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Rules Proposals for the International Rules for Seed Testing 2025 Edition

This document was prepared by the Technical Committees (TCOMs) and the Rules Committee of the Association and has been endorsed by the ISTA Executive Committee (ECOM). The proposals are submitted to the ISTA Ordinary General Meeting 2024 for voting by the nominated ISTA Designated Members on behalf of their respective Governments.

It is submitted to all ISTA Designated Authorities, ISTA Members and ISTA Observer Organizations for information two months prior to the ISTA Ordinary General Meeting 2024.

It contains proposed amendments and changes for the ISTA *International Rules for Seed Testing* and will be discussed and voted on at the Ordinary General Meeting 2024 to be held on 04 July, 2024, in Cambridge, United Kingdom.

Introduction to the ISTA Rules Proposals to become effective 1 January 2025

The current version of the ISTA International Rules for Seed Testing (ISTA Rules) is the 2024 edition.

The ISTA Rules are only available electronically as a printable pdf file and are available for free download by ISTA members from the Ingenta website: http://www.ingentaconnect.com/content/ista/rules

The electronic version also includes the French, German, and Spanish versions of the ISTA Rules. If there are any questions on interpretation of the ISTA Rules the English version is the definitive version.

For further information on the ISTA Rules, see: https://www.seedtest.org/en/publications/international-rules-seed-testing.html

The effective dates are changed annually. The changes from the previous edition of the ISTA Rules can be displayed as yellow highlighted text as a 'layer' within the electronic copy with comments on what has changed.

The ISTA Rules are the result of the work of the ISTA Technical Committees (TCOMs) with input from many different sources. Thanks go to all the Technical Committee members and the ISTA Secretariat for their help with the annual proposals.

The following Rules Proposals will be discussed at the ISTA Ordinary General Meeting in Cambridge, United Kingdom on 04 July, 2024, and may be amended without changing the intent of the proposal. If the proposals are accepted by the membership, amendments will be issued, and they will become the 2025 edition of the ISTA Rules.

Please let us know about any problems with these proposals.

Many thanks.

Ernest Allen and Sue Alvarez

Chair and Vice-Chair of ISTA Rules Committee

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Key to text changes:

~~Deleted text~~

New text

New text in large blocks, not underlined for ease of reading

Any changes made after the proposals were published to the membership

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PART A. INTRODUCTION OF EDITORIAL CHANGES

A.1. Editorial corrections

Editorial change required due to inconsistency in guidance between section 1.5.2.3 and other sections throughout the rules regarding reporting to calculated totals to one hundred percent.

This proposal was submitted by an ISTA member laboratory and approved by the ISTA rules committee.

CURRENT VERSION	PROPOSED VERSION
1.5.2.3 Purity tests on coated seeds The result of a purity test on coated seeds must be reported as follows: • Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered. • The results must be reported to one decimal place, and the percentage of all components must total 100 %. Components amounting to less than 0.05 % must be reported as 'Trace' or 'TR' (for 'Trace'). If no inert matter or other seeds are found, this must be reported as '0.0'. • In the case of pelleted seeds only, ….	**1.5.2.3 Purity tests on coated seeds** The result of a purity test on coated seeds must be reported as follows: • Following the species name, the words 'seed pellets', 'encrusted seeds', 'seed granules', 'seed tapes' or 'seed mats', as applicable, must be clearly entered. • The results must be reported to one decimal place, and the percentage of all components must total 100.0 %. Components amounting to less than 0.05 % must be reported as 'Trace' or 'TR' (for 'Trace'). If no inert matter or other seeds are found, this must be reported as '0.0'. • In the case of pelleted seeds only, ….

When going through the new rules 2024, it was noticed that there is a small error in Chapter 1 p 1-9. In the sentence on how to report moisture of pelleted seed 'seed' is missing after '… that of the combined unit comprising seed and pelleting material. Chapter 9 of moisture p 9-9 is fine.

CURRENT VERSION	PROPOSED VERSION
1.5.2.12 Moisture content	**1.5.2.12 Moisture content**

• In the case of pelleted seeds (see Chapter 11), the following statement must be entered: 'The seeds of the submitted moisture sample were pelleted, and the moisture content reported is that of the combined unit comprising and pelleting material.'	• In the case of pelleted seeds (see Chapter 11), the following statement must be entered: 'The seeds of the submitted moisture sample were pelleted, and the moisture content reported is that of the combined unit comprising seed and pelleting material.'

Wording updated to be clear that the information in the ISTA Handbook on Seedling Evaluation is a guide and not the ISTA Rules. Update requested by the ISTA Accreditation Department.

This proposal is supported by the Germination Committee.

CURRENT VERSION	PROPOSED VERSION
5.4.5 Quality control	**5.4.5 Quality control**
New deliveries of growing media must meet the requirements for the principal physical characteristics and be free of negative effects due to toxic substances or noxious micro-organisms. The characteristics composition, water retention, pH, cleanness and innocuity (freedom from phytotoxic effects and negative effects due to micro-organisms) must be checked. Alternative measurements: it may be difficult to check all the specifications or to get growing media from suppliers with the requested specifications. It is permissible to replace the measurements of pH and conductivity with biological tests, such as a test for phytotoxicity. Examples of media quality control tests are given in the *ISTA Handbook on Seedling Evaluation*. Quality control tests can be performed by the seed testing laboratory or subcontracted to laboratories specialising in soil analyses or microbiology tests.	New deliveries of growing media must meet the requirements for the principal physical characteristics and be free of negative effects due to toxic substances or noxious micro-organisms. The characteristics composition, water retention, pH, cleanness and innocuity (freedom from phytotoxic effects and negative effects due to micro-organisms) must be checked. Alternative measurements: it may be difficult to check all the specifications or to get growing media from suppliers with the requested specifications. It is permissible to replace the measurements of pH and conductivity with biological tests, such as a test for phytotoxicity. Examples of media quality control tests are given in the *ISTA Handbook on Seedling Evaluation*. These examples may be used as a non-obligatory guide for laboratories to meet the requirements of the ISTA Rules. Quality control tests can be performed by the seed testing laboratory or subcontracted to laboratories specialising in soil analyses or microbiology tests.

Editorial correction to make clear that top of sand (TS) and top of organic growing medium (TO) cannot be used if not prescribed in Table 5A. This came from discussion between the ISTA Accreditation Department and the Germination Committee.
This is supported by the Germination Committee.

CURRENT VERSION	PROPOSED VERSION
5.6.2.1.2 Methods using sand or organic growing media Sand and organic growing media are used as follows: Top of sand (TS), top of organic growing medium (TO): the seeds are pressed into the surface of the sand or the organic growing medium. Sand (S), organic growing medium (O): the seeds are planted on a level layer of moist sand or the organic growing medium and covered with 10–20 mm of uncompressed substrate, depending on the size of the seed. To ensure good aeration it is recommended that the bottom layer be loosened by raking before sowing. Sand or organic growing media may be used instead of paper, even if not prescribed in Table 5A: • when the evaluation of a diseased sample proves impracticable because of the spread of infection between seeds and seedlings on paper substrate; • for investigative purposes and to confirm evaluation of seedlings in cases of doubt; • when seedlings show phytotoxic symptoms.	**5.6.2.1.2 Methods using sand or organic growing media** Sand and organic growing media are used as follows: Top of sand (TS), top of organic growing medium (TO): the seeds are pressed into the surface of the sand or the organic growing medium. TS and TO must not be used instead of sand (S) or organic growing media (O) substrates unless they are prescribed in Table 5A. Sand (S), organic growing medium (O): the seeds are planted on a level layer of moist sand or the organic growing medium and covered with 10–20 mm of uncompressed substrate, depending on the size of the seed. To ensure good aeration it is recommended that the bottom layer be loosened by raking before sowing. Sand or organic growing media may be used instead of paper, even if not prescribed in Table 5A: • when the evaluation of a diseased sample proves impracticable because of the spread of infection between seeds and seedlings on paper substrate; • for investigative purposes and to confirm evaluation of seedlings in cases of doubt; • when seedlings show phytotoxic symptoms.

Editorial change to make the rules clearer when information is requested on other categories of ungerminated seeds, ISTA Rules, 5.6.5.3, Ungerminated seeds.

This change was requested by a member and is supported by the Germination Committee.

CURRENT VERSION	PROPOSED VERSION
5.6.5.3 Ungerminated seeds **…..** **Other categories:** Upon request of the customer, the number of empty, embryoless or insect-damaged seeds may be determined and reported under 'Other determinations' on the ISTA Certificate. To detect these other categories of seeds, the following methods may be used: Before the germination test: • X-ray test, which is conducted on the replicates used for the germination test; • cutting test, which is performed on four separate replicates of 100 seeds, soaked for up to 24 h at room temperature. Each seed is cut along its longitudinal axis and the content examined and classified as full, empty, embryoless or insect-damaged; ~~b. After the germination test:~~ • cutting test or X-ray test of apparently fresh ungerminated seeds. ….	**5.6.5.3 Ungerminated seeds** **…..** **Other categories:** Upon request of the customer, the number of empty, embryoless or insect-damaged seeds may be determined and reported under 'Other determinations' on the ISTA Certificate. To detect these other categories of seeds, the following methods may be used: a. Before the germination test: X-ray test, which is conducted on the replicates used for the germination test; b. Independent of/additional to a germination test: cutting test, which is performed on four separate replicates of 100 seeds, soaked for up to 24 h at room temperature. Each seed is cut along its longitudinal axis and the content examined and classified as full, empty, embryoless or insect-damaged; c. End of the germination test: cutting test or X-ray test of apparently fresh ungerminated seeds. ….

Editorial change to correct reference in Section 5.7(e) due to rules change approved at the 2023 OGM. In 2023, the letter (a) was deleted in this section which required the remaining sections to be renumbered. As a result, the reference in 5.7(e) must be corrected to reflect the change.

This change was requested by an ISTA member laboratory and approved by the ISTA rules committee.

CURRENT VERSION	PROPOSED VERSION
5.7 Retesting **…..** e. When the range for the replicates exceeds the maximum tolerated range in Table 5B, a retest must be carried out using the same test method or an alternative method. If the results of the retest using the same method are compatible with the first (i.e. the difference does not exceed the tolerance indicated in either Table 5C, 5D or 5E), the average of the test results must be reported on the ISTA Certificate (see 5.8.1 Tolerances). If an alternative method is used and if the results are better and within accepted tolerances, then these results must be reported on the ISTA Certificate (see 5.8.1 Tolerances) and must not be averaged with the previous test results. When retesting is carried out under the circumstances a., b., ~~c.~~ or ~~e.~~, the best results achieved must be indicated on the ISTA Certificate. The results of the other tests do not have to be reported on the ISTA Certificate, except on specific request by the applicant. f. When due to counting errors…	**5.7 Retesting** **…..** e. When the range for the replicates exceeds the maximum tolerated range in Table 5B, a retest must be carried out using the same test method or an alternative method. If the results of the retest using the same method are compatible with the first (i.e. the difference does not exceed the tolerance indicated in either Table 5C, 5D or 5E), the average of the test results must be reported on the ISTA Certificate (see 5.8.1 Tolerances). If an alternative method is used and if the results are better and within accepted tolerances, then these results must be reported on the ISTA Certificate (see 5.8.1 Tolerances) and must not be averaged with the previous test results. When retesting is carried out under the circumstances a., b., or d., the best results achieved must be indicated on the ISTA Certificate. The results of the other tests do not have to be reported on the ISTA Certificate, except on specific request by the applicant. f. When due to counting errors…

Editorial changes needed to update species names and processes throughout several methods within the Seed Health Chapter.

Changes approved by majority vote of the SHC.

Method 7-001a Critical control points (CCP) Samples may be difficult to examine due to the growth of contaminants, especially *Alternaria tenuis*, and/or *A. radicina*. Experience and great care is required for the detection of all occurrences (ISTA 1984) (Step 7). Methods 7. Examine seeds under a stereoscopic microscope at ×30 for fungal growth and up to ×80 for identification of conidia. Compare with positive control. Record the number of infected seeds in each plate. Conidiophores are simple or occasionally branched, arising usually singly from the surface of the seed, on the emerging radicle or on aerial mycelium (Fig. 1). Conidia are produced singly or in ~~chains~~ of 2 or rarely 3, ellipsoidal or barrel shaped, with little evidence of beak, up to 75 µm long, olivaceous brown, (Ellis, 1971). Under the stereoscopic microscope, conidia appear blackish and glossy (Fig. 1). Methods 5. Examine plates visually, and under a stereoscopic microscope at ×30 magnification, for fungal growth. Use a magnification of ×50 to ×80 for identification of conidia. Colonies of Alternaria radicina are irregular to circular with luxurious aerial mycelium, dark olive grey to greyish-black from above, bluish-black from below (Meier, *et al.,* 1922). Conidiophores are simple or occasionally branched, arising usually singly from the surface of the seed, on the emerging radicle or on aerial mycelium (Fig. 1). Conidia are produced singly or in ~~chains~~ of 2, or rarely 3, ellipsoidal or barrel shaped, with little evidence of beak, up to 75 µm long, olivaceous brown, (Ellis, 1971). Under the stereoscopic	Method 7-001a Critical control points (CCP) Samples may be difficult to examine due to the growth of contaminants, especially *Alternaria tenuis / alternata*, and/or *A. radicina*. Experience and great care is required for the detection of all occurrences (ISTA 1984) (Step 7). Methods 7. Examine seeds under a stereoscopic microscope at ×30 for fungal growth and up to ×80 for identification of conidia. Compare with positive control. Record the number of infected seeds in each plate. Conidiophores are simple or occasionally branched, arising usually singly from the surface of the seed, on the emerging radicle or on aerial mycelium (Fig. 1). Conidia are produced singly or in groups of 2 or rarely 3, ellipsoidal or barrel shaped, with little evidence of beak, up to 75 µm long, olivaceous brown, (Ellis, 1971). Under the stereoscopic microscope, conidia appear blackish and glossy (Fig. 1). Methods 5. Examine plates visually, and under a stereoscopic microscope at ×30 magnification, for fungal growth. Use a magnification of ×50 to ×80 for identification of conidia. Colonies of *Alternaria radicina* are irregular to circular with luxurious aerial mycelium, dark olive grey to greyish-black from above, bluish-black from below (Meier, *et al.,* 1922). Conidiophores are simple or occasionally branched, arising usually singly from the surface of the seed, on the emerging radicle or on aerial mycelium (Fig. 1). Conidia are produced singly or in groups of 2, or rarely 3, ellipsoidal or barrel shaped, with little evidence of beak, up to 75 µm long, olivaceous brown, (Ellis, 1971). Under the stereoscopic

microscope, conidia appear blackish and glossy (Fig. 1). Compare with positive control. Record the number of infected seeds in each plate (*CCP*).	microscope, conidia appear blackish and glossy (Fig. 1). Compare with positive control. Record the number of infected seeds in each plate (*CCP*).
Method 7-004 7-004: Detection of *Leptosphaeria maculans* and *Plenodomus biglobosus* in *Brassica* spp. Seed	Method 7-004 7-004: Detection of *Leptosphaeria maculans* (*Plenodomus lingam*) and *Plenodomus biglobosus* in *Brassica* spp. seed
Pathogen(s): *Leptosphaeria maculans* (Tode ex Fr.) Ces. & de Not (previously *Phoma lingam*) or *Plenodomus biglobosus* (Shoemaker & H. Brun) (previously *Leptosphaeria biglobosa*).	Pathogen(s): *Leptosphaeria maculans* (Tode ex Fr.) Ces. & de Not (previously *Phoma lingam*) New, *Plenodomus lingam* (Tode) Höhnel or *Plenodomus biglobosus* (Shoemaker & H. Brun) (previously *Leptosphaeria biglobosa*).
Background *Phoma lingam* is no longer the officially accepted name for the causal agent of black leg, stem canker and dry rot in *Brassica* species and other crucifers, the currently correct pathogen names are *Leptosphaeria maculans*(Tode ex Fr.) Ces. & de Not and *Plenodomus biglobosus* (Shoemaker & H. Brun) (previously *Leptosphaeria biglobosa*). *Leptosphaeria maculans*) has historically colonised countries where *Plenodomus biglobosus* is prevalent, such as Poland and central Canada (Fitt *et al*., 2008). PCR and pathogenicity testing were performed during the comparative test (Orgeur *et al*., 2015) to distinguish *L. maculans* from *P. biglobosus*.	Background *Phoma lingam* is no longer the officially accepted name for the causal agent of black leg, stem canker and dry rot in *Brassica* species and other crucifers, the currently correct pathogen names are *Leptosphaeria maculans* (Tode ex Fr.) Ces. & de Not (*Plenodomus lingam* (Tode) Höhnel) and *Plenodomus biglobosus* (Shoemaker & H. Brun) (previously *Leptosphaeria biglobosa*). *Leptosphaeria maculans* (*Plenodomus lingam*) has historically colonised countries where *Plenodomus biglobosus* is prevalent, such as Poland and central Canada (Fitt *et al*., 2008). PCR and pathogenicity testing were performed during the comparative test (Orgeur *et al*., 2015) to distinguish *L. maculans* (*Plenodomus lingam*) from *P. biglobosus*.
Materials	Materials Reference material: known strain of *Leptosphaeria maculans* (*Plenodomus lingam*),

Reference material: known strain of *Leptosphaeria maculans*, or *Plenodomus biglobosus* or standardised reference material	or *Plenodomus biglobosus* or standardised reference material
Method 7-005 Host: *Pisum sativum* L.~~s.l.~~	Method 7-005 Host: *Pisum sativum* L.
Materials *Media:* malt agar or potato dextrose agar	Materials *Media:* malt agar + streptomycin or potato dextrose agar + streptomycin
Petri dishes: When sowing density is given by ~~a~~ number of seeds per Petri dish, a diameter of 90 mm is assumed.	*Petri dishes:* When sowing density is given by the number of seeds per Petri dish, a diameter of 90 mm is assumed.
Methods 4. Under stereomicroscope (STM) at ×20–25 magnification, using both transmitted and incident light, hyphae are curled, often several running together, typically with moisture drops (although these evaporate easily) (Fig. 2).	Methods 4. Under stereomicroscope (STM) at ×20–25 magnification, using both transmitted and incident light, hyphae are curled, often several running together, typically with moisture drops (although these evaporate easily) (Fig. 2).
	Quality assurance Specific training This test should only be performed by persons who have been trained in fungal identification or under their direct supervision.
Media and solutions Malt agar Preparation	Media and solutions Malt agar + streptomycin Agar: 20 g Malt: 10 g Distilled/deionised water: 1000 ml Streptomycin sulphate*: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.

5. Allow the agar to cool to approx. 50 °C.	* Added after autoclaving. Streptomycin sulphate can be dissolved in water. Filter sterilisation is required. Preparation 5. Allow the agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in sterile distilled/deionised water.
Potato dextrose agar + streptomycin Preparation 5. Allow the agar to cool to approx. 50 °C.	Potato dextrose agar + streptomycin Potato dextrose agar (CCP): make according to specification of supplier Distilled/deionised water: 1000 ml Streptomycin sulphate*: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered * Added after autoclaving. Streptomycin sulphate can be dissolved in water. Filter sterilisation is required Preparation 5. Allow the agar to cool to approximately 50 °C and add streptomycin sulphate dissolved in sterile distilled/deionised water.
Method 7-009 *Pathogen(s): Fusarium circinatum* Nirenberg & O'Donnell (syn. *Fusarium subglutinans* f. sp. *Pini* Hepting, syn. *Fusarium lateritium* f. sp. *Pini* Hepting, syn. *Gibberella circinata*)	Method 7-009 *Pathogen(s): Fusarium circinatum* Nirenberg & O'Donnell, syn. *Fusarium subglutinans* f. sp. *pini* Hepting, syn. *Fusarium lateritium* f. sp. *pini* Hepting, syn. *Gibberella circinata*
Method 7-011 *Pathogen(s): Magnaporthe grisea* (Hebert) Barr (Imperfect state *Pyricularia oryzae* Cavara, syn. P. *grisea*)	Method 7-011 *Pathogen(s): Magnaporthe grisea* (Hebert) Barr (imperfect state *Pyricularia oryzae* Cavara), syn. P. *grisea*
Method 7-013a Materials Brass sieves: 1 mm mesh (2 additional sieves of larger mesh size can be useful; see point 2.3)	Method 7-013a Materials Brass sieves: 1 mm mesh (2 additional sieves of larger mesh size can be useful; see point 1.3)

Methods 2.2 Mycelium is approximately 3 µm thick, is golden brown in colour and visible without a stain (Fig. 1). Infection may vary from a few strands of short hyphae to complete invasion of the scutellum tissues. Occasionally fungi other than U. nuda occur in the scutellum but are usually darker in colour and quite distinct. When cell walls become discoloured they may be confused with mycelium of U. nuda, but this can be checked by examination at ×50 or higher magnification (Fig. 2). Compare with positive control (reference material).	Methods 2.2 Mycelium is approximately 3 µm thick, is golden brown in colour and visible without a stain (Fig. 1). Infection may vary from a few strands of short hyphae to complete invasion of the scutellum tissues. Occasionally fungi other than *U. nuda* occur in the scutellum but are usually darker in colour and quite distinct. When cell walls become discoloured, they may be confused with mycelium of *U. nuda*, but this can be checked by examination at ×50 or higher magnification (Fig. 2). Compare with positive control (reference material).
Method 7-016 *Pathogen(s): Phomopsis longicolla* Hobbs, *Diaporthe phaseolorum var. sojae* (Lehm.) Wehm. (Imperfect state *P. phaseoli* (Desm.) Sacc., syn. *P. sojae* Lehmann~~)~~; *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. *F. sp. Caulivora* (DPC), syn. *D. phaseolorum var. caulivora* Athow & Caldwell	Method 7-016 *Pathogen(s): Phomopsis longicolla* Hobbs, *Diaporthe phaseolorum* var. *sojae* (Lehm.) Wehm. (Imperfect state *P. phaseoli* (Desm.) Sacc., syn. *P. sojae* Lehm.; *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. f. sp. *caulivora* (DPC), syn. *D. phaseolorum* var. *caulivora* Athow & Caldwell
Sample preparation It is vital to exclude any possibility of cross-contamination between seed samples. This can be achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol.	Sample preparation It is vital to exclude any possibility of cross-contamination between seed samples. This can be achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol or 1 % NaOCl.
Methods 2.2 Positive control (reference material): Aseptically place seeds in an appropriate number of plates to obtain the reference culture, or plate a reference culture on ~~media~~. The number of plates required will depend on the level of contamination of the positive-control seed lot. 3. Incubation: Incubate plates for 7 d at 25 ±2 °C in the dark. ~~4. *Control:* Subculture a reference culture onto a plate of acidified PDA and incubate with the test plates. Alternatively, a sample of seed known to be infested may be surface sterilised, plated on acidified PDA and incubated under the same conditions as the test samples.~~	Methods 2.2 Positive control (reference material): Aseptically place seeds in an appropriate number of plates to obtain the reference culture, or plate a reference culture on PDA medium. The number of plates required will depend on the level of contamination of the positive-control seed lot. 3. Incubation: Incubate plates for 7 d at 25 ±2 °C in the dark.

~~5~~. *Examination:* Examine the plates after 3 and 7 d incubation using a dissecting microscope or hand lens at ×5 to ×10 magnification. Compare with control and record the number of infected seeds on each plate. Infected seeds are usually overgrown by a dense, white, floccose mycelium which often contains black, globose fruiting bodies (pycnidia) and/or black stromatic bodies (Fig. 1).	4. *Examination:* Examine the plates after 3 and 7 d incubation using a dissecting microscope or hand lens at ×5 to ×10 magnification. Compare with control and record the number of infected seeds on each plate. Infected seeds are usually overgrown by a dense, white, floccose mycelium which often contains black, globose fruiting bodies (pycnidia) and/or black stromatic bodies (Fig. 1).
Method 7-019a *Pathogen(s): Xanthomonas campestris* pv. *Campestris* (Pammel) Dowson and *Xanthomonas campestris* pv. *raphani*	Method 7-019a *Pathogen(s): Xanthomonas campestris* pv. *campestris* (Pammel) Dowson and *Xanthomonas campestris* pv. *raphani* (White) Dye
Delete figure 1	Replace figure 1 by added process flowchart
Sample size The minimum sample size ~~should be~~ 30 000 seeds and the maximum subsample size ~~should be~~ 10 000 seeds.	Sample size The minimum recommended working sample size is 30 000 seeds and the maximum subsample size must be 10 000 seeds.
Materials Brassica seedlings: susceptible to all races of the pathogen (e.g. B. oleracea ‘Wirosa’) for pathogenicity test	Materials Brassica seedlings: Use a cultivar proven to be susceptible to all races of the pathogen (e.g. *B. oleracea* ‘Wirosa’) for pathogenicity test
Method 7-019b Sample size The minimum sample size ~~should be~~ 30 000 seeds and the maximum subsample size ~~should be~~ 10 000 seeds.	Method 7-019b Sample size The minimum recommended working sample size is 30 000 seeds and the maximum subsample size must be 10 000 seeds.
Materials Brassica seedlings: susceptible to all races of the pathogen (e.g. B. oleracea ‘Wirosa’) for pathogenicity test General methods	Materials Brassica seedlings: Use a cultivar proven to be susceptible to all races of the pathogen (e.g. *B. oleracea* ‘Wirosa’) for pathogenicity test

Sectored plates: Using a laboratory marker pen draw lines on the base of a standard 90 mm plate (Petri dish) to divide it into six equal sectors.	General methods Sectored plates: Using a laboratory marker pen, draw lines on the base of a standard 90 mm plate (Petri dish) to divide it into six equal sectors.
Method 7-020 Sample size The minimum sample size ~~should be~~ 30 000 seeds and the maximum subsample size ~~should be~~ 10 000 seeds.	Method 7-020 Sample size The minimum recommended working sample size is 30 000 seeds and the maximum subsample size must be 10 000 seeds.
Material Carrot seedlings: ~~susceptible to the pathogen for pathogenicity test, e.g. 'Napoli'~~	Material Carrot seedlings: Use a cultivar proven to be susceptible to all races of the pathogen (e.g. 'Napoli') for pathogenicity test. 6.7 All negative PCR results must be confirmed with a pathogenicity test in order for the PCR results to be valid. All PCR positive results are valid and a pathogenicity test is optional.
Method 7-021 New taxonomical names of the pathogens *Title* Detection of *Xanthomonas axonopodis* pv. *Phaseoli* and *Xanthomonas axonopodis* pv. *Phaseoli* var. *fuscans* in *Phaseolus vulgaris* (bean) seed …	Method 7-021 New taxonomical names of the pathogens *Title* Detection of *Xanthomonas axonopodis* pv. *phaseoli* (*Xanthomonas phaseoli* pv. *phaseoli*) and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (*Xanthomonas citri* pv. *fuscans*) in *Phaseolus vulgaris* (bean) seed …
Pathogen(s): *Xanthomonas axonopodis* pv. *Phaseoli* (Smith) Vauterin, Hoste, Kersters & Swings, ~~syn.~~ *X. campestris* pv. *Phaseoli* (Smith) Dye. *Xanthomonas axonopodis* pv. *Phaseoli* var. *fuscans* Vauterin, Hoste, Kersters & Swings, ~~syn.~~ *X. campestris* pv. *Phaseoli* var. *fuscans* (Burkholder) Starr & Burkholder	*Pathogen(s):* *Xanthomonas phaseoli* pv. *phaseoli* (Smith) Constantin, Cleenwerck, Maes, Baeyen, Van Malderghem, De Vos, Cottyn, syn. *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Vauterin, Hoste, Kersters & Swings; *X. campestris* pv. *phaseoli* (Smith) Dye. *Xanthomonas citri* pv. *fuscans* (Schaad et al.) Constantin et al. syn., *Xanthomonas axonopodis*

	pv. *phaseoli* var. *fuscans* Vauterin, Hoste, Kersters & Swings; *X. campestris* pv. *phaseoli* var. *fuscans* (Burkholder) Starr & Burkholder
Background The two media, XCP1 and MT, have been chosen for their ease of use and selectivity for *X. axonopodis pv. Phaseoli* (Xap). In addition both media can be used to detect both *X. axonopodis pv. Phaseoli* and *X. axonopodis pv. Phaseoli var. fuscans* . Although initially the morphology of ~~*fuscans*~~ *and* ~~*non-fuscans*~~ *strains of X. axonopodis pv. Phaseoli.* Appear to be similar on the media, after a longer incubation the ~~fuscans~~ colonies are distinguished by a distinct brown pigmentation. A further advantage of MT medium is that it can also be used for identifying other bacterial seed-borne pathogens of beans, e.g. *Pseudomonas savastanoi pv. Phaseolicola* and *Pseudomonas syringae pv. Syringae.*	*Background* The two media, XCP1 and MT, have been chosen for their ease of use and selectivity for *X. axonopodis* pv. *phaseoli* (*X. phaseoli* pv. *phaseoli*) (*Xap*). In addition both media can be used to detect both *X. axonopodis* pv. *phaseoli* (*X. phaseoli* pv. *phaseoli*) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (*X. citri* pv. *fuscans*) (*Xff*). Although initially the morphology of *Xap* and *Xff* strains appear to be similar on the media, after a longer incubation the *Xff* colonies are distinguished by a distinct brown pigmentation. A further advantage of MT medium is that it can also be used for identifying other bacterial seed-borne pathogens of beans, e.g. *Pseudomonas savastanoi* pv. *phaseolicola* and *Pseudomonas syringae* pv. *syringae.*
A ~~new~~ pathogenicity assay was developed at INRA to allow a reliable characterisation of the aggressiveness of ~~X. axonopodis pv. Phaseoli~~ wild type strains and mutants (Darsonval et al., 2009). A comparison study of the new pathogenicity test and primers specific for ~~X. axonopodis pv. Phaseoli fuscans~~ and ~~non-fuscans~~ isolates (Audy et al.,1994; Boureau et al., 2012) was carried out as a collaboration between ISTA, ANSES, INRA and ISHI-Veg.	A pathogenicity assay was developed at INRA to allow a reliable characterisation of the aggressiveness of *Xap* wild type strains and mutants (Darsonval et al., 2009). A comparison study of the new pathogenicity test and primers specific for *Xap* and *Xff* isolates (Audy et al.,1994; Boureau et al., 2012) was carried out as a collaboration between ISTA, ANSES, INRA and ISHI-Veg.
Option 1: Pathogenicity assay, for laboratories not equipped or experienced with PCR. In this case, CCP must be followed and target and non-target controls added (*X. vesicatoria*, *Xap,* water). This option is also valuable and less time consuming when few suspect isolates have been detected but requires a growth chamber or greenhouse equipped for high relative humidity (RH). *Materials*	Option 1: Pathogenicity assay, for laboratories not equipped or experienced with PCR. In this case, CCP must be followed and target and non-target controls added (*X. vesicatoria*, *Xap*, *Xff,* water). This option is also valuable and less time consuming when few suspect isolates have been detected but requires a growth chamber or greenhouse equipped for high relative humidity (RH).

Reference material: a known strain ~~of fuscans or non-fuscans types of~~ X. axonopodis pv. Phaseoli (positive control) and of X. vesicatoria (negative control) or standardised reference material	*Materials* Reference material: a known strain of both *X. axonopodis* pv. *phaseoli* (*X. phaseoli* pv. *phaseoli*) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (*X. citri* pv. *fuscans)* (positive controls) and of *X. vesicatoria* (negative control) or standardised reference material
70 % ethanol~~: for disinfection of surfaces, equipment~~	70 % ethanol or an equivalent disinfecting product
2.3 Pipette 100 µl of each dilution and the undiluted seed extract onto plates of each of the selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod (see General methods).	2.3 Pipette 100 µl of each dilution and the undiluted seed extract onto plates of each of the semi-selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod or equivalent (see General methods).
3. Positive control (culture or reference material)	3. Positive controls (culture or reference material)
3.1 Prepare a suspension of a known strain of ~~X. axonopodis pv. Phaseoli, fuscans and non-fuscans,~~ in sterile saline or reconstitute standardised reference material according to the supplier's instructions.	3.1 Prepare a suspension of a known strain of *Xap* and *Xff* in sterile saline or reconstitute standardised reference material according to the supplier's instructions.
3.2 Dilute suspension sufficiently to obtain dilutions containing approximately 10^{-2} to 10^{-4} cfu/ml. This may require up to seven ten-fold dilutions from a turbid suspension.	3.2 Dilute suspension sufficiently to obtain dilutions containing approximately 10−2 to 10−4 colony forming units (cfu) /ml. This may require up to seven ten-fold dilutions from a turbid suspension.
3.3 Pipette 100 µl of appropriate countable dilutions onto plates of each of the selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod.	3.3 Pipette 100 µl of appropriate countable dilutions onto plates of each of the semi-selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod or equivalent (see General methods).
4.1 Prepare a dilution series from a sample of the extraction medium (i.e. saline plus Tween™ 20), containing no seeds, and plate on each of the media as for samples.	4.1 Prepare a dilution series from a sample of the extraction medium (i.e. saline plus Tween™ 20), containing no seeds, and plate on each of the semi-selective media as for samples.
5.1 Examine sterility check and recovery of positive control on semi-selective medium (CCP).	5.1 Examine sterility check and recovery of positive controls on both semi-selective medium (MT and XCP1)(CCP).

5.2 Examine the sample plates for the presence of typical ~~X. axonopodis pv. Phaseoli~~ colonies by comparison with the positive control plates.	5.2 Examine the sample plates for the presence of typical *Xap* and/ or *Xff* colonies by comparison with the positive control plates. If necessary, estimate the number of cfu.
5.3 After 4–5 d on MT, ~~*X. axonopodis* pv. *Phaseoli*~~ colonies are yellow distinguished by two zones of hydrolysis; a large clear zone of casein hydrolysis and a smaller milky zone of Tween™ 80 lysis (Fig. 1a, b). ~~The *fuscans* of *X. axonopodis* pv. *Phaseoli*~~ colonies produce a brown diffusible pigment. If not visible after 4 d ~~inclubate~~ for an additional day. Often the ~~*fuscans*~~ type colonies show Tween™ 80 lysis.	5.3 After 4–5 d on MT, *Xap* colonies are yellow - distinguished by two zones of hydrolysis; a large clear zone of casein hydrolysis and a smaller milky zone of Tween™ 80 lysis (Fig. 1a, b). *Xff* colonies produce a brown diffusible pigment. If not visible after 4 d incubate for an additional day. Often the *Xff* type colonies show Tween™ 80 lysis.
5.4 After 4–5 d on XCP1, ~~*X. axonopodis* pv. *Phaseoli*~~ colonies are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Fig. 2b). ~~The *fuscans* of *X. axonopodis* pv. *Phaseoli*~~ colonies produce a brown diffusible pigment after 5 d of incubation (Fig 2a). Often the ~~*fuscans*~~ type colonies show Tween™ 80 lysis.	5.4 After 4–5 d on XCP1, *Xap* colonies are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Fig. 2b). *Xff* colonies produce a brown diffusible pigment after 5 d of incubation (Fig 2a). Often the *Xff* type colonies show Tween™ 80 lysis.
6.2 Subculture ~~the~~ positive control isolate to a sectored plate for comparison (Fig. 3).	6.2 Subculture both *Xap* and *Xff* positive controls isolates to a sectored plate for comparison (Fig. 3).
6.4 Compare appearance of growth with positive control. On YDC ~~*X. axonopodis* pv. *Phaseoli*~~ colonies are yellow and mucoid in appearance (Fig. 3)(CCP).	6.4 Compare appearance of growth with positive control. On YDC *Xap* and *Xff* colonies are yellow and mucoid in appearance (Fig. 3)(CCP).
7.1 Grow seedlings of a bean cultivar known to be highly susceptible to *Xap* (e.g. ‘Flavert’ or ‘Michelet’) at 20–30 °C in small pots until the first trifoliate leaf stage (approximately 16 days after sowing).	7.1 Grow seedlings of a bean cultivar known to be highly susceptible to *Xap* and *Xff* (e.g. ‘Flavert’ or ‘Michelet’) at 20–30 °C in small pots, containing peated potting soil, until the first trifoliate leaf stage (approximately 16 days after sowing).
7.5 Inoculate plants with one positive *Xap* isolate, and 2 negative controls: *X. vesicatoria* and distilled/deionised water.	7.5 Inoculate plants with one positive *Xap* isolate, one positive *Xff* isolate, and 2 negative controls: *X. vesicatoria* and distilled/deionised water.

7.7 Record symptoms from 5 to 11 days (depending on when symptoms begin) after inoculation. Compare with positive and negatives controls. Typical *Xap* symptoms are water-soaked spots. Necrosis of lesions can develop and in case of very aggressive isolates lead to death of tissues (Fig. 5a–d). No lesions occur on negative controls (Fig. 6)	7.7 Record symptoms from 5 to 11 days (depending on when symptoms begin) after inoculation. Compare with positive and negatives controls. Typical *Xap / Xff* symptoms are water-soaked spots. Necrosis of lesions can develop and in case of very aggressive isolates lead to death of tissues (Fig. 5a–d). No lesions occur on negative controls (Fig. 6)
8.2 Use the following ~~*X. axonopodis* pv. *Phaseoli*~~ specific pair of primers from Audy *et al.* (1994) that will give a product of 800bp:	8.2 Use the following *Xap / Xff* specific pair of primers from Audy *et al.* (1994) that will give a product of 800bp:
8.6 Analyse the amplification products for a ~~*X. axonopodis* pv. *Phaseoli*~~ specific product of 800 bp (CCP) = positive identification of ~~*X. axonopodis* pv. *Phaseoli*,~~ no band = negative identification (Fig. 7). In case of a positive identification of ~~*X. axonopodis* pv. *Phaseoli*~~, as a low risk of false positive result is present (Audy *et al.,* 1994) primers detect *X. axonopodis* pv. *Dieffenbachiae* which are not supposed to be present on bean seeds), a pathogenicity test can be performed as complementary information.	8.6 Analyse the amplification products for a *Xap / Xff* specific product of 800 bp (CCP) = positive identification of *Xap / Xff*, no band = negative identification (Fig. 7). In case of a positive identification of *Xap / Xff* , as a low risk of false positive result is present (Audy *et al.,* 1994) primers detect *X. axonopodis* pv. *dieffenbachiae* which are not supposed to be present on bean seeds), a pathogenicity test can be performed as complementary information.
Critical control points (CCP) The numbers of colonies on dilution plates prepared from the positive control isolate(s) or reference material should be similar on both media (Step 5.1). Note that recovery of the ~~*fuscans* type *X. axonopodis* pv. *Phaseoli*~~ is in general lower on MT than on XCP1.	*Critical control points (CCP)* The numbers of colonies on dilution plates prepared from the positive control isolate(s) or reference material should be similar on both media (Step 5.1). Note that recovery of *X. axonopodis* pv. *phaseoli* var. *fuscans* (*X. citri* pv. *fuscans*) is in general lower on MT than on XCP1.
The preparation of PCR mixture (Step 8.1, 8.4), and the preparation of agarose gel for electrophoresis (Step 8.5) should be adapted to available material and equipment of individual laboratories testing for of ~~*X. axonopodis* pv. *Phaseoli*~~ under the condition that results will be validated on PCR controls.	The preparation of PCR mixture (Step 8.1, 8.4), and the preparation of agarose gel for electrophoresis (Step 8.5) should be adapted to available material and equipment of individual laboratories testing for of *Xap / Xff* under the condition that results will be validated on PCR controls.
	References

	Constantin EC, Cleenwerck I, Maes M, Baeyen S, Van Malderghem C, De Vos P & Cottyn B (2016) Genetic characterization of strains named as *Xanthomonas axonopodis* pv. *dieffenbachiae* leads to a taxonomic revision of the *X. axonopodis* species complex. Plant Pathology 65(5), 792–806.
Figures Figure 1. *Xanthomonas axonopodis* pv. *Phaseoli* colonies on MT plates after 4 d indicated by a large clear zone of casein hydrolysis (a) and a smaller milky zone of Tween™ 80 lysis (b). Figure 2. ~~*Xanthomonas axonopodis* pv. *Phaseoli* colonies, *fuscans*~~ (a) and ~~non-*fuscans*~~ (b), on XCP1 plates, showing a clear zone of starch hydrolysis and ~~*fuscans*~~ on XCP1 showing a milky zone, after 4 d.	*Figures* Figure 1. *X. axonopodis* pv. *phaseoli* (*X. phaseoli* pv. *phaseoli*) colonies on MT plates after 4 d indicated by a large clear zone of casein hydrolysis (a) and a smaller milky zone of Tween™ 80 lysis (b). Figure 2. *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (*Xanthomonas citri* pv. *fuscans*) (a) and *Xanthomonas axonopodis* pv. *phaseoli* (*Xanthomonas phaseoli* pv. *phaseoli*) (b), on XCP1 plates, showing a clear zone of starch hydrolysis and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (*Xanthomonas citri* pv. *fuscans*) on XCP1 showing a milky zone, after 4 d.
Figure 3. ~~*Xanthomonas axonopodis* pv. *Phaseoli* colonies, *fuscans*~~ (a) and ~~non-*fuscans*~~ (b), on YDC plates after 2 d are brown and yellow in appearance.	Figure 3. *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (*Xanthomonas citri* pv. *fuscans*) (a) and *Xanthomonas axonopodis* pv. *phaseoli* (*Xanthomonas phaseoli* pv. *phaseoli*) (b), on YDC plates after 2 d are brown and yellow in appearance.
Method 7-023 Background Both ISTA working sheets are based on dilution plating, although an immunofluorescence (IF) prescreening is part of working sheet No. 65. The present version (Kurowski & Remeeus, 2007) abandons this IF prescreening. Instead of plating in triplicate on modified sucrose peptone (MSP), as in working sheet No. 66, two plates of MSP and two plates of an additional medium, milk Tween™ (MT; Goszczynska & Serfontein,	Method 7-023 Background Both ISTA working sheets are based on dilution plating, although an immunofluorescence (IF) prescreening is part of working sheet No. 65. The present version (Kurowski & Remeeus, 2007) abandons this IF prescreening. Instead of plating in triplicate on modified sucrose peptone (MSP), as in working sheet No. 66, two plates of MSP and two plates of an additional medium, milk Tween™ (MT; Goszczynska & Serfontein, 1998), are used. MT can complement MSP, and has the advantage of being able to detect *Xanthomonas axonopodis* pv. *phaseoli* (*Xanthomonas phaseoli* pv. *phaseoli*) *and*

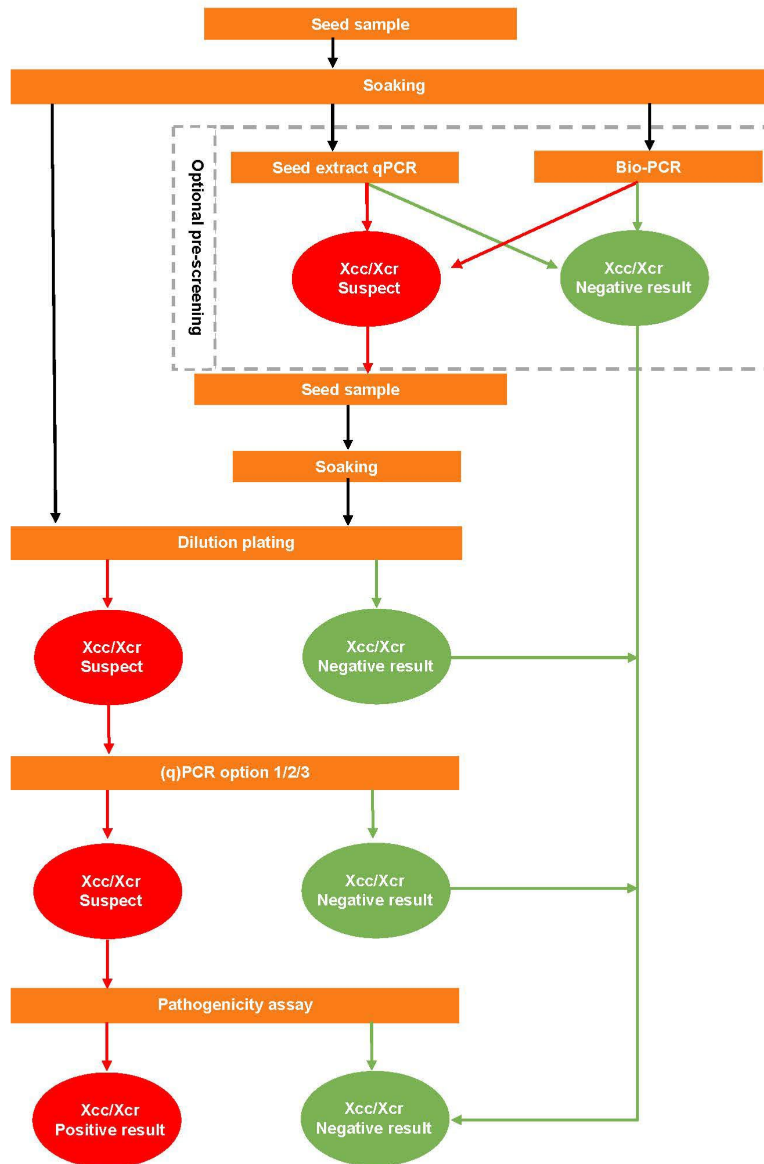
1998), are used. MT can complement MSP, and has the advantage of being able to detect *Xanthomonas axonopodis* pv. *Phaseoli* (*Xap*), which is not possible on MSP. Furthermore, MT allows *Pseudomonas savastanoi* pv. *Phaseolicola* to be distinguished from colonies of *Pseudomonas syringae* pv. *Syringae*. The value of MT for detection of *Psp and Xap* has been demonstrated in practice and in method validation studies for both pathogens (Kurowski & Remeeus, 2007; Sheppard & Remeeus, 2006).	*Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* (*Xanthomonas citri* pv. *fuscans*) (*Xap* & *Xff*), which is not possible on MSP. Furthermore, MT allows *Pseudomonas savastanoi* pv. *phaseolicola* to be distinguished from colonies of *Pseudomonas syringae* pv. *syringae*. The value of MT for detection of *Psp*, *Xap* and *Xff* has been demonstrated in practice and in method validation studies for both pathogens (Kurowski & Remeeus, 2007; Sheppard & Remeeus, 2006).
Method 7-024	Method 7-024
Host: *Pisum sativum* L.~~s.l.~~	Host: *Pisum sativum* L.
Method 7-026	Method 7-026
Host: ~~*Cucurbits*~~	Host: Cucurbitaceae
Background	Background
Squash mosaic virus (SqMV), *cucumber green mottle mosaic virus* (CGMMV) and *melon necrotic spot virus* (MNSV) are seed-transmissible viruses of ~~cucurbits~~, and therefore the detection of these viruses in seeds of ~~cucurbits~~ is an important tool in control strategies. Enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of plant viruses (Clark & Adams, 1977). ELISA methods have also been described for the detection of PEBV and PSbMV (Hamilton & Nichols, 1978; Van Vuurde & Maat, 1985, Maury *et al.*, 1987).	*Squash mosaic virus* (SqMV), *cucumber green mottle mosaic virus* (CGMMV) and *melon necrotic spot virus* (MNSV) are seed-transmissible viruses of Cucurbitaceae, and therefore the detection of these viruses in seeds of Cucurbitaceae is an important tool in control strategies. Enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of plant viruses (Clark & Adams, 1977). ELISA methods have also been described for the detection of PEBV and PSbMV (Hamilton & Nichols, 1978; Van Vuurde & Maat, 1985, Maury *et al.*, 1987).
Grow-out confirmation method for *squash mosaic virus*	Grow-out confirmation method for *squash mosaic virus*
Host: ~~Cucurbits~~	Host: Cucurbitaceae
Background	Background
The ISTA Seed Health Method 7-026 provides the possibility of simultaneous detection of *squash mosaic virus* (SqMV), *melon necrotic spot virus* (MNSV) and *cucumber green mottle mosaic virus* (CGMMV) on seed of ~~cucurbits~~ from a single seed extract with a DAS-ELISA test.	The ISTA Seed Health Method 7-026 provides the possibility of simultaneous detection of *squash mosaic virus* (SqMV), *melon necrotic spot virus* (MNSV) and *cucumber green mottle mosaic virus* (CGMMV) on seed of Cucurbitaceae from a single seed extract with a DAS-ELISA test.
	The grow-out test method was derived from a peer validation study between four laboratories

The grow-out test method was derived from a peer validation study between four laboratories organized by the International Seed Health Initiative for Vegetables, ISF (ISHI-Veg). In that study, melon seed was evaluated, but in general cucurbits can be evaluated. However, the ELISA confirmation test on plant tissue samples should be evaluated and verified for the various ~~cucurbits~~ being evaluated in the grow-out test before the results are accepted.	organized by the International Seed Health Initiative for Vegetables, ISF (ISHI-Veg). In that study, melon seed was evaluated, but in general cucurbits can be evaluated. However, the ELISA confirmation test on plant tissue samples should be evaluated and verified for the various Cucurbitaceae being evaluated in the grow-out test before the results are accepted.
Materials SqMV-infected and dehydrated melon leaves: Approx. 1 g fresh weight (equivalent)(for preparation of SqMV inoculum). Melon is used for SqMV inoculum because the titre of the virus is highest and more consistent in melon than in other ~~cucurbits~~. Known SqMV-free ~~cucurbit~~ seeds: For growing healthy plants; one part will be used as the negative control and the other part will be mechanically inoculated to be used as the positive control. The species of ~~cucurbit~~ controls must match the species of ~~cucurbit~~ being evaluated in the grow-out test.	Materials SqMV-infected and dehydrated melon leaves: Approx. 1 g fresh weight (equivalent)(for preparation of SqMV inoculum). Melon is used for SqMV inoculum because the titre of the virus is highest and more consistent in melon than in other Cucurbitaceae. Known SqMV-free Cucurbitaceae seeds: For growing healthy plants; one part will be used as the negative control and the other part will be mechanically inoculated to be used as the positive control. The species of cucurbit controls must match the species of Cucurbitaceae being evaluated in the grow-out test.
Method 7-029 Host: *Pisum sativum* L.~~*s.l*~~*.*	Method 7-029 Host: *Pisum sativum* L.
Materials Cold room or refrigerator: operating at 4 °C Sterile bent glass rods 70 % ethanol or equivalent disinfecting product Materials for oxidase tests: 1 % aqueous N,N-dimethyl paraphenylene diamine oxalate solution or ready to use tests ~~(e.g. Bactident Oxidase, Merck, 1.13300.0001)~~	Materials Cold room or refrigerator: operating at 4 °C ± 3 °C Sterile bent glass rods (or equivalent) 70 % ethanol or an equivalent disinfecting product Materials for oxidase tests: 1 % aqueous N,N-dimethyl paraphenylene diamine oxalate solution or ready to use tests
1.2 Soak the subsamples overnight (18–24 h) at 4 °C under agitation.	1.2 Soak the subsamples overnight (18–24 h) at 4 °C (±3 °C) under agitation.

2.4 Incubate inverted plates at 28 ±2 °C) and examine after 4–5 days (see step 5).	2.4 Incubate inverted plates at 28 ±2 °C and examine after 4–5 days.
3.2 Dilute the suspension sufficiently to obtain dilutions containing approximately 10^{-2} to 10^{-4} cfu/ml. This may require up to seven ten-fold dilutions from a turbid suspension.	3.2 Dilute the suspension sufficiently to obtain dilutions containing approximately 10^{-2} to 10^{-4} colony forming units (cfu)/ml. This may require up to seven ten-fold dilutions from a turbid suspension.
4.1 Plate a dilution series from a sample of the extraction medium (i.e. sterile saline), containing no seeds, and plate on each of the semi-selective media as for samples.	4.1 Prepare a dilution series from a sample of the extraction medium (i.e. sterile saline), containing no seeds, and plate on each of the semi-selective media as for samples.
5.3 On KBBCA after 4 days, *P. syringae* pv. *Pisi* colonies are creamy and half-translucent (Fig. 1). 5.4 On SNAC after 4 days, *P. syringae* pv. *Pisi* colonies are circular, white to transparent, mucoid, dome shaped and levan positive. (Fig. 2).	5.3 After 4 days on KBBCA , *P. syringae* pv. *pisi* colonies are creamy and half-translucent (Fig. 1). 5.4 After 4 days on SNAC, *P. syringae* pv. *pisi* colonies are circular, white to transparent, mucoid, dome shaped and levan positive. (Fig. 2). 5.5 The colony size and colour can differ within a sample. 5.6 Record the presence of suspect colonies (see General methods). If necessary, estimate the number of cfu of suspect and other colonies.
6. Identification of suspect colonies	6. Confirmation/identification of suspect colonies
6.4 Incubate sectored plates at 28 ±2 °C) for 2–3 days.	6.4 Incubate sectored plates at 28 ±2 °C for 2–3 days.
8.6 Incubate the inoculated seedlings at 20 ±5 °C) with saturating humidity.	8.6 Incubate the inoculated seedlings at 20 ±5 °C with saturating humidity.
	Recording of dilution plates: Record the results for all dilution plates. The most accurate estimate of bacterial numbers should be obtained from spread plates with total number between 30 and 300 colonies. However, this

	may be further complicated depending on the relative numbers of suspect pathogen and other colonies. To minimise effort, start recording with the highest dilution (most dilute) and count the number of suspect and the number of other colonies. If the total number of colonies on a plate greatly exceeds 300 there is little value in trying to make a precise count if a more reliable count has already been obtained from a more dilute plate, in which case it is sufficient to record the number of colonies as 'm' (many) if they are still separate or 'c' (confluent) if they have run together.
Figure 2. Plate of SNAC medium after 4 days of incubation at 28 ±2 °C) showing typical colonies of *P. syringae* pv. *Pisi* that are levan positive.	Figure 2. Plate of SNAC medium after 4 days of incubation at 28 ±2 °C showing typical colonies of *P. syringae* pv. *pisi* that are levan positive.

Flow chart referred to in editorial correction section 7-019a (Current chart will be replaced)



When going through the new rules 2024, it was noticed that *Carica papaya* was accidentally omitted from Table 9A. (This was added in June by the MOI.)

Table 9A Details of methods for moisture determination

Species	Grinding/cutting (9.2.5.4, 9.2.5.5)	Drying Temp. High: 130°C Low: 103°C	Drying time (h)	Tolerances of replicates (9.2.6.2)	Predrying requirement (9.2.5.6) / Remarks
1	2	3	4	5	6
. . . . . .	. . . . . .	. . . . .	. . . . . .	. . . . . .	. . . . . .
Caragana arborescens	Coarse	Low	17	Table 9B	--
Carica papaya	No	Low	17	Table 9B	-- /High oil content
Carpinus betulus	Coarse	Low	17	Table 9B	--
. . . . . .	. . . . . .	. . . . . .	. . . . . .	. . . . . .	. . . . . .

ACCEPTED BY VOTE	RESULT
34	Pass

PART C. RULES CHANGES AND NEW METHODS REQUIRING A VOTE

Chapter 1: ISTA Certificates

C.1.1 Revised conditions for issuing OICs when sampling in different countries WITHDRAWN

The ISTA Executive Committee (ECOM) and Head of the ISTA Accreditation & Technical Department (HoA&T) of ISTA reviewed the link between section 3.1 of the ISTA Accreditation Standard and the sampling and testing of seed lots in different countries.

The ISTA Accreditation Standard states "...3. Management Requirements. An accredited ISTA member must: 3.1. be an entity that can be held legally responsible for its work...".

It is the opinion of the ECOM and HoA&T that ISTA laboratories can **only** be legally responsible for samplers within their own country, or samplers employed by them to sample in another country, unless covered by the ISTA company multi-site accreditation directive.

This is consistent with the established principle that the ISTA sampling can be under the responsibility of an ISTA laboratory/sampling entity in one country and the testing can be in the same or a different country. Details of where the sample was taken and under whose authority, as well as where the sample was tested, go onto the Orange International Certificate (OIC) see the existing ISTA Rules 1.3j. Note: until 2007 if sampling was in one country and testing in a different country the results would have gone on a Green International Certificate (GIC).

If the following proposal is accepted by the membership, it means that:

1) sampling from a seed lot located in country A must be done by samplers from an accredited laboratory/sampling entity within country A,
2) a sampler from an accredited laboratory/sampling entity from country A can be sent into country B to take samples from a seed lot located in country B, and that
3) an accredited laboratory/sampling entity from country A **cannot** have authorised ISTA samplers located in country B, unless the laboratory/sampling entity is accredited under the multi-site accreditation directive. Note: some restrictions apply about off continent locations, see the ISTA website for the directive.

There is no similar rule to consider in the AOSA Rules for harmonisation.

The Rules change proposal comes from the ISTA ECOM as it affects ISTA policy and how the ISTA Accreditation Standard is applied.

CURRENT VERSION	PROPOSED VERSION
1.3 Conditions for issuance of ISTA Certificates	**1.3 Conditions for issuance of ISTA Certificates**

…………	…………
j. For an Orange International Seed Lot Certificate, the submitted sample must be tested by an accredited laboratory. The issuing laboratory must ensure that sampling, sealing, identification, testing and issuance of the certificate is in accordance with the ISTA Rules, although subcontracting of sampling and/or testing to another accredited laboratory is permissible. The laboratory which carries out sampling must provide all the information that is necessary to complete the Orange International Seed Lot Certificate. The seed lot identification ('Marks of the lot'; see 2.2.11) may take the form of a sequential series of characters or a single reference character. Each container within the lot or sublot must be identified in such a way that the containers can be readily recognised by the information provided on the certificate issued. Each container of a sublot must be marked with the identification of the original seed lot. A sublot-specific identification is not necessary unless the seed owner requests this. When the seed lot is located in a different country to the sampling laboratory, the country where the seed lot has been sampled must be reported either under	j. For an Orange International Seed Lot Certificate, the submitted sample must be tested by an accredited laboratory. The issuing laboratory must ensure that sampling, sealing, identification, testing and issuance of the certificate is in accordance with the ISTA Rules, although subcontracting of sampling and/or testing to another accredited laboratory is permissible. The laboratory which carries out sampling must provide all the information that is necessary to complete the Orange International Seed Lot Certificate. The seed lot identification ('Marks of the lot'; see 2.2.11) may take the form of a sequential series of characters or a single reference character. Each container within the lot or sublot must be identified in such a way that the containers can be readily recognised by the information provided on the certificate issued. Each container of a sublot must be marked with the identification of the original seed lot. A sublot-specific identification is not necessary unless the seed owner requests this. ISTA samplers can only be authorised to sample for issuance of an Orange International Seed Lot Certificate by a laboratory or sampling entity located in their own country, unless the laboratory or sampling entity is accredited under the Directive for "Accreditation of Laboratories and Sampling Entities with Sampling Units at Multiple Sites". ISTA samplers authorised in their own country can travel into another country to take samples for issuance of an Orange International Seed Lot Certificate. When the seed lot is located in a different country to the sampling laboratory, the country where the seed lot has been sampled must be reported either under

‘Sampling by’ or under ‘Additional observations’.	‘Sampling by’ or under ‘Additional observations’.

VOTE TO ACCEPT ITEM	**YES VOTES**	**NO VOTES**	**RESULT**
C.1.1			**WITHDRAWN**

Chapter 2: Sampling

C.2.1. Revision of Table 2C to include minimum moisture weights

The current Table 2C for lot sizes and sample sizes is divided into 3 parts: part 1 for agricultural and vegetable seeds; part 2 for tree and shrub seeds and part 3 for flower, spice, herb and medicinal species. It was not always clear in which part a species should be listed (e.g. *Helianthus annuus* in part 1 or 3 or both).
From some genera, species are listed in different parts (Examples *Thymus serpyllum* in part 3, *Thymus vulgaris* in part 1) which is not in line with crop groups for accreditation given on a genus level. In the other way around, some genera are listed in several crop groups but not listed in the corresponding parts of Table 2C.
To remove these inconsistencies, the Bulking and Sampling Committee proposes to merge all three parts of the Table 2C in only one table with all species listed in alphabetical order. In addition, it is proposed on this occasion to add a new column showing the submitted sample size for moisture testing, since in Table 2 this information is directly available to seed samplers preparing those samples.
Because of merging, in Chapter 2 and other Chapters various cross references to the former Table 2C Part 1 need to be adapted.

The proposal was approved by a majority vote of the BSC, with the agreement of TEZ (6.8) and MOI (9.2.5.1).

CURRENT:

Table 2C Part 1. Lot sizes and sample sizes: agricultural and vegetable seeds

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working samples (g)	
			Purity analysis (3.5.1)	Other seeds by number (4.5.1)
1	2	3	4	5
Abelmoschus esculentus (L.) Moench	20000	1000	140	1000
Achillea millefolium L.	10000	5	0.5	5

Table 2C Part 2. Lot sizes and sample sizes: tree and shrub seeds

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Abies alba Mill.	1000	240	120
Abies amabilis Douglas ex J.Forbes	1000	200	100

Table 2C Part 3. Lot sizes and sample sizes: flower, spice, herb and medicinal species

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Minimum working sample for purity analysis (3.5.1) (g)
1	2	3	4
Abutilon ×hybridum hort. ex Voss	5000	40	10
Achillea clavennae L.	5000	5	0.5

PROPOSED (*Note to Ness: Please Italicize the scientific names in the proposed table and place "N/A" in the empty column 6 cells*)

Table 2C. Lot sizes and sample sizes

(…)

Species	Maximum weight of lot (kg) (except see 2.8 Note 2)	Minimum submitted sample (g)	Purity analysis (3.5.1)	Other seeds by number (4.5.1)	Minimum submitted sample for moisture testing (g)
1	2	3	4	5	6
Tanacetum coccineum (Willd.) Grierson	5 000	30	8	–	
Tanacetum parthenium (L.) Sch. Bip.	5 000	20	5	–	
Taraxacum officinale F.H.Wigg., s.l.	10 000	30	3	30	
Taxodium distichum (L.) Rich.	300	500	250	–	50
Taxus spp.	1 000	320	160	–	100
Tectona grandis L. f.	1 000	2 000	1 000	–	50
Tetragonia tetragonoides (Pall.) Kuntze	20 000	1 000	200	1 000	
Thinopyrum elongatum (Host) D.R.Dewey (previously Elytrigia elongata (Host) Nevs)	10 000	200	20	200	
Thinopyrum intermedium (Host) Barkworth & D.R.Dewey (previously Elytrigia intermedia (Host) Nevski)	10 000	150	15	150	
Thuja occidentalis L.	1 000	25	4	–	50
Thuja plicata Donn ex D.Don	1 000	10	3	–	50
Thunbergia alata Bojer ex Sims	5 000	200	50	–	
Thymus serpyllum L.	5 000	5	0.5	–	
Thymus vulgaris L.	10 000	5	0.5	5	
Tilia cordata Mill.	1 000	180	90	–	100
Tilia platyphyllos Scop.	1 000	500	250	–	100
Torenia fournieri Linden ex E.Fourn.	5 000	5	0.2	–	

(…) *The whole table is presented in an additional document.*

CURRENT VERSION	PROPOSED VERSION
2.5.2.1 Minimum size of working sample … The sample weights in column 5 of Table 2C~~, Part 1,~~ for other seed determination (OSD) are 10 times the weights in column 4, subject to a maximum of 1000 g. …	**2.5.2.1 Minimum size of working sample** … The sample weights in column 5 of Table 2C for counts of other species are 10 times the weights in column 4, subject to a maximum of 1000 g. …

CURRENT VERSION	PROPOSED VERSION
2.5.4.5 Submitted sample … c) ~~For moisture determination of species that must be ground (see Table 9A): 100 g. For all other species: 50 g.~~	**2.5.4.5 Submitted sample** … c) When moisture meters are to be used for testing, a larger sample size may be necessary. Contact the accredited ISTA laboratory for specific instructions. …

When moisture meters are to be used for testing, a larger sample size may be necessary. Contact the accredited ISTA laboratory for specific instructions. …	

Current version	Proposed version
2.8 Tables for lot size and sample sizes … Note 2 (…) c. species of Poaceae listed in Table 2C ~~Part 1~~ (see 2.5.4.2). For production plants approved under 2.5.4.2, the maximum seed lot weight for Poaceae species listed in Table 2C ~~Part 1~~ is 25 000 kg (with a 5 % tolerance).	**2.8 Tables for lot size and sample sizes** … Note 2 (…) c. species of Poaceae listed in Table 2C (see 2.5.4.2). For production plants approved under 2.5.4.2, the maximum seed lot weight for Poaceae species listed in Table 2C is 25 000 kg (with a 5 % tolerance).

Current version	Proposed version
4.5.1 Working sample a. The size of the working sample must be either a weight estimated to contain at least 25 000 seed units or not less than the weight prescribed in Table 2C ~~Part 1~~, column 5. …	**4.5.1 Working sample** a. The size of the working sample must be either a weight estimated to contain at least 25 000 seed units or not less than the weight prescribed in Table 2C, column 5. …

Current version	Proposed version
4.5.3.2 Submitted subsample … The size of the submitted subsample must either be a weight estimated to contain at least 25 000 seed units or not less than the weight prescribed in Table 2C ~~Part 1~~, column 5 (Other seeds by number) for the crop species under test. …	**4.5.3.2 Submitted subsample** … The size of the submitted subsample must either be a weight estimated to contain at least 25 000 seed units or not less than the weight prescribed in Table 2C, column 5 (Other seeds by number) for the crop species under test. …

Current version	Proposed version
6.8 Standard procedures for tetrazolium testing … **Column 1: Species** Where methods are described for a group of species, only those species specifically listed in Table 2C ~~Part 1~~ may be considered to be covered. …	**6.8 Standard procedures for tetrazolium testing** … **Column 1: Species** Where methods are described for a group of species, only those species specifically listed in Table 2C may be considered to be covered. …

Current version	Proposed version
17.7 Species for which these rules apply These rules apply only to species of the Poaceae and Fabaceae listed in Table 2C ~~Part 1~~ with a maximum lot size in Table 2C of 10 000 kg.	**17.7 Species for which these rules apply** These rules apply only to species of the Poaceae and Fabaceae listed in Table 2C with a maximum lot size in Table 2C of 10 000 kg.

CURRENT VERSION	PROPOSED VERSION
9.2.5.1 General directions and precautions … The submitted sample (see 2.5.1.5–2.5.1.6) may be accepted for moisture determination only if it has the required sample size (2.5.4.5c ~~and Table 9A~~), and is in an intact, moisture-proof container (or, if issuing a Blue International Seed Sample Certificate, apparently moisture-proof) from which as much air as possible has been excluded. …	**9.2.5.1 General directions and precautions** … The submitted sample (see 2.5.1.5–2.5.1.6) may be accepted for moisture determination only if it has the required sample size (Table 2C, 2.5.4.5c), and is in an intact, moisture-proof container (or, if issuing a Blue International Seed Sample Certificate, apparently moisture-proof) from which as much air as possible has been excluded. …

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.2.1	**32**	**0**	**PASS**

Chapter 3: The purity analysis

C.3.1 Table 3B Part 3: Revised for the definition of "Seed Unit" WITHDRAWN

The definition of "Seed Unit" could be further improved for its clarity for applying the ISTA Rules. Whether dispersal tuber is a "seed unit" was debated and highlighted in ISTA PT22-2 with a natural vegetative tuber propagule of *Cyperus esculentus* (yellow nutsedge), an objectionable weed in agricultural situations in many countries.

The proposed definition for seed unit is aimed at improving clarity, being more inclusive, and aligning better with the widely accepted term as plant dispersal unit for seed testing.
The proposal was discussed in PUR and voted in the PUR committee with a majority vote.

CURRENT VERSION	PROPOSED VERSION
Table 3B Part 3. Glossary **seed unit:** ~~commonly found~~ dispersal unit, ~~i.e.~~ achenes ~~and similar fruits~~, schizocarps, florets ~~etc.,~~ as defined for each genus or species in the Pure Seed Definitions in Table 3B Parts 1 and 2	**Table 3B Part 3. Glossary** **seed unit:** dispersal unit for plant reproduction, such as true seeds, achenes, schizocarps, florets, bulbil, tuber, etc., as defined for each genus or species in the Pure Seed Definitions in Table 3B Parts 1 and 2.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.3.1			**WITHDRAWN**

C.3.2 Table 3B Part 2 PSD 36. Revised to remove the requirement to examine the caryopsis of Megathyrsus, Panicum and Digitaria in PSD 36

PSD 36 mentions "no need to check for the presence of a caryopsis" only for *Megathyrsus*, *Panicum* and *Digitaria*. The remaining genera included in PSD 36 have the same characteristics: it is exceptionally difficult to differentiate between pure seed and florets without a caryopsis. All these genera being chaffy dispersal units, are also difficult to blow as their seeds tend to be tacky and stick together. To not have to check for the presence of a caryopsis would make the purity test easier for the analyst but maintaining the consistency of the results as for *Megathyrsus*, *Panicum* and *Digitaria*.

The proposal was discussed in PUR and voted in the PUR with a majority vote.

CURRENT VERSION	PROPOSED VERSION
Table 3B Part 2. Numbered pure seed definitions 36. Spikelet, with or without pedicel, with glumes, lemma and palea ~~enclosing a caryopsis~~, plus attached sterile lemma. Floret, with lemma and palea ~~enclosing a caryopsis~~. Caryopsis. Piece of caryopsis larger than one-half the original size. *Axonopus:* spikelet, with single glume, lemma and palea ~~enclosing a caryopsis~~, plus attached sterile lemma. *Echinochloa* and *Melinis:* attached sterile lemma with or without awn. ~~*Megathyrsus*, *Panicum* and *Digitaria*: no need to check for the presence of a caryopsis~~. …	**Table 3B Part 2.** Numbered pure seed definitions 36. Spikelet, with or without pedicel, with glumes, lemma and palea, plus attached sterile lemma. No need to check for the presence of a caryopsis. Floret, with lemma and palea. No need to check for the presence of a caryopsis. Caryopsis. Piece of caryopsis larger than one-half the original size. *Axonopus:* spikelet, with single glume, lemma and palea, plus attached sterile lemma. *Echinochloa* and *Melinis:* attached sterile lemma with or without awn. …

Vote to accept item	Yes votes	No votes	Result
C.3.2	**2**	**17**	**FAIL**

C.3.3. Table 3B Part 2 and 3. Revised to add PSD 64 for dispersal tuber and its definition (glossary) WITHDRAWN

The tuber is pathway of plant vegetative reproduction and in some cases, it is how invasive species spread.

ISTA PT22-2 highlighted the issues with a natural vegetative propagule of *Cyperus esculentus* (yellow nutsedge), an objectionable weed in agricultural situations in many countries. This proposal is to classify tubers, like those of *Cyperus esculentus,* as other seeds, rather than inert matter in seed testing. Inert matter is recorded as a % weight, OSD is recorded as the number of propagation units. When dealing with objectionable weed propagules, the number of units found is more informative to seed testing stakeholders.

The proposal was supported by a majority vote of the Purity Committee.

CURRENT VERSION

Table 3B Part 2. Numbered pure seed definitions

63. Bulbil.

Piece of bulbil larger than one-half the original size.

Table 3B Part 1. Pure seed definition numbers and chaffiness of seeds, listed by genus

Genus	Family	PSD no.	Chaffiness
Cynosurus	Poaceae	28	C
Cytisus	Fabaceae	50	

Table 3B Part 3. Glossary

PROPOSED VERSION

Table 3B Part 2. Numbered pure seed definitions

63. Bulbil.

Piece of bulbil larger than one-half the original size.

64. Tuber

Piece of tuber with at least one node.

Table 3B Part 1. Pure seed definition numbers and chaffiness of seeds, listed by genus

Genus	Family	PSD no.	Chaffiness
Cynosurus	Poaceae	28	C
Cyperus (vegetative)	Cyperaceae	64	
Cytisus	Fabaceae	50	

Table 3B Part 3. Glossary

Tuber: a type of enlarged structure used as a storage organ for nutrients and asexual reproduction, which could be a stem tuber or root tuber (as in *Cyperus esculentus*).

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.3.3			WITHDRAWN

C.3.4. Revision of 1.5.2.2; 3.2; and 3.7 to clarify special test conducted on component purity parts

Laboratories are often requested to separate, weigh, and record the presence, absence, or percentage of inert matter such as soil, sclerotia, ergot, smut balls & nematode galls in the more than purity working weight (e.g., 25 000 seeds weight as specified in the ISTA Rules Chapter 2 Table 2C Part 1, Column 5). Currently, if the inert matter, such as soil, is removed and reported as a percentage on the OIC from other amounts, "... not in the rules" statement must be noted on the certificate.

The proposed changes clarify that testing in excess of the minimum purity working weight is allowed **for customer requested calculations of component parts.** The ISTA Rules currently allows this but it is not clearly stated.

This proposed amendment harmonizes ISTA with AOSA Rules.

The proposal was discussed in PUR and voted in the PUR committee with a majority vote.

CURRENT VERSION	PROPOSED VERSION
1.5.2.2 Upon request, the following information must be reported under 'Other determinations' as follows: • Other seeds may be divided into 'other crop seeds' and 'weed seeds'. In this case, the words 'Other crop seeds' must be entered, followed by the percentage by weight of other crop seeds and the name(s) of the species found. This procedure must also be used for 'Weed seeds'. • Multiple seed units must be reported as '% MSU'. • Seeds with appendages attached must be reported as '% seeds with appendages attached'. • The percentage by weight of broken pure seed.	**1.5.2.2.** Upon request, the following information must be reported under 'Other determinations' as follows: • Other seeds may be divided into 'other crop seeds' and 'weed seeds'. In this case, the words 'Other crop seeds' must be entered, followed by the percentage by weight of other crop seeds and the name(s) of the species found. This procedure must also be used for 'Weed seeds'. • Multiple seed units must be reported as '% MSU'. • Seeds with appendages attached must be reported as '% seeds with appendages attached'. • The percentage by weight of broken pure seed. • The percentage by weight of specified inert matter e.g., soil, sclerotia, ergot, smut balls, nematode galls. • Absence or presence of specified inert matter, e.g., soil, sclerotia, ergot, smut balls, nematode galls.

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The percentages may be reported to more than one decimal place if requested.

3.2.3 Inert matter

……………..
8. All material left in the light fraction when the separation is made by the uniform blowing method (3.5.2.5) except other seeds (as defined in 3.2.2). In the heavy fraction, broken florets, and caryopses half or less than half the original size, and all other matter except pure seed (3.2.1) and other seed (3.2.2).

3.7 Reporting results

Upon request, the following information must be reported under ‘Other determinations’ as follows:

• Other seeds may be divided into ‘other crop seeds’ and ‘weed seeds’. In this case, the words ‘Other crop seeds’ must be entered, followed by the percentage by weight of other crop seeds and the name(s) of the species found. This procedure must also be used for ‘Weed seeds’.
• Multiple seed units must be reported as ‘ % MSU’.
• Seeds with appendages attached must be reported as ‘ % seeds with appendages attached’.
• The percentage by weight of broken pure seed.
</td>
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The percentages may be reported to more than one decimal place if requested.

3.2.3 Inert matter
…………………..
8. All material left in the light fraction when the separation is made by the uniform blowing method (3.5.2.5) except other seeds (as defined in 3.2.2). In the heavy fraction, broken florets, and caryopses half or less than half the original size, and all other matter except pure seed (3.2.1) and other seed (3.2.2).

Upon request, specified inert matter e.g., soil, sclerotia, ergot, smut balls, nematode galls, may be separated on purity working weight, or on a weight as specified by the customer more than the minimum working weight of Table 2C column 4. For reporting the absence, presence, or percentage by weight refer to 3.7.

3.7 Reporting results

Upon request, the following information must be reported under ‘Other determinations’ as follows:

• Other seeds may be divided into ‘other crop seeds’ and ‘weed seeds’. In this case, the words ‘Other crop seeds’ must be entered, followed by the percentage by weight of other crop seeds and the name(s) of the species found. This procedure must also be used for ‘Weed seeds’.
• Multiple seed units must be reported as ‘% MSU’.
• Seeds with appendages attached must be reported as ‘ % seeds with appendages attached’.
• The percentage by weight of broken pure seed.
• The percentage by weight of specified inert matter e.g., soil, sclerotia, ergot, smut balls, nematode galls.
• Absence or presence of specified inert matter, e.g., soil, sclerotia, ergot, smut balls, nematode galls.
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The percentages may be reported to more than one decimal place if requested.	The percentages may be reported to more than one decimal place if requested.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.3.4	2	14	FAIL

Chapter 5: The germination test

C.5.1. Clarifying the use of various paper growing mediums in germination testing

Revised to improve the wording that paper can be used as a base medium with any other combination of growing media prescribed in Table 5A for a particular species. This issue was raised by a member and is supported by the Germination Committee.

This proposal originates from and is supported by the Germination Committee.

CURRENT VERSION	PROPOSED VERSION
5.4 Growing media **5.4.1 Definition** Growing media used for germination tests are products which provide sufficient pore space for air and water, for the growth of the root system and for contact with solutions (water) needed for plant growth. ~~With paper as the base medium (see 5.6.2.1.1), any combination of growing media prescribed in Table 5A for that species is allowed, provided that each growing medium is~~ verified and meets the specifications prescribed in 5.4.2.	**5.4 Growing media** **5.4.1 Definition** Growing media used for germination tests are products which provide sufficient pore space for air and water, for the growth of the root system and for contact with solutions (water) needed for plant growth. Additional paper growing media types listed in 5.4.3.1 (i.e. filter papers, blotters, towels and crepe cellulose) are allowed to be used as a base medium, to help ensure adequate moisture is provided during the germination test. Each paper growing medium type must be verified and meet the specifications prescribed in 5.4.2.

Consequential change if the above editorial correction is accepted at 5.4.3.1

CURRENT VERSION	PROPOSED VERSION
5.4.3 Growing media characteristics **5.4.3.1 Paper growing media** The paper must be wood, cotton, crepe cellulose paper or other purified vegetable cellulose. The paper may take the form of filter papers, blotters ~~or~~ towels. The paper should be such that: • the roots of the seedlings will grow on and not into it; however, it is acceptable that roots grow into the media as long as the seedling can be pulled out of the media without breaking any roots; • it possesses sufficient strength to enable it to resist tearing when handled during the test.	**5.4.3 Growing media characteristics** **5.4.3.1 Paper growing media** The paper must be wood, cotton, crepe cellulose paper or other purified vegetable cellulose. The paper may take the form of filter papers, blotters, towels or crepe cellulose. The paper should be such that: • the roots of the seedlings will grow on and not into it; however, it is acceptable that roots grow into the media as long as the seedling can be pulled out of the media without breaking any roots; • it possesses sufficient strength to enable it to resist tearing when handled during the test.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.1	**32**	**0**	**PASS**

C.5.2. Expansion of list of species that exhibit hard seeds

The primary purpose of this proposal is to expand the list of Families in the ISTA Rules, 5.2.10.1 that have hard seeds. The proposal also includes some of the families that do not exhibit hard seeds.
The secondary purpose is to remove the reference to Fabaceae in ISTA Rules 6.5.2.1.2.

This proposal harmonizes with the AOSA Rules.

This proposal was developed within the Germination Committee and is supported by the Germination Committee.

CURRENT VERSION	PROPOSED VERSION
5.2.10.1 Hard seeds Hardseededness is a form of dormancy. It is common in many species of ~~the~~ Fabaceae ~~but may also occur in other families~~. These seeds are not able to imbibe water under the conditions set out in Table 5A and remain hard.	**5.2.10.1 Hard seeds** Hardseededness is a form of dormancy. It is common in many species of Bixaceae, Cannaceae, Cistaceae, Fabaceae, Geraniaceae and Malvaceae. Other families reported to contain a few hard-seeded species include Anacardiaceae, Cochlospermaceae, Convolvulaceae, Dipterocarpaceae,

	Nelumbonaceae, Rhamnaceae, Sapindaceae and Sarcolaenaceae. These seeds are not able to imbibe water under the conditions set out in Table 5A and remain hard. Families for which there is no evidence of hard seeds include Amaryllidaceae, Brassicaceae, Chenopodiaceae, Cucurbitaceae (Note 1), Poaceae, Rosaceae, and Solanaceae. In addition, seeds of gymnosperms do not exhibit hard seed dormancy. Note 1: Very few wild species of this family have been reported to have hard seeds. However, none of the cultivated species of this family that are listed in Table 5A produce hard seeds. Seeds of those species that appear as 'hard seeds' are rigid due to physical and physiological constraints, but the seed coat is permeable to water.

Consequential change if C.5.1 is accepted:

CURRENT VERSION	PROPOSED VERSION
6.5.2.1.2 Soaking in water **…** If the percentage of hard seeds of ~~the Fabaceae~~ is to be determined for the purpose of issuing an ISTA Certificate, the seed should be soaked in water at 20 °C for 22 h. Other procedures may lead to excessive variability in results. **6.5.4 Evaluation** … Hard seeds are seeds with water-impermeable seed coats (~~e.g. Fabaceae~~) and remain hard even after premoistening **6.7 Reporting results** … In addition, in the case of species ~~of Fabaceae~~, one of the following, and only one, must be reported:	**6.5.2.1.2 Soaking in water** **…** If the percentage of hard seeds of species of those families listed in 5.2.10.1 is to be determined for the purpose of issuing an ISTA Certificate, the seed should be soaked in water at 20 °C for 22 h. Other procedures may lead to excessive variability in results. **6.5.4 Evaluation** … Hard seeds are seeds with water-impermeable seed coats (e.g. see 5.2.10.1) and remain hard even after premoistening **6.7 Reporting results** … In addition, in the case of species with hard seeds, one of the following, and only one, must be reported:

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.2	**32**	**0**	**PASS**

C.5.3. Addition of pre-soaking method for *Beta vulgaris*

Addition of presoaking method for *Beta vulgaris* as an additional method for removing inhibitory substances. This method greatly reduces the amount of water required to remove the inhibitory substances from *Beta vulgaris* before germinating.

This proposal harmonizes with the AOSA Rules.

This proposal is supported by the Germination Committee and a Method Validation Study.

CURRENT VERSION	PROPOSED VERSION
5.6.3.3. Procedures for removing inhibitory substances	**5.6.3.3. Procedures for removing inhibitory substances** Naturally occurring substances in the pericarp or seed coat which act as inhibitors of germination may be removed by soaking or washing the seeds. **Presoaking:** Soak seeds in water for 2 hours, 250 ml of water per 100 seeds. Rinse in running water and blot the surface dry. Temperature of soaking and rinsing water must be 20 to 25 °C. Pelleted seed must not be pre-soaked.
Prewashing: ~~Naturally occurring substances in the pericarp or seed coat which act as inhibitors of germination may be removed by washing~~ the seeds in running water at a temperature of 25 ±2 °C before the germination test is made. After washing, the seeds must be dried at a temperature of 20 to 25 °C (e.g. *Beta vulgaris*). Pelleted seed must not be prewashed.	**Prewashing:** Wash the seeds in running water at a temperature of 25 ±2 °C before the germination test is made. After washing, the seeds must be dried at a temperature of 20 to 25 °C (e.g. *Beta vulgaris*). Pelleted seed must not be prewashed. ....

Table 5A Part 1.

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice	Seedling Evaluation Group
1	2	3	4	5	6	7	8	9
Beta vulgaris	TP; BP; S	20⇔30; 15⇔25; 20	4	14	Presoak (2 h; 250 ml per 100 seeds) Prewash (multigerm: 2 h; genetic monogerm: 4 h). Dry at max. 25 °C	–	–	A-2-1-1-1

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.3	33	0	PASS

C.5.4. Merger of Table 5A Part 1 and Part 3 into Table 5A Part 1

Merger of Table 5A Part 1 and Part 3 being renamed Table 5A Part 1. Table 5A Part 2 will remain unchanged. This proposal comes from discussion during the ISTA Annual Meeting 2023 held in Verona, regarding the merger of other tables within the ISTA Rules (e.g. Table 9A).

The proposal is supported by the Germination Committee.

CURRENT VERSION

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice	Seedling Evaluation Group
1	2	3	4	5	6	7	8	9
Abelmoschus esculentus	TP; BP; S	20⇔30	4	21	–	–	–	A-2-1-1-2
Achillea millefolium	TP	20⇔30	5	14	–	–	–	A-2-1-1-1

….

Table 5A Part 3. Detailed methods for germination tests: flower, spice, herb and medicinal species

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Seedling Evaluation Group
1	2	3	4	5	6	7
Abutilon ×hybridum	TP; BP	20⇔30; 20	5–7	21	–	A-2-1-1-2
Achillea clavennae	TP; BP	20⇔30; 20	5	14	Light	A-2-1-1-1

….

PROPOSED VERSION

Table 5A Part 1. Detailed methods for germination tests: agricultural, vegetable, flower, spice, herb and medicinal species

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice	Seedling Evaluation Group
1	2	3	4	5	6	7	8	9
Abelmoschus esculentus	TP; BP; S	20⇔30	4	21	–	–	–	A-2-1-1-2
Abutilon ×hybridum	TP; BP	20⇔30; 20	5–7	21	–	-	-	A-2-1-1-2
Achillea clavennae	TP; BP	20⇔30; 20	5	14	Light	-	-	A-2-1-1-1
Achillea millefolium	TP	20⇔30	5	14	–	–	–	A-2-1-1-1

…..

Consequential changes if accepted.
Contents page, 5.6.5.3 Ungerminated seeds and 5.10 Germination Methods would need to be updated:

CURRENT VERSION	PROPOSED VERSION
Contents page **…** 5.10 Germination methods…………………………..5-20 Table 5A Part 1. Detailed methods for germination tests: agricultural ~~and~~ vegetable seeds … 5-22 Table 5A Part 2. Detailed methods for germination tests: tree and shrub seeds…. 5-34 ~~Table 5A Part 3. Detailed methods for germination tests: flower, spice, herb and medicinal species … 5-44~~ 5.11 Tolerance tables…………………………………… 5-55	**Contents page** **…** 5.10 Germination methods………………………..5-20 Table 5A Part 1. Detailed methods for germination tests: agricultural, vegetable, flower, spice, herb and medicinal species seeds … 5-22 Table 5A Part 2. Detailed methods for germination tests: tree and shrub seeds…. 5-34 5.11 Tolerance tables……………………………… 5-55

CURRENT VERSION	PROPOSED VERSION
5.10 Germination methods …… Abbreviations …… KNO_3 use solution of 0.2 % potassium nitrate instead of water TTZ tetrazolium test Table 5A Part 1. Detailed methods for germination tests: agricultural ~~and~~ vegetable ~~seeds~~ Table 5A Part 2. Detailed methods for germination tests: tree and shrub seeds	5.10 Germination methods …… Abbreviations …… KNO_3 use solution of 0.2 % potassium nitrate instead of water TTZ tetrazolium test Table 5A Part 1. Detailed methods for germination tests: agricultural, vegetable, flower, spice, herb and medicinal species Table 5A Part 2. Detailed methods for germination tests: tree and shrub seeds

~~Table 5A Part 3. Detailed methods for germination tests: flower, spice, herb and medicinal species~~	

CURRENT VERSION	PROPOSED VERSION
5.6.5.3 Ungerminated seeds … Fresh seeds: When 5 % or more of fresh seeds are believed to be present, their potential to germinate must be determined by dissection, tetrazolium or excised embryo. Tetrazolium may be used to determine the germination potential of all species listed in Table 5A Parts 1~~,~~ 2 ~~and 3~~, regardless of whether these species are listed in Table 6A Part 1 or 2. Those determined to have the potential to germinate are reported as fresh. Those determined not to have the potential to germinate are reported as dead. After this determination, if there is any doubt as to whether the seed is fresh or dead, it must be classified as dead. When a fuller germination assessment is required by the laboratory or upon the request of the customer, retesting utilising a procedure for removing dormancy described in 5.6.3 is essential.	**5.6.5.3 Ungerminated seeds** … Fresh seeds: When 5 % or more of fresh seeds are believed to be present, their potential to germinate must be determined by dissection, tetrazolium or excised embryo. Tetrazolium may be used to determine the germination potential of all species listed in Table 5A Parts 1 and 2, regardless of whether these species are listed in Table 6A Part 1 or 2. Those determined to have the potential to germinate are reported as fresh. Those determined not to have the potential to germinate are reported as dead. After this determination, if there is any doubt as to whether the seed is fresh or dead, it must be classified as dead. When a fuller germination assessment is required by the laboratory or upon the request of the customer, retesting utilising a procedure for removing dormancy described in 5.6.3 is essential.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.4	**31**	**0**	**PASS**

C.5.5. Additional advice for testing *Spinacia oleracea*

ISTA Proficiency Test ISTA PT22-2 *Spinacia oleracea* highlighted a higher level than expected of C and BMP ratings for the germination. Further investigation showed that the amount of water added to the media was an important aspect for germination as *Spinacia oleracea* is water sensitive. The 'Additional advice' column has been updated to recommend 'low moisture level advisable'. A report showing the evidence to support this wording will be published in Seed Testing International, April 2024.

This proposal harmonizes with the AOSA Rules.

This proposal was developed in the Germination Committee is supported by the Germination Committee.

CURRENT VERSION

Table 5A Part 1. Detailed methods for germination tests: agricultural and vegetable seeds

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice	Seedling Evaluation Group
1	2	3	4	5	6	7	8	9
Spinacia oleracea	TP; BP	15; 10	7	21	Prechill	–	–	A-2-1-1-1

PROPOSED VERSION

Table 5A Part 1. Detailed methods for germination tests: agricultural, vegetable, flower, spice, herb and medicinal species

Species	Substrate	Temperature* (°C)	First count (d)	Final count (d)	Recommendations for breaking dormancy	Additional directions	Additional advice	Seedling Evaluation Group
1	2	3	4	5	6	7	8	9
Spinacia oleracea	TP; BP	15; 10	7	21	Prechill	–	Low moisture level advisable	A-2-1-1-1

VOTE TO ACCEPT ITEM	**YES VOTES**	**NO VOTES**	**RESULT**
C.5.5	**33**	**0**	**PASS**

C.5.6. Clarifying temperature variation requirements for germination **WITHDRAWN**

Change is to make it clearer and more precise that the temperature range allowed is ±2.0 °C.

This change was requested by a member and is supported by the Germination Committee.

Current version	Proposed version
5.6.2.3 Temperature …… For any test, whether in darkness or under artificial light or indirect daylight, variation from the prescribed temperature must not be more than ±2°C. …….	**5.6.2.3 Temperature** …… For any test, whether in darkness or under artificial light or indirect daylight, variation from the prescribed temperature must not be more than ±2.0 °C …….

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.5.6			**WITHDRAWN**

Chapter 7: Seed Health Testing Methods

C.7.1 Detection of *Ascochyta rabiei* in *Cicer arietinum* (chickpea) seed (7-033)

Ascochyta rabiei (syn. *Phoma rabiei*), also known by its teleomorph name *Didymella rabiei* (syn. *Mycosphaerella rabiei*) is a very aggressive pathogen responsible for *Ascochyta* blight on chickpea seeds. This method includes a detection method to detect and identify *Ascochyta rabiei* and an optional pathogenicity test to confirm the pathogenicity of the isolates.

There is a method validation study to support this proposal. ***The entire method (7-033), as proposed for the Seed Health Methods, can be found in Appendix 1 of this document.***

AOSA Rules does not have seed health testing within its scope.

This proposal was submitted by the SH Committee.

CURRENT VERSION	PROPOSED VERSION
NONE …	**7-033: Detection of *Ascochyta rabiei* in *Cicer arietinum* (chickpea) seed** **Host:** *Cicer arietinum* (chickpea) **Pathogen(s):** *Ascochyta rabiei* (Pass.) Labrousse syn. *Phoma rabiei* (Pass.) Khune ex Gruyter. **Authors:** Le Daré ,L[1]., Sérandat, I.[1], Le Guisquet, C.[1], Penant, A.[2], Lambert, Q.[2], Grimault, V.[1] and Asaad, S.[3] [1] *GEVES, 25 rue Georges Morel, CS 90024, 49071 Beaucouzé, France* [2] *Terres Inovia, 270 Avenue de la Pomme de Pin 45160 ARDON, France.* [3] *ICARDA, Dahlia building, Bachir El Kassar Street, Verdun, Beirut 1108-2010, Lebanon* **Revision history** Version 1.0

The new method, in its final format within the Seed Health Methods, is in Appendix 1 to this document.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.7.1	**32**	**0**	**PASS**

Chapter 9: Determination of moisture content

C.9.1 Adding Table 2C to Chapter 9 as a point of reference

If the weight of the moisture sample drawn by the sampler is added to Table 2C, then “Table 2C” should be included in the reference in 9.2.5.1. The MOI suggests including "Table 2C" in the reference, and deleting the reference to Table 9A.

This proposal came from the BSC and MOI. This proposal has been voted for and approved by a majority of the MOI.

CURRENT VERSION	PROPOSED VERSION
9.2.5.1 General directions and precautions See Table 9A for directions for individual species. The submitted sample (see 2.5.1.5–2.5.1.6) may be accepted for moisture determination only if it has the required sample size (2.5.4.5c ~~and Table 9A~~), and is in an intact, moisture-proof container (or, if issuing a Blue International Seed Sample Certificate, apparently moisture-proof) from which as much air as possible has been excluded. …	**9.2.5.1 General directions and precautions** See Table 9A for directions for individual species. The submitted sample (see 2.5.1.5–2.5.1.6) may be accepted for moisture determination only if it has the required sample size (2.5.4.5c and Table 2C), and is in an intact, moisture-proof container (or, if issuing a Blue International Seed Sample Certificate, apparently moisture-proof) from which as much air as possible has been excluded. …

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.9.1	**31**	**0**	**PASS**

C.9.2 Clarification on testing ISTA recognized *Lupinus* species for Moisture Testing

The *Lupinus* genus was added to the ISTA Rules, in 2023, under agricultural species as *Lupinus* spp. (Table 9A Part 2). In the 2024 edition of the ISTA Rules, this listing is now valid for flower and other *Lupinus* species which may have smaller seeds than those intended for the current coarse grinding method for *Lupinus* spp. As a result, the MOI in consultation with the BSC, proposes to name each individual *Lupinus* species appropriate for coarse grinding to avoid confusion and testing error.

This proposal was approved by majority vote within the MOI.

Proposed Version

Table 9A Details of methods for moisture determination

The oven method must be used as specified for the species in this Table.

Species	Grinding/cutting (9.2.5.4, 9.2.5.5)	Drying Temp. High: 130°C Low: 103°C	Drying time (h)	Tolerances of replicates (9.2.6.2)	Predrying requirement (9.2.5.6) / remarks
1	2	3	4	5	6
~~Lupinus spp.~~	~~Coarse~~	~~High~~	~~1~~	~~0,2 %~~	~~To 17% moisture content or less~~
Lupinus albus L.	coarse	High	1	0.2 %	To 17 % Moisture content or less
Lupinus angustifolius L.	coarse	High	1	0.2 %	To 17 % Moisture content or less
Lupinus luteus L.	coarse	High	1	0.2 %	To 17 % Moisture content or less

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.9.2	34	0	PASS

Chapter 15: Seed vigour testing

C.15.1 Addition of a new species for the Radicle emergence test

It is proposed to add *Glycine max* as a species to which the radicle emergence test can be applied. The radicle emergence test has been shown to predict differences in the field emergence of soybean seed lots, i.e. to identify differences in seed vigour (*Seed Science and Technology*, **51**, 3, 493-503, December 2023).

This proposal was approved by the Vigour committee.

Table 15B. Specific conditions for the radicle emergence test procedures; all assessments of radicle emergence should be made by eye and without magnification

Species	Germination medium	Replication	Germination temperature	Criterion of radicle emergence	Timing of radicle emergence count
Brassica napus	Pleated papers	2 replicates of 100 seeds	20 ± 1 °C	Appearance of a radicle after breaking through the seed coat. Seeds in which the seed coat has split, but no radicle	30 h ± 15 min

				has emerged, must not be included.	
Glycine max	Between paper	4 replicates of 50 seeds	20 ± 1 °C	Production of 2mm radicle	48 h ±15mins
Raphanus sativus	Top of paper	4 replicates of 50 seeds	20 ± 1 °C	Production of 2mm radicle	48 h ±15 min
Triticum aestivum	Between paper	4 replicates of 50 seeds	15 ± 1 °C	Production of 2mm radicle Radicle includes parts that are within the coleorhiza as well as those that have emerged through it	48 h ±15 min
Zea mays	Between paper	8 replicates of 25 seeds	20 ± 1 °C or 13 ±1 °C	Production of 2mm radicle Radicle includes parts that are within the coleorhiza as well as those that have emerged through it	66 h ± 15 min at 20 ± 1 °C 144 h ± 1 h at 13 ± 1 °C

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.15.1	**31**	**2**	**PASS**

C.15.2 The effect of temperature on final radicle emergence percentages

Temperature is an important variable in the radicle emergence test. The test may for some species be conducted at one of two validated temperatures e.g. for *Zea mays*, at 20 °C or 13 °C. Testing at both temperatures will result in the same ranking of seed lots in terms of their vigour, but the final radicle emergence percentage will differ. We have added the proposed text to clarify this.

This proposal has been accepted unanimously by the Vigour Committee.

CURRENT VERSION	PROPOSED VERSION
15.8.4.4.2 Temperature for the test The radicle emergence test must be conducted at the temperature prescribed for the species in Table 15B. Temperature is the most important variable in the test, and each seed lot must be transferred to the test	**15.8.4.4.2 Temperature for the test** The radicle emergence test must be conducted at the temperature prescribed for the species in Table 15B. Temperature is the most important variable in the test, and each seed lot must be transferred to the test

temperature within 15 minutes after being set to germinate. Monitoring of temperature is desirable and rotation of seed lots and replicates is advised at time intervals of 24 h	temperature within 15 minutes after being set to germinate. Monitoring of temperature is desirable and rotation of seed lots and replicates is advised at time intervals of 24 h When two temperatures have been validated for a species, be aware that results will depend on temperature: vigour ranking will be the same, but the final percentage radicle emergence will be different.

VOTE TO ACCEPT ITEM	YES VOTES	NO VOTES	RESULT
C.15.2	**30**	**2**	**PASS**

End of Rules Proposals.

Appendix documents on next page

Appendix 1:

7-033: Detection of *Ascochyta rabiei* in *Cicer arietinum* (chickpea) seed

Le Daré ,L[1]., Sérandat, I.[1], Le Guisquet, C.[1], Penant, A.[2], Lambert, Q.[2], Grimault, V.[1] and Asaad, S.[3]

[1] GEVES, 25 rue Georges Morel, CS 90024, 49071 Beaucouzé, France
[2] Terres Inovia, 270 Avenue de la Pomme de Pin 45160 ARDON, France.
[3] ICARDA, Dahlia building, Bachir El Kassar Street, Verdun, Beirut 1108-2010, Lebanon

Background

Ascochyta rabiei (syn. *Phoma rabiei*), also known by its teleomorph name *Didymella rabiei* (syn. *Mycosphaerella rabiei*) is responsible for *Ascochyta* blight on chickpea seeds.
In 2019, the French project AsCoLuP started with the ambition to provide a better management and understanding of two diseases on chickpea and lupin. One of the aims of this project was to provide a validated method to detect, identify and test the pathogenicity of *Ascochyta rabiei* on chickpea.
Chickpea blight, caused by the fungus *Ascochyta rabiei* is one of the most serious diseases of the crop and severe epidemics have been reported worldwide (Nene, 1982; Nene and Reddy, 1987; Collard et al., 2001). This pathogen is very aggressive on chickpea crops and can spread quickly in the field once established and when weather conditions are suitable (Pearse, 2005). The crop reaction is based on the weather conditions, specific cropping practices and cultivar (Pearse, 2005).
The fungus is selectively attacking chickpea, then persists in the crop's residues, seeds, and weeds.
There is a high rate of seed-to seedling transmission of *Ascochyta* in chickpea, even a small number of infected seed can result in significant seedling infection in the field, and seed-to – seedling transmission is high. Report indicated that a 0.1 per cent *Ascochyta*-infected seed lot (one infected seed in 1000 seeds), could potentially result into 175 infected seedlings per acre (Pearse, 2005).
The use of *Ascochyta* blight-free seed and seed treatment with effective fungicides reduces the probability of transmitting seed-borne disease to the seedlings (Gan, et al. 2006). The infected seeds are often symptomless; therefore, a reliable seed health detection method is crucial to avoid plant infection.
Initial crop infection is due to the introduction of either infected seed or from movement of infected plant debris, with windborne spores (ascospores), machinery or animals. Spores of the fungus can survive for a short time on skin, clothing as well as machinery. Subsequent in-crop infection occurs when inoculum is moved higher in the canopy or to surrounding plants by wind or rain splash during wet weather (Cumming, et al. GRDC, 2009).
This method includes a detection method to detect and identify *Ascochyta rabiei* and an optional pathogenicity test to confirm the pathogenicity of the isolates.

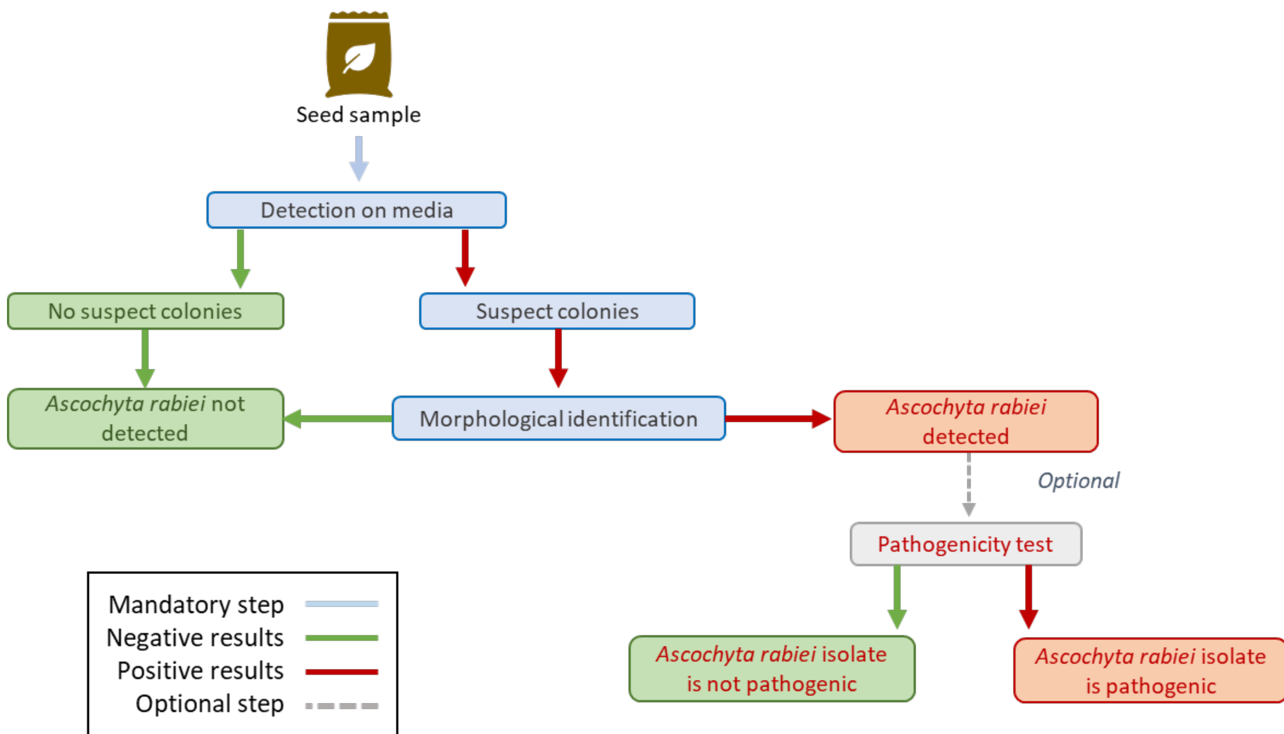

Figure 1. Process flow diagram explaining method assays and decisions taken depending on intermediate results until final result, for detection and pathogenicity of *Ascochyta rabiei* in *Cicer arietinum* (Chickpea) seed.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during preparation of media, autoclaving and weighing out of ingredients. It is assumed that this procedure is being carried out in a microbiological laboratory by persons familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic technique. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Treated seed

This method has not been validated for the detection of *Ascochyta rabiei* on treated seed. (Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3.)

Sample size

The sample (total number of seeds tested) and subsample size to be tested depend on the desired tolerance standard (maximum acceptable number of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). The minimum recommended sample size is 400 seeds.

Materials

Reference material: reference cultures or other appropriate material to use as PPC (Positive Process Control)
Media: Malt-agar or Potato dextrose agar (for detection), CSMDA (for pathogenicity test)
Autoclave
1% NaOCl (Sodium hypochlorite): For seed disinfection
Sterile distilled/deionised water
Sterile blotter paper: For blotting seed dry and germination of seedlings for pathogenicity test
Plates: 90 mm sterile Petri dishes (one per ten seeds)

Sterilizer
Tweezers
Incubator: operating at 20 ± 2ºC darkness (incubation of samples)
Incubator: operating at 20 ± 2ºC NUV (growing of reference material)
Stereomicroscope (×6.5 – 50 magnification)
Compound microscope (×100 – 400 magnification)
Chickpea seedling: susceptible to all races of the pathogen for pathogenicity test (e.g. 'Benito')
Filter
Beakers
Microscopic slides
Sterile blades
Malassez cell (Hemacytometer)
Sterile pipettes
Plastic bags
Steamed potting soil
Trays, cups, and cover
Growth chamber: operating 20 ± 2 °C, 80% RH

Methods
Note: All the steps described here are important and should be followed as written. Critical control points are indicated by CCP.
Overview of methods

1. Detection method
    1. Pre-treatment: Immerse seeds in a 1% sodium hypochlorite (NaOCl) solution for 10 min, then drain, rinse well in sterile water and drain again. Dry the seeds on sterile blotter paper.
    2. Plating: Aseptically place a maximum of 10 seeds, evenly spaced around the perimeter of the plate, onto the agar surface of each PDA or MA plate.
    3. Positive control (reference material): Aseptically plate a reference culture on media.
    4. Incubation: 7 days at 20 ±2˚C, darkness.
    5. Examination (CCP): After 7 days, examine each seed by naked eye (**Figure 2** a, b). *Ascochyta rabiei* is a slow growing fungus and might be located on the seed coat or under the seeds. To allow a correct notation, it is important to move the seeds away to reveal the presence of mycelium and pycnidia. On both media, white to green coloured mycelium develops slowly, development of pale brown to dark brown pycnidia could occurs on surface or immersed. At 7 days, colony diameter varies between 10-35 mm on both media. Pycnidia usually release orange mucilaginous mass of pycnidiospores (cirrus). Doubtful colonies may be confirmed by the presence of pycnidia on seed coat or on media surface when examined at x25 magnification, using stereomicroscope: Pycnidia are 65-245 µm diameter, ostiole 30-50µm wide. Further confirmation can be made by examining pycnidiospores present in pycnidia using compound microscope (x100-1000 magnification): pycnidiospores are hyaline, straight or slightly curved (0-)1 septate, some unicellular, slightly, or not constricted at the septum, rounded at each end, mostly 7-16 x 3-5 µm. (**Figure 3** and **Figure 4**)
2. Pathogenicity test (optional)
    1. Inoculum multiplication: Plate the isolates on CSMDA media (**Figure 5**), incubate at 20°C, NUV for at least 7 days.
    2. Pre-treatment: Immerse seeds of a chickpea cultivar know to be highly susceptible to *Ascochyta rabiei* (e.g. 'Benito') in a 1% sodium hypochlorite (NaOCl) solution for 10 min, then drain, rinse well in sterile water and drain again. Dry the seeds on sterile blotter paper.

3. Germination: Place the disinfected seeds on a wet blotter paper. Roll the paper with the seeds and place it in a plastic bag. Incubate the closed bags at 20°C, darkness for 2-3 days to allow seed germination. Make sure to germinate enough seeds for all the suspect colonies to be tested.
4. Inoculum preparation: Pour 2 mL of sterile water on the CSMDA plates containing the grown isolate and scrap the surface with a microscopic slide. Filter the inoculum and estimate the concentration using Malassez cell. Dilute, if necessary, in sterile water to obtain a concentration from 1.10^4 to 1.10^5 conidia/mL. Make sure to have a sufficient volume of inoculum to soak the seeds.
5. Inoculation: Cut the root tips (approx. 1cm) of 2-3-days-old germinated chickpea seeds (**Figure 6**) and soak 3 seeds in each inoculum for 10 min (**Figure 7**). Sow the seeds in potting soil.
6. Negative control: Repeat with soaking of 3 seeds in sterile water
7. Incubation: 10 days at 20 ± 2˚C; RH = 100% (using a cover), 12h light, 12h dark.
8. Examination: After 10 days, take the plantlets out, check the presence of symptoms and compare to the positive (**Figure 8**) and negative controls (**Figure 9**) (CCP). Symptoms caused by *Ascochyta rabiei* are necrosis on the stem (all the time) (**Figure 10**), seed blackened (most of the time), leaf wilting (often), plantlet rotting (sometimes).
9. Record the suspect colonies as pathogenic if at least one of the seedlings present black necroses on the stem.

General methods

Checking tolerances: Tolerances provide a means of assessing whether the variation in result within or between tests are sufficiently wide as to raise doubts about the accuracy of the results. Suitable tolerances, which can be applied to most direct seed health tests, can be found in Table 5B Part 1 of Chapter 5 of the *ISTA Rules* or in Table G1 in Miles (1963).

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

The report must indicate the number of seeds tested.

For detection method:

In the case of a negative result (pathogen not detected), the results must be reported as "not detected".

In the case of a positive result, the report must indicate "detected" and the percentage of infected seeds.

For pathogenicity rest:

In the case of a negative result (absence of symptoms on all seedlings), the results must be reported as "not pathogenic".

In the case of a positive result (presence of symptoms on at least one of the seedlings), the results must be reported as "pathogenic".

Quality assurance

Specific training

This test should only be performed by persons who have been trained in fungal identification or under the direct supervision of someone who has.

Critical control points (CCP)

When the wording of the original Working Sheet suggests that an action is critical this has been marked with CCP.

- The examinations should be carried out by trained technical staff, as morphological identification of *Ascochyta rabiei* and its symptoms require experience. (Step 1.5 and 2.8)
- The plantlets of the positive control seed sample should give typical symptoms (at least black necrosis on the stem) and the plantlets of the negative control sample should give no symptoms (at least absence of black necrosis on the stem). (Step 2.8)

Media and solutions

Sodium hypochlorite solution

Sodium hypochlorite for pretreatment of seed can be prepared from commercial bleach diluted to 1 % available chlorine. The concentration of chlorine in commercial bleach varies considerably and declines with storage. Use the formula:

$V_{stock} = V_{final} \times C_{final} / C_{stock}$

(where V = volume and C = % available chlorine) to calculate the volume of commercial bleach stock solution required to prepare sodium hypochlorite solutions for use in seed pretreatment.

To prepare a 1L solution of sodium hypochlorite containing 1 % chlorine from a stock of commercial bleach containing 12 % available chlorine:

$V_{stock} = 1 \times 1/12 = 0.083$

Thus add 83mL of the 12 % stock to 917 mL water. The percentage of active chlorine decreases rapidly in solution so, NaClO 1 % solution must be stored in the dark and used within 3 days of preparation. It is possible to check chlorine concentration with chlorine strip tests.

Malt agar (MA)

Malt agar + streptomycin:

Agar: 20 g

Malt: 10 g

~~According to manufacturer's instructions~~

Distilled/deionised water: 1000 mL **Streptomycin sulfate:** may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.

Added after autoclaving. Streptomycin sulfate can be dissolved in water. Filter sterilisation is required.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 mL of water.
3. Dissolve completely the ingredients in water by stirring.
4. Autoclave at 15 psi and 121 °C for 15 min.
5. Allow agar to cool to approximately 50 °C and add Streptomycin sulphate dissolved in sterile water.
6. Pour 18-20 mL of malt agar into 90 mm Ø Petri dish and allow to solidify before use.

Storage

Prepared plates may be stored at 4°C for up to 6 weeks.

Potato Dextrose Agar (PDA)

Potato Dextrose Agar: According to manufacturer's instructions

Distilled/deionised water: 1000 mL

Streptomycin: may be used between 50 mg and 130 mg, depending on the level of saprophytic bacterial contamination commonly encountered.

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 mL of distilled/deionised water.
3. Dissolve completely the ingredients in water by stirring.
4. Autoclave at 15 psi and 121 °C for 15 min.
5. Allow agar to cool to approximately 50 °C and add ~~S~~streptomycin sulphate dissolved in sterile distilled/dionised water.
6. Pour 18-20 mL of PDA into 90 mm Ø Petri dish and allow to solidify before use.

Storage

Prepared plates may be stored at 4°C for up to 6 weeks.

Chickpea Seed Meal Dextrose Agar (CSMDA)
Chickpea seed meal :40 g
D-glucose: 20 g
Agar: 14 g
Distilled/deionised water: 1000 mL

Preparation

1. Weigh out ingredients into a suitable autoclavable container.
2. Add 1000 mL of distilled/deionised water.
3. Dissolve completely the ingredients in water by stirring.
4. Autoclave at 15 psi and 121 °C for 15 min.
5. Allow agar to cool to approximately 50 °C.
6. Pour 18-20 mL of CSMDA into 90 mm Ø Petri dish and allow to solidify before use.

Storage

Prepared plates may be stored at 4°C for up to 6 weeks.

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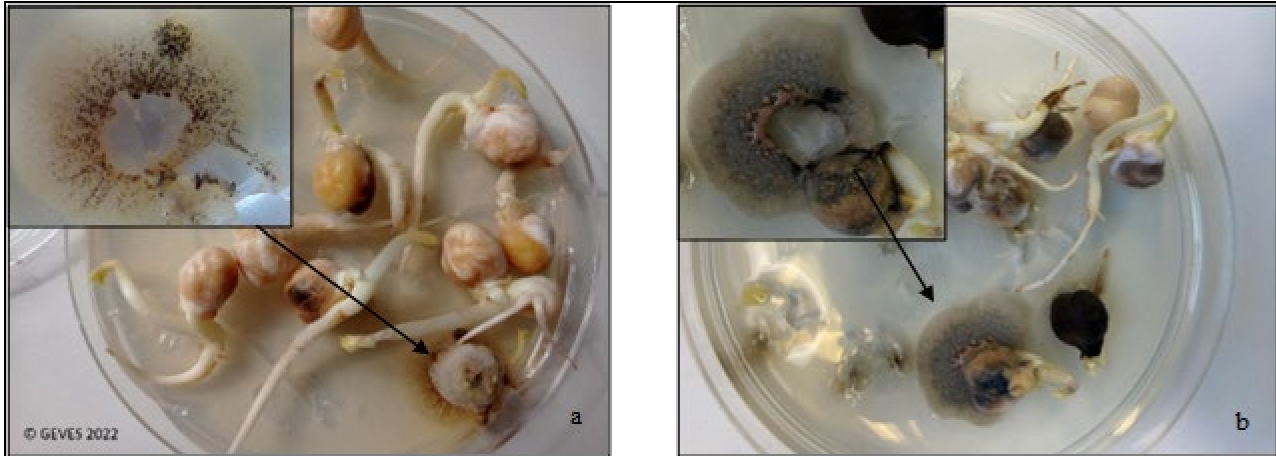

Figure 2. Colonies of *Ascochyta rabiei* after 7 days, incubation in darkness **a.** MA **b.** PDA

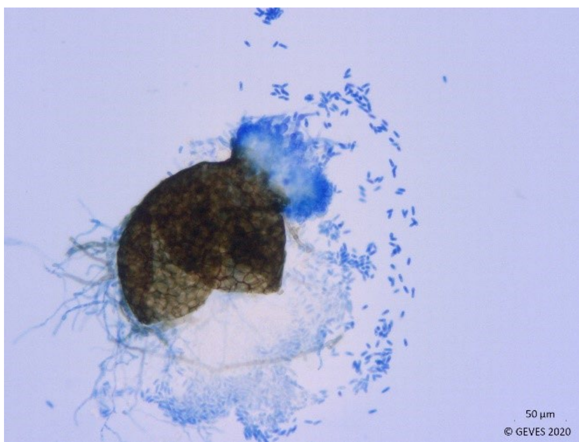

Figure 3. Pycnidia and pycnidiospores of *Ascochyta rabiei* (x200)

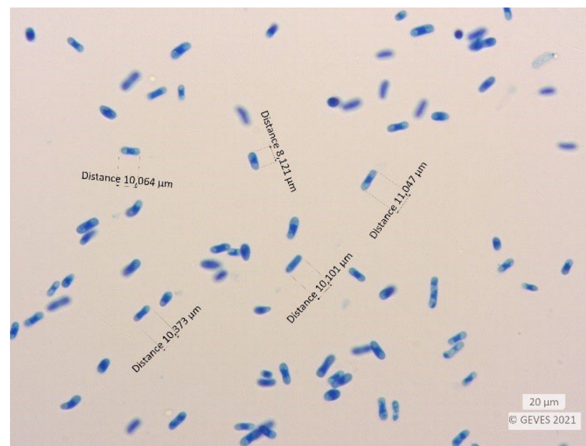

Figure 4. Pycnidiospores of *Ascochyta rabiei* (x400)

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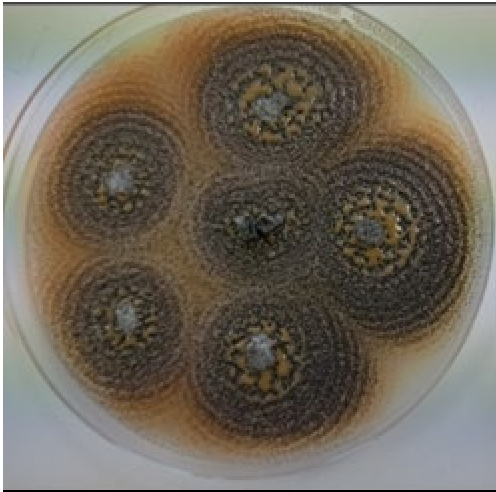
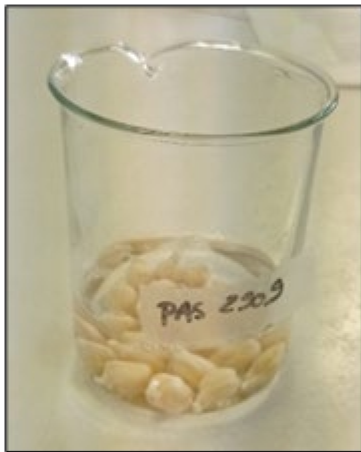
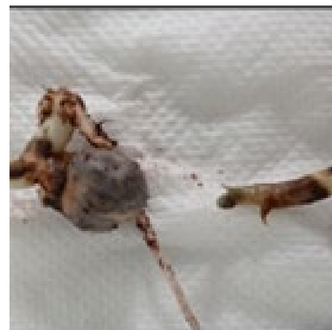
<tr><td></td><td> </td></tr>
<tr><td>Figure 5. Colonies of Ascochyta rabiei on CSMDA</td><td>Figure 6. Cutting of root-tips of 2-days-old germinated seeds</td></tr>
<tr><td>
</td><td></td></tr>
<tr><td>Figure 7. Inoculation by soaking cutted germinated seeds into 1.10^5 conidia/mL Ascochyta rabiei suspension</td><td>Figure 8. Cicer arietinum seedlings 10 days after inoculation with pathogenic isolate of Ascochyta rabiei</td></tr>
<tr><td> </td><td> </td></tr>
<tr><td>Figure 9. Cicer arietinum seedlings 10 days after inoculation with negative control</td><td>Figure 10. Example of stem necrosis due to Ascochyta rabiei on Cicer arietinum seedling</td></tr>
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