

Effect of arbuscular mycorrhiza on the growth and development of micropropagated *Annona cherimola* plants

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Annona cherimola Mill., cherimoya, is a tropical plantation crop of interest in fruit culture. Micropropagation techniques have been developed due to the need to increase productivity through clonal selection. Because of the mycorrhizal dependence exhibited by this crop for optimal growth and the recognized role of mycorrhiza establishment for the survival and development of most of the plants produced *in vitro*, the effect of mycorrhiza inoculation on the development of micropropagated plants of *Annona cherimola* was investigated.

Mycorrhizal inoculation was assayed at two different stages of the micropropagation process: (i) immediately after the *in vitro* phase, before starting the acclimatization period, and (ii) after the acclimatization phase, before starting the post-acclimatization period under greenhouse conditions. Plantlet survival was about 50 % after the acclimatization period. Plant growth and development profited remarkably from mycorrhiza establishment. Most of the arbuscular mycorrhizal fungi (AMF) assayed greatly increased shoot and root biomass and leaf area. Micropropagated *Annona* plants seem to be more dependent on mycorrhiza formation for optimal growth than plants derived from seeds. The greatest effects of AMF on plant growth were observed when they were introduced after the acclimatization period.

Key words: mycorrhizal dependence, micropropagation, *Glomus* species

Introduction

Annona cherimola Mill., cherimoya, a tropical tree native to South America, is of particular interest in fruit culture. It is well adapted to the subtropical conditions of Southern Spain where it is successfully cropped in the Granada and Málaga provinces (MORTON 1987). It belongs to the Annonaceae family of the Magnoliales (GAUSSEN et al. 1982) and, as most members of this order, it has a relatively unbranched root system, with thick roots lacking root hairs. This suggests the mycotrophic nature of this species. This fact has

been corroborated in a preliminary study in which *Annona* showed a strong dependence on arbuscular mycorrhiza formation for optimal growth and development (AZCÓN-AGUILAR et al. 1994).

The mycorrhiza formed by *Annona* plants show an exclusively intracellular hyphal development, with a cell-to-cell colonization pattern, and abundance of arbuscules and coiled hyphae within the cells (AZCÓN-AGUILAR et al. 1994). These characteristics correspond to those described for the *Paris* species (BRUNDRETT and KENDRICK 1990a). Although it has been argued that this type of associations may be less efficient with respect to

plant growth (BRUNDRETT and KENDRICK 1990b), the results obtained for *Annona* do not support this assumption (AZCÓN-AGUILAR et al. 1994).

Research programmes for the improvement of *Annona* productivity include clonal selection involving micropropagation techniques. Because of the mycorrhizal dependence exhibited by this plant for optimal growth (AZCÓN-AGUILAR et al. 1994) and the importance of mycorrhiza establishment for the survival and development of most of the plants produced *in vitro* (GIANINAZZI et al. 1990), the role of mycorrhiza in the development of micropropagated *Annona* plants was further investigated.

Material and methods

Cultures of *Annona cherimola* Mill. cv Fino de Jete were initiated from nodal segments of semi hardwood *Annona* cuttings using procedures and environmental conditions such as those described by PLIEGO-ALFARO and MURASHIGE (1987). Shoots were incubated in a basic medium consisting of MS (MURASHIGE and SKOOG 1962) salts supplemented with (mg/l): i-inositol (100), thiamine (100), pyridoxine (50), nicotinic acid (50), glycine (200), benzilaminopurine (0.15), 30 g/l sucrose and 8 g/l TC agar. Axillary and terminal shoot cuttings (2.5–3.0 cm long) were removed from the proliferative clumps and prepared for rooting. Axillary buds and basal leaves of the bottom 1 cm of the shoots were discarded.

The shoots selected were then placed in 25 x 150-mm test tubes containing 25 ml of the autoclaved basic medium, to which 1 g/l activated charcoal was added. They were incubated in light for three days. Afterwards, they were subcultured in a root induction medium similar to the basic one but supplemented with 15 g/l sucrose, 100 mg/l indolebutyric acid and 200 mg/l citric acid. The cultures were then incubated for seven days in darkness and a further three-day period in light. Finally, shoots were subcultured in a root initiation/elongation medium which differed from the basic one in that the MS macroelement amounts were halved and the medium was supplemented

with 20 g/l sucrose and 200 mg/l citric acid. Cultures were maintained for approximately two weeks in light.

During rooting, cultures were grown at 26±1°C, with 16-h light exposure to 2000 lux illumination (Sylvania regular spectrum Gro-Lux lamps). All media were adjusted to pH 5.7 prior to autoclaving at 121°C for 15 minutes.

At the end of the root initiation-elongation phase, most plantlets had some roots about 1 cm long. At this stage, they were brought into acclimatization. Plantlets were individually transplanted to 100 ml open pots containing a mixture of sterile soil-sand (1/1, v/v) and placed in a misting tunnel (100 % relative humidity) for four weeks. Afterwards they were transferred to another tunnel without mist for another two weeks. At the end of this period, plants were transplanted to 250 ml pots and transferred to normal greenhouse conditions (25/19°C day/night temperatures, 16/8 photoperiod, 75/90 % relative humidity). Hoagland nutrient solution (HOAGLAND and ARNON 1983) at 25 % strength was used to feed the plants at the rate needed to maintain a suitable soil water content.

Mycorrhizal inoculation was assayed at two different stages: (i) immediately after the *in vitro* phase, before starting the acclimatization period, with the aim of establishing the mycorrhizal symbiosis as soon as possible in the *ex vitro* phase (Experiment 1), and (ii) after the acclimatization phase, before starting the post-acclimatization period under greenhouse conditions (Experiment 2). Mycorrhizal inoculum consisted of thoroughly mixed rhizosphere samples of stock cultures, containing spores, hyphae and mycorrhizal root fragments of the corresponding arbuscular mycorrhizal fungi (AMF).

In Experiment 1, the AMF tested were *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe from Rothamsted Experimental Station (England), *Glomus deserticola* (Trappe, Bloss & Menge) from Santiago de Compostela (Spain) and a *Glomus* sp. from Dijon (France). Inoculation was done by mixing the mycorrhizal inoculum (10 g) with the soil-sand mixture used to fill the 100 mL pots. Twenty plantlets per treatment were assayed

and those that survived after the acclimatization period were allowed to grow for ten weeks.

In Experiment 2, inoculation was done on already acclimatized plantlets (51.7 % survival rate after acclimatization). *Glomus deserticola* and *Glomus intraradices* (Schenck & Smith) were tested and mycorrhizal inoculation was done when transplanting the plants to the 250 mL pots. The corresponding inoculum (10 g/pot) was mixed with the soil-sand mixture used as substrate. Ten replicate plantlets per treatment were prepared and allowed to grow for 12 weeks.

During the post-acclimatization phase, time-course records were kept on the height of the plants and on the number of leaves produced. At harvest, the fresh and dry weights of leaves, stem and roots and the leaf area were recorded and data analysed by ANOVA and Tukey's test. Mycorrhizal colonization was measured on representative stained samples (PHILLIPS and HAYMAN 1970) by the gridline intersect technique (GIOVANNETTI and MOSSE 1980).

Results

Inoculation with *G. deserticola* and *Glomus* sp. immediately after the *in vitro* phase (Exp. 1) significantly increased plant height, shoot and root biomass, number of leaves and leaf area (Figs. 1 and 2). However, it did not significantly improve survival of the plantlets *ex-vitro*. The mycorrhizal effect on leaf number, observed at the end of the acclimatization period (six weeks), appeared before its effect on plant height (Fig. 1). *Glomus mosseae*, however, did not significantly stimulate plant development (Figs. 1 and 2).

The percentage of mycorrhizal colonization was quite similar for both *G. deserticola* and *G. mosseae* (Fig. 2). However, in some plants *G. mosseae* induced abnormal infections characterized by many aborted entry points, which consisted of apparently normal appressoria, but without hyphal penetration of the root tissues. Sometimes few short infective hyphae were produced from the appressoria, but they rapidly aborted, and retracted their cytoplasm, showing septa and emp-

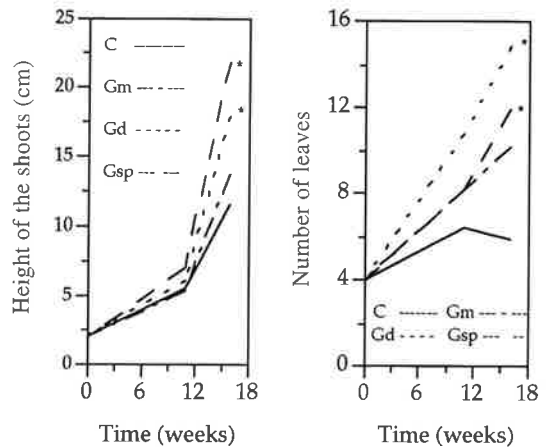
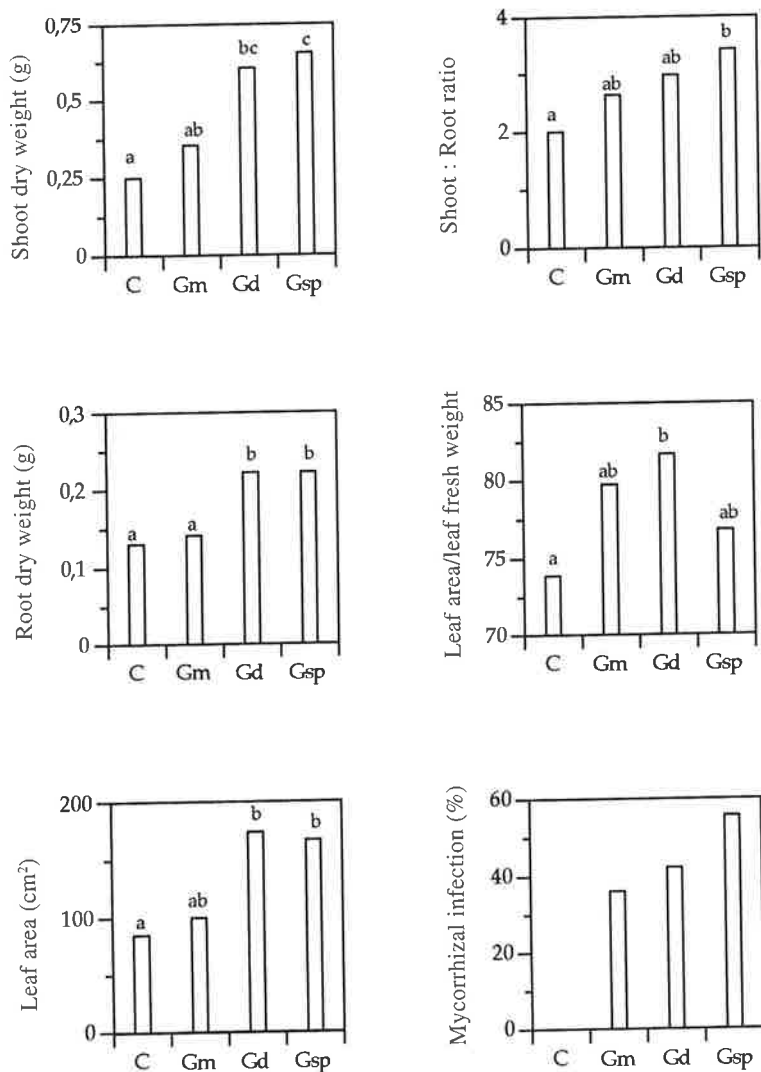


Fig. 1. Shoot height and number of leaves of mycorrhizal-inoculated (Gm = *Glomus mosseae*, Gd = *Glomus deserticola* and Gsp = *Glomus* sp.) and non-inoculated (C) micropropagated *Annona cherimola* plants throughout the growth period. Inoculation was done at the beginning of the acclimatization period. * Indicates a significant difference ($P < 0.05$) according to Tukey's test.

ty hyphal tips. These infective hyphae were unable to spread in the root cortex and to establish arbuscules. In some other cases, a very intense, and disorganized fungal colonization of the root was observed. This infection seemed to be outside host control because some meristematic and vascular tissues appeared to be colonized by the fungus. Not all plants colonized by *G. mosseae* exhibited these abnormal patterns of colonization. In some cases, as in those of mycorrhizal associations with *G. deserticola* and *Glomus* sp., the colonization pattern showed a morphology similar to the one described for plants derived from seeds, with the cell-to-cell passage, and the abundance of arbuscules and coiled hyphae within cells, which are typical of this host species.

The influence of *G. deserticola* when increasing the leaf area/leaf fresh weight ratio (Fig. 2) is noteworthy. This effect was not significantly induced by *Glomus* sp., although the effects of both fungi on plant growth were quite similar.

The mycorrhiza-induced increases in plant growth and development were even higher when mycorrhizal endophytes were inoculated after the acclimatization period (Exp. 2, Figs. 3 and 4). As in Experiment 1, the mycorrhizal effect on the



Treatments

Fig. 2. Biomass production and distribution, leaf area and mycorrhizal infection of inoculated (Gm = *Glomus mosseae*, Gd = *Glomus deserticola* and Gsp = *Glomus* sp.) and non-inoculated (C) micropropagated *Annona cherimola* plants. Inoculation was done at the beginning of the acclimatization period. For each parameter, values sharing the same letter did not differ significantly ($P < 0.05$) according to Tukey's test.

leaf number was detected before its effect on plant height (Fig. 3). All of the growth parameters measured significantly increased with mycorrhizal inoculation in all of the assayed endophytes (Figs. 3 and 4). As in Experiment 1, *G. deserticola* increased the leaf area/leaf fresh weight.

If all the data from these experiments and from others carried out on the same plant (AZCÓN-AGUILAR et al. 1994 and unpublished results), were pooled, it would be observed that the effect of mycorrhizal inoculation on plant height, given as percentage increase over control, is much higher

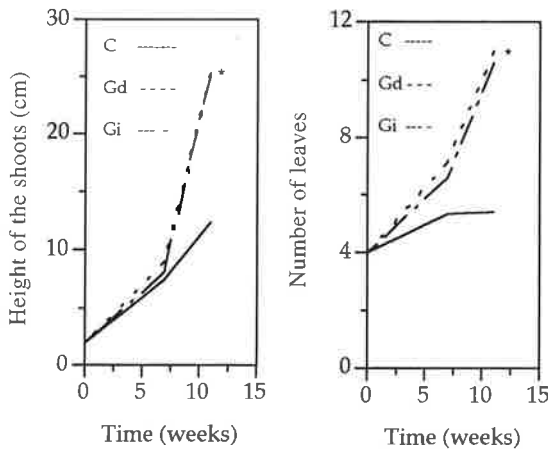


Fig. 3. Effect of different mycorrhizal fungi (Gd = *Glomus deserticola*, Gi = *Glomus intraradices* and C = non-inoculated control) on shoot height and number of leaves in micropropagated plants of *Annona cherimola* throughout the growth period. Inoculation was done at the end of the acclimatization period. * Indicates a significant difference ($P < 0.05$) according to Tukey's test.

for micropropagated plants than for those propagated from seeds (Fig. 5). This suggests that micropropagated *Annona* plants are even more dependent on mycorrhiza formation for optimal growth than plants derived from seeds.

Discussion

Most of the AMF assayed substantially increased shoot and root biomass and leaf area, corroborating the strong dependence of *Annona cherimola* on mycorrhiza for optimal growth (AZCÓN-AGUILAR et al. 1994). In addition to the general effect of improving plant growth, *G. deserticola* consistently enhanced the leaf area/leaf fresh weight ratio. This suggests that other mechanisms, apart from the increased nutrient uptake, could be involved in the effect that *G. deserticola* exerts on plant development. Plant hormone production (BARREA and AZCÓN-AGUILAR 1982) or changes in-

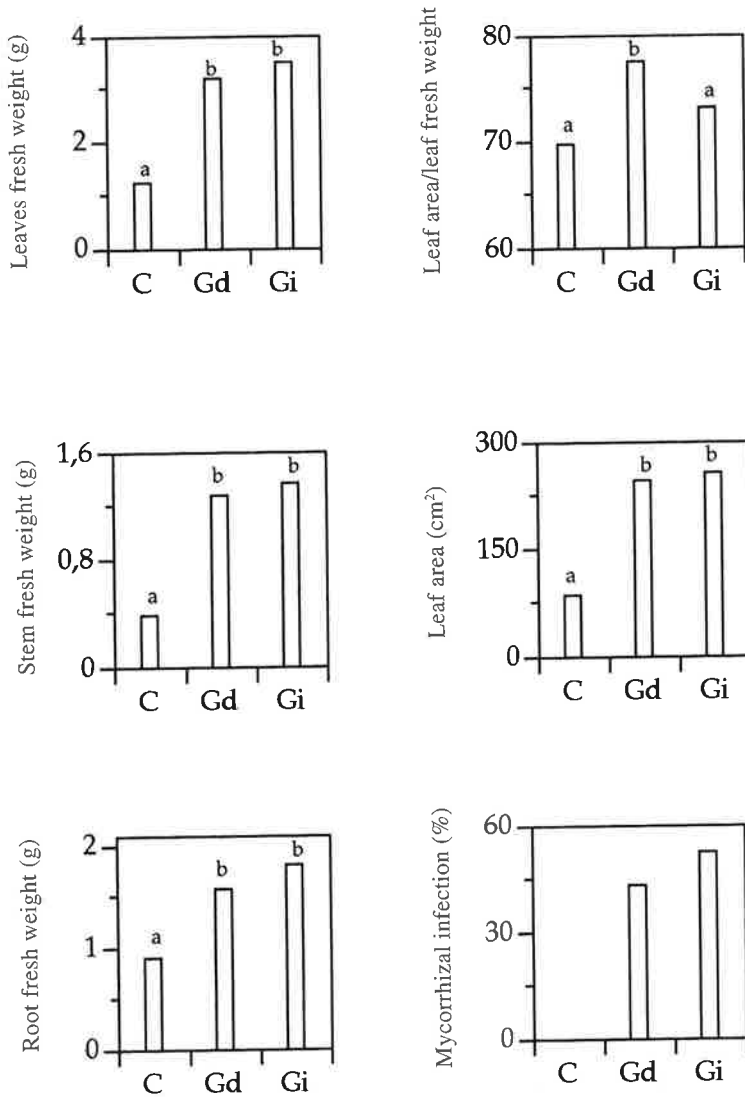
duced in the hormonal balance of the plant (DIXON 1990) may underly some of these mechanisms.

The fact that mycorrhizal dependence is greater for micropropagated plants than for those propagated from seeds can be explained on the basis of the different types of stresses (nutritional, drought, etc.) to which a micropropagated plant is subjected. In order to cope with these stresses, they depend to a greater extent on mycorrhiza during the post-vitro growth.

In relation to the inoculation time, plants benefited more from mycorrhiza inoculation when the AMF were introduced after the acclimatization period. When AMF were inoculated immediately after the *in vitro* phase, mycorrhizal colonization showed, in some cases, either aborted infection or very intense colonization, apparently outside the control of the host. It seems as if some roots (or some plantlets) were not yet mature enough to control the growth of the AMF in the root tissues and, consequently, to establish the symbiosis. Thus, they react against the fungus (abortive infection) and, if they fail to avoid infection, it appears rather disorganized and uncontrolled. Under these conditions, mycorrhiza formation could not induce beneficial effects on plant development, or it might even provoke detrimental ones. This reaction was found for *G. mosseae*-inoculated plants in some cases, although, in others, plants showed normal colonization patterns and growth increases. This suggests that this effect appears as a consequence of the physiological status of the plantlets and not as a specific reaction against *G. mosseae*. In fact, *G. mosseae* improved *Annona cherimola* growth in other circumstances (AZCÓN-AGUILAR et al. 1994).

Studies are now underway to confirm these facts and to determine the physiological status required for the root system to be able to establish a functional mycorrhizal symbiosis in micropropagated plants.

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Fig. 4. Biomass production, leaf area and mycorrhizal infection of inoculated (Gd = *Glomus deserticola* and Gi = *Glomus intraradices*) and non-inoculated (C) micropropagated *Annona cherimola* plants. Inoculation was done at the end of the acclimatization period. For each parameter, values sharing the same letter did not differ significantly (P < 0.05) according to Tukey's test.

References

AZCÓN-AGUILAR, C., ENCINA, C.L., AZCÓN, R. & BAREA, J. M. 1994. Mycotrophy of *Annona cherimola* and the morphology of its mycorrhizae. *Mycorrhiza* 4: 161-168.

BAREA, J. M. & AZCÓN-AGUILAR, C. 1982. Production of plant growth-regulating substances by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *Applied and Environmental Microbiology* 43: 810-813.

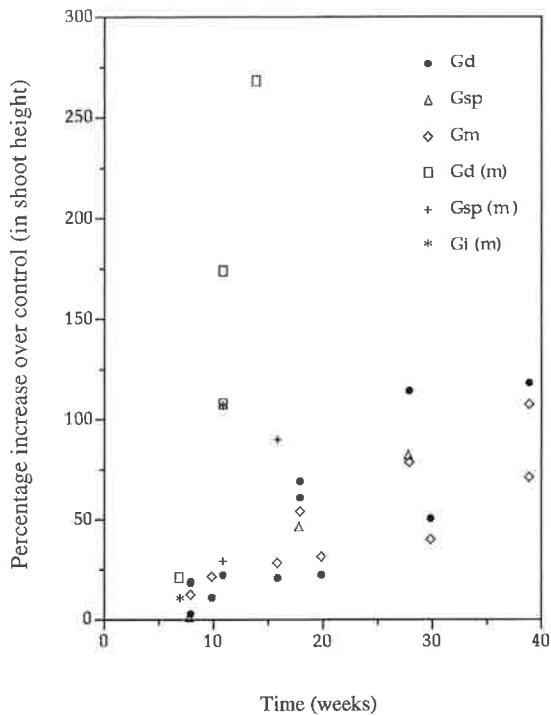


Fig. 5. Percentage increase of shoot height induced over control by different mycorrhizal fungi (Gm = *Glomus mosseae*, Gd = *Glomus deserticola* and Gsp = *Glomus* sp.) in different experiments, using micropropagated (m) and seed-propagated *Annona cherimola* plants.

- BRUNDRETT, M. & KENDRICK, B. 1990a. The roots and mycorrhizas of herbaceous woodland plants. I. Quantitative aspects of morphology. *The New Phytologist* 114: 457–468.
- BRUNDRETT, M. & KENDRICK, B. 1990b. The roots and mycorrhizas of herbaceous woodland plants. II. Structural aspects of morphology. *The New Phytologist* 114: 469–479.
- DIXON, R.K. 1990. Cytokinin activity in *Citrus jambhiri* seedlings colonized by mycorrhizal fungi. *Agriculture, Ecosystems and Environment* 29: 103–106.
- GAUSSEN, H., LEROY, J. F. & OZENDA, P. 1982. Précis de Botanique. In: Grassé, P. P. (ed.). *Végétaux supérieurs*. Masson, Paris. p. 221–230.
- GIANINAZZI, S., GIANINAZZI-PEARSON, V. & TROUVELOT, A. 1990. Potentialities and procedures for the use of endomycorrhizas with special emphasis on high value crops. In: Whipps, J.M. et al. (eds.). *Biotechnology of fungi for improving plant growth*. University Press, Cambridge. p. 41–54.
- GIOVANETTI, M. & MOSSE, B. 1980. An evaluation of tech-

- niques for measuring vesicular-arbuscular mycorrhizal infection in roots. *The New Phytologist* 84: 489–500.
- HOAGLAND, D.R. & ARNON, D. I. 1935. The water culture method for growing plants without soil. 347 p. Calif. Agr. Exp. Sta. Cir. Berkeley, California.
- MORTON, J.F. 1987. Fruits of warm climates. In: Dowling, C. F. (ed.). Florida. 505 p.
- MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* 15: 473–497.
- PHILLIPS, J.M. & HAYMAN, D. S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* 55: 159–161.
- PLIEGO-ALFARO, F. & MURASHIGE, T. 1987. Possible rejuvenation of adult avocado by graftage onto juvenile rootstocks in vitro. *HortScience* 22: 1321–1324

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SELOSTUS

Arbuskelimykorrhizasienten vaikutus mikrolisätyn annoonan (*Annona cherimola*) kasvuun ja kehitykseen

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Annona eli kirimoija (*Annona cherimola* Mill.) on kiinnostava trooppinen hedelmäkasvi. Espanjassa kehitettiin kasvin mikrolisäystä, että kasvin kloonivalintaa voitaisiin tehdä sadontuottokyvyn parantamiseksi. *Annona* tarvitsee mykorrhisaa saavuttaakseen optimaalisen kasvun. Aikaisemmissa kokeissa on todettu mykorrhisasiirrostuksen lisäävän annoonan pikkutaimien henkiinjäämistä ja kehitystä.

Tämän tutkimuksen tavoitteena oli selvittää arbuskelimykorrhizasientien siirrostuksen vaikutusta mikrolisätyn annoonan kasvuun ja kehitykseen. Kasvit siirrostettiin kah-

tena ajankohtana, joko välittömästi taimien *in vitro* -vaiheen jälkeen ennen sopeuttamista jatkoviljelyolosuhteisiin tai sopeuttamisjakson jälkeen. Sopeuttamisvaiheen jälkeen taimien eloonjäämisprosentti oli vain n. 50. Mykorrhitsa paransi taimien kasvua ja kehitystä. Useimmat käytetyt sienikannat lisäsivät huomattavasti versojen ja juurten biomassaa sekä lehtialaa. Mikrolisätyillä *Annona*-taimilla näyttää olevan suurempi riippuvuussuhde mykorrhitsan kanssa kuin siemenistä kasvatetuilla taimilla. Mykorrhizasientien vaikutus oli vahvin silloin, kun siirrostus tehtiin sopeuttamisjakson jälkeen.