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Short communication

Simultaneous elimination of *Cucumber mosaic virus* and *Cymbidium mosaic virus* infecting *Vanilla planifolia* through meristem culture

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ABSTRACT

Vanilla (*Vanilla planifolia* G. Jackson), a perennial fleshy vine infected by *Cucumber mosaic virus* (CMV) and *Cymbidium mosaic virus* (CymMV) was freed from infection through meristem culture. Apical meristem measuring 0.1–0.25 mm were isolated and cultured in Murashige and Skoog (MS) medium supplemented with 0.45 μM thidiazuron for 40–45 days to initiate the growth. Following enlargement of meristem, it was transferred to MS medium supplemented with 4.43 μM 6-benzyl aminopurine (BAP) and 2.68 μM α-naphthalene acetic acid (NAA) for regeneration. The regenerated plantlets were hardened in insect-free glasshouse and confirmed for the elimination of viruses using reverse transcription PCR (RT-PCR) with virus specific primer pairs. The frequency of CMV elimination was 79.4% while that of CymMV was 82.4% when tested individually. We obtained a frequency of 75% simultaneous elimination of both viruses. This is the first report of meristem culture and virus elimination in vanilla.

1. Introduction

Vanilla, the prince of spices used in food and cosmetic industry is obtained from fully-grown fruit of the orchid *Vanilla planifolia* G. Jackson (Family – Orchidaceae). It is propagated vegetatively by stem cuttings and lacks natural pollination (rostellum prevents contact between stamen and stigmata). Hand-pollination results in fruit (capsule) set, but the seeds produced have an undifferentiated embryo, little reserve matter, very hard and waxy teguments containing germination inhibitors and hence rarely germinate (Dequaire, 1976). Viral diseases have been reported from most of the vanilla growing countries across the world. Some of the viruses were known to cause very little damage while the others are known to severely infect the vines there by causing considerable yield reduction. So far 10 viruses belonging to different genera and families have been reported to infect vanilla worldwide (Pearson et al., 1993; Grisoni et al., 2004; Richard et al., 2009). However, only four viruses *viz*., *Bean common mosaic virus* (BCMV), *Bean yellow mosaic virus* (BYMV), *Cucumber mosaic virus* (CMV) and *Cymbidium mosaic virus* (CymMV) have been reported in India so far (Madhubala et al., 2005; Bhat et al., 2006; Bhadramurthy, 2008) and among them CMV is the most serious virus disease causing mosaic and deformation of leaves and stunting of vine while CymMV is known to cause only mild mottle or mosaic, but is well-distributed in vanilla plantations. Recent report suggested high prevalence of CMV (over 30% of the plots studied) and could severely damage up to 50% of the vanilla vines before blossom in French Polynesia (Richard et al., 2009).

When a plant is infected with virus, the titre of the virus within the plant is highly variable and most of the viruses fail to reach the meristematic tissue. Morel and Martin (1952), for the first time, reported successful application of meristem culture to produce virus-free *Dahlia* from the infected stock. Since vanilla is propagated vegetatively, presence of viruses is a matter of concern, as viruses could be transmitted to generations. Perennial nature of the crop still aggravates the situation as the virus inocula remain in the field for long, rendering the whole plantation vulnerable to disease spread by insect vectors. So far, there is no report of any attempt to regenerate vanilla from meristem or to eliminate viruses infecting the crop. Here, we report the regeneration of vanilla plantlets from meristem and simultaneous elimination of CMV and CymMV from the naturally infected vanilla vines by shoot apical meristem culture.

2. Materials and methods

Vanilla vines showing symptoms of viral infection such as mosaic, mottling and stunting were collected from farmers' fields and maintained in insect-proof glasshouse. The plants were tested for the presence of CMV and CymMV by reverse transcription

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Table 1
Primers used for detection of CMV and CymMV in vanilla by RT-PCR.

Primer	Sequence	Expected amplicon (bp)	Reference
CMV forward	5′ ATGGACAAATCTGAATCAAC 3′	657	Madhubala et al., 2005
CMV reverse	5′ TCAAACTGGGAGCACCC 3′		
CymMV forward	5′ ATGGGAGAGCCCACTCC 3′	672	Bhat et al., 2006
CymMV reverse	5′ TTATTCAGTAGGGGGTGC 3′		

polymerase chain reaction (RT-PCR) using specific primer pairs as described below. Those vines tested positive for both CMV and CymMV were vegetatively propagated by stem cuttings in glasshouse and served as mother plants for meristem culture.

Preliminary experiments revealed that MS medium (Murashige and Skoog, 1962) supplemented with 0.45 μM thidiazuron (TDZ) could effectively promote the growth of excised meristem *in vitro* when the cultures were incubated in dark. Based on this, Murashige and Skoog (MS) medium supplemented with 0.45 μM TDZ (growth induction medium) or 4.43 μM 6-benzyl aminopurine (BAP) and 2.68 μM α-naphthalene acetic acid (NAA) (regeneration medium) was used. Both media contained 3% (w/v) sucrose as carbon source and 0.3% phytagel as solidifying agent. The cultures were incubated at 25 ± 1 °C either under dark or 16 h photoperiod as applicable.

Shoot tips of 3–4 cm length were collected from the mother plants and surface-sterilized in 0.1% (w/v) mercuric chloride for 3 min followed by three rinses with sterile distilled water in a laminar air flow cabinet. Apical meristem (0.1–0.25 mm long) with 1–2 leaf primordia were excised and placed in the medium in a Petri dish. Initially the meristem was cultured in MS medium supplemented with 0.45 μM TDZ for 25–30 days in dark for growth induction. Subsequently, the enlarged meristem were transferred to regeneration medium (MS supplemented with 4.43 μM BAP and

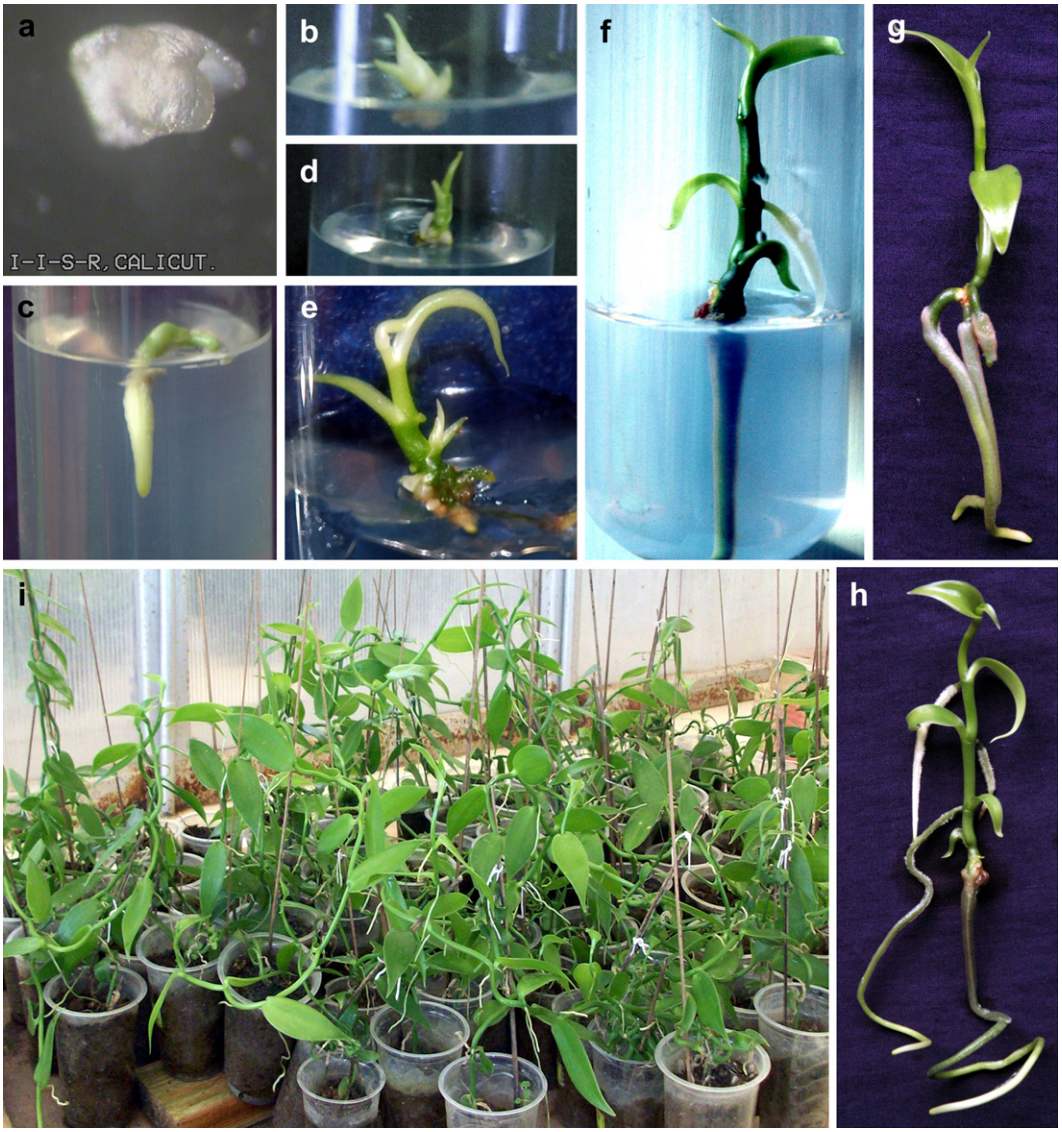

Fig. 1. Various stages in meristem culture of vanilla. (a): Freshly isolated meristem with a single leaf primordium; (b): after 30 days of culture in induction medium; (c), (d), (e), and (f): cultures 45, 60, 75 and 90 days after culturing on regeneration medium, respectively; (g) and (h): fully regenerated plantlets ready for hardening; and (i): plants in insect-free glasshouse.

Table 2
Percentage elimination of CMV and CymMV from vanilla by meristem culture.

Virus-free plants	No. of virus-free plants as tested by RT-PCR/total number	Percent of virus elimination
CMV-free	54/68	79.4
CymMV-free	56/68	82.4
Both CMV and CymMV-free	51/68	75.0

2.68 μM NAA) and incubated at 25 ± 1 °C under 16 h photoperiod with a light intensity of 40 μM m^{-2} s^{-1} provided by cool white fluorescent tubes. The regenerated plantlets with well-differentiated shoot and root system and 4–5 fully developed leaves were transplanted in small plastic cups containing sterilized potting mixture (garden soil: sand: dry, powdered cow dung: 1:1:1) and maintained in insect-free glasshouse.

Total RNA was isolated from 50 mg leaf tissue (third leaf from top in the case of meristem-regenerated plantlets and symptomatic tender leaf of mother plant) as described by Chomczynski and Sacchi (1987). RT-PCR was carried out for each virus separately in different reaction tubes. The reagent composition and RT-PCR profile were identical for the detection of both the viruses unless it is specified. The reaction was carried out in 1× Taq assay buffer (Fermentas) and contained 1.5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 400 μM dNTP mixture, 10 pM each of forward and reverse primers specific for CMV (or CymMV) (Table 1), 1 U of RNase inhibitor (Fermentas), 1.25 U of MuMLV reverse transcriptase (Fermentas), 0.75 U of *Taq* DNA polymerase (Fermentas) and 1 μg total RNA as template with a final volume of 25 μl. Prior to the addition of RNA template to the reaction mixture, it was heated to 80 °C for 10 min and rapidly cooled down in ice for 3 min to make RNA linear. Single-step RT-PCR (Madhubala et al., 2005; Bhat et al., 2006) was carried out in Eppendorf's Mastercycler Gradient by initially holding the sample at 42 °C for 45 min (cDNA synthesis) followed by 94 °C for 30 s (denature), 50 °C (for CMV) or 54 °C (for CymMV) for 1 min (primer annealing) and 72 °C for 1 min (DNA synthesis). The whole process except cDNA synthesis was repeated for another 39 cycles and a final extension was allowed at 72 °C for 10 min. The reaction products were subjected to electrophoresis in 1% agarose gel and visualized under UV light after staining with ethidium bromide. Positive reactions were identified by the presence of 657 bp product specific for CMV and 672 bp product specific for CymMV as already reported (Madhubala et al., 2005; Bhat et al., 2006). Absence of the expected amplicon indicated a negative reaction and thus the absence of the respective virus. The plants were tested two times (three and six months) of hardening in glasshouse.

3. Results and discussion

Nine plants with symptoms of viral infection, originally collected from farmers' fields were tested by RT-PCR using specific primer pairs for CMV and CymMV in separate experiments. Out of the nine plants, four produced both CMV-specific 657 bp and CymMV-specific 672 bp amplicons indicating that the plants were infected with both viruses. The identities of the amplicons were confirmed by directly sequencing the gel-purified DNA bands (data not shown). The remaining five were positive for either CymMV or CMV alone and excluded from the study as elimination of both these viruses individually from different hosts have been reported by earlier workers (Morel, 1960; Verma et al., 2004). Symptomatically, plants with double infection showed mild mottling, mosaic and leaf deformation. Plants infected with CMV alone showed leaf deformation and mosaic while that of CymMV alone showed mild chlorotic mottling.

Shoot apical meristem from naturally infected vanilla plants harbouring both CMV and CymMV (as confirmed by RT-PCR) were used for raising virus-free plantlets. Of the total 293 meristem isolated and cultured, 68 (23.2%) responded well by enlargement and elongation. All the 68 explants regenerated a single shoot in 60–75 days after transfer to regeneration medium. In about 90–100 days from transfer, the plantlets produced 3–4 well developed leaves. Rhizogenesis was also initiated in the same medium at 70–80 days from transfer. Each regenerated shoot produced 2–3 well developed adventitious roots from the base and 2–3 roots from the node, opposite to the leaf. Root, both from the base or the node, measured 9–28 cm in length and was covered with spongy tissue, a characteristic typical of orchids (Fig. 1). All plantlets with well developed shoot and root system were transplanted in sterilized potting mixture and acclimatized in insect-free glasshouse for confirmation of virus elimination. The excised meristem that did not respond to the culture medium became necrotic and subsequently turned brown and died in 5–15 days from inoculation in the induction medium. A total of 68 plants were established in green house and all were tested twice for both the viruses by RT-PCR and yielded same results as presented in Table 2. Figs. 2 and 3 show elimination of CMV and CymMV, respectively, as confirmed by RT-PCR. Each of the four infected plants regenerated 12, 14, 9 and 16 meristem-derived plantlets thus making a total of 51 healthy plants.

The use of meristem and shoot-tip cultures for pathogen-free plants is a common practice in the production of virus-free stocks of vegetatively propagated plants. Virus elimination depends on various factors such as size of meristem, the virus concerned and physiological condition of plants. In the present study low frequency (23.2%) of meristem regeneration obtained may be due

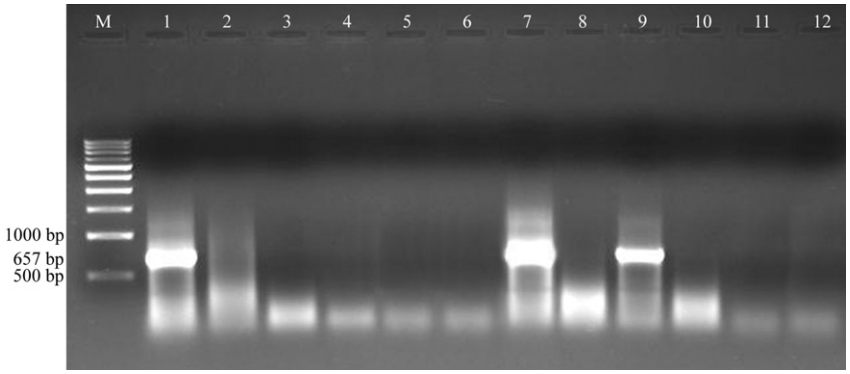

Fig. 2. Elimination of CMV as confirmed by RT-PCR. Lane M: molecular weight marker. Lanes 1 and 7: CMV-positive mother plants and their meristem-derived plantlets, lanes 2–6 and 8–12, respectively. Note that CMV is not eliminated in the plantlet shown in lane 9.

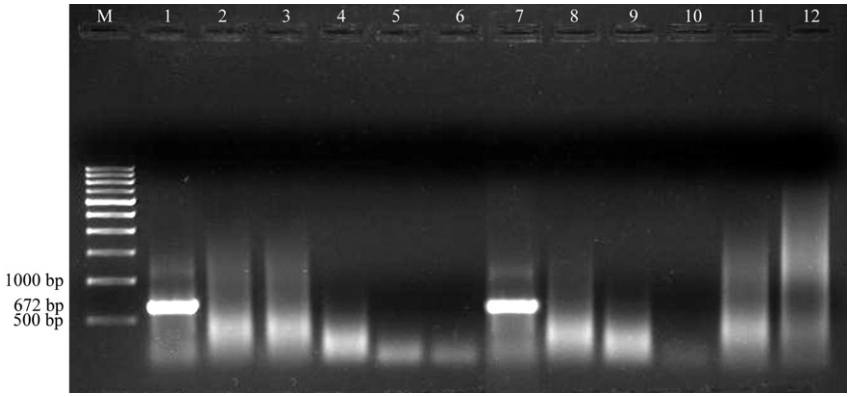

Fig. 3. Elimination of CymMV as confirmed by RT-PCR. Lane M: molecular weight marker. Lanes 1 and 7: CymMV-positive mother plants and their meristem-derived plantlets, lanes 2–6 and 8–12, respectively.

to short length of meristem (0.1–0.25 mm) used. This has led to high frequency of virus elimination (79.4–82.4%). Poor development of vascular tissue in meristem and slow cell-to-cell movement of viruses compared to growth of meristem tips might contribute for virus elimination in meristem tips (Parmessur et al., 2002). Virus elimination up to 46.7% in bamboo (Hsu et al., 2000), 73.7% in *Alstroemeria* (Chiari and Bridgen, 2002); 92% and 64% in sugarcane (Chatenet et al., 2001; Parmessur et al., 2002) were reported. Similarly simultaneous elimination of more than one viruses have also been reported in hosts such as chrysanthemum and sweet potato (Wang and Valkonen, 2008; Kumar et al., 2009). In our study, initially, all meristem-regenerated plants appeared healthy without any visual symptoms. But plants tested as positive for viruses by RT-PCR started showing symptoms after 6 months of growth in glasshouse. This confirms that RT-PCR can detect virus at very low titre even before the appearance of symptoms. Although this study covered only two viruses, the technique demonstrated in this paper may be used to eliminate other viruses infecting vanilla. The study clearly showed the possibility of getting virus-free plants from infected plants through meristem culture. This is the first report of meristem culture and virus elimination in vanilla.

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