Web Ecology 7: 77–86.

Nutritional effects of indigenous arbuscular mycorrhizal associations on the sclerophyllous species *Agathosma betulina*

K. J. Cloete, A. J. Valentine, L. M. Blomerus, A. Botha and M. A. Pèrez-Fernández

Cloete, K. J., Valentine, A. J., Blomerus, L. M., Botha, A. and Pèrez-Fernández, M. A. 2007. Nutritional effects of indigenous arbuscular mycorrhizal associations on the sclerophyllous species *Agathosma betulina*. – Web Ecology 7: 77–86.

Relatively little is currently known about the seedling physiology of arbuscular mycorrhizal (AM) *Agathosma betulina*, a sclerophyllous crop plant cultivated for its high-value essential oils and food additives. In addition, virtually nothing is known about the AM associations of this plant. Consequently, the effect of an indigenous community of AM fungi on P nutrition and C economy in seedlings, grown in nursery conditions, was determined during different stages of host and AM fungal establishment. AM fungal ribosomal gene sequence analyses were used to identify some of the fungi within the roots, responsible for the nutritional changes. During the early stages of host and AM fungal establishment (0 to 77 days after germination), host growth was reduced, whereas the rate of P-uptake and growth respiration was increased. Beyond 77 days of growth, the rate of P-uptake and growth respiration declined. These findings, together with results obtained after molecular analyses of root associated fungal DNA, indicate that AM fungi belonging to the genera *Acaulospora* and *Glomus*, improve P-uptake and costs of utilization during the early stages of seedling establishment in a nutrient-poor soil.

– K. J. Cloete and A. Botha, Dept of Microbiology, Univ. of Stellenbosch, Private Bag X1, Matieland, SA - 7602, South Africa, – A. J. Valentine, South African Herbal Science and Medicine Inst., Faculty of Science, Univ. of the Western Cape, Private Bag X17, Bellville, SA - 7535, South Africa, – L. M. Blomerus, Agricultural Research Council-Roodeplaat, Vegetable and Ornamental Plant Inst., Private Bag 5026, Stellenbosch, SA - 7599, South Africa, – M. A. Pèrez-Fernández (maperfer@upo.es), Ecology Area, Faculty of Exp. Sciences, Univ. Pablo de Olavide, Carretera a Utrera, ES - 41013, Seville, Spain.

Agathosma betulina (syn. *Barosma betulina*), a member of the *Rutaceae*, is a slow-growing, sclerophyllous scrub which is endemic to the mountains of the southwestern Cape, South Africa, situated in a Mediterranean climatic region (Spreeth 1976, Versfeld et al. 1992, Collins et al. 1996, Lubbe et al. 2003). The plant is part of the Fynbos vegetation type within the Cape Floristic Region (CFR) and is cultivated for its essential oils which find application in the food, cosmetic and pharmaceutical industries (Collins et al. 1996, Lis-Bachin et al. 2001, Lubbe et al. 2003). Plantations of *A. betulina* are mostly obtained by transplanting five-month-old seedlings, germinated in nurseries, to the field.

A. betulina mainly thrives in podzolic soils derived from quartzites (Spreeth 1976, Kruger 1979). These acidic sandy soils, with a pH ranging from 3.5 to 5.5, are strongly leached, while the nutrients are bound in detritus resulting in oligotrophic soil conditions (Kruger 1979, Stock and Allsopp 1992). The organic C content of these soils were found to be ca 1%, while the available P concentrations may be as low as 3 to 40 μg per gram soil (Kruger 1979, Fry 1987). CFR plants have adapted to these conditions by forming mutualistic symbioses with arbuscular mycorrhizal (AM) fungi (Allsopp and Stock 1993b). Surveys of the AM status of plants in the CFR revealed that 62% of indigenous sclerophyllous shrubs form arbuscular mycor-

Accepted 24 September 2007

ISSN 1399-1183

rhizae (Allsopp and Stock 1993a). However, as AM fungi are patchily distributed in Fynbos soils, soil inoculum levels may limit initial colonization, but once colonization occurs, roots rapidly become AM (Allsopp and Stock 1994).

Arbuscular mycorrhizal fungi may supply up to 80% of the P requirements of the host plant by increasing the absolute volume of soil available to the plant (Marschner and Dell 1994). However, for some plant and fungal species, there is a positive correlation between the effectiveness of AM nutrient uptake and the demand for host-derived organic C by the AM fungus (Mortimer et al. 2005). Therefore, the net benefit of the AM symbiosis to the plant depends on the benefit derived from uptake of mineral nutrients against the cost of maintaining the mycosymbiont. The latter is calculated in terms of the C supplied by the plant, which is transported below ground due to the sink effect of the AM fungus (Kucey and Paul 1982, Snellgrove et al. 1982, Koch and Johnson 1984, Jakobsen and Rosendahl 1990). A factor that contributes to this sink effect is the higher respiration rate and hence metabolic activity of AM colonized roots compared to uncolonized roots (Pearson and Jakobsen 1993). It must also be noted that AM species are functionally distinct in, for example, P-uptake and this may in turn impact on the net benefit of the AM association to the host (Jakobsen et al. 1992). Thus, AM fungal inoculation may have a parasitic or mutualistic effect on plant growth (Klironomos 2003). Although AM fungi are not currently used in the propagation of this plant, AM fungi native to the plant's roots would be the preferred fungi to be used as AM inocula in nurseries for the large scale propagation of this plant. However, so far very little is known about the identity of these fungi or their nutritional effects on *A. betulina* during seedling development.

We hypothesize that AM fungi modulate host P nutrition and C economy at different stages of host and AM fungal establishment. The aim of the study was thus to evaluate the effect of indigenous AM fungi on plant growth and C economy as well as the C-costs of P-uptake at different stages of host and AM fungal establishment within a controlled environment. The second part of the study was aimed at determining the identity of indigenous AM fungi able to colonize the roots of *A. betulina* in this environment.

Material and methods

Rhizosphere soil and root sampling

Rhizosphere soil samples (ca 400 g each), as well as root samples were randomly taken from *A. betulina* within pristine Fynbos vegetation at a sampling site comprising an area of ca 500 m^2, situated on the southern slopes of the Piketberg mountain, at an altitude of 334 to 668 m (32°45′S, 18°45′E). The soil samples were collected at depths ranging from 20 to 45 cm, air dried, sieved using a 2 mm mesh, and pooled resulting in a composite sample consisting of ca 7 kg soil. The bulk of the composite sample was subsequently subjected to cold shock treatment at 4°C for 6 weeks to enhance the germination of AM spores dormant at the time of field collection (Vimard et al. 1999).

Plant growth

Pot cultures were prepared by planting germinated *A. betulina* seeds in free-draining plastic pots, each containing 650 ml potting mixture. The seeds were surface sterilized with 0.05% (w/v) sodium hypochlorite and germinated according to Blommaert (1972). The potting mixture for AM plants (n = 50) consisted of the composite soil sample containing indigenous populations of AM fungal propagules and *A. betulina* roots (healthy AM spores g^{-1} soil: 250 ± 42; AM root colonization levels: 37 ± 2.89%) and autoclaved inert silica sand (grain size 0.5 mm) mixed in a 1:3 ratio. Control non-AM plants (n = 50), prepared in a similar manner, except that the soil was gamma-irradiated [minimum absorbed dose, 25 kGy (kGy = 0.1 megarad) per kg soil], were also included in the experimentation. To reduce potential phytotoxic effects, the irradiated soil was allowed to equilibrate at room temperature for one week before planting (Bryla and Duniway 1997). Reconstitution of the associated soil microflora, without AM propagules, in the controls was done during planting by the addition of 100 ml soil filtrate (Schubert and Hayman 1986), prepared by suspending 50 g rhizosphere soil in 1 l of distilled water and filtering it using a sieve (pore diameter, 53 μm). The potting mixtures had a pH of ca 5.

Pots were arranged in a random-block design on benches in a well-ventilated glasshouse with 14 h photo-periods (photosynthetic photon flux density 1000 to 1100 μmol m^{-2} s^{-1}). The average day/night temperatures and relative humidities were 23/15°C and 50/80% respectively. The plants were wetted to field capacity (ca 40 ml) on a weekly basis with half-strength Long Ashton nutrient solution, pH 6 (Hewitt and Smith 1975) containing 0.026% N and 0.0047% P, representative of the N and P concentrations in the field (Kruger 1979, Fry 1987).

Harvesting and nutrient analyses

Harvesting took place after 77, 157 and 224 days of growth, which represented the different phases of host and fungal establishment. Plants (n = 4) were randomly harvested and separated from the potting mixture using a gentle spray of tap water and patted dry with paper towels. Random samples of harvested roots (n = 4) were taken

prior to drying for the determination of AM colonization. The harvested material was dried at 80°C for one week, after which the dry weight (DW) was measured. The dried plant material (shoots and roots) was milled using a 0.5 mm mesh. Milled samples were analysed for their respective C, N and P concentrations, using the method described by Allison (1965), a LECO FP528 Nitrogen Analyzer with Spectrascan standards and a Vista inductively coupled plasma-atomic emission spectrometer (Munter and Grande 1981), respectively.

Staining of intraradical AM structures

Root samples were obtained by cutting the sampled root material (n = 4) into 1 cm strips and randomly selecting 1 cm root lengths for fixation in 50% (v/v) ethanol. Each root sample fixed in 50% (v/v) ethanol, was carefully rinsed in distilled water and stained using 0.05% (w/v) aniline blue (Sigma, color index no. 42755) or 1% (w/v) chlorazol black E (Sigma; color index no. 30235, Brundrett 1994a). The % AM colonization was determined using the grid-line intersect method (Brundrett 1994b).

Calculations

(a) Relative growth rate (RGR; mg g^{-1} day^{-1}): RGR over the study period was determined by measuring the change in biomass, relative to the initial biomass.

$$RGR = (H2 - H1/H3) / T2 - T1 \quad (1)$$

where T2 – T1 represents the time period (days) between harvesting periods, with H2 representing the final dry mass (mg), H1 the initial dry mass (mg) and H3 the initial dry mass in grams (g) of the harvested plant material.

(b) P-uptake rate (mol P mg^{-1} day^{-1}) is the rate at which P was taken up by roots into the whole plant: the rate of P-uptake for each growth period was calculated from the changes in P concentration (mol P g^{-1}) divided by the RGR (mg g^{-1} day^{-1}), to represent the P inflow that was required during a particular growth period.

(c) Efficiency of P utilization (ΔC: ΔP) is the C cost of the whole plant associated with the uptake of P, and was expressed as described previously (Koide and Elliott 1989). The quantity of C accumulated in the plant divided by the quantity of P accumulated in the plant was calculated for a given period of time:

$$\Delta C^{r}: \Delta P^{r} \quad (2)$$

where ΔC^{r} is the total C accumulated in the plant over a given time period and ΔP^{r} is the total P accumulated in the plant over the same time period. It should be noted that a low efficiency value indicates that less C is required for the given amount of P utilized by the plant.

(d) Root growth respiration (mmol CO_2 g^{-1} DW) represents the C respired for the biosynthesis of new tissue and was modified from Peng et al. (1993) as previously described by Mortimer et al. (2005):

$$Rg(w) = Rg(t)\ (i)/root\ gr \quad (3)$$

where Rg(w) represents growth respiration based on DW, while root gr is the root growth rate (g DW day^{-1}).

Rg(t) is the daily growth respiration (μmol CO_2 day^{-1}): (i) $Rg(t) = C_t - \Delta W_c$ where C_t (μmol CO_2 day^{-1}) is the C required for daily construction of new tissue, while the change in root C content, ΔW_c (μmol day^{-1}) was calculated by multiplying the root C content and the root growth rate.

The variable, C_t was calculated by multiplying the root growth rate by tissue construction cost (mmol C g^{-1} DW), modified from an equation previously described in literature (Peng et al. 1993):

$$Cw = \{C + [(kN/14) \times (180/24)]\}\ (1/0.89)(6000/180)$$

where Cw is the construction cost of the tissue (mmol C g^{-1} DW), C is the carbon concentration of the tissue (mmol C g^{-1} DW), k is the reduction state of the N substrate in the nutrient solution (NH_4 was used, therefore k is +3) and N is the organic nitrogen content of the tissue (g^{-1} DW) (Williams et al. 1987). The constant (1/0.89) represents the fraction of the construction cost that provides reductant that is not incorporated into biomass (Williams et al. 1987, Peng et al. 1993) and (6000/180) converts units of g glucose g^{-1} DW to mmol C g^{-1} DW.

Experimental design and statistical analyses

The treatments were composed of the factors inoculation and harvest times and 4 replicates were used for each treatment. All the percentage data were arcsine transformed (Zar 1999) prior to statistical analysis. For roots, the differences in % AM colonization between harvests (n = 4 for each treatment) were assessed by means of post hoc Student Newman Kuels multiple comparison test, Statgraphics ver. 7 ($p \leq 0.05$). For each harvest, the difference between the means of AM and non-AM plants was separated using a Student's t-test, Statistica ver. 6.0 for independent samples by groups ($p \leq 0.05$).

Genomic DNA extraction and amplification of nuclear rRNA genes

Genomic DNA was extracted according to Hoffman and Winston (1987) from pooled *A. betulina* pot cultured root

samples (n = 5). The DNA samples (n = 5) was then purified using Microspin S-300 HR columns and subjected to nested PCR.

A nested PCR procedure was subsequently used to amplify a fragment of the nuclear rRNA gene complex, that includes a section of the small subunit (SSU) or 18S rRNA gene, the internal transcribed spacer region (ITS1, ca 113 to 121 bp), 5.8S rRNA gene (ca 161 bp) and ITS2 region (ca 222 to 230 bp; Lloyd-Macgilp et al. 1996). Amplification of the above-mentioned nuclear rRNA genes was performed in a GeneAmp PCR system 2400 in a total volume of 25 µl containing 0.8 µl Takara EX Taq DNA polymerase (5U μl^{-1}), 0.08 mM dNTP, 20 pmol of each primer, 2.4 mM $MgCl_2$, 80 mM KCl, 16 mM Tris-HCl (pH 8.3), 5 µl formamide [5% (v/v)], and 0.9 µl genomic DNA (10-fold diluted in milliQ water). A similar procedure was followed for control reactions, where an equal volume of milliQ water was added to the reaction mixture instead of the template DNA. The PCR program comprised the following cycles: An initial denaturation step for 5 min at 94°C; 5 cycles (45 s at 94°C; 30 s at 61°C; 2 min at 72°C), and 25 cycles (45 s at 94°C; 30 s at 60°C; 2 min at 72°C). A final elongation of 7 min at 72°C followed the last cycle.

A universal fungal primer pair was used in the first reaction of the nested PCR. These primers were NS5 (5'-AACTTAAAGGAATTGACGGAAG-3'; White et al. 1990) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990). After the first PCR, the reaction mixtures were diluted 1000-fold and used as templates in the next amplification step. The second reaction of the nested PCR was performed with various AM specific primers in combination with universal fungal primers (ITS1 and ITS4). Primer pairs for the second amplification reaction consisted of the universal fungal primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'; White et al. 1990) in combination with the reverse AM specific primer GIGA5.8R (5'-CACATGCTTGAGGGTCAGT-3'; Redecker 2000) and the universal fungal primer ITS4 in combination with each of the following forward AM specific primers (Redecker 2000): ACAU1660 (5'-TGAGACTCTCGGATCGGG-3'), ARCH1311 (5'-TGCTAAATAGCTAGGCTGY-3'), GLOM1310 (5'-AGCTAGGCTTAACATTGTTA-3') and LETC1670 (5'-ATCGGCGATCGGTGAGT-3').

In order to estimate the quantity of PCR product, the amplification products were separated electrophoretically on 1% (w/v) agarose gels, containing ethidium bromide (1 $\mu g\ ml^{-1}$) at 90 mV for 1 h. A 21 kb ladder was used as size marker. Prior to cloning, bands (ca 500 bp) obtained during the second reaction of nested PCR were visualized by UV trans-illumination and excised. Excised DNA fragments were immediately purified by means of a high pure PCR product purification kit.

Cloning and sequencing

The purified PCR products were cloned into the pDRIVE cloning vector by means of the Qiagen PCR cloning plus kit and transformed into EZ chemically competent *Escherichia coli* (XL1-blue) cells. Positive transformants were selected, recombinant plasmid DNA isolated by means of QIAprep spin miniprep kit and the plasmid DNA digested with EcoRI to verify the presence of the expected PCR fragment.

Sequencing was done by the dideoxy chain termination method, using an ABI Prism model 377 automated DNA sequencer and T7/SP6 as sequencing primers. The sequences were analyzed and edited using the PC-based software program DNAMAN ver. 4.13. Homologues to the isolated sequences were identified using the software program BLASTn ver. 2.2.6; Altschul et al. 1997).

Phylogenetic analyses

Only the 5.8S rRNA subunit was used in the phylogenetic analyses. Full length 5.8S nuclear rRNA gene sequences representing families within the phylum Glomeromycota from GenBank and this study, was manually aligned in BioEdit ver. 7.0.1; Hall 1999. The alignment was submitted to TreeBASE [P.I.N.7432]. *Russula turci* was included as an outgroup. Phylogenetic trees were inferred using the parsimony criterion implemented in PAUP ver. 4.0. beta 10, with all characters equally weighted. Nodal support was assessed using 1000 bootstrap replicates (Felsenstein 1985) with simple taxon addition and tree-bisection-reconnection branch swapping.

As inclusion or exclusion of a few characters can greatly affect the bootstrap proportions of maximum-parsimony trees derived from limited datasets (Koper et al. 2004), we also conducted a model-based search by subjecting the aligned 5.8S nuclear rRNA gene dataset to a Bayesian analysis, using Monte Carlo Markov Chains (MCMC) as implemented in the software program MrBayes ver. 3.0. beta 4; Huelsenbeck and Ronquist 2001).

In developing a Bayesian phylogenetic hypothesis, a model of evolution (DNA substitution) needs to be adopted (Baldi and Brunak 2001). Consequently, the software program Modeltest ver. 3.6; Posada and Crandall 1998) was used to select the most suitable model of evolution. Modeltest selected the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with gamma distributed rate variation among sites (Yang 1996) as optimal model for the aligned 5.8S nuclear rRNA gene dataset. This model was thus implemented in MrBayes and the program executed following the same procedures described in Wubet et al. (2003). To test the reproducibility of the results, computations were repeated three times using the parameter for DNA site substitution, a random starting tree and default parameters settings.

Phylogenetic trees were viewed with the software program TreeView ver. 1.6.6.; Page 1996) and edited with CorelDraw 10 for Windows.

Nucleotide sequence accession numbers

Sequences obtained from AM colonized roots of *A. betulina* were deposited in the GenBank data library under the following accession numbers: AY856457 (sequence 1), AY856454 (sequence 2), AY856455 (sequence 3) and AY856456 (sequence 4).

Results

Arbuscular mycorrhizal colonization of pot grown *A. betulina*

All stages of arbuscular mycorrhizae (appressoria, intraradical hyphae, arbuscules, and vesicles) were present at 77 days after inoculation. For the duration of the experiment, none of these structures were detected in the controls (non-AM plants). The growth period 0 to 77 days showed the highest rate of AM colonization (Fig. 1). Following this period, AM colonization leveled off (days 77 to 157) and then declined towards the end of the experiment, after 224 days of growth (Fig. 1). Changes in the percentage arbuscular colonization followed the same trend as the percentage hyphal colonization, while the percentage vesicular colonization constantly increased during the experiment (Fig. 1).

Growth and nutrition

Between 0 and 77 days of cultivation the growth and RGR of AM plants were lower than non-AM plants (Fig. 2a, 3a), which resulted in smaller biomasses of AM plants at the 77 days harvest. From days 77 to 224, the RGR of AM plants increased to similar rates as the non-AM plants (Fig. 3b, 3c). This resulted in higher growth for AM plants at day 157 (Fig. 2b), and an eventual similar biomass to non-AM plants at day 224 (Fig. 2c). Plant P-uptake rate was higher in the AM plants up to day 77 and then declined to equal levels with non-AM plants at days 157 to 224 (Fig. 4a–c).

Respiratory C-costs

The efficiency of P utilization, which expresses the efficiency of C used for P acquisition, was lower in the AM plants than the non-AM plants at days 0 to 77 and days 77 to 157 (Fig. 5a). Subsequent to these growth periods, the efficiency of P utilization for AM plants were significantly

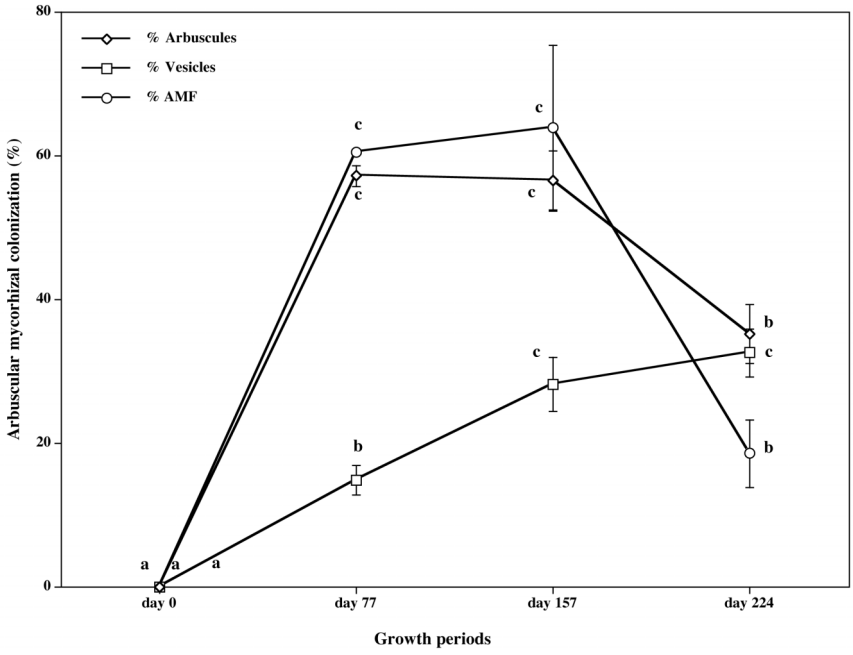

Fig. 1. Percentage (%) root length of *Agathosma betulina* colonized by arbuscular mycorrhizal fungi (% arbuscular-, % vesicular-, and % hyphal colonization) belonging to the genera *Acaulospora* and *Glomus*. The differences between harvests (n = 4) were separated using a post hoc Student Newman Kuels, multiple comparison test ($p \leq 0.05$). Statistically significant differences ($p \leq 0.05$) between each harvest are indicated by different letters. Error bars represent mean ± SE (n = 4).

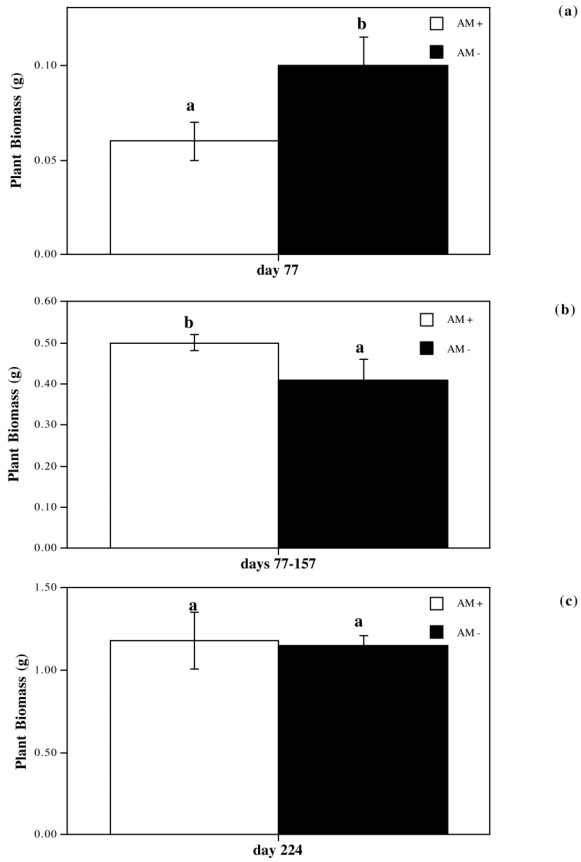

Fig. 2. Plant biomass (g) of *Agathosma betulina* for (a) the growth period 0–77 days, (b) the growth period 77–157 days and for (c) the growth period 157–224 days. Differences between arbuscular mycorrhizal inoculated (+AM) and non-AM (–AM) plants were determined by t-test for independent samples by groups. Statistically significant differences ($p \leq 0.05$) between AM inoculated and non-AM plants for each growth period are indicated by different letters. Error bars represent mean ± SE (n = 4).

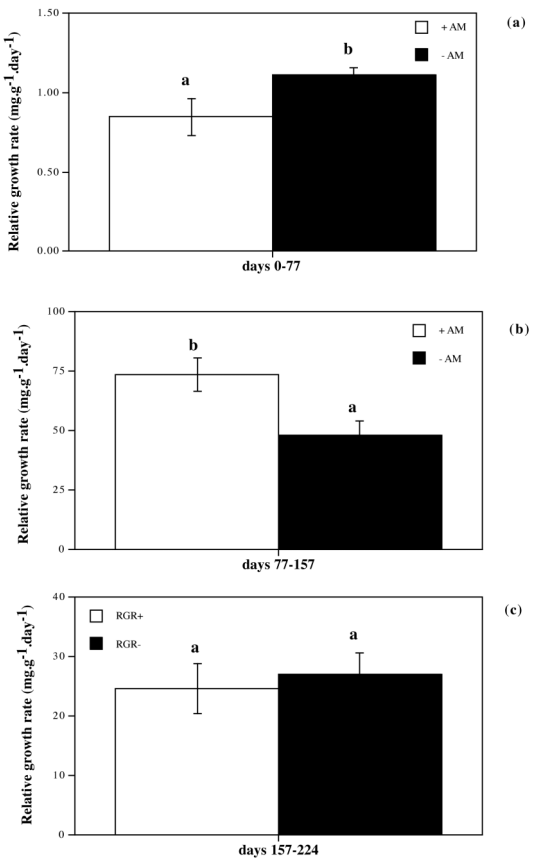

Fig. 3. Relative growth rate (mg g^{-1} day^{-1}) of *Agathosma betulina* for (a) the growth period 0–77 days, (b) the growth period 77–157 days and for (c) the growth period 157–224 days. Differences between arbuscular mycorrhizal inoculated (+AM) and non-AM (–AM) plants were determined by t-test for independent samples by groups. Statistically significant differences ($p \leq 0.05$) between AM inoculated and non-AM plants for each growth period are indicated by different letters. Error bars represent mean ± SE (n = 4).

($p \leq 0.05$) higher, compared to non-AM plants at days 157 to 224 (Fig. 5a). The root growth respiration of AM roots was significantly ($p \leq 0.05$) higher than the non-AM control roots at day 77 (Fig. 5b). However, this higher root growth respiration reached a level equal to non-AM roots at days 157 to 224.

Molecular analyses

Amplification products (ca 1 kb bands) were consistently amplified incorporating the nested primer pairs GLOM1310/ITS4 and ACAU1660/ITS4, whereas negative results were obtained in other nested PCR reactions.

Four sequences were obtained (see Nucleotide sequence accession numbers), that varied in length from 780 bp (*Acaulospora* sp. sequence) to 966 bp (*Glomus* sp. sequences), when the PCR products from the second reaction of nested PCR were cloned and sequenced. A BLASTn search indicated that the sequences were of glomalean origin.

Phylogenetic analyses

Phylogenetic analyses were done to verify the glomalean origin of the 5.8S nuclear rRNA gene sequences obtained during the study and to show the systematic position of these new sequences. We restricted the analyses to full length 5.8S nuclear rRNA gene sequences representing seven AM genera (*Acaulospora, Archaeospora, Entrophospora, Gigaspora, Glomus, Paraglomus* and *Scutellospora*), the representative sequences obtained during this study, and their corresponding closest matches from GenBank.

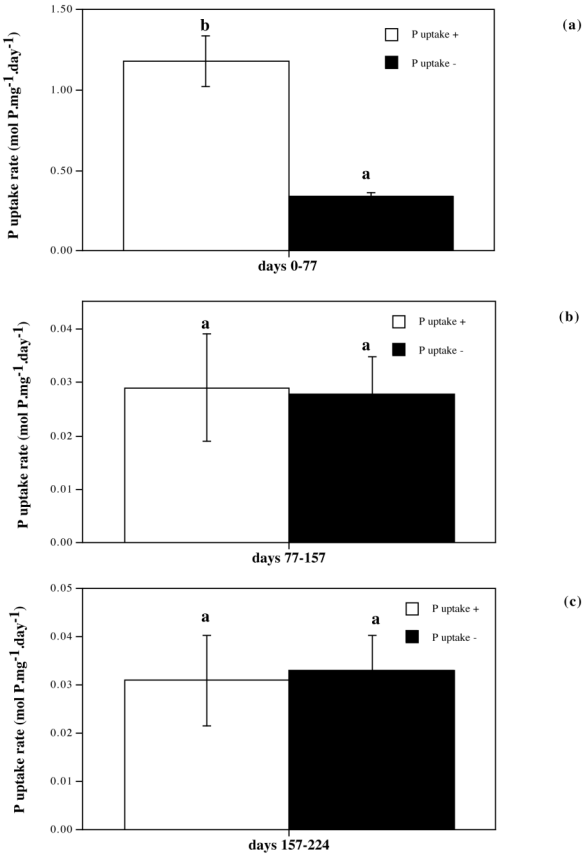

Fig. 4. P-uptake rate (mol P mg^{-1} day^{-1}), of *Agathosma betulina* for (a) the growth period 0–77 days, (b) the growth period 77–157 days and for (c) the growth period 157–224 days. Differences between arbuscular mycorrhizal inoculated (+AM) and non-AM (–AM) plants were determined by t-test for independent samples by groups. Statistically significant differences ($p \leq 0.05$) between AM inoculated and non-AM plants for each growth period are indicated by different letters. Error bars represent mean ± SE (n = 4).

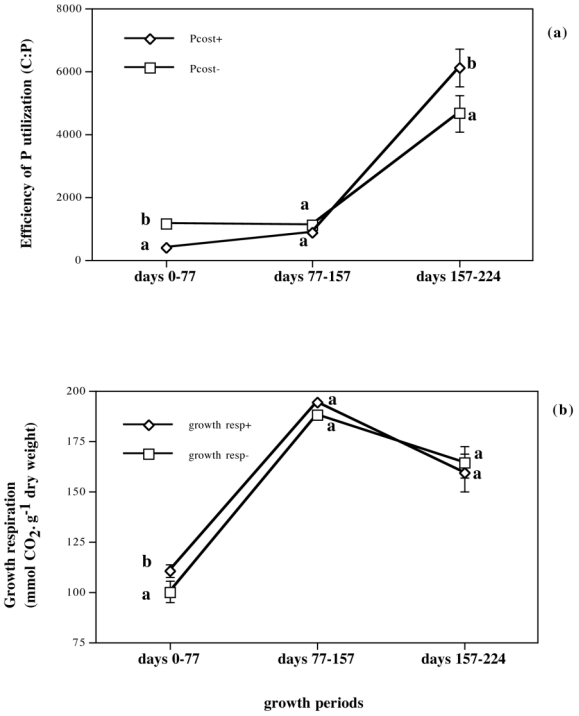

Fig. 5. (a) Efficiency of P utilization (C:P), and (b) growth respiration (mmol CO_2 g DW^{-1}) of *Agathosma betulina* colonized by arbuscular mycorrhizal (AM) fungi. Differences between AM inoculated (+AM) and non-AM (–AM) plants were determined by t-test for independent samples by groups. Statistically significant differences ($p \leq 0.05$) between AM inoculated and non-AM plants for each growth period are indicated by different letters. Error bars represent mean ± SE (n = 4).

The phylogenetic analyses showed that the root-derived sequence 1 clustered within the *Acaulospora* lineage, while sequence 2–4 clustered within the *Glomus* group 1 lineage (Fig. 6). In addition, repeated Bayesian phylogenetic analysis yielded consistent tree topologies, with the Bayesian consensus topology similar to the strict consensus topology obtained from parsimony analysis, although nodal support based on the Bayesian approach was higher.

Discussion

Soil P is considered to be one of the limiting factors for early seedling growth in ecosystems such as Fynbos and the Australian heathlands (Brown and Mitchell 1986). The mobility, and hence bio-availability, of P in these soils is low, which in turn may impact on early seedling growth (Allsopp and Stock 1995). In addition, wild plants rely on the rapid acquisition of nutrients, in excess of immediate needs, during periods of availability (Allsopp and Stock 1993b). This ensures an adequate supply of P to support growth during periods when nutrients are generally unavailable. Low levels of available P present in Mountain Fynbos soil (Kruger 1979) would thus maintain high levels of AM colonization to enhance the efficiency of AM fungal transfer of P to the host (Douds and Schenck 1990).

For a slow growing species such as *A. betulina*, this initial AM dependence on P-uptake may prevail only until the roots are established. This concurs with the current findings, where the initial growth phase (0 to 77 days after germination) was characterized by high AM colonization levels. During the same period, the increased root growth respiration of the AM plants indicated that AM colonization came at increasing C-costs to the hosts (Mortimer et al. 2005). This was evident from the lower RGR recorded for the AM plants, compared to the non-AM plants during this period, and concurs with previous studies of AM colonization C-costs (Cavagnaro et al. 2003, Mortimer et al.

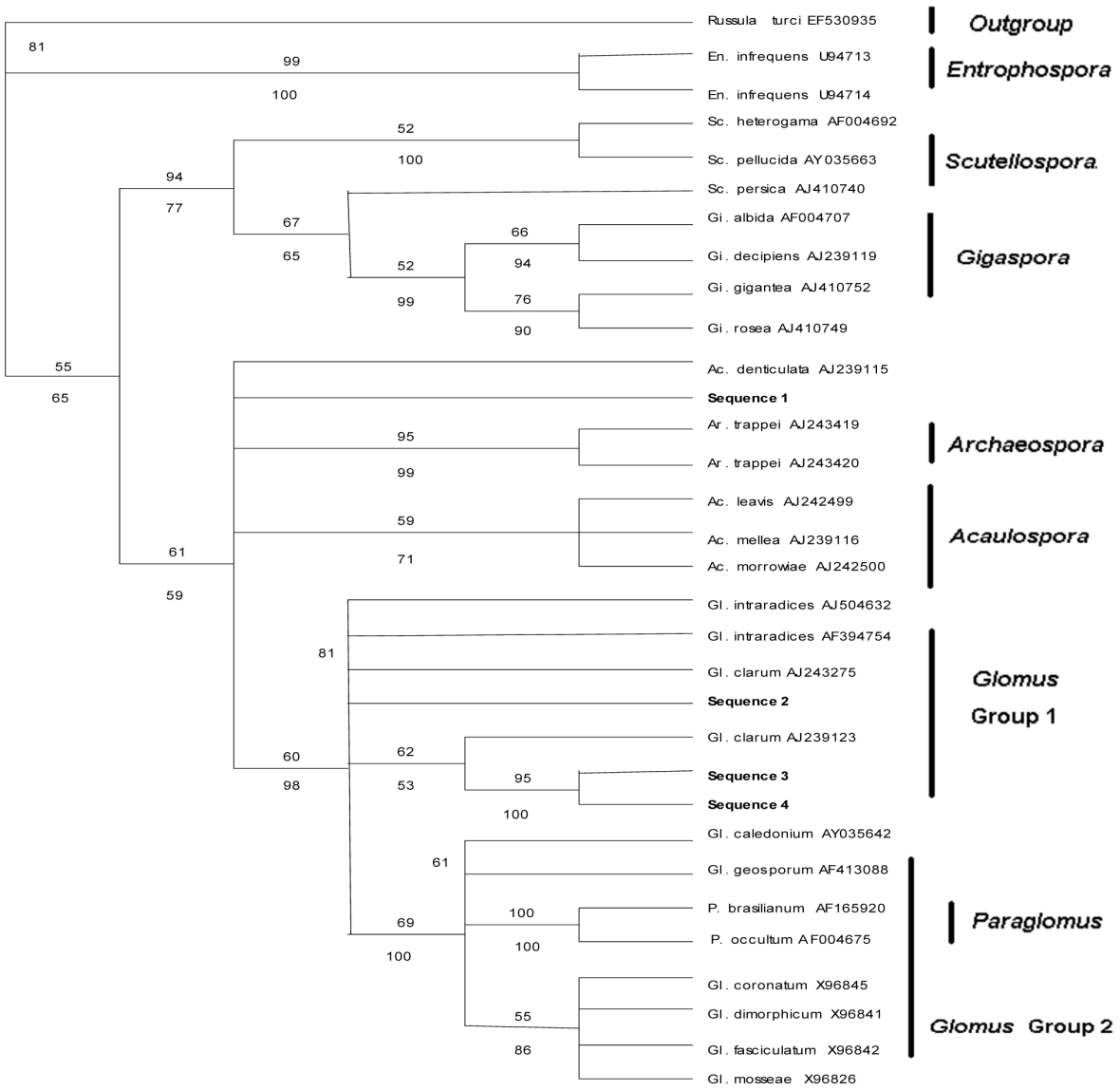

Fig. 6. Maximum parsimony tree based on full length 5.8S nuclear rRNA gene sequences of the phylum Glomeromycota. Numbers above branches refer to bootstrap percentages. Numbers below branches are Bayesian posterior probability estimates, which are expressed as percentages. Only bootstrap and posterior probability support over 50% are shown. The topology was rooted with *Russula turci.* Bold names are representatives of arbuscular mycorrhizal 5.8S nuclear rRNA gene sequences obtained during this study. GenBank accession numbers are provided for sequences of known species.

2005). However, the C-costs incurred by the host were balanced by P-uptake.

It is known that AM fungi may enhance plant P-uptake and growth at low soil P (Graham et al. 1996). In our study, total P-uptake rate was significantly ($p \leq 0.05$) higher in AM than in non-AM plants during the initial stages of growth (days 0 to 77), showing a clear positive AM effect on P accumulation. In addition, the C-costs of the enhanced AM P-uptake rate were lower in the AM roots during this period, due to the higher efficiency of P acquisition as also indicated by a previous study (Mortimer et al. 2005). However, the lower initial P utilization cost to AM plants increased as AM colonization receded during the later stages of plant growth (days 157 to 224). The plant thus has to invest more C for root growth to aid with P acquisition, increasing the C-costs of P acquisition in the plant. Concordant with results obtained in this study, a previous study showed that colonization levels of established Fynbos vegetation are usually low (37%), indicating that AM colonization of roots is mainly restricted

to the first phase of the growth period (Allsopp and Stock 1994).

The genera *Acaulospora* and *Glomus* were the dominant fungi isolated from colonized *A. betulina*. These AM fungi are indigenous to acidic, low nutrient soils, such as the soil type investigated in this study (Clark 1997) and would therefore be the preferred fungi to be used as AM inocula in nurseries for the large scale propagation of this Fynbos plant. In addition, AM inoculation may also afford the plant with a competitive advantage following transplantation into an exotic environment (Allen 1991). However, it must be borne in mind that the AM specific primers employed in the second reaction of nested PCR only amplify nuclear rRNA sequences (partial SSU; full length ITS1, 5.8S, and ITS2) of six of the known clades within the phylum Glomeromycota, with the exception of the *Glomus vesiforme* clade (Wubet et al. 2003). Therefore, our results should only be considered as a survey of these clades within the roots of *A. betulina* grown in a controlled environment, bearing in mind that other AM fungi might also have been present.

To conclude, the P nutritional benefits of the symbiosis coincided with a phase of rapid AM development during the early stages of host growth (0 to 77 days after germination). This may be typical of a sclerophyllous seedling in field-conditions, where the plant takes advantage of pulsed nutrient flushes (Allsopp and Stock 1993b) ensuring an adequate supply of P to support growth during periods when nutrients are unavailable (Chapin 1988), where after the host's reliance on AM fungi for P nutrition declines. These smaller seeded plants thus seem to be more dependent on becoming AM rapidly under low nutrient conditions in order to be competitive and thus show greater response to AM fungi, in terms of P acquisition.

Acknowledgements – We hereby acknowledge the financial assistance of the Dept of Labour of the South African Government, The South African Natl Res. Foundation and the United States Agency for Int. Dev.

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