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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 3/84 (1)

Procedures phytosanitaires
Phytosanitary procedures

PM 3/84 (1) Inspection of places of production for '*Candidatus* Phytoplasma pyri'

Specific scope

This Standard describes the procedures for inspection of places of production of plants for planting which are susceptible to '*Candidatus* Phytoplasma pyri'. The scope of a place of production inspection may be for export, for internal country movements of materials or as an element of a national survey. Further inspections would be needed to determine freedom of a country or area from the pest concerned. This Standard does not cover eradication or containment measures in infested areas or measures needed to establish and maintain pest-free places of production within areas where the pest is known to occur. It provides more detailed guidance to supplement EPPO Standard PM 3/76 (1) *Trees of* Malus*,* Pyrus*,* Cydonia *and* Prunus *spp. – inspection of places of production*.

Specific approval and amendment

First approved in 2018-09.

1. Introduction

'*Candidatus* Phytoplasma pyri' (EPPO Code PHYPPY), the pathogen which causes the disease known as pear decline, is on the EPPO A2 List of pests recommended for regulation as a quarantine pest. It is regulated in several EPPO countries, including those of the European Union (EU) (EU, 2000), and is listed as a quarantine pest in Jordan, Norway and Israel. In Turkey it is included in the A1 List (see EPPO Global Database, 2018).

'*Candidatus* P. pyri' belongs to the apple proliferation group (AP 16SrX group) which includes two additional phytoplasmas, namely '*Ca*. Phytoplasma mali' and '*Ca*. Phytoplasma prunorum' (see EPPO, 2017). The pest is widespread throughout Europe, where it causes damage on plant species from several genera and is recognized as an economically important disorder of fruit trees of the genus *Pyrus* (Seemüller & Schneider, 2004). '*Candidatus* P. pyri' inhabits phloem sieve tubes and is transmitted from plant to plant by phloem-feeding insects (Seemüller *et al.*, 2002).

The severity of the disease can depend on a number of factors, including the sensitivity of the cultivar, rootstock, variety and the age of the tree (EPPO, 2016; 2017). In particular, the grade of resistance of rootstocks and the level of vector control achieved may explain the occurrence of slow or quick decline (Seemüller *et al.,* 1986; Giunchedi *et al.*, 1995; Pastore *et al.*, 1997).

For details on the biology of '*Ca.* P. pyri', see the EPPO Datasheet on pear decline phytoplasma (EPPO, 1997). For additional information on distribution, host plants and categorization refer to the EPPO Global Database (2018).

The most important pathway for the introduction of '*Ca*. P. pyri' in new areas is with infested pear trees, scionwood (for grafting) and rootstock, and possibly via introduction of infected vectors. The outbreaks in various European countries are generally ascribed to the introduction of infested plant material.

A positive finding may mean that phytosanitary measures will be applied to ensure that the plants do not present any risk of spreading the pest. These measures may include eradication or containment measures for the lot concerned and possibly for other material in the place of production.

1.1 Vectors of '*Ca.* P. pyri'

'*Candidatus* P. pyri' is transmitted between plants by psyllids, including the species *Cacopsylla pyricola* (Davies *et al.*, 1992) and *Cacopsylla pyri* (Carraro *et al.*, 2001). Spread by vectors is generally only over a short distance, for example from tree to tree or from wild hosts in the near vicinity (Trapman & Blommers, 1992; Hodgson & Mustafa, 1984).

Vectors feed on the phloem tissues of infected plants, picking up the phytoplasmas and transmitting them to the

next plant on which they feed (Weintraub & Beanland, 2006). Phytoplasmas may overwinter in insect vectors or perennial plants.

1.2 Host plants concerned

'*Candidatus* P. pyri' is mainly known to attack plants of the genus *Pyrus*, especially *Pyrus communis* (EPPO, 2016; Seemüller & Schneider, 2004). In addition, '*Ca*. P. pyri' can also be transmitted to other species within the genus including *Pyrus betulifolia*, *Pyrus calleryana*, *Pyrus pyrifolia* var. *culta* and *Pyrus ussuriensis*. Outside of the genus *Pyrus*, other species susceptible to '*Ca*. P. pyri' include *Cydonia oblonga* and *Catharanthus roseus* (in the case of the latter the species is regarded as an artificial host) (EPPO, 2018). Many of these hosts are widely distributed in the EPPO region. *Cydonia oblonga*, which is used as root stock, is described as an incidental host (EPPO, 2018).

1.3 Symptom description

It should be noted that the distribution of the pathogen in the tree is uneven and not constant over the year or between years (EPPO, 2017). In some years the disease may be asymptomatic.

As detailed in EPPO (2017), in general the first visual symptoms occur in late summer when the leaves of affected trees develop a premature red colour followed by early leaf fall (Figs 1 and 2). Occasionally, some cultivars may develop premature yellowing of the leaves (EPPO 2017). There may be some leaf cupping or curling (Fig. 3) and there is usually premature leaf drop. It should be noted that the autumn symptoms associated with pear decline may be caused by other biotic or abiotic causes (EPPO, 2017).

The second symptoms are seen in the following spring when developing leaves remain small and pale with little or no shoot growth and no fruit production (EPPO, 2016; 2017). Spring symptoms can vary in severity from death or severe stunting (Figs 4 and 5) to a complete absence of symptoms (EPPO, 2017).

Fig. 1 Premature reddening of leaves with '*Candidatus* Phytoplasma pyri'. Photo: EPPO Global Database.

Fig. 2 Reddening of the foliage of trees with '*Candidatus* Phytoplasma pyri' (left) and healthy trees (right). Photo: EPPO Global Database.

Fig. 3 Japanese pear (nashi) cv. Hosui showing enlarged vein with leaf curl induced by '*Candidatus* Phytoplasma pyri'. Photo: L. Giunchedi, University degli Studi, Bologna (IT).

Westwood & Cameron (1978) suggested that if diseased trees are not exposed to repeated infestations of psyllids, the symptoms become milder over time. The disease needs

Fig. 4 Pear tree showing severe symptoms of decline caused by '*Candidatus* Phytoplasma pyri'. Photo: Biologische Bundesanstalt (DE).

Fig. 5 Pear tree cv. Abate Fetel grafted on quince BA29 showing little terminal growth with sparse light green and slightly rolled leaves (spring symptoms). Photo: L. Giunchedi, University degli Studi, Bologna (IT).

repeated infestation by the infected vectors, especially in trees grafted on quince rootstocks.

A typical diagnostic symptom of pear decline is a dark phloem ring immediately below the graft union in bark sections from infected trees, visible by microscopic examination of stained transverse sections.

2. Phytosanitary inspections

General background information on phytosanitary inspection of places of production is given in EPPO Standard PM 3/72 (2) *Elements common to inspection of places of production, area-wide surveillance, inspection of consignments and lot identification* (EPPO, 2009).

Inspecting of places of production (to establish freedom from '*Ca.* P. pyri') coupled with sampling for laboratory testing is one of the most effective measures to prevent the spread of the pest. The procedures described in this Standard are specific to the inspection of places of production, but may also be applicable for export inspection, when the requirements of the importing country are similar, or for internal movement of plants for planting or surveys (FAO, 2005). EPPO Standard PM 4/27 *Pathogen-tested material of* Malus*,* Pyrus *and* Cydonia (EPPO, 1999) provides guidance for the production of plant material free from '*Ca.* P. pyri'.

2.1 Inspection and sampling period

Visual inspection should be conducted at the most appropriate time of year for spotting visual symptoms. The most easily recognized symptoms occur in late summer (development of premature autumn leaf colour on affected trees) and then in the following spring (developing leaves remain small and pale with little or no shoot growth and no fruit production).

The distribution of '*Ca.* P. pyri' in the tree is uneven and is not constant within or between years. The distribution pattern in the tree is also dependent on temperature. During winter, the content of phytoplasmas in the above-ground part of the trees declines due to sieve-tube degeneration and the pathogen concentrates in the roots (EPPO, 2017).

Phytoplasmas are detected in phloem tissues in shoots from midsummer to the end of sap flow. Detection on roots is possible throughout the year, although uneven distribution also applies here (Schaper & Seemüller, 1982; Seemüller *et al.*, 1984). This should be considered when looking for symptoms or taking samples.

2.2 Inspection procedure

Inspections are carried out after checking the list of host plants and their location with the nursery supervisor and assessing the regulations or requirements of the relevant national plant protection organization for the purpose of the inspection. This may be for monitoring or survey purposes, for issuing a phytosanitary certificate or for internal movement certification, such as issuing an EU plant passport.

Phytosanitary inspection should start with an overall examination of the place of production in order to check

the physical condition of the plants. If there is abnormal die-off in a place or lot, or if there are other anomalies within the crop (e.g. abnormal growth, differences in colour, leaf curl, see Fig. 3), these lots should be checked with particular attention. If no symptoms are seen, a systematic inspection of the field should be made. Each lot should be examined as a separate unit because it will have different visual characteristics, such as size of individual plants and foliar morphology. Each lot may have potentially different levels of infection depending on variety and rootstock combinations, origin and previous treatments.

Within this Standard a lot should be defined as a number of plants of the same type (variety, rootstock variety) from the same origin and planted at the same time.

The inspection should start with lots of established mother plants, moving to rootstock beds and grafted ('finished') fruit trees.

It is also necessary to inspect host plants in the vicinity of the place of production. Also, inspection of the waste tip of discarded plants may give an indication if the disease is present.

2.3 Selection of plants for visual inspections

An adequate proportion of plants should be subjected to a thorough examination in order to detect the presence or signs of '*Ca*. P. pyri'.

The size of the unit of inspection or sample (the minimum number of individual plants to be examined) should be determined on the basis of lots undergoing inspection, taking into account the statistical background provided in ISPM No. 31 *Methodologies for sampling of consignments* (FAO, 2008).

From a lot of 10 000 plants, 3689 plants need to be inspected to provide 99% confidence of detecting visible symptoms present in 0.1% of the plants, provided the infection is uniformly distributed and the plants are selected at random. In practice inspection of whole rows randomly or evenly chosen across the field is usually carried out. For smaller lots, the number required will often mean that all plants should be inspected.

General background information on lot identification is given in EPPO Standard PM 3/72 *Elements common to inspection of places of production, area-wide surveillance, inspection of consignments and lot identification* (EPPO, 2009).

3. Sampling for laboratory testing

Visual observations alone are not always sufficient for the detection of '*Ca*. P. pyri': latent infections can be present or secondary infections caused by other organisms may hide the symptoms of the pest. In conditions when symptoms are unlikely to be seen, or to meet quarantine requirements of importing countries, sampling of asymptomatic plants for laboratory testing may be required.

3.1 Sample collection

'*Candidatus* P. pyri' is found in mature sieve tubes in the phloem of affected trees during growing months; in the winter it can be found in the roots. During late spring to early autumn (June to the end of September) leaves or shoots can be sampled and the DNA extracted from leaf midribs, petioles or phloem tissue for testing.

For symptomatic and asymptomatic plants, twenty leaves and petioles (with symptoms if available) should be sampled from each tree and sent to the laboratory. Shoot samples for phloem tissue should be taken from around the tree to obtain a representative sample because the phytoplasma may be unevenly distributed.

At all times of the year '*Ca*. P. pyri' may also be detected in the roots of affected trees if the trees are grafted onto *Pyrus* rootstocks or are growing on their own roots [if the trees are grafted on the more widespread *Cydonia oblonga* (quince) rootstocks, detection in the roots is unreliable]. Root samples could be taken from around the tree to obtain a representative sample because the phytoplasma may be unevenly distributed. Testing roots on plants growing in the field is very time-consuming and is usually only done in very specific situations.

3.1.1 Sampling of symptomatic plants

In general, samples of shoots or leaves with petioles for '*Ca*. P. pyri' should be taken from individual plants showing symptoms (but in good condition with no necrotic areas), and these should be kept separate in order to aid diagnosis and obtain a measure of the number of infected plants. Samples should not be affected by other pests. EPPO (2017) highlights that the phytoplasma may be unevenly distributed throughout the tree and thus several (at least three) different parts of the tree should be examined. In addition, a small branch should also be collected from each symptomatic part of the tree (EPPO, 2017).

If there are large numbers of plants in a lot with similar symptoms, sampling may be limited to a small number of trees with representative symptoms to confirm the presence of the pathogen in the lot.

3.1.2 Sampling of asymptomatic plants

For the purpose of declaring a place of production free of '*Ca*. P. pyri' or for the purposes of export sampling of asymptomatic plants and vectors may be required to detect latent or hidden infection. Sample size should be increased if varieties known to be susceptible to latent infection are present or their origins include potential high-risk areas or areas with high vector populations.

It should be noted that within the EPPO region there is limited experience of testing asymptomatic plants (EPPO, 2017). In Slovenia, testing in nurseries is performed on small roots sampled from at least three different root areas of the tree. Root parts should each be 10 cm long (EPPO, 2017).

Roots for *Pyrus* species trees grafted on *Cydonia oblonga* are not recommended for sampling as *C. oblonga* is not sensitive to '*Ca*. P. pyri'.

Sampling and testing of leaves or shoots can be performed as detailed in 3.1 and 3.2.

3.2 Sample preparation

The samples should be placed in a plastic bag (without any paper) that is then inflated slightly and sealed, then packed in strong containers such as cardboard or plastic boxes and padded with paper or similar to prevent movement.

Each sample should be individually labelled with nursery name, nursery reference number, date, variety and, if necessary, a way of identifying the individual tree or length of rootstock hedge so follow-up action can be taken if necessary.

Samples should be kept cool (e.g. in an icebox) and can be stored at 4°C for no more than a few weeks before processing.

Further details are available in EPPO Standard PM 7/62 (2) *Diagnostic protocol for regulated pests: '*Candidatus *Phytoplasma mali', '*Ca*. P. pyri' and* Ca. *P. prunorum* (EPPO, 2017).

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Appendix 1 – Short procedure for inspectors

These short procedures include the main elements for the practical work of inspectors when carrying out inspections at the place of production for '*Candidatus* Phytoplasma pyri'.

Time of inspection

Visual inspection should be conducted at the most appropriate time of the year. The most easily recognized symptoms occur in late summer when leaves of affected trees develop a premature red colour followed by early leaf fall. Some cultivars develop a premature yellow colour. There may be

Table 1. Phenology of symptoms of '*Candidatus* Phytoplasma pyri'

Time of inspection	Possible symptoms
Late summer–early autumn	Premature red (or yellow colour) of leaves Curling or cupping of leaves Early leaf fall
Winter	No visible symptoms (testing of roots is possible)
Spring	Small and pale leaves Little or no shoot growth No fruit production

some leaf curling or cupping. Inspections should be completed before general senescence commences in the autumn to prevent disguising of symptoms.

The second symptom is seen in the following spring when leaves remain small and pale, there being little or no shoot growth and no fruit production.

In winter, the content of phytoplasmas in the aboveground part of the tree declines due to sieve-tube degeneration and the phytoplasmas concentrate in the roots, so even if symptoms are not seen detection of the pest is possible by testing the roots of suspected plants; however, testing of quince rootstocks is regarded as unreliable (Table 1).

Where possible, inspections should be undertaken during overcast days because symptoms of viruses and phytoplasmas may be obscured by bright sunlight.

Hygiene measures

In order not to spread and increase infections, adequate precautions should be taken during inspections and sampling, such as wearing protective clothes (coat, overshoes, gloves, etc.). Gloves should be changed between different lots. All equipment for sampling must be decontaminated between different samples.

Lot identification

For *Pyrus* spp. a lot should be defined as a number of plants of the same combination of graft × rootstock variety, origin and year of planting.

Visual inspection

Phytosanitary inspection should start with an overall examination of the place of production to check the physical condition of the plants. Host plants in the vicinity of the place of production should also be inspected.

A thorough examination of lots with an abnormal level of dying off, with differences in colour, plants with an abnormal growth, stunted plants and plants with leaves reduced in size should be performed.

When infected, most cultivars develop a premature red colour, but some may develop a premature yellow colour. There may be some leaf cupping or curling and there is usually premature leaf drop. The following spring, affected trees suffer from weak growth and sparse pale foliage. The severity of the spring symptoms can vary from absence to death. There may be a line of necrotic tissue in the bark at the graft union between the scion and rootstock.

Table 2. Susceptibility of pear cultivars (from Németh, 1986; Davies *et al.*, 1992; Giunchedi *et al.*, 1995)

Susceptibility	Pear cultivars
High	var. Abate Fetel var. Conference var. Hardy Burré var. Kaiser var. Magness var. Max Red Bartlett var. Precocious var. Williams
Medium	var. Comice var. Concorde var. Montecosa Precoz

The susceptibility varies with the variety. The pear cultivars listed in Table 2 have been tested and showed differences in their susceptibility.

Sampling for laboratory testing

Visual observations alone are not always sufficient for the detection of *Ca.* P. pyri due to the fact that latent infections can be present and secondary infections caused by other organisms may hide the symptoms of the pest.

Sampling and testing techniques vary depending on the time of year and the diagnostic methods available. During late spring to early autumn (June to the end of September) leaves or shoots can be sampled and the DNA extracted from leaf midribs, petioles or phloem tissue for testing.

For symptomatic and asymptomatic plants, twenty leaves and petioles (with symptoms if available) should be sampled from each tree and sent to the laboratory. Shoot samples for phloem tissue should be taken from around the tree to obtain a representative sample because the phytoplasma may be unevenly distributed.

Sampling of symptomatic plants

In general, samples of shoots or leaves with petioles for '*Ca.* P. pyri' detection should be taken from individual plants showing symptoms (but in good condition with no necrotic areas), and these should be kept separate in order to aid diagnosis and obtain a measure of the number of plants that are infected.

If there are large numbers of plants in a lot with similar symptoms, sampling may be limited to a small number of trees with representative symptoms to confirm the presence of the pathogen in the lot.

Sampling of asymptomatic plants

Sample size should be increased if varieties known to be susceptible to latent infection are present or origins include potential high risk or areas with high vector populations.

Testing in nurseries, particularly when trees are lifted, is performed on small roots sampled from at least three different root areas of the tree. Root parts should each be 10 cm long (EPPO, 2017).

Sampling of roots of trees of the genus *Pyrus* grafted on *Cydonia oblonga* is not recommended as *C. oblonga* is not sensitive to '*Ca*. P. pyri'.

Sampling and testing of leaves or shoots can be performed as detailed above.

Sample preparation

The samples should be placed in a plastic bag (without any paper) that is then inflated slightly and sealed, then packed in strong containers such as cardboard or plastic boxes and padded with paper or similar to prevent movement.

Each sample should be individually labelled with nursery name, nursery reference number, date, variety and, if necessary, a way of identifying the individual tree or length of rootstock hedge so follow-up action can be taken if necessary.

Samples should be kept cool (e.g. in an icebox) and can be stored at 4°C for no more than a few weeks before processing.