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Detection of *Botrytis allii* in Onion Seed by Plating Assay

Validation report, November 2023

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ISHI VALIDATION REPORTS

This ISHI validation study has been conducted to determine the fitness of the described method for its intended purpose according to the ISHI Guidelines for the Validation of Seed Health Methods[1] and followed by an independent review of its outcome.

DISCLAIMER

ISF cannot guarantee that laboratories following the protocol described herewith will obtain similar results. Many factors, such as staff skills, laboratory equipment and conditions, reagents and sampling methods can influence the results. Consequently, in case of any litigation ISF will not accept any liability on the use of these tests.

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[1]Available at: https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/

Contents

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SUMMARY

Botrytis allii is the causal organism of neck rot in onion (*Allium cepa*) and is transmitted by seeds. Seed health testing has an important role in disease control, but there have been no international standard methods to date for the detection of *B. allii* in onion seeds. This report demonstrates the validity of the method for testing onion seeds for the presence or absence of *B. allii*. Extensive research, validation and comparative testing were carried out from 2008 to 2017. As a general principle, seeds are surface sterilised with sodium hypochlorite and then placed onto a half strength lactic acid agar. In 2015, reproducibility of the method was validated in a comparative test performed involving eight laboratories using healthy, medium and highly infected seeds, according to ISHI validation guidelines available at the time. Statistical analysis showed that the infection rates cannot be reproduced quantitatively in a sufficiently reliable manner, and that quantitative variations occur among laboratories. However, the presence of *B. allii* can be identified with high reproducibility in each laboratory for all infection levels qualitatively. The comparative test also revealed that diagnostic performance of the method was reliable. Therefore, the method is deemed fit-for-purpose for testing onion seeds for the presence of *B. allii*.

1. INTRODUCTION

Botrytis allii is an important seed-borne fungal pathogen and the causal organism of neck rot in onion (*Allium cepa*) (Koike *et al*., 2007; Lacy and Lorbeer, 1995; Neergaard, 1977). Plant infections are mostly asymptomatic (Du Toit *et al*., 2004) and become primarily observed during onion storage (Maude and Persley, 1977a; Maude and Persley, 1977b). The use of healthy seed is an important aspect to be considered as part of a successful disease control program (Koike *et al*., 2007). To date, no ISHI method has yet been published for the detection of *B. allii*. However, within the ISHI participants, the need for a method to be developed that can act as the industry standard was expressed.

In this report, results from multiple experiments and a comparative test (CT) carried out in 2015, as well as additional validation data are presented. The method is based on the developmental work of seed companies and information described in the literature reviewed by Chilvers and du Toit (2006) and Metcalf (1998). The protocol presented in the current test plan is based on Maude (1996). The method encompasses plating seed onto a semi-selective medium. This semi-selective medium, composed of half strength lactic acid potato dextrose agar (L-PDA) that contains pentachloronitrobenzene (PCNB) for saprophytic control, has been evaluated through CTs organized by ISHI in 2009 (DiNitto and Politikou, 2010) and onwards. Seeds are surface sterilised to reduce surface contaminants allowing for the detection of an internal infection of the target pathogen by its typical morphological characteristic exhibited on the semi-selective medium. Seeds are visually evaluated after a 12-day incubation period.

The protocol was developed for the detection of *B. allii* in untreated or physically disinfected seed lots *i.e*. hot water, heat. The method is compatible with NaOCl surface sterilisation, which is part of the method, but application of chemical sanitation of seeds and follow up testing must be validated by the user. This method is to be carried out by laboratories experienced with testing and identifying seed for fungal seed-borne pathogens, and equipped with the necessary equipment and supplies to conform to the requirements of the method.

2. OBJECTIVES

In this report, data demonstrating the validity of the method for testing onion seeds for the presence or absence of *B. allii* based on the validation criteria described in ISHI Validation Guidelines, Version 3 (ISHI, 2020) is presented. Data was collected during experiments and routine seed health testing in the period 2008-2017. Data from a comparative test in 2015 is included in this report.

3. METHOD VALIDATION

3.1. Analytical specificity

Definition ISHI guidelines: *The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity).*

The analytical specificity requirements will be met when *B. allii* strains from different geographical locations and onion varieties are detected and can be discriminated from non-target fungi.

Experimental approach

The half strength L-PDA medium amended with PCNB used to detect *B. allii* on seeds is a semi-selective medium (Annex A). It has been in use in the seed industry for more than 10 years (source: Enza Zaden BV, pers. comm. G. Hiddink, 2018).

Botrytis allii appears as grey fast-growing colonies that sporulate profusely. On half strength L-PDA semi-selective medium, *B. allii* develops tall slender branched conidiophores bearing clusters of single celled ovoid conidia 5-6 × 7-11 µm (Figure 1). This particular morphological characteristics can be used to distinguish *B. allii* from other fungi, capable of growing on this medium, by a trained technician. The medium itself does not exclude the growth of all other fungi.

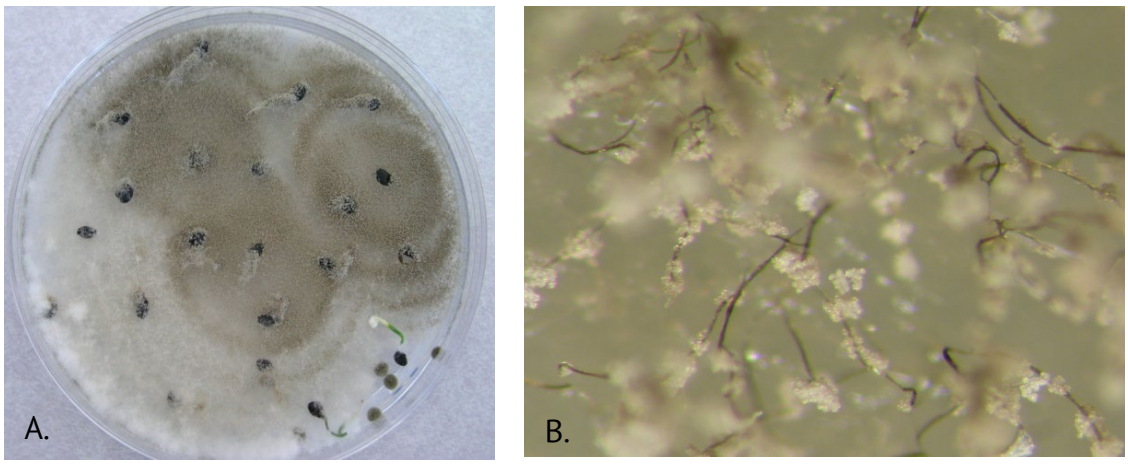


Figure 1. A. Onion seeds infected with *B. allii* grown on half strength L-PDA, 12 days after plating. **B.** Typical *B. allii* conidiophores at 25× magnification.

The semi-selective medium half strength L-PDA with PCNB was selected through a comparative test organized by ISHI in 2009 (Presentation ISHI, DiNitto, 2008). Different batches of seed were tested for *B. allii* growth on L-PDA. Analytical specificity was also analysed in the 2015 CT (Annex A). Eight independent laboratories participated in this comparative test, of which seven laboratories were growing their own positive controls that likely originated from different

locations, representing global diversity of *B. allii*. The detected saprophytes were also recorded in this 2015 CT, which demonstrates the 'exclusivity' of the method.

Results

From seven out of 13 tested seed lots from different geographical locations and consisting of different onion varieties, *B. allii* was able to grow on L-PDA after surface sterilisation (DiNitto, 2008). The seven positive controls used on the 2015 CT, that likely originated from different locations and represented global diversity of *B. allii*, were appropriately growing on L-PDA.

Regarding exclusivity, at least, the following fungi have been detected on seeds plated onto this medium: *Aspergillus niger, B. allii, Botrytis cinerea, Botrytis squamosa, Mucor spp., Penicillium spp., Rhizopus spp.,* and several other unidentified fungi (data not shown). These fungi can be clearly distinguished visually from the target *B. allii* based on their morphological appearance (Figure 2A and 2B). In the 2015 CT, *Botrytis cinerea* and species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum, Fusarium, Penicillium*, *Rhizopus*, *Stemphylium* and *Verticillium* were detected without interfering with the identification of *B. allii* (Annex B).

It is recommended to include controls of both *B. cinerea* and *B. squamosa* on separate L-PDA plates as reference to distinguish between the three fungi. *Botrytis squamosa* can be easily distinguished as it does not produce conidia easily (Steentjes *et al.*, 2021) and produces much larger conidia when sporulating (Chilvers and du Toit, 2006).

Compared to *B. cinerea*, *B. allii* produces more abundant and smaller conidiophores (1 mm), while *B. cinerea* has dematiaceous and branched conidiophores (up to 2 mm long) (Chilvers and du Toit, 2006). *Botrytis cinerea* shows a faster growth compared to *B. allii*. Additional descriptive photographs can be found in Chilvers and du Toit (2006).

These data are supported by a review of 131 *B. allii* isolates obtained from seed of 38 different onion varieties grown in four different geographical locations (Table 1) around the globe (Enza Zaden, pers comm. G. Hiddink, 2018).

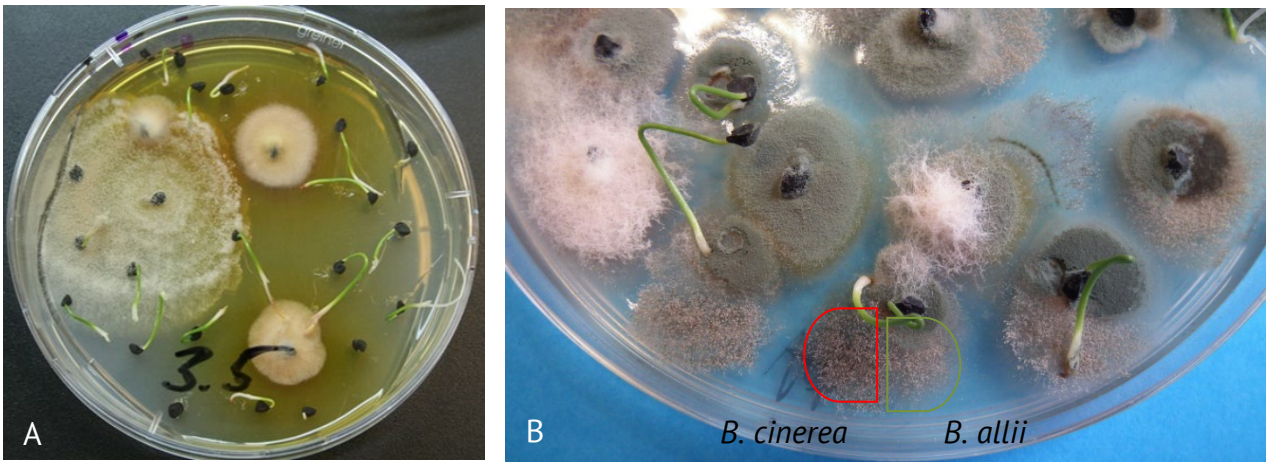


Figure 2. A. Onion seeds with *B. allii* and two saprophytes grown on half strength L-PDA, and **B.** Onion seeds with *B. allii (circled in green)* and *B. cinerea (circled in red)*.

Conclusion

Combined together, these data show that the method is able to detect *B. allii* strains from different geographical locations and onion varieties, which can be discriminated from other non-target fungi based on a morphological observation. Analytical specificity of this method was deemed acceptable.

3.2. Analytical sensitivity

Definition ISHI guidelines: *Smallest amount of the target pathogen that can be detected i.e., the limit of detection (LOD).*

For fungal plating assays the sensitivity requirements will be met when one infected seed can be detected by the assay on one Petri dish. *Botrytis allii* can be present as spores or as mycelium on the seeds. Surface disinfection as used in the protocol will remove external fungal spores and reduce saprophytes but will not kill the deep-seated *B. allii* mycelium. The number of 10 seeds per Petri dish is recommended as it allows for readability of the plate at both 7 and 12 days and for preventing saprophytic overgrowth of *B. allii* colonies that could mask its presence. Presence of mycelium in one seed will therefore result in the detection of *B. allii* with the proposed method.

The analytical sensitivity of the method is therefore deemed fit for purpose.

3.3. Selectivity

Definition ISHI guidelines: *The effect of different seed matrices on the ability of the method to detect target pathogen(s).*

The selectivity requirements will be met when *B. allii* can be detected from onion seeds with different backgrounds and geographical origins.

Experimental approach

Botrytis allii has been detected and identified on seeds with different backgrounds and produced in different geographical origins (Table 1).

Results

From 2015 to 2017 131 *B. allii* infected seed lots were detected coming from 38 different onion varieties and onion types, including red and yellow, short, intermediate and long day onions (Table 1).

Table 1. Number of *B. allii* infected onion varieties per year and area. The number of varieties presented in the table can encompass multiple seed lots per variety.

Region	Production year		
	2015	2016	2017
Africa	1	3	9
Australasia	2	7	8
Europe	0	2	5
South America	1	4	14

Conclusion

The results clearly indicate that the method is able to detect *B. allii* from different geographical origins on different varieties and onion types. It is therefore concluded that the selectivity of the method is fit for purpose.

3.4. Repeatability

Definition ISHI guidelines: *Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single laboratory.*

The repeatability requirements will be met when the measure for this performance criteria, i.e., accordance within the analyses performed at different time points with minimal variations in a single laboratory, is >90%.

Experimental approach

Results obtained for the assessment of homogeneity, stability and CT, by GEVES only, were used to evaluate the repeatability of the method. See Annex A for the comparative test plan.

In the CT, five subsamples of 100 seeds for each seed lot, healthy, medium and highly infected, were tested in July 2015. For the evaluation of homogeneity, 10 subsamples of 100 seeds were tested by the organizer for each seed lot in June 2015, prior to sending the samples to participants. To determine the stability, three subsamples of 100 seeds per seed lot were tested in August 2015 (Table 2), once all participants started testing their samples.

Statistical analysis

Data was analysed according to Langton (Langton *et al*., 2002) at the qualitative level.

Results

Qualitative results of homogeneity and stability tests together with the CT results for the three seed lots used for the 2015 CT are shown in Table 2. These tests were performed by GEVES only. The raw CT data can be found in Annex B. The homogeneity and stability test results can be found in Annex C.

Table 2. Qualitative *B. allii* detection results for the homogeneity, comparative test and stability tests per seed lot tested by GEVES. Each subsample is composed of 100 seeds.

Infection level	Homogeneity test	Comparative test	Stability test
Healthy	10 negative/10	5 negative/5	3 negative/3
Medium	10 positive/10	5 positive/5	3 positive/3
High	10 positive/10	5 positive/5	3 positive/3

The homogeneity, CT and stability tests results of the healthy seed samples gave all a negative result, and of the infected seed samples all samples gave a positive result, which corresponds to 100% accordance by Langton analysis for all three infection levels.

Conclusion

The qualitative data of the stability, comparative test and the homogeneity test showed that the accordance for the healthy and infected seed samples is above the minimum requirement of 90%. Therefore, the repeatability of the method is deemed fit for purpose at the qualitative level.

3.5. Reproducibility

Definition ISHI guidelines: *Degree of similarity in results when the method is performed across laboratories with replicates of the same subsamples.*

The reproducibility requirements will be met when the Langton analysis (Langton *et al*., 2002) of the results of the 2015 CT shows a concordance >90%.

Experimental approach

Eight laboratories from five countries participated in the 2015 CT. The set of seed lots used in this CT included five samples each of healthy, medium and highly infected seeds.

Participants also received 400 seeds of a surface sterilised seed as a positive seed control. Samples were tested according to the protocol described in Annex A. A reference strain of *B. allii* from the participants' individual collection was also included and plated separately.

At 7 and 12 days post incubation, seeds were examined for the presence of *B. allii*, using a stereomicroscope with a 25 × magnification to observe for typical morphology of *B. allii*. Typical *B. allii* colonies appear as greyish fast-growing mycelium, which sporulates profusely (Figure 1A and 1B). Laboratories compared suspect colonies detected on seeds of tested subsamples against colonies identified on seeds of the positive control sample and their reference culture plates.

Data was recorded as the number of infected seeds per sample (quantitative data) and as the health status of the seed sample (qualitative data). Under normal conditions, *B. allii* incidence at 12 days is considered as the final result. However, growth of saprophytes, which are not all eliminated by surface sterilisation, can impede the growth of *B. allii* and hamper the scoring of *B. allii* on the plates leading to a lower scoring of *B. allii* after 12 than 7 days. Therefore, in case that *B. allii* presence at 7 days is higher than at 12 days, the results obtained 7 days should be considered as the final results.

Statistical analysis

To calculate the capability of laboratories to detect *B. allii* with the current method, the Langton's analysis was carried out for a qualitative data analysis (Langton *et al.*, 2002). Boot strap method was set at 'fixed' and a bootstrap value of 5,000 was used, in the Langton-Accordance and concordance tool from the ISTA Seed Health Toolbox.

Results

Laboratories counted the number of *B. allii* infected seeds and identified saprophytes present per plate in the tested samples. Laboratories were also asked to record the number of *B. allii* positive seeds in the positive control subsample. However, laboratory 2 did not test the seeds of the positive control subsample and laboratories 5 and 7 tested only 100 positive control seeds. The raw data of the CT are presented in Annex B.

Results of this analysis revealed that there is a 100% concordance between expected results and obtained results (Table 3). These values are above the requirements set for concordance (>90%). This analysis thus showed that at the qualitative level the method is reproducible among laboratories.

Quantitative results were also assessed and showed a variability between laboratories too high to be able to determine level of contaminations of seed lots (data not shown). It was thus concluded that a quantitative data analysis would not bring value to the method.

Conclusion

The data showed that the method is reproducible for the presence and absence of *B. allii* on onion seeds at a qualitative level. For the quantitative data, which has not been statistically analysed, too much variability among laboratories to determine an infection level in contaminated samples was detected.

Table 3. Number of seed lots tested positive for *B. allii* in the 2015 CT.

Laboratory	Healthy	Medium	High	Total samples tested
1	0/5	5/5	5/5	15
2	0/5	5/5	5/5	15
3	0/5	5/5	5/5	15
4	0/5	5/5	5/5	15
5	0/5	5/5	5/5	15
6	0/5	5/5	5/5	15
7	0/5	5/5	5/5	15
8	0/5	5/5	5/5	15
Expected value	0/5	5/5	5/5	
Deviation	0	0	0	

3.6. Diagnostic performance

Definition ISHI guidelines: *An evaluation of the ability of the method to discriminate between positive and negative seed lots*

The diagnostic performance requirements will be met when diagnostic sensitivity, and specificity are >95%.

Experimental approach

The diagnostic performance was calculated based on the qualitative CT results (positive *vs.* negative). Samples were processed by each laboratory following the protocol presented in Annex A. Diagnostic sensitivity, and specificity of the method, (diagnostic performance of the assay) were calculated according to the mathematical formulas presented in Table 4.

Table 4. Formulas used for calculating the diagnostic sensitivity (Dsen) and diagnostic specificity (Dspec).

	Expected result +	**Expected result -**
Obtained result +	True positive (TP)	False positive (FP)
Obtained result -	False negative (FN)	True negative (TN)
	DSen = TP / (TP+FN) × 100	Dspec = TN / (FP+TN) × 100

Results

All laboratories correctly identified the healthy and infected lots (Table 3). No differences were observed in the number of positive seed lots detected within the medium and highly infected seed lots (Table 5).

Table 5. Analysis of qualitative results obtained in the 2015 CT.

	Expected result +	**Expected result -**
Obtained result +	80 (TP)	0 (FP)
Obtained result -	0 (FN)	40 (TN)

Diagnostic sensitivity = TP / (TP+FN) x 100 = (80/(80+0)) × 100 = 100%

Diagnostic specificity = TN / (FP+TN) x 100 = (40/(0+40)) × 100 = 100%

Conclusion

Diagnostic sensitivity and diagnostic specificity are both at 100% and above the set threshold of 95% for qualitative test results. The test meets the requirements for diagnostic performance.

4. CONCLUSION

Results presented in the present report reveal that the method is fit-for-purpose at the qualitative level and can be used for testing onion seeds for the determination of *presence* or *absence* of *B. allii*. However, at the quantitative level, variability among laboratories was too high to determine the infection levels in a reproducible manner.

Therefore, the method presented in this report is deemed fit-for-purpose for testing in a qualitative manner, but not in quantitatively.

5. ACKNOWLEDGEMENTS

The authors would like to thank all the people who have contributed over the years to the development and validation of the *Botrytis allii* onion seed test protocol. In particular, they are grateful for the extensive contributions of L. diNitto (Nunhems, USA) and L. Poulitikou (ISF) for the organisation, reporting and analysis of the comparative tests. Also, they wish to acknowledge the contribution of F. Quadt for the statistical analysis of the 2015 comparative test.

6. REFERENCES

Chilvers, M. I. and du Toit, L. J. (2006). Detection and identification of *Botrytis* species associated with neck rot, scape blight, and umbel blight of onion. *Plant Health Progress*. DOI:10.1094/PHP-2006-1127-01-DG.

DiNitto, L. (2008). Presentation: Proposal for an ISHI approved method for the detection of *Botrytis aclada* on onion seed. ISHI, Tzuba, Israel.

Du Toit, L. J., Derie, M. L. and Pelter, G. Q. (2004). Prevalence of *Botrytis* spp. in onion seed crops in the Columbia Basin of Washington. *Plant Disease,* **88,** 1061-1068.

ISHI (2020). Guidelines for the Validation of Seed Health Tests. Version 3, November 2020. Available at: https://worldseed.org/our-work/seed-health/ishi-method-development-and-validation/.

Koike, S. T., Gladders, P, and Paulus, A. O. (2007). Vegetable Diseases, A color handbook. Academic Press, Cambridge, MA, USA.

Lacy, M. L. and Lorbeer, J. W. (1995). Botrytis neck rot. Pages 18-19 in: Compendium of Onion and Garlic Diseases. H.F.Schwartz and S. K. Mohan editors. American Phytopathological Society Press, St. Paul, MN, USA.

Langton, S. D., Chevennement, R., Nagelkerke, N. and Lombard, B. (2002). Analysing collaborative trials for qualitative microbiological methods: accordance and concordance. *International Journal of Food Microbiology*, **79**, 175-81.

Maude, R. B. and Persley, A. H. (1977a). Neck rot (*Botrytis allii*) of bulb onions. I. Seedborne infection and its relationship to the disease in the onion crop. *Annuals of Applied Biology*, **86**, 163-180.

Maude, R. B. and Persley, A. H. (1977b). Neck rot (*Botrytis allii*) of bulb onions. II. Seedborne infection in relationship to disease in store and the effect of seed treatment. *Annuals of Applied Biology*, **86**, 181-188.

Maude R. B. (1996). Seed-borne Diseases and Their Control: Principles & Practice. CAB International, Wallingford, UK. Page 189

Metcalf, D. (1998). *Botrytis* seed testing - what the results mean? *Onions Australia*, **15**, 33-34.

Neergaard, P. (1977). Seed Pathology. John Wiley & Sons, New York, USA.

Politikou, L. and DiNitto, L. (2010)T. ISHI-Veg Comparative Test for *Botrytis aclada* (*alli*) Detection on Onion Seeds.

Steentjes, M. B. F., Scholten, O. E. and Kan van, J. A. L. (2021). Peeling the onion: Towards a better understanding of *Botrytis* diseases of onion. *Phytopathology,* **111**, 464-473. https://doi.org/10.1094/PHYTO-06-20-0258-IA

6. ANNEXES

Annex A: Comparative Test plan - 2015

ISHI-ISTA test plan for the detection of *Botrytis allii* in Onion seed lots

March 2015

1. ORGANIZATION AND DESIGN

1.1. Test Organizer

Louie Di Nitto, Nunhems USA

1.2. Pathogen

Botrytis allii Munn (syn. *Botrytis aclada)*

1.3. Crop

Allium cepa L. (onion)

1.4. Participating laboratories and contact persons

Bactochem, Israel	Ludmila Guzev
Bejo Zaden B.V., The Netherlands	Margreet Asma
GEVES, France	Valérie Grimault
Hazera Genetics	Roni Sussman
Microlab (99) LTD., Israel	Isaac Assouline
Nunhems, USA	Louie Di Nitto
Sakata Seeds, USA	Philip Brown
Takii & Co., Ltd., Japan	Shintaro Kusano

2. INTRODUCTION AND SCOPE OF THE METHOD

2.1 Background

Botrytis allii (Ba) is an important seed-borne fungal pathogen and is the causal organism of neck rot in onion (*Allium cepa*) (Koike *et al*., 2007; Lacy and Lorbeer, 1995; Neergaard, 1977). Plant infections are mostly asymptomatic (Du Toit *et al*., 2004) and become primarily observed during onion storage (Maude and Persley, 1977a; Maude and Persley, 1977b). The use of healthy seed is an important aspect to be considered in a successful disease control program (Koike *et al*., 2007). No ISHI method has been published to date for the detection of *Botrytis allii*. In the present report, a method of plating seed on a semi-selective medium will be evaluated. Seeds will be surface sterilised to reduce surface contaminants allowing for the detection of an internal infection of the target pathogen by its typical characteristic exhibited on the semi-selective medium.

This method is based on the developmental work of seed companies and information described in the literature. The protocol presented in the current test plan is based on Maude (1996). The semi-selective medium half strength Lactic Acid PDA that contains PCNB for saprophytic control, has been evaluated through a comparative test organized by ISHI in 2009.

The protocol was originally developed for the detection of *Botrytis allii* in untreated seed or physically disinfected seed lots *i.e*., hot water, heat. This method is however not intended for the testing of chemically treated seed. This method is to be carried out by laboratories experienced with the testing of seed for fungal seed-borne pathogens and equipped with the needed equipment and supplies necessary to conform to the requirements of the method.

2.2 Sensitivity of the proposed method

In cases that result observation is hampered by the overgrowth of saprophytes, this test will need to be repeated after the seed lot is subjected to a physical treatment, such as hot water or heat. Seed surface-sterilisation is not required in such case. The suitability of the method has been tested on physically treated seed but has not been part of the validation.

2.3 Scope

The scope of this comparative test is to evaluate the proposed method for *Botrytis allii* recovery on half strength lactic acid potato dextrose agar semi-selective medium containing pentachloronitrobenzene (PCNB).

2.4 Objective

The objective of this comparative test is to provide an ISHI validated method for the detection of *Botrytis allii* in onion untreated seed lots.

3. STATISTICAL ANALYSIS

The data will be analysed with JMP®software and ISTA developed tools as appropriate.
The analysis as a minimum will cover the following:

- Homogeneity of results within and over laboratories using a modified homogeneity test based on a Chi-Square method
- Analysis Of Variance to test for differences between laboratories and possible level*laboratory interaction.

The above does not exclude other analyses if appropriate.

4. MATERIALS AND METHODS

4.1 Seed lots and seed subsamples

In this comparative test, two naturally infected seed lots, each with a different infection level and one pathogen-free seed lot will be used. All three seed lots will be untreated. The seed lots will be selected based on their infection level by GEVES-SNES laboratory.

Each participating laboratory will test the following number of subsamples per lot:

1. One highly infected seed lot (five subsamples of 100 seeds each)
2. One medium infected seed lot (five subsamples of 100 seeds each)
3. One pathogen free (healthy) seed lot (five subsamples of 100 seeds each)

All subsamples will be coded randomly and their correspondence to seed lots will be known only to the test coordinator.

Following the ISTA ”Guidelines for organizing and analysing results of Proficiency tests (PT) and interlaboratory tests for validation of methods (CT)” (ISTA, 2012), the GEVES-SNES laboratory will prepare the subsamples and run the homogeneity and the stability tests.

The homogeneity test will be performed just before shipping the subsamples to participants on 10 untreated and on 10 surface sterilised subsamples of each lot.

The stability test will be performed just after the deadline to complete the test on three subsamples of untreated and on three surface sterilised subsamples of each seed lot.

4.2 Positive controls

4.2.1 Positive seed sample

One sample of 400 seeds from the high contamination seed lot will be surface sterilised by the GEVES-SNES laboratory and distributed to each participant. Seeds of this sample will be plated on semi-selective medium as other tested subsamples.

4.2.2. Reference culture

Each participating laboratory will use its own *B. allii* reference strain on separate plates of the semi-selective medium as a reference culture.

4.3 Materials needed to perform the test

- *B. allii* reference strain
- Container for sterilizing seed in NaOCl
- NaOCl (1% (v/v) active ingredient)
- 0.2 µm pore size filter
- Sterilised forceps
- Autoclaved blotter papers for the drying of seeds
- Laminar flow hood
- Distilled or de-ionized H_2O
- Chemicals needed for medium preparation
- Magnetic stirrer
- Autoclave
- Suitable equipment to cool down medium to 50 °C before adding PCNB
- Incubator capable of operating at 25 °C, with near ultraviolet (NUV) light Stereo microscope capable of magnifying at ×25

4.4. Medium preparation

In this comparative test, half strength lactic acid PDA semi-selective medium containing PCNB will be used to plate seed subsamples and controls. The minimum number of plates of approximately 90 mm size for a laboratory to prepare is:

- 190 plates for plating the tested subsamples and the positive control seed sample,

- 2 plates for the reference culture.

This quantity corresponds to approximately 3.84 L of medium. However, laboratories should consider preparing a higher number of plates to ensure the minimum required number.

Ingredients for half strength lactic acid PDA medium preparation:

Potato Dextrose Agar (*e.g.* Sigma or equivalent. *Please report brand used*)	19.5 g
Agar (*Please report brand used)*	5.0 g
Distilled H_2O	up to 1000 mL
Lactic acid (85%)	1.4 mL
PCNB	2 mL stock solution PCNB pentachloronitrobenzene (Aldrich P220-5, 10, or 100). (Note: Stock solution is prepared by dissolving 10 mg PCNB in 1 mL methanol. Autoclaving is not necessary).

- In an appropriate flask add PDA and agar. Fill up the flask to 1000 mL with distilled H_2O and dissolve with a magnetic stirrer.
- Seal the flask and sterilize by autoclaving at 121 °C,15 psi for 30 min. Cool down to 50 °C.
- Add 1.4 mL of filter sterilised (0.2 µm pore size) lactic acid to the autoclaved agar and PDA. Continue by adding 2 mL of PCNB stock solution.
- Mix the medium with a magnetic stirrer and pour approximately 20 mL into each plate. Pour from 1 L medium approximately 50 plates.
- If possible, leave plates at room temperature for 72 hours to allow for excess moisture to evaporate and check for contamination before use.
- Store prepared plates inverted at 4-7 °C and use within 4 weeks to ensure activity of PCNB.

4.5 Seed sterilisation and plating

- Place each onion seed subsample (codes 1-15) in a labeled perforated sterilisation container (e.g., an enclosed tea strainer made of woven screen).
- Place the container for 2 min in NaOCl solution (1% (v/v) active ingredient, check concentration with test strips: e.g., activate or equivalent (10.000 ppm)). Shake a few times and rinse in sterile H_2O for 30 sec. (For example, swirling the strainer in a 1 L beaker of sterile water under a laminar flow hood).
- While still under the laminar flow hood, dry seeds on sterile labeled blotter papers. Use sterile forceps to place aseptically 10 seeds per medium plate and space them evenly.
- Label plates with the corresponding code, plate number and date.
- Continue by plating seeds of the positive control sample. DO NOT surface-sterilize seeds of this sample as this has already been done by GEVES-SNES laboratory.
- Transfer a pure culture of Botrytis allii onto two plates of the same semi selective medium.
- Incubate all plates to 20-23 °C with 12h NUV light/12h dark until the end of the incubation period.

4.6 Evaluation of plating

- Count the number of suspect *B. allii* colonies on each plate of tested subsamples and positive control sample at 7 and 12 days. In case of highly developed saprophytic growth in plates count the number of suspect *B. allii* colonies at 7 days and use this as the final reading.

- Use a stereomicroscope with × 25 magnification to identify suspect colonies. *B. allii* colonies appear as grey fast growing and sporulate profusely (Picture 1, 2A and 2B). *B. allii* on half strength lactic acid PDA semi-selective medium, develops tall slender branched conidiophores bearing clusters of single celled ovoid conidia 5-6 × 7-11 µm.

    a. It is recommended to include controls for both *B. cinerea* and *B. squamosa* as reference for discrimination. *B. squamosa* can easily be distinguished as it does not form conidia easily (Steentjes *et al*., 2021) and shows much larger conidia when sporulating (Chilvers and du Toit, 2006)

    b. Compared to *B. cinerea*, *B. allii* has abundant and smaller conidiophores (1mm), while *B. cinerea* has dematiaceous and branched conidiophores, up to 2 mm in length (Chilvers and du Toit, 2006). *B. cinerea* shows a faster growth compared to *B. allii*. For additional descriptive photographs see Chilvers and du Toit (2006).

- Compare suspect colonies detected in plates of tested subsamples to colonies identified in plates of the positive control sample and reference culture.
- Ensure the reference culture has grown properly onto the semi-selective medium.
- Compare suspects to the reference culture.
- If there are no suspect colonies on the plate record number as “zero”.
- At 7 and 12 days of incubation, identify and count the number of developed saprophytes and other fungi on each plate.

NOTE: Under normal conditions, *B. allii* incidence at 12 days is considered as the final result. However, growth of saprophytes, which are not all eliminated by surface sterilisation, can impede the growth of *B. allii* and hamper the scoring of *B. allii* on the plates leading to a lower scoring of *B. allii* after 12 than 7 days. Therefore, in case that *B. allii* presence at 7 days is higher than at 12 days, the results obtained 7 days should be considered as the final results.

- Fill in the data record sheets provided with the test plan for each seed subsample and observation date. For the reference culture, indicate in the “Comments/Observations” column if the fungus has developed a proper growth pattern onto the semi-selective medium.

5. PARTICIPANT SCHEDULE AND INSTRUCTIONS

5.1 General schedule

- Receive comparative test program
- Accept obligations in participating in this comparative test
- Receive the seed sub-samples
- Secure all ingredients, equipment needed
- Perform the test
- Send results and comments to technical coordinator (Liana Politikou, email: liana.politikou@ufs-asso.com).

5.2 Expected time of work for each participating laboratory

The time needed to perform the test depends on the experience of participating laboratories. An estimation is given in the table below.

Time needed for 16 seed sub-samples

Day	Activity	Time needed
0	Preparation of medium, NaOCl solution, other materials	3 hours
1	Seed plating on medium	2 hours
7, 12	Evaluation of plates Recording of results on data record sheet	1 hour each evaluation day 20 min each evaluation day

5.3 Critical points

- Storage of seed samples at 5-7 °C.
- Good quality of medium ingredients for medium preparation.
- Cold storage of medium before use.
- Elimination of cross contamination during seed plating: materials of use should be sterilised with 70% (v/v) ethanol and flamed between seed subsamples.
- Utilization of aseptic techniques for all aspects of the procedure.
- Proper evaluation of plates for validation of a negative result.

5.4 Safety precautions

Principles of Good Laboratory Practice and aseptic technique should be followed during the whole testing procedure. Disposal of waste materials in an appropriate way and in accordance with local health, environment and safety regulations is considered essential. Handle all chemicals according to required country health and safety risk assessments. Special care should be taken during the preparation of PCNB stock solution since it has been characterized carcinogenic and mutagenic, and under a fume hood when dissolving with methanol.

6. References

Du Toit, L. J., Derie, M. L., and Pelter, G. Q. (2004). Prevalence of *Botrytis* spp. in onion seed crops in the Columbia Basin of Washington. *Plant Disease,* **88**, 1061-1068.

Koike, S. T., Gladders, P. and Paulus, A. O. (2007). *Vegetable Diseases, A color handbook*. Academic Press, USA.

Lacy, M. L. and Lorbeer, J. W. (1995). Botrytis neck rot. Pages 18-19 in: *Compendium of Onion and Garlic Diseases*. H.F.Schwartz and S. K. Mohan editors. American Phytopathological Society Press, St. Paul, MN, USA.

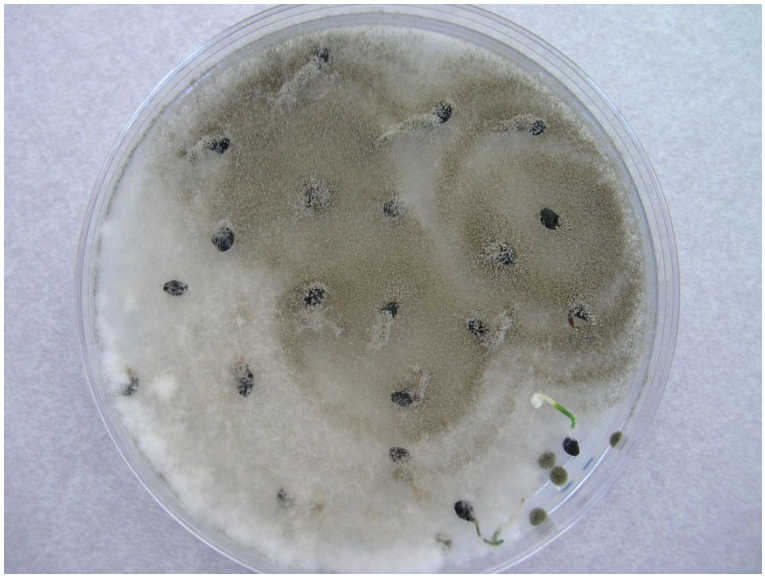
Maude, R. B., and Persley, A. H. (1977a). Neck rot (*Botrytis allii*) of bulb onions. I. Seedborne infection and its relationship to the disease in the onion crop. *Annuals of Applied Biology*, **86**, 163-180.

Maude, R. B. and Persley, A. H. (1977b). Neck rot (*Botrytis allii*) of bulb onions. II. Seedborne infection in relationship to disease in store and the effect of seed treatment. *Annuals of Applied Biology*, **86**, 181-188.

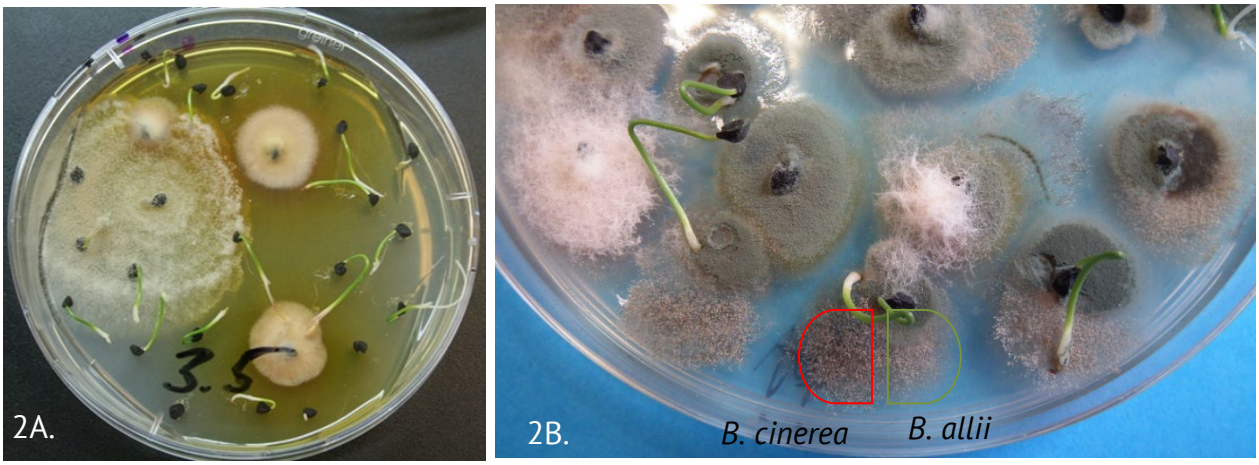
Maude, R. B. (1996). Seedborne Diseases and Their Control: Principles & Practice. CAB International, Wallingford, UK. Page 189.

Neergaard, P. (1977). Seed Pathology. John Wiley & Sons, New York.

Appendix I



Picture 1. Onion seeds with *Botrytis allii* fungus as viewed on half strength Lactic Acid Potato Dextrose Agar medium.



Picture 2. A. Onion seeds with *B. allii* and two saprophytes grown on half strength L-PDA, and **B.** Onion seeds with *B. allii (circled in green)* and *B. cinerea (circled in red)*. More *Botrytis* species are in more detail described in for example Chilvers and du Toit (2006).

Annex B: Raw Comparative Test data

See excel document: 210708 Raw CT data

210708 Botrytis raw data file overview.xlsx

Annex C: Homogeneity and stability results.

Table C.1. Homogeneity and stability test results per seed lot. For each seed lot ten subsamples were tested for homogeneity and three subsamples for stability. Each subsample is composed of 100 seeds. In the table is indicated the number of infected seeds out of 100 tested seeds. A green cell indicates a negative result, a red cell indicates a positive result.

Infection level	Homogeneity test results		Stability test results	
	7 days	12 days	7 days	12 days
Healthy	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0		
	0	0		
	0	0		
	0	0		
	0	0		
	0	0		
	0	0		
Medium	25	27	17	18
	17	18	22	22
	45	45	14	14
	28	28		
	19	20		
	10	15		
	24	24		
	11	12		
	17	17		
	9	9		
High	27	31	16	17
	28	31	25	25
	32	33	28	28
	23	24		
	30	32		
	32	32		
	19	22		
	27	28		
	24	25		
	23	27		
	27	31		