Arbuscular mycorrhizas in phosphate-polluted soil: interrelations between root colonization and nitrogen

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Abstract

Aims: To investigate whether arbuscular mycorrhizal fungi (AMF) – abundant in a phosphate-polluted but nitrogen-poor field site – improve plant N nutrition, we carried out a two-factorial experiment, including N fertilization and fungicide treatment.

Methods: Percentage of root length colonized (% RLC) by AMF and tissue element concentrations were determined for four resident plant species. Furthermore, soil nutrient levels and N effects on aboveground biomass of individual species were measured.

Results: Nitrogen fertilization lowered % RLC by AMF of *Artemisia vulgaris* L., *Picris hieracioides* L. and *Poa compressa* L., but not of *Bromus japonicus* Thunb. This – together with positive N addition effects on N status, N:P-ratio and aboveground biomass of most species – suggested that plants are mycorrhizal because of N deficiency. Fungicide treatment, which reduced % RLC in all species, resulted in lower N concentrations in *A. vulgaris* and *P. hieracioides*, a higher N concentration in *P. compressa*, and did not consistently affect N status of *B. japonicus*.

Conclusions: Evidently, AMF had an influence on the N nutrition of plants in this P-rich soil; however – potentially due to differences in their mycorrhizal responsiveness – not all species seemed to benefit from a mycorrhiza-mediated N uptake and accordingly, N distribution.

Keywords

arbuscular mycorrhiza, Benomyl, element concentrations, nitrogen fertilization, phosphate pollution, root colonization

Abbreviations

AM = arbuscular mycorrhiza

AMF = arbuscular mycorrhizal fungi

% RLC = percentage of root length colonized

66

Introduction

Arbuscular mycorrhizas (AM) are generally considered to be mutualistic symbioses: The fungus supplies its host plant with nutrients, in particular phosphorus, and in return for this receives carbohydrates (Smith and Read 2008). For establishment and functioning of the symbiosis, the nutritional status of the host is important as roots of phosphorus- as well as nitrogen-deficient plants release more exudates into the soil than those of non-deficient plants, which stimulates root colonization by arbuscular mycorrhizal fungi (AMF) (Schwab et al. 1991; Harrison 2005; Yoneyama et al. 2007). Further on, a low plant nutrient status positively affects carbon allocation to the fungus within the root (Olsson et al. 2002; Olsson et al. 2005), which in turn increases nutrient uptake by the fungus and transfer to the host (Bücking and Shachar-Hill 2005).

Thus, root colonization by AMF, often quantified as percentage of root length colonized (% RLC), usually decreases after nutrient addition; this has frequently been shown for P fertilization, after which plants allocate less C to the fungi (e.g. Daft and Nicolson 1969; Sanders and Tinker 1973; Olsson et al. 2010), and was also found for N fertilization (Chambers et al. 1980; Jensen and Jakobsen 1980; Olsson et al. 2005). However, % RLC may remain high or even increase after fertilization, if a nutrient other than the one added is limiting plant performance. The importance of relative availabilities of N and P in regulating the symbiosis has been demonstrated by showing that root colonization was reduced only when both elements were available in sufficient concentrations for the plants (Sylvia and Neal 1990; Johnson et al. 2003; Blanke et al. 2005), because not until then was belowground carbon allocation in plants reduced (Treseder and Allen 2002; Johnson et al. 2010).

Although AMF have been shown to take up and transfer significant amounts of nitrogen to plants (Govindarajulu et al. 2005; Tian et al. 2010), reports about fungal effects on plant N status are controversial: In some greenhouse studies, mycorrhizal plants had higher N levels

than non-mycorrhizal plants (e.g. Frey and Schüepp 1993; Tobar et al. 1994; Leigh et al. 2009) but not in others (Hawkins and George 1999; Hawkins et al. 2000). Field studies are considerably less frequent, but similarly, reductions of AMF abundance by fungicide treatments have been found to decrease (Dhillion and Gadsjord 2004), increase (Karanika et al. 2008) or not to change plant N concentrations (Grogan and Chapin 2000).

Cost-benefit analyses for natural communities are more complex: Influences of AMF on plants may differ from those found in experiments using single species, since there might be density- or species-dependent effects (Hart et al. 2003). As plant species vary in their mycorrhizal responsiveness (sensu Janos 2007; see also Hetrick et al. 1992; van der Heijden 2002; but note that various terms were used in the cited literature), AM can influence interspecific competition by differently affecting individual plant species (Francis and Read 1995; Moora and Zobel 1996; Wilson and Hartnett 1998; Hartnett and Wilson 1999; Hart et al. 2003; Scheublin et al. 2007; Cameron 2010). Plant responsiveness to AM has been found to vary between taxonomic groups (Francis and Read 1995), with life history traits (Wilson and Hartnett 1998), and with root system architecture (e.g. Baylis 1975; Newsham et al. 1995).

In the present study, we investigated the interrelation between % RLC by AMF and N concentration of several plant species growing at a site that had been polluted by emissions of phosphate fertilizer production, and thus, is characterized by exceptionally high phosphorus levels. Therefore, plants should be abundantly supplied with P, without recourse to mycorrhizas. Nevertheless, most species at the site are strongly colonized by AMF, although fungal diversity is low compared to similar but unpolluted field sites within the same region (Renker et al. 2005). In a previous N fertilization experiment, we found evidence that root colonization of the resident plant *Artemisia vulgaris* was positively correlated with the degree of nitrogen deficiency (Blanke et al. 2005). In this extended follow-up study, we used

four resident plant species and combined N fertilization with application of the fungicide Benomyl in a full factorial design to address the following two questions:

(1) Do well-developed arbuscular mycorrhizas – suppressed by the fungicide – actually improve plant N status at the field site?

(2) Do the four plant species – two forbs and two grasses – react similarly to fertilization and fungicide application or are there species-specific differences?

Materials and methods

Field site

The Steudnitz field site, a south-east facing calcareous slope with thin-layered rendzina soil, located 13 km north of Jena (Thuringia, Germany) on the western side of the Saale River Valley (51°01' N, 11°41' E), was exposed to emissions of a nearby phosphate fertilizer factory from 1960 to 1990. Alkaline dust deposition strongly enriched the site's topsoil with phosphorus, sodium, calcium, cadmium and fluorine, and caused soil pH to increase up to 10. As a result, the slope was largely devoid of vegetation from ca. 1980 onwards, and most nitrogen was lost from the ecosystem, resulting in a low soil nitrogen level (0.1-0.2%), which has persisted to the time of this study. (Metzner et al. 1997; Heinrich et al. 2001; Blanke et al. 2005; Held and Baldwin 2005)

After decommissioning of the factory in 1990, ecosystem regeneration set in very quickly (Heinrich et al. 2001). Within a few years, the main contaminants were either leached out (Na, F) or immobilized (Cd) due to the high regular pH (~ 8) of the calcareous soil (Langer and Günther 2001; Wagner 2004a). However, total soil P (up to 120 g kg^{-1}; Metzner et al. 1997; Langer and Günther 2001) and P availability (CAL (calcium-acetate-lactate)-method; 4 to 12 g kg^{-1} soil; Wagner 2004a; Blanke et al. 2005; Held and Baldwin 2005) were still markedly raised at the time of this study. Vegetation by then had become relatively diverse, consisting of ca. 60 species, mostly ruderal herbs and grasses, with woody plants only slowly gaining ground (Wagner et al. 2006). For a more detailed review of the regeneration of this ecosystem see Blanke et al. (2007).

Experimental design

151 To investigate the influence of plant nitrogen status on the percentage of root length colonized
152 by arbuscular mycorrhizal fungi and vice versa, we carried out a two-factorial experiment
153 combining N fertilization and fungicide application. In 2000, six experimental blocks had
154 been set up (Wagner 2004b), each containing an unfertilized control plot (-N plots) and an
155 N-fertilized plot (+N plots) with plot sizes of 2 m x 2 m. Every year +N plots received
156 8.5 g N m^{-2}, applied in March in form of slow-release pellets containing ammonium nitrate
157 (Osmocote^{TM}). In 2004, each plot was divided into two subplots of 1 m x 2 m and from March
158 onwards, one of them was treated biweekly with the fungicide Benomyl (Methyl 1-
159 (butylcarbamoyl)-2-benzimidazole carbamate; Benlate, DuPont Iberica, Barcelona, Spain),
160 applied as a soil drench (-N+B and +N+B subplots). For application to 1 m^2, 3 g Benomyl
161 (active ingredient) were dissolved in 5 litres water (slightly modified from Smith et al. 1999;
162 Grogan and Chapin 2000). Untreated subplots (-N-B or +N-B) received the same amount of
163 water to prevent confounding of fungicide and moisture effects.
164
165 Soil parameters
166
167 In June 2004, 20 soil cores of 2 cm diameter and 10 cm depth were taken in each subplot,
168 pooled and air-dried. Samples were analyzed according to DIN (Deutsches Institut für
169 Normung)- and TGL (Technische Güte- und Liefervorschriften)-instructions (VDLUFA
170 1991). Total concentrations of P, Na, K, Mg, Ca and Cd were determined from an aqua regia
171 digestion and total N according to Hendershot (1985). Soil acidity (pH) was measured in H_2O.
172
173 Plant material
174
175 Concurrent with the soil cores, we collected samples from four plant species, *Artemisia*
176 *vulgaris* L. (Asteraceae, perennial hemicryptophyte), *Picris hieracioides* L. (Asteraceae,

177 biennial hemicryptophyte), *Poa compressa* L. (Poaceae, perennial hemicryptophyte) and
178 *Bromus japonicus* Thunb. (Poaceae, annual; Schmeil and Fitschen 1993;
179 http://www.ecoflora.co.uk/), to determine tissue element concentrations and % RLC by AMF.
180 Two individuals per species were sampled in each subplot. Aboveground plant parts for
181 element analyses – complete shoots of *P. compressa* and *B. japonicus* and pooled samples
182 consisting of one basal, one intermediate and one apical leaf of *A. vulgaris* and
183 *P. hieracioides* – were washed with tap water and stored at -80°C. Root systems were fixed in
184 FAA (formaldehyde-acetic-acid: 6.0% formaldehyde, 2.3% glacial acetic acid, 45.8% ethanol,
185 45.9% H_2O (v/v)) for determination of % RLC (Schmitz et al. 1991).
186 As fine roots, where AMF are active and were examined, are short-lived (<1 year, Hodge et
187 al. 2009), we can assume that regardless of plant phenology, most of them – and accordingly
188 colonization by AMF – were newly formed during the experiment, which started in the
189 beginning of the growing season. This is important, because Benomyl inhibits fungal cell
190 division and growth (Kahiluoto and Vestberg 2000), but does not kill fungi already present.
191 Moreover, arbuscules – sites of nutrient transfer to the plant – have a short turnover time
192 (around one week, Smith and Read 2008), which means that their growth was surely affected.
193
194 Percentage of root length colonized by arbuscular mycorrhizal fungi
195
196 For staining of fungal structures, fixed roots were incubated in 10% KOH for 2 x 15 min. at
197 90°C, rinsed with tap water, acidified with 3.7% HCl for 10 min., and dyed in lactophenol
198 blue solution (Merck 113741) for 90 min. For decolourization of plant cells and storage, roots
199 were washed several times with and stored in 50% lactic acid (Phillips and Hayman 1970;
200 modified after Schmitz et al. 1991).
201 Percentage of RLC was determined with a Zeiss Axioplan light microscope using a magnified
202 intersections method (McGonigle et al. 1990; modified after Schmitz et al. 1991) and was

assessed separately for entire internal mycelium, arbuscules and vesicles. For each root sample, a minimum of 300 fields of view were counted.

Plant element concentrations

Frozen plant samples were freeze-dried, weighed and finely ground in a pebble mill. To determine N content, 2 mg subsamples of the homogenized material were analyzed with a Carbon-Hydrogen-Nitrogen-Sulfur-Determinator (Type Leco CHNS-932). For a determination of P, Na, K, Mg, Ca and Cd, 200 mg subsamples were digested in a microwave autoclave (1200 mega, MLS, Leutkirch, Germany) using 6 ml HNO_3 and 4 ml H_2O_2, and analyzed in an ICP-OES (Inductively Coupled Plasma with Optical Emission Spectrometer; Type IRIS Intrepid, Thermo Elemental, Franklin, MA, USA) with CID (charge injection device) semiconductor detectors.

Biomass data

Standing biomass of the vegetation at control and N-fertilized plots was determined annually from 2000 to 2003. Every July, plants from alternating 0.33 m^2 areas in each plot were cut at ground level, biomass was sorted to species and dried to constant weight at 80°C. Here, we only present data for *A. vulgaris*, *P. hieracioides*, *P. compressa* and *B. japonicus*.

Data analyses

Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA), R 2.1.0 (R Development Core Team 2005) or PASW 18 (IBM Corporation, Somers, NY, USA). Data collected from the two individuals per plant species sampled in each subplot in 2004 were

averaged to avoid pseudoreplication, tested for normal distribution using the Kolmogorov-Smirnov test and for variance homogeneity with Levene tests. When necessary, data were square-root or power transformed to achieve variance homogeneity. Linear mixed-effects models (LMEs) with nitrogen fertilization and fungicide treatment as fixed factors and block identity as random factor were fitted to assess nitrogen and fungicide effects on soil and plant element concentrations and on % RLC. A subsequent ANOVA tested whether model terms were significant. In case of a significant ($P < 0.05$) fungicide effect on plant N or P concentrations, Spearman correlations were calculated between % RLC by arbuscules and the respective element concentration in individual plants across all treatments. For significant correlations regression curves were fitted with % arbuscules as independent variable and tissue N concentration as dependent variable, followed by ANOVAs testing for model significance. Significant regression models with the highest R^2 were chosen to reflect the relation between the respective data.

Biomass data of individual species collected yearly from 2000 to 2003 were tested for normal distribution and variance homogeneity and power transformed to achieve the latter. Fertilizer and time effects were assessed with LMEs (fixed factors: N fertilization and time, random factor: block identity), followed by ANOVAs testing for model term significance.

Results

Percentage of root length colonized by arbuscular mycorrhizal fungi

In *A. vulgaris*, *P. compressa* and *P. hieracioides*, N fertilization reduced % RLC by internal mycelium, arbuscules and vesicles (Fig. 1a,b,c), with the reduction being significant except for arbuscules in *P. hieracioides* (Table 1). By contrast, there was no N addition effect on % RLC of *Bromus japonicus* (Fig. 1d; Table 1). Benomyl application decreased % RLC in all four species, and in most cases (except of vesicles in *B. japonicus*), we found significant interactions between fertilizer and fungicide addition, with Benomyl effects being smaller in when N was added, and correspondingly, N effects being smaller when the fungicide was applied (Fig. 1; Table 1).

Plant element concentrations

N concentrations in leaves of *A. vulgaris* were increased by N fertilization and decreased by fungicide application (Fig. 2a; Table 1), and both treatments interacted to affect leaf P and N:P (Table 2). In *P. hieracioides,* N addition significantly increased leaf N:P ratio (Table 2), and fungicide application decreased leaf N (Fig. 2b; Table 1). *P. compressa* shoot N:P was increased by N addition (Table 2), and shoot N was increased by fungicide application (Fig. 2c; Table 1). P concentrations in this species were reduced by N fertilization and increased by fungicide treatment (Table 2). We found no main treatment effects on N and P concentrations in *B. japonicus* (Table 1; Table 2); however, fertilization and fungicide application interacted to affect shoot N (Fig. 2d; Table 1). For treatment effects on tissue concentrations of K, Mg, Ca, Na and Cd see Online Resource 1.

In *A. vulgaris*, *P hieraciodes* and *P. compressa* % RLC by arbuscules was significantly correlated to tissue N – positively in the two forbs (Fig. 3a,b) and negatively in the grass (Fig. 3c). Shoot P in *P. compressa* did not show a significant correlation to arbuscule frequency (Fig. 3d). Subsequent regression analyses indicated a significant dependency of tissue N in these three species from % RLC by arbuscules across all treatments (Fig. 3a,b,c).

Soil parameters

N fertilization significantly increased soil N, whereas it did not affect P and pH (Table 3). Fungicide application did not affect any soil parameter measured, neither on its own, nor in interaction with fertilization.

Results from analyses of additional elements (K, Mg, Ca, Na and Cd) are listed in Online Resource 2.

Biomass data

In the period 2000 to 2003, standing biomass of *A. vulgaris* and *P. hieracioides* was positively affected by N fertilization, whereas that of *P. compressa* was negatively affected (Table 4). Significant interactions with time indicate that in the case of *A. vulgaris* and *P. hieraciodes*, fertilization effects varied between sampling years; additionally, biomass of *P. hieracioides* decreased over the years, as indicated by a significant time effect. There was no main effect of N addition on *B. japonicus* biomass, but a significant effect of time and an interaction between fertilization and time, resulting from a strongly increased biomass in N-fertilized plots only in 2002.

Discussion

Increased N availability at the P-rich Steudnitz field site led to a decrease in the percentage of root length colonized by arbuscular mycorrhizal fungi in *Artemisia vulgaris*, *Picris hieracioides* and *Poa compressa*. This supports the hypothesis that plants at this site are considerably colonized by AMF because of N deficiency, and that, when this is alleviated, root colonization is reduced (Blanke et al. 2005). These results fit well with a model by Treseder and Allen (2002) proposing that, as long as a plant is limited by either P or N, it allocates carbon (C) to the fungi and in turn is provided with the limiting nutrient; when both elements are sufficiently available, C allocation is decreased, which in turn reduces fungal growth. Our results might as well be explained by an extension of the functional equilibrium model (Brouwer 1983), i.e. that if belowground competition for nutrients is reduced, plants allocate less C to roots, including mycorrhizas (Johnson et al. 2008).

The estimation that plants at the Steudnitz site are N-deficient is supported by comparatively low tissue N concentrations (ca. 0.6%-1.8%) and particularly N:P-ratios (ca. 4-8) (see Marschner 2002; Tessier and Raynal 2003; Güsewell 2004; for a more detailed discussion regarding tissue N and N:P and N limitation at the field site also see Blanke et al. 2005), which increased following N-fertilization, as illustrated by leaf N in *A. vulgaris* and N:P ratios in leaves of *P. hieracioides* and shoots of *P. compressa*. Also aboveground biomass of *A. vulgaris* and *P. hieracioides* was higher in N-fertilized plots than in controls, which furthermore suggests that N is limiting growth. This assumption is supported by missing effects of additional nutrient treatments (NPK and micronutrients) in the original fertilizer study (Wagner et al. 2004b, data not shown here). Biomass of *P. compressa*, by contrast, reacted negatively to N fertilization, which is unexpected as N addition tends to favour grasses at the expense of forbs (e.g. Tilman 1987; Bobbink 1991). In our case, the more stress-tolerant *P. compressa* (SR/CSR strategist *sensu* Grime et al. 2007) may be less able to

translate increased nutrient supply into higher growth than *P. hieracioides* (R/CSR) or *A. vulgaris* (C/CR), and thus, be out-competed at higher N availability.

Benomyl application lowered % RLC by AMF in all four plant species. At the same time, it caused a reduction of leaf N in *A. vulgaris* and *P. hieracioides*, which may suggest that well-developed arbuscular mycorrhizas did improve the capability of these species to take up N. Alternatively, Benomyl could have reduced leaf nitrogen levels via stimulation of plant growth, which however was not observed in the field (V. Blanke, personal observation) and the majority of Benomyl studies (e.g. Paul et al. 1989, West et al. 1993, Hartnett and Wilson 2002). Furthermore, such a dilution effect would only occur, if not all limiting nutrients were supplied to the plant, i.e. if Benomyl addition alleviated another limiting factor without supplying N. This seems to be very unlikely, as first, in our case the limiting nutrient clearly was N and second, the only fertilization effect of Benomyl sporadically reported is that of N (e.g. Kahiluoto and Vestberg 2000; Chen et al. 2001). Moreover, soil parameters were unaffected by the fungicide treatment, and a direct N effect of Benomyl on plant N concentrations would have resembled that of N fertilizer, which was not the case. Benomyl effects on other fungi than AMF were not analyzed in this study. In the root samples largely AMF structures were visible, so that these – and manipulations of their abundance – most likely had stronger influences on plant performance than other intraradical fungi and alterations in their abundance. Generally, fungicides may lead to an increase of bacteria at the cost of fungi in soil and thus, to increased bacterial activity, N mineralization and N availability (Chen et al 2001). However, this hypothetic N fertilization effect of Benomyl has already been excluded.

The assumption that fungicide effects on tissue N concentrations of *A. vulgaris* and *P. hieracioides* are due to reductions in % RLC by AMF is supported by significant positive correlations between leaf N and the frequency of arbuscules, the sites where nutrients are

transferred from fungi to plants, and subsequent regressions of leaf N on % arbuscules. The non-linearity of regression curves and relatively low R^2 values are probably based on the fact that there is also an opposite influence of plant N status on root colonization, and that plant N nutrition is not a function of arbuscule frequency alone.

In contrast to leaf N of *A. vulgaris* and *P. hieracioides*, shoot N of *P. compressa* was positively affected by fungicide application, suggesting that AMF had a negative influence on N nutrition of this species. These findings would be consistent with those of van der Heijden et al. (2006) which indicated that total N in the biomass of co-occurring plants in microcosms was not affected by AMF, whereas the distribution of N among species was.

An explanation for the opposite response of plant N in different species to a reduction of root colonization by AMF may be provided by possible differences in their mycorrhizal responsiveness. Small, poorly branched root systems and thick roots are more responsive to mycorrhizas, whereas extensive, strongly branched root systems and fine roots are better adapted to direct nutrient uptake and thus less responsive in terms of nutrient aquisition (Baylis 1975; Newsham et al. 1995). Wilson and Hartnett (1998) discovered a positive correlation between responsiveness and root colonization and further, they assumed perennial plants to be more reponsive than biennials or annuals, because they had to develop long term strategies for nutrient competition, like carbon allocation to mycorrhizas, which would apply to competitive strategists in general. Correspondingly, they found species adapted to disturbed sites (i.e. ruderal strategists) to be less responsive. Such differences in mycorrhizal responsiveness can influence competitive interactions within plant communities: more responsive species should benefit more from the presence of AMF than less responsive species, whereas the latter should be superior competitors for nutrients when none of the plants can benefit from mycorrhizas (Moora and Zobel 1996; Hartnett and Wilson 1999; Smith et al. 1999; Scheublin et al. 2007; Stein et al. 2009).

374 The same may apply in our study: *A. vulgaris* and *P. hieracioides* were characterized by less
375 extensive root systems compared to *P. compressa* (V. Blanke, personal observation) and
376 strongly colonized by AMF in untreated plots, which suggests that they were more responsive
377 to the fungi. Correspondingly, the two forb species had higher leaf N concentrations in
378 untreated than in fungicide-treated subplots, indicating that they were better able to forage for
379 this nutrient when fully mycorrhizal. Leaf N i appeared to be more closely linked with
380 % RLC by arbuscules in *A. vulgaris* than in *P. hieracioides*, suggesting a higher mycorrhizal
381 responsiveness in the former species. This may be due to the perennial life history of
382 *A. vulgaris*, which is classified by Grime et al. (2007) as a competitive C/CR strategist,
383 whereas the biennial P. hieracioides is a more ruderal R/CSR strategist. *P. compressa*, another
384 perennial that as an SR/CSR strategist includes both stress-tolerant and ruderal traits in its life
385 history and that possesses the most extensive root system of all four investigated species, was
386 significantly less colonized by AMF under control conditions than the three other species
387 (exact Friedmann-test for plants at untreated (-N-B) subplots; $\chi^2 = 15.8$, 15.0 and 13.8 for
388 internal mycelium, arbuscules and vesicles, respectively; in each case $P < 0.001$; followed by
389 Wilcoxon-tests for pairwise differences). This suggests a lower mycorrhizal responsiveness of
390 this species. Accordingly, shoot N of *P. compressa* was higher in fungicide-treated subplots,
391 indicating that this species may compete better for nitrogen in the absence of AMF.
392 It has also been proposed that nutrient transfer from plant to plant via mycorrhizal networks
393 (see e.g. Simard et al. 2002) is directed from less to more responsive species (van der Heijden
394 2002; Wilson et al. 2006). In this case, nitrogen might have been transferred from
395 *P. compressa* to more responsive species, such as *A. vulgaris* and *P. hieracioides*. Thus,
396 *P. compressa* may have benefited from a destruction of mycorrhizal networks by Benomyl
397 and retained more N. As in the two forb species, tissue N of *P. compressa* was significantly
398 correlated to % RLC by arbuscules – in this case negatively – and shoot N was a regression

399 function of arbuscule frequency, which might indicate that this species is indeed losing N via
400 mycorrhizal networks.
401 If, as in our field site, % RLC by AMF is reduced by N fertilization, species with a low
402 mycorrhizal responsiveness – which often are nitrophilic and tend to allocate relatively more
403 C above- instead of below-ground (Johnson et al. 2008) – should be at an advantage. In this
404 study, however, aboveground biomass of the presumably more responsive species *A. vulgaris*
405 and *P. hieracioides* was increased by N fertilization, whereas that of less responsive
406 *P. compressa* was decreased. Thus, although AM appeared to be important for N nutrition of
407 some plant species, they did not appear to be the driving force behind the observed shifts in
408 plant community composition following N addition, whose main effects on plant performance
409 appear to have been more direct.
410
411 *Bromus japonicus* was strongly colonized by AMF in spite of its fine, graminoid root system.
412 However, % RLC as well as tissue N did not respond to N fertilization, and there was no clear
413 response in plant N concentration to reduction of mycorrhizal root colonization by the
414 fungicide. This suggests that for *B. japonicus*, N limitation might not be the central reason for
415 investing in mycorrhizas, and that AMF do not contribute to N nutrition of this species at our
416 site. Evidence for a less important role of N limitation for the performance of *B. japonicus* is
417 also provided by the absence of a consistent N fertilization effect on aboveground biomass in
418 several years. This may be due to the recurring need of this annual, ruderal (R/CR-strategist,
419 K. Stephan, unpublished data, according to Hodgson et al. 1999) species to establish from
420 seed, with establishment success likely depending on factors other than nitrogen availability,
421 such as drought stress, tolerance of which may be increased by mycorrhizal root colonization
422 (Al-Karaki 1998).
423

Soil analyses confirmed that P was still extremely high in 2004, with amounts between 70 and 80 g kg^{-1} soil. N was still rather low, and it was slightly increased by N fertilization, while a large part of added N seemed to have been directly taken up by plants.

When compared to soil P and standard values in literature, plant P concentrations in our study were not particularly high, ranging from ca. 2100 to 4000 ppm in leaves of the two forbs and from 1000 to 1800 ppm in shoots of the two grasses (Marschner 2002). An explanation for this could be that plants may not take up much P under N-deficient conditions to avoid an overly unbalanced N:P ratio. This would be in line with observations by Smith (1962), showing that critical P concentrations in leaves drop with decreasing N concentrations; and also with Tilman's resource ratio model (e.g. Tilman 1982), suggesting that plants take up nutrients in proportions required, irrespective of the supply ratio.

Main treatment effects on tissue P were only obvious in *P. compressa*: P concentrations increased in response to fungicide application and decreased after N-addition. Elevated plant P through reduced % RLC may be due to the same mechanisms as hypothesized for N, although the correlation between shoot P and arbuscule frequency was not significant, and it is surprising that plant P levels in this P-rich soil were affected at all. We do not have an explanation for the decrease in shoot P of *P. compressa* following N fertilization.

Conclusions

Results of our study indicate that arbuscular mycorrhizas can indeed improve plant N nutrition in the field, and suggest that, under conditions where N is more limiting to plant growth than P, plant N status can in turn feed back on root colonization by AMF. At the same time it is shown that these findings cannot be generalized for all species, as the fungicide treatment had both positive and negative effects on plant N concentration. These species-specific differences might be due to variations in mycorrhizal responsiveness, with more

450 responsive species benefiting from high percentages of mycorrhizal root colonization, and
451 less responsive species being more successful when AMF abundance is reduced. However,
452 reduced root colonization following N fertilization at best appeared to have played only a
453 negligible role in the observed shifts in plant community composition.
454
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Figure legends

Fig. 1 Percentage of root length colonized (% RLC) by arbuscular mycorrhizal fungi (AMF) for (a) *Artemisia vulgaris*, (b) *Picris hieracioides*, (c) *Poa compressa* and (d) *Bromus japonicus* in the different treatment combinations (averaged across blocks). % RLC is illustrated separately for internal mycelium, arbuscules and vesicles; means (n = 6) and standard errors of the mean shown. +N = N-fertilized plots, -N = unfertilized plots, +B = fungicide (Benomyl) treated subplots, -B = untreated subplots

Fig. 2 Nitrogen concentration in leaves of (a) *Artemisia vulgaris* and (b) *Picris hieracioides*, and in shoots of (c) *Poa compressa* and (d) *Bromus japonicus* in the different treatment combinations (averaged across blocks). Means (n = 6) and standard errors of the mean shown. +N = N-fertilized plots, -N = unfertilized plots, +B = fungicide (Benomyl) treated subplots, -B = untreated subplots

Fig. 3 Tissue N and P concentrations, significantly affected by Benomyl, plotted against the percentage of root length colonized (% RLC) by arbuscules in the respective plant species across all treatments. Spearman's rho correlation coefficients (ρ) and sample size (n) are given, plus regression curves (in case of a significant correlation, % RLC by arbuscules as independent variable and leaf or shoot N as dependent variable), R^2 and ANOVA F-statisctics testing for regression model significance. Asterisks indicate significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

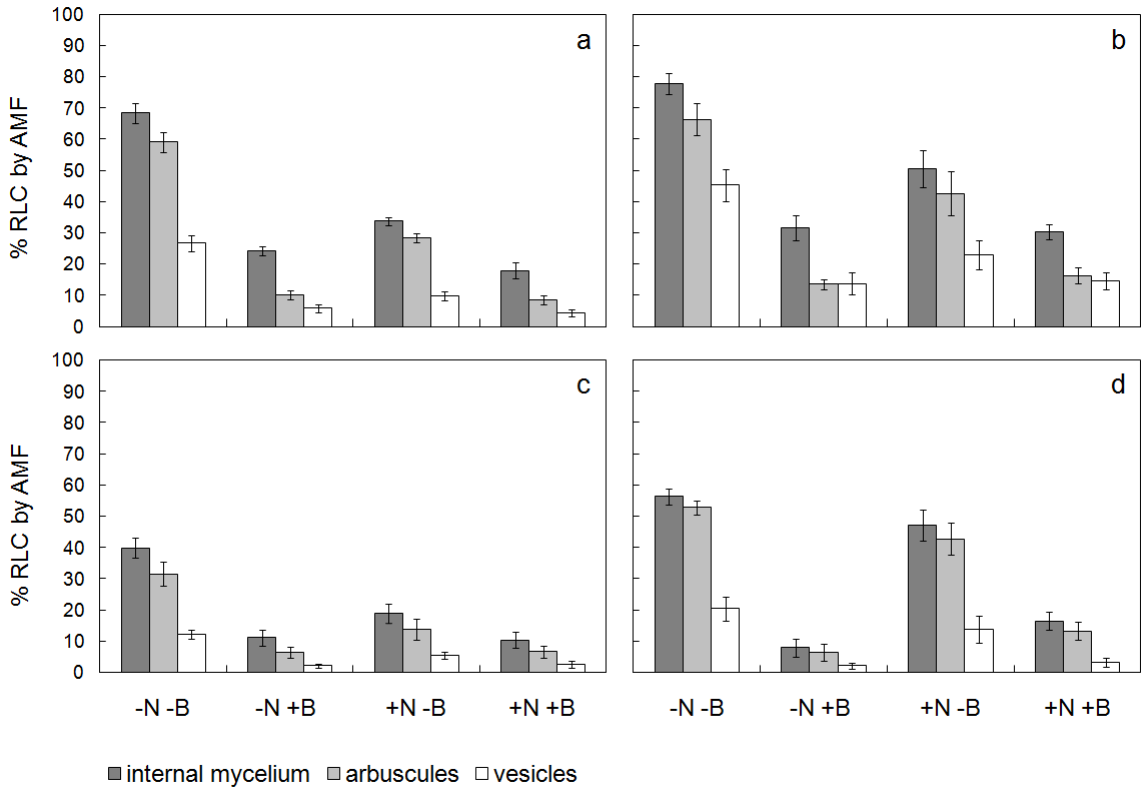

Fig. 1

(created with MS excel and Irfan View)

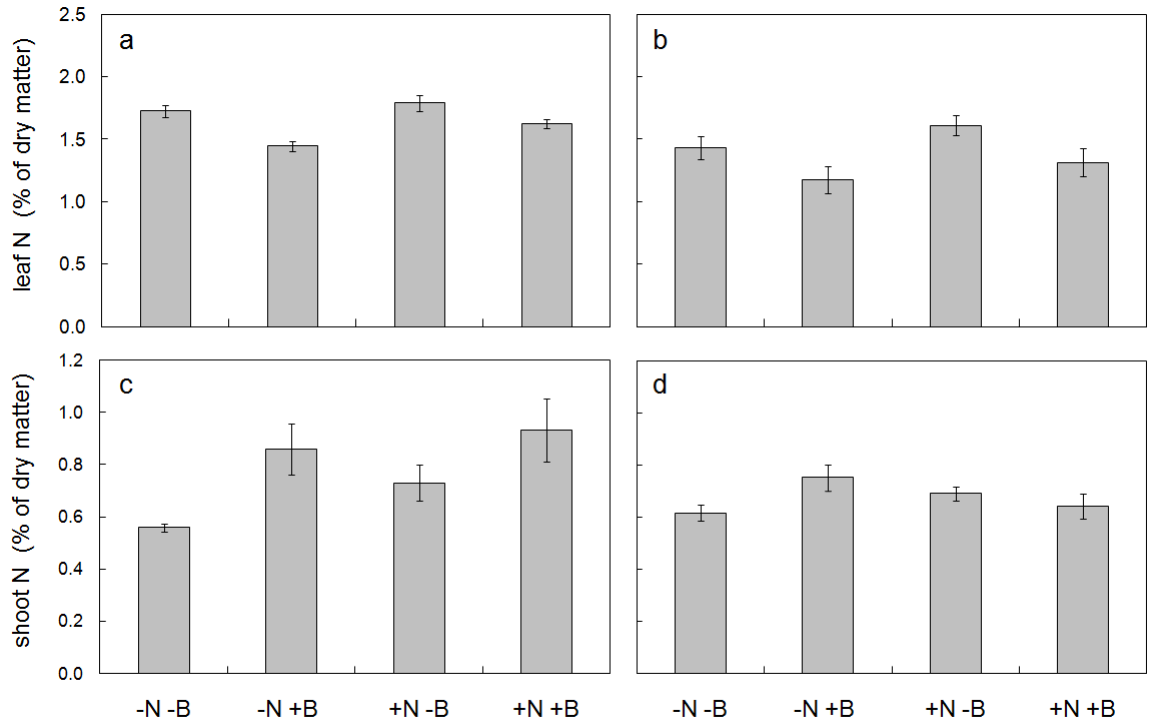

Fig. 2

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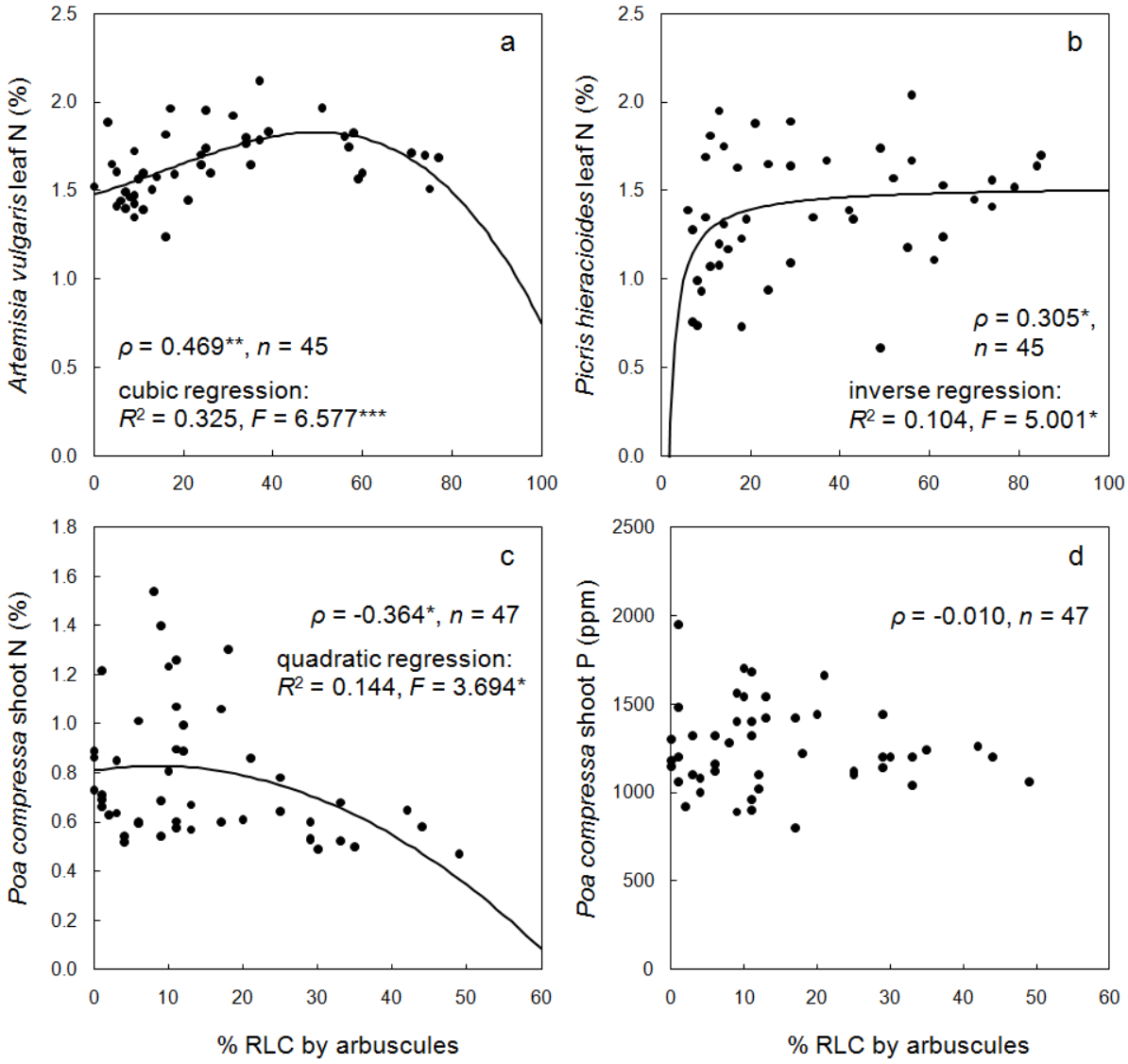

Fig. 3

(created with MS excel and Irfan View)

Table 1 Statistical results for fertilizer and fungicide effects on percentages of root length colonized by arbuscular mycorrhizal fungi and on nitrogen concentrations of *Artemisia vulgaris*, *Picris hieracioides*, *Poa compressa* and *Bromus japonicus* (depicted in Figs. 1 and 2)

F-statistics	internal mycelium (%)	arbuscules (%)	vesicles (%)	leaf N (%)
Artemisia vulgaris				
n	54.6 ***	35.5 **	44.3 **	7.3 *
b	133.0 ***	316.3 ***	92.0 ***	25.5 ***
n:b	15.7 **	27.3 ***	31.7 ***	1.7
Picris hieracioides				
n	14.8 *	4.2	8.7 *	3.2
b	79.4 ***	103.6 ***	29.9 ***	10.2 **
n:b	12.3 **	9.1 *	10.2 **	0.1
				shoot N (%)
Poa compressa				
n	14.2 *	9.7 *	9.0 *	3.5
b	42.2 ***	32.1 ***	39.8 ***	15.0 **
n:b	12.3 **	9.9 *	11.5 **	0.6
Bromus japonicus				
n	0.01	0.3	0.4	0.4
b	155.2 ***	146.5 ***	28.3 ***	1.8
n:b	7.9 *	7.3 *	1.7	7.6 *

ANOVA *F*-statistics following LMEs were used to test for significances of model terms, which are given as follows: n = N-fertilization factor, b = Benomyl application factor, n:b = interaction factor. Asterisks indicate significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 2 Tissue P concentrations and N:P-ratios of *Artemisia vulgaris, Picris hieracioides*, *Poa compressa* and *Bromus japonicus* for the different treatment combinations (averaged across blocks)

tissue elements		-N-B mean	SEM	-N+B mean	SEM	+N-B mean	SEM	+N+B mean	SEM	n *F* =	b *F* =	n:b *F* =
Artemisia vulgaris leaves												
P	(ppm)	2758	± 219	2129	± 101	2271	± 93	2604	± 231	0.001	1.1	11.8 **
N:P		6.52	± 0.48	6.83	± 0.31	8.05	± 0.42	6.56	± 0.45	1.6	3.6	8.5 *
Picris hieracioides leaves												
P	(ppm)	4063	± 300	3483	± 570	3381	± 197	3119	± 305	2.3	2.2	0.3
N:P		3.62	± 0.23	3.62	± 0.33	4.94	± 0.40	4.50	± 0.43	22.5 **	0.9	0.9
Poa compressa shoots												
P	(ppm)	1230	± 48	1405	± 68	1083	± 72	1310	± 55	6.8 *	18.7 **	0.3
N:P		4.57	± 0.10	6.12	± 0.57	6.82	± 0.41	7.13	± 0.94	11.7 *	3.8	1.7
Bromus japonicus shoots												
P	(ppm)	1563	± 54	1584	± 97	1790	± 124	1599	± 137	0.9	1.0	1.5
N:P		4.03	± 0.14	4.90	± 0.49	4.08	± 0.32	4.34	± 0.29	0.4	2.9	0.1

Element concentrations were determined for leaves of *A. vugaris* and *P. hieracioides* and for shoots of *P. compressa* and *B. japonicus.* Means (n = 6) and standard errors of the mean (SEM) shown. +N = N-fertilized plots, -N = unfertilized plots, +B = fungicide (Benomyl) treated subplots, -B = untreated subplots. ANOVA F-statistics following LMEs were used to test for significances of model terms, which are given as follows:

n = N-fertilization factor, b = Benomyl application factor, n:b = interaction factor. Asterisks indicate significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3 Soil data for the different treatment combinations (averaged across blocks). Parameters include total P and N and soil acidity (pH)

soil parameters		-N-B mean	SEM	-N+B mean	SEM	+N-B mean	SEM	+N+B mean	SEM	n *F* =	b *F* =	n:b *F* =
P	(g kg^{-1})	73.1	± 6.8	80.1	± 9.4	78.2	± 7.3	78.5	± 6.6	0.1	0.5	0.4
N	(%)	0.19	± 0.011	0.17	± 0.007	0.20	± 0.007	0.20	± 0.008	8.4 *	3.3	3.3
pH		7.5	± 0.12	7.6	± 0.13	7.5	± 0.13	7.5	± 0.13	0.01	4.7	0.9

Means ($n = 6$) and standard errors of the mean (SEM) shown. +N = N-fertilized plots, -N = unfertilized plots, +B = fungicide (Benomyl) treated subplots, -B = untreated subplots. ANOVA *F*-statistics following LMEs were used to test for significances of model terms, which are given as follows: n = N-fertilization factor, b = Benomyl application factor, n:b = interaction factor. Asterisks indicate significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 4 Aboveground biomass data for *Artemisia vulgaris*, *Picris hieracioides*, *Poa compressa* and *Bromus japonicus* from 2000 to 2003 averaged across blocks

biomass (g m^{-2})		2000		2001		2002		2003		n	time	n:time
		-N	+N	-N	+N	-N	+N	-N	+N	*F* =	*F* =	*F* =
Artemisia vulgaris	mean	14.8	18.3	7.1	102.4	7.9	36.1	3.5	19.3	6.2 *	1.3	4.8 **
	± SEM	± 8.1	± 13.4	± 4.3	± 34.4	± 5.4	± 22.5	± 2.7	± 8.0			
Picris hieracioides	mean	51.1	94.8	63.0	95.8	45.9	39.3	17.7	64.1	370 ***	4.0 *	6.1 **
	± SEM	± 14.1	± 28.3	± 14.4	± 21.6	± 8.6	± 9.3	± 4. 0	± 21.3			
Poa compressa	mean	11.6	8.5	35.3	11.6	32.6	13.0	17.1	5.8	9.8 *	2.9	0.6
	± SEM	± 6.6	± 7.5	± 14.3	± 5.3	± 14.0	± 6.5	± 10.3	± 2.9			
Bromus japonicus	mean	2.5	0.5	15.4	15.4	7.7	129.1	3.1	11.6	0.1	41.4 ***	16.8 ***
	± SEM	± 1.3	± 0.2	± 8.6	± 7.8	± 2.4	± 33.1	± 1.5	± 4.6			

Means (n = 6) and standard errors of the mean (SEM) shown. +N = N-fertilized plots, -N = unfertilized plots. ANOVA F-statistics following LMEs were used to test for significances of model terms, which are given as follows: n = N-fertilization factor, time = time factor, n:time = interaction factor. Asterisks indicate significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$