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Encapsulated somatic embryos of grape (*Vitis vinifera* L.): An efficient way for storage and propagation of pathogen-free plant material

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Summary

Cotyledonary-stage somatic embryos (5-7 mm in length) originating from leaf explants of grape (*Vitis vinifera* L.) cv. Pusa seedless were encapsulated individually in 2 % alginate gel. The encapsulated somatic embryos (ESEs) germinated successfully on 0.7 % agar medium containing B5 macrosalts (half strength), Murashige and Skoog microsalts (full strength), 3 % sucrose and 2.9 µM gibberellic acid. The percentage of germination of ESEs was higher than that of non-encapsulated somatic embryos (NSEs) of the same size on the same medium. The percent germination of ESEs increased (69.2 ±2.8) on medium supplemented with quarter strength B5 macrosalts. Of the germinating ESEs, 36 % developed into plantlets. Abscisic acid at 0.004 and 0.02 µM had no significant influence on the frequency of germination and plantlet development, however resulted in a 4-week delay in germination. Transferring the embryos onto the full-strength B5 medium containing sucrose and ABA (0.04 µM) for 4-6 weeks prior to encapsulation resulted in extended storage of up to 90 d without loss of the germination potential and the capacity to regenerate into plantlets. Normally developed plantlets regenerated from ESEs were successfully adapted to soil.

K e y w o r d s : Encapsulation, germination, plantlet regeneration, somatic embryos.

A b b r e v i a t i o n s : 2,4-D: 2,4-dichlorophenoxyacetic acid, ABA: abscisic acid, GA_3: gibberellic acid, IBA: indole 3-butyric acid, MS medium: Murashige and Skoog (1962) medium, NN medium: Nitsch and Nitsch (1969) medium, ESEs: encapsulated somatic embryos, NSEs: non-encapsulated somatic embryos, B5 medium: Gamborg *et al.* (1968) medium, EDTA: ethylene diamine tetra acetic acid.

Introduction

Grape (*Vitis vinifera* L.) is the most important fruit crop cultivated worldwide. Generally grapes are vegetatively propagated through nodal woody cuttings but cuttings cannot be stored over long periods due to fungal infection. For a rapid clonal multiplication and for the genetic improvement of grapes, several groups have reported somatic embryogenesis in grape cultivars (Mullins and Srinivasan 1976, Reisch and Roberts 1985, Gray and Mortensen 1987, Hirabayashi 1985, Martinelli *et al.* 1991, 1993, Robacker 1993, Salunkhe *et al.* 1997, 1999; Das *et al*. 2002). Protocols for transformation of grape have also been developed (Mullins *et al.* 1990, Julie and Reisch 1996, Perl *et al*. 1996, Scorza *et al*. 1996, Franks *et al*. 1998, Iocco and Thomas 2001, Das *et al*. 2002, Mezzetti *et al*. 2002). Long-term storage of clones in the form of cuttings often encountered the problem of infection. Therefore, in order to circumvent these problems development of synthetic seeds appears to be a better alternative. An important application of somatic embryos is their use in the production of synthetic seeds (Merkle *et al*. 1990). Encapsulation of embryos is the first major step for synthetic seed production. Alginate gel capsules provide protection for naked somatic embryos (NSEs) and facilitate handling (Redenbaugh and Walker 1990, Redenbaugh *et al*. 1993). Artificial seed technology provides an alternative system for propagation of transgenic plants, non-seed producing plants, polyploids with elite traits and plants with problems in seed propagation. The technology may also be of interest for storage of germplasm and transportation of elite genotypes. The synthetic seed technology is best suited for the maintenance of genetic constitution of regenerated plants and can be stored for a long time. The application of synthetic seed technology has been demonstrated for many plant species (Bapat and Rao 1988, Padmaja *et al*. 1995, Onay *et al.* 1996, Ara *et al.* 1999) and the role of abscisic acid (ABA) is implicated as a controlling factor for germination and dormancy in somatic embryos and seeds (Senaratna *et al*. 1995, Bewley 1997).

In this paper we demonstrate the development and utility of artificial seeds in seedless grape varieties. We have also studied the effect of ABA on germination and plantlet regeneration from encapsulated somatic embryos. We have encapsulated 5-7 mm long immature cotyledonary stage somatic embryos (originating from leaf explants of cv. Pusa seedless), as mature somatic embryos are longer than 2 cm and difficult to encapsulate. We report the parameters for the production of somatic embryos in grape, their encapsulation, germination and regeneration into plantlets.

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Material and Methods

Production of somatic embryos: Leaves of grape (*Vitis vinifera* L.) cv. Pusa seedless were obtained from the Horticulture Division, IARI, New Delhi. Primary somatic embryos were induced in leaf discs as mentioned in the protocol of Das *et al*. (2002, 2005). Repetitive secondary somatic embryogenesis occurs if primary somatic embryos were cultured on NN basal or NN basal medium supplemented with IBA (0.1 mg l^{-1}), and both primary and secondary somatic embryos could be used for encapsulation. In order to ensure the production of a large number of somatic embryos, secondary somatic embryos were cultured in a fresh medium at an interval of two weeks. Culturing of primary somatic embryos on NN basal or NN basal medium supplemented with IBA (0.1 mg l^{-1}) led to the production of multiple microcalli from base to apex of primary somatic embryos. These microcalli differentiated into secondary somatic embryos. At the cotyledonary stage, somatic embryos were white opaque in appearance; 5-7 mm long embryos were harvested and used for encapsulation.

Encapsulation of somatic embryos: To encapsulate somatic embryos in calcium-alginate capsules, a 2 % sodium alginate (Sigma) gel (prepared in a nutrient solution containing one quarter strength of B5 macrosalts and MS organics) and 100 mM $CaCl_2$ solution (in double distilled water) were used. For encapsulation isolated somatic embryos from embryogenic calli were suspended in 2 % sodium alginate solution for 2 min and then dropped one by one through a Pasteur pipette into $CaCl_2$ solution kept on a low speed Vortex mixer (about 100 rpm). The resulting beads were kept for 40-45 min for hardening. Encapsulated embryos were washed with sterile distilled water for 5 min to remove remnants of $CaCl_2$ from the surface of capsules. All the steps for encapsulation were done under aseptic conditions.

Germination and plantlet development from ESEs: The effect of sucrose and ABA on germination of somatic embryos was assessed. For each treatment, 6 petri dishes (each with either ESEs or NSEs) sealed with parafilm were maintained in a 16-h-photoperiod (60 µmol m^{-2} s^{-1} light intensity) at 25 ±2 °C. The number of dead, arrested (remaining green without showing any activity at the radicular or plumular end) and germinating (showing emergence of tap root and shoot meristem) somatic embryos were counted for each treatment after 4 weeks in the first and second experiments, and after 8 weeks in the third experiment. The percentage of dead, arrested, and germinating somatic embryos was calculated against the total number of encapsulated (408) and non-encapsulated (499) somatic embryos cultured. Number of embryos for each treatment ranged from 71 to 88 with a mean value of 81.6 for encapsulated embryos and 80 to 112 with a mean of 99.8 for non-encapsulated embryos. Each experiment was repeated three times.

Subsequently, the germinating somatic embryos (6 per flask) were transferred to 250 ml Erlenmeyer flasks, each with 50 ml liquid medium containing half-strength B5 macrosalts with full-strength MS microsalts, iron-EDTA and organics, 2.74 mM L-glutamine (added before pH adjustment) and 3 % (w/v) sucrose. Glutamine is known to play a regulatory as well as a nutritive role in somatic embryo maturation and during autoclaving it is converted to 5-oxoproline (Lai *et al*. 1992). The cultures were maintained in a 16-h-photoperiod as described earlier. After 4 weeks the elongated somatic embryos were cultured on MS basal semi-solid (0.7 % agar) medium for complete development of plants and percentage of plantlets was calculated for each treatment in each of the experiments.

Establishment of plantlets in soil: Normally developed plantlets with roots were transferred to plastic pots containing moistened agro peat soil and kept in artificial light (60 µmol m^{-2} s^{-1} light intensity provided by cool white fluorescent tubes) at a temperature ranging between 25 and 30 °C. The plantlets were initially covered with polythene bags to maintain humidity and were irrigated with tap water. After 20 d, the pots were transferred initially to sunlight for a short duration (30 min). This period was gradually increased and polythene bags were removed. The plants were transferred to garden soil in clay pots after 3-4 months and kept outdoor under natural light conditions.

Storage of embryos in synthetic seeds: The embryogenic calli were cultured on different concentrations of sucrose (2, 3, 6, 9 %) with ABA (0.004, 0.02, 0.04, 0.2, 0.4 µM) for 4-6 weeks followed by encapsulation of somatic embryos as described above. The synthetic seeds were kept in an open Petri dish (sterile) under the laminar flow for 4 h to dry. Following drying, the synthetic seeds were kept in a bottle at room temperature up to three months. After storage, the synthetic seeds were cultured on NN or MS basal medium supplemented with 2.9 µM GA_3 for checking their viability and germination.

Results and Discussion

Primary somatic embryos were generated from leaf explants as reported earlier (Das *et al*. 2002); they were subcultured on either NN basal or NN medium supplemented with IBA and within 4–6 weeks embryogenic callus was formed (Fig. 1 A), which differentiated into many globular to cotyledonary stage secondary embryos. The globular stage somatic embryos reached the cotyledonary stage in 4 weeks (Fig. 1 B). The cotyledons (closed) of most of the somatic embryos became opaque and milky-white in colour (Fig. 1 C) to light-green or green after transfer to light.

Germination and plantlet development from ESEs: For germination of encapsulated (Fig. 1 D) or naked white, opaque, cotyledonary-stage somatic embryos were cultured on MS agar medium containing B5 macrosalts, MS microsalts, iron EDTA, organics with 3 % sucrose and 0.7 % agar supplemented with 2.9 µM GA_3. During germination, both ESEs and NSEs turned light-green or green within a week of inoculation on agar medium. Germination was initiated with the emergence of tap roots (Fig. 2 A). Roots from ESEs became visible within 1-2 weeks of culture on agar medium, whereas root

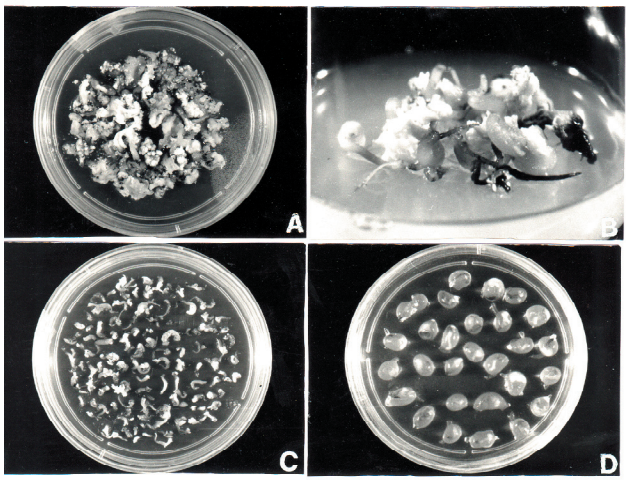

Fig. 1: Encapsulation of somatic embryos of grape (*Vitis vinifera* L.). **A**: Embryogenic callus originated from leaf explants (cv. Pusa seedless); **B**: Different stages of somatic embryos produced from the embryogenic callus; **C**: Cotyledonary stage somatic embryos; **D**: Encapsulated somatic embryos.

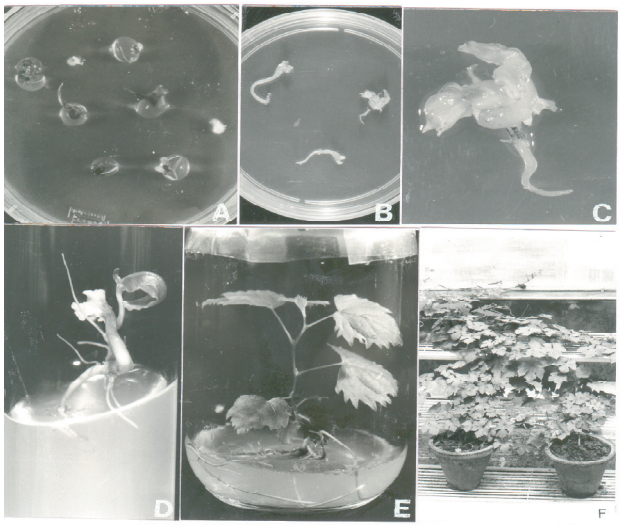

Fig. 2: Germination and plantlet regeneration from encapsulated somatic embryos. **A**: Germinating ESEs showing emergence of tap root; **B**: Germinating somatic embryo emerging in liquid culture. **C**: Emergence of shoot meristem at the tip of elongated somatic embryo in liquid culture. **D**: Plantlet developed from ESEs in semi-solid culture. **E**: Fully grown plantlet in semi-solid culture. **F**: Acclimatized plants grown in field soil.

initiation from NSEs became visible only after 2 weeks of culture in the same medium. Further root elongation and proliferation of leaves from shoot meristem were observed only after transfer to MS liquid medium containing glutamine. Roots continued to grow up to 5-10 cm within 3 weeks of culture before shoot emergence (Fig. 2 B and C). The shoot elongation of such rooted embryos became visible within 2-3 weeks by the development of small leaves (Fig. 2 D). The proliferation of shoot meristems, enlargement of leaves and formation of plantlets (Fig. 2 E) were observed only after 4-6 weeks of culture on MS basal semi-solid medium. In this way, both NSEs and ESEs developed into complete plantlets and there were no differences in the morphology of plantlets raised from ESEs and NSEs. However, after 4-6 weeks of culture, percent germination and plantlet development from ESEs was higher than that of NSEs (Tab. 1). NSEs with arrested germination showed cotyledon growth up to 2 weeks of culture in liquid medium and no growth was observed on semi-solid medium. A lower percentage of dead embryos was observed in ESEs than NSEs (Tab. 2). The reason for this may be attributed to the protection provided by capsules as well as the presence of nutrients in the gel matrix which apparently served as a nutrient bed around the somatic embryos. This facilitated growth and survival and allowed embryos to germinate as described by Redenbaugh *et al*. (1987, 1990). Our observations with grape are more or less similar to mango (Ara *et al*. 1999) but in contrast to those reported for *Santalum album* (Bapat and Rao 1988), *Solanum melongena* (Lakshmana *et al.* 1991) and *Asparagus cooperi* (Ghosh and Sen 1994), for which the frequency of germinating ESEs was lower than that of NSEs. In all these examples, however, mature somatic embryos were used in encapsulation.

Effect of concentration of B5 macrosalts on germination and plantlet development from ESEs: The strength of B5 macrosalts in the agar medium affected significantly the percent ESEs germination; the highest percentage was observed in medium supplemented with quarter strength B5 macrosalts. The ratio of germination to conversion into plantlets increased in the B5 macrosalts up to one fourth strength in MS medium containing B5 macrosalts, MS microsalts, iron EDTA, organics and 3 % sucrose supplemented with 2.9 µM GA_3 (Tab. 2).

Effect of ABA on germination and plantlet development from ESEs: In general, ESEs (no ABA treatment) started germination after 1-2 weeks of culture, whereas ABA-treated ESEs showed germination after 3-4 weeks irrespective of the ABA concentration. However, ABA concentration affected the percent response (Tab. 3). ESEs treated with 0.004 or

Table 1

Responses of NSEs and ESEs on agar medium*

Exp. No.	Condition of somatic embryos	Germination (%)	Dead (%)	Arrested germination (%)	Plantlets (%)
1	NSEs	23.8 ± 3.7	31.0 ± 6.5	45.2 ± 3.1	8.0 ± 1.6
2	ESEs	44.4 ± 4.5	19.0 ± 3.8	18.2 ± 4.3	16.0 ± 1.6

* Mean values of three independent experiments ± SD

Table 2

Influence of B5 macrosalts concentration on germination and plantlet development from ESEs*

Exp. No.	Concentration of B5 macro salt in MS medium supplemented with gibberellic acid (2.9 μM)	Germination (G) (%)	Conversion (C) of plants (%)	G/C
1	Full strength	10.0	6.65	1.50
2	Half strength	21.8	14.0	1.56
3	Quarter strength	69.2	36.0	1.92
4	One eight strength	35.8	24.4	1.47

*Values are means of three independent experiments

Table 3

Responses of ABA-treated and non-treated ESEs on agar medium*

Exp. No.	Concentration of ABA (μM)	Germination (%)	Dead (%)	Arrested germination (%)	Plantlet (%)
1	0.0	78.0 ± 3.1	17.6 ± 1.7	4.0 ± 1.6	42.2 ± 1.9
2	0.004	72.0 ± 2.1	20.6 ± 1.1	5.6 ± 1.1	39.2 ± 1.9
3	0.02	69.9 ± 3.0	20.6 ± 2.9	6.2 ± 1.5	36.2 ± 3.4
4	0.04	23.4 ± 3.2	31.4 ± 2.3	39.6 ± 2.1	12.4 ± 1.1
5	0.2	8.0 ± 1.6	41.6 ± 2.3	38.6 ± 1.1	3.0 ± 1.6
6	0.4	0	64.8 ± 1.5	40.0 ± 1.6	0

* Mean values of three independent experiments ±SD

0.02 μM ABA showed a slight decrease in percent germination and plantlet development relative to the control, but the decrease was not significant (Tab. 3). The ESEs treated with higher concentrations (0.04, 0.2, or 0.4 μM) of ABA showed a significant difference in percent response in comparison with the control, with a large number of ESEs either dying or showing arrested germination (Tab. 3). With increasing ABA concentration (0.04 to 0.2 μM), percent germination and conversion into plantlets decreased gradually, and no ESEs germinated if they were treated with 0.4 μM ABA (Tab. 3).

It is evident from the results that ABA at lower concentrations (0.004 or 0.02 μM) had no influence on embryo germination or conversion into plantlets although it caused a delay of up to 3-4 weeks in germination (recorded on MS medium containing B5 macrosalts, MS microsalts, MS iron-EDTA, MS organics and 3 % sucrose supplemented with 2.9 μM GA_3). Inhibition of germination was significant when the ESEs were treated with higher concentrations (0.04, 0.2 and 0.4 μM) of ABA. Exogenous ABA treatment prevented precocious germination of immature somatic embryos in several species (Bapat and Rao 1992, Attree *et al*. 1994).

Effect of sucrose and ABA on survival of somatic embryos: Germination and survival of somatic embryos were assessed in the medium containing different concentrations of sucrose with or without ABA. At lower concentrations of sucrose (1-3 %) embryos remained hydrated and started germination without any delay. As the concentration of sucrose was increased to 6 or 9 %, the embryos became more and more dehydrated. If the somatic embryos were first encapsulated and then cultured for 4-6 weeks in medium containing 9 % sucrose and ABA, the synthetic seeds remained viable at least up to three months at room temperature (Tab. 4). Survival of somatic embryos in synthetic seeds was checked through germination on MS medium containing B5 mac-

Table 4

Effect of sucrose with or without ABA (0.04 μM) in NN basal medium on the survival of somatic embryos after 4-6 weeks of culture

Exp. No.	Sucrose concentraion (%)	Survival of somatic embryos (%)	
		without ABA	with ABA
1	1.0	5	7.0
2	3.0	15	56.7
3	6.0	30	46.7
4	9.0	61	92.3

rosalts, MS microsalts, iron-EDTA, organics with 3 % sucrose and 0.7 % agar supplemented with 2.9 µM GA_3.

Transfer of plantlets to soil: In the first two weeks after adaptation to soil, the plantlets showed no further development. After two weeks, some plantlets showed blackening at the shoot tips, and died gradually. However, plantlets, which did not exhibit the symptoms, survived and grew in agropeat (Fig. 2 E). Growth was initially very slow. Out of 20 plantlets, 15 reached an age of 4 months in field soil (Fig. 2 F). The plantlets obtained from ESEs and NSEs were transferred into the field and no marked difference in their growth was observed. Earlier transfer of plants to soil, obtained from somatic embryos of some grape cultivars has also been reported by Das *et al*. (2002).

Our study demonstrates successful plant regeneration from *in vitro*-raised somatic embryos encapsulated in calcium alginate. The synthetic embryos are capable to germinate and regenerate into plants. The main advantage of encapsulated embryos will probably be their use as an alternative for long-term storage and mass propagation of planting material. Thus, it is expected that with further refinement of the techniques for production of somatic embryos, synthetic seeds will be used in grape biotechnology.

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