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High-frequency plant regeneration through cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.)

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Abstract A high-frequency plantlet regeneration protocol was developed for black pepper (*Piper nigrum* L.) through cyclic secondary somatic embryogenesis. Secondary embryos formed from the radicular end of the primary somatic embryos which were originally derived from micropylar tissues of germinating seeds on growth regulator-free SH medium in the absence of light. The process of secondary embryogenesis continued in a cyclic manner from the root pole of newly formed embryos resulting in clumps of somatic embryos. Strength of the medium and sucrose concentration influenced the process of secondary embryogenesis and fresh weight of somatic embryo clumps. Full-strength SH medium supplemented with 1.5% sucrose produced significantly higher fresh weight and numbers of secondary somatic embryos while 3.0 and 4.5% sucrose in the medium favored further development of proliferated embryos into plantlets. Ontogeny of secondary embryos was established by histological analysis. Secondary embryogenic potential was influenced by the developmental stage of the explanted somatic embryo and stages up to "torpedo" were more suitable. A single-flask system was standardized for proliferation, maturation, germination and conversion of secondary somatic embryos in suspension cultures. The system of cyclic secondary somatic embryogenesis in black pepper described here represents a permanent source of embryogenic material that can be used for genetic manipulations of this crop species.

Keywords Direct somatic embryogenesis · Piperaceae · Recurrent embryogenesis · Spice · Suspension culture

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Abbreviations PGR: Plant growth regulator · PE: Primary somatic embryo · SE: Secondary somatic embryo · SH: Schenk and Hildebrandt (1972)

Introduction

Black pepper (*Piper nigrum* L., *Piperaceae*), a popular spice crop grown in India and traded throughout the world plays an important role in the country's agrarian economy by contributing an export earning around 35 million US dollars annually. Among the major weaknesses responsible for low productivity of black pepper, non-availability of healthy planting materials and crop losses due to biotic and abiotic stresses are of foremost importance (Sarma and Kalloo 2004). To circumvent the above production crises, modern methods of plant propagation and gene transfer may be helpful.

Development of an advanced propagation technology such as somatic embryogenesis will assist the biotechnological improvement of black pepper. Somatic embryogenesis has great potential to aid crop improvement through large-scale clonal propagation, genetic transformation and synthetic seed production (Cervelli and Senaratna 1995). Large-scale somatic embryogenesis through cyclic secondary embryogenesis preferably in liquid culture is the most attractive method for mass cloning of plants, since very large numbers of somatic embryos can be produced in a short period of time in a limited volume of the medium with a possibility of mechanization (Akita and Takayama 1988; Raemakers et al. 1993b; Denchev and Atanassov 1995; Raemakers et al. 1995; Jayasankar et al. 1999; Choi et al. 2002a).

Secondary somatic embryogenesis is a process whereby new somatic embryos are initiated from originally formed somatic embryos or primary somatic embryos. As an experimental system it has certain advantages compared to primary somatic embryogenesis such as very high multiplication rate, independence of an explant source and

repeatability. Additionally, embryogenicity can be maintained for prolonged periods of time by repeated cycles of secondary embryogenesis. Furthermore, in many species the efficiency of explants in primary embryogenesis is lower than in secondary embryogenesis (Raemakers et al. 1995). Epidermal and single-cell origin of somatic embryos favors the use of secondary somatic embryogenesis for plant transformation. The chimeric nature of transformed embryos was changed after several cycles of secondary embryogenesis under selective conditions into completely transformed embryos (Raemakers et al. 1995). High-frequency plant regeneration systems through cyclic secondary embryogenesis were reported in several plant species of interest (Merkle et al. 1990; Raemakers et al. 1993a, 1993b; Weissinger II and Parrott 1993; Choi et al. 1997; Das et al. 1997; das Neves et al. 1999; Chen and Chang 2004; Giridhar et al. 2004).

Somatic embryogenesis in black pepper has been documented from zygotic embryo-derived callus (Joseph et al. 1996) and directly from maternal tissues at the micropylar region of germinating seeds (Nair and Dutta Gupta 2003). The ability of black pepper somatic embryos to produce secondary embryos has also been indicated.

The present study reports the establishment of a high-frequency plant regeneration system of black pepper through cyclic secondary embryogenesis. Histological evidence for secondary embryo formation and potential of scale-up utilizing liquid culture in single-flask is also described.

Materials and methods

Plant materials

Mature seeds of black pepper (*Piper nigrum* L.) cv. "Karimunda" collected from a plant grown in the germplasm repository at the Indian Institute of Spices Research, Calicut were used for establishing primary somatic embryogenic cultures. Karimunda is the most popular cultivar grown throughout Kerala, the major black pepper growing state of India. This is a prolific and regular bearer, having medium-sized berries, good dry recovery and yields good quality black pepper. The cultivar is of medium maturity and is suitable for inter cropping as well as high-density cropping (Ravindran and Nair 1984).

Establishment of primary somatic embryogenic cultures

Primary embryogenic cultures were established following the method described by Nair and Dutta Gupta (2003). The surface sterilized seeds were cultured on agar gelled full-strength, PGR-free SH (Schenk and Hildebrandt 1972) medium containing 3.0% (W/V) sucrose (SHS30) under darkness. Primary somatic embryos (PEs) derived from micropylar tissues of germinating seeds after 90 d were utilized for inducing secondary somatic embryogenic cultures.

Induction of secondary somatic embryogenesis

Primary somatic embryo clumps having pre-globular to torpedo shaped embryos (5–6 visible embryos per seed) were carefully detached and inoculated on half- or full-strength, PGR-free SH medium containing 1.5, 3.0 or 4.5% sucrose and gelled with 0.8% agar (Bacteriological grade, Hi-media). The pH of the medium was adjusted to 5.9 prior to autoclaving. Cultures were maintained at darkness at a temperature of 25±1°C. The culture conditions remained the same for all further experiments unless otherwise specified. While inoculating, the PEs were uniformly spread on the surface of the medium. There were 10 replicates (Borosil culture tubes of 25×150 mm containing 20 ml of medium) each containing the PEs from a single seed and the experiment was repeated 5 times.

The percentage of cultures that produced secondary somatic embryos (SEs) and fresh weight of the embryos per tube were recorded after 20 d of culture. The number of somatic embryos of different developmental stages per 50 mg of SE clumps was also recorded by counting under a stereo-zoom microscope (Nikon, SMZU). Total number of embryos per treatment was recorded at the end of experiment. Secondary embryogenic cultures were further maintained by sub-culturing on SH medium containing 1.5% sucrose (SHS15), at intervals of 20 d. The proliferating SEs were spread periodically on the surface of the medium, to facilitate proliferation.

Ontogeny of SEs was studied by histological analysis at different stages of secondary embryogenesis. For histological analysis, the PE explants along with developing SEs were fixed in 1:1:18 mixture of formalin–glacial acetic acid–50% ethanol (FAA) for 24 h, dehydrated in an ethanol-TBA (tertiary butyl alcohol) 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.

Rate of proliferation of secondary embryos

To study the rate of proliferation, SE clumps (25 mg fresh weight) of common lineage were cultured on 50 ml agar-gelled SHS15 medium in 250 ml Erlenmeyer flasks. There were 35 flasks at the onset of the experiment. At an interval of 5 d, five flasks were sampled at random, and weight of embryo clumps was recorded from each flask and the mean increase in weight was calculated. The observation was continued up to 35 d.

Influence of the developmental stage of explanted somatic embryos on secondary embryogenesis

Embryo clumps from primary cultures were spread on 5 ml of SHS15 liquid medium in a sterile Petri dish inside the laminar flow hood and different stages of embryos

(globular, heart, torpedo and cotyledonary) were separated out under a dissection microscope. Five embryos from a particular stage were inoculated on 20 ml agar-gelled SHS15 medium in a culture tube. There were 10 replicates per treatment and the experiment was repeated 5 times. After 20 d of culture, the percentage of secondary embryogenesis and mean number of embryos produced per tube were recorded.

Germination, conversion and ex vitro establishment of SE-derived plantlets

Secondary embryos were germinated and converted into plantlets in liquid SHS30 medium, following the method standardized by Nair and Dutta Gupta (2003) for primary somatic embryos of black pepper.

For germination, 25 secondary embryos at the cotyledonary stage were cultured in 250 ml Erlenmeyer flasks containing 30 ml liquid SHS30 medium under darkness. The medium was agitated in a gyratory shaker (B-Braun Certomat-R) at 110 rpm. Germination was recorded after 20 d. There were five flasks per experiment and the experiment was repeated 4 times. An embryo was considered as germinated only when it produced a clear taproot and distinct cotyledonary leaves.

For conversion, germinated somatic embryos were grown under 16 h/d diffuse light (10 μmol m^{-2} s^{-1}) on filter paper bridges dipped in static liquid SHS30 medium in test tubes (one germinated embryo per tube). The medium was replaced every 5 d by decanting consumed medium and adding fresh medium. Conversion was recorded after 20 d. Regenerated plants were acclimatized and transferred to greenhouse as described previously by Nair and Dutta Gupta (2003).

Secondary embryo proliferation and regeneration of plants in single-flask liquid cultures

Embryogenic clumps (25 mg each) from secondary embryogenic cultures were inoculated into five 250 ml flasks containing 25 ml of liquid SHS15 medium and incubated under darkness in gyratory shakers. After 3 days, the cultures from all the flasks were pooled together and sieved through a mesh of 500 μm to discard embryos beyond the torpedo stage. From the filtrate 5 ml was sampled to count the number of embryos at different stages. The remaining quantity was divided into 10 equal parts (12 ml approximately) and inoculated into two sets of 5 flasks containing 20 ml of either SHS30 or SHS15 medium. Cultures were maintained as described previously. Every 5 days, 20 ml of medium was withdrawn and replaced with the same quantity of fresh medium. After 20 d of culture, 5 ml of suspension was drawn from each flask and the number of embryos of each developmental stage was recorded under a stereo-zoom microscope. Then, embryos were allowed to develop to cotyledonary stage, germinate and grow further in liquid SHS30 medium. Medium replenishment was done at 5 d intervals. As the flasks became crowded with growing plantlets, they were equally distributed into more flasks to facilitate normal growth. At this stage the cultures were maintained under 16 h/d diffuse light (10 μmol m^{-2} s^{-1}). The plantlets were maintained as stated, until they grew erect and produced two to three well-developed green leaves.

Statistical analysis

Means and standard errors were calculated for frequency of secondary embryo induction as well as number of secondary embryos. The data collected were subjected to analysis of variance in the case of secondary embryo induction, and the effect of the developmental stage of somatic embryos on secondary embryogenesis, using MSTATC software package. Percentage data were subjected to angular transformation before analysis. Means were separated using Duncan's Multiple Range Test at $P=0.05$.

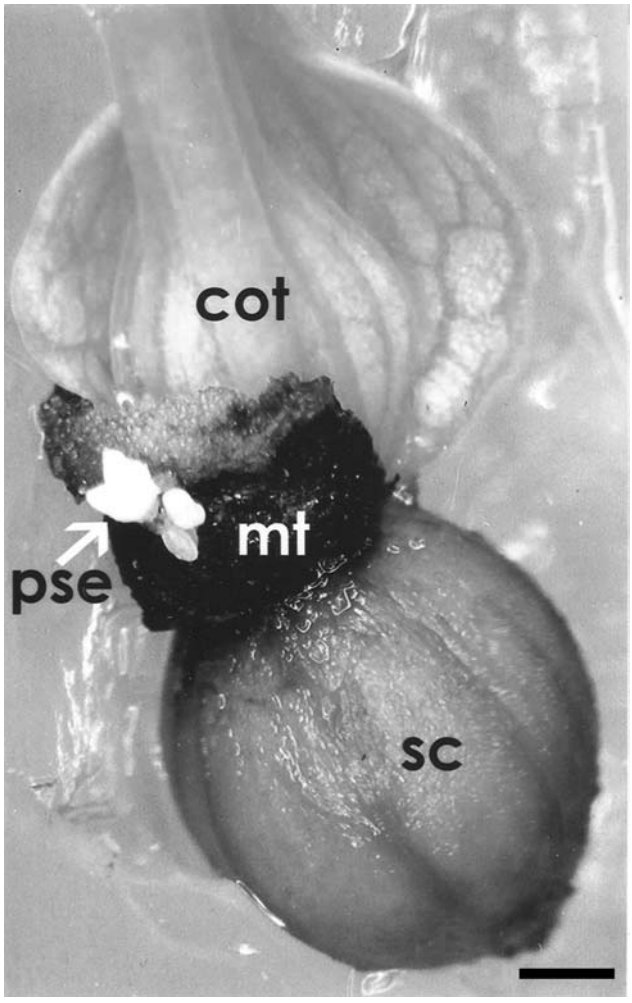

Fig. 1 Primary somatic embryogenesis from micropylar tissue of a germinating seed of black pepper after 90 d of culture on growth regulator-free SH basal medium at 24 h dark period (*Bar*=1 mm). **cot-** Cotyledon, **mt-** Micropylar tissue, **pse** (→)**-** Primary somatic embryos, **sc-** Seed coat

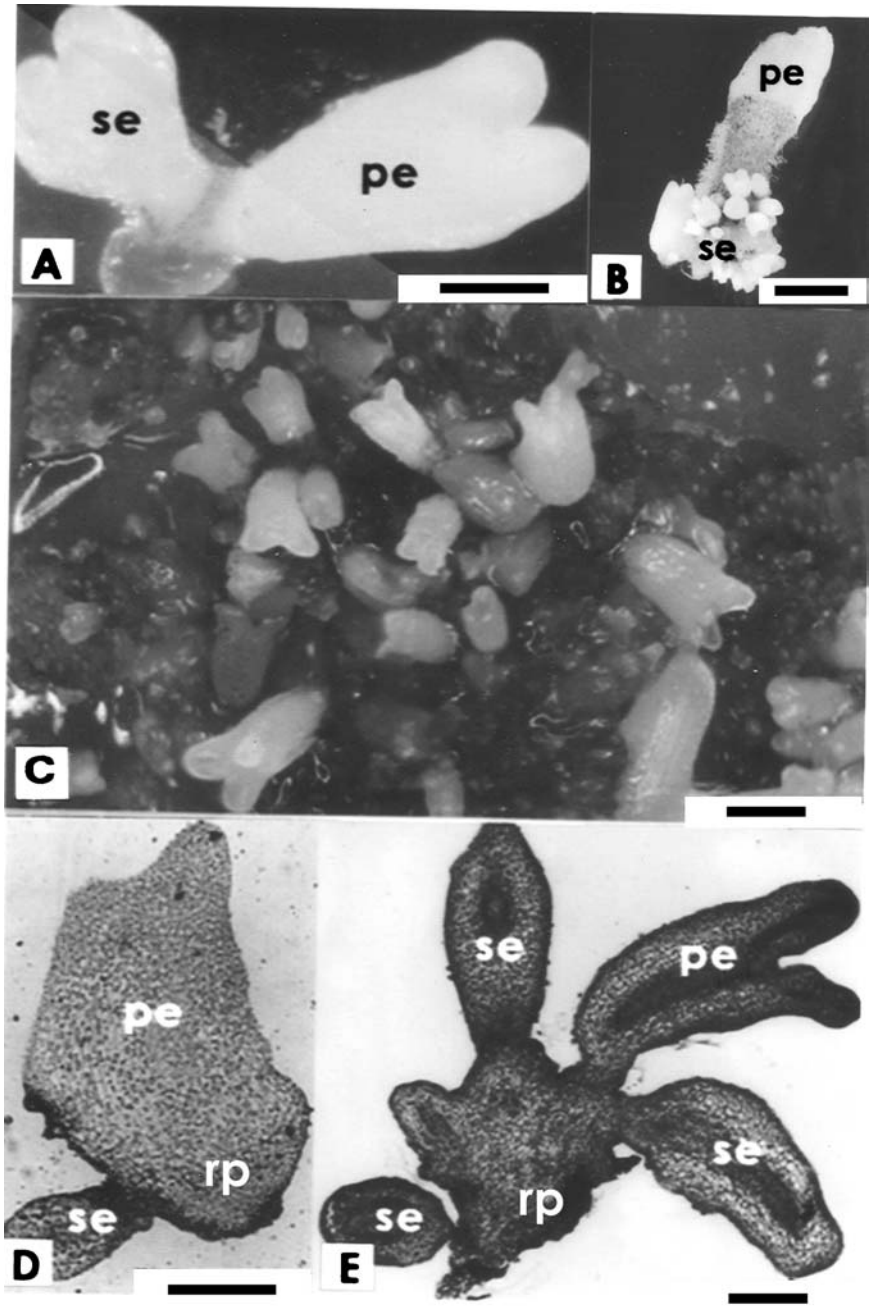

Fig. 2 Secondary and cyclic somatic embryogenesis in black pepper and histology of secondary embryogenesis. **A** Emergence of a secondary embryo from the root pole of a primary somatic embryo cultured on growth regulator-free SH medium (*Bar*=0.5 mm). **B** Several somatic embryos formed after 10 d of culture (*Bar*=0.5 mm). **C** A cluster of secondary somatic embryos resulted from cyclic secondary embryogenesis after 20 d of culture (*Bar*=0.5 mm). **D** Histological section (LS) of a primary somatic embryo showing emergence of a secondary embryo from the embryogenic tissue at the root pole (*Bar*=0.25 mm). **E** Histological section (LS) of a late torpedo stage primary embryo with secondary embryos at different stages, originated from the tissue at root pole (*Bar*=0.25 mm). **pe-** Primary somatic embryo, **se-** Secondary somatic embryo, **rp-** Root pole

Results

Primary somatic embryogenesis

More than 20% of germinating seeds cultured on PGR-free SHS30 medium in darkness produced PEs directly from the micropylar tissue (Fig. 1) by 90 d of culture.

Induction of secondary somatic embryogenesis

Secondary embryos were visible from the root pole of the PEs within 10 days of culture (Fig. 2A). The brownish-yellow tissue at the root pole of the PE proliferated into a small mass of tissue from which several SEs emerged (Fig. 2B). The process continued in a recurrent manner and by the end of 20 d of culture masses of SEs at different developmental stages were observed (Fig. 2C).

Among the media strengths and sucrose concentrations tested, full-strength SH medium supplemented with 1.5% sucrose produced significantly higher fresh weights (296.54 mg) and numbers of SEs (726) than any other treatment (Table 1). When sucrose concentration was 3.0 or 4.5% proliferation of SEs was associated with further development of embryos. At 1.5% sucrose embryogenic cultures predominantly consisted of globular embryos. Higher

Table 1 Effect of medium strength and sucrose concentration on secondary embryogenesis from primary somatic embryos of black pepper cv. Karimunda after 20 days of culture

Strength of SH medium	Sucrose conc. (%)	Percent secondary embryogenesis (Mean±SE)	Fresh weight of Sec. Emb. Clumps (mg) (Mean±SE)	Number of embryos at different developmental stages per 50 mg of secondary embryo clumps (Mean±SE)				Total number of embryos (Mean±SE)
				Globular	Heart	Torpedo	Cotyledonary	
Half	1.5	96.0±2.45 AB*	97.46±0.78 C*	54.98±0.67	41.37±0.42	31.90±0.24	6.95±0.27	676±14.25 B*
	3.0	90.0±3.16 B	59.54±0.64 D	25.56±0.56	21.16±0.34	20.73±0.27	23.12±0.89	452±13.70 D
	4.5	66.0±4.00 C	44.36±1.86 F	15.89±0.24	15.49±0.35	28.74±0.56	32.69±0.80	464±13.61 CD
Full	1.5	100.0±0.00 A	296.54±2.14 A	61.76±0.13	51.52±0.66	24.72±0.50	7.42±0.16	726±6.60 A
	3.0	96.0±2.45 AB	125.00±1.32 B	23.32±0.46	19.86±0.42	23.64±0.24	33.16±0.38	499±6.94 C
	4.5	70.0±3.16 C	50.02±0.68 E	15.37±0.34	15.58±0.27	22.71±0.55	35.88±0.38	448±9.88 D

The primary embryos were cultured on growth regulator-free, half- and full-strength SH medium under 24 h dark period. SE- standard error

* Means followed by the same letters are not significantly different at P=0.05 of Duncan's Multiple Range Test

numbers of cotyledonary embryos were observed at 3 and 4.5% sucrose. At a sucrose concentration of 4.5% some of the embryos germinated precociously. Analysis of variance indicated that medium strength and sucrose concentration significantly influenced the percentage of secondary embryogenesis and fresh weight of somatic embryo clumps (Table 2). Lengths of SEs ranged from 0.45 mm at the globular stage to 1.91 mm at the cotyledonary stage.

Histological analysis revealed that SEs originated from the embryogenic tissue proliferated at the root pole of explanted PE (Fig. 2D). The embryos budded off from the peripheral region of this tissue and SEs at different stages could be observed in the histological sections (Fig. 2E). The SEs were similar to the PEs, showing clear bipolarity and independent vascular systems. Origin of SEs from the root poles of the explanted PEs was clearly distinguishable in late-globular to cotyledonary stage explants and it was difficult in the case of early-globular embryos, as polarity was not distinct.

Proliferation of SEs

The fresh weight of secondary embryogenic clumps increased steadily between 5–25 d of culture. After 25 d the increase was slow. The highest percentage of increase was recorded between 5–10 d of culture and lowest between 30–35 d (Fig. 3).

Influence of the developmental stage of explanted somatic embryos on secondary embryogenesis

Developmental stage of primary embryos at the time of inoculation had a significant effect on secondary embryogenesis. The early developmental stages of embryos such as globular and heart stage showed significantly higher potential for secondary embryogenesis compared to torpedo and cotyledonary embryos (Fig. 4). After 20 days of culture globular PEs showed the highest percentage of secondary embryogenesis (98.4) and number of SEs (47.6) followed by heart (97.4; 46.6) and torpedo (84.4; 29.2). Cotyledonary embryos were poor in their ability to induce secondary embryogenesis and showed very low percent response (22.2) as well as number of SEs (18.4). The process of secondary somatic embryogenesis continued in a cyclic manner and resulted in clumps of SEs within 15–20 d.

Germination and conversion of SEs

Secondary somatic embryos showed a high percentage of germination (96.9) in liquid SHS30 medium under 24 h darkness and germinated SEs converted at a rate of 86.3%. Regenerated plantlets were hardened and established in soil with 75% efficiency. The plants were found to be morphologically uniform and grew vigorously under ex vitro conditions (Fig. 5 A and B).

Table 2 Analysis of variance for data in Table 1

Source of variation	*df*	Mean Square		
		Percent secondary embryogenesis	Fresh weight of secondary embryo clumps	Total number of embryos
Replication	4	21.67 NS[a]	16.96*	139.17 NS[a]
Strength of medium (A)	1	292.66*	60840.03*	5467.50*
Sucrose conc. (B)	2	2525.75*	59072.69*	185425.83*
AxB	2	28.55 NS[a]	24517.35*	3472.50*
Error	20	66.63	7.845	737.57

[a]Non Significant
*Significant at $P \leq 0.05$

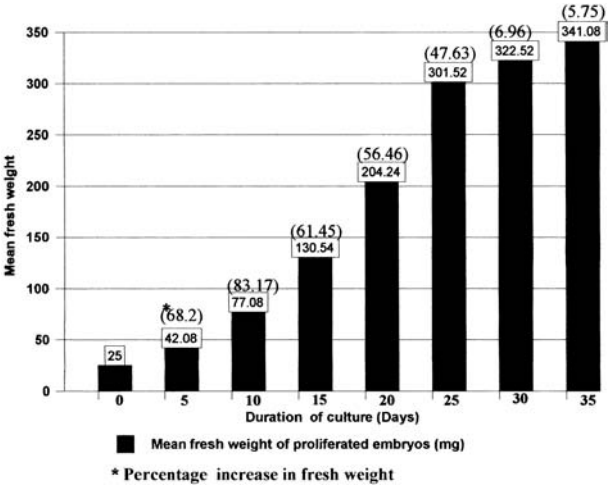

Fig. 3 Proliferation of secondary embryogenic clumps as expressed in fresh weight gain on hormone-free SH medium containing 1.5% sucrose under 24 h darkness

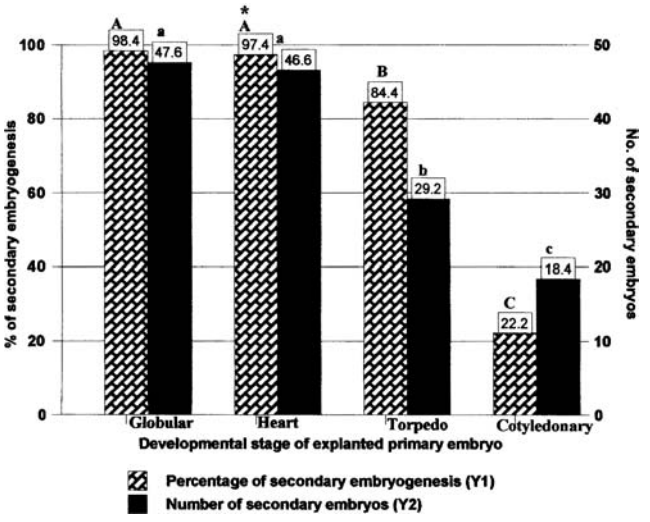

Fig. 4 Influence of the developmental stage of primary embryos of cv. Karimunda in the formation of secondary embryos after 20 days of culture on hormone-free SH medium containing 1.5% sucrose. * Means followed by the same letter are not significantly different at $P=0.05$ of Duncan's multiple range test

Secondary embryo proliferation and regeneration of plants in single-flask liquid cultures

The initial inoculum consisted mostly of globular, heart and torpedo stage embryos. A very few cotyledonary embryos (0.6 ml^{-1}) observed in the initial inoculum may have been escapes during filtration. A comparison of the number of embryos at different stages in the initial inoculum with those present after 20 d culture in SHS30 and SHS15 medium clearly showed that the SH medium with 3% sucrose favored embryo development whereas medium with 1.5% sucrose favored proliferation of SEs (Fig. 6). Cotyledonary embryos were predominant in cultures from SHS30 medium and number estimated was 36.4 ml^{-1}. Globular (49.8 ml^{-1}) and other early stages of embryos were common in cultures raised on SHS15 medium. However, replacement of SHS15 medium with SHS30 favored development of embryos into cotyledonary stage. Repeated medium replenishment and subculturing to new flasks resulted in more than 2,000 well-developed plantlets from the original inoculum within a period of 60 days (Fig. 7A–D).

Discussion

In the present study, we have described an efficient protocol for high-frequency regeneration of black pepper through cyclic secondary embryogenesis. Scaling up of the process of somatic embryogenesis and its utilization for mass propagation, synthetic seed production or genetic transformation has secondary embryogenesis as the basis. Secondary embryogenesis, also termed as recurrent, repetitive, accessory or proliferative embryogenesis, usually occurs when PEs fail to mature normally into plantlets and instead give rise to successive cycles of embryos, most commonly from superficial cells of the cotyledons or hypocotyl (Merkle et al. 1990). In black pepper, SEs originated directly from the PEs in PGR-free SH medium and proliferated further in a cyclic manner. Occurrence of direct secondary somatic embryogenesis in PGR-free media is well established in several crop plants (Smith and Krikorian 1988, Merkle et al. 1990; Parra and Amo-Marco 1998; das Neves et al. 1999; Vasic et al. 2001).

Proliferation and development of SEs were strongly influenced by the strength of medium and sucrose concentration. Optimum secondary embryogenesis was observed in full-strength SH medium containing 1.5% sucrose. Joseph et al. (1996) observed callus-mediated secondary somatic embryogenesis from PEs of black pepper in full-strength SH medium devoid of growth regulators. In contrast, half-strength SH medium favored primary embryo formation

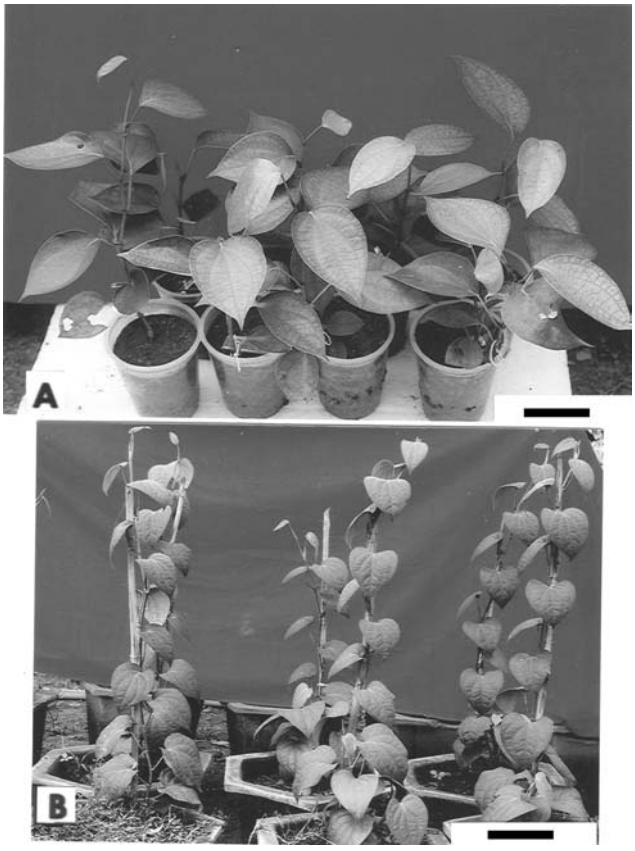

Fig. 5 Ex vitro establishment of plants regenerated from secondary somatic embryos of black pepper. **A** Plants established in plastic cups filled with sterile sand (*Bar*=6.0 cm). **B** Regenerated plants growing in pots filled with garden soil (*Bar*=10.0 cm)

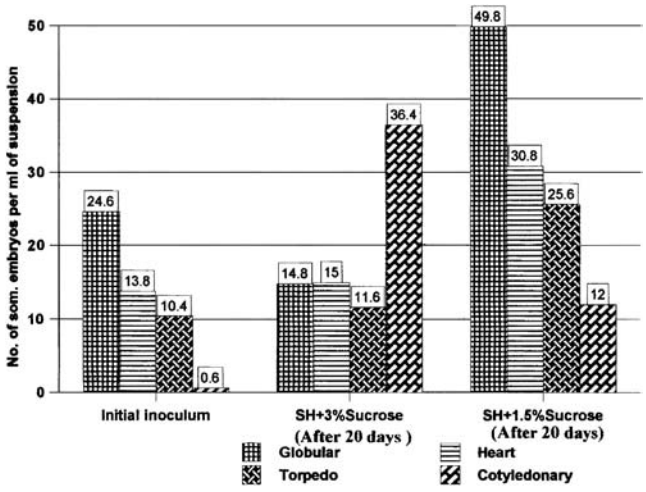

Fig. 6 Effect of SH liquid medium containing 3% and 1.5% sucrose on proliferation and differentiation of somatic embryos, after 20 days of culture

from callus without any SE formation. However, we observed induction as well as development of SEs without any active callus phase in half and full-strength SH medium.

Sucrose concentration in the medium influenced the proliferation of SEs as well as embryo differentiation into various developmental stages. Sucrose at 1.5% stimulated the proliferation but restricted the development of globular embryos to further advanced stages. At sucrose concentrations of 3.0 or 4.5% proliferation of SEs was associated with development of somatic embryos. This was evident from the number of embryos of each developmental stage in medium containing respective sugar concentrations. Sucrose at 4.5% did not impart any desirable advantage over 3.0%. Joseph et al. (1996) observed that, in callus-mediated somatic embryogenesis of black pepper, the rate of embryo differentiation was enhanced in half-strength basal medium with the sucrose level from 3.0 to 1.5%. The influence of sucrose concentration on somatic embryogenesis has also been reported in other species (Chee and Tricoli 1988; Smith and Krikorian 1988; Eapen and George 1993; Weissinger II and Parrott 1993; Choi et al. 2002a).

The average length of SEs (0.45 mm at globular to 1.91 mm at cotyledonary stage) was slightly less than that of PEs (0.50 mm at globular to 2.0 mm at cotyledonary) reported earlier (Nair and Dutta Gupta 2003). This may be attributed to the higher frequency of SEs per culture than PEs, making the nutrient availability per SE less. Also, PEs had the support of original seed explant, which stores reserve food materials.

Secondary somatic embryogenesis has been reported from several plants such as *Glycine max* (Finer 1988), *Juglans regia* (Tuleke et al. 1988), *Manihot esculenta* (Raemakers et al. 1993), *Trifolium repens* (Weissinger II and Parrott 1993), *Dalbergia sisso* (Das et al. 1997), *Medicago* sp. (das Neves et al. 1999), *Arachis hypogaea* (Little et al. 2000), *Phalaenopsis amabilis* (Chen and Chang 2004) and *Coffea* sp. (Giridhar et al. 2004). In most of the above examples, SEs originated from the regions such as cotyledons and hypocotyls of PE, which affected the normal development of PE. In the present study, the SEs appears to originate from the multiseriate suspensor-like region of the PEs at the root pole, which was originally derived from the micropylar ring tissue of the seed explants. The presence of a prominent suspensor-like structure was already recorded in early stages (globular and early heart) of PEs of black pepper (Nair and Dutta Gupta 2003). Detachment of PEs from the original explant might have stimulated the proliferation of small quantities of the embryogenic tissue attached to its root pole to give a mass of embryogenic tissue or a proembryogenic mass, which ultimately gave rise to further embryos in a cyclic manner. Histological evidence also indicates the origin of SEs from the root pole of PEs. Earlier observations showed that secondary embryogenesis occurred more frequently from PEs which were detached from the original explant than the ones remaining attached to it (Nair and Dutta Gupta 2003).

Proliferation of SE clumps was rapid and within 20 d of culture the weight of original inoculum increased from 25 to 204.4 mg. After 20 d the proliferation decreased, suggesting this period as the ideal subculture interval for long-term maintenance of embryogenic cultures.

Among the various developmental stages of PEs, globular and heart shaped embryos showed the highest response for secondary embryogenesis. The responses of the

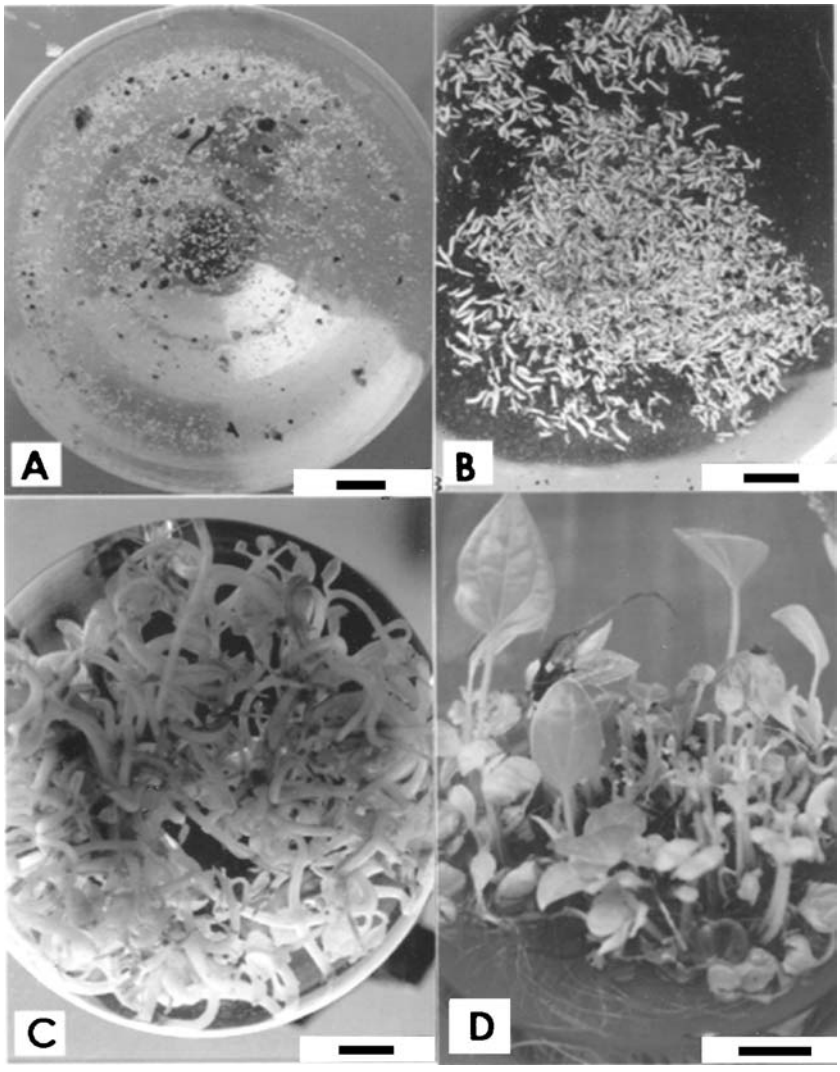

Fig. 7 Single-flask proliferation and regeneration of plantlets from secondary somatic embryos of black pepper in liquid suspension cultures. **A** Proliferating secondary embryogenic clumps dispersed in the agitating liquid SH medium containing 1.5% sucrose. **B** Cotyledonary embryos formed after 15 d of culture in medium containing 3.0% sucrose. **C** Germinated embryos after 30 d in the medium of same composition. **D** Well developed plantlets ready for ex vitro establishment. (*Bar*=10.0 mm in A–D)

torpedo-shaped and cotyledonary embryos were comparatively poor. A similar report in *Medicago trancatula* revealed that secondary embryogenesis from globular embryos was higher than that of torpedo-shaped embryos (das Neves et al. 1999).The reduction in secondary embryogenic potential in later stages of development may be due to the fact that the developing root tissues gradually replaces the embryogenic cells. It appears that in black pepper the potential for secondary embryogenesis was negatively related with the developmental stage of PEs.

A high percentage of germination and conversion of SEs and ex vitro establishment of regenerated plantlets were obtained in the present study, indicating the efficiency of the process. This makes the system useful for its further utilization in large-scale multiplication as well as genetic manipulation studies in black pepper.

A single-flask protocol for proliferation, maturation, germination and conversion of black pepper SEs was established using liquid cultures. The regulatory role of sucrose in proliferation and development of SEs was evident in this case also. The SE clumps were proliferated in PGR-free liquid SH medium containing 1.5% sucrose and after attaining sufficient proliferation, embryos were developed, germinated and converted in medium containing 3.0% sucrose. Very-high frequencies of proliferation and plantlet regeneration were attained in a single flask by simple medium replenishment. Subculture was only necessary when flasks became crowded with regenerated plants. Such a system of cyclic secondary embryogenesis and plantlet regeneration is highly desirable for scaling up in bioreactors. Large-scale plant regeneration in bioreactors utilizing somatic embryogenesis has been reported in crop plants of commercial importance such as coffee (Etienne-Barry et al. 1999; Etienne and Berthouly 2002) and Siberian ginseng (Choi and Jeong 2002; Choi et al. 2002b).

Regeneration of plants using cyclic secondary embryogenesis is also ideal for gene transfer using microprojectile-bombardment (Merkle et al. 1990; Ellis 1995; Liu et al. 1996) and through *Agrobacterium*-mediated DNA delivery (Merkle et al. 1990; Ellis 1995), as large numbers of transformed somatic embryos can be obtained by embryo cycling.

In conclusion, the present study successfully describes the establishment of cyclic secondary embryogenesis of

a cultivar of black pepper and demonstrates the potential of the system for scale-up indicating direct origin of SEs apparently from the maternal tissue. However, genetic fidelity of the regenerated plants and the origin of the SEs from the maternal tissue are to be confirmed through RAPD or AFLP.

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