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Differential benefits of arbuscular mycorrhizal and ectomycorrhizal infection of *Salix repens*

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Abstract The functional significance of arbuscular mycorrhiza (AM) and ectomycorrhiza (EcM) for *Salix repens*, a dual mycorrhizal plant, was investigated over three harvest periods (12, 20 and 30 weeks). Cuttings of *S. repens* were collected in December (low shoot P) and March (high shoot P). *Glomus mosseae* (an arbuscular mycorrhizal fungus, AMF) resulted in low AM colonization (<5%), but showed large short-term (<12 weeks) effects on shoot growth and root length. *Hebeloma leucosarx* (an ectomycorrhizal fungus, EcMF) resulted in high EcM colonization (70%), but benefits occurred over a longer term (>12 weeks). Furthermore, *G. mosseae* colonization resulted in higher shoot P uptake, shoot growth, root growth and response duration for *S. repens* collected in December than for those collected in March, whereas with *H. leucosarx* and the non-mycorrhizal treatment there were no differences between cuttings collected on different dates. Low AMF colonization was effective in the short term for cuttings at both collecting dates. Low AMF colonization of *S. repens* occurred irrespective of the amount of AMF inoculum used. The intensities and relative amounts of AMF structures in *S. repens* and *Trifolium repens* were compared over three harvest periods (12, 20 and 30 weeks) to assess plant species effects on AM colonization patterns.

Keywords Arbuscular mycorrhiza · Ectomycorrhiza · *Salix repens* · Internal P-status

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Introduction

Mycorrhizal fungi form mutualistic associations with plant roots. The two most widespread types are arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EcMF). Even though most plants form only one type of mycorrhiza, some species of *Alnus* (Molina et al. 1994), *Populus* (Lodge 1989; Lodge and Wentworth 1990), *Salix* (Lodge 1989; Dhillion 1994) and *Eucalyptus* (Lapeyrie and Chilvers 1985; Adjoud-Sadadou and Halli-Hargas 2000) form both arbuscular mycorrhiza (AM) and ectomycorrhiza (EcM). AM structures have also been observed in supposed EcM tree genera, i.e. *Abies* (Cázares and Trappe 1993), *Pseudotsuga* and *Tsuga* (Cázares and Smith 1996), indicating that dual mycorrhiza may be a common but overlooked phenomenon.

Salix repens L. is one of these so-called dual mycorrhizal plant species and is a common and widespread shrub in a great variety of Western European plant communities, in particular in the coastal dunes of the Wadden Isles. Its habitats range from dry to wet and from calcareous, humus-poor to acidic, humus-rich soils. In addition, these habitats are strongly influenced by wind and water level fluctuations. As a dual mycorrhizal plant *S. repens* may have a selective advantage in these highly dynamic ecosystems.

The gradient from the drift line to the stabilized back-dunes is characterized by decreasing pH and increasing soil organic matter content. Read (1989) has discussed the importance of mycorrhizas in the sand dune ecosystem. As the relative availability of nitrogen and phosphorus changes with succession, the occurrence of both mycorrhizal types may also change. Read (1989) proposed that AMF will predominate in the dry foredunes, where phosphorus is the main growth-limiting nutrient. In contrast, EcMF will prevail in the dune slacks due to their ability to take up nitrogen. The different dune successional stages, therefore, should be characterized not only by a typical nutrient status but also by a dominant mycorrhizal type. Contrary to this

hypothesis, *S. repens* was consistently found to be slightly AM and highly EcM in 16 field sites comprising successionally different stages of *S. repens* communities on the Dutch Wadden Isles (van der Heijden and Vosatka 1999).

The present study focusses on differences in functional significance of AM versus EcM for *S. repens* during a growing season. Two fungi were selected representing one species of each functional type, i.e. *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (AMF) and *Hebeloma leucosarx* P.D. Orton (EcMF). The experiment was performed under very nutrient-poor conditions (similar to a primary dune successional stage). Previous field observations indicated that cuttings collected in spring had a higher internal P concentration than cuttings collected in autumn and winter (van der Heijden and Vosatka 1999). March cuttings (N/P ratio = 5) are N limited and P sufficient, whereas December cuttings (N/P ratio = 13) are N sufficient and P limited (Verhoeven et al. 1996). Therefore, the experiment was performed with cuttings from both sampling dates. The objectives of this study were (1) to investigate whether *H. leucosarx* and *G. mosseae* have different effects on the performance of *S. repens,* (2) to determine whether these effects show different dynamics over time (during a growing season), (3) to assess whether *H. leucosarx* and *G. mosseae* affect plant parameters (survival, shoot biomass, N and P uptake, root length) differently, and (4) to investigate how internal shoot P concentration could affect the outcome of the mycorrhiza effect.

Difficulties arise when comparing the significance of AM and EcM. AM inocula are supplied as roots containing the fungus whereas EcM inocula are supplied as mycelial agar plugs. *G. mosseae* previously induced much faster responses in *S. repens* than *H. leucosarx* (van der Heijden 2000). To investigate whether a nutritional effect (caused by supplying mycorrhizal clover roots) might be responsible for the fast short-term effect in *S. repens*, an experiment was set up using different amounts of AMF inoculum. The ability of the AMF to form mycorrhizas with *S. repens* and with a well-known test plant, *Trifolium repens* L., was also compared.

Materials and methods

Plant and fungal material

Cuttings of (male) *S. repens* (twig ends) were collected in March 1996 (experiment 1) and December 1996 (experiments 1 and 2) from the wet, calcareous field site "Schoenus I" on the isle of Terschelling (53 23′50"N, 5 13′45" E). All cuttings were collected from the same plant in order to obtain genetic homogeneity.

H. leucosarx (strain L1, voucher specimen preserved at WAG.), collected in the autumn of 1994 from *S. repens* on Terschelling, was used in this experiment. Sporocarps were surface sterilized with alcohol (70%) and sliced in half under sterile conditions. Fungal tissue was cut from the innerside of the cap and maintained on solid alternative Melin Norkrans (AMN) medium containing (in $g \times l^{-1}$) agar (15), malt extract (3), glucose$\cdot H_2O$ (10), $(NH_4)_2HPO_4$ (0.25), KH_2PO_4 (0.5), KNO_3 (0.5), $MgSO_4 \cdot 7H_2O$ (0.15), $CaCl_2 \cdot H_2O$ (0.67), NaCl (0.025), $FeCl_3$ (0.0012) and thiamine × HCl (100 μg). As *G. mosseae* was identified in these dune sites (van der Heijden and Vosatka 1999), this fungus (BEG 12) (kindly provided by the European Bank of Glomales) was used and was maintained on *T. repens* in a sandy soil with P-poor Hoagland solution containing (in $mg \times l^{-1}$) $Ca(NO_3)_2 \cdot 4H_2O$ (590), KNO_3 (253), $MgSO_4 \cdot 7H_2O$ (246), KH_2PO_4 (7.8), $FeCl_3 \cdot 6H_2O$ (1.5), H_3BO_3 (0.610), $MnSO_4$ (0.234), $CuSO_4$ (0.055), $ZnSO_4$ (0.055), $Al_2(SO_4)_3$ (0.055), H_2MoO_4 (0.028), KI (0.028) and KB_2 (0.028).

Inoculation and plant growth conditions

For both experiment 1 and 2, the cuttings of *S. repens* were trimmed to 4 cm after storage at 4 C for 1 month, surface-sterilized twice in freshly prepared 6% H_2O_2 for 1 min (in series of 20 in 300 ml), and rinsed three times in fresh demineralized water (1 l) for 10 min. Each cutting was placed individually in a culture tube containing 20 ml sterile water agar (1%). After a 10-week rooting period in a climatized chamber (photon flux density: $120\ \mu E \times m^{-2}.s^{-1}$, 16-h day at 20 C and 8-h night at 16 C, relative air humidity 70%), equally developed cuttings were selected for the experiment.

The isolate of *H. leucosarx* was precultured twice (successively) for 3 weeks on solid media in order to obtain sufficient actively growing fungal material. *G. mosseae* was precultured on roots of *T. repens* for 15 weeks (inoculum density: roots and soil with AMF to sterilized soil, 10% v/v). The substrate used was a sand-perlite mixture 1:3 (v/v). For logistical reasons, it was not possible to collect soil from the field sites and these experiments were performed on sand with a soil chemical composition similar to the younger successional stages. The sand pH (measured in 0.01 M $CaCl_2$; Houba et al. 1990) was 5.8, nutrient contents were 70 mg N kg^{-1}, 20 mg P kg^{-1}, and 0.17% organic matter. No nutrient solution was supplied during the experiment. Demineralized water was added to the substrate (1:3:1 v/v/v sand:perlite:water) 24 h prior to autoclaving. The substrate was autoclaved twice (1 h, 121 C, 1 atm) with a 48-h interval and left for 1 week. Root growth chambers (vertically placed Petri dishes of 15 cm diameter with a slit in the edge) were filled with the sterile substrate. Each root growth chamber contained 300 ml substrate, of which 75 ml (ca. 120 g) was sand. The cuttings of *S. repens* were transferred to the root growth chambers (1 cutting per growth chamber), each cutting was inoculated with five mycelial plugs cut from the edge of a precultured EcM fungal colony, and the root system was covered with a water agar (1%) layer (5 cm in diameter) to prevent excessive loss of water from the roots. The root growth chambers were sealed with tape and sterilized anhydrous lanolin to prevent contamination and loss of water. The control plants were supplied with five plugs (0.15 ml) of solid medium without fungus. Inoculation of the AMF was performed by mixing 33.3 g freshly washed root material of *T. repens* containing *G. mosseae* with the sterile substrate (16.6 l). Cuttings were transferred to the root growth chambers, the root systems were covered with water agar, and the growth chambers were closed and sealed. As a previous experiment with addition of roots of *T. repens* without AMF to the control plants showed no effect on plant performance, no attempt was made to provide control plants with roots of *T. repens* without AMF. All soil compartments were shielded from daylight, i.e. the root growth chambers were wrapped in aluminium foil, placed vertically in transient propagators (relative air humidity almost 100% in the first week) and placed in the climate chamber. Growth conditions were photon flux density: $350\ \mu E \times m^{-2} \times s^{-1}$, 16-h day at 20 C, and 8-h night at 16 C and 60% relative air humidity.

Experiment 1: Significance of AMF and EcMF

The experiment was set up as a complete randomized block design with two factors. One factor, harvest, contained four levels,

and the second factor, fungal treatment, contained three levels. A third factor, collection date of cuttings, contained two levels. Cuttings collected in March 1996 (high P; part 1) and December 1996 (low P; part 2) were compared. Each treatment was replicated 10 times. For both collecting dates, the inoculations with *G. mosseae* and *H. leucosarx* were performed in 50 replicates, and 60 plants were supplied with non-inoculated agar plugs serving as a control for *S. repens.* After 12 weeks ($t=12$), 10 plants of each combination were harvested randomly. The experiment was continued in 2-lL pots. These pots contained 1800 ml substrate of which 450 ml (ca. 720 g) was sand. Randomly chosen specimens (25) of the remaining *S. repens* inoculated with *G. mosseae* and *H. leucosarx* and all remaining 20 specimens of non-mycorrhizal *S. repens* (control) were individually transferred to these 2-l pots. Plants were watered sufficiently over the 30-week period. At $t=20$ and 30, 10 plants were again harvested randomly.

Experiment 2: Different amounts of AM inoculum

The experiment was set up in a complete randomized design with two factors. One factor, AMF inoculum amount, contained three levels, and the second factor, harvest, contained four levels, making a total of 12 treatment combinations. *S. repens* was inoculated with different amounts of clover roots colonized by *G. mosseae*, i.e. 0.67, 1.34 and 3.35 g, in 45 replicates of each amount of inoculum. After 12 weeks, 10 plants of each combination were harvested randomly. The experiment was continued in 2-l pots, as in experiment 1. Randomly, 25 replicates (+ inoculum) were individually transferred to these pots. Plants were watered sufficiently over the 30-week period. Ten plants were harvested randomly at $t=20$ and 30. In addition, AMF inoculum viability and colonization in *T. repens* was examined when grown on the substrate containing the lowest content of inoculum colonized by *G. mosseae* (conditions as described above), and harvested after 12, 20 and 30 weeks.

Plant performance, EcM and AMF colonization, soil analysis

Length of shoots, number of shoots, and number of leaves were determined every 3 weeks. Shoot weight was determined after 72 h at 70 C at the final harvest. The plants were individually digested in sulphuric acid, salicylic acid and 30% hydrogen peroxide (Novozamsky et al. 1988). Total N and P were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard (Novozamsky et al. 1988).

The roots were immersed in water over a 2-mm sieve to remove most of the soil and rinsed gently to avoid damaging mycorrhizas. The cleaned ectomycorrhizal roots were stored in glutaraldehyde buffer (Alexander and Bigg 1981) and roots of the control plants and the AM plants were stored in 50% alcohol until they could be processed. For each sample, root length was determined according to Newman (1966) and the EcM root tips and total root tips were counted. Root biomass was not determined, since different EcM-, AM- or non mycorrhizal root systems contain different (not removable) proportions of sand (van der Heijden 2000). EcM frequencies were calculated (number of EcM root tips/total number of root tips as percent). EcM root length was measured according to Giovannetti and Mosse (1980). All root samples that had been inoculated with AMF were cleared with 10% KOH for 3 h in a water bath at 90 C, bleached in 10% H_2O_2 for 1 h, acidified in 1% HCl for 15 min and stained with trypan blue (Phillips and Hayman 1970) in lactophenol for 30 min. AMF colonization was estimated by a modified line intersect method (McGonigle et al. 1990), where a minimum of 100 line intersections per root sample (replicated three times per sample) were scored for the presence of AMF structures. AMF root length colonization (RLC) and root length colonization intensity (RLCI) (RLC × percentage of the root cross section covered by AMF structures) were calculated. Hyphal length of the extramatrical mycelium was not determined because of the presence of different amounts of hyphae of non-mycorrhizal fungi, which cannot always be separated unambiguously from hyphae of mycorrhizal fungi.

At $t=0$, and after 12, 20 and 30 weeks, plant-available nutrients in the soil were analysed using the $CaCl_2$ method described by Houba et al. (1990). $N_{dissolved}$ and $P_{dissolved}$ were analysed in 0.01 M $CaCl_2$ extracts. The $CaCl_2$ extracts were analysed spectrophotometrically with a continuous flow analyser using samples with known chemical composition as standard.

Statistical analysis

For the experiments set up in a complete randomized block design, the position of both the blocks and the pots (root growth chambers) within these blocks were randomized every 3 weeks. Significant block effects did not occur for any of the variables and no correction for block effect was made. Data were analysed by Analysis of Variance using the statistical package STATISTICA (StatSoft). Growth measurements were analysed using repeated measures. Prior to analysis, proportional mycorrhizal colonization values were arcsine square root transformed; all other data were logarithmically transformed. Bartlett's test (Sokal and Rohlf

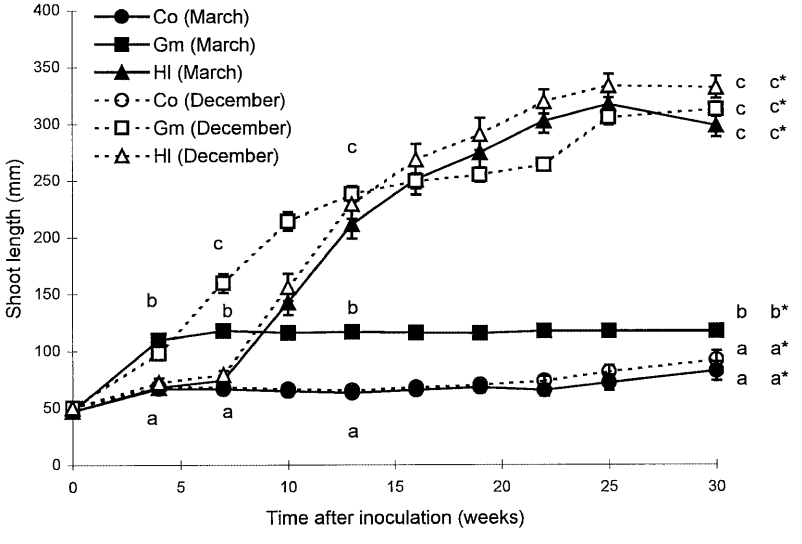

Fig. 1 Mean shoot length after 30 weeks of *Salix repens* (March 1996: *continuous line,* December 1996: *dotted line*) inoculated with *Glomus mosseae* or *Hebeloma leucosarx,* or not inoculated (control). *Bars* represent standard error. Different letters next to points at 4, 7, 10, 13 and 30 weeks after inoculation indicate significantly different shoot length, and different letters and asterisks indicate significantly different growth rates over 30 weeks between mycorrhizal treatments ($P<0.05$) according to LSD tests

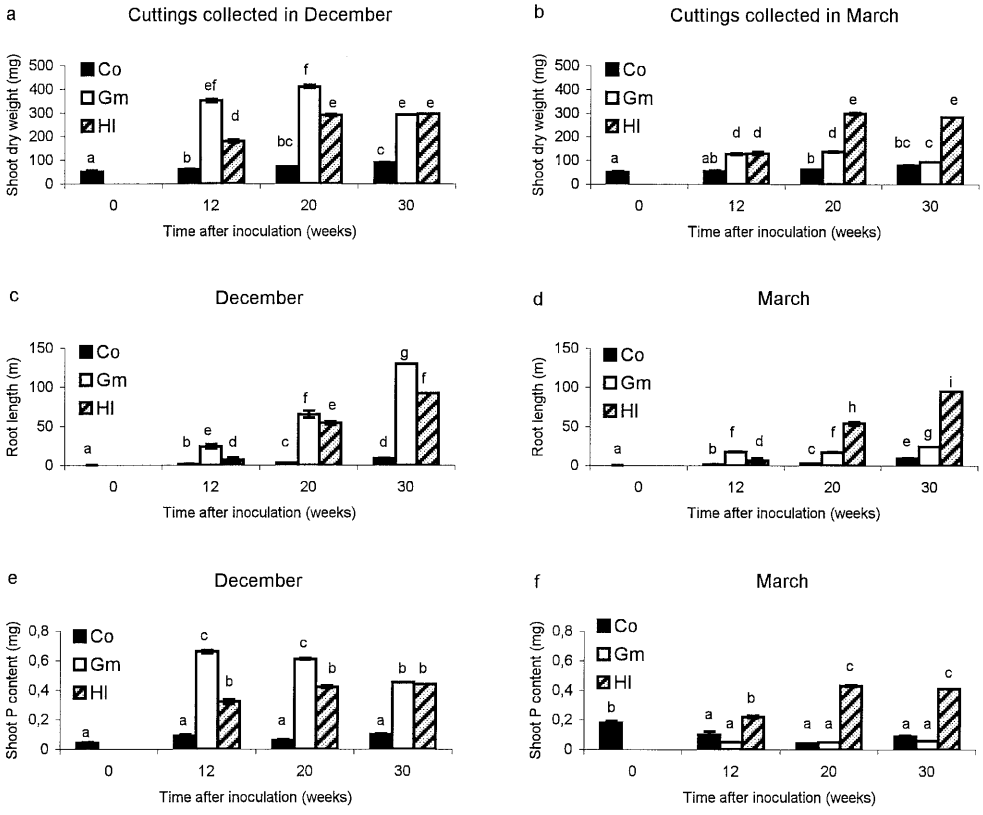

Fig. 2 Mean (**a, b**) shoot dry weight, (**c, d**) root length and (**e, f**) shoot P content of *S. repens* (collected in March and December 1996) inoculated with *G. mosseae* or *H. leucosarx,* or not inoculated (control) and harvested after 0, 12, 20 and 30 weeks. *Bars* represent standard error. Significant differences are indicated by different letters ($P<0.05$) according to LSD tests

1995) was used to determine whether variances were equal between treatments. Differences among means were evaluated with the LSD test (Sokal and Rohlf 1995). Correlations between plant parameters were tested using Spearman's rank tests (Siegel and Castellan 1988).

Results

Experiment 1: Significance of AMF and EcMF

In both experiments, only 50% of the control plants survived, compared with 90% of the mycorrhizal plants (data not shown), irrespective of the mycorrhizal type involved.

All parameters measured were significantly affected by fungus, harvest time and collecting date, i.e. time when cuttings were collected (Figs. 1, 2; Tables 1, 2). Growth responses of *S. repens* inoculated with *G. mosseae* showed large differences with respect to the collecting date of cuttings (Fig. 1), whereas *S. repens* inoculated with *H. leucosarx* and the control plants were not affected by the collecting date. Comparisons (planned contrasts) between collecting dates for plant parameters showed that the non-mycorrhizal plants differed only at $t=0$. Similarly, no differences occurred (plant parameters and mycorrhizal colonization) between collecting dates for plants inoculated with *H. leucosarx*. However, for *G. mosseae*-inoculated plants, all plant parameters were significantly different between March and December at $t=12$, 20 and 30 (Figs. 1, 2, Tables 1, 2). AM colonization (%) was different at t=20 and 30, while AM root length differed at all harvest periods.

Shoot lengths and dry weights of cuttings collected in March were generally higher for plants inoculated with *G. mosseae* than for the control, but those inoculated with *H. leucosarx* performed still better. Cuttings collected in December responded better to *G. mosseae* than to *H. leucosarx* in the short term, whereas in the long term *G. mosseae* or *H. leucosarx* showed equal benefits in terms of shoot length and dry weight (Table 1, Fig. 2a, b). Root length for both March and December cuttings responded better to *G. mosseae* in the short term, whereas in the long term *H. leucosarx* had best effects on cuttings collected in March and *G. mosseae* had best effects on cuttings collected in December (Table 1, Fig. 2c, d). For both March and December cuttings, mycorrhizal colonization by *H. leucosarx* decreased after the first harvest period. Colonization by *G. mosseae* did not change significantly over time, but at t=20 and 30 colonization of cuttings collected in December was lower than those collected in March. In contrast, AM root length was lower for cuttings collected in March than those collected in December (Table 1).

At $t=0$, shoot P concentration differed between cuttings collected in March and December ($2.0\ g\cdot kg^{-1}$ and $0.9\ g\cdot kg^{-1}$, respectively), whereas shoot N concentration was similar. Shoot N and P concentrations of cut-

Table 1 Shoot length, shoot dry weight, root length, mycorrhizal colonization, and mycorrhizal root length (shown as means ± 1 standard error with $n \geq 10$) of *Salix repens* (collected in March 1996 and December 1996) inoculated with *Glomus mosseae, Hebeloma leucosarx* or not inoculated (control) and grown for 30 weeks. Values within a column followed by a different letter differ at $P < 0.05$ according to an LSD test following a two-factor ANOVA (*df*: fungus 2, harvest 3, *df* Error 125 or *df*: fungus 1, harvest 2, *df* Error 71)

Fungal treatment and harvest time	December					March				
	Shoot length (mm)	Shoot dry weight (mg)	Root length (m)	Mycorrhizal colonization (%)	Mycorrhizal root length (m)	Shoot length (mm)	Shoot dry weight (mg)	Root length (m)	Mycorrhizal colonization (%)	Mycorrhizal root length (m)
Control ($t=0$)	50.0 ± 1.7 a	51.1 ± 9.2 a	0.03 ± 0.00 a	0 a	0 a	47.0 ± 2.5 a	50.2 ± 8.7 a	0.03 ± 0.00 a	0 a	0 a
Control ($t=12$)	64.9 ± 11.0 b	59.1 ± 3.8 b	1.31 ± 0.29 b	0 a	0 a	50.7 ± 9.5 b	54.1 ± 7.9 ab	1.36 ± 0.31 b	0 a	0 a
G. mosseae ($t=12$)	238.7 ± 9.3 d	350.6 ± 11.1 ef	24.49 ± 2.11 e	3.1 ± 0.2 b	0.76 ± 0.11 b	110.9 ± 8.7 e	127.6 ± 12.5 d	17.51 ± 2.38 f	3.2 ± 0.3 b	0.57 ± 0.10 b
H. leucosarx ($t=12$)	209.3 ± 15.6 d	179.9 ± 10.4 d	6.68 ± 1.32 d	75.4 ± 2.3 e	5.04 ± 0.76 e	166.6 ± 21.7 f	129.9 ± 18.4 d	6.18 ± 1.01 d	70.9 ± 3.3 d	4.57 ± 0.87 d
Control ($t=20$)	68.9 ± 1.9 c	71.3 ± 1.9 bc	2.87 ± 0.23 c	0 a	0 a	62.2 ± 2.5 bc	61.3 ± 2.0 b	2.90 ± 0.13 c	0 a	0 a
G. mosseae ($t=20$)	295.4 ± 16.4 e	410.2 ± 13.2 f	65.1 ± 5.1 f	2.3 ± 0.1 b	1.50 ± 0.09 c	105.2 ± 8.3 de	136.8 ± 7.8 d	16.86 ± 0.75 f	3.4 ± 0.1 b	0.58 ± 0.04 b
H. leucosarx ($t=20$)	291.1 ± 15.4 e	290.1 ± 11.7 e	54.62 ± 5.64 e	59.1 ± 3.8 c	32.28 ± 3.1 f	284.5 ± 19.3 g	297.3 ± 14.3 e	53.73 ± 5.75 h	53.4 ± 2.6 c	28.67 ± 2.8 e
Control ($t=30$)	91.5 ± 5.2 c	88.5 ± 3.6 c	8.71 ± 0.81 d	0 a	0 a	89.6 ± 8.4 cd	78.5 ± 6.0 bc	9.30 ± 0.90 e	0 a	0 a
G. mosseae ($t=30$)	313.6 ± 12.1 e	292.3 ± 12.8 e	130.11 ± 8.51 g	2.5 ± 0.9 b	3.25 ± 0.17 d	107.1 ± 3.5 e	94.9 ± 3.2 c	24.27 ± 0.60 g	4.3 ± 0.4 b	1.05 ± 0.08 c
H. leucosarx ($t=30$)	323.1 ± 19.3 e	295.2 ± 7.9 e	92.32 ± 3.97 f	66.9 ± 2.1 d	61.76 ± 2.2 g	292.1 ± 10.0 g	282.9 ± 9.2 e	95.14 ± 4.00 i	64.6 ± 1.1 c	61.45 ± 2.8 f
Fungus[a]	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001
Harvest[a]	≪0.001	≪0.001	≪0.001	0.009	≪0.001	≪0.001	≪0.001	≪0.001	0.008	≪0.001
Fungus × Harvest[a]	≪0.001	≪0.001	≪0.001	0.003	≪0.001	≪0.001	≪0.001	≪0.001	0.004	≪0.001

[a] ANOVA (*P* value)

Table 2 Shoot N concentration, shoot P concentration, total shoot N content, total shoot P content and shoot N/P ratio (shown as means ± 1 standard error with $n \geq 10$) of *S. repens* (collected in March and December 1996) inoculated with *G. mosseae, H. leucosarx* or not inoculated (control) and grown for 30 weeks. Values within a column followed by a different letter differ at $P < 0.05$ according to an LSD test following a two-factor ANOVA (*df* : fungus 2, harvest 3, *df* Error 125)

Fungal treatment and harvest time	December					March				
	N (g/kg)	P (g/kg)	N total (mg)	P total (mg)	N/P ratio	N (g/kg)	P (g/kg)	N total (mg)	P total (mg)	N/P ratio
Control ($t=0$)	10.09 ± 0.74 d	0.88 ± 0.06 a	0.52 ± 0.17 a	0.04 ± 0.01 a	13.0 ± 0.9 d	10.15 ± 0.74 d	2.01 ± 0.06 e	0.88 ± 0.19 c	0.18 ± 0.02 b	5.0 ± 0.6 b
Control ($t=12$)	7.89 ± 0.12 b	1.59 ± 0.69 a	0.47 ± 0.05 a	0.09 ± 0.02 a	5.0 ± 0.7 a	7.65 ± 0.21 b	1.90 ± 0.69 cd	0.41 ± 0.06 a	0.10 ± 0.04 a	7.4 ± 1.3 c
G. mosseae ($t=12$)	10.01 ± 0.43 d	1.88 ± 0.12 d	3.51 ± 0.16 e	0.66 ± 0.02 d	5.2 ± 0.5 a	7.67 ± 0.14 b	0.41 ± 0.02 a	0.97 ± 0.09 c	0.05 ± 0.01 a	19.0 ± 0.7 e
H. leucosarx ($t=12$)	14.98 ± 0.17 e	1.76 ± 0.13 d	2.69 ± 0.20 d	0.32 ± 0.03 b	8.5 ± 0.6 c	15.31 ± 0.97 ef	1.74 ± 0.11 de	1.86 ± 0.20 d	0.22 ± 0.03 b	9.0 ± 0.5 d
Control ($t=20$)	7.43 ± 0.26 bc	0.78 ± 0.02 a	0.53 ± 0.02 ab	0.06 ± 0.01 a	9.5 ± 0.7 c	7.27 ± 0.16 b	0.80 ± 0.02 b	0.44 ± 0.02 ab	0.04 ± 0.00 a	9.1 ± 0.2 d
G. mosseae ($t=20$)	8.62 ± 0.19 c	1.48 ± 0.07 bc	3.54 ± 0.08 e	0.61 ± 0.02 d	5.5 ± 0.6 ab	7.11 ± 0.19 b	0.37 ± 0.01 a	0.98 ± 0.07 c	0.05 ± 0.00 a	18.9 ± 0.5 e
H. leucosarx ($t=20$)	8.47 ± 0.21 c	1.44 ± 0.03 bc	2.46 ± 0.09 d	0.42 ± 0.03 c	5.9 ± 0.4 b	8.52 ± 0.24 c	1.42 ± 0.03 cd	2.43 ± 0.09 e	0.43 ± 0.02 c	5.7 ± 0.3 c
Control ($t=30$)	8.18 ± 0.27 c	1.15 ± 0.04 b	0.72 ± 0.04 b	0.10 ± 0.01 a	7.1 ± 1.3 b	8.38 ± 0.19 c	1.16 ± 0.03 c	0.65 ± 0.04 b	0.09 ± 0.01 a	7.3 ± 1.6 cd
G. mosseae ($t=30$)	7.23 ± 0.14 b	1.54 ± 0.06 cd	2.11 ± 0.05 c	0.45 ± 0.01 c	4.6 ± 0.2 a	6.35 ± 0.12 a	0.70 ± 0.02 b	0.59 ± 0.02 b	0.06 ± 0.00 a	9.2 ± 0.2 d
H. leucosarx ($t=30$)	6.32 ± 0.10 a	1.48 ± 0.02 c	1.87 ± 0.05 c	0.44 ± 0.02 c	4.3 ± 0.1 a	6.14 ± 0.10 a	1.45 ± 0.03 cd	1.72 ± 0.05 d	0.41 ± 0.01 c	4.2 ± 0.1 a
Fungus[a]	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001
Harvest[a]	≪0.001	≪0.001	0.018	≪0.001	≪0.001	≪0.001	≪0.001	0.018	≪0.001	≪0.001
Fungus × Harvest[a]	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001	≪0.001

[a] ANOVA (*P* value)

tings collected in March were generally highest for *H. leucosarx*-inoculated plants at $t=12$ and 20, whereas cuttings with *G. mosseae* showed the lowest concentrations. Cuttings collected in December showed positive responses compared with controls in N concentration at $t=12$ and in shoot P concentration for both mycorrhizal fungi at all three harvest periods (Table 2). Shoot N content of cuttings collected in March was positively affected by both mycorrhizal fungi, but to a greater extent by *H. leucosarx*: shoot P content was only positively affected by *H. leucosarx*. However, for cuttings collected in December, both N and P contents were positively affected by both fungi (Table 2, Fig. 2e, f).

With regard to short-term effects (<12 weeks), both mycorrhizal fungi were associated with increased shoot length and dry weight compared with controls, irrespective of the collecting date of cuttings. Root length was significantly increased by both mycorrhizal fungi (Fig. 2c, d). Even though *G. mosseae* gave the lowest % mycorrhizal colonization ((Table 1), it showed large positive effects on short-term shoot growth rate (Fig. 1) and root length (Fig. 2c, d). Shoot N concentration was highest in the presence of *H. leucosarx* (Table 2). *G. mosseae* inoculation resulted in the highest shoot P content for cuttings collected in December (Fig. 2e, f). Shoot N/P ratio was around 10 for cuttings with *H. leucosarx*, whereas those with *G. mosseae* showed a shoot N/P ratio of 5 for cuttings collected in December and 19 for those collected in March (Table 2).

In the long term, (30 weeks), plant growth response was significantly affected by the mycorrhizal treatments (Fig. 1, repeated measures ANOVA: all factors and their interactions, $P<0.001$). Similar positive effects for both collecting dates occurred by inoculation with *G. mosseae* in the first 7 weeks of the experiment, while large differences occurred in shoot length of cuttings collected in March and December over the longer term. Cuttings from December inoculated with *G. mosseae* continued to increase in shoot length over time, similarly to those with *H. leucosarx,* whereas cuttings collected in March showed no further increased shoot length after 7 weeks when inoculated with *G. mosseae* (Fig. 1). Growth measurements over 30 weeks showed that non-mycorrhizal plants had the lowest growth rate and plants inoculated with *H. leucosarx* had the highest; the growth curve of cuttings with *G. mosseae* depended on whether they had been collected in March or December (Fig. 1).

Over 30 weeks, fungal treatments of cuttings collected in December caused significant differences (Tables 1, 2) in shoot length, shoot dry weight, root length, mycorrhizal colonization, mycorrhizal root length, shoot N/P ratio, shoot N concentration, shoot P concentration, shoot N content, and shoot P content (Tables 1, 2, $P<0.001$). Interaction with harvest was also significant ($P<0.001$). The significant interaction with harvest for all parameters indicated differential effects of the treatments over the harvest periods. In contrast to the very fast growth response and shoot P uptake by *G. mosseae*-inoculated cuttings in the short term, effects were smaller in the long term (Fig. 2). For cuttings collected in March, all treatments also showed significant differences in plant parameters measured over 30 weeks (Tables 1, 2). Factors and their interactions showed significant effects at $P<0.001$ on all plant parameters, except mycorrhizal colonization and shoot N total.

Nutrient uptake versus nutrients in the soil available pool with two mycorrhizal types

Shoot N and P uptake were high for both March and December cuttings in the presence of *H. leucosarx*.

Table 3 Available $P_{(dissolved)}$ and $N_{(dissolved)}$ in soils in the pots of *S. repens* (collected in March and December 1996) inoculated with *G. mosseae* or *H. leucosarx* or not inoculated (control) after harvesting at 0, 12, 20 and 30 weeks (shown as means ± 1 standard error with $n \geq 10$). Significant differences between fungal treatments or time are indicated by different letters (Kruskal Wallis and Mann-Whitney-U)

Fungal treatment and harvest time	Available nutrients (mg/kg)			
	December		March	
	$P_{dissolved}$	$N_{dissolved}$	$P_{dissolved}$	$N_{dissolved}$
$t=0$	0.29 ± 0.02 d	11.86 ± 0.02 e	0.31 ± 0.01 e	11.94 ± 0.04 e
Control $t=12$	0.30 ± 0.09 d	9.73 ± 1.02 d	0.27 ± 0.10 d	9.87 ± 1.20 d
G. mosseae $t=12$	0.06 ± 0.01 b	2.60 ± 0.07 ab	0.06 ± 0.02 ab	12.80 ± 0.59 e
H. leucosarx $t=12$	0.15 ± 0.07 bc	7.47 ± 1.46 cd	0.26 ± 0.17 d	9.48 ± 0.47 d
Control $t=20$	0.17 ± 0.02 c	4.04 ± 0.17 bc	0.07 ± 0.02 b	4.94 ± 0.24 ab
G. mosseae $t=20$	0.00 a	2.33 ± 0.15 a	0.00 a	4.04 ± 0.16 a
H. leucosarx $t=20$	0.07 ± 0.01 b	3.23 ± 0.23 b	0.05 ± 0.02 b	4.24 ± 0.29 a
Control $t=30$	0.10 ± 0.01 b	4.99 ± 0.21 c	0.09 ± 0.01 b	5.19 ± 0.25 bc
G. mosseae $t=30$	0.00 a	2.28 ± 0.07 a	0.00 a	4.04 ± 0.35 a
H. leucosarx $t=30$	0.17 ± 0.03 c	5.12 ± 0.19 c	0.14 ± 0.04 c	5.72 ± 0.27 c
Kruskal-Wallis test	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$

However, shoot N and P uptake over the 0–12 weeks period was highest in the presence of *G. mosseae*, but only for cuttings collected in December (Table 2).

Available N and P in the soil after harvesting differed between fungal treatments (Table 3). P_s significantly decreased over time. In the treatment with *G. mosseae,* all available P was removed (March and December cuttings), whereas P_s was higher at the final than at the second harvest in the treatment with *H. leucosarx*.

Experiment 2: Different amounts of AM inoculum

A fast growth response was associated with *G. mosseae* (growth similar to experiment 1, December cuttings), irrespective of the amount of inoculum; the growth response of *S. repens* did not last longer when supplied with a higher amount of inoculum. No significant differences were found in plant parameters and mycorrhizal colonization (colonization levels similar to experiment 1, December cuttings) for *S. repens* supplied with three different amounts of inoculum; the interaction amount of inoculum × harvest was also not significant (Table 4).

In *S. repens,* the structures mainly consisted of internal hyphae, a few vesicles, with very occasional or no hyphal coils. In contrast, AM colonization in roots of *T. repens* showed a complete spectrum of structures, including internal hyphae, vesicles, and arbuscules (no hyphal coils were observed). Although RLC in *Trifolium* remained at around 90%, RLCI changed with time (40, 90, 60%, respectively). This coincided with a change in structures from mainly internal hyphae and arbuscules (few vesicles) to many vesicles and internal hyphae (few arbuscules), and finally mainly internal hyphae and many external spores attached to the external mycelium. Furthermore, AM colonization by *G. mosseae* in *T. repens* showed dynamic development over 30 weeks, whereas for *S. repens* RLC, RLCI and the relative amount of (few) structures remained the same throughout the different harvest periods (data not shown).

Discussion

The mycorrhizal benefits to host plants are multiple. The ultimate criterion should be the increased fitness of a mycorrhizal compared to a non-mycorrhizal plant. However, direct assessment of fitness is usually replaced by parameters such as (above-ground) performance or growth, expressed in terms of either C, N or P nutrition. Further benefits, which may not be expressed as increased growth, can also occur, such as increased survival, changes in root architecture or increased pathogen resistance. Although mycorrhizal benefit or responsiveness may be expressed as a single parameter, benefits expressed in various terms do not necessarily correlate. Such differential effects give rise to the concept of multifunctionality of the mycorrhizal symbiosis. A number of plant parameters should, therefore, be assessed before demonstrating or refuting a mycorrhizal effect.

Only 10% of *S. repens* mycorrhizal plants, compared with half of the control plants, died during the first weeks of the present experiment. Over a relatively short period (12 weeks), the two types of mycorrhiza (AM and EcM) had very different effects on plant performance, and these effects varied in time, duration, magnitude and nature over a longer period (30 weeks). For example, *G. mosseae* was very beneficial in the short term (12 weeks) for shoot and root growth and nutrient uptake, whereas *H. leucosarx* was very beneficial over the long term (7 months). The very fast growth response and P uptake shortly after inoculation with *G. mosseae* was not caused by a nutritional bias from clover roots, since no additional benefits occurred when the amount of inoculum (roots) was raised. These results agree with those of Lapeyrie and Chilvers (1985), who concluded that most of the initial growth promotion of *Eucalyptus* in calcareous soils was due to AM, which were later replaced by EcM.

The magnitude of plant responses in relation to the degree of root colonization differed between the mycorrhizal types, and low root colonization did not necessarily imply low plant response. Unlike in *Eucalyptus* (Jones et al. 1998), root colonization and mycorrhizal

Table 4 Results of ANOVA (*P* values) for shoot length, shoot biomass, root length, mycorrhizal colonization, mycorrhizal root length, shoot N and P concentration and content and shoot N/P ratio of *S. repens* ($n \geq 10$) inoculated with *G. mosseae.* The factor 'inoculum' represents three different amounts of inoculum (0.67, 1.34 or 3.35 g fresh wt. of clover roots with *G. mosseae*), and the factor 'harvest' represents three harvest periods (12, 20 and 30 weeks)

Response variable	Source of variation		
	Inoculum ($df=2$)	Harvest ($df=2$)	Inoculum × harvest ($df=4$)
Shoot length	0.875	0.019	0.754
Shoot biomass	0.896	0.004	0.898
Root length	0.256	<0.001	0.170
Mycorrhizal colonization	0.527	0.041	0.053
Mycorrhizal root length	0.894	<0.001	0.431
Shoot N concentration	0.612	<0.001	0.871
Shoot N total	0.848	<0.001	0.548
Shoot P concentration	0.617	<0.001	0.408
Shoot P total	0.560	<0.001	0.354
Shoot N/P ratio	0.269	<0.001	0.891

root length of *S. repens* varied enormously between the two mycorrhizal fungal types. Only 5% of roots were colonized by *G. mosseae* but there were very strong effects on short-term shoot dry weight, P translocation into the shoot and root length of *S. repens*. *G. mosseae* strongly increased root length of *S. repens* in the first 12 weeks (and even for 30 weeks in the *S. repens* collected in December), compared with both *H. leucosarx* inoculation and the control. This is in agreement with Hooker et al. (1992), who also reported increased lateral root branching of *Populus* after infection with three AMF species. These alterations to root system architecture would clearly have a major impact on root system function and long-term development.

The mycorrhizal fungi appeared to be very effective in nutrient uptake during the first 12 weeks. The high nutrient translocation into shoots of *S. repens* may result from the extremely low root/shoot ratio of cuttings at the start of the experiment and the greater root production by mycorrhizal than control plants. *H. leucosarx* improved P accumulation in shoots of March cuttings of *S. repens* over 30 weeks more than *G. mosseae,* although P remained detectable in the available pool of the EcM treatment but not in the AM treatment. Mycorrhizas can alter the chemistry of soil in their vicinity so that more exchangeable N or P comes into solution, or increase the volume of the soil to which roots have access via their external hyphae (Tinker 1975). These aspects warrant further research in order to assess differences in soil P assimilation of *S. repens* root systems with *G. mosseae* and *H. leucosarx.*

For *S. repens* collected in March (already high in P), plant growth rates and nutrient uptake were also stimulated more by *H. leucosarx* than by *G. mosseae* over the 30-week period. This observation generally agrees with the conclusions of Jones et al. (1998) after studying AM and EcM colonization in *Eucalyptus*, i.e. greater plant benefits from two EcMF than from three different species of AMF. However, the study by Jones et al. lasted only for 13 weeks. In my experiments, *G. mosseae* showed growth effects similar to those of the EcM fungus in the first 12 weeks. Moreover, when *S. repens* was collected in December (low P), *G. mosseae* showed similar benefits to *H. leucosarx* also in the long term. Benefits of AM and EcM for dual mycorrhizal plant species, therefore, not only depend on the plant species (and of course the fungal species) involved, but also on the physiological state of the plant (both the initial physiological state and the duration of the experiment).

Jones et al. (1998) recorded similar root lengths and root colonization by AM and EcM in *Eucalyptus*, whereas in the present study the two mycorrhizal types not only colonized different proportions of the root system but also altered root architecture differently. The paradoxical result occurred that mycorrhizal root system efficiency (P translocation into the shoot per unit root length) was higher for *H. leucosarx*, but the net amount of P translocated into the shoot (mycorrhizal root system effectiveness) was higher for *G. mosseae* over the first 12 weeks. Furthermore, in this study, growth responses and nutrient contents were related in the short term (12 weeks) [as found by Jones et al. (1998)], but not over 30 weeks. Thus experiments measuring mycorrhizal significance experiments should last longer than 10–12 weeks. The response to mycorrhizal colonization could be a function of the developmental stage of the mycorrhizal association (McGonigle and Fitter 1988). AM fungi can go through cycles of activity, with many arbuscules being produced during periods of growth in young roots and vesicles predominating at other times (Douds and Chaney 1982). It is likely that cycles of activity similarly occur in EcM (Downes et al. 1992). In the study with *S. repens, G. mosseae* showed no differences in relative amounts of structures (i.e. hyphae, vesicles) over time (or between *S. repens* cuttings with different internal shoot P-status). The initial rapid translocation of P to the shoot could, therefore, not be related to particular structures (arbuscules).

The contrasting effect of *G. mosseae* on *S. repens* cuttings collected in March or December was striking. At the start of the experiments, the cuttings looked alike (twig with four buds) and, of the parameters measured in this study, only differed in shoot P concentration. The results of the experiments, however, suggest that their physiological state was certainly not the same. While the physiological mechanism is not understood and warrants further investigation, such differential behaviour could be ecologically meaningful. Apparently, P-demand of *S. repens* is very high at the start of the growing (and flowering) season. This produces a higher dependency on AM during that time, which was also concluded for flowering strawberry (Dunne and Fitter 1989). This may indicate that the supposedly non-functional low AM colonization, previously reported for ectomycorrhiza in tree species (Cázares and Trappe 1993), can provide a functional symbiosis depending on internal nutrient status or season (plant/tree nutrient demand). P accumulation in shoots of *S. repens* colonized by *H. leucosarx* was about 50% of that in *G. mosseae*-inoculated plants in the short term. As a flush of soil nutrients in natural systems is released in spring, the relatively higher colonization by AMF in *S. repens* observed by van der Heijden and Vosatka (1999) early in spring together with rapid P uptake by AM roots, as observed in this study, could provide a functional significance to AM in P-limited *S. repens*. Furthermore, whereas Read (1989) discussed a shift in relative importance from AM towards EcM over a dune successional gradient, this study rather indicates a shift in relative importance from AM towards EcM over a growing season. The functional significance of AMF and EcMF diversity for *S. repens,* therefore, deserves further research.

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