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International Plant Protection Convention
Protecting the world's plant resources from pests

ISPM 27
ANNEX 12

ENG

DIAGNOSTIC PROTOCOLS INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DP 12: Phytoplasmas

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ISPM 27
Diagnostic protocols for regulated pests

DP 12: Phytoplasmas

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1. Pest Information

Phytoplasmas were first discovered by Doi *et al.* (1967) during their search for the agent of aster yellows. The unicellular organisms were called mycoplasma-like organisms because of their morphological similarity to animal mycoplasmas and their sensitivity to tetracycline antibiotics (Ishiie *et al.*, 1967). Phytoplasmas are obligate prokaryotic plant pathogens that do not possess cell walls, and they are pleiomorphic in profile, with a mean diameter of 200–800 nm. They inhabit the phloem sieve cells of their plant hosts. Phytoplasmas have genomes ranging in size from around 550 to 1 500 kb – a relatively small genome compared with other prokaryotes – and they lack several biosynthetic functions (Marcone *et al.*, 1999; Davis *et al.*, 2005; Bai *et al.*, 2006; Oshima *et al.*, 2013).

Phytoplasmas are associated with a wide variety of symptoms in a diverse range of plant hosts (Lee *et al.*, 2000). Characteristic symptoms associated with phytoplasma infection include virescence (the development of green flowers and the loss of normal flower pigments); phyllody (the development of floral parts into leafy structures); witches' broom (proliferation of auxiliary or axillary shoots) and other abnormal proliferation of shoots and roots; foliar yellowing, reddening and other discoloration; reduced leaf and fruit size; phloem necrosis; and overall decline and stunting (Davis and Sinclair, 1998). Some plant species are tolerant or resistant to phytoplasma infections; when infected, these plants may be asymptomatic or exhibit mild symptoms (Lee *et al.*, 2000).

Seemüller *et al.* (2002) estimated that about 1 000 plant species are affected by phytoplasmas. Most of the phytoplasma host plants are dicotyledons. Fewer phytoplasmas have been detected in monocotyledons; such hosts are mainly from the Palmae and Poaceae families (Seemüller *et al.*, 2002).

Phytoplasmas occur worldwide. The geographical distribution and impact of phytoplasma diseases depends on the host range of the phytoplasma as well as on the presence and the feeding behaviour of the insect vector. Some phytoplasmas have a broad range of plant hosts and polyphagous vectors and therefore have a wide distribution. Other phytoplasmas have restricted host ranges and oligophagous or monophagous insect vectors, which restrict their geographical distribution. For a review of the geographic distribution of the main phytoplasma taxonomic groups, see Foissac and Wilson (2010).

Phytoplasmas can be transmitted by insect vectors, dodders and grafting and can be spread by vegetative propagation of infected plant parts. Insect vectors of phytoplasmas, responsible for much of their natural spread, are restricted to phloem-feeding leafhoppers, plant hoppers and psyllids (Hemiptera, Auchenorrhyncha). They transmit the pathogen in a persistent manner. Weintraub and Beanland (2006) list more than 90 species that are known to be vectors, some of which are capable of vectoring more than one phytoplasma. Other methods of transmission of phytoplasmas include dodder and graft transmission. Dodders (*Cuscuta* and *Cassytha* spp.) are parasitic vines that develop vascular connections with their hosts through haustoria. When a bridge is established between a healthy plant and a phytoplasma-infected plant, the phytoplasma will transfer to the healthy plant via the connecting phloem elements. Graft transmission and micropropagation of plants in tissue culture can be used to maintain phytoplasmas for reference purposes (IPWG, n.d.).

Further information on phytoplasmas, including photos showing disease symptoms, a list of insect vectors and a phytoplasma classification database, can be found at the following websites: COST Action FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems (http://www.costphytoplasma.ipwgnet.org/) and Phytoplasma Resource Center (http://plantpathology.ba.ars.usda.gov/phytoplasma.html).

2. Taxonomic Information

Name: Phytoplasma

Synonyms: Mycoplasma-like organism (MLO), mycoplasma

Taxonomic position: Bacteria, Firmicutes, Mollicutes, Acholeplasmatales, Acholeplasmataceae, '*Candidatus* Phytoplasma'

The International Research Programme on Comparative Mycoplasmology (IRPCM) Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group has published guidelines for the description of '*Candidatus* (*Ca.*) Phytoplasma' species (IRPCM, 2004). Delineation of '*Ca.* Phytoplasma' species is based on 16S ribosomal (r)RNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species are ≥97.5% identical over ≥1 200 nucleotides of their 16S rRNA gene. When a '*Ca.*' species includes phytoplasmas with different biological characteristics (vectors and host plants) they can be taxonomically distinguished following specific rules reported in IRPCM (2004). Descriptions of '*Ca.* Phytoplasma' species are published in the *International Journal of Systematic and Evolutionary Microbiology* and as of March 2015, 37 '*Ca.* Phytoplasma' species have been described.

3. Detection and Identification

Polymerase chain reaction (PCR) techniques are the method of choice for phytoplasma detection. Successful molecular detection of phytoplasmas is dependent on appropriate sampling of plant tissue and reliable nucleic acid extraction methods (Palmano, 2001; Firrao *et al.*, 2007). Phytoplasmas can be unevenly distributed and in an uneven titre throughout a plant, particularly in woody hosts, and symptomatic tissue is optimal for phytoplasma detection (Constable *et al.*, 2003; Garcia-Chapa *et al.*, 2003; Christensen *et al.*, 2004; Necas and Krska, 2006). Symptomless infection can occur in some plant hosts and if this is suspected it is important to thoroughly sample different tissues of the plant.

Phytoplasma titre in the plant host affects the reliability of the PCR test (Marzachì, 2004). Phytoplasma titre can be affected by phytoplasma strain or species, host plant species, timing of infection and climatic conditions. The timing for sampling plant tissues is important as location in the plant and titre of phytoplasmas may be affected by seasonal changes (Seemüller *et al.*, 1984; Jarausch *et al.*, 1999; Berges *et al.*, 2000; Constable *et al.*, 2003; Garcia-Chapa *et al.*, 2003; Prezelj *et al.*, 2012).

For most phytoplasma diseases, leaves with symptoms are the best sources of samples for diagnosis. Phytoplasmas reside in the phloem sieve elements of infected plants and therefore the leaf petioles and midveins, stems or inner bark are often used for DNA extraction. In some cases (e.g. X-disease phytoplasma), fruit peduncles contain the highest phytoplasma titre (Kirkpatrick, 1991). Although phytoplasmas can be detected in roots and bark scrapings of dormant trees, generally it is best to test for phytoplasmas at the end of summer. Collected plant samples can be stored at −20 °C for up to six months before testing. Longer term storage is at −80 °C, or the plant material can be freeze-dried or dried over calcium chloride and stored at 4 °C.

Various nucleic acid extraction methods have been reported for phytoplasma detection by PCR. A number of methods use an enrichment step to concentrate the phytoplasmas before nucleic acid extraction (Kirkpatrick *et al.*, 1987; Ahrens and Seemüller, 1992; Prince *et al.*, 1993). These techniques can be useful for hosts in which phytoplasmas are found in low titre, such as woody perennial plants, or for "difficult" hosts from which high levels of compounds such as polysaccharides and polyphenols that can inhibit PCR are often co-extracted with the nucleic acid. In some simplified methods, plant tissue is ground directly in a commercially available lysis buffer or in cetyl trimethylammonium bromide (CTAB)-based buffer. Typically, a 2% CTAB buffer is used (it has been shown that a 3% solution is more reliable for grapevines) (Daire *et al.*, 1997; Angelini *et al.*, 2001). The DNA is then extracted directly from the lysate using commercially available silica spin columns (Green *et al.*, 1999; Palmano, 2001) or magnetic beads (Mehle *et al.*, 2013), or with organic solvents

(Daire *et al*., 1997; Zhang *et al*., 1998). The method of using magnetic beads is generally performed on an automated nucleic acid extraction instrument (e.g. KingFisher from Thermo Scientific[1]). Most extraction methods are well validated for a variety of plant host species. The choice of method is dependent on the host being tested and the availability of facilities and equipment. It may be practical to use a method incorporating a phytoplasma enrichment step for woody perennial hosts and a simplified method for herbaceous hosts. For routine diagnostics it is important to validate an extraction method for a particular host to ensure reliability.

A number of universal PCR primers have been designed that allow amplification of the 16S rRNA gene of any known phytoplasma. The most commonly used primers are the P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and R16F2n/R16R2 (Lee *et al*., 1993; Gundersen and Lee, 1996) primer pairs, which can be used in a nested PCR protocol. The P1/P7 primer pair amplifies a PCR product that contains the entire 16S rRNA gene as well as the 16S/23S rRNA spacer region. Real-time PCR has been reported to be more than or as sensitive as nested PCR, depending on the host–phytoplasma combination (Christensen *et al.*, 2004), and is more amenable to high throughput analysis because post-amplification processing is not required. Real-time PCR using TaqMan probes is also more specific and there is less chance of cross-contamination than with conventional PCR, especially nested PCR. False positives with closely related bacteria can occur with the PCR assays recommended in this protocol – a necessary compromise for a universal assay (Fránová, 2011; Pilotti *et al*., 2014). It is possible to run more specific PCR assays or if the outcome is critical (e.g. post-entry quarantine samples, new host record, new distribution), the conventional PCR product should be sequenced.

As well as amplification of the 16S rRNA gene, PCR methods have also been used to amplify other genome regions for phytoplasma detection and classification, including ribosomal protein genes (Lim and Sears, 1992; Jomantiene *et al.*, 1998; Lee *et al*., 1998; Martini *et al*., 2007), the *tuf* gene (Schneider *et al*., 1997; Makarova *et al*., 2012), the 23S rRNA gene (Guo *et al*., 2003) and the *secY* gene (Lee *et al*., 2010; Davis *et al*., 2013; Quaglino *et al*., 2013). These primers may be useful when a second independent region of the phytoplasma genome is required.

Samples may contain compounds that are inhibitory to PCR depending on the host species and type and age of the tissue. Therefore it is important to check the PCR competency of the DNA extractions using internal control primers that amplify a gene from the plant host. Inhibitory effects of the host can be overcome by further purifying the DNA through a sephacryl spin column or by adding bovine serum albumin (BSA) to the PCR mixture to a final concentration of 0.5 mg/ml (Kreader, 1996).

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Conventional nested PCR

The PCR primers used in this assay are P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al*., 1995) for the first-stage PCR:

P1 (forward): 5′-AAG AGT TTG ATC CTG GCT CAG GAT T-3′

P7 (reverse): 5′-CGT CCT TCA TCG GCT CTT-3′

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

The second-stage PCR primers are R16F2n (Gundersen and Lee, 1996) and R16R2 (Lee *et al*., 1993):

R16F2n (forward): 5′-GAA ACG ACT GCT AAG ACT GG-3′

R16R2 (reverse): 5′-TGA CGG GCG GTG TGT ACA AAC CCC G-3′

The 20 µl reaction mixture consists of 1× Taq DNA polymerase buffer containing 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM dNTPs, 1 U Taq DNA polymerase and 2 µl DNA template. The amplification conditions are an initial denaturation step of 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 53 °C (P1/P7 primers) or 50 °C (R16F2n/R16R2 primers) for 30 s and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. For nested PCR, 1 µl of the first-stage PCR products is used either undiluted or at up to a 1:30 dilution as the template for the second-stage PCR. The PCR products are analysed by gel electrophoresis. The P1/P7 and R16F2n/R16R2 primers produce a 1 800 base pair (bp) and 1 250 bp amplicon, respectively.

The presence of PCR-competent DNA in the extracts is confirmed using the universal eukaryotic 28S rRNA gene primers of Werren *et al*. (1995):

28Sf (forward): 5′-CCC TGT TGA GCT TGA CTC TAG TCT GGC-3′

28Sr (reverse): 5′-AAG AGC CGA CAT CGA AGG ATC-3′

The reaction mixture for the 28S rRNA assay has the same components and is cycled under the same conditions as the phytoplasma assay, so that the two assays can be run simultaneously in separate tubes. The 28Sf/28Sr primer pair produces a 500–600 bp amplicon.

Other primer pairs can also be used to check that the DNA is PCR-competent.

3.2 Real-time PCR

Real-time PCR is performed using the TaqMan assay designed for the 16S rRNA gene by Christensen *et al.* (2004):

Forward primer: 5′-CGT ACG CAA GTA TGA AAC TTA AAG GA-3′

Reverse primer: 5′-TCT TCG AAT TAA ACA ACA TGA TCC A-3′

TaqMan probe: 5′-FAM-TGA CGG GAC TCC GCA CAA GCG-BHQ-3′

Alternatively, the real-time PCR of Hodgetts *et al*. (2009) designed for the 23S rRNA gene can be used:

JH-F 1 (forward primer): 5′-GGT CTC CGA ATG GGA AAA CC-3′

JH-F all (forward primer): 5′-ATT TCC GAA TGG GGC AAC C-3′

JH-R (reverse primer): 5′-CTC GTC ACT ACT ACC RGA ATC GTT ATT AC-3′

JH-P uni (TaqMan probe): 5′-FAM-MGB-AAC TGA AAT ATC TAA GTA AC-BHQ-3′

The 25 µl reaction mixture consists of 1× TaqMan real-rime PCR master mix, 300 nM forward primer, 300 nM reverse primer, 100 nM FAM probe and 2 µl DNA template. All samples are tested in duplicate. The amplification conditions are an initial denaturation step of 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. These cycling conditions may vary depending on the type of master mix used (e.g. some mixes require a polymerase activation step at 95 °C for 10 min and mixes that contain uracil-DNA glycosylase (UDG) require an initial hold at 50 °C for 2 min). Real-time PCR results are analysed with the manufacturer's software provided with the instrument.

The real-time PCR assay of Christensen *et al*. (2004) uses 900 nM of the reverse primer, and this was updated to 300 nM in a later report (Christensen *et al*., 2013). This assay will work equally well with either concentration of reverse primer.

The 16S rRNA real-time PCR method was evaluated by testing phytoplasmas from 18 subgroups and was found to be as sensitive as or up to ten times more sensitive than conventional nested PCR, depending on the host–phytoplasma combination (Christensen *et al.*, 2004). A ring test for the detection of fruit tree phytoplasmas involving 22 laboratories suggested that the Christensen *et al.* (2004) and Hodgetts *et al.* (2009) assays are similar in terms of sensitivity and specificity (EUPHRESCO FruitPhytoInterlab Group, 2011).

The presence of PCR-competent DNA in the extracts is confirmed using the COX assay of Weller *et al.* (2000), which amplifies the cytochrome oxidase gene:

COX-F (forward primer): 5′-CGT CGC ATT CCA GAT TAT CCA-3′

COX-R (reverse primer): 5′-CAA CTA CGG ATA TAT AAG AGC CAA AAC TG-3′

COX-P (TaqMan probe): 5′-FAM-TGC TTA CGC TGG ATG GAA TGC CCT-BHQ-3′

Alternatively, the 18S rRNA gene assay of Christensen *et al.* (2004) can be used to confirm that the DNA is PCR-competent and is recommended for monocotyledons, for which the COX assay is less efficient:

Forward primer: 5′-GAC TAC GTC CCT GCC CTT TG-3′

Reverse primer: 5′-AAC ACT TCA CCG GAC CAT TCA-3′

TaqMan probe: 5′-FAM-ACA CAC CGC CCG TCG CTC C-BHQ-3′

The reaction mixtures for the COX and the 18S rRNA gene assays have the same components and are cycled under the same conditions as the phytoplasma real-time assay, so that the two assays can be run simultaneously in separate tubes. Alternatively, the internal control assay can be multiplexed in the same tube as the phytoplasma assay if the probe is labelled with a different reporter dye and the primer and probe concentrations have been optimized to prevent low phytoplasma levels being outcompeted by high levels of plant DNA used as the internal control.

3.3 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest nucleic acid. For PCR a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Phytoplasma DNA extracted from an infected plant, whole genome amplified DNA or a synthetic control (e.g. a cloned PCR product) may be used.

Internal control. For conventional and real-time PCR, a plant housekeeping gene such as the universal eukaryotic 28S rRNA gene (see section 3.1 for its use in the conventional nested PCR) or the COX gene (see section 3.2 for its use in the real-time PCR) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Positive extraction control. This control is used to ensure that phytoplasma nucleic acid is of sufficient quantity and quality for PCR and that the pathogen is detected. Phytoplasma DNA is extracted from infected host tissue or healthy plant tissue that has been spiked with the phytoplasma.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. If bulking of samples is done, then the quantity of positive control should be adjusted accordingly (e.g. ten lots of 20 mg sample bulked for DNA extraction, 2 mg infected leaf + 198 mg healthy plant tissue). If the positive control is not detected then the test should be repeated or the bulking rate reduced until reliable detection is achieved.

For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. The positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control may be the extraction buffer or may comprise a nucleic acid that is extracted from uninfected host tissue and subsequently amplified. In cases where large numbers of positive samples are expected, it is recommended that negative extraction controls are included between the samples for testing.

3.4 Interpretation of results from PCR

3.4.1 Conventional nested PCR

The pathogen-specific PCR will be considered valid only if:

- the positive control produces the correct size amplicon for the target pathogen
- the negative extraction control and the negative amplification control produce no amplicons of the correct size for the target pathogen.

For internal controls targeting plant DNA, the healthy control (if used), positive control and each of the test samples must produce the amplicon of the expected size. Failure of the samples to amplify with the internal control primers suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.

The test on a sample will be considered positive if it produces an amplicon of the correct size. To identify the phytoplasma present in positive samples, the amplicon will need to be sequenced (see section 3.5). In some cases, more specific PCR assays are available.

3.4.2 Real-time PCR

Real-time PCR will determine if a sample is positive or negative for phytoplasma. To identify the phytoplasma present in positive samples, a conventional PCR will need to be performed to obtain at least the 1 250 bp length of the 16S rRNA gene generated from the R16F2n/R16R2 primer pair for sequence analysis (see section 3.5). Alternatively, for some phytoplasmas it may be possible to use specific real-time PCR assays; for example, 16SrX (apple proliferation) group (Torres *et al*., 2005) and flavescence dorée (Pelletier *et al*., 2009).

3.5 Sequence analysis

PCR products should be sequenced either directly or by first cloning them into a PCR cloning vector. Sequence data can be analysed using the Basic Local Alignment Search Tool, BLASTN, available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). If the sequence shares less than 97.5% identity with its closest relative, the phytoplasma is considered to be a new '*Ca.* Phytoplasma' species. In this case, the entire 16S rRNA gene should be sequenced and phylogenetic analysis performed. Sequencing a separate region of the genome such as the 16S/23S rRNA spacer region, *secY* gene, ribosomal protein genes or the *tuf* gene is also desirable.

4. Records

Records and evidence should be retained as described in ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the phytoplasma is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- The original sample, kept frozen at −80 °C, or freeze-dried or dried over calcium chloride and kept at 4 °C.
- If relevant, DNA extractions should be kept at −20 °C or at −80 °C. Plant extracts spotted on membranes should be kept at room temperature.
- If relevant, PCR amplification products should be kept at −20 °C or at −80 °C.

5. Contact Points for Further Information

Further information on this protocol can be obtained from:

Plant Health and Environment Laboratory, Ministry for Primary Industries, PO Box 2095, Auckland 1140, New Zealand (Lia W. Liefting; e-mail: lia.liefting@mpi.govt.nz; tel.: +64 9 9095726; fax: +64 9 9095739).

Department of Economic Development, Jobs, Transport and Resources, Victoria, AgriBio, 5 Ring Road, Bundoora, VIC 3083, Australia (Fiona Constable; e-mail: fiona.constable@ecodev.vic.gov.au; tel.: +61 3 9032 7326; fax: + 61 3 9032 7604).

Department of Territory and Sustainability, Av. Diagonal 525, 08029 Barcelona, Spain (Ester Torres; e-mail: ester.torres@gencat.net).

Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection and Fruit Crops, Schwabenheimer Str. 101, D-69221 Dossenheim, Germany (Wilhelm Jelkmann; e-mail: wilhelm.jelkmann@jki.bund.de).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

6. Acknowledgements

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7. References

The present annex refers to international standards for phytosanitary measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

International Plant Protection Convention (IPPC)

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