In vitro propagation and life cycle of the arbuscular mycorrhizal fungus Glomus etunicatum

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Progress in understanding the biology of arbuscular mycorrhizal (AM) fungi (Glomales, Zygomycetes) is hampered by the limited number of species that can be successfully propagated and studied in vitro. We report the establishment of monoxenic cultures of Glomus etunicatum in association with excised Ri T-DNA transformed carrot roots. The fungus can be propagated in vitro using monoxenically formed resting spores and/or colonized root fragments. Modified White’s medium buffered with 10 mM MES (pH 6) or MOPS (pH 6.5) was most optimal for the host root growth as well as for G. etunicatum spore germination and mycorrhiza formation. The number of resting spores formed in vitro correlated positively with the length of roots occupied by arbuscular mycorrhizal structures, including arbuscules and vesicles. Spores first appeared in dual cultures within two weeks of root inoculation. Sporulation was asynchronous and continued until root senescence. Under applied culture conditions, spores achieved mature appearance within 5–7 d after their initiation. Approximately 6% of monoxenous spores were aborted at different stages of their development. Although G. etunicatum spores formed in vitro exhibited general morphological and anatomical similarity to soil-borne inoculum, they were significantly smaller and had thicker spore walls than their soil-borne counterparts. Caution should, therefore, be exercised in utilizing the in vitro system as a model of growth and development of glomalean fungi in soil.

Progress in understanding the biology of arbuscular mycorrhizal (AM) fungi (Glomales, Zygomycetes) is hampered by the limited number of species that can be successfully propagated and studied in vitro. Systems utilizing excised roots of various host species as well as different media formulations have been developed to culture glomalean fungi monoxenically (Mosse & Hepper, 1975; Miller-Wideman & Watrud, 1984; Mugnier & Mosse, 1987a, b; Bécard & Fortin, 1988). Less than 5% of currently known arbuscular mycorrhizal species have, however, been successfully cultivated using such a dual culture approach. Gigaspora margarita (Miller-Wideman & Watrud, 1984; Bécard & Fortin, 1988), Glomus fasciculatum (Declerck, Strullu & Plenchette, 1998), G. intraradices (Chabot, Bécard & Piché, 1992; St-Arnaud et al., 1996; Declerck et al., 1998), G. macrocarpum (Declerck et al., 1998) and G. versiforme (Diop, Plenchette & Strullu, 1994; Declerck, Strullu & Plenchette, 1996; 1998) have been maintained and sporulated in association with excised tomato roots or roots of carrot transformed by ‘hairy root’ inducing T-DNA from Agrobacterium rhizogenes. Glomus mosseae has been shown to colonize excised roots of tomato and clover (Mosse & Hepper, 1975) as well as Ri T-DNA transformed roots of bindweed (Convolvulus sepium) and carrot (Mugnier & Mosse, 1987b; Douds, 1997). No sporulation has, however, been observed in G. mosseae while in dual culture. Formation of hyphal networks close to the roots, and appressoria on the root surface were reported for Glomus etunicatum spores germinated near Ri T-DNA-transformed carrot roots (Schreiner & Koide, 1993).

Applicability of excised root culture systems to study diverse aspects of arbuscular mycorrhizal biology has been widely demonstrated. Host-symbiont interactions (Balaji et al., 1995; Nagahashi, Douds & Abney, 1996; Douds et al., 1998), mineral uptake by arbuscular mycorrhizal fungi (Bago et al., 1996; Villegas et al., 1996), or interactions of glomalean fungi with plant pathogenic fungi (St-Arnaud et al., 1995) have been investigated in vitro using different combinations of host roots and AM species. Additionally, in vitro cultures constitute invaluable sources of contaminant-free glomalean mycelium and spores that can be used for nucleic acid preparations (Gadkar, Adholeya & Satyanarayana, 1997; Zézé et al., 1997). This aspect is particularly important as AM fungi from field collections or pot cultures may harbour hyperparasitic fungi (Rousseau et al., 1996), or spore-wall-associated bacteria that are recalcitrant to decontamination (Walley & Germida, 1996).

We report the establishment of in vitro cultures of Glomus etunicatum using Ri T-DNA-transformed carrot roots as host. Glomus etunicatum is one of the most frequently recorded species from nature and is often used in laboratory studies.
Culturing procedures developed by Bécard & Fortin (1988) were customized for *G. etunicatum*. A description of a complete life cycle and spore developmental sequence in *G. etunicatum* in the *in vitro* system are presented. In addition, morphological and anatomical characteristics of spores formed *in vitro* are compared to the characteristics of soil-borne spores.

**MATERIALS AND METHODS**

**Inoculum preparation**

*Glomus etunicatum* W. N. Becker & Gerd. from St Paul, MN, was propagated in greenhouse soil–sand (1:1) pot cultures with maize (*Zea mays* L.) as host. Cultures were harvested after 16 wk, when the plants senesced. Air dried soil containing *G. etunicatum* spores was stored for 12 mo at 4 °C before being used.

*Glomus etunicatum* spores were extracted from soil by wet sieving and decanting followed by sucrose centrifugation modified from Daniels & Skipper (1982). Within 24 h from extraction, spores were sonicated for 5 min in sterile 0.05% (v/v) Tween 20 using a microcentrifuge spin column (Qiagen, Chatsworth, CA) where the membrane was replaced by 60 μm nylon mesh. After rinsing off the debris with a jet of sterile 0.05% Tween 20 solution, spores were transferred to a 1.5 ml microcentrifuge tube, soaked in filter sterilized 2% (w/v) chloramide T for 10 min and rinsed three times for 10 min each in a solution of 2% (w/v) streptomycin sulphate and 1% (w/v) gentamycin sulphate (Chabot *et al.*, 1992). Chloramide treatment and antibiotic rinses were repeated twice. After soaking for 24 h in the antibiotic solution at 4 °C, spore decontamination was repeated without sonication. Spores were used immediately after the sterilization.

**Effect of decontamination procedure on spore viability**

The effect of the decontamination procedure on *G. etunicatum* spore viability, expressed as their ability to germinate, was assessed by plating surface decontaminated and non-decontaminated spores on 0.5% agarose medium (Sigma, St Louis, MO) and incubated for 8 wk in the dark at 25 °C. Additionally, spore germination was also tested in the soil native for the studied *G. etunicatum* ecotype using a method modified from Hepper (1979). Each treatment of 100 spores was replicated three times. For incubation in soil, spores were sandwiched between two cellulose nitrate filters (Sartorius, Göttingen, Germany) held together by a photographic slide mount, and buried at a depth of 2 cm in steam sterilized soil–sand mix (1:1). Ten 6-wk old big bluestem seedlings (*Andropogon gerardii* Vitt.) were planted above each germination unit. Big bluestem is an obligate mycorrhizal host and was expected to positively affect spore germination (Daniels Hetrick, Gerschefske Kitt & Thompson Wilson, 1988). The germination experiment was maintained in a growth chamber at 25 °C, 100 μE m⁻² s⁻¹ light intensity, 16 h day – 8 h night photoperiod, and was watered with deionized water as needed. Germination units were recovered from big bluestem rhizosphere after 8 wk incubation, gently rinsed, stained in 0.05% (w/v) trypan blue in acid glycerol, opened and examined for spore germination using a dissecting microscope.

**Efficiency of spore decontamination, germination *in vitro* and initiation of dual cultures**

A modified minimal White’s medium, M, (Bécard & Fortin, 1988) solidified with 0.3% gellan gum (‘Phytagel’, Sigma) was used in the subsequent experiments. Incorporation of various organic buffers into the M medium was tested to determine conditions optimal for *G. etunicatum* culture establishment. The following buffers were added to the M medium at 10 mM concentrations: Bis-Tris (bis-[2-hydroxyethyl]limino-tris-[hydroxymethyl]methane), MES (2-[N-morpholino]ethane-sulphonic acid), or MOPSO (3-[N-morpholino]-2-hydroxypropanesulphonic acid). The pH was adjusted to 6 prior to autoclaving in the media amended with Bis-Tris or MES, and to 6.5 in the medium amended with MOPSO. The pH was adjusted to 5.5 in the medium without the organic buffer amendment. No pH changes were observed after autoclaving.

Excised Ri T-DNA transformed carrot roots (DC1 clone), developed by G. Bécard, were used as host for *G. etunicatum*. Roots were propagated on modified White’s medium (Bécard & Fortin, 1988). Two-week-old root cultures (initiated by transplanting three 5 cm long root tips) were inoculated each with 50 decontaminated *G. etunicatum* spores. Spores were dispensed on the surface of culture medium alongside the actively elongating root regions, 2–3 mm from the root surface. Decontaminated *G. etunicatum* spores were also dispensed in plates of modified M media without roots to assess the effects of root presence in the germination environment. Each treatment was replicated four times. Plates were incubated in the inverted position, in the dark at 30 ± 2 °C. Spore germination as well as development of contamination on spore surfaces were monitored using a dissecting and an inverted microscope. Root length was estimated using a line intersect method (Tennant, 1975). Development of mycorrhizal colonization was assessed after roots were retrieved from the media, fixed, cleared and stained with 0.05% (w/v) trypan blue in acidic glycerol (Koske & Gemma, 1989).

**Dual culture propagation**

Three 5 cm long Ri T-DNA transformed carrot root segments were explanted on M medium buffered with 10 mM MES at pH 6. After 2 wk, 40 root cultures were each inoculated with a 1 cm² plug of culture medium from 6-mo-old *G. etunicatum* dual cultures initiated from surface decontaminated spores (10 mM MES, pH 6). The inoculum plug contained monoxenic spores and colonized root segments. Six of these initiated cultures developed bacterial contamination and were discarded. After 12 wk of incubation in the dark at 30 ± 2 °C, resting spores formed in each culture were counted and the root colonization by *G. etunicatum* was assessed using a magnified intersections method (McGonigle *et al.*, 1990) after roots were fixed and stained as described previously. The entire root length from each culture was examined, which enabled us to calculate the root lengths occupied by the AM structures.
Spore development in vitro

The development of 30 resting spores of *Glomus etunicatum* was followed at 24 h intervals over a period of 8 d in dual cultures maintained in M medium buffered with 10 mM MES at pH 6.

Comparison between monoxenic and soil-borne spores

Monoxenic *G. etunicatum* spores were retrieved from a dual culture (10 mM MES, pH 6) and mounted in polyvinyl alcoholaetic acid-glycerin (PVLG) with Melzer’s reagent (Morton, Bentivenga & Wheeler, 1993). Soil-borne spores were extracted from the same batch of soil as the original inoculum for excised root cultures described before, and also mounted in PVLG with Melzer’s reagent. Spore diameter and wall thickness in at least 100 spores of each type were measured using an ocular micrometer.

RESULTS

Effects of decontamination procedure on spore viability

*Glomus etunicatum* spore viability (expressed as their ability to germinate) was not affected by the decontamination procedure. There was no difference in germination rates of decontaminated and non-decontaminated spores when they were incubated in the *A. gerardii* rhizosphere (Table 1). Both types

Table 1. Effect of decontamination procedure on germination of *G. etunicatum* spores in two different environments (Andropogon gerardii rhizosphere or 0.5% agarose medium)

<table>
<thead>
<tr>
<th>Environment</th>
<th>Decontaminated spores</th>
<th>Non-decontaminated spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. gerardii rhizosphere</td>
<td>62.5 ± 9.4</td>
<td>66.3 ± 4.2</td>
</tr>
<tr>
<td>0.5% agarose medium</td>
<td>0.0 ± 0.0</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

Germination rates between decontaminated and non-decontaminated spores did not differ significantly using an unpaired t-test at P = 0.05.

Table 2. ANOVA table for the effects of the germination environment (with and without host roots) and the medium composition (buffer type) on *G. etunicatum* spore germination rates

<table>
<thead>
<tr>
<th>Source</th>
<th>D.F.</th>
<th>Mean square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environment</td>
<td>1</td>
<td>8153</td>
<td>49.9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Medium</td>
<td>3</td>
<td>1142</td>
<td>6.99</td>
<td>0.0015</td>
</tr>
<tr>
<td>Environment × Medium</td>
<td>3</td>
<td>3316</td>
<td>20.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>24</td>
<td>163</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Summary of the *G. etunicatum* dual culture propagation experiment where roots were inoculated with media plugs containing spores and mycorrhizal root segments from a previously established dual culture

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of cultures</td>
<td>34</td>
</tr>
<tr>
<td>Cultures with visually active inoculum (%)</td>
<td>79.4</td>
</tr>
<tr>
<td>Cultures with root colonization (%)</td>
<td>52.9</td>
</tr>
<tr>
<td>Mean root length at harvest (cm)</td>
<td>94.6 ± 3.6</td>
</tr>
<tr>
<td>Mean root colonization – arbuscules (%)</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>Mean length of roots occupied by arbuscules (mm)</td>
<td>28.1 ± 5.5</td>
</tr>
<tr>
<td>Mean root colonization – vesicles (%)</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Mean length of roots occupied by vesicles (mm)</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td>Sporulating cultures (%)</td>
<td>44.1</td>
</tr>
<tr>
<td>Mean spore number produced per culture</td>
<td>152 ± 37.6</td>
</tr>
<tr>
<td>Mean spores aborted per culture (%)</td>
<td>61 ± 1.5</td>
</tr>
</tbody>
</table>

* Means ± s.e.m. where appropriate.

of spores germinated at about 60%. Decontaminated spores failed to germinate in agarose medium, whereas non-decontaminated spores, which supported prolific growth of contaminant microorganisms, germinated at 1%. The latter germination rate was not statistically greater than zero.

Spore decontamination efficiency

The decontamination procedure applied to *G. etunicatum* spores enabled about 90% of the spores plated on M media
Glomus etunicatum monoxenic culturing

Figs 3–6. *In vitro* culture of *Glomus etunicatum* in association with excised Ri T-DNA transformed carrot roots. **Fig. 3.** Distinct colony within a host root visualized by staining cleared roots with trypan blue. Bar, 100 µm. **Fig. 4.** Arbuscules. Bar, 10 µm. **Fig. 5.** Resting spores in a 12-wk-old culture. Bar, 1 mm. **Fig. 6.** Extraradical mycelium with resting spores. Bar, 100 µm.

with and without host roots to remain contaminant-free for a period of 8 wk. Spores that developed signs of bacterial contamination were immediately removed from the plates. After 8 wk all remaining ungerminated spores were taken away from the plates as experience from other experiments indicated that beyond this period all spores eventually developed bacterial contamination.

**Glomus etunicatum spore germination in vitro and initiation of dual cultures**

*Glomus etunicatum* spore germination *in vitro* was observed 4–10 d after plating. Germination always occurred through the subtending hypha. Germ tubes branched and grew in all directions.

Presence of host roots significantly increased *G. etunicatum* spore germination rates in the M media with incorporated organic buffers (Fig. 1, Table 2). In contrast, spores plated on the unbuffered M medium germinated at similar rates regardless of whether host roots were present or not. The largest increase in spore germination was observed in the M medium buffered with Bis-Tris despite the fact that root growth in the Bis-Tris buffered medium was strongly inhibited (Fig. 2). They formed abnormally large numbers of lateral root primordia which did not elongate further, resulting in roots that, at the inoculation time were significantly shorter than roots grown on other media.

*Glomus etunicatum* germ tubes usually contacted host roots within 24 h after spore germination. Hyphae proliferated abundantly near and on the surface of roots forming variously shaped-appressoria. Colonization of roots followed by resting spore formation was observed in roots grown in all media except M medium buffered with Bis-Tris, where root colonization was suppressed as the level of appressoria formation.

**Dual culture propagation**

In Ri T-DNA transformed root cultures inoculated with media plugs containing *in vitro* formed spores and colonized root fragments, active growth of the mycelium from the plugs was observed in almost 80% of plates (Table 3). Both germination of monoxenic spores and outgrowth of hyphae from mycorrhizal root fragments contributed to this mycelial growth. Establishment of arbuscular mycorrhizae was observed in about 50% of root cultures. *Glomus etunicatum* formed distinct colonies in Ri T-DNA transformed carrot roots (Fig. 3). Each fungal colony was usually initiated by several branches of one hypha that proliferated near the root. Hyphae penetrating epidermal and cortical cells immediately underlying entry points were usually coiled. Arbuscules formed in the deeper layers of cortical cells and were finely branched (Fig. 4). Arbuscular colonization averaged about 3% of the total root length (Table 3). Vesicles were small and intracellular.
Levels of vesicular root colonization were usually lower than those of arbuscular colonization and averaged about 1% of the total root length.

Formation of the first resting spores was observed within 2 wk of inoculation of root cultures and continued until roots were harvested at their senescence (12 wk) (Figs 5–6). Spores ultimately were formed in about 40% of the inoculated root cultures (Table 3). Approximately, 150 new spores were produced per root culture. There was a positive correlation ($r = 0.8$, $P < 0.0001$) between the number of spores produced per culture and the length of roots occupied by AM fungal structures (Fig. 7). No interaction was observed between the number of new spores and total root length.

Approximately 6% of spores formed monoxenically were visibly aborted at various developmental stages as often manifested by leakage of contents spilled into the surrounding medium.

**Glomus etunicatum spore development in vitro**

New spores formed singly as terminal swellings on hyphae from mycelial network originating from the inoculum or extending from the colonized root segments (Figs 5–6). Spore formation was asynchronous. Spores achieved their mature size within 48 h of initiation (Figs 8–17). Thickening of a hyaline spore wall resulted in formation of the layered wall that constituted of a hyaline outer layer up to 3 µm thick and a yellow-orange laminated inner layer 2–12 µm thick. The outer hyaline layer was characteristically uneven on the surface and appeared patchy when visualized with Melzer’s reagent. Development of lamination in the inner spore wall was accompanied by increase in the inner wall pigmentation. Development of the spore wall structure was usually completed within 72–96 h from spore initiation. The opaque milky-white colouration of a young spore corresponded to densely granular composition of spore cytoplasm (Figs 8, 13). Spore contents coalesced into discernible oil droplets with progressing pigmentation of the inner wall layer, giving mature spores an almost clear appearance (Figs 12, 17).
Comparison between monoxenic and soil-borne spores

Spores produced in monoxenic culture exhibited general morphological similarity to soil-borne spores. On average, however, the diameter of mature spores formed in vitro was significantly smaller than that of spores retrieved from pot cultures and used as inoculum (Table 4). The monoxenic spores ranged from 48–130 µm diam. whereas soil-borne spores were 84–132 µm diam. About 2% of the mature-looking monoxenic spores were smaller than 68 µm, the lower size limit in the species description (Becker & Gerdemann, 1977). The spore wall in monoxenic spores was thicker than that of soil-borne spores. This increased wall thickness was related to the increase in the width of the inner laminated wall layer. The inner layer was 2–12 µm thick in the monoxenic spores and 2–5–8 µm in the soil-borne spores. There was no difference in the thickness of the outer hyaline layer. The mature monoxenic spores tended to retain their outer hyaline wall layer, although frequently in the form of irregular patches, whereas the majority of the examined soil-borne spores lacked this layer. The spore wall thickness observed in monoxenic spores corresponded to the dimensions described in the species description (Becker & Gerdemann, 1977).

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**DISCUSSION**

Glomus etunicatum spore germination in vitro

Spore germination in *G. etunicatum* appeared to be induced by various abiotic and biotic factors. Decontaminated spores did not germinate in highly purified agarose medium. This was not related to the impaired viability because similar germination rates for decontaminated and non-decontaminated spores were found when incubated in big bluestem rhizosphere.

Germination of decontaminated *G. etunicatum* spores in M medium solidified with gellan gum and buffered with the organic buffers was significantly enhanced by the presence of excised host roots. No such effect was observed in the unbuffered M medium where spores germinated at a similar rate with or without host roots. Plant derived exudates and volatiles including carbon dioxide have modifying effects on AM fungal spore germination (Graham, 1982; Le Tacon, Skinner & Mosse, 1983; Gianinazzi-Pearson, Branzanti & Gianinazzi, 1989). Schreiner & Koide (1993) demonstrated that excised roots of both host and nonhost plants can stimulate germination in *G. etunicatum*, increasing it by about 10% in comparison to the less than 5% germination rate in the control treatment. Flavonoids that are naturally released by plants have been identified as possible factors involved in regulation of spore germination in AM fungi (Gianinazzi-Pearson et al., 1989). For example, germination of *G. etunicatum* and *G. macrocarpum* was significantly increased by exposure to the seed derived flavonoids, quercetin and quercetin-3-O-galactoside, as well as to the root exuded flavonoids, 4',7-dihydroxyflavone and liquiritigenin (Tsai & Phillips, 1991).

The lack of stimulatory effect of host roots on germination of decontaminated *G. etunicatum* spores that we observed in the unbuffered M medium, although contradictory to other findings, may indicate that this specific medium formulation created an environment where the roots did not synthesize sufficient amounts of stimulatory substances. It is also possible that the roots affected the composition of the unbuffered medium in such a way that it exerted an inhibitory effect on spore germination and thus obscured the stimulatory root effects that we observed on the buffered media. The latter explanation is supported by a great sensitivity exhibited by glomalean spore germination to adverse conditions created by unfavourable concentrations of inorganic ions, especially hydrogen, as well as various organic compounds (Green, Graham & Schenck, 1976; Hepper & Smith, 1976; Siqueira, Hubbel & Schenck, 1982).

Glomus etunicatum culture initiation and propagation

*G. etunicatum* spore germination in M media buffered with MES, MOPSO or in the unbuffered medium led to root colonization and spore production. In contrast, no root colonization beyond appressoria formation was observed in the roots propagated in the M medium buffered with Bis-Tris even though these roots exerted a highly stimulatory effect on spore germination. Root growth was inhibited in the Bis-Tris buffered medium; however, they initiated abnormally large numbers of lateral root primordia comprised of meristematic cells. Whether the lack of susceptibility to fungal colonization exhibited by roots grown in the Bis-Tris buffered medium is related to altered auxin/cytokinin balance or some other phenomenon remains to be determined.

The number of new spores produced *in vitro* was positively correlated with the length of roots occupied by AM fungal structures, including arbuscules and vesicles. This finding indicates that maximum production of monoxenic spores of *G. etunicatum* can be achieved by optimizing inoculation regimes to yield high levels of root colonization.

The aborted spore development observed *in vitro* is an interesting and not fully understood phenomenon. It remains to be investigated whether this is limited to *in vitro* conditions
or also occurs in nature. Spore abortion may be a possible mechanism of cleansing detrimental mutations from the glomalean genome. Such a mechanism would be of prominent value for the evolutionary survival of these presumably clonal organisms (Rosendahl & Taylor, 1997).

**Spore development in vitro and comparison with soil-borne spores**

*Glomus etunicatum* spore development observed under *in vitro* conditions corresponded to the developmental sequence reconstructed by Stirnér & Morton (1997) based on examination of spores of different ages extracted from soil. This indicates that, in general, spore development *in vitro* is similar to that in the natural soil environment.

Although monoxenic and soil-borne spores of *G. etunicatum* exhibited general morphological and anatomical similarities, spores formed *in vitro* were smaller in diameter and had thicker walls than spores formed in soil. The increase in spore wall thickness was related to the increase in the width of the inner laminated wall layer. Mature spores produced *in vitro* retained the outer wall layer, frequently absent from soil-borne spores most likely due to microbial activity and abrasion against soil particles. Approximately 2% of the mature-looking monoxenic spores were smaller than the lower size limit in the species description (Becker & Gerdemann, 1977). The differences in spore dimensions between soil-borne inoculum and monoxenically reproduced spores demonstrated the effects of environmental conditions on taxonomically important morphological characters of glomalean spores. *In vitro* systems, although indispensable for some purposes, provide only an approximation of the conditions natural for mycorrhizal functioning. Caution should therefore be exercised when extrapolating results observed *in vitro* to explain phenomena detected in nature.

We would like to thank Dr Guillaume Bécard for providing the excised Ri T-DNA transformed carrot roots (DC1 clone). Funding for this research was made possible by the Minnesota Legislature (ML 1995, Chp. 220, Sect. 19, Subd. 13(C)) as recommended by the Legislative Commission on Minnesota Resources from the Minnesota Environment and Natural Resources Trust Fund, Minnesota Department of Transportation, and University of Minnesota Graduate School Doctoral Dissertation Fellowship to T.E.P.

**REFERENCES**


