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RESEARCH

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Detection of mixed infection of *Prunus necrotic ringspot virus* and phytoplasma in peach trees in Egypt

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ABSTRACT

Background: Prunus necrotic ringspot virus (PNRSV), family Bromoviridae, genus Ilarvirus is known to infect stone fruit trees and causes extensive economic losses in Egypt. Distinctive symptoms with PNRSV are necrotic ring spots on leaves, bud failure and poor quantity and quality of fruits. Upon continuous observation of peach trees previously known to be infected with PNRSV, additional suspected symptoms of phytoplasma infection were detected. These involved leaf yellowing and withering, reduction in fruit size, malformation of fruits (where two or three fruits were attached together), and tree death were observed.
Objective: The objective of this study is to characterize the nature of this disease complex through examining the incidence of PNRSV and phytoplasma in these trees.
Methods: The incidence of PNRSV in the peach trees understudy was confirmed with DAS-ELISA using an authentic antiserum for PNRSV. The incidence of phytoplasma as a second pathogen in the trees was PCR tested using the universal primers for phytoplasma detection, P1/P7, in the first step. The PCR products were re-amplified with nested-PCR to verify phytoplasma incidence using the nested primers R16F2/R2.
Results: DAS-ELISA confirmed the presence of PNRSV in the tested peach trees. For phytoplasma incidence, the universal primers P1/P7 amplified one fragment of about 1800 bp in length. Nested PCR amplified amplicons of 1,200 bp; hence confirming the presence of phytoplasma in these trees. Negative results were obtained when total DNA obtained from healthy peach leaves was used as a control.
Conclusion: The present study confirms the incidence of mixed infection with both PNRSV and phytoplsama(s). Due to limited information on the infection of peach with phytoplasmas in Egypt, larger surveys should be carried out to study the occurrence of PNRSV-phytoplasma disease complex and their corresponding insect vectors.

Keywords: PNRSV; phytoplasma; DAS-ELISA; mixed infection; PCR

BACKGROUND

Stone fruit trees are the hosts of more than 30 viruses and virus-like pathogens. Both *Prunus necrotic ring spot virus* (PNRSV) and *Prune dwarf virus* are the most common worldwide viruses infecting stone fruits (Cieślińska and Morgaś, 2010). PNRSV, family *Bromoviridae*, genus *Ilarvirus* is known to cause extensive economic losses in Egypt. Distinctive symptoms with PNRSV are necrotic ring spots on leaves, bud failure and poor quantity and quality of fruits (Adel-Salam *et al*., 2008).

Phytoplasma are obligate endocellular parasitic bacteria which lack cell walls and associated with diseases affecting hundreds of plant species. Infected plants exhibit dramatic changes in organ morphology including yellowing, stunting, proliferation, phyllody and witches' broom.

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Phytoplasma infection disturbs hormonal balance in host plants (Hoshi *et al*., 2009; Gai *et al*., 2014).

Several phytoplasmas infect stone fruits. Though, the most common one in Europe is '*Candidatus* Phytoplasma prunorum' ('*Ca*. P. prunorum'), associated with European stone fruit yellows (ESFY) disease. This phytoplasma is related to '*Candidatus* Phytoplasma mali' ('*Ca*. P. mali') and '*C.* Phytoplasma pyri' ('*Ca*. P. pyri'), which cause apple proliferation and pear decline diseases, respectively (Seemüller and Schneider 2004). Further, Aster yellows phytoplasma was detected on peach trees in Jordan (Anfoka and Fattash, 2004).

Very little is known about the presence of phytoplasma-infecting stone fruits in Egypt. Preliminary attempts to test for the presence of phytoplasma in peach and apricot using polymerase chain reaction (PCR) and the R16F2n/R16R2 primers indicated the presence of unknown phytoplasma similar in its induced symptoms to those caused by the *Ca*. P. prunorum', associated with the ESFY disease (Al khazindar and Abdel-Salam, 2010). However neither restriction digestion nor sequence analysis was done on the PCR-DNA products to confirm the nature of this phytoplasma. Additionally, Steffek *et al.* (2012); however, negated the presence of ESFY in the southern European countries including Italy, Spain, Portugal, Greece, Malta, and Cyprus.

Mixed infection between viruses in stone fruits is common (Serçe and Ertunc, 2008). Very few citations; however, describing the presence of mixed infection between viruses and phytoplasmas were reported in papaya (Arocha *et al*., 2009), in tomato and pepper (Hernandez-Gonzalez *et al*., 2011), and in apple (Liebenberg, 2013).

Recently observation of peach trees, previously known to be infected with PNRSV in Giza governorate (Abdel-Salam *et al*., 2008), additional suspected symptoms of phytoplasma infection were detected. These involved leaf yellowing and withering, reduction in fruit size, malformation of fruits, and partial or complete death of peach trees were.

The present study is mainly concerned with confirming the phenomenon of mixed infection of virus and phytoplasma on peach. Interaction between the two pathogens is the scope of discussion in another coming publication.

MATERIALS AND METHODS

Plant materials:

The present study was conducted on peach trees at the experimental Farm of the Faculty of Agriculture, Cairo University, Egypt. A total of twelve trees were chosen as the subject of analysis in the present study. These included three trees for each of: single infection with phytoplasma, single infection with PNRSV, mixed infection with both phytoplasma and PNRSV, and three asymptomatic, presumably healthy, trees.

Leaf samples from symptomatic or asymptomatic peach trees were collected in the autumn of 2015. From each tree, eight leaf samples were collected. Samples were kept in plastic bags at 4^{o}C. A compound sample was made for each tree by collecting the midribs of leaves, chopping them with a sterile razor blade, and mixing them randomly.

Each compound sample was equally divided into two halves; one was used for PCR analysis and the second half for DAS-ELISA analysis.

DNA extraction:

For DNA extraction, the silica based method of Echevarría-Machado et al. (2005) was followed. Tissues (0.1 g/sample) were pulverized in liquid N_2 using mortars and pistol. The

powder was suspended in 1 ml extraction buffer (10 mM Tris-HCl, 50 mM EDTA, 500 mM sodium chloride, 10 mM ß-mercaptoethanol, pH 7.0) and 100 µl of 20% SDS were added. After mixing, samples were incubated at 65^{o}C for 10 min. For each sample, 500 µl of 5 M potassium acetate were added and tubes were shaken vigorously and incubated for 20 min in ice. Tubes were spun 14,000 g for 20 min, supernatant was transferred to new tube, and DNA was bound to 300 µl sterilized washed silica suspension through manual mixing for 3-5 min. DNA was precipitated with low speed centrifugation (1 min/14,000 g), washed twice with 70% ethanol, and pellet was air dried. DNA pellet was suspended in 50 µl of distilled water and incubated at 55^{o}C for 5 min. The DNA suspension was spun (14,000 g/2 min), transferred to new 500 µl tube, and frozen at -86^{o}C for further analysis.

PCR analysis for phytoplasma presence:

DNA samples were adjusted with sterile water to100 ng/µl for PCR analysis. The primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) was used to amplify ca 1800 bp of the 16S rRNA gene. PCR amplification was performed in 25 µl reaction mixture containing 1 ul of DNA template , 2 mM $MgCl_2$, 1X GoTaq DNA polymerase reaction buffer (Promega, WI, USA), 200 µm dNTPs, 0.4 pmol of each primer, 1.25 U of Taq polymerase (Promega, WI, USA), and water.

PCR parameters included denaturation at 95^{o}C for 1 min, 35 cycles each with denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min, and extension for 2 min at 72^{o}C, and a final extension at 72^{o}C for 10 min.

For nested PCR, The universal primers R16F2/R2 (Lee et al., 1993) designed to amplify 1200 bp of the 16S rRNA gene resulting from the 1800 bp of the P1/P7-PCR products. Concentrated DNA products, or diluted 1/10 with water, were used for PCR amplificaton. One µl of DNA template was used in the 25 µl reaction mixture containing 1X reaction buffer (Promega WI, USA), 200 µm dNTPs, 0.4 µm of each primer, 1.25 U of Taq polymerase, and water. PCR parameters were similar as above except that annealing temperature was at 50^{o}C for 2 min, and extension at 72^{o}C for 3 min, and a final extension at 72^{o}C for 10 min.

The amplified products were electrophoresed in 1% agaraose gel prepared in 1X TAE buffer. Gel was stained with ethidium bromide (0.5 µg/ml) and examined with UV illuminator.

DAS-ELISA:

Antiserum for PNRSV (Abdel-Salam et al., 2008) produced for the local Giza- isolate of the virus was used. The technique of DAS-ELISA described by Clark and Adams (1977) was followed. Samples were diluted 1/20 (w/v) with PBST buffer. IgG was used at 1/000 dilution in coating buffer. While IgG-alkaline phosphatase conjugate was diluted upon use to 1/500 dilution with enzyme conjugate buffer. Microplates were read at 405 nm.

RESULTS

Symptomatology

The primary infection of PNRSV to peach trees induced chlorotic spots which developed to necrotic spots and shoot holes (Fig. 1-A). In dual mixed infection of peach trees with phytoplasma and PNRSV leaf yellowing was developed on leaves (Fig. 1-B). Fruits developed on phytoplasma-infected trees are usually slightly smaller than normal (Fig.2-A). In mixed infection of peach trees with PNRSV and phytoplasma, the formed fruits were misshapen where two to four fruit were attached together (Fig. 2-B). Upon disease progress, general yellowing

appeared on leaves, and leaf become severely necrotic (Fig. 2-C). Branches tend to wither and dry out (Fig. 2-D). Usually trees may die out within 6 to eight years.

Fig. 1: Symptoms of PNRSV-single infection (A) and mixed infection, PNRSV &Phytoplasma, on peach leaves (B).

Fig. 2: Development of symptoms of infected peach trees with phytolasma alone (A), phytoplasma and PNESV (B, C, D)

PCR detection of phytoplasma:

Nested PCR detected amplified DNA products with the size of ca 1200 bp from both singly infected with phytoplasma as well as trees with mixed infection with PNRSV and Phytoplasma). In asymptomatic peach trees, no PCR products were detected with PCR (Fig.3).

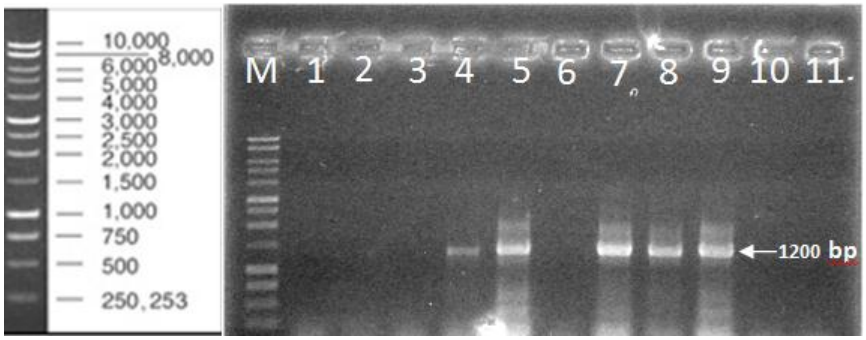

Fig. 3: Agarose (1%) gel electrophoresis of nested PCR using R16F2/R2 primers with 1200 bp amplicons recovered from peach trees infected with phytoplasma alone (lanes 4, 5) or with mixed infection with phytoplasma and PNRSV (lanes 7, 8, 9). Controls involved trees infected with PNRSV (lanes1, 2, 3) and asymptomatic trees (lanes 6, 10, 11). M = 1 kb DNA ladder (Gene Direx)

DAS-ELSA:

Results in Table 1 indicated that the antiserum of PNRSV detected PNRSV in peach trees showing symptoms of PNRSV infection alone or in trees with mixed infection with both PNRSV and Phytoplasma. The $O.D_{405nm}$ values in mixed infection were significantly higher than corresponding values in single infection with PNRSV. Negative results were obtained from asymptomatic peach trees as well as from trees infected with phytoplasma alone taken as negative control to PNRSV antiserum.

Table 1: DAS- ELISA for testing infected peach leaves against the presence of PNRSV

Optical density at 405 nm*												
Tested peach trees with symptoms of phytoplasma, PNRSV or both pathogens**												
Phytoplasma			**PNRSV**			**Phytoplasma + PNRSV**			**Controls**			
									Asymptomatic			**Buffer**
T 1	**T 2**	**T 3**	**T 1**	**T 2**	**T 3**	**T 1**	**T 2**	**T3**	**T1**	**T 2**	**T 3**	
0.06	0.05	0.05	0.73	0.60	0.91	1.60	2.17	2.01	0.05	0.06	0.04	0.04

Twelve peach trees were the subject of DAS-ELISA testing where three trees were tested for each of the four treatments as indicated in the section of Material and Methods. •Tested samples with reading values 3 times more the healthy (asymptomatic) control were considered positive. ••Data represent the mean of eight replicates per tree (T) used for DAS-ELISA testing. LSD (least significant difference) measured at $p = 0.05$ using the ANOVA: Single Factor Program = 0.123

DISCUSSION

The present study discusses the presence of mixed infection in peach trees with PNRSV and phytoplasma. The peach trees, at the present study, had a history of PNRSV presence. PNRSV was isolated from some of these trees and identified biologically, serologically, and at the molecular level (Abdel-Salam *et al*., 2008). Recently some of the PNRSV-infected peach trees started to show aggravated symptoms of leaf-shoot holes with yellowing and fruit malformation. The presence of the yellowing symptoms suggested the presence of phytoplasma associated with PNRSV infection. Additionally, other asymptomatic tree showed typical phytoplasma-yellows

symptoms alone. DAS-ELISA confirmed the presence of PNRSV in both single infection of peach with PNRSV and in dual infection of peach with PNRSV and phytoplasma. PCR detected phytoplasma in both singly and doubly infected peach trees.

Very little is known about the presence or the nature of phytoplasma-infecting stone fruits in Egypt. Preliminary study done on some peach and apricot trees in Giza governorate suggested the presence of the European stone fruit yellows (ESFY) phytoplasma '*Ca*. P. prunorum' causing the ESFY disease (Al khazindar and Abdel-Salam, 2010). However neither restriction digestion (RD) nor sequence analysis was done on the PCR-DNA products to confirm the nature of this phytoplasma. Steffek *et al.* (2012) also negated the presence of ESFY in the southern European countries including Italy, Spain, Portugal, Greece, Malta, and Cyprus.

Mixed infection between ilarviruses in stone fruits is common (Serçe and Ertunc, 2008; Pallas *et al*., 2012). Host response to *Ilarvirus* infection has recently been studied. Bellés *et al.* (2006, 2008) reported the accumulation of gentisic acid and the activation of phenylpropanoid pathway as defense responses of susceptible cucumber plants to PNRSV infection. In some cases, mixed infection between unrelated pathogens may lead to synergism and exacerbation of disease symptoms and increases in titer of both pathogens (Rentería-Canett *et al*. 2011). On the other hand, Liebenberg (2013) measured an antagonistic interaction between *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV) upon co-infection of apple (*M. domestica*). The measured titer of ASGV was decreased by half; while the ASPV titer kept unchanged. Interaction between viruses in mixed infection can be measured by quantitative Real-Time PCR (qRT-PCR) and could be used to determine pathogen titer in plant cell (Bustin, *et al*., 2009).

Very few citations described the presence of mixed infection between viruses and phytoplasma. Mixed infection was reported in papaya infected with both a *potyvirus* and a phytoplasma (Arocha *et al*., 2009). Lebsky *et al.* (2011) described the dual infection of tomato and pepper plants with a phytoplasma and two begomoviruses.

Mixed infection with virus and phytoplasma was also reported in lily infected with lily symptomless virus where infected plants had mixed infection with two phytoplasma belongs to the 16SrI-B and the 16SrXII-A groups (Bertaccini *et al*., 2002).

The qRt-PCR was used to measure the titer of ASGV and the phytoplasma, '*Ca*. P. mali', causing the apple proliferation in mixed infection. The results indicated an antagonistic interaction between ASGV and '*Ca*. P. mali'. Titer of '*Ca*. P. mali' was significantly increased, while the ASGV was significantly decreased when compared to the titer value estimated from single infection. However, the mechanism of disease interaction between ASGV and phytoplasma was not clearly illustrated. A possible explanation for the increase in '*Ca.* P. mali' titer in association with ASGV- could be that both the virus and '*Ca.* P. mali' activate salicylic acid response and that these responses are milder in single infections, compared to the response activated in the co-infection (Liebenberg, 2013).

In the present study, there was an observed increase in symptom severity, yellowing, necrotization, branch withering, and fruit malformation in mixed infection with PNRSV and phytoplasma; indicating the presence of a synergism situation (Rentería-Canett *et al*. 2011) compared to single infection with either PNRSV or phytoplasma. Such symptom aggravation would be the sum of cumulative effect of both pathogens in induction of one of the pathogen-driving resistance mechanisms (Bellés *et al.,* 2006, 2008; Liebenberg, 2013). Fruit malformation in peach trees with dual infection with PNRSV and phytoplasma, in the present study, may be attributed to severely disturbed host hormonal imbalances (Lee *et al*., 2000; Hoshi *et al*., 2009; Gai *et al*., 2014).

The present study showed a significant increase in PNRSV concentration in mixed infection when measured with DAS-ELISA. Regular PCR was not able to measure concentration of phytoplasma in single and double infection. Therefore qRT-PCR should be used in future experiments to determine concentrations of both pathogens accurately in mixed infection.

Establishment of *in vitro* tissue culture (TC) system to study interaction of single and mixed infection in host would be more accurate and more controlled than measuring interactions directly from the field. Production of antisera for phytoplasma would considerably facilitate detection of phytoplasma as well as determine pathogen concentration in host. This subject has long been non-voluntarily ignored. Recent study by Khankahdani and Ghasemi (2011) showed the possibility of production of an antiserum for the phytoplasma responsible for the Bermuda grass white leaf (BGWL), a destructive disease to Bermuda grass (*Cynodon dactylon*), present in Asia, Africa, Italy and some Latin American countries. This antiserum detected BGWL phytoplasma with Plate-Trapped Antigen Enzyme-Linked Immunosorbent Assay and dot and tissue immuno-blotting assays. Further this antiserum showed no cross reactions with other phytoplasmas causing symptoms in almond, lime, periwinkle, and sugar cane plants. Thus, should prepared antisera for two pathogens, virus and phytoplasma per se, are available; interactions between single and mixed infection can be measured correctly using *in vitro* TC controlled conditions.

Mixed infection with more than one pathogen is common as stated above. Attempts are now in progress to, *in silico*, design transgenic small interfering RNAs (siRNAs) against hotspots in pathogen genes encoding protein suppressors to host siRNAs; especially in mixed infection situation (Sharma *et al*., 2015). In other words, these siRNAs designed for combating more than one pathogen in mixed infection would facilitate development of transgenic plants having wide spectrum of resistance against multiple pathogen infection.

CONCLUSION

The present study confirms the incidence of mixed infection with both PNRSV and phytoplasma(s). Due to limited information on the infection of peach with phytoplasma in Egypt, larger surveys should be carried out to study the occurrence of PNRSV-phytoplasma disease complex and their corresponding insect vectors.

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