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Application of mycorrhizal roots improves growth of tropical tree seedlings in the nursery: a step towards reforestation with native species in the Andes of Ecuador

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Abstract Most tree species in tropical mountain rain forests are naturally associated with arbuscular mycorrhizal fungi. Previous studies in southern Ecuador of 115 tree species revealed that only three species were not associated with arbuscular mycorrhizal fungi. Seedlings of tropical tree species raised in the nursery may need to be associated with arbuscular mycorrhizal fungi to survive transplantation shock in higher numbers. Methods for establishing plantations with native tree species are not yet established for Ecuador. Thus, we investigated the feasibility of using mycorrhizal roots of seedlings of *Inga acreana*, *Tabebuia chrysantha*, *Cedrela montana* and *Heliocarpus americanus* that had trapped mycorrhizal fungi from forest humus in the nursery to inoculate *C. montana* and *H. americanus* with native arbuscular mycorrhizal fungi. Inoculation with either a mixture of mycorrhizal roots from the four species or only with mycorrhizal roots from the same tree species were compared with effects of moderate fertilization. Assessment of plant

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growth and mycorrhizal status of 6-months-old *Cedrela montana* and *Heliocarpus americanus* revealed an improvement in growth and diverse associated fungi through mycorrhizal root inoculation in comparison with moderate fertilization. Moderate fertilization did not suppress mycorrhization.

Keywords Native mycorrhizal fungi · Nursery · *Cedrela montana* · *Heliocarpus americanus* · Ecuador · Tropical mountain rain forest

Introduction

Ecuador is considered a "hotspot" of biodiversity (Brummitt and Lughadha 2003) but actually is facing the highest deforestation rate (1.7%) in South America (FAO 2006). The rapid loss of tropical mountain rain forests with their extraordinary richness in tree species calls for recruitment by plantations. Despite the outstanding number of more than 2,700 native tree species described for Ecuador (Jørgensen and León-Yánez 1999), the existing plantations consist almost exclusively of introduced species from the genera *Eucalyptus* and *Pinus*. Raising tropical tree seedlings for plantations is still challenging (Stimm et al. 2008) and introduction of arbuscular mycorrhizal fungi (AMF) to the nursery substrate appears to be an important step for AMF-dependent tree species (Allen et al. 2003, 2005).

Application of commercially available AMF spore inoculum and the need for habitat- and tree-specific fungi is controversial (Klironomos 2003; Gianinazzi and Vosatka 2004; Wubet et al. 2006). However, beside costs, many aspects as reviewed by Schwartz et al. (2006) argue against the use of commercially available AMF inoculum and in favor of local inoculum production for reforestation of the tropical mountain rain forest. The main arguments are that when inoculated seedlings are planted into the field, exotic fungi often have difficulty in establishing within the pre-existing fungal community, and if they do establish they may become fast spreading invaders out-competing the local fungal community. Thus, local fungi were found to be finally more effective (Allen et al. 2005).

Previous investigations in the tropical mountain rain forest of southern Ecuador revealed that, except for tree species in the family *Nyctaginaceae*, all the trees formed arbuscular mycorrhizas with a multitude of forest-specific fungi shared among the diverse tree species (Kottke et al. 2004, 2008). We, therefore, hypothesized that restoring the native forest on degenerated pastures would need reintroduction of a diverse, native AMF inoculum by the nursery grown seedlings. Since spores of native fungi were not available in sufficient amounts for inoculation of nursery seedlings, we tested the feasibility of improving mycorrhization and maintenance of seedlings through inoculation by mycorrhizal roots obtained from trap plants grown on forest humus.

Four native tree species from the tropical mountain rain forest in the tropical Andes of southern Ecuador, *Inga acreana* Harms (Fabaceae), *Tabebuia chrysantha* (Jacq.) G. Nicholson (Bignoniaceae), *Cedrela montana* Moritz Ex Turcz (Meliaceae), and *Heliocarpus americanus* L. (Tiliaceae), were used as trap plants and as suppliers of mycorrhizal roots to *H. americanus* and *C. montana*. Species were selected according to acceptance by local people, valuable wood (*H. americanus*, *C. montana*) or multi purpose use (*T. chrysantha*, *Inga acreana*) and access to germinating seeds at the time of sowing, a special problem in the wet tropics (Stimm et al. 2008). *H. americanus* is a fast growing, early-succession species, while *C. montana* is a mid-successional species. Both species are used in reforestation programs in Ecuador (Weber et al. 2008), but without inoculation

with mycorrhizal fungi the development is only adequate under certain site conditions (Aguirre 2007). Some authors argue that fertilization may improve the growth of seedlings (Remes et al. 2005). However, fertilization may suppress mycorrhization (Smith and Read 2008) and thus could be counter-productive because of the increased risk of seedling mortality.

A nursery experiment was set up to address the following hypotheses:

Application of mycorrhizal roots will improve mycorrhization and maintenance of seedlings as compared to the application of fertilizer.
Mixed mycorrhizal inoculum from four native trap plants will be superior to that of the same species.
Low fertilization will not suppress mycorrhization but will improve plant growth and survival.
Moderate fertilization may yield equivalent growth as inoculation with AMF.
Early- and mid-succession plants may react differently to mycorrhizal application and fertilization.

Type and amounts of fertilizer were chosen according to previous experiences from a nursery experiment carried out on European beech (*Fagus sylvatica* L.) in Germany (Kottke and Hönig 1998). Here we report first results obtained from 6-months-old, nursery-grown seedlings of *H. americanus* and *C. montana* in the tropical Andes.

Materials and methods

Experimental setting

The nursery experiments were conducted in Loja, Ecuador, at the Universidad Nacional de Loja. The nursery of the Forestry School is situated at 2,160 m a.s.l. (04°02′09″S, 79°11′49″W). Average temperature in the greenhouse ranged from 15 to 21°C. Only natural sunlight was available. There was a preliminary trial to obtain mycorrhizal rootlets from four native tree species: *T. chrysantha*, *H. americanus*, *I. acreana* and *C. montana*. These tree species were chosen because of their ecological and economical importance for local people and availability of seeds.

Seeds of all four species were collected in the forest bordering Podocarpus National Park, Loja and Zamora-Chinchipe Provinces, Southern Ecuador and pre-germinated in a mixture (1:1) of steam-sterilized mine sand and steam-sterilized agricultural soil. Seedlings were transplanted into individual 500-ml plastic bags 5 weeks after germination and used as trap plants in a substrate consisting of humus from the tropical mountain rain forest mixed with steam-sterilized mine sand (1:3). Plant roots were harvested after 6 months of growth. Microscopic observation of the roots indicated colonization of the seedlings by AMF species of *Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora*. The mycorrhizal roots were used immediately as inoculum source for the next experiment on *H. americanus* and *C. montana*.

One hundred and fifty plants of *H. americanus* and *C. montana* were germinated as mentioned above and transferred to individual 500-ml plastic bags filled with a mix of soil from tropical mountain rain forest with steam-sterilized sand (1:3). Seedlings were either inoculated with mycorrhizal roots of the same species (*C. montana* or *H. americanus*) or by a mixture of mycorrhizas from the four trap species (*T. chrysantha*, *H. americanus*, *I. acreana* and *C. montana*) or not inoculated. Twenty centimeters of mycorrhizal roots

Table 1 Treatments of *Cedrela montana* and *Heliocarpus americanus* seedlings in the nursery experiment

Code	Tree species	Description of treatments
C0	*C. montana*	Not inoculated, no fertilizer
C1	*C. montana*	Inoculated with mycorrhizal roots from *C. montana* seedlings, no fertilizer
C2	*C. montana*	Mixed inoculum[a], no fertilizer
C3	*C. montana*	Mixed inoculum[a], Osmocote® fertilizer 0.25 g (0.5 kg/m^3)
C4	*C. montana*	Inoculated with mycorrhizal roots from *C. montana*, Osmocote® fertilizer 0.25 g (0.5 kg/m^3)
C5	*C. montana*	Not inoculated, Osmocote fertilizer® 0.50 g (1 kg/m^3)
H0	*H. americanus*	Not inoculated, no fertilizer
H1	*H. americanus*	Inoculated with mycorrhizal roots from *H. americanus* seedlings, no fertilizer
H2	*H. americanus*	Mixed inoculum[a], no fertilizer
H3	*H. americanus*	Mixed inoculum[a], Osmocote® fertilizer 0.25 g (0.5 kg/m^3)
H4	*H. americanus*	Inoculated with mycorrhizal roots from *H. americanus*, Osmocote® fertilizer 0.25 g (0.5 kg/m^3)
H5	*H. americanus*	Not inoculated, Osmocote fertilizer® 0.50 g (1 kg/m^3)

[a] Mycorrhizal roots obtained from *I. acreana*, *T. chrysantha*, *H. americanus* and *C. montana*

were added in the planting hole. Osmocote® fertilizer (nitrogen 15%, phosphorus P_2O_5 8%, potassium K_2O 11%, magnesium 0.9%, sulfur 1.9%, boron 0.002%, iron 0.4%, manganese 0.005%, molybdenum 0.018% and zinc 0.017% coated to provide slow-release nutrients over 6 months) was added to the planting hole of every inoculated plant (treatments C3, C4, C5 and H3, H4, H5) in two amounts: high, 0.5 g/plastic bag (1 kg/m^3), or low, 0.25 g/plastic bag (0.5 kg/m^3). In the case of the control treatments, neither mycorrhizal roots nor fertilizer were added.

Standard substrate analysis was conducted at Universidad Nacional de Loja. Soil pH was 5.0; organic matter content 3.5% (combustion), and phosphorus 30 μg P_2O_5 ml^{-1} (colorimetric). The conditions were the same for all the treatments.

The experiment consisted of six treatments (Table 1), five seedlings per five replicates, 25 seedlings per tree species and treatment, resulting in a total of 150 seedlings for *C. montana* and *H. americanus*, respectively. The bags were randomized with respect to tree species and treatments in the nursery.

Evaluation of plant growth and mycorrhizal status

After 6 months of growth 20 plants per treatment were randomly harvested. Plant height, root collar diameter (RCD), fresh and dry weight of aboveground (leaves and shoots) and belowground biomass of 15 plants was determined. Five plants were used to observe mycorrhizal status by microscope after staining. To prepare for mycorrhiza evaluation fine roots were cleaned under tap water and fixed in 50% ethanol. Later, randomly selected parts of the fine roots were cut in 2 cm pieces, treated in 10% KOH at 60°C in a water bath for 24–48 h, washed twice in tap water, acidified in 10% HCl for 2–3 min and stained by 0.05% methyl blue in 90% lactic acid for 8 h at 60°C in a water bath. Rootlets were partly destained in 90% lactic acid overnight (modified from Grace and Stribley 1991). Fifteen

root pieces, 2 cm in length each, were observed by use of light microscope at 400× magnification. Colonization by AMF was estimated according to amount of cells and intercellular space containing hyphae and classified on a six step scale: $0 = 0\%$, $1 < 1\%$, $2 < 10\%$, $3 < 50\%$, $4 > 70\%$, $5 > 90\%$ colonization. An average score (0–5) was assigned to each root piece and the final colonization score of each plant was calculated as average of the 15 root pieces.

Morphological differences of the AM fungi, such as size, form and diameter of vesicles, diameter of hyphae, inter- and intracellular hyphae, simple and branched coils, presence or absence of anastomoses, regular or irregular arbuscules were considered to obtain preliminary identification of fungal genera (INVAM 1998 http://www.invam.caf.wvu.edu/fungi/taxonomy/classification.htm).

Spore sampling and identification

Substrates were collected from the bags of the sampled plants, pooled from the respective species and kept in a refrigerator at 4°C for 8 months. The separation of AMF spores was carried out by wet sieving and decanting method (Gerdemann and Nicholson 1963). Apparently viable spores were extracted manually by Pasteur pipette and transferred to Petri dishes with distilled water and observed under a stereomicroscope at 32× magnification. Viable spores found in four samples of 100 g of dry substrate, respectively, were counted and numbers averaged. Twenty spores of each morphotype were collected, half placed in PVLG (Polyvinyl lacto-glycerol), and the other half in PVLG + Melzer's reagent, covered by a cover glass, and crushed to observe the numbers of wall layers and staining reactions at 100×, 400× and 1000× magnification. Morphological characters, such as shape, size, ornamentation, number of wall layers, presence or absence of subtending hyphae, and color of the outer layer, were compared with descriptions available on the INVAM website. Similar spores were grouped as morphotypes. Selected germinated spores of each morphotype were mounted on glass slides and stored as vouchers in the Laboratorio de Microbiología of the Universidad Técnica Particular de Loja.

Statistical analysis

GraphPad Prism® software package (http://www.graphpad.com/prism/Prism.htm) was used to analyze the plant growth and spore data combined with box and whiskers graphs. When two groups had equal variances, a standard *t* test was used to compare corresponding samples, based on least significant differences at $\alpha = 0.05$, and one-way ANOVA. Mann–Whitney test was applied to evaluate statistical differences among spore frequencies in substrates of *C. montana* and *H. americanus* at $\alpha = 0.05$.

Results

Growth of *Cedrela montana* and *Heliocarpus americanus* seedlings

Performance of *C. montana* and *H. americanus* in non-inoculated and non-fertilized plants was lower in general in comparison with inoculated and/or fertilized plants. Those plants which caused the large standard deviation in root collar diameter in *C. montana* (Fig. 1a) were later found to be mycorrhizal. Application of 0.50 g Osmocote per seedling to the

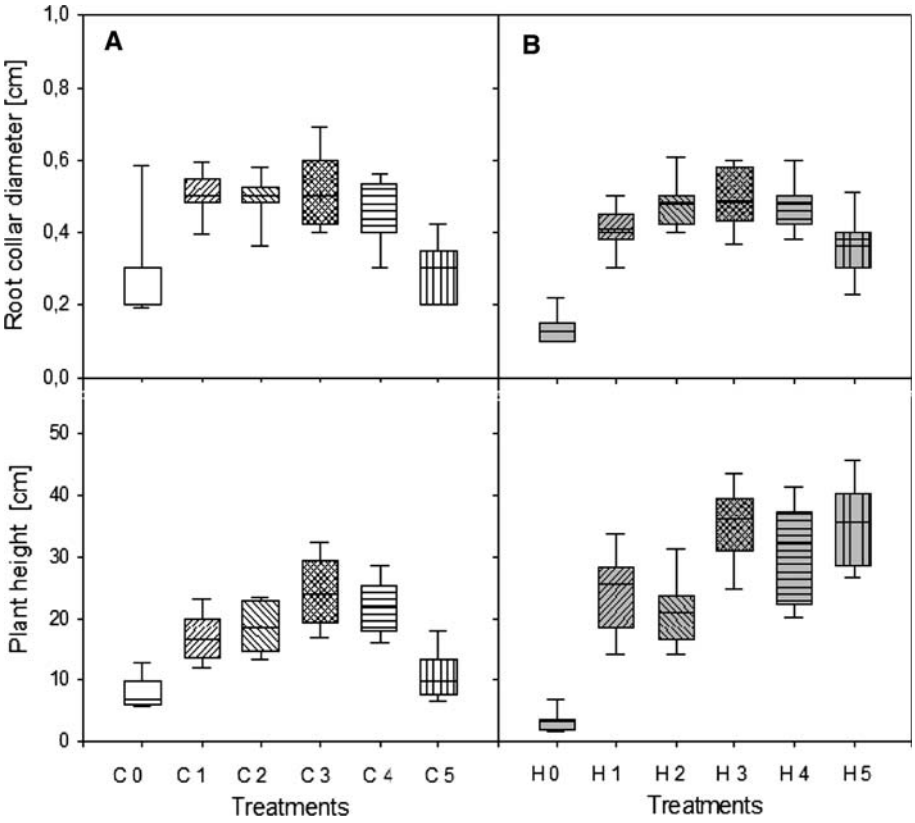

Fig. 1 Root collar diameter and height of 6-months-old *Cedrela montana* (**a**) and *Heliocarpus americanus* (**b**) seedlings. *Different letters* indicate significant differences ($P < 0.05$) between treatments. The *box* extends from the 25th percentile to the 75th percentile, with a *line* at the median (the 50th percentile). The whiskers show the highest and lowest values

non-inoculated plants did not stimulate growth of *C. montana*, on the other hand, height and biomass of *H. americanus* plants were significantly improved but root collar diameter was lower (Figs. 1b, 2b). Both tree species reacted positively to inoculation in comparison with the control treatments.

Use of mycorrhizas obtained from *C. montana* seedlings or mixed inoculum from the four forest species did not result in different root collar diameter or plant height in *C. montana* but biomass production was higher with the mixed inoculum. Application of 0.25 g Osmocote per inoculated *C. montana* seedling resulted in improved plant height but not in larger root collar diameter, leaf production, or root dry weight (Figs. 1a, 2a). Inoculation by mycorrhizas from *H. americanus* significantly improved root collar diameter, plant height and biomass of *H. americanus* when compared with the non-inoculated, no-fertilized plants, and inoculation with mixed mycorrhizas from the four tree species was even more effective for root collar diameter and biomass production. Higher values were obtained for plant height and biomass by application of 0.25 g Osmocote to inoculated

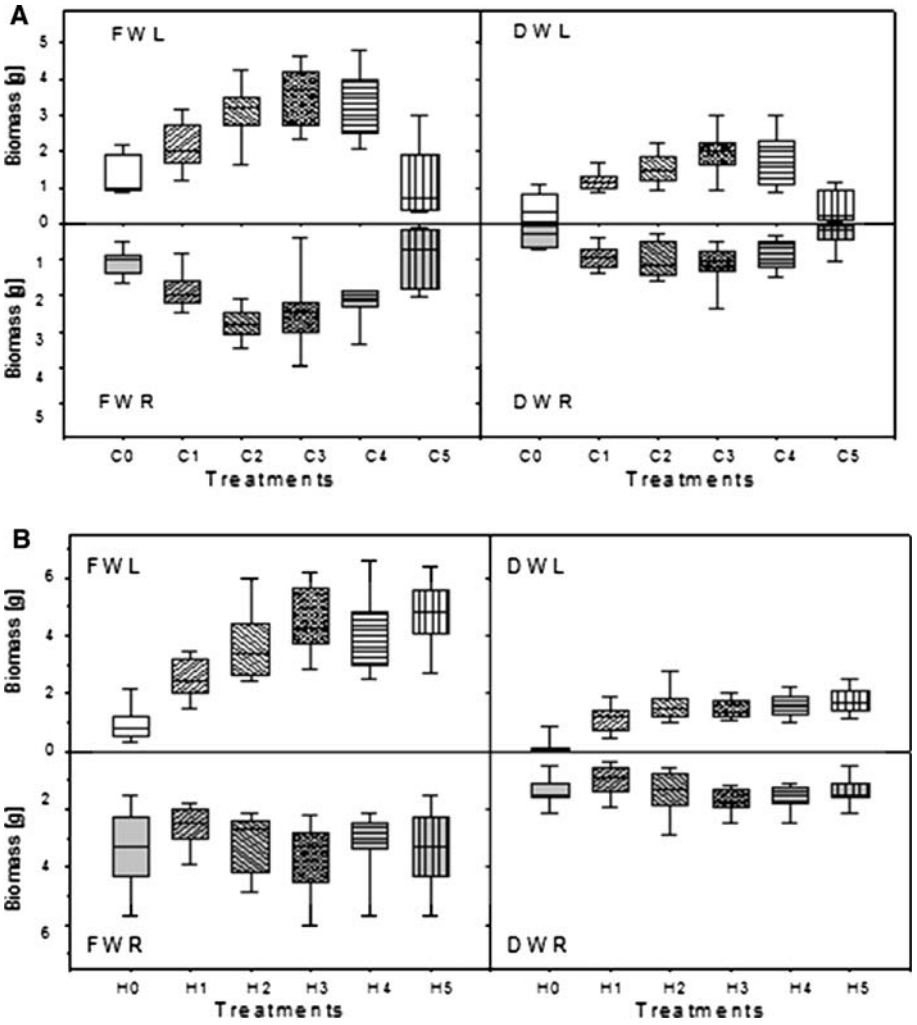

Fig. 2 Fresh and dry weight of leaves (FWL/DWL) and roots (FWR/DWR) of 6-months-old *Cedrela montana* (**a**) and *Heliocarpus americanus* (**b**) seedlings. *Different letters* indicate significant differences ($P < 0.05$) between treatments. The *box* extends from the 25th percentile to the 75th percentile, with a *line* at the median (the 50th percentile). The whiskers show the highest and lowest values

seedlings of *H. americanus*, but root collar diameter reached only the level of treatment (Figs. 1b, 2b).

Mycorrhization of 6-months-old seedlings

All inoculated plants were highly colonized by arbuscular mycorrhizal fungi as displayed by intracellular hyphal coils, intercellular hyphae, frequent vesicles, spores and arbuscules. Estimated degree of mycorrhizal colonization showed that inoculated plants had at least 30% and up to 70% of their root cortical cells colonized (Fig. 3). Negligible fungal

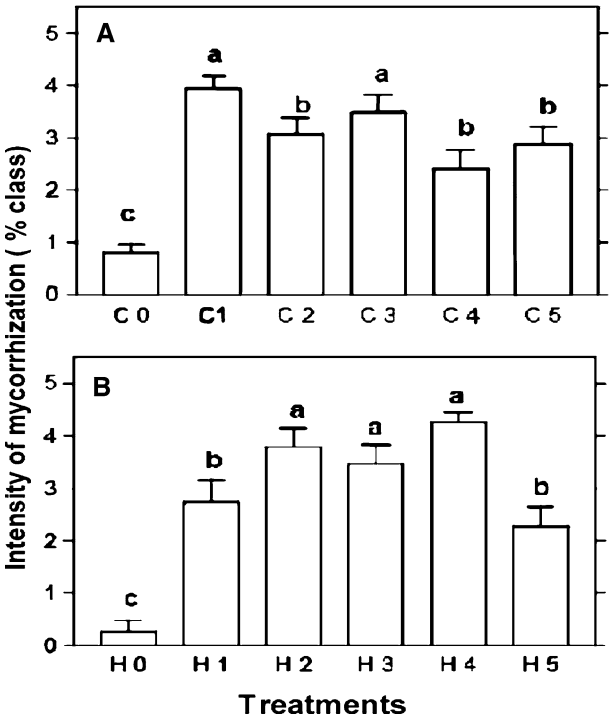

Fig. 3 Estimated rate of mycorrhization of 6-months-old *Cedrela montana* and *Heliocarpus americanus* seedlings; class 0 = 0%; class 1 < 1%; class 2 < 10%; class 3 < 50%; class 4 > 70% and class 5 > 90% of cortical cells colonized. Based on a six level scale of percentage values

colonization was observed in the non-fertilized, non-inoculated treatments. Colonization by mycorrhizal fungi was observed in the tall plants of non-inoculated but fertilized treatments, whereas small sized seedlings did not show mycorrhization. Staining intensity, morphology of vesicles, inter- and intra-cellular hyphae and auxiliary cells indicated members of *Glomus*, *Acaulospora*, *Scutellospora* and *Gigaspora*.

Spore production and identity

On average, 272 spores were found in 100 g substrate of *C. montana* and 175 spores in the substrate of *H. americanus*. No significant difference in spore production was observed between the two tree species (Mann–Whitney at $P = 0.2$). Three morphotypes (A1 to A3) were determined as *Acaulospora* species, two as belonging to *Glomus* (G1 and G2. Fig. S1 in electronic appendix). G1 and G2 were morphologically very similar. Four spore morphotypes (A1, A2, A3, G1) were discerned from *C. montana* substrate and one was found in *H. americanus* substrate. G1 and G2 were frequent spore types, while the other morphotypes occurred at low frequencies (Fig. S1 in electronic appendix).

Discussion

Although it is well established that tropical tree seedlings need mycorrhizal fungi to withstand natural stress conditions (Alexander and Lee 2005), techniques for application of these fungi to nursery grown seedlings are still underdeveloped. Mixing humus or soil from

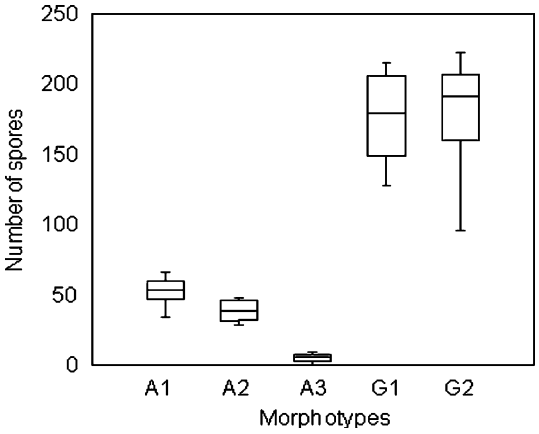

Fig. 4 Frequency of spore morphotypes in 100 g dry substrate of *Cedrela montana* (A1, A2, A3, G1) and *Heliocarpus americanus* (G2). *Acaulospora* species are represented with A and *Glomus* species with G

natural forest stands to the nursery substrate may be the simplest technique (Allen et al. 2003). By this method we obtained the mycorrhizal trap plants that served as providers of inoculum. However, as shown by the results from control treatments, growth improvement and mycorrhization rates were low on the very nutrient-limited substrate in our experiment. In the case of *C. montana*, only those plants that found mycorrhizal fungi in the forest humus could grow. Moderate fertilization improved growth of *H. americanus* but not of *C. montana*. Thus the early-successional species *H. americanus* could make better use of added nutrients than the mid-successional species *C. montana*, and the latter appeared to be more mycorrhiza-dependent (Janos 2007).

Despite no addition of mycorrhizal roots, *H. americanus* displayed mycorrhizas when treated with fertilizer. Fungi were obviously attracted by *H. americanus* from the forest humus probably due to larger root development than with *C. montana*. Another possible explanation could be that *C. montana* may exhibit some specificity for fungi not found in the substrate. The moderate fertilization (0.5 g Osmocote), thus, did not suppress mycorrhization. According to literature, high soil fertility levels, in particular high P fertility, stimulate plant growth but repress AMF development (Smith and Read 2008). Maximizing AMF contributions through precise fertilization allows the production of good yields in soils maintained at lower fertility levels, reducing nutrient loss to the environment while improving soil physical and biological quality (Hamel and Strullu 2006). Low amounts of fertilizer (0.25 g Osmocote) slightly improved growth of seedlings inoculated with AMF. For practice, low fertilization combined with inoculation is, however, not recommended because of low effect in relation to costs.

Significant differences in mycorrhizal colonization seemingly corresponded to growth of *H. americanus*, but not in the case of *C. montana*. This observation can be interpreted as high responsiveness of *C. montana* to low colonization (Janos 2007). Presence of mycorrhizas may be more important than colonization level for the growth of mid-succession tropical tree seedlings.

The inoculation technique applied here helped to select native fungi that may be well adapted to the local nursery conditions. The survey of mycorrhizal structures revealed a multitude of AMF. A higher number of AMF species has been shown to be favorable for plant growth (Lovelook and Ewel 2005; van der Heijden et al. 2008) and is also indicated here by the fact that best growth results were obtained by use of mixed mycorrhizal

inoculum. The risk of using mycorrhizal roots as a source of inoculum is the possibility of carrying pathogens but the effect of these appears to have been negligible in this experiment.

Frequency of spores in the substrates was very low when compared to results of similar studies but other trap plants and substrates (Molina et al. 2006; Eom et al. 2000). One reason for the low number of spores found in this study may be due to lack of water stress for trap plants. In other studies watering was continuously reduced to promote sporulation of associated AMF fungi. We did not reduce watering because we wanted to use the mycorrhizas as inoculum for a subsequent experiment (Kottke, unpublished). Different frequencies of spore morphotypes in both substrates and the low number of spores found in *H. americanus* substrate are in agreement with previous findings of highly variable spore production rate of AMF species with different host plants (Bever et al. 1996). Interestingly, the less mycorrhiza-dependent *H. americanus* promoted spore production less than the highly mycorrhiza dependent and responsive *C. montana*.

Results showed for the first time for Ecuador that maintenance of tree seedlings was significantly improved by inoculation with mycorrhizas of either the individual tree species or a mixture of mycorrhizas from the four trap species. The technique is much easier to handle and with lower costs than spore production for a tropical country with limited facilities for storage of inoculum. High diversity of AMF associated with seedlings may also be advantageous after planting to the field, as stress conditions will select for the best adapted species (Allen et al. 2003, 2005). Inoculation techniques for native tree species will help to provide alternatives to the predominant monocultures with *Eucalyptus* spp. and *Pinus* spp, and thus contribute to restoration of biodiversity in the Ecuadorian Andes.

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