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Somatic embryogenesis and plant regeneration in black pepper (*Piper nigrum* L.): I. Direct somatic embryogenesis from tissues of germinating seeds and ontogeny of somatic embryos

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SUMMARY
A protocol was developed for induction, maturation and germination of somatic embryos from the tissues of germinating seeds of black pepper (*Piper nigrum* L.). Explants were cultured on growth regulator – free solid SH medium maintained in the dark. The first somatic embryos developing directly from the explant tissue were noticed after 60 d of culture. Somatic embryos originated from a ring-like tissue on the micropylar region of the seeds. Sucrose concentration of the medium was found to be crucial for the induction of somatic embryos, and 30 g l^{-1} was found to be the optimum. Maturation and germination of somatic embryos were achieved on the same medium. Suspension culture enhanced the process of maturation and germination. Regenerated plants were established in soil. Histology confirmed the ontogeny and each stage of development. Growth regulators were found to inhibit the induction of somatic embryogenesis. Cytological analysis of the regenerated plants revealed the normal chromosome number of 2n=52.

Black pepper (*Piper nigrum* L., *Piperaceae*) is the most important of all the spices grown in India and is widely used in culinary preparations, food processing, perfumery and also as a condiment throughout the world. India is a major producer and exporter of black pepper, the annual export being over Rs. 4000 million. In spite of its great economic importance, the productivity of black pepper in India is low compared with that of other pepper producing countries, mainly because of nonavailability of adequate planting materials of high yielding varieties and losses due to diseases, nematodes and insect pests. Vegetative multiplication through conventional methods is unable to meet the demand for planting material. Propagation through seeds is cumbersome, uncertain and yields only heterogeneous progenies (Ravindran *et al.*, 2000). An efficient and reproducible *in vitro* plant regeneration system, preferably from single cells, is a prerequisite for applying modern methods of gene transfer aided by *Agrobacterium* based vectors or by particle bombardment. Such a system may also be helpful for the multiplication and supply of the elite genotypes on a large scale.

Micropropagation of black pepper through organogenesis using various explants from mature vines (Philip *et al.*, 1992; Bhat *et al.*, 1995) and from seedlings (Mathews and Rao, 1984) has been reported. A micropropagation system using somatic embryogenesis is usually preferred over organogenesis, as more regenerants can be obtained from few or single cells which increases the scope for getting transformed plants in gene transfer experiments. Direct somatic embryogenesis from vegetative and seed tissues other than zygotic embryos and endosperm can be utilized as a method for large-scale propagation and multiplication of elite genotypes or high yielding varieties. Encapsulated somatic embryos can be used as artificial seeds for storage and safe exchange of germplasm.

The only published report available on somatic embryogenesis of black pepper was from the callus derived from zygotic embryos of mature seeds (Joseph *et al.*, 1996). The present study reports an effective protocol for direct somatic embryogenesis and whole-plant regeneration in black pepper.

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MATERIALS AND METHODS

Establishment of embryogenic cultures

Fresh, healthy, fully ripe seeds of *Piper nigrum* L. 'Karimunda' were collected from plants grown in the germplasm repository at the Indian Institute of Spices Research, Calicut. The seeds were washed under running tap water and surface sterilized with 0.1% mercuric chloride solution for 10 min, followed by repeated washings (3–4 times) with sterile double-distilled water. The seeds were inoculated on 50 ml each of half and full-strength SH (Schenk and Hildebrandt, 1972) medium containing 0.8% (w/v) agar (Bacteriological grade, Hi-media) and sucrose 15 g l^{-1}, 30 g l^{-1} and 45 g l^{-1}. The pH of the medium was adjusted to 5.9 prior to autoclaving. Cultures were maintained separately in three light conditions: 16 h normal light (36 μmol m^{-2} s^{-1}), 16 h diffused light (10 μmol m^{-2} s^{-1}) and 24 h dark at a temperature of 25 ± 1°C. There were three replications (Erlenmeyer flasks, 250 ml) each containing 30 seeds. The experiment was repeated five times.

After germination the seedlings with attached seed coats were rearranged on the medium in such a way that the micropylar region of the seeds were partially immersed in the medium. Cultures were regularly observed for somatic embryogenesis. The cultures were transferred to fresh medium of the same composition, intervals of 30 d. Percentage of seeds with somatic embryos and number of embryos in each responding seed were recorded after 90 d of culture. The seeds sowing somatic embryogenesis were confirmed by observing them under stereo-zoom microscope (Nikon, SMZU). The induced somatic embryos were allowed to mature in the induction medium.

Germination and conversion

Germination of somatic embryos was tested in solid as well as agitating liquid media under the three light conditions described. Twenty five embryos at the cotyledonary stage were placed for germination in wide mouthed 250 ml Erlenmeyer flasks containing 30 ml of half-strength and full-strength SH medium having 30 g l^{-1} sucrose (SHS30). The liquid medium was agitated in a gyratory shaker (B-Braun Certomat-R) at 110 rpm. Germination was recorded after 20 d. There were five replications per treatment. An embryo was considered as germinated perfectly, only when it produced a clear taproot and distinct cotyledonary leaves.

For conversion, germinated somatic embryos were grown under diffused light on filter paper bridges dipped in static liquid SHS30 medium in test tubes. The medium was replenished every 5 d and conversion was recorded after 20 d.

Acclimatization and establishment of regenerated plants in soil

Plantlets with well-developed roots (3–4 cm) were taken out of the culture vessel, washed thoroughly under running tap water and planted in plastic pots filled with moistened sterile sand and covered with polythene bags having 4–5 holes for aeration. After successful establishment in the sand they were transferred to small earthen pots (30 cm diameter) filled with garden soil and kept in a greenhouse for further growth.

Statistical analysis

Mean and standard error were calculated for embryogenesis, germination and conversion. LSD was used to compare treatment means at the >95% level of probability in the case of embryogenic induction.

Effect of growth regulators

Growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4–D) and α-naphthalene acetic acid (NAA) at concentrations 0.5, 2.0 and 5.0 mg l^{-1} were used either singly or in combination with 6-benzylaminopurine (BAP) at 0.1, 0.5 and 1.0 mg l^{-1}. The explants were cultured for one month on growth regulator containing media and then transferred to growth regulator-free SHS30 medium for somatic embryogenesis.

Ontogeny and development of somatic embryos

Ontogeny and sequential development of somatic embryos were studied by histological analysis of seed explants at different stages of somatic embryogenesis. For histological analysis, the explants and somatic embryos were fixed in 1:1:18 mixture of formalin-glacial acetic acid-50% ethanol (FAA) for 24 hours, dehydrated in an ethanol-TBA (tertiary butanol) series and infiltrated with and embedded in paraffin (56–58°C, Merck, India). Sections of 8–10 μm were cut using a Leica rotary microtome and stained with Heidenhain's iron-alum-haematoxylin (Johansen, 1940). The sections were observed and photographed under appropriate magnifications using a Leica DMRB research microscope.

Chromosome number of regenerated plants

Chromosome analysis was carried out following the procedure standardized for black pepper (Nair *et al.*, 1993). Twenty regenerated plants were randomly selected and analysed for chromosome number. Chromosome number was counted from at least five well spread mitotic metaphase plates per plant. Photomicrographs were taken under the 100× oil immersion objective of a Leica DMRB research microscope using an 8× photolens.

RESULTS

Induction of somatic embryogenesis

Seed germination started within 15 d of culture under all light conditions and continued up to 30 d. By this time most of the normal seeds had germinated raising the small cotyledons and seed coats away from the medium, as germination is epigeal. Freshly germinated black pepper seed consists of a pair of small pale green folded cotyledons attached to the seed coats containing other seed tissues inside, a small slender hypocotyl and a primary root with one or two lateral roots. The point of attachment of seed coat with cotyledons is surrounded by a ring-like brownish translucent tissue that was originally covering the micropylar region of intact seed, like a dome shaped projection.

After 60 d of culture, a few seeds on the full strength SHS30 medium maintained in 24 h darkness, exhibited a swelling of the ring-like tissue surrounding the micropylar region and within a few days small globular structures appeared on the edge of this tissue (Figure 1A). As days progressed, these structures exhibited clear bipolarity. Examination of cultures under stereomicroscope confirmed these as somatic embryos (Figure 1B). Somatic embryos at different developmental stages could be observed on the same seed explant (Figure 1C). Lengths of somatic embryos ranged from 0.5 mm at the globular stage to 2.0 mm at the cotyledonary stage. By 90 d of culture, more seeds started to produce somatic embryos. Among the three sucrose concentrations tested, 15 g l^{-1} and 30 g l^{-1} produced somatic embryogenesis from seeds. Medium containing 45 g l^{-1} sucrose failed to give any response. The somatic embryogenesis occurred under 24 h darkness, 8 h darkness and 16 h diffused light but not under 16 h of normal light. A combination of sucrose in the medium at 30 g l^{-1} and culture maintenance under 24 h darkness induced a significantly high percentage of seeds with somatic

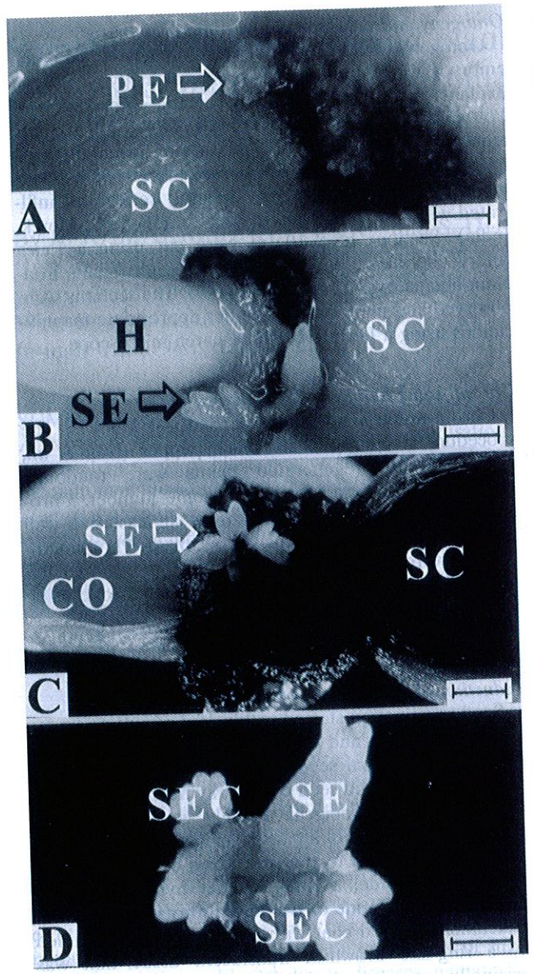

FIG. 1
Somatic embryogenesis from germinating seeds of black pepper. (A) Initiation of proembryos from micropylar ring tissue of germinating seed (arrowed) after 60 d of culture. (B) Emergence of early stages of somatic embryos after 90 d. (C) Many primary embryos of different developmental stages after 120 d. (D) Secondary embryogenesis from the root pole of a primary embryo. Bars represent 1 mm in A, B and C and 0.5 mm in D. (SC – Seed coat; H – Hypocotyl; CO – Cotyledon; PE – Proembyros; SE – Somatic embryos; SEC – Secondary embryos).

embryos (23.3). However, the number of somatic embryos per responding seed was not significantly different (Table I). Somatic embryos matured on the induction medium itself. A few even started germination on the same medium. Some primary embryos, which had become detached from the seed explant by chance and fallen on the medium, were found to produce secondary embryos from their root pole (Figure 1D).

Germination, conversion and ex vitro *establishment of plantlets*

Somatic embryos germinated readily in almost all the treatments tested and gave more than 65% germination. Maximum germination (97.6) was obtained in full-strength liquid medium under 24 h darkness (Table II). The germinated somatic embryos had well-developed cotyledons and taproots (Figure 2A, 2B). Conversion rate of germinated somatic embryos ranged from 54.9 to 85.2%. In general conversion rate was higher in somatic embryos germinated in the liquid medium. Further growth of embryos germinated in solid medium was very slow. Somatic embryos successfully germinated and converted into plantlets were hardened and established in soil with 80% efficiency. The plants were found to be morphologically uniform and grew vigorously under *ex vitro* conditions (Figure 2C, 2D).

Effect of growth regulators

Growth regulators in the medium were found to be highly inhibitory for the induction of somatic embryos. No somatic embryogenesis was observed on explants cultured in medium supplemented with NAA or 2,4-D either alone or in combination with BAP. Callusing was observed from the micropylar ring tissue in the medium supplemented with 0.5 mg l^{-1} 2,4-D alone. This callus gradually became necrotic and turned brownish without giving any embryogenic response. No callusing was observed with other concentrations of 2,4-D as well as other growth regulators.

Ontogeny and development of somatic embryos

Histological analysis revealed the single cell origin of somatic embryos. The outermost layer of the micropylar ring tissue proliferated on contact with the culture medium. This tissue was slightly brown. Certain cells in the peripheral region of this tissue became densely cytoplasmic and divided repeatedly to form masses of

TABLE I
Effect of light and sucrose concentration on induction of somatic embryogenesis from seeds of black pepper 'Karimunda' on growth regulator-free SH medium after 90 d of culture

Photoperiod	Sucrose (g l^{-1})	Percent somatic embryogenesis ± SE	No. of somatic embryos per responding seed ± SE
16 hours normal light	15	0.00	0.00
	30	0.00	0.00
	45	0.00	0.00
16 hours diffused light	15	10.00±1.05	5.60±0.245
	30	14.67±1.70	6.00±0.550
	45	0.00	0.00
24 hours darkness	15	12.00±1.70	5.40±0.510
	30	23.34±2.11	6.00±0.548
	45	0.00	0.00
SE standard error	LSD (P = 0.05)	3.678	0.308

TABLE II
Effect of the nature of medium, medium strength and light on germination of somatic embryos of black pepper 'Karimunda'

Nature of medium	Medium strength	Light condition	% Germination	% Conversion ± SE
Solid	Half	24 h dark	80.8±2.65	61.2±4.53
		16 h diffused	82.4±2.71	58.3±3.04
		16 h normal	66.4±2.71	70.4±2.76
	Full	24 h dark	82.4±2.71	55.9±2.77
		16 h diffused	80.8±5.12	54.9±4.10
		16 h normal	70.4±0.98	64.2±2.36
Liquid (suspension)	Half	24 h dark	88.8±2.65	79.9±1.17
		16 h diffused	88.8±2.33	77.3±0.83
		16 h normal	80.8±1.50	76.6±2.23
	Full	24 h dark	97.6±0.98	85.2±2.51
		16 h diffused	96.0±1.26	83.3±2.67
		16 h normal	87.2±2.33	84.5±2.84

Data on germination were collected 20 d after culture and conversion after 20 d of germination; SE = standard error.

small densely cytoplasmic cells with deeply stained nuclei (Figure 3A, 3B, 3C). Embryos originated from the outermost layer of these cell masses with a small multiseriate suspensor-like structure (Figure 3D). An active callus stage was not observed at any phase of development. The micropylar ring tissue from which somatic embryos originated is an extension of the inner layer of inner integument tissue which forms the outer layer of the dome-like projection at the micropylar region of mature intact seed.

Histological studies of somatic embryos confirmed a clear bipolarity right from the globular stage (Figure 4A). Vascular differentiation was prominent from the heart shaped stage towards (Figure 4B, 4C, 4D, 4E, 4F). Root and shoot meristems were observed from the late torpedo to the cotyledonary stage (Figure 4E, 4F).

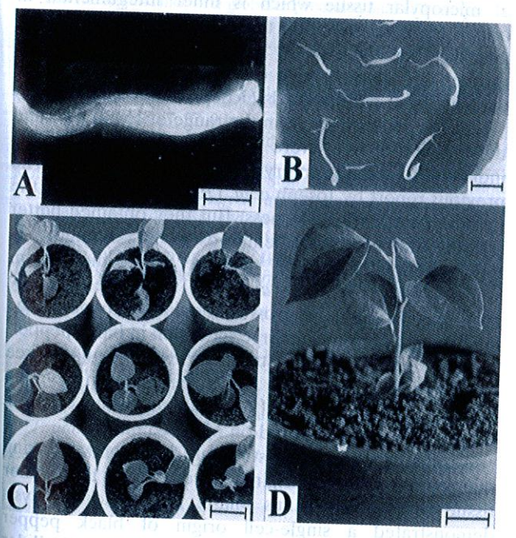

FIG. 2
Germination of somatic embryos and *ex vitro* establishment of regenerated plants. (A) Early stage of germination showing tap root and small cotyledons. (B) Germinated somatic embryos showing well developed cotyledons, taproot and lateral root initials. (C) Plantlets established in plastic cups filled with sterile sand. (D) A regenerated plant growing in pot filled with garden soil. Bars rpresent 1 mm in A, 10 mm in B, 20 mm in C and 4 cm in D.

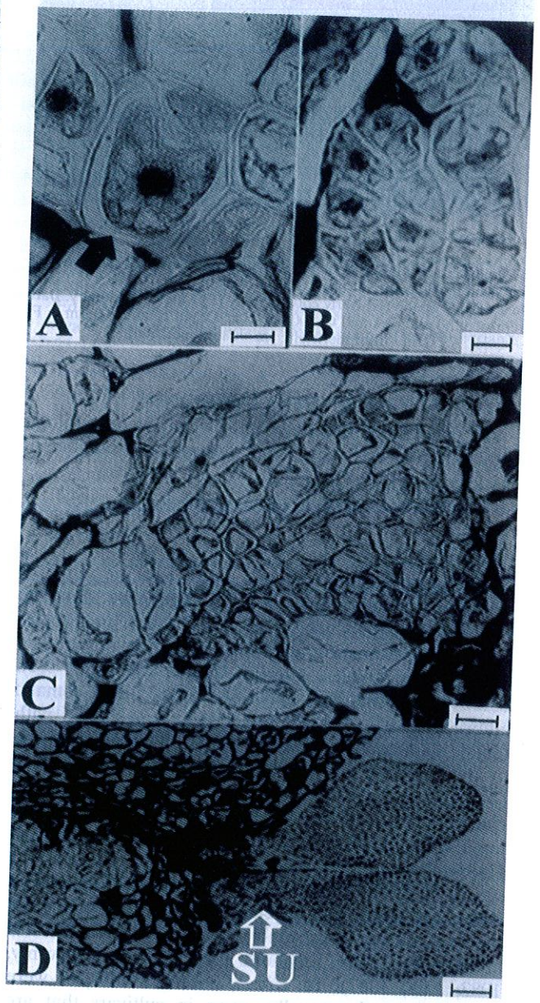

FIG. 3
Histological sections (LS) of micropylar tissue of black pepper seeds showing embryogenesis. (A) Single densely cytoplasic embryogenic cell (arrowed). (B) Multicellular embryonal complex. (C) Cluster of embryogenic cells. (D) Somatic embryos formed from the embryogenic cell clusters on the peripheral region of micropylar tissue. Bars represent 25 μm in A and B, 50 μm in C and 130 μm in D. (su – suspensor like structure).

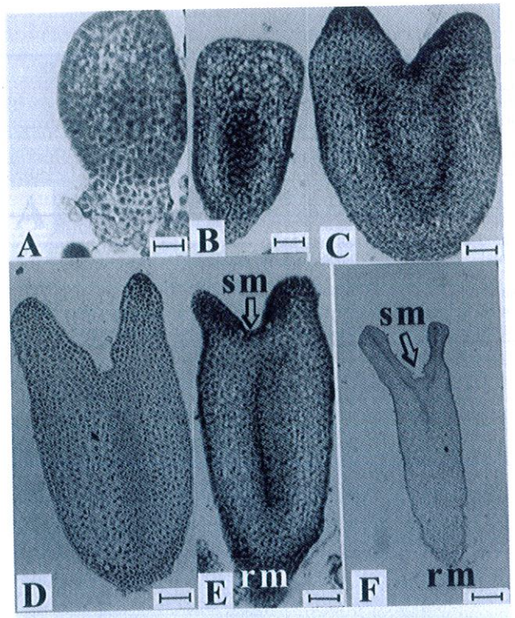

FIG. 4
Histological sections (LS) of the somatic embryos of black pepper at different developmental stages. (A) Globular (B) Early heart (C) Late heart (D) Torpedo (E) Early cotyledonary (F) Late cotyledonary. Bars represent 50 μm in A and C, 100 μm in B, D and E, and 200 μm in F. (rm – root meristem; sm – shoot meristem)

Chromosome number of regenerated plants

All twenty regenerated plants which were cytologically analyzed had the normal chromosome number of black pepper (2n=52) (Figure 5).

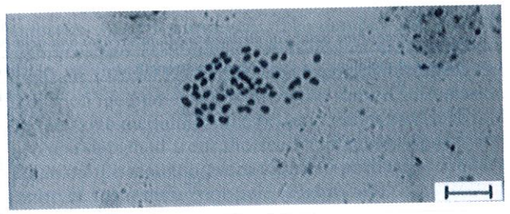

FIG. 5
Mitotic metaphase cell from the root tip of a plantlet regenerated from somatic embryo showing 2n=52. Bar represents 5 μm.

DISCUSSION

The present study reports direct somatic embryogenesis in black pepper for the first time. Somatic embryos were produced from the tissue at micropylar region of germinating seeds, on growth regulator-free SH medium. Ovules, nucellar tissues and other seed tissues are particularly liable to display direct somatic embryogenesis, as they are closely associated with female gametophyte (George, 1993). This may be due to the presence of pre-embryogenic determined cells (PEDCs), as stated by Sharp *et al.* (1982). In plants like *Carica* and *Hevea*, somatic embryos originate from the inner integuments of ovules (Litz and Conover, 1981; El Hadrami *et al.*, 1991) and in carrot, tissue of mericarp seed coat produce somatic embryos directly (Smith and Krikorian, 1988). The nucellar tissue of many plants has the capacity for direct embryogenesis *in vitro* (Haccius and Hausner, 1976; Eichholtz *et al.*, 1979; Rangaswamy, 1982; Litz, 1987). In *Citrus*, somatic embryos were formed from the nucellus even in cultivars that are normally monoembryonic (Kunitake and Mii, 1996). It was reported that somatic embryos arise from the micropylar end of *Citrus* nucellus. In the present study, primary somatic embryos were obtained directly from the outer layers of ring-like micropylar tissue in the germinating seeds. The tissue layers covering the micropyle of black pepper seed is composed of the innermost layer of inner integument above and the nucellar epidermis below (Kanta, 1962; Ravindran *et al.*, 2000). Kanta (1962) reported the densely cytoplasmic nature of radially elongated cells of the innermost layer of inner integument at the micropylar region of developing seeds of black pepper. This is a characteristic of cells having embryogenic potential (Williams and Maheswaran, 1986). Even though most of these cells get filled with tannin as the seed matures (Kanta, 1962), it appears that some retained their original state and might have expressed their embryogenic potential on contact with the culture medium in ideal conditions. It is logical to think that the origin of somatic embryos in this case is not from nucellar epidermis or other nucellus related tissue, in which case the frequency of primary embryogenesis would have been very high, as the major part of black pepper seed is occupied by nucellar tissue in the form of perisperm. Moreover histological evidence indicates that embryos originate from outermost region of micropylar tissue which is inner integumental in origin. Plant regeneration through somatic embryogenesis has been previously reported in black pepper through the mediation of callus using zygotic embryo explants from mature seeds (Joseph *et al.*, 1996).

Sucrose concentrations in the range of 15–30 g l^{-1} and the absence of light were found to be critical for the induction of somatic embryos, in the present study. The previous report by Joseph *et al.* (1996) also supports the favourable effect of darkness. But they obtained better proliferation of somatic embryos in half-strength SH medium containing 15 g l^{-1} sucrose, unlike the present study. Similar to Joseph *et al.* (1996), in the present study also, the presence of growth regulators found to be inhibitory for somatic embryo induction in black pepper. Somatic embryogenesis using growth regulator-free medium was reported in *Panax ginseng* using zygotic embryos (Choi and Soh, 1996) and cotyledon explants (Choi *et al.*, 1998) cultured on MS medium.

Results of the present study are important, showing that somatic embryos originate directly from the mother tissue without any callus phase. Histological analysis demonstrated a single-cell origin of black pepper somatic embryos for the first time. Such unicellular origin of somatic embryos has been widely described in other species (Button *et al.*, 1974; Maheswaran and Williams, 1984; Nagmani *et al.*, 1987; Vieitez *et al.*, 1992; Toonen and De Vries, 1996). The previous report on somatic embryogenesis in black pepper (Joseph *et al.*, 1996) provided no details of ontogeny.

Changes in ploidy level have been reported in many culture systems leading to mixed populations of polyploids and aneuploids; plants regenerated from such cultures often show a range of chromosome complements (Sunderland, 1977; D'Amato, 1978). Such variations are not desirable in the regeneration systems used for micropropagation. The chromosomal status of regenerated plants in the present study was found to be normal (2n=52), indicating the stability of regenerated plants at the chromosomal level. This can be attributed to the direct formation of embryos without an active callus phase.

The origin of somatic embryos from the integumental layer of seed which is part of the mother plant and high germination and conversion obtained in the present study indicate the feasibility of the system for commercial micropropagation. Secondary embryogenesis may be helpful in scaling up the process.

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