In vitro REGENERATION OF *Didymopanax morototoni*

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ABSTRACT

The present study aimed at establishing a complete plant regeneration protocol for *Didymopanax morototoni* (matchwood), a native Brazilian forest species. Four types of explants (root, shoot, node, and cotyledonary leaves) were obtained from *in vitro* germinated seeds. In the first step, woody plant medium (WPM) with casein hydrolysate (250 mgL^{-1}) and 2,4-D (1.0 and 5.0 mgL^{-1}) were used combined with kinetin (0.1 and 1.0 mgL^{-1}). Twenty days after inoculation, the material was evaluated. Embryogenic calli were split, transferred to expression medium with several combinations of NAA and KIN, and moved to fresh medium after 60 days. Light did not interfere in embryo expression. Somatic embryos were formed either from individual cells or cell clusters. Plantlets were obtained in WPM medium and 10 gL^{-1} of sucrose with no plant regulator, or using 0.1 mgL^{-1} BAP and 0.5 mgL^{-1}GA. Plantlets from somatic embryos of *D. morototoni* developed in 33% of the cases.

Keywords: somatic embryo, woody plants, embryogenesis, plantlets, *Schefflera* (*Didymopanax*), *Araliaceae*.

RESUMO

Regeneração *in vitro* de *Didymopanax morototoni*

O presente estudo visou o estabelecimento de um completo protocolo de regeneração para *Didymopanax morototoni* (morototó, caixeta) uma espécie florestal nativa do Brasil. Quatro tipos de explantes (raiz, caule, nódulo foliar e folha cotiledonar) foram obtidos a partir de sementes germinadas. Na primeira etapa, meio WPM com caseína hidrolisada (250 mgL^{-1}) e 2,4D (1,0 e 5,0 mgL^{-1}) foram usados em combinação com cinetina (0,1 ou 1,0 mg L^{-1}). Vinte dias depois de inoculado, o material foi avaliado. Calos embriogênicos foram divididos e transferidos para meio de expressão com várias combinações de ácido naftaleno-acético e cinetina, e repicados a cada 60 dias para meio novo. A luz não interferiu na expressão embriogênica. Embriões somáticos foram formados ou de células individuais ou de agregados de células. As plântulas foram obtidas no meio WPM com 10 g L^{-1} de sacarose e sem reguladores de crescimento ou com 0,1 mg L^{-1} de Benzil-adenina e 0,5 mg L^{-1} de giberelina. O desenvolvimento das plântulas a partir de embriões somáticos de *D. morototoni* foi alcançada em 33% dos casos.

Palavras-chave: embrião somático, plantas lenhosas, embriogênese, *Schefflera* (*Didymopanax*), *Araliaceae*.

INTRODUCTION

Didymopanax morototoni (Aublet) Decaisne et Planchon, known in Brazil as *morototó* or *caixeta* is potentially important as a reforestation species (Daniel *et al*., 1994). Besides fast growth, it offers several characteristics recommending its use in mass culture such as straight cylindrical trunks, sought after as timber and by craftworkers.

In spite of its plentiful fruit production, seeds do not germinate easily, and the species itself is

somewhat rare in natural formations. The conventional methods of propagation by cutting stocks and grafting sections are difficult and not very often successful, thus limiting nursery plant production and, consequently, supply for reforestation programs. However, micropropagation of this species has been done (Mantovani *et al*., 1999).

Somatic embryogenesis is another useful technique since large quantities of plants can be produced in limited spaces at relatively low cost (Gratapaglia & Machado, 1999). But micrografting in the rooting stage of woody plants is a possible obstacle.

Somatic embryo development can take place either directly (without intervening calli), or indirectly (with an initial callus phase). The presence of competent cells, which is basically expressed by the physiological state (De Jong *et al*., 1993) may be the beginning of somatic embryo development (Raemakers *et al*., 1995). Thus, cells detached from surrounding areas could be stimulated to begin embryogenesis pathway, and the presence of plant growth regulators could change cell sensitivity and differentiation (Trewavas, 1991; Guerra & Handro, 1998).

For successful *in vitro* somatic embryogenesis, selecting the right explant is crucial, and in the case of some species has been enough to obtain somatic embryos (Ammirato, 1983). But in some species almost all tissue is responsive, *e.g*., tobacco, carrot, and potato, whereas in others, only embryonic tissue is (Bajaj, 1995). Furthermore, according to Haccius (1978), somatic embryos can begin from a meristematic cell layer. In any case, explant origins can be revealed by histological studies at various developmental stages of somatic embryogenesis (Canhoto & Cruz, 1996). In any case, explant origins can be revealed by histological studies at various developmental stages of somatic embryogenesis.

There are several formulations for *in vitro* culture, among which woody plant medium (WPM) (Lloyd & McCown, 1980) and liquid proliferation medium (LPm) (Von Arnold & Eriksson, 1981) are widely used. Microcuttings of *D. morototoni* have been grown when using WPM (Mantovani & Franco, 2000).

Plant regeneration via somatic embryogenesis may be considered successful when complete plantlets are produced and adequately acclimatized to *ex-vitro* conditions.

The purpose of this investigation was to: a) determine the most suitable explant of *D. morototoni* for somatic embryogenesis; b) develop a protocol for somatic embryogenesis in this species; c) establish the histological origin of somatic embryos; and d) regenerate complete plantlets acclimated to *ex-vitro* conditions.

MATERIALS AND METHODS

Seeds of *Didymopanax mortotoni* (Aubl.) Dcne & Planch (= *Schefflera morototoni* (Aubl.) Macguire, Steyerm. & Frodin) were collected on the ground under the canopies of several trees in the central part of Rio Grande do Sul state, Brazil. They were disinfected and germinated *in vitro* (Franco & Ferreira, 2002). Explant sources were fifty-day-old seedlings split into root, stem, stem node, and blade.

The explants were cultured for twenty days in WPM (Lloyd and McCown, 1980) solidified in 0.65 (% w/v) agar medium (Difco-Bacto), supplemented with 250 mgL^{-1} casein hydrolysate and 30 gL^{-1} sucrose. The medium was autoclaved and immediately thereafter, 1 or 5 mgL^{-1} 2,4 filter-sterilized dichlorophenoxyacetic acid (2,4-D) and 0.1 or 1.0 mgL^{-1} kynetin (Kin) were added. The calluses obtained were uniformly split and cultured in the same medium to which had been added 0., 0.01, 0.1, and 1.0 mgL^{-1} naphthalaneacetic acid (NAA) and 0., 0.01, 0.1, 1.0, and 10.0 mgL^{-1} kinetin. The material was cultured in a growth room either at 14.3 $\mu Em^{-2}.s^{-1}$ PPFD for a 16 h light photoperiod or in darkness at 25 ± 2 °C. All experiments were arranged in a randomized layout with 10 replications.

To obtain complete plant regeneration, four somatic embryos/petri plate of similar size and weight and at the same developmental stage were placed in ½ WPM or full strengh WPM, plus sucrose 10, 20, and 30 gL^{-1} and 1.5 gL^{-1} of activated charcoal. Agar and hydrolysated casein were used as previously described for somatic embryogenesis. No phytoregulators were used in this period. Growth, rooting, and multiplication rates were recorded after 60 days. Number of leaves, and root and shoot length were also noted. Each treatment was replicated ten times.

For histological studies the explants were fixed in 3% glutaldehyde in a 0.1 M phosphate

buffer, pH 7.2, for 24 h (Gabriel, 1984). Dehydration was obtained through standard ethanol series (1 h per step). The samples were pre-embedded in hydroximethylmetacrilate and ethanol (1:1), followed by infiltration (Jung's hystoresin). For general examination, semi-thin (3-5 μm) sections were cut on a 781 ANCAP mycrotome and stained with 0.2% toluidine blue buffered at pH 4.0 (O'Brien & McCully, 1981).

The experimental design was completely randomized with ten replicates per treatment. Data were subjected to analysis of variance; means were compared by Duncan's test ($p \leq 0.01$)

RESULTS

Explants from root, stems, cotyledonary leaves, and leaf nodes of fifty-day-old seedlings generated calli after cultured for 20 days in the presence of growth regulators. Calli were (color) white to green, (texture) compact to friable, and (surface) smooth to rough. Callus induction in darkness was more efficient than that in light (Figs. 1a and 1b). Leaves and nodes were the most responsive explants in this phase.

Sixty percent of the embryos were induced in the treatment with 5.0 mgL^{-1} 2,4-D plus 0.1 mgL^{-1} Kin. Five mgL^{-1} 2,4-D plus 1.0 mgL^{-1} Kin yielded 20% of the embryos; another 20% were obtained by adding 1.0 mgL^{-1} 2,4-D plus 1.0 mgL^{-1} Kin.

When these calli were split and cultured for 4 months in the presence of NAA and Kin more calli tissue was produced. Low phytoregulator concentrations applied to calli derived from shoot and leaf node sources were the most efficient combinations, producing friable, yellowish embryogenic calli. These were most apt to produce embryos (Figs. 2a and b), although more compact,

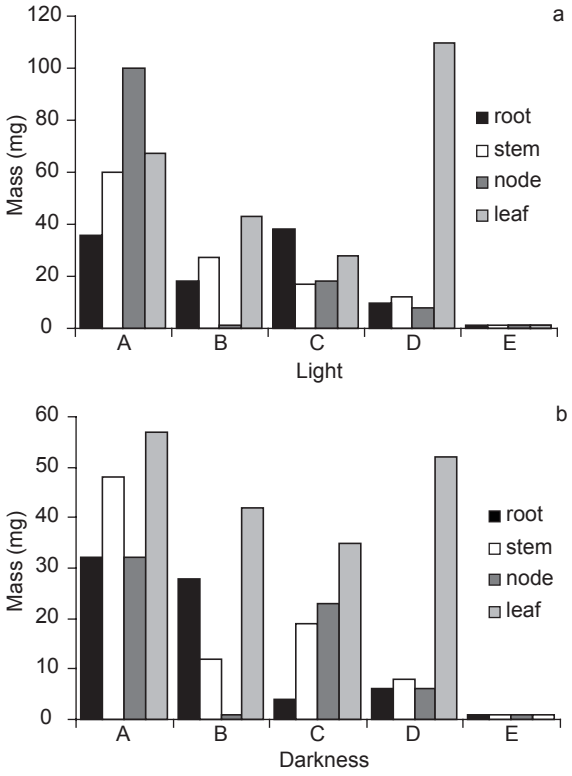

Fig. 1 — Dark-grown (a) and light-brown (b) callus weight induced by 2,4 dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) from root, shoot, and cotyledonary leaves and node leaves of *Didymopanax morototoni*. A = 1.0 mgL^{-1} 2,4-D + 0.1 mgL^{-1} Kin; B = 1.0 mgL^{-1} 2,4D + 1.0 mgL^{-1} Kin; C = 5.0 mgL^{-1} 2,4-D + 0.1 mgL^{-1} Kin; D = 5.0 mgL^{-1} 2,4-D + 1.0 mgL^{-1} Kin; and E = control (no plant growth regulator).

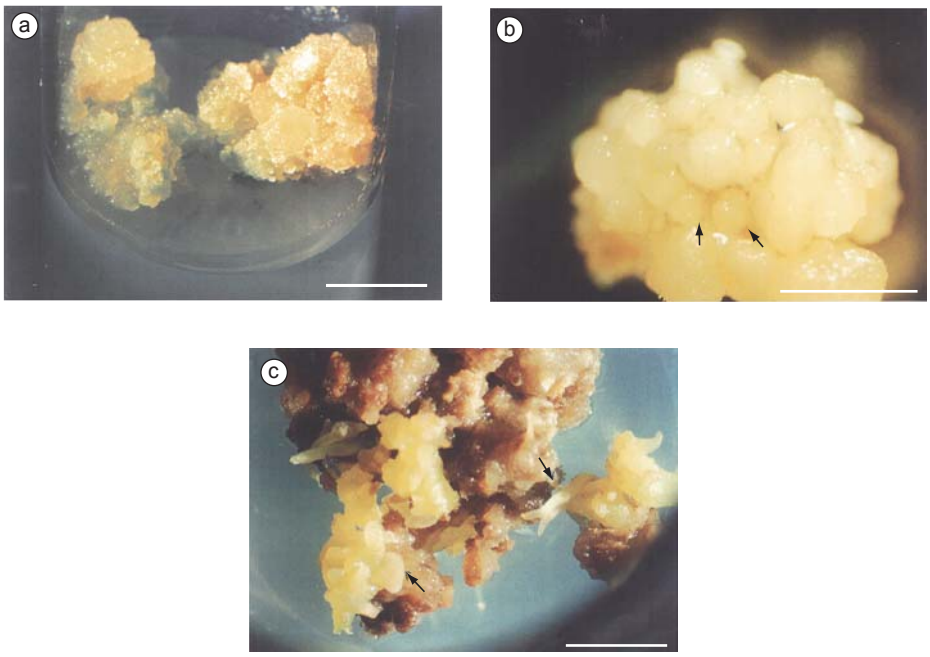

Fig. 2 — *D. morototoni* callus morphology. a: Non-uniform light-cultivated callus with greenish compact zones and more friable, granular, and yellowish parts; b: Cluster of embryos at globular stage; and c: Friable calli covered by embryos at several developmental stages. The arrow indicates an embryo at cotyledonary stage. Bar = 1 cm.

greenish calli were also capable of embryo production (Fig. 2c)

No embryos were produced directly, except those derived from or adjacent to cambial tissue and pericycles (Fig. 3a). Centers of competent cells had been observed in a few compact calluses (Fig. 3b), but usually friable calli (Fig. 3c) gave rise to an indefinite number of embryos (Fig. 3d).

There was a conversion to plantlets of somatic embryos, demonstrating their physiological and morphological maturity (Fig. 4a). Plantlet survival in pots was around 33%, which can be considered reasonable (Fig. 4b).

Plantlet size (Fig. 5a), root numbers (Fig. 5b), and leaf number (Fig. 5c) were lower at highest concentration of plant growth regulators (BAP and GA_3) used. The results for the control without plant growth regulators did not differ from those for the lowest concentration of plant growth regulators.

The rooting and multiplication rates were significantly higher when full-strength WPM medium was used (Fig. 6). Sucrose reduction increased leaf number but did not affect root and shoot length (Fig. 7).

DISCUSSION

Since young tissues have higher meristematic activity, higher surviving rate and better *in vitro* growth (Litz & Jarret, 1991; Tisserat *et al*., 1979), they are used in converting somatic cells into embryogenic cells. This process begins with an induction phase, followed by the expression phase, in which competent cells are triggered by various factors. Cells in different differentiation stages and determination degrees are, mediated by phytoregulators, able to acquire competence (Sharp *et al*., 1980; Christianson & Warnick, 1988). According to Vasil (1982), embryonic competence induction occurs in the presence of high auxin concentration, the most commonly used being 2,4-D (Jimenez, 2001; Mathur *et al*., 2001). However, morphogenic structure expression should occur in the absence, or at lower auxin levels. If 2,4-D is replaced by a weaker auxin such as NAA in a relatively low concentration (between 0.1 and 0.01 mgL^{-1}), somatic embryo expression is possible. For *D. morototoni*, kinetin applied at the same low concentration as NAA, appears to benefit the expression phase. The substitution of 2,4-D by

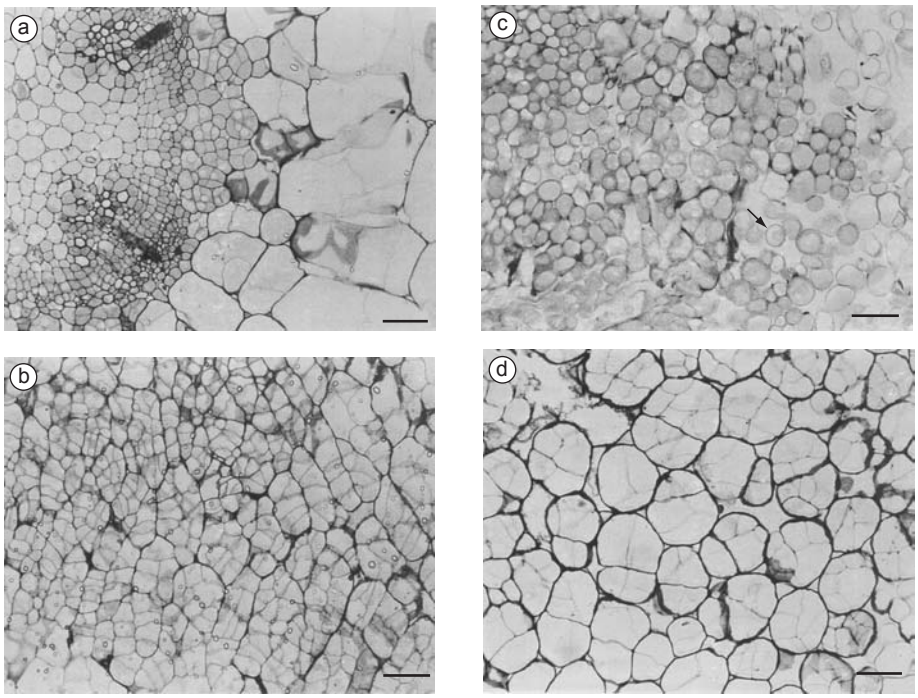

Fig. 3 — Histological section of *D. morototoni* explants. a) Cotyledonary node explant showing a sprout of cells with dense cytoplasm and thick walls; arrow indicates an early globular embryo near the pericycle; b) Section through a dense callus, showing cells with thick walls, a typical somatic embryo precursor; c) Sectioned friable, typically embryogenic callus; and d: Group of cells with thick walls in a pre-globular stage in embryos originating in parenchyma tissue. Bar = 60 µm.

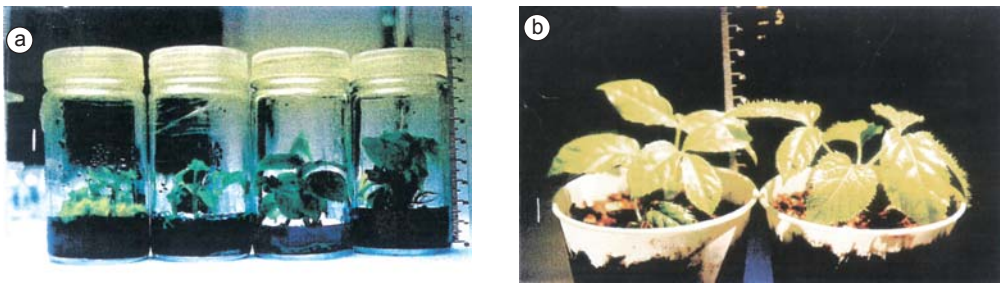

Fig. 4 — a) *D. morototoni* plantlets in axenic conditions just before transferal to *ex-vitro* conditions; and b) Potted plantlets (scale cm).

NAA in the second step furthered expression in several other species, *e.g.*, *Olea europea* (Shibli-Rida *et al*., 2001), *Simmondsia chinensis* (Hamama *et al*., 2001), and *Quercus robur* (Zegzouti *et al*., 2001).

Histological study of the *D. morototoni* explants indicates that somatic embryos may begin in two ways: 1) in the pericycle layer; and 2) in parenchymal cells of the explant callus. In the first case, embryos seem to originate from either a single cell or a few competent cells, whereas those developed from calli appear to have a multicelular origin. These observations differ from those made by Canhoto & Cruz (1996). But embryo origin is difficult to determine exactly (Faure *et al*., 1996), in spite of the numerous anatomical slices analyzed. In any case, a dual origin has been already been suggested for somatic embryos (Barciela & Vieitz, 1993; Puigderrojols *et al.*, 2001; Williams & Maheswaran, 1986).

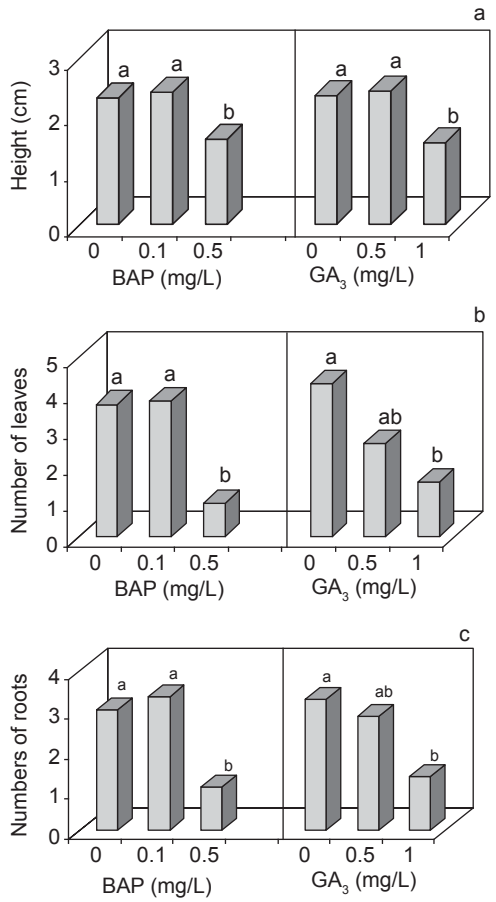

Fig. 5 — Effects of BAP and GA_3 concentrations on height of *D. morototoni* plantlets derived from somatic embryos; b) Effect of BAP and GA_3 concentrations on the average number of roots/plantlet of *D. morototoni*; and c) Effect of BAP and GA_3 concentrations on the number of leaves/plantlet. The same letter within the same parameter does not differ statistically by Duncan's test ($p \leq 0.01$).

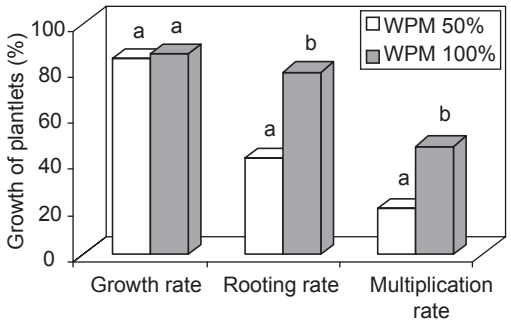

Fig. 6 — Growth, rooting. and multiplication rates of *D. morototoni* plantlets in ½ WPM and full-strength WPM medium. The same letter within the same parameter does not differ statistically by Duncan's test ($p \leq 0.01$).

Embryogenic cells were minute, resembled meristematic cells, displayed more densely staining nuclei and nucleoli, denser cytoplasm and starch grains, and a thick cell wall. Such walls are characteristic of somatic embryo formation (Canhoto & Cruz, 1996; Canhoto *et al.*, 1999;

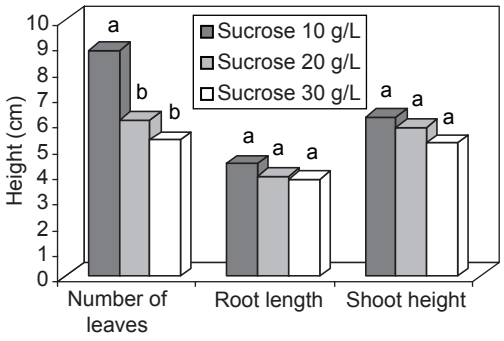

Fig. 7 — Evaluation of *D. morototoni* growth after 60 days of culture in three sucrose concentrations. Root lengths and shoot heights in centimeters. The same letter within the same parameter does not differ statistically by Duncan's test ($p \leq 0.01$).

Verdeil *et al*., 2001), which is quite clear in Fig. 3b, 3d.

Of the four kinds of explants tested, only the shoot and nodal segments allowed somatic embryo induction and development. This is critical, in some species, for explants that in spite of friable calli, root and leaf explants do not complete embryogenesis, and for other species might be due to not only to physiological differences among explant sources, but also to conditions under which development took place (Harry & Thorpe, 1991; Jimenez, 2001). Cytokinin presence can stimulate somatic embryo maturation (Evans *et al*., 1981) and make plantlet regeneration feasible, as was the case for *Myrtus comunis* (Canhoto *et al*., 1999). But absence of plant growth regulators generally yielded better developmental responses for *Eucalyptus* (Muralidharam & Mascarenhas, 1995), *Juglans cinerea* (Pijut, 1993), and *Simarouba glauca* (Rout & Das, 1994). Addition of plant growth regulators should not be recommended for *D. morototoni* after the conversion stage.

Sucrose increase in the medium may have inhibited carbon metabolism and chlorophyll amount, and reduced the activity of D-ribulose-1-5-biphosphate carboxylase/oxygenase and, consequenly, carbon uptake. Hdider & Desjardins (1994) demonstrated that strawberry plants grown at 0.1% sucrose had a higher photosynthesis rate, hence were more autotrophic. For *D. morototoni* sucrose decrease produced more vigorous plantlets.

Through the procedure outlined here, a degree of success was reached for *D. morototoni* comparable to that obtained for *Panax ginseng*, another Araliaceae (Mathur *et al*., 2001).

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