PEST SURVEY CARD

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Pest survey card on *Phyllosticta citricarpa*

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Abstract

The European Commission requested EFSA (M-2017-0137) to assist the Member States to plan annual survey activities of quarantine organisms using a statistically sound and risk-based pest survey approach, in line with current international standards. *Phyllosticta citricarpa* is a Union quarantine pest and the causal agent of citrus black spot (CBS). It is not known to occur in the EU so special requirements for the import of its host plants and fruit from third countries are in place to prevent its introduction and spread. *P. citricarpa* produces asexual pycnidia and pycnidiospores on fruit lesions, twigs and leaf litter, but it can also reproduce sexually, giving rise to pseudothecia and ascospores, which are particularly important for long-range dissemination. Most commercial citrus species are susceptible to CBS. Due to the variety of environments in which the fungus is currently distributed, all citrus-growing areas in the EU are potentially suitable for the establishment of the pest. The main pathways for its introduction and spread include production, handling and transport of infected plants for planting or fruit. The goal of the visual examination is to detect the symptoms caused by *P. citricarpa*. Since symptoms are non-specific and may be highly variable, visual examination should be followed by laboratory testing. Moreover, CBS has a long incubation period and infected fruit may still be asymptomatic at maturity. In this case, symptom induction methods or other early detection approaches (e.g. airborne spore traps or rain collectors for splash-dispersed conidia) could be further explored. For symptomatic fruit, three main methods for identifying *P. citricarpa* in the laboratory are presented. Based on the analyses of the information on the pest–host plant system, the various units that are needed to design a survey should be defined and tailored to the situation of each Member State.

Keywords: CBS, citrus black spot, *Citrus* spp., delimiting survey, detection survey, *Guignardia citricarpa*, risk-based surveillance

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Introduction

The information presented in this survey card was summarised from recent publications on *Phyllosticta citricarpa*, mainly EFSA Opinions (EFSA PLH Panel, 2009, 2014a, 2014b, 2014c, 2016, 2018), the European and Mediterranean Plant Protection Organization (EPPO) standards on phytosanitary measures (PM7/17(2)) (EPPO, 2009), International Standards for Phytosanitary Measures (ISPM) (no. 27) (FAO, 2016a, 2019), a risk assessment (USDA APHIS, 2010), the EPPO Global Database and the Centre for Agriculture and Bioscience International (CABI) datasheet (CABI, 2019).

The objective of this pest survey card is to provide the relevant biological information that is needed to prepare surveys for *P. citricarpa* in EU Member States (MSs) following the methodology described by EFSA (2018). This document is part of a toolkit that is being developed to assist and support the MSs in the planning of a statistically sound and risk-based pest survey approach in line with International Plant Protection Convention (IPPC) guidelines for surveillance (FAO, 2016b). The toolkit consists of pest-specific documents and more general documents relevant for all pests to be surveyed:

i. Pest-specific documents:
   a. The pest survey card on *Phyllosticta citricarpa*[1]
   b. The guidelines for statistically sound and risk-based surveys of *Phyllosticta citricarpa* that guide the reader through the entire process of survey design including the sample size calculations.

ii. General documents:
   a. The general survey guidelines
   b. The RiBESS+ manual[2]
   c. The statistical tools RiBESS+ and SAMPELATOR[3].

1. The pest and its biology

1.1. Taxonomy

Scientific name: *Phyllosticta citricarpa* (McAlpine) Van der Aa

Species: *Phyllosticta citricarpa*

Synonym(s): *Guignardia citricarpa* Kiely, *Phoma citricarpa* McAlpine, *Phyllostictina citricarpa* (McAlpine) Petrák, *Leptodothiorella* sp. (spermatial state)

EPPO Code: GUIGCI

Common name of the pest: Citrus black spot, CBS

Taxonomy: Class: Dothideomycetes **Order:** Botryosphaeriales **Family:** Phyllostictaceae **Genus:** *Phyllosticta* **Species:** *Phyllosticta citricarpa*

Citrus black spot (CBS) is a fungal disease caused by *Phyllosticta citricarpa* (McAlpine) Van der Aa. According to the current *Melbourne Code* (2012), the anamorph name *P. citricarpa* has been given priority over the teleomorph name *Guignardia citricarpa* Kiely. Morphologically, pycnidiospores (conidia) and ascospores of *P. citricarpa* are nearly indistinguishable from those of other *Phyllosticta*

[1] The Pest survey card will be updated in the form of Story Map that will be available in the Plant Pests Story Maps Gallery available online: https://efsa.maps.arcgis.com/apps/MinimalGallery/index.html?appid=f91d6e95376f4a5da206eb1815ad1489

[2] https://zenodo.org/record/2541541/preview/ribess-manual.pdf

[3] https://websso-efsa.openanalytics.eu/auth/realms/efsa/protocol/openid-connect/auth?response_type=code&client_id=shiny-efsa&redirect_uri=https%3A%2F%2Fshiny-efsa.openanalytics.eu%2Fsso%2Flogin&state=d6f7f997-d09f-4bb0-afce-237f192a72d5&login=true&scope=openid

species such as the endophyte *P. capitalensis*, which is widespread in CBS-affected areas (EFSA PLH Panel, 2014a). Molecular methodologies prove, however, that *P. citricarpa* is a clearly distinguishable taxonomic entity.

1.2. EU pest regulatory status

Phyllosticta citricarpa is a Union quarantine pest listed in Annex II Part A of Commission Implementing Regulation (EU) 2019/2072[4], which includes harmful organisms not known to occur in the EU, whose introduction into, and spread within, all Member States shall be banned. Commission Delegated Regulation (EU) 2019/1702[5] also lists *P. citricarpa* as a priority pest.

Import of plants for planting from third countries of *Citrus* L., *Fortunella* Swingle, *Poncirus* Raf., and their hybrids is prohibited as laid down in Commission Implementing Regulation (EU) 2019/2072. Fruit belonging to *Citrus* spp., *Fortunella* spp., *Poncirus* spp. and their hybrids, other than fruit of *C. aurantium* (L.) and *C. latifolia* (Tanaka) free from leaves and peduncles, imported from third countries must originate in countries, areas, places or sites of production free from *P. citricarpa*. In the case of places or sites of production free from *P. citricarpa*, fruit must: (i) be found free of symptoms of *P. citricarpa* by official inspection of a representative sample; or (ii) have been subjected to appropriate treatments and cultural measures against *P. citricarpa*, and have been found free of symptoms of the fungus by official inspections at the site of production.

Moreover, additional measures in the EU (Commission Implementing Decision (EU) 2016/715 last amended by Commission Implementing Decision (EU) 2019/449[6]) are laid down for the import of citrus fruits from Argentina, Brazil, South Africa and Uruguay, distinguishing the end uses (industrial processing into juice and other uses). Fruit of *Citrus sinensis* (L.) Osbeck 'Valencia' originating in South Africa and Uruguay should be tested for latent infections of *P. citricarpa* at origin. Fruit destined for industrial processing must be found free of symptoms of *P. citricarpa* by official inspection and must originate from a site of production subjected to appropriate treatments against *P. citricarpa* carried out at the appropriate time. Also, their movement, storage and processing, transport and labelling should take place under specified conditions.

The general requirements for survey of quarantine organisms in the EU territory are laid down in Regulation (EU) 2016/2031[7].

1.3. Pest distribution

EPPO (online) lists *Phyllosticta citricarpa* to be present in Angola, Ghana, Kenya, Mozambique, Namibia, South Africa, Tunisia, Uganda, Zambia, Zimbabwe, Argentina, Brazil, Cuba, USA (Florida), Uruguay, Bhutan, China (Fujian, Guangdong, Guangxi, Jiangsu, Sichuan, Xianggang (Hong Kong), Yunnan, Zhejiang), India (Maharasthra), Indonesia (Java), Philippines, Taiwan and Australia (New South Wales, Queensland, Victoria) (Figure 1). In particular, *P. citricarpa* was recently reported in India (Das et al., 2018) and Tunisia (EPPO, 2019; Boughalleb-M'Hamdi et al., 2020). It is to be noted that Guarnaccia et al. (2017) reported *P. citricarpa* from leaf litter of smallholdings or gardens in four locations in Europe (in Italy, Malta and Portugal). These findings were discussed by the EFSA PLH

[4] Commission Implementing Regulation (EU) 2019/2072 of 28 November 2019 establishing uniform conditions for the implementation of Regulation (EU) 2016/2031 of the European Parliament and the Council, as regards protective measures against pests of plants, and repealing Commission Regulation (EC) No 690/2008 and amending Commission Implementing Regulation (EU) 2018/2019. OJ L 319, 10.12.2019, p. 1–279.

[5] Commission Delegated Regulation (EU) 2019/1702 of 1 August 2019 supplementing Regulation (EU) 2016/2031 of the European Parliament and of the Council by establishing the list of priority pests. OJ L 260, 11.10.2019, p. 8–10.

[6] Commission Implementing Decision (EU) 2016/715 of 11 May 2016 setting out measures in respect of certain fruits originating in certain third countries to prevent the introduction into and the spread within the Union of the harmful organism *Phyllosticta citricarpa* (McAlpine) Van der Aa (notified under document C(2016) 2684), OJ L 125, 13.5.2016, p. 16 last amended by Commission Implementing Decision (EU) 2019/449 of 18 March 2019 amending Commission Implementing Decision (EU) 2016/715 (notified under document C(2019) 2024). OJ L 77, 20.3.2019, p. 76–77.

[7] Regulation (EU) 2016/2031 of the European Parliament of the Council of 26 October 2016 on protective measures against pests of plants, amending Regulations (EU) No 228/2013, (EU) No 652/2014 and (EU) No 1143/2014 of the European Parliament and of the Council and repealing Council Directives 69/464/EEC, 74/647/EEC, 93/85/EEC, 98/57/EC, 2000/29/EC, 2006/91/EC and 2007/33/EC. OJ L 317 23.11.2016, p. 4.

Panel (2018) in relation to the survey and sampling methodology used. The reports have so far not been confirmed in surveys conducted by the competent National Plant Protection Organisations in the same locations.

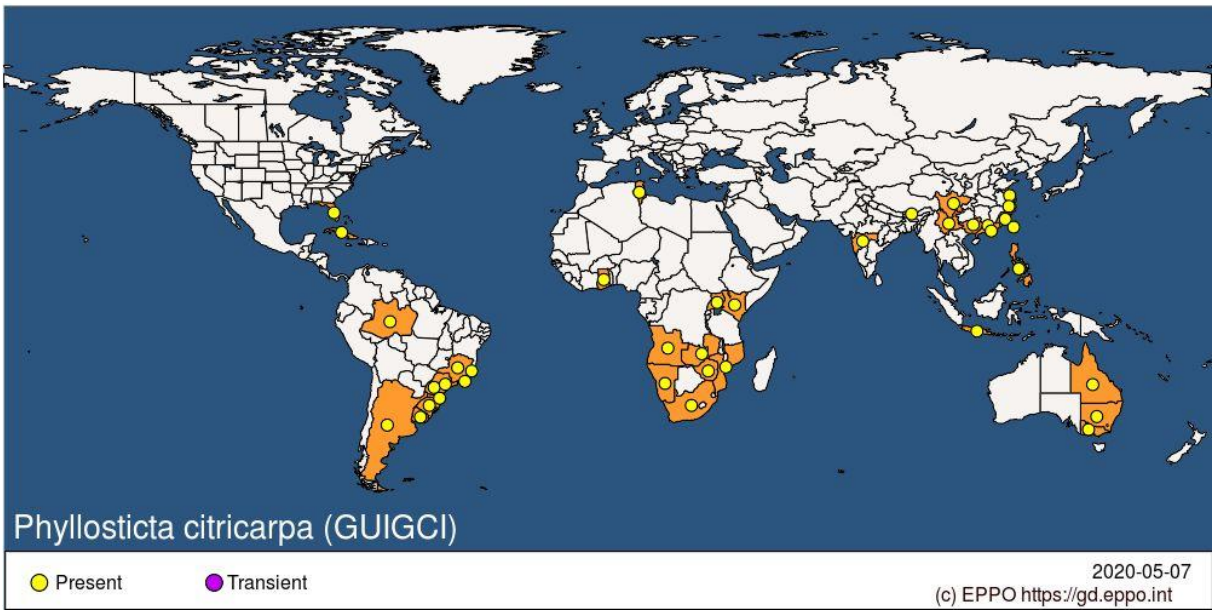

Figure 1: Global distribution of *Phyllosticta citricarpa* (Source: EPPO Global Database, https://gd.eppo.int/)

1.4. Life cycle

Asexual pycnidia and pycnidiospores of *Phyllosticta citricarpa* are produced on fruit lesions, twigs and leaf litter (Kotzé, 2000, Wang and Dewdney, 2018). Information on the seasonality of pycnidiospore production needs to be further explored. The pycnidiospores can be splash-dispersed and washed off onto other plant parts such as twigs, young fruit and leaves. The fungus can also reproduce sexually when two complementary mating types are present. Sexual reproduction gives rise to pseudothecia which bear ascospores (Tran et al., 2017). These are particularly important for long-range dissemination. Pseudothecia may develop on the leaf litter within 40–180 days of leaf drop, depending on the environmental conditions (Figure 2) (EPPO, 2009). Studies conducted in South Africa indicated that the production of ascospores by *Phyllosticta* spp. is concentrated mainly in spring and summer (Fourie et al., 2013).

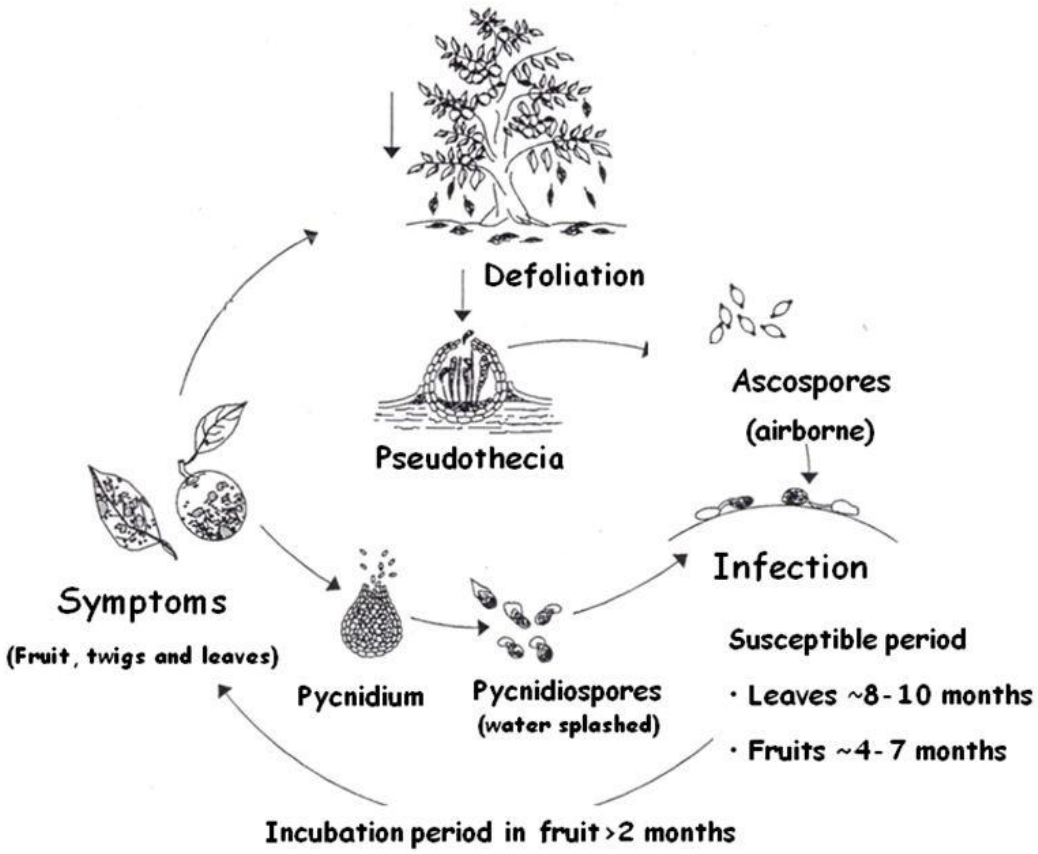

Figure 2: Life cycle of *Phyllosticta citricarpa* (extracted from EFSA PLH Panel, 2014a) and adapted from a drawing by D. Drouillard in Timmer (1999) © American Phytopathological Society and modified according to Aguiar et al. (2012), Brentu et al. (2012), Lanza et al. (2018), Reis et al. (2003) and Truter (2010). *Phyllosticta citricarpa* has two infection cycles: a primary cycle driven by ascospores in the leaf litter and a secondary cycle involving pycnidiospores produced on lesions in fruit, twigs and leaves

Citrus leaves are susceptible to *P. citricarpa* infection for up to 10 months (Truter et al., 2004; Truter, 2010). In South Africa, the critical period for fruit infection starts at fruit set and lasts 4–5 months. (Kotzé, 1981). However, data from Ghana and Brazil indicated that sweet orange fruit are susceptible for 7 months after fruit set (Brentu et al., 2012; Lanza et al., 2017). Following infection, the fungus remains in a quiescent state until the fruit becomes fully grown and mature. The symptoms could appear 2–5 months after infection, often coinciding with fruit ripening and are generally influenced by physiological and environmental factors, such as temperature, rain, light and tree age (Kotzé, 1981; Frare et al., 2019). Therefore, the optimal period for visual examination to detect CBS-like symptoms on fruit will depend on the ripening calendar for each citrus species and cultivar (EFSA PLH Panel, 2014a). An example of the ripening calendar for citrus fruit for the fresh market from Spain is shown in Figure 3. This calendar is in line with the fruit maturity criteria as set out by the international standards for citrus fruit (OECD, 2010) and could be used as a proxy for other citrus-growing areas in the EU, since these belong to similar climatic zones within the Mediterranean Basin.

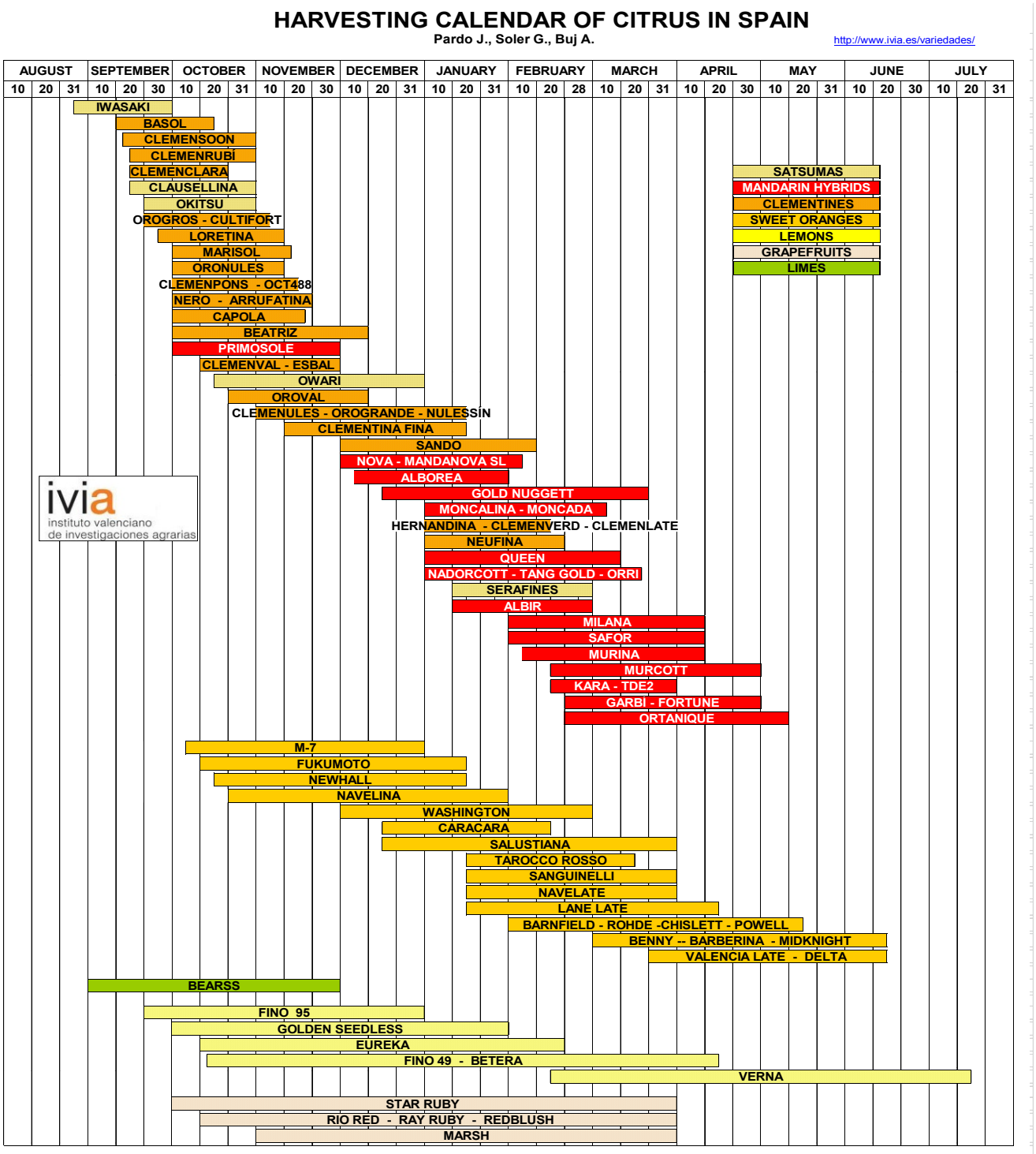

Figure 3: Example of a ripening calendar of different citrus species/cultivars in Spain (Source: Istituto Valenciano de Investigaciones Agrarias, http://www.ivia.gva.es/va/)

1.5. Host range and main hosts

Except for sour orange (*Citrus aurantium* L.) and Tahiti lime (*Citrus latifolia* Tanaka), all commercial citrus species and cultivars are susceptible to *Phyllosticta citricarpa* (EFSA PLH Panel, 2014a; Kotzé, 1981; Baldassari et al., 2008). In the EUROPHYT database (online)[8], interceptions of *P. citricarpa* have been reported on *Citrus maxima* from China in 2020. However, recent studies with artificial inoculations defined pomelo (*Citrus maxima*) and finger lime (*Citrus australasica*) as being immune to *P. citricarpa* (Miles et al., 2019). The authors also indicate that the endophytic *Phyllosticta* spp. can be commonly misidentified as *P. citricarpa*.

Table 1 shows the lists of the known susceptible citrus species for *P. citricarpa* adapted from EFSA PLH Panel (2014a) and updated as per Miles et al. (2019).

Table 1: Citrus species susceptible to citrus black spot, caused by *Phyllosticta citricarpa*. The first five species are relevant for surveillance in the EU (adapted from Table 3 of EFSA PLH Panel (2014a) updated as per Miles et al. (2019))

Host plants of *Phyllosticta citricarpa*

Citrus limon (L.) Burm.f.	Lemon
Citrus sinensis (L.) Osbeck	Sweet orange
Citrus reticulata Blanco	Mandarin
Citrus unshiu (Swingle) Marcow	Satsuma mandarin
Citrus paradisi Macfad.	Grapefruit
Fortunella spp.	Kumquat
Poncirus trifoliata (L.) Raf.	Trifoliate orange
Citrus medica L.	Citron
Citrus aurantifolia (Christm.) Swingle	Key lime
Citrus limettioides Tanaka	Sweet lime
Citrus hystrix DC	Kaffir lime

EFSA PLH Panel (2014a) indicates that *C. limon* is the most susceptible citrus species, followed by (late-maturing) *C. sinensis*. It has been observed that the first disease outbreaks in a region usually occurred in lemon (*C. limon*) orchards and later spread to adjacent citrus orchards (Kotzé, 1981). However, CBS emerged in Florida (USA) directly in sweet orange (*C. sinensis*) orchards (Schubert et al., 2012). In addition, late-maturing cultivars of sweet orange were considered more susceptible than early-maturing ones (Timmer, 1999). However, cultivar field trials conducted in Brazil, as well as studies comparing the rate of disease progress, indicated that disease expression depends on environmental factors and the ripening stage (Spósito et al., 2004; Sousa and de Goes, 2010).

Regarding the other host plants subject to the prohibition of importation of plants for planting as laid down in Commission Implementing Regulation (EU) 2019/2071, Annex VI point 11 (see Section 1.2), kumquat (*Fortunella*) was recorded by Kiely (1948) in Australia as moderately susceptible to CBS under conditions of natural infection, but no further experimental information is available on this species. No conclusive information has been found on the susceptibility of *Poncirus trifoliata* to *P. citricarpa*.

In conclusion, all susceptible citrus species cultivated in the EU are considered relevant for *P. citricarpa* surveillance. This is mainly the case for *C. limon* (lemon) and *C. sinensis* (sweet orange) and to a lesser extent for *C. reticulata* (mandarin), *C. unshiu* (satsuma mandarin) and *C. paradisi* (grapefruit).

[8] The EUROPHYT (European Union Notification System for Plant Health Interceptions) database was accessed on 21 April 2020.

The other susceptible species listed in Table 1 are not relevant for annual surveys because they are either not cultivated in the EU (*C. medica*, *C. aurantifolia*, *C. limettioides* and *C. hystrix*) or there is high uncertainty on their host status (*Fortunella* spp., *Poncirus trifoliata*).

1.6. Environmental suitability

The potential establishment of *Phyllosticta citricarpa* in the EU will be influenced by climate conditions. The extent of temperature and wetness duration relevant for pycnidiospore or ascospore infection has not been determined experimentally and the only data available in the literature are the rate of spore germination and some limited field data. Kotzé (1963) stated that the conditions required for ascospore germination varied from 15°C to 29.5°C and from 15 to 38 hours of wetness. McOnie (1964) found that ascospores were able to infect fruit with 15 or more hours of continuous wetness.

Subtropical citrus-growing regions with a summer rainfall pattern and a high annual precipitation are known to be areas prone to CBS (Kotzé, 1981, 2000). However, the disease is also present in arid and semi-arid areas such as Eastern Cape Province and the north of Limpopo Province in South Africa (Paul et al., 2005). The disease has recently been reported in Tunisia, under Mediterranean-type climate conditions (EPPO, 2019). Simulations performed for previous EFSA Opinions concluded that the various climates of the EU citrus-growing areas are potentially suitable for the establishment of *P. citricarpa* (EFSA PLH Panel, 2014c, 2008). Establishment was rated as moderately likely because susceptible hosts are widely available and the environmental conditions in many EU citrus-growing areas are suitable (with high uncertainty) for *P. citricarpa* ascospore production, dispersal and infection (EFSA PLH Panel, 2014c). Therefore, all citrus-growing areas in the EU are considered in this survey card, namely production areas in Cyprus, Spain, France, Greece, Croatia, Italy, Malta and Portugal (Figure 4).

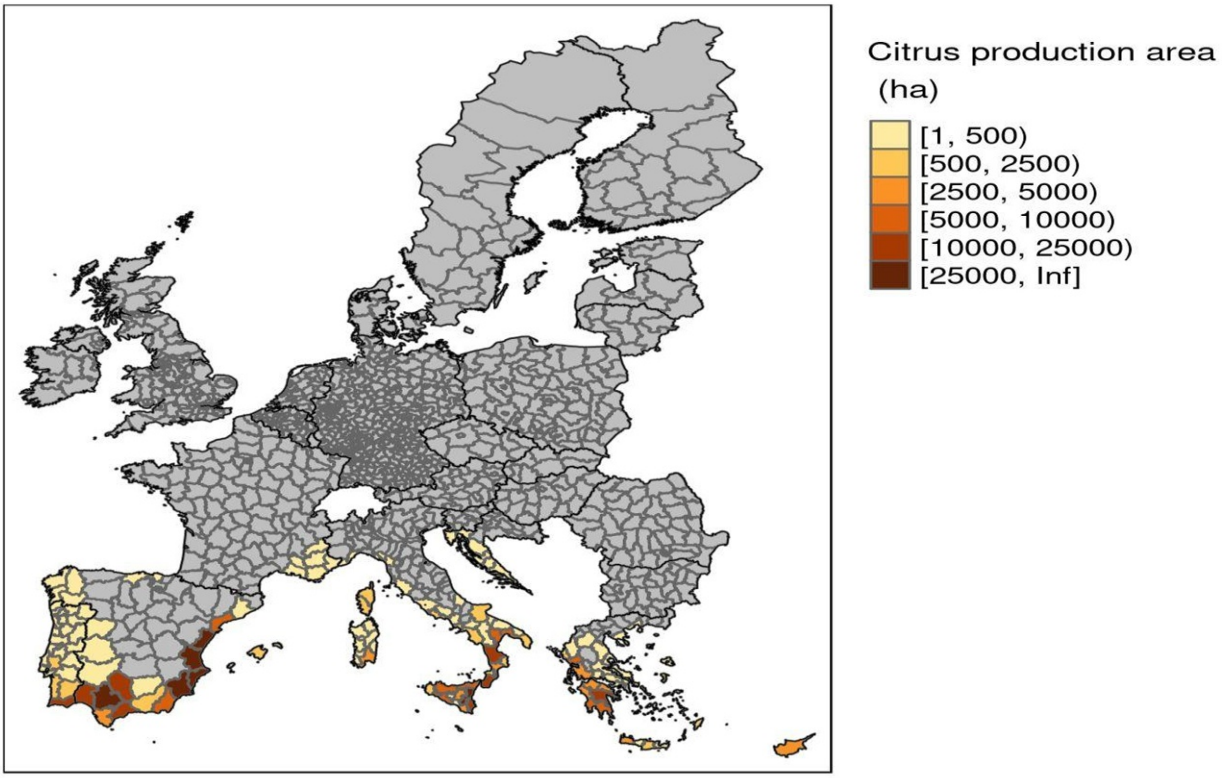

Figure 4: Citrus-growing regions based on citrus production data from national statistical databases of Cyprus, Spain, France, Greece, Croatia, Italy, Malta and Portugal at NUTS3 level (EFSA PLH Panel, 2014c)

1.7. Spread capacity

The pathogen is very likely to enter and spread with human assistance by means of infected plants for planting or fruit (EFSA PLH Panel, 2014a). Natural spread of *Phyllosticta citricarpa* is known to mainly happen by dispersal of airborne ascospores, but there is limited knowledge of the potential distance of such spread. Nonetheless, the ascospore inoculum of the fallen, decomposing citrus leaves is considered the greatest spread risk for *P. citricarpa* (USDA APHIS, 2010). In addition to airborne ascospores, infected leaf litter could also be dispersed by the wind over relatively long distances. Considering the presence of ascospore inoculum, in EFSA (2019) the maximum spread rate for *P. citricarpa* was estimated by expert knowledge elicitation to be approximately 800 m per year (with a 95% uncertainty range of 56 - 3,562 m). Therefore, 800 m is the potential spread distance of the airborne ascospores used for the purpose of the delimiting surveys when the presence of the two mating types cannot be excluded.

Pycnidiospores of *P. citricarpa* were typically regarded to be of minor epidemiological relevance when compared with the airborne ascospores (Kotzé, 1981, 2000), although they were considered relevant during the first stages in recently established outbreaks (Garrán, 1996; Whiteside, 1967). Recent CBS epidemics in Florida, caused by a clonal (asexual) population of *P. citricarpa* (Wang et al., 2016; Hendricks et al., 2017), thus unable to produce the (sexual) ascospores, showed that pycnidiospores can play a role in epidemics. The pycnidiospores produced on fruit lesions, twigs and leaf litter can be splash-dispersed or washed off by rain to relatively short distances, infecting other susceptible plant parts (Kotzé, 1981). Recent studies under laboratory conditions demonstrated that *P. citricarpa* pycnidiospores could reach longer distances than previously thought, particularly under wind-driven rain conditions. According to the studies by Perryman et al. (2014) and Perryman and West (2014), pycnidiospores of *P. citricarpa* can be splashed from pycnidia in fruit lesions by simulated wind-driven rain at least 8 m. That was the longest horizontal distance evaluated in those experiments, but it was also suggested that *P. citricarpa* pycnidiospores might also be dispersed much further when rain droplets become aerosolised. Similarly, splash-dispersed conidia of *Plenodomus tracheiphilus* were able to disseminate from pycnidia on infected citrus tissue up to 16 m, which was again the maximum distance evaluated (Laviola and Scarito, 1989; EFSA PLH Panel, 2014b). Based on this, a 20 m radius was established for the eradication of *P. tracheiphilus* in Spain (MAPAMA, 2016). This is also the potential spread distance used for the annual detection surveys.

1.8. Risk factor identification

The identification of the risk factors and their relative risk estimation is essential for performing a risk-based survey. It needs to be tailored to the situation in each Member State (MS). The proportion of the target population for each risk factor needs to be known or estimated by each MS. This section presents examples of risk factors that could be relevant for some MSs, but other factors might be more relevant for others.

A risk factor is a biotic or abiotic factor that increases the probability of infestation by the pest in the area of interest. The risk factors that are relevant for the surveillance are those that have more than one level of risk for the target population. The risk factors that will be considered for the surveys need to be characterised by their relative risk and the proportion of the overall plant population to which they apply.

Example 1: Risk activities, locations and areas

For the identification of the risk areas to be surveyed as a priority, it is necessary to first identify the risk activities that could contribute to the introduction or the spread of *Phyllosticta citricarpa*. These activities should then be connected to specific locations, called 'risk locations'. In consideration of the spread capacity of the pest and the availability of host plants around these locations, high-risk areas can be defined.

Risk activity

Surveillance efforts should concentrate on activities that could potentially result in the introduction and spread of the pathogen.

EFSA PLH Panel (2014a) lists the possible pathways for the entry of *P. citricarpa* into EU territory and addresses both the trade of citrus plants for planting and citrus fruit. Both of these are identified as relevant risk activities for *P. citricarpa* surveillance.

The major pathways of entry are closed for plants for planting, based on the current legislation in place (see Section 1.2 and Annex VI point 11 of Commission Implementing Regulation (EU) 2019/2072). Nonetheless, these pathways should be taken into consideration in a survey design as the movement of plants for planting is a very efficient means for spread and further establishment of the fungus in the EU's citrus-growing areas.

The movement of citrus fruit through trade is considered a risk activity for introduction of the pest. The import of infected fruit by passenger traffic is possible but is much less important in the risk of introduction.

Moreover, according to the EFSA PLH Panel (2014a), under the current EU regulation, the potential transfer of *P. citricarpa* to citrus host plants is mainly related to splash dispersal from discarded unmarketable whole fruit, peel or citrus by-products, which may be produced in facilities growing or handling citrus fruit (Figure 5). Thus, a second relevant risk activity is that of the production and handling of citrus fruit. A third type of activity can be distinguished; namely, non-commercial production. This is defined as any production that is not subject to systematic monitoring and agricultural practices.

Risk location

In relation to transport, the end points of import pathways should be identified and considered. These would commonly be nurseries or garden centres. Geo-localising the nurseries of an area enables a detection survey to be designed and targeted accordingly even if the nurseries themselves are not going to be subject to that survey. The time when these risk locations are surveyed (e.g. expected time when the nurseries have the most imports of citrus trees) is a critical factor to be considered. Historical data on *P. citricarpa* interceptions from import inspections on citrus fruit can also be used to prioritise locations. Pathways where interceptions of the pest have occurred might have a higher risk. The end points of these pathways at destination can also be expected to be higher risk locations and consequently could have a higher probability of becoming infected.

In relation to production and handling of citrus plants for planting or fruit, these activities occur in locations such as nurseries, garden centres, packing houses, processing plants, outdoor fruit-drying facilities, fresh fruit markets and livestock feeding areas. Non-commercial production locations (not subject to systematic monitoring and agricultural practices) include neglected orchards as well as residential trees from backyards and gardens. From these, the locations that can be geo-localised serve as risk locations and can inform the survey design.

Figure 5: Processing of citrus pulp residue and whole citrus fruit in close proximity to citrus orchards (top panels). Uncontrolled citrus waste discharged in the vicinity of neglected citrus trees (bottom left panel). Sweet orange orchard with low-hanging branches and fruit (Valencia, Spain) (bottom right panel) (Pictures from EFSA PLH Panel, 2014a)

Different locations corresponding to the risk activities for *P. citricarpa* are summarised in Table 2. Different levels of risk could also be assigned to these locations depending on whether or not they are involved in international trade.

Table 2: Risk activities and corresponding risk locations relevant for the surveillance of *Phyllosticta citricarpa* in all EU Member States

Risk activity	Risk locations
Production and transport of citrus plants for planting	Nurseries and garden centres cultivating citrus plants
Production, storage and handling of citrus fruit	Packing houses, processing plants, outdoor fruit-drying facilities, fresh fruit markets and livestock feeding areas
Non-commercial production (not subject to systematic monitoring and agricultural practices)	Neglected orchards and residential trees from backyards and gardens

Risk areas

The risk areas can be defined as specific areas in a set of epidemiological units (see Glossary) adjacent to the risk locations defined above. As an example, in Spain an epidemiological unit in citrus-growing areas can be defined as orchards of ½ hectares as these are a homogeneous unit in terms of cultural practices, property and cultivars grown. This can vary from one region to another. The definition of risk areas around a certain risk location takes into consideration the spread capacity of *P. citricarpa* and the availability of host plants, suggesting that areas of citrus orchards are of key interest.

- For detection surveys, i.e. where no positive case has yet been reported, the objective of the survey is to substantiate pest freedom or to detect the fungus if it is present. The smaller the risk areas, the higher the number of risk areas that can be surveyed for the same level of surveillance efforts. For *P. citricarpa*, three different risk areas can be defined (high, medium or baseline risk) where the survey efforts can be distributed differently. Considering that only *P. citricarpa* pycnidiospores are present on fruit lesions, a distance of 20 m (see Section 1.7) is considered sufficient to define the high-risk and medium-risk areas for *P. citricarpa* in the area contiguous to the risk locations. Figure 6 shows the relative risk estimated by expert knowledge on the three different types of risk area.

Risk areas contiguous to the risk locations	Relative risk
High Risk Areas (HRA): a band 20 m wide in the citrus orchards adjacent to risk locations, consisting of packhouses or facilities that are located in a citrus-growing area and that also process imported fruit from third countries	2
Medium Risk Areas (MRA): a band 20 m wide in the citrus orchards adjacent to risk locations, consisting of packhouses or facilities that are located in a citrus-growing area and that do not process imported fruit from third countries (garden centres and nurseries)	1.5
Baseline area: all other citrus orchards	1

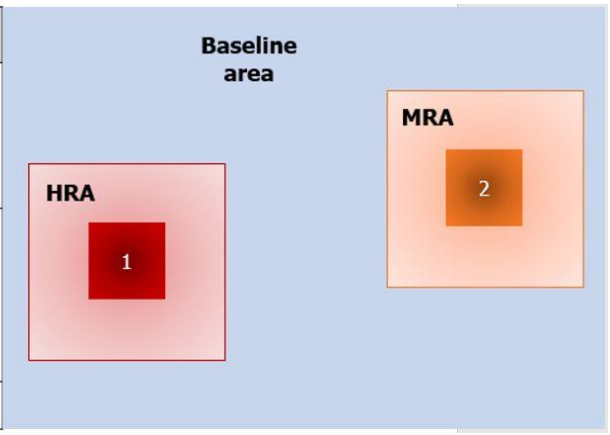

Figure 6: Example of relative risks estimated by expert knowledge for different risk areas for risk-based annual detection surveys of *Phyllosticta citricarpa* in a Member State. Locations 1 and 2 are risk locations, with location 1 also processing imported fruit from non-EU countries and location 2 not doing so. The 20 m band adjacent to location 1 is designated a high-risk area, while the 20 m wide band adjacent to location 2 is designated a medium-risk area. The blue area is the baseline area

- For a delimiting survey, i.e. when a first positive finding has been reported, the first action should be to trace the introduction site of the pest (risk location). The mating type(s) of the first finding(s) could then be identified (see Section 2.2.2) as the potential spread distance of the fungus depends on them. If the delimiting survey is conducted in an area that has been surveyed yearly, then the probability of finding the two different mating types in the same year is negligible. In this situation the natural spread is estimated to be about 20 m. Whereas, if the surveys in the area have not been conducted in recent years, the possibility of having introduced the two mating types in two different introduction events, resulting in the production of airborne ascospores, cannot be excluded. In this case, the potential spread distance with airborne ascospores is estimated to be about 800 m (see Section 1.7). This is shown in Figure 7 where the potentially infected zones to survey depend on how long it has been since the last detection survey was performed. Figure 7 also shows that if all risk locations are surveyed on a yearly basis, when there is a positive finding, the efforts of the related delimiting survey are reduced considerably. In the delimiting survey, the strategy being to determine the smallest area where the pest is contained, it is recommended to survey concentric bands around the risk location from the periphery to the risk location itself. Therefore, a band of 800 m can be defined around a risk location considering that a detection survey was conducted at the site the previous year.

Additional bands of 800 m should be considered for each additional year since the last detection survey (Figure 7). The delimiting activity would always start from the outer band working inwards.

Year since the last detection survey at the site	Potential spread distance from the risk location	Potentially infected zone for delimiting survey
Year 0 (finding of the pest)	0	20
Year 1	0 – 20 m	800
Year 2	20 – 800 m	1,600
Year 3	800 – 1,600 m	2,400
Year 4	1,600 – 2,400 m	

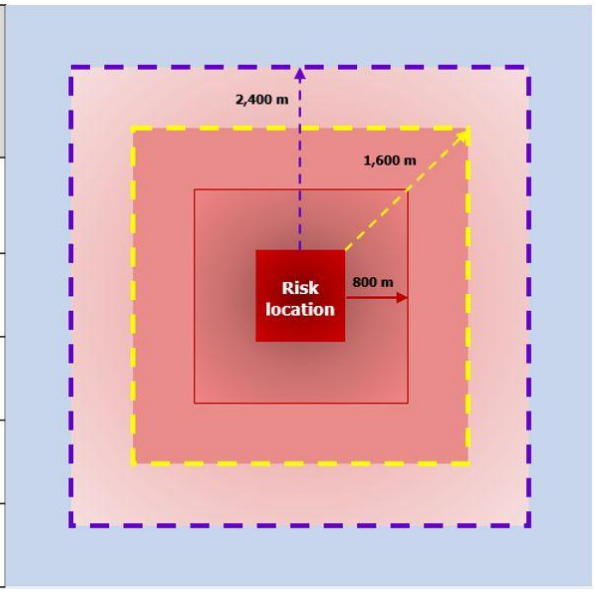

Figure 7: Potential infected zone for a delimiting survey of *Phyllosticta citricarpa* depending on the last detection survey that was conducted at the site

Example 2: Susceptible species

The EFSA PLH Panel (2014a) listed the known susceptible citrus species that are relevant and indicated that *C. limon* is known to be much more susceptible to CBS than other *Citrus* spp., followed by late-maturing cultivars of *C. sinensis* (Table 2). Consequently, a second risk factor could be defined as the presence of hosts with increased susceptibility in comparison with other hosts.

Based on the literature review provided in EFSA PLH Panel (2014b) on empirical observations, the relative risk in terms of the most susceptible species has been estimated for the various main hosts of *P. citricarpa* as indicated in Table 3 below. The proportion of the target population for each category needs to be calculated by each Member State.

Table 3: Example of relative risks for the various hosts of *Phyllosticta citricarpa*

Botanical name	Common name	Relative risk (most susceptible)
Citrus limon (L.) Burm.f.	Lemon	1.5
Citrus sinensis Osbeck	Sweet orange (late-maturing cultivars)	1.4
Citrus sinensis Osbeck	Sweet orange (other cultivars)	1
Citrus reticulata Blanco	Mandarin	1
Citrus unshiu (Swingle) Marcow	Satsuma mandarin	1
Citrus paradisi Macfad.	Grapefruit	1

2. Detection and identification

2.1. Visual examination

The goal of the visual examination is to detect the symptoms caused by *Phyllosticta citricarpa*. Details of the procedure for visual examination and detection of CBS can be found in the official protocols available on the identification of *P. citricarpa* on symptomatic fruit (EPPO, 2009; FAO, 2019), and on twigs or leaves (EPPO, 2009).

2.1.1. Symptoms

The symptoms of *Phyllosticta citricarpa* can potentially be found on the fruit, pedicels, leaves and twigs of *Citrus* species and related genera such as *Poncirus*, *Fortunella* and their hybrids (FAO, 2019). Symptoms caused by *P. citricarpa* are non-specific and may be highly variable. Thus, the observed symptoms are designated as CBS-like symptoms that should consistently be followed by laboratory testing for *P. citricarpa* identification (see Section 2.2).

Symptoms on leaves

Symptoms on leaves are rarely observed; only in highly susceptible species such as *C. limon* or on poorly managed trees. Consequently, the sampling effectiveness (see Glossary) of visual examination for detecting CBS-like symptoms on leaves is likely to be very low.

If symptoms do appear, they start as pinpoint spots visible on both leaf surfaces and may increase in size up to 3 mm in diameter. They are circular, with centres becoming grey or cinnamon brown in colour, surrounded by a dark brown to black margin and a yellow halo (Kotzé, 2000). Pycnidia may occasionally be present in the centre of the lesions on the adaxial leaf surface (FAO, 2019).

Symptoms on twigs

On small twigs, more commonly on *C. limon* than on other citrus species, round, slightly sunken lesions (0.5–2 mm in diameter) with a brown to black margin and a grey to light brown centre may be found. Pycnidia are occasionally present in the centre of the lesions (FAO, 2019).

Symptoms on fruit

The symptom expression on fruit depends on the host species and cultivar, the ripening stage of the fruit, the time of the year and the climate conditions (mainly temperature and humidity). Fruit symptoms of CBS caused by *P. citricarpa* can be detected by visual examination. However, due to the long incubation period characteristic of *P. citricarpa*, CBS-like symptoms on fruit are visible only at maturity, several months after infection (EFSA PLH Panel, 2014a). Hence, visual examination of fruit should be conducted to coincide with the ripening stage for each *Citrus* species and cultivar (Table 1). Further, climatic conditions at the time of maturity of the fruit may have an influence on the severity of the symptoms.

Various types of symptoms can appear on the fruit, e.g. hard spot (Figure 8), freckle spot, false melanose (Figure 9), and virulent spot (FAO, 2019). As described by Kotzé (1981), hard spot lesions on green fruit may appear with a surrounding yellow halo. On mature fruit, the lesions have a depressed light brown to grey-white centre, which is surrounded by a dark brown circle and a green halo. Black pycnidia may also be distinguishable in the centres of these lesions. This type of lesion is present more on the sunlight-exposed side of the fruit. Freckle spots may initially be similar to freckles, yet with time, it is possible for individual spots to merge into one big lesion. These may eventually become virulent spots during storage, when the fruit has fully matured, and the temperature is higher than in the field. The lesions of virulent spots are depressed, irregular and have a brown to brick red halo. Overall, however, it is hard to distinguish between different types of spot and, therefore, it is advised to take a sample of any suspicious symptom, regardless of its severity.

The sampling effectiveness of detecting these CBS-like symptoms on an infected symptomatic fruit by a trained inspector is estimated by experts to be 0.9. In other words, this indicates that only for 10% of the cases a symptomatic, infected fruit will not be recognised.

Figure 8: Hard spot symptoms caused by *Phyllosticta citricarpa* on sweet orange (left) and lemon (centre) fruit. Lesions caused by *P. citricarpa* on sweet orange leaves (right). Photos: A. Vicent

Risk of misidentification

The symptoms of CBS are variable in appearance and often resemble those caused by other citrus pathogens or by insects, mechanical damage or cold damage, particularly in the case of freckle spot and false melanose (EPPO, 2009; FAO, 2019). Figure 9 shows false melanose symptoms caused by *P. citricarpa* and melanose symptoms caused by another fungal pathogen of citrus, *Diaporthe citri.* Symptoms that might be confused with those of CBS have previously been reported in the Mediterranean Basin, but these were caused by other pathogens, pests or abiotic disorders (Amat, 1988; Agustí, 2012; Agustí et al., 2004; Vacante and Calabrese, 2009).

Figure 9: Symptoms of false melanose caused by *Phyllosticta citricarpa* on sweet orange (left) and melanose caused by *Diaporthe citri* on grapefruit (right). Photos: A. Vicent

2.1.2. Asymptomatic plant parts

Phyllosticta citricarpa is characterised by a long incubation period, and asymptomatic infections may occur both in fruit and leaf tissue. Infected fruit may still be asymptomatic at maturity and could

escape detection by visual examination (EFSA PLH Panel, 2014a). In situations where the incidence of fruit with CBS-like symptoms is typically low or even below the detection level by direct visual examination, the EPPO (2009) and FAO (2019) protocols do not provide enough information to conclude on pest presence. In those situations, field inspectors are likely to also collect samples of asymptomatic fruit for laboratory testing. This is particularly relevant for survey programmes that target early detection of the disease. This could be the case for the surveys conducted in the highest risk areas and when climatic conditions are more favourable for the development of the fungus. In the absence of symptoms, in order to increase the chances of performing the laboratory test on the infected tissue of the fruit, symptom induction methods could be further explored. As an example, in Brazil, symptom induction is routinely applied on non-mature fruit to confirm that the fruit can be exported (Normative Instruction Nº 3, January 8th, 2008[9]). Thirty days before the beginning of the harvest, citrus fruit is sampled for induction of symptoms in the laboratory. The samples consist of at least one fruit from 1% of the trees of each production unit with a minimum of 20 fruits. If all samples are negative after 30 days, the fruit can receive a permit for exportation.

In particular, Baldassari et al. (2007) reports that dipping fruit of 'Pera-rio' sweet orange in an ethephon solution and incubating it under continuous light at 25ºC for 15 days can induce symptom expression in asymptomatic fruit. In addition, treatment of non-mature citrus fruit with an ethephon solution (750 ppm) followed by incubation at 25°C with constant light for 28 days is also described by Silva et al. (2016). Symptoms start to appear in positive citrus fruit two weeks after the treatment, but most samples will turn symptomatic after three weeks.

Symptom induction treatments are particularly suggested for a delimiting survey where the presence of the pest has already been confirmed. Figure 10 shows the CBS-like symptoms after treatment of asymptomatic infected fruit with ethephon.

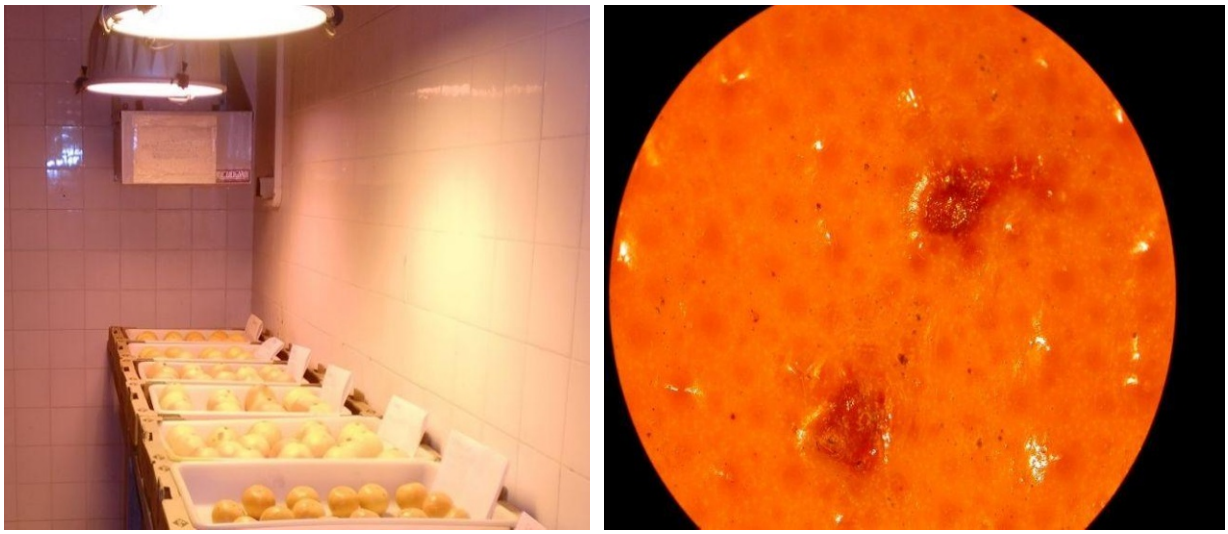

Figure 10: Asymptomatic citrus fruit treated with ethephon and incubated under continuous light (left). Lesions developed on previously asymptomatic citrus fruit, infected by *Phyllosticta citricarpa* after the ethephon treatment (right). Photos: A. Vicent

In this particular case, the sampling effectiveness of detecting CBS-like symptoms on an infected asymptomatic fruit after ethephon treatment is estimated by experts at 0.8 based on empirical observations. This value is lower than that for infected fruit with symptoms that have developed naturally, indicating that ethephon is estimated to reveal symptoms of asymptomatic infections in

[9] Instrução Normativa nº 3 de 08/01/2008 / MAPA - Ministério da Agricultura, Pecuária e Abastecimento (D.O.U. 09/01/2008). Available online: http://www.diariodasleis.com.br/busca/exibelink.php?numlink=1-77-23-2008-01-08-3 [Accessed: 21 April 2020].

80% of the treatments of infected asymptomatic fruit. However, further studies are required to confirm the effectiveness of ethephon in inducing CBS-like symptoms in mature citrus fruit and the time required after dipping for the visual examination. No studies were found on the effect of the ethephon dipping technique on different citrus species at different stages of maturity.

It is important to mention that the current emergency measures make testing for latent infections compulsory for South Africa and Uruguay, where the pest is known to occur. However, the logistical constraints linked to the application of such procedures for *P. citricarpa* also need to be assessed in the context of detection and delimiting surveys.

Table 4 summarises the estimated sampling effectiveness of the detection using visual examination of CBS-like symptoms on infected fruit.

Table 4: Sampling effectiveness for *Phyllosticta citricarpa* using visual examination on mature citrus fruit

Type of mature fruit	Sample selection	Sampling effectiveness
With CBS-like symptoms	Selection of fruit with CBS-like symptoms. Examination with a magnifying lens or a dissecting microscope (FAO, 2019)	0.9
Asymptomatic	Random selection	0
	Random selection and symptom induction. CBS-like symptoms are induced on fruit by ethephon treatment at 25°C under continuous light (Baldassari et al., 2007). Post-induction examination with a magnifying lens or a dissecting microscope (FAO, 2019)	0.8

2.1.3. Alternative detection methods

Given the asymptomatic period of *Phyllosticta citricarpa*, an alternative approach for early detection could be to capture the airborne or rain-splashed spores. These techniques are currently being investigated in Malta and Italy using, for example, a Hirst spore sampler as seen in Figure 11. However, results of this research are not yet available. New generation technology for airborne spore trapping, including molecular methods, could also be considered. A rain collector for splash-dispersed conidia is shown in Figure 11.

Figure 11: Hirst spore sampler for airborne spore trapping (left); Rain-splash collector (right) Photos: A. Vicent

Additionally, leaf litter can be used in the detection process since sporulation can be induced under laboratory conditions. Artificial wilting of asymptomatic green citrus leaves will facilitate the detection of *P. citricarpa* (Truter, 2010). For the leaf litter, alternate wetting and drying enhances the production of pycnidia and pseudothecia (McOnie, 1964; Meyer et al., 2012). However, regarding the sampling effectiveness of detection of CBS-like symptoms on leaves, leaf litter and twigs, the uncertainty is very high as very little information is available, and the samples for identification by laboratory testing will include asymptomatic plant parts with latent infections for which no official diagnostic protocols are currently available.

2.2. Laboratory testing and identification

For symptomatic fruit, three major approaches for identification of *Phyllosticta citricarpa* are described and recommended in FAO (2019) and EPPO (2009):

- Incubation of lesions followed by molecular methods
- Isolation and culturing followed by molecular methods
- Molecular methods applied directly on lesions.

Regarding asymptomatic fruit and plant parts, no official protocols are currently available, and in this case, sampling potentially infected tissue has a very low detection sensitivity when using the recommended laboratory tests and protocols from FAO (2019) and EPPO (2009). In this case the tissue for laboratory testing is randomly selected with a low probability of finding the pathogen.

If it is considered necessary to sample leaf litter, this should be ideally done during spring and summer (see Section 1.4) and relies on the laboratory testing of randomly collected samples.

2.2.1. Incubation, isolation and culturing

For incubation, fruit with CBS-like lesions or rind pieces carrying those lesions are surface disinfected with 70% ethanol. The disinfected fruit should be incubated under high relative humidity in constant light at 27ºC and periodically checked for the presence of newly formed pycnidia (Bonants et al., 2003). If CBS-like symptoms appear, molecular methods can be applied directly for an accurate identification of *Phyllosticta citricarpa* among other pycnidia-forming fungi.

For isolation, small pieces of lesions or pycnidia are selected from the fruit rind, they are surface disinfected and placed on agar media. *P. citricarpa* cultures growing in agar media are very similar to those of other *Phyllosticta* species described on citrus (FAO, 2019). Detailed information about the pest identification can be found in EPPO (2009), FAO (2019) and EFSA PLH Panel (2014a). Bonants et

al. (2003) indicated a sensitivity of 10% and 50% for the isolation and incubation methods, respectively, but even if it was higher, it would not be as sensitive as molecular testing. In addition to cultural and morphological characteristics (Figure 12), molecular methods are necessary for an accurate identification of *P. citricarpa* in cultures.

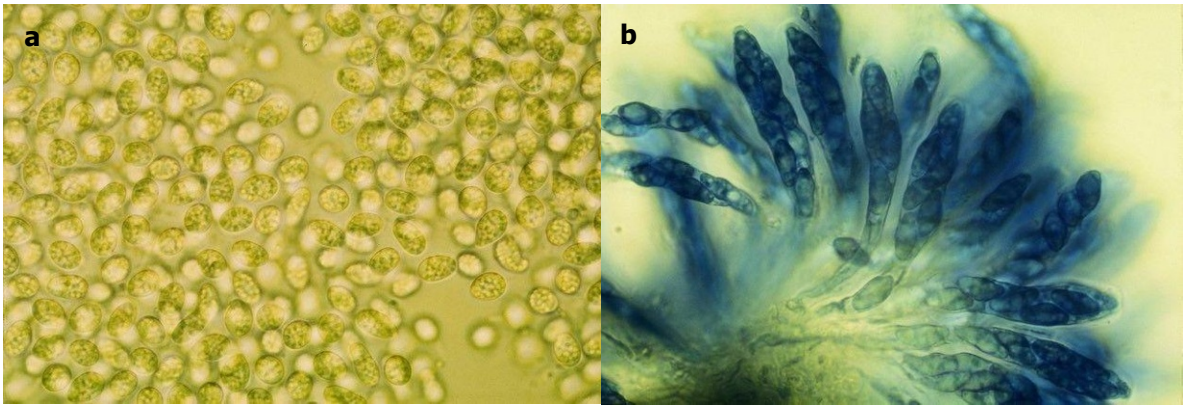

Figure 12: Spores of *Phyllosticta citricarpa*: (a) asexual (conidia), (b) sexual (pseudothecia with ascas) (Courtesy of E. Feichtenberger and H. Della Coletta-Filho)

2.2.2. Molecular methods for identification

For the identification of *Phyllosticta citricarpa* by molecular assay, two methods have been referred to by EPPO (2009) and FAO (2019): conventional PCR assay by Peres et al. (2007) with a detection limit of 1 pg DNA/µl, and real-time PCR assay by Gent-Pelzer et al. (2007) with a detection limit of 0.01 pg DNA per reaction. Real-time PCR will generate a positive signal from a single CBS lesion on a fruit, while conventional PCR may give inconclusive results in a few cases (FAO, 2019), in which case it is necessary to pursue the identification by real-time PCR or sequencing methods. However, Coletta-Filho et al. (2016) report that both sets of primers may also amplify non-pathogenic species such as *P. citribraziliensis* and *P. capitalensis*.

In addition to those molecular methods, Meyer et al. (2006) described another conventional PCR for the detection of *P. citricarpa*. Hu et al. (2014) developed a qPCR method that could quantify *P. citricarpa* in plant tissue. Additionally, Tomlinson et al. (2013) developed a loop-mediated isothermal amplification method which could be deployed in the field. Recently, Schirmacher et al. (2019) developed a new species-specific real-time PCR for diagnosis of *P. citricarpa* on citrus species.

However, none of the methods indicated above can differentiate between *P. citricarpa* and its sister species *P. paracitricarpa*, which is pathogenic to sweet orange (Guarnaccia et al., 2019). Sequencing is thus required for an accurate identification of *P. citricarpa*. According to EPPO (2009) and FAO (2019), Internal Transcribed Spacer (ITS) sequencing can be used to confirm a positive result by conventional PCR. However, Glienke et al. (2011) and Wang et al. (2012) indicated that other loci may be added for an accurate identification of *Phyllosticta* species. Several loci are also needed to differentiate between *P. citricarpa* and *P. paracitricarpa* (Guarnaccia et al., 2019). Multilocus sequencing can be performed using pure cultures, i.e. after isolation of the fungus. Research is being conducted to develop new PCR methods to differentiate between *P. citricarpa* and *P. paracitricarpa*. Until they are available, the methods currently indicated in the official protocols for *P. citricarpa* are considered here (EPPO, 2009; FAO, 2019).

On symptomatic citrus fruit, by their nature, the molecular methods have a very high sensitivity. The sensitivity of the real-time PCR method is given at 90% per lesion and the pathogen can be detected rapidly within 1 day. By independent testing of multiple lesions, sensitivity above 99% can be attained (EPPO, 2009).

The three types of identification method are shown in Table 5 along with indicative specificity and sensitivity values and information about the choice of available methods for performing the diagnostic test on the detected fruits with CBS-like symptoms.

Research is ongoing on the development of laboratory methods for the identification of *P. citricarpa* on citrus leaf litter and is not addressed in this survey card.

Amorim et al. (2017) developed a multiplex PCR protocol for the identification of the two *P. citricarpa* mating types that can be applied to determine the possibility of sexual reproduction for a delimiting survey.

Table 5: Methods of identification of *Phyllosticta citricarpa* on symptomatic mature citrus fruit (EPPO, 2009; FAO, 2019; Bonants et al., 2003)

Method	Reference	Sensitivity
Molecular methods	Conventional PCR combined with real-time PCR (EPPO, 2009; FAO, 2019)	Nearly 1.00
Incubation	Incubated in constant light for five days, and checked for pycnidia (Bonants et al., 2003), followed by molecular methods	0.50
Isolation and culturing	Fruit lesions are disinfected with ethanol, cut out from the peel and placed on agar media (Bonants et al., 2003); identification followed by molecular methods	0.10 to 0.6 depending on culturing conditions

Summary of the processes for detecting and identifying *Phyllosticta citricarpa* on fruit

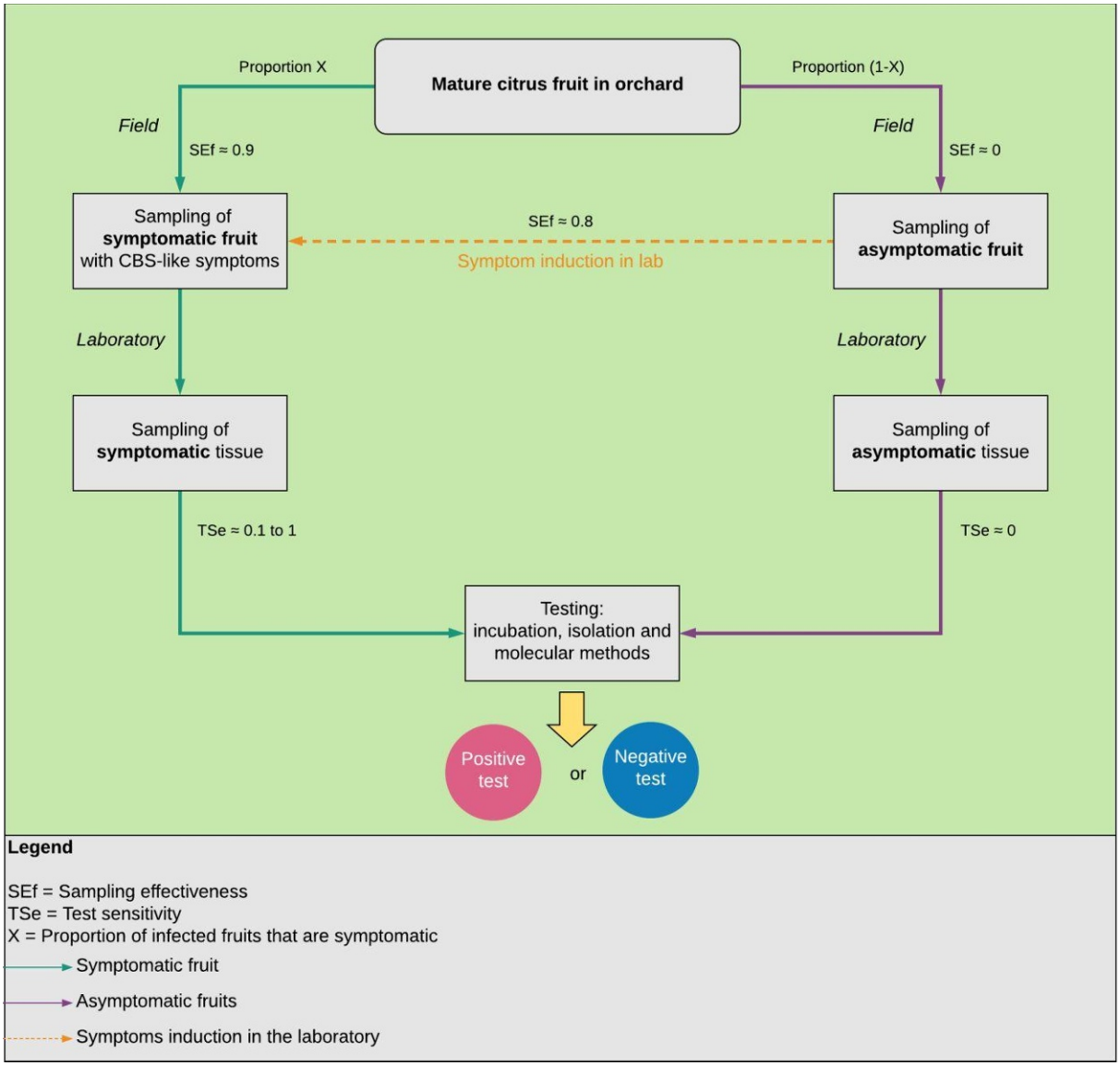

Figure 13: Flow chart for the processes to detect and identify *Phyllosticta citricarpa* on citrus fruit, indicating the respective sampling effectiveness, sensitivities and specificities

Figure 13 illustrates the different steps in the *P. citricarpa* detection and identification processes, indicating the respective sensitivities and specificities as detailed in the previous sections.

The initial assumption in the survey is that infected fruit could be asymptomatic at the time of the survey. The main steps that can be distinguished are the visual detection and sampling of fruit in the field and the sampling of tissue followed by the identification methods in the laboratory. Several important factors are shown in the flow chart:

i. The proportion of infected fruit that is expressing CBS-like symptoms (X) is unknown and it is not possible to estimate this value as the pest is not known to occur in the EU. When sampling only the symptomatic fruit in the field (Figure 13, green line), only an unknown proportion of the infested fruit is considered in the survey. Consequently, the overall method sensitivity of the detection process is also undetermined as it depends on X. However, at fruit maturity, symptoms of CBS are expected to be expressed on infected fruit, and consequently the amount of infected fruit escaping the detection process is low. Nevertheless, the detection process of *P. citricarpa* should not focus only on the symptomatic fruit as the sampling of asymptomatic fruit is also necessary.

ii. For the visual examination of asymptomatic infected fruit (Figure 13, purple line), the sampling effectiveness for visual detection is close to zero (SEf≈0). For the laboratory testing of the selected parts of asymptomatic tissue, the test sensitivity is also close to zero (TSe≈0). This indicates that it is not a reliable approach to detect CBS and identify an infection of *P. citricarpa* when only testing asymptomatic fruit.

iii. In order to detect *P. citricarpa* on asymptomatic fruit, it is necessary to use a method with a higher likelihood of detecting the infected fruit. A method that needs further exploration and confirmation is the symptom induction by the use of ethephon as discussed in the sections above. This method could induce symptoms on the infected asymptomatic fruit (Figure 13, orange line). In addition, the current diagnostic protocols only address the laboratory tests performed on symptomatic fruit or tissue. When inducing symptoms, these laboratory tests can also be performed on sampled tissue material with CBS-like symptoms. A robust method could allow for better use of resources in terms of the amount of fruit inspected.

3. Key elements for survey design

Based on the analyses of the information on the pest–host plant system, the different units that are needed to design the survey have to be defined and tailored to the situation in each Member State. The size of the defined target population and its structure in terms of number of epidemiological units need to be known.

When several pests have to be surveyed in the same crop, it is recommended that the same epidemiological and inspection units are used for each pest in order to optimise the survey programme as much as possible. This would optimise field inspections since they are organised per crop visit and not by pest. Table 6 shows an example of these definitions.

Table 6: Example of definitions of the target population, epidemiological unit and inspection unit

	Definition
Target population	Citrus plants growing in orchards, backyards and gardens in each Member State
Epidemiological unit	A single homogeneous area that contains at least one individual host plant (e.g. citrus orchard, backyard or garden)
Inspection unit	A host plant with mature fruits

The general guidelines (EFSA, in preparation-a) for the risk-based statistically sound surveillance are presented in a separate document and describe the use of the toolkit for survey design, including the reasoning for choosing the type of survey to design depending on its objectives, the manual for guiding the user through the statistical tools for sample size calculations, essential considerations when choosing the sampling sites and taking the samples, collecting the data and reporting the data and the survey results.

The specific guidelines for the survey of *Phyllosticta citricarpa* are also presented in a separate document (EFSA, in preparation-b) and describe step-by-step the process of the sample size calculation for risk-based surveillance. Two cases are addressed, a detection survey for pest-freedom demonstration and a delimiting survey in the event of a first positive detection. For both cases, the description of the different surveillance components required to determine statistically sound sample sizes is provided.

The steps that will generally be necessary are the following:

1/ Determine the type of survey based on its objectives. For *P. citricarpa*, the type of survey will depend on the pest status (according to International Standards for Phytosanitary Measures (ISPM)

No. 8) in the area of interest. The objective could be to substantiate pest freedom, to delimit an outbreak area following an infection or to determine the pest prevalence. The next steps deal with the example of substantiating pest freedom.

The overall confidence level and design prevalence of the survey have to be decided by the risk managers before designing the surveys as they reflect the acceptable level of the risk of infestation of the host plants by *P. citricarpa*. The general guidelines for pest surveillance provide further details on the choice of these values and the related consequences in terms of pest surveys.

2/ Define the target population and its size. When determining the target population for surveillance of *P. citricarpa*, the host plants that are relevant for the survey area have to be selected. The size of the target population should be determined. For example, the target population could be all host trees in a Member State.

3/ Define the epidemiological units. The epidemiological units should be single homogeneous areas that each contains at least one individual host plant.

4/ Determine the inspection unit. For an orange orchard, for example, the inspection unit is a single orange tree.

5/ Determine the number of inspection units per epidemiological unit. For an orange orchard, this is the average number of orange trees per epidemiological unit.

6/ Implement the inspections and, if appropriate, the sampling, following the procedures suggested by the competent authorities, within the epidemiological units and estimate the method's effectiveness in order to determine the overall method sensitivity (sampling effectiveness × diagnostic sensitivity). A representative number of plants should be examined and if there are suspicious symptoms they should be sampled. RiBESS+ can be used to calculate how many inspection units need to be examined or sampled when using a predefined prevalence level (e.g. 1%) to obtain a particular confidence level. This confidence level is in turn needed to calculate the number of sites to be inspected (Step 8). Note that the more units that are inspected, the higher the confidence will be. The competent authorities need to align the survey efforts with the resources available.

7/ Define the risk factors. A risk factor affects the probability that a pest will be present or detected in a specific portion of the target population. It may not always be possible to identify or include a risk factor in the survey design. Risk factors can only be included when both the relative risk and the proportion of the overall plant population to which they apply are known or can be reliably estimated.

8/ Determine the number of epidemiological units to survey. RiBESS+ can be used to determine the number of epidemiological units to survey in order to achieve the objectives of the survey set at Step 1 in terms of confidence level (e.g. 95%) and design prevalence (e.g. 1%), while also including the method sensitivity from Step 6 and the risk factors identified in Step 7. As a result, considering, for example, fields where host plants are present, the number of fields that need to be surveyed is estimated for a Member State in order to state with 95% confidence that the prevalence of *P. citricarpa* will be at 1% or below.

9/ Summarise and evaluate the survey design. At this stage, it is necessary to evaluate whether the above steps have resulted in a survey design that matches the available resources, meaning that a feasible number of inspections can be performed within an acceptable time frame per inspection, and resulting in a feasible number of samples. If not, available resources should be adjusted. This adjustment would result in a modified survey design using different input parameters of the statistical tool RiBESS+ (e.g. varying the number of components, method sensitivity, etc.).

10/ Integrate the pest-based survey into a crop-based survey (optional).

11/ Allocate the calculated survey effort. In the survey area, the output of RiBESS+ should be allocated proportionally to the host plant population or to the number of epidemiological units. In addition, the survey size should be selected from the list of available locations.

12/ Data collection and survey reporting. Consider which data are needed and how these data will be reported together with the related assumptions.

13/ Plan, develop or update the specific instructions for the inspectors. These activities are not addressed by EFSA and fall within the remit of the competent national authorities.

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General glossary for pest survey

Term	Definition*
Buffer zone	An area surrounding or adjacent to an area officially delimited for phytosanitary purposes in order to minimise the probability of spread of the target pest into or out of the delimited area, and subject to phytosanitary or other control measures, if appropriate (ISPM 5: FAO, 2020).
Component (of a survey)	A component is a survey entity which can be distinguished based on its target population, the detection method (e.g. visual examination, laboratory testing, trapping) and the inspection unit (e.g. vectors, branches, twigs, leaves, fruits). A pest survey comprises various components. The overall confidence of the survey will result from the combination of the different components.
Confidence	The sensitivity of the survey is a measure of reliability of the survey procedure (Montgomery and Runger, 2010). The term **confidence level** is used in 'Methodologies for sampling of consignments' (ISPM 31: FAO, 2016b).
Delimiting survey	Survey conducted to establish the boundaries of an area considered to be infested by, or free from, a pest (ISPM 5: FAO, 2020).
Design prevalence *analogous to the term **level of detection** used in 'Methodologies for sampling of consignments' (ISPM 31: FAO 2016b)*	It is based on a pre-survey estimate of the likely actual prevalence of the pest in the field (McMaugh, 2005). The survey will be designed in order to obtain at least a positive test result when the prevalence of the disease will be above the defined value of the design prevalence. In 'freedom from pest' approaches, it is not statistically possible to say that a pest is truly absent from a population (except in the rare case that a census of a population can be completed with 100% detection efficiency). Instead, the maximum prevalence that a pest could have reached can be estimated, this is called the 'design prevalence'. That is, if no pest is found in a survey, the true prevalence is estimated to be somewhere between zero and the design prevalence (EFSA, 2018).
Detection survey	Survey conducted in an area to determine whether pests are present (ISPM 5: FAO, 2020).
Diagnostic protocols	Procedures and methods for the detection and identification of regulated pests that are relevant to international trade (ISPM 27: FAO, 2016a).
Epidemiological unit *analogous to the term **lot** used in 'Methodologies for sampling of consignments'*	A homogeneous area where the interactions between the pest, the host plants and the abiotic and biotic factors and conditions would result in the same epidemiology should the pest be present. The epidemiological units are subdivisions of the target population and reflect the structure of the target population in a geographical area. They are the units of interest to which statistics are applied (e.g. a

(ISPM 31: FAO 2016b)	tree, orchard, field, glasshouse, or nursery) (EFSA, 2018).
Expected prevalence	In prevalence estimation approaches, it is the proportion of epidemiological units expected to be infested or infested.
Expert knowledge elicitation	A systematic, documented and reviewable process to retrieve expert judgements from a group of experts in the form of a probability distribution (EFSA, 2014).
Host plant	A host plant is a plant species belonging to the host range on which the pest could find shelter, feed or subsist at least for a period of time.
Host range	Species capable, under natural conditions, of sustaining a specific pest or other organism (ISPM 5: FAO, 2020). This definition is limited to array of host plants species and does not include the commodities other than plants or plant parts.
Identification	Information and guidance on methods that either used alone or in combination lead to the identification of the pest (ISPM 27: FAO, 2016a).
Infected versus infested	Infected is used when a pathogen is referred to in relation to its hosts (e.g. the trees are infected by the bacterium). Infested is used when an insect is referred to in relation to its hosts (e.g. the trees are infested by beetles). Infested is used when the pest is mentioned in relation to an area (e.g. an infested zone).
Inspection	Official visual examination of plants, plant products or other regulated articles to determine whether pests are present or to determine compliance with phytosanitary regulations (ISPM 5: FAO, 2020).
Inspection unit *analogous to* ***sample unit*** *used in 'Methodologies for sampling of consignments' (ISPM 31: FAO 2016b)*	The inspection units are the plants, plant parts, commodities or pest vectors that will be scrutinised to identify and detect the pests. They are the units within the epidemiological units that could potentially host the pests and on which the pest diagnosis takes place (EFSA, 2018).
Inspector	Person authorised by a national plant protection organisation to discharge its functions (ISPM 5: FAO, 2020).
Method sensitivity *analogous to the term* ***efficacy of detection*** *used in 'Methodologies for sampling of consignments'*	The conditional probability of testing positive given that the individual is diseased (Dohoo et al., 2010). The method sensitivity (MeSe) is defined as the probability that a truly positive host tests positive. It has two components: the sampling effectiveness (i.e. probability of selecting infested plant parts from an infested plant) and the diagnostic sensitivity (characterised by the visual inspection

(ISPM 31: FAO 2016b)	and/or laboratory test used in the identification process). The diagnostic sensitivity is the probability that a truly positive epidemiological unit will result positive and is related to the analytical sensitivity. It corresponds to the probability that a truly positive inspection unit or sample will be detected and confirmed as positive. The sampling effectiveness depends on the ability of the inspector to successfully choose the infested plant parts in a host plant. It is directly linked to the sampling procedure itself and on the training of the inspectors to recognise the symptomatology of the pest. Furthermore, symptom expressions are dependent, among other factors, on the weather conditions as well as on the physiological stage of the host plant when the sample is taken.
Pest diagnosis	The process of detection and identification of a pest (ISPM 5: FAO, 2020).
Pest freedom	Pest freedom can be defined, for a given target population, in a statistical framework, as the confidence of freedom from a certain pest against a pre-set design prevalence (threshold of concern).
Population size	The estimation of the number of the plants in the region to be surveyed (EFSA, 2018).
Relative risk	The ratio of the risk of disease in the exposed group to the risk of disease in the non-exposed group (Dohoo et al., 2010).
Representative sample	A sample that describes very well the characteristics of the target population (FAO, 2014).
RiBESS+	Risk-based surveillance systems. This is an online application that implements statistical methods for estimating the sample size, global (and group) sensitivity and probability of freedom from disease. Free access to the software with prior user registration is available at https://shiny-efsa.openanalytics.eu/
Risk assessment	Evaluation of the probability of the introduction and spread of a pest and the magnitude of the associated potential economic consequences (ISPM 5: FAO, 2020).
Risk factor	A factor that may be involved in causing the disease (FAO, 2014). It is defined as a biotic or abiotic factor that increases the probability of infestation of the epidemiological unit by the pest. The risk factors relevant for the surveillance should have more than one level of risk for the target population. For each level, the relative risk needs to be estimated as the relative probability of infestation compared with a baseline with a level 1. Consideration of risk factors in the survey design allows the survey efforts to be enforced in those areas, where the highest probabilities

	exist to find the pest.
Risk-based survey	A survey design that considers the risk factors and enforces the survey efforts in the corresponding proportion of the target population.
SAMPELATOR	Sample size calculator. This is an online application that implements statistical methods to estimate the sample size for pest prevalence estimation surveys. Free access to the software with prior user registration is available at https://shiny-efsa.openanalytics.eu/
Sample size	The sample size refers to the output of the statistical tools for survey design (RiBESS+ and SAMPELATOR). 'A well-chosen sample will contain most of the information about a particular population parameter but the relation between the sample and the population must be such as to allow true inferences to be made about a population from that sample.' (BMJ, online). The survey sample consists of the required number of 'inspection units' or samples thereof to be examined and/or tested in the survey to retrieve sufficient information on the pest presence or prevalence in the total population. In the case of risk-based surveys, the sample size is calculated on the basis of statistical principles that integrate risk factors. If the examination for pest presence is performed by laboratory testing, at least one sample is taken from each inspection unit. These samples will undergo relevant laboratory testing.
Sampling effectiveness	For plants, it is the probability of selecting infested plant parts from an infested plant. For vectors, it is the effectiveness of the method to capture a positive vector when it is present in the survey area. For soil, it is the effectiveness of selecting a soil sample containing the pest when the pest is present in the survey area.
Specified plant	The plant species known to be susceptible to the pest. For example, for *Phyllosticta citricarpa*, the list of specified plants, which includes host plants and all plants for planting, other than seeds, belonging to the genera or species, can be found in Annex I of Decision (EU) 2015/789.
Survey	An official procedure conducted over a defined period of time to determine the characteristics of a pest population or to determine which species are present in an area (ISPM 5: FAO, 2020).
Target population *analogous to* ***consignment*** *used in 'Methodologies for*	The set of individual plants or commodities or vectors in which the pest under scrutiny can be detected directly (e.g. looking for the pest) or indirectly (e.g. looking for symptoms suggesting the presence of the pest) in a given habitat or area of interest. The different components pertaining to the target population that need to be specified are:

sampling of consignments' (ISPM 31: FAO 2016b)	• definition of the target population: the target population has to be clearly identified; • target population size and geographic boundary. (EFSA, 2018)
Test	Official examination of plants, plant products or other regulated articles, other than visual, to determine whether pests are present, identify pests or determine compliance with specific phytosanitary requirements (ISPM 5: FAO, 2020).
Test specificity	The conditional probability of testing negative given that the individual does not have the disease of interest (Dohoo et al., 2010). The test diagnostic specificity is the probability that a truly negative epidemiological unit will give a negative result and is related to the analytical specificity. In freedom from disease it is assumed to be 100%.
Visual examination	The physical examination of plants, plant products, or other regulated articles using the unaided eye, lens, stereoscope or microscope to detect pests or contaminants without testing or processing (ISPM 5: FAO, 2020).

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