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Molecular Analysis of Alternaria Populations Early Blight Causal Agents in Potato Plants

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SUMMARY

Early blight of potato can be caused by the two fungi *Alternaria solani* and *Alternaria alternata*, with significant impact observed on crop productivity in the field from the last past years in Europe. However, it is impossible to determine visually if the necrotic symptoms are caused by one or the other species. On the other hand, the differentiation of the two species based on the observation of the morphology of the spores is not considered as fully reliable because this spore diagnostic first means that the fungi remain alive despite of the collect conditions (treatments and drying situations) in the field. Secondly, *Alternaria alternata* differentiates spores much faster than *Alternaria solani* when isolated on artificial medium in Petri dish introducing a bias in this morphological diagnostic when both are present on the same sample.

Finally the efficacy of fungicides has been demonstrated dependent on the fungal species especially for the QoI fungicides. In fact, the use during these last years of QoI fungicides has shown the development of resistance due to the respective F129L and G143A mutations according the species in the genes encoding the cytochrome bc1, Qo site of the respiration complex III.

To improve the diagnostic of both *Alternaria* species and the detection of the point mutations conferring the resistance to the respiration complex III inhibitors, Bayer SAS has developed molecular tools to support *Alternaria* sp. monitoring and appropriate fungicide recommendation in the field.

The fine analysis of the sequences of the genes encoding the cytochrome bc1 reveals the presence of not yet described sequences in the introns of several *Alternaria* isolates. The presence of these particular sequences will be discussed in term of *Cyt bc1* gene organization, fungal population evolution, and horizontal gene transfer.

KEYWORDS:

Potato, early blight, *Alternaria solani*, *Alternaria alternata*, molecular analysis, qPCR

INTRODUCTION

When Early blight in US is reported to be mainly due to *Alternaria solani,* the agents responsible of Early blight in European countries are belonging to the two species *Alternaria solani* and *Alternaria alternata*. The occurrence of the disease was correlated to significant yield losses even if the economic input is difficult to evaluate. Based on the disease frequency monitoring, Early blight was mentioned to become more and more important in the last years in Germany and in Poland for example. In regards of more and more specific fungicides against Late blight reaching the market, of the reduction of the mancozeb rates, and of the potential climatic changes favourable for *Alternaria sp.*

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in potato growing area, it could be expected that problems related to *Alternaria* sp. infection could continue to increase in the future in Europe. On potato, both *Alternaria* sp. cause necrotic lesions which are definitively not possible to be distinguished between spots caused by *Alternaria solani* or *Alternaria alternata*. The biological spore diagnostic after subculture on an artificial medium is time consuming and not fully reliable since *Alternaria alternata* is able to produce spores more rapidly and easily than *Alternaria solani* in such conditions. Therefore in a mixed population of spores, mainly *A. alternata* can be detected.

The widely used fungicides designed as Qo inhibitors (QoIs) inhibit mitochondrial respiration by binding to the Qo site of the cytochrome *bc1* enzyme complex. This blocks the electron transfer process in the respiration pathway leading to the death of the treated fungi (1). Resistance to QoI is often conferred by single amino acid exchanges in cytochrome *bc1*, either in position 143 where a glycine is replaced by an Alanine (G143A; 2), or in position 129 for which a phenylalanine is substituted by a leucine (F129L; 3, 4). Cytochrome *bc1* gene structure has been studied in several fungal species and the position of the intron close to the codon 143 seems to protect the sequence from possible mutations at this site in some species (5).
These limitations and the advances which occurred this last years in molecular phyto-diagnostic (6) drive us to establish methods based on Polymerase Chain Reaction (PCR) to quantitatively assess the presence of each *Alternaria* species and the cytochrome *bc1* gene appeared to be a good candidate to quantify at the same time the fungal species and the point mutations conferring resistance to Qo inhibitors.

MATERIALS AND METHODS

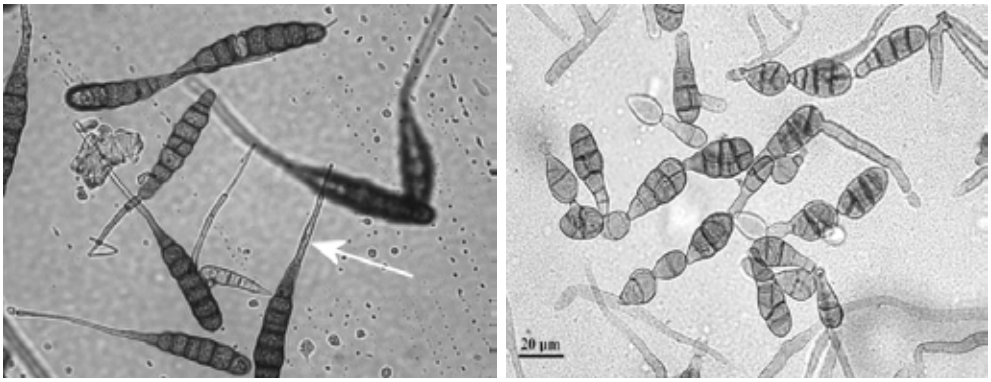
Alternaria sp. strains
Alternaria solani and *Alternaria alternata* strains were provided respectively by N.C. Gudmestad and J.S. Pasche for US references and collected randomly from across Europe fields respectively in Germany, Netherlands and Poland during the 2007 and 2008 monitoring campaigns (table 1).

Table 1: *Alternaria samples collected from across Europe in 2007 and 2008*

Sample code	Expectation/ Field symptôms	Conidia diagnostic
07GE051 A	?	No spores
07NL020	?	No spores
07NL024	solani	alternata
07NL029	solani	alternata
07PL008	?	alternata
07PL009	?	alternata
07PL010		alternata
07PL011		alternata
07PL059A		alternata
07PL059B	?	solani
07PL060B	?	alternata
08GE028	solani	alternata
08GE029	alternata	alternata
08BE041	solani	solani
08GE029	alternata	alternata

Mycelium and spore morphology study
The fungi are readily cultured on artificial media such as V-8 juice agar (7). The observation was done under a binocular magnifier to distinguish the size and morphology of conidia (Fig.1 & 2). *A. solani* has conidia (15-19 X 150-300 µm) with 9-11 transverse septa and few if any longitudinal septa. Spores are usually borne singly. Spores of *A. alternata* (20-63 X9-18 µm) are smaller than those of *A. solani*, are formed in chain and lack the typical long beak

Figure 1&2: *Long conidia (15-19 X 150-300 µm) of A. solani with a typical long beak and smaller conidia of A. alternata (20-63 X9-18 µm) in chain without long beak*



In vitro cultivation of fungal strains and mycelium preparation
Purified strains of *Alternaria solani* and *Alternaria alternata* were maintained in Petri dishes on Potato Dextrose Agar (PDA) medium at 21°C. 100 ml of liquid Potato Dextrose Broth (PDB) medium are inoculated with 10 agar plugs (5x5mm) infected with mycelium and cultivated at 21°C for 72h under agitation at 110 rpm. Mycelium is collected on 100µm nylon mesh, blended for 30 sec at maximum speed in a Warring Blendor in 50ml of PDB. 5 ml of this solution is used to inoculate 100 ml of PDB and further cultivated for 48h at 21°C under agitation 110 at rpm. Mycelium is then collected by centrifugation 30 min at 3200g, frozen in liquid nitrogen and lyophilized for 24h and stored at -80°C.

DNA preparation
For the preparation of fungal strains genomic DNA, 30mg of lyophilized mycelium was used as a starting material. For plant infected by fungi, ten leaf disks of 10 mm diameter were frozen in liquid nitrogen and further lyophilized. Plant material was grinded 2 x 1min at 30Hz in a RetschMM301 mixer mill. DNA was extracted using QiaPreP miniprep (Qiagen) according to the manufacturer protocol with the following modifications: the mix is vortexed for 5 min after the addition of 250 µl P1 buffer, lysis is allowed for 5 min, let sit 5 min after addition of PE buffer. Elution from column is performed by adding 25µl of hot water (70°C), let sit 1 min and centrifuge to collect eluted DNA. Extracted DNA quantity was measured by OD260nm using a Nanodrop spectrophotometer (Thermo Scientific).

Sequencing of CytB gene
Genes were amplified by PCR from genomic DNA using species specific primers based on published sequences (5). For *A. solani* the forward primer was AS5F 5'-AGAACTCTAGTATGAACTATTGG and the reverse primer Asint143a_R 5'-CACAGTGGCTATGTGCTTGG leading to a theoretical 2216bp amplicon. Amplification was performed using Advantage2PCR system (Clontech) with 1

min at 95°C, 30 cycles of 30 sec at 95°C, 1min at 60°C, 3 min at 68°C and 13 min at 68°C for elongation. For *A. alternata* the forward primer was DTRcytb2: 5'-CTAGTATGAACTATTGGTAC and the reverse primer was DTRcytb2Rmodified 5'-GGAGCAAAAGATATTCTTTC leading to theoretical 338 bp amplicon. Amplification was performed using Phusion high fidelity enzyme (Finnzyme) with 1 min at 98°C, 30 cycles of 30 sec at 98°C, 30 sec at 57°C, 30 sec at 72°C and 5 min at 72°C for elongation. Resulting PCR fragments were cloned using pGEM-T easy for *A. solani* (Promega) or pCR4Blunt Topo (Invitrogen) for *A. alternata* and transformed into E coli Top10 by the heat shock procedure. Positive cloned were send for sequencing at MWG Biotech using M13 universal forward or reverse primers and SP-sol-1868Reverse 5'- TGGTGGAAAAGGCAGGGTAT for *A. solani* or SP-alt-1302Reverse 5'- CGAGCTTATTGTTGGTATTACTCACTCA for *A. alternata*.

Quantitative PCR

Primers were designed using the Primer Express software and all the Q-PCR experiments were performed in an ABI Prism7900HT cycler (Applied Biosystems). For each species to be analyzed, 4 PCR primer sets were designed. Primers concentrations were optimized, primer specificity was tested on the non targeted *Alternaria* species, and primer set amplification efficacy was calculated. The following primers set were selected: for *A. solani* forward primer is SP-sol-1658F (5'-GTAGAGTATGTTGAATACTCTAACCAGACAA) and reverse primer is SP-sol-1759R (5'-ATGTTAAGAATTTGTCCTGAACAGTTT) respectively used at 900nM and 50nM in the reaction mix; for *A. alternata* forward primer is SP-alt-153F (5'-CTTATGAGTGCTATACCTTGAGTAGGTCA) and reverse primer is SP-alt-376R (5'-TCTCCGTCTATCAATCCTGCTAAA), respectively used at 300nM and 50nM in the final reaction mix. The Q-PCR reaction mixture contained 10µl Master mix sybergreen (AppliedBiosystem), the corresponding primers, 5µl of diluted DNA and water to 20 µl. The Q-PCR cycle was the following: 2 min at 50°C, 10 min at 95°C followed by 50 cycles of 15 sec at 95°C and 1 min at 60°C, and ended by 15 sec at 95°C, 15 sec at 60°C, 15 sec at 95°C.

Quantification of Alternaria species present on leaf infected material

Serial dilutions at 1/10; 1/100 and 1/1000 of the extracted DNA were amplified by Q-PCR. Dilutions of *A. alternata* and *A. solani* purified DNA between 10^{-2} to 10^{-7} µg/µl were used to plot standard curves. Leaf disks artificially infected by *A. solani* or *A. alternata* were mixed together in various proportions to determine standard curves of amplification. Primers efficacies were calculated with the SDS software (Applied biosystems). The fungal presence detection limit was set to Ct equivalent to non infected plant samples. Differences of Ct (cycle threshold) were used to determine the relative proportion of *A. alternata* and *A. solani* in the samples.

RESULTS

Cytochrome bc1 gene structure and species specific sequences

Primers as previously described (5) were used to amplify the corresponding sequence of the cytochrome *bc1* gene of five European strains of *Alternaria alternata*. After having sequenced the amplicons, it appeared that a novel intron was found downstream of the position 143. The codons related to the amino acids 129 and 143 remained localized on the same exon (Fig. 3). A similar approach was done on four European and 2 American strains of *Alternaria solani* (Fig. 4). The gene structure of two European strains was similar to that previously described (5). The sequences obtained for the two American strains and the other two European strains showed that the first intron differed from the one known. The sequence comparison showed 88% homology with the

corresponding intron of *Pyrenophora teres*. In addition the novel intron is localized between the codon encoding the amino acid 129 and that encoding the amino acid 143. In addition the size of the exons was modified.

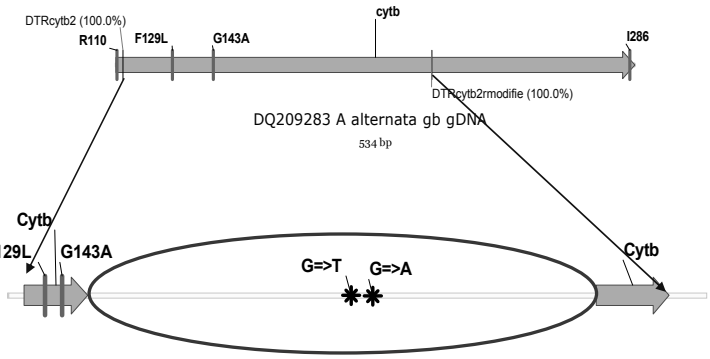


Figure 3: *A .alternata cytochrome bc1 gene structure. The upper part corresponds to the known structure (5), the lower part corresponds to those found in this study with a novel intron surrounded in red.*

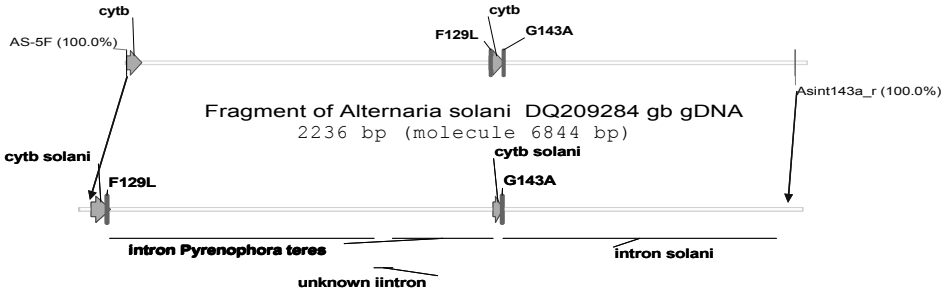


Figure 4: *A. solani cytochrome bc1 gene structure. The upper part corresponds to the known structure (5) found in two strains, the lower part corresponds to those found in this study with a novel intron with a significant homology to that found in P. teres.*

Quantitative PCR and quantification of Alternaria species present on infected leaves

For each fungal species four PCR primer sets were designed (cf. Material and Methods). After the optimization of their concentrations, primer specificity was assessed on the non targeted *Alternaria* species as well as the amplification efficacy of each couple of primers. DNA prepared from the mycelium was used. This allowed the selection of the final sets of forward and reverse primers for respectively *A. alternata* and *A. solani* as described in Materials and Methods.

DNA from potato leaves infected with either one or the other fungal species was then used to validate the selected primer sets. As control, DNA from non infected leaves was isolated. Using the primers specific for *A. alternata* a Ct of 31 was found whereas a Ct of 37 was observed with the *A. solani* specific primers. This showed that the plant DNA was not significantly interfering in the quantification of fungal DNA. Then 10 infected leaf discs infected with either *A. solani* or *A. alternata* were mixed in different ratio from respectively 10/0, 9/1, 8/2 to 0/10 (Table 2). Quantitative PCR on isolated DNA was performed to determine the ratio of each fungal species. The more accurate results (Table 2) were obtained using a dilution 1/10 of the DNA template.

Table 2*: % of DNA of each fungal species found by qPCR in different ratio of infected leaf tissues*

Number of infected leaf discs		% of DNA of each species	
A. alternata	*A. solani*	*A. alternata*	*A. solani*
0	10	0	100
1	9	30	70
2	8	56	44
3	7	65	35
4	6	72	28
5	5	82	18
6	4	86	14
7	3	88	12
8	2	91	9
9	1	95	5
10	0	100	0

The data obtained showed that the selected primers are specific to detect the DNA of each fungal species in extracts from infected leaf tissues. Only *A. alternata* was detected in the leaf samples not infected with *A. solani* and vice versa. Whereas it is not a linear relationship, when the proportion of leaf tissues infected with a given fungal species is increasing, the proportion of DNA corresponding to this species was also increasing. This led us to analyze samples harvested in the fields and compare the qPCR data obtained with the data obtained by biological analysis of the spore phenotype(s) and the field data observations (Table 3).

Table 3: *comparison of the fungal species characterization from the field symptoms, the spore phenotype analysis in the laboratory and the qPCR molecular diagnostic*

Sample code	Expectation/ Field symptôms	Conidia diagnostic	Molecular diagnostic % A. alternata - % A. solani	
07GE051 A	?	No spores	50% alt	50% sol
07NL020	?	No spores	93% alt	10% sol
07NL024	solani	alternata	100% alt	
07NL029	solani	alternata	100% alt	
07PL008	?	alternata	50% alt	50% sol
07PL009	?	alternata	85%alt	15%s ol
07PL010		alternata	100% alt	
07PL011		alternata	100% alt	
07PL059A		alternata	100% alt	
07PL059B	?	solani	14%alt	86% sol
07PL060B	?	alternata	80%alt	20% sol
08GE028	solani	alternata		100% sol
08GE029	alternata	alternata	95% alt	5% sol
08BE041	solani	solani		100% sol
08GE029	alternata	alternata	95% alt	5% sol

On the 15 representative samples analyzed, only 6 were characterized in the fields as having "typical" symptoms of *A. alternata* or *A. solani*. Two samples suspected as *A. solani* were identified as *A. alternata* only. Two were confirmed by qPCR as *A. solani* infected when 2 others were found a mixture *A. alternata* and *A. solani*.

In the laboratory, isolation of the fungi to be cultured on an artificial medium was successful for 13 samples on the 15. For each sample, the fungal species was determined by analysing the phenotype(s) of the spores. The qPCR analysis confirmed the data for 6 samples only. For the other 6 samples, whereas one species was clearly identified by biological analysis, the qPCR data clearly showed that a mixture between *A. solani* and *A. alternata* was present.

CONCLUSION AND PERSPECTIVE

Molecular diagnostic methods are necessary to improve the detection of plant pathogens (6). Our data showed that it is possible to set up a molecular based PCR diagnostic method using primers designed from the cytochrome *bc1* gene to determine the presence of *A. alternata* and *A. solani* in infected potato leaf tissues. The non linear relationships found by mixing known ratio of infected leaf tissues can be explained by the fact that the two fungal species showed different time course and ability to infect and multiply in the leaves. Nevertheless the technology appears to be robust, fast and reliable. Using the adequate primers, either by performing a second PCR or by establishing a multiplex approach, both the species and the mutation G143A and F129L could be assessed. With the evolution of the PCR technology, *e.g.* the geneXpert technology (8), it is possible to foresee applications at the field level.

The finding of the presence of an intron in the cytochrome *bc1* gene of *A. solani* closely related to the sequence of the intron found in *P.teres* suggests that a transfer of genes between the two species could have occurred. Whereas the relevance of Horizontal Gene Transfer (HGT) in eukaryotes is still under discussion, examples have been described in some species like *Candida parapsilosis* (9) and gene transfer between prokaryotes and fungi seems to occur relatively frequently as it has been recently reviewed (10). This suggests that exchange of gene between fungi is also possible and indeed it has been described (10, 11). What is the consequence of this exchange of genetic material on the evolution of the recipient species or on its pathogenic behaviour or its fitness? Are differences related to HGT between fungal clades? The development of molecular diagnostic will offer the possibility to find more of such events which will provide biological tools to study in more details and to better understand the evolution of fungal population.

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