Molecular detection and identification of a phytoplasma associated with cinnamon (*Cinnamomum cassia* B.) witches' broom disease in Quang Ngai province, Vietnam

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Received 30 May 2017; accepted 5 September 2017

Abstract:

Cinnamon (*Cinnamomum cassia* B.) is an important crop in Vietnam for domestic consumption and exportation. In recent years, a disease known as cinnamon witches' broom (CinWB) has been discovered on cinnamon grown in Tra Bong district - Quang Ngai province. The typical symptoms of CinWB were the formation of small tumors on the stems, branches, petioles, and veins of plants. The tumors become long squid-like tassels giving the appearance of a witches' broom. Infected plants are stunted, with delayed growth and development, causing a high reduction in the yield and quality of farmed cinnamon. In the present study, nested-PCR was applied with the universal primer pairs P1/P7 and R16F2n/R16R2. PCR products of an approximate size of 1200 bp were amplified from the twelves CinWB-showing samples collected from Tra Bong district, Quang Ngai province. All PCR products were directly sequenced in both directions using R16F2n and R16R2 primers. A BLAST search indicated that DNA sequences of all 12 PCR products were identical and show 99% identity with phytoplasma sequences of the 16SrXIV group. And the CinWB phytoplasma isolated from the CinWB-showing cinnamon from Tra Bong district - Quang Ngai province (QQNVN) was deposited in GenBank under an accession number JX413793.

Keywords: ***cinnamon witches' broom, nested-PCR, phytoplasma.***

Classification number: ***3.1***

Introduction

Chinese cinnamon (*Cinnamomum cassia* B.) is among the oldest spices, reaching ancient Egypt, by the seventeenth century B.C. [1]. Cinnamon is grown wild and is also cultivated in South-East Asia, south China (Kwangxi and Kwangtong provinces), Burma (Myanmar), Laos and Vietnam. It was introduced into Indonesia, Sri Lanka, South America and Hawaii. In Vietnam, it is found in many provinces from the North to the South, but is concentrated in the provinces of Quang Ninh, Yen Bai, Tuyen Quang, Ninh Binh, Thanh Hoa, Nghe An, Thua Thien - Hue, Quang Nam, Quang Ngai and in the Western Highland plateau. In Quang Ngai province, cinnamon is widely grown in mountainous districts of Tra Bong, Tay Tra, Son Ha, and Son Tay. The total area of land used for cultivating cinnamon is 3,000 ha in this region, with Tra Bong accounting for about 1,000 ha.

In a poor mountainous district such as Tra Bong, cinnamon is a very important crop helping small farmers to overcome their difficulties in life. However, in recent years, cinnamon production has been affected by disease and insect infestations, among them the CinWB was one of the most important factors causing the severe yield loss. The disease significantly reduced the quality and yield of cinnamon, directly taking from the livelihood of growers. More than 30% of farm land has been infected by the disease, with 3-year-old plants in the Tra Son, Tra Hiep and Tra Thuy communes of Tra Bong district most affected.

A significant amount of CinWB was found in nurseries, and on both young and old trees in Tra Giac hamlet (Tra Mi district, Quang Nam province) - considered a hot spot for this disease [2]. It has been found that CinWB is caused by a phytoplasma which can be combated with preventive measures including soaking cinnamon seeds in warm water (70°C) containing an antibiotic before sowing [1]. However, the mode of transmission, vector(s) and other aspects of this disease are not fully understood; and only a single measure, phytosanitation, has been identified as effective at reducing its incidence. In addition, no studies on methods of detection and identification of a CinWB phytoplasma on a molecular scale have been conducted in Vietnam.

Nested-PCR techniques in combination with DNA sequencing and phylogenetic analysis are currently the best methods for differentiation, characterization and classification of

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phytoplasmas associated with plant diseases [3-5]. The 16S rDNA gene, 16S-23S rDNA intergenic spacer region and 23S rDNA gene are the targets for detecting and identifying different phytoplasmas [6, 7]. In Vietnam, nested-PCR and phylogenetic analysis have also been used for detection and identification of many other phytoplasmas associated with plant diseases in recent years [8-11].

In this paper, a DNA-based approach and phylogenetic analysis based on 16S rDNA gene sequencing confirmed a phytoplasma strain of 16SrXIV group is associated with CinWB in Tra Bong district, Quang Ngai province.

Fig. 1. Typical symptoms of cinnamon witches' broom disease collected from Tra Bong district, Quang Ngai province, Vietnam (Photo source: Quang Ngai plant protection sub-department).

Materials and methods

Plant materials

CinWB - infected samples were collected from different fields in the Tra Bong district - Quang Ngai province of Vietnam by the Quang Ngai plant protection sub-department and one sample was collected from an asymptomatic cinnamon plant in the North of Vietnam as the first negative control. Another sample devoid of DNA template was used as the second negative control.

DNA extraction and nested-PCR assay

Total genomic DNAs were extracted from 1 gr of CinWB-showing plant tissues and an asymptomatic sample using DNeasy plant mini kit (QIAGEN) according to the manufacturer's instructions. The extracted DNAs were quantified with a UV-Vis Spectrophotometer Optima SP-3000 nano (Indonesia) and subjected to nested-PCR assays.

Fifty nanograms of the extracted DNA were used for PCR amplification using P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3')/P7(5'-CGT CCT TCA TCG GCT CTT-3') primers [12, 13] in a 25 µl reaction. The PCR reaction included 0.4 µM of each primer, 0.2 µM of each dNTP, 1.25 U DreamTaq DNA polymerase (Fermentas, Vilnius, Lithuania) and 1×Dream Taq polymerase buffer. The first round of PCR assays were 35 cycles of: 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min in a Mastercycler Pro (Eppendorf, Germany). In the nested-PCR assay, 1 µl of the first PCR product was used as the DNA template in a mixture containing R16F2n (5'-GAA ACG AGT GCT AAG ACT GG-3') and R16R2 (5'-TGA CGG GCG GTG TGT ACA CCC G-3') primers [6] and other PCR components - as in the first round PCR assay. Water and DNA extracted from the symptomless cinnamon plant were used as negative controls in all PCR reactions. Six microliters of the nested-PCR products were separated in 1% agarose gel containing 0.5 µg/ml ethidium bromide and visualized with GelDoc-It® 310 Imaging System (United Kingdom).

Phylogenetic analysis

The nested-PCR products were purified and directly sequenced with both R16F2n and R16R2 primers with an ABI3100 sequencer. The DNA sequences were subjected to a BLAST search tool https://blast.ncbi.nlm.nih.gov/Blast.cgi [14] to identify the closest match. The 22 phytoplasma 16S rDNA sequences were obtained from GenBank (Table 1). Phylogenetic analysis was conducted using the Neighbor-Joining method in MEGA 6.0 [15] with default values and 1,000 bootstrap analysis replications, and *A. laidlawii* was used as an outgroup.

Results and discussion

CinWB symptoms

CinWB usually affects cinnamon seedlings in nurseries, and both young and old trees in the field (Fig. 1). Symptoms usually appear on the stems, branches, petioles and veins of cinnamon. Firstly, tumors appear on the stems and branches of plants. These tumors develop long squid-like tassels. The infected plants become stunted, and their development is delayed; if plants are infected at an early stage of development, there is a significant risk of death, leading to yield loss for farmers. The disease damages plants throughout the year; however, the new infection starts developing from September to March of the next year; and the growth rate of tassels then increases rapidly from November to December.

Detection and identification of CinWB phytoplasma

In the first round of PCR assay using P1/P7 primers, there was no DNA

Table 1. Phytoplasma strains, associated diseases and accession numbers of their 16S rDNA sequences used for phylogenetic analysis.

Phytoplasma strain	16 SrDNA group/ sub-group	Associated disease	Geographical location	GenBank accession No.	Reference
LDN	XXII-A	Awka wilt of	Nigeria	Y14175	Tymon, et al. (1998)
LYJ-C8	IV	Coconut lethal yellowing	Jamaica	AF498307	Harrison, et al. (2002)
SorBS	XXIV-A	Sorghum bunchy shoot	Australia	AF509322	Blanche, et al. (2003)
Pin127S	XXI-A	*Cand.* Phytoplasma pini	Spain	AJ632155	
CnWB	VI	Japanese chestnut trees witches broom	South Korea	AB054986	Namba, et al. (2002)
LfWB	VIII	Loofah witches broom	Taiwan	AF353090	Dally, et al. (unpublished)
EY1	V-A	Elm yellows	USA	AY197655	Lee, et al. (2004)
CPR	VI	Clover proliferation	USA	AY390261	Hiruki, Wang (2004)
AshY1	VII	Ash yellows	USA	AF092209	Griffiths, et al. (1999)
RYD-Th	XI	Rice yellow dwarf	Thailand	AB052873	Jung, et al. (2003)
BGWL	XIV	Bermuda grass white leaf	Iran	EF444485	Salehi, et al. (unpublished)
BGWL-C1	XIV	Bermuda grass white leaf	Italia	AJ550984	Marcone, et al. (2004)
CinWB	XIV	Cinnamon witches broom	Vietnam	JX413793	This study
CYD	XIV	Coconut yellow decline	Malaysia	EU328159	Nejat, et al. (2009)
BGWL	XIV	Bermuda grass white leaf	Malaysia	EU294011	Nejat, et al. (2009)
CYD	XIV	Coconut yellow decline	Malaysia	EU636906	Nejat, et al. (2009)
BGWL	XIV	Bermuda grass white leaf	Thailand	AF248961	Davis, Dally (unpublished)
Cand. Phytoplasma phoenicium	IX	Lethal disease of almond trees	Lebanon	AF515636	Verdin, et al. (2003)
WX	III	Western X-disease	-	L04682	Schneider, et al. (unpublished)
WTTWB	XXV-A	Weeping tee tree witches broom	Australia	AF521672	Davis, et al. (unpublished)
WBDL	II	Lime witches broom	United Arab Emirates	U15442	Zreik, et al. (1995)
HibWB	XV	Hibiscus witches broom	Brazil	AF147708	Montano, et al. (2001)
A. laidlawii		-	-	M23932	

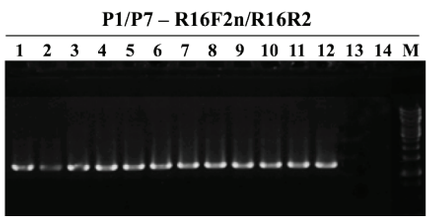

Fig. 2. Nested-PCR assay of phytoplasma isolated from the CinWB-showing cinnamon plants collected from Tra Bong district, Quang Ngai province, Vietnam (M: 1 kb DNA ladder; lanes 1 to 12: CinWB-infected samples collected from Tra Bong district, Quang Ngai province; lane 13: symptomless cinnamon plant as the first negative control; lane 14: No DNA as the second negative control).

observed in electrophoresis (data not shown). This was due to the fact that phytoplasma distributes un-uniformly in infected plant tissue therefore low DNA volume was amplified from the first round of PCR.

In the second round of PCR (nested-PCR), amplicons of about 1.2 kb in length were obtained from all 12 DNA templates isolated from the CinWB-showing cinnamon plants (Fig. 2 lanes 1-12), but the two negative controls produced no amplification (Fig. 2 lanes 13-14).

The twelve nested-PCR products were directly sequenced from both directions using two primers, R16F2n and R16R2, which were used in the second round of PCR. All 12 DNA sequences were identical. The consensus sequence of CinWB phytoplasma obtained in cinnamon grown in Quang Ngai province, Vietnam (QQNVN) was deposited in GenBank under accession number JX413793 (Fig. 3, shown in bold letters). A phylogenetic tree was constructed using 22 phytoplasma 16S rDNA sequences (Table 1). The QQNVN strain shared a high sequence similarity with a number of phytoplasmas classified in the 16SrXIV group and the phylogenetic tree confirmed this homology (Fig. 3).

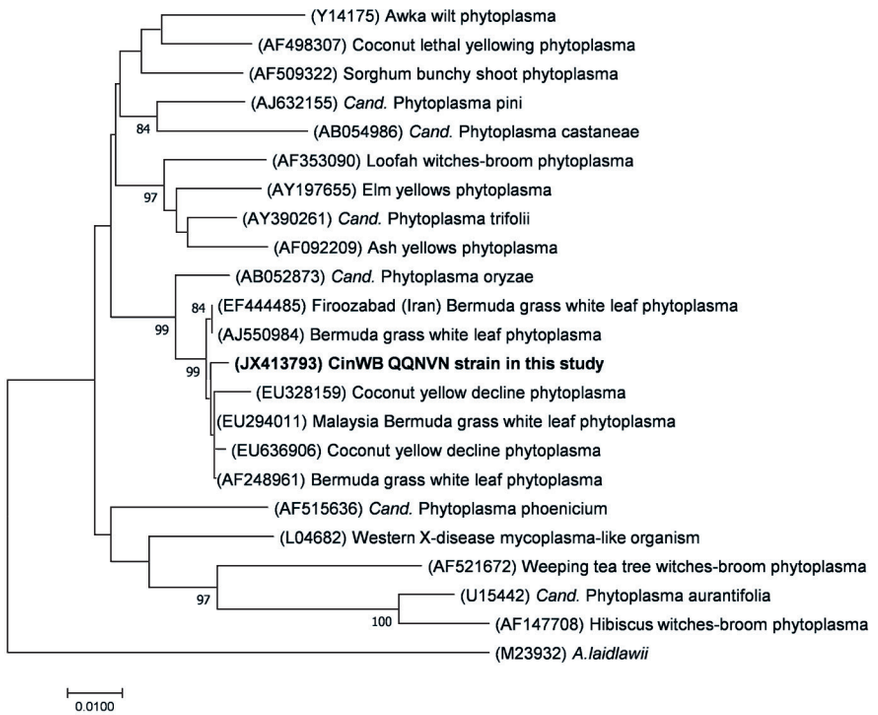

Fig. 3. Phylogenetic distance tree constructed by the neighbour-joining method, comparing the 16S rDNA sequence of QQNVN with other phytoplasmas from GenBank. Accession numbers are shown in parentheses. The number of branches is confidence percentages obtained from 1,000 bootstrap replicates (only values above 80% are shown). *A. laidlawii* is an outgroup.

Conclusions

In the present study, a combination of nested-PCR assays, DNA sequencing and phylegenetic analysis was applied. The results have confirmed the association of a phytoplasma strain with cinnamon plants showing witches' broom symptoms grown in Tra Bong district, Quang Ngai province. Through these approaches, for the first time the presence of a phytoplasma that belongs to a 16SrXIV group was demonstrated. Further studies are needed for fully understanding the causal agent(s) and their transmission manners - basic information for accurate management of such a disease.

ACKNOWLEDGEMENTS

Many thanks to the Quang Ngai plant protection sub-department for their support and assistance in collection of samples.

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