'Candidatus Phytoplasma mali'

Scientific Name
'Candidatus Phytoplasma mali' Seemüller & Schneider, 2004

Synonyms:
AP Phytoplasma, AP-MLO

Common Name(s)
Apple proliferation, apple witches' broom

Type of Pest
Phytoplasma

Taxonomic Position
Class: Mollicutes, **Order:** Acholeplasmatales, **Family:** Unassigned

Reason for Inclusion in Manual
CAPS Target: AHP Prioritized Pest List – 2010 through 2011; Pests of Economic and Environmental Importance List 2012 through 2017

Pest Description
Mollicutes are prokaryotes that have small genomes (530 bp to 1350 kbp), lack a cell wall, are pleomorphic, and have a low G + C content (21-29 mol%). They belong to the class Mollicutes and are the putative causal agents of yellows diseases that affect at least 1,000 plant species worldwide (McCoy et al., 1989; Seemüller et al., 2002; Bertaccini et al., 2014). 'Phytoplasma', formerly known as mycoplasma-like organism (MLO), has been adopted to collectively name the wall-less, non-helical prokaryotes that reside in the phloem tissue of the infected plant host and are transmitted primarily by insect vectors. The vectors are principally leafhoppers and planthoppers, although psyllids have been shown to vector these organisms as well (Carraro et al., 1998; White et al., 1998; IRPCM, 2004; Weintraub and Beandland, 2006). Although phytoplasmas have been detected in affected plant tissues and insects with the use of technologies based on transmission electron microscopy, antibodies, and nucleic acids, they are currently unable to be routinely cultured *in vitro*. Phytoplasmas cannot be morphologically or ultrastructurally distinguished from one another using either electron or light microscopy (CABI, 2009). '*Candidatus'* in scientific classification is a formal word that is placed before the genus and species name of bacteria that cannot be maintained in a Culture Collection. '*Candidatus'* status may be used when a species or genus is well characterized but unculturable (IRPCM, 2004).

Apple proliferation (AP) is a severe disease of apple caused by a phytoplasma in the apple proliferation group (group 16SrX) of phytoplasmas (Seemüller et al., 1994). Pathogens of this group have occasionally been detected in stone fruits as well (Lee et al., 1995; Navratil et al., 2001; Paltrinieri et al., 2001; Mehle et al., 2006; Cieslinska and

Morgas, 2011). The group/cluster also includes phytoplasmas associated with other perennial fruit tree diseases, including European stone fruit yellows ('*Candidatus* Phytoplasma prunorum'), peach yellow leaf roll, and pear decline ('*Candidatus* Phytoplasma pyri') (Seemüller et al., 1994). In contrast, most phytoplasmas infecting stone fruit in North America (X-diseases), including '*Candidatus* Phytoplasma pruni' (Davis et al., 2013), are members of the Western-X disease group (Poggi Pollini et al., 2001).

'Candidatus Phytoplasma mali' is found in the sieve tubes of the current season's phloem. The phytoplasma is highly pleomorphic, approximately 200 to 800 nm in diameter. The phytoplasma is bounded with a trilaminar cytoplasmic membrane but lacks a rigid cell wall (Seemüller, 1990).

Analyses of a non-ribosomal DNA fragment, composed of three putative open reading frames (ORFs) (in particular ORF2 coding for a protein significantly homologous with a bacterial nitroreductase), proved the existence of at least three different AP phytoplasma subtypes named AT-1, AT-2, and AP-15 (Jarausch et al., 1994, 2000). Molecular characterization of the genes coding the ribosomal proteins L22 and S3 revealed the presence of higher genetic heterogeneity within strains of '*Ca.* P. mali' and led to the proposal of four subtypes rpX-A, rpX-B, rpX-C, and rpX-D (Martini et al., 2005, 2008). Analyses of ribosomal and non-ribosomal DNA fragments of '*Ca*. P. mali' populations from northwestern Italy revealed the presence of three AP phytoplasma subtypes (AT-1, AT-2, and AP-15), and reported the identification of at least two phytoplasma genetic lineages, designated AT-1a and AT-1b, among the AP phytoplasma strains of the AT-1 subtype (Casati et al., 2010).

Figure 1. Witches' broom on apple due to the AP phytoplasma. Image courtesy of Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau Archive, www.bugwood.org.

Biology and Ecology

Transmission of AP phytoplasma has been reported to occur via natural root fusions/root grafts in medium-aged and old apple orchards (Bliefernicht and Krczal, 1995; Vindimian et al., 2002; Baric et al., 2008; Ciccotti et al., 2008). Psyllids and leafhoppers have been reported as vectors of the disease (Seemüller, 1990). There is no reported seed or pollen transmission (Seidl and Komarkova, 1974). '*Ca.* P. mali' has been commonly transmitted by grafting. There are also reports of its transmission to *Catharanthus roseus* (Madagascar periwinkle) using the parasitic plant *Cuscuta* spp. (dodder) (Marwitz et al., 1974; Heintz,

1986). Apple proliferation is often disseminated in scion wood; although it is primarily systemic, aerial parts of the trees are not always evenly colonized and may, thus, yield uninfected buds.

Figure 2. Healthy apple leaf (top) compared to AP phytoplasma infected leaf (bottom) showing reduced lamina size, chlorosis, and enlarged stipules. Image courtesy of Biologische Bundesanstalt für Land-und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau Archive, www.bugwood.org.

The AP phytoplasma is transmitted in a persistent-propagative manner by the insect vectors *Cacopsylla picta* and, *C. melanoneura*. *Cacopsylla picta* has been proven by transmission trials as vector in Italy (Frisinghelli et al., 2000; Carraro et al., 2008) and in Germany (Jarausch et al., 2003; 2011), and is regarded as the main vector. *C. melanoneura* has only been shown as a vector in Northern Italy (Tedeschi et al., 2002; 2003) and is regarded as a nonvector in Germany (Mayer et al., 2009). Adults of both species migrate from their overwintering shelter plants into the apple orchards between the end of January until February and from the end of March to April for *C. melanoneura* and *C. picta*, respectively. Both species reproduce on apple and the springtime generation stays on the host plant until June or July (Tedeschi et al., 2002; Mattedi et al., 2008; Mayer et al., 2011; Jarausch et al., 2011). As both species are univoltine, they aestivate and overwinter as adults on shelter plants, mainly conifers (Lauterer 1999, Čermák and Lauterer, 2008; Mayer et al., 2011; Pizzinat et al., 2011; Jarausch and Jarausch, 2014). The insects transmit the phytoplasmas as both adults and nymphs (Tedeschi and Alma, 2004; Carraro et al., 2008; Jarausch et al., 2011). In the spring when the vectors move to the apple trees from shelter plants, they are infective. The vectors remain infective during the entire period when they are present (Jarausch et al., 2011). Tedeschi et al. (2003) demonstrated in particular the importance of the overwintered adults of *C. melanoneura* in vectoring apple proliferation. In Italy the most important vector of AP phytoplasma appears to be *C. melanoneura* (Tedeschi et al., 2002, 2003, 2004, 2012), while in Germany *C. picta* has a major role in '*Ca.* Phytoplasma mali' transmission (Jarausch et al. 2003, 2004, 2008; Mayer et al. 2009).

The distribution of AP phytoplasma in the tree was shown to be inconsistent throughout the year (Schaper and Seemüller, 1982). Seemüller et al. (1984) showed that during the winter months (December to March), the AP phytoplasma population decreases markedly in aerial parts in response to the degeneration of sieve tube elements. In contrast, the organism could be found in the roots of all disease trees at relatively high numbers throughout the year. Baric et al. (2011) also found, however, that there was

seasonality in the phytoplasma concentration in the roots with the highest populations detected from December through May. The 'reinvasion' of the branches from the roots occurred in April or May and increased in summer to its highest level in late summer or early fall. Pedrazzoli et al. (2008a) found that the phytoplasma was not detectable from aerial parts of the plants in late spring, it reappeared progressively during the summer, and the maximum peak occurred between the middle of July and the end of December. Seemüller et al. (1984) did not attribute this reaction to low temperature sensitivity, because the survival rate of the organism in diseased trees exposed to temperatures between +4°C (39°F) and -20°C (-4°F) was similar.

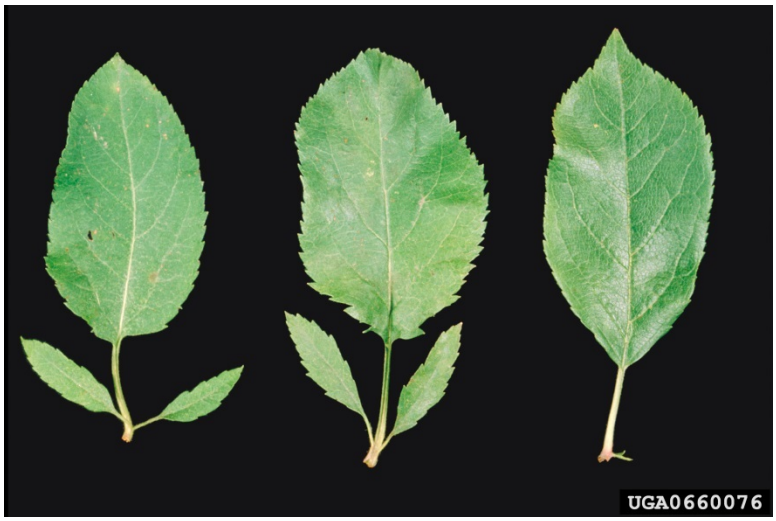

Figure 3. Healthy apple leaf (right) compared to AP phytoplasma infected leaves (left, middle showing enlarged stipules. **Note:** Stipules are enlarged and resemble small leaflets at the base of the leaf. Image courtesy of Biologische Bundesanstalt für Land-und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau Archive, www.bugwood.org.

The distribution pattern of phytoplasmas in trees was also dependent upon temperature. In France, phytoplasmas could be found throughout the tree at temperatures of 21-25°C (70-77°F), causing symptoms; at 29-32°C (84-90°F) symptoms were inhibited and phytoplasmas were found only in the roots but reinvaded the branches when plantlets were stored at the lower temperatures (Ducroquet et al., 1986).

Pedrazzoli et al. (2008b) found the highest percentage of graft transmission occurred between June and August (12 to 30%); while those conducted from March to May were very low (0-0.08%). The authors concluded that the most suitable period for collecting scions was in the springtime when the probability of AP transmission was at the lowest. Grafting of stem scions removed during dormancy prevented transmission or yielded only a low transmission rate. Transmission by root grafting in winter was generally successful and for this reason has become an established method for indexing trees (Seemüller et al., 1984).

Recovery is the spontaneous remission of symptoms in plants that have been symptomatic. This phenomenon has been observed with the AP phytoplasma. Carraro et al. (2004) showed that plants that 'recovered' were still infected and had high populations of the AP phytoplasma in their roots. For symptoms to develop in above ground tissues, the phytoplasma had to be present in that tissue. Musetti et al. (2004)

found that the production of hydrogen peroxide appears to be involved in the recovery phenomenon by counteracting pathogen virulence. Calcium-dependent callose synthesis/deposition and phloem-protein plugging of the sieve tubes may form a physical barrier to prevent recolonization of the crown during the following spring may also play an important role in the recovery phenomenon (Musetti et al., 2010).

Figure 4: Greatly enlarged flower with numerous petals on apple tree infected with apple proliferation. Image courtesy of Loschi. DBADP, University of Udine, Italy. http://www.cabi.org/compendia/cpc/.

The most promising approach to controlling AP would be the use of resistant plants. The fluctuation in the colonization pattern in apple by the AP phytoplasma discussed above has led to the presumption that growing scion cultivars on resistant rootstocks can prevent the disease or reduce its impact. Phytoplasma concentration in AP-susceptible, *Malus* x *domestica*-based rootstocks are much higher than in AP-resistant rootstocks that were derived from *Malus sieboldii* (Seemüller et al., 1984). Seemüller et al. (2008) examined open-pollinated seedlings of *Malus sieboldii* and *M. sargentii* and 22 rootstock selections with either *M. sieboldii*, *M. sargentii*, or *M. hupehensis* in their parentage for AP resistance in comparison to the clonal *M.* x *domestica*-based rootstocks M 9, M 11, M 13, stocks of the B (Budagovski) and the Polish P series, and *M. robusta* seedlings. Inoculation and natural infection results showed that Golden Delicious-grafted trees on most of the *M. sieboldii*-derived progenies showed a high level of AP resistance expressed by low cumulative disease indices, a high percentage of non- or barely-affected trees, low incidence of the small fruit symptom, and no or little effect on vigor. Trees on M 9, M 11, B 118, and *M. robusta* seedlings were moderately susceptible; while trees on progenies with *M. sargentii* and *M. hupehensis* parentage, rootstocks of the P series, B 9, B 490, and M 13 proved to be highly susceptible.

Symptoms/Signs

Trees infected by apple proliferation often occur in clusters, and these clusters grow (expand) year by year (Bliefernicht and Krczal, 1995). Symptoms are unevenly distributed on the plants. Additionally, there is considerable variability in virulence in '*Ca.* P. mali'. Based on symptomatology,

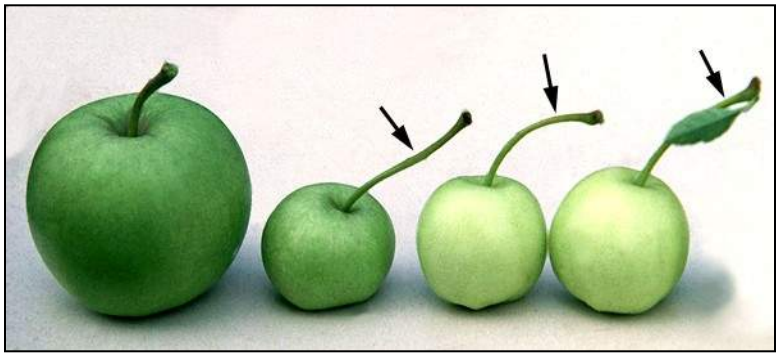

Figure 5: Small fruits with elongated, narrow peduncles (arrowed) from AP infected trees alongside a large healthy fruit (far left). Image courtesy of Loschi. DBADP, University of Udine, Italy. http://www.cabi.org/compendia/cpc/.

the phytoplasma strains can be defined as avirulent to mildly, moderately, or highly virulent. In addition, trees can be simultaneously affected by more than one strain of the apple proliferation phytoplasma (Seemüller and Schneider, 2007; Seemüller et al., 2010).

Apple: In general, trees affected by the AP phytoplasma lack vigor. Trunk circumference and crown diameter are reduced compared to healthy trees. Shoots are thin and the bark, which is sometimes fluted lengthwise, has a reddish-brown color. Necrotic areas appear on the bark, and some branches may wither. Diseased trees may die, but they often can recover if adequately fertilized (EPPO, 1997).

Figure 6: Wilting (top) and dying (bottom) cherry trees infected with the AP phytoplasma. Images from Mehle et al. (2006).

Late growth of terminal buds during autumn is usually the first noticeable symptom. A rosette of terminal leaves, which often become infected with powdery mildew, sometimes develops late in the season in place of the normal dormant bud. A more reliable symptom, however, is the premature development of axillary buds, which give rise to secondary shoots/shoot proliferation (witches' brooming) (Fig. 1). These abnormal secondary shoots are usually numerous near the apex of the main shoot, whereas normal laterals of healthy trees arise nearer the base of the shoots. The angle between these secondary shoots and the main shoots is abnormally narrow on infected trees (Bovey, 1963). The witches' brooms do not develop repeatedly on the same branch. They may appear successively on various parts of the tree, or all at once over the whole tree, but usually develop only during the first two or three years following infection.

Leaves appear earlier than normal. Leaves of infected plants roll downward and become brittle, they are finely and irregularly serrated and are smaller than normal (Fig. 2). They also tend to turn red in autumn in contrast to the green coloration of healthy plants. Summer leaves are chlorotic (Fig. 2). Early defoliation may occur.

Stipules are abnormally enlarged (long) (Fig. 2, 3) while petioles are rather short (an

important symptom in nursery surveys). Leaf rosette may appear on the shoot ends or the shoot tips may die (an important symptom in nursery surveys). Flowering is delayed, sometimes until late summer or autumn, but most blossoms on infected trees are normal. In some cases, flowers show numerous petals (Fig. 4), and the fruit peduncles are abnormally long and thin (Fig. 5). The calyx end and peduncular cavities are shallower and broader, giving the fruit a flattened appearance. Fruit fail to set, may stay on the tree for a long period, and are reduced in size (Fig. 5) with incomplete coloration and poor flavor. Seeds and seed cavities are smaller. Root weight is reduced; the fibrous root system of infected trees forms compact felt-like masses of short roots so that the larger ones are unable to develop (a fine hairy root system).

Figure 7: Floral (top) and phloem necrosis (bottom) in cherry trees infected with the AP phytoplasma. Images from Mehle et al. (2006).

Cherry: Symptoms of AP in cherry include wilting, dying, and floral and phloem necrosis (Fig. 6, 7) (Mehle et al., 2006).

Apricot: Symptoms of AP in apricot include stem necrosis and leaf wilting (Mehle et al., 2006).

Plum: The primary symptom of AP in plum is late blooming (Mehle et al., 2006).

Dahlia: Symptoms of AP in dahlia include bushy growth accompanied by shoot proliferation, narrowed leaves, and flower bud deficiency (Kaminska and Sliwa 2008a). **Note:** plants in this study were co-infected with apple proliferation and aster yellows phytoplasmas.

Rose: Symptoms of AP in rose include dieback, witches' broom, bud proliferation, stunted growth, leaf and flower malformation, and shoot and flower proliferation (Kaminska and Sliwa, 2004). **Note:** plants in this study were co-infected with apple proliferation and aster yellows phytoplasmas.

Lily: Symptoms of AP in lily include leaf scorch/leaf burn, leaf malformation and necrosis; flower bud abscission (Kaminska and Sliwa, 2008b).

Pest Importance

Apple proliferation is one of the most important vector-borne, graft-transmissible diseases of apple trees in Europe. The disease has spread epidemically in northern Italy and in Germany, causing serious damage to several traditional apple growing areas (Bliefernicht and Krczal, 1995; Carraro et al., 2004). The disease causes significant damage to apple trees and may cause a 50% reduction in fruit size, a reduction of sugar and acid content of fruit, an overall reduction of tree growth and vigor, and death of young trees in severe cases (Fialova et al., 2003; Baric et al., 2008; Al-Jabor and Gharz Eddin, 2009). Reports of up to 60% or more loss in fruit weight have been observed as well (Tedeschi et al., 2002). Overall, the commercial value of fruit is decreased 30 to 100% based on poor quality (Tedeschi et al., 2002). Apple proliferation is classified as a quarantine organism in Europe and North America. There are essentially no effective controls for this disease, but spraying insecticides to reduce vector populations and the use of healthy replanting material are the primary management options (Galetto et al., 2005). Host resistance is also being explored to manage apple proliferation (Seemüller et al., 1984, 2008).

Trees infected with apple proliferation are particularly sensitive to powdery mildew (*Podosphaera leucotricha*) (EPPO, 1997).

'Candidatus Phytoplasma mali' is listed as a harmful organism in Chile, Colombia, Japan, and Peru (USDA-PCIT, 2014). There likely will be trade implications with these countries if this pathogen becomes established in the United States.

Known Hosts

Apples are the main host, and most cultivars are susceptible. Apple cultivars known to be affected by the AP phytoplasma are included in Table 1.

Table 1: Susceptibility of apple cultivars to apple proliferation phytoplasma.

Rating	Cultivar	Source
Highly Susceptible	Florina	Loi et al., 1995; Osler et al., 2001
Highly Susceptible	Prima	Loi et al., 1995
Highly Susceptible	Priscilla	Loi et al., 1995
Medium Susceptibility	Idared	Nemeth, 1986
Medium Susceptibility	McIntosh	Nemeth, 1986; Kartte and Seemüller, 1991
Medium Susceptibility	Starking Delicious	Nemeth, 1986; Kartte and Seemüller, 1991; Rumbou et al. 2008
Medium Susceptibility	Starkrimson	Nemeth, 1986
Susceptible	Belle de Booskop	EPPO, 1997
Susceptible	Canadian Renette	Osler et al., 2001
Susceptible	Granny Smith	Osler et al., 2001
Susceptible	Gravenstein	EPPO, 1997

Susceptible	Golden Delicious	EPPO, 1997; Kartte and Seemüller, 1991; Osler et al., 2001
Susceptible	Jonathan	Kartte and Seemüller, 1991
Susceptible	Rome Beauty	Kartte and Seemüller, 1991
Susceptible	Winter Banana	EPPO, 1997
Tolerant	Roja de Benejama	EPPO, 1997
Tolerant	Antonokova	Nemeth, 1986
Tolerant	Cortland	Nemeth, 1986
Tolerant	Spartan	Nemeth, 1986
Tolerant	Yellow transparent	Nemeth, 1986
Tolerant	Wealthy	Nemeth, 1986

Major Hosts:
Malus domestica (apple) and *Malus pumila* (crab apple).

Other Hosts:
Catharanthus roseus (Madagascar periwinkle)*, *Convolvulus arvensis* (field bindweed), *Corylus avellana* (hazel), *Crategus monogyna* (hawthorne), *Cynodon dactylon* (Bermuda grass), *Dahlia cultorum* (dahlia), *Lilium* spp. (lily), *Prunus avium* (cherry), *Prunus armeniaca* (apricot), *Prunus domestica* (plum), *Prunus persica* (peach/nectarine), *Prunus salicina* (Japanese plum), *Pyrus communis* (European pear), and *Vitis vinifera* (grapevine) (Lee et al., 1995; Marcone et al., 1996a; Schneider et al., 1997; Del Serrone et al., 1998; Seemüller et al.,1998; Navratil et al., 2001; Paltrinieri et al., 2001; Mehle et al., 2006; Kaminska and Sliwa, 2008ab; CABI, 2009; Tedeschi et al., 2009).

*Experimental host.

The phytoplasma can be artificially inoculated to *Malus baccata*, *M. coronaria*, *M. domestica*, *M. floribunda*, *M. fusca*, *M. gloriosa*, *M. ionensis*, *M. platicarpa*, *M. purpurea*, and *M. robusta* (Nemeth, 1986).

Magnolia spp. (magnolia) and *Rosa* spp. (rose), have been found to be hosts for phytoplasmas in both the apple proliferation and asters yellows phytoplasma groups, although the specific phytoplasma within the AP group/clade was not identified (Kaminska and Sliwa, 2003; Kaminska and Sliwa, 2004). Similar results were observed for *Spartium junceum* (Spanish broom), which was found to be a host for phytoplasmas in both the apple proliferation and elm yellows group (Marcone et al., 1996b).

Known Vectors (or associated insects)

Primary vectors: *Cacopsylla picta* (*C. costalis)*, *C. melanoneura*, and *Fieberiella florii.*

Tedeschi et al. (2002; 2012) and Frisinghelli et al. (2000) reported that the psyllid *Cacopsylla melanoneur*a and *C. picta* (synonym *C. costalis*) are vectors of apple proliferation in Italy. Jarausch et al. (2003) and Carraro et al. (2008) also confirmed *C. picta* as a vector of apple proliferation in Germany and Italy, respectively. In Germany, the leafhopper *Fieberiella florii* has been implicated as a vector of apple proliferation

phytoplasma (Bliefernicht and Krczal, 1995; Krczal et al., 1988; Tedeschi and Alma, 2006). *F. florii* occurs in both Europe and North America and would likely vector the AP phytoplasma if the pathogen were introduced into the United States. Hegab and El-Zohairy (1986) suggested *Philaenus spumarius*, a spittle bug, and *Artianus interstitialis*, a leafhopper, transmits apple proliferation, but these results could not be confirmed by other authors (Krczal et al., 1988; Refatti et al., 1986).

Trees infected with apple proliferation are particularly sensitive to powdery mildew (*Podosphaera leucotricha*) (EPPO, 1997). There appears to be an interaction between apple rubbery wood disease and apple proliferation, the former promoting transmission of the latter (Bovey, 1963).

Known Distribution

Africa: Tunisia. **Asia:** Syria, Turkey. **Europe:** Albania, Austria, Belarus, Belgium, Bosnia-Hercegovina, Bulgaria, Croatia, Czech Republic, Finland, France, Germany, Greece, Hungary, Italy, Moldova, Netherlands, Norway, Poland, Romania, Serbia, Slovakia, Slovenia, Spain, Switzerland, and Ukraine (Avinent and Llacer, 1995; EPPO, 1997; Del Serrone et al., 1998; Fialova et al., 2003; Myrta et al., 2003; Mehle et al., 2006; Canik and Ertung, 2007; Delic et al., 2007; Duduk et al., 2008; Rumbou et al., 2008; Al-Jabor and Gharz Edin, 2009; CABI, 2009; Krizanac et al., 2010; Ben Khalifa and Fakhfakh, 2011; EPPO Reporting Service, 2011; Lemmetty et al., 2013; ProMed-mail, 2013; USDA, 2013b; EPPO, 2014; Valasevich and Schneider, 2016).

The “possible detection” of apple proliferation from Nova Scotia, Canada (USDA, 2013b) cannot be confirmed after further testing and analysis (CFIA, 2013). Thus, Canada has been removed from the distribution listing.

Apple proliferation has been found but not established in Denmark (Bovey, 1963). Apple proliferation was found and eradicated from the United Kingdom in 1985 (Davies et al., 1986). There are unreliable records of this phytoplasma in Cyprus, India, Russia, and South Africa (EPPO, 2014).

Pathway

According to Federal Order DA-2013-18, effective May 20, 2013, the import of both *Malus* and *Prunus* spp. propagative material is currently prohibited from all countries except Canada and the Netherlands to restrict import of host material of *Anoplophora chinensis* (Chinese longhorned beetle) and *Anoplophora glabripennsis* (Asian longhorned beetle) (USDA, 2013). Prior to this Federal Order, import of *Malus* and *Prunus* spp. propagative material was allowed from the following countries known to have AP: Belgium, Canada, France, Germany, and Netherlands (USDA, 2013a).

Since 2004, there have been shipments of *Malus* spp. plant material from the following known AP host countries: Canada (561), Czech Republic (8), France (57), Germany (23), and Italy (7) (AQAS, 2014). One shipment from France contained over 2.2 million plant units. Since 2004, there have also been shipments of *Prunus* spp. plant material from the following known AP host countries: Canada (573), Czech Republic (2), France

(31), Germany (1), Hungary (1), Italy (1), Netherlands (13), Spain (2), and the United Kingdom (7). The largest of these shipments was from Canada and contained 49,222 Plant Units (AQAS, 2014).

There have been 37 interceptions of *Prunus* spp. plant material intended for propagation from eleven different host countries since 2004 (AQAS, 2014). There have also been 124 interceptions of *Malus* spp. plant material intended for propagation from 10 different host countries since 2004 (AQAS, 2014).

The vector *Fieberiella florii* is not reportable at U.S. ports of entry (AQAS, 2014). There have been interceptions of *Cacopsylla* spp. from Canada (4) and Italy (1). It is not known whether or not these interceptions were of a species known to vector AP.

Potential Distribution within the United States

This phytoplasma has a relatively narrow host range. Survey efforts should be focused in apple growing states/regions.

Survey

Approved Method for Pest Surveillance*: The CAPS-approved survey method is to collect symptomatic plant tissue by visual survey. For 2017 surveys, follow instructions in Phytoplasma sample submission for Cooperative Agricultural Pest Survey (CAPS) Program and Farm Bill Goal 1 surveys FY 2017.

If you have taken the hands-on phytoplasma specific training at CPHST Beltsville, you can screen your own phytoplasma samples. **Note:** You will still have to follow the protocol in the linked document for confirmations.

*For the most up-to-date methods for survey and identification, see Approved Methods on the CAPS Resource and Collaboration Site, at https://caps.ceris.purdue.edu/approved-methods

Literature-Based Methods:

Delic et al. (2007) carried out surveys during autumn (October) and spring (April). Several hectares of orchards were visually inspected, and stone fruit, apple, and pear trees were checked for symptoms of phytoplasma infection. The symptoms considered were witches' broom, small leaves, enlarged stipules, and reddening. Fialova et al. (2003) visually examined apple trees from intensive orchards, nurseries, and private gardens for the presence of proliferation symptoms and enlarged stipules. Samples were taken during the vegetative season from all trees with symptoms. Three shoot samples per tree were used for the molecular analyses. Ermacora et al. (2008) conducted two field inspections annually in September and December to identify AP symptoms. In September, plants were monitored for witches' brooming, small leaves with enlarged stipules, and reddish leaves. In December, only witches' brooming was monitored.

Rekab et al. (2010) sampled trees at the beginning of June (spring), the end of July (summer), and the beginning of October (fall). Each tree was subdivided into three or four homogeneous sampling zones, depending on tree size, and from each zone three samples were taken. Each sample was composed of 8 leaf disks, about 1.4 cm in diameter (0.35 mg total on average), including the middle of leaf midribs. Phytoplasma presence in the trees was uneven. Samples collected in mid-summer had significantly lower phytoplasma concentrations, as measured by quantitative PCR, than those collected in the spring or fall. Samples collected in the fall were less variable than those collected in other sampling dates and a lower number of samples were negative. The experiment also showed that a sample of 8 leaf disks is too small, but a sample of 24 leaf disks, particularly if collected in the fall, may provide a reliable means to estimate differences in phytoplasma concentration, which were two orders of magnitude different in these experiments. If less pronounced differences are observed, the sample size required would be much larger (Rekab et al., 2010).

Avinent and Llacer (1995) took bark samples from diseased trees and used bark from one or two year old branches for phloem extraction of the phytoplasmas occurring in fruit trees in Spain.

Due to the large percentage of latent infections, observation of symptoms will not always reveal the 'true disease status" of an orchard. Baric et al. (2007) sampled each tree from eight adjacent rows representing one-third of the orchard by collecting at least three pencil-thick root pieces. The AP phytoplasma was detected using the Baric and Dalla Via (2004) quantitative PCR assay.

Insect vectors, *Cacopsylla picta* overwintered adults and nymphs, were shaken from apple trees onto an underlying net and grouped using an aspirator (Carraro et al., 2008). The population of the vector in the orchards was high, however, in this site. Yellow sticky traps, sweep netting, and a beat tray methodology have also been used to sample insect vectors (Tedeschi et al., 2002; Galetto et al., 2005; Fialova et al., 2008; Tedeschi et al., 2009; Casati et al., 2010).

Key Diagnostics/Identification

Approved Method for Pest Surveillance*:

Molecular: For 2017 surveys, follow instructions in Phytoplasma sample submission for Cooperative Agricultural Pest Survey (CAPS) Program and Farm Bill Goal 1 surveys FY 2017.

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Literature-Based Methods:

Culture: The phytoplasma that is associated with apple proliferation is obligate and was not cultured on microbiological growth media.

Serological: ELISA is available. Loi et al. (2002) developed monoclonal antibodies against apple proliferation phytoplasma. Brzin et al. (2003) showed that an ELISA procedure was very sensitive and reliable compared to PCR. The phytoplasma could be reliably detected in samples of leaves, shoots, and roots during the growing season and also in dormant bud-wood and roots.

This DAS-ELISA is commercially available from Bioreba AgG (http://www.bioreba.ch/files/Product_Info/ELISA_Reagents/ApP_DAS_ELISA.pdf). Some isolates of the AT-1 subtype, however, may not be recognized with this test (Martini et al., 2005), and a comparison was carried out in order to compare the sensitivity of this method to the one of nested PCR with diverse groups of primers (Bertaccini et al., 2008).

Biological Indexing: Greenhouse indexing, which consists of graft-transmission onto a woody indicator is a time-intensive method. For apple proliferation phytoplasma, this process can take up to two years. The best woody indicator for fast diagnostic detection of the AP phytoplasma is *Malus* x *dawsoniana* (EPPO, 1997). If the very sensitive indicator *Malus* x *dawsoniana* is grafted directly in June on the scion, it develops a leaf reddening during the following autumn and bark splitting and scaling during the next spring (EPPO, 1997).

Fluorescence Microscopy: For large scale diagnosis, the DAPI (4', 6'-diamidino-2-phenilindole, 2HCl) staining method (Seemüller, 1976) can be used, although the percentage of false negative can reach high levels. False negatives generally occur when phytoplasma colonization of plants is poor or uneven. This test detects fluorescence of phytoplasmas in the sieve tubes of the leaf veins.

Biological indexing and DAPI staining are time-consuming and do not often allow specific identification of phytoplasmas (Poggi Pollini et al., 2001).

Molecular: Seemüller and Schneider (2004) offer a summary of the molecular studies conducted on the apple proliferation, European stone fruit yellows, and pear decline phytoplasmas. The authors conclude that the phytoplasmas are coherent and discrete taxa and can be distinguished as distinct species with the proposed names '*Ca.* P. mali' (apple proliferation), '*Ca.* P. prunorum' (European stone fruit yellows), and '*Ca.* P. pyri' (pear decline). Specific primers for molecular identification of '*Ca.* P. mali' are available (Jarausch et al., 1994). A chromosome map of the apple proliferation phytoplasma is available (Lauer and Seemüller, 2000). Seemüller et al. (2010) used single-strand conformation polymorphism (SSCP) and sequence analysis to examine strain similarity of '*Ca.* Phytoplasma mali'. Recently the nucleotide sequence of the entire chromosome of '*Ca*. P. mali' strain AT was determined (Kube et al., 2008).

DNA extraction and enrichment: Kirkpatrick et al. (1987), Ahrens and Seemüller (1992), and Maixner et al. (1995) developed a procedure to enrich DNA of phytoplasmas using axial phloem tissue of apple trees. Most authors working with the AP phytoplasma used these procedures or some modification (*e.g.,* Malisano et al., 1996) of these procedures (Jarausch et al., 1994; Avinent and Llacer, 1995; Fialova et al., 2003; Baric and Dalla Via, 2004; Delic et al., 2007; Carraro et al., 2008). The method of Doyle and Doyle (1990) was also employed for isolating DNA from the insect vector (Carraro et al., 2008) and plant samples (Firraro et al., 1994; Kison et al., 1994; Delic et al., 2007; Bisognin et al., 2008). Green et al. (1999) developed an 'easy and efficient' DNA extraction method from woody plants for detection of phytoplasmas by PCR.

PCR: PCR amplification is now widely used for the sensitive and reliable diagnosis of phytoplasmas in fruit trees. Due to the close genetic relatedness of the apple proliferation group of phytoplasmas, specific identification often requires the digestion of the amplicons with various endonucleases and subsequent RFLP analysis or sequencing (Deng and Hiruki, 199; Ahrens and Seemüller, 1992; Gundersen and Lee, 1996; Lee et al., 1993; Lee et al., 1995; Schneider et al., 1995; Smart et al., 1996; Kison et al., 1997; Gibb et al., 1999; Jarausch et al., 2000; Heinrich et al., 2001; De Salvador et al., 2007). Firrao et al. (1994) developed a 'rapid' PCR protocol for the apple proliferation organism without the need for a restriction digestion or hybridization step.

Restriction analysis of P1A/P7a amplicons using *Hpa*II and *Fau*I endonucleases allows us to 1) distinguish AT-1 from AT-2 and AP-15; 2) differentiate the genetic lineages AT-a1 and AT-1b, and 3) discriminate AT-1 isolates from Czech Republic, Germany, Hungary, Italy, and Serbia (Casati et al., 2010; Paltrinieri et al., 2010; Franova et al., 2013).

Nested PCR: Nested PCR has been employed for the detection of phytoplasmas both in plants and psyllids using universal primers (Deng and Hiruki, 1991; Gundersen and Lee, 1996) and/or 16SrX phytoplasma group specific primer pairs (Lee et al., 1995; Lorenz et al., 1995).

Immunocapture PCR (IC-PCR): Rajan and Clark (1995) use immunocapture-PCR to detect apple proliferation in apple bark. They used rabbit polyclonal antibodies to capture the phytoplasma and then amplified with universal PCR primers.

Real-time PCR: Jarausch et al. (2004) developed a quantitative real-time PCR for apple proliferation phytoplasma in plants and insects from a nitroreductase gene sequence. Galetto et al. (2005) developed an apple proliferation specific real-time PCR assay from the same nitroreductase gene sequence. These authors also developed a universal assay for detection of phytoplasmas belonging to groups 16SrV, 16SrX, and 16SrXII. Torres et al. (2005) developed a real-time PCR that will detect '*Ca.* P. mali', '*Ca.* P. prunorum', and '*Ca.* P. pyri' (three phytoplasmas in apple proliferation group of quarantine importance).

Baric and Dalla-Via (2004) developed a real-time PCR for apple proliferation

phytoplasma in apple plant material. The assay also amplified a host gene from apple as an internal control. Baric et al. (2006) compared the Baric and Dalla Via (2004) real-time PCR with four conventional PCR assays. The real-time procedure had the highest sensitivity and specificity and was not susceptible to PCR inhibition. The one downfall was the high cost of the procedure.

Monti et al. (2013) validated and implemented an EvaGreen Real-time PCR protocol based on ribosomal protein genes for sensitive and specific quantification of '*Ca.* Phytoplasma mali' in insects. This method, compared to the use of probes, is less expensive and easier to use because it only requires the design and synthesis of two PCR primers, which decreases assay setup and costs. Moreover, EvaGreen technology provides a higher reproducibility, a less PCR inhibition effect, weak binding for short dsDNA fragments as well as higher and narrowed melting curves compared with SYBR Green protocols.

Easily Confused Pests

The apple proliferation (AP) phytoplasma is phylogenetically closely related to the European stone fruit yellows (ESFY) and pear decline (PD) phytoplasmas. These three phytoplasmas belong to the 16SrX group and have nearly identical 16S rDNA sequences (Seemüller and Schneider, 2004).

The peach yellow leaf roll (PYLR) phytoplasma from California was found by Kison et al. (1997) to also be closely related to AP, PD, and ESFY. The PYLR agent could clearly be distinguished from the AP and ESFY phytoplasmas by Southern Blot hybridization with DNA fragments from the AP phytoplasma and by RFLP analysis of ribosomal DNA employing *SSp*I, *Bsa*AI, and *Rsa*I restriction endonucleases. The PYLR phytoplasma, however, was indistinguishable from the PD phytoplasma by PCR-amplified ribosomal DNA (Kison et al., 1997).

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Update History:

April, 2014: Updated distribution, added pathway section.

July, 2014: Added Davis, 2013 reference. Incorporated comments from Bertaccini, Jarausch, Seemueller, and Tedeschi, and added their suggested references.

June, 2016: Added new phytoplasma sample submission information to survey and diagnostic sections.

October, 2016: Added Belarus to distribution section.