Introduction

Electronic phytosanitary certificates are the electronic equivalents of the wording and data of phytosanitary certificates in paper form. Electronic certificates should be issued by the national plant protection organization (NPPO) of the exporting or re-exporting country and made directly available to the NPPO of the importing country without any intermediate step.

If using electronic phytosanitary certificates, NPPOs should develop and use systems that generate certificates using standardized language, message contents and exchange protocols. This appendix provides guidance on these elements and refers to an ePhyto homepage (http://ePhyto.ippc.int), which is located on the IPPC website. The ePhyto homepage provides further details and links to information indicated in this appendix by "Link 1" etc.

All the requirements and procedures laid down in this standard apply to electronic certification unless more specific provisions are provided in this appendix.

1. XML message and XML schema

NPPOs should use the World Wide Web Consortium (WC3) Extensible Markup Language (XML) (Link 1) as the standardized language for exchange of electronic certificate data between NPPOs; the data should be standardized in the format of the United Nations Centre for Trade Facilitation and Electronic Business (UN/CEFACT) Sanitary and Phytosanitary (SPS) XML schema (Link 2), as adjusted and further specified for phytosanitary electronic certification on the ePhyto homepage. This pertains to the IPPC ePhyto certificate for export (Link 3) and to the IPPC ePhyto certificate for re-export (Link 4).

2. Electronic contents of the XML schema: harmonization by coding

To standardize the XML schema and to allow for automatic electronic processing of data, elements of the phytosanitary certificates should be coded as follows.

2.1 Country names

The names of countries referred to in the certificate (i.e. the country of origin, of export, of re-export and of destination and transit) should be coded using the International Organization for Standardization (ISO) two-digit country codes (Link 5).

2.2 Scientific names

The names of the certified plants and plant products and the names of regulated pests should be coded using the European and Mediterranean Plant Protection Organization (EPPO) codes as available in EPPO Plant Protection Thesaurus (EPPT) (Link 6).

2.3 Description of consignment

Commodity classes and type of packaging are included in the description of the consignment. The commodity class is essential to further characterize plants and plant products beyond the level of the scientific name and should be coded using the IPPC commodity class codes (Link 7). The type of packaging should be coded using the United Nations Economic Commission for Europe (UNECE) recommendation 21 (which includes codes for packages and packaging materials) (Link 8). Other elements of the description of the consignment may include weight, volume and height, and should be coded using UNECE recommendation 20 (codes for units of measure used in international trade) (Link 9). There should also be the possibility to use free text for any description of the consignment. However, to facilitate electronic processing of data, the use of free text should be limited.

2.4 Treatments

Any treatment types that need to be specified should be coded using the IPPC treatment type codes (Link 10). Active ingredients should be coded using the coding system of Codex Alimentarius (Link 11). Other parameters (i.e. concentration, dosage, temperature and duration of exposure) should be coded using UNECE recommendation 20 (Link 12).

2.5 Additional declarations

For 2012 member consultation, please indicate any commodity class you consider missing from Link 7. For 2012 member consultation, please indicate any treatment types you consider missing from Link 10.
Standardized wording for additional declarations are recommended in Appendix 2 and these should be coded using IPPC additional declaration codes (Link 13). There should also be the possibility to insert free text, either to supplement the coded additional declaration as indicated on the ePhyto homepage, or to allow for additional declarations that are not yet coded or standardized.

2.6 Further elements

The date and time format should be coded following UNECE recommendation 7 (numerical representation of dates, time and periods of time) (Link 14).

Point of entry and point of destination should be coded using the United Nations Code for Trade and Transportation Locations (UN/LOCODE) code (Link 15).

2.7 Name of authorized officer

The name of the officer issuing the phytosanitary certificates should be automatically inserted on the certificate by the system.

3. Exchange mechanisms

To ensure that the electronic exchange of data is secure and authentic, the certificate data should be encrypted. Countries are encouraged to use the encryption system recommended by UN/CEFACT (Link 16), which is widely available and considered sufficiently secure.

The method of transmission over the Internet should be through secured login with user name and password from the NPPO of the exporting country to the NPPO of the importing country. The NPPO of the exporting country should inform the exporter of the actual certificate number covering the consignment concerned.

4. Re-export

The phytosanitary certificate for re-export should have the original phytosanitary certificate for export as an attachment. In the situation where paper and electronic certificates are both in use, provisions are needed to fulfill this requirement when a phytosanitary certificate for re-export is issued in the following situations.

4.1 Re-export in a full electronic system

In the situation where both the phytosanitary certificate for export and for re-export are electronic certificates, the electronic phytosanitary certificate for export can be attached to the electronic phytosanitary certificate for re-export.

4.2 Re-export with electronic certificate while export with paper certificate

In cases where the original phytosanitary certificate for export is a paper certificate and the phytosanitary certificate for re-export is an electronic certificate, a scan of the original phytosanitary certificate for export (preferably in PDF format) can be incorporated into the electronic phytosanitary certificate for re-export.

4.3 Re-export with paper certificate while export with electronic certificate

In cases where the original phytosanitary certificate for export is an electronic document and the phytosanitary certificate for re-export is a paper document, the phytosanitary certificate for export can be printed in a certificate format and validated by the NPPO of the country of re-export by stamping, dating and countersigning. The printed version of the electronic phytosanitary certificate of export becomes a certified copy and can then, in paper form, be attached to the paper phytosanitary certificate for re-export.

5. Replacement of phytosanitary certificates

In cases where phytosanitary certificates need to be replaced, the IPPC replacement of phytosanitary certificates code should be used (Link 17).

6. Details for filling out electronic certificates

Owing to the nature of electronic certificates and to the fact that electronic certificates are exchanged directly between NPPOs, some details for filling out electronic certificates differ from those for filling out paper certificates.

Declared name and address of consignee

In the case of paper certificates, the term “To order” may be used in particular instances where the consignee is not known and the NPPO of the importing country permits the use of this term (“Declared name and address of consignee” in section 5 of this standard under “Description of Consignment”). In the case of electronic certificates, instead of “To order”, the name and address of the contact person responsible for the consignment should be inserted in sufficient detail. This enables the NPPO of the importing country to contact the consignee (or its representative) if necessary.
**INTRODUCTION**

**Scope**

The standard provides guidelines for the determination of the host status of fruits and vegetables (hereafter referred to as fruit) to fruit fly infestation and describes three categories of host status for fruit flies.

**References**


ISPM 30. 2008. *Establishment of areas of low pest prevalence for fruit flies (Tephritidae)*. Rome, IPPC, FAO.

ISPM 35. 2012. *Systems approach for pest risk management of fruit flies (Tephritidae)*. Rome, IPPC, FAO.

Further references are listed in Appendix 1.

**Definitions**

Definitions of phytosanitary terms can be found in ISPM 5 (*Glossary of phytosanitary terms*). For purposes of member consultation the following term and definition are presented in this draft ISPM but will be moved to ISPM 5 after adoption:

<table>
<thead>
<tr>
<th>Host status</th>
<th>the condition of a plant as a host for a pest.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural host</td>
<td>a plant species or cultivar that has been found to be infested under natural field conditions by the target fruit fly species and to sustain the production of reproductive adults.</td>
</tr>
<tr>
<td>Non natural host</td>
<td>a plant species or cultivar that is not a natural host but has been scientifically demonstrated to be infested and to sustain the production of</td>
</tr>
</tbody>
</table>

**Notes**

Note that indented lists in this draft are numbered for purposes of MC and will become bulleted lists in the final draft. This status box is not an official part of the draft standard and will not be translated.

Historical evidence, pest interception records and scientific literature on host status may provide accurate information so that host status determination based on field evaluations is not required. However, historical records and literature may sometimes be unreliable, for example:

1. Fruit fly and plant species or cultivars may be incorrectly identified and reference specimens may be unavailable for verification.
2. Collection records may be incomplete, incorrect or of dubious value (e.g. host status based on the catch from a trap placed in a fruit plant or based on infested fallen or damaged fruit).
3. Important details may have been omitted, for example, cultivar and stage of maturity, physical condition of fruit at the time of collection or the orchard sanitary condition.

Outline of Requirements

This standard describes requirements for determining the host status of a fruit for a particular fruit fly (Diptera: Tephritidae) species and designates three categories of host status (natural host, non-natural host and non-host).

The host status category for hosts of fruit flies is fundamental for pest risk assessment and for determining pest risk management options. Hence, categories of and procedures for determining the host status should be harmonized and applied to fruit fly risk analysis.

Requirements include:

1. proper identification of the fruit fly species, test fruit (including cultivar and stage of maturity) and, for field trials, control fruit
2. the specification of parameters for adult and larval fruit fly surveillance and field trial design to determine host status and specify the defined condition(s) of the fruit to be evaluated as a host
3. biological stages of the fruit fly (larvae, pupae or adults) to be used as the basis for determination of host status
4. holding and handling of the fruit to rear fruit flies after exposure
5. evaluation of collected data and interpretation of results.

Further, this protocol recommends that laboratory trials should not be used as the basis for determination of host status of fruits to fruit fly infestation.

BACKGROUND

Fruit flies (Diptera: Tephritidae) are economically important pests that often require the application of phytosanitary measures for movement of host commodities in trade. The host status of commodities (fruit) produced from a particular plant species is an important element of pest risk analysis (PRA) for a particular fruit fly species for assessing the likelihood of pest introduction and spread as well as determining appropriate risk management options (ISPM 11:2004).

The host status of fruits for fruit flies is a fundamental concept for pest risk analysis and the subsequent decision to take measures against fruit flies. Hence, categories of and procedures for determining the host status should be harmonized (ISPM 28:2006, ISPM 30:2008 and ISPM 35:2012).

Some host records listed in the scientific literature are flawed with respect to host status. Such host records have, in some cases, resulted in the imposition of unnecessary or overly restrictive phytosanitary measures by national plant protection organizations (NPPOs) on some fruit commodities. Given this, there is a need for an international guidance that helps NPPOs to determine host status in order to avoid unnecessary trade restrictions.

30. Historical evidence, pest interception records and scientific literature on host status may provide accurate information so that host status determination based on field evaluations is not required. However, historical records and literature may sometimes be unreliable, for example:

1. Fruit fly and plant species or cultivars may be incorrectly identified and reference specimens may be unavailable for verification.
2. Collection records may be incomplete, incorrect or of dubious value (e.g. host status based on the catch from a trap placed in a fruit plant or based on infested fallen or damaged fruit).
3. Important details may have been omitted, for example, cultivar and stage of maturity, physical condition of fruit at the time of collection or the orchard sanitary condition.

32. More detailed information on one approach to reporting of host status determinations from natural infestations can be found in Aluja and Mangan (2008).

33. Protocols and comprehensive trials to determine fruit fly host status have been documented in the scientific literature. However, inconsistencies in terminology and methodology contribute to variations in interpretation of fruit fly risk and in application of inadequate phytosanitary measures. Harmonization of terminology, protocols and evaluation criteria for determination of fruit fly host status will promote consistency among countries and scientific communities. When clear evidence of host status is not available, then host status field trials, detailed experimental design, and the acceptable level of effectiveness and statistical confidence for trials may be required.

34. GENERAL REQUIREMENTS

Three categories of host status (natural host, non-natural host and non-host) can be determined using the following steps, as also outlined in the flow chart (Figure 1).

36. A. In cases where, from existing biological or historical information, the evidence is very clear that the fruit do not allow infestation leading to the production of reproductive adults, no further surveys or field trials may be required and the fruit should be categorized as a non-host.
37. B. In cases where, from existing biological and historical information, the evidence is very clear that the fruit allows infestation leading to the production of reproductive adults, no further surveys or field trials may be required and the fruit should be categorized as a natural host.
38. C. In cases where additional information is required, extensive larval (fruit sampling) and adult field surveillance (trapping) or field trials are necessary to determine fruit infestation or non-infestation.
39. C1. In cases where no infestation is found after conducting extensive larval and adult field surveillance, the fruit may be categorized as non-host.
40. C2. In cases where infestation is found by surveillance, the fruit should be categorized as a natural host.
41. C3. In cases where no infestation is found, additional field trials may be needed under semi-natural conditions to assess whether the target fruit fly can successfully produce reproductive adults on the particular fruit species or cultivar.
42. C3a. If the target fruit fly species cannot produce reproductive adults, the fruit should be categorized as a non-host.
43. C3b. If the target species can produce reproductive adults, the fruit should be categorized as a non-natural host.
**SPECIFIC REQUIREMENTS**

Host status can be determined through historical production or trade data revealing natural infestations, through surveillance by extensive fruit sampling to gather evidence of natural infestations, or through trials under semi-natural field conditions. Where historical data do not provide clear evidence, surveillance through fruit sampling may be sufficient to determine host status. Field trials under semi-natural conditions may be conducted in cases where host status has not been scientifically determined by surveillance, or when, based on PRA, there is a need to determine if a particular fruit is a non-natural host.

Because extreme artificial conditions are inherent in laboratory tests under which flies are presented with harvested fruit that undergoes rapid physiological changes with respect to resistance to infestation, the results obtained may be meaningless from a biological and regulatory perspective for determination of host status. As has been widely documented, under artificial conditions, females of polyphagous species will lay eggs in almost any substrate presented to them and, in most cases, larvae develop into viable adults. As a result, highly biased results may be obtained and, therefore, laboratory tests are not recommended. Therefore, host status determinations should be based on fruit sampling and, where needed, on additional trials under semi-natural field conditions (i.e. field cages, fruit-bearing bagged branches or greenhouses).

If field trials are required, they should focus on the specific physiological condition of the fruit and target fruit fly incidence over the entire growing area, relevant harvest and export periods. Any field trials should be replicated, statistically analysed, and the levels of confidence reported based on sample size so that data are verifiable and replicable.

**Figure 1.** Flow chart indicating proposed steps for fruit fly host status determination.

1. Natural Host Status Determination by Surveillance Using Fruit Sampling

Host status can be determined and designated based on confirmation of natural infestation during the harvest period (fruit sampling) without any field trials. Natural infestation samples should be representative of the range of production areas and environmental conditions, maturity stages and natural damage levels. Surveillance by fruit sampling is the most reliable method to determine natural host status because it

1. does not interfere with the natural behaviour of fruit flies
2. accounts for high levels of variability in the fruit, fruit fly behaviour and periods of activity.

However, disadvantages of the surveillance of natural infestation by fruit sampling include the facts that

1. variability in fruit fly behaviour is not completely known or controlled
2. variability in the fruit is not completely known or controlled.

2. Host Status Determination with Field Trials under Semi-natural Conditions

The objective of host status field trials is to demonstrate host status of a specified fruit under specific defined conditions based on statistically valid data. Trials may include field cage, fruit-bearing bagged branches and greenhouse (including glass, plastic and screen houses).

Advantages of semi-natural field trials include:

1. Fruit flies are allowed to exhibit natural oviposition behaviour.
2. The fruit remains attached to the plant and does not degrade during the trials.

Disadvantages of semi-natural field trials include:

1. Field trials can be resource intensive.
2. Environmental variables may compromise the trials.

The following subsections outline elements to take into account when designing field trials.

2.1 Fruit samples

The following requirements apply to fruit sampling in the trials:
1. Sampling protocols should be based on principles of independence and randomness and be appropriate for the statistical analysis to be performed.
2. Period of time, the number of repetitions per growing season and the number of replicates should account for the variability of target fruit flies and fruit over time and over the production area. This should account for early and late harvest conditions and be representative of the range of actual production and growing conditions. The weight and number of fruit required and replicates per trial to determine effectiveness and confidence level should be specified. Sample size should provide a confidence level of at least 95%.
3. To determine host status and confidence level, the number of eggs oviposited and resulting immatures or adults should be determined from controls. Infestation level should be measured by determining the proportion of infested fruit and the number of larvae, pupae or adults yielded per individual fruit and per kilogram of fruit.

**2.2 Fruit flies**

The following operational procedures are recommended:

1. Basic information on target fruit fly species and their known hosts from the determined production area should be compiled.
2. The use of wild populations for the trials is desirable. If wild flies cannot be obtained in sufficient numbers, the colony should be no older than three generations at the initiation of the trial, when possible, and maintained on natural hosts to ensure normal oviposition behaviour.
3. When possible, the fruit fly colony should originate from the same area as the target fruit.
4. Prior to the field trials, the pre-oviposition, oviposition and mating periods should be determined so that sexually mature, mated females are exposed to the fruit at the peak of their reproductive potential.
5. The age of the adult females and males used in the trials should be recorded at the mating date and at the beginning of the trials.
6. The number of gravid females required per trial should be determined according to fruit size and trial conditions. The number of fruit flies per replicate should be determined according to fruit fly biology, amount of fruit to be exposed, and other trial conditions.
7. The exposure time of the fruit to the target fruit fly species should be between 24 and 72 hours.
8. The number of dead adults occurring during the field trials should be recorded and dead fruit flies should be replaced with live adults of similar physiological conditions. High adult mortality may indicate that unfavourable conditions (e.g. excessive temperature) or contamination of trial fruit (e.g. residual insecticides) has occurred. In such a case, the trials should be repeated. It should be noted if an individual female is used in more than one trial.

**2.3 Fruits**

The fruit used in the host status field trials should be:

1. the same cultivar and from the same production area as that to be exported
2. free from contaminants, pesticides, dirt, fruit flies and other pests
3. fruit of defined physiological condition
4. commercial grade of a defined colour, size and physiological condition from which the host suitability should be evaluated
5. at an appropriate defined stage of maturity measured by dry matter or sugar content.

**2.4 Controls**

As controls, known natural hosts are required for all cage or glasshouse trials. Fruit should be free of prior infestation (e.g. by bagging, from a pest free area). Fruit flies used in control and trial replicates should all come from the same cohort.

**2.5 Field trials**

For this standard, field trials include field cage, fruit-bearing bagged branch and greenhouse trials. Trials may be conducted in sequence. However, it may be more practical to conduct trials simultaneously while the fruit is available. Trials should be appropriate for the evaluation of the physiological condition(s) of the fruit as a potential host for fruit fly infestation.

Mesh field cages may enclose whole fruit-bearing plants (large field cages) or parts of plants including the plants for the trials. It is important to note that, because the fruit fly females are artificially confined with the specific fruit under observation, they may be forced to lay eggs in a non-natural host.

**2.6 Field cage trials**

Mesh field cages may enclose whole fruit-bearing plants (large field cages) or parts of plants including the plants for the trials. It is important to note that, because the fruit fly females are artificially confined with the specific fruit under observation, they may be forced to lay eggs in a non-natural host.

**2.7 Fruit Handling for Insect Emergence**

As controls, known natural hosts are required for all cage or glasshouse trials. Fruit should be free of prior infestation (e.g. by bagging, from a pest free area). Fruit flies used in control and trial replicates should all come from the same cohort.

**3. Field trials**

For this standard, field trials include field cage, fruit-bearing bagged branch and greenhouse trials. Trials may be conducted in sequence. However, it may be more practical to conduct trials simultaneously while the fruit is available. Trials should be appropriate for the evaluation of the physiological condition(s) of the fruit as a potential host for fruit fly infestation.

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**3.1 Fruit Handling for Insect Emergence**

As controls, known natural hosts are required for all cage or glasshouse trials. Fruit should be free of prior infestation (e.g. by bagging, from a pest free area). Fruit flies used in control and trial replicates should all come from the same cohort.
Fruit collected from natural and semi-natural conditions, as well as control fruit, must be held until larval development is complete. Fruit holding conditions should maximize fruit fly survival and be specified in the experimental design.

Fruit must be held in an insect-proof facility or container under conditions that ensure pupal survival. Holding conditions that should be considered include, but are not limited to:

1. temperature
2. relative humidity
3. availability and suitability of pupation medium
4. facilitation of accurate recording of the number of larvae, pupae and adults emerging from fruit sampled. (A subsample of fruit may be taken to calculate the percentage of fruit infestation.)

Data to be recorded include, but are not limited to:

1. physical conditions (e.g. temperature, relative humidity) in the fruit holding facility, daily during the period of fruit holding;
2. date and number of collected larvae and pupae from the test fruit and the controls:
   - The medium may be sieved at intervals before all larvae have left the fruit and at the end of the holding period (which varies with temperature and host status).
   - The normal period of development for target fruit fly species should be determined from the controls and colony. At the end of the holding period, the fruit should be dissected before being discarded, to determine the presence of live and dead larvae or pupae remaining inside if larvae have had enough time to emerge. If live larvae are present, the fruit should be held until all mature larvae have exited or been removed.
   - All or a subsample of pupae should be weighed and abnormalities recorded.
3. number and emergence dates of adults by sex:
   - All emerging adults should be identified to species and sex, and counted. Abnormalities should be recorded.
4. ability to reproduce and produce reproductively viable progeny.

Data from samples obtained by fruit sampling should be analysed individually to determine the significance of experimental variables.

The following procedures apply to data collection and analysis:

1. The percentage of emergence should be determined.
2. Calculate levels of infestation and levels of confidence that will support host status determination.
3. The sample size used to ascertain the confidence level should be determined by scientific reference.
4. Parameters such as the level of infestation, time of development of larvae and pupae, and number of viable adults should be considered to define the level of host infestation.

Emergence of a reproductively viable adult from field trials under semi-natural conditions in any one replicate indicates that it is a non-natural host.

6. Record-Keeping

The NPPO should keep appropriate records of host status field trials for a period of at least five years. Information kept should be appropriate for the intended purpose of determination of host status. Information in the records should include, but is not limited to:

1. scientific name of the target fruit fly
2. scientific name, cultivar, and origin of the fruit
3. location of identified reference specimens (which should be kept in an official collection)
4. records on the origin and rearing of the fruit fly colony
5. physiological condition of the fruit for fruit fly infestation
6. trials conducted, experimental design, dates, locations, raw data, statistical calculations and results
7. key scientific references used
8. additional information, including photographs, that may be specific to the fruit fly, the fruit or host status.

Records should be kept and made available upon request of the NPPO of the importing country.

APPENDIX 1: Additional references


Follett, P.A. & Hennessey, M.K. 2007. Confidence limits and sample size for determining nonhost status of
fruits and vegetables to tephritid fruit flies as a quarantine measure. *J. Econ. Entomol.*, 100: 251–257.


NAPPO RSPM No. 30. 2008. Guidelines for the determination and designation of host status of a fruit or vegetable for fruit flies (Diptera: Tephritidae). Ottawa, NAPPO.


ANNEX Z: Establishment of fruit fly quarantine areas within a pest free area in the event of an outbreak (Year)

Background

A fruit fly outbreak detected in a fruit fly-pest free area (FF-PFA) may pose a risk for those importing countries where the fruit fly species is considered a regulated pest. Phytosanitary actions for quarantine purposes are needed because movements of regulated articles from and through quarantine areas pose a risk of spreading the target fruit fly species.

1. Establishment of a quarantine area

When a fruit fly outbreak is detected within an FF-PFA, a quarantine area should be established. The NPPO of the exporting country should determine the criteria for the declaration of an outbreak in accordance with this standard and other relevant ISPMs.

The quarantine area should cover at least the actual infested area as determined by delimiting surveys plus the dispersal capability of the fruit fly species.

A circle delimiting the minimum size of the quarantine area should be drawn, centred on the actual pest finding and with the radius determined by the NPPO of the exporting country and based upon all relevant biological factors of the pest, ensuring that this delimiting circle includes the fruit fly’s dispersal capability. In the case of several pest findings, several (possibly overlapping) circles should be drawn accordingly, as in Figure 1.

If necessary for the practical implementation of the quarantine area, the NPPO of the exporting country may decide to expand the area to follow administrative or topographical boundaries.

A global positioning system (GPS) device or map with geographical coordinates may be used for delimiting and recognizing the quarantine area. Signposts may be placed along boundaries and on roads to alert the public and notices published to facilitate public awareness.

Notes

2. Phytosanitary actions

Each stage of the production chain (e.g. growing, packing, transporting, shipping) may lead to fruit fly spread from the quarantine area into the FF-PFA. Appropriate phytosanitary measures should be applied to mitigate this risk to the surrounding FF-PFA and to the importing country.

Any movement of regulated articles from these areas should follow the phytosanitary regulations established by the NPPO of the exporting country.

Phytosanitary measures already accepted in endemic fruit fly infested areas may also be implemented in the quarantine area.

2.1 Production

There may be non-infested hosts or production sites within the quarantine area because of host status or physiological stage of host development. In these cases, preventive measures such as fruit bagging, fruit stripping, protein bait sprays and netting may be applied to avoid infestation, if approved by the NPPO of the exporting country and agreed to by the NPPO of the importing country.

2.2 Movement of regulated articles

Movement of regulated articles into, from, through or within the quarantine areas should follow phytosanitary regulations to maintain phytosanitary security.

Regulated articles should be moved in safeguarded conditions and with the necessary documentation to certify the origin and destination. Verification of secure transport arrangements may be done using various methods including NPPO audits.

2.3 Packing

Packing facilities may be either within or outside the quarantine area. Facilities should be registered with the NPPO of the exporting country. The facility may pack regulated articles grown in or outside the quarantine area. Safeguarding measures should be taken into account in each case. The NPPO of the exporting country should approve the methods of disposal of rejected produce from the quarantine area to reduce the risk of spread of the pest.

2.3.1 Packing facilities located within the quarantine area

For facilities packing produce grown exclusively within the quarantine area, safeguarding measures are required at the facility to prevent the spread of the target fruit fly species.

For facilities packing plant products grown outside the quarantine area, it is essential to establish physical isolation by using insect-proofed packaging and separate storage for different consignments or lots within the facility. This is required to protect the plant products from infestation or cross-contamination.

Flow charts and signage should clearly indicate the movement of plant products through the secure areas of reception, packing and dispatch. They should be maintained by the facility and available for audit.

2.3.2 Packing facilities located outside the quarantine area and packing plant products from the quarantine area

In the case of packing facilities located within the FF-PFA but outside the quarantine area, the main objective is to protect the FF-PFA from the target fruit fly species. It is essential to ensure physical isolation through insect-proofed packaging and separate storage of different consignments or lots within the facility (ISPM 34:2010).

Separate areas for reception, processing and dispatch are required to maintain segregation of the produce.

Monitoring for the target fruit fly species should be conducted at the facility and adjacent area to ensure FF-PFA status while the quarantine area is in force (Appendix 1 (2011) of ISPM 26:2006).

2.4 Storage

Cold storage or other storage facilities may be located within or outside the quarantine area. Facilities normally provide adequate safeguarding. However, some additional considerations should be taken into account.

These may include registration with the NPPO of the exporting country, and management of rejected plant products if inspection or quality control activities occur.

Plant products sourced from the quarantine area should be stored separately.

2.5 Processing

If the processing facility is located within the quarantine area, host produce destined for processing (such as juicing, canning, pureeing) does not pose a fruit fly risk and does not require phytosanitary security.

If the facility is located outside the quarantine area, it will require physical isolation through an insect-proofed reception area and storage prior to processing (ISPM 34:2010).

Approved disposal of rejected plant products or waste is essential to reduce the risk of spread of the pest in either situation.
2.6 Phytosanitary treatments

Treatment facilities should be registered by the NPPO of the exporting country. Regulated articles, moving into a FF-PFA or for export to countries where the target species of fruit fly is regulated, may require a post-harvest phytosanitary treatment (cold treatment, heat treatment, fumigation, irradiation etc.). Safeguarding measures may be required for treatment facilities located within the FF-PFA if treating host produce from quarantine areas. Approved pre-harvest treatments, including bait sprays and fruit bagging, may be used in lieu of post-harvest treatments if agreed to by the importing country. The NPPO of the exporting country should approve the method of disposal of rejected produce from the quarantine area to reduce the risk of spread of the pest.

2.7 Shipping for export

Inspection for phytosanitary certification of regulated articles originating in the quarantine area may occur at airports or seaports. It should be conducted in a facility approved by the NPPO of the exporting country to ensure the phytosanitary integrity of the consignment and to prevent the spread of the pest. Plant products should be packed with safeguarded material.

2.8 Retail and commerce

Regulated articles being sold within the quarantine area are at risk of infestation if exposed prior to being sold (i.e. on display in an open air market). Such regulated articles should be safeguarded while on display and being stored.

3. Documentation and record-keeping

The phytosanitary measures used for the quarantine area should be adequately documented. They should be reviewed and updated regularly, including corrective actions, if required (see also ISPM 4.1995).

4. Removal of the quarantine status within the FF-PFA

Eradication of the target fruit fly species from the quarantine area should meet the criteria for reinstatement of FF-PFA after an outbreak, according to ISPM 26:2006. Removal of the quarantine status and reinstatement of FF-PFA status should coincide with a declaration of eradication. This declaration of eradication is based on no further detections of the target fruit fly species for a period determined by the biology of the species and prevailing environmental conditions as confirmed by surveillance (ISPM 26:2006). Phytosanitary measures for FF-PFAs should be reinstated.

5. References and additional resources


ISPM 4. 1995. Requirements for the establishment of pest free areas. Rome, IPPC, FAO.


This diagnostic protocol was adopted by the Xth Session of the Commission on Phytosanitary Measures in 20--.

1. Pest Information

Guignardia citricarpa Kiely, the causal agent of “citrus black spot” disease, is a leaf-spotting and fruit-blemishing pest affecting Citrus, Poncirus, Fortunella and their hybrids. Except for Citrus aurantium L. and its hybrids and C. jambhiri Tan., all commercially grown Citrus species are susceptible (Kotzé, 2000; Aguilar-Vidoso et al., 2002). Citrus limon L. is particularly susceptible and, thus, in an unaffected area, the disease usually appears first on C. limon (Kotzé, 2000).

Citrus black spot was first recorded in Australia in 1895 on C. sinensis (Linnaeus) Osbeck (Benson, 1895). It is now present in some citrus-producing areas in Asia, Africa, Australia and South America (CABI, 2011). In March 2010, G. citricarpa was detected for the first time in a few citrus groves in Florida (USA) (NAPPO, 2010). Surveys on the distribution of the organism in the area are ongoing (USDA-APHIS, 2010). The organism has not been reported from Europe, Central America or the Caribbean region (EPPO/CABI, 1997; CABI/EPPO, 1998; CABI, 2011; NAPPO, 2010).

G. citricarpa has significant economic impact mainly because of the external blemishes it causes, which make citrus fruit unsuitable for the fresh market. Severe infections may cause premature fruit drop (Kotzé, 2000). Some losses due to fruit drop occur in years favourable for pest development and when fruit is held on the trees past peak maturity (CABI, 2011). In addition, latently infected (asymptomatic) fruit at harvest may still develop symptoms during transport or storage (Kotzé, 1996).

The epidemiology of citrus black spot is influenced by the availability of inoculum, the occurrence of environmental conditions favourable for infection (warm, wet and humid conditions), the growth cycle of the citrus tree and the age of the fruit in relation to its susceptibility to infection (Kotzé, 1981, 2000). In areas where rain is confined to a single season, pseudothecia with ascospores, produced exclusively on leaf litter, are the main source of inoculum. Where rain is not confined to a single season, where out-of-season fruit with lesions remain on the trees after flowering and fruit set, or where successive and irregular flowering occurs in the cultivated citrus species and varieties, pyecnidia with conidia of the anamorph Phylosticta citricarpa (McAlpine) Aa are also important as inoculum sources (Kotzé, 1981; Spósito et al., 2008).

Pseudothecia develop 40–180 days after leaf drop, depending on the frequency of wetting and drying as well as on the prevailing temperatures (Kotzé, 1981). Citrus leaves drop all year round in some countries and seasonally in others, and this affects the availability of inoculum. The optimum temperature for pseudothecial formation is 21–28 °C and no pseudothecia are formed below 7 °C or above 35 °C (Lee and Huang, 1973). Ascospores release takes place during rainfall and occasionally during irrigation or heavy dew (Kiley, 1949a; Kotzé, 2000). Ascospores are forcibly released up to a height of 1.2 cm above pseudothecia and are carried by air currents throughout the canopy and over long distances (Kiley, 1949a). Ascoспорophore discharges are closely influenced by the rainfall pattern (Kotzé, 1981). The critical period for infection starts at fruit set and lasts 4–6 months, whereas the first symptoms on fruit do not appear until more than 6 months after fruit set (Baldassari et al., 2006). In Brazil, fruit of C. sinensis of “Valencia” and “Natal” varieties are susceptible until the end of the season: a macroconidial state in the genus Phylosticta and a microconidial in the genus Leptodothiorella (Kiely, 1949a). Pycnidia with conidia are produced on fruit, leaves, dead beigs, fruit pedicels and in abundance on leaf litter (Kotzé, 2000). They may be splash-dispersed onto the canopy or washed off from infected late-hanging fruit onto younger fruit and leaves that are still at the susceptible stage (Agostini et al., 2006; Spósito et al., 2008). The microconidial state, Leptodothiorella sp., is also referred to as “sporangial” state (Kiely, 1949a), usually appears on fallen leaves before pseudothecia develop. However, the role of...
microconidia in the biology of *G. citricarpa* is still unclear.

Symptom development on mature fruit is enhanced by rising temperatures, high light intensity, drought and poor tree vigour. Older trees usually have more citrus black spot than younger trees (Kotzé, 2000). Spread of *G. citricarpa* to new areas is assumed to have taken place through infected nursery stock or other planting material rather than through citrus fruit (Kotzé, 2000; Timmer, 2004).

It should be noted that in symptomless citrus fruit or fruit with minute spots (<2 mm in diameter) without pycnidia, the non-pathogenic endophyte *Guignardia mangiferae* A.J. Roy (anamorph *Phylosticta capitatus*) was recorded in many plant families, may be present. The cultural, morphological and molecular characters that differentiate *G. mangiferae* from *G. citricarpa*, the species pathogenic to citrus, have been described by Baayen et al. (2002). Furthermore, symptoms of *G. citricarpa* may be confused with those caused by *Phylllosticta citiansana* Wulandari, Cron & Gruyter, a newly described pathogen that has so far been found only on *Citrus maxima*. The pathogenicity of *P. citiansana* to other citrus species is unknown. The cultural, morphological and molecular characters that differentiate *P. citiansana* from *G. citricarpa*, the species pathogenic to the citrus, have been described by Wulandari et al. (2009).

2. Taxonomic Information

Name: *Guignardia citricarpa* Kieky (teleomorph)

Anamorph: *Phylllosticta citricarpa* (McAlpine) Aa (macroconidial state)

Synonyms: *Phylllostictina citricarpa* (McAlpine) Petr.

*Phoma citricarpa* var. *mikan Hara*

*Phoma citricarpa* McAlpine

Synanamorph: *Leptodothiorella* sp. (microconidial state)

Taxonomic position: Eukaryota, Fungi, Ascomycota, Pezizomycotina, Dothideomycetes

Common names:

- English: citrus black spot
- French: taches noires des agrumes; maladie des taches noires des agrumes
- Spanish: mancha negra de los Citrus; mancha negra de las frutas de citricos
- Portuguese: pinta preta dos citros

Reference: MycoBank 286752

3. Detection

Fruit, pedicels, leaves and twigs of *Citrus*, *Poncirus*, *Fortunella* and their hybrids may potentially harbour *G. citricarpa* (CAABI, 2011).

3.1 Symptoms on fruit

Several types of symptoms (e.g. hard spot, freckle spot, false melanose, virulent spot) appear on fruit, depending on the temperature and fruit maturity (Kotzé, 2000). The presence of *G. citricarpa* on fruit is unlikely to be accurately confirmed based only on visual examination, as symptoms are variable in appearance and can easily be confused with those caused by other citrus pathogens, mechanical or insect damage (Snowdon, 1990; Kotzé, 2000). The following four main types of symptoms, described by Kieky (1949a, 1949b, 1960), are widely recognized.

**Hard spot**: the most typical symptom of citrus black spot, consisting of shallow lesions, 3–10 mm in diameter, with a grey to tan centre and a dark brown to black margin (Figure 1A). At advanced stages of symptom development, the centre of the lesions becomes crater-like. Individual hard spot lesions may either remain small or coalesce to form larger lesions. A yellow halo, when the fruit is green, or a green halo, when the fruit is yellow or orange, may appear around these lesions. Quite often, pycnidia of the anamorph *P. citricarpa* are produced in the centre of these spots (Figure 1a) and can be detected by using a hand lens or a dissecting microscope. Hard spot usually appears when fruit starts maturing, even before colour change and on the side of the fruit most exposed to sunlight (Kotzé, 1981, 2000). Hard spot symptoms with pycnidia would be the most easily identified as citrus black spot.

**Freckle spot**: grey, tan, reddish or colourless spots, 1–3 mm in diameter, slightly depressed at the centre and with no halo around them (Figure 1B). The spots turn brown with age and are almost always devoid of pycnidia (Figure 1b). Freckle spots mostly develop after the fruit has changed colour and may also appear as satellite spots around hard spot lesions (Bonants et al., 2003) (Figure 1C). Individual freckle spots may coalesce to form larger lesions that turn into virulent spots (Figure 2C), especially during storage (Kotzé, 1981, 2000).

**False melanose or speckled blotch**: usually appears on green fruit as small, raised, dark brown to black lesions often surrounded by dark specks (FUNDECITRUS, 2005) (Figure 2A, 2a, 2B). The lesions are devoid of pycnidia and may coalesce as the season progresses (CAABI, 2011). This type of symptom is observed in citrus-growing areas where *G. citricarpa* has been present for a long time (FUNDECITRUS, 2005).

**Virulent spot** or spreading spot or galloping spot: sunken, irregular, red to brown or colourless lesions, which appear on heavily infected mature fruit towards the end of the season (Figure 2C). Numerous pycnidia eventually develop in these lesions under conditions of high humidity (Kotzé, 2000). Virulent spots grow rapidly covering two-thirds of the fruit surface within 4–5 days. It is the most damaging symptom, because, unlike the other types of symptoms, it extends deeply into the mesocarp (albedo), occasionally involving the entire thickness of the rind, causing premature fruit drop and serious post-harvest losses (Kotzé, 1981).

Two additional types of symptoms (below) have also been reported to occur on citrus fruit, though infrequently.

**Lacy spot**: superficial, yellowish lesions with a dark yellow to brown centre, a smooth texture and no defined margins (Aguilar-Vildoso et al., 2002) (Figure 2D). This type of symptom appears on green fruit and may cover a big part of its surface (Goes, 2001). The lesions are devoid of pycnidia and frequently appear as brown netting on a yellow background. Fruit showing lacy spot usually appear aggregated in the tree canopy (Dr M. Spósito, personal communication).

**Cracked spot**: superficial, slightly raised, variable in size, dark brown to black lesions with a cracked surface and irregular margins (Goes et al., 2000) (Figure 2E). The lesions are devoid of pycnidia and appear on fruit older than six months. This type of symptom has been associated with infection by *Phyllosticta olivae* Ashmead (FUNDECITRUS, 2005).

It should be noted that more than one of the above described types of symptoms or intermediate stages between one type of symptom and another may be observed on the same fruit (Figure 1C, 1c).

In areas with high inoculum pressure, symptoms may also appear on small fruit, calyxes and pedicules. The symptoms on calyxes are red to dark brown lesions similar to freckle spots. On small fruit and pedicules,
3.2 Symptoms on leaves and twigs

Citrus black spot usually occurs on leaves as quiescent infections without any visible symptoms (Sutton and Waterston, 1966). If symptoms appear, they start as pinpoint spots visible on both leaf surfaces. The spots, which may increase in size up to 3 mm in diameter, are circular with their centres becoming grey or light brown in colour surrounded by a dark brown to black rim and a yellow halo (Kotzé, 2000) (Figure 3A).

Lesions similar to those on leaves may also occur on small twigs, more commonly on C. limon than on other citrus species (Dr M. Truter, personal communication). Symptoms are small (0.5–2 mm in diameter), round, slightly sunken lesions with a brown to black margin and a grey to light brown centre (Figure 3B). Pyrosoria may occasionally be present in the centre of the lesions.

3.3 Comparison of citrus black spot symptoms with those caused by other organisms or abiotic factors

Symptoms on fruit are variable in appearance and often resemble those caused by other citrus pathogens (such as P. citri, Diaporthe citri, Mycosphaerella citri, Alternaria alternata pv. citri, Septoria spp., Colletotrichum spp.), insect or mechanical damage (Snowdon, 1990; Bonants et al., 2003) or cold damage, particularly in the case of freckle spot (Dr L. Diaz, personal communication).

As the symptoms caused by G. citricarpa on citrus fruit are similar to those caused by other pathogens, reliable diagnosis can be made only by using the methods described below.

4. Identification

This protocol describes the detection and identification of G. citricarpa on citrus fruit. Citrus fruit should be inspected for the detection of any symptoms typical of citrus black spot (see section 3). If suspected symptoms are present in the form of spots or lesions, they are examined with a magnifying lens or a dissecting microscope for the presence of pycnidia. However, as the pycnidia and spores of P. citriasiana (anamorph of G. citricarpa) are very similar to those of P. citri, the recently described pathogen on C. maxima (Wulandari et al., 2009), the identity of G. citricarpa has to be confirmed by applying the diagnostic methods described below (Figure 4). The diagnostic Method A (isolation and culturing) is used for the identification of G. citricarpa on citrus fruit, but can also be used on leaves, twigs and pedicels, whereas Method B (molecular assays) is applied on citrus fruit only.

If after applying Method A the cultural characteristics of the colonies grown on cherry decoction agar (CHA) and oatmeal agar (OIA) media are not consistent with those of G. citricarpa (see section 4.2, requirements (i), (ii) and (iii)), then the plant material is considered free of G. citricarpa. On G. citricarpa-like cultures that do not produce mature pycnidia within 14 days, application of real-time polymerase chain reaction (real-time PCR) to sections of 4.3.2 or internal transcribed spacer (ITS) sequencing (see section 4.3.3) is recommended. However, isolation and culturing of the organism on appropriate media followed by a direct molecular test of the cultures is a time-consuming procedure and thus undesirable in time-critical diagnosis of consignments.

There are two PCR methods (conventional and real-time) available for the detection and identification of G. citricarpa on citrus fruit (see sections 4.3.1, 4.3.2). However, it has been recently observed during routine testing of C. maxima fruit showing typical symptoms, that the real-time PCR method developed by Geni-Pelzer et al. (2007) gives no amplification (Dr J.P. Meffert, personal communication). The reason is that the citrus black spot-like symptoms on C. maxima are caused by P. citriasiana, a newly described species, closely related to G. citricarpa (Wulandari et al., 2009). As it is not clear whether G. citricarpa is able to cause typical symptoms on C. maxima, fruit of this Citrus species showing such symptoms should also be tested for the presence of G. citricarpa.

The real-time PCR method, developed by Geni-Pelzer et al. (2007) (see section 4.3.2), can be used for a positive diagnosis of G. citricarpa, as it will give a positive signal only when G. citricarpa is present, and not with any of the related species. The conventional PCR method (as described in section 4.3.1) will give no amplification when either G. citricarpa or P. citriasiana is present. In this case, after a positive signal, isolation and culturing (see section 4.1) or a real-time PCR (see section 4.3.2) or ITS sequencing (see section 4.3.3) should be performed to discriminate between the two species.

It should be noted that occasionally acervuli of the common endophytic fungi Colletotrichum spp. may be present and may look similar to pycnidia of G. citricarpa. However, Colletotrichum spp. can be differentiated by the presence of setae in their acervuli, the production under humid conditions of pink- or salmon-coloured masses of conidia on the surface of the lesions, and the morphology of their conidia (Kotzé, 2000).

In the present protocol, methods (including references to brand names) are described as published, as these define the original level of specificity achieved. Laboratory procedures presented may be adjusted to the standard of individual laboratories, provided that they are adequately validated.

4.1 Method A: Isolation and culturing of G. citricarpa

Fruit lesions are excised with a cork borer or scalpel, dipped in 70% ethanol for 30 s, surface disinfested with 1% sodium hypochlorite (NaOCl) for 2 min, rinsed twice in sterilized distilled water, and blotted dry (Peres, 2007). For increasing the isolation frequency, lesions must be excised carefully with any asymptomatic tissue being removed prior to plating (Dr N.A. Peres, personal communication). Subsequently, the lesions are transferred both to CHA dishes for testing the growth rate of the colonies and to OA (see below, "Culture media") or PDA amended with 50 µg ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin (OEPP/EPPO, 2003). If PDA is used and slow-growing, dark G. citricarpa-like cultures develop on it, they are subsequently transferred both to CHA dishes for testing the growth rate of the colonies and to OA (see below, "Culture media") dishes for evaluating the yellow pigment production. At the same time, the cultures grown on PDA medium are placed under near-ultraviolet (NUV) light at 22 °C to induce pycnidium formation. Cultures that (i) grow slowly on CHA medium (see below, "Cultural characteristics") and (ii) produce the characteristic pycnidia and conidia of the anamorph P. citriasiana (see below, "Cultural characteristics") are identified as belonging to G. citricarpa.

The method has the following shortcomings: (a) G. citricarpa is a rather slow-growing fungus and often overgrown by other fungi in culture (e.g. G. gloeosporioides) (Peres et al., 2007), as none of the culture media used is selective for G. citricarpa, and (b) it is a time-consuming method, as it requires 7–14 days for the production of pycnidia.

4.2 Culture media

Cherry decoction agar (CHA). Cherry juice is made by boiling 1 kg of cherries, free of stones and petioles, in 1 litre of tap water for approximately 2 h. The extract is filtered, poured into bottles, sterilized for 30 min at 110 °C and stored until use. In a bottle containing 0.8 litres of distilled water, 20 g of technical agar no. 3 is added and sterilized for 15 min at 121 °C. Immediately after sterilization, 0.2 litres of the sterilized cherry extract (pH 4.5) is added, mixed well and sterilized for 5 min at 102 °C (Gams et al., 1998).

Oatmeal agar (OA). 30 g of oatmeal flakes is placed into cheesecloth and suspended in a pan containing tap water. After simmering for approximately 2 h, the flakes are squeezed, filtered through cheesecloth and the extract is sterilized for 15 min at 121 °C. In a bottle containing 1 litre of the oatmeal extract, 20 g of technical agar no. 3 is added and sterilized for 15 min at 121 °C (Gams et al., 1998).

Potato dextrose agar (PDA). PDA is commercially available. Alternatively, it can be prepared according to the
Cultures of G. citricarpa are very similar to those of the endophytic, non-pathogenic to citrus G. mangiferae (anamorph P. capitalesis) and P. citriasiana, a species recently described on C. maxima fruit (Wulandari et al., 2009).

Identification of G. citricarpa colonies is possible by combining (i) the colony growth on CHA medium (the average colony diameter after 7 days at 22 °C in darkness is 25–30 mm for G. citricarpa, >40 mm for G. mangiferae and 18–20 mm for P. citriasiana (average of two isolates), although the ranges may overlap), (ii) the thickness of the mucoid sheath surrounding the conidia (<1.5 μm for G. citricarpa and P. citriasiana, >1.5 μm for G. mangiferae) (Figures SC, SD, 6B, 6C), and (iii) the length of the conidial appendage (4–6 μm for G. citricarpa and G. mangiferae, 7–14 μm for P. citriasiana) (Dr J.P. Melfert, personal communication).

Different molecular methods have been developed for the identification of G. citricarpa directly on pure cultures and fruit lesions (Bonants et al., 2003; Meyer et al., 2006; Gent-Pelzer et al., 2007; Peres et al., 2007; Stingari et al., 2009). However, none of these methods has been validated on plant material other than fruit (e.g. leaves, twigs). Two methods, a conventional PCR assay, developed by Peres et al. (2007), and a real-time PCR assay, developed by Gent-Pelzer et al. (2007), are recommended for the identification of G. citricarpa. It should be noted that a real-time PCR will pick up a signal from a single lesion, which is uncertain with the conventional PCR.

Spermatial state: in the form-genus Leptodothiorella, formed both on host and in pure culture; spermatia dumbbell-shaped, rarely cylindrical, straight or slightly curved, 5–8 μm × 0.5–1 μm.

DNA is extracted either from fungal cultures grown for 7 days in potato-dextrose broth or from single fruit lesions. In the second case, the symptomatic tissue is dissected out, leaving behind as much pith and outer

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DNA is extracted either from fungal cultures grown for 7 days in potato-dextrose broth or from single fruit lesions. In the second case, the symptomatic tissue is dissected out, leaving behind as much pith and outer
rind as possible.

[85] Specificity (analytical specificity) was assessed with the G. citricarpa reference strain CBS 111.20 (representative for 10 G. citricarpa isolates ITS sequence group I: Baayen et al., 2002), the G. mangiferae reference strain GC14 (representative for 22 G. mangiferae isolates ITS sequence group II: Baayen et al., 2002), other citrus pests (Alternaria spp., Penicillium spp., Colletotrichum spp.), Phytophthora citricarpa and Guignardia bidwellii. Only G. citricarpa gave a positive reaction. The sensitivity (analytical sensitivity; detection limit) is 10 fg DNA per reaction and the diagnostic sensitivity is 100% (Gent-Pelzer et al., 2007).

4.3.2.1 General information

[87] The protocol was developed by Gent-Pelzer et al. (2007). The nucleic acid source is mycelium or diseased fruit lesions. The assay is designed to amplify part of the ITS region producing an amplicon of 69 bp. The oligonucleotides used are:

Forward primer: GcF1 (5'-GGT GAT GGA AGG GAG GCC T-3')
Reverse primer: GcR1 (5'-GCA ACA TGG TAG ATA CAC AAG GGT-3').

4.3.2.2 Methods

[92] Nucleic acid extraction and purification

DNA is extracted either from plugs of mycelium (0.5 cm in diameter) taken from the edges of a colony grown on CZA (see section 4.1, "Culture media") at 22 °C in darkness or from fruit lesions. Lesions are dissected from the peel, removing as much as possible of the surrounding albedo and peel tissue. Mycelium plugs or lesions are cut into small pieces and placed in a 1.5-ml microcentrifuge tube with a secure-fitting flat-top cap containing a stainless steel bead (3.2 mm in diameter) and 125 µl of extraction buffer (0.02M phosphate-buffered saline (PBS), 0.5% Tween 20, 0.2% bovine serum albumin). The tube is shaken in a bead beater for 80 s at 5 000 bpm. The mixture is centrifuged for 5 min at 4 °C or at ~20 °C for longer periods.

After amplification, 10 µl of the reaction mixture is loaded along with a molecular weight marker (100 bp DNA Ladder) onto a 1% agarose gel, separated by electrophoresis, stained with ethidium bromide or alternative reagents, viewed and photographed under UV light (Sambrook et al., 1989).

[84] Polymerase chain reaction

The master mix (concentration per 20 µl single reaction) is composed as follows: 0.5 µl of MGW, 8 µl of 2.5× Eppendorf MasterMix (Taq DNA polymerase at 0.06 U µl-1), 2.5× Taq reaction buffer (4 mM Mg2+, 500 µM of each dNTP), 0.8 µl of each primer at 10 µM (final concentration 0.4 µM) and 2 µl of template DNA. The PCR cycling parameters are 94 °C denaturation for 2 min, 39 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 1 min and 72 °C extension step for 10 min. A PCR product of 300 bp indicates the presence of G. citricarpa DNA.

4.3.2.3 Essential procedural information

DNA from a reference strain of G. citricarpa (positive control) must also be included as an additional sample to ensure that amplification has been successful. PCR amplification must also be performed on a sample, where the G. citricarpa DNA extract has been replaced with the DNA extract of other related species or with a sample run using healthy exocarp (negative control). To monitor possible reagent contamination and false positives, a sample must be substituted by water (reaction control). An internal amplification control (IAC) to check for false negative reactions caused by inhibition of the amplification reaction is not described by Peres et al. (2007), and must therefore be developed and tested.
The PCR cycling parameters are 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A cycle threshold (Ct) value <40 indicates the presence of G. citricarpa DNA.

### 4.3.2 Essential procedural information

**DNA from a reference strain of G. citricarpa (positive control) must also be included as an additional sample to ensure that amplification has been successful. PCR amplification must also be performed on a sample, where the G. citricarpa DNA extract has been replaced with the DNA extract of other related species or with a sample run using healthy exocarp (negative control). To monitor possible reagent contamination and false positives, a sample must be substituted by water (reaction control).**

To check for false negative reactions caused by inhibition of the amplification reaction, 12.5 fg of an internal amplification control, 75 nM of IAC forward primer FIAC (5'-TGG CCC TGT CCT TTT ACC AG-3'), 75 nM of IAC reverse primer RIAC (5'-TTT TCG TTG GGA TCT TTC GAA-3'), 50 nM of IAC MGB Taqman probe (5'-ACA CAA TCT GCC-3'), VIC™ label and quencher dye Eclipse Dark Quencher can be added to the reaction mixes. The IAC is a plasmid, containing a green fluorescent protein gene (GFP) that can be obtained from Dr P.J.M. Bonants (Plant Research International, Wageningen, the Netherlands).

**To defrost extracted DNA, if necessary; prepare enough reaction mix for testing at least one sample of the unknown isolate, a positive control containing amplifiable DNA and negative control reactions of reaction mix mixes. The IAC is a plasmid, containing a green fluorescent protein gene (GFP) that can be obtained from Dr P.J.M. Bonants (Plant Research International, Wageningen, the Netherlands).**

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### 4.3.3 Identification of G. citricarpa by ITS sequencing

#### 4.3.3.1 General information

The identity of positive samples obtained by conventional PCR can be confirmed by sequencing. The method for sequencing of the ITS 1 and 2 regions of the fungal ribosomal RNA gene is described below.

#### 4.3.3.2 Methods

**Nucleic acid extraction and purification**

DNA should be extracted from a 1 cm² plug taken from a pure culture of the test isolate. A suitable DNA extraction kit, is used or DNA is extracted following a more traditional method, such as that described in Hughes et al. (2000). Extracted DNA should then be stored at 4 °C for immediate use or at −20 °C if testing is not to be performed on the same day.

**Polymerase chain reaction**

Total reaction volume of a single PCR reaction is 50 µL, and is composed as follows: 37.5 µl of molecular grade water, 5.0 µl 10× PCR reaction buffer (+15 mM MgCl₂) (Roche), 4.0 µl dNTPs (10 mM each), 0.6 µl primer ITS1 (10.0 µM), 0.6 µl primer ITS4 (10.0 µM), 0.3 µl DNA Taq-polymerase (5 U/µl) (Roche) and 1.0 µl of DNA extract.

The PCR cycle parameters are: 94 °C for 30 s, 40 cycles (94 °C for 15 s, 55 °C for 60 s, 72 °C for 30 s), 72 °C for 5 min, 20 °C for 1 min.

**Sequencing of amplicons**

Run 5 µl of the amplified mixture on a 1.5% agarose gel to check for positive test reactions. Purify the remaining 45 µl from positive test reactions using a suitable PCR purification kit, following the manufacturer’s instructions. Sequence with forward primer ITS1 and reverse primer ITS4.

**Amplification and analysis**

DNA from a reference strain of G. citricarpa DNA extract has been replaced with the DNA extract of other related species or with a G. citricarpa to ensure that amplification has been successful. PCR amplification must also be performed on a sample, where the sample run using healthy exocarp (negative control). To monitor possible reagent contamination and false positives, a sample must be substituted by water (reaction control).

Amplification and analysis

To check for false negative reactions caused by inhibition of the amplification reaction, 12.5 fg of an internal amplification control, 75 nM of IAC forward primer FIAC (5'-TGG CCC TGT CCT TTT ACC AG-3'), 75 nM of IAC reverse primer RIAC (5'-TTT TCG TTG GGA TCT TTC GAA-3'), 50 nM of IAC MGB Taqman probe (5'-ACA CAA TCT GCC-3'), VIC™ label and quencher dye Eclipse Dark Quencher can be added to the reaction mixes. The IAC is a plasmid, containing a green fluorescent protein gene (GFP) that can be obtained from Dr P.J.M. Bonants (Plant Research International, Wageningen, the Netherlands).

**DNA from a reference strain of G. citricarpa (positive control) must also be included as an additional sample to ensure that amplification has been successful. PCR amplification must also be performed on a sample, where the G. citricarpa DNA extract has been replaced with the DNA extract of other related species or with a sample run using healthy exocarp (negative control). To monitor possible reagent contamination and false positives, a sample must be substituted by water (reaction control).**

To check for false negative reactions caused by inhibition of the amplification reaction, 12.5 fg of an internal amplification control, 75 nM of IAC forward primer FIAC (5'-TGG CCC TGT CCT TTT ACC AG-3'), 75 nM of IAC reverse primer RIAC (5'-TTT TCG TTG GGA TCT TTC GAA-3'), 50 nM of IAC MGB Taqman probe (5'-ACA CAA TCT GCC-3'), VIC™ label and quencher dye Eclipse Dark Quencher can be added to the reaction mixes. The IAC is a plasmid, containing a green fluorescent protein gene (GFP) that can be obtained from Dr P.J.M. Bonants (Plant Research International, Wageningen, the Netherlands).

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**To defrost extracted DNA, if necessary; prepare enough reaction mix for testing at least one sample of the unknown isolate, a positive control containing amplifiable DNA and negative control reactions of reaction mix mixes. The IAC is a plasmid, containing a green fluorescent protein gene (GFP) that can be obtained from Dr P.J.M. Bonants (Plant Research International, Wageningen, the Netherlands).**

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Dr Irene Vloutoglou, Benaki Phytopathological Institute, 8, St. Delta street, GR-145 61 Kifissia, Athens, Greece (Tel.: +30 210 8180201; Fax: +30 210 8077506; e-mail: lykoufioglou@bpi.gr).

Dr Johan Meffert, Plant Protection Service, 15, Geertjesweg, 6706 EA, Wageningen, The Netherlands (Tel.: +31 417 496837; e-mail: j.meffert@wur.nl).

Dr Luis E. Diaz, Ministry of Husbandry, Agriculture and Fisheries, General Directorate of Agricultural Services, Mycology Department, Av. Millán 4703, CP 12900, Montevideo, Uruguay (Tel.: +598 2 3043992; Fax: +598 2 3043992; e-mail: ldiaz@migap.gub.uy).

8. References


9. Figures
Figure 2: False melanose, virulent spot, lacy spot and cracked spot symptoms caused by *Guignardia citricarpa* on sweet orange (*Citrus sinensis*) and lemon (*C. limon*) fruits: (A) false melanose lesions on mature sweet orange; (a) mature sweet orange showing false melanose lesions surrounded by dark specks; (B) false melanose lesions on a green sweet orange; (C) virulent spot lesions on sweet orange; (D) lacy spot symptoms on a green sweet orange; (E) cracked spot type of lesions on sweet orange (the lesions are slightly raised, cracked with irregular margins and devoid of pycnidia).

Courtesy: FUNDECITRUS (photos A, B, C, D, E) and E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil (photo a).

Figure 3: Symptoms of citrus black spot caused by *Guignardia citricarpa* on lemon (*C. limon*) leaves (A) and twigs (B).

Courtesy: E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil (photo A) and M. Truter, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa (photo B).
Figure 4: Flow diagram for the diagnosis of *Guignardia citricarpa* on citrus fruit

1. The molecular assays have been validated for the identification of the organism on pure cultures and fruit lesions and not on any other plant material (e.g. leaves, twigs).

Figure 5: Colony characteristics of *Guignardia citricarpa* and conidial morphology of the anamorph *Phyllosticta citricarpa*: (A) colony with irregular margin surrounded by a translucent zone of colourless submerged mycelium (arrow) after 30 days of growth on potato dextrose agar (pH 5.5) at 25 °C and a 12-h photoperiod; (B) conidial slime oozing from mature pycnidia; (C, D) conidia with a thin mucoid sheath (C, arrow) and a colourless subulate appendage (D, arrow, magnification 1,000× with immersion oil)

Courtesy: L.E. Diaz, Ministry of Husbandry, Agriculture and Fisheries, Montevideo, Uruguay.
Figure 6: Conidia morphology and cultural characteristics of Guignardia citricarpa and G. mangiferae: (A) conidia of P. citricarpa, anamorph of G. citricarpa, with thin (<1.5 μm) mucoid sheath; (B, C) conidia of P. capitatusis, anamorph of G. mangiferae, with thick (>1.5 μm) mucoid sheath (scale bar = 10 μm) (photo C was taken under a light microscope equipped with differential interference contrast); (D, E) colonies of G. citricarpa (D) and G. mangiferae (E), after 7 days of growth on (top row) oatmeal agar, (middle row) malt extract agar, and (bottom row) cherry decoction agar (Note production of a yellow pigment around the colony of G. citricarpa grown on oatmeal agar (D, arrows) and absence of this pigment in cultures of G. mangiferae grown on the same medium (E).)

Courtesy: G. Verkley, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (photos A, B, C) and W. van Lienden, Plant Protection Service, Wageningen, The Netherlands (photos D, E).

Footnote 1 The use of the brand Eppendorf® for PCR amplification in this diagnostic protocol implies no approval of it to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

Footnote 2 The use of the brand Takara Bio Europe S.A.S for the 2× Premix Ex Taq master mix in this diagnostic protocol implies no approval of it to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

Footnote 3 The use of the brand Roche for the PCR reaction buffer and the DNA Taq-polymerase in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.
The protocol is quite complicated because of the many different methods used by countries testing their harvested wheat, and when importing from other countries.

Notes

Adoption

This diagnostic protocol was adopted by the Commission on Phytosanitary Measures in 20-.

1. Pest Information

Tilletia indica Mitra causes the disease Karnal bunt, or partial bunt, of wheat (Triticum spp.). Karnal bunt was first described in Karnal, India, in 1931. T. indica is a serious pest for international trade. It is of economic importance because it reduces grain quality; and its restricted distribution makes it a concern for those countries currently free from the pest. The pathogen is widespread in parts of South Asia and Southwest Asia (Wiese, 1987; USDA, 2007). It has also been detected in North America, including the south-western United States, and South Africa (Fuentes-Davila, 1996; Crous et al., 2001).

Hosts include Triticum aestivum, Triticum durum and Triticum aestivum × Secale cereale are rare; however, Secale spp. have been shown to have the potential to be a host (Sareeford et al., 2008). Tilletia indica has been shown to infect other grass species under glasshouse conditions but has never been detected in the field in these alternative hosts (Inman et al., 2003).

T. indica is a floret-infecting smut pathogen. Seeds are infected through the germinal end of the kernel and the fungus develops within the pericarp where it produces a powdery, brown-black mass of teliospores. When fresh, the spore masses produce a foetid, decaying fish-like smell (trimethylamine). Unlike systemic smuts, it is not usual for all the seeds on an ear to be infected with T. indica, and heads with infected seeds do not differ in appearance from healthy heads (Figure 1). Seeds are usually only partially colonized, showing various degrees of infestation (Figure 2). Therefore it is very difficult to detect the disease in the field. The symptoms are not usually seen until after harvest, unless infestation levels are high.

T. indica reduces grain quality by discoloring and imparting an objectionable odour to the grain and products made from it. It also causes a small reduction in yield. Generally Tilletia aestivum containing more than 3% bunted kernels is considered unsatisfactory for human consumption.

There are other Tilletia species that can be confused with T. indica and are commonly found in harvested grain or seeds. These include T. walkeri (a pathogen of Lolium perenne and L. multiflorum), T. horrida (a pathogen of Oryza spp.) and T. ehrhartiae (a pathogen of Ehrhartia calycina). In Australia, T. walkeri and T. ehrhartiae are found to contaminate harvested seed of Triticum aestivum, T. walkeri and T. horrida are present in the United States and are detected in harvested seed of Triticum aestivum especially where Oryza spp. and Lolium spp. are grown in rotation with Triticum aestivum (Castlebury, 1998; Castlebury and Carris, 1999; Pascoe et al., 2005). Because of the morphological similarity of these pathogens, accurate identification of the different pathogens is important.

2. Taxonomic Information

Name: Tilletia indica Mitra

Synonyms: Neovossia indica (Mitra) Mundkur

Taxonomic position: Fungi: Basidiomycota, Ustilaginomycotina, Exobasidiomycetes, Exobasidiomycetidae, Tilletiales, Tilletiaceae

The following experts were involved in ring-testing of the original protocol: A. Radova (State PhytoSanitary Administration, Olomouc, Czech Republic); I. Voutioglou (Benaki Phytopathological Institute, Athens, Greece); A. Porta-Puglia (Istituto Sperimentale per la Patologia Vegetale, Rome, Italy); C. Montuschi (Servizio Fitosanitario-Regionale, Bologna, Italy); I. van Brouwershaven (NPPO, Wageningen, The Netherlands); M. de Jesus Gomes, E. Diogo & M.R. Malheiros (Direcção-Geral de Protecção das Culturas, Lisboa, Portugal); V. Cockrell (Science and Advice for Scottish Agriculture, Edinburgh, UK); A. Barnes (Food and Environment Agency, York, UK).

This draft protocol has further been commented upon by: Valerie Cockrell (Science and Advice for Scottish Agriculture, Edinburgh, UK).

It was sent for comment to members of the QUADS (Quadlateral Scientific Collaboration in Plant Biosecurity) working on exchange of diagnostic tools for plant pests.
Before beginning it is important that all equipment is soaked for 15 minutes in a bleach solution (1.6% NaOCl active ingredient), to eliminate the risk of false positives by cross-contamination from previous samples. The bleach kills teliospores and makes them appear hyaline compared with the normally dark, pigmented teliospores. All equipment is rinsed with tap water after soaking.

The 50 g sample is placed in an Erlenmeyer flask (250 ml) with a 100 ml of 0.01% Tween 20 aqueous solution. The samples are then placed on a shaker for three minutes at 200 rpm to release the teliospores from the seeds. The solution and seeds are then poured onto a 53 µm sieve, which is sitting on top of a 20 µm sieve, which is sitting inside a funnel on top of another flask (500 ml). The flasks that contained the seeds are then rinsed twice (approximately 100 ml) with sterile tap water and this is then poured over the seeds sitting in the sieve. The seeds are further washed with sterile tap water (approximately 200–300 ml) using an aspirator bottle to ensure a good removal of the teliospores from the seed. The seeds and the 53 µm sieve are removed. The 20 µm sieve is tilted to a 45° angle and, using an aspirator bottle filled with sterile tap water, the debris is washed on the sieve from the top to the bottom with a sideways sweeping motion going backwards and forwards. This is to wash all teliospores recovered from the seeds into the lower side of the sieve. The teliospores and debris are then washed into a 15 ml conical centrifuge tube. It is important that....
When suspect teliospores are found in a wash test, the seeds in both the washed subsample(s) and the larger sample could be re-examined for symptoms. If symptoms are found, these should be confirmed by microscopic examination of the teliospores. Any grass seeds found in the sample should also be examined for signs of bunt infestation and, if found, the associated teliospores should be examined microscopically. If the teliospores found in the wash test are the same as those found on bunted kernels a diagnosis can be made. If, however, there are no bunted kernels found in the larger sample, molecular tests are recommended for identification.

Table 2 lists morphological characteristics of Tilletia indica teliospores and includes the common Tilletia species that can be found in seeds or grain shipments and confused with T. indica.

### 4.1.1 Morphological identification

T. indica teliospores are globose to subglobose, sometimes with a small hyphal fragment (more common on immature teliospores, but occasionally on mature teliospores), mostly 22–47 μm in diameter, occasionally larger (mean 35–41 μm); pale orange/brown to dark, reddish brown; mature teliospores are black and opaque (Figures 4 and 5); densely ornamented with sharply pointed to truncate spines, occasionally with curved tips, 1.5–5.0 μm high, which in surface view appear as either individual spines (densely echinulate) or as closely spaced, narrow ridges (finely cerebriform) (Figures 4 and 5); the spines are covered by a thin hyaline membrane (CMI, 1983; Carris et al., 2006).

Sterile cells of T. indica are globose, subglobose to lachrymiform (tear-shaped), yellowish brown, 10–28 μm × 48 μm, with or without an apiculus (short stalk), with smooth walls up to 7 μm thick and laminated. Sterile cells are likely to be uncommon in sieved washings (CMI, 1983; Carris et al., 2006).

If < 10 teliospores are present in a wash test, the morphological identification can be confirmed. If < 10 teliospores are detected, morphological characters are not considered totally reliable for confident discrimination between T. indica and the morphologically similar species T. walkeri, T. horrida and T. ehrhartiae. It is then recommended that the sample is resampled by preparing new subsamples from the original 1 kg and tested.

### 4.1.2 Morphological comparisons with other Tilletia species

Other tubercule-spored Tilletia species may be confused with T. indica (Durán and Fischer, 1961; Durán, 1987; Pimentel et al., 1998). These species are less likely to be found as contaminants of Triticum aestivum, but they include Tilletia barclayana sensu lato (sensu of various Poaceae, e.g. Panicum and Paspalum), Tilletia aliena (on Eragrostis), Tilletia ehrhartiae (on Ehrhartia calycina), Tilletia inolens (on Lachnagrostis filiformis), Tilletia rugospora (on Paspalum), Tilletia bouteilouae (on Bouteiloua gracilis). However, Pascoe et al. (2000) showed that in Australia, T. walkeri and T. ehrhartiae are common contaminants of harvested Triticum aestivum. None of these morphologically similar species have been found to naturally infect Tilletia aestivum. In the United States, the morphologically and genetically similar fungus Tilletia walkeri and also Tilletia horrida are known contaminants of harvested Triticum aestivum (Smith et al., 1996; Castelbury and Carris, 1999; Cunfer and Castlebury, 1999). The most important morphological characters that discriminate T. indica, T. walkeri, T. horrida and T. ehrhartiae are teliospore size (range and mean), exospore ornamentation and colour (Table 2; Figures 4–8). The literature on spore sizes is often variable. Spore size is affected by the mounting medium and by heating treatments.

The median profiles can be enhanced by bleaching the teliospores in 10% sodium hypochlorite for 15–20 min. If necessary, teliospores can then be rinsed twice in water and stained, for example with trypan blue or cotton blue in lactoglycerol (Figure 8).

### 4.2 Isolation and germination of teliospores

There are now two methods available to confirm the identification of spores detected in the sieve wash test. There is the standard procedure of recovering the spores from the slide (section 4.2.1) and a new procedure developed by Tan et al. (2009) which enables PCR to be done directly on a single spore (section 4.2.3).

<table>
<thead>
<tr>
<th>Species</th>
<th>Spore size range (μm)</th>
<th>Spore size mean (μm)</th>
<th>Spore colour</th>
<th>Spore shape</th>
<th>Spore sheath</th>
<th>Spore spines</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. indica</td>
<td>28–54</td>
<td>30–41</td>
<td>Brown to dark reddish brown, opaque</td>
<td>Globose to subglobose</td>
<td>Present</td>
<td>1.4–5 μm in surface view, densely echinulate or as closely spaced, narrow</td>
<td>Triticum spp.</td>
</tr>
</tbody>
</table>

T. indica is a facultative biotroph. To produce cultures, teliospores are soaked in water, quickly surface-sterilized and then germinated on water-agar plates. The teliospores can be recovered from the slides and cover slips by washing them with distilled water over the 20 μm sieve and then into a clean sterile conical centrifuge tube (as in section 3.2). The volume should be approximately 3–5 ml. The tubes are incubated overnight at 21 °C to hydrate the teliospores and make fungal and bacterial contaminants more susceptible to subsequent surface sterilization. After incubating overnight, pellet the teliospores by centrifuging at 1200 g for 3 min.

The teliospores are then sterilized by removing the supernatant and suspending the pellet in 5 ml of bleach (0.3–0.5% active NaOCl) and inverting the tube quickly three times and centrifuging at 1200 g for 1 min. Some teliospores can be killed if the total time in the bleach exceeds 2 min. Instead of bleach, teliospores can be surface sterilized for 30 min in 5–10 ml of acidified electrolyzed water (AEW). AEW effectively surface sterilizes teliospores but, compared with a 1–2 min bleach treatment, stimulates rather than reduces telospore germination (Bondre et al., 1999). The teliospores are then washed twice by removing the supernatant, resuspending the pellet in 1 ml sterile distilled water (SDW) and centrifuging at 1200 g for 5 min.

The pellet is then plated onto 2% water agar with antibiotics (WA+A) by resuspending in 1 ml of SDW and placing 200 μl of the telospore suspension aseptically onto the plates and spreading with a sterile spreader. Incubate the WA+A plates at 21 °C with a 12 h light cycle. Leave for about 5 days before sealing plates or placing the plates inside clean polyethylene bags.

After 7–14 days, non-dormant teliospores produce a promycelium bearing 32–128 or more basidiospores (primary sporia) at its tip. These colonies produce secondary sporia typically of two types: filiform and allantoid. These can then be cultured directly on solid or liquid nutrient media, such as potato dextrose broth (Figure 9). Small blocks of agar (1 cm × 1 cm) bearing germinated teliospores or colonies are cut out, and then stuck to the underside of a Petri dish lid so that the germinated teliospore is facing the surface of the broth. This allows the sporeidia to be released onto the broth surface. The dishes are incubated at 21 °C with a 12 h light cycle. After 2–3 days, basidiospores deposited onto the broth surface produce small mats of mycelia approximately 0.5–1.0 cm diameter. Each mycelial mat is removed with a sterile dissecting needle, and touched onto sterile filter paper to remove excessive broth. The mycelium is placed in suitable vials (e.g. 1.5–2.0 ml microcentrifuge tubes) for immediate DNA extraction, or stored at −80 °C for subsequent DNA extraction.

Germination of teliospores for molecular analysis may not always be possible, for example if seeds are treated with NaOH as in the case of fungicide-treated grain. Increasing the number of sieved replicates may increase the number of teliospores recovered and hence the number of teliospores that can be germinated. Teliospores can have a period of dormancy, which can effect germination (Carris et al., 2006); it should not be assumed that teliospores that do not germinate are not viable. This can be resolved by carrying out direct real-time PCR on individual teliospores (see section 4.3.4).

Table 2. Morphological characteristics of Tilletia indica, Tilletia walkeri, Tilletia horrida and Tilletia ehrhartiae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Spore size range (μm)</th>
<th>Spore size mean (μm)</th>
<th>Spore colour</th>
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<td>Present</td>
<td>1.4–5 μm in surface view, densely echinulate or as closely spaced, narrow</td>
<td>Triticum spp.</td>
</tr>
</tbody>
</table>
In median view, smoother more complete outline due to spines being densely arranged occasionally with curved tips.

| T. walker² | 28–35 | 30–31 | Pale yellow to dark reddish brown (never black/opaque) | Globose | Present | Extending to tips of projections, hyaline to yellowish brown | 3–6 µm Coarse+/− cerebriform. Wide incompletely cerebriform ridges in surface view. In median view, profile is irregular with gaps between spines | Lolium perenne and L. multiflorum |

| T. horrida² | 14–36 | <25 | Light to dark chestnut brown, can be semi-opaque | Globose to subglobosa | Present | Extending to the ends of the spines, hyaline to tinted | 1.5–4 µm Frequently curved, and appear as polygonal scales in surface view | Oryza spp. |

| T. ehhartae | 17–25 | | Very dark olivaceous brown when mature. Can be opaque because of melanization of the scales. | Globose to subglobosa | Present | Extending to the apex of the spines or slightly beyond | 1–2.5 µm Cylindrical or slightly tapered spines. In surface view, rarely cerebriform. Larger, acute polygonal scales | Ehrharta calycina |

Notes: *Based on Inman et al. (2003). †Based on Castlebury, 1998; Milbrath et al., 1998; Castlebury & Carris, 1999; Culter & Castlebury, 1999. ‡As T. janczykii: Durán & Fischer, 1961; CMII, 1965; Durán, 1987; Castlebury & Carris, 1999; or as T. horrida: Khanna & Payak, 1968; Aggarwal et al., 1995; Castlebury, 1998. §Pascoe et al., 2005.

4.2.2 Germination of similar Tilletia species

In culture, T. walker and T. indica produce very similar colonies. On potato dextrose agar (PDA) after 14 days at 19 °C with a 12-h light cycle, both species typically produce white to cream-coloured, slow-growing, irregular, crustose colonies, about 4–6 mm in diameter (Figure 9). In contrast, comparable cultures of T. horrida grow significantly more slowly (colonies only 2–3 mm in diameter) because of their higher temperature optima. T. horrida isolates may also produce a reddish-purple pigment (Figure 9), both on PDA and potato dextrose broth.

4.2.3 Recovery of single teliospores

After examination of the teliospores and recording of the morphology, the slide is allowed to dry out, either with or without the cover slip on. When removing the cover slip this is placed on the slide upside down so it can be checked for teliospores adhering to it.

On another slide place a single piece of a cover slip (1 × 1 mm) that has been sterilized (autoclave at 121 °C for 15 min). Place a 1 µl drop of Tris-EDTA (TE) buffer onto this piece of cover slip. Under either a compound or dissecting microscope, single teliospores are picked off with a very fine needle and placed into the TE buffer droplet. The teliospores will transfer to the droplet. Using forceps place another small piece of a cover slip on top to make a sandwich. The teliospores are crushed by using the forceps to press down on the cover slip, and then the glass sandwich is transferred into a 0.2 ml PCR tube. The procedure then followed is as described in section 4.3.4.1.

4.3 Molecular identification

There are a number of molecular methods available for T. indica. The three main protocols described rely upon the germination of the teliospores, so that DNA can be extracted from the mycelial mat produced. The germination of the teliospores can take up to three weeks. Any one of the methods described below may be used. It is essential that reference material (positive controls) have been obtained from experts in this area (refer to section 6). These methods work well, but are dependent upon the germination of the teliospores so that enough DNA can be extracted for the protocols to work. Peterson et al. (2000) found that the average telospore germination rate was 55%, which severely reduces the chances of identifying the teliospores by molecular methods.

Diagnostically significant differences exist between T. indica, T. walker and T. horrida in their nuclear and mitochondrial DNA. Interspecific polymorphisms have been identified using various PCR methods, including random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) methods (Lanneche et al., 1998; Pimentel et al., 1998). In the nuclear ribosomal (DNA) internal transcribed spacer (ITS) ITS1 and ITS2 regions, there is a > 98% similarity.
between T. walkeri and T. indica sequences (Levy et al., 1998). However, within the ITS1 region, T. walkeri has a diagnostically important restriction enzyme site (Sca 1) that is not present with T. indica, T. horrida or other closely related species (Levy et al., 1998; Pimentel et al., 1998). With MinDNA, sequence differences have enabled species-specific primers to be designed for T. indica and T. walkeri (Frederick et al., 2000). These primers can be used in conventional PCR assays or in a TaqMan system in conjunction with a probe (Frederick et al., 2000).

### 4.3.1 Restriction enzyme analysis of the ITS1 region

The target gene region of the ITS1 is the nuclear ribosomal RNA gene (Pimentel et al., 1998). The PCR amplicon produced includes both ITS1 and ITS2 and the conserved fragment 5.8 S. This amplicon is approximately 670 base pairs (bp) including primer sequences. Oligonucleotides used are:

- **Forward primer ITS1** (5′-TCC GTA GGT GAA CCT GCG G-3′)
- **Reverse primer ITS4** (5′-TCC TGG TAT TGA TAT GC-3′) (White et al., 1990).

DNA is extracted from mycelium. This is done by grinding up the mycelium using a mortar and pestle or by placing approximately 0.1 g of mycelium in a sterile 2 ml microcentrifuge tube one-third full with sterile 0.5 mm glass beads and 1 ml molecular grade water (MGW). Seal tube with screw lid containing an o-ring and oscillate the tube in a beadbeater, or tissue lyser on ¼ power for 5 min. Allow the ground sample to stand for 30 s, then extract its DNA using a proprietary DNA extraction kit for fungi. No DNA cleanup or lysis buffer is added before extracting DNA using a proprietary DNA extraction kit for fungi. No DNA cleanup is required. Either use extracted DNA immediately or store overnight at 4 °C or at −20 °C for longer periods.

PCR for this assay uses the following mastermix (concentration per 25µl single reaction): 20.2 µl of MGW, 2.5 µl of 10X PCR buffer containing 15 mM MgCl₂ (Applied Biosystems), 1.0 µl each of dNTPs (10 mM) (final concentration of 0.1 mM each), 0.1 µl AmpliTaq (5 U/µl) (Applied Biosystems), 0.1 µl of each primer (25 µM), 1.0 µl neat extracted DNA obtained as described above. PCR cycling parameters: 94 °C denaturation for 1 min, 25 cycles of 94 °C for 15 s, 65 °C for 15 s, 72 °C for 15 s, and then a 72 °C extension step for 6 min.

- **As required 10 µl of reaction products is mixed with a running marker and run on a 2% agarose gel.**

When testing for T. walkeri the following primer is used. Replace Tin 3 with 0.1 µl of forward primer Tin 11 (5′-TAA TGT TGG CGT GGC GGC AT-3′) (25 µM). This produces an amplicon of 163 bp.

- **Positive reactions produce a single amplicon of 414 bp for both for T. indica (primers Tin 3/Tin 4) and T. walkeri (primers Tin 11/Tin 4).** If the T. walkeri and T. indica specific primers do not produce positive results for the test samples (but positive control DNA samples are positive), then the sample extractions belong to another Tilletia species, such as T. horrida. Restriction enzyme analysis may enable further species identification of these samples if required (section 4.3.1). Alternatively no amplification can result from poor-quality DNA. This can be checked by testing extracts with the universal primers (ITS1 and ITS4) described in section 4.3.1. If the samples contain good-quality DNA, and hence test samples are not T. indica or T. walkeri but another Tilletia species, then a single band (≤ 670 bp) will be produced when PCR amplions are run out on an agarose gel. However, if amplification is still not produced, fresh DNA should then be extracted and retested.

### 4.3.3 PCR assay using species-specific primers and a fluorescent probe in a TaqMan system

The assay was designed by Frederick et al. (2000) using the mitochondrial DNA producing an amplicon of 163 bp. Oligonucleotides used are:

- **Forward** for Taq 1 produces four DNA fragments (60, 110, 150 and 335 bp) and Sca 1 produces no cuts. Other Tilletia species give different restriction patterns with these or other enzymes (see Pimentel et al., 1998).

- **Restriction of PCR amplicon is done as follows. Restriction mix (concentration per 20µl single reaction): 7.3 µl MGW, 2.0 µl of restriction buffer (Promega), 0.2 µl of bovine serum albumin (10µg/µl), 0.5 µl of restriction enzyme, either Taq 1 or Sca 1 at 1 unit/µl (Promega), and 10.0 µl neat DNA amplicon solution as produced above (> 50 ng/µl DNA). This is then incubated for three hours at 37 °C, gently mixing the reaction by inversion during incubation. If required, store restricted products at 4 °C before visualizing on a gel. When required, mix 10 µl of reaction products with a running marker and run on a 2% gel.

- **The assay is positive for T. indica if amplified test samples are cut with restriction enzyme Taq 1 to give five products (individual products occur at 60, 70, 110, 170 and 260 bp respectively) and there is no cut with Sca 1. A positive for T. walkeri is obtained if amplified test samples are restricted with Taq 1 to give the same five fragments as with T. indica, but Sca 1 restricts amplified products to give two fragments at 140 bp and 520 bp respectively. If the amplified product comes from T. horrida, Taq 1 produces four DNA fragments (60, 110, 150 and 335 bp) and Sca 1 produces no cuts. Other Tilletia species give different restriction patterns with these or other enzymes (see Pimentel et al., 1998).**

### 4.3.2 Conventional PCR assay using species-specific primers

The assay was designed by Frederick et al. (2000) using the mitochondrial DNA producing an amplicon of 163 bp. Oligonucleotides used for T. indica are:

- **Forward primer Tin 3** (5′-CAA TGG TGG GCT GGC GGC GC-3′)
- **Reverse primer Tin 4** (5′-CAA CTC CAG TGA TGG CTC CG-3′).

DNA is extracted from mycelium. This is done by grinding 0.5–1.0 g of mycelium in a 1.5 ml microcentrifuge tube with 75 µl lysis buffer and grinding with a sterile pestle attached to a power drill. An additional 75 µl lysis buffer is added before extracting DNA using a proprietary DNA extraction kit for fungi. No DNA cleanup is required. Either use extracted DNA immediately or store overnight at 4 °C or at −20 °C for longer periods.

PCR for this assay uses the following mastermix (concentration per 25µl single reaction): 20.2 µl of MGW, 2.5 µl of 10X PCR buffer containing 15 mM MgCl₂ (Applied Biosystems), 0.25 µl of each dNTPs (10 mM) (final concentration of 0.1 mM each), 0.1 µl AmpliTaq (5 U/µl) (Applied Biosystems), 0.1 µl of each primer (25 µM), 1.0 µl neat extracted DNA obtained as described above. PCR cycling parameters: 94 °C denaturation for 1 min, 25 cycles of 94 °C for 15 s, 65 °C for 15 s, 72 °C for 15 s, and 72 °C extension step for 6 min.

- **As required 10 µl of reaction products is mixed with a running marker and run on a 2% agarose gel.**

When testing for T. walkeri replace Tin 3 with 1.0 µl of forward primer Tin 11 (5′-TAA TGT TGG CGT GGC GGC AT-3′) (25 µM) to test for T. walkeri, which produces an amplicon of 163 bp.

- **12.5 µl of 2X universal TaqMan master mix, 1.0 µl of each primer (10 µM), 1.0 µl of TaqMan probe (10 µM), and 1.0 µl neat extracted DNA (obtained as in section 4.3.2). PCR cycling parameters: 50 °C for 2 min, 95 °C for 10 min, 34 cycles of 95 °C for 15 s and 60 °C for 1 min.**

Optical-grade reaction tubes and caps should be used to allow real-time amplification to be monitored.

- **When testing for T. walkeri, replace Tin 3 with 1.0 µl of forward primer Tin 11 (5′-TAA TGT TGG CGT GGC GGC AT-3′) (25 µM) to test for T. walkeri, which produces an amplicon of 163 bp.**

- **T. indica produces amplification with primers Tin 3/Tin 4 while T. walkeri needs primers Tin 11/Tin 4. If neither primer set produces amplification but control samples react as expected, then the sample extractions belong to another Tilletia species, such as T. horrida. For example, when testing for T. indica and the threshold cycle (Ct) of the sample is > 40, the result indicates that it is negative for T. indica and is highly likely to be another species of Tilletia. Likewise, when testing for T. walkeri and the Ct > 40, the result indicates that it is negative for T. walkeri and is highly likely to be another species of Tilletia. Restriction enzyme analysis may enable further species identification of these samples if required (section 4.3.1).**
Alternatively no amplification can result from poor-quality DNA. This can be checked by testing extracts with the universal primers (ITS1 and ITS4) described in section 4.3.1. If the samples contain good-quality DNA, then a loss in genetic material. The specificity of the probe for T. indica was investigated in a DNA mixture of T. indica, T. walkeri, T. caries, T. laevis, T. controversa or T. fusca. None of these isolates amplified in either the T. indica or T. walkeri-specific assays (Fredrick et al., 2000).

4.3.4 Direct real-time PCR on teliospores

This assay was designed by Tan et al. (2009) to use the ITS region that occurs between the nuclear small- and large-subunit ribosomal DNA (rDNA). It was found that the Tilletia species have two variable regions (ITS1 and ITS2) separated by the conserved 5.8S rRNA gene (Levy et al., 2001; Tan and Murray, 2006). Each PCR reaction is performed in 20 µL (single reaction). The mixture consists of 20 µL of 1.5 mM MgCl2, 200 µM of each of the four deoxynucleotides dATP, dTTP, dCTP and dTTP, 0.5 µM of each of the primer pair and 0.5 U Taq DNA Polymerase (Invitrogen4, USA) in 1X buffer L (50 mM Tris, pH 9.5, 20 mM NaCl, 1% Triton X-100, 0.1% gelatin).

The thermal cycling parameters included an initial cycle of 95 °C for 3 min, followed by 20 cycles of 94 °C for 20 s, 63 °C for 30 s, 72 °C for 30 s, with the annealing temperature decreased by 1 °C per cycle for 5 cycles to 55 °C, and finally followed by a 10 minute and 1 minute incubation at 72 °C and 4 °C respectively.

If required, the restricted products are stored at 4 °C before visualizing on a gel. When required, mix 10 µl of reaction products with a running marker and run on a 2% gel. The expected fragment size is 260 bp. This fragment will not be visible if the PCR is done on a single teliospore, as there will not be enough DNA present.

4.3.4.2 Real-time five-plex fluorescent PCR assay for identification of PCR

Real-time PCR assays with the dual-labelled probes and oligonucleotide primers (Table 3) in 20 µL reactions in 0.1 ml microfuge tubes are performed in the Rotorgene 6000 instrument (Qiagen, Australia). The five-plex reaction mixture consists of 1X ImmoBuffer (Bioline6, UK, 5 mM MgCl2, 200 µM of each of the four deoxynucleotides dATP, dTTP, dCTP and dTTP, 1 U Immolase DNA Polymerase (Bioline6, UK) and 0.2 µM, 0.4 µM and 0.9 µM of each of the dual-labelled probes, the four forward primers and the four reverse primers respectively (Table 3). The template DNA is 1 µl of PCR product from the PCR amplification of Tilletia-specific DNA or a known DNA concentration of a Tilletia spp.

The thermal cycling parameters included an initial cycle of 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 60 s with the annealing temperature decreased by 1 °C per cycle for 6 cycles to 60 °C. Dynamic tube normalization option was used to determine the average background of each individual sample before the amplification. Fluorescence data was recorded to five channels: green, yellow, orange, red and crimson.

The sensitivity of the test for single spores was 10–40% (i.e. out of known positive T. indica spores only 10–40% gave positive PCR results) (Tan and Wright, 2009). This is because of a number of reasons, including all T. indica spores and bunted grain had to be autoclaved twice for the work to be done, so there may have been a loss in genetic material. The specificity of the probe for T. indica was investigated in a DNA mixture of T. indica, T. walkeri or T. ehrhartae or T. caries, in ratios of 1:0.1 pg and 0.1:1 pg (appropriate concentration range indicated from single-spore analysis). The specificity of the primers was found and not to react with other Tilletia species.

Table 3. Sequences and modifications of the primers and probes used in the multiplex diagnostic assay for T. indica and other related Tilletia spp.

<table>
<thead>
<tr>
<th>Primer pairs (sequence 5'-3')</th>
<th>Probes (modifications 5', 3')</th>
<th>Channel</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-DL-For: CTTCGGAAGAGTGCTCCTT (nt. 64–81)</td>
<td>ACCGAGAAAGGAGGCGC (nt. 105–120) (6-FAM, BHQ1)</td>
<td>Green</td>
<td>T. indica</td>
</tr>
<tr>
<td>KB-DL-Rev: CCGGAACGGGACTCG (nt. 127–142)</td>
<td>ACGGAAAGGAGGCC (nt. 67–82)</td>
<td>Yellow</td>
<td>T. walkeri</td>
</tr>
<tr>
<td>Hor-DL-For: GCCCAATCTTCTCTCAGTAC (nt. 87–102)</td>
<td>CAACCCAGAATCGAGGAGTGA (nt. 60–81) (CAL Fluor Red 610, BHQ2)</td>
<td>Orange</td>
<td>T. horrida</td>
</tr>
<tr>
<td>Hor-DL-Rev: CCGGGACGGGAGCATA (nt. 87–102)</td>
<td>AGAGGTCCGCTTATCCACCA (nt. 75–97) (Quasar 705, BHQ2)</td>
<td>Red</td>
<td>Broad range</td>
</tr>
<tr>
<td>Tri-DL-For: ATGGCGCCGTCTCTC (nt. 56–73)</td>
<td>GTAGTGCTTGTGGTTGGAATAG (nt. 99–112)</td>
<td>Quasar 670, BHQ2</td>
<td></td>
</tr>
<tr>
<td>Tri-DL-Rev: CCGCATTTATCTCTTG (nt. 72–90)</td>
<td>CAGACGGAGGCTTCTCGGAGGAC (nt. 104–126) (Quasar 705, BHQ2)</td>
<td>Quasar 705, BHQ2</td>
<td></td>
</tr>
<tr>
<td>Ehr-DL-For: CGCGATTTATGCTGTT (nt. 72–90)</td>
<td>Ehr-DL-Rev: GTTGAAGACCAAGCCATC (nt. 128–146)</td>
<td>Quasar 705, BHQ2</td>
<td></td>
</tr>
</tbody>
</table>

Notes: GenBank No. Af398434, Af310180, Af310171, Af398447, Ay770433. The list of the reference material and used and place of origin is in Tan et al. (2009) and material is held at EMU in Australia (refer to contacts list, section 6 of this diagnostic protocol). nt. = nucleotide.


5. Records

Please refer to section 2.5 in ISPM 27:2006 for the list of information that needs to be recorded and kept.
number of teliospores detected in each positive subsample. Cultures should be kept (mycelium from broths or mycelial plugs from agar plates can be stored frozen at −80 °C).

6. Contact Points for Further Information

Further information on this organism can be obtained from:

- Department of Agriculture and Food, Western Australia. South Perth 6151 Australia (Ms Domine Wright; email: domine.wright@agric.wa.gov.au; Tel: +61 8 9368 3875; Fax: +61 8 474 2658).
- NSW Dept. of Primary Industries, Camden, NSW, 2570 Australia (Dr Mui-Keng; email: mui-keng.tan@industry.new.gov.au).
- Laboratory of Plant Inspection and Quarantine, Shenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen City, 518045 Guangdong Province, China (Dr Guiming Zhang; email: zgm2001cn@yahoo.com.cn; Tel: +86 755 8211 1148; Fax: +86 755 2508 8630).
- USDA ARS NAA, Fort Detrick MD 21702 USA (Mr Gary Peterson; email: gary.peterson@ars.usda.gov).
- Food and Environment Agency, Sand Hutton, York YO41 1LZ, United Kingdom (Dr Kelvin Hughes; email: Kelvin.Hughes@fera.gov.uk).

7. Acknowledgements

This basis of this protocol was originally drafted by A.J. Inman, K.J.D. Hughes and R.J. Bowyer (2003), Food and Environment Agency, York, UK. That protocol was ring-tested in different European laboratories (Riccioli et al., 2002), and has formed the basis of the EPPO protocol PM 7/29(1) (EPPO, 2004).

The protocol has been enhanced by D.G. Wright, Department of Agriculture and Food, Western Australia, Australia; K.J.D Hughes, Food and Environment Agency, Sand Hutton, York, United Kingdom; and Guiming Zhang, Laboratory of Plant Inspection and Quarantine, Shenzhen City, China. V. Cockrell, Science and Advice for Scottish Agriculture, Edinburgh (United Kingdom) reviewed the protocol.

8. References


9. Figures
Figure 3. Flow diagram showing the process to be used for the detection and identification of Tilletia indica in grain samples.

[Secretariat notes that the reference to section 4.2.2 in the lower right-hand box should be 4.2.3; this will be modified before being sent for member consultation]
Figure 4. Pictorial key to teliospore ornamentation. Use in conjunction with Table 2 (section 4.1).

Photographs courtesy of Dr. Alan Inman, Central Science Laboratory, York.

Figure 5. Teliospores of Tilletia indica showing surface ornamentation patterns. Spines are densely arranged, either individually (densely echinulate) or in closely spaced, narrow ridges (finely cerebriform).
Figure 6. Teliospores of *Tilletia walkeri* showing surface ornamentation patterns. Spines are coarsely arranged and forming wide, incompletely cerebriform to coralloid ridges or thick clumps. Scale: 10 mm = 17 µm. Photographs courtesy of Dr Alan Inman, Central Sciences Laboratory, York.

Figure 7. Teliospores of *Tilletia horrida* showing surface ornamentation patterns. Polygonal scales or, occasionally, with cerebriform ridges. Scale: 10 mm = 17 µm. Photographs courtesy of Dr Alan Inman, Central Sciences Laboratory, York.
A: *Tilletia indica*

B: *Tilletia walkeri*

Figure 8. Teliospores of *Tilletia indica* (top) and *T. walkeri* (bottom) showing teliospore profiles in median view after bleaching and then staining with lactoglycerol-trypsin blue. Note the smoother outline on *T. indica* teliospores compared with the more irregular outline of *T. walkeri* teliospores with more obvious gaps between spines.

Figure 9. Colonies of *Tilletia indica* (right), *T. walkeri* (centre) and *T. horrida* (left) after 7 days (top), 10 days (centre) and 14 days (bottom) on PDA at 19 °C and a 12-hour dark/light cycle. Note slower growth, and purple pigmentation after 14 days, for *T. horrida* colonies.

Photographs courtesy of Dr Alan Inman, Central Sciences Laboratory, York.

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[Footnote 1] The term "seeds" is used in the rest of the protocol but it also represents "grain" in case a sample of a commodity class for seeds intended for processing or consumption is involved.

[Footnote 2] The use of products of the brand Applied Biosystems in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.
[Footnote 3] The use of products of the brand Promega in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[Footnote 4] The use of products of the brand Invitrogen in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[Footnote 5] The use of products of the brand QIAGEN in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[Footnote 6] The use of products of the brand Bioline in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[Footnote 7] A. Radova, State Phytosanitary Administration, Olomouc (CZ); I. Vloutoglou, Benaki Phytopathological Institute, Athens (GR); A. Porta-Puglia, Istituto Sperimentale per la Patologia Vegetale, Rome (IT); C. Montuschi, Servizio Fitosanitario Regionale, Bologna (IT); I. van Brouwershaven, NPPO, Wageningen (NL); M. de Jesus Gomes, E. Diogo & M.R. Malheiros, Direcção-Geral de Protecção das Culturas, Lisboa (PT); V. Cockerell, Science and Advice for Scottish Agriculture, Roddinglaw, Edinburgh (GB); A. Barnes, FERA, Sand Hutton, York (GB).