted in extensive development of root nodules (50.61±14.05 mg dry weight root nodules per plant, mean ± SD; n=6 plants). The root nodules showed reddish to pinkish color, indicating active N_2 fixation (Blauenfeldt et al. 1994). We observed no contamination of control plants. Plant performance differed between treatment groups. Rhizobia-colonized plants had a significantly higher above-ground biomass than controls (one-way ANOVA, $F_{1,10}$=11.77, P=0.006; Fig. 1a), but root biomass did not differ between colonized plants and controls ($F_{1,10}$=1.50, P>0.05; Fig. 1b). At time of harvest plants with rhizobia were significantly taller (one-way ANOVA, $F_{1,10}$=11.22, P=0.007; Fig. 1c) and had developed a significantly higher number of secondary leaves than control plants ($F_{1,10}$=64.70, P<0.001; Fig. 1d). In addition, leaf area of rhizobia-colonized plants was generally enhanced (split-plot ANOVA, $F_{1,10}$=20.26, P=0.001; Fig. 2a). Leaf area was lowest for young leaves ($F_{2,20}$=12.02, P<0.001) but did not differ significantly between intermediate and mature leaves (linear contrast, P> 0.05). The effect of rhizobia was consistent across leaf stages (interaction leaf stage x rhizobia: $F_{2,20}$=0.99, P> 0.05). Leaf mass per area (LMA) increased with leaf age (Fig. 2b, $F_{2,20}$=28.34, P<0.001) but was not affected by rhizobial colonization ($F_{1,10}$=0.04, P> 0.05; interaction: $F_{2,20}$=0.01, P>0.05).

Leaf chemistry

The symbiosis with rhizobia led to a considerable increase of the cyanogenic potential (HCNp) of plants (split-plot ANOVA, $F_{1,10}$=25.01, P=0.001, Fig. 3a). In general, HCNp decreased with increasing leaf age ($F_{2,20}$=40.19, P<0.001; Fig. 3a). This effect, however, differed between experimental groups (interaction leaf stage x rhizobia: $F_{2,20}$=22.55, P<0.001). In the group of rhizobia-inoculated plants leaves showed a gradual decrease of HCNp with increasing age (linear contrasts between all means with P<0.05), whereas there were no significant differences between leaf stages in rhizobia-free plants (linear contrast, P> 0.05). The positive effect of rhizobia on HCNp remained significant across all leaf stages (all linear

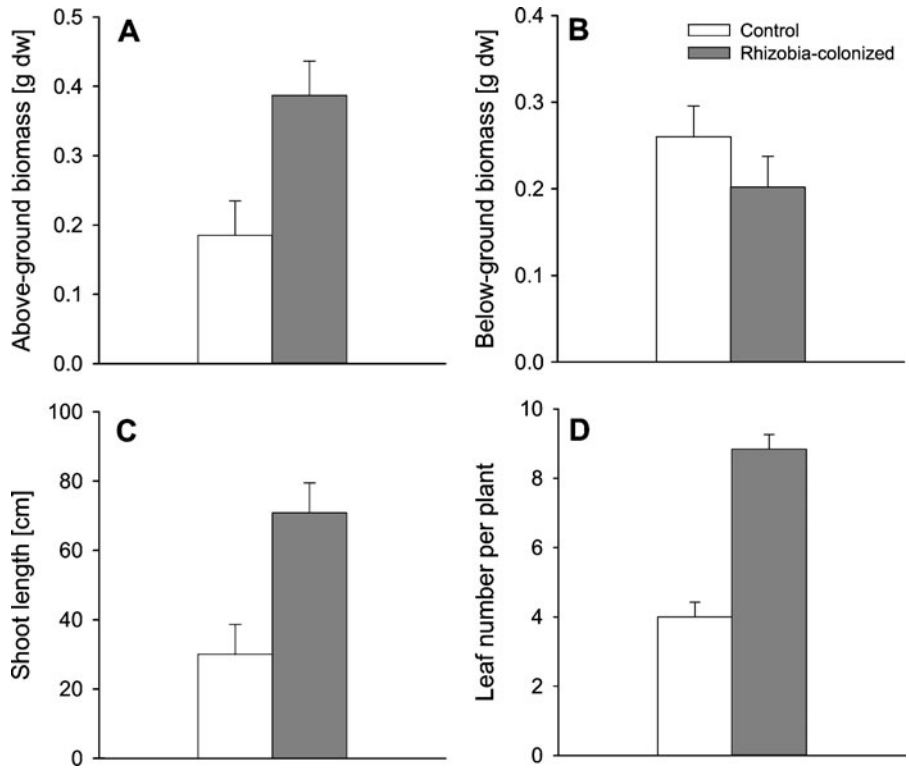

Fig. 1 Effects of rhizobia on plant performance traits. Above-ground biomass (**a**) and root biomass (**b**) of rhizobia-colonized and rhizobia-free plants was determined on dry weight basis after 5 weeks of cultivation. In addition, shoot length (**c**) and leaf number (**d**) of plants was evaluated. Values given in the figure are means + SE; n=6 plants per treatment

contrasts with $P<0.05$). Rhizobia colonization resulted in an increase of cyanide by the factor 8.4 in young, 10.5, and 10.4 in mature leaves. Soluble protein concentration did not differ between inoculated and control plants (split-plot ANOVA, $F_{1,10}=0.08$, $P>0.05$), but decreased with leaf age in both groups (split-plot ANOVA, $F_{2,20}=22.59$, $P<0.001$; no significant leaf stage x rhizobia-interaction: $F_{2,20}=0.03$, $P>0.05$; Fig. 3b). Young leaves in both treatment groups showed a significantly higher protein content than intermediate and mature leaves (linear contrasts, $P<0.05$). Protein concentration did not differ between intermediate and mature leaves (linear contrast, $P>0.05$).

The quantitative relation of cyanide per protein resembled the observed pattern of HCNp observed for leaf stages and treatment groups (Fig. 4) as HCNp showed variation depending on rhizobia colonization while concentration of soluble proteins did not. Thus, the cyanide per protein ratio was increased for rhizobial plants ($F_{1,10}=34.95$, $P<0.001$) but not affected by leaf age ($F_{1,20}=1.77$, $P>0.05$; interaction: $F_{1,20}=1.00$, $P>0.05$), since both proteins and cyanide decreased with leaf age.

Feeding trials

In general, Mexican bean beetles significantly preferred leaves of uncolonized plants over leaves from rhizobia-inoculated plants both in the light (split-plot ANOVA, $F_{1,15}=15.57$, $P=0.0013$) and in the dark experiment ($F_{1,15}=22.89$, $P=0.0002$; Fig. 5a, b). In both experiments increasing leaf age had a positive influence on leaf consumption by beetles (light: $F_{2,10}=4.08$, $P=0.05$: dark: $F_{2,10}=5.88$, $P=0.02$). However, for the light experiment there was a marginally significant ($F_{2,15}=2.95$, $P=0.08$) and for the dark experiment a significant interaction ($F_{1,15}=3.74$, $P=0.048$) between rhizobia colonization and leaf age. In both experiments, consumption increased with increasing leaf age only for control plants (Fig. 5a, b), but remained constant for different leaf stages of the rhizobia-colonized plants. As a consequence, consumption of leaves of the control plants was significantly higher only for intermediate and mature leaves (linear contrasts, $P<0.05$, see Fig. 4a, b). Beetles consumed slightly more under light conditions (mean 10.54 mm^2) than in the dark (mean 9.51 mm^2) but this difference was not significant (paired t-test).

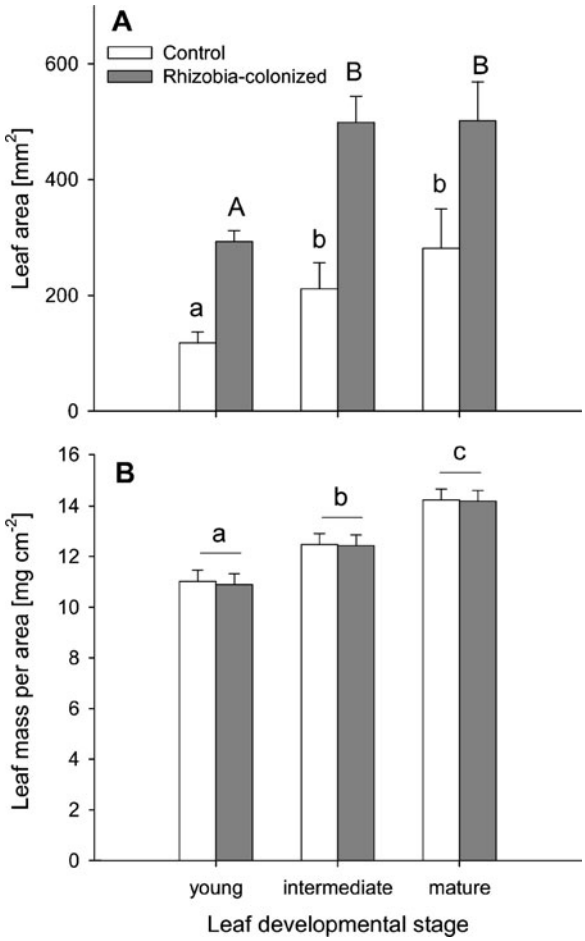

Fig. 2 Effects of rhizobia on leaf area (**a**) and leaf mass per area (**b**) of different leaf developmental stages. Values given in the figure are means + SE; n=6 plants. Different letters (*lower case for control plants, upper case for inoculated plants*) indicate statistically significant different means following linear contrasts (P<0.05). Leaf area was increased for rhizobia-colonized plants across all leaf developmental stages whereas leaf mass per area was not affected by rhizobia (see text)

Discussion

In this study we demonstrated that plant colonization by rhizobia resulted in substantial effects on herbivore food selection. In controlled laboratory experiments we showed that rhizobia not only increased plant growth and biomass production (Sprent and Sprent 1990) but also quantitatively altered plant defense (cyanogenic glycosides) of lima bean. Additional nitrogen provided by rhizobia to the host plant increases the nitrogen content in plant tissue (Sprent 2001), which is a crucial parameter determining food plant quality to insect herbivores (Schädler et al. 2007). However, analyzing total N does not provide information on which compounds contribute to the overall nitrogen pool of a given plant and which compounds are quantitatively affected by additional nitrogen provided by rhizobia. With respect to higher trophic levels, the outcome of increased levels of amino acids and proteins should differ from the effects of enhanced concentrations of toxic nitrogen containing metabolites such as alkaloids or cyanogenic glycosides (Johnson and Bentley 1991; Ball et al. 2000; Awmack and Leather 2002). In this study, we observed increased levels of cyanide in all leaf stages due to additional nitrogen supplied by the

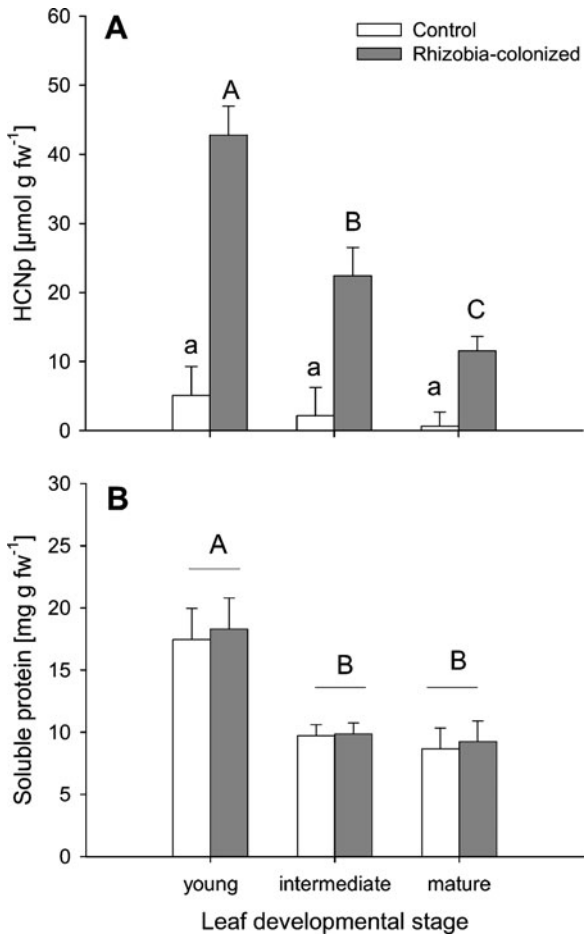

Fig. 3 Effects of rhizobia on leaf cyanogenic potential (**a**) and soluble protein (**b**). Values given in the figure are means + SE; n=6 plants. Different letters (*lower case for control plants, upper case for inoculated plants*) indicate statistically significant different means following linear contrasts (P<0.05). HCNp was increased for rhizobia-colonized plants across all leaf developmental stages (**a**), whereas soluble protein was not affected by rhizobia (**b**)

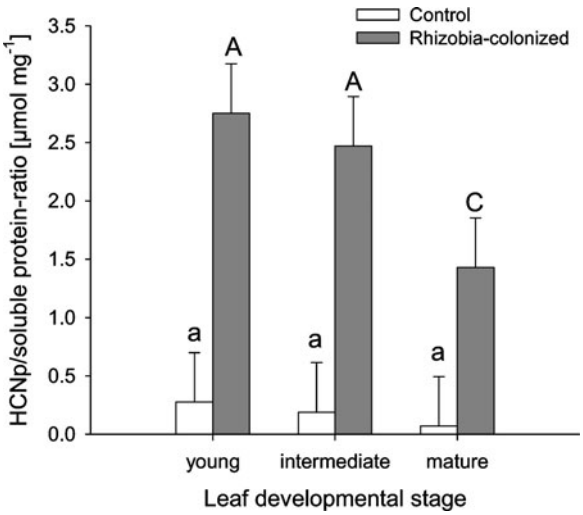

Fig. 4 Effects of rhizobia on cyanogenic potential per unit soluble protein. Values given in the figure are means + SE; $n=6$ plants. Different letters (*lower case for control plants, upper case for inoculated plants*) indicate statistically significant different means following linear contrasts ($P<0.05$). HCNp/soluble protein-ratio was increased for rhizobia-colonized plants across all leaf developmental stages

bacteria. In contrast to defense-associated compounds, soluble protein concentration was not significantly affected by rhizobia-colonization and apparently did not drive the observed changes in feeding choice by Mexican bean beetles (Fig. 4). This effect observed on the lima bean systems provides a mechanistic explanation for the different effects of rhizobia on herbivores, which had been feeding on cyanogenic and acyanogenic strains of white clover (*Trifolium repens*) observed by Kempel et al. (2009). In this study, Kempel and co-workers demonstrated that for *T. repens* rhizobia increased plant growth and the performance of *Spodoptera littoralis* on acyanogenic plants, whereas this positive effect of rhizobia on the caterpillars did not occur in a cyanogenic clover strain.

For wildtype lima bean, leaf area consumption of herbivores decreased with enhanced leaf cyanide in rhizobia-colonized plants (Figs. 3 and 4). Cyanogenesis is an effective feeding inhibitor across a wide range of invertebrate and vertebrate herbivores affecting both generalists and specialists (Zagrobelny et al. 2004; Ballhorn et al. 2007). Soluble protein concentration represents an important nutritive trait (Ganzhorn 1992), but we found cyanide rather than soluble protein concentration to be the factor determining consumption by beetles. With focus on cyanogenesis, the quantitative relationship of cyanide to protein—especially to proteins with high amounts of the *sulphur*-containing amino acids cysteine and methionine—is an important measure determining plant's overall food quality, as these amino acids are required for enzymatic detoxification of cyanide by rhodanese or β-cyanoalanine synthase (e.g., Urbańska et al. 2002; Ballhorn et al. 2009b). While the contribution of *sulphur*-containing amino acids to the pool of soluble proteins in lima bean leaves remains elusive so far, we could show clear effects of rhizobia-colonization on the ratio of cyanide per protein (Fig. 4). In our study, the quantitative relation of cyanide per protein resembled the observed pattern of HCNp observed for leaf stages and treatment

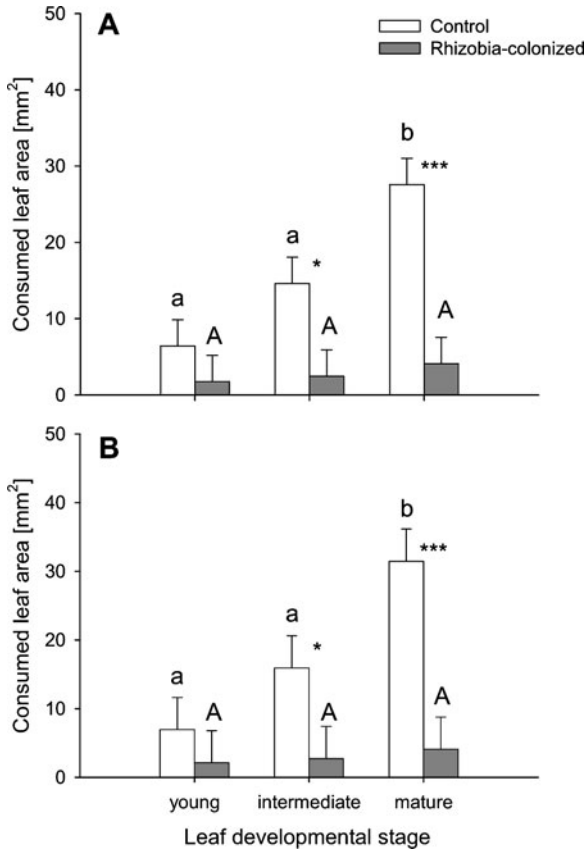

Fig. 5 Effects of rhizobia on leaf consumption by leaf beetles under light (**a**) and dark (**b**) conditions. Values are means + SE; $n=6$ plants. Different letters (*lower case for control plants, upper case for inoculated plants*) indicate statistically significant different means following linear contrasts ($P<0.05$). *Asterisks* indicate statistically different means between the rhizobia treatments following linear contrasts (* for $P<0.05$, *** for $P<0.001$)

groups as HCNp showed variation depending on rhizobia colonization while concentration of soluble proteins did not.

In addition to cyanogenesis, lima bean shows a range of other defense-associated chemical leaf traits that have not been measured in this study such as phenolics and polyphenol oxidase (PPO) activity. However, these traits are primarily associated to resistance to pathogens than playing a central role in herbivore defense (Ballhorn et al. 2010a,b). Besides chemical traits, physical leaf properties such as leaf toughness and tissue water content can crucially affect attractiveness and palatability of leaves for insect herbivores (Coley et al. 1985). In the present study, we used individual leaves for chemical analyses and feeding trials to exclude uncontrolled variation among leaves. As all three leaflets of each leaf had to be used in fresh form for the different experiments we measured leaf mass per area (LMA) on the fresh weight basis of leaf material. Therefore, we cannot differentiate between tissue water content and dry matter per leaf area, which represents the typical measure for LMA (Ballhorn et al. 2007). However, for each leaf stage we observed no significant differences of LMA between rhizobia free and rhizobia-colonized plants indicating similar physical leaf characteristics of both treatment groups. Results of this study support the suggestion that increased cyanide levels in rhizobia-colonized plants are the driving force behind the observed effects on the herbivores. In contrast to soluble protein and LMA, leaf cyanide showed significant treatment dependent variation which corresponded to the deterrence of herbivores in feeding trials. This is in line with own previous studies on lima bean, which demonstrated the importance of cyanogenesis as defense against generalist and specialist herbivores. For example, lima bean's cyanogenesis quantitatively deterred generalist desert locusts (Ballhorn et al. 2005, 2010a) and affected food plant choice and oviposition preference (Ballhorn and Lieberei 2006; Ballhorn et al. 2010a) as well as larval performance and reproduction of specialist Mexican bean beetles (Ballhorn et al. 2007).

In the present study, in addition to better overall performance, plants treated with rhizobia had clearly darker green leaves. Many herbivorous insects use visual cues to locate suitable host plants. For example, coleopteran herbivores have been repeatedly reported to orientate on color (Szentesi et al. 2002). Thus, we conducted additional feeding trials in the dark to exclude the factor 'leaf color'. Beetles showed higher activity under light conditions (i.e. movement in the Petri dish, cleaning of antennae, legs, and mouth parts), but there was only a weak tendency toward higher leaf consumption. Choice behavior of insects observed under light and dark conditions was statistically not different indicating importance of variation of leaf quality due to enhanced nitrogen-availability rather than variation in color.

Factors driving plant-microbe and plant-herbivore interactions are complex and we are only beginning to understand their functional interplay in nature (Bennett and Bever 2007; Ballhorn et al. 2009a; Yi et al. 2009). In addition to variation in plant primary and secondary metabolites mediated by rhizobia, attractiveness of plants to herbivores is influenced by multiple factors, such as occupation by other herbivores or pathogens, presence of predators or parasitoids, microclimatic conditions, as well as by plant architecture, distribution and availability (e.g., Denno et al. 1995; Rostás et al. 2003; Bonte et al. 2010). Under natural conditions belowground plant-associated species often show distinct spatial structure, although detailed knowledge on the scales of spatial variation in most natural systems is largely lacking (Ettema and Wardle 2002). Studies on wild lima beans in Mexico revealed strong quantitative variability of root nodulation ranging from <5 nodules per plant to over 400 (DJB, unpublished data) making variation in nitrogen-availability of individual plants at natural sites likely. In consequence, as we could demonstrate in the present study, differences in the allocation of nitrogen to cyanogenic glycosides and thus resistance to herbivores should be expected. Substantial differences in cyanogenesis and herbivore resistance among individual lima bean plants in nature have been illustrated (Ballhorn et al. 2009a). While this variation of cyanogenesis was largely genetically controlled additional variation may arise from different degrees of rhizobia-association.

The relative allocation of resources towards defense traits is regarded as constrained by trade-offs with plant growth (Herms and Mattson 1992). Here we could show that rhizobia promoted growth and at the same time increased defense against herbivores. This in consequence represents a sub-

stantial fitness benefit that reaches beyond current insights of increased N-uptake and supports the view of Bennett et al. (2006) that root symbionts may interfere with these trade-offs and allow plants to enhance both defense and growth. However, since insects have repeatedly been reported to select larger plants for feeding and reproduction these benefits might be outweighed in natural systems (Bolter et al. 1997; Hoy et al. 2000). Since, in contrast to HCNp, soluble protein in lima bean was not affected by inoculation, additional nitrogen made available by rhizobia is selectively channeled to specific physiological pathways. From mycorrhizal fungi it is known that they may activate the plant-defense system (Pozo and Azcon-Aguilar 2007) and given common signaling pathways for both forms of symbiosis (Marx 2004) a similar effect seems to plausible for rhizobia. We therefore conclude, that plant symbionts importantly mediate physiological trade-offs with implications for ecological strategies. To our knowledge this is the first study demonstrating quantitative effects of rhizobia on a nitrogen-based plant defense and resulting defensive effects on a natural insect herbivore. Considering the wide distribution and ecosystemic importance of legume-rhizobia interaction, our findings add a new dimension concerning the ecological impact of rhizobia in multi-species networks.

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