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MYCORRHIZATION OF MICROPROPAGATED APPLE ROOTSTOCKS

Micorrización de portainjertos de manzano micropropagados

Alicia Castillo Sallé[1]✉, Adriana Montañez Massa[2], Roberto Docampo Romero[3], Pablo Rodríguez Bruno[4], Danilo Cabrera Bologna[4] and Roberto Zoppolo Goldschmidt[4]

ABSTRACT. Micropropagation through *in vitro* plant cultivation allows large-scale production of identical individuals genetically to the starting material. Woody species have difficulties in the acclimatization stage due to their slowness in the development of physiological response to environmental changes. The ultimate success of *in vitro* propagation depends on the capacity of plants to adapt in the moment of transfering from the laboratory to the greenhouse conditions. One of the tools to offset losses during acclimatization is the use of arbuscular mycorrhizal fungi (AMF), which sets mutualistic symbiotic associations unspecific with 90 % of vascular plants. AMF, because of their action as agents of growth bioregulation as bio-fertilizers or biocontrollers have received special attention in handling and propagation of fruit plants. In this work the effects of inoculation with AMF at the start of acclimatization are presented to mycorrhization. Inoculation with one type of AMF over two rootstocks of apple was done in a clone of M9 and one rootstock of the Cornell-Geneva series (RN29 and Geneva®41 respectively) set in three different substrates. Seedlings inoculated with AMF when compared to the control, presented further expansion of their leaves, bigger diameter and greater height, all significantly different. Acclimatization period was reduced from 60 to 40 days. The incorporation of this type of technologies could generate a more sustainable management of plant production with less use of agrochemicals.

Key words: adaptation, vegetative propagation, simbiosis

RESUMEN. La micropropagación mediante cultivo *in vitro* de plantas permite la producción a gran escala de individuos genéticamente idénticos al material de partida. Las especies leñosas tienen dificultades en la etapa de aclimatación por la lentitud que presentan en el desarrollo de respuestas fisiológicas y morfológicas a los cambios de ambiente. El éxito final de la propagación *in vitro* depende de la capacidad de transferencia de las plantas desde el laboratorio a las condiciones de invernáculo. Una de las herramientas para contrarrestar las pérdidas durante la aclimatación, es la utilización de hongos micorrízicos arbusculares (HMA), que establecen asociaciones simbióticas mutualistas no específicas con el 90 % de las plantas vasculares. Los HMA, por su acción como agentes de biorregulación del crecimiento, biofertilizantes o biocontrol, han tenido especial atención en el manejo y propagación de las plantas frutícolas. En este trabajo se presentan los efectos de la inoculación con HMA en el inicio de la aclimatación. Para la micorrización se empleó un solo tipo de inóculo de HMA sobre dos portainjertos de manzano: un clon de M9 y otro de la serie Cornell-Geneva (RN29 y Geneva®41, respectivamente) en tres sustratos diferentes. Los plantines inoculados con micorrizas presentaron mayor expansión de sus hojas, mayor diámetro y mayor altura, respecto al control, mostrando diferencias significativas. La aclimatación se redujo de 60 a 40 días. La incorporación de este tipo de tecnología, podría generar beneficios orientados a un manejo sustentable de la producción de plantas con menor uso de agroquímicos.

Palabras clave: adaptación, propagación vegetativa, simbiosis

INTRODUCTION

Uruguay produces high quality fruit that is destined to the domestic market and to export in variable volume every year, representing about 5 % of the gross annual production value (VBPA according its acronyms in English). To compete in export markets with a high level of demand, it is necessary to achieve high efficiency in all phases of

[1] Unidad de Biotecnología INIA "Las Brujas" Ruta 48, km 10, Canelones Uruguay

[2] Laboratorio de Microbiología de Suelos, Instituto de Ecología y Ciencias Ambientales (IECA) Facultad de Ciencias. Mataojo 2055

[3] Suelos y Riego, INIA "Las Brujas" Ruta 48, km 10 Canelones Uruguay.

[4] Programa de Investigación en Producción Frutícola, INIA "Las Brujas" Ruta 48, km 10, Canelones. Uruguay

✉ acastillo@inia.org.uy

the production process. For this reason, it is essential to install forests with excellent quality plants from the nursery, since the plant is the first link in the fruit production chain (1).

Modern fruit-growing is based on plantations that use clonal dwarfed rootstocks that allow the cultivation of high-density crops. From the use of this type of rootstock, an increase in the efficiency of the production is sought, increasing the volumes and unifying the quality of the fruit, as well as offering resistance to certain pests and diseases of the soil (2). These rootstocks are vegetatively propagated in the field by the use of pile caps (bark) and for this purpose it is necessary to have blocks of mother plants that supply important volumes of rooted seedlings.

The mother plants of the pile cap must be controlled annually, to know and to assure that the sanitary state is maintained free of pathogens and diseases. Micropropagation is another way of obtaining high quality clonal plants (3). With the application of this technique it is possible to obtain a large number of plants in a few months, preserving the health and the genetic identity of the material (4).

Micropropagation consists of producing plants from portions of the mother plant. In general, vegetative meristematic apices, cultivated aseptically in a test tube, where environmental and nutritional conditions can be controlled strictly through the culture medium (5).

This technique, which has become an important alternative within propagation methods in a wide range of species (6),it is composed of a series of sequential stages: establishment, proliferation or multiplication, rooting and acclimatization (7). However, some difficulties arise because a plant that has originated *in vitro* differs in many respects from those formed *in vivo* (8), because the conditions, both environmental and the substrate, in which they grow, are very different. Also, it is important to note that *in vitro* plant growth is heterotrophic in that *in vivo* it is autotrophic. Acclimatization proves to be the bottleneck of the entire micropropagation process, due to the low survival of seedlings that occurs in the passage from *in vitro* to *in vivo* growth. The ultimate success of *in vitro* propagation depends on the transferability of plants, from the laboratory environment to the greenhouse conditions.

The *in vitro* environment, with a high relative humidity, low or zero gas exchange, CO_2 shortage during almost all the period, ethylene production and low photosynthetic density induces morphological and physiological changes in the plants developed under this condition. The anatomy of the leaf *in vitro* is influenced by light and moisture, differing anatomically from those originated *in vivo* (8, 9). After transferring the plants to the *ex vitro* environment, they have to correct all these abnormalities to acclimate to the new greenhouse environment.

In order to increase the efficiency of acclimatization, the application of arbuscular mycorrhizal fungi (AMF) was evaluated as a way of conferring greater resistance to environmental stressors and their establishment in the field (10). Several authors have used inoculation with mycorrhizae or plant growth promoting bacteria as a tool to counteract losses during acclimatization (11, 12). The objective of the present work was to evaluate the effect of the inoculation with AMF in micropropagated plants of apple rootstock in the acclimatization phase.

MATERIALS AND METHODS

As plant material two apple rootstocks were selected: a M9 clone, designated RN29, of Belgian origin and a rootstock of a Cornell-Geneva series, Geneva® 41, from Cornell University. Both rootstocks were multiplied and rooted *in vitro*. The rooted plants were removed from the flasks and carefully washed to remove the residues from the culture medium. At the time of transplantation, the seedlings were inoculated with mycorrhiza AMF *Glomus mossae monosporic*, obtained by multiplication in trap cultures of *Paspalum dilatatum*.

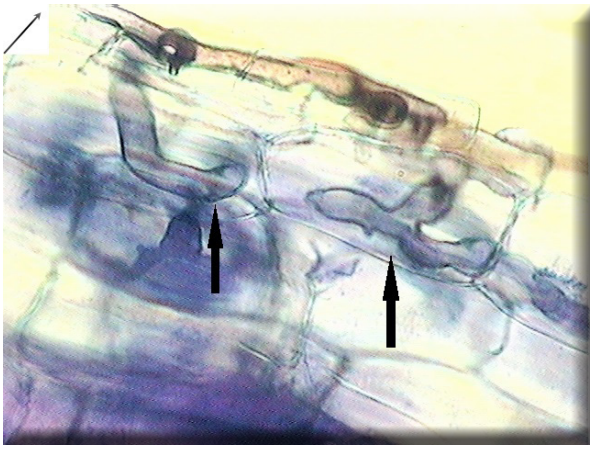
1 g of inoculum was placed in the substrate, under the plant, at the transplantation time. All substrates used were autoclaved before use, 1) horse bed compost; 2) mixture of ¼ commercial peat, ¼ of sand, ¼ pad of rice and ¼ of pine mulch; 3) commercial peat. The P content of the substrate was 1,9, 15,6 and 49,6 mg L^{-1}, respectively (saturated pulp extract analysis by colorimetry) (13). In all three cases, each substrate was evaluated under two conditions: with mycorrhizae and without inoculation, as a control treatment (six treatments). A total of 150 plants were evaluated per treatment, 20 plants were used in each acclimatization vessel, the experiment being repeated twice. Root samples were taken to determine the presence of mycorrhizae at 20 days after transplantation. The roots were stained with trypan blue at the rate of 0,5 g L^{-1} in 1: 1 solution of lactic acid: glycerol with modifications (14).

[A] Durán, F. V. I. ''Situación y perspectivas de las cadenas agroindustriales 2014-2015'' [en línea]. En: Anuario OPYPA 2014, edit. MGAP, Montevideo, 2014, pp. 15-38, [Consultado: 30 de junio de 2015], Disponible en: <http://www.mgap.gub.uy/OpypaPublicaciones/ANUARIOS/Anuario2014/pdf/Anuario_2014_web.pdf>.

At the vegetative level, growth parameters were measured: plant height, number of leaves and diameter of the neck in different stages of acclimatization. To avoid dehydration, the plants were covered during the first 15 days, then the ventilation was gradually increased until leaving them uncovered. At this stage no fungicides or fertilizers were applied.

RESULTS AND DISCUSSION

In the treatments inoculated with AMF the first structures were observed in the establishment of the symbiosis in the samples taken 20 days after the transplant; as shown in Figure 1, appressors were observed within the cells of the radicular tissue.



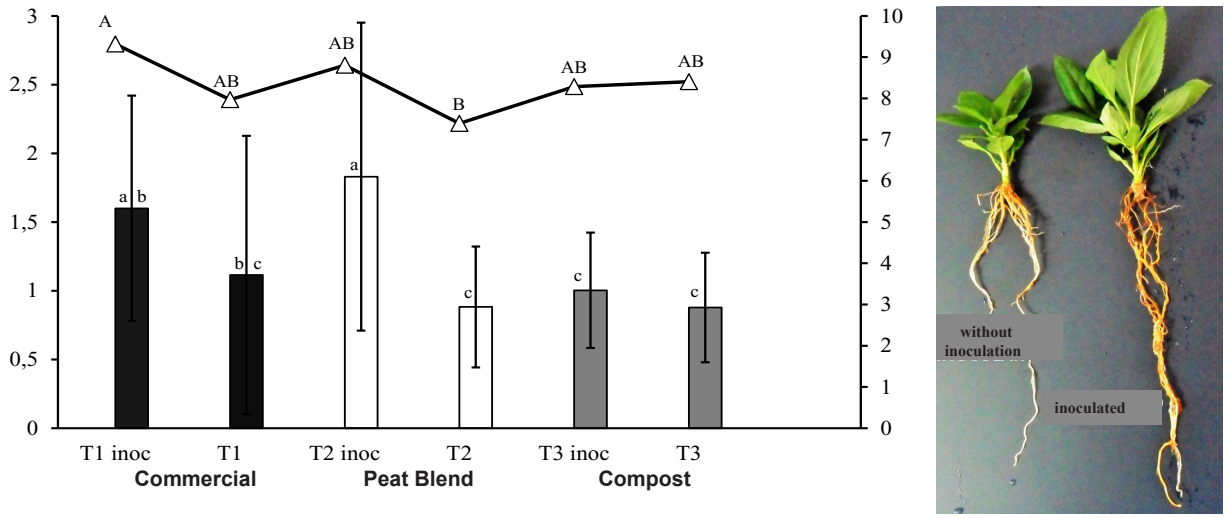
The arrows indicate the presence of appressoria within 20 days of acclimatization in the roots of inoculated plants.

Figure 1. Development of the first structures in the establishment of symbiosis (appressoria) at 20 days of acclimatization

Figure 2 shows the evaluation results of the vegetative growth parameters in the six treatments, showing the same behavior in both rootstocks. The inoculated plants showed significant difference in height, reaching the highest height in the compost substrate of horse bed (T1inoc) and in the substrate mixture of peat, sand, rice husk and mulch (T2inoc). Between these two treatments inoculated with AMF there was no significant difference. In the plants transplanted in commercial peat, the lowest height of the plants was obtained and there was no significant difference between the inoculated and the non-inoculated plants (T3inoc and T3 treatment).

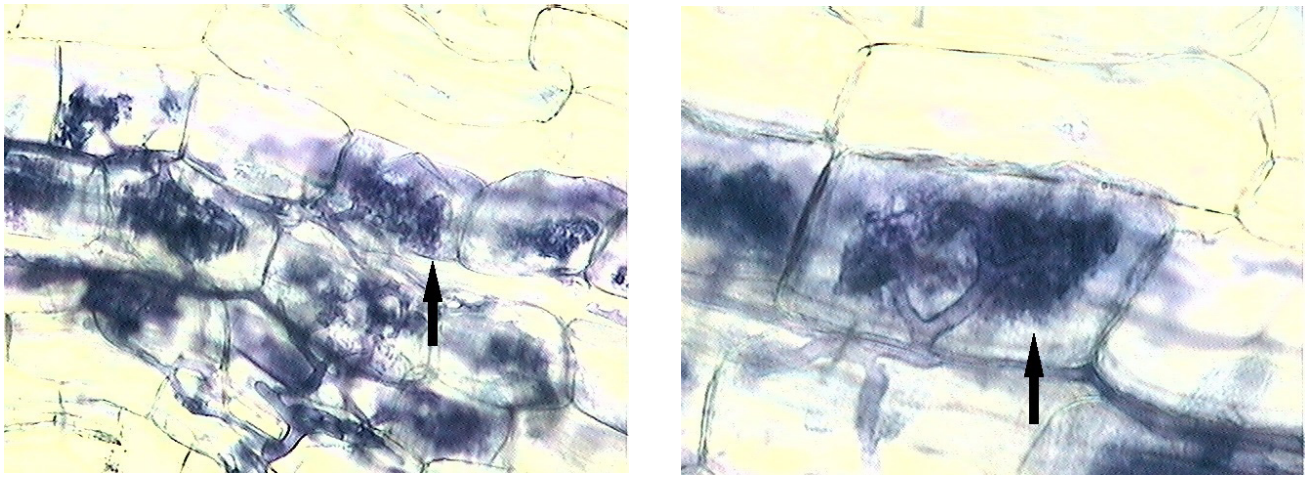
In the number of leaves it was observed a significant difference between treatments 1A (inoculated), with an average value of nine and treatment 2B (uninoculated mixed substrate), with seven leaves on average. The rest of the treatments showed an intermediate value. Although the leaf area was not measured, it was possible to observe a greater expansion of the leaves in the treatments with inoculated plants.

At 40 days after acclimatization, a second evaluation under the microscope of the root cells was performed. The development of structures called arbuscules, corresponding to the mycelium of the mycorrhiza, was observed in all treatments with inoculation (Figure 3). *AMF* are associated with most plant species under natural conditions (15), colonize the roots and develop a network or strands of external hyphae that extend from them, thereby increasing the contact surface between the Plant and soil. This external mycelium acts as a complementary radical system, of extraordinary importance for the absorption of nutrients and water by plants (16).



Different letters indicate significant differences according to LSD 95%

Figure 2. Height of plants (bars) and number of leaves (line) of Geneva®41 at 20 days of inoculation with mycorrhizae (a); Plants of the RN29 rootstock at 20 days of acclimatization with and without inoculation (b)



Arrows point to the arbuscules

Figure 3. Evaluation of root cells at 40 days of acclimatization

These beneficial microorganisms have been extensively studied and employed; however, its application in micropropagated plants is scarce. The results show the colonization by the mycorrhiza in early during the inoculation of the root system at 20 days after the acclimation and the consequences of the symbiosis were evident in the growth parameters evaluated.

To varying degrees, all plants grown *in vitro* are susceptible to transplant crisis or stress, a phase that is the bottleneck of the entire micropropagation process. The ultimate success of *in vitro* propagation depends on the ability of plants to transfer outside the laboratory environment to hothouse conditions, which vary among the herbaceous species have a rapid response, while the woody species require more time for their acclimatization. Knowledge of the physiological and morphological characteristics of plants growing *in vitro* is essential to minimize losses and ensure a high transplant survival. The inoculation facilitated the initiation of the physiological changes required, in order to favor the adaptation of the seedlings to autotrophy and subsequent *ex vitro* growth (17).

Both the heterotrophic nutrition is given *in vitro* and the poor mechanism to control water loss due to poor stomatal functionality, mean that the micropropagated plant is unbalanced in its ability to absorb and replenish transpired water (18). Due to the lack of wax in the cuticle compared to plants grown in greenhouse or field, the transpiration rate of these is significantly higher when they are growing *in vitro*.

In the first 25 days of the acclimatization process, no changes are observed at the morphological level, as there is a slow transition of physiological processes to changes in environmental conditions. However, in the inoculated plants, growth and development of new leaves were observed in the early stages. In this symbiotic association the fungus feeds on carbohydrates stored in plant cells in simple forms of fructose, glucose and sucrose, as well as the radical exudates of plants. It is for this reason that the inoculation of micropropagated plants in early stages confers greater resistance to environmental stressors in the acclimatization phase (19).

Phosphorus is the main nutrient, which has positive effects for the association of plants with AMF since it is relatively immobile in the soil, but there are also benefits in the dynamics of other nutrients such as N, Zn, Mg and Ca (20). In the choice of substrates to be evaluated, the sensitivity of mycorrhizae to phosphorus levels was considered, two substrates with low P content (<1 ppm) were selected. No fertilization regimen was used in seedlings inoculated by the inverse relationship between the availability of P and the colonization of mycorrhizal fungi (21, 22). To this is attributed that substrate 3, with high content of P, showed no significant difference among the inoculated and uninoculated plants. Mycorrhizal activity was inhibited by the presence of P.

In the substrates with low P content in which the inoculation with *AMF* resulted in greater growth, allowed to shorten the acclimation stage of 40 to 60 days.

CONCLUSIONS

- By evaluating development parameters of plants during acclimation, the action of arbuscular mycorrhizal fungi as agents of growth biofeedback, biofertilizers and biocontrol was demonstrated.
- The results indicate that its application in the acclimatization and nursery phase can represent benefits for producers, reduction of production costs, handling of organic products and the obtaining of plants with superior vigor and quality in a shorter time.
- The incorporation of beneficial microorganisms into intensive agriculture is important for achieving sustainable production systems and ecosystems with greater resilience to adverse conditions.

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During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial

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