

International Seed Testing Association Secretariat, Zürichstrasse 50, CH-8303 Bassersdorf, Switzerland Phone: +41 44 838 60 00 Fax: +41 44 838 60 01

Email: ista.office@ista.ch - http://www.seedtest.org

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Diagnostic protocol 7/91 for *Gibberella circinata* on *Pinus* spp. (pine) and *Pseudotsuga menziesii* (Douglas-fir) seed

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Diagnostics Diagnostic

Gibberella circinata

Specific scope

This standard describes a diagnostic protocol for *Gibberella circinata*¹.

Specific approval and amendment

Approved in 2009-09.

Introduction

Gibberella circinata is the causal agent of pitch canker disease. The disease almost exclusively affects *Pinus* sp., but was also described on Douglas-fir (*Pseudotsuga menziesii*). This disease is a serious threat to the pine forests wherever it occurs (especially on plantations of *Pinus radiata*), due to extensive tree mortality, reduced growth and timber quality. Multiple branch infection may cause severe crown dieback and eventually lead to the death of the tree. This aggressive fungus may also cryptically infect the *Pinus* seeds and may cause damping-off in seedlings. Conifer seeds can be colonized by *G. circinata* internally (where it can remain dormant until seed germination) and externally (Storer *et al.*, 1998).

Fusarium circinatum, anamorph of *G. circinata*, is predominantely a wound pathogen and enters the host tree through mechanical wounds or feeding holes caused by woodboring insects. The fungus may move from tree to tree by aerial dispersion of the conidiospores or through vectoring by feeding insects (Gordon *et al.*, 2001; Schweigkofler *et al.*, 2004). However, long-range dispersal of the pathogen from affected areas to disease-free areas may be driven by infected seed movement and movement of infected plant material (Storer *et al.*, 1998).

The fungus is officially reported in the USA, Mexico, Haiti, South Africa, Japan, Chile (OEPP/EPPO, 2005) and has been officially reported in the EPPO region only recently: Spain (Landeras *et al.*, 2005; under eradication), Italy (Carlucci *et al.*, 2007 eradicated), France (OEPP/EPPO, 2008 under eradication). In most instances of introduction into new areas the pest was first found in nurseries.

The fungus has gone through a number of anamorph name changes, with the same teleomorph Gibberella fujikuroi. Originally described as Fusarium lateritium f. sp. pini (Snyder et al., 1949), it was successively renamed Fusarium moniliforme f. sp. subglutinans (Kuhlman et al., 1978), F. subglutinans f. sp. pini, 'H' mating population (Correll et al., 1991, 1992) and was finally ranked at the species level as F. circinatum, with a new teleomorph recognition: G. circinata (Nirenberg & O'Donnell, 1998). The G. fujikuroi sp. complex encompasses at least 36 anamorphic Fusarium sp. (O'Donnell et al., 1998), from which the new species G. circinata is now excluded. Numerous Fusarium sp. residing in this complex, already fully described or still poorly documented, are morphologically similar and identification may require several techniques to be undertaken. In particular, Steenkamp et al. (1999) report that some of the distinguishing morphological characters may be inadequate or insufficient to make a definite identification of F. circinatum (Nirenberg & O'Donnell, 1998).

Although an official ISTA method was published in 2002 to detect *F. moniliforme* f. sp. *subglutinans* in seeds of *Pinus taeda* and *Pinus elliotii* (ISTA, 2002), the latter is not recommended as the morphological features indicated as typical for *F. moniliforme* f. sp. *subglutinans* in this method are based on a substrate not showing the characteristic sterile hyphae of this pathogen and are not sufficient to ensure a reliable identification of *F. circinatum* Nirenberg & O'Donnell (anamorphic stage of *G. circinata*).

Identity

Name: Gibberella circinata Nirenberg & O'Donnell Anamorph: Fusarium circinatum Nirenberg & O'Donnell Synonyms: Fusarium subglutinans f. sp. pini Hepting; F. moniliforme Sheldon var. subglutinans Wollenweber F. lateritium

¹Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

f. sp. *pini* Hepting; *Fusarium subglutinans* (Wollenweber & Reinking) Nelson *et al.* f. sp. *pini* Correll *et al.*

Taxonomic position: Fungi: Ascomycota: Hypocreales: Nectriaceae

Notes on taxonomy and nomenclature: For many years, the pitch canker pathogen was known only as an anamorph (form or pathotype of *F. subglutinans*; Correll *et al.*, 1991). Since its teleomorph was discovered following *in vitro* crosses, it is known as *G. circinata* (Nirenberg & O'Donnell, 1998). However, since only the anamorphic form of *G. circinata* will be observed in pure culture after isolation, *F. circinatum* will consistently be used in the morphological descriptions throughout this protocol. **EPPO code**: GIBBCI

Phytosanitary categorization: EPPO A1 list no. 306.

Detection

Symptoms

The fungus causes cankers that girdle branches, aerial roots and even trunks of *Pinus* sp., often associated with conspicuous and sometimes spectacular resin exudates ('pitch') in response to the fungal infection (Fig. 1A,B). *Gibberella circinata* may also be soil-borne, and can infect seeds cryptically (Storer *et al.*, 1998). The fungus can also cause root rot (Coutinho *et al.*, 1997). Symptoms may be observed at any time of the year.

Infected seedlings show usual damping off symptoms, but are not distinctive to a *G. circinata* infection: needles turn red, brown or chlorotic and die from the base upwards or the seedling dies (Fig. 2).

Root infections are most often observed on seedlings in nurseries but can also occur on exposed roots of larger trees in landscape plantings. Symptoms on roots are brown discoloration and disintegration of the cortex and are similar to symptoms caused by other root rot pathogens. Consecutive above-ground symptoms are generally not apparent until the pathogen reaches the crown after it girdles the stem, causing yellowing of the foliage. Resin-soaked tissue may then be observed after removal of the bark on the lower part of the stem.

Aerial infection symptoms include yellowing of the needles, which turn red in time and finally drop, and dieback of the shoots. Multiple branch tip dieback, due to repeated infections, may lead to a significant crown dieback. The female cones on infected branches may also become affected and abort before reaching full size. Cankers may thereafter appear on the shoots (Fig. 3), on the main stems and even on the trunk, associated with the typical resin bleeding (Fig. 1A,B). However, the symptoms in older trees can be mistaken with those caused by Sphaeropsis sapinea (Fr.) Dyco & Sutton (synonym Diplodia pinea), therefore the diagnosis should be based on testing. Sometimes, this resin bleeding may coat the trunk and lower branches for several metres below the infection level. The stem cankers are flat or slightly sunken and may sometimes affect large surfaces of cortical and subcortical tissue of the trunk. Removal of the bark shows subcortical lesions with brown and resin-impregnated tissues.

Sampling procedure

Despite the fact that they may exhibit different susceptibility levels to *G. circinata*, all the *Pinus* sp. along with douglas-fir (*Pseudotsuga menziesii*), may be potentially attacked by this fungus. There are two methods to be used, depending of the type of material to be sampled (plant tissue/seeds).

Plant tissue (except seeds)

For trunk or branch cankers, the inner bark in the area directly around the visible lesion should be cut repeatedly with a sterile blade until a canker margin is observed. Pieces of tissue, including phloem and xylem, should be removed to try to collect portions of the lesion edge, where the fungus is the most active. The pieces of tissue should be wrapped with sheets of sponge towels or newspapers and placed in a sealed plastic bag.



Fig. 1 (A,B) Copious resin exudates (pitch) beneath cankers caused by *Gibberella circinata* (courtesy of J Armengol Instituto, Agroforestal Mediterraneo, Universidad politeenica de Valencia/CNRS and R Ioos, Station de Mycologie, Malzéville, FR).



Fig. 2 Seedling damping-off caused by *Gibberella circinata* in nursery (courtesy of E Landeras, Laboratorio de Sanidad Vegetal, Oviedo, ES.).



Fig. 3 Shoot cankers caused by *Gibberella circinata* (courtesy of J Armengol, Institute Agroforestal Mediterraneo. Universidal Politeenica de CNRS.).

Sections of shoots, twigs or aerial roots should be collected by visual inspection upon observation of the symptoms indicated above. The sample should include the lesion edge and a few centimeters of healthy-looking tissue ahead of the lesion. The pieces of tissue are wrapped with sheets of sponge towels or newspapers and placed in a sealed plastic bag.

All samples of plant material should be sent to the laboratory as soon as possible after sampling, or refrigerated until transfer. When received in the laboratory, the samples should be kept in a refrigerator until analysis. The sample should be analysed within 8 days.

Seeds

Depending on the method chosen for the identification, the number of seeds to be analyzed per lot may be different. The total number of seeds is to be tested in order to detect the pest at different infection levels in a lot needs to be determined statistically (useful guidance is given in Tables 1 and 2 of ISPM no. 31 *Methodologies for sampling of consignments* (IPPC, 2008). Levels of infection in seeds can be very low (AM Pérez-Sierra, pers. comm.). Sample size recommended by ISTA is 400 seeds for plating (ISTA, 2009). However, larger samples (e.g. 1000 seeds) can easily be processed by biological enrichment before DNA analyses (Ioos *et al.*, 2009). As no symptoms can be seen on seeds, the lot should be sampled randomly. As counting of seeds may be laborious in some cases, the sampled seeds may be weighed instead of being counted. One thousand seeds may be collected in accordance with Table 1, which gives examples of mean thousand-seed-weight for the major *Pinus* or *Pseudotsuga* sp. The seeds will be subsequently analyzed without any surface disinfection, as *G. circinata* may be present on the seed husk, as well as inside the seed.

Isolation on semi-selective medium

Plant tissue except seeds

Isolations from symptomatic conifer trees or seedlings are made onto media including Komada, Dichloran Chloramphenicol

 Table 1 Mean thousand seed weight for the major Pinus and Pseudotsuga sp.

 (source: French Forestry Board 'Office National des Forêts'). TSW, thousand seed weight

| Species | Indicative TSW (g) | Species | Indicative TSW (g) |
|---------------------------------|-----------------------|----------------------------------|-----------------------|
| Pinus aristata | 22 | Pinus mugo subsp pumilio | 6 |
| Pinus armandi | 245 | Pinus nigra subsp koekelare | 21 |
| Pinus banksiana | 4 | Pinus nigra var. austriaca | 20 |
| Pinus bungeana | 130 | Pinus nigra var. calabrica | 18 |
| Pinus brutia | 53 | Pinus nigra var. corsicana | 15 |
| Pinus canariensis | 120 | Pinus nigra subsp. salzmannii | 16 |
| Pinus cembra | 350 | Pinus palustris | 75 |
| Pinus contorta var latifolia | 5 | Pinus parviflora | 125 |
| Pinus coulteri | 330 | Pinus pinaster | 55 |
| Pinus eldarica | 62 | Pinus pinea | 895 |
| Pinus densiflora | 18 | Pinus ponderosa | 42 |
| Pinus gerardiana | 295 | Pinus pumila | 105 |
| Pinus griffithi | 58 | Pinus radiata | 29 |
| Pinus halepensis | 18 | Pinus rigida | 7 |
| Pinus jeffreyi | 110 | Pinus strobus | 14 |
| Pinus koraiensis | 460 | Pinus sylvestris | 7 |
| Pinus lambertiana | 300 | Pinus tabuliformis | 32 |
| Pinus leucodermis | 25 | Pinus taeda | 27 |
| Pinus montana uncinata | 9 | Pinus thunbergii | 14 |
| Pinus uncinata | 19 | Pinus wallichiana | 50 |
| Pinus mugo subsp mugo | 7 | Pseudotsuga menziesii | 13 |

Ioos et al. (2009)

| | 8 I I | | | |
|---------|--|--------|-----------------|-----------------------------|
| | Sequence | Size | | |
| Primer | (5'-3') | (bp) | Target | Reference |
| H3-1a | ACT AAG CAG ACC GCC CGC AGG | ca 520 | Histone H3 gene | Steenkamp et al. (1999) |
| H3-1b* | GCG GGC GAG CTG GAT GTC CTT | | | |
| CIRC1A | CTT GGC TCG AGA AGG G | 360 | IGS rDNA region | Schweigkofler et al. (2004) |
| CIRC4A* | ACC TAC CCT ACA CCT CTC ACT | | | |
| FCIR-F | TCG ATG TGT CGT CTC TGG AC | 146 | IGS rDNA region | Ioos et al. (2009) |
| FCIR-R* | CGA TCC TCA AAT CGA CCA AGA | | | |
| FCIR-P | FAM-CGA GTC TGG CGG GAC TTT GTG C-BHQ1 | | | |
| ITS1 | TCC GTA GGT GAA CCT GCG G | ca 580 | ITS rDNA region | White et al. (1990) |
| ITS4* | TCC TCC GCT TAT TGA TAT GC | | - | |

150

18S rDNA

Table 2 Sequence and target of the PCR primers and probes combinations

GCA AGG CTG AAA CTT AAA GGA A

JOE-ACG GAA GGG CAC CAC CAG GAG T-BHO1

CCA CCA CCC ATA GAA TCA AGA

*reverse primers.

18S uni-F

18**S** uni-R* 18S uni-P

Peptone Agar (DCPA) or onto Potato dextrose agar supplemented with streptomycin sulphate (PDAS) (see Appendix 1). Plant material should be surface-sterilized for up to 1 min in a 1.5% solution of sodium hypochlorite, and rinsed in sterile distilled water to eliminate saprophytic organisms from the plant material which would otherwise overgrow any Fusarium. On seedlings the pathogen is isolated from the lower part of the stem and from the roots. On mature trees, isolations are made from cankers. The cankers are washed thoroughly with water, and isolations are made from wood-chips taken from the edge of the lesion found beneath the affected bark (Fig. 4). During incubation, the plates are observed periodically and all the Fusarium spp. colonies are transferred to Potato dextrose agar (PDA) and to Spezieller-Nährstoffarmer Agar (SNA) (Appendix 1) for morphological identification. This method is very efficient and reliable to isolate any Fusarium spp. from infected tissue and does not require expensive equipment. However, the correct morphological identification of F. circinatum in pure culture requires experience and a molecular confirmation should be carried out in case of uncertainty.

Seeds

Seeds are directly plated onto Fusarium semi-selective media (e.g. Komada's medium. DCPA medium see Appendix 1) without previous surface disinfection. Plates are incubated at room temperature ($22 \pm 6^{\circ}$ C). During incubation, the plates are observed periodically and all the Fusarium spp. colonies are transferred to Potato dextrose agar (PDA) and to Spezieller-Nährstoffarmer Agar (SNA) (Appendix 1) for morphological identification. This method is efficient and reliable to isolate any Fusarium spp. from the seeds and does not require expensive equipment, though time- and space-consuming when serial analyses are conducted. However, the correct morphological identification of F. circinatum in pure culture requires experience and in case of uncertainty a molecular confirmation should be carried out. In addition, Storer et al. (1998) have demonstrated that agar plating of pine seeds may not be able to detect dormant (quiescent) propagules of F. circinatum, thus leading to an unknown risk of false negative results.

Direct detection in planta using molecular techniques (plant tissue, including seeds)

See Identification section for description. These methods are fast, efficient and reliable in detecting G. circinata specifically, without previous agar plating, thus saving a lot of space and time, but require molecular biology facilities and instruments. In addition, as these techniques target the DNA of the fungus, active and quiescent forms of the pathogen should be equally detected. However, positive conventional PCR requires confirmation as crossreaction with phylogenetically closely related species might occur and lead to false positive results.



Fig. 4 Sub cortical necrosis caused by Gibberella circinata beneath a canker observed on a stem (courtesy of E Landeras, Laboratorio de Sanidad Vegetal, Oviedo, ES).



Fig. 5 Flow diagram for diagnosis of Gibberella circinata on plant tissue and on seeds.

Identification

The procedures for the identification of *G. circinata* on *Pinus* spp. and *Pseudotsuga menziesii* will consist of (i) isolating the fungus from the plant tissue on semi-selective culture media followed by morphological and, in the case of uncertainty, molecular identification, or (ii) directly detecting the fungus *in planta* by conventional PCR, SyBr green real-time PCR or dual-labelled probe real-time PCR. A flow diagram indicating the different combinations of methods is shown in Fig. 5.

Morphological characteristics in pure culture

For morphological identification, the isolates are grown on PDA to study colony morphology and pigmentation, and on SNA (Appendix 1) to study formation and type of microconidia and conidiogenous cells. SNA and PDA plates are incubated at room temperature. All isolates are examined after 10 days and confirmed as *F. circinatum* based on the morphological features described by Nirenberg & O'Donnell (1998) and Britz *et al.* (2002). On PDA, *F. circinatum* grows relatively rapidly (average growth of 4.7 mm/day at 20°C; Nirenberg & O'Donnell, 1998). After 10 days, the colony should have an entire margin, white



Fig. 6 Cultural aspect of the anamorphic stage of *Gibberella. circinata* (*F. circinatum*) on potato dextrose agar (left: *Fusarium circinatum* MAT-1; right: *Fusarium circinatum* MAT-2). MAT-1 mating type produces typical coiled sterile hyphae on Spezieller-Nährstoffarmer Agar (SNA), whereas MAT-2 mating type produces not distinctively coiled or even uncoiled sterile hyphae (courtesy of A. Pérez-Sierra, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia (ES)) (see also Fig. 9A,B).



Fig. 7 (A) Erect conidiophores bearing microconidia arranged in false heads of *Fusarium circinatum*, observed directly on Spezieller-Nährstoffarmer Agar (SNA) medium (×200 magnification) (courtesy of R. Ioos, Station de Mycologie, Malzéville (FR)) and (B) on a microscopic slide (×400 magnification) (courtesy of A. Pérez-Sierra, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia (ES)).

cottony or off-white aerial mycelium with a salmon tinge in the middle or with a purple or dark violet pigment in the agar (Fig. 6). On SNA, microconidia are aggregated in false heads (Fig. 7A,B), with branched conidiophores, mono and polyphialidic- conidiophores (Fig. 8A,B), obovoid microconidia in aerial mycelium, mostly nonseptate or with occasionally 1-septum. Chlamydospores are absent. The sterile hyphae (coiled/not



Fig. 8 Mono- and polyphialidic conidiophores of *Fusarium circinatum* observed on Spezieller-Nährstoffarmer Agar (SNA) medium (courtesy of J. Armengol).



Fig. 9 (A) Coiled and not distinctively coiled sterile hyphae produced on Spezieller-Nährstoffarmer Agar (SNA) medium by MAT-1 (left) and MAT-2 (right) mating type isolates of *Fusarium circinatum*, respectively (courtesy of A. Pérez-Sierra, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia (ES)). (B) Groups of coiled sterile hyphaes and polyphialidic conidiophores produced on Spezieller-Nährstoffarmer Agar (SNA) (courtesy of R. Ioos, Station de Mycologie, Malzéville (FR)).

distinctively coiled) are characteristic of *F. circinatum* and are observed clearly on this medium (Fig. 9A,B). The epithet '*circinatum*' refers to these typical coiled hyphae, also called 'circinate' hyphae.

Molecular methods

There are several molecular methods currently available to confirm the identity of the anamorphic stage of *G. circinata* isolated in pure culture or to detect and identify directly *G. circinata in planta*.

- A PCR-RFLP (Restriction Fragment Length Polymorphism) test, with primers and RFLP pattern developed by Steenkamp *et al.* (1999) is presented in Appendix 2 and is appropriate for identification of the anamorphic stage of *G. circinata* in pure culture only as contaminants or host material may affect the quality and numbers of PCR amplicons.
- SyBr green real-time PCR or conventional PCR tests with primers designed by Schweigkofler *et al.* (2004) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in seeds, and is presented in Appendix 4. However, when carried out on plant samples DNA, verification of the nature of the PCR amplicon should be carried out by sequencing for conventional PCR, or by melting analysis for SyBr green real-time PCR (Appendix 5). Indeed, infection by other *Fusarium* spp. is frequent and cryptic speciation was reported in the *Gibberella fujikuroi* sp. complex (Steenkamp *et al.*, 2002). PCR cross-reaction might occur with phylogenetically close *Fusarium* sp., especially with high amounts of *Fusarium* template DNA
- Method for real-time PCR with primers and a dual-labelled probe designed by Ioos *et al.* (2009) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in plant tissue, including seeds, and is presented in Appendix 6. This method proved to be more sensitive than the conventional PCR (diagnostic sensitivities of 79.1% and 58.6%, respectively; Ioos *et al.*, 2009) described in Appendix 4 and its specificity is strengthened thanks to the combination of specific primers and probe.

Another conventional PCR test has been developed by Ramsfield *et al.* (2008) but there is no experience with this test in the EPPO region, therefore it is not described in the protocol.

DNA extraction from pure culture

Fungal DNA should be extracted using an appropriate standard method for DNA extraction from fungi e.g. regular commercial plant DNA extraction kits (or other methods reviewed in Irlinger *et al.*, 2008) and analyzed following any of the tests presented in Appendices 2, 4 or 6.

DNA extraction from plant tissue (except seeds)

Total DNA from potentially infected plant tissue should be extracted as described in Appendix 3 and analyzed following any of the tests presented in Appendices 4 or 6.

DNA extraction from seeds

Total DNA from potentially infected seeds should be extracted as described in Appendix 3 and analysed following any of the tests presented in Appendices 4 or 6.

Reference cultures

The type strain of *G. circinata* (CBS 405.97) and other strains (CBS 117843, Spain; CBS 119864, South Africa; CBS 100197, USA) are available from CBS, Utrecht (NL).

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO standard PM 7/77 *Documentation and reporting of a diagnosis.*

Further information

Further information on this organism can be obtained from:

Ioos R, Laboratoire National de la Protection des Végétaux (LNPV), Station de Mycologie, Domaine de Pixérécourt, BP 90059, F54220 Malzéville (FR), Tel: +33 (0) 383338662, fax: +33 (0) 383338652; e-mail: renaud.ioos@agriculture.gouv.fr

Pérez-Sierra AM, Grupo de Investigación en Hongos Fitopatógenos, Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia (ES), Tel: (+34) 963879254, fax: (+34) 963879269; e-mail: aperesi@eaf.upv.es

Acknowledgements

This protocol was originally drafted by:

Ioos R, Laboratoire National de la Protection des Végétaux, Station de Mycologie, Malzéville (FR) and A. M. Pérez-Sierra, Grupo de Investigación en Hongos Fitopatógenos, Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Valencia (ES).

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Appendix 1 – Composition of the different culture media

Komada medium (Komada, 1975):

This medium is suitable for isolation of *Fusarium circinatum* from plant tissue, including seeds, but not for identification. The basal medium contains:

- K₂HPO₄: 1.0 g
- KCl: 0.5 g
- MgSO₄ 7H₂O: 0.5 g
- Fe-Na-EDTA: 10 mg
- L-Asparagine: 2.0 g
- D-Galactose: 20.0 g
- Technical agar: 15.0 g
- Distilled water to 1.0 L

The pH is adjusted to 3.8 ± 0.2 with 10% phosphoric acid. The basal medium is autoclaved and slightly cooled before adding the following filter-sterilized supplemental solutions:

- Pentachloronitrobenzene (PNCB, 75% w/w): 1.0 g
- Oxgall: 0.5 g
- Na₂B₄O₇ 10H₂O: 1.0 g
- Streptomycin: 6 mL/L of stock solution (5 g of streptomycin in 100 mL distilled water).

PDAS

Potato dextrose agar supplemented with 0.5 mg/mL of streptomycin sulphate salt (775 units/mg solid).

Dichloran Chloramphenicol Peptone Agar (DCPA)

(slightly modified by Ioos et al., 2004; after Andrews & Pitt, 1986)

This medium is suitable for isolation of *Fusarium circinatum* from plant tissue, including seeds, but not for identification. The medium contains:

- Bacteriological peptone, 15.0 g
- KH₂PO₄, 1.0 g
- MgSO₄(7H₂O): 0.5 g
- Chloramphenicol: 0.2 g
- 2.6-dichloro-4-nitroanilin (dichloran) (0.2% W/V in ethanol, 1.0 mL): 2 mg
- Violet crystal (0.05% W/V in water, 1.0 mL): 0.0005 g
- Technical agar: 20.0 g
- Distilled water: to 1.0 L

Spezieller-Nährstoffarmer Agar (SNA) (Gerlach & Nirenberg, 1982)

This medium should be mandatory used for identification of *F. circinatum*, based on morphological features. The medium contains:

- KH₂PO₄: 1.0 g
- KNO3: 1.0 g
- MgSO₄ 7H₂O: 0.5 g
- KCl: 0.5 g
- Glucose: 0.2 g
- Sucrose: 0.2 g
- Technical agar: 20.0 g
- Distilled water to 1.0 L

Optionally, two 1-cm² square pieces of sterile filter paper may be laid on the surface of the agar since *Fusarium sporodochia* are sometimes more likely to be produced at the edge of the paper.

Appendix 2 – Identification at species level by PCR-RFLP (Steenkamp *et al.*, 1999)

(1) General information

Steenkamp *et al.* (1999) described a technique based on a PCR-RFLP carried out on the histone H3 gene to identify the anamorphic stage for *G. circinata* from pure culture. Total DNA extracted from a pure *Fusarium circinatum* (anamorphic stage of *G. circinata*) culture is the nucleic acid source.

The PCR test targets the histone H3 gene and produces a 515 bp amplicon for *G. circinata* (sequences of partial *G. circinata* H3 gene may be retrieved from Genbank, accessions AF150847 to AF150853). The histone H3 gene is first amplified in *Gibberella* spp. using the primer pair H3-1a (forward) and H3-1b (reverse) (Table 2).

(2) Methods

Nucleic acid extraction and purification

Fungal DNA should be extracted using an appropriated standard method for DNA extraction from fungi (Irlinger *et al.*, 2008). Purified DNA should be frozen until analysis.

PCR reaction

The PCR reaction mixture includes:

- 1× PCR buffer (supplied with the DNA polymerase),
- 0.25 mM each dNTP,
- 2.5 mM MgCl₂,
- 0.2 µM of each H3-1a and H3-1b primers,
- 0.05 U/µL DNA polymerase
- 25–50 ng of template DNA,
- Molecular grade water (MGW) is added to reach the final reaction volume (20 μL).

The PCR reaction conditions are carried out on a thermocycler equipped with a heated lid and include an initial denaturation at 92°C for 1 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 63°C for 1 min and elongation at 72°C for 1 min. A final elongation step is done at 72°C for 5 min. The PCR product is kept at 5°C or less, until restriction analysis.

Enzymatic digestion

Histone H3 PCR products obtained are thereafter consecutively digested with restriction enzymes *CfoI* and *DdeI*, respectively.

Consecutive enzymatic digestion is performed by addition of 5 U *CfoI* to 15 μ L of unpurified PCR product followed by incubation at 37°C for 3 h. Subsequently, the sodium chloride concentration is adjusted to 100 mM and 5 U of *DdeI* is added to the reaction mixture. This is followed by further 5-h incubation at 37°C. However, Pérez-Sierra *et al.* (2007)showed that it was possible to add both enzymes simultaneously without adjustment of the sodium chloride concentration, and to simply incubate the mixture for 5 h at 37°C.

The digested amplicons are separated by electrophoresis in 2-3% agarose gels followed by ethidium bromide staining.

The PCR-RFLP pattern of *G. circinata* (refered to as the anamorph *F. subglutinans* f. sp. *pini*, mating population 'H', by Steenkamp *et al.* (1999) consists of bands of 250, 232, and 33 (not observed on the gel) bp.

(3) Essential procedural information

Total DNA obtained from a reference culture of *G. circinata* should be processed in parallel with the DNA samples to be analyzed in order (i) to have a reference control pattern on the final electrophoresis gel and (ii) to ensure that the PCR/enzymatic process was correctly achieved.

Appendix 3 – DNA extraction for *in planta* detection of *Gibberella circinata*

(1) Plant tissue (except seeds)

Grinding

Potentially infected plant tissues are collected from the sample and first roughly cut using a sterile scalpel blade, without prior surface disinfection step. Small pieces of approximately 0.5– 1 cm^2 should be first collected then subsequently cut into smaller pieces (<2–3 mm, each side) into a sterile plastic Petri dish.

The sample is then transferred into a 2 mL microcentrifuge tube corresponding to approximately 200 μ L and ground for 2 min with two 3-mm steel or tungsten carbide beads and 400 μ L of the lysis buffer provided by the DNA extraction kit, at a frequency of 30 Hz with a bead beater (Tissuelyser[®], Qiagen, or equivalent). The samples may also be ground in a mortar by a pestle with liquid nitrogen, or using other efficient grinding techniques.

DNA extraction and purification

Total DNA should be extracted preferably following the extraction protocol described by Ioos *et al.* (2009) using the commercial DNA extraction kit Nucleospin Plant II[®] miniprep (Macherey-Nagel, Hoerdt, France), which proved to be efficient. However, other DNA extraction protocols may be used providing that they proved to yield total DNA at least equivalent with at least similar quality and quantity.

Total DNA is extracted following the manufacturer's instructions with slight modifications. First, the chemical lysis incubation step is extended to 20 min, using the PL1 lysis buffer. After this incubation step, the sample is centrifuged

for 5 min at approximately 11 000 g to compact the debris and only the supernatant is recovered to be further processed following the manufacturer's instructions. Total DNA is finally eluted with 100 μ L of the elution buffer provided by the manufacturer and stored frozen until analysis. Total DNA is directly used as a template for conventional or real-time PCR (Appendices 4 and 6).

(2) Seeds

Biological enrichment

This procedure was initially described by Ioos *et al.* (2009) and should be followed when the presence of *G. circinata* is checked by a conventional or real-time PCR test carried out directly on a seed DNA extract (Appendix 4 and 6). The purpose of this preliminary biological enrichment step is to increase the biomass of viable *G. circinata* propagules, prior to DNA extraction and molecular testing.

As recommended by ISTA for agar plating technique (ISTA, 2002), at least 400 seeds per seed lot are incubated at $22 \pm 3^{\circ}$ C for 72 hrs in a cell culture flask with potato dextrose broth (PDB, Difco, Beckton, Dickinson and Co, Sparks, MD, USA). However, larger sample sizes (e.g. 1000 seeds in Ioos *et al.*, 2009) can easily be processed by this test and may increase the chance to detect the fungus when present at low infection levels. The flask's size should be chosen so that the entire seed sample can be spread more or less as a 'single seed'-thick layer. Depending on the species of *Pinus*, the average size of the seed may vary greatly and the quantity of PDB per flask should be manually adjusted in a way that the seed layer is almost completely overlaid by the liquid medium.

Grinding

After incubation, the whole content of the flask (seeds and PDB) is transferred aseptically into a decontaminated mixer bowl of appropriate volume, and is subsequently ground with a mixer mill till a homogenous solution is obtained. Sterile water or sterile PDB may be added at this step in case the ground sample remains too thick. Two sub-samples of approximately 500 μ L are then collected and transferred aseptically into individual 2-mL microcentrifuge tubes for DNA extraction.

DNA extraction and purification

Total DNA should be extracted preferably following the extraction protocol described by Ioos *et al.* (2009) using the commercial DNA extraction kit Nucleospin Plant II[®] miniprep (Macherey-Nagel, Hoerdt, France), which proved to be efficient, but other DNA extraction protocols may be used providing that they proved equivalent in yield and quality of DNA.

Total DNA is extracted individually from the two 500 μ L sub-samples following the manufacturer's instructions with slight modifications. Proceed as described above for plant tissue.

Appendix 4 – Identification at species level by conventional or SyBr green real-time PCR (Schweigkofler *et al.*, 2004)

(1) General information

Schweigkofler *et al.* (2004) described a technique based on a conventional or a SyBrgreen real-time PCR designed from the rDNA IGS (Inter Genic Spacer) region to identify the anamorphic stage of *G. circinata* in pure culture or in trapped airborne spores, but may be adapted to the analysis of seeds following the biological enrichment step (See *Identification* section, Ioos R., pers. comm.).

The PCR test targets a region of the IGS and produces a 360 bp amplicon for *G. circinata* (sequences of the IGS region for *G. circinata* may be retrieved from Genbank, accessions AFAY249397 to AY249403). A specific region of the IGS is amplified with *G. circinata* DNA using the primer pair CIRC1A (forward) and CIRC4A (reverse) (Table 2).

(2) Methods

Nucleic acid extraction and purification See Appendix 3.

Conventional PCR reaction

A *G. circinata*-specific IGS portion is amplified by PCR as follows.

The PCR reaction mixture includes:

- $1 \times PCR$ buffer supplied with the DNA polymerase,
- 0.25 mM each dNTP,
- 2 mM MgCl₂,
- 0.5 μM of each CIRC1A and CIRC4A primers,
- 0.05 U/µL DNA polymerase
- 6.25 µL of template DNA,
- Molecular grade water is (MGW) added to reach the final reaction volume (25 μL).

Each DNA extract should be tested by at least two replicate reactions.

The PCR reaction conditions should be carried out in a thermocycler equipped with a heated lid and include an initial denaturation at 94°C for 3 min, followed by 45 cycles for denaturation at 94°C for 35 s, annealing at 66°C for 55 s and elongation at 72°C for 50 s. A final elongation step is done at 72°C for 12 min.

The PCR products are separated by electrophoresis in a 1% agarose gel followed by ethidium bromide staining. A DNA template containing amplifiable *G. circinata* DNA will yield a 360-bp fragment after a CIRC1A/CIRC4A PCR.

SyBr green real-time PCR reaction

A *G. circinata*-specific IGS portion is amplified by PCR as follows.

The PCR reaction mixture includes:

1× PCR buffer supplied with the DNA polymerase,

- 0.25 mM each dNTP,
- 5 mM MgCl₂,
- 0.5 μM of each CIRC1A and CIRC4A primers,
- SyBrgreen dye (concentration to be adjusted following the manufacturer's recommendation)
- 0.05 U/µL DNA polymerase
- 6.25 µL of template DNA,
- Molecular grade water is (MGW) added to reach the final reaction volume (25 μL).

Each DNA extract should be tested by at least two replicate reactions.

The real-time PCR reactions are carried out in a suitable PCR instrument equipped with a system capable of fluorescence monitoring.

The PCR reaction conditions include an initial denaturation at 95° C for 3–10 min (according to the type of DNA polymerase), followed by 45 cycles for denaturation at 94°C for 35 s, annealing at 66°C for 55 s, and extension at 72°C for 50 s. The fluorescence of the reporter dye is monitored at the end of each extension step.

The accumulation of *G. circinata* PCR amplicons is monitored in real-time by the measurement of the specific fluorescence of the SyBr green dye incorporated into the PCR product. A DNA template containing amplifiable *G. circinata* DNA will yield a Cycle threshold (Ct) value. The Ct value represents the estimated cycle number from which the level of fluorescence becomes significantly superior to the background fluorescence level.

The nature of the amplicons should be checked by yielding melting curves at the end of the amplification process and by comparison to the melting curves yielded with the PCR positive control.

(3) Essential procedural information

A **DNA extraction negative control** (blank tube) should be included for each DNA extraction series in order to ensure the absence of contamination during this step.

A **PCR negative control** containing no target DNA should be included in every test in order to ensure the absence of contamination during PCR.

A **PCR positive control** should be used (genomic DNA from a reference strain of *G. circinata*, or subcloned *G. circinata* CIR-C1A/CIRC4A PCR product). When testing plant and seed samples, the positive control should correspond to the limit of detection of the test (LOD). This **LOD positive control**² should

be included in order to assess the performance of the PCR run and to ensure that the negative results are caused by an absence or a too low level of the PCR target in the DNA sample, rather than by an insufficient PCR efficiency.

The quality of the DNA extract should be assessed by a relevant means e.g. by spectrophotometry, by using an *ad hoc* **internal amplification control** or by testing the extract in PCR with the fungal ribosomal genes primers ITS1 and ITS4 (White *et al.*, 1990). In the latter case, the PCR conditions are those described above, simply replacing the FCIRC1A/-CIRC4A primers with ITS1 and ITS4 primers (Table 2), and decreasing the annealing temperature to 50°C. A positive signal (approximately 600 bp) following this test would mean that the DNA extract was amplifiable: DNA was successfully extracted and the level of co-extracted inhibiting compounds was sufficiently low.

Interpretation of results:

- A sample will be considered positive if it produces amplicons of 360 bp and provided that the contamination controls are negative.
- A sample will be considered negative if (i) it produces no band of 360 bp; (ii) provided that the sample DNA extract proved to be amplifiable and that no significant inhibition occurred; and (iii) if used, that the LOD positive control tested in the PCR run yielded a 360 bp amplicon.
- Plant samples whose DNA extract yields a Ct inferior or equal to Ct_{LOD} should be considered as infected by *G. circinata*, provided that the negative controls (PCR and DNA extraction) do not yield Ct.
- Plant samples whose DNA extract does not yield a Ct inferior or equal to Ct_{LOD} should be considered as non-infected by *G. circinata*, or infected below the detection threshold of the technique, provided that the sample DNA extract proved to be amplifiable and that no significant inhibition occurred.
- Tests should be repeated if any contradictory or unclear results are obtained.

Appendix 5 – Confirmation of *Gibberella circinata* by sequencing

As the CIRC1A/CIRC4A conventional PCR test might cross react with phylogenetically close species (including non- or ill-described species of the *G. fujikuroi* complex), especially when testing high amount of *Fusarium* template DNA, the nature of the CIRC1A/CIRC4A amplicon can be verified by sequencing. Send an appropriate CIRC1A/CIRC4A PCR product for two-way sequencing with primer CIRC1A and CIRC4A as forward and reverse primer, respectively. The consensus sequence, from which the primers' sequences are trimmed prior to this, is compared by BLAST with those deposited in Genbank for numerous phylogenetically close *Fusarium* sp. (http://www.ncbi.nlm.nih.gov). The sequence lying between CIRC1A and CIRC4A on the IGS region is sufficiently discriminant to identify *G. circinata*.

²LOD positive control is made of diluted genomic DNA from a reference strain of *G. circinata*, or diluted subcloned *G. circinata* CIRC1A/CIRC4A PCR product. It can be defined as the lowest target amount giving positive result in at least 95% of the times, thus ensuring a \leq 5% false negative rate.

Appendix 6 – Identification at species level by dual-labelled probe real-time PCR (loos *et al.*, 2009)

(1) General information

Ioos *et al.* (2009) described a technique based on a real-time PCR designed from the rDNA IGS (Intergenic spacer region) to identify the anamorphic stage of *G. circinata* (*F. circinatum*) in pure culture or directly in plant samples.

The PCR test targets a region of the IGS and produces a 149 bp amplicon for *G. circinata* (sequences of the IGS region for *G. circinata* may be retrieved from Genbank, accessions AFAY249397 to AY249403). A specific region of the IGS is first amplified with *G. circinata* DNA using the primer pair FCIR-F (forward) and FCIR-R (reverse) and detected by a fluorescent probe FCIR-P (Table 2).

(2) Methods

Nucleic acid extraction and purification See Appendix 3.

Real-time PCR reaction

A *G. circinata*-specific IGS portion is amplified by real-time PCR as follows.

The real-time PCR reaction mixture includes:

- $1 \times PCR$ buffer supplied with the DNA polymerase,
- 0.20 mM each dNTP,
- 5 mM MgCl₂,
- 0.2 μM of each FCIR-F and FCIR-R primers,
- 0.1 µM of FCIR-P probe,
- 0.025 U/µL Hotstart DNA polymerase,
- 25–50 ng of template DNA,
- Molecular grade water (MGW) is added to reach the final reaction volume (20 μL).

Each DNA extract should be tested by at least two replicate reactions.

The real-time PCR reactions are carried out in a suitable PCR instrument equipped with a system capable of fluorescence monitoring.

The PCR reaction conditions include an initial denaturation at 95°C for 10 min, followed by 40 cycles for denaturation at 95°C for 15 s, annealing/extension at 70°C for 55 s. The fluorescence of the reporter dye is monitored at the end of each annealing/extension step.

The accumulation of *G. circinata* PCR amplicons is monitored in real-time by the measurement of the specific fluorescence of the reporter dye cleaved from the FCIR-P probe. A DNA template containing amplifiable *G. circinata* DNA will yield a Cycle threshold (Ct) value. The Ct value represents the estimated cycle number from which the level of fluorescence becomes significantly superior to the background fluorescence level.

(3) Essential procedural information

A **DNA extraction negative control** should be included for each DNA extraction series in order to ensure the absence of contamination during this step (blank tube containing sterile MGW, or 500 μ L of PD Broth for seed samples).

A **PCR negative control** (no template control, containing for instance MGW) should be included in every experiment to check the absence of contamination during PCR.

A **PCR limit of detection (LOD) positive control**³ should be used in order to assess the performance of the PCR run and to ensure that the negative results are caused by an absence or a too low level of the PCR target in the DNA sample, rather than by an insufficient PCR efficiency.

The quality of the DNA extract should be assessed by a relevant means, e.g. by spectrophotometry, by testing the extract in conventional PCR, with the universal fungal ribosomal genes primers ITS1 and ITS4 (See Appendix 4) or in real-time PCR, with other universal plant and fungal primers and probe such as 18S uni-F/-R/-P (Ioos *et al.*, 2009) or other universal tests described in the scientific literature. A positive signal (approximately 600 bp) following ITS1/ITS4 PCR or a Ct yielded with 18S uni-F/-R/-P real-time PCR test would mean that the DNA extract was amplifiable: DNA was successfully extracted and the level of co-extracted inhibiting compounds was sufficiently low.

Interpretation of results:

- Plant samples whose DNA extract yields a Ct inferior or equal to Ct_{LOD} should be considered as infected by *G. circinata*, provided that the negative controls (PCR and DNA extraction) do not yield Ct.
- Plant samples whose DNA extract does not yield a Ct inferior or equal to Ct_{LOD} should be considered as non-infected by *G. circinata*, or infected below the detection threshold of the technique, provided that the sample DNA extract proved to be amplifiable and that no significant inhibition occurred.
- Tests should be repeated if any contradictory or unclear results are obtained. Doubtful or borderline results should be re-analyzed using the same or another technique (e.g. sequencing).

³LOD positive control is made of diluted genomic DNA from a reference strain of *G. circinata*, or diluted subcloned *G. circinata* FCIR-F/FCIR-R PCR product. It can be defined as the lowest target amount giving positive result in at least 95% of the times, thus ensuring a \leq 5% false negative rate.

Proposal for replacement of mCS20ABN and FS media recipes in ISTA Rule 7-019a (*Xanthomonas campestris* pv. *campestris* detection in *Brassica* spp. seed lots) by adapted versions

Sato, M., Seed Health Testing Laboratory, National Center for Seeds and Seedlings (NCSS), Fujimoto 2-2, Tsukuba, Ibaraki, 305-0852, Japan (sweet@affrc.go.jp)

Asma, M., Bejo Zaden B.V, Seed Technology Laboratory, P.O. Box 50, 1749 ZH Warmenhuizen, The Netherlands, (m.asma@bejo.nl)

Politikou, L., ISF, 7 Chemin du Reposoir, 1260 Nyon, Switzerland, (liana.politikou@ufs-asso.com)

The recipes of the FS and mCS20ABN semi-selective media described in the ISTA Rule 7-019a were adapted to increase their performance regarding the recovery and recognition of *Xanthomonas campestris* pv. *campestris* (Xcc) on the plates as well as the safety and practicality in their preparation. The adapted and ISTA Rule7-019a media recipes were compared between 5 seed health testing laboratories in an ISHI-Veg peer validation study that was organised in parallel to the ISTA proficiency test. Extracts of three 10,000-seed subsamples of a low, medium, high and Xcc-free cabbage lot were plated on media plates prepared with the adapted and the ISTA Rule 7-019a recipes. Results comparison showed no difference between the two recipes. The benefits of the adapted FS and mCS20ABN media recipes suggest the replacement of the ISTA Rule 7-019a recipes by the former.

Introduction

Xanthomonas campestris pv. *campestris* (Xcc) is an important seed-borne bacterial pathogen and the causal organism of black rot in Brassicas. The use of healthy seed is critical to control this bacterial disease. The ISTA Rule 7-019a published in 2005 for the detection of Xcc is a seed-washing and liquid-plating assay (1). In this assay, bacteria are first extracted from seeds by shaking and then the seed extract is diluted and plated on semi-selective media. The suspect Xcc colonies are confirmed in a pathogenicity test. A PCR test was adopted in this method in 2013 as an alternative to the pathogenicity test for the confirmation of Xcc suspects.

The ISTA Rule 7-019a is based on the results from a comparative test in 13 laboratories organized by the International Seed Health Initiative for Vegetables, ISF (ISHI-Veg) in 2005 (2). However, the composition of the semi-selective media mCS20ABN and FS in ISTA Rule 7-019a does not correspond to the composition of mCS20ABN and FS used in the underlying comparative test.

In the mCS20ABN medium the amount of KH_2PO_4 was increased in the ISTA Rule 7-019a from 1.0 g/L to 1.59 g/L and the amount of $(NH_4)_2HPO_4$ was lowered from 0.8 g/L to 0.33 g/L. Moreover, evidence was obtained that the sensitivity of Xcc with respect to neomycin activity is pH dependent (3). After the ISHI-Veg comparative test in 2005, the mCS20ABN medium recipe was further optimized by increasing the amount of KH_2PO_4 from 1.0 g/L to 2.8 g/L that ensured stable pH in the medium. This increase in conjunction with the reinstatement of the amount of $(NH_4)_2HPO_4$ to 0.8 g/L resulted in a better buffered medium. Finally, the amount of agar was increased from 15.0 g/L to 18.0 g/L as it showed better absorption of the seed extract (pers. com. Dr. H. Koenraadt, NAKTuinbouw, The Netherlands).

The FS medium recipe included in the ISTA Rule 7-019a also differed from the recipe used in the ISHI-Veg comparative test in 2005. The concentration of starch was reduced from 25.0 g/L to 10.0 g/L and this hampered the recognition of Xcc colonies by the typical halo (pers. com. Dr. H. Koenraadt, NAKTuinbouw, The Netherlands). Also, the KNO₃ was omitted and gentamycin was added at 0.4 mg/L. It was suggested that these changes could result in a low or no recovery of Xcc colonies and therefore were not accepted in the adapted recipe. Finally, the expensive and very toxic cycloheximide was replaced in both adapted media recipes by nystatin for safety reasons and improvement of fungal control.

For these reasons, many seed health testing laboratories use the adapted version of mCS20ABN and FS media recipes and not the recipes described in the ISTA Rule 7-019a.

Aim of the peer validation study

The aim of this ISHI-Veg peer validation study was to compare the performance of the mCS20ABN and FS media adapted recipes to the media recipes described in ISTA Rule 7-019a. Provided that the performance of the media from the adapted recipe is better or equivalent to the latter, the data generated by this study will provide scientific evidence to back up a proposal to replace the mCS20ABN and FS media recipes in ISTA Rule 7-019a with the adapted recipes.

This study was organised and performed in parallel to the ISTA proficiency test on ISTA Rule 7-019a. Participants were five seed-health-testing laboratories from Japan, France, The Netherlands, USA and Israel.

Materials and Methods

Seed lots and seed subsamples

Four, untreated, cabbage (*Brassica oleracea*) seed lots with different levels of Xcc natural contamination and saprophytes were selected by the Seed Health Testing Laboratory, National Center for Seeds and Seedlings (NCSS) in Japan. Prior to the peer validation study, fifteen 10,000-seed subsamples from each seed lot were tested in a heterogeneity test following the detection method and media recipes described in ISTA Rule 7-019a. However, no heterogeneity test was performed on the seed lots with the adapted media recipes.

A subsample was considered positive if at least 1 YDC positive colony isolated *either* from FS *or* mCS20ABN medium was confirmed in the pathogenicity test. Eleven, 15, 15 and 0 seed subsamples of the 15 tested in total were found Xcc positive from the No. 1, No. 2, No. 3 and No. 4 seed lots respectively. The confirmed Xcc cfu/seed values were analysed using a Generalized Linear Model assuming a Poisson distribution. The mean cfu/seed of the No. 1, No. 2, No. 3 and No. 4 seed lots were 4, 358, 34.7 and 0 respectively. Based on these results the No. 1, No. 2 and No. 3 seed lots were characterised having a low, high and medium Xcc contamination level respectively whereas the seed lot No. 4 was characterised being healthy.

Each participating laboratory received three 10,000 seed-subsamples per seed lot that were prepared based on the thousand seed weight (TSW) of their corresponding lot. All 12 in total seed subsamples were coded randomly and their correspondence to the seed lots was not revealed to ensure a blind test.

Media preparation

Based on the 12 received seed subsamples, participating laboratories prepared an adequate number of mCS20ABN and FS media plates following the recipes described in ISTA Rule 7-019a. Additionally, they prepared an equal number of mCS20ABN and FS media plates following the below described adapted recipes (2):

mCS20ABN adapted recipe: The final concentration of the compounds per litter of distilled water was: 2.8 g KH₂PO₄, 0.8 g (NH₄)₂HPO₄, 0.4 g MgSO₄.7H₂O, 2.0 g bacto tryptone, 2.0 g soya peptone, 6.0 g L-glutamine, 1.0 g L-histidine, 1.0 g dextrose, 25.0 g soluble starch (Merck 1252) and 18.0 g agar. The pH was adjusted to 6.5 and the medium was autoclaved at 121°C for 15 min. When the mixture cooled to 50°C the following sterile antibiotics were added: 35 mg nystatin, 40 mg neomycin sulphate and 100 mg bacitracine.

FS adapted recipe: The final concentration of the compounds per litter of distilled water was: 0.8 g K₂HPO₄, 0.8 g KH₂PO₄, 0.5 g KNO₃, 0.1 g MgSO₄ 7H₂O, 0.1 g yeast extract, 25.0 g soluble starch (Merck 1252), 15.0 g agar along with 1.5 ml methyl green (1% aq.) which the laboratories added. The pH was adjusted to 6.5 and the medium was autoclaved at 121°C for 15 min. When the mixture cooled to 50°C the following sterile antibiotics were added: 35 mg nystatin, 3 mg D-methionine, 1 mg pyridoxine-HCl, 50 mg cephalexine, 30 mg trimethoprim.

All medium plates were stored at 4°C for at least 4 days prior to use to become opaque due to the retrogradation of the starch. This practice facilitated the recognition of starch hydrolysis by Xcc colonies. The extracts of the 12 seed subsamples were plated in parallel on plates of 7-019a and adapted recipes of mCS20ABN and FS media and results were compared.

Reference culture

Participating laboratories used a known Xcc strain grown on YDC medium and suspended in sterile saline (0.85% NaCl) as a positive control.

Xcc detection method description

The Xcc detection method described in ISTA Rule 7-019a was followed to extract bacteria from the subsamples, prepare sterility check plates, dilutions of seed extracts and positive control, incubate and examine plates for Xcc suspect colonies, check Xcc suspects morphology on YDC medium plates and perform the pathogenicity test for their confirmation.

Data analysis

Following the seed lots' characterisation in the heterogeneity test, the SeedCalc Version 8 (4) spreadsheet application was used to calculate the Xcc contamination rate in the low contamination level lot. The number of expected positive subsamples from this lot - plated on the 7-019a media recipes - in the peer validation study at a probability higher than 5% was then calculated with the spreadsheet application developed by J. L. Laffont (ISTA Statistics Committee Chair). The 11 positive 10,000-seed subsamples of the 15 tested revealed a contamination rate of 0.01% and 0-3 expected positive subsamples of the 3 distributed to laboratories. In the medium and high contamination level lots, as all 15 subsamples were positive in the heterogeneity test, it was expected that all 3 distributed subsamples per lot would be found positive in the peer validation study when plated on 7-019a media plates. Regarding the healthy level lot, as all 15 subsamples were Xcc negative in the heterogeneity test, it was expected that all 3 distributed subsamples per lot would be found positive subsamples were positive in the heterogeneity test, it was expected that that all 3 distributed subsamples per lot would be found positive in the peer validation study when plated on 7-019a media plates. Regarding the healthy level lot, as all 15 subsamples were Xcc negative in the heterogeneity test, it was expected that that all 3 distributed subsamples per lot would be found negative on the 7-019a media plates.

In the peer validation study, the Xcc suspects were recorded in cfu/ml for each (laboratory x contamination level x subsample x semi-selective medium x recipe x dilution x plate) combination. The positive colonies in the pathogenicity test determined the subsamples that were Xcc positive on the (medium x recipe) combination. A subsample was considered negative in this combination if there were no Xcc suspects recorded or if the Xcc suspects were negative on YDC medium and no pathogenicity test was performed. The case of Xcc suspects being positive on YDC medium but not tested in the pathogenicity test was considered to be a missing value (laboratory 4, high contamination level, subsample 2, mCS20ABN medium, 7-019a recipe).

Results of laboratory 5 were not included in the analysis as there was a positive result in the healthy level lot and a lower number of positive subsamples in the medium level lot than in the rest laboratories (Tables 1, 2 and 3). Laboratory 5 recorded Xcc suspects in one subsample from the healthy level lot in both 7-019a and adapted recipes of FS medium which were confirmed being Xcc in the pathogenicity test (raw data not shown). The Xcc suspects were recorded only in the plates of (10^{0}) undiluted extract and not in the 10^{-1} or 10^{-2} dilutions. As the results of the heterogeneity test and of the rest laboratories in the peer validation study showed no Xcc presence in the tested subsamples, this result of laboratory 5 could be attributed either to a cross contamination or to a very low Xcc presence in the lot that became apparent due to a sampling effect.

Results

A variable number of Xcc suspects was recorded by all 1-4 laboratories in the subsamples of the healthy level lot in plates of both media recipes (raw data not shown). However, all suspects were

confirmed negative in the pathogenicity test. Regarding the sterility control plates no Xcc suspects were recorded in any laboratory (raw data not shown).

In Table 1, the number of final positive detected subsamples - positive in either one or both FS and mCS20ABN media of the same recipe - is presented per laboratory, contamination level and recipe. The expected number of positive subsamples plated on the 7-019a media recipes was detected by all 1-4 laboratories in the healthy, low, medium and high level lots (Table 1).

Results of the peer validation study showed that the final number of positive detected subsamples in the healthy, low, medium and high level lots was the same in 7-019a and adapted media recipes and that was in each laboratory (Table 1).

The number of positive subsamples detected in each FS medium recipe was the same in all contamination levels in laboratories 1 and 2 (Table 2). A slightly smaller number of positives was detected in the adapted than in the 7-019a FS medium recipe in laboratories 3 and 4 which varied on the contamination level lots (Table 2).

The number of positive subsamples detected in each mCS20ABN medium recipe was the same in all contamination levels in laboratories 1 and 2 (Table 3). Regarding the rest of laboratories the detected number of positives in the adapted compared to the 7-019a recipe was equal or higher varying on the contamination level lot (Table 3).

Discussion and Conclusions

The adapted recipes of mCS20ABN and FS semi-selective media showed to perform equally to the recipes described in the ISTA Rule 7-019a for the Xcc contaminated and pathogen free cabbage seed lots.

The benefits of the adapted mCS20ABN recipe are summarised as follows: better buffered medium that ensures pH stability due to the increase of KH_2PO_4 and $(NH_4)_2HPO_4$ amounts and better absorption of the seed extract as a result of the agar amount increase in the medium. Regarding the FS medium, the clear formation of typical halos around the Xcc suspect colonies that is a result of the starch amount increase improves their recognition while the KNO₃ amount increase and omission of gentamycin improves the Xcc recovery. Finally, the nystatin replaces the toxic cycloheximide and improves the fungal control in both semi-selective media. The benefits of the adapted recipes of FS and mCS20ABN semi-selective media over the 7-019a recipes support the suggestion for replacement of the 7-019a recipes with the former.

Moreover, the adapted recipes of mCS20ABN and FS media are available in a ready-to-use package that allows for a standardised and easy preparation. Already, many seed health testing laboratories on an international level use the adapted recipes for the routine detection of Xcc on untreated and on disinfested/disinfected *Brassica* spp. seed.

In addition, the adapted recipe of both media have been described in the ISTA Rule 7-019b for the detection of Xcc on disinfested/disinfected *Brassica* spp. seed with grinding.

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| | Healthy level | | Lov | Low level | | um level | High level | |
|--------------|------------------|----------------|------------------|----------------|------------------|----------------|------------------|----------------|
| Laboratories | 7-019a recipe | Adapted recipe |
| 1 | 0+/3 | 0+/3 | 2+/3 | 2+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 2 | 0+/3 | 0+/3 | 1+/3 | 1+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 3 | 0+/3 | 0+/3 | 1+/3 | 1+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 4 | 0+/3 | 0+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 5 | 1+/3 | 1+/3 | 1+/3 | 1+/3 | 2+/3 | 1+/3 | 3+/3 | 3+/3 |

Table 1. Number of final positive subsamples detected per laboratory, contamination level and recipe of the total tested.

<u>Table 2</u>. Number of positive detected subsamples of total tested on 7-019a and adapted recipe of <u>FS</u> medium per contamination level and laboratory.

| | Heal | Healthy level | | Low level | | lium level | High level | |
|--------------|------------------|----------------|------------------|----------------|------------------|----------------|------------------|----------------|
| Laboratories | 7-019a recipe | Adapted recipe |
| 1 | 0+/3 | 0+/3 | 2+/3 | 2+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 2 | 0+/3 | 0+/3 | 1+/3 | 1+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 3 | 0+/3 | 0+/3 | 1+/3 | 0+/3 | 2+/3 | 1+/3 | 3+/3 | 3+/3 |
| 4 | 0+/3 | 0+/3 | 2+/3 | 1+/3 | 2+/3 | 2+/3 | 3+/3 | 2+/3 |
| 5 | 1+/3 | 1+/3 | 1+/3 | 1+/3 | 2+/3 | 1+/3 | 3+/3 | 3+/3 |
| | | | | | | | | |

<u>Table 3</u>. Number of positive detected subsamples of total tested on 7-019a and adapted recipe of <u>mCS20ABN</u> medium per laboratory and contamination level.

| Healthy level | | vel | Low level | | Mediu | m level | High level | |
|-----------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--|----------------------------|
| boratories 7- | 019a recipe | Adapted recipe | 7-019a recipe | Adapted recipe | 7-019a recipe | Adapted recipe | 7-019a recipe | Adapted recipe |
| 1 | 0+/3 | 0+/3 | 2+/3 | 2+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 2 | 0+/3 | 0+/3 | 1+/3 | 1+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 3 | 0+/3 | 0+/3 | 0+/3 | 1+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 |
| 4 | 0+/3 | 0+/3 | 3+/3 | 3+/3 | 3+/3 | 3+/3 | 2+/3 * | 3+/3 |
| 5 | 0+/3 | 0+/3 | 1+/3 | 1+/3 | 1+/3 | 0+/3 | 3+/3 | 3+/3 |
| 1 2 3 4 5 | 0+/3 0+/3 0+/3 0+/3 0+/3 | 0+/3 0+/3 0+/3 0+/3 0+/3 | 2+/3 1+/3 0+/3 3+/3 1+/3 | 2+/3 1+/3 1+/3 3+/3 1+/3 | 3+/3 3+/3 3+/3 3+/3 1+/3 | 3+/3 3+/3 3+/3 3+/3 0+/3 | 3+/3 3+/3 3+/3 2+/3 * 3+/3 | 3+ 3+ 3+ 3+ 3- |

* One seed subsample was recorded as missing value.

Validation of a new method for the detection of Acidovorax valerianellae on Corn salad (Valerianella locusta)

Grimault, V., GEVES-SNES, 25 Rue Georges Morel, CS 90024, 49071 Beaucouze, Cedex, France (valerie.grimault@geves.fr)

Politikou, L., ISF, 7 Chemin du Reposoir, 1260 Nyon, Switzerland (liana.politikou@ufs-asso.com)

The performance of an ISHI-Veg developed method for the detection of the seed-transmitted pathogen *Acidovorax valerianellae* (Av) on corn salad (*Valerianellae locusta*) seed was evaluated in an international comparative test between eight laboratories organized by ISHI-Veg. The method includes a grow-out test performed in a sweatbox followed by PCR confirmation of Av on symptomatic/doubtful corn salad cotyledons at 14 or 21 days after sowing. One pathogen-free untreated, and two (low and medium infected) naturally contaminated seed lots with variable saprophytic loads were compared. Each seed lot was tested in six blind replicates of 5,000-seed. One 1,000-seed sample from the pathogen-free and one 1,000 seed sample of the medium contaminated seed lot served as negative and positive control, respectively. In the same comparison a potting soil-vermiculite mixture showed comparable results to vermiculite alone as substrate and is therefore considered an alternative. No significant effect of Thiram treatment was shown on the saprophytic load of seed lots. Yet, its application in the recommended ratio can ensure discernible Av symptoms on corn salad cotyledons. The detection method showed high values of accordance (repeatability), concordance (reproducibility), sensitivity, specificity and accuracy for all three contamination levels. Therefore, it is considered to be a reliable method for the detection of Av on corn salad seed and is highly recommended in routine seed health testing.

Introduction

Acidovorax valerianellae (Av) is a gram-negative bacterium causing circular water-soaked spots that turn black on cotyledons and leaves of corn salad (*Valerianella locusta*) plants (Gardan *et al.*, 2003). It is one of the major pathogens of corn salad and is soil transmitted (Grondeau *et al.*, 2003).

Seed transmission of Av was demonstrated through grow-out tests originally carried out for the detection of *Peronospora valerianellae* (Grimault *et al.*, 2006). Isolated strains from diseased corn salad cotyledons grown from contaminated seeds were compared and found identical to *Acidovorax valerianellae* strains described by Gardan *et al.* (2003). Grondeau and Samson (2009) described a detection method for Av on corn salad seeds based on the use of a semi-selective medium. However, this method proved to be inefficient in routine testing of corn salad seed samples. Av grows slowly on media and its identification was difficult due to the presence of fast- growing saprophytes.

A grow-out test using a sweat box assay was developed by Rijk Zwaan NL in 2005 (H. Linders, personal communication). It comprised sowing seeds on a substrate in a sweat box, observation of Av symptoms that developed on cotyledons, foci formation, and finally, confirmation of suspect Av isolates in a PCR test. Av foci develop from one single contaminated seed. Foci have a round to confluent shape. One focus includes one to several seedlings with Av symptoms on the cotyledons.

Cotyledons damping-off which interfered with the observation of Av symptoms and recording was frequently observed in initial tests. GEVES-SNES compared blotter, sand, soil and vermiculite substrates for Av disease development and saprophytic growth levels. Although in most substrates damping-off was observed, development and observation of Av symptoms were best when seeds were sown on vermiculite. Suspect isolates were confirmed to be Av in a PCR test (V. Grimault, personal communication, 2008).

The effect of two different incubation conditions on the development of Av symptoms in sweat boxes was subsequently compared (Grimault *et al.*, 2008). Sweat boxes were incubated at 20°C with 12 h light/12 h dark and at 25°C with 12 h light, 18°C with 12 h dark. Both sets of conditions

were found to be equivalent in the development of typical Av disease symptoms on corn salad cotyledons. In the case of dormant seeds, an incubation period of 7 days at 5° C in the dark was applied post sowing. A grow-out was then performed with the seeds in the above mentioned incubation conditions.

Saprophytic growth in sweatboxes was, nevertheless, found to cause cotyledons damping-off and mask Av symptoms. The efficacy of fungicides applied as a substrate treatment to control saprophytic growth without inhibiting the development of Av was tested in a peer validation study with 3 laboratories. The highest saprophytic control in the sweatboxes was seen with the application of the fungicide Thiram at a rate of 4 g/kg of seeds on vermiculite. Moreover, Thiram didn't inhibit disease development at this concentration (Serandat, 2008) but did so at concentrations above 12 g/kg seeds (G. Hiddink, personal communication, 2007).

Specific primers for the detection of Av were developed by Enza Zaden NL in 2004 (J. Heldens, personal communication) and several studies were carried out to validate their specificity. Pathogenicity and PCR tests were performed on a collection of 28 Av isolates and saprophytes at GEVES-SNES to validate these primers. A correlation of 100% was shown between the two tests (Mazouni, 2007). Additional validation of the specificity of these primers was done by UMR Pavé (INRA-Angers-Nantes) by comparing 41 Av strains, 8 *Acidovorax* spp. strains and 3 strains of closely related genera as part of the BASELE project (Portier, 2010). The expected PCR amplification products were generated with these primers for all Av strains but not for the other bacteria.

The proposed method for the detection of Av on corn salad seed is based on a grow-out test in a sweat box and confirmation of Av cotyledons with a PCR test. The method has been in use by several public and company seed health testing laboratories since 2006. It can be used to test untreated seed, seeds treated with fungicides, and seeds that have been chemically or physically disinfected.

Aim and objective of the comparative test

The aim of this comparative test was to evaluate the proposed method for the Av detection on corn salad seed. The ultimate objective is to develop an internationally accepted seed health testing method for detecting Av on corn salad seed lots.

Materials and Methods

Seed lots and subsamples

Three untreated corn salad (*Valerianella locusta*) seed lots with variable levels of natural Av contamination and saprophytes were selected by the GEVES-SNES Laboratory in France. The seed lots (O 086397, O 086398 and O 083751) were characterized prior to the comparative test based on the average number of Av foci that developed in the sweatboxes. They were sown on a Thiramtreated substrate and tested using the proposed method to characterise them. From each seed lot 2 subsamples of 5,000 seeds were tested. In the lots O 086397, O 086398 and O 083751 an average of 16, 6.5 and zero foci were recorded. Thus, contamination in the seed lots was characterized as being medium, low and healthy, respectively.

The distribution of Av in the low and medium contamination-level seed lots was then evaluated in a homogeneity test that ran on 10 and 15 subsamples of 5,000 seeds using the Thiram-treated substrate. The "Homogeneity Test" Excel spreadsheet application developed by the ISTA Statistics Committee showed that the Av distribution was homogeneous in both seed lots (Appendix I).

For the comparative test each participating laboratory received six 5,000-seed subsamples from each of the three seed lots. These 18 subsamples were randomly coded to ensure a blind comparative test. One 1,000-seed subsample from the medium seed lot and one 1,000-seed subsample from the healthy seed lot were included as positive and negative controls, respectively. These controls were made known to the participating laboratories. Laboratories 1 and 4 received an

extra set of 20 seed subsamples to be tested with a combination of soil and vermiculite substrate. All seed subsamples were prepared by the sampling department of GEVES-SNES with the use of the rotary sample divider apparatus based on the thousand-seed weight of each seed lot.

All available seed material was utilised in the homogeneity and comparative tests. Therefore, no "Stability Test" was performed by the test organiser.

Grow-out method

Treated and untreated substrates

One chlorine disinfected sweatbox (*e.g.* Rotho, Ref. 4045, 10 L, 40 cm x 33.5 cm x 17 cm) was labelled with the number corresponding to each of the 20 seed subsamples. Three from the six coded seed subsamples per seed lot were randomly selected by the test organiser to be sown on to Thiram-treated and untreated substrates. The seed samples coded 2, 3, 5, 6, 7, 10, 13, 16 and 18, and the positive and negative control seed subsamples were sown on vermiculite treated with Thiram 80% WP (bis(dimethylthiocarbamoyl) disulphide, C6H12N2S4) at a rate of 4 g Thiram / kg of seeds.

Laboratories weighted each seed subsample that was to be grown on Thiram-treated substrate. The corresponding amount of Thiram was then measured based on each subsample's weight. The measured quantity of Thiram was then dissolved in 1L de-ionised water and poured into the corresponding sweatbox. During this process the laboratories took appropriate safety precautions (European chemical Substances Information System (ESIS) http://ecb.jrc.ec.europa.eu/esis/). In the remaining sweatboxes 1L of de-ionised water was added. Finally, all sweatboxes were filled with 2L of medium-sized vermiculite.

Laboratories 1 and 4 prepared 20 additional sweatboxes for the extra set of seed subsamples they received. The seed subsamples coded 2, 3, 5, 6, 7, 10, 13, 16 and 18 from the extra set of seeds were sown on potting soil treated with Thiram following the above mentioned procedure.

Seed sowing, incubation and inspection of symptoms

The seeds of each subsample were spread evenly over the whole surface of the vermiculite substrate into the corresponding sweatbox. Laboratories 1 and 4 sowed the extra set of 20 seed subsamples over the 2 L potting soil substrate and covered the seeds with 1 L of medium-sized vermiculite. Laboratory 1 added 0.5 L of vermiculite under the seeds to further impede the growth of saprophytes in the sweatboxes and covered them with 0.5 L of vermiculite.

All sweatboxes were then closed with a lid and incubated in one of the two conditions: i) 20°C with 12 h light/dark and ii) 25°C with 12 h light, 18°C with 12 h dark.

On the 14th and 21st days of incubation corn salad cotyledons were inspected for typical Av symptoms: small, circular, black water soaked lesions. Development of Av foci and saprophytic growth were also inspected and recorded in the provided data record sheet.

PCR confirmation

Collecting and grinding symptomatic cotyledons

On the 21st day, from every sweatbox 1-8 Av-symptomatic cotyledons from different foci were collected with aseptic tweezers and placed in containers (*e.g.* Extraction bags from Universal, Art. No. 430 100, BioReba, Switzerland) with a maximum of four cotyledons per container. A similar number of Av-symptomatic and symptomless cotyledons were collected from the positive and negative control sweatboxes, respectively. In sweatboxes where no Av symptoms were observed no cotyledons were collected and no PCR confirmation test was performed.

Each sample of collected cotyledons was ground in 3 ml of 0.85% sterile saline (8.5 g NaCl/1,000 mL de-ionised water, autoclaved at 121°C, at 15 psi for 15 min) with the use of a press

grinder (*e.g.* hand homogeniser) or equivalent tool and 1mL of the extract was collected in a separate Eppendorf tube. One (1) mL of the 0.85% sterile saline used for grinding was also collected in a separate Eppendorf tube and served as "process control".

Av DNA extraction from collected cotyledon samples

The cotyledon extracts and the process control were centrifuged at 6000-7000 x g for 5 min. The supernatant was discarded and the pellet re-suspended with 200 μ L sterile water. The suspensions were incubated at 100°C for 5 min and on ice for 5 min. A 10-fold dilution with sterile water was prepared from each suspension in a separate Eppendorf tube.

PCR primers, positive and negative controls, reaction mixture, profile and products visualisation

Laboratories used DNA of their own reference Av strain as PCR positive control and sterile water as PCR negative control. The primers set developed by Enza Zaden with DNA amplification product size of 346 bp targeting Av were used. The primers were:

ZTO57-F: GAT CGT GGG GGA TAA CGG A ZTO58-R: GTC ATG AGC TCT CTT TAT TAG AAA CAG

The PCR was carried out in a 25 μ L reaction mixture containing 1 μ L of DNA extract in 19.8 μ L sterile Milli Q, 1 x Buffer (10x concentrated PCR reaction buffer with Mg+, from Roche Cat. Number: 11271318001 with composition: 10mM Tris-HCl, 15mM MgCl₂ and 500mM KCl), 0.1mM dNTP's, 0.4 pmol/ μ L each of upstream and downstream primers and 0.05 U/ μ L of Taq polymerase.

Laboratories that didn't use the recommended mixture from Roche used their own in-house recipe. These PCR mixes contained a 1.5 mM final concentration of MgCl₂ and 1x final concentration of the Buffer.

The PCR profile was 2 min incubation at 94°C followed by 35 cycles of 30 sec at 94°C, 1 min at 63°C and 1 min at 72°C. A final 5 min incubation at 72°C and infinity at 4°C. Ten (10) μ L of each PCR-sample and of the PCR negative control (sterile saline) were run on a 1.5% agarose gel in 1x TAE buffer. A 100bp DNA ladder was included. The samples ran on the agarose gel by electrophoresis in 1x TAE buffer. The gel was stained with ethidium bromide. The amplification products were analysed for an Av specific product of 346 bp under UV-light. The PCR result of each tested sample was recorded next to the corresponding sweatbox in the data record sheet.

Data analysis

All 8 participating laboratories submitted the results they had generated. For each (laboratory x contamination level x treatment x sweatbox/seed subsample) combination the number of Av foci and the saprophytic growth rated on a scale of 1-3 were recorded on the 14th and on the 21st days. Values of the same variables including the number of symptomatic and/or doubtful cotyledons in those collected and the PCR positives of the total tested samples were additionally recorded per combination on the 21^{st} day.

The decision on whether a seed sample and the combinations (laboratory x contamination level x treatment x sweatbox/seed subsample) were Av positive or negative was determined based on either the grow-out result or the PCR result on the 21^{st} day. A seed subsample was considered Av negative if there were no symptomatic cotyledons observed *or* if the samples of collected cotyledons – symptomatic or doubtful – were found negative in the PCR test. A seed subsample was considered Av positive if at least one sample of collected cotyledons - symptomatic or doubtful – gave a PCR positive result.

The statistical analysis was performed on the final results (binary data; positive or negative) of the 1-6 laboratories. Results of Laboratories 7 and 8 were not included in the statistical analysis as there was a low number of positive seed subsamples found in the two contaminated seed lots and a positive result in the healthy (zero-contamination) lot (Table 1). The unexpected results of the Laboratories 7 and 8 were attributed to their lack of experience with identifying Av symptoms and performing the PCR test.

The norm NF EN ISO 16140 (AFNOR, 2003) was followed to evaluate the performance criteria - sensitivity, specificity and accuracy - for contamination levels and treatments. This evaluation was performed by comparing the expected results of all laboratories with those obtained. The results were in the form of positive and negative agreements and deviations (see Appendix II).

For each contamination level and treatment, accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) was evaluated using the definitions developed by Joesefsen *et al.* (2004), viz. the percentages of finding the same result positive or negative from two similar samples analysed in the same or different laboratories respectively and under standard repeatability conditions. The method and tool developed by Langton *et al.* (2002) was used to evaluate accordance and concordance.

The effect of Thiram-treated substrate on saprophytic growth in the sweatbox was studied in a χ^2 test. The test was performed on the ratings for saprophytic growth (on the scale 1-3) for all three seed lots recorded on the 21st day. Data generated by Laboratories 1, 2, 3, 5, and 6 were analysed. As Laboratory 4 rated saprophytic growth in all 18 sweatboxes as 2 its data was not included in the analysis.

No statistical analysis was performed on the results of the extra set of seed subsamples that Laboratories 1 and 4 received and sowed on soil-vermiculite combination.

Results

Laboratory 4 recorded zero foci and zero doubtful cotyledons in all sweatboxes with seed subsamples from the healthy seed lot (data not shown). Thereafter, no PCR test was performed and the final result for these seed subsamples was recorded based on the grow-out, where the absence of symptomatic cotyledons was considered to be a 'negative' result.

Laboratories 1, 3, 5 and 6 recorded zero number of collected Av symptomatic cotyledons but a variable number of cotyledons with doubtful symptoms in sweatboxes 1, 4, 4 and 2, respectively. Cotyledons with doubtful Av symptoms were confirmed negative in the PCR test by Laboratories 1, 3 and 6.

However, for one subsample from a healthy lot (no. 16) Laboratory 5 confirmed the pure extract of 1 cotyledon with doubtful symptoms as being Av positive, whilst its dilution gave a doubtful result. This seed subsample was finally considered to be positive based on the PCR result of the pure cotyledon extract (data not shown). For another subsample of the same lot (no. 8) Laboratory 5 reported a negative PCR result for the pure extract of 1 cotyledon with doubtful symptoms but a doubtful PCR result on its dilution. The subsample no. 8 was considered to be negative.

The remaining seed subsamples from the healthy seed lots from Laboratories 1, 3, 5 and 6 were Av negative based on the grow-out.

Av cotyledons and cotyledons with doubtful symptoms collected by Laboratory 2 in 4 sweatboxes containing healthy seed subsamples were confirmed Av negative in the PCR test (data not shown). The remaining healthy seed subsamples of Laboratory 2 were Av negative as no symptoms were recorded.

Regarding the low and medium contamination levels all 6 laboratories observed Av symptoms on the cotyledons and recorded a variable number of Av foci in all subsamples sown on both treated and untreated substrates. All collected Av cotyledon samples were confirmed PCR positive. Laboratories 2 and 5 collected a variable number of cotyledons with doubtful symptoms. Three out of 5 samples that Laboratory 2 tested were found to be PCR positive. Laboratory 5 confirmed all its doubtful samples as being PCR negative (data not shown).

The expected and the obtained results from Laboratories 1-6 are presented for contamination levels and substrate treatment in Table 1. The sensitivity of the evaluated grow-out and PCR confirmation method was 100% for the low, medium and the low+medium contaminated seed lots and treatments (Table 2). In other words, no false negative results were obtained.

The specificity of the method was 100% for the combination healthy x untreated substrate as there were no false positives and 94.44% for the healthy x Thiram- treated substrate (Table 2).

The accuracy was 100% for the low, medium and the low+medium contaminated seed lots and treatments and for the healthy x untreated substrate combination. It was 94.44% for the healthy x Thiram-treated substrate. Accordance and concordance values were high in all cases (Table 2). The values that were less than 100% in the specificity, accuracy, accordance and concordance criteria in the healthy x Thiram-treated combination were due to the decision to consider seed subsample no. 16 of Laboratory 5 to be PCR positive.

No significant difference was shown between the Thiram-treated and the untreated substrate on the level of saprophytic growth based on the test (Tables 3 and 4).

The results obtained by the PCR test for seed subsamples sown by Laboratories 1 and 4 on soilvermiculite were identical to those of seed subsamples sown on vermiculite substrate (Table 5). Both laboratories recorded zero Av symptomatic cotyledons in the seed subsamples from the healthy lots and a variable number of Av foci in the seed subsamples from the contaminated lots (data not shown). However, Laboratory 4 didn't record any Av symptoms on the cotyledons from the positive control seed subsample.

Discussion and conclusions

No PCR test was performed if there were no Av symptoms observed on the cotyledons. The PCR test confirmed typical and doubtful Av symptoms that were recorded on the cotyledons of seed subsamples from the three seed lots.

In this comparative test symptoms were confirmed on the last day of incubation. Typical Av symptoms can also be clearly observed on the 14th day of incubation. In such cases symptoms can be confirmed in a PCR test without waiting to day 21. A PCR positive result would bring the test to an end. In case of a negative PCR result or no symptoms observation on the 14th day, incubation must be extended to the full 21 days where the final recording and PCR confirmation are performed.

Although the healthy seed lot was identified as being Av negative by the test organiser, the possibility that it was very weakly contaminated by Av cannot be excluded (as it wasn't tested in the homogeneity test). This might explain the PCR positive result that Laboratory 5 found on a doubtful symptomatic cotyledon from a healthy seed subsample.

The evaluated grow-out and PCR confirmation method for the detection of Av on corn salad seeds was shown to be repeatable (high accordance values) and reproducible (high concordance values) with high sensitivity, specificity and accuracy at all three contamination levels. Therefore, it is highly recommended for routine seed health testing for the detection of Av on corn salad seed. The minimum recommended sample size to be tested for the detection of Av is 10,000 seeds divided in 2 subsamples of 5,000 seeds.

The results of this comparative test didn't show a significant effect of Thiram on saprophytic growth, which is opposed to findings of previous studies (Serandat, 2008). However, in case of high (suspected) saprophytic load in the seed subsamples it is recommended to treat the substrate with Thiram as it will reduce damping-off on corn salad cotyledons and ensure discernible Av symptoms in the sweatbox. Yet, if more than 20% of the corn salad seedling population is damped-off due to fungal infection and <u>no</u> Av symptoms can be observed in the sweatbox this must be indicated in the test result. In this case the test result is considered invalid and it is recommended to repeat the test

with sufficient seeds to compensate for seedling loss with the option of a Thiram-treated substrate. If, however, Av symptoms are observed on the cotyledons and confirmed in the PCR, the test result is valid and the seed-subsample is infected.

The comparable final results obtained from seed subsamples grown on a soil substrate and on vermiculite support the use of the former as an alternative in the grow-out test.

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| | | | | No. of obtained positive seed subsamples | | | | | | | |
|----------|----------------------------|------------------------|--|--|---|---|------|-------|------|----------------|----------------|
| | | | | | | | Labo | orato | ries | | |
| Seed Lot | Contamination Level | Treatment ^a | No. of expected positive seed subsamples | 1 | 2 | 3 | 4 | 5 | 6 | 7 ^b | 8 ^b |
| O 083751 | Healthy | Т | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| | | N/T | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| O 086398 | Low | Т | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 | 1 |
| | | N/T | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 1 |
| O 086397 | Medium | Т | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 3 |
| | | N/T | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 |

Table 1. Final results of participant laboratories of the grow-out and PCR confirmation method for the detection of Av on cornsalad seeds.

^{*a*} T= Treatment with Thiram fungicide, N/T=No treatment applied.

^b Results were excluded due to lack of experience with the method.

| Table 2. Statistical evaluation of 1-6 participant laboratories final results of the grow-out and PCR confirmation method for the detection of Av on cornsal | id seeds |
|--|----------|
|--|----------|

| Seed Lot | Contamination Level | Treatment | Sensitivity (%) | Specificity (%) | Accuracy | Accordance (%) | Concordance (%) |
|---------------------|----------------------------|----------------|------------------|------------------|----------|----------------|-----------------|
| | | T ^a | N/A ^b | 94.44 | 94.44 | 88.9 | 88.9 |
| O 083751 | Healthy | N/T^{a} | N/A ^b | 100 | 100 | 100 | 100 |
| | | T ^a | 100 | N/A ^b | 100 | 100 | 100 |
| O 086398 | Low | N/T^{a} | 100 | N/A ^b | 100 | 100 | 100 |
| | | T ^a | 100 | N/A ^b | 100 | 100 | 100 |
| O 086397 | Medium | N/T^{a} | 100 | N/A ^b | 100 | 100 | 100 |
| All positive levels | | T ^a | 100 | N/A ^b | 100 | 100 | 100 |
| | | N/T^{a} | 100 | N/A ^b | 100 | 100 | 100 |

^{*a*} T= Treatment with Thiram fungicide, N/T=No treatment applied.

^b N/A= Not applicable.

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| | 0 | bser | ved va | Expected values | | | |
|--------------------------|---------------|------|--------|-----------------|----|--------|------|
| | Classes Total | | | Total | | Classe | es |
| Treatments | 0 | 1 | 2-3 | | 0 | 1 | 2-3 |
| Thiram treated substrate | 12 | 23 | 10 | 45 | 10 | 20.5 | 14.5 |
| Untreated substrate | 8 | 18 | 19 | 45 | 10 | 20.5 | 14.5 |
| Total | 20 | 41 | 29 | 90 | | | |

Table 3. Observed and expected values of saprophytic scale (0-3) of all but Laboratory 4 and all three seed lots.

<u>**Table 4**</u>. Statistical output of χ^2 test on values of saprophytic scale.

| Pearson's Chi-squared test | | | | | | | |
|----------------------------|----|--------|--|--|--|--|--|
| χ^2 | df | Р | | | | | |
| 4.2029 | 2 | 0.1223 | | | | | |

Table 5. Final results of cornsalad seed subsamples sown on vermiculite and on soil-vermiculite substrates.

| | | | Vermiculite Soil-Vermicu | | miculite | |
|----------|----------------------------|------------------------|--------------------------|----------|-------------|------------|
| | | | | Labor | atories | |
| | | | 1 | 1 | 4 | 4 |
| | Contamination Level | | No. of obtain | ned posi | tive seed s | subsamples |
| Seed Lot | | Treatment ^a | | - | | - |
| O 083751 | Healthy | Т | 0 | 0 | 0 | 0 |
| | | N/T | 0 | 0 | 0 | 0 |
| O 086398 | Low | Т | 3 | 3 | 3 | 3 |
| | | N/T | 3 | 3 | 3 | 3 |
| O 086397 | Medium | Т | 3 | 3 | 3 | 3 |
| | | N/T | 3 | 3 | 3 | 3 |

^{*a*} T= Treatment with Thiram fungicide, N/T=No treatment applied.

Appendix I – Homogeneity test results for the Av contaminated seed lots.

Low level lot

| homogeneity test - seed h | ealth | | | | | | | | | | |
|------------------------------------|-------|-------|------|-------|----------------|-------|-------|-------|-------|------|---------|
| | | | | | | | | | | | |
| Sample size | 5000 | | | | | | | | | | |
| Samples | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| infected seed nb. | | 2 | 5 | 4 | 4 | 2 | 1 | 8 | 4 | 10 | Average |
| healthy seed percentage Average | 100 | 99.96 | 99.9 | 99.92 | 99.92 99.92 | 99.96 | 99.98 | 99.84 | 99.92 | 99.8 | 99.92 |
| Tolerance | | | | | 1.41 | | | | | | |
| H value | | | | | 1.39 | | | | | | |
| Homogeneity check | | | | | OK | | | | | | |

Medium level lot

| homogeneity test - seed l | health | | | | | | | | | | | | | | | |
|---------------------------|--------|-------|-------|------|-------|-------|-------|------|------|-------|-------|-------|-------|-------|-------|---------|
| | | | | | | | | | | | | | | | | |
| Sample Size | 5000 | | | | | | | | | | | | | | | |
| Samples | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | |
| | | | | | | | | | | | | | | | | |
| infected seed nb. | 23 | 19 | 19 | 15 | 12 | 13 | 13 | 20 | 15 | 21 | 14 | 18 | 17 | 18 | 12 | Average |
| healthy seed percentage | 99.54 | 99.62 | 99.62 | 99.7 | 99.76 | 99.74 | 99.74 | 99.6 | 99.7 | 99.58 | 99.72 | 99.64 | 99.66 | 99.64 | 99.76 | 99.67 |
| Average | | | | | 99.67 | | | | | | | | | | | |
| Tolerance | | | | | 1.41 | | | | | | | | | | | |
| H value | | | | | | | | | | | | | | | | |
| Homogeneity check | | | | | OK | | | | | | | | | | | |

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Appendix II – Definitions and mathematical formulas of sensitivity, specificity and accuracy criteria as determined by the norms NF EN ISO 16140.

| | expected result + (target) | expected result - (non target) |
|-------------------|-----------------------------|--------------------------------|
| Obtained result + | positive agreement +/+ (PA) | positive deviation -/+ (PD) |
| Obtained result - | negative deviation +/- (ND) | negative agreement -/- (NA) |

* PA = positive agreement, ND = negative deviation, NA = negative agreement, PD = positive deviation, N = total number of possible agreement

These definitions allowed the calculation of sensitivity, specificity and accuracy according to the following mathematical formulas:

Sensitivity = $\Sigma PA/(\Sigma PA + \Sigma ND)x100$

Specificity = $\Sigma NA/(\Sigma NA + \Sigma PD) \times 100$

Accuracy = $(\Sigma NA + \Sigma PA)/(\Sigma PA + \Sigma NA + \Sigma PD + \Sigma ND)x100$

Appendix III – Statistical output of the excel application developed by Langton *et al.* (2002).

Healthy level with Thiram treatment

Healthy level without Thiram treatment

| Accordance an | d concordance worksheet | Clear data Clear results Run bootstrap | | | | | |
|---|--|---|--|--|--|--|--|
| Clear data | r data Clear results Run bootstrap | | | | | | |
| | Confidence limits: 95 Bootstrap samples: 3000 Bootstra | | | | | | |
| | Bootstrap method: representative | Number of Bootstrap 95% limits | | | | | |
| Number of Laboratory samples positives | Bootstrap 95% limits Estimate Bootstrap s e lower upper | Laboratory samples positives Estimate Bootstrap s.e. lower upper | | | | | |
| 1 3 0 | Within lab pairs 18 | 2 2 0 Within lab matched pairs 16 | | | | | |
| 2 3 0 | Within lab matched pairs 16 Accordance (propp) 0.889 0.1021 0.6666667 1 | 3 3 0 Accordance (proph) 1.000 0.0000 1 1 4 3 0 Accordance (proph) 1.000 0.0000 1 10 | | | | | |
| 4 3 0 | Accordance (percentage) 88.9% 10.21% 66.67% 100.00% | 5 3 0 Total pairs 136 | | | | | |
| 5 <u>3</u> 1 6 <u>3</u> 0 | Total pairs 153 Total matched pairs 136 | 6 3 0 Total matched pairs 136 | | | | | |
| 7 | Between pairings 135 | 7 Between pairings 120 8 same between 120 | | | | | |
| 9 | Concordance (propn) 0.889 0.0909 0.7111111 1 | 9 Concordance (propn) 1.000 0.0000 1 1 | | | | | |
| 10 | Concordance (percentage) 88.9% 9.09% 71.11% 100.00% | 10 Concordance (percentage) 100.0% 0.00% 100.00% 100.00% | | | | | |
| 12 | Above results are based on 3000 bootstrap samples of 6 labs using representative method | 12 Above results are based on 3000 bootstrap samples of 6 labs using representative met | | | | | |
| 13 | | 13 | | | | | |

Low level with/without Thiram treatment and Medium level with/without Thiram treatment.



All positive levels with/without Thiram treatment.



Proposal for the addition of *Cicer arietinum* (Kabuli type) as a species to which the conductivity test for seed vigour can be applied

Mohammad Khajeh-Hosseini¹, Carina Gallo² and Hulya Ilbi³

¹Department of Crop Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran. agr844@gmail.com or saleh@ferdowsi.um.ac.ir

². National Institute of Agricultural Research, Oliveros Experimental Station, Oliveros, Argentina

³ Seed Technology Centre, Department of Horticulture, Faculty of Agriculture, Ege University, Izmir, Turkey

Summary

Six seed lots of *Cicer arietinum* (Kabuli type), all having a laboratory germination of >80%, were tested by three laboratories using the electrical conductivity test, as described in the ISTA Rules for *Pisum sativum* for 24h in two runs in each laboratory. All laboratories consistently identified the same significant differences in the seed lot conductivity and the data was repeatable within laboratories and reproducible between laboratories. This provides evidence in support of the addition of *Cicer arietinum* (Kabuli type), to the ISTA Rules as a species for which the conductivity test can be applied.

Introduction

The conductivity test is validated in the ISTA Rules as a test that can be applied to species of Pisum sativum (garden pea only, excluding petit-pois varieties), Phaseolus vulgaris and Glycine max. This test is based on the leakage of solutes that occurs from all seeds that are soaked in water. These solutes include sugars, amino acids and most importantly for the test, electrolytes. Thus the incidence of leakage can be detected by measurement of the electrical conductivity (EC) of the seed soak-water. The test was developed following the observation of the correlation between solute leakage and field emergence in wrinkled-seeded vining peas (Pisum sativum). Low leakage and therefore low conductivity was associated with seeds that emerged well, that is seeds with high vigour; whereas low vigour seeds with poor emergence had high levels of leakage and conductivity (Matthews and Whitbread, 1968). The conductivity test has also been used as an indicator of field emergence in field beans (Vicia faba, Hegarty, 1977), Phaseolus beans (Matthews and Bradnock, 1968; Powell et al., 1986), soybean (Oliveria et al., 1984; Yaklich et al., 1984) and long bean (Vigna sesquipedalis; Abdullah et al., 1991). Leakage has also been related to emergence in the light-coloured, larger seeded Kabuli type chickpea (Cicer arietinum) (Khajeh-Hosseini et al., 2007; Khajeh-Hosseini and Rezazadeh, 2011). Investigations are currently in progress using the coloured, smaller-seeded Desi type (ISTA Vigour Committee, 2013-2016). The test identifies where solute leakage occurs as a result of decreased membrane integrity and the death of tissue during the ageing of seeds. The objective of this study was to demonstrate that the conductivity test applied to Cicer arietinum (Kabuli type), is both repeatable within laboratories and reproducible between laboratories.

Materials and Methods

Samples of six seed lots of *Cicer arietinum* were obtained from Plant Research Institute, Ferdowsi University of Mashhad. The seeds originated from Iran and all had a normal germination above 80%. Coded samples of the seed lots were sent from Mashhad, Iran to the participating laboratories, namely Department of Crop Science, Ferdowsi University of Mashhad, Mashhad, Iran, Department of Horticulture, Ege University, Izmir, Turkey; National Institute of Agricultural Research, Oliveros Experimental Station, Oliveros, Argentina. Each laboratory completed two runs of the conductivity

test using the same method as that described for peas in the ISTA Rules (ISTA, 2013) i.e. four replicates of 50 seeds in each run, each soaked in 250 ml deionised/ distilled water for 24 h at 20°C. The conductivity was measured after 24 h and expressed as μ S cm⁻¹ g⁻¹ of seed.

The data from each run of conductivity was analysed separately using

(a) Analysis of Variance: Data were analysed by two way analysis of variance using the statistical package SAS (Version 9.1) followed by the calculation of the LSD.

(b) calculation of z-scores and

(c) the statistical tool developed by S. Grégoire according to ISO 5725-2 to calculate h-values and k-values. The statistical tool is available for download at the ISTA website:

http://www.seedtest.org/upload/cms/user/ISO572511.zip

Results

The means for each of the seed lots in both runs of the test of measurements of the conductivity showed clear and significant differences in seed leachate conductivity and hence vigour (Tables 1, and 3). Overall, seed lots of A, D and F had the highest conductivity in both runs of measurements, i.e. lowest vigour, followed by lot C, while lots B and E had the lowest conductivity indicating the highest vigour.

Application of Table 15B from the ISTA Rules (ISTA, 2013) showed that, the replicate data (Appendix 1 and 2) for lots B, C and E in each laboratory were in tolerance with one another. The mean conductivity measurements for lots A, D and F were all greater than 53.9, the highest value for which there is a tolerance value in Table 15B (ISTA, 2013). However, if the value for a mean of 53.9 μ S cm⁻¹ g⁻¹ i.e. 13.8 is applied, the data for these lots is in tolerance, with the exception of lot D in the first run in Lab 2. The range in the replicate data for lot D in Lab 2, run 1 was clearly different from that seen in all other tests on this lot, suggesting that there was a particular problem during testing.

There were small, but significant, differences in the overall means from the three laboratories (Tables 1 and 3). However, the lot means from individual labs were in tolerance for lots B, C and E in both runs. Since the overall means for lots A, D and F were above 53.9, the maximum tolerance value in Table 15D (ISTA, 2013) was applied i.e. 12.4. The lab means for lot A were therefore in tolerance in Run 1 and means for all three lots (A, D and F) were in tolerance in Run 2.

The Coefficient of Variation for the comparative test was 5.9 a value comparable with that reported (6.4%) for the method validation of conductivity for *Glycine max* (Powell, 2012).

Calculation of the z-scores (Tables 2 and 4) revealed that all data fell within the values that are acceptable within ISTA proficiency tests.

Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire, based on ISO 5725-2; this allows the calculation of h- and k-values. The h-values show the tendency for a laboratory to give over-estimations or under-estimations compared to the mean of all the results available whereas the k-values give a measure of the variability of the repeats. Higher values indicate greater under- or over-estimations (h-values) or greater variability between replicates (k-values).

No significant h-values were found in both runs (Figure 1 and 3), indicating that the measurements were not over or underestimated. There was only one significant k-value, namely for lot D in lab 2 in the first run (Figure 2) indicating that there was greater variability between replicates.

Repeatability and reproducibility values are affected by the examined species and the seed quality of the lots tested, with low vigour seeds often having higher values. It is therefore not possible to compare directly the data from comparative tests using different seed lots. The values obtained for

repeatability (Tables 5 and 6) were similar to those for both *Glycine max* (Powell, 2012) and *Phaseolus vulgaris* (Powell, 2009) although the reproducibility values were higher.

Discussion

The conductivity test consistently identified differences between seed lots in each of three laboratories. The test was both repeatable within laboratories and reproducible in different laboratories. In addition, where the conductivity measurements of lots fell within the range of the tolerance tables (ISTA, 2013), the replicates within the laboratories and the mean values obtained for each lot in different laboratories were within tolerance. In three lots (A, D and F) seed vigour was particularly low, giving conductivity measurements higher than those in the tolerance tables. Nevertheless, application of the highest tolerance value from Tables 15B and D (ISTA, 2013) revealed that only in lot D, Run 1 were the replicate means out of tolerance, and only two out of six lab means (2 runs x 3 labs) were out of tolerance. This provides evidence in support of the addition of *Cicer arietinum* (Kabuli type), to the ISTA Rules as a species for which the conductivity test can be applied.

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Table 1: Comparison of laboratory and seed lot means of six lots of chickpea (Kabuli type) tested by three laboratories using the conductivity test for 24 hours (first run)

| Lah | Lot | | | | | | | |
|-----------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|--------------------|--|
| Lau | Α | В | С | D | Ε | F | | |
| 1 | 65.24 ^C | 40.37 ¹ | 49.53 ^G | 58.75 ^{DE} | 40.88 ¹ | 55.43 ^{EF} | 51.69 ^b | |
| 2 | 75.56 ^{AB} | 44.17 ^{HI} | 50.12 ^G | 76.89 ^A | 50.66 ^{FG} | 71.33 ^B | 59.81 ^a | |
| 3 | 64.04 ^C | 40.56 ¹ | 47.61 ^{GH} | 62.96 ^{CD} | 41.88 ¹ | 63.26 ^{CD} | 53.38 ^b | |
| Lot means | 68.28 ^a | 41.69 ^d | 49.08 ^c | 66.20 ^a | 44.47 ^d | 63.34 ^b | | |

For lot and lab means, different lower case letters indicate that values are significantly different using LSD at the 5% level.

Within a row (laboratory), different upper case letters indicate that values (lots) are significantly different using LSD at the 5% level

| Table 2: Comparison | of means, standard devia | ations (SD) and z- | scores for six seed | lots of chickpea |
|------------------------|-----------------------------|--------------------|-----------------------|------------------|
| (Kabuli type) tested b | by three laboratories using | g the conductivity | y test for 24 hours (| first run) |

| Lot | | | | | | | | |
|----------|--------|--------|-------------|--------|--------|--------|--|--|
| Lab | Α | В | С | D | E | F | | |
| a) means | | | | | | | | |
| 1 | 65 24 | 40 37 | 49 53 | 58 75 | 40 88 | 55 43 | | |
| 2 | 75.56 | 44.17 | 50.12 | 76.89 | 50.65 | 71.33 | | |
| 3 | 64.04 | 40.56 | 47.61 | 62.96 | 41.88 | 63.26 | | |
| | | | 1 | | 1 | | | |
| Mean | 68.28 | 41.69 | 49.08 | 66.20 | 44.47 | 63.34 | | |
| SD | 6.33 | 2.14 | 1.31 | 9.49 | 5.38 | 7.95 | | |
| | | | b) z-scoros | | | | | |
| 1 | -0 480 | -0.616 | 0.343 | -0 785 | -0.667 | -0 994 | | |
| 2 | 1,150 | 1.158 | 0.786 | 1.125 | 1.148 | 1.00 | | |
| 3 | -0.669 | -0.528 | -1.122 | -0.341 | -0.481 | -0.010 | | |

Table 3: Comparison of laboratory and seed lot means of six lots of chickpea (Kabuli type) tested by three laboratories using the conductivity test for 24 hours (second run)

| Lah | Lot | | | | | | |
|-----------|---------------------|---------------------|---------------------|---------------------|--------------------|---------------------|--------------------|
| Lab | Α | В | С | D | Е | F | |
| 1 | 63.73 ^A | 42.08 ^{DE} | 45.74 ^D | 55.74 ^C | 40.81 ^E | 57.98 ^{BC} | 51.01 ^a |
| 2 | 60.08 ^{AB} | 34.89 ^G | 41.36 ^E | 58.80 ^{BC} | 40.23 ^E | 60.37 ^{AB} | 49.29 ^b |
| 3 | 58.01 ^{BC} | 36.39 ^{FG} | 39.60 ^{EF} | 59.97 ^B | 34.99 ^G | 56.83 ^{BC} | 47.63 ^b |
| Lot means | 60.60 ^a | 37.79 ^d | 42.23 ^c | 58.17 ^b | 38.67 ^d | 58.39 ^b | |

For lot and lab means, different lower case letters indicate that values are significantly different using LSD at the 5% level.

Within a row (laboratory), different upper case letters indicate that values (lots) are significantly different using LSD at the 5% level

Table 4: Comparison of means, standard deviations (SD) and z-scores for six seed lots of chickpea (Kabuli type) tested by three laboratories using the conductivity test for 24 hours (second run)

| | Lot | | | | | | | |
|----------|--------|--------|-------------|--------|--------|--------|--|--|
| Lab | Α | В | С | D | E | F | | |
| a) means | | | | | | | | |
| 1 | 63.73 | 42.08 | 45.74 | 55.74 | 40.81 | 57.98 | | |
| 2 | 60.06 | 34.89 | 41.36 | 58.80 | 40.22 | 60.36 | | |
| 3 | 58.01 | 36.39 | 39.60 | 59.97 | 34.98 | 56.83 | | |
| | | | • | | | | | |
| Mean | 60.60 | 37.79 | 42.23 | 58.17 | 38.67 | 58.39 | | |
| SD | 2.90 | 1.06 | 3.16 | 2.18 | 3.21 | 1.80 | | |
| | | | b) z-scores | 6 | | | | |
| 1 | 1.079 | 1.051 | 1.110 | -1.114 | 0.666 | -0.227 | | |
| 2 | -0.182 | -2.731 | -0.275 | 0.288 | 0.482 | 1.094 | | |
| 3 | -0.893 | -1.320 | -0.832 | 0.825 | -1.149 | -0.866 | | |

Table 5: Values for repeatability and reproducibility of results from the conductivity test for 24 hours (first run) on *Cicer arietinum* (Kabuli type)

| Lot | Repeatability | Reproducibility |
|-----|---------------|-----------------|
| Α | 2.9413 | 6.8287 |
| В | 2.6171 | 3.1164 |
| С | 3.1967 | 3.0626 |
| D | 5.1384 | 10.4841 |
| Ε | 2.0213 | 5.6599 |
| F | 3.7064 | 8.5755 |

Table 6: Values for repeatability and reproducibility of results from the conductivity test for 24 hours (second run) on *Cicer arietinum* (Kabuli type)

| Lot | Repeatability | Reproducibility |
|-----|---------------|-----------------|
| Α | 1.3704 | 3.1318 |
| B | 3.1056 | 4.6488 |
| С | 3.0902 | 4.1419 |
| D | 2.8252 | 3.2793 |
| Ε | 1.8312 | 3.5785 |
| F | 3.0154 | 3.1754 |



Figure 1: h-values for six seed lots of *Cicer arietinum* (Kabuli type) tested using the conductivity test for 24 hours (first run) in three laboratories.



Figure 2: k-values for six seed lots of *Cicer arietinum* (Kabuli type) tested using the conductivity test for 24 hours (first run) in three laboratories



Figure 3: h-values for six seed lots of *Cicer arietinum* (Kabuli type) tested using the conductivity test for 24 hours (second run) in three laboratories



Figure 4: k-values for six seed lots of *Cicer arietinum* (Kabuli type) tested using the conductivity test for 24 hours (second run) in three laboratories

Appendix 1:

Data for each replicate conductivity reading for each of six lots taken in each of three laboratories (first run)

| | | Lab | | | | |
|-----|------|-------|-------|-------|--|--|
| Lot | Rep | 1 | 2 | 3 | | |
| Α | 1 | 62.86 | 77.03 | 66.08 | | |
| | 2 | 64.44 | 70.87 | 65.33 | | |
| | 3 | 65.43 | 76.91 | 65.67 | | |
| | 4 | 68.23 | 77.44 | 59.08 | | |
| | Mean | 65.24 | 75.56 | 64.04 | | |
| В | 1 | 42.39 | 46.10 | 36.57 | | |
| | 2 | 40.37 | 45.52 | 42.13 | | |
| | 3 | 38.69 | 45.74 | 40.91 | | |
| | 4 | 40.02 | 39.30 | 42.64 | | |
| | Mean | 40.37 | 44.17 | 40.56 | | |
| С | 1 | 47.02 | 50.94 | 47.19 | | |
| | 2 | 46.24 | 55.08 | 46.83 | | |
| | 3 | 52.65 | 49.71 | 49.29 | | |
| | 4 | 52.19 | 44.75 | 47.14 | | |
| | Mean | 49.53 | 50.12 | 47.61 | | |
| D | 1 | 57.02 | 64.93 | 63.13 | | |
| | 2 | 58.16 | 75.82 | 63.49 | | |
| | 3 | 60.36 | 83.57 | 61.65 | | |
| | 4 | 59.46 | 83.22 | 63.56 | | |
| | Mean | 58.75 | 76.89 | 62.96 | | |
| Е | 1 | 38.71 | 52.44 | 39.67 | | |
| | 2 | 41.77 | 49.54 | 43.41 | | |
| | 3 | 39.86 | 50.02 | 44.74 | | |
| | 4 | 43.16 | 50.63 | 39.69 | | |
| | Mean | 40.88 | 50.66 | 41.88 | | |
| F | 1 | 57.53 | 65.99 | 61.37 | | |
| | 2 | 56.57 | 77.27 | 64.63 | | |
| | 3 | 56.03 | 73.04 | 59.97 | | |
| | 4 | 51.58 | 69.03 | 67.07 | | |
| | Mean | 55.43 | 71.33 | 63.26 | | |

Early counts of radicle emergence as a vigour test for oil seed rape

A.A. Powell¹, S. Matthews¹, L. Kerr³, G. McLaren⁴ and M.-H. Wagner²

1 School of Biological Sciences, Cruickshank Building, 23 St Machar Drive, University of Aberdeen, AB24 3UU, UK (agr791@abdn.ac.uk) 2 GEVES, Station Nationale d'Essais de Semences (SNES) Angers, France 3 Alexander Harley Seeds (Milnathort) Ltd, Milnathort, Tayside, KY13 7RF, UK 4 SASA, Roddinglaw Rd, Edinburgh, EH12 9FJ, UK

Summary

Radicle emergence (RE) of nine seed lots of oilseed rape was assessed after 30h at 20° C by each of three laboratories. Clear and significant differences were observed between lots in all laboratories. All results were within tolerance and both repeatability and reproducibility were good, there being no evidence of over-dispersion. It is proposed that the RE test be validated as a vigour test for oilseed rape.

Introduction

Early counts of radicle emergence of maize (after 6d at 13°C or 66 h at 20°C), have been shown to be closely related to the rate of germination, as expressed by the mean germination time (MGT) and to vigour, reflected in the rate of and final field emergence (Matthews *et al.*, 2011a,b). This resulted in the introduction of the radicle emergence (RE) vigour test for maize to the ISTA Rules in 2010. Similar highly significant relationships have been seen in several other crop species (Matthews and Powell, 2011). Recent work on oilseed rape (*Brassica napus*) (Matthews *et al.*, 2012) has shown that single counts of radicle emergence of oilseed rape, taken after 30h at 20°C are also indicative of the rate of germination and vigour. Thus, single counts of radicle emergence predicted the MGT (R^2 = 0.920), 7 day field emergence (R^2 = 0.961) and maximum field emergence (R^2 = 0.713). In addition, the results were highly consistent between three laboratories (Matthews *et al.* 2012). The aim of this report is to analyse the data from the three laboratories to illustrate the repeatability and reproducibility of the test method.

Materials and Methods

Seed Material

Nine seed lots of the winter oilseed rape cultivar Vision were selected from samples of seed lots sent to Alexander Harley (Milnathort) Seeds Ltd., Scotland for routine testing by their associated company Senova Ltd. The seed had been produced in the south of England. The standard germinations of the lots were all high, 95% or above for all except one lot which had a standard germination of 90% (table 1).

<u>Table 1:</u> Details of nine lots of winter oilseed rape cv. Vision compared in three laboratories: standard germination (mean of 200 seeds in each of three laboratories) and year of production.

| Seed lot | Year of production | Standard germination (normal seedlings%) |
|----------|--------------------|---|
| А | 2010 | 99 |
| В | 2009 | 97 |
| С | 2009 | 95 |
| D | 2010 | 90 |
| Е | 2010 | 98 |
| F | 2010 | 98 |
| G | 2009 | 96 |
| Н | 2010 | 98 |
| Ι | 2010 | 98 |

Participating laboratories

The participating laboratories were the Official Seed Testing Stations in Scotland (SASA, Edinburgh) and France (SNES, Angers, France); and the Alexander Harley Seeds laboratory (Scotland). Samples of the lots were distributed in September 2011. On receipt the laboratories were directed to store the seed in moisture-proof containers or bags at low temperature (4 to 10°C). The samples were coded independently of the test participants. After preliminary work, standardised methods were agreed and the laboratory tests reported were concluded in January and February 2012.

Single counts of RE

Two replicates of 100 seeds were tested for germination following the ISTA Rules (ISTA, 2012). In each of the three laboratories, seeds were placed onto blue pleated paper in plastic boxes using their routine germination method. The seeds were held at 20°C in 12 / 12 hour light / dark cycles in two laboratories and in continuous light in the third. Counts of radicle emergence (RE) were made after 30 hours and normal seedlings (ISTA, 2012) were evaluated at seven days. Radicle emergence was defined as the appearance of a radicle after breaking through the seed coat. Seeds in which the seed coat was split, but no radicle had emerged, were not included.

Previous work by these participating laboratories (unreported data) had identified that the above method resulted in repeatable results'

Statistical analysis

Possible outliers were assessed using side by side boxplots (figure 1) and by computing tolerances for germination test replicates. The performance of the method was assessed through the estimation of repeatability and reproducibility parameters in the context of binomial data. The Generalised Linear Model was fitted with the SAS GLIMMIX procedure

Results

Radicle emergence counts after 30h at 20°C revealed clear differences between the seed lots in all laboratories (table 2)

<u>Table 2:</u> Radicle emergence (%) after germination 30h at 20°C of nine lots of oilseed rape in each of two 100-seed replicates in three laboratories. Seed lots are placed in order of their overall mean RE test results, with the highest test result placed at the top of the table.

Data in columns having the different upper case letters are significantly different (p< 0.05).

| Lot | Lab 1 | | Lab 2 | | Lab 3 | | | Overall lot | | |
|---------------------|-------|-----|-----------------|-----|-------|-----------------|-----|-------------|-----------------|------------------|
| | Rep | Rep | Mean | Rep | Rep | Mean | Rep | Rep | Mean | mean |
| | 1 | 2 | | 1 | 2 | | 1 | 2 | | |
| 1 | 97 | 93 | 95 | 97 | 90 | 94 | 97 | 97 | 97 | 95 ^A |
| 2 | 96 | 92 | 94 | 93 | 99 | 96 | 94 | 91 | 91 | 94 ^A |
| 3 | 93 | 88 | 91 | 83 | 86 | 85 | 92 | 90 | 91 | 89 ^B |
| 4 | 88 | 91 | 90 | 83 | 84 | 84 | 78 | 75 | 77 | 83 ^C |
| 5 | 85 | 87 | 86 | 87 | 88 | 88 | 83 | 90 | 87 | 87 ^{BC} |
| 6 | 76 | 76 | 76 | 74 | 63 | 69 | 66 | 61 | 64 | 70 ^D |
| 7 | 77 | 74 | 76 | 70 | 67 | 69 | 70 | 59 | 65 | 70 ^D |
| 8 | 59 | 59 | 59 | 53 | 46 | 50 | 55 | 48 | 52 | 53 ^E |
| 9 | 58 | 56 | 57 | 50 | 50 | 50 | 47 | 45 | 46 | 51 ^E |
| Overall lab mean | | | 80 ^A | | | 76 ^в | | | 74 ^B | |

Data exploration with side-by-side boxplots

There was a wide range in the mean radicle emergence assessments from the nine seed lots (figure 1A), but there were no outliers. There were small differences between the median values obtained by the three laboratories (figure 1B). No seed lot x lab interaction was exhibited in the side-by side boxplots (figure 1C)



Figure 1: Comparisons of the mean RE data from seed lots (A), laboratories (B) and seed lot x laboratory (C)

Data checking

All the data was in tolerance (table 3)

<u>Table 3:</u> Mean germinations and tolerance ranges (2 replicates x 100 seeds) for nine lots of oilseed rape tested in three laboratories.

| Seed lot | | Lab 1 | Lab 2 | Lab 3 |
|----------|-------------------------|-------|-------|-------|
| 1 | Maximum tolerance range | 7 | 8 | 6 |
| | Observed range | 4 | 7 | 0 |
| | Mean | | 93.5 | 97 |
| | | | | |
| 2 | Maximum tolerance range | 8 | 6 | 9 |
| | Observed range | 4 | 6 | 3 |
| | Mean | 94 | 96 | 92.5 |
| | | | | |
| 3 | Maximum tolerance range | 9 | 11 | 9 |
| | Observed range | 5 | 3 | 2 |
| | Mean | 90.5 | 84.5 | 91 |
| | | | | |
| 4 | Maximum tolerance range | 9 | 11 | 13 |
| | Observed range | 3 | 1 | 3 |
| | Mean | 89.5 | 83.5 | 76.5 |
| | | | | |
| 5 | Maximum tolerance range | 11 | 10 | 11 |
| | Observed range | 2 | 1 | 7 |
| | Mean | 86 | 87.5 | 86.5 |
| | | | | |
| 6 | Maximum tolerance range | 13 | 15 | 15 |
| | Observed range | 0 | 11 | 5 |
| | Mean | 76 | 68.5 | 63.5 |
| | | | | |
| 7 | Maximum tolerance range | 13 | 15 | 15 |
| | Observed range | 3 | 3 | 11 |
| | Mean | 75.5 | 68.5 | 64.5 |
| | | | | |
| 8 | Maximum tolerance range | 15 | 16 | 16 |
| | Observed range | 0 | 7 | 7 |
| | Mean | | 49.5 | 51.5 |
| | 1 | | | |
| 9 | Maximum tolerance range | 15 | 16 | 15 |
| | Observed range | 2 | 0 | 2 |
| | Mean | 57 | 50 | 46 |

Repeatability

Let:

- . I be the total number of samples
- . J be the total number of labs
- . K_{ij} be the number of reps of *m* seeds in sample *i* and lab *j*
- . p_{iik} be the percentage for sample *i*, lab *j* and rep *k*

The repeatability standard-deviation is computed as:

$$S_r = \sqrt{f_r^2 \frac{\overline{p}_{\dots} \left(100 - \overline{p}_{\dots}\right)}{m}}$$

where:

. \overline{p} is the overall average percentage.

. f_{r}^{2} is an estimate of the dispersion parameter:

$$f_r^2 = \frac{1}{IJ} \sum_{i,j} \frac{var_obs_{ij}}{var_bin_{ij}}$$

where:

$$var_obs_{ij} = \frac{1}{K_{ij} - 1} \sum_{k} (p_{ijk} - \overline{p}_{ij.})^2$$
 and $var_bin_{ij} = \frac{\overline{p}_{ij.} (100 - \overline{p}_{ij.})}{m}$ with $\overline{p}_{ij.}$ being the average percentage is seemala, i and lab.

in sample *i* and lab *j*

If $f_r^2 > 1$ one speaks of overdispersion because the data have larger variance than expected under the assumption of a binomial distribution.

Results:

| $\overline{P}_{}$ | S _r | f _r |
|-------------------|----------------|----------------|
| 76.78 | 4.16 | 0.98 |

<u>There is no evidence for overdispersion as f_r^2 is not > 1.</u>

Reproducibility

The reproducibility standard-deviation is computed as:

$$S_{R} = \sqrt{\frac{1}{I} \sum_{i} \sum_{j} \frac{\left(\overline{p}_{ij.} - \overline{p}_{i..}\right)^{2}}{J - 1}}$$

where:

$$i = 1, 2, ..., I$$
 $j = 1, 2, ..., J$

. \overline{p}_{ii} is the percentage in sample *i* and lab *j*

$$. \ \overline{p}_{i..} = \frac{\sum_{j} \overline{p}_{ij.}}{J}$$

Assuming a binomial distribution, the variance of \overline{p}_{ii} is:

$$\operatorname{Var}\left(\overline{p}_{ij.}\right) = \frac{\overline{p}_{ij.}\left(100 - \overline{p}_{ij.}\right)}{n}$$

where n = 200. We then compute the following quantity to characterize overdispersion when Lab and Sample by Lab variations are considered:

$$f_R^2 = \frac{n S_R^2}{\overline{p}_{...} (100 - \overline{p}_{...})} \text{ where } \overline{p}_{...} = \frac{\sum_{i,j} \overline{p}_{ij.}}{IJ}$$

The square root of f_R^2 is then compared to the f value defined by Miles (1963) in equation AG4 and which is used to develop ISTA tolerance tables for comparing germination results from different labs. Results:

| \overline{p}_{\dots} | S _R | f _R | f |
|------------------------|----------------|----------------|------|
| 76.78 | 4.58 | 1.53 | 1.74 |

<u>The f_R value is smaller than the f value, therefore there is no evidence of overdispersion.</u>

Generalized Linear Model to test whether lab, lot, or lab x lot effects are significant or not.

*Radicle_emergence_counts*_{*ijk*} ~ Binomial(100, π_{ijk})

$$logit(\pi_{ijk}) = log\left(\frac{\pi_{ijk}}{1 - \pi_{ijk}}\right)$$
$$= \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij}$$

where:

- . μ is the general effect.
- . α_i is the fixed effect of sample *i*
- . β_j is the fixed effect of lab j
- . $(\alpha\beta)_{ij}$ is the interaction effect between sample *i* and lab *j*

This model has been fitted with the SAS GLIMMIX procedure.

Results:

Table 4: Results of the generalised Linear Model

| Type III Tests of Fixed Effects | | | | | | |
|---------------------------------|--------|--------|---------|--------|--|--|
| Effect | Num DF | Den DF | F Value | Pr > F | | |
| Seed lot | 8 | 27 | 76.40 | <.0001 | | |
| Lab | 2 | 27 | 4.07 | 0.0285 | | |
| Seed lot x Lab | 16 | 27 | 1.31 | 0.2579 | | |

The seed lot and the lab main effects are significant (table 4); significant differences between lot means and lab means are exhibited in table 2; the seed lot x lab interaction is not significant.

Discussion

The significant differences in the radicle emergence of seed lots, revealed here across three laboratories have previously been shown to predict both the rate of, and final, emergence of the lots in the field (Matthews *et al.* 2012) and therefore predict differences in seed vigour of the lots. The standard germination (%) was also significantly correlated with the rate of, and final, emergence. However, the difference between the lowest and highest standard germination was small, only 9% when all lots were considered, and only 5% different for eight of the nine lots. Clear differences in vigour between the lots would therefore not be identified. In contrast, the range of RE test results was from 51 to 95%, clearly distinguishing differences between the lots. Thus, lots E and F were clearly identified as having higher vigour than lots I and H even though they all had the same standard germination, 98% (Table 1). In addition the repeatability and reproducibility of the data supports the proposal that the radicle emergence test can be used routinely to reveal differences in seed vigour of oilseed rape

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