ISOLATION, CULTURE, AND REGENERATION OF MOTH BEAN 
VIGNA ACONITIFOLIA LEAF PROTOPLASTS

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SUMMARY

Mesophyll protoplasts, isolated with purified Driselase from a drought resistant food crop (moth bean, Vigna aconitifolia), were cultured in standing microdrops. High division rates and plating efficiency were obtained by purifying the enzyme, by adding glutamine and asparagine to the culture medium, and by gradually reducing the levels of osmoticum. On auxin free medium, the protoplast-derived calli produced embryoids which developed into complete plants.

Key words: Vigna aconitifolia — Leaf protoplasts — Somatic embryogenesis — Regeneration of plant from protoplast

INTRODUCTION

In recent years, many attempts have been made to isolate and culture the protoplasts of leguminous plants, but success in regenerating entire plants has been limited to two genera of forage legumes Medicago sps. [1—4] and Trifolium repens [5,6]. We report here the successful regeneration of plants from isolated mesophyll protoplasts of an important legume used for human consumption.

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Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; GA, gibberellic acid; NAA, naphthaleneacetic acid.

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V. aconitifolia (Jacq.) Marechal, (moth bean) is a highly drought tolerant food crop grown widely in India [7]. Well suited to arid and semi-arid areas of the tropics, it grows well where soils are sandy and poor in nutrients, and the rainfall scarce. V. aconitifolia is used both for food and fodder, has a high quantity and quality of seed proteins, fixes atmospheric nitrogen, and is resistant to diseases and pests. It has been accordingly singled out as worthy of further study [8].

MATERIALS AND METHODS

Plant material

Seeds of V. aconitifolia 'Jadia' obtained from the National Bureau of Plant Genetic Resources, New Delhi-110 013, India, were planted in vermiculite, subirrigated three times a week with Hyponex (1.2 g/l of a 7-6-19 NPK analysis, Copley Chemical Co., Copley, OH), in a controlled environment growth chamber under 260 μE m⁻² s⁻¹ photon flux density (combined fluorescent-incandescent light) in a 16-h photoperiod at 30°C.

Isolation of protoplasts

The first true leaves of 14-day-old seedlings were harvested 1–2 h into the photoperiod and washed several times with tap water. The leaves were surface sterilized for 5 min in 5.0% of commercial bleach (final concentration of sodium hypochlorite 0.26%) containing a few drops of surfactant Tween-80. After 5–6 washings of the sterilized leaves in sterile distilled water, the lower epidermis was peeled off with a fine forceps. The leaves were floated with the peeled surface down on 4.0 ml (3 leaves/dish) of protoplast isolation medium in 60 X 15 mm plastic petri dishes. The protoplast isolation medium consisted of: desalted (see below) 1.0% Driselase (Kyowa Hakko Kogyo Co., Ltd., Japan); mannitol, 9.0%; salts (KH₂PO₄, 27.2 mg/l; KNO₃, 101.0 mg/l; CaCl₂·2H₂O, 148 mg/l; MgSO₄·7H₂O, 240.0 mg/l and KI, 0.16 mg/l) and MES (2(1-N-morpholino) ethanesulphonic acid), 3.1 mM. The pH was adjusted to 5.8 with 0.1 N KOH before filter sterilization with a 0.2-μm pore size Nalgene filter. Commercial crude Driselase is toxic to leaf tissues and released protoplasts. Accordingly, desalted and purified enzyme was used for protoplast isolation. Other cellulolytic enzymes such as Cellulase Onozuka R-10, Kinki Yakult Manufacturing Co.; Cellulysin, CalBiochem., and Meicelase, Meiji Seika Kaisha Ltd. were ineffective alone or in combination with pectinases.

Five grams of crude Driselase were dissolved in 20.0 ml of 1.0 mM phosphate buffer (pH 6.0). After 2 h the solution was centrifuged at 23 000 × g for 10 min to remove the insoluble impurities, and the supernatant fluid fraction loaded onto a Sephadex G-50 column (40 × 20 cm), and twenty 10-ml eluate fractions collected. Protein was estimated in each fraction using Bradford's Coomassie Blue method [9]. Eight fractions, containing 75% of the protein were collected and stored at 4°C in the cold room. This
enzyme solution was used for a month, after suitable dilution (equivalent to 1.0% of crude Driselase on a protein basis) without loss of activity. A CaCl₂ concentration of more than 2 mM was inhibitory to the release and survival of protoplasts; we therefore used 1 mM of this salt, which aided protoplast stability. The digestion and regeneration of the cell wall were monitored with Calcofluor White, as described by Nagata and Takebe [10].

The leaves were incubated in the isolation medium at 30°C for 5 h and were then placed on a gentle reciprocating shaker (40 rev./min) for 1 h to release the protoplasts. The undigested material, (mostly vascular tissue and upper epidermis) was removed gently with a forceps and the enzyme-protoplast suspension filtered through nylon gauze, 55-μm pore size, to remove debris. The resulting crude suspension of protoplasts and subcellular particles was centrifuged for 5 min at 100 × g; the supernatant fluid discarded, the protoplast pellet resuspended in protoplast culture medium and again pelleted by centrifugation. The final pellet was resuspended in 10 ml of 21% (w/v) sucrose solution containing the same salts (but with CaCl₂ ⋅ 2H₂O, now 900 mg/l) as used in the isolation medium. The pH of the sucrose solution was adjusted to 5.8, after which it was overlaid by 2.0 ml of protoplast culture medium and was again centrifuged at 100 × g for 5 min. The intact protoplasts floated on the sucrose layer, forming a green ring in the culture medium layer. These protoplasts were removed gently with a Pasteur pipette and washed once again with culture medium. After repelleting, the protoplasts were resuspended in culture medium for further studies of growth and development.

Culture of protoplasts

The protoplasts were cultured in 25.0-μl standing drops on the bottom of 60 × 15 mm plastic petri dishes (10 drops/dish). The culture dishes were sealed with Parafilm and kept in humid plastic boxes (20 × 15 × 10 cm) at 26°C in diffuse light for the first 10 days and subsequently, at 30 or 50 μE m⁻² s⁻¹ photon flux density (Daylight fluorescent tubes). The compositions of culture media used are given in Table 1.

The protoplasts were cultured for the first 4 days in GS-1 medium; on the 5th day of culture 25 μl of GS-2 medium was added to each culture drop. On the 10th day, 25 μl of GS-3 medium was added. Colonies derived from 3–4-week-old protoplasts were transferred to GS-3 medium with 0.6% purified agar. The localized green areas which developed at the surfaces of calli were transferred to GS-4 agar (0.6% agar) medium and kept under 200 μE m⁻² s⁻¹ photon flux density at 27°C. The small plants with two cotyledons were transferred to GS-5 agar (0.8% agar) medium and kept at 30°C under 200 μE m⁻² s⁻¹ photon flux density. The plants thus raised were then transferred to coarse vermiculite for further development.

RESULTS

With the exception of Driselase, none of the cellulolytic enzymes was
<table>
<thead>
<tr>
<th>Constituents</th>
<th>GS-1</th>
<th>GS-2</th>
<th>GS-3</th>
<th>GS-4</th>
<th>GS-5</th>
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<td>Macro- and micronutrients</td>
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<td>MS</td>
<td>MS</td>
<td>MS</td>
<td>1/2 MS</td>
</tr>
<tr>
<td>CaCl₂ · 2H₂O (mg/l)</td>
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<td>900</td>
<td>960</td>
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<tr>
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<td>KM</td>
<td>KM</td>
<td>NO*</td>
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<td>5</td>
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<td>Zeatin (mg/l)</td>
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<tr>
<td>GA₃ (mg/l)</td>
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<td>Others:</td>
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<td>L-Arginine (mg/l)</td>
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<tr>
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<tr>
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<td>5.8</td>
<td>5.8</td>
<td>5.8</td>
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</tbody>
</table>

*NO = no addition.

effective in releasing protoplasts from the leaves either alone (0.1–5.0%) or in combination with pectinases. Such enzyme mixtures produced isolated cells whose walls were still not digested after 20 h. In contrast, protoplast formation with Driselase (0.25–2.5%) started after 1.5 h and continued for up to 12 h, depending on the enzyme concentration. Driselase concentrations above 1.5% caused lysis of protoplasts, resulting in a drastic reduction in the yield of protoplasts. The average protoplast yield with 1% crude Driselase was 1–2 x 10⁶/g leaf tissue; with desalted and purified enzyme, this could be increased up to 5–7-fold over a shorter incubation period.

The diameter of the protoplasts ranged from 25–45 μm. They were bright green, with chloroplasts regularly arranged along the periphery (Fig.1), and without Calcofluor White fluorescence around the plasma membrane, indicating complete cell wall digestion. Addition of selected salts to the isolation medium affected the quality and yield of protoplasts, calcium
Fig. 1. Freshly isolated leaf protoplasts of *Vigna aconitifolia*. Bar = 25 \( \mu \text{m} \).

Figs. 2 and 3. First and second cell divisions of protoplast, respectively. Bar = 25 \( \mu \text{m} \).

Figs. 4 and 5. Protoplast-derived colony (Bar = 25 \( \mu \text{m} \)) and callus (Bar = 1 cm), respectively.
chloride concentration being most critical. The absence of Ca$^{2+}$ from the isolation medium reduced the net yield of viable protoplasts because of the instability of the released protoplasts during washing and centrifugation. The optimum concentration for protoplast release was 1 mM; more than 2 mM not only inhibited the formation of protoplasts but also caused excessive

Fig. 6. Cotyledons coming from embryoid like structure. Bar = 500 μm.
Figs. 7 and 8. Embryo with two cotyledons and root (Bar = 1 cm) and complete moth bean plant derived from leaf protoplast with extensive root system. Bar = 1 cm.
browning of tissues. No protoplasts were formed within 20 h in media containing 5 mM calcium chloride. During culture, the protoplasts increased as much as twice or more in diameter, and became highly vacuolated within 24–48 h. The chloroplasts became light green in color and became unevenly distributed. Cell wall regeneration occurred in about 5% of the protoplasts in patches around the periphery during the first 24 h and was completed in the next 24 h in all the viable protoplasts. Cell plate formation and the first division were first recorded after about 60–70 h (Fig. 2) in 2–10% of the cells derived from protoplasts; after this, many cells elongated and became dumbbell shaped. About 50% of the cells underwent their first cell division by the 5th day of culture and 60–70% by the 6th day.

The average plating efficiency never reached more than 10% in protoplasts isolated with crude Driselase. Such protoplasts and the culture drops became dark brown, and 80–90% of the protoplasts died. No such symptoms were observed in protoplasts isolated with purified enzyme. Exposure of the protoplasts to a high light intensity during the initial 4 days also caused browning and deterioration, even of dividing cells.

On the 5th day, GS-2 medium with reduced osmoticum was added, for if the cultures are kept in GS-1 medium for a longer period, the 2nd and 3rd divisions are delayed and their frequency reduced. After addition of GS-2 medium, the cells underwent their 2nd and 3rd divisions on the 7th and 9th days, respectively (Fig. 3). Similarly, on the 10th day of culture, GS-3 medium was added. The rapid cell divisions resulted in the prompt formation of multicellular colonies (Fig. 4). The overall average plating efficiency was 60%. On transfer to GS-3 agar medium the colonies developed into rapidly growing, yellow-green and watery calli (Fig. 5), whose healthy growth and subsequent differentiation was greatly aided by the addition of asparagine and glutamine. Within 2–3 weeks, localized greenings (1–3/callus) appeared at the surfaces of 90% of these calli. Such green initials developed into embryoids on transfer to GS-4 medium at 27°C. The embryoids had two cotyledons (Fig. 6) and often obvious roots (Fig. 7). Plants were recovered from these embryoids on transfer to hormone-free MS medium. The roots formed on hormone-free GS-4 and MS media grew very rapidly for one day only, then showed browning of tips and deterioration. However, on GS-5 medium the plants developed roots very rapidly (Fig. 8); these grew rapidly and continued their growth when the plants were transferred to vermiculite. Of 206 plants obtained in this way, 30 survived when transferred to vermiculite, and all produced normal plants.

DISCUSSION

Driselase, a product of the Basidiomycete fungus *Irpex lacteus*, was the only enzyme capable of releasing protoplasts from *V. aconitifolia* leaves. The crude enzyme contains various impurities, including low molecular compounds, such as salts and phenolics, which produce harmful effects on the
protoplasts and result in low protoplast yield and plating efficiency. Purification of the enzyme with Sephadex removed virtually all impurities imparting the dark brown color, as well as the toxic effect. This was evident from the high yield and plating efficiency of protoplasts in culture. Patnaik et al., [13] earlier found that purification of Meicelase similarly improved the plating efficiency of Petunia parodii protoplasts manyfold. The deleterious effects of high light intensity seemed to be correlated with a high bleaching of chlorophyll and the appearance of brown precipitates in the culture drops.

The high plating efficiency of protoplasts in our experiments resulted in part from (i) serial addition of fresh medium which gradually diluted the osmoticum and (ii) addition of asparagine and glutamine to the culture medium. Jha and Roy [14] reported rapid and vigorous growth of protoplast-derived calli after a short interval following reduction of the mannitol levels in the media; similar observations were reported for pea mesophyll protoplasts [15]. Donn [16] found that the addition of amino acids enhanced the rate of division and shortened the time between protoplast isolation and the first cell division in Vicia narbonensis.

In V. aconitifolia, the development of green meristematic areas on the protoplast derived calli on GS-3 medium and the subsequent development of embryoids with two cotyledons from such green islands on GS-4 medium (without auxin but with low cytokinins) supports the idea that the protoplasts regenerated into plants through somatic embryogenesis, as in Medicago sps. [2,4]. The plants grew vigorously at 30°C and very poorly at lower temperatures, reflecting the natural growth of this plant at high temperatures.

We are now using these methods routinely in our laboratory for culture and regeneration of Trigonella foenumgraecum [17] and Vigna unguiculata.

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