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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