Axenic germination of *Scutellospora erythropa* and *Scutellospora nigra* in *in vitro* conditions

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Received 21 September 1999; revised 21 June 2000

Spores of *Scutellospora erythropa* and *Scu. nigra* isolated from neem rhizosphere soils from coastal regions of Chennai were tested for axenic germination in *in vitro* conditions. They showed positive results in media of different composition using root exudates, soil extract, thiamine HCl and inositol. The combined medium increased the spore germination in *Scu. erythropa* and in *Scu. nigra* over water agar control. The germ tube often grew up to 3.8 cm on combined media but no vegetative spores and extramatrical auxiliary cells were observed during the experiment. There was significant increase in hyphal growth when the roots were introduced into the medium, 3 days after spore germination.

Axenic cultivation of Vesicular Arbuscular Mycorrhizal (VAM) fungi has so far not been achieved. Spores of most VAM fungi readily germinate *in vitro* with hyphae elongating for a relatively short period on various media. Hyphal growth from germinating spores ceased before exhaustion of the spore reserves. This has motivated investigations on nutritional, physiological and genetic aspects. Germination studies on VAM suggested that storage was necessary to overcome dormancy in freshly harvested spores. Several factors reported to affect spore germination like nutrients, root exudates, soil temperature, moisture, pH and light. In the present paper we have examined the germination of *Scutellospora erythropa* and *S. nigra* in different media to assess the effect of these factors.

Materials and Methods

**Multiplication and maintenance of VAM spores** —
Spores of *Scu. erythropa* and *Scu. nigra* were isolated from rhizosphere soils (pH 7.8, N-123 kg/acre, P-24 kg/acre, K-100 kg/acre, EC=0.5 m mhos/cm², texture — sandy) of neem trees in coastal area near Chennai. The VAM spores were isolated by wet sieving and decanting method and modified sucrose centrifugation method. Isolated VAM fungi were multiplied in pot cultures on *Sorghum vulgare* and stored for 3 months at 10°C.

**Surface sterilization of spores** — Spores were surface sterilized using a modification of the two-step procedure. The steps were carried out aseptically in a laminar flow cabinet, except for centrifugation. Only young spores with brownish and red brown colour were selected and picked up using a fine sterile brush. Spores were transferred to petri plates (5 or 9 cm diam.) containing different agar media.

**Collection of neem root exudate** — Neem seeds were germinated in sterilized mixture of soil-sand (2:1) (10 µg/g of available phosphorus) and the seedlings were given 1/4 strength Hoagland's solution minus phosphorus. Five months after planting, 20 neem seedlings of uniform size were collected. Roots were pretreated for 2 hr in aerated deionised water containing antibiotics to control bacterial growth during the subsequent period for exudate collection. Roots were allowed to stand for 22 hr in fresh aerated deionised water under low light condition. The exudate solution of 200 to 250 mL was filter sterilized before storage at 5°C.

**Preparation of soil extract** — Soil extracts were prepared by addition of 200 g of soil in 300 mL deionized water and agitation for 48 hr. The extract was freeze-dried and added to 1 L of water agar media. Initially freeze-dried extract was sterilized by filtration (0.2 µm Millipore), then it was dissolved in water.

Thiamine HCl and inositol were used at 0.1, 0.5 mg/L, respectively. Root exudate and soil extract was added at 5 mL/L each. Water agar was prepared by dissolving 1g of agar in 100 ml of deionised water. In all combinations of the media, the pH was adjusted to 6.8.

The spores were incubated in dark at 28°C. Germination was assessed every 7 days up to 30 days. Spores were considered germinated when the germ
tube was visible. Germ tube growth was assessed at the end of each experiment by grading individual spores on a scale of 0 to 4 according to the length of the longest hypha: 0 = no growth; 1 = less than 5 mm; 2 = 5 to 10 mm; 3 = 10 to 15 mm; 4 = greater than 15 mm.

The results were averaged per treatment by dividing the sum of all grades by the number of germinated spores in a given treatment. Percentage of spore germination and percentage germ tube growth from the control of 2 VAM species were recorded.

The per cent of change in germ tube growth between treatment and the control was calculated by the following equation:

\[
\text{Per cent germ tube growth} = \frac{100 \times (\text{Average grade value of germtube for each treatment} - \text{Average grade value of germtube of control})}{\text{Average grade value of germtube of control}}
\]

**Results**

Characteristic features of VAM spores are summarized in Table 1. Per cent germination, change in germ tubes of *Scu. erythropa* and *Scu. nigra* in different media composition are presented in Table 2. Root exudate mixed with water agar increased the germination of *Scu. erythropa* (37%) compared with water agar control (29%). Soil extract (36%), thiamine HCl (32%) and inositol (30%) also increased germination compared to control. Root exudates increased germination of *Scu. nigra* by 34% followed by soil extract (33%), thiamine HCl (32%), inositol (29%) over water agar control (25%). In the new medium (soil extract + root exudate + thiamine HCl + inositol), spore germination of *Scu. erythropa* increased by 70% and in *Scu. nigra* by 65% respectively compared to water agar control. Root exudate increased germ tube growth by 28% over water agar. Soil extract increased germ tube growth by 35% over control. Thiamine HCl increased by 10% and inositol by 3% over water agar control. The combined medium increased 31% of germ tube growth in *Scu. erythropa* (Fig.1) and 48% in *Scu. nigra* (Figs 2,3) over water agar control. Surface sterilized azygosporous of *Scu.erythropa* and *Scu. nigra* germinated within 5-8 days on agar media. A germ tube emerged directly through the spore wall near the hyphal attachment. The germ tube often grew up to 3.8 cm on the agar based media but no vegetative spores and extramatrical auxiliary cells were observed. Root exudates stimulated the branching of germ tubes. The stimulation of germination and germ tube development by root exudate occurred in the absence of other microbial contamination. Soil extract also induced the germination and germ tube growth. Thiamine HCl significantly increased germination and germ tube growth of *Scu. erythropa* and *Scu. nigra*. Inositol also increased the spore germination. Fungal growth was...
completed in 5 weeks after germination with a maximum of 3.8 cm of hyphae extending from the spores.

In the absence of roots, hyphal elongation was slow and reached a maximum of 3.8 cm in 10 days after germination. Germ tubes either produced some branches extended up to 20-30 mm or without branches. There was a significant increase (20-fold) in hyphal growth when the roots were introduced into the medium 3 days after spore germination, without establishment of physical contact. Under *in vitro* condition, the hyphae demonstrated spontaneous growth with rapid increase in growth rate at the start (3-6 days) and then decreased progressively (15-58 days). No apparent correlation between length of elongated hyphae and spore size was observed.

When spore was severed from the germ tube after 5 days of germination, hyphal extension gradually stopped within 2 days. The freshly excised root bits of *Allium cepa*, surface sterilized and placed near the germinated (4-7 days) VAM spores with the germ tube in contact with roots were colonised by VAM fungal hyphae at 15 days (Figs 4,5). Complete hyphal network in the excised roots was observed. In addition, extramatrical hyphal elongation persisted even after removal of the spore. The hyphae in the root extended to medium but no spores were produced in the medium or inside the root bits.

**Discussion**

The axenic cultivation of members of the family Endogonaceae forming VAM symbioses is an important challenge from both the scientific and practical point of view. Empirical methods using diverse culture media including root extracts and root exudates indeed stimulate the growth of the fungi but have not facilitated the establishment of permanent pure cultures. In the present work, VAM germination showed positive results in media of different composition using root exudates, soil extract, thiamine HCl and inositol.

Root exudates mixed with water agar increased the germination of *Scu. erythropa* and *Scu. nigra* spores. Root exudates mixed with soil extract, thiamine and inositol significantly improved germination. Root exudates influence nutrient availability and microbial activity in the rhizosphere soil and they may influence nutrient solubility and uptake indirectly through their Table 1 - Characteristic features of VAM spores used for germination experiments

<table>
<thead>
<tr>
<th>Spore characters</th>
<th><em>Scutellospora erythropa</em></th>
<th><em>Scutellospora nigra</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Habitat</td>
<td>Red sandy soils</td>
<td>Red sandy soils</td>
</tr>
<tr>
<td>Spore size</td>
<td>298-503 µm</td>
<td>300-851 µm</td>
</tr>
<tr>
<td>Shape</td>
<td>Red-brown</td>
<td>Dark brown-black</td>
</tr>
<tr>
<td>No. of wall groups</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Wall width</td>
<td>6-15 µm</td>
<td>7-9 µm</td>
</tr>
<tr>
<td>Wall Lamellation</td>
<td><em>A(UU'U</em>)B(LM)*</td>
<td>Nr</td>
</tr>
<tr>
<td>Ornamentations</td>
<td>Laminations</td>
<td>Pores in the outer wall</td>
</tr>
<tr>
<td>Suspensor size</td>
<td>70-118 x 33-573 µm</td>
<td>43-55 x 85-111 µm</td>
</tr>
<tr>
<td>Hyphal attachment</td>
<td>Terminal</td>
<td>Lateral</td>
</tr>
<tr>
<td>Nature of hyphae</td>
<td>Septate</td>
<td>Septate</td>
</tr>
<tr>
<td>Auxiliary Cells</td>
<td>Clustered, Knobby</td>
<td>Clustered, Knobby</td>
</tr>
</tbody>
</table>

Nr — Not reported

*Identification of above VAM fungi following the manual of Schenck and Perez*.
effects on microbial activity\textsuperscript{18}. Several authors have suggested that specific compounds contained in root exudates are capable of stimulating hyphal growth of VAM fungi\textsuperscript{19-22}. Spore germination of \textit{G. fasciculatum} was stimulated by cell free extracts of non-symbiotic nitrogen fixers like \textit{Azotobacter brasilense} and \textit{A. lipoforum}\textsuperscript{23}.

Root exudates and CO\textsubscript{2} which induced hyphal growth from spores may also be pre-requisite factors for growing VAM fungi in pure culture. Nevertheless the mechanisms of fungal growth stimulation by root exudates and CO\textsubscript{2} are not known\textsuperscript{24}. Some authors found that root exudates of host plants not only stimulated hyphal growth, but also exerted some morphogenetical effects on the fungus\textsuperscript{9,20,24-26}.

The results are in accordance with the report of Danials and Graham (1976) where soil extract improved the germination of spores of \textit{G. mossea}\textsuperscript{9}.

Thiamine increased spore germination and germ tube growth as it is components of root exudates and may play an important role in the early events of mycorrhiza formation\textsuperscript{10}.

Even though inositol had low effect on germination of \textit{Scu. nigra} and \textit{Scu. erythropa}, it may serve as a carbon source for VAM fungi and increase mycorrhizal conditions in \textit{in vitro} condition. Hence the combination of root exudates, soil extract, thiamine and inositol is a potential medium for germination of VAM spores in \textit{in vitro} condition to achieve the pure culture of VAM fungi. Further research on these lines should bring to light some interesting data on obtaining pure culture of VAM fungi.

**Acknowledgement**

The authors are thankful to Prof.D.Lalithakumari, Director, Centre for Advanced Study in Botany, University of Madras for providing lab facilities and encouragement. One of the authors (K.S) would like to thank CSIR, New Delhi for providing financial assistance.

**References**

RAMAN & SAMBANDAN: AXENIC GERMINATION OF VAM