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Study of the epidemiology of *Alternaria alternata* on potato

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SUMMARY

The involvement *of A. alternata* in early blight is discussed controversially. Brown spots developing on leaves of potato cultivar Markies are nevertheless often attributed to an *A. alternata* infestation. These symptoms appeared on Markies plants on two independent occasions in the greenhouse without any inoculation. In 75% of the necroses, neither *A. alternata* nor *A. solani* were present, which was confirmed by qPCR. Inoculation of Markies plants with *A. alternata* isolates derived from the same cultivar failed. Brown spots were also observed in field trials with Markies, but qPCR data did not indicate *A. alternata* as the causal agent of these necroses, either in the untreated control, in an artificial inoculation with *A. solani*, or in a double Ortiva® treatment. DNA amounts for this fungus were always low (< 2ng fungal DNA/µg total DNA) and *A. solani* predominant (up to 138 ng fungal DNA/µg total DNA). The Markies brown spots may have physiological causes.

KEYWORDS

Alternaria alternata, *Alternaria solani*, pathogenicity, disease progress, qPCR, potato cultivar Markies

INTRODUCTION

Early blight caused by *Alternaria* spp. is a problem in many potato growing areas of the world. In Germany, both *A. solani* and *A. alternata* can be found on early blight symptoms of potato leaves (Leiminger *et al.*, 2014). Although there is consensus about *A. solani* being a causal pathogen, the impact of *A. alternata* is discussed controversially. Stammler *et al.* (2014) doubt its role as a causal agent, and suggest it being rather a secondary invader which lives saprophytically on lesions and is therefore often isolated from leaf spots. In contrast, Droby *et al.* (1984) and Shtienberg (2014) state the pathogenicity of *A. alternata* towards potato leaves and the development of brown spots on the lower side of potato leaves after inoculation.

The potato cultivar Markies is a widely-used starch potato cultivar in Germany and there are many reports about the sudden appearance of a large number of small brown spots on its

leaves. Since *A. alternata* can almost always be detected on them, they are often attributed to an *A. alternata* infection, and Markies is considered to be highly susceptible to this fungus.
Markies was therefore used for inoculation experiments with *A. alternata* in the greenhouse. The disease progression of both *A. solani* and *A. alternata* was compared in a field trial with a naturally infected control, an artificial inoculation with *A. solani* and a fungicide treatment. To quantify the presence of both species in the crop, leaves were analyzed with a qPCR.

MATERIALS & METHODS

Artificial inoculation in greenhouse and field trials
Isolates of *A. solani* and *A. alternata* were grown on SN-Agar (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4$ * $7H_2O$, 0.5 g KCl, 0.2 g Glucose, 0.2 g Saccharose, 0.6 ml 1n NaOH, pH 5.5, 20 g Agar-Agar, 1 l dist. H_2O) at 22°C and 12 h NUV-light for two weeks. For greenhouse experiments, eye cuttings of cultivar Markies were planted in Einheitserde Typ T, 12 cm pots and grown at ca. 20–22 °C in a greenhouse cabin. Spray inoculation was carried out with a spore solution (dist. H_2O + 0,05% Tween 20) containing 10^5 conidia/ml, applied till run off.
For field trial inoculation, sterilized shredded rye kernels were inoculated with spore solution, incubated for two weeks at 22°C and 12 h NUV-light, and scattered between the potato plants (cultivar Markies) on the hills.

Isolation from potato leaves
To verify the presence of *A. solani* and *A. alternata* on leaves showing symptoms, they were surface sterilized in 3% NaOCl for 1 min, washed in sterile distilled water, and pieces of leaf tissue showing necroses were cut out and placed on SN-Agar. After a 2–3 day incubation at 22°C and 12 h NUV-light, plates were examined for conidia formation.

Genomic DNA extraction
For DNA extraction, 10 leaves/plot and leaf level (middle and upper) were randomly collected, washed for 3 min in running tap water and ground in liquid nitrogen. DNA was extracted according to Fraaije *et al.* (1999) with modifications.

Real-time PCR
The primer pairs used for quantification of *A. solani* and *A. alternata* DNA were developed by Leiminger *et al.* (2014) and Schuhegger *et al.* (2006), respectively. The real-time PCR reaction mixture consisted of 10 µl Maxima SYBR Green qPCR Master Mix (2x) (Thermo Scientific), 0.3 µM each primer, 0.5 µl BSA (20 µg/µl) and 40 ng DNA in a 20 µl volume for both species. The 2-step PCR was performed in a Mx3005P QPCR system (Stratagene) with an initial step of 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 61°C for 1 min.

RESULTS

Greenhouse experiments (2013)
For the *A. alternata* inoculation experiments, 6-week-old eye cuttings of Markies should be used. However, shortly before the time of inoculation, around the beginning of flowering, the plants suddenly showed lesions on the middle and upper leaves that looked like an *Alternaria*

infestation (Fig. 1a,b). As the temperature in the greenhouse cabin in which they were grown was regulated by opening and closing the windows, a natural infection of the possibly highly susceptible cultivar by windspread inoculum couldn't be excluded.
Consequently, a second batch of Markies eye cuttings was grown in a closed cabin with air conditioning. But again, the upper and middle leaves of the nearly 6-week-old plants suddenly developed symptoms similar to early blight before they could be inoculated (Fig. 1c).

Figure 1a, b. *Symptoms on 6-week-old, non inoculated Markies plants in an open greenhouse cabin (11 June 2013)*
c. *Symptoms on 6-week-old, non-inoculated Markies plants in a closed greenhouse cabin with air conditioning (10 July 2013)*

As the involvement of relevant amounts of external inoculum could be excluded in the second batch, 40 leaf areas with symptoms were cut out, put on SN-agar and incubated at 22°C and 12 h NUV-light, to check if *Alternaria* spp. were actually present on them. However, only on 10 out of the 40 necroses *A. alternata* could be found, and *A. solani* on 5. Thus 75% of the symptoms were completely free of *Alternaria spp.*
A qPCR analysis of the leaf material confirmed these results: for *A. alternata* an extremely low DNA content of 1.48 pg DNA/µg total DNA was detected, and for *A. solani* a very low content of 88.58 pg DNA/µg total DNA.
It is discussed that wounds could provide an entrance for *A. alternata* and therefore enhance the infestation with the fungus. To test this hypothesis, the plants showing symptoms were inoculated with *A. alternata* isolates gained from the first batch of Markies plants. However, no

increase in leaf necrotisation could be observed; both inoculated and non-inoculated plants had 17% necrotic leaf area (NLA).

Field trials (2014)

To compare the disease progression of *A. alternata* and *A.solani*, a field trial with cultivar Markies was conducted.

This consisted of three treatments: control (natural infection, no fungicide treatment against early blight), artificial inoculation with *A. solani* (27 June, no early blight specific fungicide treatment) and fungicide treatment with Ortiva® (0.5 l/ha, 27 June + 31 July).

Visual assessment and rating of the necrotic leaf area was done at weekly intervals for the middle and upper leaf level. The real extent of leaf tissue colonization by both fungi was determined by qPCR for three timepoints.

The often reported sudden appearance of brown "A. *alternata*" spots on the leaves of Markies was observed in this trial (Fig. 2), independent of the treatment.

Figure 2a. *Brown spots on leaves of cultivar Markies in the field (5 July 2014, Ortiva® treatment 27 June 2014)*

b. *Brown spots on leaves of cultivar Markies in the field (11 Sept 2014, Ortiva® treatment 27 June and 31 July 2014)*

The *A. solani* qPCR results of the first date (Table 1, 31 July) show that both the artificial inoculation and the fungicide treatment were successful: the *A. solani* DNA content in the middle leaf level was highest in the inoculated plots (100.13 ng/µg total DNA) and very low in the fungicide treatment (0.5184 ng). 4.39 ng of *A. solani* DNA in the control indicates that the natural infection hasn't reached its epidemic phase yet, and was still at a low level. DNA contents of 0.0287, 0.0441 and 0.1174 ng in the upper leaves show that the fungus hasn't spread to the upper parts of the plants yet.

Table 1. *qPCR and visual necrotisation assessment data, field trial, cultivars Markies and Jelly, 2014 (DNA = ng fungal DNA/µg total DNA, % NLA = % necrotic leaf area, middle = middle leaf level, upper = upper leaf level, n.a. = not analyzed)*

	31.7.			14.8.			4.9.		
Cultivar, treatment, leaf level	A. a. DNA	A.s. DNA	% NLA	A. a. DNA	A.s. DNA	% NLA	A. a. DNA	A.s. DNA	% NLA
Markies, control, middle	1,1738	4,3900	14,5	0,7553	75,13	23,8	n.a.	n.a.	78,5
Markies, control, upper	0,0304	0,1174	1,4	0,1771	0,9265	6,3	1,1903	35,43	23
Markies, inoculated, middle	0,4681	100,13	23,3	1,0391	138,75	54	n.a.	n.a.	94
Markies, inoculated, upper	0,0071	0,0441	0,8	0,1570	0,6971	11,4	1,6003	57,13	30
Markies, fungicide, middle	1.9270	0,5184	10,1	1,1019	3,1798	16,6	n.a.	n.a.	49,5
Markies, fungicide, upper	0,0066	0,0287	0,8	0,1927	0,0525	8,7	0,9226	5,6118	25
Jelly, middle							0,2903	201,63	24,5
Jelly, upper							0,1682	114,38	12,5
Markies, greenhouse	0,0015	0,0886	17						

Two weeks later (Table 1, 14 August) the DNA amounts in the middle leaf level have increased in all treatments, but were still highest in the inoculation (138.75 ng/µg total DNA) and lowest in the fungicide treated plot (3.1798 ng/µg total DNA), indicating the continuing effect of the inoculation and the efficacy of the second Ortiva® treatment. On the upper leaves the colonization with *A. solani* was still low and on almost the same level in the control and the artificial inoculated plots (0.9265 ng/µg respectively 0,6971 ng/µg total DNA). This shows that both natural and artificial infection haven't progressed to the upper leaves yet. The second Ortiva® treatment resulted in the lowest DNA content in the fungicide treatment (0.0525 ng/µg total DNA).

A further two weeks later (Table 1, 4 Sept) the middle leaves couldn't be analyzed anymore. Due to their progressed necrotisation it wasn't possible to extract sufficient clean DNA. However, *A. solani* had spread to the upper leaves by this time. A slightly higher DNA amount of 57.13 ng/µg total DNA in the inoculated treatment compared to the control (35.43 ng/µg total DNA) indicated the continuing impact of the artificial inoculation. With 5.6118 ng/µg total DNA, the effect of the double Ortiva® treatment was still clearly visible in the fungicide plots.

The course of the leaf necrotisation progress (% NLA, Table 1) was similar to that of the *A. solani* DNA content. On 31 July for the middle leaves the highest value was found in the inoculated plots (23.3% necrotic leaf area), and the lowest in the fungicide treatment (10.1% NLA). However, one wouldn't expect a 10% leaf area necrotisation in a fungicide treated crop, compared to 14.5% in the untreated control. In addition, an *A. solani* DNA content of 0.5184 ng/µg total DNA was clearly indicating that the *A. solani* leaf infestation was negligible as a cause of this necrosis. The extent of necrotisation on the upper leaves was equally low in all three treatments (1.4, 0.8 and 0.8% NLA), but to high to be caused by the low *A. solani* colonization detected with the qPCR.

By 14 August (Table 1), an increase in necrotisation could be observed in all plots and leaf levels. In the middle leaves, the highest amount was still found in the inoculation treatment (54% NLA) and the lowest in the fungicide treatment (16.6% NLA). Similar to 31 July, the low colonization of the upper leaves and the high level of necrotisation, even in the fungicide treated variant, clearly showed that neither *A. solani* nor *A. alternata* was involved in the formation of these lesions.
By 4 September, necrotisation had reached 94% in the inoculation treatment and 49.5% in the fungicide treatment on the middle leaves. The upper leaves did not differentiate: with 23, 30 and 25% NLA the level was almost uniform. But, as seen previously, the DNA amount in the fungicide treatment was still low and, additionally, much lower than in the other two treatments. So again, *A. solani* can be excluded as a possible source of these necroses.

Regarding *A. alternata* disease progress (Table 1), the DNA amounts of this fungus found in every leaf level and every treatment were very low, never exceeding 2 ng/µg total DNA. With the exception of two dates (31 July, fungicide treatment, middle leaves and 14 August, fungicide treatment, upper leaves) they were distinctly lower than that of *A. solani*, which rose up to 138.75 ng/µg total DNA. Consequently, *A. alternata* can be excluded as the cause of the necroses found on the fungicide treated Markies leaves.
Comparing the results of cultivar Markies with an untreated, non-inoculated control of cultivar Jelly (Table 1, 4 September), located at the same field site, the degree of necrotisation in both the middle and upper leaf levels was much higher in all variants of Markies: 49.5 - 94% and 23–30% compared to 24.5 and 12.5% for middle and upper leaves in Jelly. The lower necrotisation in Jelly was associated with DNA contents of 201.63 and 114.38 ng/µg total DNA for *A. solani* and 0.2903 and 0.1682 ng/µg total DNA for *A. alternata* in the middle and upper leaves, respectively. The former amounts were much higher than the amounts connected with a similar necrotisation in Markies, and clearly reveal *A. solani* as the cause of the lesions in Jelly. The latter were lower, and maybe hint at a possible higher colonization due to the higher necrotisation in Markies.
The qPCR results of the greenhouse trials with cultivar Markies (Table 1, 0.0015 ng *A.a.* DNA/µg total DNA and 0.0886 ng *A.s.* DNA/µg total DNA), compared to field trial leaves with similar necrotisation (17% NLA), demonstrate that the lesions on the greenhouse plants were hardly colonized by either fungi, and thus must be caused by something else.

DISCUSSION

The pathogenicity of *A. alternata* towards potato is discussed controversially in the literature. In Germany, starch potato cultivar Markies is considered as highly susceptible, because brown spots can be regularly observed on its leaves and *A.alternata* can be isolated from them most of the time.
In greenhouse trials, different inoculation methods for *A.alternata* inoculation should be tested with Markies eye cuttings. However, the almost 6-week-old plants suddenly developed necrotic lesions resembling early blight symptoms, without any inoculation, on two independent occasions. The presence of *A.solani* and *A.alternata* in the necroses was tested mycologically and by qPCR. 75% of the symptoms were free of *Alternaria* spp. and qPCR showed that DNA amounts in the leaves were correspondingly low. Therefore both *Alternaria* spp. could be excluded as the cause of these symptoms. Furthermore, an artificial inoculation of Markies plants showing symptoms with *A. alternata* isolates derived from the same cultivar was not successful.

This is in accordance with Stammler *et al.* (2014), who found a low or no virulence at all for *A. alternata* in potato.

The progression of both *A. solani* and *A. alternata* under natural conditions was compared in a field trial, also with cultivar Markies. Visual assessment (% necrotic leaf area) and qPCR (ng fungal DNA/µg total DNA) data were collected for three dates in a non-inoculated, non fungicide (early blight) treated control, an artificial inoculation with *A. solani* and a double fungicide treatment with Ortiva®. The typical sudden appearance of brown spots on the leaves of Markies could be observed in all treatments, even in the fungicide treated. However, the *A. alternata* DNA content of the leaves never exceeded 2 ng DNA/µg total DNA and was, with two exceptions, always lower than the corresponding amount of *A. solani* DNA, which reached a maximum of 138 ng DNA/µg total DNA. Thus, an important impact of *A. alternata* in the formation of the necrotic lesions is unlikely. As the colonization of the leaves by both *A. alternata* and *A. solani* was marginal in the fungicide treatment (0.0066–1.9270 and 0.0287–5.6118 ng DNA/µg total DNA, respectively), *Alternaria* spp. could be excluded as the cause of the brown spots there.

The *A. solani* DNA amounts detected in the untreated control of the comparison cultivar Jelly were much higher than in Markies (201 and 114 ng DNA/µg total DNA compared to n.a. and 35 ng DNA/µg total DNA). In contrast, Jelly had a lower necrotisation level than Markies (24.5 and 12.5% NLA compared to 78.5 and 23% NLA). Together with the small amounts of *A. alternata* DNA, the lesions in Jelly could be clearly related to the *A. solani* infestation.

In summary, *A. alternata* can be excluded as the causal agent of the typical "Markies brown spots". They are possibly caused physiologically; in the greenhouse, they occurred with the beginning of flowering, i.e. the switch from the vegetative to the generative phase of the plant. Additionally, Markies reacts strongly to ozone exposure (data not shown); at low doses with development of necrotic lesions, at higher doses with defoliation.

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