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PROTOPLASMA

Plant Regeneration Through Somatic Embryogenesis in Protoplast Cultures of Sandalwood (*Santalum album* L.)

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Summary

Protoplasts were isolated from embryogenic cell suspension cultures derived from proliferating shoot segments of a 20-year-old sandalwood tree (*Santalum album* Linn.). Under appropriate conditions, isolated protoplasts divided in liquid culture medium and produced embryogenic cell aggregates and globular embryos. Plating of cell aggregates on a fresh medium facilitated the differentiation of somatic emryos which further developed into plantlets.

Keywords: *Santalum album* L.; Sandalwood; Somatic embryogenesis; tree protoplasts.

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; GA_3, Gibberellic acid; IAA, indoleacetic acid; IBA, indolebutrytic acid; MES, 2-(N-morpholino)ethane sulfonic acid; MS Murashige and Skoog's medium as modified in the text.

1. Introduction

The possibilities of plant improvement through somatic cell hybridization and genetic manipulation have focussed much research on protoplasts (Schieder and Vasil 1980). For woody plant improvement in particular, protoplast technology provides new options for genetic modification or limited gene transfer which may complement conventional breeding programs.

Sandalwood (*Santalum album* L.) is a woody species of great commercial value in India and Southeast Asia and is the source of fragrant wood and its oil. The species is raised conventionally by seeds, and vegetative propagation has not been successful. The trees are often affected by the lethal "spike" disease and clonal multiplication of mature, resistant genotypes is desirable and has proved to be feasible. Stem segments and shoot tip explants from 20-year-old trees (Lakshmi Sita *et al.* 1979, Rao and Raghava Ram 1983, Rao *et al.* 1984) seedling tissues (Rao and Bapat 1978, 1980, Bapat and Rao 1979, 1984), embryo (Rao 1965, Rao and Rangaswamy 1971) as well as endosperm (Rangaswamy and Rao 1963, Lakshmi Sita *et al.* 1980a, Rao and Raghava Ram 1983) have produced cultures *in vitro* which were capable of regeneration either through shoot formation or somatic embryogenesis. In this communication we describe the regeneration of sandalwood plants through protoplasts.

2. Materials and Methods

2.1. Cell Suspension Culture

Embryogenic cell suspension cultures were established from 3-year-old callus cultures of *Santalum album* L. (which originated from proliferated shoot segments from mature, 20-year-old trees) on liquid medium containing Murashige and Skoog's (1962) macro- and micronutrients and vitamins plus folic acid (1 mg/l), sucrose (34 g/l) and 2,4-D (0.5, 1, and 2.5 mg/l). Suspensions were initiated by adding a relatively large inoculum of friable callus to 25 ml medium. After 7–8 days, the small cell clumps and single cells were pipetted from the top into a new flask and allowed to settle. Approximately half of the medium was discarded and an equal volume of fresh medium was added. For another 4 weekly transfers, the small cell clusters were repeatedly selected and diluted as above. After 2 months the established cell suspension could be routinely subcultured every 4–5

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days, 6–8 ml suspension per 14 ml fresh medium in 100 ml Erlenmeyer flasks on a gyrotary shaker at 150 rpm under diffuse light.

In order to determine the morphogenic capacity of the suspensions, cells were plated on various liquid and MS agar media either without hormones or containing zeatin (0.2, 0.35 mg/l), BAP (0.1, 0.5, 1.0, 2.0 mg/l) or BAP (0.1, 0.5, 1.0, 2.0 mg/l) + 2,4-D (0.01 mg/l). Callus and/or embryos which developed were transferred subsequently to MS or half-strength MS (half the normal concentrations of macro- and micronutrients, normal concentration of iron, vitamins and sucrose) without hormones or with BAP (0.1, 1.0 mg/l), IAA (1.0 mg/l) or GA_3 (1.0 mg/l) + BAP (0.2 mg/l). Secondary, dicotyledonous somatic embryos were subcultured onto MS basal medium (agar) or filter-paper bridges in a modified White's medium (DODDS and ROBERTS 1982) with IAA (0.5 mg/l) or a combination of IAA (1.0 mg/l), IBA (0.5 mg/l) and GA_3 (0.5 mg/l).

2.2. *Protoplast Culture*

A 4–5-day-old suspension served as a source for protoplasts. After draining off the nutrient solution, the remaining settled cells were combined with 50 ml of filter-sterilized enzyme solution in a roller flask and placed on a cell production roller apparatus (Bellco Glass Inc., New Jersey, U.S.A.) for 2–6 hours at 25 °C at 1.5 rpm. Initially, combinations of macerozyme (0.2–1%), cellulase (1%) and pectolyase (0.01–0.05%) or cellulase (1%), hemicellulase (1%) and pectinase (0.5%) were used.

However, the most suitable enzyme solution consisted of 1.0% Onozuka cellulase RS, 1.0% Onozuka macerozyme R-10 and 0.5% driselase in 0.55 M mannitol, pH 5.7. Protoplasts were separated from undigested cell groups by filtration through 100, 50, and 25 µm sieves and were diluted with an equal volume of seawater (seawater was diluted with distilled water to ca. 720 mOs/kg H_2O) and sedimented at 70 g for 3 minutes, washed once more with seawater and floated on 0.6 M sucrose (120 g). After a final seawater wash, protoplasts were plated as thin liquid layers into 6 cm plastic petri dishes (2 ml per plate) at a density of 1–5 × 10^5 protoplasts/ml in V 47 basal medium (BINDING 1974) containing 3 mM MES, 2,4-D (0.5 mg/l) and BAP (0.5 mg/l) and adjusted to 750 mOs/kg H_2O with mannitol, pH 5.8. The plates were sealed with parafilm and incubated at 26 °C in continuous, diffuse light. After 2–3 weeks, cultures were diluted at 1-week intervals with V 47 medium containing 2,4-D and BAP (0.1–1.0 mg/l) (600 or 400 mOs/kg H_2O) at ratios of 4:1, 3:1, 2:1, 1:1, 1:2 (protoplast culture: fresh medium). Five weeks after isolation, protoplast-derived colonies were transferred to agar plates containing MS medium fortified with various growth regulators. Somatic embryos developed from the callus masses after 3 weeks. These were further transferred to fresh medium to induce the germination of embryos and the development of plantlets.

3. Results

3.1. *Cell Suspension Cultures*

Stable cell suspensions consisted of groups of starch-filled cells along with a small proportion of elongated or enlarged single cells and chains of cells (Fig. 1). Suspensions could be most easily maintained at the lower 2,4-D concentrations (0.5 and 1.0 mg/l). The addition of 5% coconut milk to the medium caused a rapid decline in the cultures.

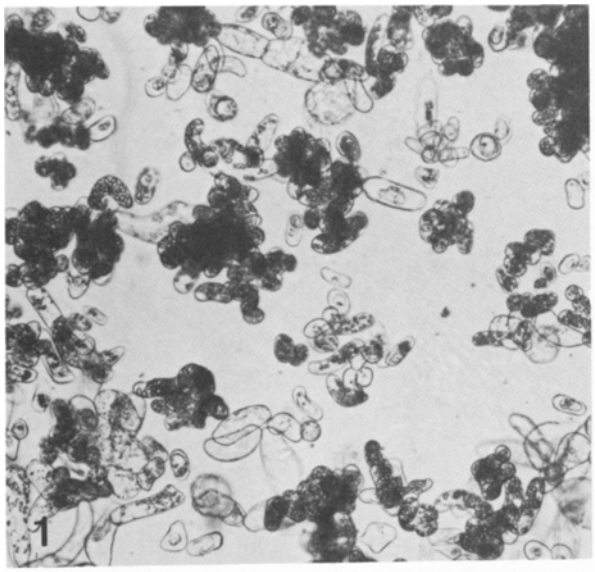
It was determined after plating on various media that the suspensions were indeed embryogenic. The nature of the suspension had an effect on the rapidity with which embryos were formed. From one cell line, callus without apparent organization developed upon plating and embryos appeared only after subsequent transfer. From other cell lines, club- and heart-shaped embryos were formed within 10 days after plating on liquid and agar media with different concentrations of BAP, with and without 2,4-D (Fig. 2). The hormone concentrations tested did not appear to be critical for embryo formation, although slightly more organized embryos were observed on media with low BAP (1.0, 0.5 mg/l) and no 2,4-D. Often more than one (2–4) somatic embryo developed from a proembryogenic mass (Fig. 2). In liquid media, the embryos did not develop past the early cotyledonary stage; however, on agar media many pluricotyledonous structures were observed (Fig. 3). The embryos did not germinate after transfer to MS and half-strength MS, but instead, became grossly enlarged, accumulated chlorophyll and anthocyanin and began to produce secondary embryos on all media except MS + IAA (1.0 mg/l) and MS + GA_3 (1.0 mg/l) + BAP (0.2 mg/l). The secondary embryos were quite normal in development, and all stages from filamentous proembryos to dicotyledonous structures with prominent suspensors were observed (Fig. 4). Mature embryos formed *in vitro* (Fig. 5) developed into plantlets on White's medium + 0.5 mg/l IAA (Fig. 6).

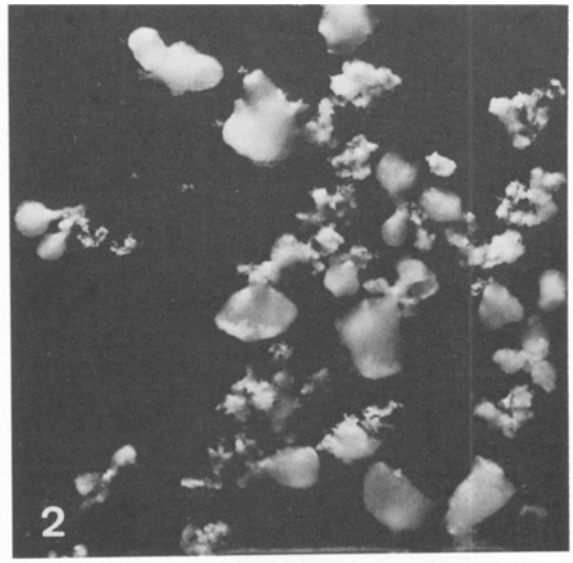
3.2. *Protoplast Culture*

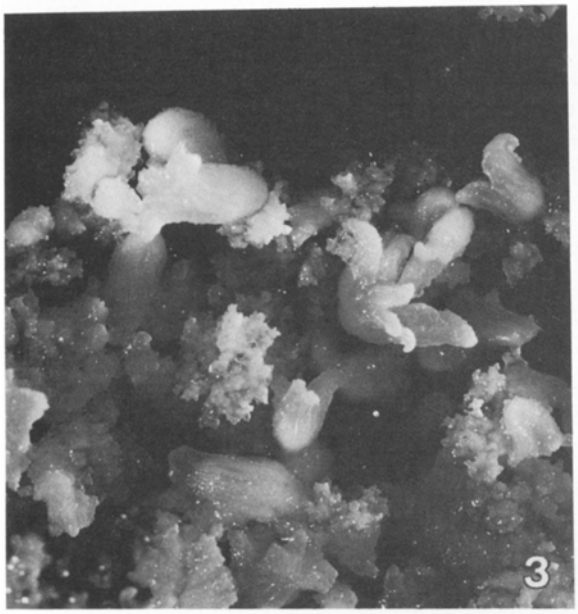
A satisfactory yield of protoplasts was recorded after 4–5 hours when macerozyme and driselase were used in combination with cellulase R-10. However, if cellulase R-10 was replaced with cellulase RS, there was a substantial increase in the number of protoplasts liberated within the same time period.

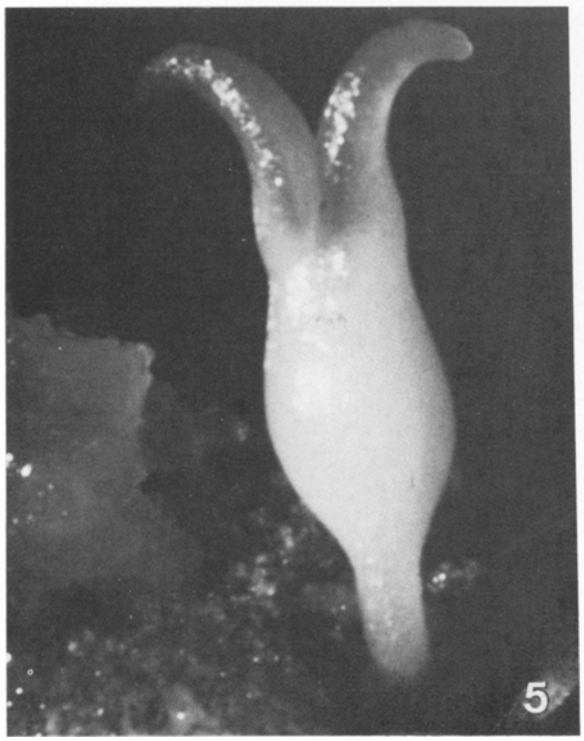
The protoplasts ranged in size from 30 µm to more than 80 µm (Fig. 7); the larger probably represented spontaneous fusion bodies which never divided but formed instead enormous single cells after several days. Budding also occurred frequently. Various basal media with BAP and 2,4-D in different concentrations and combinations were tested for their ability to support cell wall formation and divisions. Divisions did occur to a limited extent in B 5 medium (GAMBORG *et al.* 1968) and slightly more frequently in MS medium, both, however, were surpassed by V 47 medium.

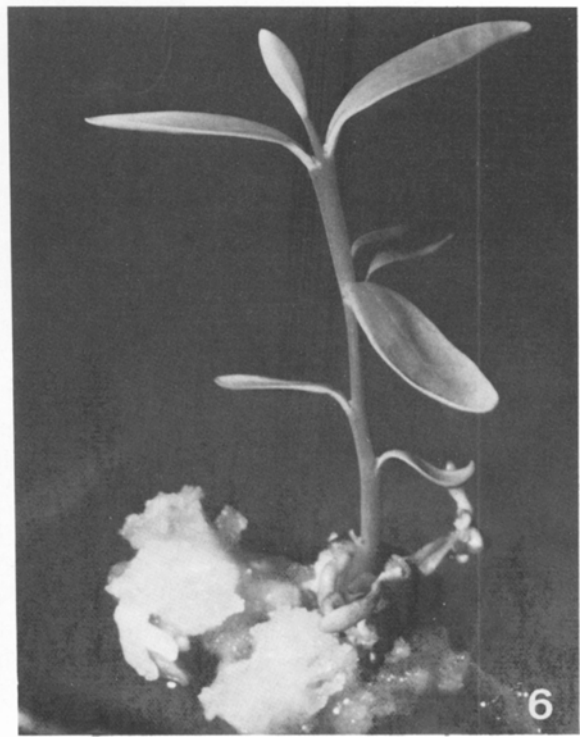
The first division in the regenerated protoplasts occurred on the third day (Fig. 8), and by the fifth day 3 or 4 cells could be observed. Small protoplast-derived











Figs. 1–6

colonies had developed by the third week (Fig. 9) and these grew rapidly when diluted with fresh medium. However, cultures frequently became necrotic after 3 weeks unless all culture media were buffered with MES. The dilution series tested indicated that degeneration of the cultures proceeded rapidly when the osmolality was reduced by more than 100 mOs/kg H_2O at one step. Cell colonies were compact, small aggregates, remarkably similar to the embryogenic cell aggregates observed in the suspension cultures. Cells were so profusely loaded with starch grains that vacuoles and nuclei were obscured. The macroscopic colonies and globular embryos resulting from protoplast cultures (Fig. 10) proliferated further and began to form somatic embryos on MS medium supplemented with various growth substances. IAA (1 mg/l) in combination with BAP (1 mg/l) or casamino acids (400 mg/l) caused prolific embryo formation. The callus was pale yellow and was so soft and friable that embryos dropped off easily when the tissues were lifted. If casamino acid was replaced with yeast extract (400 mg/l) differentiation of embryos tended to be less intense and the callus assumed deep yellowish or brown coloration. Reducing the BAP concentration to 0.5 mg/l also brought down the quantum of embryo differentiation. An interesting observation was that on MS + BAP (1 mg/l) cotyledon differentiation was more vigorous. No significant differences were observed between two levels of osmolality (400 and 700 mOs/kg H_2O) tested with respect to embryo differentiation. Many embryos were produced in scattered clusters upon a large matrix of soft, embryogenic callus (Fig. 11). Although embryos of all developmental stages could be recognized there was a predominance of globular embryos which arose simultaneously and each one of them had a clearly discernible suspensor giving it a club-shaped appearance. Altogether the embryos formed *in vitro* showed the characteristic features of dicotyledonous embryos including well defined cotyledons, shoot apex and root (Fig. 12).

Many atypical somatic embryos were found randomly mixed with normal embryos, a phenomenon which was observed also from suspension-derived cultures. Some of these embryos showed development of only the root or shoot region, others exhibited anisocotyly and pluricotyly with deeply lobed or fringed cotyledons. Such anomalies appeared to be an inherent tendency of the embryogenic callus and were not reflections of the changes in the milieu. In addition to normal and atypical embryogenesis, the somatic embryos showed a strong tendency for further cell proliferation or secondary embryogenesis. Generally secondary embryos, which originated from peripheral portions of the primary embryos, never attained maturity but invariably showed a tendency towards hypertrophy or proliferated into a callus tissue which in turn produced more embryos. Thus the cycle of differentiation, dedifferentiation and redifferentiation of embryos went on in an uninterrupted sequence in the protoplast derived callus.

To induce the germination of embryos and the development of plantlets, the embryogenic callus containing predominantly mature embryos was transferred to MS basal medium with half-strength of major elements or White's medium and supplemented with various growth regulators, 4% or 5% sucrose and 0.6% agar. Plantlet development (Fig. 13) was observed after 3 weeks on both basal media supplemented with IAA (1), IBA (0.5), and GA_3 (1 and 3 mg/l). Embryo germination was also observed on MS basal medium containing IAA (1), IBA (0.5) and coconut milk (10%). A sucrose concentration of 4–5% proved beneficial since most embryos developed leafy cotyledons. Root development in general tended to be restricted; however, the plantlets obtained did possess a strong tap root. Further, a strategy has to be worked out to initiate root development in each embryo in order to exploit this system to its best advantage.

4. Discussion

Many reports exist on the regeneration of plants from protoplasts of herbaceous plants (Vasil and Vasil 1980, Rao 1982). However, relatively few attempts have been made on the culture of protoplasts of woody species and most of these studies are preliminary (Redenbaugh *et al.* 1981, Smith and McCown 1982). *Citrus* is the only arborescent genus in which plant

Figs. 1–6. Development of somatic embryos and plantlet in cell suspension cultures of *Santalum album* L.

Fig. 1. Cell suspension showing compact aggregates of embryogenic cells and many vacuolated and elongated non-embryogenic cells

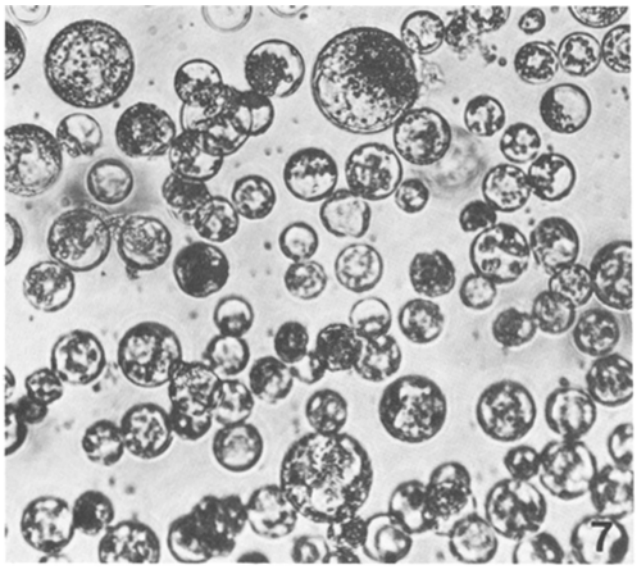
Fig. 2. Club- and heart-shaped embryos observed in suspension cultures on MS + BAP

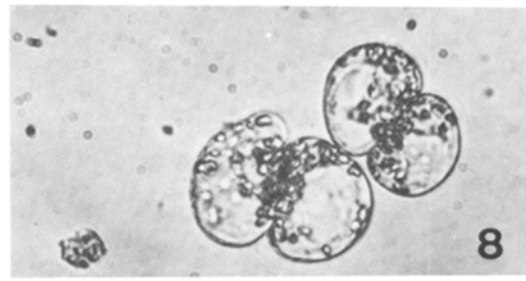
Fig. 3. Pluricotyledonous embryos observed in embryogenic callus

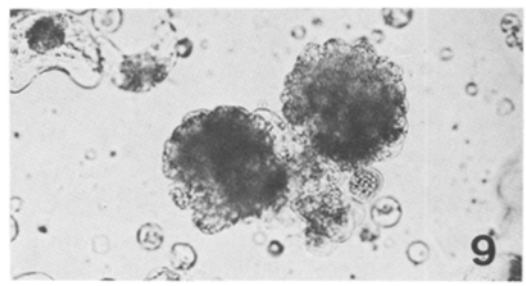
Fig. 4. Formation of secondary embryos, note the different developmental stages

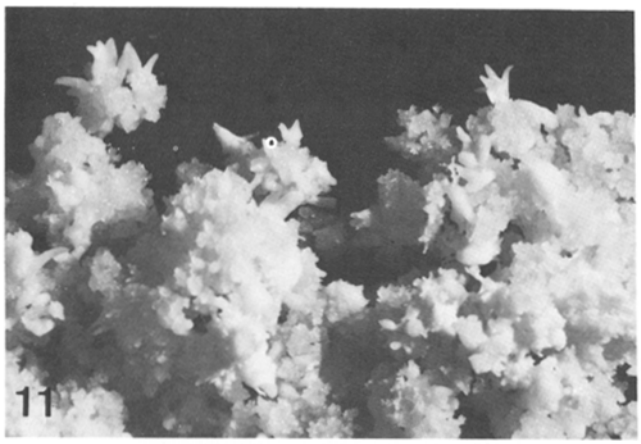
Fig. 5. Mature dicotyledonous embryo

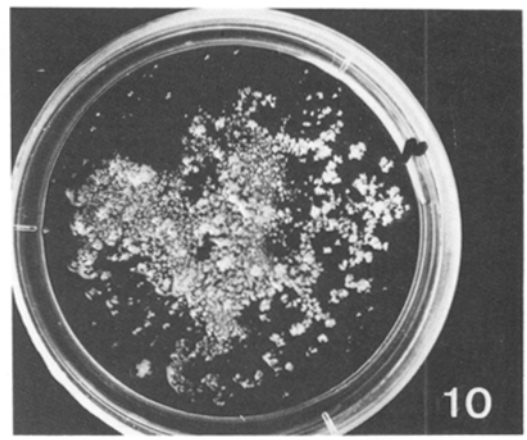
Fig. 6. Plantlet obtained on White's medium supplemented with 0.5 mg/l IAA

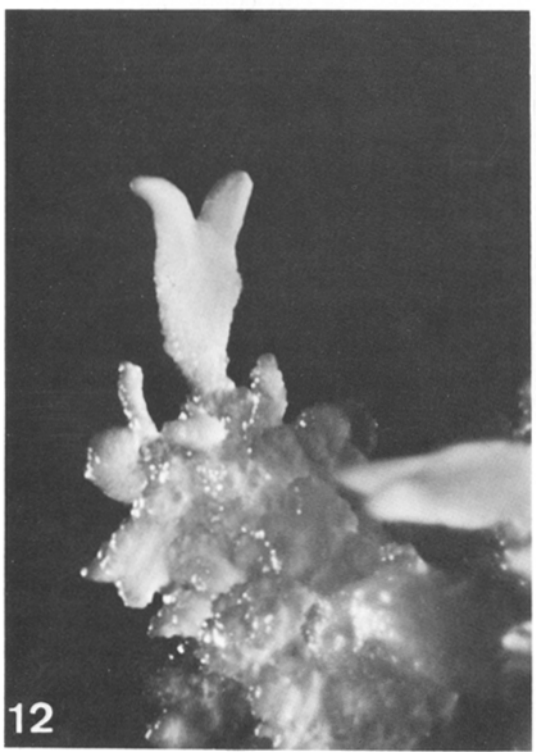


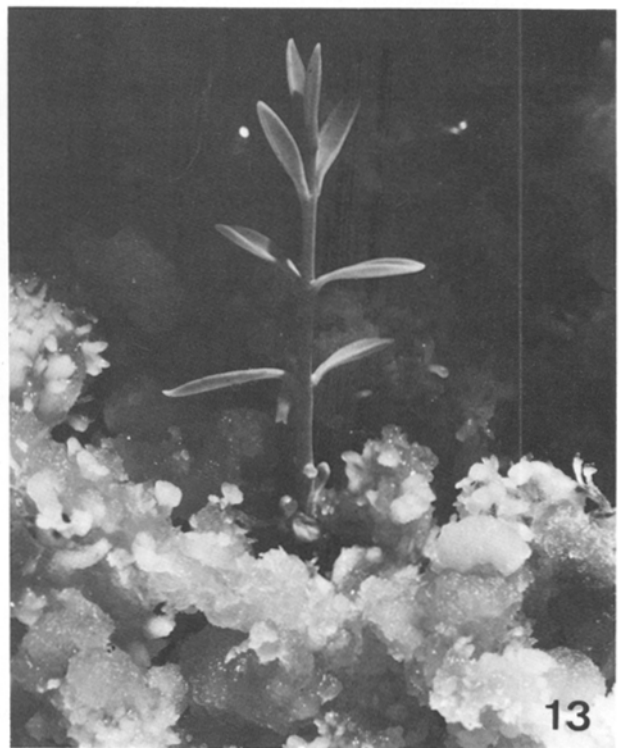












Figs. 7–13

regeneration has been reported from protoplasts (VARDI *et al.* 1975, GALUN *et al.* 1977, VARDI and SPIEGEL-ROY 1982).

Santalum album (Sandalwood) is a tree species which is exceptionally amenable for *in vitro* studies. Although plant regeneration from sandalwood suspension cultures has been reported previously (LAKSHMI SITA *et al.* 1980b), no clear evidence of embryogenesis is reported. Our results differ further in that suspension-derived callus transferred to a medium containing GA_3 became soft and watery and no embryoids were observed, whereas the previous work indicated that GA_3 could induce embryogenesis in callus (LAKSHMI SITA *et al.* 1979) or plated suspensions (LAKSHMI SITA *et al.* 1980b). In the present investigation, morphogenetically capable cell suspensions were established and from such cell suspensions, protoplasts were isolated which developed into embryogenic cell aggregates. By suitable manipulation of the hormonal levels, somatic embryogenesis was induced in protoplast-derived cell aggregates.

Somatic embryogenesis *in vitro* has been recorded in a number of plant species and there is strong evidence that the somatic embryos originate from single cells (HACCIUS 1978). This has been demonstrated in *Daucus carota* (BACKS-HÜSEMANN and REINERT 1970), *Ranunculus sceleratus* (KONAR *et al.* 1972) and *Citrus sinensis* (BUTTON *et al.* 1974). In the present study the small, richly cytoplasmic cells observed in protoplast cultures are indeed the progenitors of somatic embryos and form embryos either directly or develop into cellular aggregates from which embryos subsequently arise.

The formation of mature, dicotyledonous embryos in the protoplast-derived callus was so rapid and prolific that it was almost impossible to handle them and nurture them to complete plants. An efficient protocol needs to be worked out so that somatic embryos can be harvested at the appropriate stage and routinely developed into complete plants. Such cultures would offer a good, dependable system for cloning which would certainly be a preferable method for propagation over shoot/root regeneration which is considered to be multicellular in origin.

The formation of atypical embryos observed in cultures in the present investigation was not unexpected. Such a phenomenon has been frequently described in most species in which somatic embryogenesis has been reported (TISSERAT *et al.* 1979, VASIL and VASIL 1982). Our investigations on sandalwood have established that embryogenic cell suspensions could be developed, maintained and used for protoplast technology so that the techniques of parasexual hybridization and genetic modification can be adopted for tree breeding programs.

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Figs. 7–13. Somatic embryogenesis and plant regeneration in isolated protoplast cultures of *Santalum album* L.

Fig. 7. Freshly isolated protoplasts

Figs. 8 and 9. First and subsequent divisions in isolated protoplasts

Fig. 10. Macroscopic colonies developed in plated protoplasts

Fig. 11. Differentiation of somatic embryos in protoplast-derived callus tissue

Fig. 12. A fully developed dicotyledonous embryo obtained in protoplast-derived callus

Fig. 13. Development of a plantlet from a somatic embryo

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