Development of an autotrophic culture system for the in vitro mycorrhization of potato plantlets

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Abstract

An autotrophic culture system was developed for the in vitro mycorrhization of potato plantlets. Roots of micropropagated plantlets were associated to an arbuscular mycorrhizal fungus under in vitro conditions, while shoots developed under open air conditions. Several thousand spores, an extensive extraradical mycelium and an abundant root colonization were obtained. Spores were able to colonize new plantlets under the same conditions. These results support the capacity of the autotrophic culture system to continuously culture arbuscular mycorrhizal fungi and may serve as a powerful tool to investigate various aspects of the symbiosis for which a source–sink relationship and photosynthetic active tissues are necessary.

Keywords: Arbuscular mycorrhizal fungi; Continuous culture; Monoxenic; Solanum tuberosum; Sporulation dynamics

1. Introduction

The technique of in vitro cultivation of root organs has been developed over the last decades [1–3] opening important avenues for studying plant–fungi interactions. It is found especially useful for the study of arbuscular mycorrhizal (AM) fungi since these obligate biotrophs rely on plant tissues to complete their life cycle. All areas of the AM fungi biology per se, as well as of the biology of the symbiotic relationship, have been revisited using in vitro cultures [4].

Despite the large array of applications, the use of excised root organs to study the AM symbiosis presents some major limitations, materialized by the absence of photosynthetic tissues, a normal hormone balance and physiological source–sink relationships [5]. For instance physiological studies, such as those involving the mechanisms of transport of minerals and carbohydrates from fungus to plant shoots and inversely, may suffer the absence of photosynthetic tissues. Identically, the addition of sucrose to the growth medium, to compensate for the absence of photosynthates, may modify the biochemistry of the plant–fungal interaction [5]. In this context, the development of in vitro cultivation systems in which autotrophic plants are associated to AM fungi appears essential.
The first in vitro system associating an AM fungus with a whole plant dates back to the early sixties. The fungus *Endogone* sp. was associated to different plantlets (white clover, corn, onion, cucumber, orchard grass) on an inorganic salt medium [6]. In the following two decades several authors experienced the in vitro association of an AM fungus with a host plant in liquid or agar medium [7–11]. Plants originating from somatic embryos were also demonstrated to form typical AM fungal structures in vitro [12]. Unfortunately, and perhaps due to the difficulty to reproduce and utilize these systems and to the limited quantitative and qualitative data generated on the growth and development of the symbiotic partners, little attention has been paid to these in vitro culture systems.

In the mid-nineties a tripartite (strawberry plant – mycorrhizal carrot root) [13] and a bipartite (strawberry – AM fungal isolated spores) [14] in vitro culture system were developed. With these systems, studies were performed on the water uptake and stomatal conductance [15,16], on the effect of light and CO₂ on the growth and photosynthesis of mycorrhizal plants [17] and on the use of mycorrhizal vitroplants in the micropropagation industry [18]. However, these systems presented some drawbacks. In the tripartite culture system [13], three partners were associated (the vitroplant, the AM fungus and the root organ culture). Therefore, the strict one-to-one organism interaction could be disturbed. In the bipartite culture system on polyurethane foam [14] the development of the extraradical mycelium as well as its micro-morphology, i.e., the architecture of the colony [19] is difficult to investigate non-destructively.

In the present study we developed a novel and easy to use culture system, in which the plant roots are associated to an AM fungus and develop on a gelled medium under strict in vitro conditions, while the photosynthetic shoot develops under open air conditions. Spore production dynamics, intraradical root colonization, germination capacity of the newly produced spores and their capacity to reproduce the fungal life cycle were assessed.

2. Materials and methods

2.1. Potato stock plantlets

In vitro propagated potato plantlets of *Solanum tuberosum* cv. Désirée, Kennebec, Pink Fir Apple and Rosa were supplied by the Station de Haute Belgique in Libramont, Belgium and *S. tuberosum* andigena cv. Waycha by the Scottish Agricultural Science Agency, Scotland. The cultivar *Solanum phureja* (CGN 18281) was supplied as true potato seeds by the Center for Genetic Resources, Wageningen, The Netherlands. The true potato seeds were surface sterilized with NaClO for 10 min, then rinsed 3 times during 10 min with deionized sterilized (121 °C for 15 min) water. After surface sterilization, the seeds were plated in Petri plates (90 mm diameter, 10 seeds per plate) containing the Modified Strullu Romand (MSR) medium ([20] modified from [11]) and were kept in the dark at 27 °C. After germination, nodal cuttings were placed in sterile culture boxes (90 × 60 × 50 mm, 20 cuttings per box), filled with 50 ml of 4.412 g l⁻¹ Murashige and Skoog (MS) medium [21], supplemented with 20 g l⁻¹ sucrose, 3 g l⁻¹ Gel Gro™ (ICN, Biomedicals, Inc., Irvine, CA, USA) and adjusted to pH 5.9 before sterilization (121 °C for 15 min). Plantlets were kept in a growth chamber at a constant temperature of 22 °C and illuminated for 16 h d⁻¹ under a photosynthetic photon flux of 15 µmol m⁻² s⁻¹. The plantlets of the other cultivars were also sub-cultured in vitro using nodal cuttings as described above. The process was repeated every 6–8 weeks.

2.2. Arbuscular mycorrhizal fungi cultures

Transformed carrot roots (*Daucus carota* L.) colonized with *Glomus intraradices* Schenck & Smith (MUCL 43194) were purchased from GINCO (http://www.mbla.ucl.ac.be/ginco-bel). Cultures were provided in Petri plates (90 mm diameter) on the MSR medium. The Petri plates were incubated 4 months in an inverted position in the dark at 27 °C. Several thousand spores were produced during this period.

2.3. Autotrophic culture system for the in vitro mycorrhization of potato plantlets

A hole (±2 mm diameter) was made in the side and the lid of Petri plates (90 mm diameter) containing 40 ml of MSR medium lacking sucrose and vitamins, and solidified with 4 g l⁻¹ Gel Gro™. Seven days-old rooted nodal explants, cultured on the MS medium, as described above, were transferred to the Petri plates. One plantlet was inserted in each Petri plate, with the roots placed on the surface of the medium and the shoot extending beyond the hole (Figs. 1 and 2).

Spores of *G. intraradices* from the AM fungal cultures described above were isolated by solubilization of the medium [22] and maintained in deionized sterilized (121 °C for 15 min) water before inoculation. An approximate of 150 spores were placed in the vicinity of the roots of the potato plantlets in each Petri plate. The Petri plates were then closed and the hole cautiously plastered with sterilized (121 °C for 15 min) silicon grease (VWR International, Belgium) to avoid contaminations. The Petri plates were then sealed with Parafilm (Pechiney, Plastic Packaging, Chicago, IL 60631), covered with an opaque plastic bag and incubated vertically in a growth chamber set at 22/18 °C (day/night) with 70% relative humidity, a 16 h photoperiod and a
photosynthetic photon flux (PPF) of 300 μmol m⁻² s⁻¹. The lamps used in the growth chamber covered a spectrum from λ = 400–700 nm. At week 4, 9 and 15 (according to the duration of the experiments described below), 10 ml of sterilized (121 °C for 15 min) MSR medium lacking sucrose and vitamins (cooled to 40 °C in a water bath) was added to the Petri plates to maintain an adequate level of MSR medium and to supply the plantlets with renewed nutrients. All the operations described above were conducted under a horizontal laminar hood.

Each autotrophic culture system was considered as an experimental unit in the experiments described below.

2.4. Experiment 1: Dynamics of spore production and development of plant and fungus

Eight experimental units consisting of *S. tuberosum* L. cv. Rosa plantlets inoculated with *G. intraradices* were non-destructively monitored to establish the dynamics of spore production. The total number of newly produced spores was counted 5, 6, 7, 8, 10, 13, 17 and 22 weeks after the association of the AM fungal propagules with the potato plantlets. The extraradical hyphal length was estimated at week 22. Spore number and hyphal length were estimated under a stereo microscope at 10–40× magnification. A grid of lines was marked on the bottom of each Petri plate. Spores were counted in each cell formed by the grid and summed over the entire Petri plate [23]. For the estimation of the hyphal length [24], the presence of hyphae was recorded at each point where they intersected a line and the total number of intersects between hyphae and gridlines was included in the formula of Newman [25], used to relate the total length of roots (or hyphae) [26] in a given area to the number of times they intersected a number of straight lines placed within this area. Plantlets were harvested at week 22. Shoot height was measured and microtubers counted. Roots were cleared in 10% KOH and stained with 0.2% Trypan blue [27]. Sixty
randomly selected root pieces (10 mm length) were mounted on microscope slides and examined under a bright-field light microscope at 40 or 125× magnification. The frequency (%F) and intensity (%I) of AM fungal root colonization were estimated [23].

The experiment was repeated with the same cultivar and fungal inoculum and under the same growth conditions for a period of 6 weeks in order to assess the reproducibility of the results obtained. Eight experimental units were considered. Spore production and hyphal length were estimated as above.

2.5. Experiment 2: spore germination and continuous culture

Newly produced spores, isolated from experiment 1, were tested for their capacity to germinate and ability to reproduce the fungal life cycle. Fifty spores were isolated from the Petri plates with forceps under laminar flow hood and placed singly in Petri plates (90 mm diameter) containing the MSR medium without sucrose and vitamins. Germination was observed every 4 days during 16 days under stereo microscope at 10–40× magnification. An approximate of 50 spores were also associated to S. tuberosum cv. Rosa plantlets in the autotrophic culture system to test their ability to reproduce the fungal life cycle. Eight experimental units were considered. After 6 weeks of culture the number of newly produced spores, extraradical hyphal length and %F and %I of root colonization were estimated as above.

2.6. Experiment 3: suitability of the autotrophic culture system for the in vitro mycorrhization of different potato cultivars

Five cultivars were tested: S. tuberosum L. cvs. Désirée, Kennebec, Pink Fir Apple, S. tuberosum andigena cv. Waycha and S. phureja. Eight experimental units were considered for each cultivar. The newly produced spores were counted after a period of 6 weeks as described above.

2.7. Statistical analysis

Data analysis was performed with the statistical package Statistica® for Windows [28]. Data that were normally distributed and had homogeneous variances were subjected to an analysis of variance (ANOVA). The Tukey HSD (honest significant difference) test was used to identify the significant differences (p ≤ 0.05).

3. Results

3.1. Experiment 1: dynamics of spore production and development of plant and fungus

Spores started to germinate within 7 days following their association with the potato plantlets. Hyphal growth was observed emerging through the lumen of the subtending hyphae and extended straight in the medium with several short ramifications formed. After 2 weeks, the first contact points between hyphae and roots were observed. At that time, hyphae started to spread more profusely into the medium. The first newly produced spores were observed at week 3. The number of spores produced was low during the 5 initial weeks of culture and increased thereafter (Fig. 3). After 4–6 weeks of culture, a network of hyphae covered the whole volume of the medium. Numerous branched absorbing structures were observed and anastomoses between primary hyphae and between secondary and primary hyphae were noticed. Arbuscules and vesicles (Fig. 4) were detectable in the roots starting from week 6. Culture medium was added in the Petri plates at week 4, 9 and 15 but did not impact spore production dynamics. A mean number of 940 ± 334 spores per week was formed during the last 5 weeks of the experiment, i.e., from week 17 to week 22. At the end of the experiment, an average of 12,264 ± 4745 spores was produced on a mean hyphal length of 1300 ± 244 cm. Fully developed spores were yellow-brown and contained numerous lipid droplets. Their mean diameter averaged 60–90 μm. Spores were terminal or intercalary and appeared single or in clusters containing up to 15 individuals. Some empty spores were detected at all observation times. At the end of the experiment, no plateau phase was
reached and spores were still in the phase of abundant production (Fig. 3).

Root colonization at week 22, estimated by %F and %I, reached a value of 74 ± 7% and 29 ± 4%, respectively. The roots contained many hyphae, vesicles and arbuscules. The vesicles and arbuscules were observed in the cortex of the main and lateral roots.

After transfer to the autotrophic culture system, the potato plantlets continued their growth. Primary roots developed on the surface and into the culture medium. They ramified profusely after a few days and, within 4–5 weeks, nearly the complete volume of the medium was occupied by thin roots. After 10 weeks of culture, plantlets started to form stolons with microtubers produced inside and outside the Petri plates. At the end of the experiment, plant shoots were about 15–20 cm high with 0–3 microtubers (8–10 mm diameter) produced.

The experiment was repeated under the same growth conditions as above for a period of 6 weeks. Spore production reached a value of 404 ± 378 which was statistically not different (p = 0.70) to the number of spores (483 ± 421) produced after 6 weeks in the first part of the experiment (see Fig. 3). The extraradical hyphal length reached a value of 125 ± 96 cm. The architecture of the extraradical mycelium as well as the growth characteristics of the plantlets was similar as above.

3.2. Experiment 2: spore viability and continuous culture

Ninety-eight percent of the newly produced spores germinated within 4 days. No subsequent germination was noted during the 16 days observation. Hyphae growth was observed emerging through the lumen of the subtending hyphae. Long hyphae were formed with several short ramifications. The spores were able to form associations with the roots of the potato plantlets in the autotrophic culture system and to produce new spores. An average of 944 ± 815 spores were produced after 6 weeks. This value did not significantly differ (p = 0.12 and p = 0.11, respectively) from the number of spores produced in experiment 1.

A network of extraradical hyphae covered the whole Petri plate with the formation of numerous branched
absorbing structures and anastomoses. Hyphal length averaged 255 ± 200 cm. This value did not significantly differ \( (p = 0.12) \) from the hyphal length measured in the first experiment. The internal root colonization was estimated at \( F = 23 ± 19\% \) and \( I = 11 ± 2\% \).

3.3. Experiment 3: suitability of the autotrophic culture system for the in vitro mycorrhization of different potato cultivars

Whichever the potato cultivar considered, spores were able to germinate, producing long hyphae that grew into the medium with several ramifications formed. The production of new spores followed the same dynamic as in experiment 1 with few spores produced until week 4 followed by a fast increase thereafter. The number of newly produced spores varied between the different cultivars (Fig. 5). The highest number was obtained with \( S. tuberosum \) cv. Désirée as host and the lowest with \( S. phureja \). Differences were significant between these two cultivars. No differences in spore production were further observed between the other cultivars.

4. Discussion

In the present study, we developed an easy to use and reproducible autotrophic culture system for the in vitro mycorrhization of potato plantlets.

The mineral composition of the growth medium was identical to the MSR medium used to grow AM fungi on excised transformed carrot roots [20], but without sucrose and vitamins supplemented to the medium. Shoots developed under high light intensity \( (PPF = 300 \mu\text{mol m}^{-2} \text{s}^{-1}) \), allowing plant photosynthesis [29] and therefore production of carbon and vitamins, while roots developed under dark growth conditions. As with the classical MS medium, the potato plantlets belonging to six different cultivars, grown on the MSR medium lacking sucrose and vitamins, developed actively [30]. The duration of experiment 1 (22 weeks) also supported the formation of stolons and microtubers.

Spore production exceeded 12,000 individuals after 22 weeks of culture. Data reported with the same AM fungal strain (MUCL 43194) [31] and with two different strains, MUCL 41833 [32] and MUCL 43204 [31] of the same AM fungal species, associated to excised transformed carrot roots on the MSR medium, gave comparable results. Only one study, to our knowledge, reported higher spore production with \( G. intraradices \) (MUCL 41833) in mono-compartmental Petri plates [33]. However in all these experiments, spore productions were recorded at the plateau phase [32], while in our experiment spores were still in the phase of heavy production with a weekly increase of 940 ± 334 individuals during the last 5 weeks. The absence of a plateau phase during this 22 weeks experiment could be related to the addition of MSR medium at fixed intervals, supplying the potato host plantlets with nutrients and therefore the fungal symbiont with carbon issued from the photosynthesis. Addition of growth medium was earlier tested [34] with \( G. intraradices \) associated to excised transformed carrot roots on the Minimal medium [35]. This author observed that the addition of glucose but also of mineral nutrients increased the spore production as compared to cultures not receiving supplemental C and/or minerals.

Whichever the experiment considered, spore production highly varied between replicates of the same cultivar. The coefficient of variation was 39% after 22

Fig. 5. Spore production of \( G. intraradices \), 6 weeks after association to the roots of different potato cultivars in autotrophic, in vitro conditions. Histograms represent means of eight replicates ± SE. Statistically significant differences \( (p \leq 0.05) \) are indicated by different letters within the histograms.
weeks of culture in experiment 1 and even higher in experiments 2 and 3. Such observation confirmed previous results obtained with in vitro cultures of various AM fungi [36,26]. Specifically focusing on G. intraradices, coefficients of variation calculated from different experiments ranked from 11% [37] to 22% [31], 38% [38] and even 61% [33]. The high variability observed in our experiment could be attributed to the host–fungus interaction and to both the fungal and plant physiology. Indeed, partner communication in the pre-symbiotic and symbiotic phase is a complex multi-step process under genetic control, regulated by an intimate molecular dialog between the host root and the obligate symbiont [39]. Such multi-step process could impact root colonization and subsequent spore production. Initiation of the symbiotic phase (i.e., root colonization) in our experiment varied noticeably between replicates. In some experimental units, AM fungi colonized roots within 3 weeks while in others it took more than 4 weeks. As a result, and specially for the experiments restricted to 6 weeks, the spore counts highly varied between the experimental units. It is assumed that such variability could partly level off at the later stage of culture development and probably when cultures reach the plateau phase. Such assumption should be considered in further experiments.

The length of extraradical hyphae measured in our study was similar to the values recorded in experiments conducted on excised transformed carrot roots with three different strains of G. intraradices [31,33]. Identically, the intraradical root colonization values estimated in our system were equivalent with the ones obtained on excised transformed carrot roots associated with G. intraradices, using the same method of estimation [33].

The newly produced spores were able to germinate, colonize a new host and reproduce the fungal life cycle. Spore production was close to the number obtained in the mother generation. Hyphal length was important and root colonization abundant. This supports the capacity of the autotrophic culture system to maintain AM fungi under continuous culture.

Spore production was obtained on four European and two Andean potato cultivars demonstrating the suitability of the system to cultivars differing in genotype and phenotype. Spores numbers, however, differed between the cultivars as earlier reported with tomato excised root lines [40] and different cultivars of carrot and clover excised roots [41]. This suggested that responsiveness of AM fungi varied between cultivars of a same species. Further experimentations are expected to continue in order to decipher the factors, at the level of the cultivar, which result in high rates of spore production.

The autotrophic culture system developed in the present study allowed the AM fungus to develop, under in vitro conditions, typical colonization structures and to produce an abundant extraradical mycelium with spores morphologically similar to those observed in the classical monoxenic culture systems on excised root organs [20] and in pot cultures. Spore production, extraradical hyphal length and intraradical root colonization were close to the values recorded with the same strain or different strains of the same species cultured in association with excised transformed carrot roots [31–33]. The newly produced spores were capable to form associations with a new host in vitro, following sub-cultivation, indicating that the AM fungus is able to complete its life cycle through successive generations. The autotrophic culture system was successfully applied to different potato cultivars (several European and Andean cultivars) and to Medicago truncatula L. (data not shown). Such system facilitates the study of various aspects regarding biochemistry, genetics and physiology of the symbiosis for which a source–sink relationship and photosynthetic active tissues are necessary.

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