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APPLICATION OF MYCELIUM DONOR PLANT (MDP) SYSTEM ON *In vitro* MYCORRHIZATION OF POTATO

Aplicación del sistema de Planta Donante de Micelio (PDM) en la micorrización *in vitro* de papa

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ABSTRACT. *In vitro* mycorrhization of plants is a highly complex process that depends on ensuring the needs of plants and fungi, organisms with different nutritional requirements. Today doesn´t exist an *in vitro* culture system which guarantee the mycorrhization of *Solanum tuberosum* L. (potato), although some authors have made several attempts to achieve it. The aim of this study was to evaluate the applicability of Mycelium Donor Plant (MDP) system for *in vitro* mycorrhization of potato plants in order to get mycorrhized plants in a short period, with proven practical use. Experimentally, a similar system to MDP, in which *S. tuberosum* plants (seven days old) were associated with an extensive network of mycelium coming from *Medicago truncatula* plants, previously mycorrhized with the AMF *Rhizoglomus intraradices* was design. High levels of potato plant colonization (55 %), just 12 days after contact the mycelium networks, were obtained. Potato plants were in excellent conditions to be transplanted and they were able to reproduce the fungal colony when they were replanted in fresh media, producing a large number of extraradicals structures (mycelium and spores), demonstrating the applicability of MDP system for *in vitro* mycorrhization of *S. tuberosum*.

Key words: extraradical micelium network, in vitro culture, *Rhizoglomus intraradices, Solanum tuberosum*

RESUMEN. La micorrización de plantas *in vitro* es un proceso altamente complejo que depende de garantizar las necesidades de plantas y hongos, organismos que tienen requerimientos nutricionales diferentes. En la actualidad, aún no se ha diseñado un sistema de cultivo *in vitro* que garantice la micorrización eficiente de plantas de *Solanum tuberosum* L. (papa), aunque algunos autores han realizado varios intentos por lograrlo. El objetivo de este estudio fue evaluar la aplicabilidad del sistema Planta Donante de Micelio (PDM) a la micorrización *in vitro* de este cultivo para obtener plantas micorrizadas en un corto periodo, con probada utilidad práctica. Experimentalmente se trabajó con un sistema diseñado de forma similar al PDM en el cual se asociaron plantas de *S. tuberosum*, de siete días de subcultivadas, con una extensa red de micelio proveniente de plantas de *Medicago truncatula* Gaertn., previamente micorrizadas con el hongo micorrízico arbuscular *Rhizoglomus intraradices*. Se obtuvieron elevados niveles de colonización (55 %) de las plantas de papa a solo 12 días de haber entrado en contacto con las redes de micelio. Las plantas de papa se encontraban en condiciones óptimas para ser trasplantadas y fueron capaces de reproducir la colonia fúngica cuando se replantaron en medios frescos, produciendo un elevado número de estructuras extraradicales (micelio y esporas), demostrando la aplicabilidad del sistema PDM en la micorrización *in vitro* de *S. tuberosum*.

Palabras clave: cultivo *in vitro*, redes de micelio extraradical, *Rhizoglomus intraradices*, *Solanum tuberosum*

INTRODUCTION

Over the last three decades, different lines of research have been developed in Cuba to study and introduce AMFs in agricultural production systems,

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based on the broad spectrum of arbuscular mycorrhizal symbiosis (1, 2). Due to the importance of this topic, the search for new inoculation pathways is a priority objective, mycorrhization of in vitro plants being one of the greatest aspirations.

Mycorrhization of in vitro plants is a highly complex process, which depends on guaranteeing the needs of plants and fungi (3), organisms with different nutritional requirements, and in the specific case of AMF, only limited information is available. The culture media, the culture systems and the inoculant source used are the factors that determine its success (4).

At present, an in vitro culture system has not been designed to guarantee the efficient mycorrhization of S. tuberosum plants. Although some authors (4, 5) have made several attempts to achieve this and several systems and culture media have been used, it has not been possible to accelerate the mycorrhizal process of the plants, nor to homogenize the germination of the propagules used. These authors have also shown that the use of spores as inoculum does not guarantee the root system colonization in a short period nor the production of a large number of extraradical structures, because these propagules require a long time to germinate and colonize, in addition they are influenced by diverse factors, difficult to control in such conditions.

In 2009, an in vitro mycorrhizal system of plants called the Mycelium Donor Plant (MDP) was published, which exploits for the first time the colonizing capacity of the mycorrhizal extraradical and in which large amounts of mycorrhizal inoculum are obtained in vitro, The time that mycorrhizal plants of M. truncatula are managed under autotrophic conditions (6).

This system was based on the low specificity of mycorrhizal associations in the wild, where hyphae that emerge from live mycorrhized roots are able to connect plants of the same or different species through extensive mycelial networks (6, 7).

Taking into account the precedents related to in vitro mycorrhization of S. tuberosum, it was decided to evaluate the applicability of the MDP system to the in vitro mycorrhization of this culture to obtain mycorrhizal plants in a short period with proven practical usefulness.

MATERIALS AND METHODS

In this study, the MDP system (6) was used for the rapid and homogeneous production of mycorrhizal potato plants in vitro, using extraradical mycelial networks as an inoculum source. Preliminary results (4) were taken into account, in which spores were inoculated as inoculum, which are not adequate propagules for mycorrhizal poplar plants in such conditions, since they require a prolonged time to germinate and establish colonization in the roots.

On the other hand, due to the high nutritional requirements of the potato and its rapid development under in vitro conditions, it is necessary to control other factors (eg, nutritional, environmental) that make the management of these systems complex. In this respect, reference is made in an earlier study (4) in which different systems and culture media were evaluated to guarantee *in vitro* mycorrhizae of potato plants and spores were used as inoculant, making clear the need to search for other propagules for ensure rapid colonization of *S. tuberosum*.

Biological material

It was used vitroplants of *Solanum tuberosum* L. cv. Desirée supplied by the laboratories of the Haute station, Belgium. The multiplication of the plant material was done by micropropagation of the nodal sections and 20 explants were placed in sterile culture boxes (90 x 60 x 50 mm) containing 50 mL of MS solid medium (Murashige and Skoog) (8) (Duchefa, Biochemie) supplemented with 20 g L^{-1} sucrose and 3 g L^{-1} Gel Gro® (ICN, Biomedicals, Inc., Irvine, USA) as the gelling agent, adjusted to pH 5,7 before sterilization. The culture boxes were transferred to the growth chamber under controlled conditions (22 °C, photoperiod of 16 h day^{-1} and photosynthetic photon flux of 225 µmol m^{-2} s^{-1}) to guarantee the development of the explants.

It also worked with seeds of *Medicago truncatula* Gaertn. Cv. Jemalong, strain A-17 (SARDI, Australia), which were sterilized by immersion in sodium hypochlorite with 8 % active chlorine for 10 minutes. They were washed with sterile deionized water and incubated in the dark (27 °C) to germinate in 90 mm diameter Petri dishes, containing 35 mL of SRM (Strullu and Romand modified) (9) medium without sugars and without vitamins, solidified with 3 g L^{-1} Gel Gro®. In each plate 25 seeds were placed. The plants were used four days after germination.

The fungal inoculum (Rhizoglomus intraradices Schenck & Smith, MUCL-41833), from four months of culture, was purchased from GINCO (In vitro collection of Glomeromycota, BCCM/MUCL, Microbiology Unit, Catholic University of Louvain, Louvain la Nueva, Belgium). The strain was supplied in a 90 mm diameter in Petri dish, in association with transformed roots (Ri T-DNA) of carrot (Daucus carota L.) in SRM medium, solidified with 3 g L^{-1} of Gel Gro®. The inoculum was subcultured using the methodology described by Cranenbrouck et al. (10) and incubated at 27 °C in the dark in inverted position.

Procedure to establish the system MDP

To establish the PDM system, the system published by Voets (6) was used as the basis, using the same methodology in its design.

In the root compartment of each Petri dish was also placed a plant of M. truncatula of four days of sprouting. The small roots were placed on the surface of the culture medium and the air system extended out of the plate.

Fifty spores of R. intraradices were inoculated on the roots, after extraction of a monoxenic culture of four months of age, by solubilization of the gel (10); in addition the system was closed and sealed (6). They were transferred to the growth chamber under controlled conditions (22 °C, photoperiod of 16 h day^{-1} and photosynthetic photon flux of 225 µmol m^{-2} s^{-1}) and maintained in a vertical position.

Weekly, 5 mL of SRM medium was added at a temperature of about 35 °C with 4 g L^{-1} of Gel Gro®. In this way, an adequate level of the medium was also guaranteed over the dividing wall (±2mm), to facilitate passage of the hyphae into the hyphal compartment (HC). After eight weeks of culture the number of hyphae crossing the partition wall between the radical compartment (RC) and the HC, the length of the mycelium and the number of spores produced in the HC were determined.

The number of hyphae crossing over the dividing wall was quantified using a bright field composite microscope (125-250X, Olympus BH-2, Olympus Optical GmbH, Germany). The mycelial length and number of spores developed in HC were evaluated using a dissecting microscope (10-40X, Olympus SZ40, Olympus Optical GmbH, Germany) (5).

Two groups of 25 experimental units were taken (each system was considered an experimental unit) to continue with the next phase of the study. Subsequently, two new holes (± 2mm diameter) were made in the HC of the systems, as described by other researchers (6).

The plates were carefully opened and two potato plants of seven days of subculture were placed, presenting approximately 3 cm in length and three to four leaves per plant. The roots were placed gently on the culture medium and the stem was positioned towards the outside of the plate. All systems were carefully sealed, the plates were covered with black polyethylene bags, leaving the aerial system of the plants exposed to growth chamber conditions similar to those mentioned above, but in a horizontal position.

Evaluations

At this time of the study, the 25 experimental units were divided into five groups, so that each group composed of five units was only kept in contact with the mycelial network on the corresponding days to complete the dynamics.

After 3, 6, 9, 12 and 15 days, according to established dynamics, one plant per experimental unit was extracted from their systems; the remaining plants were used for replanting. Subsequently, the roots of the extracted plants were stained (11) and the frequency and intensity of the colonization were estimated (12).

At 3, 6, 9, 12 and 15 days after contact with the mycelial network, the remaining plants of the five replicates were carefully extracted and transferred to Petri dishes of 90 mm diameter. The plants were transferred to E medium (modified MS) (4) without sugar or vitamins. The production of spores per petri dish and the development of the mycorrhiza extraradical, were measured weekly for four weeks (5).

Statistical analysis

After normality and homogeneity of variance (Brown-Forsythe test) were tested), using the statistical package SPSS Version 21 for Windows (13), the data were subjected to simple classification Variance Analysis. The values of the frequency and intensity variables were transformed according to the expression $\arcsen\sqrt{x}/100$ and checked their normal distribution. Variance Analysis and Tuckey Test (14) were performed to form the two groups of 25 MDP systems that would later be used to colonize the S. tuberosum seedlings, taking into account that there were no significant differences for $p < 0,05$ in the three variables evaluated (number of hyphae crossing the dividing wall between compartments, mycelial length in the hyphal compartment and number of spores in the hyphal compartment). With the spore production and mycelial length values at different sampling times (3 to 12 days), in the different weeks after the association, the confidence intervals from the mean to 95 % probability were calculated, taking into account the number of replicates and the reproducibility of the data. The SPSS Version 21 package for Windows (13) was also used.

RESULTS AND DISCUSSION

Between 10 and 12 days after the beginning of the experiment the first contact points between the germination hyphae from the spores and the roots of the plants were observed and the mycelium began to develop profusely within the medium in the radical compartments. This behavior was similar to that reported in the study, which proposes the system for mycorrhizal M. truncatula, using the same fungal species (6).

After six weeks, the hyphae began to cross the dividing wall between the RC and the HC, accounting for about 463±28 hyphae that had crossed to the HC at the end of this period.

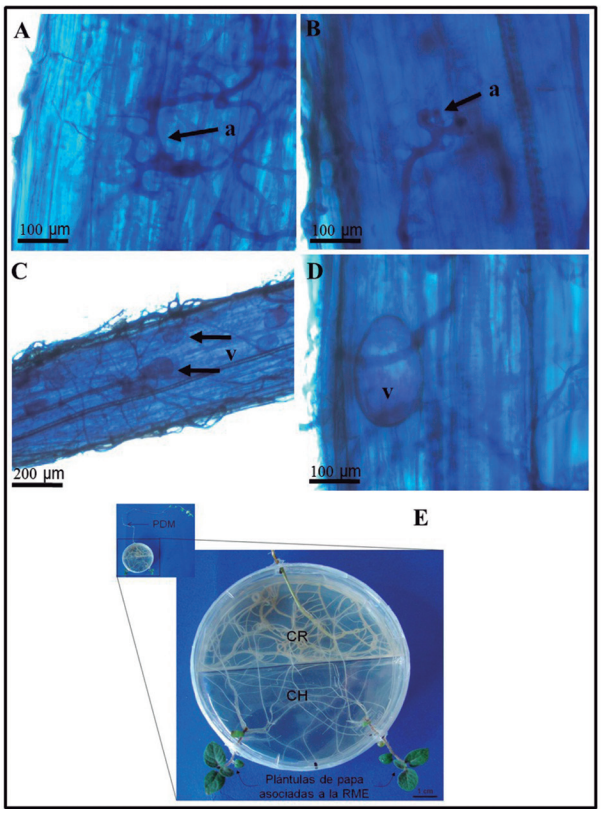
In both compartments the mycelium was characterized by abundant primary or running hyphae and low order hyphae bearing spores, branched absorption structures (ERA) and spores associated with ERA; typical behavior of the *R. intraradices* colony under in vitro conditions (6, 15).

Two weeks later, the extracellular mycelium had intensely colonized almost all HC and an average of 1338±186 cm of hyphae and more than 1 400 spores (1 420±238) had occurred. In the 25 experimental units that were selected to associate potato plants, divided into five groups of five systems, no significant differences were observed among the groups for the different variables analyzed (Table), appreciable in the p values.

Table. Significance values (p) determined by Tukey's test for the variables related to the fungal development present in MDP systems

Variables	Values of signification
Systems to associate plants of *S. tuberosum*	
Number of hyphae crossing over the dividing wall between CH and CR	0,173
Mycelial length in CH	0,235
Number of spores in CH	0,487

CH: hyphal compartment, CR: radical compartment,



Stain in Parker blue ink. A and B: appressoria formation (a) on the surface of potato roots at three days of contact with the mycorrhizal extracellular networks. C and D: production of vesicles (v) inside the cortical cells of roots of potato seedlings after nine days. E: Seedlings of S. tuberosum (16 days of age) after nine days of contact with RME. Petri dishes 90 mm dia. RC: radical compartment, HC: hyphal compartment

Figure 1. Colonization of potato seedlings associated with the mycorrhizal extracellular network (RME)

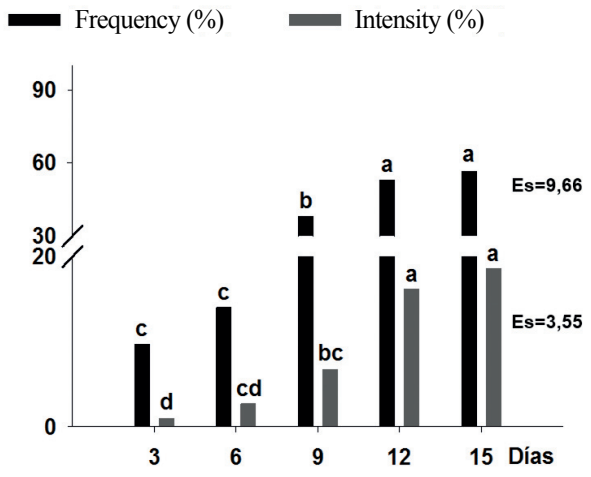
After the first three days of associating potato plants with RME, numerous appressoria were observed on the root surface of both plants (Figure 1A and B), located in the apical and subapical areas. These structures are the first that are observed after the contact between the hyphae of germination and the surface of the radical system is also the one that allows the infective propagule to adhere to the root to, from there, begin to penetrate the epidermis and settle in the radical interior (16).

On the sixth day, some vesicles were observed in the interior of the roots that began to be numerous from the ninth day (Figure 1C and D), being more frequent in the plants that were 12 and 15 days associated to the systems.

In Figure 1 is shown the healthy status of potato plants growing in SRM medium after nine days associated with the mycelial network present in the PDM system hyphal compartment. The SRM medium is characterized by having very low nutritional contents (3), since it is designed to guarantee the fungal establishment and is not suitable for the development of potato plants in vitro; however, as the plants were growing only 12 days in this medium, no symptoms of nutritional deficiency were observed, contrary to what other authors reported (4) when they inoculated the plants with spores in that same medium and the experiment had a duration of 40 days.

The evaluations carried out during the association days of the plants at the EMRs (3, 6, 9, 12 and 15 days) that reflect the establishment of mycorrhization is shown in Figure 2.

The levels of mycorrhizal colonization, expressed by the frequency and intensity variables, started to increase after the first three days of association with SMR until reaching a maximum value, close to 60 %, at 12 days. From that moment they remained constant until the end of the dynamics, showing no differences among the plants associated to the 12 and 15 days.



Equal letters in the same variable did not differ significantly for p <0.05 according to Tukey's test, n = 5

Figure 2. Dynamics of frequency and intensity of fungal colonization during 15 days of association between *S. tuberosum* plants and the extraradical mycelium networks

According to the analysis carried out in the scientific literature, such high colonization values have not been obtained so far in *S. tuberosum* under *in vitro* conditions (5) and they are higher than those reached by field culture (17, 18) , which do not exceed 30 %.

The high percentage of colonization reached in the plants is due to the dense mycelial network composed of hundreds of actively growing hyphae that emerged from the previously colonized roots and dispersed around the roots of the associated plants, as described by the authors of system used (6). In the experimental system, the mycelium from the roots of the donor plants occupied almost completely the hyphal compartment, producing about 1340 cm of hyphae, reason why the access of these hyphae to the roots of the newly associated plants was extremely simple.

Although the values obtained in this study, regarding the length of hyphae in the hyphal compartment, are lower than those reported by Voets (6), who reported having quantified about 4000 cm of hyphae, were sufficient for the plants will reach such high values of colonization in a period of time of only 12 days.

The results differ from previously published (5,19) in in vitro culture systems in which *S. tuberosum* and M.truncatula plants were inoculated, respectively, using a high dose of spores (100 per petri dish) as a source of inoculum.

These authors reported levels of colonization near 50 %, after eight weeks of cultivation and plants with severe symptoms of nutritional deficiency, showing the inconvenience of using spores as inoculum in these systems.

As discussed above, when spores are used as a source of inoculum the colonization process can be delayed, not only in occurrence, but also in extending in the radical system (4). This may be due to the fact that the spores have a lack of homogeneity in germination, limited capacity for independent growth and the colonization points that originate are local and are conditioned by the proximity to the root (20, 21).

When potato plants were removed and transferred to fresh medium E, a differentiated fungal behavior could be observed, depending on the days that the plants associated with SMRs had been (Figure 3A and B).

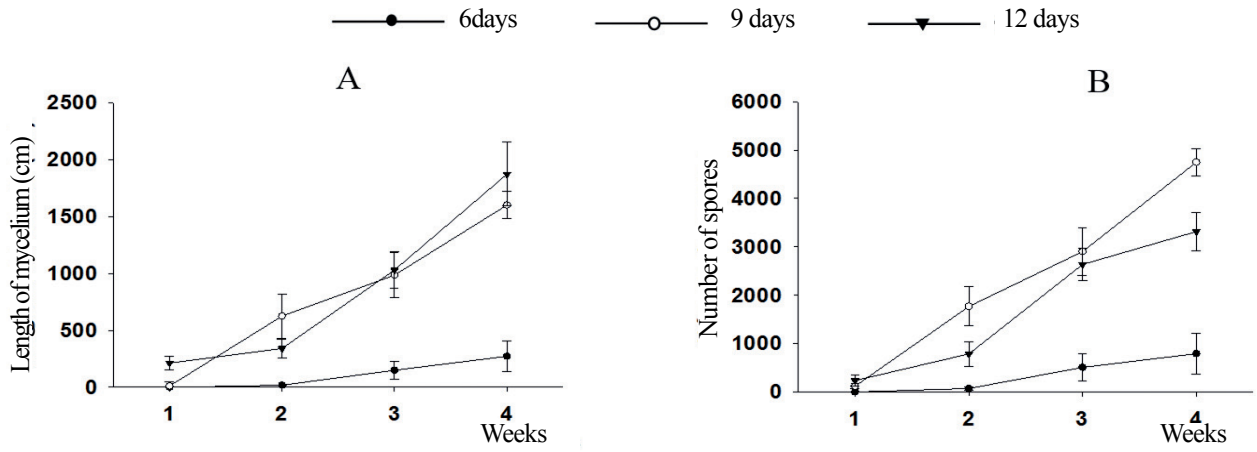
In the plants that remained only three days associated with the SMR, no new mycelial growth was observed during the four weeks of sampling, whereas in the case of the associated plants six days or more, a marked mycelial growth was observed, intact or damaged hyphae that were attached to the root surface, such as hyphae emerging from the radical interior (6). This growth was gaining in intensity as the plants were longer (9 and 12 days) (Figure 3A and B) in contact with the mycelial networks and therefore had a greater number of intraradicular fungal structures.

It was observed that the hyphae emerged from within the roots from the 48 hours of transplanted in the plants that were 9 and 12 days (Figure 4A and B) associated to the SMR and were characterized by a straight and profuse growth within the medium.

Numerous vesicles were also observed inside the roots (Figure 4D) and after the first week the mycelium had been intensively dispersed in the culture medium. Spore production was in correspondence with mycelial development and about 100 spores per Petri dish had already been produced during the first week of sampling.

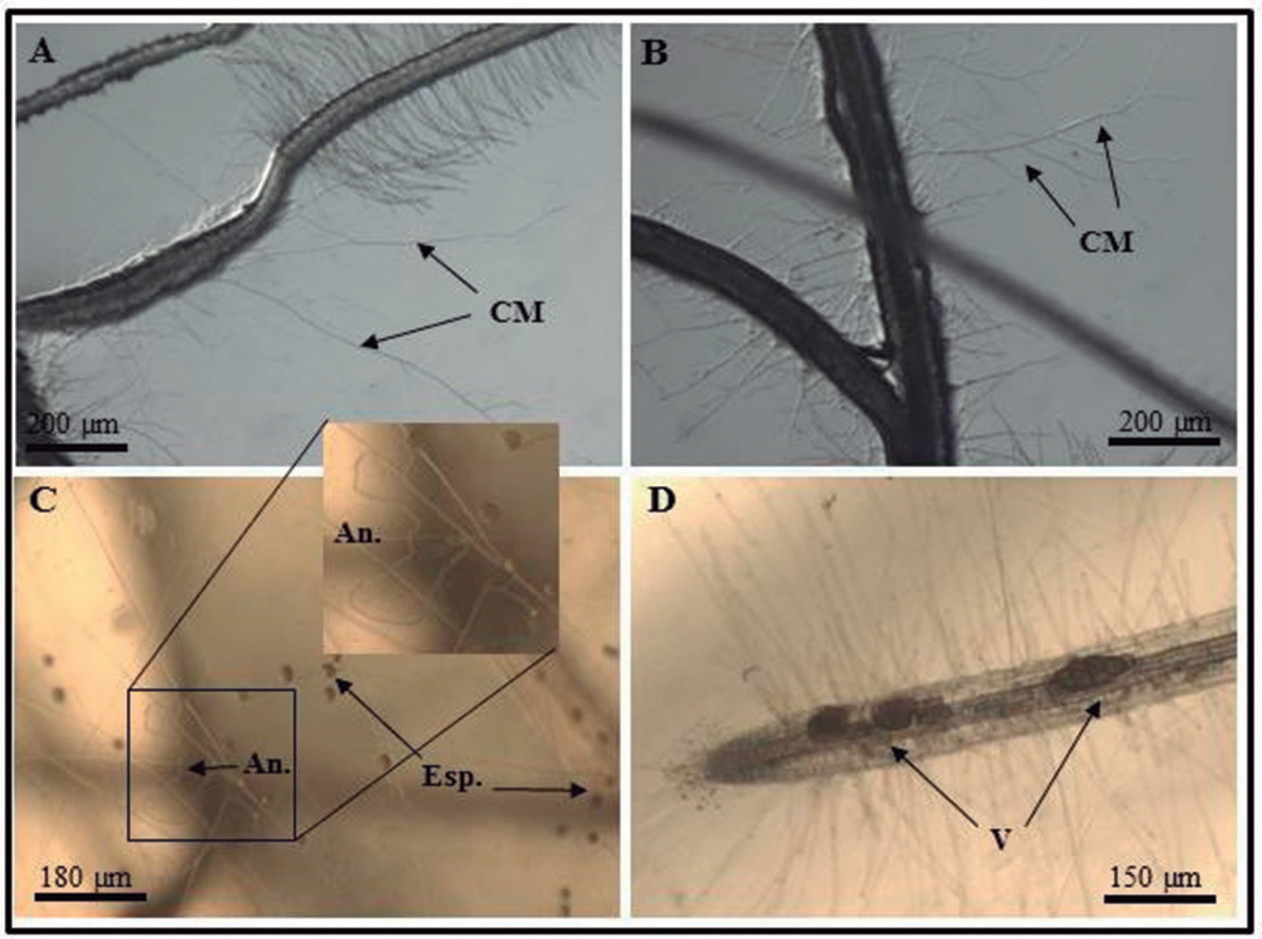
After four weeks, mycelium showed the typical growth of a colony of R. intraradices, with numerous anastomosis (Figure 4C) and a considerable number of spores per petri dish (Figure 4C and Figure 3B), with values close to 5,000.

Plants that stayed 12 days in contact with SMRs showed a new mycelial growth pattern similar to the one described above for nine-day plants, although they produced a smaller number of spores per petri dish.



The bars represent means of five replicates ± the confidence intervals for p <0.05

Figure 3. Dynamics of mycelial growth (A) and spore production (B) by Petri dish (90 mm diameter) of R. intraradices associated with S. tuberosum plants after transplantation to fresh medium E



Photos taken under the dissection microscope (4 -40x)

Figure 4. A andy B: Mycelial growth (CM) from nine - day plant roots of S. tuberosum associated with the extraradial mycelium networks, after 48 hours of transfer to the new medium (E). C: Developed mycelium and spores produced (Esp.) In potato plants after nine days associated with SMR, at 4 weeks of transfer to the new medium. An: anastomosis interconnecting hyphae in the colony. D: Presence of abundant vesicles (V) inside potato roots

When analyzing the values of colonization intensity, characterized by the presence of vesicles and arbuscules was confirmed, that the state in which the mycorrhiza is found, it is of vital importance to guarantee an abundant growth of the mycelium. These structures were only observed in the plants that were more than six days associated with the mycelial network and, for their part, the intensity values were significantly higher after the 12 days of association (Figure 2).

In the plants that were associated in the MPD system was not possible to relate the hyphal re-growth with the presence of arbuscules, because the first vesicles were also observed after six days of the plant roots coming into contact with the mycelium. These thick-walled structures, besides being important warehouse organs, play a significant role as propagules in root fragments (5, 10) and possibly also in living roots. On the other hand, the possibility that mycelial growth may have also occurred from the intraradical hyphae, since the AMF hyphae have the capacity to grow out of the roots and explore new environments (3), as it was also observed in this experiment.

In the plants that were associated with the mycelial networks, it was also observed that extraradical hyphae attached to the roots (removed from the initial medium for transfer to fresh media) continued to grow through intact or damaged sections. This could represent another mechanism of importance for the new fungal growth in transplanted plants. According to some authors (21), when AMF hyphae suffer damage naturally, they are able to regrow, repair, explore the surrounding environment and also colonize new roots, because they have efficient repair mechanisms.

Other authors have also shown that by cutting and extracting the culture medium from a hyphal compartment and adding fresh medium, the growth of new mycelium is promoted in a synchronized way, through the regrowth of cut hyphae or the formation of new hyphae (22).

The abundant number of spores (~5,000) produced by *R. intraradices* when colonizing *S. tuberosum* (Figure 3B) also reflects the advantages of the MDP system, as reported in this study the largest amount of spores obtained in vitro in a period of four weeks in colonized potato plants, in relation to the rest of the systems cultivation that have been published so far for this plant species.

Mycorrhizal potato plants can be obtained when using the MPD system, within a few days associated with the system (between 9 and 12 days), with high levels of colonization, able to reproduce the fungal colonies when transplanted to new media. In addition, if careful management of the systems, avoiding possible microbial contaminations, these can be reused allowing to associate two new plants in each system. These mycorrhizal plants at such a young age are in a physiological stage that allows their transplantation to greenhouse with the subsequent benefit that represents the mycorrhization in that phase and the massive production of mycorrhizal inoculum in culture plates or in bioreactors.

CONCLUSIONS

The inoculation of potato plants with Rhizoglomus intraradices extracellular mycelium from a donor plant allows to obtain high levels of in vitro colonization and to reduce the time of the colonizing process of S. tuberosum. These plants only four weeks after transplantation reproduce the fungal colony in fresh media. A large number of extraradical structures (mycelium and spores) are produced and the number of spores produced is approximately 100 per cubic centimeter.

As it has been exposed through this study the MPD system can be adapted to other species of plants and could be applied in diverse fields of investigation related to the AMF, not only from the point of view of the basic investigation, but also practice. On the other hand, the system constitutes the basis for the development of a future in vitro mycorrhizal system that allows the introduction of AMFs into current plant production practices and the production of efficiently mycorrhized in vitro plants, which will probably be more apt to face the stress that represents the adaptation to the edaphoclimatic conditions.

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