Emerging phytoplasma diseases of stone fruits and other crops and their possible impact on EU Countries

Istanbul, Turkey

December 1st and 2nd 2011

COST Action FA0807

Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Program

December 1th

04:00 pm – Arrival of participant and preliminary plenary discussion

07:00 pm – Social dinner

December 2nd

09:00 am – Welcome addresses

09:30 am – **A. Bertaccini and B. Duduk**. Taxonomy of phytoplasmas associated with emerging diseases.

10:00 am – **A. Alma and R. Tedeschi**. Emerging phytoplasma diseases: research of the insect vectors.

10:30 am - 11:00 am Coffee break

11:00 am – **K. Çağlayan, M. Gazel, Ç. Ulubas Serçe, İ. Adem Bozkurt and E. Elçi**. Phytoplasma disease of stone fruit trees in Turkey and their containment.

11:30 am – **D. Canik, F. Ertunc, S. Paltrinieri, N. Contaldo and A. Bertaccini.** Current status of grapevine phytoplasma infections in Turkey.

12:00 am – **M. Molino-Lova, C. Mahfoud, Y. Abou Jawdah, E. Choueiri, H. Abdul-Nour, R. Fakr, R. al Achi, A. Alma, L. Picciau and P.A. Bianco.** Results of last surveys for stone fruit phytoplasma disease management in Lebanon.

12:30 am – **Y. Abou Jawdah, E. Choueiri, P. Bianco, M. Molino-Lova, S. Hajj-Hassan and R. al Achi.** Almond witches'-broom phytoplasma: situation in Lebanon and action of the ministry of agriculture for the disease eradication during the 2010-2011 seasons.

01:00 pm - 02:30 pm Lunch

02:30 pm – **N. Salem.** Virus and phytoplasma diseases of stone fruits in Jordan.

03:00 pm – **M. Al Khazindar and A. Abdel Salam**. Phytoplasma in stone fruits and date palm in Egypt.

03:30 pm – **C. Ikten, E. Yol, M. Catal and B. Uzun.** Frequency distribution of sesame phyllody infected by phytoplasmas in Antalya, Turkey.

04:00 pm - 04:30 pm Coffee break

04:30 pm – **P. A. Bianco, D. Bulgari, P. Casati and F. Quaglino**. Conventional and novel strategies for the phytoplasma diseases containment.

05:00 pm – Round table discussion

06:30 pm – Conclusions

07:00 pm Closing of the meeting

Contents

Taxonomy of phytoplasmas associated with emerging diseases

Assunta Bertaccini[1] and Bojan Duduk[2]
[1]DiSTA, Plant Pathology, *Alma Mater Studiorum*, University of Bologna, Bologna, Italy
assunta.bertaccini@unibo.it
[2]Institute of Pesticides and Environmental Protection, Belgrade, Serbia
bojan.duduk@pesting.org.rs

Summary
Numerous phytoplasma-associated diseases are causing severe epidemics that are in some cases emerging diseases in several parts of the world. Detection and fine molecular characterization of associated-phytoplasmas is a key for understanding disease cycle and for planning the correct disease management strategies. Molecular diversity of phytoplasmas is achieved by multilocus identification using, besides the 16S rDNA gene, ribosomal proteins S3, tuf, SecY, amp, imp and GroEl genes. Phytoplasmas associated with emerging diseases mainly belong to ribosomal groups 16SrII and 16SrIX although phytoplasma strains belonging to other groups such as 16SrIII, 16SrVI, 16SrVII and 16SrXII in different area of the world can also be regarded as associated with possible emerging diseases.

Key words: phytoplasma, epidemic, detection, classification, plant diseases.

Introduction

Diseases associated with phytoplasma presence occur worldwide in many crops and in some cases they are able to completely destroy cultivations in their epidemic phases. Plants infected by phytoplasmas exhibit symptoms suggesting profound disturbances in the normal balance of growth regulators. These include virescence/phyllody (development of green leaf like structures instead of flowers), sterility of flowers, proliferation of axillary buds resulting in a witches' broom behavior, abnormal internodes elongation, generalized stunting (Bertaccini and Duduk, 2009). Recent molecular data on phytoplasmas have provided considerable insights into their molecular diversity and genetic interrelationships that are the basis for several comprehensive studies on phytoplasma phylogeny and taxonomy (Hogenhout *et al.*, 2008). Sensitive and accurate detection of these micro-organisms is a prerequisite for the study and management of phytoplasma-associated diseases. Detection is routinely done by different nucleic acid techniques mainly based on polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism analyses (RFLP) and/or sequencing.

Phytoplasma diseases of emerging importance

The list of diseases associated with phytoplasma presence continues to grow; many newly emerging diseases have been identified in the last ten years. One of the most interesting cases is the citrus huanglongbing disease that was associated with aster yellows-related phytoplasmas in China (16SrI) (Chen *et al.*, 2008) and with pigeon pea witches' broom-related phytoplasmas (16SrIX) in Brazil (Teixeira *et al.*, 2009). These kind of findings confirm the non rare occurrence of similar symptoms associated with diverse phytoplasmas and the need of pathogen identification as first step to perform then identification of insect vector, of transmission ways and alternate host plants: all these information are needed to efficiently contain the disease impact on agriculture and environment. A list of the phytoplasma associated disease that are severely emerging worldwide is given in table 1.

In several regions of the Middle East citrus species are affected by witches' broom disease that almost eliminated the traditional lime production in Iran and neighbouring countries and also affects the citrus production (Mardi *et al.*, 2011). The disease can be associated with diverse phytoplasmas, however the '*Ca*. P. aurantifolia'-related phytoplasmas are very often detect in several other species worldwide and also in Europe with reports on flowers (Davino *et al.*, 2007), in insects (Parrella *et al.*, 2008) besides the one on cactus pear (Granata *et al.*, 2006). Grapevine is also a species that is infected by different phytoplasmas worldwide and recent identification of aster yellows and '*Ca*. P. fraxini' in Italy, South Africa (Alma *et al*., 1996; Engelbrecht *et al.*, 2010) and in Chile (Gajardo *et al.*, 2009) respectively, represents potential emerging diseases, important is also the detection in Iran of a new stolbur strain infecting this crop (Karimi *et al.*, 2009). The frog skin disease of cassava in Colombia (Alvarez *et al.*, 2009) and a cherry decline in Chile (González *et al.*, 2011) are both related to X-disease phytoplasmas and are emerging diseases in these areas.

Table 1. Classification of phytoplasmas associated with emerging diseases with RFLP analyses of 16S rRNA gene.

Phytoplasma disease	16Sr group/subgroup	'*Candidatus* Phytoplasma' species	Geographic distribution
Almond witches' broom	**16SrIX-B**	'*Ca*. P. phoenicium'	Lebanon
Cassava frog skin	**16SrIII-L**		Colombia
Grapevine yellows	**16SrI-B**	'*Ca*. P. asteris'	Italy, South Africa
Grapevine yellows	**16SrVII-A**	'*Ca*. P. fraxini'	Chile
Grapevine yellows	**16SrXII**		Iran
Jujube witches' broom	**16SrV-B**	'*Ca*. P. ziziphi'	China, Korea, Italy
Lime witches' broom	**16SrII-B**	'*Ca*. P. aurantifolia'	Oman/Iran
Potato purple top wilt	**16SrVI-A**	'*Ca*. P. trifolii'	USA
Potato purple top wilt	**16SrXVIII-A**	'*Ca*. P. americanum'	USA

The jujube witches' broom disease is quite widespread in jujube as well as other crops in China and Korea (Jung *et al.*, 2003; Fan *et al.*, 2008) but the same phytoplasmas was recently detected in cherry in a few cases also in Italy (Paltrinieri *et al.*, 2008). Last is potato that was reported infected by several phytoplasmas (Paltrinieri and Bertaccini, 2007; Munyaneza, 2010) some of them, see table 1, only reported in USA but group 16SrVI phytoplasmas were also identified elm in USA (Jocobs *et al.*, 2003), in *Vaccinium* sp Europe (Borroto Fernandez *et al.*, 2007), in sesame in Turkey (Sertkaya *et al.*, 2007) and very recently in grapevine in Syria (Contaldo *et al.*, 2011). Almond witches' broom and the group 16SrIX phytoplasmas are the emerging phytoplasmas in Lebanon (Abou-Jawdah et al., 2009), but phytoplasma belonging to the same group were already reported in other areas worldwide such as Colombia (Duduk *et al.*, 2008), Italy (Bertaccini *et al.*, 2009) and in coniferous trees in USA (Davis *et al.*, 2010).
Very recently also phytoplasma detection in new species such as kiwi (*Actinidia* spp) very often in double infection with bacterial opens new perspectives in concept of emerging diseases since the presence of these prokaryotes could increase the plant sensitivity to other pathogens changing the epidemic cycle of known diseases (Bertaccini *et al.*, 2011).

Molecular tools for phytoplasma strain differentiation
The use of primers based on 16S rRNA gene allow identification of phytoplasmas subgroup (Lee *et al.*, 1998a; 1998b). Finer differentiation of phytoplasmas for better characterization of emerging phytoplasma diseases is necessary for epidemiological purposes therefore additional markers such as ribosomal protein (*rp*), *secY*, *tuf*, and *GroEl* gene should been used as supplementary tools (Lee *et al.*, 2004a; 2004b; 2006a; Martini *et al.*, 2002; 2007; Schneider *et al.*, 1997; Marcone *et al.*, 2000; Duduk *et al.*, 2009, Mitrović *et al.*, 2011a; 2011b). Finer subgroup delineation achieved by combining RFLP analyses of 16S rRNA and other genes different according with phytoplasma group considered in multiple gene analyses is therefore desirable for correct disease management.

Management of phytoplasma diseases

Control of epidemic outbreak of phytoplasma diseases are carried out by controlling the vector, eliminating the infected plants and producing pathogen-free and possible resistant plants (Bertaccini, 2007). However phytoplasmas may overwinter in insect vectors or in perennial plants and interact in various manners with insect hosts: examples of both reduced and increased fitness were described (Christensen *et al.*, 2005, Sugio *et al.*, 2011). Transovarial transmission of some phytoplasmas can also be relevant in introducing emerging diseases in new environments (Bertaccini, 2007). The most common way for dissemination of emerging phytoplasma diseases is the propagation or micropropagation of infected material (Bertaccini *et al*., 1992; 2011; Tian *et al.*, 2000).

Recently also the possibility of phytoplasma transmission by seed was reported in several species such as alfalfa and lime in which the presence of phytoplasmas belonging to ribosomal groups 16SrI, 16SrXII and 16SrII was demonstrated (Khan *et al*., 2002; Botti and Bertaccini, 2006). Similar results were reported in tomato from Italy and Bulgaria, and corn from Italy and Serbia (Calari *et al.*, 2011); these findings represents new pathways for phytoplasma dissemination as well as increases the possibility of emerging diseases.

Figure 1. Left micropropagated lime from seed infected by 16SrII phytoplasmas, center alfalfa from seed left seedling infected by 16SrII phytoplasmas; right, on the right cassava roots infected by frog skin disease (picture Juan F Mejia).

Knowledge about the mechanisms of plant host resistance to phytoplasmas is little, but the paucity of effective disease management strategies for these diseases lends a high priority to these questions. Efforts continue to identify germplasm encoding natural resistance to *Mollicutes*, and to incorporate such genes *via* selection and breeding programs into various crops and trees, involving resistance to either the pathogen itself or to the insect vector are of very urgent need.

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Emerging phytoplasma diseases: research of the insect vectors

Alberto Alma and Rosemarie Tedeschi
DIVAPRA, Entomologia e Zoologia applicate all'Ambiente "C. Vidano", Facoltà di Agraria, Università di Torino, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy
alberto.alma@unito.it
rosemarie.tedeschi@unito.it

Summary
In the last few years many reports focused on the emergences related to new phytoplasma diseases or new outbreaks of already known ones. Some of these diseases were recorded in Europe and gave cause of concerns because of their possible wide spreading throughout the continent, damaging forest trees or economically important crops. The purpose of the present work is to provide useful information for an appropriate and efficient identification of the insect vectors.

Key words: phytoplasma, epidemic, insect vector, identification.

Introduction

In the last few years many reports focused on the emergences related to new phytoplasma diseases or new outbreaks of already known ones. Some of these diseases were recorded in Europe and gave cause of concerns because of their possible wide spreading throughout the continent, damaging forest trees or economically important crops. It is the case of phytoplasmas affecting conifers such as '*Candidatus* Phytoplasma pini' (Sliwa *et al.,* 2007; Valiunas *et al.*, 2010) that represent a possible treat to timber industry, or the case of the maize redness associated with stolbur phytoplasma that can provoke severe losses (Duduk and Bertaccini, 2006; Bekavac *et al.*, 2008; Jóvic *et al.*, 2009). On the contrary other phytoplasma diseases are emerging in the Mediterranean Basin and in the Middle East and they are particularly worrying for their possible impact on EU countries. Recently '*Ca.* P. phoenicium' strains were associated with diseases affecting different important crops such as almond, peach and nectarines in Lebanon and Iran (Verdin *et al.*, 2003; Abou-Jawdah *et al.*, 2003; Abou-Jawdah *et al.*, 2009) and causing the death of more than 100,000 plants.
In all these situations the knowledge of the insect vectors is crucial for well-timed and efficient control strategies, to avoid further spreading of the pathogens.
In the case of new phytoplasma diseases, the vectors are always unknown and big works are required to identify these insects. Likewise, when new outbreaks occur in new geographical areas, the already known vectors may not have, there, the same role and the presence of other possible vectors should be investigated. Recent studies carried out in Lebanon to find the possible vectors of '*Ca.* P. phoenicium' highlighted some difficulties that are usually encountered when new zones are investigated. The purpose of the present work is to provide useful information for an appropriate and efficient identification of the insect vectors.

Insect samplings for vector search

The study of the entomophauna related to the crop of interest is not always easy and often different sampling techniques should be combined, due to the different life cycle of the insects. Moreover the search should not be restricted only to the crop, but it should be extended to the surrounding weeds and shrubs. The phytoplasma vectors are often polyphagous and they can feed on the crops just occasionally. Moreover once a putative vector has been identified, the knowledge of the association with some wild plants is fundamental to understand the epidemiology of the disease and to the pest management.
When nothing is known in a certain area, the first step is to carry out samplings that enable the capture of a high number of insects belonging to different species. The Malaise traps are quite

suitable for these kind of study because they are able to catch a wide range of insects that are present in the study area independently to their association with the crop or the wild vegetation. With the indications obtained by these samplings, more detailed ones should be carried out with the beat-tray method or the sweep net on both the crop and the surrounding vegetation. These two methods allow the capture of alive specimens that can be properly stored in the laboratory for further molecular analyses. On the contrary the insects captured with the Malaise traps are not suitable for this purpose, because, even if the preservation liquid contains ethanol, the insects remain in the field for several days and often at high temperatures. Moreover the possibility to catch alive specimens is very important for mass rearing setup or further transmission trials.
When these samplings are carried out, a detailed collection of informative data is recommended: collector, place and date of collection, host plant, etc.
Both sweep net and beat trays allow to investigate plant species selectively providing interesting data on host plant associations.
Sticky traps can support the Malaise traps for a preliminary screening of the local entomophauna, but they are particularly useful in a second step to study the population dynamics of a selected group of possible insects vectors. They should be replaced at least every fortnight. Sometimes the captured insects are removed for subsequent molecular analyses, but in that case the exposure time should not exceed one week; anyhow it is not the best method to gather specimens for molecular analyses in particular in warm regions.
Finally the use of vacuum insect collectors (D-VAC) is very useful to sample dense vegetation or the soil surface. These instruments are usually powered by gasoline engines and the insects are collected live in organdy bags and may be taken to the laboratory for separation and further trials. If alive specimens are not required, the collected insects can be placed in alcohol by gathering up and inverting the bag into a jar.

Collected insects handling

After preliminary wide range samplings the collected material should be well identified using morphological tools and following dichotomous keys. A quite good expertise is required when the captured material has to be identified. For this purpose an appropriate grounding of the technicians is desirable to discriminate the taxa and to focus on the ones that include phytoplasma vectors, that are Auchenorrhyncha and Sternorrhyncha (mainly leafhoppers, planthoppers and psyllids). Preliminary training schools are recommended if the personnel is not familiar with such entomological groups. The possibility to encounter criptic species is not rare and in such situation, molecular tools, when available (Bertin *et al.*, 2010a; 2010b; Tedeschi and Nardi, 2010), are recommended to discriminate them. These tools are also particular useful for the identification of females and young stages, considering that often the main taxonomic characteristics concern male genitalia.
If the collected insects are bound to be used in further trials (molecular analyses, mass rearings or transmission trials), good practices should be followed for their transfer from the field to the laboratory and for their preservation.
Collected insects in the field should be placed in vials containing some leaves or a small twig, to keep them alive, and preferably transferred in a cool box. Once in the laboratory, they should be immediately observed for identification and then used for laboratory trials, otherwise stored in pure ethanol or at -20°C to preserve total DNA.

Molecular analyses to assess the presence of phytoplasmas in the insects

All the captured insects belonging to the Auchenorrhyncha or the Sternorrhyncha can be analysed with molecular tools to assess, in a preliminary screening the presence of phytoplasmas. Different kind of molecular analyses can be done (predominantly PCR and RFLP) and in the literature the proper protocols for each situation can be found.
To achieve valuable results it is very important to stress the fact that if an insect results positive for a phytoplasma it does not mean that it is a vector; but it helps the search of the vectors to be

narrowed. Indeed molecular tools are so sensitive that they allow to detect small phytoplasma quantities ingested, even by non-vector species, during the plant sap sucking. The only prove of vector activity comes from transmission trials.

Transmission trials

Experimental transmission essays are fundamental to ascertain the vector ability of putative vector species (Weintraub and Beanland, 2006). To carry out them, naturally infected insects or experimentally infected ones can be used. In the first case healthy insects are caged on infected plants for an acquisition access period (AAP), then they are moved to a healthy suitable plant to complete the latency period (LP), and finally transferred to healthy plants for a inoculation access period (IAP). Alternatively infected insects collected on crops with a quite high infection level can be used for the trials. At the end of the experiments the plants should be observed for symptom appearance and molecular analysed for phytoplasma presence. In the case of woody plants we must keep in mind that symptoms often require some time (even more than one year) to appear, and likewise the phytoplasma titre can be very low, thus not detectable, for months. Although biological transmission trials are the best way to ascertain phytoplasma transmissibility, these procedures are very laborious requiring big amounts of healthy and infected insects and of healthy plants on which make them survive and transmit. Moreover sometimes they are not feasible because we do not know the host plants of the insect we want to study. It happens often with species that colonise the crop just occasionally and for which the host range is not already well know or even in the case of new species never described before for which, of course, nothing is known about the biology.
Transmission to an artificial feeding medium is a good and practical alternative to test the inoculative ability of candidate insect vectors (Tanne *et al.*, 2001; Bressan *et al.*, 2006, Pinzauti *et al.*, 2008). Normally microcentrifuge tubes are used as insect chambers: the cavity of the cap is filled with a liquid feeding medium (a sucrose or sucrose+sorbitol solution) and sealed with a parafilm membrane. After a IAP, the insects are removed and tested for phytoplasma presence. Likewise the feeding medium is removed and frozen or immediately molecularly analysed. This procedure allows a large-scale study of phytoplasma transmission giving valuable information on the epidemiology of new phytoplasma diseases.

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Phytoplasma diseases of stone fruit trees in Turkey and their containment

Kadriye Çağlayan, Mona Gazel, Çiğdem Ulubas Serçe, İ. Adem Bozkurt and E. Elçi
Mustafa Kemal University, Faculty of Agriculture, Plant Protection Department, 31034 Antakya/Hatay, Turkey
kcaglayano@yahoo.com

Abstract
Although fruit tree phytoplasmas were studied since 1999 in Turkey, there have been very limited studies and records on stone fruit tree phytoplasmas. The main symptoms on apricot, plum, peach and almond were chlorosis between veins, off season flowering and fruiting as a result of early bud breaking, longitudinal leaf rolling and quick die-back. More than 500 cultivated and wild *Prunus* plants in or nearby germplasm nurseries and commercial orchards during 2002-2009 were tested by using universal primers P1/P7 and fU5/rU3 for direct and nested PCR, respectively. Amplification products were digested with the *Rsa*I and *Ssp*I enzymes. The average incidence of '*Candidatus* Phytoplasma prunorum' was detected as 10.19%. The most infected stone fruit species were apricot and plum fallowed by almond and peach. No phytoplasma was found in cherries and wild *Prunus* species yet.

Key words: '*Ca.* P. prunorum', cultivated and wild *Prunus*, PCR-RFLP, Turkey.

Introduction

Turkey is the center of wide range of fruit trees cultivation because if its environmental conditions. Most of the fruit species like apricot, apple, plum and cherries are indigenous to the area. '*Candidatus* Phytoplasma prunorum' associated with of European stone fruit yellows (ESFY) disease is the quarantine phytoplasma infecting trees of the *Prunus* genus worldwide (Seemüller and Schneider, 2004). It causes serious economic losses in cultivated *Prunus* species. Susceptible young apricot and plum trees infected with '*Ca.* P. prunorum' die quickly within 1-2 years after inoculation, and the pathogen also causes yield and quality losses on trees older than five years (Nemeth, 1986). Recently some phytoplasmas-like symptoms such as off-season flowering, deformation and rolling of the leaves, reduced yield, reddening of leaves and decline on apricot, plum and almond were observed in both germplasm and commercial orchards located main fruit growing regions, such as Meditarranean, Marmara and Aegean. The first report of ESFY symptomatology in Turkey was reported by Çağlayan and Gazel (1999) and then molecular identification studies were performed by different workers (Jaraush *et al*., 2000; Çağlayan *et al*., 2004; Sertkaya *et al*., 2005; Ulubaş *et al*., 2006). The objective of the present study was to determine the incidence of '*Ca.* P. prunorum' mainly in mother blocks, nurseries and commercial orchards in several stone fruit growing regions of Turkey during 2002-2009.

Materials and methods

Surveys were carried out during 2002-2009 in mother blocks, nurseries, commercial orchards and wild *Prunus* plants in several stone fruit growing regions of Turkey (Meditarranean, Marmara and Aegean regions). More than 500 cultivated and wild *Prunus* species were collected for PCR-RFLP analyses.

Total DNA was extracted from one gram of fresh leaf midribs and phloem tissue of branch by using chloroform/phenol procedure (Prince *et al*., 1993). Samples were resuspended in TE buffer [10 mM Tris-HCl, 1 mM EDTA, (pH 8.0)] and diluted to adjust the final concentration of 20 ng/µl. The universal primers for phytoplasma detection, P1/P7 (Deng and Hiruki, 1991; Smart *et al*., 1996) were used in the first step, amplifying one fragment of about 1800 bp in length. The second step was performed with the fU5/ rU3 specific primers (Lorenz *et al*., 1995). All obtained amplicons were subjected to the RFLP analysis with *Rsa*I, *Ssp*I (MBI, Fermentas, GmbH, Germany). The digested products were analyzed by electrophoresis using 2% agarose gel and stained with ethidium bromide, DNA bands were photographed under UV light.

Results and discussion

Symptoms typical of ESFY infection were observed mainly on apricot and plums. The main symptoms on apricots were off season flowering in winter and leaf rolling whereas decline was most obvious symptom on plums (Figure 1). High correlation between early bud break symptoms on apricots and phytoplasma detection by PCR is already reported by Laimer *et al*. (2001) and Jarausch *et al*. (2008).

Figure 1. Leaf rolling, and off-season flowering of apricot (left and center); decline of plum trees (right) infected by '*Ca.* P. prunorum'.

Fragments of the expected size (850 bp) were amplified both from symptomatic and asymptomatic plants and also positive controls, but were not produced from healthy samples or water used as negative controls (Figure 2). Patterns obtained from RFLP of amplified sequences from phytoplasma infected trees were indistinguishable from each other, and were identical to those of the ESFY phytoplasma strain (Figure 3).

The overall incidence of phytoplasma infection in tested samples was 10.19%. However 35.89% of samples from germplasm orchards was infected by '*Ca.* P. prunorum' whereas disease incidence was 5.55% in commercial orchards. Highest infection rate was found in apricot (12.65%), followed by plum trees (11.60%), almond (5.74%) and peach (2.43%). No phytoplasma was found in cherries and wild *Prunus* species yet. The high incidence of '*Ca.* P. prunorum' in germplasm orchard can be taken as an indication that imported plant material is largely responsible for its introduction in the country. Since there is no established certification programme for fruit trees in Turkey, the propagation of the infected trees might cause increased infection in the future.

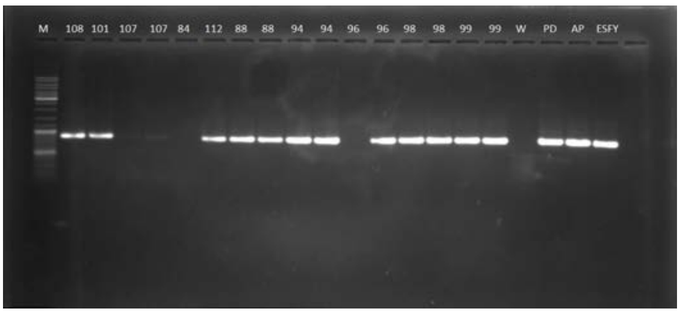

Figure 2. Nested PCR amplification of phytoplasma ribosomal DNA from symptomatic apricots (84, 88, 101, 107, 108, 112); plums (94, 96); almond (98); peach (99) and water control, using the universal primers P1/P7 followed by the specific primers fU5/rU3. M: Marker marker, #SMO331 (MBI Fermentas), positive controls: (PD: pear decline, AP: apple proliferation, ESFY: European stone fruit yellows).

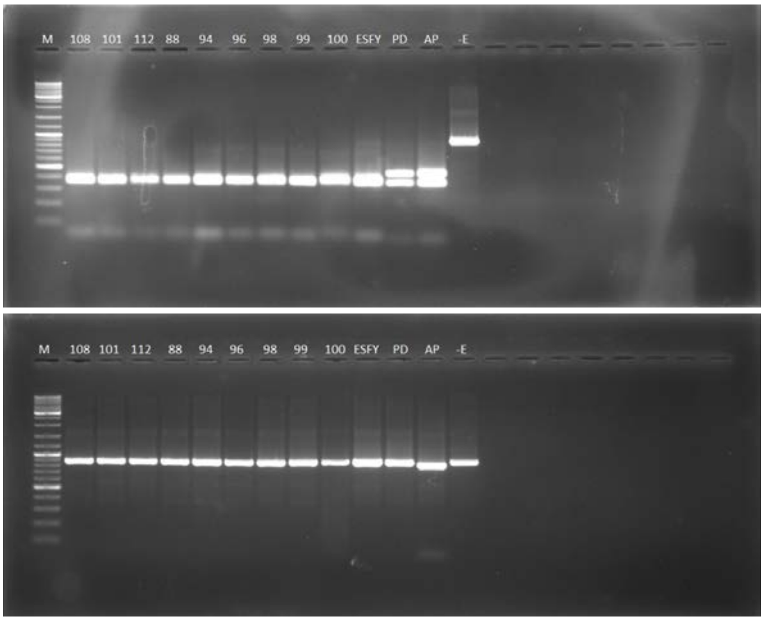

Figure 3. Results of RFLP analyses of fU5/rU3 nested PCR products from apricots (88, 101, 108, 112); plums (94, 96); almond (98, 100); peach (99) using *Rsa*I (top) and *Ssp*I (bottom) restriction enzymes. M: Marker marker, #SMO331 (MBI Fermentas), positive controls: (PD: pear decline, AP: apple proliferation, ESFY: European stone fruit yellows), -E: control uncut.

Our survey carried out for several years showed that the ESFY pathogen is widely spread in all important stone fruit growing regions of Turkey. All cultivated stone fruit species were found infected but *Prunus armeniaca* is the most affected species as reported before (Jarausch *et al*., 2008). *Prunus* species with no typical phytoplasma symptoms were also often found infected by phytoplasma. This observation imply that the latent infections in nurseries and orchards can play important role for spreading of ESFY phytoplasma and associated disease in Turkey.

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Current status of grapevine phytoplasma infections in Turkey

Didem Canik[1], Filiz Ertunc[1], Samanta Paltrinieri[2], Nicoletta Contaldo[2] and Assunta Bertaccini[2]
[1]Faculty of Agriculture Plant Protection Department, Ankara University, 06110, Diskapi, Ankara, Turkey
didemcanik@gmail.com dcanik @agri.ankara.edu.tr
[2]Dipartimento di Scienze e Tecnologie Agroambientali, Patologia vegetale, *Alma Mater Studiorum*-University of Bologna, Bologna, Italy
assunta.bertaccini@unibo.it

Abstract
Grapevines with severe redness and inward curling of leaves were collected from the main Turkish viticulture production areas. Nucleic acid extraction followed by nested PCR/RFLP analyses and sequencing allowed phytoplasma identification in symptomatic grapevines. The majority of samples were infected with 'bois noir' phytoplasmas, while in some samples 16SrIX or 16SrI-B phytoplasmas were identified.

Key words: grapevine, PCR/RFLP analyses, sequencing, phytoplasma identification.

Introduction

Turkey is one of the nations native to grapevine in the middle east and table and vine grape varieties have been grown in the majority of Turkey regions. Grapevines with severe redness and inward curling of leaves were observed in the main viticulture production areas of Turkey therefore surveys were carried out to verify phytoplasmas presence and identity and several phytoplasmas were preliminary identified (Canik *et al.*, 2011a; 2011b). Relevance and incidence of these phytoplasma are under study.

Materials and methods

The main viticulture production areas were surveyed. Severe redness and inward curling of leaves were the major symptoms of the collected plants. Nucleic acid was extracted from midribs according to a chloroform/phenol protocol (Prince *et al*, 1993). The phytoplasma strains stolbur (STOL, ribosomal subgroup 16SrXII-A), aster yellows (PRIVA, ribosomal subgroup 16SrI-B) and Naxos (ribosomal subgroup 16SrIX-C) maintained in collection in periwinkle were employed as reference strains in restriction fragment length polymorphism (RFLP) analyses. Direct PCR with ribosomal P1/P7 universal primer pair, followed by nested PCR with R16F2n/R2 (Gundersen and Lee, 1996), and R16(I)F1/R1 and R16(V)F1/R1 (Lee *et al.*, 1994) primer pairs were carried out. RFLP analysis were performed by *Tru*I to R16(I)F1/IR1 products. Further molecular characterization was carried out on one uncloned R16F2/R2 amplicon purified using Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions with R16F2 and R16R2 primers, using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The obtained sequence was aligned by using Clustal W and BioEdit (Hall, 1999) softwares and deposited in GenBank.

Results and Discussion

The majority of positive samples resulted as phytoplasmas affiliated to 16SrXII ribosomal group. These phytoplasmas are also referred to as stolbur phytoplasmas, and reported to be associated in grapevine to 'bois noir' disease. Using specific primers R16(I)F1/R1 in a few cases also phytoplasmas belonging to aster yellows group (16SrI-B) were identified. In some of the symptomatic samples 16SrIX phytoplasmas were identified: one of these was employed for sequencing. This sequence show 99% identity with phytoplasmas assigned to group 16SrIX.
'Bois noir' disease is widespread and stolbur group related grapevine phytoplasmas have also

recently been reported from Iran (Karimi *et al.*, 2009) and China (Duduk *et al.*, 2010). Aster yellows phytoplasmas were reported in grapevine in several countries after the first finding in Italy (Alma *et al.*, 1996). The 16SrIX group phytoplasmas are severely infecting plants in different regions, especially in those bordering Turkey (Choueiri *et al.*, 2001; Abou-Jawdah *et al.*, 2002) so their identification in grapevine for the first time indicates the susceptibility of the species to this pathogen and the urgent need to further verify its presence in grapevine to avoid its possible epidemic diffusion.

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Results of last surveys for stone fruit phytoplasma disease management in Lebanon

Marina Molino Lova[1], C. Mahfoud[2], Yusuf Abou Jawdah[3], Elia Choueiri[4], H. Abdul-Nour[5], R. Fakhr[6], R. al Achi[7], Alberto Alma[8], Luca Picciau[8] and Pier Attilio Bianco[1]

[1]Di.Pro.Ve. Sezione Patologia Vegetale, Università degli Studi di Milano, Milan, Italy
[2]AVSI Foundation, Jounieh Ghadir, Lebanon
[3]Faculty of Agricultural and Food Sciences (FAFS), American University of Beirut, Beirut, Lebanon
[4]Department of Plant Protection, Lebanese Agricultural Research Institute (LARI), Tal Amara, Zahlé, Lebanon
[5]Faculty of Science, Lebanese University (LU), Fanar, Lebanon
[6]Facultè des Sciences Agronomiques, Universitè Saint Esprit de Kaslik (USEK), Kaslik, Lebanon
[7]Ministry of Agriculture, Bir Hassan, Beirut, Lebanon
[8]Di.Va.P.R.A. Sezione Entomologia e Zoologia applicate all'ambiente, Università degli Studi di Torino, Turin, Italy
piero.bianco@unimi.it

Key words: Almond witches' broom (AlmWB), survey, phytoplasma, management.

The stone fruit production in Lebanon is threatened by the alarming spread of almond witches' broom (AlmWB), a very dangerous disease associated with the presence of '*Candidatus* Phytoplasma phoenicium' in almond trees. The disease was firstly reported in Lebanon on 2000, responsible for the death of more than 100,000 almond trees in the Country (Choueiri *et al.*, 2001; Abou Jawdah *et al.*, 2002; Verdin *et al.*, 2003). After a decade, the disease spread all over the cultivated regions, affecting also peaches and nectarines, representing a risk for the stone fruit production not only in Lebanon but also in the Middle East and Mediterranean regions (Abou Jawdah *et al.*, 2009).
A joint work of academic research, rural development and international cooperation has been carried out by the Italian NGO AVSI, in order to create an Italian-Lebanese task-force able to detect the pathogen, define its spread and study its epidemic features (Molino Lova *et al.*, 2010).
A national survey has been conducted in 495 Lebanese villages, monitoring about 900 orchards. On the basis of symptom observation, 368 plant samples have been collected and analyzed. Ninety-five percent of symptomatic almonds and 100% of symptomatic peach/nectarine samples tested positive to the analysis, whereas 99.1% of asymptomatic almond and 100% of asymptomatic peach/nectarine samples tested negative to the analyses. The disease was detected in 16 out of 26 Lebanese districts, at different rate of infection, on almond peach and nectarine samples. On the basis of results from the molecular analysis, a GIS (Geographic Information System) map has been drawn describing the spread of the disease in the Country. The map, presented to the Lebanese Ministry of Agriculture, is a useful tool to show the extent of the endemic regions, as well as the *foci* of infection and, above all, is a basis for political decisions about the disease management at a national level. The map is constantly updated, according to the further monitoring carried out in the Lebanese orchards, allowing locating new infected orchards in different regions.
Moreover, a large insect collection, carried out over two years, allowed achieving a deeper knowledge on the fauna biodiversity in the Lebanese stone fruit orchards, finding some putative phytoplasma vectors that will be investigated in further researches. The insect collecting confirmed the presence of the insects already indicated by Dakhil and co-workers (2011) as positive to the specific analysis for '*Ca*. P. phoenicium' detection.
Lebanese and Italian researchers and technicians were involved in the project that provided a solid support in capacity building, conducted training in molecular techniques and entomological knowledge (identification of phytoplasma insect vectors), and created four working teams in Lebanon, with the aim to establish a durable collaboration. An important aspect of this work has been the strong and direct link created between the rural and the academic institutions, through

awareness campaigns and workshops held in order to share with the Lebanese farmers the scientific findings achieved during the project development.
Further actions will be taken by the Lebanese Ministry of Agriculture, in order to reduce the disease spread, to continue the research about the pathogen epidemic and to develop a pilot area for the disease control and trees replacement, as an example for the neighbouring regions.

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Almond witches' broom phytoplasma: situation in Lebanon and action of the ministry of agriculture for the disease eradication during the 2010-2011 seasons

Yusuf Abou Jawdah[1], Elia Choueiri[2], Per Attilio Bianco[3], Marina Molino-Lova[3], Salah Hajj-Hassan[4] and R. al Achi[4]

[1]Faculty of Agricultural and Food Sciences (FAFS), American University of Beirut, Beirut, Lebanon

[2]Lebanese Agricultural Research Institute, Tal Amara, Rayak, Zahlé, Lebanon

[3]Di.Pro.Ve.-sezione Patologia Vegetale, Università degli Studi di Milano, Milan, Italy

[4]Ministry of Agriculture, Beirut, Lebanon.

abujawyf@aub.edu.lb

Key words: AlmWB**,** stone fruits, phytoplasma, Lebanon.

Almond witches' broom phytoplasma (AlmWB) was first observed by farmers in North Lebanon in the early nineties. In the late nineties surveys were conducted and the first diagnostic reports on the aetiology of the disease were published in 2001-2003, a new phytoplasma belonging to the pigeon pea group IX was implicated that was later on named '*Candidatus* Phytoplasma phoenicium' (Choueiri *et al.*, 2001; Abou-Jawdah *et al.*, 2002, Verdin *et al.*, 2003).

AlmWB associated disease was localized mainly in North Lebanon where it became epidemic, and in two other villages one in the South and one in the Bekaa region. Then it spread to the other districts, due to failure of the authorities to take any control measures aimed at disease eradication or at least reducing its spread. Upon intervention of the Italian NGO (AVSI), a more or less comprehensive survey was undertaken and the farmers and Ministry officials were alerted.

The recent survey showed that AlmWB caused epidemics on almond, peach and nectarine and became more widespread: it was detected in 16 districts out of a total of 26, extending from extreme North to extreme South (Molino Lova *et al.*, 2010).

Under the patronage of HE the Minister of Agriculture, a national conference/press release was organized at the Syndicate of Engineers, whereby farmers were alerted and a national program for the management of AlmWB was promised. A committee was formed at the Ministry of Agriculture to set the national plan for management of AlmWB. National experts in cooperation with the Italian counterparts met several times in order to draft a national plan to eradicate /manage the disease.

The plan may be divided into three parts: training the extension officers, direct control measures and research on the epidemiology of the disease for an effective disease management/eradication program. The extension service will be trained, equipped and mobilized to participate in the eradication activities; an extension pamphlet on symptoms and IPM of AlmWB was prepared. For the direct control measures, the Bekaa area and the South of Lebanon followed by Mount Lebanon are considered the major areas of stone fruit production where eradication measures should proceed immediately; farmers will be compensated by granting them free seedlings as replacement crops and will be trained on IPM to mange insects and weeds. Experts in horticulture prepared a list of suitable alternative replacement crops depending on the soil characteristics, microclimate and availability of irrigation water.

Promising preliminary results, on eradication, were already obtained in Sarada plain, South Lebanon, where the farmers responded rapidly by eradicating diseased trees and the disease was apparently eradicated from that region within three years. In 2007, the workers observed only a limited number of trees (4-6) with phytoplasma symptoms but did not take any action, in 2008 approximately 90 trees were diseased, farmers responded rapidly by eradicating symptomatic trees, in 2009 only three to five infected trees were observed and quickly eradicated, in 2010 no symptoms of infection were observed in the region.

In the North, AlmWB eradication will start from the periphery of infested areas towards the inside, the reason is to prevent further spread into new areas. Since the North of Lebanon was considered the epidemic centre of AlmWB in Lebanon, studies may be conducted on alternate hosts, on identification of the disease vector(s) using molecular tools and bioassays, and on evaluation of possible sources of resistance.
National universities and research centres will cooperate with the universities of Milano and Torino in the study of disease epidemiology. Disease monitoring based on symptoms and use of molecular techniques will be carried yearly over an initial period of three years in all areas. The production of stone fruit seedlings will be authorized only in phytoplasma free areas. The execution of this plan is awaiting financial support.

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Virus and phytoplasma diseases of stone fruits in Jordan

Nida' M. Salem
Plant Protection Department, Faculty of Agriculture, University of Jordan, Amman 11942, Jordan
nmsalem@rss.gov.jo

Key words: stone fruits, virus, phytoplasma, Jordan.

Stone fruits (almonds, apricots, cherries, nectarine, peaches and plums) are important economic crops in Jordan. Stone-fruit trees are traditionally grown in about 3,530 ha in Jordan, peach being the most important and wide-spread species (1,764 ha). It is followed by apricot, plum, and almond grown on 898, 554, and 313 ha, respectively (DOS, 2010). The productivity of stone fruits in Jordan is falling behind that in many developed countries (FAO, 2010). This situation is attributed to agronomic, cultural, pathological, and entomological factors. A wide range of symptoms suggesting virus infection were observed in many growing areas in Jordan. Field surveys were therefore conducted in the main stone fruit growing areas of Jordan during 2000-2002 to assess phytosanitry status of *Prunus* in commercial orchards, a mother block, nurseries and a varietal collection (Salem *et al.*, 2003; 2004; Al-Nsour *et al.*, 2010a; 2010b). The presence of virus diseases and their identification was ascertained through field observations and serological testing using DAS-ELISA. The following viruses were identified: *Prunus necrotic ring spot* (PNRSV) and *Prune dwarf* (PDV) ilarviruses, *Apple chlorotic leaf spot trichovirus* (ACLSV), *Strawberry latent ring spot* (SLRSV), *Tomato black ring* (TBRV), *Cherry leaf roll* (CLRV), *Arabis mosaic* (ArMV) and *Tomato ring spo*t (ToRSV) nepoviruses and *Plum pox potyvirus* (PPV). A total of 208 almond, 451 apricot, 149 cherry, 250 nectarine, 1016 peach, and 478 plum trees were tested individually for PNRSV by DAS-ELISA. A round 15% of tested samples were infected with PNRSV. The virus incidence in almond, nectarine, plum, peach, cherry, and apricot was 24, 16, 16, 14, 13, and 10% of tested tress, respectively. The level of viral infection was highest in the mother block (19%), and lowest in the samples from the nurseries (10%) (Salem *et al.*, 2003; 2004). On the other hand, 16% of the tested samples were infected with ToRSV. The disease incidence percentages in apricot, almond, peach, nectarine, plum, and cherry trees were 10, 14, 15, 19, 22 and 28% of the tested trees, respectively. The level of viral infection was highest in the commercial orchards (20%) and lowest in samples obtained from nurseries (Al-Nsour *et al.*, 2010b). Recently PPV was detected in 4% of 1,847 tested samples during 2007-2008. The PPV incidence in nectarine, plum, peach, cherry and apricot was 2.4, 3.1, 2.8, 3.1, and 6.1% of the tested trees, respectively. The level of viral infection was the highest in the mother block (7.4%) and the lowest in the samples from the commercial orchards (3.5%) (Al-Nsour *et al.*, 2010a).

During 2002, disease symptoms resembled those caused by phytoplasmas were observed in Al-Jubiha and Homret Al-Sahen area. Anfoka and Fatash (2004) reported the presence of aster yellows phytoplasma (16SrI) affecting peach trees in Jordan. Phytoplasmas were detected from peaches trees by polymerase chain reaction (PCR) using universal phytoplasmas primers P1/P7 followed by R16F2/R2. Since the information on the infection of stone fruit trees with phytoplasmas is very limited, a nationwide survey should be carried out to study the occurrence and distribution of phytoplasma diseases of stone fruit trees. In addition intensive studies should be carried out to investigate the distribution of the phytoplasmas insect vectors and their natural hosts all over the country.

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Phytoplasma in stone fruits and date palm in Egypt

Maha Al Khazindar[1] and A. Abdel Salam[2]
[1]Botany Department, Faculty of Science, Cairo University, Egypt
malkhazi@aucegypt.edu
[2]Plant Pathology Department, Faculty of Agriculture, Cairo University, Egypt
ammamoun@gmail.com

Abstract
Phytoplasmas are pleomorphic wall-less prokaryote organisms of the class Mollicutes. They colonize the plant phloem and are not cultivated *in vitro*. Phytoplasma has been implicated as associated with several hundreds of plant diseases and is considered as one of the most important plant pathogens reducing the productivity of several economic crops worldwide from tropical to temperate countries. Leafhoppers (order Hemiptera) are among the most important vectors transmitting the pathogen in a persistent manner. Several methods have been used for the identification of phytoplasma presence. Transmission Electron Microscope (TEM) allows the observation of characteristic phytoplasma morphology in sieve tubes of plant host. The inability to isolate and culture phytoplasma has impeded their identification and classification, therefore PCR assays using universal primers designed based on conserved sequences, 16S rDNA gene, allow the detection of a wide array of unknown phytoplasma associated with plants and insects.

Key words: phytoplasma, date palm, Al-Wijam, stone fruits, nested PCR.

Date palm

Date Palm (*Phoenix dactylifera* L.) is one of the most important crops in Egypt. At present, Egypt is recorded as one of the top ten in date palm producing countries. Date palm plantations have been suffering from number of pests and diseases, such as Dubas Bug, Red Palm Weevil, Lesser Date moth, and Al-Wijam disease. Symptoms due to phytoplasma infection on date palm were reported in many Arab countries. Al-Wijam disease symptoms of date palms was recorded at the first time in Saudi Arabia by Nixon (1954) and Elarosi *et al.* (1983) and in Kuwait (Al-Awadhi *et al.,* 2002). Also in Sudan, Cronje *et al.* (2000) reported phytoplasma as the pathogen associated with white tip die-back disease on date palm.

Abou-El-Einin (2010) tested eighty date palm trees collected from El-Marazik, El-Badrasheen, El-Wahat, and Demiate Governorates in Egypt to study the prevalence of Al-Wijam disease using DNA hybridization technique. Symptoms including retardation in terminal bud growth, whole crown of leaves (rosetting symptoms), and yellow longitudinal line on the midribs were observed. Non-radioactive dig-labelled probes specific for 16SrDNA region of phytoplasmas was used to detect the infected trees which showed positive results for all samples.

In our investigation, witches' broom-like symptoms were detected on date palm trees in Al Badrasheen, Al Giza and Rasheed Governorates in Egypt. Symptoms vary from chlorotic streaks, leaf stunting and a marked reduction in fruit and stalk size, which leads to reduction in fruit production at harvest (Figure 1). Nested-PCR for 16S rDNA universal gene sequence amplification using a nested primer pairs R16F2n/R16R2 (Gundersen and Lee, 1996) resulted in DNA amplification of approximately 1,250 bp. Healthy date palm samples were used as controls which did not show any amplification product. Phytoplasma identification is still under investigation using sequencing for phylogeny studies.

Stone fruits

Stone fruit trees (*Prunus* spp.) are a major fruit industry in the Mediterranean area. The stone fruit cultivated area in Egypt is 49,209 ha with an approximate yearly production of 476,849 tonnes (6% of total fruit production). Apricot, peach and plum are the most abundant stone fruit crops in Egypt. Stone fruit species are affected by severe diseases associated with phytoplasma. European stone fruit yellows (ESFY) is present in several countries of Europe and the Mediterranean. In our investigation, phytoplasma symptoms were detected on apricots and peaches in Al Giza Governerate, Egypt. ESFY-like symptoms showing leaf curling, yellowing and fruit malformation causes serious economical losses (Figure 1). Nested-PCR has improved the ability to accurately identify phytoplasma. All infected samples collected from apricots and peaches showed positive results. PCR product using degenerate primers were re-amplified for nested-PCR to verify phytoplasma identity using nested primers for 16SrDNA which amplified approximately 1,250 bp. Negative results were obtained when healthy plants were used. Further studies including epidemiology and dig-labelled probes are still under investigation. There are no any previous records for the presence of phytoplasma infecting stone fruits in Egypt.

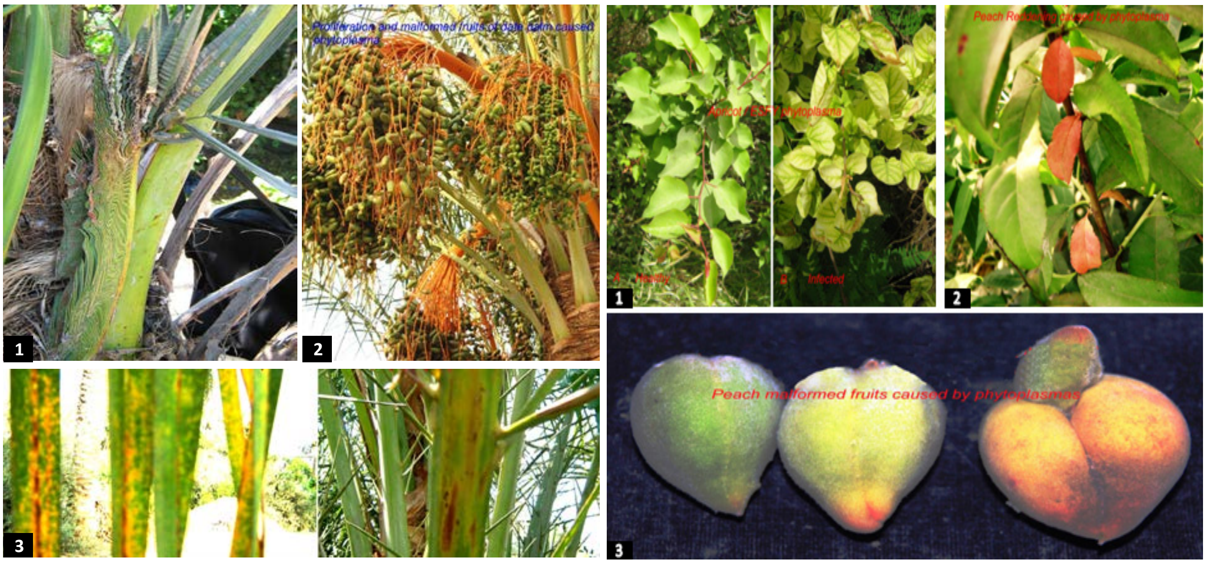

Figure 1. Left: symptoms associated with phytoplasmas on date palm trees 1. Trees showing witches' broom. 2. Reduction of fruit and stalk size 3. Leaves showing chlorotic streaks. Right: 1 and 2. Apricot plant showing reddening of leaves 3. Malformation on apricot fruits.

Control

Phytoplasma diseases are still under recognition and investigation but needs more attention as a serious non curable problem that could become uncontrollable. Fast spreading via insect vectors or by vegetative propagation and/or tissue-culture lines derived from infected tissues without notice is of great danger; therefore the use of tested-healthy plant material is of basic importance followed by vector control and weed (alternative host) control. Also, precise knowledge of the epidemiology of the disease is necessary for its control.

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Frequency distribution of sesame phyllody infected by phytoplasmas in Antalya, Turkey

Cengiz Ikten[1], Engin Yol[2], Mürsel Çatal[1] and Bülent Uzun[2]
[1]Department of Plant Protection, Faculty of Agriculture, Akdeniz University, TR-07058, Antalya, Turkey
[2]Department of Field Crops, Faculty of Agriculture, Akdeniz University, TR-07058, Antalya, Turkey
bulentuzun@akdeniz.edu.tr

Keywords: phyllody, phytoplasma, *Sesamum indicum* L., witches' broom.

Sesame (*Sesamum indicum* L.) is one of the most important oilseed crops in Turkey and it is cultivated especially in southern regions. However its production in Turkey has fallen about 40% in the last decade (FAO, 2010). Major factors that limit its production are instability in yield, non-mechanized harvesting, non-synchronous maturity, and susceptibility to diseases and pathogens. Sesame phyllody, known as a viral disease earlier, is a very serious and destructive disease and it is now associated with phytoplasmas that are uncultured wall-less bacteria (class Mollicutes) that live in the phloem of host plant and in the emolinph of insect vectors. The sesame infected plants become stunted and the floral parts are modified in to leafy structures bearing no fruits and seeds resulting in significant yield losses. Various symptoms occur according with different growing stages and time of infection.

During 2008 to 2010 growing seasons, lots of sesame plants infected with phyllody disease were observed in Antalya province located in the southern of Turkey (Figure 1). The major sesame growing areas in Antalya (Aksu, Boğazkent, Denizyaka, Beşkonak and Döşemealtı) were visited twice in two different developmental stages of plants in the farmer fields and monitored for the disease distribution and frequency (Figure 1). The disease symptoms were observed in all the farmer fields visited (Figure 2). The disease distribution is mainly located in the borders of the fields. In order to identify the severity of phytoplasma disease on sesame, the plants showing witches' broom symptoms were counted in 100 m^2 of each field. According to calculations from the data obtained from farmer fields, the frequency distribution of sesame phyllody was recorded as 62%, 59%, 42%, 37% and 56% for Aksu, Boğazkent, Denizyaka, Beşkonak and Döşemealtı, respectively. Visual overall surveys of sesame fields in Antalya province indicated a disease incidence of up to 50%.

Figure 1. The farmer fields visited in the several parts of Antalya province, Turkey: Aksu (1), Boğazkent (2), Denizyaka (3), Beşkonak (4) and Döşemealtı (5).

Figure 2. Phyllody symptoms in sesame plants collected in different fields of Antalya province.

Conventional and novel strategies for the phytoplasma diseases containment

Pier Attilio Bianco, Daniela Bulgari, Paola Casati and Fabio Quaglino
[3]*Di.Pro.Ve.-sezione Patologia Vegetale, Università degli Studi di Milano, Milan, Italy*
piero.bianco@unimi.it

Keywords: phytoplasma, endophytes, induced resistance, disease control.

Phytoplasmas are obligate bacterial plant pathogens that cause economically relevant yield losses in annual and perennial crops worldwide and they are transmitted in nature by phloem feeders, mostly leafhoppers, planthoppers and psyllids. Impossibility of cultivating phytoplasma impairs the development of efficient methods to control these pathogens. Conventional strategies for phytoplasma containment are based on pesticide application against insect vectors and the use of resistant plants (when available). Owing to the great yield losses caused by phytoplasmas, their absence from propagation materials is essential for sustainable plant production. This is particularly important for vegetatively propagated crops in which infected planting materials transmit the pathogen to the new crop. Pathogen-free plants have been obtained using many different techniques, such as shoot tip culture, thermotherapy, leaf tissue-derived somatic embryogenesis, stem culture, treatment of plant tissues with antibiotics and cryotherapy of shoot tips. Moreover, other strategies have been tested, namely: (i) production of transgenic plants expressing antibodies against the major phytoplasma membrane protein (ii) production of transgenic plants expressing antimicrobial peptides; and (iii) protecting the plants using elicitins, small proteins that stimulate P protein plugs and callose release in phloem sieve elements (Laimer *et al.*, 2009). Till today, such treatments against phytoplasmas have been proved partially ineffective.

Current studies evidenced that a promising approach concerns the use of natural or induced resistance. Different compounds tested as resistance inducers were able to suppress symptoms on specific phytoplasma strain but they have limited applications (Romanazzi *et al.*, 2009). Recently, there has been an increasing interest in the use of biocontrol agents that could be employed in different strategies: (i) study of microorganisms which are pathogenic to the insect (Schnepf *et al.*, 1998), (ii) symbiotic microorganisms able to reduce vector competence (Beard *et al.*, 1998); (iii) antagonisms mediated by the production of allelochemicals; (iv) induction of plant defense response. For example, reduced symptom expression in phytoplasma-infected plants treated with arbuscular mycorrhizal fungi (Kaminska *et al.*, 2010) and *Epicoccum nigrum* Link (Musetti *et al.*, 2011) were recently reported. Moreover, studies on bacteria as potential biocontrol agents or plant resistance inducers have given promising results (Gamalero *et al.*, 2010; Bulgari *et al.*, 2011).

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