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In vitro elimination of *Piper yellow mottle virus* from infected black pepper through somatic embryogenesis and meristem-tip culture

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ABSTRACT

Piper yellow mottle virus (PYMoV) is known to infect black pepper (*Piper nigrum*) in India and other parts of the world. In the present study, somatic embryogenesis and meristem-tip culture alone and in combination with the antiviral agent ribavirin were attempted to eliminate PYMoV from infected black pepper explants. Cyclic somatic embryos obtained from the micropylar region of matured seeds collected from PYMoV-infected black pepper plants of six varieties were regenerated and hardened in the greenhouse. Testing of somatic embryo-derived plants by PCR showed virus elimination in 55–100% of the plants of the different varieties of black pepper. PYMoV elimination was increased when cyclic somatic embryos were pre-treated with ribavirin before regeneration. A protocol for meristem-tip culture of black pepper plants was developed that consisted of excising the meristem from PYMoV-infected black pepper plants and inoculating it in the regeneration medium containing antibiotics (to remove endophytic bacterial contamination), followed by rooting and hardening of the plants. Testing of the meristem-derived plants showed PYMoV elimination in 84% of the plants. Further elimination of the virus was achieved when meristem-tip culture was combined with ribavirin treatment.

1. Introduction

Piper yellow mottle virus (PYMoV) (genus: *Badnavirus*) is an important virus that infects black pepper and related plant species in India and other parts of the world (Lockhart et al., 1997; Bhat et al., 2003). The infected plants show a diverse range of symptoms such as mosaic, chlorotic specks; vein clearing; yellow mottling; leaf size reduction and deformation; reduction in internode length; and stunting of plants (Lockhart et al., 1997; deSilva et al., 2002; Bhat et al., 2003). Black pepper is a perennial vine propagated vegetatively through stem cuttings that favor the build-up of virus concentration over time, and the virus translocates through vegetative propagules. In addition, PYMoV is also transmitted through seeds (Deeshma and Bhat, 2014). Considering the absence of a resistant variety, the production and use of virus-free plants are important for the management of the virus. Tissue culture methods such as meristem-tip culture and somatic embryogenesis alone or in combination with antiviral agents have been successfully used for the production of virus-free plants from virus-infected plant in many crops (Panattoni et al., 2013). To date, there is no report on virus elimination from black pepper. The aim of the present study was to develop tissue culture-based methods such as somatic embryogenesis and meristem-tip culture for PYMoV elimination from black pepper.

2. Materials and methods

2.1. Source of plant materials

Ten black pepper plants each from six varieties (IISR Malabar Excel, IISR Shakthi, IISR Thevam, Panniyur-1, Sreekara, and Subhakara) showing typical viral symptoms were screened for PYMoV by PCR and used as a source material for somatic embryogenesis. Shoot tips of the black pepper var. Sreekara infected with PYMoV were used as explants for meristem-tip culture. Known PYMoV-infected and healthy black pepper plants were used as controls.

Abbreviations: PYMoV, *Piper yellow mottle virus*; PCR, Polymerase chain reaction; ORF, Open reading frame; SH, Schenk and Hilberlandt; WPM, Woody plant medium; var, Variety; CTAB, Cetyl trimethylammonium bromide; BA, Benzyl adenine; KN, Kinetin; NAA, Naphthalene acetic acid.

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2.2. Identification of PYMoV-infected mother plants

PCR using primers specific to open reading frame (ORF) II of PYMoV was used to identify infected mother plants. The forward primer 5′-TTTGTCAAGCCAAGAGACCAC-3′ represented bases 1195–1215 and the reverse primer 5′-TTGAGTGATTTGGTCCTCCAC-3′ represented bases 1526–1547 of the PYMoV genome (GenBank accession number: KJ873041). Total DNA, isolated as described by Hareesh and Bhat (2008), was used as the template for PCR. Briefly, the method involved grinding leaf tissues (1:5, w/v) in CTAB buffer [100 mM Tris HCl (pH 8), 4 mM EDTA (pH 8), 1.4 M NaCl, 2% CTAB, 1% PVP, 0.5% β-mercaptoethanol] followed by extraction with phenol:chloroform:isoamyl alcohol (24:25:1). The supernatant was re-extracted after the addition of 10% CTAB and chloroform:isoamyl alcohol (24:1). The DNA in the supernatant was precipitated by adding 3 M sodium acetate and isopropanol. The precipitated DNA was collected by centrifugation, dissolved in water, quantified by spectrophotometry, and used as the template in PCR. The PCR was performed using EmeraldAmp GT PCR Master Mix (Takara Bio. Inc, Shiga, Japan), which included a 2 × premix composed of DNA polymerase (hot start), an optimized reaction buffer, dNTPs, a density reagent, and a green gel loading dye. The 25-μl PCR reaction mix contained 12.5 μl of EmeraldAmp GT PCR Master Mix (2 × Premix), 100–500 ng template, forward and reverse primers each of 0.2 μM final concentration, and sterile water to make up the final volume. The thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) was programmed for an initial denaturation step at 98 °C for 1 min, followed by denaturation at 98 °C for 10 s, primer annealing at 54 °C for 30 s, primer extension at 72 °C for 1 min (35 cycles) and finally, an extension step at 72 °C for 5 min. The PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

2.3. Somatic embryogenesis and testing of somatic embryo-derived plants for PYMoV

Somatic embryo-derived plants were produced from six varieties of black pepper by using the protocol described by Nair and Gupta (2006) and Sasi and Bhat (2016). Briefly, the method involved inoculation of the scooped-out embryo along with the micropylar region (obtained from matured seeds collected from PYMoV-infected black pepper plants) on SH30 medium (SH medium containing 3% sucrose) and incubation at 28 °C in dark for the induction of primary somatic embryogenesis. The primary somatic embryos along with the whole explant were inoculated in SH07 (for var. Panniyur-1), SH10 (for var. IISR Shakthi, IISR Thevam, Sreekara, and Subhakara), and SH15 (for var. IISR Malabar Excel) for the induction of secondary somatic embryogenesis and cyclic somatic embryogenesis. Each cyclic somatic embryo was regenerated to produce plantlets in SH35 liquid medium (SH medium containing 3.5% sucrose) with shaking at 110 rpm for 30 days. Well-differentiated plantlets were transferred to woody plant medium (WPM) containing 2% charcoal for rooting, and the rooted plantlets were hardened in the greenhouse. Somatic embryo-derived plants of all six varieties were tested for the presence/absence of PYMoV by PCR as described above along with a known positive and negative control.

2.4. Somatic embryogenesis combined with chemotherapy and testing of plants for PYMoV

The cyclic somatic embryo of var. Sreekara obtained as described above was used as the starting material for this experiment. Approximately 100 mg of cyclic somatic embryos were inoculated in SH medium containing 1% sucrose supplemented with different concentrations of ribavirin (0, 10, 20, 30, and 50 mg/l). Two subcultures were performed at a 15-day interval in the same medium. Observations such as change in color, proliferation rate, and necrosis of cyclic somatic embryos were recorded on the 30th day of incubation. Ribavirin-treated cyclic somatic embryos were then converted into plantlets in liquid SH (SH35) medium, and well-differentiated plantlets were transferred to hormone-free WPM for proper organogenesis. The well-established rooted plantlets were hardened using a sterile potting mixture in the greenhouse. The experiment was repeated thrice, and the number of plantlets that survived after hardening at each ribavirin concentration was recorded. The hardened plants were tested for the presence/absence of PYMoV by PCR.

2.5. Meristem-tip culture and testing of meristem-derived plants for PYMoV

The shoot tips of PYMoV-infected black pepper plants of var. Sreekara were dipped in 1.5% bavistin 2 days prior to explant collection. The explants were collected and treated again with bavistin for 10 min, followed by treatment with water containing 1% Tween 20 for 10 min and washed under running tap water for 10 min. The explants were surface sterilized with 0.1% mercuric chloride for 10 min and rinsed with sterile water thrice. Meristems of approximately 2–3 mm were excised from the shoot tips under a laminar air flow module by using a clean, sterile scalpel and were inoculated initially into WPM medium containing 3 mg/l benzyl adenine (BA) + 1 mg/l kinetin (KN) (Babu et al., 1997). To prevent the death of inoculated meristems due to endophytic bacterial contamination, antibiotics such as tetracycline, penicillin G, and spectromycin were tested individually and in combination to control endophytic bacterial growth. A loopful of each of the two isolated bacteria was streaked onto LB medium containing 1% of each of the antibiotics and incubated at 28 °C for 3 days.

After determining the suitable antibiotics, the meristems were finally inoculated into WPM medium containing 3 mg/l BA +1 mg/l KN and 1% each of tetracycline and spectromycin, followed by incubation at 28 °C with a 16-h photoperiod and then monitored daily. The cultures were allowed to grow for 2–3 weeks for meristem elongation. The extended meristems were then transferred to the same medium without antibiotics till shoots were formed. The shoots were then transferred to a rooting medium (WPM with 3 mg/l BA +1 mg/l NAA) (Babu et al., 1997), and the rooted plantlets were hardened in the greenhouse. The experiment was repeated thrice, and the number of the plantlets that survived in WPM and the plantlets that survived after hardening was recorded. All hardened plants were tested for the presence/absence of PYMoV by PCR.

2.6. Meristem-tip culture combined with chemotherapy and testing of plants for PYMoV

Meristems excised from the shoot tips of virus-infected plants of var. Sreekara, as described above, were inoculated in a meristem regeneration medium (WPM with 3 mg/l BA + 1 mg/l KN +1% tetracycline + 1% spectromycin) containing ribavirin at 0, 10, 20, 30, and 50 mg/l for a period of 30 days, with two subculturing in the same medium at an interval of 15 days. Ribavirin-treated meristems were then transferred to the same medium without ribavirin and antibiotics for proper shooting, followed by rooting as described previously. Well-rooted plantlets were hardened in the greenhouse. The experiment was repeated thrice, and the number of plantlets that survived in WPM and hardening in each of the different ribavirin concentration-treated meristems was recorded. All hardened plants were tested for the presence/absence of PYMoV by PCR.

3. Results and discussion

3.1. Identification of PYMoV-infected mother plants

All 10 plants from each of the six varieties were PYMoV-positive, as determined by PCR. (Supplementary Fig. 1). The identity of the amplicon was confirmed by sequencing the PCR product that showed 100% identity with ORF II of PYMoV (GenBank accession number: KJ873041).

3.2. Somatic embryogenesis and testing of somatic embryo-derived plants

Cyclic somatic embryogenesis was established by culturing scooped-out embryos along with the micropylar region from matured seeds as described by Sasi and Bhat (2016). Primary and secondary somatic embryos appeared in the different black pepper varieties after 48–85 days and 65–100 days, respectively. Cyclic somatic embryos of the different black pepper varieties that appeared 90–100 days were converted into plantlets and hardened in the greenhouse (Fig. 1). When the total DNA isolated from 227 somatic embryo-derived plants belonging to the six varieties was subjected to PCR, 65 plants showed a positive reaction, indicating that 28% of the plants were infected with PYMoV, while the remaining plants (78%) were free of PYMoV (Fig. 2; Table 1). Virus elimination through somatic embryogenesis is due to the lack of a relation between somatic embryos and the mother plant (Goussard and Wiid, 1993). Moreover, viruses are mainly restricted to the vascular tissue, while somatic embryos arise from non-vascular tissues, thus increasing the chances of virus elimination (Guiderdoni and Demarly, 1988). Many studies have reported virus elimination through somatic embryogenesis in different crops such as cocoa, garlic, grapevine, sugarcane, and potato (Quainoo et al., 2008; Panattoni et al., 2013). In the present study, the virus elimination rate varied from 55% to 100% in the different varieties of black pepper (Table 1). All somatic embryo-derived plants of var. IISR Thevam were free of PYMoV, while only 55% of plants of var. IISR Malabar Excel were free of PYMoV. Elimination of the virus increased when the cyclic somatic embryos were subjected to repeated subculturing. Thus, higher virus elimination was observed in var. IISR Thevam plants, which were obtained after several cycles of repeated subculturing of the cyclic somatic embryos. Variety-wise variation in virus elimination (87–100%) was reported for *Cacao swollen shoot virus* in cocoa by Edward and Wetten (2016). D'Onghia et al. (2001) and El-Sawy et al. (2013) reported complete elimination of *Citrus psorosis virus* from six citrus varieties through somatic embryogenesis.

3.3. Somatic embryogenesis combined with chemotherapy and testing of plants for PYMoV

Proliferation of the cyclic somatic embryo was not affected at 10 and 20 mg/l ribavirin, while at 30 mg/l ribavirin, proliferation was slow, and at 50 mg/l ribavarin, complete necrosis and death of the somatic embryo were observed. In general, regeneration of the plantlets was slower in the ribavirin-treated cyclic somatic embryo than in the control. The number of plants produced also decreased (up to 50% in 30 mg/l ribavarin) at different concentrations of ribavirin (Table 2). The phytotoxic effect of ribavirin slows the multiplication rate of the cyclic somatic embryo and kills the tissue at high concentrations. Similar results of reduced multiplication rate and mortality of grapevine shoots due to ribavirin were reported by Guţă et al. (2014).

Thirty hardened plants from each treatment were tested for the presence/absence of PYMoV by PCR. Seven of 30 plants from the 10 mg/l ribavirin treatment group tested positive for PYMoV (Fig. 3). All 30 plants each from 20 to 30 mg/l ribavirin treatment groups tested negative for PYMoV (figure not shown), indicating that treatment with 20 mg/l ribavirin is sufficient for the complete elimination of PYMoV from the cyclic somatic embryo of black pepper. Ribavirin inhibits the 5' capping of viral RNA by decreasing intracellular guanosine, thereby inhibiting the movement of the virus and resulting in its elimination (Cassells and Long, 1982). The present study is the first report of production of virus-free plants by combining somatic embryogenesis with ribavirin treatment.

3.4. Meristem-tip culture and testing of meristem-derived plants for PYMoV

PYMoV infection in black pepper plants of var. Sreekara was identified by PCR (Supplementary Fig. 2). Meristem explants collected from these plants when cultured in WPM medium containing 3 mg/l BA + 1 mg/l KN led to death of the meristem due to endogenous bacterial contamination. Of the three antibiotics tested individually and in combination, 1% each of tetracycline and spectromycin were effective in controlling endogenous bacterial

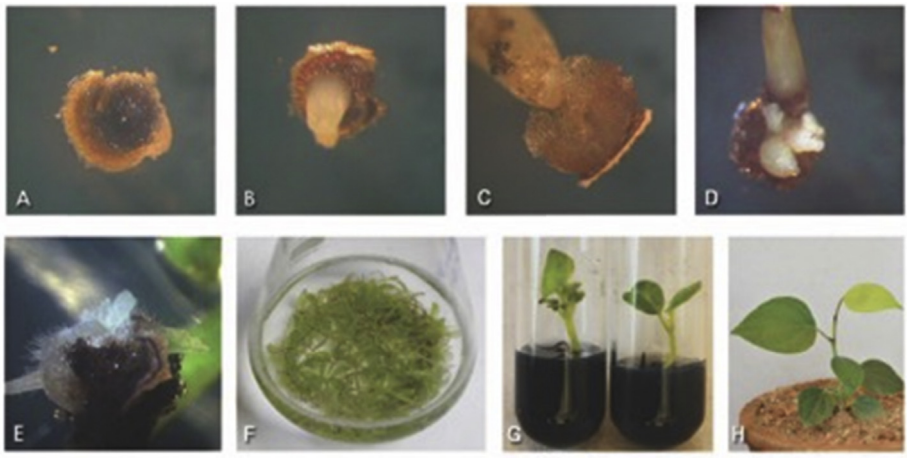

Fig. 1. Somatic embryogenesis in black pepper. (A) Scooped-out embryo with the micropylar region; (B) Germinating embryo; (C) Germinated embryo showing primary somatic embryogenesis; (D–E) Secondary somatic embryos giving rise to cyclic somatic embryos; (F) Differentiated plantlets in liquid SH medium; (G) Plantlets in WPM; (H) Hardened plantlets. Photographs A–E were taken with a stereomicroscope at 40× magnification.

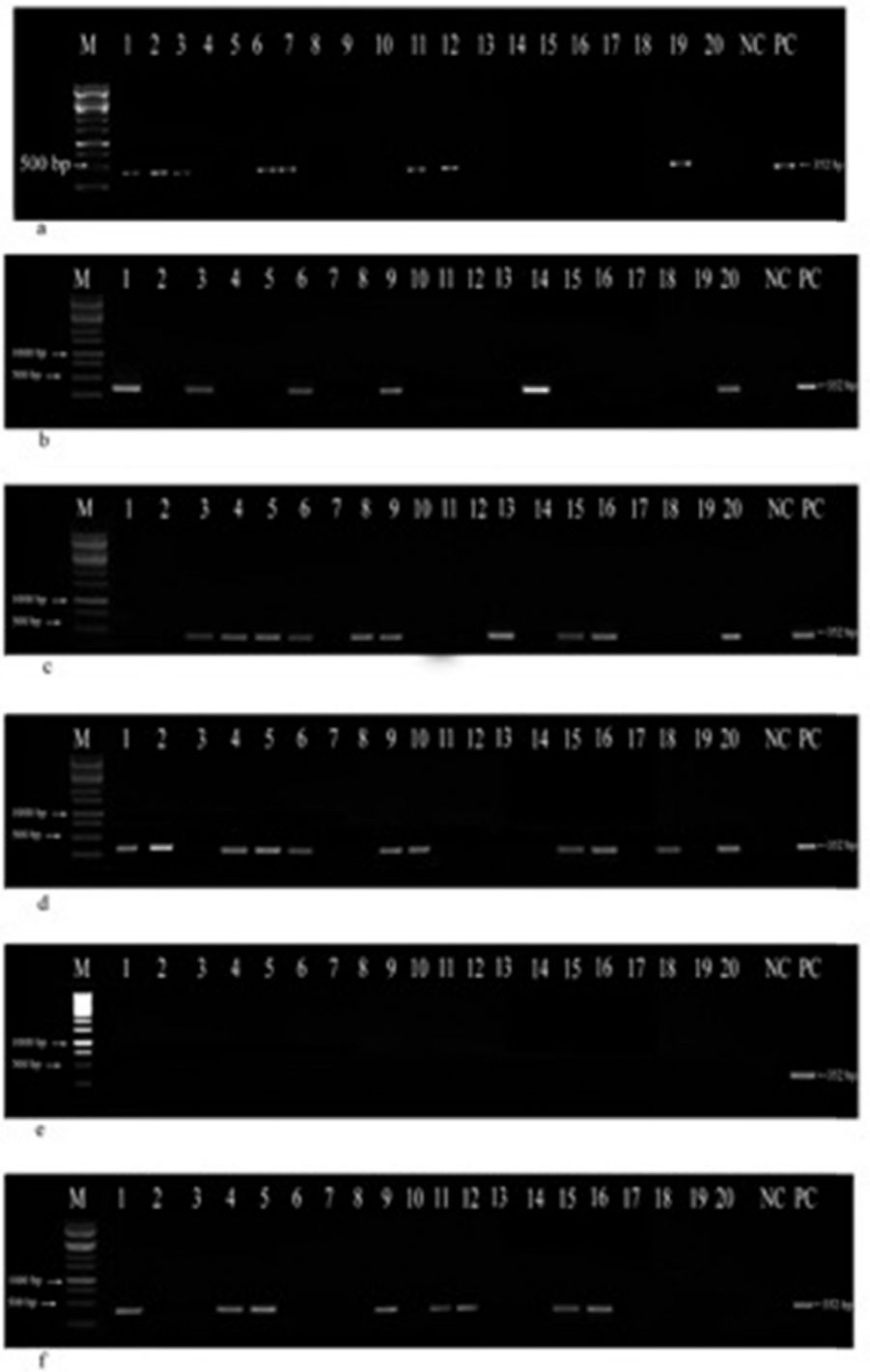

Fig. 2. Testing of somatic embryo-derived plants of the different varieties of black pepper for the presence/absence of *Piper yellow mottle virus* by PCR (a) IISR Malabar Excel, (b) Panniyur-1, (c) IISR Shakthi, (d) Sreekara, (e) IISR Thevam, and (f) Subhakara. Lane M: DNA marker (1 kb ladder), Lanes 1–20: somatic embryo-derived plants, Lane NC: negative control, Lane PC: positive control.

contamination observed in meristem-tip culture. Thus, finally, meristems were cultured in WPM containing 3 mg/l BA +1 mg/l KN + 1% tetracycline +1% spectromycin. The presence of charcoal in WPM prevents the accumulation of phenol and has been used in *in vitro* culture of black pepper (Babu et al., 1997; Nair and Gupta, 2006). The use of antibiotic for the elimination of endophytic bacteria has been reported in the tissue culture of banana (Habiba et al., 2002), yam (Wakil and Mbah, 2012), and oil palm (Eziashi et al., 2014). Kulkarni et al. (2007) identified five endophytic bacteria associated with black pepper shoot tip culture and used gentamycin to control their growth. In the present study, approximately 76% of the meristem-derived plantlets were free from bacterial contamination, while the remaining had slight endophytic contamination that did not affect the growth of the meristem. Approximately 86% of the meristems responded well by elongation and produced 2–3 leaves in 35–50 days of inoculation (Fig. 4).

Table 1
Testing of somatic embryo-derived plants of different varieties of black pepper for *Piper yellow mottle virus* (PYMoV) by PCR.

Variety	No. of plants tested	No. of plants tested positive	Percent virus elimination
IISR Malabar Excel	37	17	55
IISR Shakthi	40	14	65
IISR Thevam	30	0	100
Panniyur −1	20	6	70
Sreekara	50	14	72
Subhakara	50	14	72

Table 2
Ribavirin sensitivity assay for the regeneration of cyclic secondary somatic embryos of black pepper var. Sreekara.

Ribavirin concentration (mg/l)	No. of plants that survived in WPM	No. of plants that survived in hardening
0 (Control)	38 ± 5	32 ± 4
10	33 ± 2	31 ± 2
20	32 ± 2	30 ± 2
30	17 ± 1	15 ± 2
50	0	0

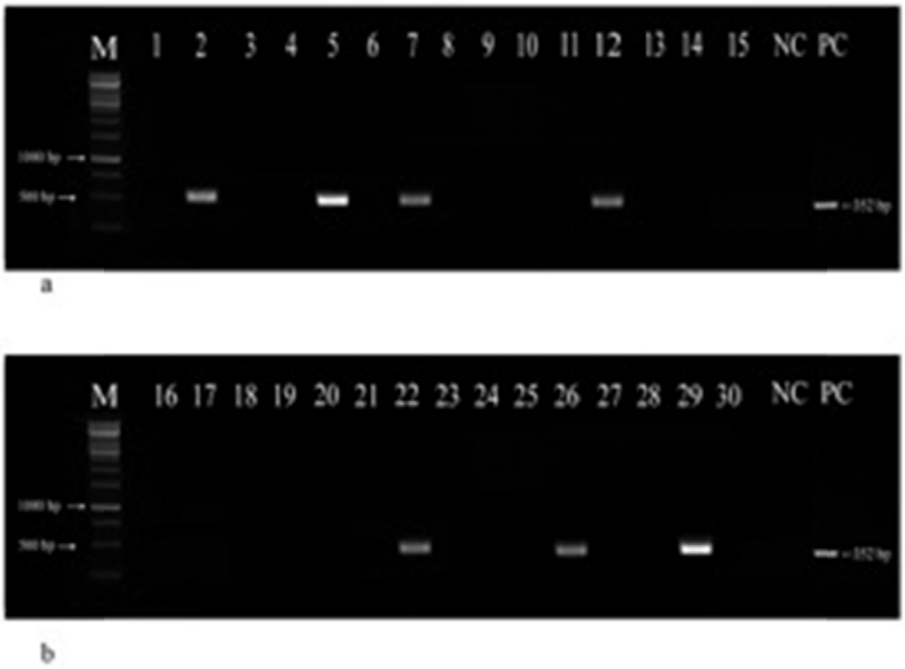

Fig. 3. Agarose gel showing the presence of *Piper yellow mottle virus* in 10 mg/l ribavirin-treated somatic embryo-derived plants. Lane M: DNA marker (1 kb ladder), Lanes 1–30: test plants, Lane NC: negative control, Lane PC: positive control.

Transferring to WPM medium containing BA and NAA initiated rooting, and the plants were ready to harden by the 90th day. Four of the 30 inoculated meristems failed to develop roots, while all other meristem-derived plants showed proper shooting and rooting by the 90th day. Twenty-six well-developed plantlets were hardened in the greenhouse in a potting mixture.

Of the 26 meristem-derived plants tested in PCR, four plants showed positive reaction, indicating that 84% of the meristem-derived plants were free of PYMoV (Fig. 5). Many studies have reported virus elimination (75–100%) through meristem-tip culture in different crops, e.g., *Grapevine leafroll associated virus* in grapevine, *Cucumber mosaic virus* and *Cymbidium mosaic virus* in vanilla, and *Potato virus X* and *Potato virus Y* in potato (Fayek et al., 2009; Retheesh and Bhat, 2010; Al-Taleb et al., 2011; Hossain et al., 2013). Slow cell-to-cell movement of the virus, rapid multiplication of meristematic cells (high amount of auxin), and presence of virus-inhibiting components in the meristematic region contributed to virus elimination (Parmessur et al., 2002).

3.5. Meristem-tip culture combined with chemotherapy

Except at 10 mg/l ribavirin, all other treatments resulted in slow elongation and regeneration of meristem. The number of days taken for the regeneration of meristem in the presence of 20 and 30 mg/l ribavirin was more (approximately 60–80 days) than that of the control (approximately 35–50 days). The number of plantlets obtained also varied among the treatments. The maximum number of plantlets (10) was obtained in 10 mg/l ribavirin treatment, while the minimum of plantlets (6) was obtained in 50 mg/l ribavirin treatment (Table 3). Similar results were observed in apple (Hansen and Lane, 1985) and potato (Parmessur and Saumtally, 2001; Nascimento et al., 2003). Mirosława (2007) found that ribavirin at

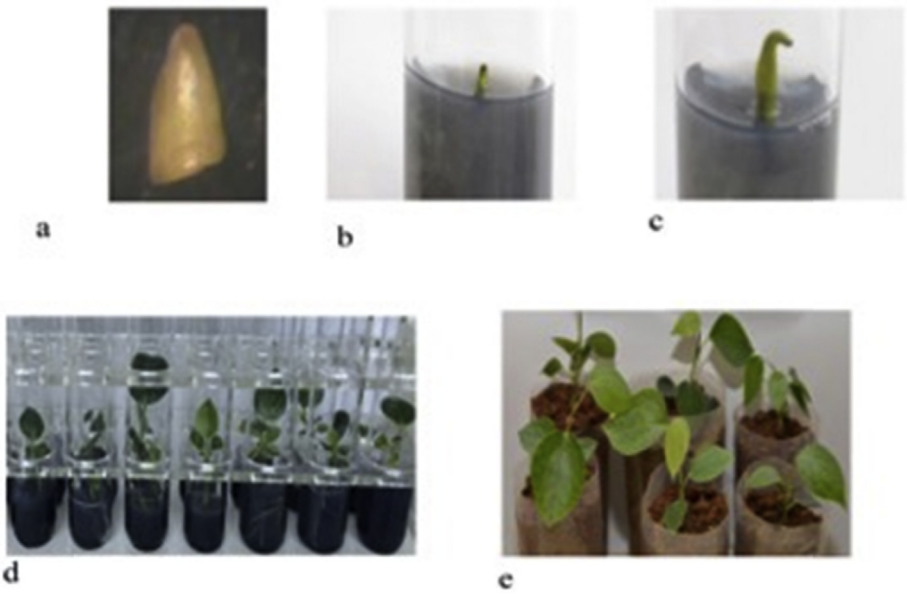

Fig. 4. Meristem-tip culture in black pepper var. Sreekara (a) microscopic view of an excised meristem at 40× magnification (0th day), (b–c) elongation of the meristem (30th and 45th day), (d) well-developed plants in WPM (90th day), and (e) hardened meristem-derived plants in the greenhouse (120th day).

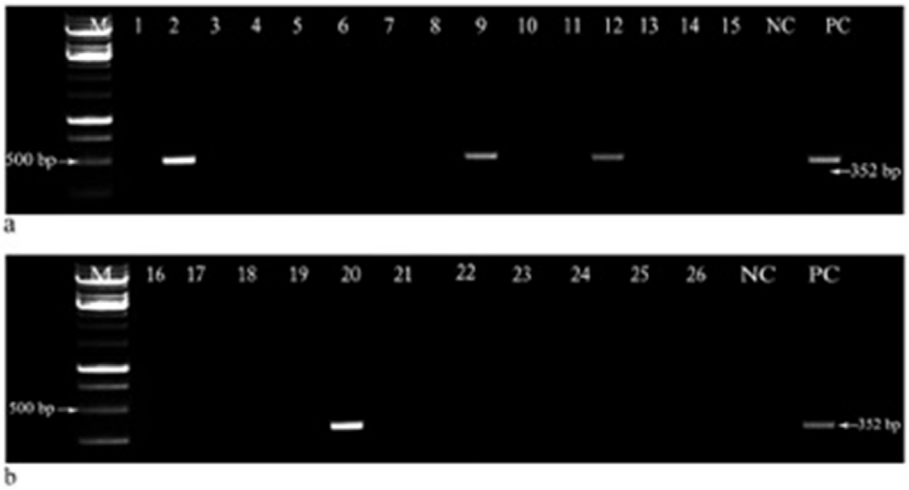

Fig. 5. Screening of the meristem-derived plants for *Piper yellow mottle virus* by PCR. Lane M: DNA marker (1 kb ladder), Lanes 1–26: Meristem-derived plants of var. Sreekara, Lane NC: negative control, Lane PC: positive control.

Table 3
Effect of different concentrations of ribavirin on the regeneration of meristem and plantlet production.

Ribavirin concentration (mg/l)	No. of meristem inoculated	No. of meristem regenerated	No. of meristem-derived plants that were hardened
0 (control)	6 × 3	6 ± 0	5 ± 1
10	6 × 3	5 ± 1	4 ± 1
20	6 × 3	4 ± 1	3 ± 1
30	6 × 3	3 ± 1	3 ± 1
50	6 × 3	3 ± 1	2 ± 1

a concentration of 50–100 mg/l was toxic to plum shoots and induced chlorosis and necrosis, leading to death of the plant. Parmessur and Saumtally (2001) and Danci et al. (2012) studied the phytotoxic effect of ribavirin on four varieties of potato and reported that potato meristems perished at a ribavirin concentration of 30 mg/l, and concentrations above 30 mg/l caused necrosis of *in vitro* plantlets. After acclimatization, the plants showed no morphological difference with respect to growth and development.

All ribavirin-treated meristem-derived plants (36) from different treatments tested negative for PYMoV, as determined by PCR, indicating complete elimination of the virus (figure not shown). Three of the 17 untreated meristem-derived plants showed the presence of PYMoV. Thus, meristem-tip culture combined with ribavirin treatment was found to be more effective than meristem-

tip culture alone for PYMoV elimination from black pepper. Supplementing the WPM medium with 10 mg/l of ribavirin was sufficient for the complete elimination of PYMoV from the meristem. Earlier studies also reported complete virus elimination when meristem culture was combined with ribavirin treatment in pear (Hu et al., 2012), sugarcane (Neelamathi et al., 2014; Dewanti et al., 2016), and garlic (Kudělková et al., 2016). The present study also concluded that meristem culture combined with chemotherapy is better than meristem culture alone for the elimination of PYMoV from black pepper.

In conclusion, the findings of this study indicate that somatic embryogenesis or meristem-tip culture alone was successful for the elimination of PYMoV from black pepper. Further improvement or complete elimination of PYMoV was possible when somatic embryogenesis or meristem-tip culture was combined with chemotherapy. For somatic embryogenesis, once cyclic somatic embryos are obtained, they can be used for the continuous production of plants at least for a year. Furthermore, increase in the number of subculturing cycles leads to increased virus elimination. Thus, the advantage of somatic embryogenesis over meristem-tip culture is its ability to produce a large number of plantlets per explant, unlike meristem-tip culture that produces only one plantlet per explant.

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Conflict of interest

Both authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cropro.2017.09.004.

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