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Efforts towards a harmonized early blight detection method, results of the first *Alternaria* ring test

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SUMMARY
Early blight is becoming an increasing problem in the potato cultivation. The aim of this research was to establish which fungi (*Alternaria* species) could be isolated from lesions in potato leaves collected in commercial fields and field experiments. By carrying out a ring test we wanted to establish whether different isolation procedures by the involved laboratories gave comparable results and thus to come to suggestions for a common EuroBlight isolation protocol for Alternaria spp.. Recovery rates of *Alternaria* spp. were high and seemed consistent for the three laboratories involved. Recommendations for an early blight isolation protocol are given.

KEYWORDS
Alternaria solani, Alternaria alternata, isolation, determination, Early Blight, potato, *Solanum tuberosum*

INTRODUCTION

Early blight is becoming an increasing problem in the potato cultivation. Within Europe there is no consensus on the causal agent of early blight. *Alternaria solani* and *A. alternata* are named the most often, although also other *Alternaria* spp. seems to be associated with early blight. Furthermore, often lesions on potato leaves are found from which no pathogen can be isolated. Several research groups collect infected potato leaves and all have their own method for isolation and determination of the fungi present in and on the potato leaves. The aim of this research was to establish which fungi (Alternaria species) could be isolated from lesions in potato leaves collected in commercial fields and field experiments. Furthermore we wanted to establish whether different isolation procedures by the involved laboratories gave comparable results and thus to come to suggestions for a common EuroBlight isolation protocol for *Alternaria* spp. Therefore potato leaf samples with lesions were taken and sent to the laboratories in München, Wijster and Wageningen.

MATERIAL AND METHODS

Potato leaves samples were taken from 13 locations and 5 countries (Table 1). Sub samples from the same location were made and sent to each of the three laboratories. The potato leaves were put between tissue paper which was put in an envelope and sent by mail. When available, information on spray schedule, cultivar and early blight severity was included.
Upon arrival each laboratory made the assessment according to their own protocol. From each sample at least 10 lesions were used to determine the causal agent.

Isolation and determination at TUM
Lesions from infected leaves were cut to a size of 0.5 to 1 cm. The pieces were surface sterilized in a 5% NaOCl solution for 1 minute followed by washing in sterile water. SN (slight nutritious) medium was used for production of spores. The samples were incubated at 20°C under UV-light with a 12/12h photoperiod during 3 to 6 days. Sporulation was checked under a light microscope and the *Alternaria* species found was established. The method can be found on the EuroBlight website http://euroblight.net/alternaria/protocols/

Isolation and determination at HLB
Lesions from infected leaves were cut. The pieces were not surface sterilized. The necrotic tissue was transferred to water agar containing 50 µg/ml streptomycin. The samples were incubated at 20°C with 16 h photoperiod during 3 to 8 days. No UV-light was used. Sporulation was checked under a light microscope and the *Alternaria* species found was established after three and seven days of incubation. A second assessment was made to check the first one.

Isolation and determination at Wageningen-UR
Lesions from infected leaves were cut to a size of 1 cm^2. The pieces were not surface sterilized. Water agar (1.5%) with ampicillin (200 mg/l) was used. Imprints of both sides of the lesions were made on the agar. Then the potato leaf cuttings with lesions were put in an upright position in the water agar. The samples were incubated at 15°C with 16 h photoperiod during two weeks. No UV-light was used. Sporulation was checked under a light microscope and the *Alternaria* species found was established. Both the imprint and the lesions were checked for sporulation. If no spores were formed the incubation was prolonged for 1 more week (usually this was not necessary). A little bit of agar with mycelium of *Alternaria* spp. was transferred to a new Petri dish with water agar to purify the sample. This was also a second check on the results. The method can be found on the EuroBlight website http://euroblight.net/alternaria/protocols/

Table 1. *Origin of samples from potato leaves with Early Blight symptoms sent to the three laboratories*

Nr.	country	sent by	field location	variety	fungicide treatment	disease severity	sampling date
1	Germany	TUM	Freising	Agila	untreated	?	15.07.2014
2	Germany	TUM	Freising	Maxilla	untreated	?	15.07.2014
3	Germany	TUM	Straßmoos	unknown	Mancozeb	moderate	25.07.2014
4	Belgium	PCA	Kruishoutem	Bintje	untreated	5%	08.08.2014
5	The Netherlands	HLB	Wijster	Festien	untreated	10%	11.08.2014
6	Sweden	SLU	Nymö	Kuras	untreated	?	31.07.2014
7	Sweden	SLU	Nymö	Kardal	untreated	?	07.08.2014
8	Sweden	SLU	Nymö	Kuras	untreated	?	19.08.2014
9	Germany	TUM	Niedersunzing	unknown	treated	moderate to high	24.08.2014
10	Belgium	PCA	Lozer	Bintje	untreated	0.5-1%	28.08.2014
11	The Netherlands	WUR	Westmaas	unknown	treated	?	01.09.2014
12	The Netherlands	WUR	Valthermond	Aveka	untreated	20%	03.09.2014
13	The Netherlands	WUR	Lelystad	Bintje	untreated	75%	02.09.2014

RESULTS

Alternaria solani was found in each sample by all laboratories, except for sample number 1 where the pathogen was missing at HLB and WUR (Table 2). *Alternaria alternata* was missing more often especially at Wageningen UR. Also when *A. alternata* was found it was less abundant than *A. solani.*

Table 2. *Alternaria identification from 13 locations at three laboratories*

Nr.	TUM	WUR	HLB	TUM	WUR	HLB
1	A.s1	missing	missing	A.a.	A.a.	A.a
2	A.s +	A.s.	A.s.	A.a.	missing	A.a.++
3	A.s ++	A.s.	A.s ++	A.a.	missing	A.a.+
4	A.s +	A.s +	A.s ++	A.a.	A.a.	A.a.++
5	A.s +	A.s.	A.s +	A.a.	A.a.	A.a.
6	A.s ++	A.s +	A.s ++	A.a.	A.a.	A.a.
7	A.s ++	A.s.	A.s ++	A.a.+	missing	A.a.+
8	A.s +	A.s +	A.s ++	A.a.	A.a.	A.a.
9	A.s +	A.s.	A.s	A.a.	missing	missing
10	A.s.	A.s.	A.s ++	missing	missing	A.a.
11	A.s +	A.s +	A.s ++	A.a.	A.a.	A.a.+
12	A.s +	A.s +	A.s ++	A.a.	A.a.	A.a.
13	A.s ++	A.s +	A.s ++	A.a.	A.a.	A.a.+

[1]: no sign means that the pathogen was found but was just present, + = moderately present, ++ abundantly present

DISCUSSION

In the first sample both HLB and Wageningen UR could not find *A. solani* whereas the pathogen was found by TUM. However this sample was taken at 17 July but arrived in the laboratories in The Netherlands more than one month later. Usually the samples arrived within one or two weeks after sampling. In the 12 other cases *A. solani* was found readily, although more abundantly when assessed by TUM and HLB compared to Wageningen UR. This could be method related, laboratory related or scale related. Nevertheless recovery rates were high and seemed consistent.

A. alternata was found in most of the samples as well, although less abundant than *A. solani.* The fungus was missing in samples 9 (HLB, WUR), 10 (TUM, WUR) and 2, 3 and 7 (WUR). The main difference in the procedure between TUM and HLB on the one hand and WUR on the other is the incubation temperature which is 20°C and 15°C respectively. Stammler (2014), showed that *A. solani* was recovered more easily when isolated at 16°C (72%), whereas *A. alternata* was more easily isolated at 22°C (86%). This could explain that *A. alternata* was not readily found by WUR.

Although it is known that UV-light can stimulate sporulation of *Alternaria solani*, in this experiment it was not a prerequisite.

CONCLUSIONS

The experiment was set-up to find the best method for determining *Alternaria* spp. on infected leaves. The following recommendations are given for isolation and determination.

- Sample leaves (app. 10 leaves), preferably from plots, which were not treated with Alternaria specific fungicides.
- In the case that leaves will be shipped, make sure that leaves are dried. Therefore leaves should be put between paper towels.
- cut out of little infection sites (1 cm^2), one lesion per leaf.
- surface sterilization is NOT obligatory, however it helps to reduce fungal/bacterial contamination (5% NaOCl, 1 min).
- Medium: no particular recommendations (water agar 1.5% with antibiotica or SNA).
- Incubation of petri dishes in climate room, temperature (15) 20°C, photoperiod (16/8h) is requested.
- UV-light is NOT obligatory but will support sporulation.
- spore formation can be visualized with a binocular within 3 to 7 days.

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