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REGULAR ARTICLE

DETECTION OF PHYTOPLASMA IN CITRUS ORCHARDS OF PAKISTAN

Shazia Mannan[1*], Muhammad Ibrahim[1], Shahid Nadeem Chohan[2,3], Paul Holford[3]

[1]*Department of Biosciences, COMSATS Institute of Information Technology, Sahiwal, Pakistan*
[2]*Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan*
[3]*Centre for Plant and Food Science, University of Western Sydney, Locked Bag 1797, Penrith South DC, NSW 1797, Australia*

SUMMARY

Citrus fruits are one of the major export commodities of Pakistan. However, being such an important crop citrus is affected by a number of destructive diseases and phytoplasmal disease is one of them. In Pakistan no significant research has been conducted on phytoplasmal diseases of citrus. Therefore, present study was conducted to confirm the presence of phytoplasmal particles in diseased samples of citrus from Sahiwal, Pakpattan, Multan and Khaenwal districts the most important citrus growing areas of Pakistan. For this purpose DNA was extracted from leaf samples collected from the three districts and single (O-MLO) and nested PCR were applied to detect phytoplasmal particles. With O-MLO primers a 558bp fragment was amplified from 16S rRNA phytoplasmal gene and 1.2kb phytoplasmal DNA fragment was amplified with nested PCR. The results revealed the presence of citrus phytoplasma in Southern Punjab region of Pakistan. In order to confirm the alternate hosts of citrus phytoplasma as well as the insect vectors involved in the transmission of the disease, weeds as well as insects were collected from citrus orchards for molecular detection of phytoplasma and their analysis are is in progress.

Keywords: Citrus Phytoplasma, Pakistan, Molecular Detection, Nested PCR.

Shazia Mannan et al. Detection of Phytoplasma in Citrus Orchards of Pakistan.. J Phytol 1 (2010) 49-54
*Corresponding Author, Email: shazia@ciitsahiwal.edu.

1. Introduction

Citrus are among the chief earning fruit crops of Pakistan and are grown in an area of 160,000 ha with production of 1.5 MMT annually (Anonymus). A majority of the farming community have small land holdings due to which adoption of the latest technologies is very difficult task; even than the farmer has been working hard to survive. Usually the inputs of an average farmer on per acre basis is about PK Rs. 14,000-15,000 with an average out put from PK Rs. 40,000-60,000 reserving a net profit of about Rs. 25,000 to 45,000 per year per acre (Azam, 2006). Most of this citrus is grown in the province of Punjab including the areas of Sargodha, Bhalwal, Jhang, Sahiwal, Pakpattan and Multan. The losses of citrus are increasing day by day due to diseases caused by different pathogens. The most destructive diseases also include phytoplasmal diseases such as witch's broom, stubborn and little leaf.

Witch's broom disease of Lime (WBDL) is a very destructive phytoplasmal disease of acid limes. The disease first appeared in Oman around 1975 and has spread extensively. It is estimated that over 98% of limes currently grown in Oman are infected with WBDL that killed lime trees in less than 5 years and has become a major limiting factor for lime

production. WBDL later appeared in the United Arab Emirates (UAE) and has recently been found in Iran and India. Typical witches' brooms were also seen on several calamondin trees in Pakistan in Lahore.

Little leaf disease of citrus is also one of the serious problems of sweet orange plants. The causal organism of citrus little leaf disease remained ambiguous for a long time. However now citrus little leaf disease is also considered to be associated with spiroplasma citri. The helical morphology of the pathogen can be easily recognized in 150–200 nm thick sections of infected plants by electron microscopy. The leafhoppers (*Euscelis plebejus*) are found to be involved in transmission of spiroplasma to sweet orange (*Citrus sinensis* cv. Valencia) (Markham *et al.,* 2008).

Stubborn disease is also one of the destructive diseases of citrus. The causal agent of Stubborn is found to be the phytoplasma known as *Spiroplasma citri*. The disease was first reported in 1915 in California. It is a damaging disease in the warm regions of Middle East California, Arizona, most of North Africa, the eastern Mediterranean Basin. The disease does not appear to be a problem in cool areas (Whitney and Rolfes, 1999).

Most citrus species and cultivars are hosts of S. *citri (*Frison and Taher, 1991*).* Sweet Oranges, grapefruit, tangelos, mandarins and mandarin hybrids are highly susceptible to infection. Acid limes, Lemons, trifoliate orange and trifoliate orange hybrids can be infected experimentally by graft inoculation, but usually exhibit milder symptoms than other varieties (Raju *et al.,* 1981).

Chapot surveyed Pakistan in 1970 and he reported wide occurrence of citrus stubborn disease symptoms (Chapot, 1970). Although the exact economic losses due to phytoplasma in citrus have not been estimated but phytoplasmas has been reported to cause losses of about 70-100% in the majority of earning crops (Armando *et al.,* 2005). The symptoms of these diseases have been observed earlier in citrus orchards of Pakistan but there was no confirmed report of phytoplasma detection until 2009 when '*Candidatus* Phytoplasma asteris' was detected in orchards close to Islamabad (Fahmeed *et al.,* 2009). However, the extent of infection is not known and the vector(s) has not been identified. Therefore, this study was conducted to detect the presence of phytoplasmas in citrus from orchards in the districts of Sahiwal, Pakpattan and Multan, some 500 km west of Islamabad in the province of Punjab, as well as to identify possible alternative hosts and the vector(s). Insects especially leafhoppers or planthoppers of the order Hemiptera have been reported worldwide to be responsible for spread of phytoplasmal diseases (Maixner, 2005). The most frequently found families of insects are *Cixiidae*, *Cicadellidae* (*Auchenorrhnycha*), and *Psyllidae* (*Sternorrhyncha*). They are all plant suckers, provided with piercing mouthparts, and feed into the plant phloem (Maurizio, 2002)

In order to confirm the involvement of insects in spreading phytoplasma in citrus orchards of Punjab, leafhoppers have been collected from Sahiwal and Sargodha orchards. Further studies are in progress.

2. Materials and Methods

Sampling

Citrus orchards in Sahiwal, Pakpattan and Multan districts were surveyed and leaves showing phytoplasma-like symptoms were collected from plants including sweet and blood oranges, grapefruit (*C.* × *aurantium* L.), mandarins (*C. reticulata*), lemons (*C.* × *limon* (L.)) and limes (*C.* × *aurantiifolia* (Christm.) [Sapindales: Rutaceae]. Survey is currently being extended to include the rest of Punjab Province to ascertain the incidence and prevalence of diseases caused by phytoplasmas.

During the surveys in the districts of Sahiwal, Pakpattan and Multan all the tehsils were visited and in each tehsil 5 orchrads were visited and from each orchard at lease 5 symptomatic samples were collected. However, the numbers of orchards for sampling depended upon cropping intensity. More

orchards were visited in tehsils with intense cropping such as in Sahiwal district. Orchards of at least one acre area were observed for phytoplasma symptoms and sample collection.

In order to identify the plants that act as alternative hosts for phytoplasma, weeds as well as grasses were collected from the infected orchards. Moreover, insects that could be involved in transmission of phytoplasma disease were collected most from around plants showing phytoplasma-like disease symptoms.

Molecular Analysis of Samples

A total of 20 samples of sweet oranges were tested from Sahiwal district, 15 from farmer orchards and 5 from the Horticulture Research Center at Sahiwal.

DNA Extraction

DNA was extracted from the petioles and midribs of samples using the method of Doyle and Doyle, 1990. 1g tissue of midrib and young stem was crushed finely. 2ml hot Cetyl ammonium bromide buffer with composition of 2% CTAB, 100 ml Tris, 20mM EDTA and 1.4M NaCl was added to the sample and were incubated for 1 hour at 65°C temperature. Then extraction was done with 24:1 ratio of chloroform & isoamyl alcohol. After that it was centrifuged at 1500g for 7 minutes and aqueous layer was eluted. Then 1/10 volumes of 3M sodium Accetate (pH 5.2) was added that was followed by 2 volumes of cold absolute alcohol. It was then incubated at -20°C for 12 hours. Precipitates of DNA were centrifuged for 10 minutes at low speed. Ethanol was added to the DNA pellet and was again centrifuged. The supernatant was discarded and the pellet was dried. Finally the DNA pellet was dissolved in 70ml sterilized nuclease free water.

PCR Reactions

In order to detect phytoplasma in suspected citrus samples single as well as nested PCR were applied. The positions as well as sequences of primers are given in detail in (Table 1 and Figure 1). For all PCR Reactions DNA of '*Candidatus* Phytoplasma aurantifolia', obtained from Central Science Laboratory, UK was used as a positive control.

Table 1: Sequences of Primers used for phytoplasmal DNA amplification

Title	Sequence (5´ to 3´)
P1	AAGAGTTTGATCCTGGCTCAGGATT
P7	CGTCCTTCATCGGCTCTT
R16F2n	GAAACGACTGCTAAGACTGG
R16R2	TGACGGGCGGTGTGTACAAACCCCG
O-MLO-F	ACGAAAGCGTGGGGAGCAAA
O-MLO-R	GAAGTCGAGTTGCAGACTTC
R16mF2	CATGCAAGTCGAACGA
R16mR1	CTTAACCCCAATCATCGAC

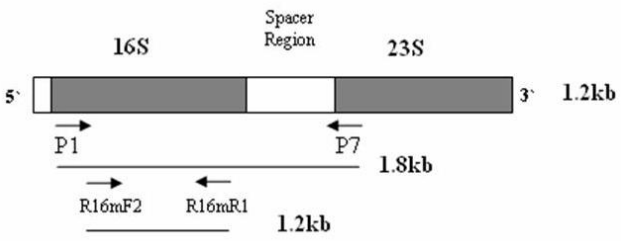

Figure 1. Illustration of a phytoplasma rRNA operon that shows the 16S and 23S genes and intergenic spacer region. The location of Oligonucleotide primers are marked with arrows

O-MLO PCR

For single PCR O-MLO primers of Doyle and Doyle (1990) were used. A total reaction mixture of 50µl contained 1µl of test DNA, 1ul of each primer, 2.5mm each of the four dNTPs, 0.5ul of Taq polymerase and 3ul of Taq buffer. The reaction was subjected to 35 amplification cycles each of 1 minute denaturation at 95°C, 45 seconds of annealing at 55°C and 45 seconds of extension at 72°C. The final extension step was for 7 minutes.

The PCR amplification products obtained from DNA of healthy and diseased citrus plants after 35 cycles were electrophoresed in 1.7% agarose gel in TBE buffer.

Nested PCR

P1/P7& R16F2n/R16R2

The P1/P7 primer pair of Deng and Hiruki (1991) and Schneider et al (1995) was used in conjunction with primers R16F2n/R16R2

(Gundersen and Lee, 1996) for nested PCR. The initial PCR with P1/P7 primers was performed in a standard 50ul reaction mixture. With initial denatuarion at 95°C for 3 minutes and 35 cycles of amplifications with 30seconds of denaturation at 94°C, 1 minute annealing at 53°C, 1.5 minutes extension at 72°C and final extension at 72°C for 10 minutes. The product of P1/P7 was diluted 1/20 before adding into the nested reaction with R16F2n/R16R2. Nested PCR was conducted at same amplification cycles with annealing at 56°C. The product was run on 1% agarose gel.

P1/P7 & R16mF2/R16mR1

The P1/P7 primer pair as mentioned above was used in conjunction with primers R16mF2/R16mR1 (Gundersen and Lee, 1996) for nested PCR. The initial reaction was carried as mentioned above however the annealing temperature was 58°C.

3. Results

Sampling

Citrus orchards in Sahiwal, Pakpattan and Multan districts were surveyed and leaves showing phytoplasma-like symptoms were collected. In this way a total of about 300 samples were collected.

During the surveys in citrus orchards weeds that could be the possible alternate hosts of Phytoplasma were identified to be couch grass *Cynodon dactylon* (L.) Pers. and wild oat *Avena fatua* L., [Poales: Gramineae], field bindweed *Convolvulus arvensis* L. [Solanales: Convolvulaceae], and fat-hen *Chenopodium album* L. [Caryophyllales: Chenopodiaceae].

While visiting the citrus orchards insects that could be the potential vectors were collected and after identification they were found to be Asiatic citrus psylla *Diaphorina citri* Kuwayama [Hemiptera: Psyllidae], and leafhoppers (possibly *Balclutha punctata* (Fabricius) and *Empoasca decipiens* Paoli [Hemiptera: Cicadellidae])

PCR Reactions

A total of 20 samples of sweet oranges from Sahiwal district were tested, 15 from farmer orchards and 5 from the Horticulture Research Center at Sahiwal.

It was observed that out of 15 samples collected from the farmers' orchards 6 samples and out of 5 samples collected from the Horticulture Research Center, 3 samples were infected with phytoplasma since specific phytoplasmal sequences were amplified from the DNA extracted from these samples.

The single PCR with O-MLO primers amplified a 558 bp sequence of phytoplasma from DNA extracted from the infected plants.

However, the nested PCR with P1/P7 in conjunction with R16F2n/R16R2 and R16mF2/R16mR1 amplified a 1.2 kb fragment with each primer set. This confirmed the presence of phytoplasmal infection (Figure 1). The PCR products are under the process of sequencing in order to determine the group of phytoplasma.

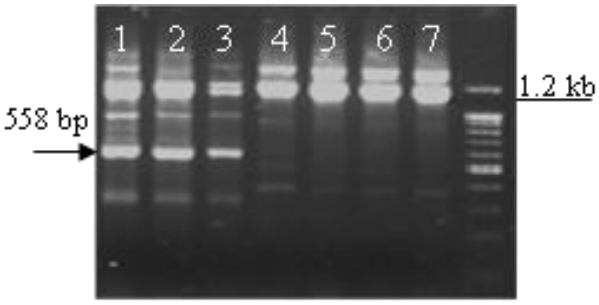

Figure 2. Typical PCR amplification products. Lanes 1-3, single PCR; lanes 4-7, nested PCR; lanes 1, 2 & 4-6, infected sweet orange; lanes 3 & 7, control ('*Ca*. Phytoplasma aurantifolia'); lane 8 molecular weight markers.

4. Discussion

In Pakistan citrus are the most important among fruits with respect to its Area and Production. Currently the area on which citrus is produced is about 162,000 hectares with 2.1 million tons estimated production. A broad range of citrus varieties are being grown in Pakistan including mandarins (Kinnow), Oranges (Blood Red and Ruby Red) Lemons & Limes; Grapefruit (Marsh Seedless, Duncan, and Foster). Mostly rough lemon and sour

orange are used as rootstocks in Punjab and NWFP. Although citrus is grown in all the four provinces of Pakistan with about 95% share of Punjab Province and more than 90% held by Kinnow only. Almost all the cultivars grown in Pakistan are self-compatible and self-fruitful so no pollinators are used normally. Different diseases including phytoplasmal diseases such as witch's broom, stubborn and little leaf as well as *Phytophthora*, gummosis wither tip and stem end rot hurt both the crop and plant health adversely (Azam, 2006).

Unless we understand a disease and its causal agent, we cannot control it. Since no data is available on status of citrus phytoplasma disease in Pakistan therefore, no proper plans can be made to control the disease. Through these studies we will be able to understand the actual status of phytoplasma in orchards of Punjab moreover the prevalent strains of phytoplasma are being identified and the factors involved in spread of the disease are being studied.

For molecular detection and characterization of phytoplasma in present studies O-MLO primers were used. Since these primers were designed from the conserved regions of the 16S rRNA gene of the Oenothera phytoplasma (O-MLO) that is located between 759 and 1359bp (Doyle and Doyle, 1990) therefore, they can be used as universal primers that can successfully detect phytoplasmal DNA in the infected samples. Being a universal feature the 16S rRNA gene can also provides taxonomic information on mollicutes and other prokaryotes. Hence this gene has widely been used to obtain phylogenetic information on non-culturable phytoplasmas (Ahrens and Seemuller, 1992). RFLP analysis of the amplified PCR products obtained during these studies is in progress to distinguish various groups and subgroups of phytoplasma prevalent in the orchards of Punjab, Pakistan.

Nested PCR was also used for detection of phytoplasma in the samples collected from the citrus orchards. The two universal primer pairs R16mF2/R16mR1 and R16F2n/ R16R2 designed for amplification of phytoplasma 16S rDNA were used to detect phytoplasma. This is a very sensitive method that can detect phytoplasma in very low concentrations. Since phytoplasma are unevenly distributed and present in low titer in the phloem tissues of infected hosts, it is important to have such highly sensitive detection methods (Khan *et al.,* 2004)

During the present studies insects that could be the possible vectors of phytoplasma were collected from the orchards. After identification they were found to be Asiatic citrus psylla and leafhoppers belonging to Cicadellidae. According to Maurizio the most studied vector species in the *Auchenorrhyncha* group belong to *Cicadellidae*, particularly of the subfamily *Deltocephalinae*. The epidemiology of phytoplasma diseases depends upon the method of their transmission by the leafhopper or psyllid vectors (Maurizio, 2002). Accurate information on the spread of phytoplasmas to far distances by leafhoppers; their survival in insect that overwinter as either nymph form, adult forms or as eggs; the transfer of phytoplasmas through the seeds of their host plants and their possible harmful effects on insect vectors; the incubation period in plants infected at various stages of their growth would be much useful to prevent the distant spread of phytoplasma as well as their introduction into new agricultural regions.

During these studies management strategies will be devised and farmers will be advised to keep the orchards free from the weeds that serve as alternate hosts for phytoplasma. Secondly control strategies will be applied against the insects identified as vectors for phytoplasma. Moreover, healthy bud wood supply will be ensured by screening the mother plots at Sahiwal and other Citrus Research Centers in Punjab. Initially, this project will help to control the disease in the few districts but ultimately the outcome from these studies can be implemented in other citrus growing areas and finally we will be able to control losses due to phytoplasma in citrus industry of Pakistan.

References

Ahrens, U and Seemuler, E., Phytopathology. Detection of DNA of Plant Pathogenic mycoplasma like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. 1992. 82, 828-832.

Anon(undated)All about – Citrus. http://www.pakissan.com/english/allabout/orchards/citrus/index.shtml (accessed 30/4/2009)

Armando García, Luis Pérez Vicente, Elizabeth Blanco. (2005). Phytoplasmas and plant quarantine in Cuba 1st Phytoplasma Workshop. Quarantine Central Laboratory, National Center of Plant Health (CNSV), Havana, Cuba. PETRIA 15 (3), 245-282.

Azam, H. 24th- 25th July, 2006. Citrus Growing in Pakistan: Growers Perspective /Economic Analysis. Session II – Citrus Growing & Management Practices, Industry & Supply Chain, Extension Services. Workshop on Citrus research, development and extension in Pakistan and Australia: Islamabad, Pakistan, ASLP Citrus Workshop Workbook Page 16

Chapot, H. 1970. Les problémes de la production des agrumes au Proche-Orient et en Afrique du Nord. UNDP/ FAO Report AT 2870. Rome, FAO.

Deng S, Hiruki D (1991) Amplification of 16S rRNA genes from cultureable and nonculturable mollicutes. J. Microbiol. Methods **14,** 53–61.

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus **12,** 13–15.

Fahmeed, F, Arocha Rosete Y, Acosta Perez K, Boa E, Lucas J (2009) First report of '*Candidatus* Phytoplasma asteris' (Group 16SrI) infecting fruits and vegetables in Islamabad, Pakistan. J. Phytopathology doi: 10.1111/j.1439-0434.2009.01549.x

Frison, E.A. and M.N. Taher. 1991. FAO/IBPGR Technical Guidelines for the safe movement of citrus germplasm. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Gundersen DE, Lee I-M (1996) Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35: 144–151.

Khan, J. A. Srivastava, P. and Singh, S. K. 2004. efficacy of Nested-PCR for the detection of phytoplasma causing spike disase of sandal.

Maixner, M., 2005. Risks posed by the spread and dissemination of grapevine pathogens and their vectors. In: Plant Protection and Plant Health in Europe: Introduction and Spread of Invasive Species, Symposium Proceedings No. 81. The British Crop Production Council, Alton, Hampshire, UK, pp. 141–146

Markham, P. G. R., Townsend, M. Bar-Joseph, M. J. Daniels, Audrey Plaskitt Brenda M. Meddins (2008). John Innes Institute, Colney Lane, Norwich, Nor 70f Annals of Applied Biology, Volume 78, Pp 49 – 57.

Maurizio, C. and Alberto A. 2002. Phytoplasma and insect vectors. Atti del "Workshop"/Proceedings of the "Workshop" Petria 12(3), 353-359.

Raju, B.C., G. Nyland, E.A. Backus, and D.L. McLean. 1981. Association of Spiroplasma with brittle root of horseradish. Phytopathology 71:1067-1072.

Schneider B, Seemuller E, Smart CD, Kirkpatrick BC (1995) Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In 'Molecular and diagnostic procedures in mycoplasmology, Vol. 1'.(Eds S Razin, JG Tully) pp. 369–380. (Academic Press: San Diego)

Whitney, E. & Rolfes, S. 1999. *Understanding nutrition*. Belmont, Ca., USA, West/Wadsworth. Eighth ed. (ed. W. Rolfes). Publication date: April 2006.