Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2016; 4(4): 109-115

Received: 25-05-2016
Accepted: 27-06-2016

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Optimization of cyclic somatic embryogenesis and assessing genetic fidelity in six varieties of black pepper (*Piper nigrum* L)

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Abstract
Embryos along with micropylar tissue dissected out from matured seeds of six varieties of black pepper were used for somatic embryogenesis. Primary somatic embryos were induced from the micropylar region after 40 to 85 days in different varieties. Sucrose concentration was found to be crucial for the production and proliferation of secondary somatic embryos (SE) and cyclic SE. SE was visible from root pole region of primary embryos within 65 to 100 days in different varieties on Schenk and Hildebrandt (SH) medium. SE gave rise to cyclic SE in the same medium within 10-20 days in different varieties. Colour and size of cyclic SE varied among varieties. Regeneration of cyclic SE into plantlets was successful in SH liquid medium and transferred into woody plant medium. The rooted plants were hardened in the green house, the number of plantlets obtained varied among varieties. Genetic fidelity testing of somatic embryo-derived plants of all varieties with corresponding mother plants using six simple sequence repeats markers, showed genetic uniformity.

Keywords: Somatic embryogenesis, cyclic somatic embryo, regeneration, somatic embryo-derived plantlets, black pepper, SSR

Abbreviations: PCR-Polymerase chain reaction, PE-Primary somatic embryo, PEI-Primary somatic embryo induction, SE-Secondary somatic embryo, SEI-Secondary somatic embryo induction, SH-Schenk and Hildebrandt, SH07, SH10, SH15, SH30, SH35, SH40 and SH45-SH medium containing 0.75%, 1.0%, 1.5%, 3.0%, 3.5%, 4.0% and 4.5% sucrose respectively, SSR-Simple sequence repeats, WPM-Woody plant medium

Introduction
Black pepper is the most important and the most widely used spice in the world. The black pepper of commerce comprises the dried fruits of the tropical, perennial climbing plant *Piper nigrum* L., which belong to the family Piperaceae. It has several medicinal properties such as defensive role against infection by microbes, insects and animals (Scott *et al*., 2008; Ahmad *et al*., 2011) [19, 2], treating digestive disorders, gastric problems, diarrhea, ingestion and respiratory problems (Parganiha *et al*., 2011) [15]. Due to their poor viability and high sterility in post-fertilization stages, seed propagation in this plant is cumbersome and yields only a few heterogeneous progenies. Black pepper, therefore, is conventionally propagated vegetatively through stem cuttings (Kanta, 1962) [10]. *In vitro* propagation methods provide a strapping tool for the mass multiplication and germplasm conservation of this economically important species. Protocols for regeneration through shoot tip (Philip *et al*., 1992; Bhat *et al*., 1995; Ahmad *et al*., 2010) [16, 4, 1] as well as through somatic embryogenesis (Joseph *et al*., 1996; Nair and Gupta, 2006) [9, 14] have been reported. Somatic embryogenesis is the most appealing method for mass cloning of plants since a very large number of somatic embryos can be produced using a limited volume of medium in a short period (Choi *et al*., 2002; Nair and Gupta, 2006) [5, 14].

The occurrence of cryptic genetic defects arising *via* somatic variation in the regenerate can limit the utility of the micropropagation system. To confirm the quality of the plants, it is important to establish genetic fidelity of micropropagated plants. Morphological descriptions, physiological observations, cytological studies and isoenzyme analysis are previously used methods for assessing the genetic purity and somaclonal variation in tissue culture raised plants. These are found to be time-consuming and do not yield authentic results. Polymerase chain reaction (PCR) in conjunction with Random Amplified Polymorphic DNA (RAPD) and

Simple Sequence Repeat (SSR) primers have become a suitable technique to determine the genetic stability of micropropagated plants of different crops (Devarumath *et al*., 2007) [6].

So far somatic embryogenesis has been reported only in cv. Karimunda of black pepper. The present study describes the production of the somatic embryo and their regeneration in six varieties of black pepper and its genetic fidelity testing using SSR markers.

2. Materials and Methods

Plant materials

Mature seeds of black pepper collected from six varieties (IISR Malabar Excel, IISR Shakthi, IISR Thevam, Panniyur-1, Sreekara and Subhakara) collected from plants grown at the ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India were used as source materials for establishing primary somatic embryogenic cultures.

Induction of primary somatic embryos (PE)

Somatic embryogenesis was carried out by slightly modifying the protocol described by Nair and Gupta (2006) [14] as described in Jiby and Bhat (2011) [8]. Seeds were soaked in tap water for overnight and on the next day outer mesocarp of the seed were removed by slight rubbing before surface sterilization. Seeds were surface sterilized with 0.1% mercuric chloride solution for 5 min, followed by repeated washing (3-4 times) with sterile double distilled water. The surface sterilized seeds were allowed to dry on sterile filter paper in laminar air flow for 30 min. Then the embryo along with micropylar tissue was dissected out aseptically with a sterile scalpel. The dissected tissues were cultured on agar gelled full-strength, hormone-free SH (Schenk and Hildebrandt, 1972) [18] medium containing 3% (w/v) sucrose (SH30) under darkness for 90-120 days for inducing primary somatic embryos (PEs). The pH of the medium was adjusted to 5.8 before autoclaving.

Induction of secondary somatic embryo (SE) and cyclic somatic embryo (cyclic SE)

The tissues showing PE on the micropylar region was inoculated on full strength, hormone-free SH medium containing different sucrose concentration (SH07, SH10, SH15) and gelled with 0.8% agar (Plant tissue culture grade, Hi-media) and incubated in complete darkness for production of secondary somatic embryo (SE) and cyclic SE. Cyclic SEs was maintained by regular subculturing at 30 days interval in the same medium. The number of cultures inoculated, PEs and SEs obtained were recorded (Table 1). Different developmental stages of somatic embryogenesis were recorded under a stereomicroscope (Leica Microsystems Ltd, Switzerland) (Fig. 1).

Table 1: Somatic embryogenesis in different varieties of black pepper

Variety	No. of seed derived explant inoculated	No. of germinated embryos	No. of explants showed P.E	Days taken for P. E. I	Percent P. E showing S. E	Days taken for S. E. I from P. E	Plantlets obtained per 100 mg of cyclic SE	Plantlets survived after hardening	Hardening efficiency
IISR Malabar Excel	1302	395	274	45±5	4.74	90±10	53±7	46±5	88
IISR Shakthi	313	75	33	45±5	12.12	90±10	30±5	25±5	83
IISR Thevam	132	45	35	45±5	5.71	90±10	32±5	26±4	87
Panniyur I	1178	303	189	80±5	13.22	90±10	12±4	10±2	83
Sreekara	397	144	124	65±5	4.03	70±5	39±5	32±4	82
Subhakara	253	42	18	65±5	26.31	70±5	28±5	22±4	78

PE-Primary somatic embryo, P. E. I-Primary somatic embryo induction, SE-Secondary somatic embryo, S.E. I-Secondary somatic embryo induction.

Conversion of cyclic somatic embryos (cyclic SE) into plantlets

Conversion of cyclic SE into plantlets was done as per the protocol of Nair and Gupta (2006) [14] after determining the optimum sucrose concentration for each variety. The cyclic SE of different varieties were inoculated into SH (liquid) containing different sucrose concentration such as 3.0% (SH30), 3.5% (SH35), 4.0% (SH40) and 4.5% (SH45) to determine the optimum sucrose concentration for regeneration. The regeneration protocol included inoculating about 100 mg of cyclic SE to hormone-free SH (liquid) medium with 3.5% sucrose for development of embryos into plantlets under dark for 20 days followed by another 10 days maintained under 16 h/day diffuse light with shaking in an orbital incubator shaker (Lab Companion, Korea) at 110 rpm with replenishment of medium at every 10 days. The well-differentiated plantlets were allowed to grow under 12 h light in the same medium until they produced two to three leaves. These well-developed plants were transferred to woody plant medium (WPM) with 3.5% sucrose, 0.8% agar and 0.2% charcoal for another 30 days maintained under 16 h/day diffuse light. The well-established rooted plantlets were hardened in the green house. The experiment was repeated three times and the number of plantlets obtained per 100 mg of cyclic SE in liquid SH, the plantlets survived in WPM and the plantlets that survived after hardening were recorded (Table 1).

Genetic fidelity testing of regenerated plants

Genetic fidelity testing of somatic embryo-derived plants with corresponding mother plants was done using SSR markers developed for black pepper by Menezes *et al*. (2009) [12]. Ten hardened plants of each variety were tested along with corresponding mother plants. Another related species of black pepper namely *Piper colubrinum* was used to compare the profile. Total DNA isolated from each of the plants using the procedure of Hareesh and Bhat (2008) [7] was subjected to PCR to amplify SSR regions using six pairs of SSR primers. The PCR reaction contained 1x PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 ng each of forward and reverse primers, 1.5 units of *Taq* polymerase, 1 µl of template DNA and sterile water to a final volume of 25 µl. The thermal cycler was programmed for initial denaturation at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 52-57°C for 1 min, synthesis at 72 °C for 1 min and a final extension for 10 min at 72 °C. The PCR products were analyzed on 1% agarose gel, and photographs were taken by using the Gel Documenting system (Alpha-Innotech Corporation, California, USA).

3. Results

Establishment of primary somatic embryos

About 28% of seed derived explant inoculated responded by germination of the zygotic embryo, rest was lost by either contamination or did not germinate. PEs was seen after 40 to 85 days in different varieties (Table 1; Fig. 1). Earliest PE production was seen in 45±5 days in the varieties IIISR Malabar Excel, IISR Shakthi and IISR Thevam while the var. Panniyur-1 showed PE after 80±5 days.

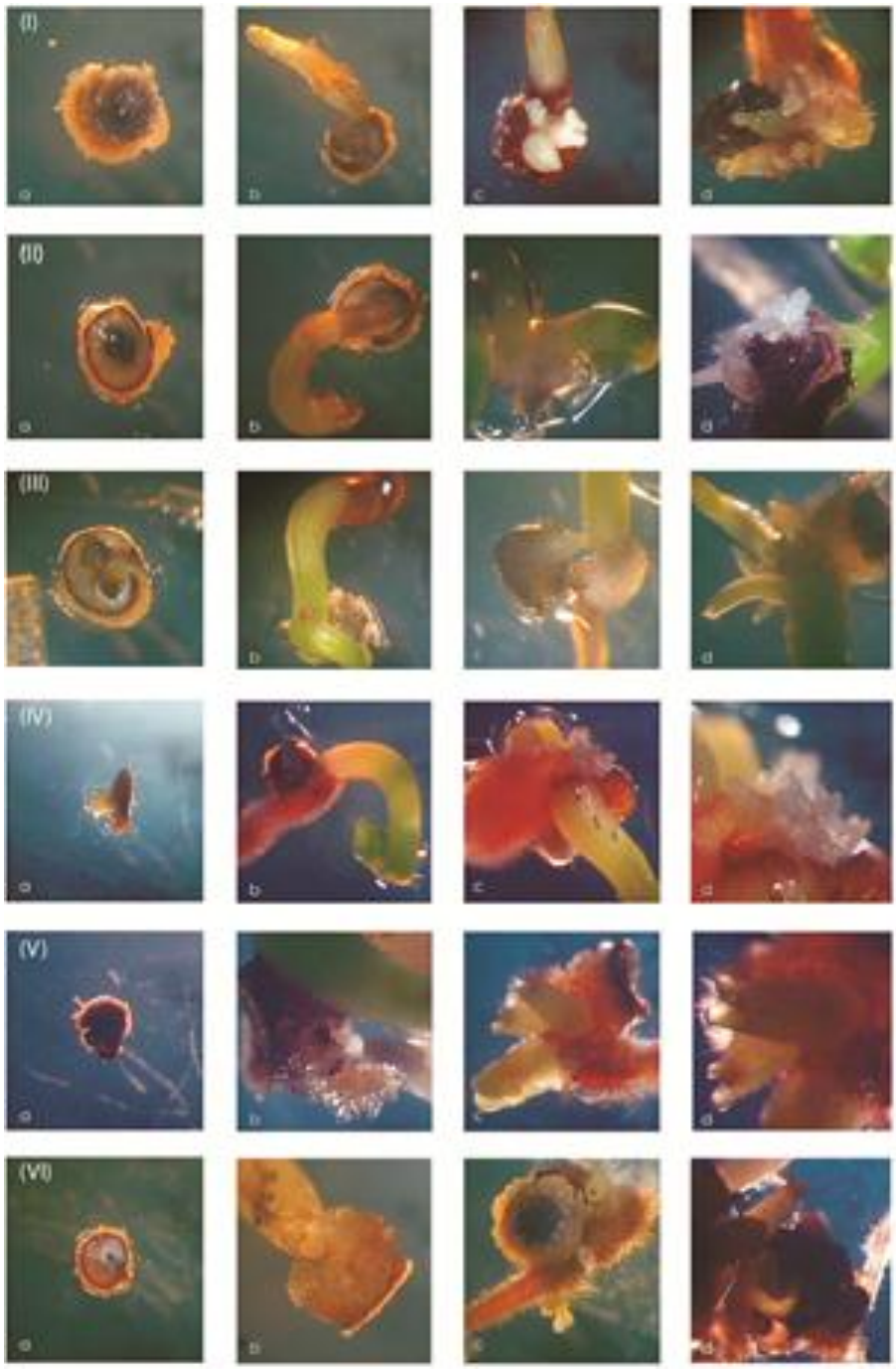

Fig 1: Somatic embryogenesis in different varieties of black pepper as seen under stereomicroscope. (i) IISR Malabar Excel (ii) IISR Shakthi (iii) Panniyur-1 (iv) Subhakara (v) Sreekara (vi) IISR Thevam. (a) Scooped out micropylar region with embryo (b-c) Germinating embryo with micropylar region showing primary somatic embryogenesis (d) Primary somatic embryos (PE) giving rise to secondary somatic embryo (SE) and cyclic SE.

Induction of secondary somatic embryo (SE) and cyclic somatic embryo (cyclic SE)

Initially when PEs were inoculated in SH15, in some varieties development of plantlets rather than SE were observed. Hence in order to determine the optimum sucrose concentration for SE production, PEs of all varieties was inoculated in SH07, SH10 and SH15. The results showed that SH07 was optimum for the var. Panniyur-1, SH10 for the varieties IISR Thevam, IISR Shakthi, Sreekara and Subhakara, and SH15 for the var. IISR Malabar Excel (Fig. 2). SE was emerged from the brownish yellow tissue at the root pole of the PE within 65 to 100 days in different black pepper varieties (Table 1; Fig. 1). SE induction was maximum in the var. Subhakara (26.31%), followed by Panniyur-1 (13.22%) and IISR Shakthi (12.12%) while it was minimum (4.03%) in the var. Sreekara. SEs of all varieties when inoculated on the corresponding medium (SH07/SH10/SH15) gave rise to cyclic SE within 10-20 days. Cyclic SE are maintained by subculturing on the respective

medium at every 30 days. Cyclic SE of Panniyur-1 was greenish white while it was pale white in Sreekara, yellowish white in IISR Malabar Excel, and yellowish brown in IISR Thevam, IISR Shakthi and Subhakara (Fig. 2). Among varieties, Panniyur-1 produced the largest sized cyclic SE while the var. IISR Shakthi produced the smallest. The rate of multiplication of cyclic SE also varied from variety to variety. Varieties such as IISR Malabar Excel, IISR Shakthi, Sreekara and Subhakara showed a higher rate of multiplication compared to the var. Panniyur-1.

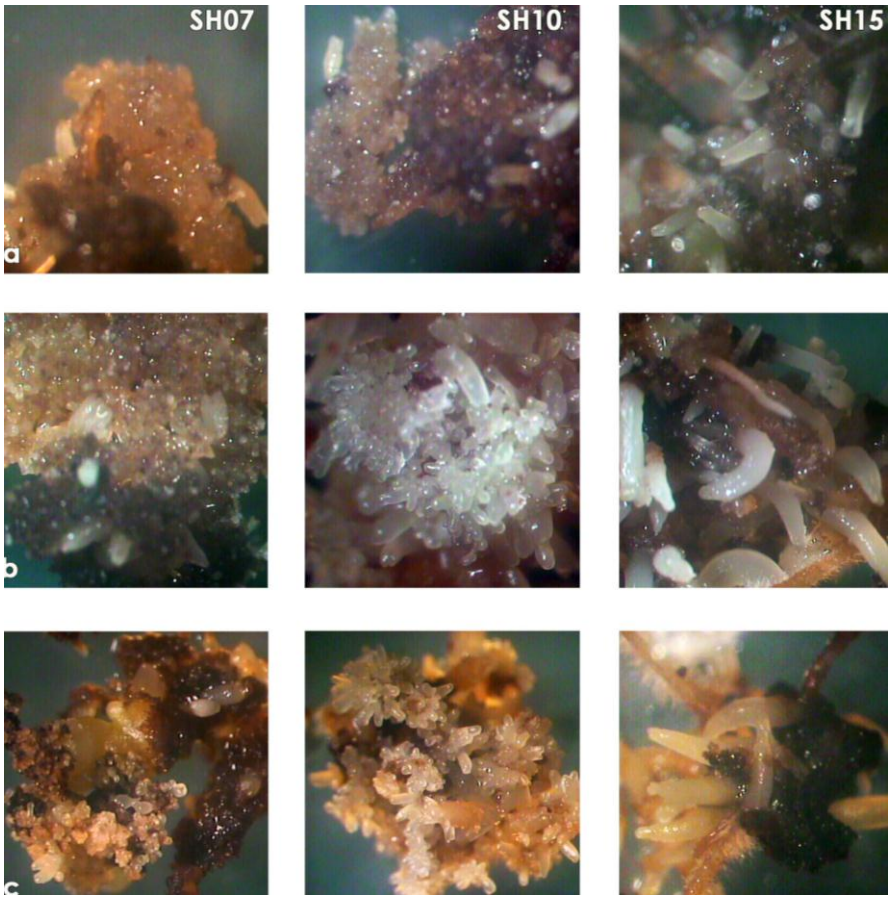

Fig 2: Determination of the optimum sucrose concentration for the production of secondary somatic embryo (SE) and cyclic SE in different varieties (a) IISR Thevam (b) Sreekara (c) Subhakara of black pepper. PEs was inoculated on to SH medium containing different concentration of sucrose such as 0.75% (SH07), 1.0% (SH10) and 1.5% (SH15).

Conversion of cyclic somatic embryo (Cyclic SE) into plantlets

As sucrose percent was increased from 3.0 to 4.5%, the rate of conversion of the cyclic SE into plantlet was higher and faster in all varieties (Fig. 3). However, when it was beyond 4.0%, it caused the reduction in number of regenerated plants. Optimum sucrose concentration for conversion of cyclic SE to plantlets was found to be 3.5% for all varieties. The cyclic SE was transferred to SH (liquid) medium with 3.5% (SH35) sucrose for further development of embryos into fully developed plantlets (Fig. 4). The number of plantlets obtained from 100 mg of cyclic SE varied from variety to variety (Table 1). The maximum number of plants (53±7) was seen in the var. IISR Malabar Excel while minimum (12±4) was observed in the var. Panniyur-1. All the rooted plantlets were successfully transferred into the soil where they developed into normal plants in the greenhouse with an average of 83% survival. Plantlets showed a remarkable growth performance after two months of acclimatization. It was observed that there was no morphological differences with respect to growth and development except for the var. IISR Thevam which showed slow growth at the initial stage after hardening than other varieties.

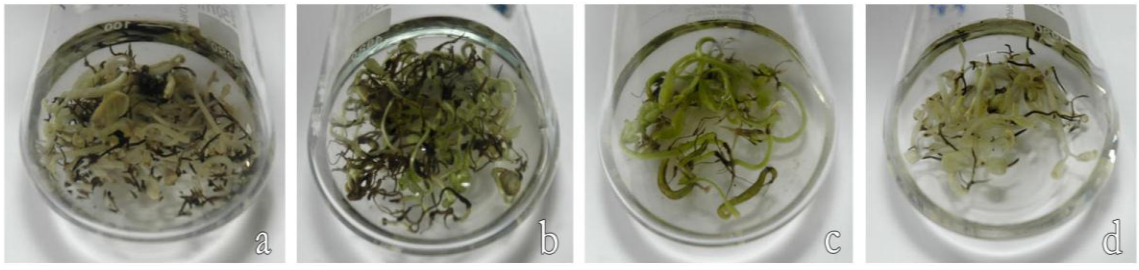

Fig 3: Determination of optimum concentration of sucrose for conversion of somatic embryos into plantlets in SH medium in the black pepper var. IISR Malabar Excel. (a) 3.0% sucrose (b) 3.5% sucrose (c) 4.0% sucrose (d) 4.5% sucrose

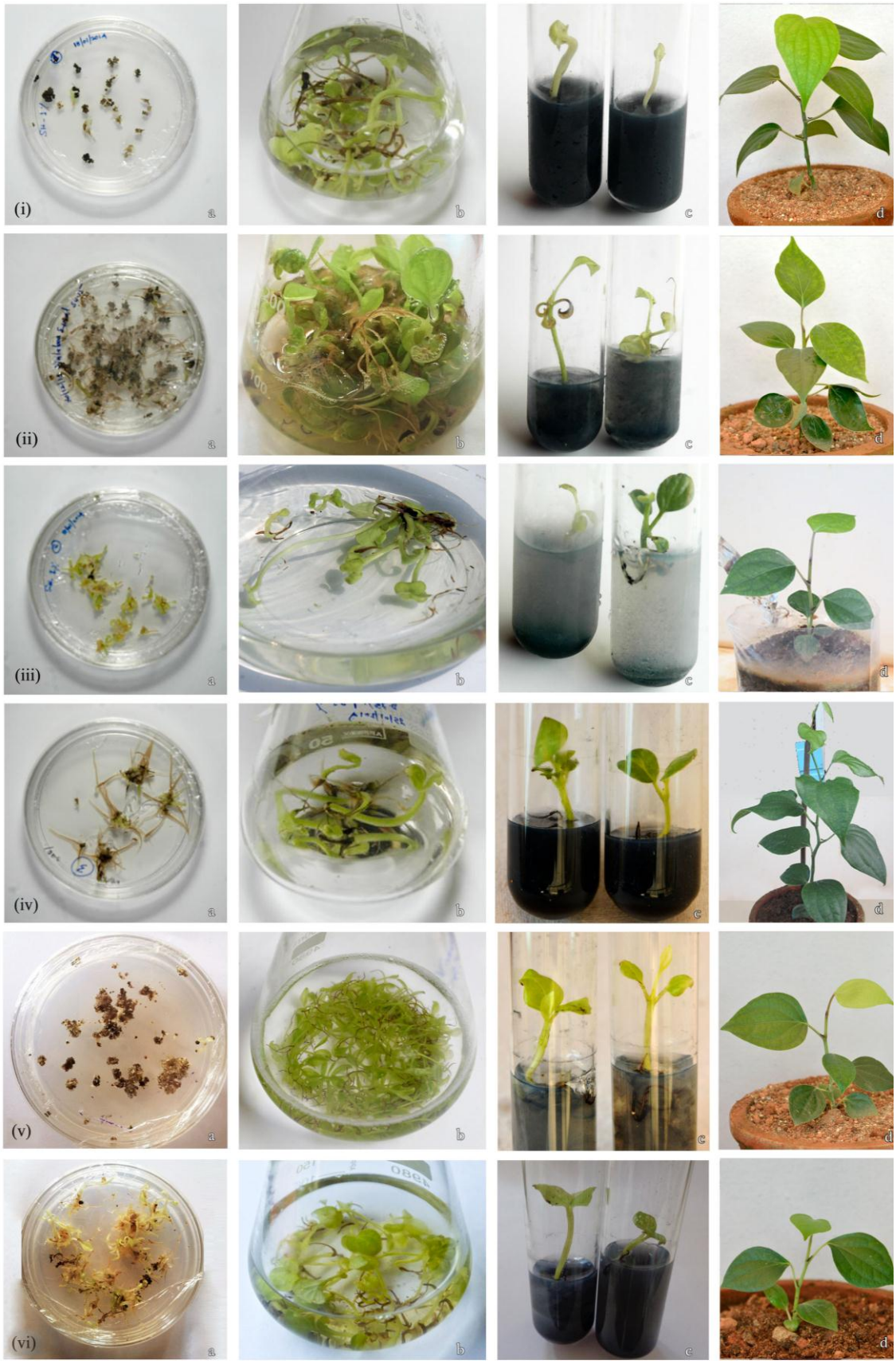

Fig 4: Regeneration of plantlets from cyclic secondary somatic embryo in different varieties (i) IISR Malabar Excel (ii) IISR Shakthi (iii) Panniyur-1 (iv) Subhakara (v) Sreekara (vi) IISR Thevam. (a) Cyclic SE in SH medium (b) Differentiated plantlets in liquid SH medium (c) Plantlets in WPM (e) Hardened plantlets.

Genetic fidelity testing of regenerated plants

Total DNA isolated from somatic embryos-derived plantlets of each variety along with corresponding mother plant and *Piper colubrinum* was subjected to SSR analysis for testing the genetic fidelity of plants. The PCR products, when analyzed on agarose gel, showed an amplicon ranging from 164 bp to 298 bp in different primer combinations (Fig. 5). The banding pattern among mother and somatic embryo-derived black pepper plants were same across different varieties indicating genetic uniformity while *P. colubrinum* either showed the band of different size or no band depending on the primer used.

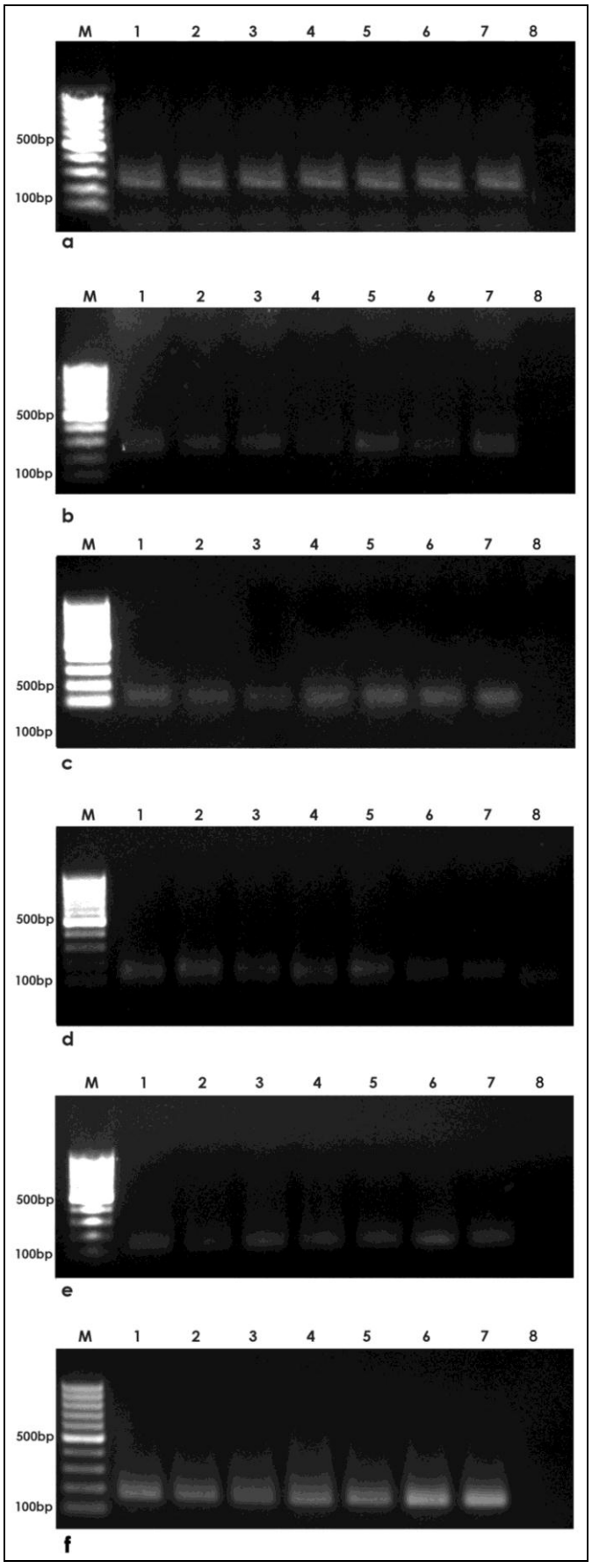

Fig 5: Genetic fidelity testing of somatic embryo-derived plants of black pepper var. IISR Malabar Excel using SSR markers. Lane M shows Marker DNA ladder, Lane 1-6 shows somatic embryo-derived plants, Lane 7 shows corresponding mother plant and Lane 8 shows *Piper colubrinum.* SSR profile obtained with (a) primer PN D10, (b) primer PN F1 (c) primer PN E3 (d) primer PN H8a (e) primer PN G11, and (f) primer A5.

4. Discussion

When the factors for inducting cyclic somatic embryogenesis and regeneration get optimized, it can be applied for micropropagation, breeding through somatic hybridization and genetic transformation. Nair and Gupta (2005) [13] studied the effect of explant and genotypes on primary somatic embryogenesis in black pepper but did not report its regeneration. Later the same authors reported cyclic SE in the black pepper cv. Karimunda (Nair and Gupta, 2006) [14] but somatic embryogenesis in other genotypes and genetic fidelity was not reported so far.

In the present study, cyclic somatic embryogenesis, regeneration and genetic fidelity of six genotype of black pepper is reported. The primary somatic embryogenesis of all six genotypes was initiated by using dissected out embryo along with micropylar region as explant. This method caused more exposure of explant to osmotic effect of the sucrose triggering the development of somatic embryos. The number of days taken for PE production varied depending on the variety. When PEs of all varieties were inoculated on SH medium containing 1.5% sucrose as reported by Nair and Gupta (2006) [14], except in the variety IISR Malabar Excel, the rest produced plantlets rather than SE, indicating that the correct sucrose concentration is crucial for the production of SE and cyclic SE. Our study determined the optimum sucrose concentration required for each of the varieties for the production of SE and cyclic SE. Sucrose plays an important role as an osmotic regulator and as a major carbon source. At a particular concentration it stimulates proliferation but restrict the development of globular embryo to further stages (Nair and Gupta, 2006) [14]. Requirement of optimum sucrose for the production of SE in other crops such as carnation and cactus are reported (Karami *et al*., 2006; Ruminska *et al*., 2013) [11, 17]. Cyclic SE is helpful in mass multiplication of somatic embryos. As reported earlier in date palm by Aslam *et al*. (2011) [3] in the present study also we could differentiate the cyclic SE of different black pepper varieties based on colour and size. Nair and Gupta (2006) [14] reported the use of 3% sucrose for conversion of cyclic SE into plantlets. We found that conversion of cyclic SE into plantlets was more and faster when sucrose concentration was increased beyond 3.0%. However it was found to have detrimental effect when sucrose concentration was beyond 4.0%. A sucrose concentration of 3.5% was found optimum for conversion of embryos to plantlets in all varieties. In general, the varieties exhibited a differential response with regard to the time taken for germination, PE, SE, cyclic SE and number of plantlets produced. This kind of differential response among different varieties was also reported in banana, date palm (Sidha *et al*., 2007; Aslam *et al*., 2011) [20, 3]. The average number of plantlets obtained from 100 mg of cyclic SE ranged from 10 to 46 in different varieties of black pepper with 83% survival and hardened plantlets showed normal growth and development. Homogeneity in amplification profile observed for all somatic embryo-derived plantlets and corresponding mother plants indicate genetic uniformity of plants suggesting that our regeneration protocol can be used for the *in vitro* propagation of black pepper with no risk of genetic variability.

In conclusion, the present study optimized the protocol for somatic embryo production and plant regeneration in six commercially grown varieties of black pepper, which can be used for micropropagation. Somatic embryogenesis is also reported to eliminate viruses in different crops such as citrus, grapevines, cocoa. As black pepper is known to be infected by *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic*

virus (CMV), the somatic embryogenesis protocol developed in this study can be attempted to produce virus-free plants. Also, as reported earlier by Jiby and Bhat (2011) [8], these somatic embryos can also serve as explant for genetic transformation of different varieties of black pepper.

5. Acknowledgements
We thank Department of Biotechnology, Government of India for the financial support (Grant no. BT/PR14813/AGR/02/761/2010), Dr. Nair RR for technical suggestions, Dr. Saji KV for seed materials, Head (Crop Protection) and Director, ICAR-Indian Institute of Spices Research, Kozhikode for facilities.

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