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Detection of the phytoplasmal agent of pear decline in Iran, Isfahan province, using nested-PCR

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Abstract

Pear decline is a very important phytoplasma disease that causes considerable quantitative and qualitative losses to this fruit crop. Due to economical importance of pear in Isfahan province, Iran, and the difficulty to determine the occurrence of the disease simply based on symptoms in orchards, a detection method for the phytoplasma causing disease in pears in the region was developed. Since the polymerase chain reaction (PCR) assay is a reliable and sensitive technique for identification of phytoplasma, nested-PCR method were employed which is included different sets of universal and specific primer pairs. Using P1/P7 together with fU5/rU3, or NPA2F/R in nested-PCR, products of the expected sizes were obtained from only 25% of the symptomatic samples. To examine the variation between phytoplasmal isolates from Isfahan and other countries, and to design a specific primer pair for the Iranian isolates, the PCR product from one of the samples, was sequenced. The BLASTN results showed high similarity to *Knautia arvensis* associated phytoplasma (99%). Significant homology also was found with phytoplasmas of almond witches' broom (96%), peach X disease (93%), pear decline (94%) and apple proliferation (92%). Having confirmed that there is a variation between the sequence of local phytoplasmas and similar pathogens deposited in the database, a pair of primers (fPD/rPD) were designed from the sequence using OLIGO software to increase the sensitivity of nested-PCR for detection of pear associated phytoplasma in Isfahan. In further experiments, using specific designed primers, the pathogen was detected in 72% of the samples. These primers are vastly introduced to improve the limit of detection and the specificity of the tests for the detection of pear phytoplasmas in the region.

Keywords: *Pear Decline; Phytoplasma; Nested-PCR*

Introduction

Pear is one of the economically important fruit crops in Iran. In recent decades pears with Pear Decline symptoms were observed in some of the main growing areas of Isfahan province. Pear decline is one of the most destructive diseases of pear trees in the world. It caused loss of more than one million trees during 1959-1962 in California (Agrios, 1997).The disease is found in most pear gardens and has been reported from different

countries such as Italy, Spain, Australia and USA. In Iran it was first reported from Fars province (Salehi et al., 2002).

The casual agent of pear decline is a kind of phytoplasma which has not been cultured *in vitro* yet. Classification of these prokaryotes was done on the basis of conserved sequences of rDNA genes, the TU elongation factor and tRNAs. Pear decline phytoplasma is a member of the 16S X group (Lee et al., 1998). They are monocellular particles that are found in phloem. The disease can be transmitted by an insect vector (*Psylla* sp.) or grafting (Avinent et al., 1997).

Symptom expression and disease severity of pear decline have been described as: 1) the quick decline less than 60 days often happens in summer and autumn, 2) a slow decline during one or more years with a reduction in terminal growth, yield and fruit size and emergence of light green leaves with rolled-up margins and 3) foliar reddening that is sometimes associated with leaf curl and thickening of leaf veins and accelerated leaf senescence. Because of high similarity of these symptoms with those for drought stress and certain mineral deficiencies, the detection of the disease on the basis of the symptoms is very difficult (Jones and Aldwinckle, 1990).

Classical assays for detection of phytoplasmas such as grafting to indicator plant (Hibben and Wolanski, 1971), and electron or fluorescent microscopy supply useful basic information but they do not have enough sensitivity to detect low-titer phytoplasmal infection in woody plants (Gibb et al., 1995 and Sinclair et al., 1990). Serological experiments also have had some problems. For example polyclonal antisera showed substantial cross-infection with antigens from healthy plants and production of monoclonal antibodies against a single phytoplasma epitope is very difficult (Lin and chen, 1986).

The amplification of phytoplasmal DNA by the polymerase chain reaction (PCR) has proved to be a highly sensitive, relatively simple and rapid method of detecting phytoplasma (Davis and lee, 1993 and Ahrens and Seemuler, 1992). Nested PCR using universal primers followed by specific primers which were located in the 16S rDNA, intergenic spacer (IS) and the 23S rDNA region of the phytoplasma genome has shown to improve the limit of detection and the specificity of the tests for phytoplasmas (Lorens et al., 1995 and Heinrich et al., 2001).

The occurrence of pears with decline symptoms, such as reddening of foliage and downward curling of the leaves that are not distinguishable from nutrient deficiency and drought stress or foliage reddening of healthy trees on quince rootstock, is very important in the commercial production areas in Isfahan. Thus the availability of an appropriate assay prompted the examination of the trees for the presence of phytoplasma.

Materials and methods

Plant materials

Samples from 50 pear trees were collected from 6 different pear cultivating region in Isfahan during 2002-2004. The trees showing general dieback, foliage reddening, leaf rolling with thickening of veins, enlargement of stipules and early blossoming were sampled. In all cases, the collected samples were transferred to the lab in sealed plastic bags. To investigate the effects of time of sampling on detection of phytoplasmas, sample

collection was done at two periods: 1) middle spring to early summer, and 2) late summer to middle autumn. For negative controls, some asymptomatic trees were sampled as healthy plants.

DNA extraction

DNA was extracted from 0.5g of leaf midribs, petiols or phloem branches powdered in liquid nitrogen using a mortar and pestle. The procedure of Murry and Thompson using CTAB buffer (1.4 mM NaCl, %2w/v CTAB, 20 mM EDTA, %0.2 β-Mercaptoethanol, 100mM Tris-HCl pH 8) was used with minor modifications (Murry and Thompson, 1989, Sharbatkhari, 2004). Each DNA extract was precipitated with isopropanol, pelleted by centrifugation, resuspended in 700µl distilled water and treated with RNase for 1 h at 37 ̊C. DNA samples were quantified at OD_{260} using a spectrophotometer and their quality was determined by electrophoresis on 0.7% agarose gels in TAE buffer.

PCR amplification

In all cases the final reaction volume was 20µl and contained 250µM dNTP, 0.2 µM of each primer, 1U Taq polymerase and 1X PCR buffer (Roche, Germany). The temperature profile for PCR amplification was 95 ̊C pre-denaturation for 2 min followed by 35 cycles of 95 ̊C for 30s, 55 ̊C for 75s and 72 ̊C for 90s, and 10 min final extension at 72 ̊C. Products from the primary PCR were diluted 1:10 to 1:40 and used in a secondary PCR. The nested PCR was used the same temperature profile except that the annealing step was at 58 ̊C, 60 ̊C or 64 ̊C for 30 s, for the primer pairs fU5/rU3, NPA2F/R and fPD/rPD, respectively. (The primer sequences have been listed in table 1).

DNA sequencing

The 485bp product using the P1/P7 primer pair in the primary PCR and the NPA2F/R primer pair in the secondary PCR from a positive sample was sequenced. It was cloned into pGEM-T vector according to PROMEGA protocol. The clones were selected on LB media containing 10µl ampicillin (100mg/ml), 500µl Xgal (20mg/ml dimethyl formamide), and 500µl IPTG (238mg/ml H_2O) per liter. After plasmid extraction, the presence of a 485 nucleotide fragment was confirmed by *EcoR*I digestion. DNA sequencing was done by the SEQLAB Company using the diedeoxy chain termination method. The sequence was compared with other sequences in Genbank by BLASTN software.

Primer design

The initial PCR was performed with the universal primers P1/P7 of phytoplasmas (Smart et al., 1996). Specific primers NPA2F/R were also selected on the basis of previous researches (Heinrich et al., 2001). The product of nested PCR was sequenced. Primer design was done on the basis of the sequence using OLIGO software. After comparison of the sequence with the plant sequences or other plant pathogenic bacteria, and confirmation

of dissimilarity between them, the forward and reverse primer pair was designed for more reliable detection of phytoplasma in pear trees of Isfahan region using nested PCR.

Results and Discussion

Amplification of phytoplasma DNA

When template DNA from pear trees was amplified with universal phytoplasma primer pair P1/P7 no visible band was detected by agarose gel electrophoresis. However when the diluted PCR products obtained with primers P1/P7, were reamplified in nested PCR with the universal primer pair fU5/rU3 that are commonly used for detection of fruit trees phytoplasma throughout the world (Lorens et al., 1995), 850 bp phytoplasmal band was detected. In all positive samples, a 450bp fragment was also amplified that was not detected in healthy samples (Figure 1). This band was also reported by other scientists (Firrao et al., 1993). Because of some weak amplifications, the specific primer pair NPA2F/R was used to increase the efficiency of PCR amplification.

Due to the high annealing temperature and no double band or non-specific bands, the NPA2F/R primer pair was selected to detect the phytoplasma disease in stone and pome fruit trees (Heinrich et al., 2001). Infection was detected in only 25% of the samples that had disease symptoms (Figure 2). In healthy plants as a negative control no amplification was observed. The inability to produce specific bands with specific primer pairs also was reported before and it seems that the genetic diversity among strains creates sufficient mismatch between specific primers and genomic DNA of phytoplasma (Schneider and Gibb, 1997).

DNA sequencing and primer design

Due to point mutation in primer binding site nucleotides, it is essential to design specific primers to detect regional phytoplasmas. Reproducible results may not obtained by using specific primers that were designed for phytoplasmal strains of other regions. Therefore, a 485bp fragment from the Iranian strain of pear decline phytoplasma was sequenced for better detection of phytoplasmal DNA and more specific amplification. Comparison of this sequence with other related sequences in the Genbank revealed the similarity of 96%-99% in the 1137-1414 16S rDNA nucleotide region with the sequences of other phytoplasma strains from different countries by BLASTN. Amplification of phytoplasmal bands in pear samples from the Isfahan state using NPA2F/R, fU5/rU3 primer pairs and the high homology of the sequenced fragment with the phytoplasmal DNA reported in the Genbank indicated the presence of phytopladmal infection in pear trees in the region. Primer design was done using the OLIGO software (the sequence shown in Table 1). On the basis of BLASTN results, the sequences of these primers did not have any homology with neither plant sequence nor other phytopathatogenic bacteria. All the amplified DNA samples from the first PCR by P1/P7 were diluted 20 times and used in nested PCR with fPD/rPD. The results showed that all positive samples and some suspicious ones with weak amplification, produced the expected fragment of 422bp by fPD/rPD, and 72% of the samples were detected as infected (Figure 3). Healthy plant as a negative control did not produce any

band in any condition. PCR/RFLP pattern of the 422bp fragment confirmed the results was the same as the pattern predicted on the basis of phytoplasmal 16S rDNA in Genbank (data not shown). Similar PCR/RFLP experiment has been performed in southern Australia to detect phytoplasmas in declining pears (Schneider and Gibb, 1997). Due to the high ability of the fPD/rPD primers to detect phytoplasma in infectious trees and no amplification in the negative controls (healthy plants), this primer pair can be used in detection of pear phytoplasmas in the region.

Effects of symptom type and time of sampling on phytoplasma detection

Most samples with symptoms of leaf rolling with reddening of leaf edges were infected but some of samples that suffered from reddening of the leaves did not produce expected bands. Thus, color change in the leaves is not an ideal symptom for this disease. On the basis of experiments done on pear decline in Spain, 21% of the samples with leaf reddening symptoms and 33% of the samples with decline symptoms did not have any phytoplasmal infection (Avinent et al., 1997). It seems that other factors such as stock or physiological stresses like drought or nutrient deficiency have a role in the expression of these symptoms. However, it could be said that there are not completely defined symptoms in Isfahan gardens for phytoplasma infection as stated (Jones and Aldwinckle, 1990). So all symptoms like leaf rolling, reddening of leaf edges, decrease in growth and leaf size and number need to be taken into consideration as phytoplasma infectious symptoms in trees. Due to the presence of different symptoms in pear gardens and the difficult identification, the detection with specific primers such as fPD/rPD are very necessary to prevent the epidemic of the disease transferred to other places by grafted seedlings.

Due to change in the phytoplasma concentration during host growth, time of sampling is an important factor for detection of pathogen. In previous experiments to detect phytoplasmal agent in fruit trees, sampling was done in the autumn (Avinent et al., 1997). Investigation of the type of symptoms and the time of sampling showed that the presence of phytoplasma in the leaf midribs collected late in the summer to middle of autumn is more reliable than other samples collected in spring and the detection of phytoplasma in midribs is stronger than bark samples. Considering that the pathogen hibernates in plant roots and is gradually transported in plant sap in the spring, the amount of phytoplasma is very low in the top of trees, while colonization of the pathogen in the plant and insect vector attack at the end of the summer can greatly increase the phytoplasma amount and disease severity (Errea et al., 2002).

Table 1. Primer pairs used in PCR reactions.

Primer	Sequence	Expected size	Reference
P1 P7	5ʹ-aagagtttgatcctggctcaggatt-3ʹ 5ʹ-cgtccttcatcggctctt-3ʹ	1784bp	Smart et al., 1996
NPA2F NPA2R	5ʹ-atgacctgggctacaaacgtga-3ʹ 5ʹ-ggtgggcctaaatggactcg-3ʹ	485 bp	Heinrich et al., 2001
fU5 rU3	5ʹ-cggcaatggaggaaact-3ʹ 5ʹ-ttcagctactctttgtaaca-3ʹ	850 bp	Lorens et al., 1995
fPD rPD	5ʹ-caatggctgttacaaagggtag-3ʹ 5ʹ-gcgtgtgctctaaccaactgag-3ʹ	422bp	Sharbatkhari et al., 2004

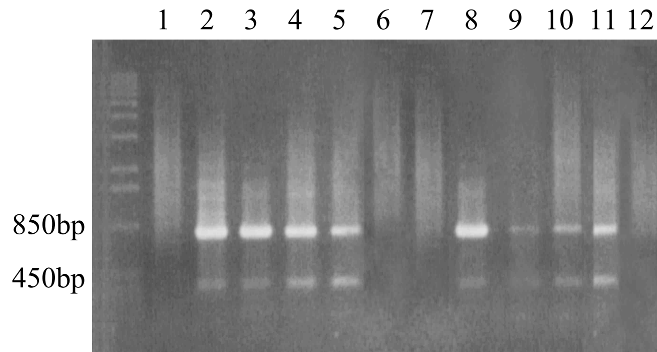

Figure 1. Agarose gel electrophoresis of PCR products from different pear samples in Isfahan using primer pairs fU5/rU3. Lanes 1: Healthy pear, 2:PD43, 3: PD45, 4: PD48, 5: PD1, 6: PD7, 7: PD8, 8: PD9, 9: PD11, 10: PD20, 11: PD22, 12: PD26.

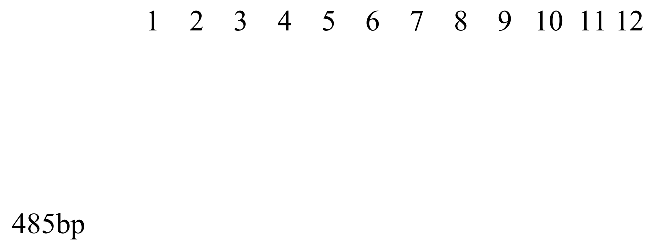

Figure 2. Polymerase chain reaction (PCR) amplification of 485 bp rDNA fragment from various pear samples in Isfahan using primer pair NPA2F/R. Lanes 1: Healthy pear, 2: PD43, 3: PD45, 4: PD48, 5: PD1, 6: PD7, 7: PD8, 8: PD9, 9: PD20, 10: PD22, 11: PD16, 12: Healthy pear.

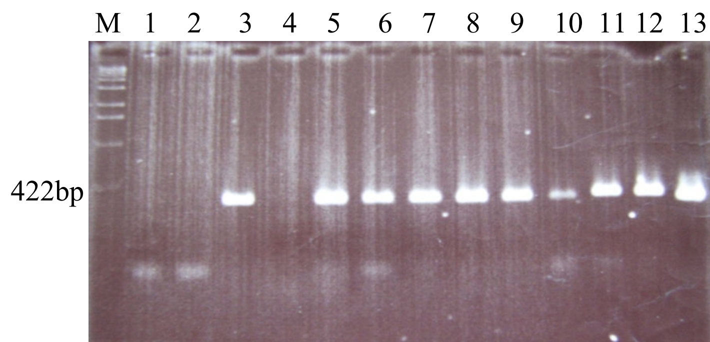

Figure 3. Nested PCR amplification of a 422bp rDNA fragment from various pear samples in Isfahan using primer pair P1/P7 followed by primer pair fPD/rPD. Lanes 1: Healthy pear, 2: PD7 3: PD11, 4: PD12, 5: PD20, 6: PD22, 7: PD26, 8: PD30, 9: PD31, 10: PD33, 11: PD35, 12: PD36, 13: PD37.

References

Agrios, G.N., 1997. Plant pathology. Academic Press, 4th Ed.
Ahrens, V., Seemuler, E., 1992. Detection of DNA of phytopathogenic mycoplasma-like organisms by a Polymerase Chain Reaction that amplifies a sequence of the 16S rDNA gene. Phytopathology. 82, 828-832.
Avinent, L., Llacer, A. G., Almacellas, J., Tora, R., 1997. Pear decline in Spain. Plant Pathol. 46, 694-698.
Davis, R.E., Lee, I.M., 1993. Cluster-specific Polymerase Chain Reaction amplification of 16S rDNA sequences for detection and identification of mycoplasma-like organisms. Phytopathology. 83, 1008-1011.
Errea, P., Aguelo, V., Hormoza, J.I., 2002. Seasonal variation in detection and transmission of pear decline phytoplasma. J. Phytopathol. 150, 439-443.
Firrao,G., Gobbi, E., Locci, R., 1993. Use of Polymerase Chain Reaction to produce oligonucleotide probes for mycoplasma-like organisms. Phytopathology. 83, 602-606.
Gibb, K.S., Padovan, A.C., Mogen, B.D., 1995. Studies on sweet potato and other plant species growing in northern Australia. Phytoplathology. 85, 169-174.
Heinrich, M., Botti, S., Caorava, L., ArthoferA, W., Sterommer, S., Hazzer, V., Ktinger, H., Bertaccini, A., Machado, M., 2001. Improved detection methods for fruit tree phytoplasmas. Plant. Mol. Biol. Rep. 10, 160-179.
Hibben, C.R, Wolanski, B., 1971. Dodder transmission of a mycoplasma from ash witches broom. Phytopathology. 61, 151-156.
Jones, A.L., Aldwinckle, H.S., 1990. Compendiom of apple and pear disease, Minnessota.
Lee, I., Gundersen, D.E., Bertaccini, A., 1998. Phytoplasma: Ecology and genetic diversity. Phytopathology. 88, 1359-1366.
Lin, C.P., Chen, T.A., 1986. Comparison of monoclonal antibodies and polyclonal antibodies in detection of the aster yellows mycoplasma-like organism, Phytopathology.76, 45-50.
Lorens, K.H., Schneider, B., Ahrens, U., Seemuler, E., 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology. 85, 771-776.
Murry, M.G., Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. Nucl Acid Res. 8, 4321-4325.
Salehi, M., Izadpanah, K., 2002. First report of pear decline in Iran. Plant disease journal.
Schneider, B., Gibb, K.S., 1997. Detection of phytoplasmas in declining pears in sauthern Australia. Plant Dis. 81, 254-258.
Sharbatkhari, M., 2004. Detection of phytoplasma agent of pear decline and apple proliferation in Isfahan province.MSc Thesis. Isfahan University of technology. P : 85.
Sinclair, W.A., Ivli, R.J., Dyer, A.T., 1990. Ash yellows: Geografic range and association with decline of white ash. Plant Dis. 74, 604-607.
Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemullar, E., Kirkpatrick, B.C., 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rDNA spacer region. Amr. Soc. Micribiol. 62, 2988-2993.