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ORIGINAL PAPER

Both the arbuscular mycorrhizal fungus *Gigaspora rosea* and *Frankia* increase root system branching and reduce root hair frequency in *Alnus glutinosa*

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Abstract *Alnus glutinosa* is an important pioneer species that forms effective symbioses with *Frankia* and ecto and arbuscular mycorrhizal fungi (AMF). There is evidence that *Frankia* and AMF interact and the focus of this study was to investigate how interactions affected root system and root hair development. *A. glutinosa* seedlings were grown in pots in soil pre-inoculated with the AMF *Gigaspora rosea*. Seedlings were inoculated with *Frankia* either immediately on transfer to AMF-inoculated pots (day 0) on day 15 or on day 30 following AMF inoculation so the effect of timing of inoculation on interactions could be determined. Seedlings were harvested in batches at intervals of 10, 15, 20, 25 and 30 days after the commencement of each treatment. Both *G. rosea* and *Frankia* increased root branching and effects were greater when both were present. By contrast, both *G. rosea* and *Frankia* decreased root hair numbers markedly. Effects on root hair development were not a consequence of phosphorous, as P levels were not changed significantly in seedlings colonised by *G. rosea* or nodulated by *Frankia*. Effects are not due to differences in root system size but conceivably could offset some of the carbon costs incurred by the symbioses.

Keywords *Alnus glutinosa* · *Gigaspora rosea* · *Arbuscular mycorrhiza* · *Frankia* · Root branching · Root architecture · Root topology · Root hairs

Christopher T. Wheeler is now retired.

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Introduction

The ability of alder to form symbiotic relationships with the actinomycete *Frankia* and ectomycorrhizal and arbuscular mycorrhizal fungi (AMF) are important characteristics that improve their growth, make them important pioneer species and encourage the planting of these trees on sites that are degenerate and nutrient poor (Gardner 1986; Wheeler et al. 1986; Wheeler and Miller 1990; Lumini et al. 1994). Several studies have shown positive effects of dual symbiosis with *Frankia* and AMF on the growth of a number of alder species and have provided evidence of a synergy between the micro-symbionts that may enhance the development and functioning of the symbioses Chartapaul et al. (1989); Fraga-Beddiar and le Tacon 1990; Jha et al. 1993; Isopi et al. 1994; Oliveira et al. 2005). Utility of such functions has also encouraged the investigation of the value of inoculating seedlings in nurseries and it has been demonstrated, for example, that inoculation of alders with *Frankia* can provide significant growth benefits (Hooker and Wheeler 1987; Wheeler et al. 1991). Inoculation with *Frankia* and mycorrhizal fungi together can improve the growth of seedlings (Isopi et al. 1994; Walker and Wheeler 1994) but it has also been demonstrated that co-inoculation of *A. glutinosa* with *Frankia* and AMF can, at least under

some conditions, inhibit early seedling growth. However, this effect is only temporary and is relieved as seedlings develop a larger shoot system (Orfanoudakis et al. 2004). It is generally accepted that the main benefit to the host plant from nodulation by *Frankia* comes from nitrogen fixation and that facilitation of nutrient availability and uptake is the major benefit to the host plant of colonisation of roots by AMF. Other benefits arise from the effects that colonisation by AMF and nodulation by *Frankia* have on root growth and architecture. Thus, changes in shoot:root ratios have been observed many times for plants supplied with mineral nitrogen compared with those reliant on nitrogen fixation, or for plants receiving supplementary phosphate compared with plants colonised by AMF (Barea and Azcon-Aguilar 1983). The lower shoot:root length ratio of non-infected *Datisca trinervis* compared with mycorrhizal and *Frankia* nodulated plants growing in the same but sterilised soil was suggested by Chaia and Raffaele (2000) to reflect resource re-allocation to root development that may facilitate uptake of soil nutrients. In a study by Isopi et al. (1994), the shoot: root weight ratio of non-nodulated *Alnus cordata* was also lower than that of nodulated plants; the generally higher specific root length of the roots of nodulated seedlings the main source of differences in shoot:root weight ratios. Colonisation by AMF can also change allocation of resources to the root system of plants in a more sophisticated way, causing alterations to the spatial pattern of root branching and significant modifications, including changes to root mortality (Atkinson et al. 2003; Hooker et al. 1995). In trees, roots of *Vitis vinifera* (Schellenbaum et al. 1991), *Populus* (Hooker et al. 1992), *Prunus cerasifera* (Berta et al. 1995) and *Olea europea* (Citernesi et al. 1998) were reported to be branched more extensively when colonised by an AMF. In an attempt to understand the precise nature of these changes and the biological and ecological implications they may have for plants Fitter and Stickland (1991) carried out mathematical studies on the data collected from the root system of plants colonised by AMF and discovered that AMF colonised root systems tended to form a rather random root distribution pattern, whereas plants grown under controlled conditions free of the mycorrhiza tended to have a herringbone growth pattern controlled largely by the plant and more efficient for exploring the soil over long distances. Initially, it was considered that these changes to root system architecture, brought about by alteration to root branching, may be due to enhanced phosphorus nutrition but the data of Fitter and Stickland (1991) had suggested this was unlikely and this was confirmed in experiments by Hooker et al. (1992). Depression of the mitotic index in apical meristems of AMF-infected roots has been identified as part of the morphogenetic programme that, at least in some cases, leads to changes in root morphology (Berta et al. 1990, 1991), but the actual mechanisms responsible are still not understood. There has been only one report of the effects of *Frankia* on root branching. In this study, Isopi et al. (1994) inoculated *A. cordata* with *Frankia* and reported no effect on root branching; but branching was increased significantly with one of two species of *Glomus*.

Root hairs are a significant anatomical and physiological feature of most plant roots. They are the main pathways for nutrient absorption from the soil with many reports from the early 1950s describing the function of root hairs and their role in plant nutrition. It is now known that root hairs are the main pathway of direct plant phosphate absorption, demonstrated by the correlation between the available nutrients at the depletion zone and numbers of root hairs (Baon et al. 1994). The relationship between root hairs, *Frankia* and AMF is complex. It is known that root hairs are often involved in nodulation of the root by *Frankia*; with *Frankia* entering via the root hairs, particularly in symbiosis with *Alnus* (Berry and Sunell 1990). In the case of AMF, plants with high numbers of root hairs generally have low dependence on AMF, whereas those with low numbers have a higher dependence (Baylis 1975). For example, in Cyperaceae, the development of AMF followed the "Baylis hypothesis", where plants growing in nutrient rich soil environment are rich in root hairs but show poor colonisation by mycorrhizal fungi (Muthukumar et al. 2004). It is clear then that plant root hair development is controlled to some extent at least by both the plant and soil environment. However, there is insufficient information available concerning how micro-symbionts interact with plant root hair development. Understanding interactions is particularly important in developing a better understanding of the processes that are involved in the tripartite symbiosis in actinorrhizal plants, where growth benefits of colonisation by *Frankia* and AMF show a cooperative action (Sempervalan et al. 1995).

The purpose of the study reported here is to investigate in more detail than previously how interactions between AMF and *Frankia* affect the branching of *A. glutinosa* roots, and consequently the development of root systems. For the first time, the effects of AMF and *Frankia* on the development of root hairs are also determined. The findings will serve to increase understanding of how partners in this tripartite symbiosis interact to affect root development and also add to the body of data from which hypotheses concerning the mechanisms that regulate changes in root architecture may be formulated.

Materials and methods

Experimental design and procedures

Seeds of *Alnus glutinosa* (L.) Gaertn. from Durham County, N.E. England, were obtained from the Forest Commission,

U.K. Seeds were surface-sterilised with 5% sodium hypochlorite for 5 min, washed with distilled water and then germinated in a glass house 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, U.K) enriched with 2.24 g Crone's (-N) nutrients/litre of perlite (Hewitt 1966; Wheeler and Miller 1990). Seedlings were used in experiments 30 days later, following emergence of the first true leaves and when approximately 2 cm high.

The AMF isolate used was *G. rosea* (Banque Europeenne des Glomeales; BEG 9), selected because it had colonised *A. glutinosa* relatively well in a previous study (Orfanoudakis et al. 2004). The culture was maintained and inoculum produced for these experiments on roots of *Plantago lanceolata* grown in pot culture.

One hundred and eighty replicate *A. glutinosa* seedlings from a batch of 360 were inoculated with *G. rosea.* Seedlings were inoculated by mixing colonised *P. lanceolata* root pieces thoroughly within the growth medium (1.2 g fresh weigh roots per seedling). Non-inoculated control seedlings were inoculated with the same weight of non-colonised *P. lanceolata* roots. Seedlings were grown in a medium consisting of 50:50 mix of loam soil and sand (the mix contained 83 ppm NO_3; 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). Pots were 5 cm in diameter and sterilised prior to use with 50% ethanol; each was filled with approximately 200 g of the growth medium. One seedling was placed in each pot.

Frankia UGL010708 originally isolated from Balmaha, Loch Lomond Scotland, was cultured in propionate medium containing casamino acids as a N source (Hooker and Wheeler 1987). *A. glutinosa* seedlings were inoculated with mycelium, previously washed free of culture medium with distilled water. The seedlings were maintained in Crone's liquid culture, in a glasshouse free of other strains of *Frankia*, and crushed nodules from these seedlings used as a source of inoculum in these experiments. Lobes of nodules were cut from the root system and surface-sterilised with 5% sodium hypochlorite for 5 min. The nodules were then washed thoroughly 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). The suspension was then filtered through a 50-μm nylon mesh (Plastok, Liverpool, UK). Each *A. glutinosa* seedling was inoculated with 5 ml of the filtrate.

Thirty AMF-inoculated seedlings were inoculated with *Frankia* on day 0, 30 on the 15th and 30 on 30th, i.e. day after inoculation with AMF. The remaining 90 seedlings received 5 ml distilled water only on the same days as the seedlings were inoculated with *Frankia*. A further thirty seedlings from the original batch of seedlings were inoculated with *Frankia* only on day 0, 15 and 30 of the experiment. The total length of the experiment was thus 60 days from the time of inoculation with AMF at day 0.

The experiment was carried out from July to September in an air-conditioned glasshouse under natural light supplemented by lights (400 W; General Electric Kolorlux H400/40, 47904, General Electric Company, USA); temperature was regulated to 27±2°C (day) and 17±2°C (night) and humidity to 60%. Pots were watered every 4 days with distilled water to field capacity.

Plant harvest and analysis at harvest

Seedlings were harvested after the appropriate period, e.g. 10, 15, 20, 25 and 30 days following inoculation with *Frankia*. The shoot was cut at the root collar and the dry weight of the shoot measured after drying to constant weight at 78°C. The roots were then removed carefully from the pots, after soaking in water. Great care was taken to avoid disturbing the integrity of the root system. Particles of soil adhering to the fine roots were removed using a wash bottle over a fine sieve.

Topological analysis of the root system was carried out for each seedling. First-order roots were those with an apical meristem, second order occurred as branches of the first orders, third order occurred as branches of the second order, and so on (Hooker and Atkinson 1996). All plant roots were excised carefully from the root system using scissors or scalpel and forceps and viewed with an illuminated magnifier, keeping roots of the same order together, and then stored in 50% (*v*/*v*) ethanol at 5°C in Eppendorf tubes or universal bottle (28 ml), until all measurements were complete. Roots were placed on a Petri dish and photographed with a CCD camera (Nikon DMX 1200, Nikon Instr. Inc, USA). The total length of the root system and the length of each root order were measured using the NIH Image Analysis Software (http:rsb.info.nih.gov/nih-image).

Root hairs on each seedling were counted and measured using the NIH Image Analysis Software, as follows. All roots in the root system were examined and root hairs along the whole length counted. Counting was achieved by mounting sections, approximately 1 cm long, on glass slides with glycerol. A calibrated digital image of each root segment was captured using a Nikon CCD camera (Nikon DXM 1200, Nikon Instruments Inc, USA), coupled to a compound microscope (Nikon E6000 Eclipse) applying ×40 total magnification. The root segment was flattened and thus root hairs could be observed at the two sides of the root image. The root hair digital images were traced with a free hand selection tool (http:rsb.info.nih.gov/nih-image/manual) and the root hairs counted.

The number and position of the nodules was assessed visually using the naked eye and binocular microscope when required. Prior to estimation of colonisation by AMF all roots had been stored in 50% (*v*/*v*) ethanol at 5°C and separated into different orders for architecture and root hair studies. For estimation of AMF, a 40% stratified random sample of roots (from all root orders) was used. Roots were incubated in 10% (*v*/*v*) KOH at room temperature for 24 h, rinsed with distilled water before staining with 0.05% (*w*/*v*) Trypan Blue in acidic glycerol over night. Stained samples were examined with binocular and compound microscopes and estimations of AMF colonisation made using a gridline intersect method. The stained roots were also used for the root hair measurements since the staining process did not damage the root hairs.

Plant material was dried at 78°C until constant weight and shoots and roots (after the completion of all the other measurements) weighed separately before combining for analysis of P and N content. Roots used for the estimation of AMF colonisation were not used for N and P measurements. Tissues were milled to <2 mm and digested with a mixture of H_2SO_4, HNO_3 and $HClO_4$. P was determined spectrophotometrically using the ammonium molybdate colorimetric method. N was determined using the Kjeldahl method.

Data analysis was conducted using one-way analysis of variance (ANOVA). When means were not equal, i.e. ANOVA identified a significant difference between them, Dunnett's procedure for multiple comparisons was applied to test which means were significantly different from the relevant control group and Tukey's procedure applied to pairs of means to test which means were significantly different from each other. All the data analysed followed a normal distribution.

Results

Plant growth

The precise effect of nodulation on plant dry weight depended upon the timing of inoculation and the length of the growth period, both before and after inoculation. Thus, the dry weights of the seedlings inoculated with *Frankia* 15 and 30 days after AMF inoculation (at day 0) were progressively greater than those co-inoculated. The degree of plant development when inoculated with *Frankia* affected the response of plants, so that the dry weights of the plants inoculated at day 30 recovered from the growth stress imposed by dual inoculation 20 days after inoculation while dry weights of the plants co-inoculated (day 0) or inoculated at day 15 were similar or less than the control plants, even 30 days after *Frankia* inoculation (Fig. 1). In detail, inoculation with AMF alone resulted in growth depressions in the early stages of growth but after 60 days there was an increase in growth of 20%. Inoculation with *Frankia* immediately or after 15 days increased growth initially by 20%, but then there was a reduction before growth recovered again. Delaying inoculation for 30 days resulted in a reduction in growth followed by an increase, but after growing plants for 30 days there was no effect on growth. Inoculating seedlings with AMF and *Frankia* together or with *Frankia* after a 15-day delay resulted in growth reductions of over 50% but when inoculation with *Frankia* was delayed for

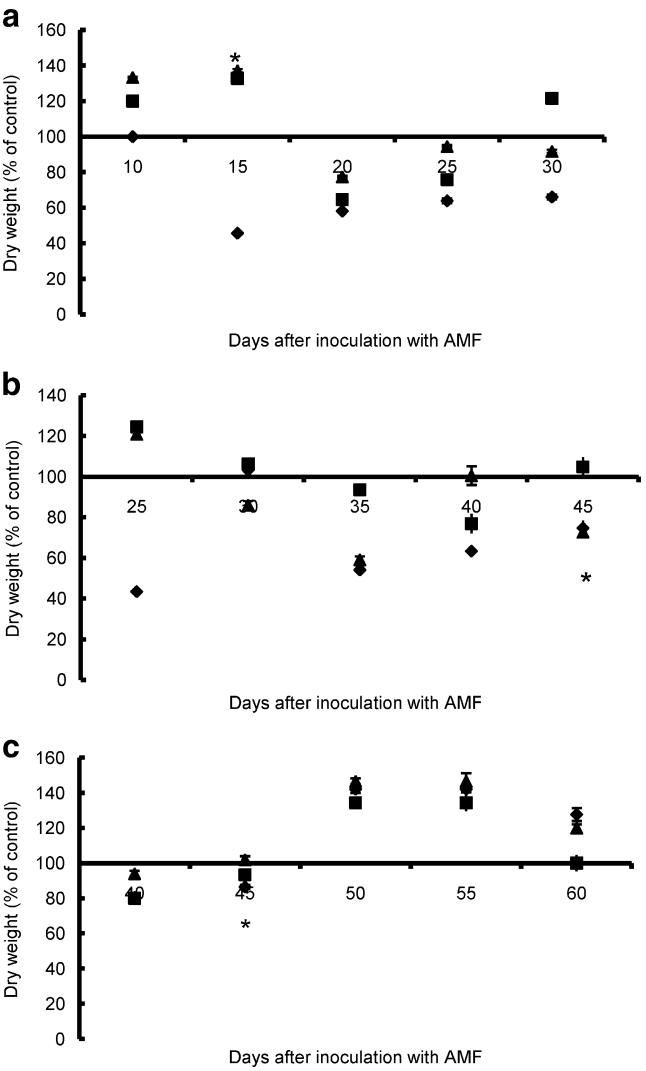

Fig. 1 *A. glutinosa* seedling roots colonised by AMF following inoculation with AMF alone (*filled upright triangle*), *Frankia* (*filled square*) or with AMF and *Frankia* (*filled diamond*) immediately or after a delay of 15 or 30 days. Seedlings in each treatment were harvested after 10, 15, 20, 25 and 30 days growth. Figures are the means of six replicate plants. Data points marked with an *asterisk* are not significantly different from each other ($P<0.05$)

30 days increases in growth of over 40% followed early depressions.

Linear regression analysis of a plot of shoot:root ratios for controls against all inoculated seedlings, irrespective of the time of inoculation and inoculant, gave a correlation coefficient of 0.72 and a value for the slope of regression line of 0.64 (graph not shown here). These data demonstrate that root growth of inoculated seedlings was favoured slightly over shoot development. The phosphorous content of plant material ranged from 0.9 to 1.2 mg g^{-1} dry weight with no significant differences due to treatment or time. Nitrogen content showed a similar trend, with the content of plant material ranging from 0.9 to 1.4 mg g^{-1} dry weight but no significant differences due to treatment or time.

Nodulation

Nodulation of seedlings inoculated with *Frankia* was highly variable (Fig. 2). There are no significant effects of co-inoculation with AMF or timing on nodulation. However, timing of inoculation with *Frankia* was important in determining the order of roots that become nodulated. When seedlings were inoculated with *Frankia* at the beginning of the experiment the data suggest, perhaps not surprisingly, that their first-order roots became nodulated whereas when inoculation was delayed for 15 days more second and third-order roots were nodulated (and no first-order roots). When inoculation was delayed for 30 days, most nodules were located on third-order roots. Thus nodules appear to be concentrated on roots most recently formed. After second- and higher-order roots had formed nodulation of first-order roots was poor compared to roots of higher orders. For example, nodules did not develop on first-order roots of seedlings inoculated with *Frankia* at Day 30 when these roots were thickened and had significant deposits of tannin. In most treatments, second- and third-order roots bore the greatest number of nodules. Nodulation was not detected in non-inoculated seedlings or those inoculated with AMF alone indicating that cross contamination between treatments did not occur.

Colonisation

Colonisation of roots by *G. rosea* was very variable between different root orders, with no trends evident.

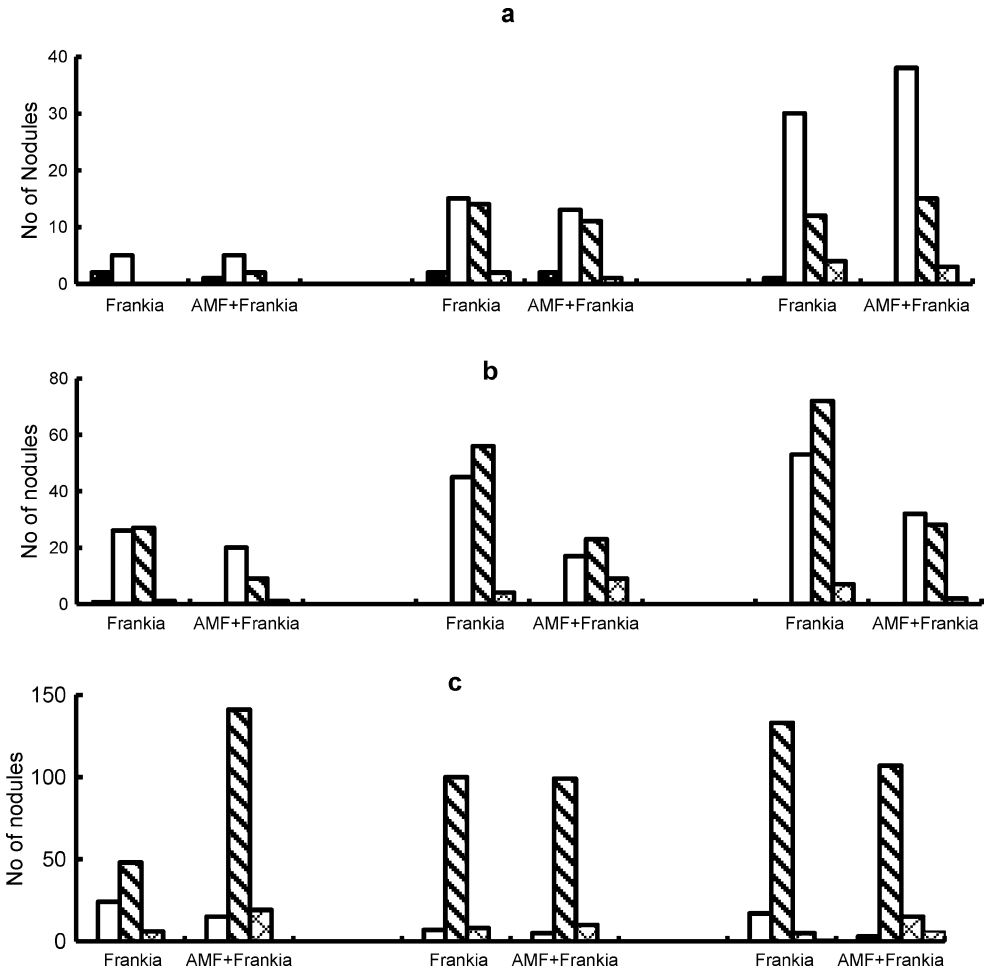

Fig. 2 Nodulation of *A. glutinosa* seedlings following inoculation with *Frankia* alone or with AMF and *Frankia* immediately (**a**) or after a delay of 15 (**b**) or 30 days (**c**). Seedlings in each treatment were harvested after 10, 15, 20, 25 and 30 days after inoculation with *Frankia*. Figures are the means of six replicate plants (first-order roots, *black bars*; second-order roots *open bars*; third-order roots *lines*; fourth-order roots *hatched bars*). Data were too variable to provide statistically meaningful data on a treatment basis and are provided so that general trends can be observed

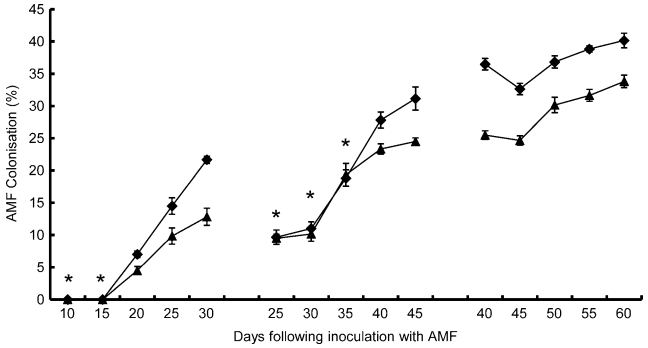

Fig. 3 Total number of roots per *A. glutinosa* seedling following inoculation with AMF alone (*filled upright triangle*) or with AMF and *Frankia* (*filled diamond*) immediately or after a delay of 15 or 30 days. Figures are the means of six replicate plants. Data points marked with an *asterisk* are not significantly different from each other ($P<0.05$)

Consequently, data is shown as the mean% colonisation of whole root systems of seedlings inoculated with AMF alone or with AMF+*Frankia* (Fig. 3). Roots of all orders became colonised by AMF, but colonisation was not apparent until approximately 20 days after inoculation. Colonisation increased with time following inoculation and roots inoculated with AMF+*Frankia* became colonised more heavily with AMF than those inoculated with AMF alone. This effect was most apparent in the roots of seedlings inoculated with *Frankia* at the same time as with AMF. Here, roots of dual-inoculated seedlings were colonised at 21.5%; more than 12% higher than roots of seedlings inoculated with AMF alone. AMF colonisation was not detected in non-inoculated seedlings or those inoculated with *Frankia* alone indicating that cross contamination between treatments did not occur.

Root system branching

Effects of nodulation on the development of the root system became more apparent as plants aged (Fig. 4). Thus, seedlings inoculated with AMF or with Frankia + AMF at Day 0 and grown for a further 30 days had significantly greater numbers of roots 30 days following inoculation.

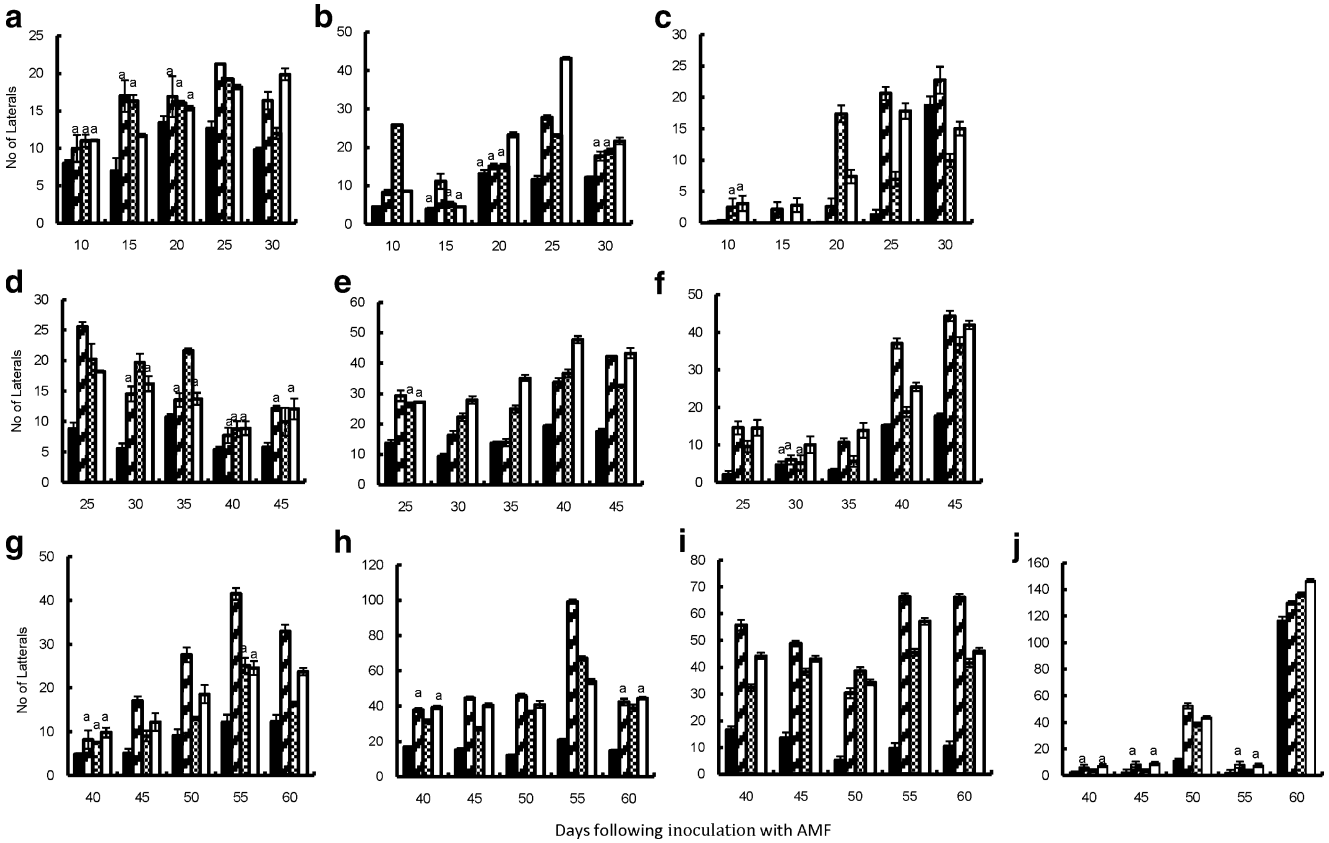

Fig. 4 Numbers of lateral roots of *A. glutinosa* seedlings following inoculation with AMF alone or with AMF and *Frankia* immediately (**a** first-order, **b** second-order, **c** third-order) or after a delay of 15 days (**d** first-order, **e** second-order, **f** third-order) or 30 days (**g** first-order, **h** second-order, **i** third-order, **j** fourth-order). Seedlings in each treatment were harvested after 10, 15, 20, 25 and 30 days growth. Figures are the means of six replicate plants (controls *black bars*, AMF alone *lines*; *Frankia grey bars*, AMF and *Frankia open bars*). Data points marked with the *same letter* are not significantly different from each other ($P<0.05$)

Similarly, when seedlings were inoculated with *Frankia* after 30 days, and grown for a further 30 days, the root numbers of inoculated seedlings were up to 200% greater than those of controls. By contrast, when seedlings were inoculated with *Frankia* after 15 days and grown for a further 30 days root numbers of inoculated seedlings were not significantly different from the non-inoculated controls. Figure 4 shows the data for individual root orders. The numbers of first- and second-order lateral roots generally increased on both AMF and *Frankia* inoculated seedlings at all times of inoculation, more second-order reflecting an increased rate of branching of first-order roots and so on. An exception was when seedlings were inoculated with *Frankia* at day 15 and grown for 30 days. In this case, there was a decrease in the number of first-order roots of more than 60% over the 30 days of growth following inoculation.

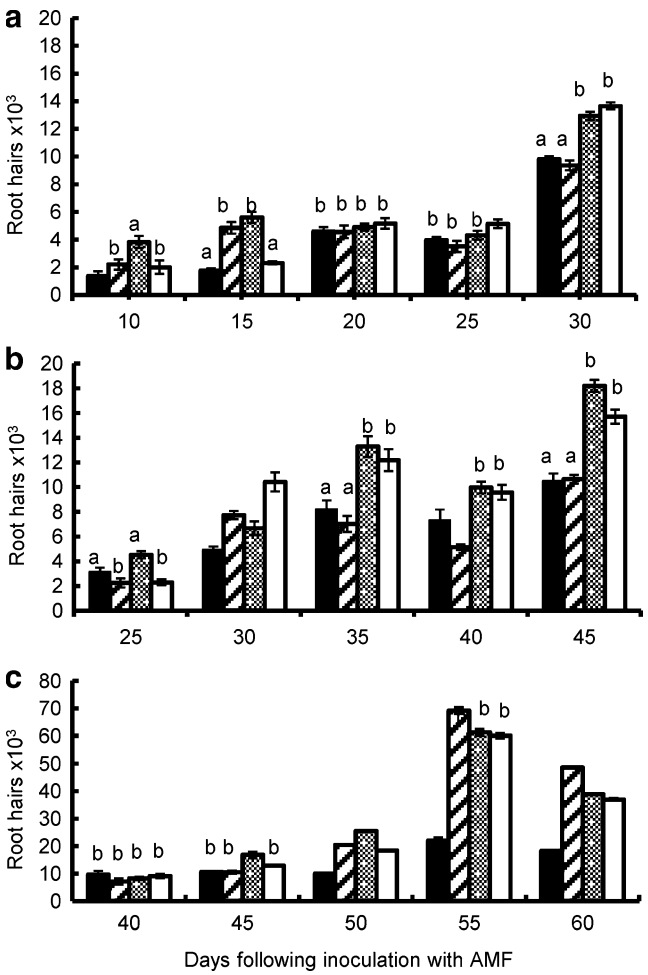

Fig. 5 Total numbers of root hairs on roots of *A. glutinosa* seedlings colonised by AMF following inoculation with AMF alone or with AMF and *Frankia* immediately or after a delay of 15 or 30 days. Seedlings in each treatment were harvested after 10, 15, 20, 25 and 30 days growth. Figures are the means of six replicate plants. Data points marked with an *asterisk* are not significantly different from each other ($P<0.05$)

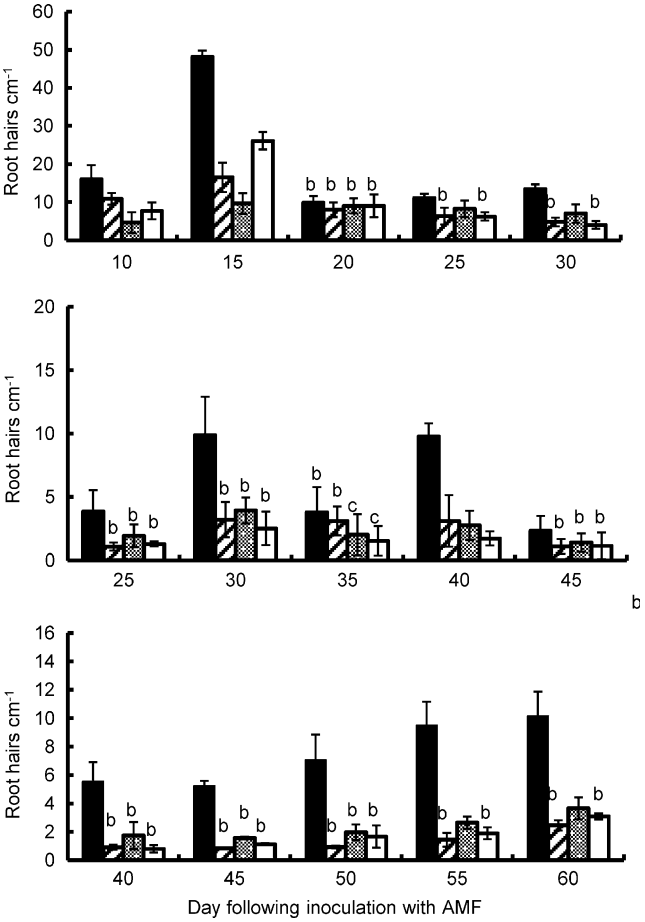

Fig. 6 Density of root hairs on roots of *A. glutinosa* seedlings colonised by AMF following inoculation with AMF alone or with AMF and *Frankia* immediately or after a delay of 15 or 30 days. Seedlings in each treatment were harvested after 10, 15, 20, 25 and 30 days growth. Figures are the means of six replicate plants (controls *black bars*, AMF alone *lines*, *Frankia grey bars*, AMF and *Frankia open bars*). Data points marked with the *same letter* are not significantly different from each other ($P<0.05$)

Numbers of third-order roots generally increased over the 30-day period following inoculation with *Frankia* at day 0 and after 15 days but numbers remained relatively constant in seedlings inoculated after 30 days. As seedlings aged, third-order roots became the largest contributor to the root system, with the total number on (30 day) inoculated seedlings after 30 days growth more than five times those of controls. At this time, significant but similar numbers of fourth-order roots were present on roots of both inoculated and control seedlings, but few or no fourth-order roots were present on seedlings younger than this.

Root hair development

Inoculation with AMF and *Frankia* alone or together resulted in an increase in the total number of root hairs (Fig. 5) but a substantial reduction in root hair density (Fig. 6); the extent

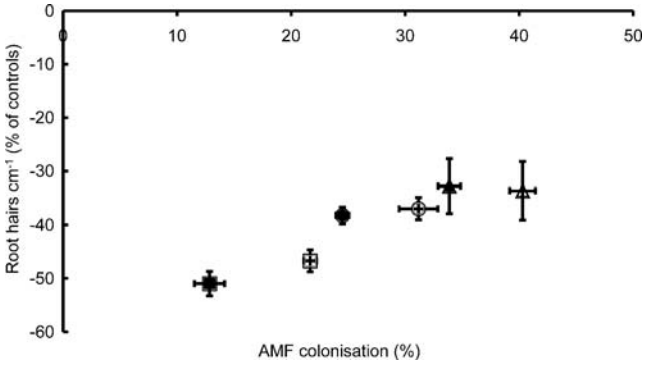

Fig. 7 Density of root hairs on roots of *A. glutinosa* seedlings colonised by AMF following inoculation with AMF alone (*filled*), *Frankia* alone (*open*) or with AMF and *Frankia* immediately (*square*) or after a delay of 15 (*circle*) or 30 days (triangle). Seedlings were harvested following 30 days growth. Figures are the means of six replicate plants. Data points marked with an *asterisk* are not significantly different from each other ($P<0.05$)

of reduction was variable with the most substantial reduction, of 85%, measured for seedlings inoculated with *Frankia* after 30 days and then grown for 25 days (55 days from AMF inoculation) and the same reduction was measured for seedlings inoculated with AMF at the same time point. In general, reductions were more consistent and greater when seedlings had been grown for longer periods. There was no correlation between the density of the root hairs and root system length for the uninoculated controls, AMF, *Frankia* or *Frankia*+AMF-inoculated seedlings (R^2 of 0.0042, 0.016, 0.0195 and 0.0003, respectively) showing that differences in root system size did not explain the differences. Comparison of % AMF colonisation with reduction in numbers of root hairs (Fig. 7) showed a significant negative correlation. A similar correlative comparison for *Frankia* was not possible due to variability in nodulation. However, AMF+*Frankia* showed the same correlation.

Discussion

These data demonstrate clearly that *Frankia* and AMF singly and in combination, can enhance the growth of *A. glutinosa* seedlings, but that reductions in growth can also occur; the precise impact dependent on the state of seedling development at inoculation, timing of inoculation and growth period. The impact of timing of inoculation and early depression of growth followed by growth enhancements has been reported previously by Orfanoudakis et al. (2004) in studies with the AMF, *Frankia*, *A. glutinosa* tripartite symbiosis. Moreover, in this previous study, the effects of time were similar to that reported here, with growth depressions 30 days following inoculation alleviated after 60 days when the seedlings had developed a shoot system with a greater photosynthetic capacity. Beneficial effects on growth have also been reported in previous studies with *Frankia* and AMF in *A. glutinosa* (Isopi et al. 1994) but there are two important factors that distinguish our findings and make them of particular interest. Firstly, substantial enhancements to growth resulted from inoculation, even though colonisation by AMF remained under 45%, and in most cases much lower than that (Fig. 2). Colonisation was thus much lower than usually observed in many typically mycotrophic species, e.g. leek or strawberry, but significant increases in growth were still observed; which suggests an effective symbiosis. Secondly, improvements in growth were achieved even when the seedlings were growing in a fertile growth medium, with phosphorus at 240 ppm and nitrogen 83 ppm; levels at which additional phosphorus and nitrogen would be unlikely to bring growth benefits. This suggests that the growth benefits observed in this study are unlikely to be due to nutritional benefits conferred by the symbioses. Further evidence for this hypothesis is provided by the plant nutrient data which showed no differences in plant tissue phosphorus or nitrogen as a result of inoculation, indicating that benefits are due to other factors. It has been hypothesised that benefits of AMF to trees frequently result from the promotion of seedling establishment through impacts on root system development (Hooker and Atkinson 1996); often very important in the early stages of tree growth under natural conditions. There is already evidence for AMF altering the development of root systems of a range of plants, including trees, usually producing a more branched system, (Berta et al. 1995). In this study, it is shown to occur also for *A. glutinosa*, following colonisation with *G. rosea*. The increases in branching reported here are not dissimilar to those reported previously for tree seedlings (Hooker et al. 1992) and it is interesting that these increases occur at such high phosphorus levels; providing further important evidence to support the hypothesis that AMF-induced changes to branching are, at least in part, mediated by non-nutritional factors. The increased allocation of resources to the root that were observed was not unusual and was measured frequently in studies where plants are inoculated with AMF (Chaia and Raffaele 2000).

What this study also demonstrates, and for the first time, is that infection by *Frankia* (nodulation) can also result in changes to root branching, of a similar magnitude to those induced by AMF, e.g. increases of up to five times in some root orders (Fig. 4). Ecologically, this is a very important finding because it suggests that as well as having a direct impact on the establishment and growth of the plant through symbiotic nitrogen fixation *Frankia* is also able to impact on the seedling indirectly by enhancing the capacity of the root system to access other nutrients and water in the soil. The combined impact of these changes is likely to facilitate significantly the survival and competitive advantage of

seedlings; particularly in nutrient- and water-deficient environments. Moreover, because roots colonised by *Frankia* are more branched and thus shorter lived, fluxes of nitrogen and carbon to the soil are also likely to be larger and occur more rapidly. From a nitrogen cost perspective, this would not be an issue as a plant nodulated by *Frankia* could fix all the nitrogen it required. However, if carbon was in short supply; under a forest canopy or in winter, for example, the costs could begin to outweigh the benefits.

The extent of changes to root architecture as a result of colonisation by AMF and the likely consequences for function have been reviewed (Berta et al. 2002). Hypotheses have been proposed for mechanisms through which AMF modify (usually increase) root branching including plant hormones, nutrients and changes to cell cycles (see Hooker and Atkinson 1996). The data in this study provide further evidence that nutrients are not entirely responsible for AMF-induced changes to root architecture but no conclusive evidence for other responsible factors. However, there have been some interesting reports identifying factors responsible for controlling root branching in *Arabidopsis* that, put together with the evidence presented here for *Frankia* nodulated seedlings, provide some clues to help identify the mechanisms involved. Two lines are of interest. The first is the effects of sugar to nitrogen ratios. Malamy and Ryan (2001) discovered that when *Arabidopsis* was grown on high ratios of sucrose to N lateral root initiation was reduced. However, when ratios of C to N were lowered, lateral root initiation, and thus branching, was restored. They suggested that the C to N ratio acts as a signal that determines root branching. However, Zhang et al. (2007) suggested it is also possible that nitrate itself could be a signal; with high sucrose and low nitrate leading to a rapidly depleted nitrate supply because of high metabolic activity driven by the high sucrose supply. If this were the case, the evidence leads us to hypothesise that *Frankia* could be depleting internal sucrose levels, through utilising plant photosynthate or enhancing endogenous nitrate levels; in both ways reducing the C:N ratio and thus enhancing branching. Another possibility is the effect of L-glutamate. In recent studies with *Arabidopsis*, Walch-Liu et al. (2006) discovered that the presence of this common organic form of nitrogen external to the root resulted in the growth of a more branched root system. Glutamate comprises 25% of the α-amino N of the nodules of *A. glutinosa* (Wheeler and Bond 1970) and although there is no evidence for significant exudation of N from nodules circulation of the products of nitrogen fixation is now held to be an important factor in regulation of nodule activity (Parsons et al. 1993).

Root hairs are involved in a wide range of different processes in plants, including infection by pathogens and symbiotic organisms such as AMF and *Frankia* and by extending the root system, facilitating the uptake of nutrients and water. The finding in this study, therefore, that AMF and *Frankia* reduced the density number of root hairs on *A. glutinosa* by up to 85% (Fig. 5), is an important discovery. This is the first report on the effects of either AMF or *Frankia* on the development of root hairs and thus represents a step change in understanding of the organismal and ecosystem level impacts of these symbioses. Importantly, there is strong evidence that effects of AMF and *Frankia* on root hair density are independent of effects on plant or root system size. Evidences for this are: the lack of any correlation between the density of root hairs and root system length, comparison of data in Fig. 4 with Fig. 5 showing that even when total numbers of root hairs in controls or inoculated seedlings are similar, differences between numbers of root hairs per unit length are maintained; that although seedlings, when inoculated, were eventually larger, most plants where reductions in root hair density was measured were much younger than this, and thus the same size or smaller than the uninoculated controls.

One factor that is known to increase the frequency of root hairs in *Arabidopsis thaliana* is low levels of phosphorus (Ma et al. 2001; Müller and Schmidt 2004) and so one potential explanation for AMF decreasing root hair density in this study is increased phosphorus levels, although this would not explain the effects of *Frankia.* However, this study was designed to minimise as far as possible nutritional impacts of the symbioses. This was manipulated by the high nutrient levels in the growth substrate already discussed and validated by the lack of differences between *A. glutinosa* tissue concentrations of phosphorus and nitrogen. Our data would thus appear to rule out increased phosphorus as a mechanism and suggest a non-nutritional basis for the measured effects on root hair density. However, given the very significant reductions measured, it is reasonable to hypothesise that the reduction in root hair development may in some way offset some of the carbon costs incurred by the symbioses. If these data demonstrating a reduction in root hair numbers induced by AMF *G. rosea* on *A. glutinosa* are typical of an AMF–host response, then this is a very significant discovery, with implications for our understanding of how AMF interacts with their plant hosts. Interestingly, if confirmed, such a discovery would shed interesting new light on the Baylis hypothesis. That arbuscular mycorrhizal plants have relatively fewer root hairs could be because of the presence of the fungus not, as originally proposed, its natural state.

To conclude, the root system of *A. glutinosa* has displayed in this study a remarkable degree of plasticity in response to colonisation by AMF or *Frankia*. Partitioning of resources to different types of roots and root hairs changed markedly in response to colonisation and nodulation, with likely impacts on the carbon costs of root system building and maintenance and the function of the root system.

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