Phytoplasma disease in date palm in Saudi Arabia

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ABSTRACT

The date palm, (*Phoenix dactylifera* L.) is one of the most important cash crops in Saudi Arabia, occupying 155,118ha in which 23 million trees produce more than 991,546t of dates annually. A disease called Alwijam (caused by phytoplasma) has lately been affecting date palms in Saudi Arabia. The main symptoms are leaf stunting, yellow streaking and a marked reduction in fruit and stalk size, which leads to failure in fruit production at harvest time. Identifying the associated phytoplasma and a putative vector are important contributions to our knowledge of the disease in Saudi Arabia and can open up a new understanding of such diseases. In order to facilitate this, date palm samples both with and without symptoms of phytoplasma were collected from different locations of Saudi Arabia. The DNA was extracted from the collected plant samples and indexed by a nested PCR reaction using different sets of phytoplasma generic primers P1/P7 and or, R16mF2/R16mR1, to amplify the 16S rDNA region. PCR products were cloned into a pGEM-T easy vector and sequenced. The sequences of the phytoplasma obtained were submitted to GenBank under the accession numbers JQ045567, JQ045570, JQ045571, KC252994, and KC252995. The sequences of 16S rDNA obtained were compared with those of other phytoplasmas in GenBank and the results obtained indicated that there are two types of the phytoplasma infected date palm: 16SrI in Alhasa and 16SrII in other locations in Saudi Arabia.

Key words: Phytoplasma, Date Palm, 16Sr I, 16Sr II, Saudi Arabia

INTRODUCATION

The date palm, (*Phoenix dactylifera* L.), is one of the most important cash crops in Saudi Arabia. The area planted with date palm is 155,118 ha, in which more than 23 million trees produce more than 991,546 t of dates annually (Statistics Book, Ministry of Agriculture, 2011). Alwijam disease has been investigated by very few scientists. The first record of the disease is recorded in a book by Badawi (1945). The disease was recorded in SA by Elbaker (1952) and Nixon (1954). Later, Elarosi et al. (1983) reported that two species of *Fusarium* were always associated with the root of the Alwijam date palm. The symptoms are characterised by a stunting and yellow streaking of the leaves, with fruits and fruit stalks reduced in size (by around 30%). The association of viral, fungal and nematode pathogens with the disease is not consistent (Abdusalam *et al.,*1992. Abdusalam *et al.,*1993. Elarosi *et al.,* 1982). A phytoplasma pathogen was suspected to cause Alwijam-affected palms, following histopathology and antibiotic therapy studies (Abdusalam *et al.,* 1993). This was further supported by El-Zayat *et al.,* 2000, who reported a similar phytoplasma causing lethal yellow coconut palm in Florida. However, Alhudaib et al (2007) were reported that association of a phytoplasma of 16SrI group, *Ca*. P. asteris, with Alwijam in Alhasa, Saudi Arabia

Phytoplasmas are prokaryote organisms of the class *Mollicutes*, which affects more than 700 plant species from tropical to temperate countries (Jones, 2002). They cannot be cultivated *in vitro* and are mainly transmitted by leafhopper or planthoppers of the order *Hemiptera* (Maixner, 2005). Phytoplasmas have been associated with diseases in date palm such as white tip die-back (WTD), slow decline in Sudan in North Africa (Cronjé *et al.,* 2000a. Cronjé *et al.,*

2000b), yellowing in Kuwait (Al-Awadhi *et al.,* 2002), and lethal decline in Texas (Harrison *et al.,* 2002).

In this paper, we report the association of a phytoplasma of 16SrI group and 16SrII group, with Alwijam in Saudi Arabia.

MATERIALS AND METHODS

Sample collection

A survey was done during 2011-2013 in different location of Saudi Arabia (Alhasa, Alkharj, Almadinah, Jouf, Qassim and Riyadh). More than 900 leaf samples were collected of date palm including some that displayed symptoms (Fig 1) and some that did not. Date palm tissue culture product was used in all our experiments as a (healthy) negative control.

DNA Extraction and PCR

The total DNA was extracted from the collected samples using CTAB with ß-mercapto-ethanol (1-2%) (Doyle and Doyle, 1990) method as follows: 200 mg of date palm tissues were homogenized to powder by adding liquid nitrogen and were homogenized using the coffee blender.

Aliquots of final DNA reparations were used as template for a nested PCR (nPCR) assay with phytoplasma 16S rDNA primers P1/P7 and or R16mF2/R16mR1 (Gundersen *et al.,* 1996). PCRs were performed in 25 µl volumes containing 2 µl of DNA template 1 µl of each primer (10 pmol), 2.5 µl of 2.5mM dNTPs, 2.5 µl of MgCl2, 2.5 µl of 10 X Taq polymerase buffer and 1.5 units of Taq DNA polymerase (Invitrogen). First round amplifications were performed in a thermocycler using an initial denaturation at 94 °C for 2min, followed by 35 cycles at 94°C for 45 sec, 55 °C for 1 min, and 72 °C for 2 min, and final extension at 72°C for 10 min. Aliquots of each reaction mixture were analyzed by 1% agarose gel electrophoresis using TBE buffer. The gel was stained in Ethedium bromide and visualized by UV transillumination and photographed (Syngene Bio Imagins, IN Genius). A DNA size marker (1kb DNA marker Promiga, USA) was used to estimate the sizes of the PCR products.

Sequencing and data analysing

Phytoplasma rDNA amplified by PCR using the primer pair P1/P7 was purified on spin columns (QIAquick gel extraction kit; QIAGEN. UK). To determine which group this phytoplasma is located in, the PCR products in each location were purified with from agarose gel as above. The purified PCR products were cloned into a pGEM-T easy vector (Promiga, USA) according to the manufacturers' instructions and then sequenced.

An Agincourt CleanSEQ™ kit was used to clean up the PCR sequencing reaction as follows: Twenty ml of PCR sequencing product were used and placed in another clean tube and 10 ml of magnetic beads were added to the PCR sequencing product. Then 80ml of 80% ethanol was added, mixed and incubated at room temperature for 3 min in a magnetic tray. The liquid was pipetted out of the tube carefully and then 150ml of 80% ethanol were added and pipetted out. Finally the tubes were placed on normal try and 50ml of water was added. Samples were heated at 90 °C for 3 minutes, then half of each sample was loaded on an ABI 377XL automated DNA sequencing instrument, using a 36 cm well to read plates and a 5% Long Ranger (FMC) acrylamide gel. The 16S rDNA sequences of phytoplasmas identified in our study were compared with others in Genbank (Table 1) by BLAST (Altschul *et al.,* 1990). Sequences were aligned and a phylogenetic tree constructed by the program MEGA version 5 (Tamura *et al.,* 2011) using 1000 bootstrap datasets to support the branch values. *Acholeplasma palmae* was used as the outgroups to root the Phylogenetic tree.

RESULTS AND DISCUSSION

Survey and PCR to identify of date palm phytoplasma:

This survey was carried out to determine the occurrence of phytoplasma in different date palm farms in Saudi Arabia. PCR was used to detect the phytoplasma in all the collected samples. PCR using different primers and nucleotide sequences were carried out to identify phytoplasma from the collected samples. All the works was done in Pests and Plant Diseases Unit at King Faisal University.

Phytoplasma rDNA was amplified more than 900 leaf samples. Samples were representative with the 876 bp expected second round PCR amplification. No PCR products were obtained from apparently healthy palms (Fig 2). Fig 3 showed that some of tested samples such as 13, 14 and 18 were negative and that other samples were positive such as 1,2,3,4,5,8,15,16,17 and 19. Also this date showed that some plants were symptomless but gave positive reactions to phytoplasma primers and the nucleotide sequence analysis confirmed.

The obtained data after PCR test as shown that in (Fig. 4), the highly percentage of infection with phytoplasma was in Alhasa and the infection percenage near to 12 %, however the percentages of infection in Alkharj, Riyadh and Qassim were 5.5%, 3.4% and 7.6% respectively, in other side there were no infected date palm samples in Almadinah and Jouf and the percentage of infect was 0% in all.

Nucleotide sequencing and alignment analysis:

Positive samples from different location (Alhasa, Alkharj, Qassim and Riyadh) were sequenced and submitted to

GenBank under accession numbers JQ045567, JQ045570, JQ045571, KC252994, and KC252995. Sequence alignment was carried out for those sequences and from the alignment data we discovered that from the phylogeny tree in Fig 4, the sequence of JQ045567 (sequence for phytoplasma isolated from *Phoenix dactylifera* from Alhasa) was almost all (87%) in a separate cluster to the other sequences identified while the sequences of JQ045571 (sequence for phytoplasma isolated from *Phoenix dactylifera* from Alkharj), JQ045570 (sequence for phytoplasma isolated from *Phoenix dactylifera* from Alkharj), KC252994 (sequence for phytoplasma isolated from *Phoenix dactylifera* from Qassim) and KC252995 (sequence for phytoplasma isolated from *Phoenix dactylifera* from Riyadh) were in same cluster and their identity ranged from 96% to 100%. This data indicates that the isolated phytoplasma from Alhasa may be in different group. Therefore, a comparative analysis of the obtained sequences and other sequences that were available in the gene bank was carried out. To determine the evolutionary relationships among those sequences, we selected 15 sequences for the phytoplasma, some belonged to 16SrI (group I) and others to 16SrII (group II) etc. Sequences are present in GenBank under accession numbers, as shown in Table 1. The data of the phylogeny tree (Fig 5) referred to the sequences of JQ045570, JQ045571, KC252994, KC252995 were in same cluster with 16SrII but only the sequence of JQ045567 (sequence for phytoplasma isolated from *Phoenix dactylifera* from Alhasa) was in the other cluster with 16SrI. These results explained why the identity of JQ045567 was 87%, because it belonged to a different group which was agreed with Alhudaib et al 2007. Taken all together, these results indicated that phytoplasma group II (16SrII) was detected on date palms in different locations in Saudi Arabia (Alkharj, Riyadh and Qassim) at the same time while the only location we detected the phytoplasma group I (16SrI) in Alhasa.

Seemüller et al. (1998) considered phytoplasmas as belonging to the same group if they showed 97% or more similarity between their sequences, while values less than 95% would place phytoplasmas in different groups. LDT and FcoLY type-diseases have been associated with phytoplasma diseases in coconut (Warokka, 2005). El-Zayat et al. (2000), identified a phytoplasma associated with Alwijam disease based on an 87% identity of 16S rDNA with that of the Florida lethal yellows phytoplasma; however, the amount of Alwijam samples where the phytoplasma was detected was not specified, and additionally it was not found in Alwijam affected date palms collected from our surveys. The 16SrI, *Ca. P. asteris* is the only phytoplasma group distributed worldwide and the most diverse in plant and insect hosts (Lee *et al.,* 2000). It has been found in sandalwood in India (Schneider *et al.,* 1993), and safflower and carrot in Israel (Orenstein *et al.,* 1999, Schneider *et al.,* 1993). Results of our experiments extend this to date palms associated in Alhasa but not in other locations in Saudi Arabia. Sequence similarity between the phytoplasma 16SrI date palm PPH (from Alhasa) to those in Table (1) in characterized phytoplasma strains *Ca. P. asteris,* AAY, AY1, MPV, OY Date Palm, BD was 95%-99%; and 51% to FCoLY and LDT. Also less than 87% with the rest of phytoplasma group 16SrII. The phylogenetic tree (Fig. 5) reveals that the phytoplasma identified in date palm is phylogenetically distant from phytoplasmas of 16SrIV group. In our survey a phytoplasma of group 16SrII was detected in date palm. Ca. Phytoplasma aurantifolia group has been recorded from weeds and herbaceous crops in areas of Southern Europe, Africa, New Zealand, Asia, Australia and America (Garnier *et al.,* 1991; Leyva-Lo´ pez *et al.,* 2002; Ghosh *et al.,* 1999; Tran-Nguyen *et al.,* 2003; Tessitori *et al.,* 2005; Tolu *et al.,* 2006). Particularly in the Gulf region, the group has been identified in lime (Zreik *et al.,* 1995), alfalfa (Khan *et al.,* 2001, 2002), sesame (Al-Sakeiti *et al.,* 2005, Esmailzadeh-Hosseini *et al.,* 2007) ; and garden beet (Mirzaie *et al.,* 2007) in Iran. Phytoplasma in date palm showed a 98% identity of 16Sr II and phylogeny results (Fig. 5) clearly support that it is a member of this group. This is the first report of the identification of the 16SrII phytoplasma in date palm, and contributes to the knowledge on the biodiversity of phytoplasmas associated with Alwijam disease in the region.

The results in this study obtained indicated that there are two types of the phytoplasma infected date palm: 16SrI in Alhasa and 16SrII in other locations in Saudi Arabia. Further studies of transmission will be conducted to confirm that vector likes leafhopper and weeds might play the role of etiology of this disease, which will allow the development of more efficient control measures, and reveal new insights into the epidemiology of Alwijam disease.

Acknowledgements

This work was supported by King Abdullaziz City for Science and Technologies (grant no. AT-28-111) and King Faisal University. Also we would like to thank Wael Alarby and Zake Abohamd at Plant Protection Department and Ministry of Agriculture, Saudi Arabia for assistance during sampling.

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Table

Table 1: Acronyms GenBank accession numbers of phytoplasma 16S rDNA sequences used to construct the phylogenetic tree

Acronym	Phytoplasma strain designation	RFLP Group	Accession number
PYC	'Papaya yellow crinkle'	16SrII	YI0097
PnWB	'Peanut witches broom'	16SrII	L33765
TBB	Tomato big bud phytoplasma	16SrII	EF193359
WBDL	*'Candidatus* Phytoplasma aurantifolia'	16SrII	U15442
TWB	Tomato witches'-broom phytoplasma (our Sequence)	16SrII	HM584815
AAY	'American aster yellows'	16SrI	X68373
AY	Aster yellows phytoplasma	16SrI	AF222063
AY1	Aster yellows phytoplasma strain AY1	16SrI	AF322644
MPV	Mexican periwinkle virescence phytoplasma	16SrI	AF248960
OY	'Onion yellows'	16SrI	D12569
Date Palm	'Date palm phytoplasma' Old Seq from 2007	16SrI	DQ913090
BD	Barley deformation	16SrI	AY734453
LDT	Coconut lethal decline	16SrIV	X80117
FcoLY	Coconut yellows	16SrIV	U18747
A. laidlawii	*Acholeplasma laidlawii*	as root	M23932
PPH	Date palm from alhasa	This study	JQ045567
KhD	Date palm from Alkharj	This study	JQ045570

Acronym	Phytoplasma strain designation	RFLP Group	Accession number
KhD2	Date Palm from Alkharj	This study	JQ045571
QassimD	Date palm from Qassim	This study	KC252994
RiyadD	Date palm from Riyad	This study	KC252995

Figures

Fig. 1: Symptoms of phytoplasma in date palms

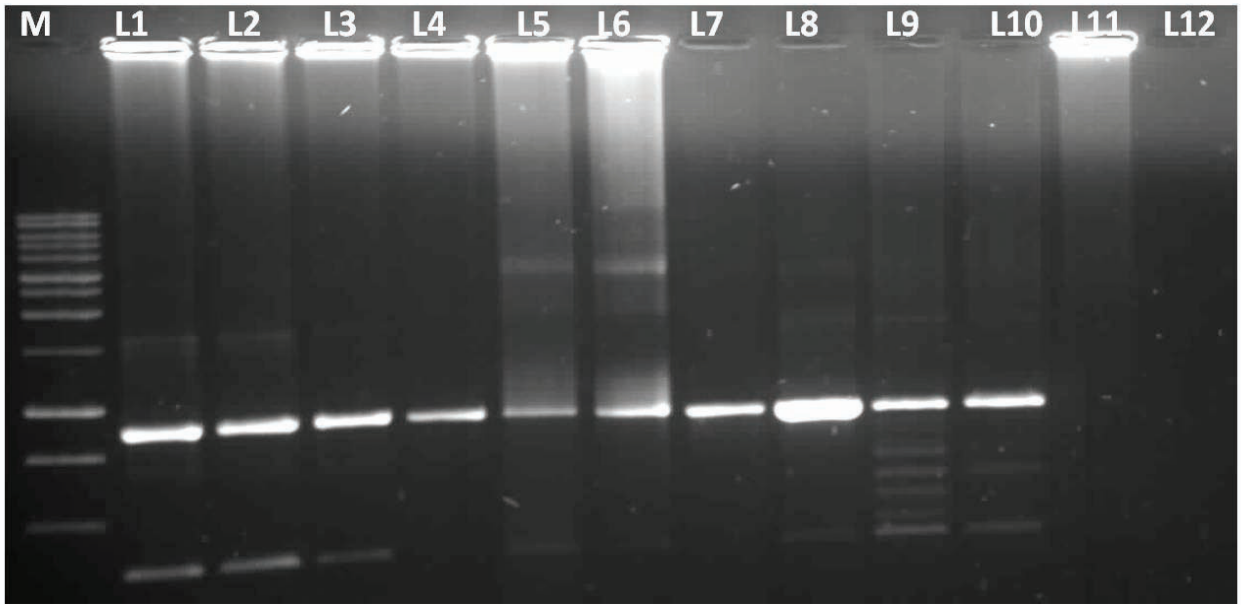

Fig. 2: Agarose gel electrophoresis analysis of nested PCR amplification products using nested primers fU5/rU3. M: 1kb DNA ladder, L1 to L4: positive control, L5 to L10: random date palm samples, L11: negative control and L12: dH2O.

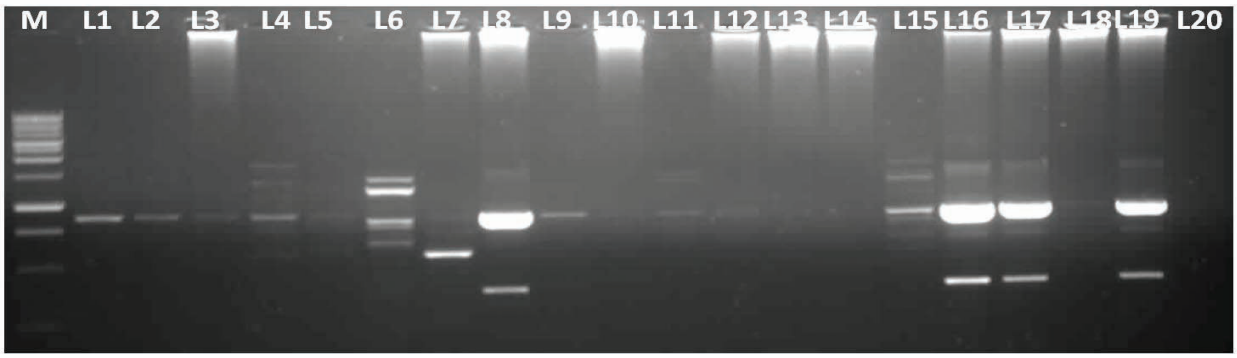

Fig. 3: Agarose gel electrophoresis analysis of PCR amplification products using nested primers fU5/rU3. M: 1kb DNA ladder, L1 to L15: Date palm samples from Alhasa, L16: Infected date palm positive control, L17: Group II positive control, L18: date palm samples from Almadinah, L19: date palm from Alkharj and L20: dH2O negative control.

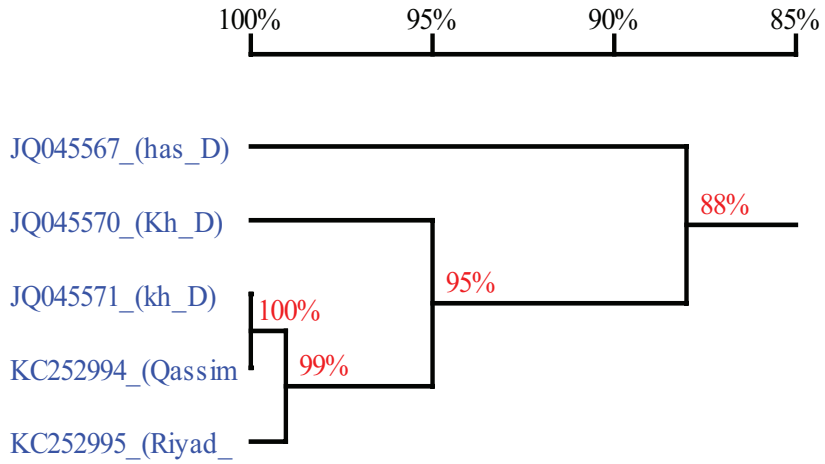

Fig. 4. Phylogenetic relationships of 16S ribosomal RNA gene, for infected date palm using nested PCR with set of primers rU3/fU5.

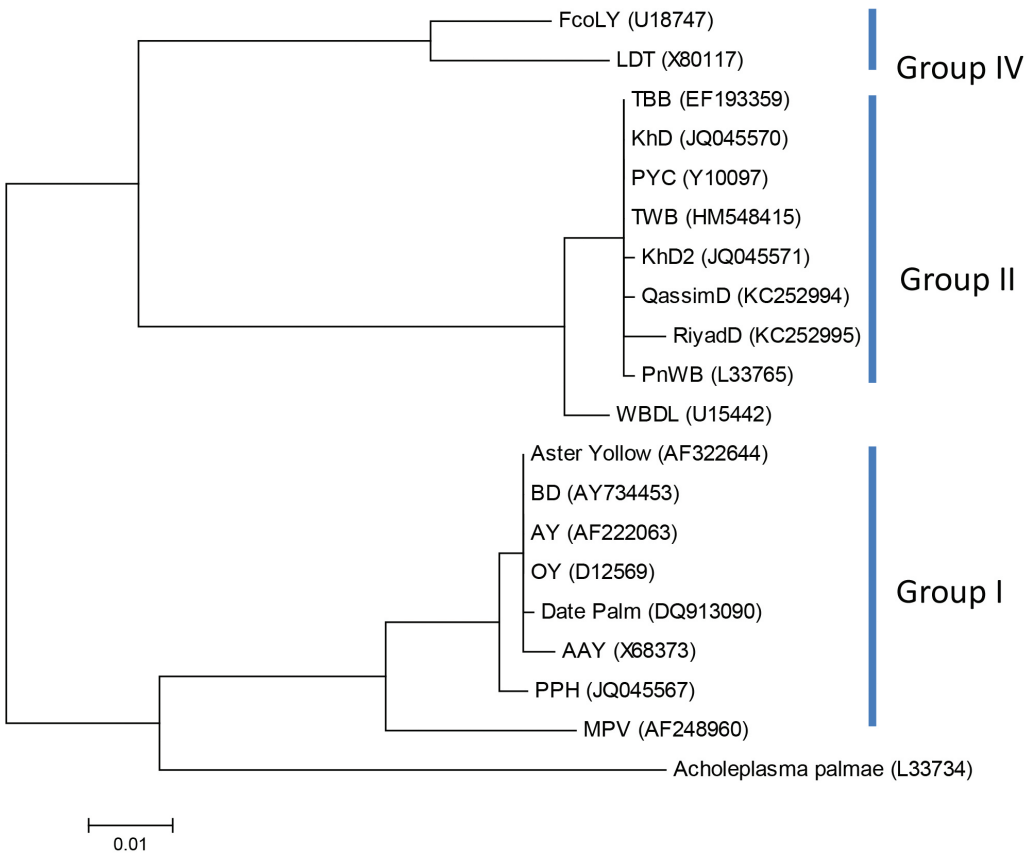

Fig. 5: Phylogenetic relationships tree of 16S ribosomal RNA gene, for infected date palm using nested PCR with the set of primers rU3/fU5 with other sequences obtained from GenBank. The 0.01 bar indicates one nucleotide change per 100 nucleotides.