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Short communication

Detection and characterization of the phytoplasma associated with a phyllody disease of black pepper (*Piper nigrum* L.) in India

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Abstract

Using polymerase chain reaction (PCR), the phytoplasma was detected in black pepper (*Piper nigrum*) with phyllody symptoms in India. A 1.20 kb DNA fragment encoding the portion of phytoplasma 16S rDNA consistently amplified by nested PCR was cloned and sequenced. The sequenced region contained 1230 nucleotides. Sequence analyses showed that the gene was most closely related to members of aster yellows group (16Sr I) of phytoplasma. The sequence identity with members of aster yellows group (16Sr I) was >98% while that with members of other groups (16Sr II to 16Sr XV and other undesignated groups) ranged from 87 to 96%. On the basis of sequence identity and phylogenetic relationship studies, it is concluded that phytoplasma infecting black pepper in India belongs to aster yellows group. This is the first report of identification of phytoplasma in black pepper.

Keywords: Black pepper; Phyllody disease; 16S rDNA sequence; Sequence analyses; Aster yellows phytoplasma

1. Introduction

Phytoplasmas are non-helical mollicutes causing diseases characterized by flower malformation, growth aberrations, yellowing and/or decline in many plant species (Seemuller et al., 1998; Lee et al., 2000). Phytoplasmal diseases are spread by leaf hoppers and plant hoppers besides spread by vegetative propagation through cuttings, storage tubers, rhizomes or bulbs. In recent years molecular methods have been used to identify and differentiate diverse phytoplasmas. Phylogenetic analysis of 16S rDNA sequences and/or RFLP analysis of PCR amplified 16S rDNA sequences were used to differentiate various phytoplasmas (Lee et al., 1993; Seemuller et al., 1998). Based on the phylogenetic analysis of 16S rDNA gene sequence, 20 distinct phytoplasma groups were identified (Seemuller et al., 1998).

Black pepper, obtained from dried berries of *Piper nigrum* L., is an important spice of international commerce for many south east Asian countries. India is a leading producer of black pepper in the world and the crop is grown in an area of 1,89,804 ha with a production of 71,160 mt (*Source*: International Pepper Community, Jakarta, Indonesia). Phyllody disease on black pepper was first reported during 1986 from Wyanad District of Kerala, India (Sarma et al., 1988). The disease was characterized by malformation of the entire spike and the affected vines showed conspicuous tufts of malformed branches giving a witches broom appearance with yellowing symptoms that were well discernible from a distance (Sarma et al., 1988). In a recent survey, the occurrence of the disease was also reported from other black pepper growing regions of Kerala state, India. The incidence of the disease was severe in areas adjacent to forests and association of two types of plant hoppers was seen with diseased vines (Anonymous, 2002). Based on the symptomatology, involvement of a phytoplasma was suspected in the disease. This paper reports the cloning and sequencing of 1.2 kb fragment of 16S rDNA of the

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phytoplasma from phyllody disease affected black pepper and its identification.

2. Materials and methods

2.1. Phyllody isolates and nucleic acid extraction

Ten phyllody disease affected black pepper samples collected from Kozhikode District of Kerala state, India were used in this study. Spikes collected from asymptomatic black pepper plants from Kannur District of Kerala state, India were used as healthy control. Total DNAs were extracted from healthy and malformed black pepper spikes using DNA Plant kit (Macherey-Nagel, Duren, Germany). Total DNAs extracted from a known phytoplasma (periwinkle little leaf) was used as positive control. Water control (without template DNA) was also included in all the PCR reaction to check for contamination if any.

2.2. Nested PCR

The total genomic DNA was subjected to nested PCR using universal primers designed to amplify a specific sequence within the 16S rDNA of phytoplasmas (Gundersen and Lee, 1996). Primers P6 (5′ CGGTAGGGATCACTTGTTACGACTTA 3′) (Deng and Hiruki, 1991) and SN910601 (5′ CGAAAAAACCTTACCAGGTCTTTG 3′) (Namba et al., 1993) were used for the first round amplification of the 16S rDNAs. For the second round, to amplify an internal fragment of the 16S rDNA, primers R16F2n (5′ GAAACGACTGCTAAGACTGG 3′) corresponding to bases 144–163 and R16R2 (5′ TGACGGGCGGTGTGTACAAACCCCG 3′) corresponding to bases 1365–1386 (Lee et al., 1993) were used. The PCR reaction (100 μl) contained 200 ng each of the primers, 2.5 units Taq Polymerase 1× PCR buffer, 1.25 mM $MgCl_2$ and 10 μM each of the dNTPs. PCR mix (27 μl) containing the above components was added to the tubes containing the template DNA (73 μl)

Table 1
GenBank accession numbers of 16S rDNA gene sequences used in this study

Phytoplasma strain	Designation	16S rDNA group affiliation	GenBank accession number
Black pepper phyllody	BPP		AY823413 (this study)
Tomato big bud	TBB	Aster yellows (16Sr I)	L33760
American aster yellows	AAY		X68373
Aster yellows	AY		AY180952
Maize bushy stunt	MBS		AF487779
Mulberry dwarf	MD		AY075038
Onion yellows	OY		D12569
White clover phyllody	KVG		X83870
Blueberry stunt	BBS		AY265220
Apricot chlorotic leaf roll	ACLR		X68338
Chrysanthemum yellows	CyB		AY265214
Peanut witches' broom	PnWB	Peanut witches' broom (16Sr II)	L33765
Western X-disease	WX	X disease (16Sr III)	L04682
Coconut lethal yellowing	LY	Coconut lethal yellows (16Sr IV)	U18747
Elm yellows	EY1	Elm yellows (16Sr V)	AF189214
Elm yellows	ULW		X68376
Lm yellows	WVEY		AF122911
Alder Yellows	ALY		Y16387
Hemp dogbane phytoplasma	HD1		AF122912
Flavescense dorée	FD		X76560
Rubus stunt	RS		Y16395
Brinjal little leaf	BLL	Clover proliferation (16Sr VI)	X83431
Ash yellows	AshY	Ash yellows (16Sr VII)	AF189215
Loofah witches broom	LFWB	Loofah witches broom (16Sr VIII)	AF248956
Pigeon pea witches' broom	PPWB	Pigeon pea witches' broom (16Sr IX)	AF248957
Apple proliferation	APU	Apple proliferation (16Sr X)	AF248958
Rice yellow dwarf	RYD	Rice yellow dwarf (16Sr XI)	D12581
Australian grapevine yellows	AGY	Stolbur (16Sr XII)	L76865
Mexican periwinkle virescence	MPV	Mexican periwinkle virescence (16Sr XIII)	AF248960
Bermuda grass white leaf	BGWL	Bermuda grass white leaf (16Sr XIV)	AF248961
Hibiscus witches broom	HibWB	Hibiscus witches' broom (19Sr XV)	AF147708
Other undesignated groups			
Italian bindweed stolbur	IBS	Italian bindweed stolbur	Y16391
Buckthorn witches' broom	BWB	Buckthorn witches' broom	X79431
Spartium witches broom	SPAR	Spartium witches broom	X92869
Italian alfalfa witches' broom	IAWB	Italian alfalfa witches' broom	Y16390
Cirsium phyllody	CirP	Cirsium phyllody	X83438
Acholeplasma laidlawii	*A. laidlawii*	NA	M23932

resulting in a final reaction volume of 100 μl. Amplification was performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) programmed for one cycle of 94 °C for 3 min followed by 35 cycle reaction profile involving 30 s of denaturation at 94 °C, 60 s of annealing at 53 °C and 90 s of extension at 72 °C and single cycle of final extension at 72 °C for 10 min for the first round amplification. The reaction product (1 μl) of first round amplification was used as template for the second round of amplification with similar reaction profile except for the annealing step, which was carried out at 56 °C (instead of 53 °C).

2.3. Cloning of PCR product and sequencing

Following PCR, reaction products were analysed by 1% agarose gel electrophoresis in Tris–acetate EDTA (TAE) buffer containing ethidium bromide (0.4 μg/ml). DNA was visualized and photographed using a UV transilluminator and a gel documentation apparatus (Alpha Innotech Corporation, CA, USA). 500 bp ladder was used as a size standard. The PCR product was purified using Strata Prep PCR purification kit (Stratagene, LaJolla, CA, USA) followed by polishing the purified PCR product using P*fu* DNA Polymerase (Stratagene, LaJolla, CA, USA) and dNTP mix. The resultant product was then cloned into pPCR Script Amp SK (+) cloning vector using pPCR Script Amp SK (+) cloning vector kit (Stratagene, LaJolla, CA, USA) and competent *Escherichia coli* (strain DH5α) were transformed by following standard molecular biology procedures (Sambrook and Russell, 2001). Recombinant clones were identified by restriction endonuclease digestion, and selected clones were sequenced with automated sequencing of Applied Biosystems (ABI prism) following dideoxy chain terminator sequencing protocol (Perkin-Elmer).

2.4. Sequence analyses

Sequence data were compiled using Seqaid Version 3.6 (Rhoads and Roufa, 1985). Multiple sequence alignments were made using Clustal W (Thompson et al., 1994). Phylogenetic tree was constructed by Neighborhood Joining Bootstrap method (Bootstrap analysis with 1000 replicates) in Clustal X (1.81) and rooted tree was generated using TREEVIEW software (Win 32) (Page, 1996). The 16S rDNA nucleotide sequences of other phytoplasma isolates used for comparison (Table 1) were obtained from GenBank (Benson et al., 1999). The BLAST programme (Altschul et al., 1997) was used to identify related sequences available from the GenBank database.

3. Results and discussion

The affected plants showed various kinds of malformation of vegetative and floral parts. The affected vines showed conspicuous tufts of malformed branches giving a witches broom appearance with yellowing symptoms (Fig. 1a). The stalk of affected spike increased in length, the bracts and the flowers were transformed into small leaf like structures and floral buds of the affected spikes were transformed into small branches with nodes and internodes similar to fruiting laterals (Fig. 1b). Malformed fruiting laterals produced aborted flower buds/small leaf like structures. DNA extracted from these malformed budtips was used as template in PCR. In the case of first round PCR, use of primers SN910601/P6 failed to yield visible amplified DNA

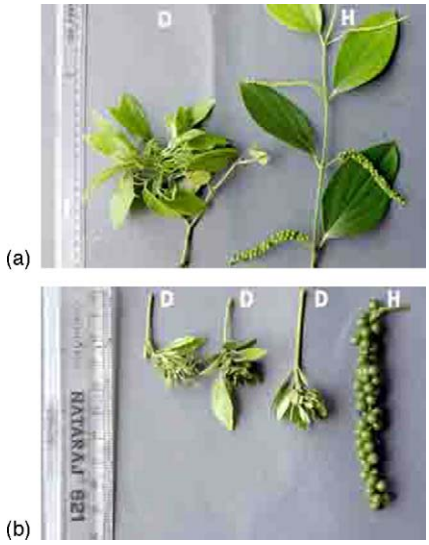

Fig. 1. (a) Lateral branch from healthy (H) and phyllody disease affected (D) black pepper showing malformed branches giving witches broom appearance. (b) Healthy (H) and phyllody disease affected black pepper spikes (D) showing malformation of bracts, flowers into small leaf like structures and floral buds into small branches.

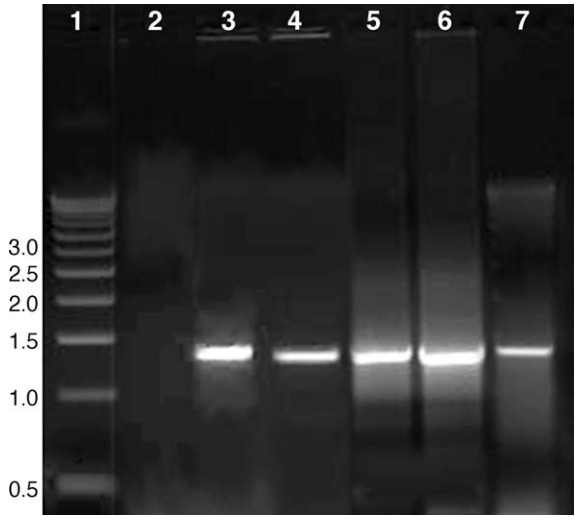

Fig. 2. Agarose gel showing nested-PCR amplification of 1.2 kb fragment of 16S rDNA. The template consisted of the product of direct PCR obtained from total DNA extracted from healthy and phytoplasma affected plants: lane 1: 500 bp DNA size ladder and numbers on the left indicate their size in kb; lane 2: healthy black pepper (negative control); lane 3: periwinkle little leaf (positive control); lanes 4–7: phyllody affected black pepper samples.

Table 2
Percent nucleotide sequence identities of 1.2 kb sequences of 16S rDNAs from selected phytoplasma isolates classified in group 16Sr I (aster yellows) and 16Sr V (elm yellows) with phytoplasma isolate from black pepper

	16Sr I (aster yellows group)										16Sr V (elm yellows group)						
	TBB	AAY	AY	MBS	MD	OY	KVG	BBS	ACLR	CyB	ALY	FD	HD1	RS	ULW	WVEY	EY1
BPP	98.8	98.9	99.4	99.4	99.3	99.4	98.7	99.0	98.8	98.7	87.4	87.0	87.2	87.6	87.6	87.9	88.0
TBB		98.9	99.4	99.4	99.3	99.4	99.0	99.2	98.8	99.6	88.3	87.8	87.9	88.3	88.0	88.4	88.5
AAY			99.4	99.4	99.4	99.5	98.8	99.1	98.7	98.5	87.6	87.2	87.3	87.7	87.7	88.1	88.1
AY				100	99.9	100	99.3	99.7	99.2	99.3	87.9	87.4	87.6	88.0	88.0	88.4	88.5
MBS					99.9	100	99.3	99.7	99.2	99.3	88.0	87.5	87.7	88.1	88.1	88.5	88.5
MD						99.9	99.2	99.6	99.2	99.2	87.9	87.4	87.6	88.0	88.0	88.4	88.5
OY							99.3	99.7	99.2	99.3	88.0	87.5	87.7	88.1	88.1	88.5	88.5
KVG								99.2	98.7	98.9	90.1	89.7	89.8	90.2	89.9	90.3	90.3
BBS									99.1	99.0	87.8	87.3	87.5	87.9	87.9	88.3	88.4
ACLR										98.7	87.9	87.4	87.6	88.0	88.0	88.4	88.5
CyB											88.0	87.5	87.7	88.1	87.7	88.1	88.2
ALY												99.6	99.4	99.6	99.7	99.7	99.8
FD													99.2	99.1	99.2	99.2	99.3
HD1														99.0	99.4	99.4	99.4
RS															99.2	99.3	99.4
ULW																99.7	99.8
WVEY																	99.9

Designation given to isolates and their GenBank accession numbers are given in Table 1.

fragments either from infected or healthy tissues of black pepper. This could be due to the presence of DNA concentration below the detection limit in ethidium bromide-stained agarose gel. When the first round PCR products were reamplified in the nested PCR using primers R16F2n/R16R2, a DNA fragment of 1.2 kb was obtained in the diseased black pepper samples and a known phytoplasma positive sample (periwinkle little leaf) but not in asymptomatic plants (Fig. 2). This DNA fragment was cloned and its nucleotide sequence determined. The sequence was deposited in GenBank and its accession number listed in Table 1. The sequenced region which contained 1230 bases was compared with corresponding region of phytoplasma isolates belonging to different groups from different hosts and regions (Table 1). Comparative sequence analyses showed that the black pepper phytoplasma shared maximum sequence identity (98.7–99.4%) with phytoplasma isolates belonging to aster yellows group (16Sr I) (Table 2). In

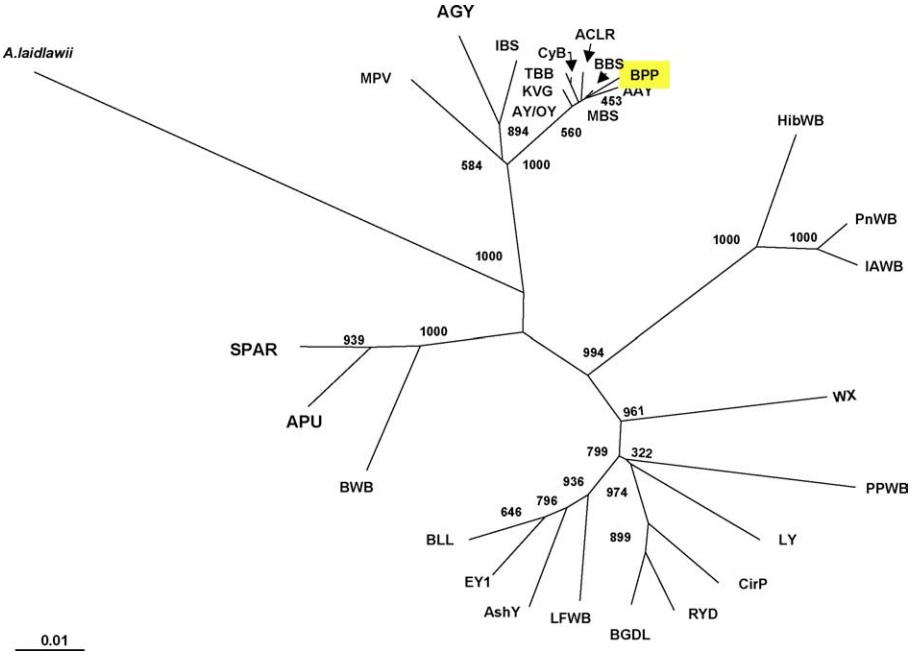

Fig. 3. Radial tree drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of 1.2 kb sequences of 16S rDNA from 29 distinct isolates of phytoplasma representing different groups and black pepper isolate (BPP). *A. laidlawii* was used as the outgroup. Sequences for comparisons were obtained from GenBank and designation given to each of the isolates and their GenBank accession numbers are given in Table 1. The bootstrap values are shown at the individual nodes.

contrast, black pepper phytoplasma showed an identity ranging from 87.0 to 88.0% with seven members of elm yellows group (16Sr V) used for comparison. Identities among different members within a group was >98% (Table 2). The identities of black pepper phytoplasma with 13 other groups and other undesignated groups (Table 1) were in the range of 88–96% (not shown). Phylogenetic tree constructed using these sequences also revealed that among phytoplasmas, black pepper phytoplasma was most closely related to the members of 16S rDNA group I (aster yellows) forming one cluster that is well separated from other groups (Fig. 3).

The 16S rDNA sequence identities are used as criteria for identifying and grouping phytoplasma isolates. Sequence similarities between two distinct 16S rDNA groups of phytoplasmas range from 88 to 94% (Lee et al., 1993, 2000). Since the 16S rDNA gene sequence similarity of black pepper phytoplasma with members of aster yellows group (16Sr I) was above the threshold level of 94%, it is proposed that black pepper phytoplasma should be regarded as a member of aster yellows group (16Sr I). This is the first report of identification and characterization of phytoplasma infecting black pepper. Except in China, India and Sri Lanka, so far there is no report of occurrence of phytoplasma on black pepper from other black pepper growing countries of the world (Sarma et al., 1988; McCoy et al., 1989). As black pepper is clonally propagated through stem cuttings, the nested PCR described in the present studies can be efficiently used to identify phytoplasma free plants. This would also facilitate detection of phytoplasma in potential vector insects and identification of other hosts of black pepper phytoplasma.

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