Monoxenic in vitro production and colonization potential of AM fungus *Glomus intraradices*

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The paper reports the establishment of mycorrhizal infection of a non-mycorrhizal Ri-T-DNA transformed carrot root when co-cultured with a surface sterilized sweet potato root segment colonized by arbuscular mycorrhizal (AM) fungus *G. intraradices* on minimal M medium. Extensive fungal hyphal emergence from each cut end of the mycorrhizal sweet potato root piece was observed in one week old cultures. These hyphae caused infection on contacting the transformed-carrot-root segment and produced many hyphae and spores both inside and outside the zone of the root after 6 week of growth. Axenically produced fungal propagules proliferated on the surface of fresh minimal M medium when sub-cultured without any root segment. On repeated sub-culturing, these propagules did not lose their ability to grow and produced many juvenile small spore-like vesicles during the non-symbiotic phase. Although these spores were morphologically and anatomically similar to their soil borne counterparts, they were much smaller. When placed in the vicinity of a fresh hairy root on the minimal medium or a Sudan grass seedling in sand culture, the axenically produced AM fungal propagules caused root infection, but the infection characteristics were significantly different to the original culture in terms of shape (spherical vs oval) and size (20 μm vs 45 μm) of the intraradical vesicles, and absence of 'H' branches. Sudan grass seedlings inoculated with the axenically cultured fungus showed significantly (P<0.05) higher dry weights plant⁻¹. When compared to the plants inoculated with sand cultures, the growth parameters and the percentage infection were not significantly different. However, when both sources of inocula were used together, a synergistic effect on plant growth as well as root infection was observed.

Glomalean fungi are universal symbionts, which form arbuscular mycorrhizal (AM) associations with most plants and are important in various ecological conditions. In vitro culturing of AM fungi is difficult due to their obligate biotrophic nature and this limits their use for commercial utilization. AMF inoculum has been produced mainly by pot cultures, soil-free methods, aeroponic systems, and hydroponics.

Axenic cultures of AMF with living roots or suspension cultured plant cells as symbiont partner are reported. Diop et al. reported in vitro culture of AMF on Ri-T-DNA transformed tomato roots producing extensive external hyphae with spores and typical AMF infection of the roots. Plenchette et al. maintained AM fungus *Glomus versiforme* under axenic in vitro conditions on modified medium over three generations without loss of infectivity. Jolicoer et al. produced propagules of AMF *Glomus intraradices* in an airlift bioreactor by culturing transformed carrot hairy roots.

The purpose of this study is to report the production of AMF inoculum by co-culturing AMF colonized root segment with a non-mycorrhizal hairy (Ri-T-DNA transformed) root on a minimal M medium to result in producing extra-matrical AMF hyphae and spores and then sub-culturing the fungus so produced in the absence of the root. Infectivity of the monoxenic subculture of the AMF so produced is also tested by MPN method.

Production of mycorrhizal roots — Mycorrhizal sweet potato roots were produced aeroponically using *Glomus intraradices* Schenck & Smith (INVAM isolate WV 994A-1) as inoculum in an ultrasonic nebuliser system under green house conditions. Mycorrhizal root pieces harboured AM fungal structures such as non-septate inter- and intra-cellular hyphae averaging 8.9 μm diameter, oval to elliptical intra-radical vesicles (20-42 μm in diam.) and arbuscules. AMF colonized root pieces were surface sterilized with 3% H₂O₂ for 15 min followed by 3-4 washings with sterilized distilled water, and then aseptically transferred to Petri dishes containing minimal M medium with 1% sucrose. The dishes were stored at 4°C until used.

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Production of hairy roots — Agrobacterium rhizogenes (UNSW) was used to produce Ri-T-DNA transformed carrot roots. The root cultures were routinely propagated on minimal M medium as per Sharp and Doran13.

Dual cultures of mycorrhizal and non-mycorrhizal roots — A 6-7 cm Ri T-DNA transformed non-mycorrhizal carrot root explant and a surface sterilized heavily AMF infected 0.5 cm piece of sweet potato root produced by improved aeroponic culture technique6 were placed in close proximity onto the minimal M medium, and incubated at 25 °C in the dark. For the control, only mycorrhizal sweet potato root pieces were allowed to grow on the minimal M medium.

Viability of monoxenic subcultures of the AMF — The viability of the spores and hyphae produced on minimal M medium in dual cultures was tested by transferring (a) an infected transformed carrot root piece, (b) a minimal M medium agar block with fungal hyphae but without transformed root piece, and (c) an AMF infested minimal M medium agar block and a non-transformed root onto the fresh minimal M medium and incubating the plates in dark at 25°C.

Infectivity of subcultures of the AMF — In order to compare the infectivity of the sub-cultures of the AM fungus so produced, five day old Sudan grass seedlings grown from surface sterilized seeds (3% H2O2 for 3 min followed by 3 washing with sterilized distilled water) on moistened filter paper, were transferred to 500 ml plastic pots filled with steam watered to field capacity and inoculated by adding (i) 10 g of the original sand culture of AM fungus (INVAM 994A-1) from the USA, (ii) 10×1 cm pieces of the transformed and mycorrhizal carrot root, (iii) 1 cm² minimal M medium block infested with the AM fungus, and (iv) a combination of (i) and (iii), into a 5 cm deep hole before inserting the radicles of 5 pre-germinated Sudan grass seedlings per pot. The pots were maintained in a glass house (temperature range 13-25°C and photoperiod 14 h). After 6 weeks, the Sudan grass seedlings were harvested and their fresh and dry weights (80°C until constant) were determined. Some fresh root segments from each treatment were cleared (2.5% KOH) and stained (acid glycerol aniline blue) before measuring the amount of AM fungal colonization7. The number of viable AM fungal propagules per g of inoculum was determined by the most probable number (MPN) technique as per Khan14.

Continuous culture of G. intraradices were maintained over a period of 1 year. Carrot discs, infected with A. rhizogenes, produced 2-3 hairy roots at the infection sites after 3-4 weeks of incubation. The excised root tips grew very well on hormone-free minimal M medium and produced a few lateral branches.

When a mycorrhizal and aeroponically produced surface sterilized sweet potato root segment was placed in close proximity of carrot hairy root on the same minimal M medium plate, fungal hyphae appeared both laterally and from the ends of the root cuttings from the sweet potato mycorrhizal root pieces. Extensive hyphal growth emerging from ends of mycorrhizal root pieces was observed in one week old cultures. These hyphae caused infection on contacting the non-mycorrhizal transformed carrot root segment placed in the close proximity on the same plate. Sporulation started after 6 day of incubation of Ri-T-DNA transformed roots. Spores were found both inside and outside the zones of root after 6 weeks of growth. External hyphae of the fungus possessed all the characteristics typical of AMF, i.e. non-septate hyphae with angular projections and terminal spores, but the spores formed on the minimal M medium were much smaller than the original fungus. (15-47 μm compared with 90 μm). Internally, stained transformed carrot roots showed relatively straight dark stained hyphae which produced ‘H’ branches and numerous intraradical oval vesicles. Arbuscules were rarely observed.

Axenically produced fungal propagules proliferated on the surface of minimal M medium and covered it 4 weeks after incubation. On repeated sub-culturing, these propagules did not lose their ability to sporulate on fresh minimal M medium. No sporocarps were observed in the present in vitro systems. When placed in the vicinity of a fresh hairy root on minimal M medium or a Sudan grass seedling in sand culture, these axinically produced AMF propagules caused root infection, but the infection characteristics were significantly different to the original axenic culture in terms of intraradical vesicles shape (spherical) and size (18.5-22 μm) and absence of ‘H’ branches.

Sudan grass seedlings inoculated with the axenically cultured fungal inoculum sources, i.e. mycorrhizal transformed roots or minimal medium block infested with axenic propagules of G. intraradices, showed significant (P<0.05) differences in plant dry weights (Table 1). Microscopic examination revealed numerous intraradical vesicles and hyphae in the root.
cortices and numerous terminal spores on the extramatrical mycelium on the root surfaces of Sudan grass seedlings. Root colonization values for seedlings inoculated with mycorrhizal transformed roots or with axenic propagules of Glomus intraradices raised on minimal M medium, were not statistically different (Table 1). When compared to the seedlings inoculated with the sand cultures of Glomus intraradices, the DW and the percentage root infection values were also not significantly different. However, when transformed mycorrhizal roots and sand culture of Glomus intraradices, were used together, a synergistic effect on plant growth as well as root infection was observed (Table 1). The infectivity of the subcultures of the axenic AMF was not reduced, as evident from the MPN data (Table 1). The MPN assay also showed that the axenic AMF inoculum had a significantly higher number of infective propagules than those produced by sand cultures (Table 1).

The present investigation reveals synthesis of AMF infection by Glomus intraradices in transformed carrot roots. The transformed carrot root was able to grow on the minimal M medium. These results are in agreement with those of previous researchers. Karandashev et al. 15 raised dual cultures of AM fungi (including Glomus intraradices) and carrot hairy roots and found well-developed mycorrhizae with typical characteristics. Pawlowska et al. 16 reported establishment of axenic cultures of Glomus etunicatum in association with excised Ri T-DNA transformed carrot roots and demonstrated that AMF can be propagated in vitro using axenically formed resting spores and/or colonized root fragments. Abdul-Khaliq and Bagyaraj 17 reported successful mycorrhizal infection of Ri T-DNA transformed tomato roots during co-cultivation with spores of Gigaspora margarita on minimal M medium. This technique provides a better way to visualize the full life cycle and the events that occur during the pre- and post-colonization phases of Glomalesan fungi.

Although Strullu and Romandi 18 reported regeneration of vigorous hyphae from three Glomus spp. from surface sterilized mycorrhizal root segment on minimal M medium, no mycorrhization from the fungus so produced was tested. This is the first known report of mycorrhization of a Ri-T-DNA transformed non-mycorrhizal root segment when co-cultured with a non-transformed mycorrhizal root segment. Earlier investigators 19,20 reported production of AM infections in the root organ cultures, but in the growing system used fungal hyphae in the rootless compartment were still physically connected with the parent root through the dividing wall. Many researchers found that growth of the AM fungi ceases when the hyphal link to the root was broken. Various nutritional and genetic factors have been suggested to explain the lack of growth of the extraradical phase of AM fungi (for references see Diop et al. 19). In contrast, we were able to achieve the axenic growth of AMF in absence of a host root after the initial stimulus has been provided by the starter culture. Ishii et al. 21 reported successful axenic culture of Gigaspora ramisporophora in the absence of a plant partner but with the addition of methanol extract from bahia grass roots to the culture medium. Recently, Karandashev et al. 15 established monoxenic cultures of AM fungi including Glomus intraradices from a sterilized root piece with mycorrhiza alongwith fungal spores.

Sudan grass seedlings inoculated with axenically produced AMF in the present study showed typical AM fungal colonization and had significantly higher DW plant 1. These results are consistent with those of Mathur and Vyas 20 who found in vitro produced G. deserticola significantly beneficial for biomass production and nutrient uptake of in vitro raised plantlets in pots. Vimal et al. 22, in contrast, found no difference in colonization potential of monoxenic in vitro produced G. intraradices spores compared with a root-segment inoculum from open pot culture.

Although G. intraradices spores, formed in vitro on minimal M medium in the absence of any host root in the present study, exhibited general morphological and anatomical similarity to soil-borne spores, they were significantly smaller than their soil borne counterparts. They may be juvenile spores as they pro-

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>DW 1plant(g)</th>
<th>% infection</th>
<th>MPN index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.18 ± 0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Sand culture</td>
<td>0.51 ± 0.00</td>
<td>3.30 ± 0.00</td>
<td>1.35 ± 0.00</td>
</tr>
<tr>
<td>Transformed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycorrhizal roots</td>
<td>0.46 ± 0.00</td>
<td>3.60 ± 0.00</td>
<td>1.86 ± 0.00</td>
</tr>
<tr>
<td>M medium block</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected with axenic AMF propagules</td>
<td>0.46 ± 0.00</td>
<td>3.55 ± 0.00</td>
<td>2.14 ± 0.00</td>
</tr>
<tr>
<td>Transformed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycorrhizal roots + sand culture</td>
<td>0.38 ± 0.00</td>
<td>3.94 ± 0.00</td>
<td>4.26 ± 0.00</td>
</tr>
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Mean values in a column followed by different letters are significantly different at P<0.05 (n=10)
duced typical AMF infection in Sudan grass seedlings when used as inoculum in the present study. de Souza and Barbera studied the ontogeny of G. clarum in Ri-T-DNA transformed clover and carrot roots during the non-symbiotic growth phase and after the colonization of the transformed root and found that sporelike small vesicles during the non-symbiotic phase were juvenile spores. Pawlowska et al. observed similar differences between soil borne and monoxenic cultures of G. etunicatum in association with excised Ri-T-DNA transformed carrot roots. The literature also reveals that the spore size and morphology of AMF differ greatly, even within the same species. This could be associated with the relief of root induced growth repression, as postulated by St Arnaud et al. It may also be that the flavinoids released by the non-transformed sweet potato root and transformed carrot root in the present study had a differential effect on the fungal growth. Piche et al. have shown that the Ri-T-DNA transformed and non-transformed carrot roots release different flavinoids which are involved in a regulatory system which controls the hyphal growth of Glomus margarita. Further, after establishing itself inside a root, AM fungus changes the exudation pattern of host roots both quantitatively and qualitatively. Glomus macrocarpum was shown to reduce the exudation of both sugars and amino acids from the fine roots. Perhaps, a stimulatory substance produced in AM-colonized sweet potato root in association with Ri-T-DNA transformed carrot root in the present study supplied its needs to grow and sporulate without any plant partner.

The root organ culture technique employed in this study provides a better way to visualize the full life cycle and the events that occur during the pre- and post-colonization phases of Glomalean fungi. Furthermore, the use of molecular techniques and DNA technology will also provide additional tools for studying genetic variations between various AM-fungi. Clapp et al. reported that the ribosomal small subunit sequence varied considerably within spores of Scutellospora spp., an AM fungus.

The two novel biotechnological approaches used in this study, i.e. the use of Agrobacterium-transformed plant roots and mycelial cultures of mycorrhizal fungi, are considered as the research tools in the environmental remediation research. The contributions of mycorrhizal symbionts to soil productivity and enhanced heavy metal uptake during phytoextraction of contaminated sites has not yet been seriously considered and require further exploitation. Efforts to phytoexmediate contaminated soil can be enhanced by inoculating hyperaccumulator plants with axenic AMF inocula most appropriate for the site.

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References


