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Sensitive detection of a phytoplasma associated with little leaf symptoms in *Withania somnifera*

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Abstract

Withania somnifera is an important medicinal plant native to the Indian-sub continent. Owing to the presence of a number of precious alkaloids, flavonoids and withanolides, it is widely used in the Indian and African systems of medicines. It is severely affected by phytoplasma present in the sieve tubes of phloem. With a view to micropropagate phytoplasma-free *W. somnifera* plants, an efficient and effective nested PCR-based system was developed for detection of associated phytoplasmas. Universal primers, designed from the 16S rDNA sequences of phytoplasmas, were applied in direct/nested-PCR. Total DNA extracts from leaf tissues of 33 suspected symptomatic and 11 non-symptomatic plants were subjected to direct PCR. The direct PCR products were subsequently employed as templates in nested PCR. The nested PCR could reamplify direct PCR products yielding a DNA fragment of 1.4 kb. A phytoplasma was detected in all the diseased plants and not from the healthy looking plants. Further, it was sensitive enough to amplify phytoplasma DNA obtained from crude DNA diluted up to 2500 times from naturally infected plants and also from various stages of *in vitro*-propagated diseased plants. Identical restriction fragment polymorphism enzyme profiles were obtained following restriction enzyme digestion of nested PCR products, obtained from five different plants, by *Eco*RI, *Alu*I and *Rsa*I restriction endonucleases. The developed nested PCR based system should facilitate indexing of the phytoplasma in different stages of *in vitro*-generated plants and probably identification of, as yet unknown, hosts and vectors of phytoplasma associated with phytoplasma disease of *W. somnifera*.

Abbreviations: PCR – polymerase chain reaction; RFLP – restriction fragment length polymorphism

Introduction

Withania somnifera is an evergreen shrub of the family Solanacea. It is cultivated as a commercial medicinal crop in India, Pakistan, Sri Lanka and Africa. Known by numerous names such as Ashwagandha, Winter cherry, Indian Ginseng, *W. somnifera* is a main source of alkaloids, flavanoids and withanolides (Anon., 1976). It possesses inhibitory activity in the growth of tumour, various gram-positive bacteria and pathogenic fungi. In India, the plant is used in more than 100 drug preparations for the cure of several important diseases like cancer, arthritis, gynaecological disorders, fungal infection, diuresis and tuberculosis (Duke, 1985; Asthana and Raina, 1989). The pharmacological activity of the roots is attributed to the presence of several alkaloids.

Besides being infected by a fungus, nematode and virus (Pathak and Raychoudhuri, 1967; Bhatti et al., 1974; Mahrshi, 1986), a phytoplasma associated with witches'-broom disease of *W. somnifera*

was demonstrated by electron microscopic studies (Zaim and Samad, 1995). However, basic information about its host range and possible insect vectors is not yet available and the causal phytoplasma still remains uncharacterised. Phytoplasmas cause changes in the concentration of important metabolites and some of these are involved in the defence mechanisms. The effect of the phytoplasma on the accumulation of total alkaloid has been studied. It was shown that clover phyllody phytoplasma infection causes an increase in alkaloid production in *Catharanthus roseus* (Favali et al., 2004).

National Botanical Research Institute (NBRI), Lucknow, India, maintains a rich collection of *W. somnifera* germplasms. Further, NBRI has undertaken a research programme aimed at eliminating phytoplasmas/viruses, associated with important medicinal plants, through tissue culture. Since phytoplasmas are unevenly distributed and present in low titres particularly in the apical meristem of their hosts, it is highly desirable to have a sensitive detection system. Electron microscopic techniques are suitable when the titre of phytoplasma is relatively high in host tissues. PCR-based amplification of 16S rDNA of phytoplasma has greatly contributed to the identification and characterisation of unidentified phytoplasmas (Lee et al., 1993, 1998; Seemüller et al., 1998; Bertaccini et al., 2005). It is more sensitive than microscopic, serological and hybridization assays. For micropropagating phytoplasma-free *W. somnifera* plants, it is a prerequisite to have a sensitive detection system. Meristem tip culture offers an effective way of eliminating phytoplasmas from infected tissues. The success of meristem culture, however, depends on the selection of meristem tips and efficient regeneration. While longer tips are likely to be infected, smaller ones are difficult to regenerate via tissue culture. Therefore a sensitive PCR is necessary to detect low levels of phytoplasmas in very small tips of plants for the elimination of phytoplasmas, and screening of the pathogen at different stages of *in vitro* culture, such as explants, calli, differentiated plantlets and mature plants before hardening. After applying *in vitro* phytoplasma elimination procedures, an effective and sensitive detection system is a must for their periodic indexing against phytoplasma infection to ensure that they remain phytoplasma free. The objective of this study was to detect phytoplasmas associated with *W. somnifera* by the use of nested PCR. This paper describes the presence of phytoplasmas in crude DNA samples prepared from (i) symptomatic and asymptomatic (healthy looking) leaves of *W. somnifera* plants, (ii) different stages of *in vitro*-grown phytoplasma culture and (iii) reports the sensitivity of nested PCR for detection of associated phytoplasma.

Materials and methods

Phytoplasma source

Detection of phytoplasma was attempted from both naturally infected as well as *in vitro*-propagated infected culture.

Naturally infected plants

Symptomatic leaves were collected from 33 phytoplasma-suspected diseased *W. somnifera* plants and 11 seemingly healthy plants from different experimental plots of NBRI, during winter time (November 2004–February 2005) when symptoms were distinct and more pronounced. One sample was taken from each plant for further processing. In summer diseased plants escaped symptoms; eight such plants were randomly selected and leaves were collected from these healthy looking plants to monitor phytoplasma infection.

In vitro *culture*

Apical meristems of naturally infected diseased plants were used for establishing an *in vitro* propagation system for rapid multiplication and maintenance of phytoplasma culture. Apical meristems (>3 mm) from phytoplasma-infected *W. somnifera* plants were excised, surface-sterilized and aseptically inoculated into Murashige and Skoog's (MS) medium supplemented with different concentrations of cytokinins and auxins (6-benzylamino purine 1.5 mg l^{-1}; indole-3-butyric acid 0.1 mg l^{-1}; Murashige and Skoog, 1962; Sen and Sharma, 1991).

Total DNA isolation

Fresh leaf tissues (0.5 g) were collected during winter time from a total of 33 diseased and 11 seemingly healthy plants of *W. somnifera*. Apart

from these, during summer time leaf samples were collected from eight apparently healthy looking plants that had exhibited symptoms during winter. Total DNA was isolated from fresh tissues of each sample (naturally infected apical meristem, leaf and stem *in vitro* culture) using a phytoplasma enrichment procedure (Ahrens and Seemüller, 1992). The resulting nucleic acid extracts were precipitated with isopropanol, resuspended in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before RNAse treatment at 37 °C for 1 h and subjected to PCR.

Nested PCR-based detection of phytoplasmas

Two sets of universal primers were used to amplify 16S rDNA of the phytoplasma associated with *W. somnifera*. The P1 and P7 primer pair amplifies a 1.8 kb fragment of the phytoplasma that includes the 16S rRNA gene, intergenic spacer and 23S rDNA region (Deng and Hiruki, 1991; Schneider et al., 1995). The second primer pair R16mF2/R16mR1 was nested within the annealing positions of primers P1 and P7 along 16S rDNA (Schneider et al., 1995). The PCR comprised of two steps i.e. direct PCR followed by nested PCR.

In direct PCR, the template consisted of total DNA (1 μl) extracted from each of symptomatic and asymptomatic leaves. A total of 50 μl PCR mixture contained 125 μM of dNTPs, 40 pmole each of the upstream and downstream primers, 10× PCR reaction buffers, 1.5 mM $MgCl_2$ and 1.5 U *Taq* DNA polymerase. The PCR parameters consisted of 25 cycles of denaturation at 94 °C for 1 min (4 min for the first cycle), annealing at 48 °C for 1 min, and extension at 72 °C for 2 min. The last cycle was extended for 5 min. An aliquot of 5 μl of each direct PCR product was analysed by electrophoresis in 1% agarose gel containing ethidium bromide and visualised with a UV transilluminator.

One μl each of direct PCR product was reamplified in nested PCR. The components of nested PCR mixtures were the same as described for direct PCR except the primers. The nested PCRs were primed using the second set of primers R16mF2/R16mR1. A total of 50 μl of PCR mix was subjected to 30 thermal cycles with denaturation at 94 °C for 1 min (5 min for the first cycle), annealing at 50 °C for 2 min, extension at 72 °C for 3 min, which was extended for 5 min in the last cycle. An aliquot of 5 μl was analysed in 1% agarose gel as mentioned in direct PCR and photographed.

Sensitivity of nested PCR

To determine the sensitivity of nested PCR, 1 μl of crude DNA from symptomatic leaf tissues was diluted with sterile deionised water in various dilutions (1:10, 1:100, 1:1000, 1:2000, 1:2500 and 1:3000) and subjected to direct PCR. One μl of direct PCR of each sample served as the template in nested PCR. The oligo primers, PCR conditions and parameters were the same as described for nested PCR. An aliquot of 5 μl was run on agarose gel, viewed and photographed.

RFLP analyses

Nested PCR-amplified DNA fragments obtained from five different diseased plants were subjected to single restriction endonuclease digestion with *Eco*RI, *Alu*I and *Rsa*I as per the manufacturer's instruction (New England Biolabs). The products of digestion were analysed through 5% non-denaturing polyacrylamide gel electrophoresis followed by staining with ethidium bromide and visualization of the DNA band by UV transillumination.

Cloning of nested-PCR product, sequencing and phylogenetic analysis

The nested PCR-amplified DNA fragment, obtained from one diseased plant, was cloned into vector pUC 19 at *Sma*I site using SureClone Ligation kit following the supplier's instructions (Amersham Biosciences). One DNA clone was sequenced by an automatic sequencer. The sequences were deposited in GenBank and aligned with those of 16S rDNA of other related phytoplasmas using BLAST searches (Altschul et al., 1997).

Multiple sequence alignment was done by Clustal W (Thompson et al., 1994) using corresponding 16S rDNA sequences derived from Aster yellows group (16SrI) phytoplasma strains (Table 2).

A phylogenetic tree was obtained by the neighbour-joining method (Saitou and Nei, 1987) from

the distance matrix using Clustal W (1.82) and drawn by NJ plot (Perrière and Gouy, 1996).

Results

Phytoplasma symptoms

The symptoms of the diseased plants consisted of reduction in leaf size, shortening of internodes and dwarfing. Affected plants showed discolouration of leaves ranging from light green to yellow. The most affected 14 plants had small, yellow leaves with necrosis and remained stunted in winter, while 30 suspected diseased plants had light green leaves, the size of the leaf/plant was not shortened and it was difficult to make clear distinctions.

Phytoplasma detection

Direct PCR did not yield any DNA fragment from all the DNA samples (results not shown). Nested PCR based assays using samples from symptomatic plants yielded a DNA fragment of 1.4 kb. A phytoplasma was detected in leaf samples of 33 phytoplasma-suspected symptomatic plants, while 11 plants were PCR negative. Positive detections were indicated by strong, moderate or weak amplification of 16S rDNA of 1.4 kb (Figure 1; Table 1). From symptomatic plants, eight plants were randomly selected for monitoring of the phytoplasma during summer time when the symptoms were absent in *W. somnifera*. Interestingly, none of the eight plants yielded the DNA fragment in nested PCR (results not shown). Apical meristems (natural infection) and leaf and stem *in vitro* culture tested positively in nested PCR (Table 1).

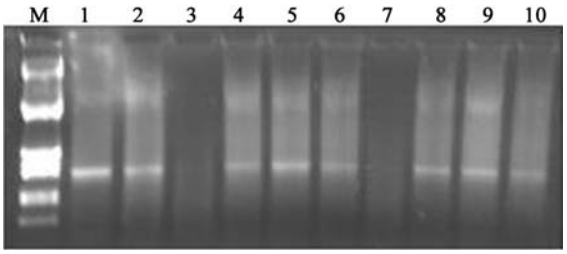

Figure 1. Agarose gel electrophoresis showing nested-polymerase chain reaction (PCR) amplification of 1.4 Kb 16S rDNA of phytoplasma, employing universal primer pair R16mF2/R16mR1. The template consisted of direct PCR product obtained from crude DNA extracts isolated from leaf tissues of different naturally infected symptomatic (lanes 1, 2, 4–6, 8–10) and asymptomatic (lanes 3, 7) *W. somnifera* plants. Positive detections are represented by DNA fragments of strong (lane 1), moderate (lanes 2, 5, 9) or weak (lanes 4, 6, 8, 10) intensities. M = λ DNA digested with *Eco*RI and *Hin*dIII, fragment sizes (bp) from top to bottom 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564.

Table 1. Phytoplasma detection in *Withania somnifera* plants by nested PCR

Sample	Total number	Positive	Negative	Figure
Naturally infected				
Yellow leaf	14	14	None	1
Light green leaf	30	19	11	1
Apical meristem (> 3 mm)	10	10	None	
In vitro *phytoplasma culture*				
Leaf	10	10	None	
Stem	10	10	None	

Sensitivity of PCR

A 1.4 kb PCR product was visualized for nested PCR products when the direct PCR products, obtained from crude DNA extracts (symptomatic leaves) diluted to 1:2500, served as templates for nested PCR. There was however, no detection with 1:3000 diluted DNA (Figure 2). Positive detections were indicated by strong, moderate or weak amplification of the 16S rDNA product depending upon its dilution. Nested PCR was sensitive enough to reamplify the direct PCR product sampled from DNA diluted 2500 times.

RFLP analyses

PCR products amplified from five different phytoplasma-affected plants after digestion with the restriction enzymes *Eco*RI, *Alu*I, *Rsa*I, resulted in identical RFLP profiles for each enzyme indicating that all five infected plants contained the same phytoplasma. Representative RFLP profiles for five 16S rDNA of *Withania* phytoplasma are shown in Figure 3.

Table 2. Name and GenBank accession numbers of phytoplasma 16S rDNA sequences used in this study

Phytoplasma strains or Associated disease	16S rRNA group/subgroup	GenBank Accession
Aconitum proliferation phytoplasma (AcP)	16SrI-A	AF510323
Withania phytoplasma (WP)	This study	DQ151998
Aphanamixis polystachya (AphY)	16SrI-B	AY495702
Aster yellows (Btsv2S)	16SrI-B	AY180943
Hydrangea phyllody (HYPh)	16SrI-B	AY265219
Clover phyllody (CP-KVG)	16SrI-C	AY265218
Japanese hydrangea phyllody (JHP)	16SrI-D	AB010425
('*Candidatus* Phytoplasma japonicum')		
Blueberry stunt (BBS1)	16SrI-E	AY265220
Silene virescence (SiVir)	16SrI-M	AY744070

AcP, AphY, Bstv2S, HYPh, CP-KVG, JHP, BBS1 and SiVir are strains of *'Candidatus* Phytoplasma asteris

Nucleotide sequencing and phylogenetic analysis

Nucleotide sequences of one 16S rDNA clone (1432 bp) have been submitted to GenBank as accession number DQ151998. Sequence identities between the 16S rDNA derived from the *Withania* phytoplasma and the corresponding regions of other phytoplasmas (listed in Table 2) were determined. A comparative sequence alignment showed that the *Withania* phytoplasma shared 99% identity with those of strains assigned to the Aster yellows group (16Sr I) (Table 3).

A phylogenetic tree, constructed using 16S rDNA sequences of one *Withania* phytoplasma isolate and eight '*Candidatus* Phytoplasma asteris' strains representing the Aster yellows group (16SrI), revealed a close relationship, thus supporting affiliation of the *Withania* phytoplasma isolate to this group (Figure 4).

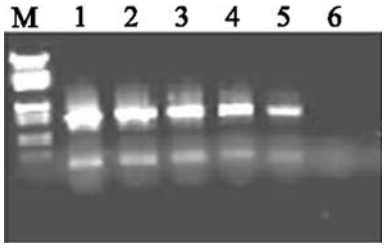

Figure 2. Agarose gel electrophoresis showing nested-polymerase chain reaction (PCR)-based amplification of 1.4 kb 16S rDNA of phytoplasma associated with *W. somnifera*, employing universal primer pair R16 mF2/R16 mR1. The template consisted of direct PCR product obtained from crude DNA extracts (isolated from infected leaves of *W. somnifera*) in dilution of 1:10 (lane 1); 1:100 (lane 2); 1:1000 (lane 3); 1:2000 (lane 4); 1; 2500 (lane 5); 1:3000 (lane 6). M = λ DNA digested with *Eco*RI and *Hind*III.

Discussion

So far little information is available on phytoplasma(s) associated with *W. somnifera*. An initial study had demonstrated its presence in phloem tissues of infected plants showing witches'-broom symptoms by means of electron microscopy (Zaim and Samad, 1995). This technique, however, has limitations due to low titre of phytoplasmas and their uneven distribution in infected plants. Sensitive detection of phytoplasmas is an important component in micropropagating phytoplasma-free plants.

A sensitive nested PCR has been developed for the detection of the *Withania* phytoplasma. It was shown that oligo primer pairs (P1/P7 and R16mF2/R16mR1) designed from 16S rDNA sequences of phytoplasmas could effectively detect the associated phytoplasma occurring in diseased *W. somnifera* plants. With this procedure, phytoplasma DNA could be detected from direct PCR product obtained from 2500 times diluted crude DNA extracts of infected leaf tissues.

It is generally assumed that the titre of phytoplasmas is very low in shoot tips of the plants and meristem remains the ideal choice for micropropagation of phytoplasma-free plants. However, the size of explants is very crucial for the success of micropropagation-based elimination programmes. Meristem tips >1 mm are likely to be infected, while those <0.3 mm are difficult to regenerate.

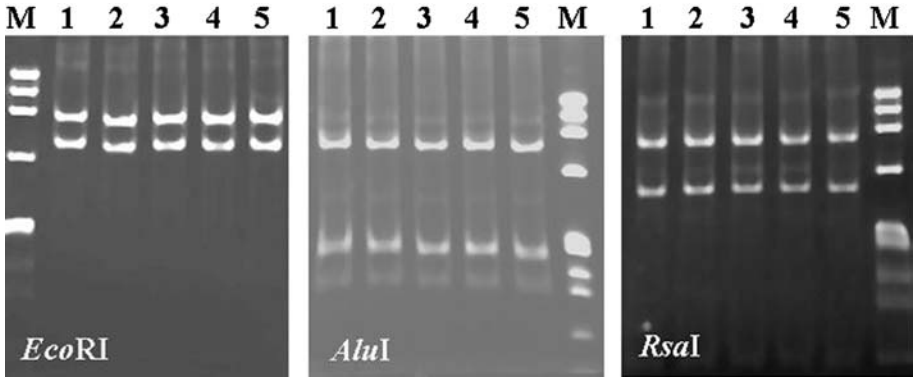

Figure 3. *Eco*RI, *Alu*I and *Rsa*I digested RFLP of nested PCR amplified 16S rDNA (1.4 kb) of phytoplasma derived from five different naturally infected *Withania somnifera* plants (lanes 1–5). M = φX 174 *Hae*III digests, fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281/271, 234, 194, 118.

Therefore, all the established meristem tips are not necessarily pathogen-free and require a sensitive detection system for their indexing (Parmessur et al., 2002). The nested PCR-based approach, developed in this study, to detect the phytoplasma associated with *W. somnifera* should allow detection of the phytoplasma in as little as 2 μg of fresh infected tissues under the experimental conditions described in this paper.

The direct PCR product did not result in apparent banding in agarose gel electrophoresis. Southern hybridization also did not reveal any positive signals when direct PCR products were probed with homologous labelled DNA (results not shown). Specific reamplification of direct PCR products in nested PCR, however, clearly demonstrates that the concentration of the 16S rDNA of the targeted phytoplasma in direct PCR-amplified product was below the detection limit in ethidium bromide-stained gels or Southern hybridization assay. A lack of visible specific PCR products from crude nucleic acid samples could be due to the presence of DNA at a concentration below the detection limit in ethidium bromide-stained agarose gels. Besides this, insufficient phytoplasma concentration in DNA template or the presence of *Taq* polymerase inhibitors in the PCR mixture may also influence the results obtained.

In this study, symptomatic plants that tested positively, had escaped symptoms in summer and yielded negative results in nested PCR. Partial recovery of *W. somnifera* phytoplasma symptoms during summer is a well-established phenomenon (Zaim and Samad, 1995).

Nucleotide sequence data and phylogenetic studies based on the single 16S rDNA clone cluster *Withania* phytoplasma together along with other characterized members of the Aster yellows group (16SrI). It is, therefore, proposed that the *Withania* phytoplasma be assigned to the Aster yellows

Table 3. Percentage nucleotide sequence identities of 16S rDNA from selected '*Candidatus* phytoplasma strains' (16SrI) with *Withania* phytoplasma isolate

	AphY	JHP	Btsv-2S	HYPh	CP-KVG	AcP	BBS1	SiVir	*A. laidlawii*
WP	**99**	94	**99**	**99**	98	98	**99**	**99**	78
AphY		95	98	**99**	98	98	**99**	**99**	79
JHP			95	95	95	95	95	95	80
Btsv2S				**99**	98	98	**99**	**99**	80
HYPh					98	98	**99**	**99**	81
CP-KVG						98	**99**	98	81
AcP							**99**	**99**	82
BBS1								**99**	80

Designation given to strains and their GenBank accessions are mentioned in Table 2.

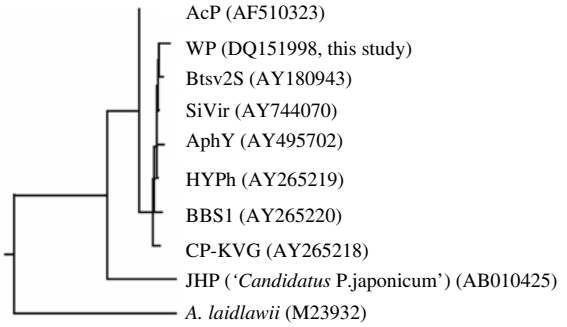

Figure 4. Phylogenetic tree of 16S rDNA sequences from selected strains of '*Candidatus* Phytoplasma asteris' (16SrI) and *Withania* phytoplasma employing *Acholeplasma laidlawii* as the outgroup, constructed by the neighbour-joining method. Designations given to strains and their GenBank accessions are given in Table 2.

group. This is the first report of the identification of a phytoplasma infecting *W. somnifera* at the group level. Nucleotide sequences and RFLP analyses of 16S rDNA of several isolates with a set of restriction endonucleases may help in classifying this phytoplasma to a subgroup level (Lee et al., 1998). Identical RFLP patterns obtained from five different diseased plants showed that a similar phytoplasma was present in all the samples tested. *Eco*RI, *Alu*I and *Rsa*I-generated profiles of 16S rDNA of *Withania* phytoplasma may help in the identification of an unknown phytoplasma associated with *W. somnifera*, together with unknown hosts and vectors in this region.

In conclusion, a phytoplasma associated with *W. somnifera* can be detected by means of nested PCR. It is sensitive enough to detect the phytoplasma in crude DNA extract diluted 2500 times. The system has the potential to detect possible infection of the phytoplasma in micropropagted phytoplasma-free *W. somnifera* plants.

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