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Differentiation of Alternaria species and quantification of disease development using real-time PCR

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SUMMARY
An Alternaria-specific real-time PCR assay was developed to clearly discriminate *A. solani* and *A. alternata*. The use of real-time PCR allowed a quantitative estimation of fungal biomass in plant tissues. With this work we report real-time PCR applications to accurately assess the extent of Alternaria spp. colonization during disease development. This assay provides a useful tool to quantify pathogen levels during initial latent stage of infection and disease development.

KEYWORDS
Alternaria solani, *Alternaria alternata*, disease quantification, molecular diagnosis,

INTRODUCTION

Early blight (EB), caused by fungi of the genus Alternaria, belongs to one of the most devastating diseases of potato. Pathogens, which are discussed to be involved in EB disease are *Alternaria solani* as well as *A. alternata*. Several studies had shown that *A. solani* and *A. alternata* could be isolated simultaneously out of EB typical symptoms (Bäßler *et al.*, 2004; Leiminger, 2009; Latorse *et al.*, 2010). Up to now, techniques of Alternaria identification mainly rely on agar plate methods, where Alternaria species are differentiated macroscopically by the morphology of their spores. Similarly, disease scorings based on the assessment of visual symptoms do not guarantee a distinct differentiation of pathogens. Although methods exist to detect Alternaria species in potato, current techniques do not allow a quantification of the fungi *in planta*. The most commonly used molecular technique for the identification of Alternaria species in general is conventional PCR with species-specific primers based on the ribosomal DNA internal transcribed spacer (ITS). The application of real-time PCR represents a highly sensitive and specific technique for the detection and quantification of nucleic acids (Taylor *et al.*, 2001). With this work we firstly describe real-time PCR applications to accurately assess the extent of Alternaria colonization of potato leaves during early disease development and to differentiate both species. The use of this assay opens the opportunity to track the specific progression of Alternaria species within the host which may contribute to a better understanding of EB

epidemiology. This may then be used in epidemiological investigations as well as in disease management strategies.

MATERIAL AND METHODS

Field studies

Leaf samples were taken from naturally infected field trials. Sampling schemes were designed as a randomized complete block and were replicated four times. Experimental plots were situated within a commercial potato field which remained untreated against EB, thus providing natural inocula for EB disease development. In 2003 and 2004 field trials were carried at Weihenstephan, in 2005 at the location Straubing. All plots were fertilized and cultivated according to general agricultural practice.

Visual evaluation of EB infection

Disease progression was observed weekly from potato emerge until death of the potato plants. Within each of the replications, 10 plants per plot were monitored for disease progression of EB or other diseases (e.g. late blight) and percentage of EB infected leaf area was assessed visually. The disease severity per plant was calculated as a mean value. For leaves which were either completely dead or where the lower leaves had senesced and dropped from the plant, the disease evaluation was omitted.

Collection of plant probes

From the onset of first visible symptoms on older leaves, leaf samples were taken within an approx. 14 day interval out of EB untreated control plots. Each plot was divided into 4 subplots and leaves were collected out of lower, middle and apex leave sections. Ten leaves were taken for each subplot and were mixed for further analysis. Each plot was repeated 4-times and was randomly distributed. Tissue was harvested and immediately frozen in liquid nitrogen at the site. For levels, which did not contain any leaves due to premature defoliation caused by EB, sampling was discontinued.

Genomic DNA extraction and PCR amplification

Samples were carefully ground in liquid nitrogen and crushed with a pestle to a fine powder. DNA was extracted out of 100 mg homogenized plant material. The final DNA was finally dissolved in 100 µl Tris EDTA (TE buffer, pH 8). 1µl part of this DNA solution was used for quantitative PCR (real-time PCR) which was performed in 25 µl reactions with Absolute QPCR SYBR Green® ROX Mix. Real-time PCR analysis has been carried out to elucidate which of both species was dominating. Therefore species-specific primers were developed. Alternaria specific primers derived from the base-sequences of the internal transcribed spacers (ITS) of ribosomal RNA (rRNA) genes. Quantitative PCR of *A. solani* produced a 152 bp fragment and a 95 bp fragment of *A. alternata*, but no amplicon was obtained from uninfected potato leaves. In order to determine the species specificity of the primers, purified genomic DNA of 50 different fungal species, including major tomato and potato pathogens were included in the PCR assays.

RESULTS AND DISCUSSION

Visual quantification of EB disease development

The infection process of Alternaria spp. was monitored by visual disease scorings as well as by real-time PCR analysis. Disease ratings revealed that first disease symptoms occurred at a very young age of potato plants. Here, EB was the primary foliar disease among all observed diseases. Over all years, disease epidemics progressed slowly till the end of July and more rapidly thereafter (Figure 1). As the season progressed, EB symptoms rapidly increased and spread onto higher leaf levels. Secondary spread of EB was observed in all years starting from the end of July or the beginning of August. Heavy EB epidemics occurred throughout all five years of investigation. EB resulted in premature defoliation of potato plants in mid September.

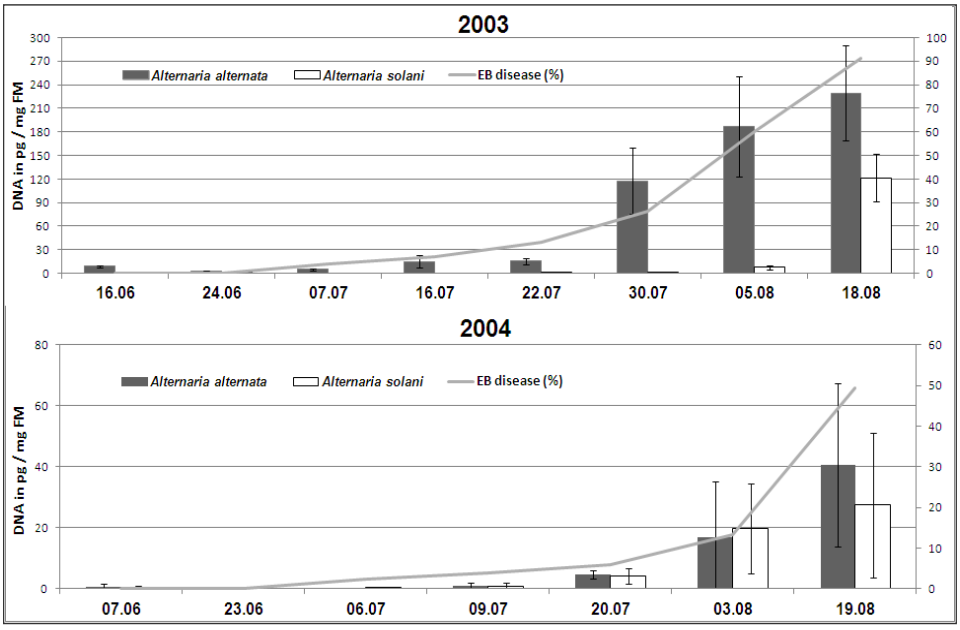

Figure 1. *Specific disease progression of A. solani and A. alternata measured by real-time PCR compared to disease progression of early blight in 2003 and 2004, location Weihenstephan*

Quantifying EB disease in leaves

Within the developed PCR system both Alternaria species could be clearly identified and differentiated. No positive amplification signals were obtained on the non-target fungal species. Primers revealed that the targets were well conserved within the repetitive DNA sequence of either *A. solani* and *A. alternata*. The development of fungal species within the leaves was monitored. The real-time PCR analysis detected the presence of Alternaria DNA in almost all samples at quite early stages of plant development. Higher amounts in DNA levels of *A. alternata* were observed in 2003, showing *A. alternata* as the predominant species from the beginning of sampling (Figure 1). In 2004, DNA of both species were found to be present at almost equal levels throughout the season (Figure 1). In 2005, *A. solani* was found to be the

predominant species with high quantities of fungal DNA. Although small amounts of DNA has been detected at the beginning of the season for both species, significantly higher amounts of *A. solani* DNA could be detected with progressing season. In contrast, in 2005 low *A. alternata* DNA values were observed which remained at low levels till the end of the season (Figure 2).
In 2005 leaf samples were taken simultaneously out of three different plant sections. Analysis of the middle and top third of the canopy revealed that Alternaria-DNA could be detected prior to the development of EB symptoms. Here, real-time PCR allowed the detection of fungal growth already during the latent phase of disease development. The largest increase of the amounts of DNA took place at the middle leaf section in 2005. The largest increase of the amounts of fungal DNA took place during the symptomatic stage of the infection with severe EB symptoms (Figure 2). Real-time PCR allowed to quantify Alternaria species in field infected potato leaves. Detection of fungal DNA failed on the very last sampling dates. Here, real-time PCR did not yield any results although leaves were heavily infected by EB. This is probably due to the lack of live mycelia which had been completely converted to spores, which had obviously not been disrupted by powdering in liquid nitrogen.

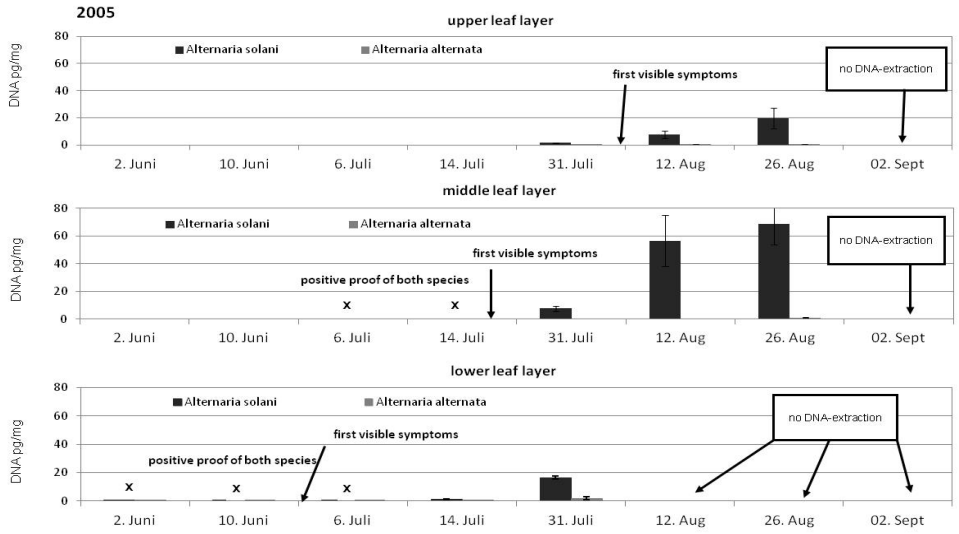

Figure 2. *Disease progression of A. solani and A. alternata measured by real-time PCR in 2005, location Straubing. To follow up species development in more detail, potato plants were divided into three leaf layers (bottom, middle and upper leaf section). Detection of fungal DNA failed on the very last sampling dates*

Analysis yielded significantly different amounts of Alternaria-DNA of both pathogens and among all investigated years. Although both pathogens were present, their intensity of infestation differed strongly in different years. Only in 2003 *A. alternata* was solely found at higher DNA levels. In the following year 2004 a more equal distribution of both species was revealed by real-time PCR. In fact, *A. solani* was found at higher DNA amounts throughout the season at the location Straubing over the years 2005 to 2007 (data not shown). Even though both species

were found in almost all years, stable and higher DNA levels were expressed by real-time PCR analysis for *A. solani* rather than for *A. alternata*. Higher DNA values of *A. solani* can be explained by its higher pathogenicity and faster disease development (Rotem, 1994, Viskonti and Chelkowski, 1992).

The sensitive real-time PCR detection technique enabled to measure the degree of fungal development at different plant ages. It could be shown that older leaves from lower leaf sections were infected earlier than other leaves from higher leaf sections. With increasing leaf age successively higher located leaves were affected by EB, reflecting higher DNA levels. These confirm older results of Harrison and Venette (1970), who found that higher leaf levels were increasingly affected as the season progressed. These results demonstrate that the developed real-time PCR protocol is a sensitive and reproducible method for *in planta* quantification of Alternaria spp. during potato leaf colonization. The gradual upward progression of EB disease can be seen in the real-time PCR results.

Although disease pressure was very low and symptom appearance was very inconspicuous, real-time PCR has proven to be very effective for the quantification of Alternaria DNA levels at very early or even latent stages of infections. Early samplings from leaves of the middle and upper leaf sections confirmed Alternaria DNA, although no visual symptoms were found. PCR analysis enabled the screening of pathogen colonization already during the initial latent stage of infection, which would help to improve diagnosis.

CONCLUSIONS

Real-time PCR has proven as diagnostic assay to investigate the impact on species development and represents a more objective manner in the observation of fungal development. This technique can now be used in routine inspections to screen material for the presence of EB relevant pathogens and would thus help in the understanding of the dynamics of Alternaria spp. in potato field grown material. The use of this technique will be a useful tool in effective EB disease management as well as in plant pathogen interaction studies.

REFERENCES

Bäßler, E., Asensio, N., Leiminger, J., Hausladen, H., Bahnweg, G., and Zinkernagel V., 2004. Investigations on the appearance and control of *Alternaria solani* and *Alternaria alternata* on potato. Mitteilungen der Biologischen Bundesanstalt f. Land- & Forstwirtschaft 396, 100-101.

Harrison, M.D., and Venette, J.R., 1970. Chemical control of potato early blight and its effect on potato yield. American Potato Journal 47, 81-86.

Latorse, M.P., Schmitt, F., Peyrard, S., Veloso, S., and Beffa, R., 2010. Molecular analysis of Alternaria populations early blight causal agents in potato plants. In: Westerdijk, C.E., Schepers, H.T.A.M., eds, PPO Special Report no. 14, 2010. Applied Plant Research BV, Wageningen, 179-186.

Leiminger, J., 2009. Alternaria spp. an Kartoffeln – Empirische Untersuchungen zur Epidemiologie, Schadrelevanz und integrierten Bekämpfungsstrategien. Ph.D. Thesis, Technische Universität München, Germany.

Rotem, J., 1994. The genus Alternaria, biology and pathogenicity. APS Press, St. Paul, Minnesota, USA.

Tylor, E., Bates, J., Kenyon, D., Maccaferri, M., and Thomas, J., 2001. Modern molecular methods for characterization and diagnosis of seed-borne fungal pathogens. J. Phytopathology 83, 75-81.

Viskonti, A., and Chelkowski, J., 1992. Alternaria Biology, plant diseases and metabolites, eds., Elsevier Verlag, Amsterdam.