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IDENTIFICATION OF PHYTOPLASMA FROM MANGO TREES SHOWING SUDDEN DEATH PHENOMENON THROUGH MOLECULAR TECHNIQUES

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Abstract

Mango (*Mangifera indica* L.) is susceptible to a number of diseases. One of the important diseases is sudden death phenomenon which is a complex of biotic and abiotic factors. Several fungi have been isolated from mango tree showing sudden death. But some of its symptoms resemble with those caused by Phytoplasma. This study has been made for the detection of phytoplasma from trees showing sudden death. The detection process involved PCR and fluorescent microscopy of samples collected from various locations in Punjab. Out of 90 samples only one sample showed the presence of Phytoplasma. This shows that phytoplasma is not directly involved in the disease development and may be contributing as a predisposing factor.

Introduction

Mango (*Mangifera indica* L.) is considered as a king of fruits because of its nutritional value and taste. It is sensitive in nature. It is being cultivated in South Asia for over 4000 years. It is susceptible to a number of diseases. The area under the cultivation of mango crop has largely increased but at the same time, its yield is very low. The area under production of mango is 1034.6 thousand tons (Anon., 2003). The Diseases of mango crop play an important role in its low yield along with other problems in the agronomic management practices. Sudden death disease of mango has become a serious problem in Pakistan. Mango Death Syndrome is a complex of both biotic and abiotic factors. Several macroscopic fungi have been frequently observed on trees showing sudden death disease symptoms. The fungi isolated from declined mango trees include *Ceratocystis fimbriata*, *Alternaria alternata*, *Cladosporium* sp., *Colletotrichum gleosporioides*, *Dothiorella dominicana*, *Fusarium* sp., *Lasiodiplodia theobromae*, *Penicillium* sp., *Pestalotiopsis* sp. and *Phomopsis* sp., (Ploetz, 2004). The abiotic factors that favor the establishment of this disease include drought, water stress, high temperature, sun scold, humidity and also the unskilled cultural practices and less attention by the farmers and growers (Munawar *et al*., 2005).

The phytoplasma, formally known as mycoplasma like organisms (MLOs), are prokaryotes that do not possess a cell wall and that cause diseases of over 300 species around the world (McCoy *et al*., 1989). The mechanisms involved in the phytopathogenicity of plant mollicutes are poorly understood. Toxins and lactic acid production have been suggested as possible factors (Daniels, 1983). The association of phytoplasma with sudden death disease of mango trees was evaluated using the ultra sensitive methods for general detection of phytoplasma.

Table 1. Detection of Phytoplasma in leaf veinal tissues and fungal pathogens on trunk of mango tree.

Location	No. of total samples	Detection of Phytoplasma through PCR	Detection of fungal pathogens
Faisalabad	10	-	*Fusarium mangifereae* (Stem, roots) *Botryodiplodia theobromae* (Bark, stem)
Jhang	10	-	*Cladosporium* sp. (Stem)
Shorkot	5	-	*Cladosporium* sp. (Stem)
Qatal Pur	10	-	*Alternaria alternata* (Bark)
Khanewal	5	-	*Alternaria alternata, Cladosporium* sp.,
Kabir Wala	10	-	*Botryodiplodia theobromae* (Stem), *Fusarium* sp.
Multan	20	-	*Ceratocystis fimbriata,Cladosporium* sp., (Stem) *Alternaria alternata* (Bark)
Muzaffar Garh	20	+	*Botryodiplodia theobromae* (Stem), *Alternaria alternata* (Bark)

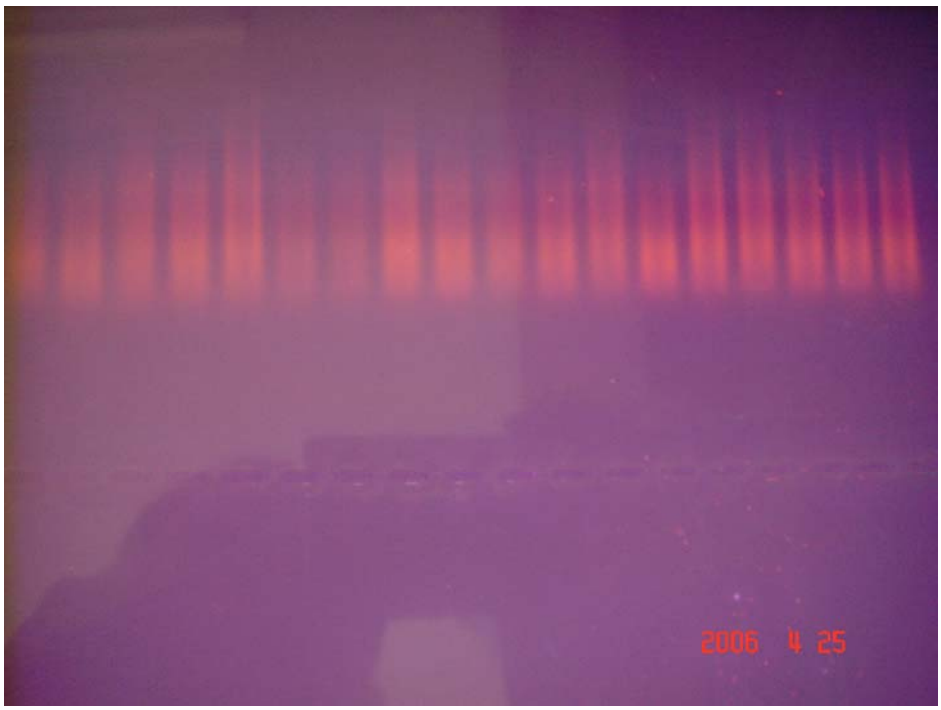

Fig. 1. PCR ampliefied 16S rRNA fragment with the primer pair of MLO

Materials and Methods

Sample collection: A total of 90 samples were collected from the mango trees showing characteristic symptoms of Sudden death phenomenon. From Faisalabad, Jhang, Shorkot, Qatal Pur, Khanewal, Kabir Wala and Multan (Table 1).

Fluorescent microscopy

Diamidino-phenylindole staining: Samples of different thickness i.e. 10, 12 and 15 µ were taken and variable dilutions (1:10, 1:25, 1:50, 1:100, and 1:200) of the stock solution [0.2 µg/ml of stain DAPI (4', 6-diamidino-2-phenylindole 2HCl)] were used.

For this purpose segments of plant twigs were fixed in 2.5% glutaraldehyde and stored at 4°C. For section cutting stem segments were rinsed in the distilled water and mounted on the stage of a freezing microtome. Longitudinal sections were cut from segments of citrus frozen tissue of the innermost secondary phloem of stems at a thickness of 10µ, then flooded with DAPI solution for 30 minutes and washed with 0.1M phosphate buffer pH 7.0 before mounting on slides. Sections were examined with a florescence microscope.

Phytoplasma DNA amplification

Phytoplasmal DNA extraction

(CTAB) method: 0.1 gram of leaf sample were ground in 0.5 ml hot (65°C) Cetyl trimethyl ammonium bromide (CTAB) buffer and incubated at 65°C for 30 minutes. Equal volume of chloroform and isoamyl alcohol (24:1) were added and suspension were centrifuged at 4000rpm for 10 minutes. Aqueous (upper) layer were removed to a clean tube. Two third volume of cold isopropanol and 1/10 volume of 3M sodium acetate were added to the aqueous layer and left over night at -20°C. Next day the mixture was spin at 10,000 rpm for 30 minutes and supernatant were poured off. Pellet was dried in vacuum desiccators. Pellet was resuspended in 0.5 µl TE buffer and transferred to microfuge tube (Daire *et al*., 1997).

NaOH Method: 0.03 gm leaf tissue was grounded with 100µl of 0.5N Sodium hydroxide, using pestle and mortar. The mixture was transferred to the Eppendorf and centrifuged at 13,000 rpm for 15 minutes in microfuge tubes. Pellet was discarded and the supernatant diluted in 100 volumes of 0.1M Tris-HCI buffer, pH 8.0.

DNA amplification: Phytoplasmal DNA was amplified by the Polymerase Chain Reaction (PCR) using the universal primer pairs derived from highly conserved ribosomal sequences and priming the 5' end of the 16s rRNA gene respectively, amplifying a 500 bp fragment, PCR and RFLP analysis was performed (Marcone *et al*., 200).

Sequence of the primers

Forward primer: 5'-ACGAAAGCGTGGGGAGCAAA-3'

Reverse primer: 5'-GAAGTCGAGTTGCAGACTTC-3'

Polymerase chain reaction: Extracted DNA of mango plants with typical symptoms of phytoplasma were taken. The 50µl of reaction mixture contained 3µl of extracted DNA, 1µl of each primer, 8µl of dNTPs, 0.5µl of Taq polymerase, 5µl of 10x PCR buffer, 3ul of 25mM $MgCl_2$ and 29.5µl of (double distilled deionized) H_2O were prepared. The Gene Amp PCR system 2400 (Perkin Elmer) were used. During first cycle denaturation was

carried out at 94°C for 5 minutes, annealing at 50°C for 1 minute and extension was conducted at 72°C for 3 minutes. After that 35 cycles were conducted at 94°C denaturation temperature for 1 minute, 52°C annealing for 1 minute and 72°C Extension for 1 minute. Post PCR extension was carried out at 72°C for 10 minutes and finally was stored at 4°C.

Gel electrophoresis: Electrophoresis of the amplified PCR products was carried on a 0.8% horizontal agarose gel in Tris borate EDTA (TBE) buffer at 510 volt and current of 50 mA for one and a half hours. The gel was visualized under UV transilluminator after staining with Ethidium bromide (EtBr).

Results and Discussion

The visual assessment of sudden death disease at different locations of Punjab were carried out during the year 2005. At all locations typical symptoms of sudden death disease were observed. The presence of phtoplasma was detected in symptomatic trees of mango trees. PCR assays detected phytoplasmas in positive control samples. In contrast, fruiting bodies of *Ceratocystis fimbriata*, *Alternaria alternata* and *Cladosporium* sp., were consistently found on trunks of symptomatic but not asymptomatic trees. They were further confirmed by PCR and DAPI fluorescent microscopy. The phytoplasma was not found in all locations except one location where phytoplasma was found. The samples were also confirmed for phytoplasma through fluorescent microscope. Specimens were fixed in 2.5% Glutaraldehyde before sectioning as fixed specimens were superior to fresh ones for the DAPI test, because DNA from nuclei often Smeared extensively in unfixed sections. This smearing sometimes made it difficult to ascertain the original location of fluorescent particles (Fauqia *et al*., 2001). Nuclei in fixed sections retained their integrity and cell outlines were clearer than in unfixed sections, which facilitated the identification of cell types.

Some samples showed complete negative results however some samples showed some fluoresce but it was not confirmly said that it was phytoplasma because DAPI test is non specific test. These samples were further processed for PCR reaction but no valuable results were found (Fig. 1).

Phytoplasma have been associated with several shade and fruit trees, including stunting, brittle leaves, general chlorosis and off-season discoloration of foliage, premature defoliation and death of affected trees within one or several years (Ahrens *et al*., 1993; Sinclair *et al*., 1989). In nature, phytoplasma induced diseases are transmitted and spread by insect vectors (McCoy *et al*., 1989). Generally, phytoplasma infection starts locally on above-ground branches and leaves and becomes systemic throughout the whole tree including the root system phytoplasma are confined to phloem tissues in affected trees and induce systemic symptoms. Sudden collapse of healthy looking trees, bark at collar region becomes dark brown and rotten with bad odour. On scrapping the affected portion a canker becomes visible in the stem, on removal of affected bark a thick liquid of light brown colour oozes out with fowl smell. Mango sudden death disease has been suspected to be caused by phytoplasma infection because some symptoms induced on affected mango trees resemble those induced by phytoplasma infection on other tree crops. Failure to confirm the presence of phytoplasma in all the tested mango trees except one tree which showed the presence of phytoplasma and also that sudden death syndrome

raises a doubt that phytoplasma actually play a role in the disease development. Phytoplasma association if any may be an occasional event unlikely to be directly correlated with sudden death of mango trees.

Fungal pathogens have been readily isolated from symptomatic but not healthy mango trees. The pathogenicity of these fungi has been demonstrated (Malik *et al*., 2005). Some predisposing factors also play an important role towards the progress of the disease like injury to plants during cultural practices and physical damage by other means; drought or water stress; sun scorch and high humidity; non adoption of recommendations of agriculture experts and presence of bark beetle (Munawar *et al*., 2005).

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