Twelfth EuroBlight workshop
Arras (France), 3-6 May 2010

Population genetics – consequences on early blight disease

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SUMMARY
Members of the genus *Alternaria* are found in a wide variety of habitats worldwide. As causal agents of early blight disease *Alternaria solani* and *Alternaria alternata* are important pathogens in potato representing a risk to crop production. Due to premature defoliation, early blight epidemics can cause major yield losses. Recent investigations have been carried out in order to achieve more detailed information about disease progress and early blight control. Epidemiologic studies showed that both species can lead to necrotic symptoms. Up to now, field observations do not ensure positive proof for the differentiation of necrotic spots either caused by *A. solani* or *A. alternata*. By the use of specific primers, both pathogens could be isolated and quantified out of leaf samples. Therefore, PCR-based methods enabled a clear and simple differentiation of both pathogens. Little is known about pathogenic specialization and the generation of fungicide resistant isolates. Therefore, genetic analyses of plant pathogen populations are important for understanding epidemiology and host-pathogen interactions. This work deals with the analysis of intra-specific diversity of different *A. solani* isolates. By the use of RAPD markers spatial genetic diversity within and among *A. solani* isolates from different fields in Southern Germany were investigated. Our results show that the fungus is highly divers. This indicates that the fungus has a high potential to adapt to different environments, potato cultivars or even to build up fungicide resistance.

KEYWORDS:
Early blight, species differentiation, PCR, genetic diversity, RAPD

INTRODUCTION

Species of the genus *Alternaria* are very common and abundant. *Alternaria* species in general, including *A. solani* and *A. alternata* have a world-wide geographic distribution (Rotem, 1994). The genus *Alternaria* includes saprophytic and pathogenic fungi. Due to its high adaptability, early blight has the potential to become a serious threat for potato cultivation in Germany. Apart from the widespread potato disease late blight, early blight is causing increasing problems (Hausladen, 2006). During the last years, early blight became more and more important in German potato

growing areas. Recognized primarily as a foliage pathogen, epidemics occur when the weather is warm and dry with short periods of high moisture. Both species infect the plant by conidia which are either wind blown or splashed onto plant surfaces. Visual analysis of the symptoms does not allow to distinguish if the necrotic spot is caused by *A. solani* or *A. alternata*. One objective of this study was thus to investigate the occurrence and distribution of both species (*A. solani* and *A. alternata*) on early blight infected leaves. For this, PCR-based methods have been used for specific molecular diagnostics (Bahnweg *et al*., 1998). Beyond morphological characteristics, PCR enables a clear and simple differentiation of both pathogens, which can be used irrespective of symptoms or typical sporulation. *Alternaria* species have no known sexual stage, which precludes genetic analyses other than parasexual. *Alternaria* pathogens should consist of many asexual lineages evolving independently with little or no genetic exchange (Peever *et al.,* 1999). Variation in neutral genetic markers (RAPD) should accumulate independently in each lineage due to mutation. Molecular analysis may demonstrate the existence of diversity in these asexual pathogens. In fungal systems, RAPD-PCR has been widely applied for the characterisation of species and isolates. RAPD analysis was used to examine the levels of genetic variation of *A. solani* isolates within and among different geographical origins. The amount and distribution of genetic variation within populations gives an indication of disease evolution (Adachi *et al.,* 1993). An improved understanding of the genetic basis of species specificity has important basic and applied implications. Genetic analyses of pathogen populations are important for understanding epidemiology and host-pathogen interactions, as well as for the development of disease control strategies (Aradhya *et al.*, 2001; Morris *et al.*, 2000). The aim of this study was to estimate the amount of intra-specific variation within 45 isolates of *A. solani* from different locations in Bavaria and Germany and to assess the potential of RAPD-PCR as a diagnostic tool to distinguish between morphologically similar or identical isolates of one species.

MATERIALS AND METHODS

EARLY BLIGHT MONITORING

A monitoring programme has been accomplished in naturally infected potato fields throughout Germany in order to document the distribution and occurrence of early blight in German potato growing areas. Early blight-infected leaves were obtained during this monitoring programme since 2005. Collected leaf samples were investigated for the occurrence of *A. solani* and *A. alternata* according to morphological characteristics. Up to now, investigations on disease incidence of early blight in Germany are only based on single observations by farmers or advisory services. The realization of this early blight monitoring enabled an outline of the distribution as well as the local occurrence of both *Alternaria* species in German potato growing areas.

ISOLATE COLLECTION

Infected leaflets were sampled at random and one isolate was taken per collected leaflet. Leaf pieces bearing a single lesion were cut from infected leaves and surface sterilized. Leaf cuts were transferred to petri dishes and incubated under UV-light. Isolates were obtained by single spore isolation and prepared for long-term storage. *A. solani* cultures were identified on the basis of morphological characteristics and spore size. Isolates of *A. solani*, 17 in 2006 and 20 in 2008, were collected from one potato field located in Weihenstephan. In addition in 2008 eight isolates have been collected from different potato fields in Bavaria and Germany (locations: Atting, Ehetal, Geltolfing, Straßmoos, Thonstetten, Uelzen).

QUANTITATIVE PCR ANALYSIS

The amplification of specific DNA-segments allowed the characterization of both species *A. solani*

and *A. alternata* out of small amounts of fungal DNA. Quantitative PCR (qPCR) enabled an overview about the distribution and quantitative amount of both species within infected leaves. Leaf sampling started 8 weeks after crop emergence and were carried out until vine death. Out of each repetition, ten potato leaves were collected every second week and stored in liquid nitrogen immediately. Specimens were homogenised with a mortar in liquid nitrogen and were used for further analysis. By the use of quantitative analysis, early blight development in the leaf could be investigated in the course of the potato vegetation cycle.

PCR-BASED GENETIC DISCRIMINATION

The RAPD technique relies on the presence of priming sites for a single primer on the genome in an inverted orientation. No prior knowledge of the genome to be analysed is required. For the RAPD technique, random 10 bp OP (Operon Technologies, Alameda, California) oligonucleotides primers were used to produce amplified DNA fragments. RAPD markers have been demonstrated to provide a quantitative assessment of genetic relationships and similarities of genotypes. Preliminary tests were performed with 5 complete sets of OP primers. Random primers yielding amplicon patterns discriminating between isolates or groups were used for further investigations. Only bands that could be clearly distinguished as present or absent were scored. Similarity coefficients between all pairs of isolates were calculated by the Sokal & Michener coefficient (simx/y = (a+d)/(a+b+c+d)). Data of all pairwise comparisons of isolates are presented as a two-way similarity matrix, which was then used to perform an average linkage cluster analysis.

RESULTS AND DISCUSSION

EARLY BLIGHT MONITORING

Over several years, *Alternaria* species were monitored and isolated out of leaf necrosis. Early blight is a destructive disease, which can cause premature defoliation of the potato plant. Morphological analysis and pathogen specific investigations showed that early blight pathogens are present in almost every potato growing area in Germany. Within the monitored years, the frequency in the occurrence of both *Alternaria* species showed differences. In 2006 *A. solani* and *A. alternata* could be isolated from almost every infected leaf sample. In the following years, mainly *A. alternata* was isolated from the first samples. In 2007 and 2008 *A. solani* was isolated out of 55 to 60% of the investigated leaf samples (Table 1). In both years, *A. solani* was missing in almost every leaf sample until mid/end of July. Weather conditions may influence the development of both fungal species and favour the development of *A. alternata* in the beginning of the potato growing period. According to Viskonti and Chelkowski (1992) weather conditions may influence the development of both species in a different way.

Table 1: *Isolation frequency of A.solani and A. alternata out of infected leaf sample*

species / Year	*A. solani*	*A.alternata*
2006	95%	98%
2007	60%	96%
2008	55%	100%

Our results show that only by regarding symptoms it is not possible to distinguish if the necrotic spot is caused by *A. solani* or *A. alternata*. Although necrotic symptoms can be truly defined as early blight symptoms there are obvious differences in the occurrence of both species. Morphological or PCR investigations should be used in order to obtain a clear differentiation and distribution of both *Alternaria* species.

QUANTITATIVE PCR ANALYSIS

Quantitative real-time PCR has been carried out over several years (2003 to 2005). Beyond morphological analysis, PCR-based methods allowed the verification of species-specific DNA fragments. Optical disease ratings only gave an idea about disease distribution and disease epidemic in the field. However, quantitative real-time PCR enabled a specific assessment of *A. solani* and *A. alternata* and could be also used to quantify the amount of fungal DNA in infected plants. Specific primers for the detection and identification of both *Alternaria* species were highly sensitive and showed a clear differentiation between both *Alternaria* species.

Figure 1 and 2 show the amount of fungal DNA, which could be verified for *A. solani* and *A. alternata* during the vegetation periods 2003 and 2005. In all investigated years, both species could be detected out of analysed leaf samples (data 2004 not shown). However, species showed a different seasonal distribution as well as different levels of DNA amounts. Early blight species were first attested at the beginning of June out of sampled leaves within all years (June 16th (2003), June 7th (2004) and June 2nd (2005)), thus showing that *Alternaria* pathogens could be detected quite early in the stage of the potato development. In general, fungal DNA amounts stayed at a very low level at the beginning and increased till the end of the season. In comparison of the years, the distribution of both species was not equal. In 2003, *A. alternata* developed much faster than *A. solani*. First observations of *A. solani* were made before the end of July. Until the end of the season, DNA levels of *A. alternata* were almost twice as much as of *A. solani*. In 2004, both species were distributed much more equal just from the beginning of the observations (data not shown). 2005 showed opposite results, as DNA amounts of *A. alternata* stayed at a very low level. Only fungal DNA of *A. solani* was detected with increasing levels until the end of the season.

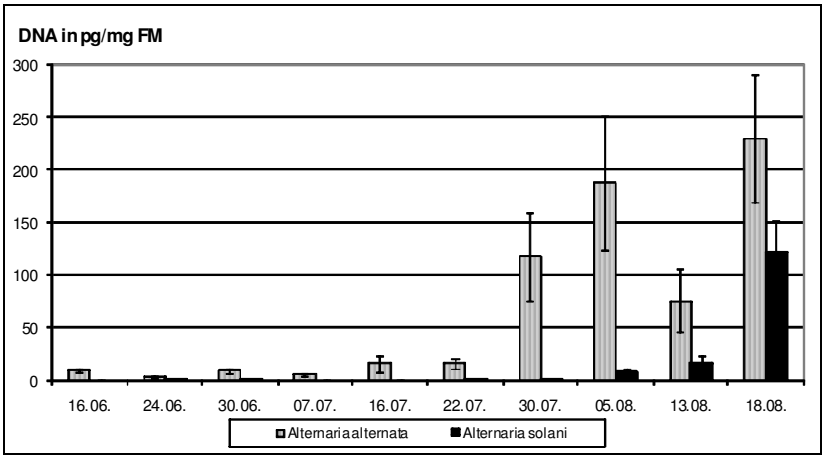


Figure 1*: Amounts of fungal DNA of A. alternata and A. solani out of sampled leaves in Weihenstephan 2003*

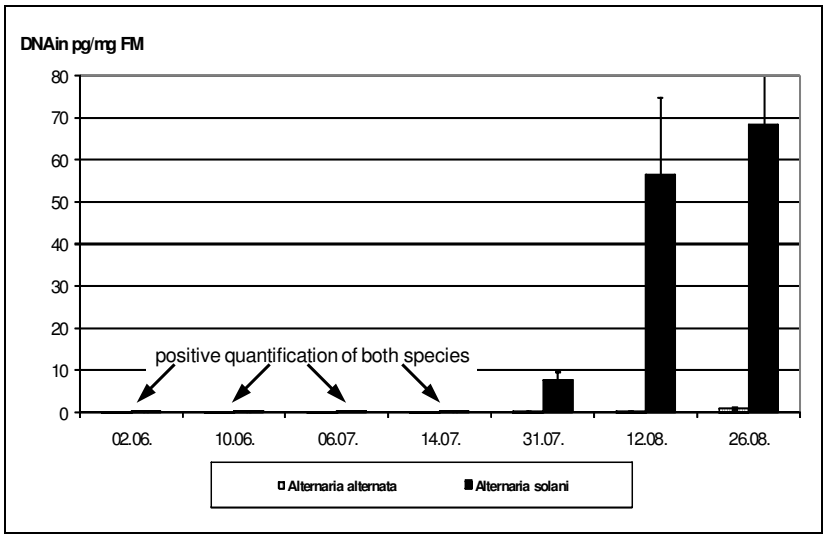
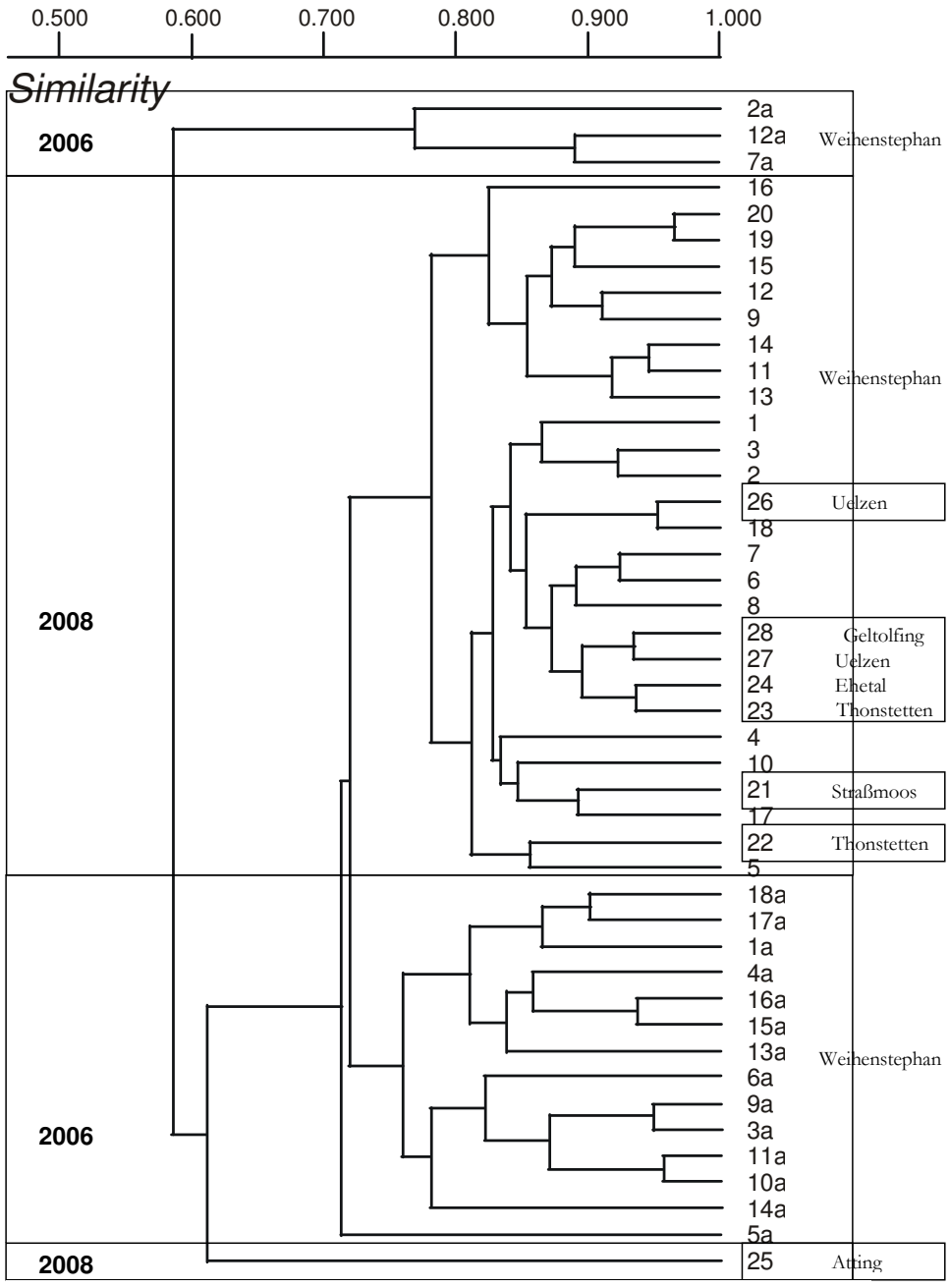


Figure 2: *Amounts of fungal DNA of A. alternata and A. solani out of sampled leaves in Weihenstephan 2005*

Our results show, that already small amounts of fungal DNA can be detected in leaves, often without any visible leaf necrosis. Therefore, molecular analysis enables a quick and safe proof of primary infections. According to the high primer specificity, which has been tested at more than 80 fungal diseases of potatoes and other crops, *A. solani* and *A. alternata* can be distinguished efficiently. According to Viskonti and Chelkowski (1992), weather conditions play a role for the development of both *Alternaria* species, which may explain the different distribution of species in comparison of the years. Our results show, that both species could be detected together in all years at different levels, indicating that both species are involved in early blight disease and could be seen as a "pathogen complex".

PCR-BASED GENETIC DISCRIMINATION

To further discriminate *A. solani* isolates, a RAPD analysis was performed. The highly polymorphic nature of RAPD markers makes them especially useful for the differentiation of clonal lineages of fungi that reproduce asexually. Ten primers were chosen based on a preliminary screening of primers. A total of 90 different amplicons were generated by the 10 different RAPD primers. Figure 3 shows the cluster analysis of all RAPD profiles. Cluster analysis of all RAPD profiles revealed high genetic diversity, not only at different locations of Bavaria (isolates 22-28) but also in samples of the same field (1a-18a (2006), 1-20 (2008)). Isolates from geographically distant fields were equally genetically divergent when compared to isolates sampled from one single field. A clustering according to geographic origin was not apparent. High differences in genetic diversity were detected between two sampling years, although isolates were collected from the same location. Weir *et al.* (1998) inferred from this high heterogeneity the possibility of pathogenic specialization. The mechanisms available for genetic change in *A. solani* are still largely unknown. Van der Waals *et al*., (2004) and Weir *et al*., (1998) hypothesized that evolutionary processes like mutation, selection, and gene flow may have influenced *A. solani* populations. The incidence of causal recombination might be the cause for the high diversity within isolates. Another cause for genetic variation may be natural mutation or large population size. The large population size of *Alternaria* species makes it more likely that new mutants with higher fitness will emerge and be able to multiply within the infected host. New alleles introduced in the population increase the chances of breakdown of resistance genes. However,


0.500
0.600
0.700
0.800
0.900
1.000
Similarity
2006
2a
12a
7a
Weihenstephan
2008
16
20
19
15
12
9
14
11
13
Weihenstephan
1
3
2
26
18
Uelzen
7
6
8
28
27
24
23
Geltolfing
Uelzen
Ehetal
Thonstetten
4
10
21
17
Straßmoos
22
5
Thonstetten
2006
18a
17a
1a
4a
16a
15a
13a
6a
9a
3a
11a
10a
14a
5a
Weihenstephan
2008
25
Atting

some of the investigated isolates, which were sampled from widely separated regions, pointed out only little genetic variation (isolates 23, 24, 27 and 28), indicating similar, if not identical isolates. These results indicate, that the various geographical subpopulations are not genetically isolated. Our results show high diversity within isolates of *A. solani*, which suggests that mechanisms for recombination and production of novel genotypes are available. How these factors may influence the population dynamics and evolution of the fungus demand further attention.

SUMMARY AND OUTLOOK

Enhanced investigations about population diversity help to determine the genetic structure of pathogen populations. This knowledge of genetic structure offers insights into the future evolutionary potential of pathogen populations. Our results indicate the presence of surprising genetic heterogeneity within the populations of *A. solani* which must be kept in mind when designing protective measures for agriculture. This may prove useful to optimize the management of fungicides to maximize their useful life expectancy and minimize the losses that result from reduced efficacy of these control methods. Understanding the genetic diversity of *A. solani* on potato will thus aid in future disease management strategies of early blight.

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