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Occurrence of *Peanut stripe virus* on patchouli and raising of virus-free patchouli plants by meristem tip culture

Vorkommen des *Peanut stripe virus* in Indischem Patschuli und Erzeugung virusfreier Patschulipflanzen durch Meristemspitzenkultur

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Abstract

Patchouli (*Pogostemon cablin*) was found infected with a viral disease showing mosaic symptoms on the leaves in the Chandpur farm at Institute of Himalayan Bioresource Technology campus, Palampur, Himachal Pradesh, India. After ELISA, RT-PCR, IC-PCR and sequencing, the virus was identified as *Peanut stripe virus* (PStV). Sequence comparison with corresponding sequences of established potyviruses showed 52–93% homology at nucleotide level. *Peanut stripe virus* was eliminated from Patchouli plants using meristem tip culture. MS medium amended with benzylaminopurine (2 mg l^{-1}) and indole3-butyric acid (0.05 mg l^{-1}) was used for shoot proliferation and rooting of plants, respectively. More than 88.89% virus-free plants were obtained from 0.2 mm of meristem as shown by indirect ELISA whereas 80.55% virus-free plants were obtained from 0.3 mm size of meristem as indicated by RT-PCR.

Key words: IC-PCR, indirect ELISA, meristem tip culture, patchouli, *Peanut stripe virus*, RT-PCR

Zusammenfassung

Auf der Chandpur Farm des Institute of Himalayan Bioresource Technology, Campus Palampur, Himachal Pradesh, Indien, wurde eine Virose mit Blattmosaik-Symptomen an Indischem Patschuli (*Pogostemon cablin*) entdeckt. Mittels ELISA, RT-PCR, IC-PCR und Gensequenzierung wurde der Erreger als *Peanut stripe virus* (PStV) identifiziert. Ein Sequenzvergleich mit bekannten Potyviren ergab Homologien von 52–93% auf dem Nucleotidlevel. Das *Peanut stripe virus* konnte mit Hilfe der Meristemspitzenkultur aus den Patschulipflanzen eliminiert werden. Zur Sproß- und Wurzelbildung der Calli wurde MS-Medium verwendet, dem 2 mg ml^{-1} Benzylaminopurin bzw. 0,05 mg ml^{-1} Indolbuttersäure zugegeben wurde. Mehr als 88,89% virusfreie Pflanzen wurden, wie die indirekte ELISA zeigte, aus 0,2 mm großen Meristemstücken gewonnen, während die RT-PCR ergab, dass sich aus 0,3 mm großen Meristemen 80,55% virusfreie Pflanzen entwickelten.

Stichwörter: IC-PCR, indirekte ELISA, Meristemspitzenkultur, Patschuli, *Peanut stripe virus*, RT-PCR

1 Introduction

Pogostemon cablin (patchouli), a member of Labiatae, is a hairless herbaceous plant of about 1 m height. Whole plant contains essential oil (3–3.5%) but oil content of leaves is higher (30–40%). The oil is used as a base material in perfumery industries as it has strong fixative properties. It is also used as flavouring ingredient in many food products. It also possesses many antifungal and bacteriostatic properties (Anonymous 1977; Sharma 2002).

Patchouli is known to be infected by a number of viruses such as *Patchouli mosaic virus* (PaMV), *Tobacco necrosis virus* (TNV), *Patchouli mild mosaic virus* (PaMMV) and *Patchouli mottle virus* (PaMoV). In the present study, we are reporting for the first time infection of patchouli plants with *Peanut stripe virus* (PStV). PStV was earlier reported on peanut (*Arachis hypogaea* L.) from China (Xu et al. 1983) and later from India (Rao et al. 1989).

PStV is considered to be a peanut-infecting strain of *Bean common mosaic virus* (BCMV) (McKern et al. 1992; Vetten et al. 1992; Saiz et al. 1994). The BCMVsubgroup was previously subdivided into two groups namely serotypes A and B based on serological difference (Vetten et al. 1992). The authors proposed that these are in fact distinct potyvirus species.

PStV in groundnut has been detected successfully by ELISA and RT-PCR techniques (Jain et al. 2000). During survey of virus-infected plants from Chandpur field in the Institute of Himalayan Bioresource Technology (IHBT) Palampur campus, we came across patchouli plants displaying characteristic mosaic on the leaves (Fig. 1). These plants were tested for the possible presence of potyvirus using ELISA and RT-PCR techniques which confirmed its presence. After sequence analysis this isolate showed similarity to *Peanut stripe potyvirus*.

Several tissue culture techniques are being utilized for the production of virus-free plants. Meristem tip culture has been used for production of PaMMV-free plants (Sugimura et al. 1995) as well as PaMV free plants (Kukreja et al. 1990). Essential oil production have been increased using virus-free patchouli plants derived from meristem tip culture (Sugimura et al. 1995).

In the present study, we report occurrence of *Peanut stripe virus* in patchouli plants as well as production of virus-free plants using meristem tip culture. Patchouli is an economically important plant and production of virus-free plants will improve the quality of planting material and oil that can be extracted.

2 Materials and methods

2.1 Plant material

Infected material of *Pogostemon cablin* (Fig. 1) was collected from the fields of Chandpur area at IHBT Palampur campus. Plant leaves were harvested and stored immediately in liquid nitrogen for RNA extraction and further stored in a −80°C deep freezer.

2.2 Virus detection using enzyme linked immunosorbent assay (ELISA)

All the buffers, antibodies, enzyme conjugates, substrate and positive control were obtained for potyvirus group from Agdia (Elkhart, IN, USA) and standard protocols were followed as

Fig. 1: *Pogostemon cablin* (patchouli) showing *Peanut stripe virus* mosaic symptoms.

per manufacturer's instructions. The procedure followed was as described by CLARK and ADAMS (1977). Absorbance was taken at 405 nm with microplate reader (Bio-Rad, Hercules, CA, USA). Only those samples were considered positive whose reading was at least three times higher than the blank or negative control.

2.3 Detection of virus by RT-PCR and immunocapture RT-PCR (IC-RT-PCR)

Infected leaves of patchouli were used for the total RNA extraction using RNA Aqueous™ kit (Ambion, Austin, TX, USA). All the buffers used were provided in the kit and standard protocols were followed (as per manufacturer's instructions). RT-PCR was performed as described by VAN DER VLUGT et al. (1999) using degenerate primer pair cp9502 (5'-GCGGATCCTTTTTTTTTTTTTTTTT-3') specific for 3' end of potyviruses genome and cpup (5'-TGAGGATCCTGGTGYATHGARAAYGG-3', where Y = C/T, H = A/T/C, R = A/G), specific for coat protein region of potyviruses. Reverse transcription reaction for cDNA synthesis was carried out in 25 µl reaction mixture using 7 µl (1–2 µg) RNA, 0.2 µM downstream primer (p9502), 2 µl of 40 mM dNTP mix, 10 U human placental RNase inhibitor, 5 µl of 5 × RT buffer and 200 U of M-MLV reverse transcriptase enzyme (USB, Cleveland, OH, USA). The reaction mixture was incubated at 42°C for 75 min. PCR amplification was carried out in GeneAmp PCR9700 system (Applied Biosystems, Foster City, CA, USA) with 50 µl reaction mixture containing 7 µl of above synthesized cDNA, 0.2 µM upstream primer, 0.2 µM downstream primer, 5 µl of 10 × PCR buffer, 3 µl of 10 mM dNTP mix and 1.5 U of *Taq* DNA polymerase. Amplification was carried out for 45 cycles with denaturation at 94°C for 1 min, annealing temperature of 54°C for 2 min and elongation at 72°C for 1 min and final elongation at 72°C for 10 min. The amplified PCR-product was run on 1% agarose gel and visualized after staining with ethidium bromide. The amplified fragment was cloned in pGEM-Teasy vector (Promega, Madison, WI, USA) and sequenced.

For IC-PCR thin walled 0.5 ml microfuge tube was first treated for 15 min each in 0.1 n HCl and 4 n NaOH. After each treatment, the tubes were rinsed with PBST (0.02 M phosphate, 0.15 M saline, 0.05% Tween-20, pH 7.5), washed with 95% ethanol for 15 min and air dried at room temperature. The tubes were then coated with 100 µl of potyvirus group-specific antibody (Agdia) diluted to 1:500 in coating buffer (0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4°C. The tubes were washed three times with sterile distilled water and used immediately or stored at 4°C till use.

2.4 Sample preparation for IC-PCR

Infected leaf tissues (1 g) from *P. cablin* (patchouli) was ground in 5 ml of extraction buffer (phosphate-buffered saline, pH 7.4 containing 0.01 M sodium sulfate, 2% polyvinyl pyrrolidone (MW 40,000), 0.02% sodium azide, 0.2% powderd egg albumin and 2% Tween-20), with a mortar and pestle. The extract was centrifuged and the supernatant (100 µl) was loaded into the antibody coated microfuge tube. The tube was incubated at room temperature (22°C) for 2 h. The tube was then emptied and rinsed three times with autoclaved water. To destabilize the antibody bound virus particles, 70 µl sterile water was added to each tube which was then subjected to two cycles of alternate freezing (−80°C) for 10 min and thawing (70°C) for 5 min. The tubes were then transferred to ice for RT-PCR.

The IC-RT-PCR procedure was first optimized for the dilution of coating antibodies, plant sap, concentration of reverse transcriptase, number of amplification cycles and annealing temperature. A dilution of antibody mixture at 1:200, plant sap at 1:10, and using 100 U of M-MLV reverse transcriptase (Sigma, Bangalore, India) in a reaction volume of 20 µl was used for reaction. Primer annealing at 54°C for an additional 30 cycles gave clear bands in agarose gel eletrophoresis. Thereafter, these parameters were used in IC-RT-PCR.

2.5 Cloning and sequencing

Amplified coat protein gene was cloned in the pGEM-Teasy vector (Promega). Sequencing was performed in automated sequencer (ABI Prism 310) with universal primers, upstream T7 5' -TAATACGACTCACTATAGGG-3' and downstream SP6 5'-ATTTAGGTGACACTATAG-3' using the Sanger's dideoxy chain termination method (SANGER et al. 1977).

2.6 Sequence analysis

Sequence analysis was carried out with corresponding sequences of other established potyviruses from the EMBL database using BLAST. BLASTP was used to find out the amino acid sequence similarities. ALIGN-2 program using the DOTHELEX algorithm (TATUSOVA and MAIDEN 1999) was used for pair wise comparisons. MULTALIN program (CORPET 1988) was used to generate multiple alignments. Phylogenetic tree was constructed with the help of ClustalW (CHENNA et al. 2003) (www.ddbj.nig.ac.jp). The tree was subjected to bootstrap (1000 replicates) and was viewed with the help of TREEVIEW (PAGE 1996).

2.7 In vitro *production of* Peanut stripe virus-*free patchouli plants*

The infected plants of the *P. cablin* were used as source of explants (1–2 cm long shoot tips having two leaf primordia). The shoot tips were washed in running tap water for about half an hour followed by washing in the double distilled water containing few drops of Tween-20. For surface sterilization 80% ethanol (1 min) and $HgCl_2$ (30 s) were used in the laminar flow hood followed by washing with autoclaved double-distilled water. The shoot tips were then trimmed using sterilized blade to 1–2 mm and implanted vertically in the MS (MURASHIGE and SKOOG 1962) medium supplemented with benzylaminopurine (BAP) (2 mg l^{-1}). The pH of the medium was adjusted to 5.6 with 1 N NaOH and autoclaved at

121°C at 15 lb pressure for about 20 min. All the cultures were maintained at 25 ± 2°C under 16 h photoperiod with 70–80% relative humidity. Seven to 8 weeks after implantation of shoot tips, shoots which developed were sub cultured on half strength MS medium containing agar (6 g l^{-1}) and indole-3-butyric acid (IBA) (0.05 mg l^{-1}) for rooting. Well developed plantlets were washed with water to remove adhering agar and transferred to plastic pots containing sterilized soil mixture. These plants were then shifted to hardening chamber (80% RH, 16 h light, 8 h dark period and 25 ± 2°C temp). After 30-40 days, the plantlets were transferred to the sterilized pots containing sterilized soil in an insect proof net house. Then, the plants were checked for the presence of virus after regular intervals.

3 Results

3.1 *ELISA*

ELISA showed positive results in case of potyvirus (tests were carried out in triplicate). Infected samples showed A_{405} value of 0.909, 0.899, 0.900, negative control of 0.035, 0.032, 0.033 and positive control of 1.033., 1.034, and 1.032, respectively (triplicate). The results of ELISA confirmed the presence of a possible potyvirus as the antisera was group-specific (Agdia).

3.2 *RT/IC-PCR*

RT-PCR/IC-RT-PCR using potyvirus group-specific primers p9502 and cpup gave an amplification of about 800 bp of partial coat protein gene and 3' UTR in patchouli sample (Fig. 2a and b). This further confirmed the presence of a potyvirus. The amplified fragment was cloned and sequenced. The sequence was submitted to EMBL database with accession number AJ851894. This sequence contains 454 bp partial 3' region of CP gene of the virus. IC-PCR also gave desired amplification of 800 bp (Fig. 2b) corresponding to partial coat protein gene and 3' UTR in the sample. After cloning and sequencing, it showed 96% similarity to PStV and 88% to *Bean common mosaic virus*. PStV is considered to be a strain of *Bean common mosaic virus* (McKern et al. 1992).

When this partial sequence of coat protein gene was aligned with equivalent sequences from other potyviruses including *Peanut stripe virus*, *Plum pox virus*, *Zucchini yellow mosaic virus* etc., the sequence showed 66–96% identity at the nucleotide level while at the protein level the similarity values ranged from 72–96% (Table 1).

3.3 *Meristem culture and shoot multiplication*

In total about 80–85% of meristem tips sprouted within about three weeks of implantation and the differentiation of the shoots occurred in about 5–6 weeks. After multiplying the shoots in the same medium, 6–7 long shoots emerged from a single cultured meristem. Callus formation started from the base of the meristem and large number of shoots appeared (Fig. 3a and b).

3.4 *Effect of meristem size on establishment and virus elimination*

A small size meristem (0.1–1 mm) used in this study resulted in callus formation only. While 0.3 mm long meristem produced callus and shoots both and 80.55% plants produced from these cultures were found to be free of *Peanut stripe virus* (index by DAS-ELISA) and of these, 66.67% were found negative for PStV as indexed by RT-PCR. The percentage of obtaining the virus-free plants decreased with increase in size and 0.7–1.0 mm long meristem were not useful to raise virus-free plants (Table 2).

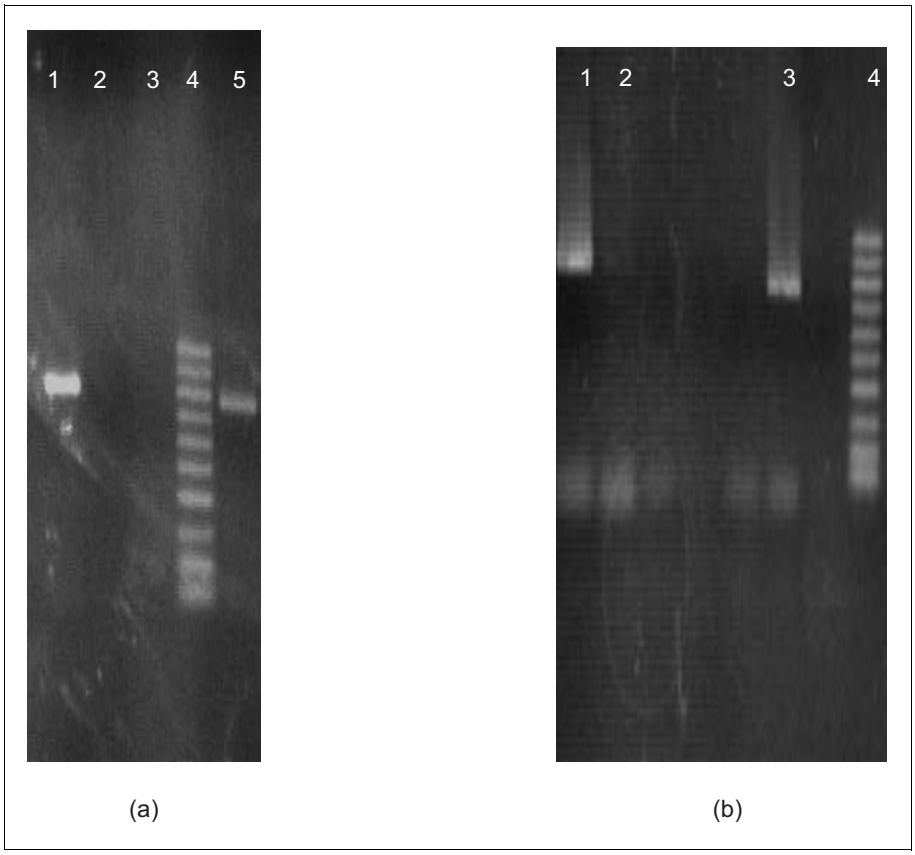

Fig. 2: IC-RT-PCR and RT-PCR analysis of *Peanut stripe virus*-infected patchouli plants. (a) Amplification of the potyvirus specific fragment from patchouli showing mosaic symptoms by IC-RT-PCR; lanes 1: positive control, 2-3: negative control, 4:100 bp marker and 5: CP gene amplified from patchouli plants. (b) Amplification by RT-PCR; lanes1: positive control, 2: negative control, 3: experimental samples, 4: 100-bp marker.

Table 1: Percent amino acid (below diagonal) and nucleotide (above diagonal) sequence similarities between partial CPs of different potyviruses

	PStV-I	PStV-A	ZYMV-H	PPV-H	NLSYV-C	DaMV-C	ABMV-J	CabMV-T	DMV-U	BCMV-M
PStV-I	X	96	76	69	67	74	91	89	88	88
PStV-A	96	X	77	69	68	74	92	91	90	90
ZYMV-H	87	88	X	66	70	75	77	77	77	72
PPV-H	72	71	73	X	72	66	68	67	67	63
NLSYV-C	72	72	78	80	X	61	69	67	67	68
DaMV-C	81	82	88	73	72	X	76	75	74	73
ABMV-J	95	97	87	71	75	84	X	93	93	91
CabMV-T	93	97	89	72	72	83	97	X	98	92
DMV-U	93	96	89	72	72	82	97	98	X	92
BCMV-M	93	96	82	63	71	80	95	95	95	X

PStV-I: *Peanut stripe virus* Indian isolate AJ851894.
PStV-A: *Peanut stripe virus* Australian isolateY11774.
ZYMV-H: *Zucchini yellow mosaic virus* Hungarian isolate AJ459955.
PPV-H: *Plum pox virus* Hungarian Isolate AJ000340.
NLSYV-C: *Narcissus late season yellows virus* Chinese isolate AJ493579.
DaMV-C: *Dasheen mosaic virus* Chinese isolate NC_003537.
ABMV- J: *Azuki bean mosaic virus* Japanese isolate AB012663.
CabMV-T: *Cowpea aphid-borne mosaic virus* Thailand isolate U72204.
DMV-U: *Dendrobium mosaic potyvirus* US isolate U23564.
BCMV-M: *Bean common mosaic virus* Mexican isolate L11890.

Fig. 3: Production of virus-free patchouli plants by meristem tip culture. (a) Initiation and production of shoots along with callus. (b) Rooting and proliferation of virus tested shoots.

3.5 Rooting, hardening and acclimatization

For rooting the shoots were transferred to MS medium containing 6 g l^{-1} agar and supplemented with IBA at the concentration of 0.05 mg ml^{-1}. Rooting was observed in all the cultures (100%). The establishment of roots in the medium took about 3–4 weeks. These rooted plants were then shifted to the hardening chamber.

4 Discussion

Basic techniques like ELISA and RT-PCR were used for the virus detection in patchouli plants showing mosaic, which confirmed the presence of a potyvirus. After cloning and sequencing, the virus isolate showed 96% identity with *Peanut stripe virus* (Australian isolate Acc. No. Y11774). Present isolate also showed 90% identity to *Bean common mosaic virus*. In earlier studies, PStV has been identified as a strain of *Bean common mosaic virus* (McKern et al. 1992). Sequence alignment at both nucleotide and amino acid level with other established potyviruses also confirmed its potyviral nature and its placement as PStV.

Tissue culture has been applied to a large variety of ornamental and medicinal plants (Brants and Vermeulen 1965). In the present study, meristem tips were cultured in MS medium supplemented with BAP (2 mg l^{-1}) for shoot proliferation and IBA (0.05 mg l^{-1}) for rooting. Several small shoots also arise from the callus formed that later on attained good growth. In an earlier study, Kukreja et al. (1990) have also used meristem tips to propagate *Pogostemon cablin* in MS medium with various growth regulators viz. BA, NAA etc. We did not examine

Table 2: Effects of different sizes of meristem tips on production of *Peanut stripe virus*-free patchouli plants

Size no.	Size of meristem (mm)	Tissue differentiation	No. of shoots grown *in vitro*	Virus indexing			
				DAS–ELISA no. of virus-free plants	% of virus-free plants	RT-PCR no. of virus-free plants	% of virus-free plants
1	0.1	callus	0	0	0	0	0
2	0.2	callus shoot	36	32	88.89 [a]	29	80.55 [a]
3	0.3	shoots	36	29	80.55 [ab]	24	66.67 [b]
4	0.4	shoots	36	23	63.89 [c]	17	44.44 [c]
5	0.5	shoots	36	18	50.00 [d]	14	38.89 [d]
6	0.6	shoots	36	3	8.34 [e]	1	2.79 [e]
7	0.7	shoots	36	0	0	0	0
8	0.8	shoots	36	0	0	0	0
9	0.9	shoots	36	0	0	0	0
10	1.0	shoots	36	0	0	0	0

Means in the column followed by different letters are statistically different according to the Duncan multiple range test ($P > 0.05$).

the essential oil production in virus-free plants in comparison to infected plants. However, studies conducted earlier on the raising of virus-free tissue cultured patchouli plants have confirmed that essential oil production has been increased by using virus-free patchouli plants (SUGIMURA et al. 1995).

Small size of meristem tips (0.2–0.3 mm) was found to be optimum for eliminating PStV from patchouli. Large sized meristems (0.7–1.0 mm) carried the virus with them and all the shoots produced from them were found to be the infected.

The RT-PCR described here is efficient technique for typing PStV. Meristem tip culture was established as a reliable approach for production of PStV-free patchouli plants.

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