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Early Interactions between Arbuscular Mycorrhizal Fungi and *Frankia* during Colonisation and Root Nodulation of *Alnus glutinosa*

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Abstract

Glasshouse experiments showed significant differences in early interactions of *Alnus glutinosa* with five different arbuscular mycorrhizal species and *Frankia*. Thirty and 60 days after inoculation of roots with AMF alone, colonisation was highest with *Glomus hoi* and *G. mosseae* followed by *Gigaspora rosea*. Lowest colonisation was with *Acaulospora scrobiculata* and *Scutellospora castanea*. In associations with AMF resulting in relatively low colonisation, e.g. *S. castanea*, plant biomass after 60 days was higher than in un-inoculated controls but when inoculated with *Frankia* also was equal to controls. In contrast, the biomass of plants with high levels of AMF colonisation e.g. with *G. hoi*, was equal to the controls when alone but was lower in dual symbiosis with *Frankia*. This effect was

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particularly marked 30 days after inoculation. There was also a depressive effect of nodulation on plant biomass 30 days after dual inoculation that was less apparent after 60 days. This effect was most marked for AMF giving rise to the highest levels of colonisation (*G. hoi* and *Gi. rosea*). The data suggest that in dual inoculated alder seedlings, inhibitory effects on early growth can result, possibly from competition with micro-symbionts for resources such as photosynthates. However, effects may only be temporary, to be relieved as seedlings develop a larger shoot system with greater photosynthetic capacity.

Keywords: Competition, symbiont, photosynthate, *Frankia*, *Glomus hoi*, *Glomus mosseae*, *Gigaspora rosea*, *Acaulospora scrobiculata*, *Scutellospora castanea*.

1. Introduction

Species of the genus *Alnus* are trees with economical and ecological importance in temperate floras of the Northern Hemisphere. They are pioneer plants with the ability to colonise sites of poor fertility and several are riverine species. They have been utilized in reforestation programs in Europe and are planted commonly for reclamation of old industrial and mine spoil sites and in urban areas such as roadsides and parks; sites that often are problematic for the growth of other broadleaf tree species (Tjepkema et al., 1986; Wheeler and Miller, 1990; Strukova et al., 1996). An important characteristic that facilitates the growth of alders on nutrient poor sites is their ability to form root nodules with the actinomycete *Frankia* (Wheeler et al., 1986). It has also been shown for alders, and some other actinorhizal species, that growth and nodulation under nutrient poor conditions is aided by the formation of symbiotic relationships with ectomycorrhizal fungi (EMF) and arbuscular mycorrhizal fungi (AMF), (Gardner, 1986; Lumini et al., 1994; Wheeler and Miller, 1990). Mineralisation of leaf and fine branch litter and root turnover of actinorhizal species has been shown to increase soil nitrogen and phosphorus status of sites where they have been planted (Arnebrant et al., 1993; Dawson, 1990; Malcolm et al., 1985; Strukova et al., 1996).

The function of root nodules in nitrogen fixation and their positive role in promoting plant growth in laboratory and field experiments is well established for several species of *Alnus* (Schwintzer and Tjepkema, 1990; Fraga-Beddiar and Le Tacon, 1990; Jha et al., 1993; Isopi et al., 1994). The symbioses formed with EMF by *A. glutinosa* are preferentially with *Pisolithus* and *Paxillus* (Molina, 1981). Early reports for dual inoculated Myricaceae showed improved growth of *Comptonia peregrina* and *Myrica gale* when inoculated with *Frankia* and AMF or with *Frankia* alone, when compared with un-inoculated plants or plants with AMF only (Berliner and Torrey, 1988). However, AMF colonisation

of the roots was not reported. Symbiosis of *A. glutinosa* and *Alnus incana* with AMF, the establishment of a tetrapartite symbiosis with EMF, AMF and *Frankia* and laboratory experiments indicating positive effects on plant growth of association with one species of AMF, *Glomus fasiculatum*, were described by Chatarpaul et al. (1989). *Alnus cordata* seedlings dual inoculated with *Frankia* and *Glomus mosseae* and *G. fasiculatum* showed increased nodulation by *Frankia*, colonisation by AMF and better plant growth five months after inoculation (Isopi et al., 1994). *A. glutinosa* also showed better growth after dual inoculation with *Frankia* and *G. fasiculatum* at two different phosphate levels (Fraga-Beddiar and le Tacon, 1990). Similar results were reported for *Alnus nepalensis* inoculated with *Frankia* and *G. mosseae* and grown under various phosphorus regimes (Jha et al., 1993).

In symbioses with Rhizobiaceae, colonisation by AMF has also been shown to enhance nodulation, nitrogen fixation, and carbon flow e.g. in *Vicia faba* (Kucey, 1982). However, reports of more detailed studies have shown that the timing of inoculation can influence the development of the symbiotic relationships in seedlings. Thus, Bethlenfalvay et al. (1985) showed that delaying inoculation of soybean with *Bradyrhizobium* after inoculation with *Glomus* inhibited nodule development (increase in weight). When plants were nodulated before inoculation with *Glomus*, the development of fungal biomass per root was also inhibited. Gardner (1986) reported that previous AMF inoculation of *Casuarina equisetifolia* inhibited nodulation by *Frankia*. These results suggest that competition between microsymbionts for nutrients and, or for infection sites may influence the development of the symbioses in seedlings. However, Sempavalan et al. (1995) showed a co-operative, not a competitive relationship between *Frankia* and *Glomus* for nodulation and colonisation of *C. equisetifolia* roots in experiments that involved different orders and time sequences of inoculation. Negative growth effects of AMF colonisation have been observed with other plant species (Hayman, 1982; Pearson et al., 1993). Variations in the responses to colonisation reported by different researchers suggest that the effects of early interactions between seedlings and microsymbionts may be dependent on the species of plant and symbiont and the environmental conditions under which experiments have been carried out.

In the current study, glasshouse experiments were carried out to determine the effects of different combinations of AMF and *Frankia* on nodulation, AMF colonisation and growth of *A. glutinosa*. The study focussed on how interactions between different species of AMF and with *Frankia* affect the early events of nodulation and colonisation. The data from such experiments may help to resolve the reasons for different reports of the growth effects of AMF colonisation on nodulated plants and enhance understanding of early interactions in the EMF, *Frankia*, AMF tetrapartite symbioses in *Alnus*.

2. Materials and Methods

Germination of seeds

Seeds of *Alnus glutinosa* (L.) Gaertn. from Durham County, N.E. England, were purchased from the Forestry Commission, U.K. The weight of one thousand seeds at sowing was 1.536 g. Seeds were surface sterilised with 5% sodium hypochlorite for 5 min, washed with distilled water and then germinated in the glasshouse under controlled temperature (day/night 27–17°C; 16 h photoperiod; with natural light supplemented with mercury vapour lighting). Seedlings were grown in seed trays, containing Perlite (L.B.S. Group, UK) enriched with 2.24 g Crone's (–N) nutrients/litre of Perlite (Hewitt, 1966; Wheeler and Miller, 1990). Seedlings were used 30 days later, following emergence of the first true leaves and when approximately 2 cm high.

Culture of Frankia and AMF and inoculation of seedlings

Frankia UGL010708, originally isolated from Balmaha, Loch Lomond (Scotland), was cultured in propionate medium containing casamino acids as N source (Hooker and Wheeler, 1987). *A. glutinosa* seedlings were inoculated with mycelium, previously washed free of culture medium with distilled water. The plants were maintained in Crone's liquid culture, in a glasshouse free of other strains of *Frankia*, and crushed nodules used as a source of inoculum.

Lobes of nodules were cut from the root system and surface sterilised with 5% sodium hypochlorite for 5 min. The nodules were washed well with distilled water, crushed in a sterile mortar and pestle and diluted with distilled water; 500 ml of water for 2.5 g nodules (Hooker and Wheeler, 1987; Jha et al., 1993). The suspension was then filtered through sterile 50 µm nylon mesh. Each *A. glutinosa* seedling was inoculated with 5 ml of the filtrate. Control (un-inoculated) and mycorrhizal only seedlings received 5 ml of distilled water.

AMF isolates *Glomus mosseae*, Banque Europeenne des Glomeales (BEG) 12; *Gigaspora rosea* BEG 9, *Scutellospora castanea* BEG 1, *Acaulospora scrobiculata* BEG 33 were maintained on roots of *Plantago lanceolata* grown in pot culture. An isolate of *Glomus hoi* (V98), was obtained from Dr. M. Vestberg (Lankaa Research and Elite Plant Unit, Finland). Prior to use as inocula cultures were maintained under identical conditions in a greenhouse in pot culture with *Plantago lanceolata*. Levels of colonisation in the *P. lanceolata* roots used as inoculum were: *Glomus mosseae* (BEG 12), 85%; *Gigaspora rosea* (BEG 9), 80%; *Scutellospora castanea* (BEG 1), 75%; *Acutelospora scrobiculata* (BEG 33), 76%; *Glomus hoi* (V98): 90%.

Experimental design

Forty replicate *A. glutinosa* seedlings were inoculated with the AMF *G. hoi*, *G. mosseae*, *Gi. rosea*, *S. castanea*, *A. scrobiculata*, respectively. Twenty replicate plants from each mycorrhizal treatment were inoculated with the *Frankia* extract. The remaining plants each received 5 ml distilled water. Twenty seedlings were inoculated with the *Frankia* extract only. *Frankia* inoculum was applied with a 5 ml sterile syringe to the base of each seedling. Control plants received 5 ml of distilled water. Plants were harvested 30 and 60 days after inoculation. *A. glutinosa* seedlings were inoculated with AMF by mixing AMF colonised root pieces of *P. lanceolata* (1.2 g fresh weight roots per seedling) into the growth medium, consisting of a 50:50 mix of loam soil (NO_3 83 ppm; NH_4 127 ppm; P_2O_5 240 ppm; K_2O 270 ppm; MgO 11 ppm; B 0.75 ppm; Cu 1.5 ppm; Fe 3.3 ppm; Mn 1.5 ppm; Mo 2.1 ppm; Zn 0.75 ppm) and sand (sterilised by autoclaving at 121°C). Pots (10 cm) were sterilised with 50% ethanol, and were filled with 323 g of the growth medium. Control plants were inoculated by mixing the same weight of non colonised root pieces of *P. lanceolata* into the growth medium. One seedling (free of Perlite) was placed in each pot. Pots with seedlings of the same treatment were placed in a trough, sterilised previously with 50% ethanol. Plants were watered every 4–5 days with distilled water to field capacity.

The experiment was carried out from July to September in a glasshouse with natural light supplemented by artificial lights (400W; General Electric Kolorlux H400/40, 47904). The temperature in the glasshouse was regulated to a maximum of 27°C (day), and a minimum of 17°C (night). Humidity was maintained at 60%.

Seedling harvest, arbuscular mycorrhizal colonisation and Frankia nodulation

Harvested seedlings were removed carefully from the pots and the soil washed gently from the roots under running tap water over a 20 µm sieve to ensure that all fine roots were recovered. For each seedling, fresh and dry weight of the shoots and roots were measured. For dry weights, plant tissues were separated into roots and shoots and dried at 60°C to constant weight. A portion (30%) of the fresh root system from each seedling was stored in 50% ethanol at 5°C for AMF colonisation and nodulation measurements.

The preserved roots were examined under a binocular microscope for nodulation. For estimation of AMF colonisation, roots were stained with 0.05% Trypan blue in acidic glycerol (modified Phillips and Hayman, 1970; Koske and Gemma, 1989). Stained samples were examined with compound microscope at 200x total magnification and percentage of AMF colonisation was recorded (Trouvelot et al., 1986).

Statistical analyses

The data were tested for normal distribution and then a Bartlett test of variances was applied to determine if the variances were similar. Analysis of Variance was then performed. If effects of treatment were identified then t tests were performed to identify the significance of differences between individual means. Experimental data were analysed utilising Excel (Microsoft) and Graph Pad Prism (Graph Pad Software, Inc., USA).

3. Results

A. glutinosa seedlings were colonised successfully by the five AMF strains tested after 30 days (Figs. 1 and 2). Seedlings inoculated with *Frankia* alone and *Frankia* plus AMF were all nodulated after 30 days (Fig. 5). Neither nodules nor AMF were detected in roots of plants not inoculated with these symbionts.

Colonisation of roots and growth of plants inoculated with AMF alone

The proportion of roots colonised by AMF alone was dependent upon the AMF species and did not change significantly between 30 and 60 days (Figs. 1 and 3). Thus, the highest percentage of roots colonised, between 18 and 31%, was evident in seedlings inoculated with *G. mosseae* and *G. hoi*, respectively. In contrast, seedlings inoculated with *A. scrobiculata* and *S. castanea* showed the lowest colonisation, between 6 and 13%, respectively, at the same harvests. *Gi. rosea* gave rise to 12 and 20% root colonisation, intermediate between the values for other strains.

Plant dry weight was not affected significantly 30 days after inoculation with the different species of AMF except for seedlings inoculated with *Gi. rosea* for which the dry weight was 42% lower than the un-inoculated controls (Fig. 1). After 60 days, the only significant differences in plant dry weights compared with controls was a small increase of 20% in plants colonised by *S. castanea* and *G. hoi* alone (Fig. 3). Neither fresh weight to dry weight ratios nor shoot to root dry weight ratios were different between treatments.

Colonisation and nodulation of roots and growth of plants inoculated simultaneously with AMF and Frankia

Compared with seedlings inoculated with AMF alone, dual inoculation of seedlings did not affect the percentage of roots colonised by AMF markedly after 30 days (Figs. 1 and 2) but after 60 days, colonisation by *A. scrobiculata*,

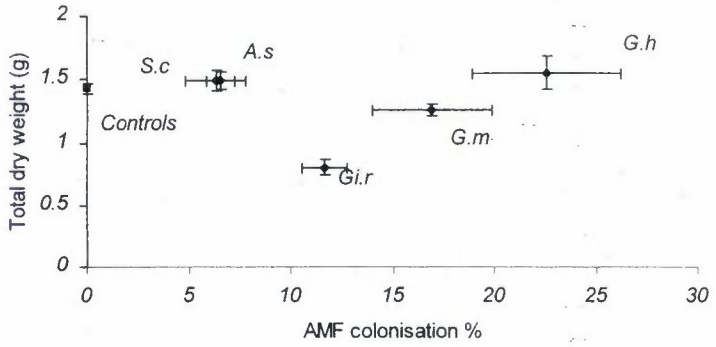

Figure 1. Growth (dry weight) and root colonisation of *A. glutinosa* 30 days after inoculation with different AMF. Seedlings were grown in a 1:1 mix of sterilised sand and soil. Data are means of 10 replicate plants. Bars indicate standard errors. Abbreviations are as follows: G.m = *Glomus mosseae*, G.h = *Glomus hoi*, Gi.r = *Gigaspora rosea*, A.s = *Acaulospora scrobiculata*, S.c = *Scutellospora castanea*.

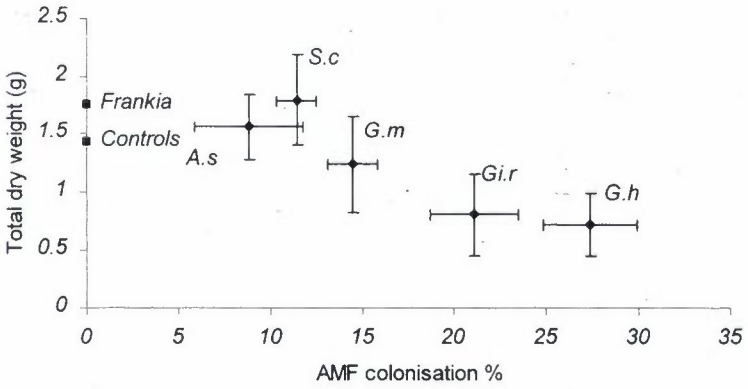

Figure 2. Growth (dry weight) and root colonisation of *A. glutinosa* 30 days after dual inoculation with *Frankia* and with different AMF. Seedlings were grown in a 1:1 mix of sterilised sand and soil. Data are means of 10 replicate plants. Bars indicate standard errors. Abbreviations are as in Fig. 1.

S. castanea and *G. mosseae* all increased significantly (Figs. 3 and 4). The least effects on colonisation of a further period of plant growth were shown for the two AMF that gave the highest levels of colonisation after 30 days (*Gi. rosea* and *G. hoi*). The largest effect of dual inoculation on colonisation was observed with *S. castanea*. Colonisation of roots inoculated with this AMF alone was among the lowest for the isolates tested after 30 days but 60 days after dual

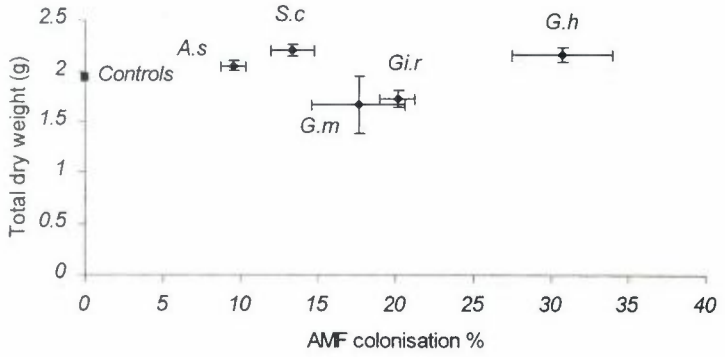

Figure 3. Growth (dry weight) and root colonisation of *A. glutinosa* 60 days after inoculation with different AMF. Seedlings were grown in a 1:1 mix of sterilised sand and soil. Data are means of 10 replicate plants. Bars indicate standard errors. Abbreviations are as in Fig. 1.

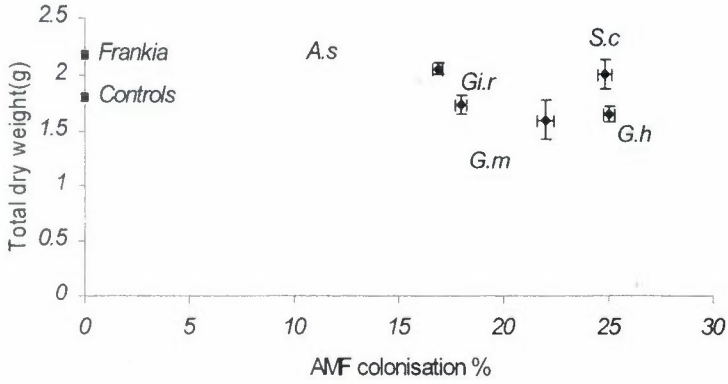

Figure 4. Growth (dry weight) and root colonisation of *A. glutinosa* 60 days after dual inoculation with *Frankia* and with different AMF. Seedlings were grown in a 1:1 mix of sterilised sand and soil. Data are means of 10 replicate plants. Bars indicate standard errors. Abbreviations are as in Fig. 1.

inoculation, seedlings showed levels of colonisation similar to that of *G. hoi*, which colonised roots to the greatest extent (Fig. 4).

There was no correlation between percentage of roots colonised by AMF and specific nodule number (nodule number g^{-1} root dry weight) for any of the mycorrhizal associations investigated. However, compared with plants inoculated with *Frankia* alone, specific nodule number was increased by colonisation by all the mycorrhizal species tested, both 30 and 60 days after dual inoculation (Figs. 5 and 6). After 30 days, the maximum increase in nodule

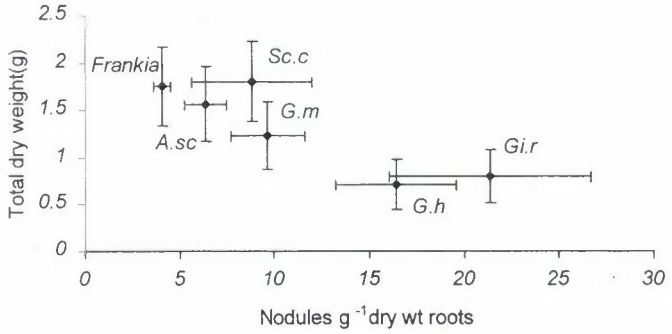

Figure 5. Effect of nodulation (nodule number g^{-1} root dry weight) on growth (total dry weight) of *A. glutinosa* 30 days after dual inoculation with *Frankia* and with different AMF. Seedlings were grown in a 1:1 mix of sterilised sand and soil. Data are means of 10 replicate plants. Bars indicate standard errors. Abbreviations are as in Fig. 1.

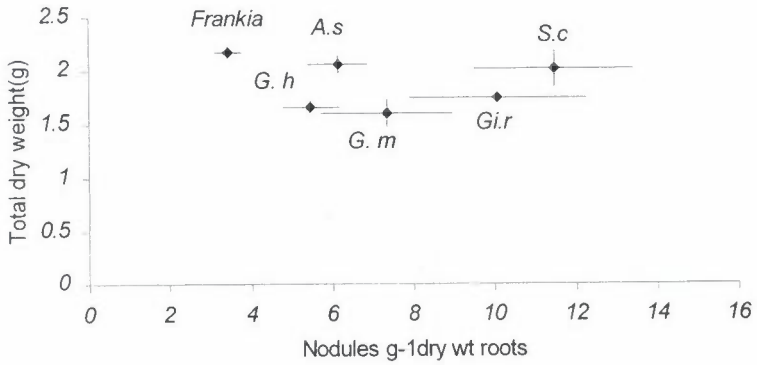

Figure 6. Effect of nodulation (nodule number g^{-1} root dry weight) on growth (total dry weight) of *A. glutinosa* 60 days after dual inoculation with *Frankia* and with different AMF. Seedlings were grown in a 1:1 mix of sterilised sand and soil. Data are means of 10 replicate plants. Bars indicate standard errors. Abbreviations are as in Fig. 1.

specific number was with *G. hoi* (75% increase) and *Gi. rosea* (81% increase). After 60 days, the increase in nodule specific number for *Gi. rosea* was 66% and 70% for *S. castanea*. The specific nodule number of plants colonised by the five AMF species decreased as root size increased over the period between 30 and 60 days. For example, the specific nodule number of plants colonised by *Gi. rosea* fell by over 50% between 30 and 60 days as root dry weight increased by 42%.

The dry weight of dual inoculated plants 30 days after inoculation was reduced, by up to 58% with *G. hoi* and *Gi. rosea* (Fig. 5) but there was no significant effect on plant weight after 60 days. Neither fresh weight to dry weight ratios nor shoot to root dry weight ratios were different between treatments.

4. Discussion

Values for the percentage colonisation of *A. glutinosa* roots by AMF alone 60 days after inoculation were between 31% and 20% respectively for *G. hoi* and *Gi. rosea* and 13% for *S. castanea* and 10% for *A. scrobiculata* (Fig. 3), showing that the aggressiveness with which AMF colonise alder roots is species dependent. These colonisation levels are relatively low compared with most herbaceous plants but are not unusual for many of the hard wood species. For example roots of *Olea europea* were colonised by 29.4% to 38.5% after inoculation with *G. mosseae* (Citernesi et al., 1998). In contrast, a relatively high percentage of AMF colonisation (80%) was recorded when *Prunella grandiflora* and *P. vulgaris* were inoculated with various *Glomus* isolates (Streitwolf-Engel et al., 1997). Root cell density and the thickness of root cell walls are among the factors that determine AMF internal hyphae development (Smith et al., 1997).

Effects of dual inoculation on nodulation were determined by counts of nodule number rather than by measurement of nodule weight, both because of the problems of separating very small nodules from underlying root tissue and in order to assess whether AMF compete for *Frankia* infection sites. Specific nodule number (number of nodules g^{-1} roots) was generally increased by colonisation by all the mycorrhizal species, both 30 and 60 days after inoculation (Figs. 5 and 6). This is indicative of a co-operative, rather than competitive interaction between *Frankia* and AMF for infection as noted previously for *Casuarina* (Sempavalan et al., 1995). It is not practical to count the number of invasion sites by AMF. However, significant, positive effects of dual inoculation on the percentage of roots colonised by AMF were apparent after 60 days, with highest levels of colonisation at this time by *S. castanea* and *G. hoi*. (Figs. 1–4). However, there was no correlation between the percentage of roots colonised by AMF and specific nodule number for any of the mycorrhizal associations investigated.

These observations of positive effects of dual inoculation with AMF and *Frankia* on colonisation of *A. glutinosa* roots by AMF and on nodulation are in keeping with previous reports for alders, where the magnitude of effect has also been found to vary with mycorrhizal species (Chatarpaul et al., 1989; Fraga-Beddiar, 1990; Isopi et al., 1994). Thus, colonisation of *Alnus cordata* by

AMF increased from 32% to 40.5% when nodulated by *Frankia* (Isopi et al., 1994). Relatively high percentages of colonisation by *G. fasiculatum* (58%) were also recorded for 4-month-old *A. glutinosa* after inoculation with *Frankia* (Fraga-Beddiar, 1990).

In the experiments described, plants were grown in media that contained sufficient available N, P and other minerals to support the growth of non-inoculated plants. Consequently, in comparison to non-inoculated controls, inoculation with the different AMF species or with *Frankia* alone had no significant effect on average plant dry weight either 30 or 60 days after inoculation (Figs. 1–4). However, plant growth was reduced in plants that were colonised heavily by AMF species (*Gi. rosea* and *G. hoi*) 30 days after dual inoculation with *Frankia* and AMF (Fig. 2). This effect was not apparent after 60 days when plants were larger, with greater leaf development (Fig. 4). The biomass of AMF in colonised roots, with their high concentrations of lipids, can be large and create a major sink for carbon assimilates in the host plant (Smith et al., 1997; Harris and Paul, 1987). AMF colonisation can also affect the growth of the plant root system, with increased branching (Berta et al., 1995; Berta et al., 2002) and decreased longevity of roots (Hooker et al., 1995). It is therefore possible that reduced growth during the early stages of the symbioses may reflect the energy demands involved in establishing and maintaining active symbioses, together with effects on the development of the root system. Colonisation of a root system by mycorrhizal fungi thus forms a significant energy cost to the host plant, which must be compensated for in some way. Consequently, inhibitory effects on plant growth of colonisation by AMF have been demonstrated clearly, particularly under conditions where photosynthesis is not high. For example, *Plantago lanceolata*, inoculated with *G. mosseae*, showed reduced growth compared with non-mycorrhizal plants at relatively low temperature (15°C) when photosynthetic rate was reduced (Forbes et al., 1996). The combination of nodulation by *Frankia* and AMF colonisation is even more costly energetically. Not only are there substantial requirements for energy to support nodule development and nitrogenase activity but the establishment of a nitrogen fixing nodule containing *Frankia* can increase AMF biomass (Isopi et al., 1994; Fraga-Beddiar et al., 1990; Jha et al., 1993) thus raising the energy requirements of the colonised root system even further.

It is possible, therefore, that in the experiments described here the size and photosynthetic capacity of seedlings inoculated with AMF alone was sufficient after 30 days to support extensive AMF colonisation but insufficient to meet the combined demands of AMF colonisation and *Frankia* nodulation. Inhibitory effects may then have been relieved at 60 days due to further growth of the host plant.

Frankia and AMF may not only compete for photo-assimilates but also for mineral nutrients. During the early stages of colonisation, the AMF external

mycelium is not developed and therefore the fungus cannot supplement the supply of nutrients, and especially phosphate, to the plant to the same extent as it is able when developed fully. Presumably, regulatory systems within the host plant normally limit the development of the micro-symbionts during these early growth stages so that the association rarely becomes pathogenic. It is of interest that changes in the relative levels of N and P circulating in the host plant have been implicated in the regulation of nodulation in actinorhizal plants (Wall and Huss-Danell, 1997).

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