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CHARACTERIZATION OF ALTERNARIA SPECIES ON POTATO

Towards a better understanding of the fungal genus in Flanders

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TABLE OF CONTENTS

TABLE OF CONTENTS **5**

ABBREVIATIONS **13**

PROBLEM STATEMENT AND DISSERTATION OUTLINE **17**

CHAPTER 1 - GENERAL INTRODUCTION **21**

Solanum tuberosum **23**

Origins, taxonomy and characteristics 23

Potato production in Belgium 25

Potato diseases and control 26

The genus *Alternaria* **33**

Taxonomy and morphological characteristics 33

Phylogeny 34

Disease cycle 36

Allergens and production of mycotoxins 38

Plant-pathogen interactions **46**

Effectors of pathogens with diverse lifestyles 46

Phytohormones and their role in plant defense 47

Phytohormones used as weapons by fungi 50

Pathogens tapping into the defense signaling network of plants *50*

Phytohormones produced by pathogens *51*

***Alternaria – Solanaceae* interactions** **54**

Disease management and fungicide resistance 54

Alternaria – tomato pathosystem 57

Symptoms and economic impact *57*

Molecular mechanisms of early blight on tomato *57*

Alternaria – potato pathosystem 59

Symptoms and economic impact *59*

Environmental influences *60*

CHAPTER 2 - ENVIRONMENTAL FACTORS ACCOUNT FOR THE DIFFERENCE IN DISEASE INCIDENCE BETWEEN TWO GROWING SEASONS 63

Abstract 65

Introduction 66

Materials and methods 68

Field monitoring 68

Environmental and window-pane analysis 70

Statistical analyses 71

Results 72

Field survey 72

Environmental analysis 73

Window-pane analysis 75

Discussion 77

Supporting information 80

Acknowledgements 81

CHAPTER 3 - THE FLEMISH ALTERNARIA POPULATION IS MORE DIVERSE THAN A TWO-SPECIES COMPLEX 83

Abstract 85

Introduction 86

Materials and methods 88

Identification of field isolates 88

PCR and sequencing 89

Diversity and phylogenetic analyses 89

Results **91**

Multi-locus sequencing analysis 91

Microscopic identification 92

Discussion **103**

Supporting information **105**

CHAPTER 4 - SPECIES PREVALENCE AND DISEASE PROGRESSION STUDIES DEMONSTRATE A SEASONAL SHIFT IN THE FLEMISH ALTERNARIA POPULATION COMPOSITION ON POTATO **109**

Abstract **111**

Introduction **112**

Materials and methods **114**

Real-time PCR 114

Identification of field isolates 114

Fitness test 115

Leaf disk virulence assay 115

Artificially inoculated field trial 116

Statistical analyses 116

Results **117**

Population composition study 117

Fitness test 118

Leaf disk virulence assay 118

Artificially inoculated field trial 122

Discussion **124**

Acknowledgements **127**

Addendum – *In vitro* virulence assay optimization **128**

Introduction 128

Methods and results 128
Trial 1 .... *128*
Trial 2 .... *129*
Trial 3 .... *129*
Trial 4 .... *129*
Trial 5 .... *130*
Conclusions 131
CHAPTER 5 - ETHYLENE PRODUCTION AND ITS ROLE DURING ALTERNARIA INFECTIONS ON POTATO 133
Abstract 135
Introduction 136
Materials and methods 139
Determining spore germination time frames and variability during infection 139
In vitro ethylene production 140
In planta ethylene production 140
Pharmacological assays 141
In vitro toxin analysis 141
In planta toxin analysis 142
Statistical analyses 144
Results 145
Variability of spore germination time frames on inoculated potato leaves 145
In vitro ethylene production 147
Ethylene production during *Alternaria* infections on potato leaves 149
Ethylene biosynthesis inhibition assay 150
In vitro toxin analysis 151
In planta toxin analysis 152
Discussion 154

Acknowledgements **159**

Addendum 1 – Optimization of the *in planta* infection technique for measuring ethylene emission **160**

Introduction 160

Methods and results 160

Infection assay 1 .... *160*

Infection assay 2 .... *161*

Infection assay 3 .... *161*

Conclusion 162

Addendum 2 – Optimization of a staining method for microscopy analysis of infected leaf discs **163**

Introduction 163

Methods and results 163

Paraffin embedding protocol 1 .... *163*

Paraffin embedding protocol 2 .... *163*

Trypan blue staining method *164*

Conclusion 164

CHAPTER 6 - GENERAL DISCUSSION AND FUTURE PERSPECTIVES **165**

General discussion **167**

Introduction 167

How much EB/BS disease incidence is there in Flanders? 167

The field survey demonstrated low disease incidence in two growing seasons *167*

How complex is the *Alternaria* population on Flemish potato fields? 168

The local Alternaria population is more elaborate than a two-species complex *168*

The multilocus sequence analysis demonstrates considerable inter- and intraspecific genetic variation *169*

What is the role of *A. alternata* and *A. solani* in the infection process? 170

Large-spored isolates are more virulent than small-spored isolates *170*

The detection of large-spored species in the field is in co-occurrence with an increase in disease incidence *171*

Alternaria species are able to produce ET in vitro via the KMBA pathway *173*

Infections with small-spored isolates cause higher ET emission levels than with large-spored isolates.. *174*

Small-spored species boost the plant ET biosynthesis, which is used by large-spored species to colonize the host faster *175*

What are the toxin profiles of *Alternaria* isolates encountered in Flanders?...................... 176

Small-spored Alternaria species display different toxin production profiles but these could not be linked with virulence or ET production *176*

Are abiotic stress factors triggers for *Alternaria* disease development?............................ 178

Sandy soils seem to be more prone to EB/BS disease *178*

Precipitation and relative humidity accounted for the differences in disease incidence between two growing seasons *179*

Future perspectives 181

Potato yield in relation to control measures.. 181

Sensitivity of the isolate collection to HGT 181

In silico and PCR analysis for ET biosynthesis enzymes and development of mutants... 182

In vitro and *in vivo* toxin production assay 182

Further exploration of the role of small- versus large-spored *Alternaria* species during an infection on potato leaves 183

Influence of nitrogen and drought stress on disease pressure in relation to sandy soils being more prone to EB/BS disease 183

Effect of temperature and relative humidity on EB/BS disease pressure 184

Correlation between temperature and concentration of small and large *Alternaria* spores in the air 184

Implement the data in existing forecasting models... 184

SUMMARY 187

SAMENVATTING 191

BIBLIOGRAPHY 195

CURRICULUM VITAE 221

DANKWOORD 227

ABBREVIATIONS

AAL-toxin	*Alternaria alternata f. sp. Lycopersici* toxin
AASC	*Alternaria arborescens* species complex
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACR-toxin	*Alternaria citri* (lemon pathotype) toxin
ACS	ACC synthase
ACT-toxin	*Alternaria citri* (tangerine pathotype) toxin
AF-toxin	*Alternaria alternata* (strawberry pathotype) toxin
AK-toxin	*Alternaria kikuchiana* (Japanese pear pathotype) toxin
ALT	Altenuene
Alt a 1	*Alternaria* major allergen gene
AME	Alternariol monomethyl ether
AM-toxin	*Alternaria mali* (apple pathotype) toxin
AOA	2-amino-oxyacetic acid
AOH	Alternariol
APLV	Andean potato latent virus
APMV	Andean potato mottle virus
APS	American Phytopathological Society
ASC	*Alternaria* stem cancer gene 1
AT-toxin	*Alternaria longipes* (tobacco pathotype) toxin
ATX	Altertoxin
AUDPC	Area under the disease progress curve
AVG	2-aminoethoxyvinyl glycine
BR	Brassinosteroid
CD	Conditionally dispensable
CK	Cytokinin
CMV	Cucumber mosaic virus
COI1	Coronatine insensitive 1
Cyt bc1	Cytochrome bc1
DAMP	Damage-associated molecular pattern

DMI	Demethylation inhibitor
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
Dpi	Days post inoculation
DSS	Decision support system
EB/BS	Early blight / brown spot disease
Ecp6	*Cladosporium fulvum* lysin motif effector 6
EDA	Epoxy-decatrienoic acid
EFE	Ethylene forming enzyme
EFSA	European Food and Safety Authority
EF-a	Translation elongation factor a
EIN2	Ethylene insensitive 2
EndoPG	Endopolygalacturonase
ERF	Ethylene response factor
ESI	Electrospray ionization
ET	Ethylene
ETS	Effector-triggered susceptibility
FAO	Food and Agriculture Organization
GA	Giberrelic acid
Gpd	Glyceraldehyde-3-phosphate dehydrogenase
HGT	Horizontal gene transfer
HR	Hypersensitive response
HST	Host-specific toxin
ICBN	International Code of Botanical Nomenclature
ICNCP	International Code of Nomenclature of Cultivated Plants
ICS1	Isochorismate synthase 1
IQR	Interquartile range
ITS	Internal transcibed spacer
JA	Jasmonic acid
K2P	Kimura-2-parameter distance
KMBA	α-keto-γ-methylthiobutyric acid
LAT	Degrees latitude
LC	Liquid chromatography
LON	Degrees longitude

LPAS	Laser-based photoacoustic spectroscopy
LS	Large-spored
LSU	Large subunit
MAPK	Mitogen-activated protein kinase
MCP	1-methylcyclopropene
MEA	Malt extract agar
MEK2	Mitogen-activated protein kinase cascade component
MET	L-methionine
ML	Maximum likelihood bootstrap support value
MLSA	Multi-locus sequence analysis
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog basal medium
MS/MS	Tandem mass spectrometry
MTA	5-methylthioadenosyl
MTR	5-methylthioribose
MTR-1P	5-methylthioribose-1-phosphate
MYC	MYC transcription factor
NPR1	Non-expressor of pathogenesis-related proteins 1
Oom	Oomycetes
OPA12-2	Anonymous *Alternaria* gene region
PAMP	Pathogen-associated molecular pattern
PAMV	Potato aucuba mosaic virus
PCA	Proefcentrum Aardappelteelt
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDB	Potato dextrose broth
Pep-13	Pattern-associated molecular peptide of 13 amino acids
PLP	Pyridoxal-5-phosphate
PP	Bayesian posterior probability
PR-protein	Pathogenesis-related protein
PRR	Pattern recognition receptor
PTI	Pattern-triggered immunity
PVA	Potato virus A
PVM	Potato virus M

PVS	Potato virus S
PVT	Potato virus T
PVX	Potato virus X
PVY	Potato virus Y
QoI	Quinone outside inhibitor
rAUDPC	Relative area under the disease progress curve
RH	Relative humidity
ROS	Reactive oxygen species
RPB2	RNA polymerase second largest subunit
R-protein	Resistance protein
SA	Salicylic acid
SAM	S-adenosylmethionine
SAMS	SAM synthetase
SAR	Systemic acquired resistance
SD	Standard deviation
SDHI	Succinate dehydrogenase inhibitor
SIPK	Salicylic acid-induced protein kinase
SL	Strigolactone
SNA	Synthetic nutrient agar
SS	Small-spored
SSU	Small subunit
STE	Stemphyltoxin
TBRV	Tomato black ring virus
TeA	Tenuazonic acid
TEN	Tentoxin
TNV	Tobacco necrosis virus
TRSV	Tobacco ringspot virus
TRV	Tobacco rattle virus
UPLC-MS	Ultra-performance liquid chromatography - mass spectrometry
VIGS	Virus-induced gene silencing
WIPK	Wound-induced protein kinase
WRKY45	WRKY45 transcription factor with a conserved WRKYGQK motif

PROBLEM STATEMENT AND DISSERTATION OUTLINE

With approximately 4 million tons produced each year, the potato is the second most important nutritional crop in Belgium, after sugar beet. Although China, India and Russia top the list in potato production, the highest yield is achieved in Belgium: around 52 tons per hectare in 2017 (PotatoPro, 2017). Moreover, 80 % of the total Belgian production is further processed or exported to other countries, a lot of those are third-world countries. Although most of the efforts made in the crop protection sector are against late blight disease caused by *Phytophthora infestans*, this disease is not the only one that endangers the yield and quality of potatoes. Disease symptoms caused by species of the fungal genus *Alternaria* are increasingly reported by local potato growers as problematic. A survey conducted in 2010 by the Flemish Agricultural Center of Potato Cultivation showed that 60 % of potato growers apply additional fungicides specifically against *Alternaria* species. However, in 2008 and 2009, disease symptoms caused by *Alternaria* species were observed by more than half of the questioned potato growers. For 20 % of the growers, symptoms were considered moderate and for 3 %, disease symptoms were reported as severe. The exact cause of the increase of *Alternaria*-caused disease symptoms remains elusive, although in professional literature a few causes are mentioned, such as climate change, the prohibition of organotin compounds and the restricted use of the fungicide mancozeb, shifts in the cultivation of specific cultivars that may be more susceptible to *Alternaria* infections, and a more economical fertilization (van der Waals, 2001).

Alternaria symptoms on potato are due to a disease complex caused by two species, *A. alternata* and *A. solani*. Previously, the former species was considered to be the causal agent of potato brown spot, while the latter causes potato early blight. However, both species are commonly found together on an infected leaf. Moreover, symptoms of both diseases are hard to discriminate without microscope analyses. For these reasons, *Alternaria* on potato is referred to as a disease complex.

Despite the increasing incidence, not much is known about the local *Alternaria* population found on potato fields, the factors contributing to the disease and its control. Indeed, some reports indicate that *A. alternata* is the first species that can be observed in the field (Hausladen et al., 2004; Hausladen, 2006; Hausladen and Leiminger, 2007; De Lange, 2010), while others claim that it is dependent on the environmental conditions of each growing season and that therefore, *A. solani* is sometimes first detected (Leiminger et al., 2010). Similarly, there exists some controversy on the pathogenicity of both species. Generally, *A. solani* is considered to be pathogenic, while *A. alternata* is reported as saprophytic and will only be pathogenic on weakened plant tissue (nutrient deficiency, drought stress, tissue damage, etc.) (Petrunak and Christ, 1992; Turkensteen et al.,

2010). Other researchers indicate *A. alternata* as a true plant pathogen (Hausladen et al., 2004). Intrinsic differences of both the fungus and host might be contributing to this ambiguity. Indeed, some authors have already reported large genetic variability among *Alternaria* species (Petrunak and Christ, 1992; van der Waals et al., 2003; Leiminger et al., 2010). Especially the intra- and interspecific diversity in toxin production seems to be considerable (Akamatsu et al., 1999; Foolad et al., 2000; Thomma, 2003; Leiminger et al., 2010). It is believed that this diversity could quickly render these pathogens more virulent and that consequently, it will become increasingly difficult to develop control strategies.

Next, little information is available on the effect of environmental factors on the disease development. While it is known that alternating dry and wet periods favor the growth of *Alternaria* hyphae (Bashi and Rotem, 1975; van der Waals et al., 2001), the effect of temperature is ambiguous (Holley et al., 1985; Ganie et al., 2015). Also, the type of soil seems to have an effect on disease incidence (Shtienberg et al., 1996). More and more publications also report a connection between *Alternaria* infections and plant hormones involved in abiotic stress. In that regard, the plant hormone ethylene was shown to be involved in *Alternaria* spore germination and mycelium growth (Kepczynska, 1994) and that ethylene production in tomato was increased after application of *Alternaria* toxins (Prasad and Upadhyay, 2010). These examples illustrate that ethylene, and possibly other stress hormones are involved in disease development caused by *Alternaria* species. However, if this is also the case in other host plants, such as potato, remains elusive.

Due to the lack of knowledge concerning genetic diversity and virulence profiling of the local *Alternaria* population on potatoes, or of environmental influencers of the disease, the current control strategies mostly imply the application of preventive fungicides. A better understanding of the disease progression, the population composition and genetic diversity and the effect of abiotic factors on disease development could ultimately lead to more integrated and sustainable disease management strategies. Indeed, most research on *Alternaria*-caused infections has been performed on tomato, whereas the interaction between these fungi and potato is largely unexplored. For these reasons, this research project aims to obtain insight in:

1. The genetic diversity, phylogenetic relationships, toxin profiles and population structure of the *Alternaria* population on Flemish potato fields.
2. The influence of environmental factors with respect to disease development and the role of abiotic stress hormones during *Alternaria* infections on potato.

To realize these objectives, the following research questions were posed:

1. How much disease incidence is there in Flemish potato fields?
2. How complex is the *Alternaria* population in the Flemish potato cultivation?
3. What is the role of *A. alternata* and *A. solani* in the infection process? Is *A. alternata* merely a saprophytic species or does it have a virulent nature?
4. What are the toxin profiles of *Alternaria* isolates encountered in Flanders? Are these related to the aggressiveness of the isolates?
5. Are abiotic stress factors triggers for *Alternaria* disease development? What is the role of abiotic stress hormones such as ethylene during an *Alternaria* infection on potato?

Throughout this manuscript, an answer to the above mentioned research questions is given (Fig. P1). Firstly, in **chapter 1**, some information on the potato plant is provided, highlighting its origin and characteristics, the production industry and its diseases. This is followed by an overview of the current knowledge on the genus *Alternaria* in which its taxonomy, life cycle and morphology is laid out. Then, some general insights are given on the interaction between plants and pathogens, concentrating on host plant immunity responses and the pathogen's virulence mechanisms before focusing on the interaction between *Alternaria* and two crops of the *Solanaceae* family, tomato and potato. Since this research project focuses on potato as the host plant, a field survey is presented in **chapter 2** in which the incidence of *Alternaria*-caused potato diseases is monitored during the growing seasons of 2014 and 2015. This survey is accompanied by an in-depth environmental analysis to reveal abiotic factors influencing disease incidence. In **chapter 3**, *Alternaria* isolates collected from the monitored field are investigated for intra- and interspecific genetic variation, while phylogenetic relations between the isolates are also inferred. This information, together with the results from the field survey fueled the study presented in **chapter 4**, where the *Alternaria* population composition during the two growing seasons is investigated, and in which an artificially infected field assay and an *in vitro* virulence analysis of the collected isolates are described. These studies elucidate the role of the different *Alternaria* species in the infection process and indicate that a difference in virulence between groups of species is the mechanism behind the observed disease progress curve in the field. In **chapter 5**, this mechanism is further explored by investigating the molecular machinery behind the difference in virulence between species. State-of-the-art hormone measurements affirm the significant role of the plant hormone ethylene during *Alternaria* infections on potato, while also revealing that virulent *Alternaria* species employ ethylene *in planta* and use it as a virulence factor. This research is concluded in **chapter 6**, where the main findings and hypotheses are discussed and some future research challenges are suggested.

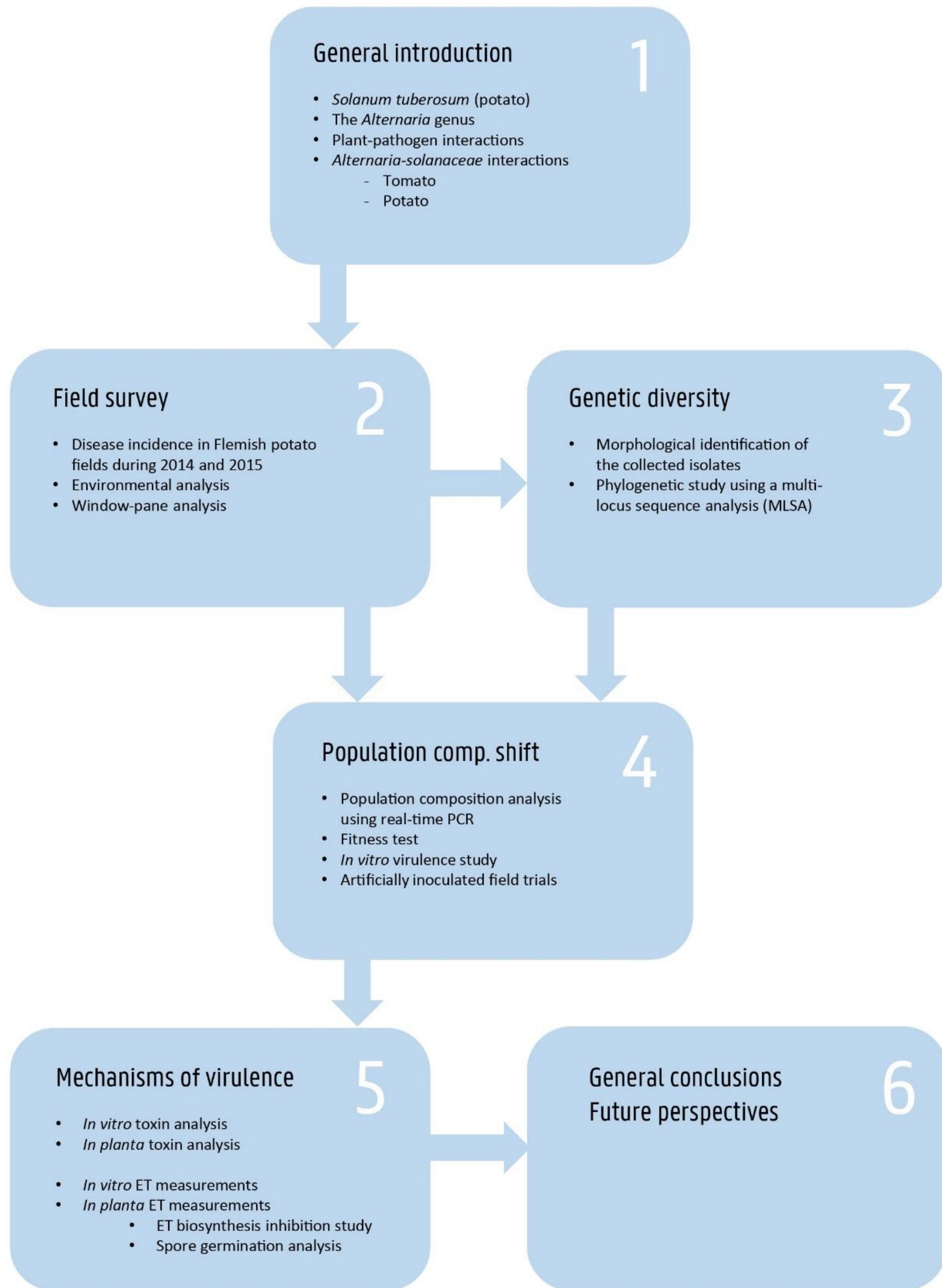

Figure P1. Schematic outline of the manuscript. Each box represents a chapter in which the most important analyses are outlined. ET: ethylene, PCR: polymerase chain reaction.

CHAPTER 1

GENERAL INTRODUCTION

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SOLANUM TUBEROSUM

Origins, taxonomy and characteristics

The potato plant, *Solanum tuberosum* L., is a tuberous crop grown in many parts of the world. It is grown in ca. 125 countries (Mullins et al., 2006) and contributes to a great share of the world's food supply since its tubers are in essence an energy reserve of carbohydrates. According to the Food and Agriculture Organization (FAO), the potato plant is the fourth most important food crop grown worldwide, after maize, wheat and rice (FAO, 2008).

The genus *Solanum*, a large and diverse group within the nightshade family (*Solanaceae*), encompasses predominantly plant species that are cultivated for their ornamental flowers. Although a significant number of edible crops can be classified within the *Solanaceae* family, only two food crops, potato and tomato, are found within the genus *Solanum*.

It is believed that the potato was originally domesticated in Peru and Bolivia, South America, approximately 8000 - 10000 years ago (Spooner et al., 2005), after which the Spanish have introduced it to Europe, following their conquest of the Inca Empire. Spooner et al. (2005) could trace its wild species progenitors to a monophyletic group of around 20 morphologically similar taxa, all part of one species complex. Today however, the cultivated potato exhibits large morphological and genetic diversity due to numerous events of introgression, interspecific hybridization or allopolyploidy, or due to the sexual compatibility between multiple species (Machida-Hirano, 2015). The taxonomy of the present cultivated species is therefore an ambiguous matter. They can be classified into separate species by the International Code of Botanical Nomenclature (ICBN) or into 'cultivar groups' by the International Code of Nomenclature of Cultivated Plants (ICNCP). If the classification by the ICBN is followed, potato plants have been classified into 21 species (Bukasov, 1971), nine species (Ochoa, 1990) or seven species (Hawkes, 1990). In contrast, Dodds (1962) classified cultivated potatoes by the ICNCP and recognized only three species with multiple 'cultivar-groups'. The term 'cultivar-group' is used by the ICNCP to delineate cultivated plants with similar traits that are of use for the agriculturist into taxonomic clades. Huamán and Spooner (2002) finally proposed a classification of all cultivated potatoes into four species: *S. tuberosum*, with two cultivar-groups, *S. ajanhuiri*, *S. juzepczuki* and *S. curtilobum*. To this day, potato taxonomists worldwide have yet to reach an agreement.

The most common cultivated species is *S. tuberosum*. Its morphology is highly dependent on variety. Flowers can be purple, white, blue or pink with yellow stamen (Winch, 2006). After flowering, green fruits are formed which contain up to 300 seeds. The fruits contain high dosages of the toxic alkaloid solanin and are not suited for consumption. Although new varieties can be

grown by planting the seeds from these fruits, potatoes are usually propagated vegetatively by planting tubers that are genetically identical to the parent (Winch, 2006). Most cultivated potato plants are tetraploid, containing 48 chromosomes, though there are varieties that are diploid (2n = 24), triploid (3n = 36) and even pentaploid (5n = 60) (Spooner et al., 2010). These improved varieties are commonly grown in the upland Andes and are thought to be more vigorous, possessing traits such as higher stress and disease tolerance, increased size and are adapted to grow at high altitudes (3000 - 4000 m above sea level).

S. tuberosum (4n = 48) commonly grows in regions with lower altitudes (height) and under moderate temperatures (optimally 18 - 20 °C) (FAO, 2008). Therefore, they are planted in early spring in temperate zones (Northern Europe and North America) and in winter in (sub)tropical regions (South America, Asia and Australia). As potatoes are very adaptable crops, they can be planted in any type of soil, except very alkaline or saline soils. A loose soil, with little resistance to tuber growth, rich in organic matter and good drainage (loamy and sandy soils) are considered as the most optimal. Soil moisture should be kept at a high level, while the frequency of applying fertilizer is dependent on available soil nutrients. When such optimal conditions are met, a healthy potato plant grows 20 to 60 cm in height with tubers extending to 300 g each, totaling approximately 25 to 35 tons per hectare (FAO, 2008). Worldwide, potato production goes up to 380 million tons each year (De Cicco and Jeanty, 2017), with China, India, Russia, Ukraine and the United States respectively being the countries with the highest production (Fig. 1.1).

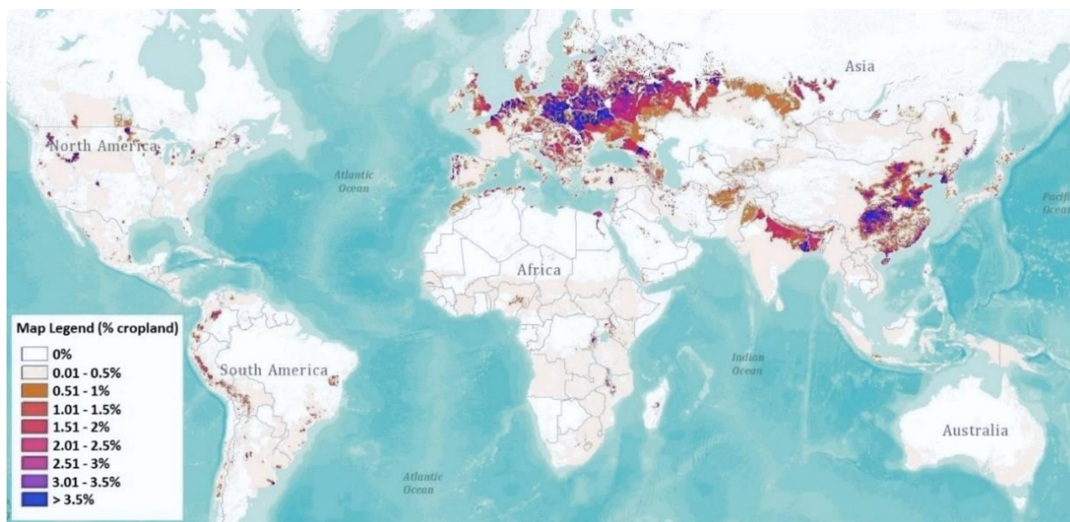


Figure 1.1. Worldwide geographical distribution of potato cultivation in 2014 (RTB-CGIAR, 2005).

These countries collectively produce ca. two-thirds of the total world production (FAO, 2008). Before the early 1990's, potato cultivation was primarily centered in Europe, North America and the former Soviet Union, with Europe being the world leader in potato production. Since then, there

has been a significant increase in Asian, South American and African production. Concerning consumption rates, Europe reaches the highest numbers compared to other continents (ca. 90 kg per capita per year) (FAO, 2008).

Potato production in Belgium

Although Belgium's potato area counts only 90 thousand hectares, the country ranks in the top 25 countries in terms of yearly potato production worldwide. In Europe, Belgium already accounts for 6.9 % of the total European potato production (Fig. 1.2), highlighting the very high yields that are achieved, bearing in mind that Belgium is only 0.2 % of the total area in Europe. Indeed, in 2017, Belgian potato yield peaked at 52 tons per hectare, making the country the highest yielding in the world (NEPG, 2018). Thanks to this, potatoes are Belgium's main food crop. Furthermore, a large portion of fresh and processed potatoes (accounting for over 1 billion euros) are being exported to other countries like France, The Netherlands and Germany (De Cicco and Jeanty, 2017).

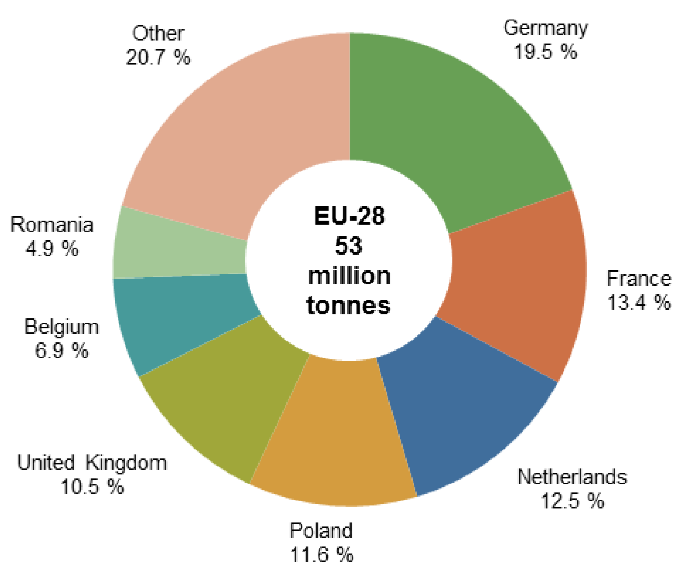


Figure 1.2. Potato production percentages for the main producing EU member States (De Cicco and Jeanty, 2017).

Two varieties, Bintje and Fontane, account for around 56 % of the cultivated potato area in Belgium (28 % each) (AHDH, 2017). The cultivation area of Bintje has declined in recent years, because growers are encouraged to move towards high yield varieties such as Innovator or Challenger. However, due to the low seed prices, Bintje is still the main variety cultivated for the fresh potato market and the processing industry. In point of fact, Belgium is the biggest exporter of frozen fries and other potato products worldwide, with more than 3.5 million tons of potatoes being processed into packaged products (Vilt, 2012). This means that, if annual potato production in Belgium

fluctuates around the four million tons mark, less than 15 % of Belgian potatoes is consumed fresh (De Cicco and Jeanty, 2017).

Potato diseases and control

The numbers in the paragraph above emphasize the need for protecting the Belgian potato industry against pathogens. On potato, around 60 diseases, either caused by fungal, oomycete, bacterial, viral or nematode pathogens (Table 1.1), are known to cause serious problems for the agriculturist. In this paragraph, the most economically important microbial diseases according to the Food and Agriculture Organization (FAO) will be discussed.

The main potato disease is undoubtedly late blight, caused by the necrotrophic oomycete *Phytophthora infestans*. Around the year 1845, this disease was responsible for the 'Great Famine', which killed millions of Irish, shrinking the population with over 50 percent. Together with poverty and political turmoil, this resulted in a large increase of emigrations to Great Britain, North America and Australia. Although not as significant as in Ireland, other Western European countries suffered from the late blight outbreak. Belgium, and in particular Flanders, lost around 100.000 lives during the same period (Vanhaute et al., 2006). More than 150 years later, late blight remains the number one potato disease worldwide, costing the potato growers an estimated 50 million dollars annually in crop protection chemicals (AHDH, 2017).
The first symptoms are characterized by dark (green) spots at leaf tips and margins that become brown or black depending on the air humidity. On the lower levels, a grey mildew surrounding the lesions indicates sporulation (Fig. 1.3). Symptoms may spread from one plant to the entire field in less than a week (Henfling, 1987). Stems can also get readily infected, resulting in the collapse of the plant. When secondary spores are washed from the foliage into the soil, the tubers can get infected. Tuber symptoms are usually visible by superficial brown, purple or reddish lesions which easily penetrate tuber tissue. Secondary soft rot bacteria can then invade the already formed lesions, producing a rotten smell (Schumann and D'Arcy, 2000).
Late blight can be kept under control by a combination of multiple agricultural practices (Schumann and D'Arcy, 2000). Pre-planting management can include the usage of more resistant cultivars (such as Sarpo Mira, Innovator or Markies), applying good drainage to reduce moisture levels, removing volunteer plants (that grow on a field despite not being deliberately planted or sown and may be susceptible to late blight) or applying non-host crop rotations. Post-planting control measures mostly encompass fungicide sprays (mainly contact fungicides such as mancozeb to prevent infection). Irrigation should be controlled carefully because it affects leaf wetness, which is known to increase the risk of infection.

Figure 1.3. Late blight symptoms on potato foliage. The white halo surrounding the black lesions indicates sporulation (Cornell, 2005).

Early blight, caused by *Alternaria solani*, is in many regions considered as the second most important potato disease, after late blight. *Alternaria*-caused diseases on potato will be discussed extensively in the following paragraphs and chapters.

Stem canker or black scurf is another widespread potato disease caused by the fungus *Rhizoctonia solani*. This disease can either be soil-borne, causing black scurf symptoms or tuber-borne, causing stem canker and generally occurs in cold soils where tuber growth is inhibited (Beagle-Ristaino and Papavizas, 1985). Indeed, planting in temperate soils to get potato plants to emerge quickly, is crucial for managing this disease as well as disinfecting tubers. Black scurf symptoms on tubers are superficial crusts (sclerotia) that can be scratched off. Stem canker can be visualized as reddish brown and sunken necrotic patches with distinct edges on the stems and stolons of infected plants. These symptoms can be easily mistaken for blackleg symptoms, caused by soft rot bacteria such as *Pectobacterium carotovora spp.*, *Pectobacterium chrysanthemi* and *Dickeya solani*.

Blackleg or soft rot disease is one of the most serious bacterial diseases on potato (Perombelon, 2002). The causal agents (*Pectobacterium spp.* and *Dickeya solani*) are opportunistic pathogens that often remain dormant in tubers at the onset of infection. Favorable conditions (water on the tubers, moderate temperature, oxygen depletion) will trigger breakdown of latency and rotting. Rotting of the stem (blackleg) usually occurs when large numbers of bacteria invade the stem after colonization of the tubers and conditions are favorable again (after heavy rainfall). Restriction of water flow in the xylem vessels subsequently results in stunting, wilting and chlorosis of the foliage (Perombelon, 2002). Management measures are mainly the use of disease-free seed potatoes and decontaminated equipment since the disease is easily transferred by contact between plant parts. Once a plant is infected, it cannot be salvaged and it has to be removed (De Boer and Rubio, 2004).

Another bacterial disease that is spread worldwide is (common) scab, caused by *Streptomyces scabies*. In contrast to the previous described diseases, scab is restricted to tuber infection. Especially in dry seasons and in regions where irrigation is not available, these pathogens can form a real problem. They form rough, scabby patches on tubers that lead to dark brown, raised or pitted lesions covering the entire tuber surface and to tuber distortions. Common scab can be kept to a minimum in acid soils (pH 5.2 and below). Powdery scab symptoms (caused by *Spongospora subterranean*) are very similar as those of common scab but tend to be smaller and lighter in color (Mulder and Turkensteen, 2005).

Fungal and bacterial pathogens are not the only threat to cultivated potato plants. Indeed, also viruses can cause real problems for the potato cultivator. Leafroll is a common viral disease that is transferred mainly by aphids. The virus is very persistent, meaning that once an aphid carries the virus, it is infective for life. As the name suggests, symptoms are the curling or ‘rolling’ of the leaves. This can be a very slight curl in the first year of infection accompanied by a mild discoloration in the upper layers of the plant. The second year, the plant will be fully infected, with rolling occurring in the lower layers of the foliage and leaves that feel dry and brittle (Taliansky et al., 2003). Leafroll can be effectively controlled by using healthy seed and applying aphicides.

Table 1.1. List of known microbial (bacterial, oomycete (oom.), fungal, nematode and viral (vir.)) diseases on potato (adopted and modified from O'Brien and Rich (1976), International Potato Center (1996), Mulder and Turkensteen (2005)).

	Disease name	Causal agent	Plant parts affected	Common modes of transmission
Bacteria	Blackleg / soft rot	*Pectobacterium spp. / Dickeya solani*	Tubers, stem, leaves	Soil, water
	Brown rot / bacterial wilt	*Ralstonia solanacearum*	Tubers, leaves	Tubers and soil
	Ring rot	*Clavibacter michiganensis*	Tubers, stem, leaves	Tubers, equipment
	Scab (common)	*Streptomyces scabies*	Tubers	Soil, seeds, rotational crops
Oom.	Late blight	*Phytophthora infestans*	Tubers, stem, leaves	Air, water, seeds
	Leak	*Pythium ultimum*	Tubers	Soil
Fungi	Black dot	*Colletotrichum coccodes*	Roots, stolons, stems, leaves	Soil
	Black rot	*Rosellinia spp.*	Roots, tubers, stems	Soil
	Brown spot	*Alternaria alternata*	Tubers, leaves	Air, soil
	Botrytis gray mold	*Botrytis cinerea*	Leaves	Air, water
	Charcoal rot	*Macrophomina phaseolina*	Tubers	Soil
	Common rust	*Puccinia pittieriana*	Leaves	Wind,
	Early blight	*Alternaria solani*	Tubers, leaves	Air, soil
	Fusarium wilt	*Fusarium spp.*	Tubers, stem, leaves	Soil, seeds
	Dry rot	*Fusarium solani*	Tubers	Soil
	Gangrene	*Phoma exigua (var. foveata)*	Tubers, stem	Soil
	Phoma leaf spot	*Phoma andina*	Leaves	Soil

(Table 1.1 continued)

	Disease name	Causal agent	Plant parts affected	Common modes of transmission
Fungi	Pink rot	*Phytophthora erythroseptica*	Tubers	Soil
	Powdery mildew	*Erysiphe cichoracearum*	Stem, leaves	Air
	Septoria leaf spot	*Septoria lycopersici*	Leaves	Soil, water
	Stem canker	*Rhizoctonia solani and Rhizoctonia spp. anastomosis groups*	Tubers, stolons, stem	Soil, seeds
	Scab (powdery)	*Spongospora subterranea*	Tubers	Soil, seeds
	Stem rot	*Sclerotium rolfsii*	Tubers, stem	Soil, plant parts
	Silver scurf	*Helminthosporium solani*	Tubers	Soil, seeds
	Skin spot	*Polyscytalum postulans*	Tubers, stem	Soil, seeds
	Tecaphora smut	*Thecaphora solani*	Tubers, stolons, stem	Soil, seeds
	Verticillium wilt	*Verticillium spp.*	Tubers, stem, leaves	Soil, tubers
	Violet root rot	*Helicobasidium purpureum*	Tubers	Soil
	Wart	*Synchytrium endobioticum*	Tubers, stolons, stem	Soil, seeds
	White mold	*Sclerotinia sclerotiorum*	Tubers, stem, leaves	Soil, air, water
Nematodes	Cyst nematode	*Globodera pallida/rostochiensis*	Roots	Soil, equipment
	False root-knot nematode	*Nacobbus aberrans*	Roots	Soil, tubers
	Lesion nematodes	*Pratylenchus spp.*	Roots, tubers	Soil, seeds
	Potato rot nematode	*Ditylenchus destructor*	Tubers, stolons, stem	Soil, seeds
	Root-knot nematode	*Meloidogyne spp.*	Roots, tubers	Soil

(Table 1.1 continued)

	Disease name	Causal agent	Plant parts affected	Common modes of transmission
Viruses	APLV	Andean potato latent virus	Leaves	Sap, beetles
	APMV	Andean potato mottle virus	Leaves	Sap, beetles
	Alfalfa (Calico)	Alfalfa mosaic virus	Leaves	Sap, aphids
	CMV	Cucumber mosaic virus	Leaves	Sap, aphids
	Deforming mosaic	Deforming mosaic virus	Leaves	Grafts
	Leafroll mosaic (PVM)	Potato virus M	Leaves	Sap, aphids, tubers
	Latent mosaic (PVX)	Potato virus X	Leaves	Sap, tubers
	Leafroll	Leafroll virus	Leaves	Aphids, tubers
	Mild mosaic (PVA)	Potato virus A	Leaves	Aphids, tubers
	Mop-top	Potato mop-top virus	Tubers, leaves	Soil, fungal vector
	Tuber blotch (PAMV)	Potato aucuba mosaic virus (F)	Tubers, leaves	Sap, aphids
	PVS	Potato virus S	Leaves	Sap, aphids
	PVT	Potato virus T	Leaves	Sap, tubers
	Rugose mosaic (PVY)	Potato virus Y	Leaves	Aphids
	Spindle tuber	Potato spindle tuber viroid	Tubers, leaves	Equipment, seeds, insects
	TNV	Tobacco necrosis virus	Tubers	Fungal vector
	TRV	Tobacco rattle virus	Tubers, stems, leaves	Nematodes
	TRSV	Tobacco ringspot virus	Leaves	Nematodes, seeds
	TBRV	Tomato black ring virus	Leaves	Soil, nematodes, weeds
	Tomato spotted wilt	Tomato spotted wilt virus	Tubers, stems, leaves	Thrips vector

(Table 1.1 continued)

	Disease name	Causal agent	Plant parts affected	Common modes of transmission
Vir.	Yellow dwarf	Potato yellow dwarf virus	Tubers, stems, leaves	Equipment, leafhoppers
	Yellow vein	Potato yellow vein disease virus	Tubers, leaves	Whitefly
	Haywire	Beet curly-top virus	Tubers, leaves	Seeds, beet leafhoppers

THE GENUS ALTERNARIA

Taxonomy and morphological characteristics

The fungal genus *Alternaria* encompasses for the most part saprophytic species that live on dead plant debris and that can be abundantly found in the soil. Some species, however, are (opportunistic) pathogens exhibiting a broad variety of host plants such as potato, tomato, carrot, broccoli, cauliflower, apple, citrus, ornamentals and cereals (Thomma, 2003).

Alternaria species are ascomycetes that were formerly classified as *Fungi imperfecti* because most of the species lack any known sexual (teleomorph) stage during their life cycle (Table 1.2). The *Fungi Imperfecti* are a non-phylogenetic class of fungi that do not fit into the common classification, which is based on morphological characteristics of sexual structures. Indeed, fungi belonging to this class, produce their spores asexually. They were also categorized as 'form taxons' because of the same reason. Since 2011, fungi are divided by the International Code of Nomenclature for algae, fungi and plants, which subsequently updated the *Alternaria* taxonomy (Table 1.2). Very few species are known to have a teleomorph (sexual) stage. Those that do, are now placed in the genus of *Pleospora* (El-Alwany, 2015; Neeraj and Verma, 2010).

Table 1.2. Former and new taxonomy of the genus *Alternaria*.

Taxonomy	Former	New
Kingdom	*Fungi*	*Fungi*
Phylum	*Fungi Imperfecti* (form-taxon)	*Ascomycota*
Class	*Hypomycetes* (form-taxon)	*Dothideomycetes*
Order	*Monilialis* (form-taxon)	*Pleosporales*
Family	*Dematiaceae* (form-taxon)	*Pleosporaceae*
Genus	*Alternaria* (form-taxon)	*Alternaria / Pleospora*

The genus *Alternaria* was first described by Nees von Esenbeck in 1816 as a single species, *A. tenuis* (now *A. alternata*) (Nees von Esenbeck, 1816). He expressed that the species was characterized by the production of large, dark and multi-cellular conidiospores (50 - 200 µm in length) exhibiting longitudinal and transverse septa (Ellis and Gibson, 1975; Neergaard, 1945). The spores have an elongated beak and gradually become broader at the base, exhibiting a drop-like or a baseball bat-like appearance. The establishment of other *Alternaria* species show that spores are produced on conidiophores as single spores or in (branched) chains, depending on the species (Fig. 1.4). This structure of spore placement is referred to as spore catenulation. *Alternaria* spores are dark-colored due to the presence of the pigment melanin in the primary cell walls and in the

septa (Thomma, 2003). Since disrupting melanin biosynthesis reduces spore size and septa, the pigment is thought to be actively involved in conidial development (Kawamura et al., 1999). Besides this, melanin also acts as a barrier against environmental stress, like extreme temperatures or UV radiation, adding to the survival of the spores (Rehnstrom and Free, 1996). In *Magnaporthe* and *Colletotrichum* species, it has even been demonstrated that melanin has a direct function in virulence (Howard and Valent, 1996). Indeed, mutants deficient in melanin accumulation did not form appressoria structures necessary for penetrating the host.

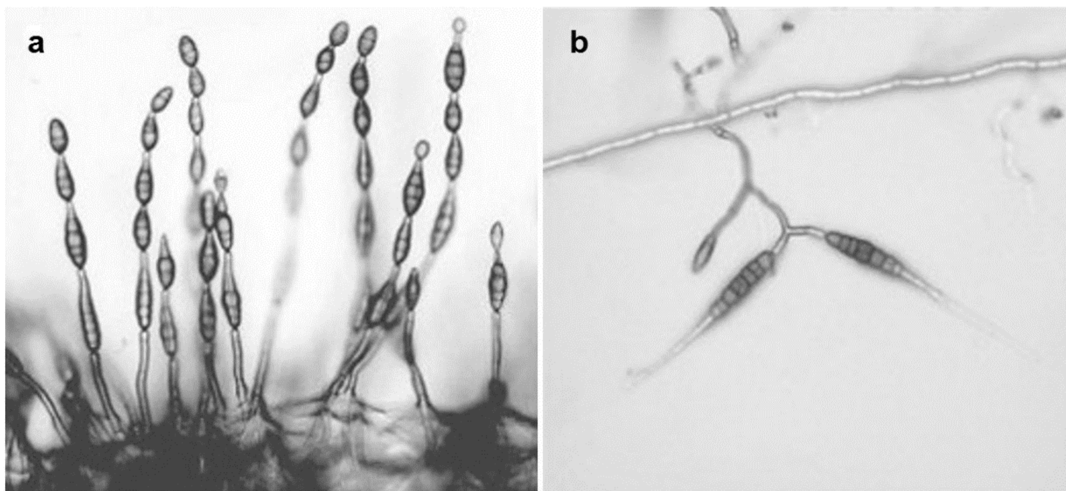


Figure 1.4. Drop-like conidia in chains produced by *A. alternata* (a) and baseball bat-like single conidia produced by *A. solani* (b). Adapted from McKenzie (2013).

Alternaria colony morphology varies widely, but usually appears as white or gray 'wool-like' molds that grow darker with age. Their mycelium is septate and generally short, branched and multinucleate (Thomma, 2003; van der Waals et al., 2001). When grown on culturing media, *Alternaria* colonies show distinct concentric rings which is inherent to growth interruptions. The 'woolly' appearance of the colony is due to the production of aerial mycelium, whereas the discoloration of the medium can be attributed to the production of dark pigments (van der Waals et al., 2001).

Phylogeny

Since the first descriptions of the genus, taxonomists have disagreed on the classification of *Alternaria* species. To illustrate this, Nees von Esenbeck (1816) was the first to describe a species of the genus *Alternaria*, namely *A. tenuis*. Shortly after, Fries (1832) was unwilling to acknowledge this species and proposed an alternative classification: *Torula alternata*. Eighty years later, von Keissler (1912) proposed to synonymize both species with *Alternaria alternata*, due to uncertainties

in the former descriptions. Due to the morphological variability, its dependency on environmental conditions and subsequent re-descriptions (Elliott, 1917; Wiltshire, 1933), a plethora of different species arose, whether or not its taxonomy was justified. In 2007, Simmons summarized 275 species based upon morphological characteristics (Simmons, 2007). Although classification methods based on morphological characteristics are still widely used, molecular methods combined with phylogenetic analyses is considered more accurate in delineating species within genera. When phylogenetic species groups correlate with groups based on morphological characteristics, it provides highly reliable evidence for the proposed species-group, although according to many reports, results from the two methods do not always correlate (Pryor and Gilbertson, 2000; Chou and Wu, 2002; Pryor and Bigelow, 2003; Wang et al., 2011; Lawrence et al., 2016).

Recently, based on multi-gene phylogeny, the *Alternaria* genus has been subdivided into 24 species clades called 'sections'. (Woudenberg et al., 2013a; Woudenberg et al., 2013b). The genomic regions used in this study were parts of the 18S nrDNA (*SSU*), 28S nrDNA (*LSU*), the internal transcribed spacer (*ITS*) regions, glyceraldehyde-3-phosphate dehydrogenase (*Gpd*), the RNA polymerase second largest subunit (*RPB2*) and the translation elongation factor a (*EF-a*). The phylogenetic tree based on sequences of these genomic regions revealed a well-supported node that distinguishes *Embellisia annulata*, together with a sister clade of *Pleospora* / *Stemphylium* species from a large *Alternaria* clade holding 24 phylogenetically distinct sections. Within these sections, many allied genera that were differently classified before are now synonymized under the *Alternaria* clade, among which are *Allewia*, *Brachycladium*, *Chalastospora*, *Crivellia*, *Sinomyces* and *Ulocladium* species (Fig 1.5). For full descriptions of the different species within *Alternaria*, we kindly refer to the study by Woudenberg et al. (2013a).

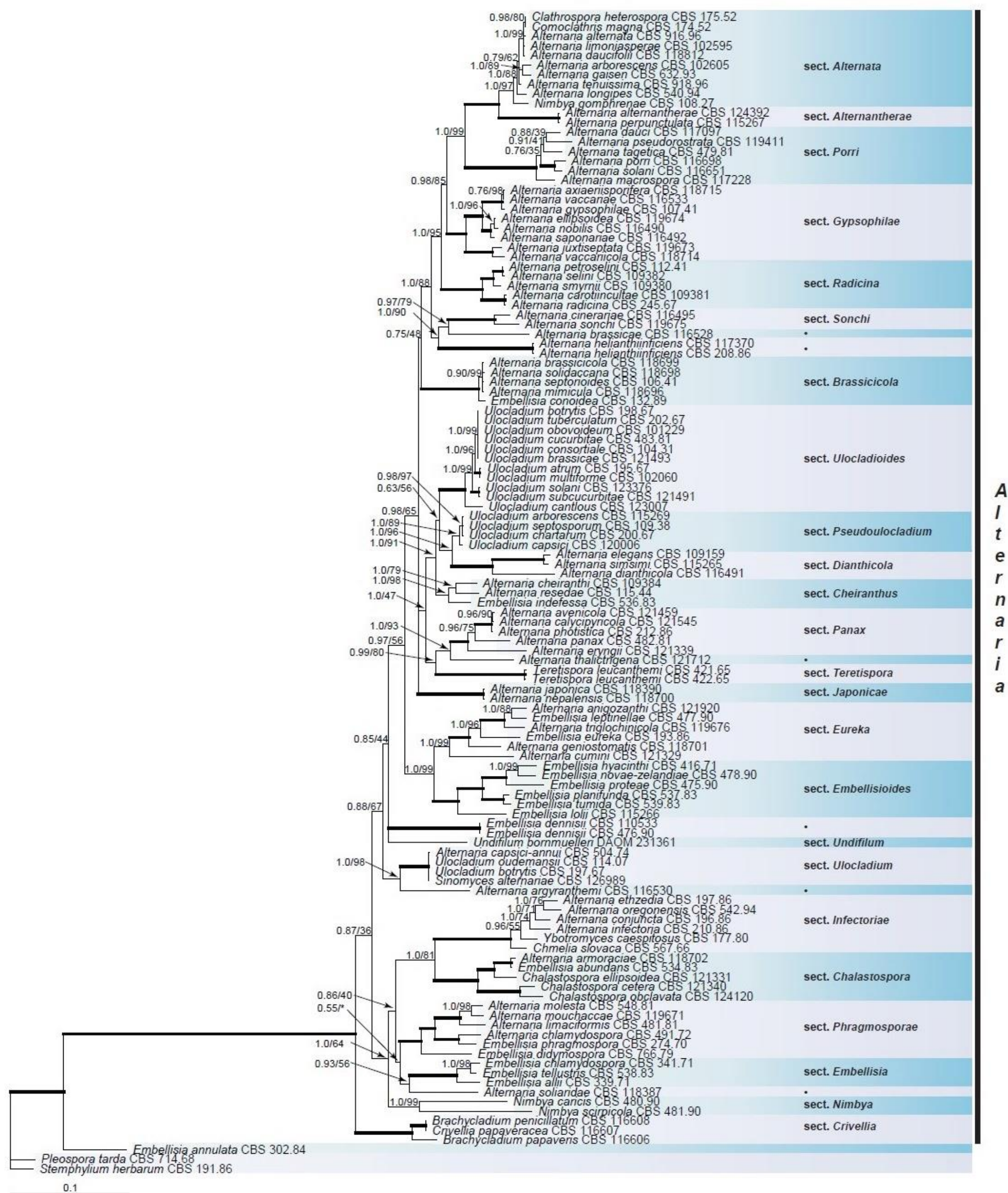


Figure 1.5. Consensus phylogeny tree of the different sections within the genus *Alternaria*. The tree is based on the *Gpd*, *RPB2* and *EF-a* sequences of 121 strains. Numbers on the nodes indicate Bayesian posterior probabilities (PP) and Maximum likelihood bootstrap support values (ML) (PP / ML). Thickened lines indicate a PP / ML ratio of 1 / 100 (Woudenberg et al., 2013a).

Disease cycle

When a plant seed is infected by *Alternaria*, the fungus can survive in the seed and will remain in a latent stage until the seed germinates. If the fungus is not seed-borne, it can persevere for extended periods of time as spores and even as mycelium on dead plant material, in the soil, on

infected tubers or other susceptible crops (Rotem, 1994; van der Waals et al., 2001; Wharton and Kirk, 2007). Once an *Alternaria* spore lands on the leaf surface after being dispersed by wind or by rain, it can infect the plant under favorable conditions. Spore germination will occur in spring when the leaves are moist and temperatures are between 20 and 30 °C. The drying of the foliage as the morning dew disappears, also stimulates infection (Rotem, 1994). This infecting spore now serves as the primary inoculum to initiate disease. The germ tubes originating from the spore will penetrate wounds or stomata, which is especially the case for less virulent species. More virulent species can penetrate directly through the cuticle by the formation of an appressorium, which is essentially a specialized ‘drilling’ structure that uses turgor pressure to serve its function (Rotem, 1994; Thomma, 2003; Wharton and Kirk, 2007). The plant’s first line of defense is generally the cuticle, which is composed of cutin, a hydroxyl fatty acid polyester, and waxes. To overcome this, appressoria also produce a combination of cutinases, lipases and galacturonidases to break down fatty acid and polysaccharide polymers respectively (Berto et al., 1997; Fan and Koller, 1998; Isshiki et al., 2001). Moreover, Eshel et al. (2002) demonstrated that, during an *A. alternata* infection, a self-induced increase in pH (by ammonia secretion) triggers the production of fungal endoglucanases. Indeed, the modulation of environmental pH is a well-studied strategy for a number of fungal pathogens (Vylkova, 2017). Next to penetration mechanisms and enzymatic processes, also the production of mycotoxins adds to weakening the host for infection (Ballio, 1991). The production and variety of *Alternaria* mycotoxins will be discussed below in the section ‘Allergens and production of mycotoxins’.

Once the pathogen is past the epidermal layer, its hyphae will first grow intercellularly before invading and depriving the host cells of nutrients. Two to four days after infection, symptoms will begin to form. An infection with *Alternaria* species causes characteristic necrotic lesions, leading to a decrease in photosynthetic capacity. The lesions are generally restricted by leaf veins and show a target-like appearance, often called the ‘bullseye-type lesion’ (Fig.1.6), which is thought to be the result of growth interruptions due to varying environmental conditions (Pscheidt, 1985; Thomma, 2003). Besides this, lesions are also identified by a yellow uninvaded halo that surrounds the necrotic tissue. This chlorotic zone is an effect of the diffusion of secondary metabolites produced by the fungus residing in the necrotic center (Agarwal et al., 1997; Tewari, 1983).

In the next stage of the *Alternaria* life cycle, secondary spores will be produced on necrotic foliar tissue. Alternating wet and dry periods, combined with an optimal temperature of around 20 °C, are ideal conditions for secondary spore production. The spores will accumulate for seven to fourteen days to be subsequently dispersed by wind or by rain splashes (van der Waals et al., 2001; Wharton and Kirk, 2007). These spores now serve as secondary inoculum that can infect neighboring plants,

thereby ending one disease cycle. *Alternaria* is indeed a polycyclic fungus, meaning that multiple rounds of secondary spore production will occur in one growing season, leading to an exponential increase of affected plants and disease symptoms. Moreover, as *Alternaria* pathogens mainly target mature or senescing plants that have been injured or that have suffered nutrient deficits (Wharton and Kirk, 2007), the incidence will escalate dramatically if no control measures are undertaken.

Figure 1.6. Necrotic 'bulls-eye' lesions and a yellow halo caused by *Alternaria* species on tomato (Kemmit, 2002).

Allergens and production of mycotoxins

Besides saprophytes or pathogens, *Alternaria* species are common allergens in humans, causing hay fever or asthma (Bush and Prochnau, 2004; Lawrence et al., 2008). The spores produced by *Alternaria* species are present in large quantities in the air during the summer with concentrations exceeding 500 spores / m^3 several times every season (AirAllergy.be, 2017). Immunocompromised patients can even develop skin infections or infections of mucous membranes, such as on eye balls, due to *Alternaria* spores. Disorders like this are commonly called alternariosis or alternariatoxicosis (Matson et al., 2010).

One of the main hazards of *Alternaria*-infected food or feed commodities is the presence of mycotoxins; low-molecular weight secondary metabolites, produced by the fungus and harmful to human and animal health. The production of such toxins can also occur at low temperatures, which can pose a major problem for refrigerated food and feed storage (Solhaug et al., 2016). Some of these mycotoxins also have phytotoxic effects (Table 1.3) and are regarded as additional virulence factors that can aid cell penetration and enhance disease symptoms, but that are not essential for the disease (Ballio, 1991). *Alternaria* species are known to produce over 70 toxins, which can be classified into diverse chemical groups (Table 1.3). *Alternaria* phytotoxins can also be divided as host-specific or non-host specific. According to EFSA, the most relevant non-host specific *Alternaria* toxins in terms of human and animal health are alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA) and altenuene (ALT) (EFSA, 2011).

AOH, AME and ALT (Fig. 1.7) belong to the group of dibenzo-α-pyrones and show high acute toxicity in mammalian cells. However, only the effects of AOH and AME have been identified on a molecular level. Indeed, they are known to form reactive oxygen species (ROS) and interact with DNA topoisomerases, thereby causing DNA strand breaks. In mammalian cells, these events trigger cell cycle arrest, leading to a reduction in cell proliferation, especially in the gastrointestinal tract and esophagus (Lehmann et al., 2006; Solhaug et al., 2016). These types of DNA strand damage and the formation of hydroxylated metabolites by toxin-induced cytochrome P450 enzymes can lead to carcinogenesis (Schreck et al., 2012), which is why these toxins are extensively studied as food and feed contaminants. In contrast, few attempts have been made to investigate the phytotoxic effect of AOH and AME. One study reported that AME inhibits the photosynthetic electron transport chain in isolated spinach chloroplasts (Demuner et al., 2013).
On the other hand, the phytotoxic effect of tentoxin (TEN) (Fig. 1.7), a cyclic tetrapeptide, is well known and focuses on the inhibition of chloroplast development rather than the electron transport chain (Klotz, 1988). Asam and Rychlik (2015) reported that TEN also inhibits photophosphorylation and induces chlorosis. For this toxin, no toxicological data is available for humans or other mammals.
Tenuazonic acid (Fig. 1.7), an amine metabolite that is considered the most acutely toxic among the *Alternaria* mycotoxins, has been shown to be toxic in several animal species like mice, guinea pigs, rabbits, chickens and monkeys (Solfrizzo et al., 2004; Asam and Rychlik, 2013; Hickert et al., 2015; Cabral et al., 2016) This mycotoxin has been investigated widely due to its inhibitory effect on cytoplasmic mRNA translation (Rychlik, 2012). In mice, its LD_{50} value is similar to the mycotoxin deoxynivalenol (DON), produced by *Fusarium* species (Asam and Rychlik, 2013), which has similar toxic effects as TeA and the capability to induce apoptosis (Pestka, 2010). Just like DON, an acute 33 day-trial on monkeys fed with 89.6 mg TeA kg^{-1} bw led to vomiting, bloody diarrhea and hemorrhagic lesions in the intestinal tract (Hickert et al., 2015). In 1976, TeA was found to be responsible for the outbreak of onyalay, a human hematologic disorder in Africa, and esophageal cancer in Linxian province in China due to the high *A. alternata* contaminations in cereal grains (Steyn and Rabie, 1976). In plants, TeA toxicity can be explained by the strong inhibition of photosystem II activity which hampers the mitochondrial electron transport chain (Chen et al., 2014).
Another group of non-host specific mycotoxins are the perylene quinones altertoxin-I, -II and -III (ATX-I, -II and -III) and stemphyltoxin-I and -III (STE I and III) (Fig. 1.7). Although very few studies have been performed due to a lack of available reference compounds, they are still regarded critical because of their mutagenic properties. Like AOH and AME, they cause DNA strand breaks, with

ATX II being even more mutagenic than the former two mycotoxins (Fleck et al., 2012). However, knowledge about the underlying modes of action is still missing.

In contrast to the non-host specific compounds, host-specific mycotoxins are pathogen effectors that cause severe effects only in certain host plants (Tsuge et al., 2013). Multiple host-specific toxins have been identified. However, one toxin, the *Alternaria alternata f. sp. lycopersici* (AAL) toxin (Fig. 1.8), is well characterized. This toxin is an aminopentol ester and is produced by *A. alternata f. sp. lycopersici*, which is pathogenic on tomatoes. AAL-toxin inhibits the biosynthesis of ceramides, which can be abundantly found in the lipid bilayer of cell membranes. This inhibition leads to an accumulation of sphingosine precursors and the subsequent depletion of sphingosines, ultimately causing cell death. A detailed description of the action of AAL-toxin on programmed cell death can be found below in the section: '*Molecular mechanisms of early blight on tomato*'. AAL-toxin is similar to fumonisin B1, which is known to cause neural tube defects and esophageal cancer in humans (Stockmann-Juvala and Savolainen, 2008). Other *Alternaria* host-specific toxins include AM-, AF-, AK-, ACT-, ACR- and AT-toxins (Table 1.3), produced by respectively *A. mali* (apple pathotype), *A. alternata* (strawberry pathotype), *A. kikuchiana* (Japanese pear pathotype), *A. citri* (tangerine pathotype), *A. citri* (lemon pathotype) and *A. longipes* (tobacco pathotype) (Tsuge et al., 2013). These make a total of seven different diseases caused by host-specific toxins that are known to this date. AM-toxins (Fig. 1.8) are cyclic tetrapeptides and bring about invaginations in the plasma membrane and a reduction in photosynthetic activity in chloroplasts (Kohmoto et al., 1982).The AF-, AK- and ACT-toxins (Fig. 1.8) are structurally analogous esters of epoxy-decatrienoic acid (EDA) and cause a rapid loss in K^+, leading to plasma membrane dysfunction (Maekawa et al., 1984; Otani, 1985; Kohmoto et al., 1993; Park and Ikeda, 2008). ACR-toxins are polyalcohols with a dihydropyrone ring (Fig. 1.8) that cause uncoupling of mitochondrial oxidative phosphorylation leading to mitochondrion dysfunction (Akimitsu et al., 1989). AT-toxin also affects the mitochondria though its chemical structure is currently not known (Tsuge et al., 2013).

During a study by Akamatsu et al. (1999), it was found that phytopathogenic strains of *A. alternata* showed small (< 2.0 Mb) chromosomes after pulsed-field electrophoresis analysis of the genome, while non-pathogenic strains did not have these small chromosomes. These chromosomes are also called supernumerary or conditionally dispensable (CD) chromosomes because they are not essential for growth or reproduction. Instead they are crucial for the disease-causing capability of the fungus (Covert, 1998). CD chromosomes have been shown for the apple, strawberry and tomato pathotypes of *Alternaria* and are known to carry gene clusters that encode for the different host-specific toxins (Johnson et al., 2001; Hatta et al., 2002; Akamatsu et al., 2003; Hatta et al.,

2006). The gene clusters for the host-specific toxins of Japanese pear, tangerine and rough lemon pathotypes are also carried by small chromosomes, suggesting that these chromosomes may also be conditionally dispensable and that they control host-specific pathogenicity (Tanaka and Tsuge, 2000; Tsuge et al., 2016). It is believed that the acquisition of these CD chromosomes occurs through horizontal gene transfer (HGT). Ma et al. (2010) co-cultivated pathogenic *Fusarium oxysporum f. sp. lycopersici* (pathogenic on tomato) with a non-pathogenic *F. oxysporum* strain and observed that the non-pathogenic strain became pathogenic after co-cultivation. This may explain how new pathogenic strains within a fungal species originate in nature. The fact that non-pathogenic strains can become pathogenic in a single HGT event can also be seen as the incentive for quick changes in epidemiology, often observed in field surveys (Thomma, 2003). Sequence analyses of essential genes from the *Alternaria* tomato pathotype such as β-tubulin, melanin biosynthesis genes and mating type genes located on the essential chromosomes, compared with sequences of CD chromosomes revealed high polymorphisms among the strains. Therefore it is speculated that the origin of CD chromosomes from the *Alternaria* tomato pathotype might be different from that of the essential chromosomes.

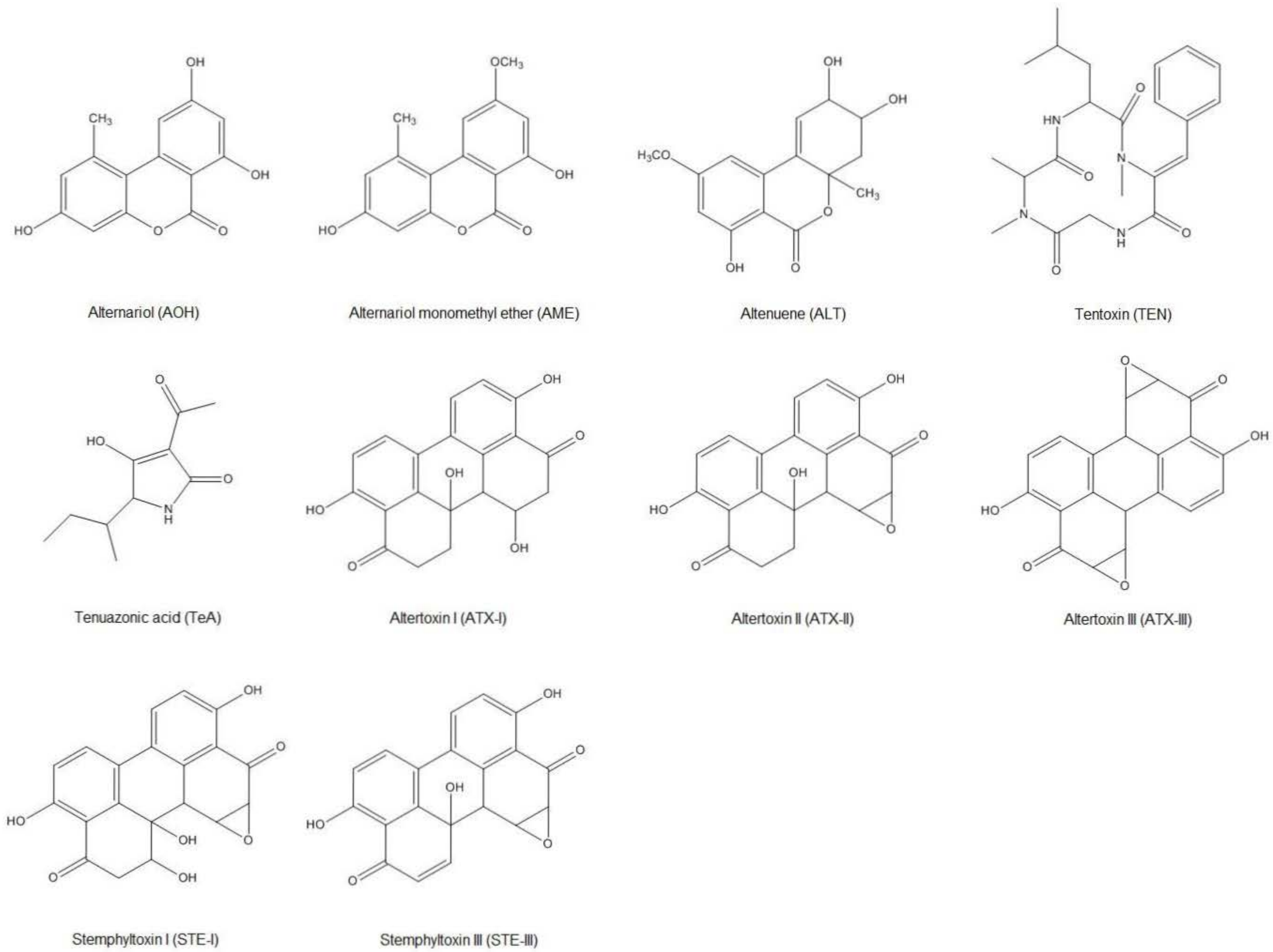


Figure 1.7. Chemical structures of relevant non-host specific toxins produced by *Alternaria* species.

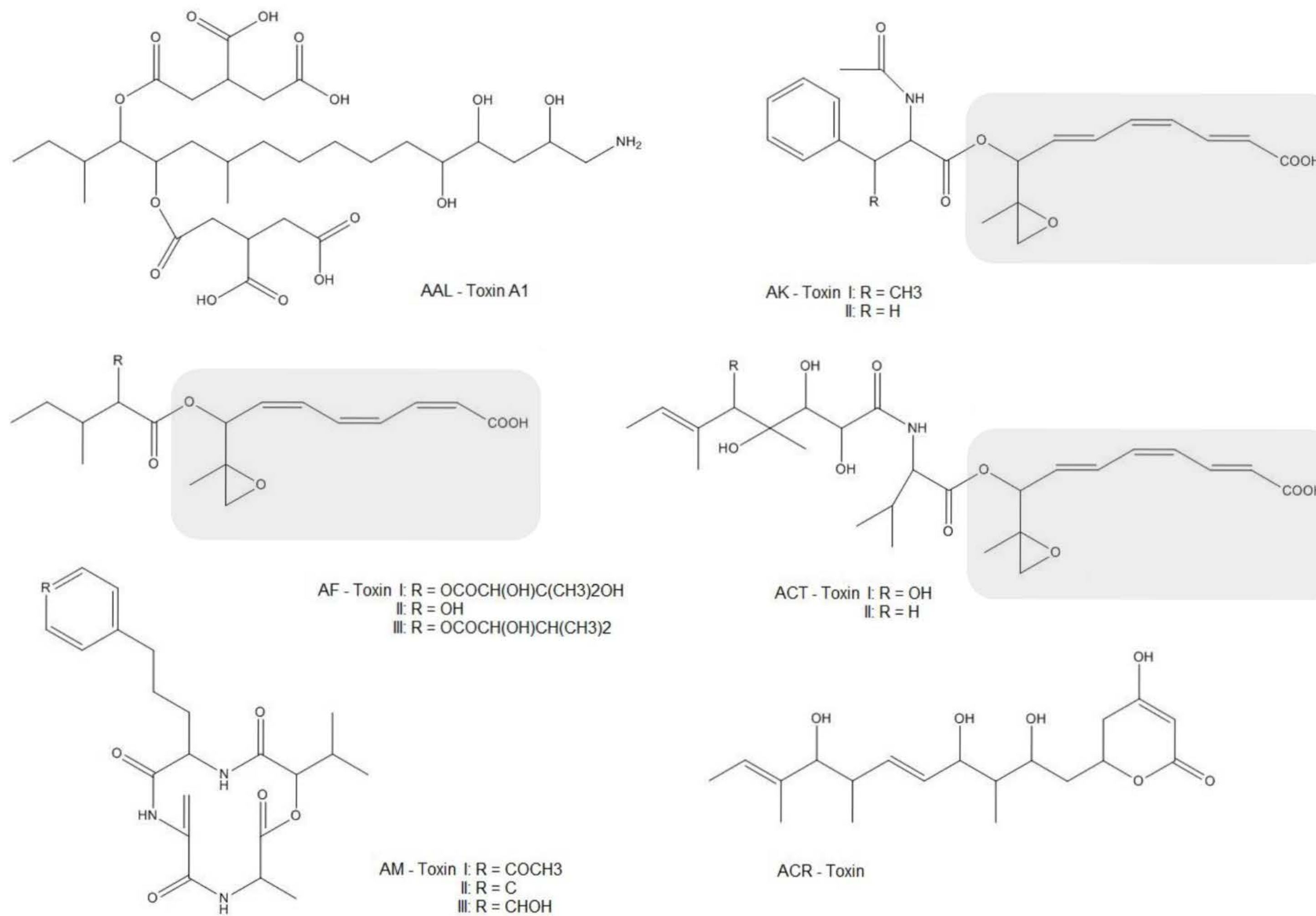


Figure 1.8. Chemical structures of relevant host-specific toxins produced by *Alternaria* species. The gray boxes indicate a shared epoxy-decatrienoic acid (EDA) moiety between AK-, AF-, and ACT-toxins.

Table 1.3. List of relevant host- and non-host specific toxins produced by *Alternaria* species.

	Chemical group	Mycotoxin	Chemical formula	Molecular weight (g mol^{-1})	Mycotoxin effect	Phytotoxic effect	Reference(s)
Non-host specific toxins	Benzopyrones	Alternariol (AOH)	$C_{14}H_{10}O_5$	258,226	ROS formation, DNA strand breaks	Inhibition electron transport chain	(Lehmann et al., 2006; Solhaug et al., 2016; Demuner et al., 2013)
		Alternariol monomethyl ether (AME)	$C_{15}H_{12}O_5$	272,253			
		Altenuene (ALT)	$C_{15}H_{16}O_6$	292,284			
	Cyclic tetrapeptides	Tentoxin (TEN)	$C_{22}H_{30}N_4O_4$	414,498	Unknown	Inhibition chloroplast development	(Klotz, 1988)
	Amine metabolites	Tenuazonic acid (TeA)	$C_{10}H_{15}NO_3$	197,231	Inhibition peptide synthesis, apoptosis induction	Inhibition photosystem III	(Solfrizzo et al., 2005; Asam & Rychlik, 2013; Chen et al., 2014; Cabral et al., 2016)
	Perylenequinones	Altertoxin-I (ATX-I)	$C_{20}H_{16}O_6$	352,337	ROS formation, DNA strand breaks	Unknown	(Fleck et al., 2012; Fraeyman et al., 2017)
		Altertoxin-II (ATX-II)	$C_{20}H_{14}O_6$	350,321			
		Altertoxin-III (ATX-III)	$C_{12}H_{12}O_6$	348,306			
		Stemphyltoxin-I (STE-I)	$C_{20}H_{14}O_7$	366,324			
		Stemphyltoxin-III (STE-III)	$C_{20}H_{12}O_6$	348,309			

(Table 3.1 continued)

	Chemical group	Mycotoxin	Chemical formula	Molecular weight (g / mol)	Mycotoxin effect	Phytotoxic effect	Reference(s)
Host-specific toxins	Aminopentol esters	*A. alternata f. sp. lycopersici* (AAL) toxin	$C_{25}H_{47}NO_{10}$	521,648	Neural tube defects, esophageal cancer	Inhibition ceramide biosynthesis	(Stockmann-Juvala and Savolainen, 2008; Mase et al., 2012)
	Cyclic tetrapeptides	AM-toxin I (Alternariolide)	$C_{23}H_{31}N_3O_6$	445,516	Unknown	Chloroplast membrane invaginations	(Kohmoto et al., 1982)
		AM-toxin II	$C_{22}H_{29}N_3O_5$	415,490			
		AM-toxin III	$C_{22}H_{29}N_3O_6$	431,489			
	Epoxy-decatrienoic acid esters	AF-toxin I	$C_{22}H_{32}O_9$	440,489	Unknown	Increase K+ efflux, plasma membrane dysfunction	(Maekawa et al., 1984)
		AF-toxin II	$C_{17}H_{24}O_6$	324,373			
		AF-toxin III	$C_{22}H_{32}O_8$	424,490			
		AK-toxin I	$C_{32}H_{27}NO_6$	413,470	Unknown	Increase K+ efflux, plasma membrane dysfunction	(Otani et al., 1985)
		AK-toxin II	$C_{22}H_{25}NO_6$	399,443			
		ACT-toxin I	$C_{26}H_{39}NO_{10}$	525,595	Unknown	Increase K+ efflux, plasma membrane dysfunction	(Kohmoto et al., 1993)
		ACT-toxin II	$C_{26}H_{39}NO_9$	509,596			
	Pyrone-ring polyalcohol	ACR-toxin	$C_{19}H_{30}O_6$	354,443	Unknown	Mytochondrion dysfunction	(Akimitsu et al., 1989)
	Unknown	AT-toxin	Unknown	Unknown	Unknown	Mytochondrion dysfunction	(Tsuge et al., 2013)

PLANT-PATHOGEN INTERACTIONS

Plants are constantly exposed to numerous biotic interactions, many of which are harmful as illustrated in the paragraphs above. Some interactions though are beneficial to the plant such as rhizosphere bacteria that can promote the plant's growth and induce plant defense. The harmful interactors, like herbivorous insects or microbial pathogens attempt to retrieve energy-rich compounds from the plant without any returning benefit for the host (Pieterse et al., 2012; Broekgaarden et al., 2015). Since plants are sessile organisms, they have evolved sophisticated defense mechanisms: the initial response is characterized by the recognition of pathogen- or damage-associated molecular patterns (P-/DAMPs) by pattern recognition receptors (PRRs) (Jones and Dangl, 2006). This so-called pattern-triggered immunity (PTI) often leads to immediate production of defense-related hormones, like jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), to the production of reactive oxygen species (ROS) and to mitogen-activated protein kinase (MAPK) signaling cascades (Boller and Felix, 2009; Broekgaarden et al., 2015). These responses ultimately induce pathogenesis-related (PR) proteins to combat the attacker. However, successful pathogens have found ways to circumvent this PTI by using pathogen effectors in what is called 'effector-triggered susceptibility' (ETS) (i.e. altering the hosts' metabolism or tapping into its signaling network), which in turn, can be counteracted by plant resistance (R) proteins that specifically recognize these effectors, inducing the plant effector-triggered immunity (ETI), leading to hypersensitive responses (HR) and subsequent apoptosis. The plants' ETI can again be counteracted by a new phase of ETS. This forms the basis of the 'zig-zag model', proposed by Jones and Dangl (2006). However, the dichotomy between PTI and ETI is being questioned recently since it was revealed that some PTI responses (triggered by PAMPs) elicit local cell death (hypersensitive response) or systemic acquired resistance (SAR), which are generally believed to be ETI responses (triggered by secreted effectors) (Thomma et al., 2011).

Effectors of pathogens with diverse lifestyles

Depending on whether the pathogen is biotrophic, hemibiotrophic or necrotrophic, ETS is differentially regulated. The action of effectors of biotrophic pathogens is generally focused on reprogramming the host immune system and obtaining nutrients from living host cells (Chaudhari et al., 2014). In that sense, biotrophs have to be subtle in manipulating the host's defense systems since they need living plant cells for growth and propagation. In the case of biotrophic fungi, effectors are believed to be secreted by specialized structures called 'haustoria' that form inside the apoplast or cytoplasm. These metabolites can generally be categorized in three classes: apoplastic, cytoplasmic and nuclear effectors (Chaudhari et al., 2014). Apoplastic effectors function

primarily against PTI through the inhibition of host lytic enzymes, such as proteases, hydrolases or glucanases. In the interaction between *Cladosporium fulvum* and tomato, the pathogen secretes the effector Ecp6, which binds selectively to chitin oligosaccharides, preventing its recognition by the host and subsequent deployment of chitinases (de Jonge et al., 2010). The action of cytoplasmic effectors is generally based on targeting defense signaling components, such as MAPK kinase activity (Lindeberg et al., 2012). Nuclear effectors act against plant defense regulation on a genetic level, as these metabolites migrate to the nucleus to suppress ETI responses such as HR and apoptosis. Indeed, it was shown for *Hyaloperonospora arabidopsidis* that 16 out of 49 putative effectors translocate to the nucleus (Caillaud et al., 2012).

On the other hand, necrotrophs secrete phytotoxins, reactive oxygen species (ROS), enzymes or a blend of these components with the aim of killing off the host cells quickly and taking up nutrients from dead plant tissue (Horbach et al., 2011). Many *Alternaria* species have a necrotrophic lifestyle and as stated above, some species produce host-specific toxins which renders the pathogen extremely virulent on that specific host plant. For example, one spore of *Alternaria kikuchiana*, a pathogen of Japanese pear, produces enough AK toxin to kill approximately 100 host cell 4 hours after inoculation (Nishimura and Kohmoto, 1983). Similar to biotrophs, necrotrophs produce a plethora of cell-wall degrading enzymes in order to support the penetration process and to obtain carbohydrates. This action elicits PTI responses, however, the necrotrophic pathogen attempts to overcome this by producing toxins that quickly cause necrosis, consequently blocking these early host defense reactions (Horbach et al., 2011). In some plant-pathogen interactions, it has been revealed that the pathogen, formerly classified as a necrotroph, does not kill its host cells immediately, but rather adopts a biotrophic lifestyle in an initial infection phase before eventually switching to a necrotrophic lifestyle. These pathogens are called hemibiotrophs and combine infection strategies of both biotrophs and necrotrophs. For example, *Magnaporthe oryzae*, which causes rice blast disease, has been considered a necrotroph, however, Kankanala et al. (2007) were able to show using live-cell imaging that the pathogen invades host cells through invasive hyphae that are not destructive. They also demonstrated that invaded plant cells could be plasmolysed in the initial stages of infection, indicating that these cells were still vital. This phenomenon is very reminiscent of obligate biotrophs, which is why this early infection stage is called the biotrophic stage of a hemibiotrophic lifestyle.

Phytohormones and their role in plant defense

Concerning the host plant, it is believed that, when PTI responses (e.g. cell wall callose depositions and secretion of lytic enzymes) are overcome by the pathogen, the plant will deploy a more sophisticated arsenal of defense responses, called ETI. The phytohormones JA, SA and ET, which

are generally seen as the major players in plant defense, regulate PTI and ETI through complex signaling pathways. Their antagonistic or synergistic interactions provide the plant with an intricate system that activates attacker-specific defenses, while minimizing the fitness cost. This communication system between phytohormones is often referred to as cross-talk. It must be noted that this signaling network is different in monocots versus dicots (De Vleesschauwer et al., 2014). In dicots, like *Arabidopsis thaliana*, the defense network is better understood than in monocots. Therefore, the dicots will be discussed first.

Figure 1.9 illustrates that, in case of a biotrophic pathogen, SA–mediated responses are needed. In case of necrotrophic pathogen, the host will activate JA signaling. Clear trade-offs between these two signaling pathways have been numerously reported. For example, Spoel and Dong (2008) reported that *A. thaliana* inoculation with *Pseudomonas syringae* induced SA signaling and repressed JA signaling, also rendering the plant more susceptible to necrotrophs. Similarly, JA responses that are activated by chewing insects like *Pieris rapae* can be suppressed by applying 1 mM of SA in *A. thaliana* (Koornneef et al., 2008).

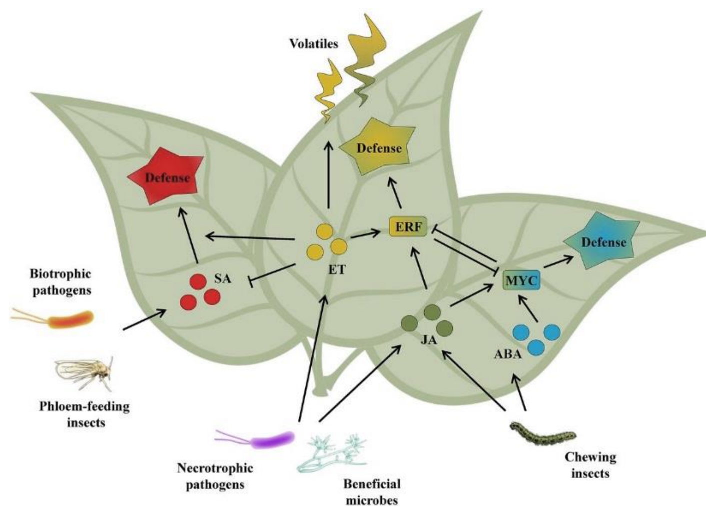


Figure 1.9. Schematic overview of phytohormone actions and -cross-talk in plant defense. Arrows represent positive regulations, end-blocked lines represent negative regulations. SA: salicylic acid, ET: ethylene, JA: jasmonic acid, ABA: abscisic acid, ERF: ethylene response factors, MYC: MYC transcription factor (Broekgaarden et al., 2015).

The fact that phloem-feeding insects and chewing insects elicit contrasting signaling pathways is illustrated by Zhang et al. (2009). This group reported that, in lima bean, JA-dependent defenses triggered by the spider mite *Tetranychus urticae*, are inhibited by SA-mediated responses induced by the phloem-feeding sweet potato whitefly, *Bemisia tabaci*. On the other hand, reports have been made of situations where the clear antagonistic trade-off between SA- and JA signaling does not apply. Indeed, treatment of *A. thaliana* with small amounts of SA and JA resulted in a synergistic induction of SA- and JA-responsive genes. When the concentrations of both hormones were

increased, the effects were antagonistic, which also illustrated that the resulting trade-off or synergistic action between SA and JA is concentration-dependent (Mur et al., 2006).

Accumulating evidence indicates a central role for ET, acting positively and negatively on both the SA and JA pathways. Indeed, on the one hand, ET is known to act positively on SA-mediated defense against *Leptosphaeria maculans* in *Brassica napus* (Sasek et al., 2012) and negatively against *P. syringae* in *A. thaliana* (Chen et al., 2009). On the other hand, ET also interacts positively with the ERF (ethylene response factor) branch of the JA-mediated defense against necrotrophs (Lorenzo et al., 2003; McGrath et al., 2005). The MYC branch of JA-mediated defense is synergized by abscisic acid (ABA) and activates defense against chewing insects. ET can also act indirectly against chewing insects by inducing the release of volatile compounds that can repel them or that can attract natural enemies of these insects (Scala et al., 2013). Interestingly, cross-talk between SA and JA pathways can also be modulated by ET concentration and production timing (not shown in the figure). SA-mediated suppression of JA-dependent defense is regulated by the transcriptional co-regulator NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) (Spoel et al., 2003). Simultaneous stimulation of the JA and ET pathways (by inoculation with a necrotrophic pathogen) in *Arabidopsis* strongly suppressed the NPR1 dependency of the SA-JA cross-talk (Leon-Reyes et al., 2010) and resulted in insensitivity to future SA-mediated suppression of JA-ET defense pathways. This observation indicates that ET steers the plant in prioritizing the JA-ET signaling (against necrotrophs) over the SA-NPR1 pathway (against biotrophs) (Leon-Reyes et al., 2010).

As stated earlier, all the findings described above apply to dicots, since most research has been done in *A. thaliana*. For monocots however, defense signaling can be very different. Indeed, JA and SA act mainly antagonistic in dicots, whereas in rice (*Oryza sativa*), a monocot, these hormones act synergistically in case of a pathogen attack, either necrotrophic or biotrophic (De Vleesschauwer et al., 2014). Also, where SA signaling is regulated by NPR1 in dicots, SA signaling in rice is controlled by NPR1 and another transcription factor, WRKY45, which acts as a positive controller for activation of SA defense genes (Ueno et al., 2013; De Vleesschauwer et al., 2014). Many aspects of monocot defense signaling are still unclear though, such as the cross-talk between JA and SA and the role of ET, which is why more research is needed to reveal its complex signaling network.

Although potato is a dicotyledonous plant, the crosstalk of its defense hormones shows more resemblance to monocots. Indeed, a study by Wiesel et al. (2015) showed that exogenous application of JA and SA on potato plants modulated gene expression of over 140 genes

synergistically, which is very reminiscent of the situation in rice, a monocotyledonous plant. Conversely, in *A. thaliana*, it is generally accepted that SA-induced transcription factors aid in suppression of the JA pathway and vice versa (Pieterse et al., 2012; Derksen et al., 2013). Moreover, Pep-13, a PAMP of *Phytophthora,* induces both JA and SA accumulation in potato, again contradicting the general situation observed in dicots (Halim et al., 2009). It should be noted that this dichotomy between monocots and dicots might be an interpretation due to inconsistent experimental approaches by which the cross-talk of phytohormones has been analyzed within or between these two types of plants. For example, a clear discrepancy exists in the way of inducing defense responses such as treatments with effectors or exogenous hormones or inoculations with the complete pathogenic species, which might elicit different reactions. Also, the timing and method of measuring the defense response is very inconsistent across reports investigating the same plant-pathogen interaction. Therefore, the interpretation of results concerning hormone cross-talk warrants caution.

Next, more and more findings reveal that other hormones like auxins (Kazan and Manners, 2009), cytokinins (CK) (Choi et al., 2011), abscisic acid (ABA) (Asselbergh et al., 2008), gibberellins (GA) (De Bruyne et al., 2014), brassinosteroids (BR) (De Bruyne et al., 2014) and strigolactones (SL) (Stes et al., 2015) have active roles in fine-tuning plant defense. Henceforth, it became clear that plant defense is not regulated by a binary regulation system, but by an intricate network of hormones that activate or inhibit downstream mechanisms and that can exert positive or negative actions on each other.

Phytohormones used as weapons by fungi

Pathogens tapping into the defense signaling network of plants

Since the activated defense pathways of the plant and its subsequent production of hormones is dependent on the type of attacker, tapping into this network can be a successful virulence strategy for either biotrophic or necrotrophic pathogens. A well-studied example is the interaction between *Botrytis cinerea* and tomato (Kettner and Dorffling, 1995; Audenaert et al., 2002). In this pathosystem, *B. cinerea* is able to stimulate the tomato ABA biosynthesis, so ABA can act as a virulence factor to increase susceptibility. Also, de Torres-Zabala et al. (2007) focused on the interaction between *Pseudomonas syringae* and *Arabidopsis*, showing that the pathogen is able to produce type III-secreted effectors that target the hosts' ABA signaling pathway, ultimately rendering susceptibility.

Another example of a pathogen exploiting its hosts' defense network is *Cochliobolus miyabeanus*, which causes brown spot disease on rice. De Bruyne (2015) was able to highlight specific ET emission patterns in inoculated rice leaves, while also showing that leaves that are compromised

in ET biosynthesis obtained strong resistance to the disease. These results indicate that *C. miyabeanus* relies heavily on ET metabolism in rice in order to cause disease.

Phytohormones produced by pathogens

Certain pathogens have evolved to alter the plant defense network by producing phytohormones or mimics thereof by themselves (Tudzynski and Sharon, 2002; Chanclud and Morel, 2016). Fungal development is not compromised when phytohormone biosynthesis is blocked in the fungus, indicating that they are not essential for survival but rather act as secondary metabolites to modulate the hormone balance in the host (Robert-Seilaniantz et al., 2007).

Although phytohormone biosynthesis in microorganisms was first discovered in gall-forming and root-modifying bacteria, numerous reports of fungi producing auxins, cytokinins or abscisic acid can be found (Kettner and Dorffling, 1995; Morrison et al., 2015). *Fusarium oxysporum* was found to produce auxins and when over-expressing the biosynthesis genes, it triggered a hypervirulent phenotype on *Orobanche* (Cohen et al., 2002). The causal agent of corn smut, *Ustilago maydis* can also produce auxins, however, they are not essential for virulence (Reineke et al., 2008). Examples of fungal cytokinins were found by Jiang et al. (2013) and Chanclud et al. (2016), though any indication for a role in virulence has not been shown. Concerning fungal ABA production, Kettner and Dorffling (1995) and Denance et al. (2013) proved that ABA is produced by *Botrytis cinerea* and *Fusarium oxysporum* respectively and that it enhances susceptibility in tomato by repressing SA-mediated defenses. Limited info is available on fungi producing the archetypal defense hormones JA and SA. However, ET has been frequently found to be produced by fungal pathogens (Chagué, 2010) (Table 1.4).

Fungi are able to produce ET through one of three different biosynthesis pathways (Fig. 1.10). Most fungi possess only one route, although there are exceptions that are able to produce ET via all three pathways (Table 1.4) (Chagué, 2010). Two biosynthesis routes start from methionine (MET) as a precursor. The first is the ACC pathway, also known as the plant biosynthesis pathway, where ET is synthesized through three enzymatic steps, generating respectively S-adenosylmethionine (SAM), 1-aminocyclopropane-1-carboxylic acid (ACC) and ultimately ET (Johnson and Ecker, 1998). This pathway is scarcely found in fungi. The action of ACC and of other enzymes depending on pyridoxal-5-phosphate (PLP / vitamin B_6) is inhibited by 2-aminoethoxyvinyl glycine (AVG) or 2-amino-oxyacetic acid (AOA), two well-known inhibitors of (plant) ET biosynthesis. The second pathway is the KMBA pathway where MET is deaminated via an unknown transaminase that converts MET into α-keto-γ-methylthiobutyric acid (KMBA). The latter is finally oxidized in the presence of hydroxyl radicals that are formed by the action of NADH:FE(III)-oxidoreductase to

release ET (Primrose and Dilworth, 1976; Fukuda et al., 1989). In *B. cinerea*, it was found that KMBA can be photo-oxidized in the presence of light, while in the dark, KMBA was directly oxidized by the former way (Chagué et al., 2002). The last ET biosynthesis pathway starts from the tricarboxylic acid (TCA) cycle intermediate, 2-oxoglutarate, and utilizes an ethylene forming enzyme (EFE) that requires the additional cofactors, arginine or lysine, oxygen and Fe^{2+} (Chagué, 2010). This pathway has been identified in several fungi. In *P. digitatum*, the ACC-, the KMBA- and the EFE pathway seem to coexist (Billington et al., 1979; Jia et al., 1999). Also *Cochliobolus miyabeanus* seems to possess multiple distinct pathways to synthesize ET, namely the EFE and KMBA pathway (De Bruyne, 2015). These findings, together with the fact that KMBA is an intermediate in a MET salvage pathway that exists in plants (Fig. 1.10), may suggest that alternative pathways for ET production might be triggered depending on available nutrients or external stimuli. The exact function of fungal ET during disease remains elusive, although it is speculated that it acts as an enhancer of disease processes that have already been triggered (De Bruyne, 2015).

Table 1.4. List of ET-producing fungal pathogens. Adapted and modified from Chagué (2010).

Species	Pathway(s)	Reference
Aspergillus terreus	KMBA	(Akhtar et al., 2005)
Botrytis cinerea	KMBA	(Chague et al., 2002)
Cochliobolus miyabeanus	KMBA	(De Bruyne, 2015)
	EFE	
Colletotrichum demathium	KMBA	(Tzeng and Devay, 1984)
Cryptococcus albidus	KMBA	(Fukuda et al., 1989)
Dictyoselium mucoroides	ACC	(Amagai and Maeda, 1992)
Fusarium oxysporum	KMBA	(Tzeng and Devay, 1984)
	EFE	(Hottiger and Boller, 1991)
Penicillium digitatum	ACC	(Jia et al., 1999)
	KMBA	(Billington et al., 1979)
	EFE	(Fukuda et al., 1986)
Penicillium cyclopium	EFE	(Pazout and Pazoutova, 1989)
Saccharomyces cerevisiae	KMBA	(Billington et al., 1979)
Verticillium species	KMBA	(Tzeng and Devay, 1984)

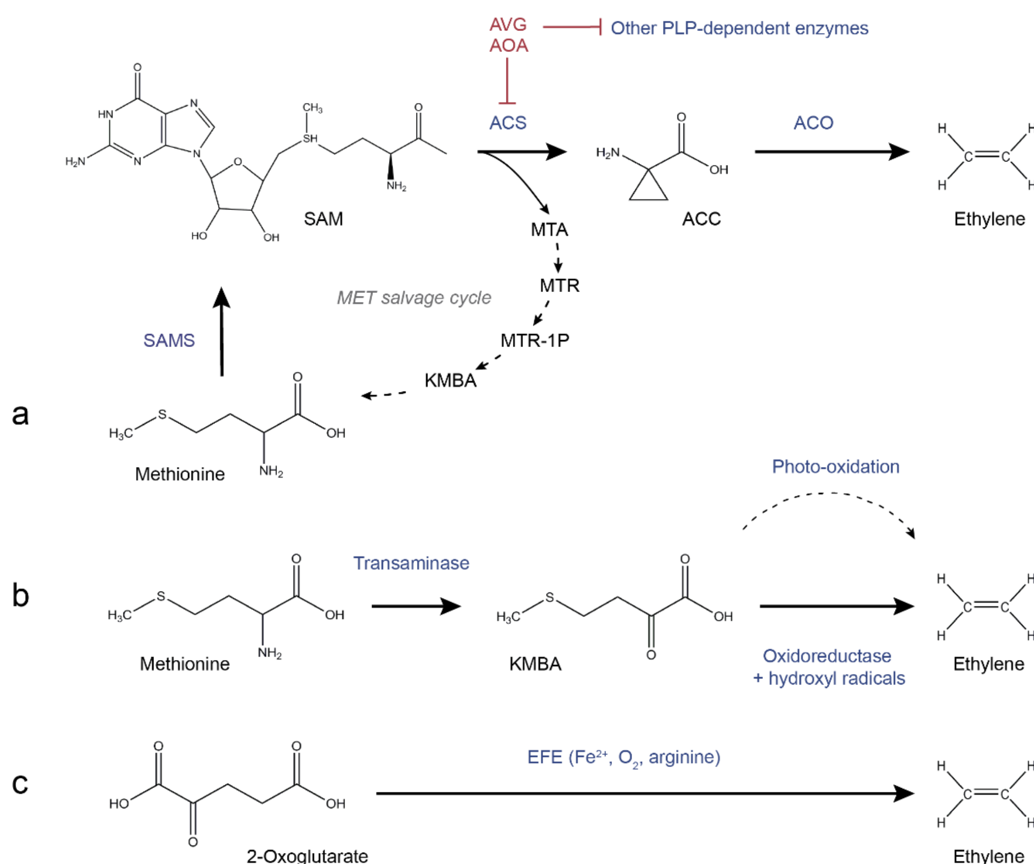


Figure 1.10. Ethylene biosynthesis pathways in microorganisms. (a) The ACC pathway, also known as the active ET biosynthesis pathway in plants. Methionine is converted to S-adenosylmethionine (SAM) by SAM synthetase (SAMS). Then, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) which also releases 5-methylthioadenosine (MTA). This compound can be reverted to methionine through the MET salvage cycle. Ethylene is ultimately synthesized from ACC by the action of ACC oxidase (ACO). The action of ACS, as well as that of other PLP-dependent enzymes, is inhibited by 2-aminoethoxyvinyl glycine (AVG) or 2-amino-oxyacetic acid (AOA). (b) The KMBA pathway also starts from methionine and utilizes an unknown transaminase to release α-keto-γ-methylthiobutyric acid (KMBA), which can then be oxidized directly to ethylene in the presense of light or by the action of an oxidoreductase in the presence of hydroxyl radicals. (c) The EFE pathway starts from 2-oxoglutarate and requires an ethylene forming enzyme (EFE) and its cofactors Fe^{2+}, oxygen and the amino acid arginine. MTR: 5-methylthioribose, MTR-1P: 5-methylthioribose-1-phosphate, PLP: pyridoxal-5-phosphate. Adapted and modified from Chagué (2010) and Depaepe and Van Der Straeten (2019).

ALTERNARIA – SOLANACEAE INTERACTIONS

Although *Alternaria* species are pathogenic on a number of crops, they are notorious as causal agents of epidemics in tomato and potato, two crops of the *Solanaceae* family. As mentioned in the previous sections, one of the most economically important diseases on potato is early blight, caused by the species *Alternaria solani*. Early blight can also be found on tomato and is one of its major destructive diseases, hence it has received considerable attention in crop protection and it is well studied. Because disease management and fungicide resistance mechanisms in *Alternaria* isolates are very similar for early blight on potatoes and early blight on tomatoes, this discussion will be combined for both crops. Subsequently, the *Alternaria*-tomato and the *Alternaria*-potato pathosystems will be examined separately.

Disease management and fungicide resistance

Full resistant tomato or potato cultivars to early blight do not exist due to a lack of resistant sources (Chaerani and Voorrips, 2006). Moreover, the fact that good agricultural practices can keep the disease to a controllable level that is economically insignificant has slowed down the breeding of resistant cultivars (Kemmit, 2002). These agricultural practices should include the removal of necrotic plant parts, fruit, tubers or susceptible volunteer weeds (non-edible *Solanaceae* such as nightshade or horse nettle) prior to tomato and potato planting, crop rotation with non-susceptible host crops to reduce inoculum buildup and ensuring that seeds or transplants are pathogen-free before planting. Also, good soil drainage is important to keep the leaf wetness period minimal (Kemmit, 2002). Because early blight is a disease of maturing, senescing crops, one should pay attention to the nitrogen level in the soil. Excessive amounts of nitrogen in the soil make crops develop too quickly which makes them weak and more prone to injury or infection (Kemmit, 2002). Even though these practices reduce the risk of *Alternaria* infections, controlling the disease still relies heavily on synthetic fungicides. Most growers apply fungicides in so-called ‘spraying programs’, which are fixed timetables that are followed in order to apply the compounds in the optimal time period and the optimal quantity. The spraying programs mainly rely on environmental conditions, which are taken up in forecasting models like FAST or P-DAY to estimate new potential outbreaks (Madden et al., 1978; Pscheidt and Stevenson, 1986; Gleason et al., 1995). Fungicides such as mancozeb and chlorothalonil form the basis of spraying programs and are applied almost every week to counter new pathogen growth (Kemmit, 2002). These fungicides have a multi-site mode of action, which reduces the risk of resistant fungi development and are therefore often used in rotation with other fungicides. The downside is that weather effects gradually remove the compounds from the leaf surface. Other fungicides have a single-site mode of action and are mainly

employed to affect the growth of pathogens that are already infecting the host. Three classes of such fungicides are generally used against early blight: the quinone outside inhibitors (QoI's), the succinate dehydrogenase inhibitors (SDHI's) and the demethylation inhibitors (DMI's). QoI's like azoxystrobin, pyraclostrobin or fluoxastrobin, all part of the strobilurin chemical group, inhibit fungal mitochondrial respiration by binding to the quinol oxidation site. This binding blocks electron transfer between cytochrome b and c1, ultimately leading to a lack of ATP production (Bartlett et al., 2002). Fenamidone and famoxadone, also QoI's, have the same mode of action as strobilurins (Fernández-Ortuño et al., 2010). Advantages of QoI's are their broad-spectrum activity, low use rates and control of resistant isolates to other fungicides. Moreover, in some crops, including wheat and barley, QoI's application can lead to greater yield and quality. It is hypothesized that this effect is due to the strong activity of QoI's on fungal development, which prevents the induction of energy-intensive defense responses. This hypothesis has not been proven though (Bartlett et al., 2002). The downside of QoI's is their single-site mode of action, which has a high risk for intrinsic resistance development in fungal strains.

The main mechanism of QoI resistance involves mutations in the fungal cytochrome b gene, which leads to peptide sequence changes and ultimately prevents fungicide binding. The most frequent mutation is an amino acid substitution in position 143, where a glycine is replaced with an alanine (therefore called the G143A mutation). This mutation confers complete resistance in several phytopathogenic fungi to QoI's (Grasso et al., 2006; Fernández-Ortuño et al., 2010). This mutation is often observed in *A. alternata* strains, but was found to be lethal in *A. solani*. On the other hand, in *A. solani*, another amino acid substitution, the F129L mutation, has been frequently reported. Here, a phenylalanine is replaced with a leucine at position 129. In contrast to the G143A mutation, the F129L mutation confers partial resistance to QoI's (Grasso et al., 2006; Fernández-Ortuño, 2010, Leiminger et al., 2014). Additionally, Leiminger et al. (2014) revealed the existence of two structurally different cytochrome b genes (genotype I and II) which differed in the presence (genotype I) or absence (genotype II) of an intron. The F129L mutation appeared only in genotype II isolates. A third, less common substitution, is the G137R mutation, where a glycine is replaced with an arginine at position 137. Similar as for the F129L mutation, the G137R mutants exhibit partial resistance to QoI's (Sierotzki et al., 2006).

The second group of fungicides with a single-site mode of action used in early blight control on tomatoes are the succinate dehydrogenase inhibitors (SDHI's). Like the QoI's, SDHI's inhibit the mitochondrial respiration chain, although the target site is different: SDHI's block the activity of the succinate dehydrogenase, also called complex II in the respiration chain. The fact that SDHI's have a different binding site than QoI's makes them excellent candidates for alternating or mixing the

two, reducing the risk for resistance development. Indeed, no cross-resistance has been observed so far between the two groups of fungicides. The binding site of SDHI's is formed by three different subunits of complex II, namely subunits B, C and D (complex II consists of four subunits in total: A, B, C and D). Mutations leading to resistance to SDHI's can occur in all three subunits (FRAC, 2014). The current generation of SDHI products are based on the compounds boscalid, penthiopyrad and fluopyram (Hirooka and Ishii, 2013).
Similar as for QoI resistance, the mechanism behind SDHI resistance is also based on point mutations leading to amino acid substitutions in the peptide chain of the target protein(s). Five different amino acid substitutions have been attributed to the reduced sensitivity towards SDHI fungicides: H278Y in *A. solani* or H277Y in *A. alternata* (histidine replaced with tyrosine at position 278 or 277) and H278R in *A. solani* or H277R in *A. alternata* (histidine replaced with arginine at position 278 or 277) in subunit B, H134R (histidine replaced with arginine at position 134) in subunit C, H133R (histidine replaced with arginine at position 133) and D123E (aspartic acid replaced with glutamic acid at position 123) in subunit D (Avenot et al., 2008a; Avenot et al., 2008b; Mallik et al., 2014; Landschoot et al., 2017a).

The third group of fungicides with a single-site mode of action are labeled as demethylation inhibitors (DMI's), which inhibit the sterol C14-α-demethylation of 24-methylenedihydrolanosterol, a precursor of ergosterol in fungi. Ergosterol is a sterol molecule found in cell membranes of fungi and protozoa and adds to the fluidity of the membrane. These DMI compounds (such as difenoconazole or propiconazole) also have a broad spectrum of activity, are excellent to be used in mixtures with QoI's and SDHI's to reduce resistance development, and have both protective and curative efficacy (He et al., 2019).
Not much is known about the molecular mechanisms of resistance against DMI's. However, isolates with reduced sensitivity towards DMI's have been detected in *Alternaria alternata* (Avenot et al., 2016). Since this species is known to quickly develop resistance against QoI's and SDHI's, care must be taken in the frequency of DMI applications. The fungicide resistance action committee (FRAC) monitors the development of fungicide resistance and classifies different fungicides in either one of three groups depending on their mode of action (low, medium or high risk for resistance development). Due to the single-site modes of action of QoI's, SDHI's and DMI's, these fungicides are classified as high, medium to high, and medium risk fungicides respectively. Therefore, the usage of these compounds should be carefully monitored and should not exceed certain guideline limits in order to extend the lifetime of these fungicides.

Alternaria – tomato pathosystem

Symptoms and economic impact

Alternaria solani can infect many parts of the tomato plant, such as foliage, collars, stems and fruit though there exists some confusion on the names of the different symptoms. Here, we refer to early blight as foliage symptoms. All other symptoms are referred to as collar-, stem- and fruit rot (Walker, 1952; Chaerani and Voorrips, 2006). As previously described, early blight appears as dark 'bullseye'-type lesions with a surrounding yellow zone (Fig. 1.6) These necrotic patches usually start as small (1 - 2 mm) brown to black spots that gradually increase in surface (ca. 10 mm) albeit they are confined between leaf nerves (Kemmit, 2002). Advanced epidemics can quickly lead to premature defoliation, which weakens the plant and exposes fruit to injury or infection. Fruit rot occurs both on green and ripe tomatoes and usually develops from mycelia extending from the stems to the calyx of the fruit. Lesions develop as dark, sunken and leathery patches that sometimes show the characteristic concentric rings ('bullseye'-type lesions) (Sherf and MacNab, 1986; Kemmit, 2002). Stem rot often occurs as sunken and lens-shaped lesions. On young seedlings, these symptoms are generally found at the ground line, leading to complete girdling of the stem which is known as collar rot. On adult plants, stem rot also expands to side branches, showing small spots that enlarge to form the typical 'bullseye'-type lesions (Walker, 1952).

Early blight occurs worldwide where tomatoes are grown but can cause serious problems in the US where tomatoes are grown in large fields rather than on a horticultural scale (Kemmit, 2002). Humid regions are generally more prone to *Alternaria* epidemics than less humid regions, though it largely depends on the season itself and on the applied control measures. Unsprayed fields can suffer yield losses up to 80 %, however, incidences of 5 % have also been reported for uncontrolled crops (Basu, 1974; Datar and Mayee, 1981; Kemmit, 2002). It is therefore difficult to accurately estimate annual crop losses to this disease. In terms of annual expenses on fungicides, it is estimated that 32 million dollars are globally spent on *Alternaria* control (Kemmit, 2002).

Molecular mechanisms of early blight on tomato

In order to reduce the usage of synthetic fungicides and to search for alternative control measures, efforts have been made to deduce the molecular mechanisms which are involved in an *Alternaria* infection on tomato (Prasad and Upadhyay, 2010; Zhang et al., 2011; Mase et al., 2012). Prasad and Upadhyay (2010) observed that, when a culture filtrate of *Alternaria alternata,* isolated from tomato, was inoculated on tomato leaves, similar necrotic lesions were produced as when a pure culture of the pathogen would be inoculated. Moreover, both pathogen and filtrate inoculation triggered H_2O_2 production and led to increased levels of ET in tomato leaves within 6 hours of

treatment. Previous research showed that an *Alternaria alternata* toxin, AAL-toxin, causes H_2O_2 production in *Arabidopsis thaliana* (Gechev et al., 2004). Also, treatment of tomato and *A. thaliana* with AAL-toxin enhanced the plant ethylene production (Moussatos et al., 1994). When Prasad and Upadhyay (2010) applied the ethylene biosynthesis inhibitor 2-aminoethoxyvinyl glycine (AVG) to inoculated tomato leaves, necrosis was significantly lowered. Because of these observations, it was presumed that AAL-toxin was present in the culture filtrate and that it was causing the observed necrosis on the tomato leaves through a downstream mechanism that involves the plant hormone ethylene. Moussatos et al. (1994) were among the first to report that ET biosynthesis and ET recognition are crucial for AAL toxin-induced cell death. Also, Gechev et al. (2004) observed that ET-responsive genes were significantly up-regulated within the first seven hours of AAL-toxin treatment. Since it was clear that ET biosynthesis and -recognition have a crucial role in AAL toxin-induced cell death, efforts have been made to elucidate the signaling pathway (Fig. 1.11). For this purpose, Mase et al. (2012) used the host plant *Nicotiana umbratica*, which can be easily manipulated by virus-induced gene silencing (VIGS) and is equally sensitive to AAL-toxin as tomato if it lacks the *Alternaria stem cancer gene 1* (*asc/asc* genotype) (Brandwagt et al., 2001). To unravel the pathway leading to this programmed cell death (PCD), several components known to be active in disease resistance were silenced by VIGS: MEK2, a component of the mitogen-activated protein kinase (MAPK) cascade in eukaryotes, salicylic acid-induced protein kinase (SIPK), and wound-induced protein kinase (WIPK). The latter two components are known to act downstream of MEK2 (Ekengren et al., 2003; Tanaka et al., 2009). Constitutive expression of MEK2 indeed leads to PCD-like cell death in tobacco (Yang et al., 2001), defense gene expression and generation of reactive oxygen species, like H_2O_2 (Asai et al., 2008). When MEK2-silenced *N. umbratica* leaves were inoculated with *A. alternata* or AAL-toxin alone, no symptoms were detectable after 6 days after inoculation. This observation indicates that MEK2 participates in PCD (Mase et al., 2012). After silencing the genes isochorismate synthase 1 (*ICS1*, a key enzyme involved in SA biosynthesis), coronatine insensitive 1 (*COI1*, active in JA signaling), and ethylene insensitive 2 (*EIN2*, active in ET signaling), they observed that PCD was compromised only in the *EIN2*-silenced leaves. This finding was backed up by the fact that leaves treated with 1-methylcyclopropene (MCP), an inhibitor of ET receptors, exhibited no necrotic symptoms, indicating a pivotal role of ET in PCD. To investigate the signaling pathway further, ET production was monitored after AAL-toxin treatment of MEK2-silenced leaves and control leaves. In the latter, ET production was markedly increased, whereas it was not in MEK2-silenced leaves. After ET application (10 µg mL^{-1}) of MEK2-silenced leaves, PCD was restored. These discoveries indicate that MEK2 regulates PCD by controlling ET production. Finally, as it is known that SIPK and WIPK are involved in stabilizing ACC synthase, a key enzyme in ET biosynthesis (Liu and Zhang, 2004; Kamiyoshihara et al., 2010), the roles of

SIPK and WIPK in PCD were also investigated. SIPK and WIPK were found to be activated by AAL-toxin in *N. umbratica* using an immunocomplex kinase assay, while they were not activated after water treatment. Also, in SIPK- and WIPK-silenced *N. umbratica* leaves, PCD was compromised and no ET accumulation was observed. These results suggest that PCD triggered by AAL-toxin requires a MAPK (MEK2-SIPK/WIPK) signaling cascade that controls ET production, leading to the activation of ethylene response factor (ERF) genes which ultimately results in cell death (Fig. 1.11) (Mase et al., 2012).

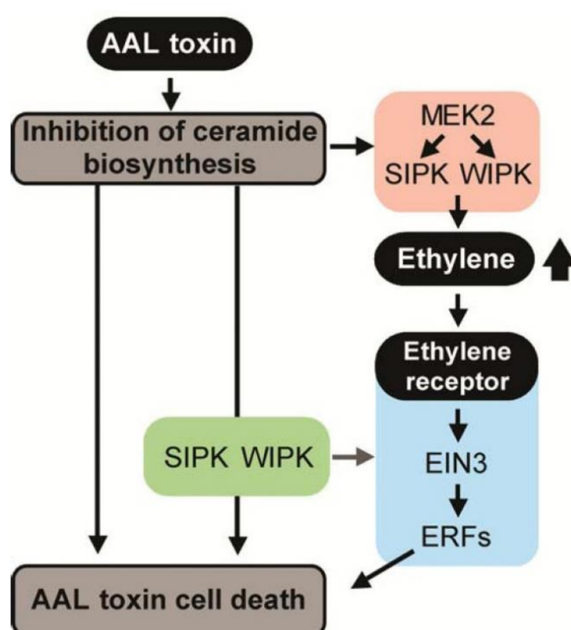


Figure 1.11. Signaling model for AAL toxin-triggered cell death. AAL-toxin inhibits ceramide biosynthesis which may activate the MAPK cascade consisting of MEK2 and its downstream components salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK). This MAPK cascade increases ethylene (ET) production which, after ET recognition, leads to activation of ethylene response factors (ERF) by ethylene insensitive 3 (EIN3) and ultimately to cell death. SIPK and WIPK also have ET-independent roles in AAL toxin-induced cell death (Mase et al., 2012).

Alternaria – potato pathosystem

Symptoms and economic impact

In the past, two potato diseases caused by *Alternaria* species were recognized and respectively described as early blight (caused by *A. solani*) and brown spot disease (caused by *A. alternata*). However, both diseases are often observed together on the same leaves and initial early blight symptoms are similar to brown spot symptoms, making them hard to discriminate (Stevenson et al., 2001). Additionally, a discrepancy exists between pathogenicity reports of either *A. alternata* and *A. solani*, which will be further discussed in chapter 4. For these reasons, this disease complex will here be referred to as early blight/brown spot (EB/BS) disease.

Symptoms of EB/BS disease are very similar than those of early blight on tomatoes, which also exhibit the characteristic 'bullseye-type' lesions surrounded by a yellow halo (Fig. 1.6). In an early stage of the disease, small brown to black spots are visible on the foliage, which gradually increase and merge to form large, brown patches (Thomma, 2003; van der Waals et al., 2001). Like early

blight on tomato, EB/BS disease on potato is more prominent in older, senescing leaves that are typically weaker and damaged by wind, rain or insects. Under conducive conditions, lesions may also spread to stems and petioles (van der Waals et al., 2001). Tuber lesions are sunken, dry and leathery and with a brown discoloration. Infection of the tubers usually occurs post-harvest due to wounds inflicted during harvest or bad storage conditions (Kemmit, 2002). Indeed, infected tubers should be removed before storage, since the infection may spread quickly if humidity becomes too high.

Uncontrolled infections cause premature defoliation, leading to compromised photosynthesis rates and eventually to reduced yields. A discrepancy often occurs between foliage damage and yield loss, which is due to the increase in foliage death when the plant reaches maturity (and most of the yield has been produced) causing the disease incidence to augment rapidly (van der Waals et al., 2001). This, combined with the fact that the disease is sometimes identified falsely, makes it hard to accurately estimate yield losses for EB/BS disease. EB/BS disease occurs wherever potatoes are grown and can be observed every season, albeit the incidence remains low in most areas. Literature states 5 - 50 % yield losses worldwide (Neergaard, 1945; van der Waals et al., 2001; Kemmit, 2002; Kapsa and Osowski, 2004). According to local surveys, EB/BS disease incidences are increasing in Belgium and in Germany during the last two decades (Hausladen et al., 2004; Hausladen and Leiminger, 2008; De Lange, 2010). The exact reason for this increase remains elusive, however in literature, a few possible reasons are proposed, namely the stronger legislations on the usage of broad-spectrum fungicides like mancozeb, the cultivation of high-yield potato varieties that may be more susceptible to *Alternaria* species, a more economical fertilization and finally, the climate change (Jilderda et al., 2006; Leiminger and Hausladen, 2007; De Lange, 2010).

Environmental influences

As previously described, early blight or EB/BS epidemics occur most during alternating periods of high humidity and moderate temperatures (Rotem, 1994). However, not only leaf wetness and temperature appear to influence disease development. Indeed, recent literature shows that rainfall, relative humidity and soil type also have an impact on EB/BS incidence (van der Waals et al., 2001; Wharton and Wood, 2013; Shtienberg, 2014).

As mentioned in the problem statement, very few information is available on the effect of environmental factors on the disease pressure in Flanders, Belgium. Also, the molecular mechanisms of EB/BS disease on potato are unknown. Therefore, in the next chapter rainfall,

relative humidity, temperature and soil type will be discussed as influencing factors on EB/BS disease, focusing on the situation in Flanders. In parallel, disease incidences are reported of the 2014 and 2015 seasons and are linked to environmental changes in order to reveal potential correlations. The following chapters will present data on genetic diversity of the local *Alternaria* population, EB/BS disease development and molecular mechanisms of virulence.

CHAPTER 2

ENVIRONMENTAL FACTORS ACCOUNT FOR THE DIFFERENCE IN DISEASE INCIDENCE BETWEEN TWO GROWING SEASONS

Adapted from:

Vandecasteele M, Landschoot S, Carrette J, Verwaeren J, Höfte M, Audenaert K and Haesaert G (2018). Species prevalence and disease progression studies demonstrate a seasonal shift in the *Alternaria* population composition on potato. Plant Pathology 67, 327-36.

ABSTRACT

To assess the incidence of early blight/brown spot (EB/BS) in Flanders (Belgium), agricultural potato fields in 22 locations were monitored and scored during the growing seasons 2014 and 2015. In general, disease incidences for both growing seasons were low, although within each season, distinct differences in EB/BS incidence were found between the monitored locations. In order to reveal possible determining factors for the difference in disease intensity between the different locations, an environmental analysis was performed in which soil type, rainfall and temperature were analyzed in relation to disease incidence. In 2014, potato plants grown in sandy soils had more EB/BS disease than those grown in clay or loamy soils. However, the low disease incidence in 2015 meant that differences in disease levels between soil types could not be discerned for that growing season. Rainfall, temperature or potato variety were not indicated as determining factors for the different disease intensities between locations. When comparing disease severity between the two growing seasons, it was found that in 2014, EB/BS incidences were higher than those monitored in 2015, although the difference was not significant. A window-pane analysis demonstrated that rainfall and humidity accounted for the differences in disease incidence between both growing seasons.

INTRODUCTION

In the past, potato diseases caused by *Alternaria* species were described as early blight (caused by *A. solani*) and brown spot (caused by *A. alternata*) disease. However, both diseases are often observed together on the same leaves and initial early blight symptoms are similar to brown spot symptoms, making them hard to discriminate (Stevenson et al., 2001). For this reason and the fact that in most reports, there is an ambiguity between the two diseases, this disease complex will be referred to here as early blight/brown spot (EB/BS) disease.

As stated in chapter 1, this disease occurs worldwide where potatoes are grown. However, since most research is conducted on the occurrence of late blight and its control, less info is available on EB/BS disease. For this reason, during the last two decades, efforts have been made to monitor the disease on agricultural fields, predominantly in Europe and the USA. In Germany, several reports were made from 2002 until 2007 to assess the incidence of EB/BS disease (Hausladen et al., 2004; Hausladen, 2006; Hausladen and Leiminger, 2007). The disease occurred in all potato production areas from the beginning of July with incidence peaks in the eastern and southern parts of Germany. One month later, disease incidences increased to 50 to 80 % leaf necrosis in all fields under study, which was imputed to the high temperatures measured in July. In Poland, a survey was conducted from 1998 until 2003 (Kapsa and Osowski, 2004). Up to 87 % of all the observed fields showed EB/BS disease symptoms. These were detectable as early as the end of May or the beginning of June. In Bonin (northern Poland), EB/BS incidence was significantly lower across the four years under study, which was attributed to varying meteorological conditions. However, no further information was provided on which environmental factors contributed significantly to the increase in disease incidence.

All reports described above score the disease as a percentage of leaf necrosis. Oppositely, some research groups indicate EB/BS incidence as a percentage of yield loss. These percentages vary strongly though, with yield losses of 20 - 30 % (Christ and Maczuga, 1989; Shtienberg et al., 1996) to up to 70 - 80 % (Stevenson et al., 2001). Interestingly, when data from both scoring methods from several growing seasons are available, combining them can be used to build prediction models that compute possible yield losses based on leaf necrosis (Yellareddygari et al., 2018). It was found that a disease severity of 100 % meant a reduction of 32 % in yield loss. In later growth stages, the reduction in yield loss was smaller (decrease of 19 %).

Besides the potato variety that is planted, the occurrence of EB/BS symptoms on potato foliage is highly dependent on environmental factors, such as wetness duration, humidity and temperature. Indeed, several research groups noted that alternating dry and wet periods favor the growth of *Alternaria* hyphae and that periods of heavy rain favor sporulation (Bashi and Rotem, 1975; van

der Waals et al., 2001; Gudmestad and Pasche, 2007). Infection mostly occurs in humid and temperate climates with temperatures of 24 - 29 °C (Rotem, 1994; Wharton and Wood, 2013). Holley et al. (1985) reported that the type of cultivar and the duration of leaf wetness are important factors determining the rate of EB/BS infections, whereas temperature on its own was negatively correlated with the infection rate, most probably due to the fact that lower temperatures lengthen the duration of leaf wetness. In contrast, Ganie et al. (2015) investigated the role of weather factors on EB/BS disease development during 2009 and 2010 and highlighted that temperature had a significant positive correlation with disease intensity. Relative humidity and precipitation also showed positive correlations, although they were not significant. In total, weather factors accounted for 65.5 % variation in disease incidence. As these environmental changes influence fungal growth, so do they affect secondary spore production and subsequent aerial spore concentrations. Indeed, in South Africa, during the growing seasons of 2001 and 2002, a distinct drop in spore numbers was detected in winter, whereas peaks in spore concentrations coincided with increased temperature and alternating wet periods. Spore dispersal also showed a diurnal pattern, with few spores detected during the night, when temperature and wind velocity were lowest (van der Waals et al., 2003). These results were similar as those reported by Harrison et al. (1965), some forty years earlier.

The present study was conducted to assess the EB/BS disease incidence in Flanders, as no previous studies had been performed and little to no information was available on the disease for this region. In parallel, the influence of potato variety on disease pressure was investigated together with environmental factors such as temperature, relative humidity, precipitation and soil type as influencing factors for EB/BS disease during the growing seasons of 2014 and 2015.

MATERIALS AND METHODS

Field monitoring

During the growing seasons of 2014 and 2015, agricultural potato fields at 22 locations across Flanders (Belgium) were monitored once a week to gain insight into the prevalence and epidemiology of EB/BS disease (Fig. 2.1). At some locations, more than one field was monitored, which resulted in a total of 30 fields and 27 fields that were scored in 2014 and 2015 respectively. Locations were selected in areas with significant potato cultivation. Two types of commonly grown cultivars, Bintje and Fontane which are both early to intermediate maturing varieties were grown. All monitored fields were managed according to common agricultural practice, i.e. all fields were treated weekly against *Phytophthora infestans* using one or a combination of the active ingredients mancozeb, azoxystrobin, difenoconazole and mandipropamid. Some of these compounds can have collateral effects against *Alternaria*. On each field, four subplots (20 m^2 each) were selected to be monitored and on each subplot, 50 plants were scored. Scoring was performed by walking around the plots, counting the plants that showed EB/BS symptoms and estimating the necrotic area of plant leaves in that subplot. Then, a percentage was allocated to the subplot according to Table 2.1. A simplified scheme of the scoring procedure is presented in figure 2.2. This was performed for each of the four subplots per field, after which the average percentage of necrosis was calculated. This procedure was performed for two months (July and August) during both growing seasons. This was performed in collaboration with the Agricultural Centre for Potato Research in Kruishoutem, Belgium and their partners and volunteers. In order to minimize variability in the scoring of EB/BS symptoms, demos of the scoring procedure were organized by the Agricultural Centre for Potato Research. To quantify the disease severity over time, the relative area under the disease progress curve (*rAUDPC*) was calculated. First the *AUDPC* was calculated using equation 1:

$$AUDPC = \sum_{i=1}^{N_i - 1} \frac{(y_i + y_{i+1})}{2}(t_{i+1} - t_i) \qquad (1),$$

where N_i is the total number of observations, y_i is the estimated percentage of leaf necrosis at the ith observation and t_i is the date at the ith observation. The *rAUDPC* was then calculated as described in equation 2:

$$rAUDPC = \frac{AUDPC}{AUDPC_{max}} \qquad (2)\,,$$

where $AUDPC_{max}$ is the maximum *AUDPC* value that would be reached if estimations of leaf necrosis at every observation were at maximum (100 %). Results are displayed in box-plots which graphically depict the data by the minimum (lowest line), the lower quartile, the median (band inside the box), the upper quartile and the maximum (highest line). Outliers (> Q3 + 1.5 IQR distance) are displayed by small dots above or below the box.

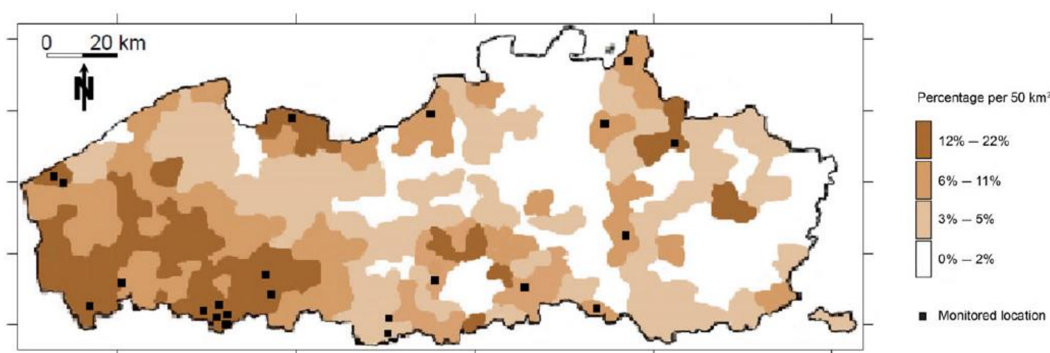


Figure 2.1. Potato acreages in Flanders. Monitored fields are highlighted with a black dot (Plattelandswijzer, 2005).

Table 2.1. Scoring table for EB/BS disease symptoms in the field. Adapted from Cox and Large (1960).

Percentage necrosis (%)	Description
0.0	No necrosis
0.2	< 25 lesions / 100 plants
0.4	< 75 lesions / 100 plants
0.6	< 2 lesions / plant
0.8	< 6 lesions / plant
1.0	< 10 lesions / plant
5.0	< 50 lesions / plant
10.0	< 100 lesions / plant
25.0	All plants affected, 75 % of leaf surface is green
50.0	All plants affected, 50 % of leaf surface is green
75.0	All plants affected, 25 % of leaf surface is green
95.0	All plants affected, 5 % of leaf surface is green
100.0	Complete defoliation

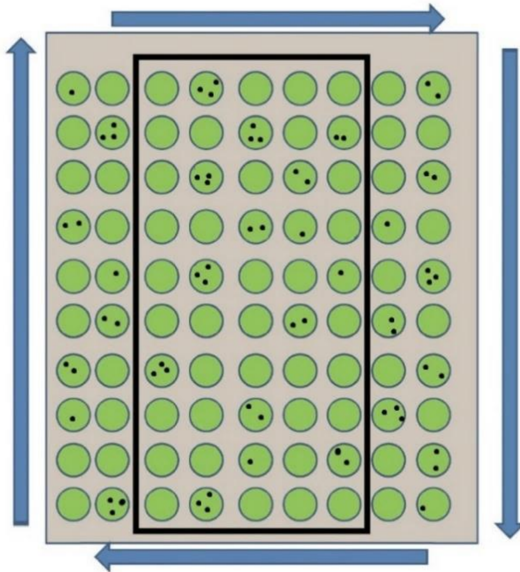


Figure 2.2. Scheme of the scoring process for the field survey. Evaluation was performed once a week by walking around each subplot, counting the plants that showed symptoms and estimating the necrotic area of those plants. A percentage was then allocated according to Table 2.1. In this example, 50 plants (highlighted by the black rectangle) are scored. Only 15 out of 50 plants show 1 - 3 spots. If an average of 2 spots per plant is accepted, this would account for 60 spots per 100 plants. According to Table 2.1, this would result in 0.4 % necrosis on this subplot. This procedure was performed 4 times on each field.

Environmental and window-pane analysis

To reveal potential correlations between differences in disease incidence and environmental factors, official data from different weather stations in Flanders for the growing seasons of 2014 and 2015 and information about soil type were obtained online. These were subsequently interpolated on maps of Flanders by the ordinary Kriging principle. *rAUDPC* values obtained for the different monitored locations were then mapped using the R v. 2.15.3 software package (R Core Team, 2014).

To investigate if periods or 'timeframes' of certain weather conditions during the growing season correlated with variations in *rAUDPC* values, an empirical method called 'window-pane analysis' was used which was formalized by Coakley and Line (1982). This type of analysis is applied to determine the length and starting time of temporal windows where environmental variables are either positively or negatively associated with disease severity. The window-pane analysis is based on the specification of a time window and the calculation of mean environmental variables (rainfall, mean temperature and mean relative humidity) during that time window. For our analysis, a time window of five days was chosen since EB/BS disease is a fast-developing disease with an infection cycle of approximately five to seven days. This time window is moved in daily increments along the duration of the growing season (July 10th - September 15th), which gives 63 time windows over a duration of 68 days. Therefore, for each weather variable, 68 correlations were estimated.

Statistical analyses

The R v. 2.15.3 software package (R Core Team, 2014) was used for statistical evaluation of the field monitoring data (to test differences in disease incidence between growing seasons) and environmental data (to test differences in disease incidence between varying environments). Statistical significance between groups of data was inferred at a significance level of $\alpha = 0.05$. Because normality assumptions of parametric tests were not met for all assays, differences between groups of data were tested for significance using a non-parametric Kruskal–Wallis test. If significant differences between the groups were found, a Dunn post hoc test at $\alpha = 0.05 / n$ with n the number of pairwise comparisons was performed.

RESULTS

Field survey

The incidence of EB/BS in potato fields at 22 locations across Flanders (Belgium) was assessed over the duration of two growing seasons. Results are shown in figure 2.3. The percentage of leaf necrosis remained at a very low level (0 - 0.5 % leaf necrosis) in the first half of the growing season (until mid-August). In 2014, a steep increase in leaf necrosis was observed from mid-August. In 2015, this increase began later, at the beginning of September. Disease incidences for most tested fields were low, with only a few locations displaying *rAUDPC* values of over 10 % (Supporting information: Fig. S2.1). When comparing disease incidences of the monitored locations in 2014 with those in 2015, it was found that the severity of leaf necrosis was greater in 2014 than in 2015, especially during September. However, the difference was not significant (p-value = 0.297, Kruskal-Wallis test).

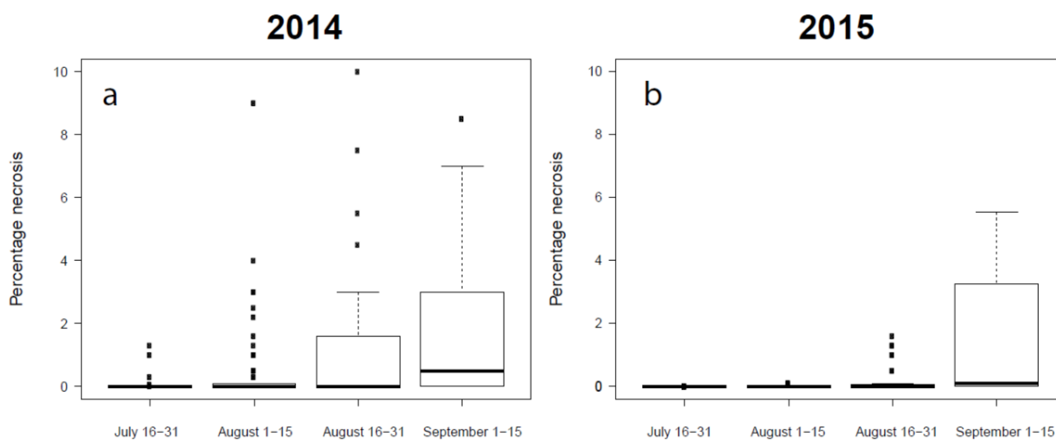


Figure 2.3. Percentage leaf necrosis due to *Alternaria* species on all monitored fields (2014: n = 30; 2015: n = 27) during four periods of the growing season for 2014 and 2015. Fields were monitored once a week during the months of July and August.

Additionally, the varietal effect was investigated (Fig. 2.4). The analysis showed that the variety Fontane is most susceptible to EB/BS disease in 2014 (p-value = 0.0223, Kruskal-Wallis test), but in 2015, the difference between the two varieties was not significant (p-value = 0.326, Kruskal-Wallis test). Fontane was also most susceptible when combining both years in the analysis (p-value = 0.0221).

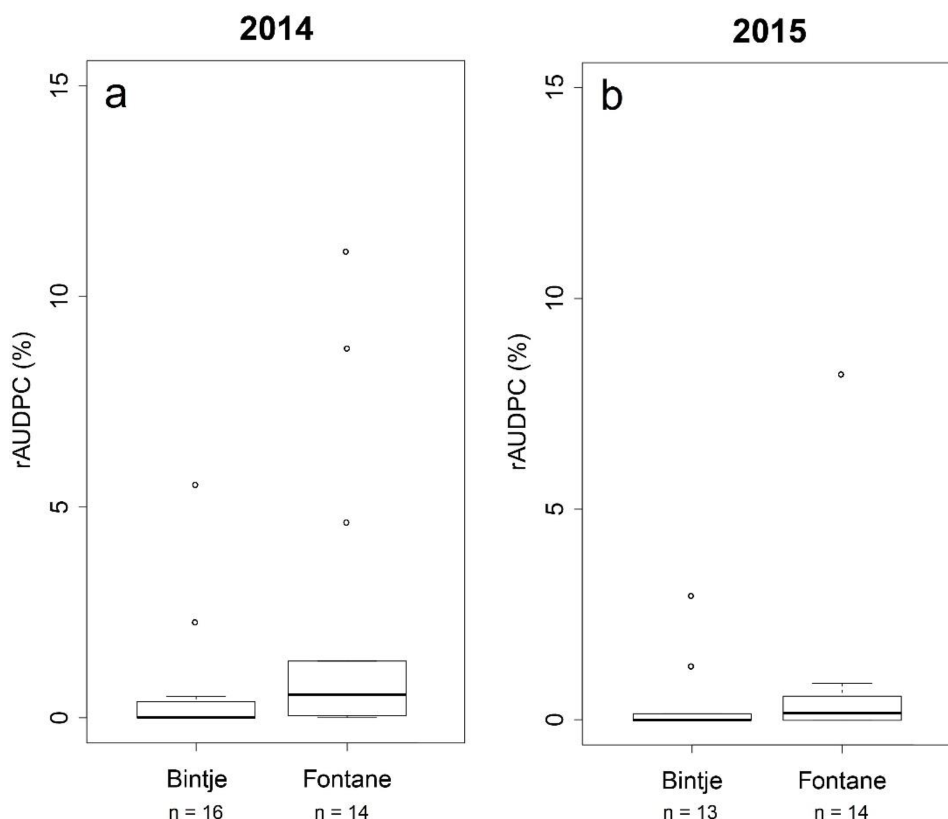


Figure 2.4. Relative Area Under the Disease Progress Curve (rAUDPC) values for all monitored cultivars for the 2014 growing season (a) and the 2015 growing season (b). The number of monitored fields that grew the cultivar Bintje or Fontane is represented by n. Fields were monitored once a week during the months of July and August.

Environmental analysis

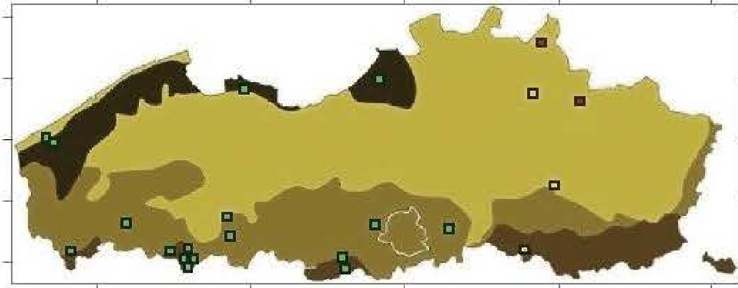
Next, an environmental analysis was performed in which the *rAUDPC* values were linked with soil type, average rainfall and temperature. For each season, the *rAUDPC* values were divided in three classes (low (0 - 1 %), medium (1 - 5 %), highest (5 - 10 %)) and the locations with their respective *rAUDPC* class were layered on maps depicting soil type, average rainfall and average temperature (Fig. 2.5). This was done for both growing seasons. It was observed that, in 2014, potato plants grown in sandy soils appear to be more prone to EB/BS disease than those grown in clay or loamy soils (p-value = 0.023, Kruskal-Wallis test) (Fig. 2.5a). However, in 2015, when the disease pressure was very low, the disease incidence levels for locations in sandy soils were not significantly different to those of fields located in clay or loamy soils (p-value = 0.340, Kruskal-Wallis test). No correlation between rainfall or temperature and EB/BS incidence per location was found in a single growing season (Fig. 2.5b, c).

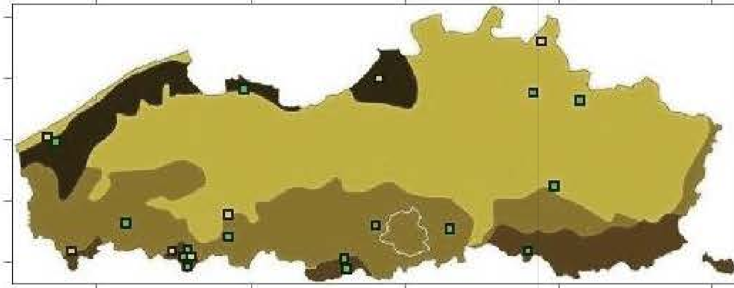
Additionally, correlations between type of variety and *rAUDPC* values monitored in different soil types were investigated, though no significant positive correlation was found.

2014

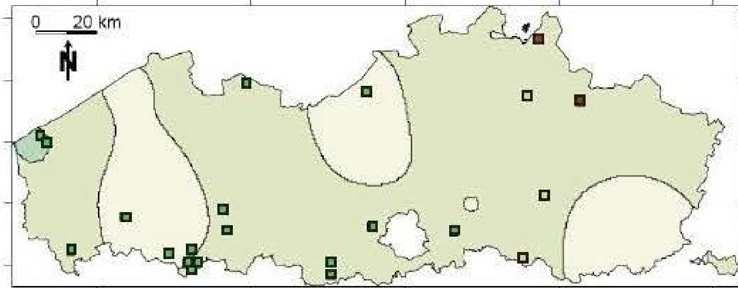
2015

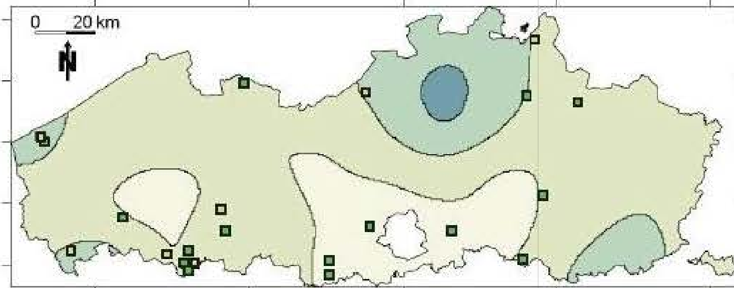
a



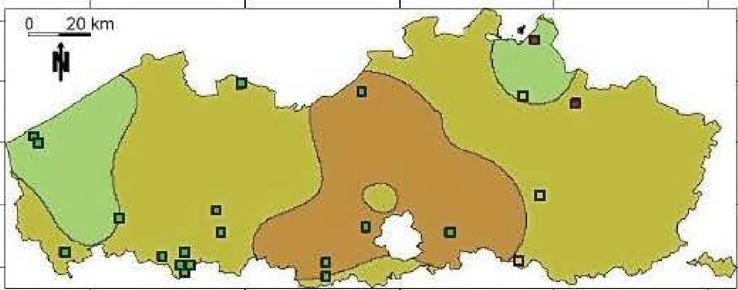


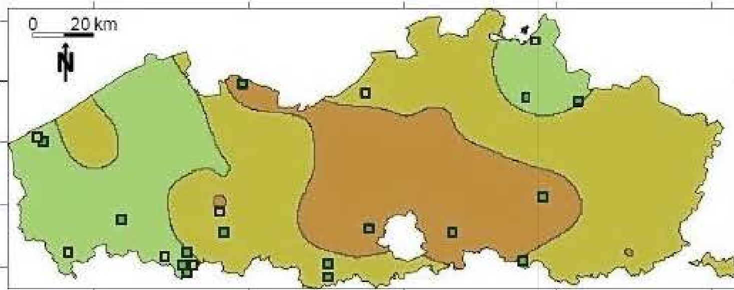
b





c





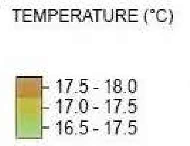


Figure 2.5. Relative Area Under the Disease Progress Curve (*rAUDPC*) values of EB/BS disease during the 2014 and 2015 growing seasons mapped on the monitored locations in Flanders according to (a) soil type, (b) average rainfall and (c) average temperature. Soil type map adapted from *www.bodemwijzer.*be. Temperature and rainfall maps are constructed through interpolation of official weather data obtained from different weather stations in Flanders during 2014 and 2015. Interpolation was performed on the basis of the ordinary Kriging principle.

Window-pane analysis

In order to further investigate the link between disease and weather variables, a window-pane approach was pursued. Figure 2.6 represents the Spearman rank correlation coefficients between weather conditions during the growing seasons of 2014 (Fig. 2.6a) and 2015 (Fig. 2.6b), and variations in *rAUDPC* values (a summary of the weather data can be found in Supporting information: Table A2.1). It can be seen from the analysis of 2014 (Fig. 2.6a) that during the first part of the growing season (before July 29th), the correlations between the *rAUDPC* and weather conditions were not significant. However, during the period between July 29th and August 29th, the *rAUDPC* was positively correlated with rainfall during two time windows (July 28th until August 5th and July 23rd until August 28th). Furthermore, the relative humidity had a significant positive correlation during the period starting from August 9th until August 18th. Temperature was never positively correlated with the *rAUDPC*. However, temperature was significantly negatively correlated with *rAUDPC* from August 4th until August 30th and at the end of the growing season (after September 10th). In contrast, no significant correlations were found between weather variables and *rAUDPC* values of the 2015 growing season (Fig. 2.6b).

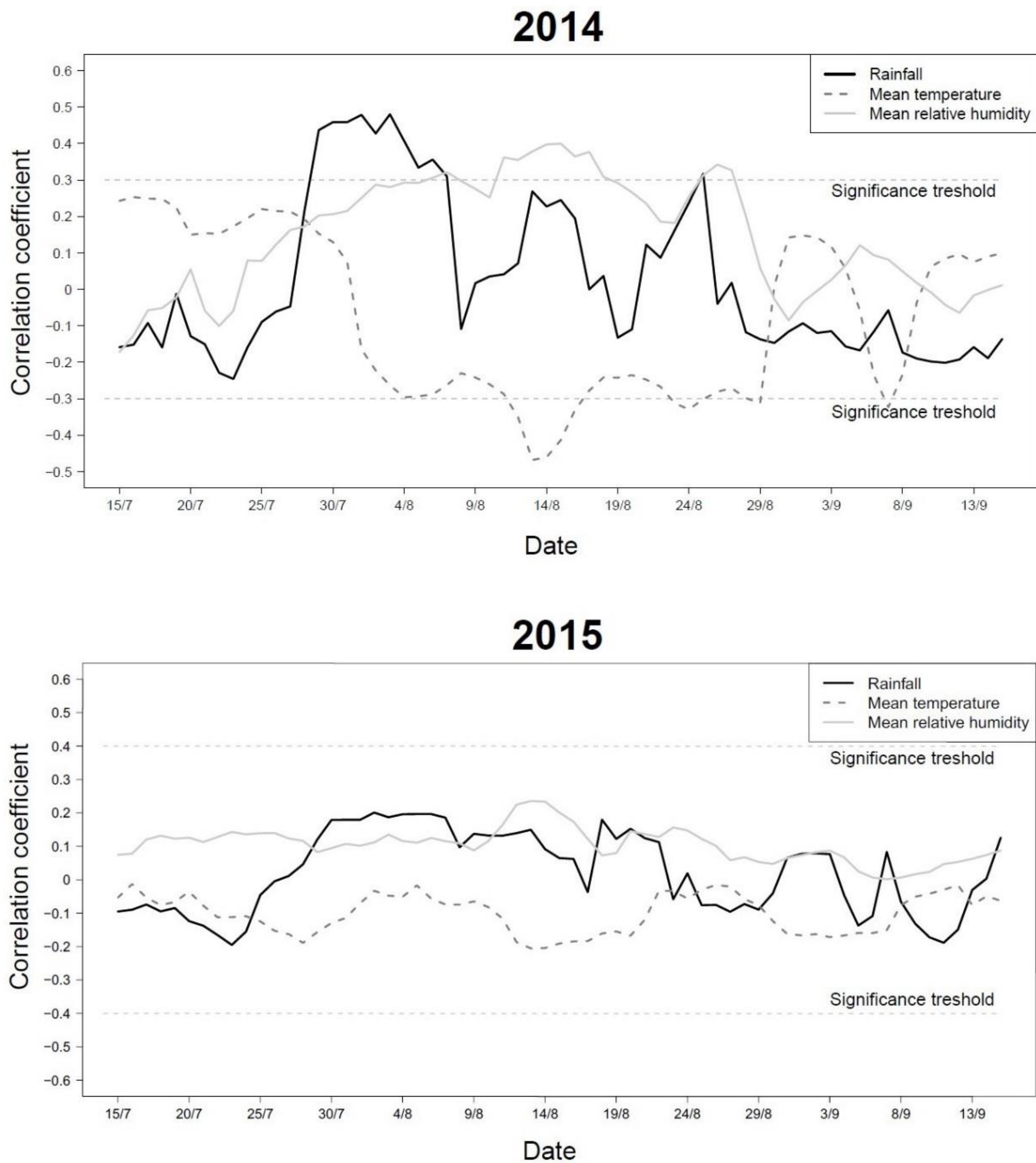


Figure 2.6. Spearman rank correlation coefficients for the association between total rainfall, mean temperature and mean relative humidity, and the *rAUDPC* values during the 2014 growing season (upper plot) and the 2015 growing season (lower plot). For this analysis, a window-length of five days was used. Mean environmental variables of all monitored fields were determined for each time window and correlations were calculated using the *rAUDPC* values of those fields. For the growing season of 2014, correlation coefficients were based on *rAUDPC* values of 30 fields, while for the growing season of 2015, *rAUDPC* values of 27 fields were taken up in the analysis. Correlations above or below the dashed vertical line are significant.

DISCUSSION

This study was undertaken because in the last two decades, EB/BS disease is increasingly observed in Flemish potato fields and is therefore labeled as an emerging problem. With the aim of assessing the disease incidence, agricultural fields at 22 locations across Flanders were monitored for EB/BS disease during the growing seasons of 2014 and 2015. Although symptoms have previously been reported to be difficult to distinguish from other stress responses such as ozone damage (Turkensteen et al., 2010), it was possible to assess the disease symptoms very precisely. By the so-called "bulls-eye" type lesions, EB/BS disease is easily distinguished from late blight, other types of infections and abiotic stress by the trained eye. In addition, field isolates (see Chapter 3) were obtained from the collected necrotic leaf samples, which caused similar symptoms if inoculated on healthy potato leaves and, in agreement with Koch's postulates, the pathogenic fungi could be re-isolated from the inoculated leaf samples. Thus, it was illustrated that the observed symptoms were caused by *Alternaria* species.

In general, our survey revealed very low incidence levels in Flemish agricultural fields in 2014 and 2015. Indeed, with a few exceptions, total *rAUDPC* values of all monitored fields remained below 10 %. In contrast, Hausladen (2006) reported epidemic levels of up to 100 % necrosis in September of 2003, 2004 and 2005. This difference in disease severity can be attributed to one of multiple factors. Firstly, there might be a difference in the applied chemical control. Similar as the fields in our study, Hausladen (2006) indicated that all agricultural fields were treated against late blight (*Phytophthora infestans*). However, since no specific active ingredient was mentioned, it cannot be excluded that the applied product in the two studies may have had an influence on disease severity. Secondly, there might have been a difference in weather circumstances. As it becomes clear that factors such as temperature, precipitation or relative humidity affect EB/BS disease incidence, the difference in disease severity between the two studies may be explained by the difference in average temperature. Indeed, Hausladen (2006) mentioned high temperatures in July of 2003, 2004 and 2005, whereas the average temperatures in Flanders in 2014 and 2015 remained mostly under 20 °C. Finally, some reports have mentioned that *Alternaria* species are prone to horizontal gene transfer (Johnson et al., 2001; Hatta et al., 2002; Akamatsu et al., 2003; Hatta et al., 2006; Ma et al., 2010), which may give rise to highly pathogenic *Alternaria* species due to the gain of conditionally dispensable chromosomes that carry genes for toxin production.

Regarding disease progression, a recurring pattern emerged. During the first half of the growing season, leaf necrosis levels were low to negligible. From mid-August, a sharp increase in leaf necrosis was observed for all monitored fields. The field surveys conducted in the present investigation also showed that the incidence of disease in 2014 was higher than in 2015, especially from September onwards. Weather data from both growing seasons (Supporting Information: Table S2.1) showed that the month of September 2014 was substantially dryer than September 2015 (0.68 vs 37.63 mm). It is known from literature that weakened plant tissue due to drought stress are more susceptible to diseases caused by *Alternaria* species (Thomma, 2003). However, from September onwards, the tubers were maturing and consequently, the foliage died rapidly. Thus, the reason for escalation of the disease may have been the necrosis of the foliage, rather than drought stress, since *A. solani* is a necrotrophic pathogen. Therefore, no clear-cut conclusions can be drawn from the precipitation data of both growing seasons.

To look deeper at the difference in disease incidence within growing seasons, an environmental analysis was performed which showed that in 2014, the eastern regions (sandy soils) of Flanders were more affected than the western (clay soils) and central regions (loamy soils). Although other research groups have reported that crops grown in sandy soils are more prone to *Alternaria* infection (Shtienberg, 2014), this hypothesis could not be confirmed in the growing season of 2015, most probably because of the low disease pressure in that season. Further research is therefore needed to substantiate this hypothesis. No apparent correlation between rainfall or temperature and EB/BS incidence per location was found in a single growing season. Indeed, most likely as a result of the small sampling area (13225 km^2), the variance between average rainfall or average temperature in sandy regions during the growing season of 2014 and the precipitation and average temperature in other regions was too small to find a correlation. The varietal effect was also analyzed, which showed that Fontane was more susceptible to EB/BS in 2014 and generally in both years of the study. However, the locations with sandy soils did not grow more of the variety Fontane than the variety Bintje. Therefore, the higher disease incidence in sandy soil regions cannot be attributed to the cultivated variety. The difference in susceptibility between Bintje and Fontane might be explained by the fact that Fontane is more susceptible to late blight than Bintje (Andrivon et al., 2010), which could have influenced the use of fungicides by the farmer to control late blight. Since the products used against late blight often have or don't have side-effects against *Alternaria* species, this might have resulted in a false interpretation of susceptibility towards EB/BS disease. A full analysis of the applied active ingredients on both cultivars could help to resolve this issue.

A window-pane analysis was performed to determine if weather variables are significantly correlated with EB/BS incidence during certain time frames. It was noticed that during two time frames in 2014 (July 28th until August 5th and August 23rd until August 28th), there were significant positive correlations between rainfall and *rAUDPC*. In addition, between these two time frames (i.e. during August 9th until August 18th), there was a positive correlation between relative humidity and *rAUDPC*. In contrast, during the 2015 growing season, no significant correlations were found between rainfall, mean temperature or mean relative humidity, and *rAUDPC* values, most probably due to the low disease pressure. Therefore, it is concluded that in 2014, but not in 2015, rainfall and relative humidity can be seen as factors influencing the increased disease pressure. Surprisingly, significant negative correlations were found between the mean temperature and *rAUDPC* in 2014. Although this observation was in agreement to what was found by Holley et al. (1985), it contradicted some other reports, stating that higher temperatures favor colonization by *Alternaria* (Degenhardt et al., 1982; van der Waals et al., 2001; Hausladen et al., 2004; Ganie et al., 2015). These ambiguities indicate that EB/BS disease development is dependent on multiple factors. Thus, if a high disease incidence is observed at a lower temperature, this may result from the amount of rainfall and relative humidity being optimal at that time point. Indeed, it can be seen in figure 2.6 that the time window where temperature is negatively correlated with disease incidence (August 4th until August 15th), is in the same time window that shows a positive correlation between relative humidity and disease incidence. Higher correlation coefficients were also found between rainfall and disease incidence, although not positively significant.

This study provides a clear view of EB/BS disease progression during two growing seasons, while also highlighting that environmental factors, such as soil type, rainfall and relative humidity are contributing factors that influence the disease progression. Due to the low disease pressure monitored in 2015, these findings could not be discerned for that growing season, indicating that more research over several years is needed to substantiate the hypotheses mentioned above. To elucidate the underlying mechanism behind the increased disease incidence from mid-August, the local *Alternaria* population was first molecularly identified in order to analyze the population composition at different time points during a growing season. These studies will be discussed in the following two chapters.

SUPPORTING INFORMATION

Table S2.1. Summary from the weather data used in the window-pane analysis.

Period	Year	Total Rainfall (mm)	Mean RH (%)	Mean Temperature (°C)
July 16-31	2014	33.83	78.30	20.89
August 1-15	2014	34.08	81.60	17.60
August 16-31	2014	49.08	85.12	14.33
September 1-16	2014	0.68	83.35	16.01
July 16-31	2015	30.27	77.60	17.30
August 1-15	2015	34.10	76.40	19.41
August 16-31	2015	58.05	83.26	17.48
September 1-16	2015	37.63	88.26	14.13

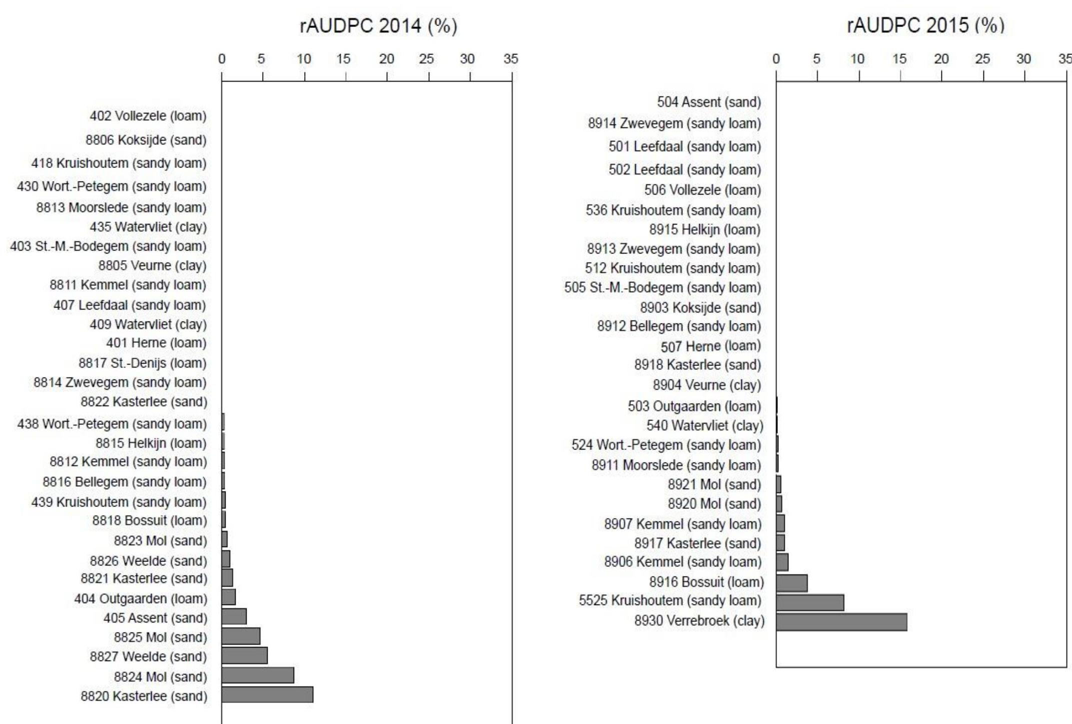


Figure S2.1. Relative Area Under the Disease Pressure Curve (rAUDPC) values for all monitored fields for the 2014 and 2015 growing seasons. Fields are ordered from least to most infected.

ACKNOWLEDGEMENTS

We would like to thank the staff from the Agricultural Centre for Potato Research (Proefcentrum aardappelteelt, PCA) in Kruishoutem (Belgium) and the staff from Inagro in Beitem (Belgium) for monitoring the potato fields.

CHAPTER 3

THE FLEMISH *ALTERNARIA* POPULATION IS MORE DIVERSE THAN A TWO-SPECIES COMPLEX

Adapted from:

Landschoot S*, Vandecasteele M*, De Baets B, Höfte M, Audenaert K and Haesaert G (2017). Identification of *A. arborescens*, *A. grandis*, and *A. protenta* as new members of the European *Alternaria* population on potato. Fungal Biology 121, 172-188.

* Both authors contributed equally to this article

ABSTRACT

Alternaria species, primarily the small-spored *Alternaria alternata* and the large-spored *Alternaria solani*, are considered a serious threat to potato cultivation. To reveal the underlying mechanisms of disease progressions and to develop adequate control strategies, it is important to gain insight into the diversity of the local *Alternaria* population. Therefore, a multiple-locus sequence analysis was approached to molecularly identify isolates that were collected from Flemish potato fields. Based on the sequence analyses of the internal transcribed spacer region and the glyceraldehyde-3-phosphate dehydrogenase gene, the small-spored and large-spored *Alternaria* isolates could be separated from each other. However, the resolution of the analysis was not sufficient to reveal phylogenetic clusters within these two groups. Sequence analyses of the calmodulin gene and the RNA polymerase second largest subunit gene showed that besides *A. solani*, also *A. grandis* and *A. protenta* were present in the Flemish large-spored *Alternaria* population. Sequence analyses of the *Alternaria* major allergen gene *Alt a 1* and the translation elongation factor-a revealed that both *A. alternata* and species belonging to the *Alternaria arborescens* species complex were present in the small-spored *Alternaria* population. Furthermore, according to the histone h3 sequence the members of the *A. arborescens* species complex could be subdivided into two distinct phylogenetic groups. Microscopic analysis of the studied isolates' catenulation revealed that isolates molecularly identified as *A. alternata* had few to no branches, whereas for most *A. arborescens* isolates, clusters of branched conidial chains could be observed. The catenulation structure of *A. arborescens* group A isolates could not be discerned from those of *A. arborescens* group B isolates. Also, this distinction in catenulation structure between isolates identified as *A. alternata* and those that were identified as *A. arborescens* was not consistent among all studied isolates, again highlighting the high morphological variety within *Alternaria* species.

INTRODUCTION

As stated in chapter 1, the taxonomy of the genus *Alternaria* is a much debated issue. Indeed, several re-descriptions and revisions of the taxonomy of these genera have complicated the matter over the years, resulting in a plethora of new species (Elliott, 1917; Wiltshire, 1938; Wiltshire, 1933; Joly, 1964). Also, the species within the genus *Alternaria* encompass a considerable morphological diversity. Therefore, in the past, a division into subgeneric groups, based on conidium characteristics has been proposed. According to Neergaard (1945), the genus *Alternaria* can be subdivided into three sections based on catenulation (chain-formation of spores). Simmons (2007) proposed an organization of the genus into 14 species-groups, each typified by a representative species, based upon characteristics of spores and catenulation. However, a major disadvantage of morphological classification is that these characteristics are not only affected by intrinsic factors, but also by environmental conditions, subsequent subculturing, cultivation medium, etc. (Thomma 2003). As a result, many research groups have approached molecular methods to delineate species clades. The downside of this new approach is that clusters of species based on molecular methods do not always correlate with groups based on morphological characteristics, suggesting that a consensus must be reached between delineations formed by the two methods (Pryor and Gilbertson, 2000; Chou and Wu, 2002; Lawrence et al., 2016).

As described in chapter 1, the genus *Alternaria* has been divided into 24 sections based on multi-gene phylogeny (Lawrence et al., 2013; Woudenberg et al., 2013a; Grum-Grzhimaylo et al., 2016). In an attempt to delineate the large-spored *Alternaria* species in the section *Porri*, Woudenberg et al. (2014) used a multi-locus sequence analysis. Based on the sequence analysis of the DNA internal transcribed spacer region (*ITS*) region, the glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) gene, the RNA polymerase second largest subunit (*RPB2*), the translation elongation factor-a (*EF-a*) and the *Alternaria* major allergen gene *Alt a 1*, the section *Porri* could be divided into 63 morphospecies (e.g. *Alternaria solani*, *Alternaria protenta*, *Alternaria grandis*, etc.).

In order to obtain a clearer and more stable species classification in the section *Alternaria*, which contains most of the small-spored *Alternaria* species, Woudenberg et al. (2015) performed whole-genome sequencing supplemented with a transcriptome analysis and multi-gene sequencing. The multigene phylogeny was based on the sequences of parts of nine genomic regions, namely the *ITS* regions 1 and 2 and intervening 5.8S nrDNA, the 18S nrDNA (*SSU*), the 28S nrDNA (*LSU*), the *Gpd* gene, *RPB2*, *EF-a*, *Alt a 1*, endopolygalacturonase (*endoPG*) and an anonymous gene region (*OPA10-2*). Based on genome and transcriptome comparisons and molecular phylogenies, these authors concluded that the section *Alternaria* consists of 11 phylogenetic species (e.g. *Alternaria tomatophila*, *Alternaria longipes*, *Alternaria gossypina*, etc.) and one species complex

(the *Alternaria arborescens* species complex (*AASC*)). A majority of morphospecies could not be subdivided with the set of nine partial gene sequences used in the study of Woudenberg et al. (2015) and are synonymized under *A. alternata*. A more detailed study, including the identification of host-specific toxin gene clusters could possibly reveal new groups within this clade (*formae speciales*).

To date, the small-spored *A. alternata* and the large-spored *A. solani* are the predominant pathogenic species reported on potato, causing premature defoliation, which has a significant impact on tuber yield and potato quality. Recently, also the large-spored species *A. grandis* (Rodrigues et al., 2010) and *A. protenta* (Woudenberg et al., 2014) have been identified as members of the *Alternaria* species complex in potatoes. Moreover, besides *A. alternata*, also the small-spored species: *Alternaria tenuissima*, *Alternaria dumosa*, *Alternaria interrupta*, *A. arborescens*, *Alternaria infectoria*, and *Alternaria arbusti* have been associated with *Alternaria*-caused diseases of potato in Iran (Taheri et al., 2009; Ardestani et al., 2010), Russia (Orina et al., 2012), China (Zheng and Wu, 2013) and the USA (Tymon et al., 2016). Woudenberg et al. (2015) synonymised *A. tenuissima*, *A. dumosa*, and *A. interrupta* under *A. alternata*, although according to Zheng et al. (2015), *A. tenuissima* can be distinguished from *A. alternata* based on the partial coding sequence of the histone h3 gene. *A. tenuissima* isolates possess a 106 bp insert in the partial coding sequence of the histone h3 gene, which is absent in the *A. alternata* isolates.

The aim of this research was to gain insight into the Flemish *Alternaria* population present on naturally infected potato leaves. Therefore, a collection of 83 isolates, obtained during three years (2012 - 2014) on different potato fields throughout Flanders (Belgium), was used. Microscopically the field isolates were divided into two groups: small-spored and large-spored *Alternaria* isolates. Gaining insight into the population diversity (i.e., which species are present and what is the genetic variability between isolates within one species) is important to unravel disease progression mechanisms and to improve control measures against EB/BS disease on potato. Therefore, a multi-locus sequence analysis was approached in which sequences of seven genomic regions, the *ITS* region, the *Gpd* gene, the *EF-a*, the histone h3 gene, the *Alt a 1* gene, *RPB2* and the calmodulin gene, were analyzed.

MATERIALS AND METHODS

Identification of field isolates

Potato leaflets, showing EB/BS symptoms, were randomly sampled from 47 agricultural fields throughout Flanders (Fig. 3.1). They were collected from the beginning of July until mid-September at regular intervals during three growing seasons (2012 - 2014). From each leaf, one necrotic lesion was excised, surface-sterilized with 5 % NaOCl for about 1 min, washed in sterile distilled water and subsequently placed on synthetic nutrient agar (SNA) medium (1 g L^{-1} KH_2PO_4, 1 g L^{-1} KNO_3, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.5 g L^{-1} KCl, 0.2 g L^{-1} glucose, 0.2 g L^{-1} saccharose, 1 M NaOH and 20 g L^{-1} agar) at 21 °C. After one week, outgrowing mycelium was transferred to new Petri dishes and incubated at 21 °C. To induce sporulation, plates were incubated at 25 °C for 2 weeks under near-UV light (12 h light and 12 h dark). To obtain single spore colonies, one spore of each plate was transferred to new SNA medium. Finally, isolated species were microscopically identified by their spore size and shape. Based on the spore size and shape of the single-spored colonies, the isolates could be divided into two categories: large-spored species (150 - 300 x 15 - 19 µm) and small-spored species (20 - 63 x 9 - 18 µm). For this study a subset of 11 large-spored and 72 small-spored *Alternaria* isolates was used. The fact that we obtained a lot more *Alternaria alternata* isolates compared to *Alternaria solani* isolates is due to the fact that mostly *A. alternata* could be isolated from the leaf samples. An overview of the isolates used in this study is listed in Supporting information: Table S3.1.

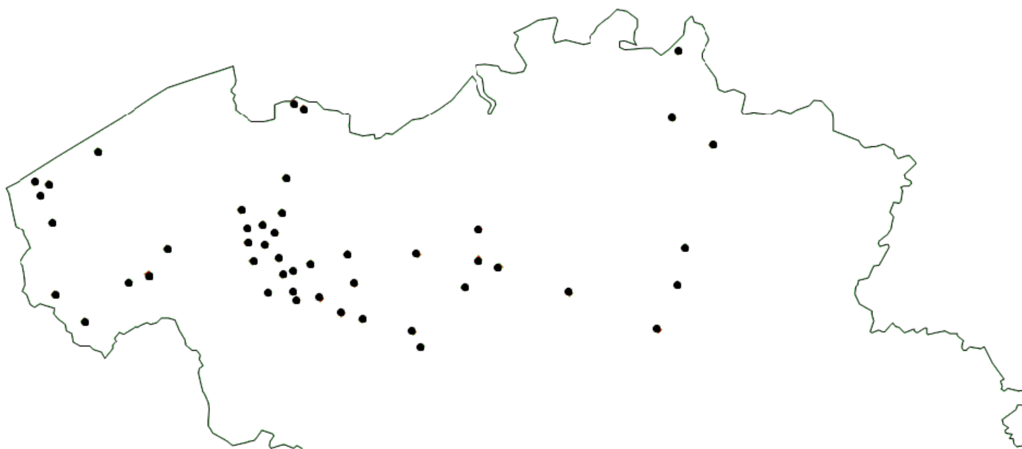


Figure 3.1. Locations of the potato fields where leaf samples with EB/BS symptoms were collected. Samples were collected from the beginning of July until mid-September at regular intervals during three growing seasons (2012 - 2014).

PCR and sequencing

DNA was extracted from 7-day-old mycelium cultured on Potato Dextrose Broth. Then the mycelium was transferred into a 1.5 mL microcentrifuge tube and lyophilized for 24 h. The mycelium was crushed under liquid nitrogen. Genomic DNA extraction was carried out using the Invisorb Spin Plant Mini Kit (Stratec) according to the manufacturer's instructions. The DNA concentration was measured using a Quantus™ Fluorometer (Promega) and adjusted to 5 ng DNA μL^{-1}. The primers and the PCR conditions for amplification are given in Table 3.1. After amplification the PCR product was purified using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-tek). Finally, the purified PCR products were sequenced in both directions by Macrogen Europe Laboratory services (The Netherlands) using the corresponding primers.

Diversity and phylogenetic analyses

To compare the genetic profile of the 83 collected field isolates with each other and with the existing sequences from reference isolates a genetic diversity study, complemented with a phylogenetic analysis was performed. The sequences of the genes in study were obtained from GenBank (*http://www.ncbi.nlm.nih.gov/genbank/*). This set of seven genes (Table 3.1) was based on the paper by Woundenberg et al. (2013) and provides enough resolution to distinguish species within small- and large-spored *Alternaria* species according to the classification proposed in the aforementioned paper. Since this allocation is now regarded as the standard and most recent classification of the *Alternaria* genus (Highly Cited Paper as of March 2019), we organized our isolates according to this classification. For the genetic diversity analysis, multiple sequence alignments were generated and manually refined in BioEdit 7.0.9. The genetic distance between the different species was evaluated by calculating the pairwise Hamming distance between sequences. The Hamming distance measures the diversity between two aligned sequences with a fixed length by calculating the number of sites at which the corresponding nucleotides differ. Hamming nucleotide distance matrices were generated for each alignment of sequences, and the average fraction of nucleotide sequence identity was calculated. Furthermore, for each set of sequences the number of variable characters and the number of parsimony informative sites was determined. All analyses were performed using the R v. 2.15.3 software package (R Core Team, 2014). The phylogenetic relationships among the isolates were inferred using maximum likelihood trees based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Bootstrap values > 70 % were considered as significant and are indicated in the phylogenetic trees.

Table 3.1. Primer sequences and corresponding references and PCR conditions.

Gene	Primer sequence	Reference	PCR conditions
ITS region	TCCTCCGCTTATTGATATGC GGAAGTAAAAGTCGTAACAAGG	(White et al., 1990)	5 min 94 °C, 35x (45 s 94 °C, 45 s 52 °C, 90 s 72 °C), 6 min 72 °C.
Gpd gene	ATGCCACAGTATCGAGCACA CAAGAGGCGTTGGAGAGAAC	(Berbee et al., 1999)	4 min 94 °C, 35x (30 s 94 °C, 45 s 60 °C, 60 s 72 °C), 5 min 72 °C.
Histone h3	ACTAAGCAGACCGCCCGCAGG GCGGGCGAGCTGGATGTCCTT	(Glass and Donaldson, 1995; Kang et al., 2002)	2 min 96 °C, 30x (15 s 96 °C, 30 s 55 °C, 35 s 75 °C), 2 min 72 °C.
Alt a 1	ATGCAGTTCACCACCATCGC ACGAGGGTGAYGTAGGCGTC	(Woudenberg et al., 2014)	5 min 94 °C, 40x (30 s 94 °C, 30 s 55 °C, 60 s 72 °C), 7 min 72 °C.
EF-a	CATCGAGAAGTTCGAGAAGG GGARGTACCAGTSATCATGTT	(Carbone and Kohn, 1999; O'Donnell et al., 1998)	5 min 94 °C, 40x (30 s 94 °C, 30 s 52 °C, 45 s 72 °C), 7 min 72 °C.
Calmodulin	AGCAAGTCTCCGAGTTCAAGG CTTCTGCATCATCAYCTGGACG	(Lawrence et al., 2013)	4 min 95 °C, 35x (30 s 95 °C, 30 s 58.5 °C, 1 min 72 °C), 5 min 72 °C.
RPB2	GGGGWGAYCAGAAGAAGGC CCCATRGCTTGTYYRCCCAT	(Sung et al., 2007; Liu et al., 1999)	5 min 94 °C, 5x (45 s 94 °C, 45 s 60 °C, 2 min 72 °C), 5x (45 s 94 °C, 45 s 58 °C, 2 min 72 °C), 30x (45 s 94 °C, 45 s 54 °C, 2 min 72 °C), 5 min 72 °C.

RESULTS

Multi-locus sequencing analysis

The summary statistics, giving phylogenetic information for the *ITS* region, the *Gpd* gene, the *EF-a*, the histone h3 gene, the *Alt a 1* gene, the calmodulin gene and *RPB2* are presented in table 3.2. The phylogenetic trees based on the *ITS* region (Fig. 3.3) and *Gpd* gene (Fig. 3.4) show that the large-spored *Alternaria* isolates are phylogenetically distinct from the small-spored *Alternaria* isolates. This separate cluster comprising the large-spored isolates is, for each gene, supported by a bootstrap value of 100 %. At first glance, the topology of the *Gpd*-based tree looks rather similar to the *ITS* tree distinguishing the small-spored and large-spored isolates. Nevertheless, there are some sub clusters that are supported by high bootstrap values, but to which none of the reference isolates belong (Fig. 3.4).

Since the polymorphisms within the *ITS* and *Gpd* regions are too low to delineate species within the small-spored or large-spored *Alternaria* isolates, additional sequence analyses of the calmodulin gene, *RPB2* (large-spored isolates), *Alt a 1* gene and *EF-a* (small-spored isolates) and the histone h3 gene (small and large-spored isolates) were performed.

The sequence analysis of the calmodulin gene (Fig. 3.5) and *RPB2* (Fig. 3.6) together with the resulting trees revealed that within the large-spored *Alternaria* isolates besides *Alternaria solani* also *Alternaria grandis* and *Alternaria protenta* are present on naturally infected potato leaves in Belgium (Fig. 3.2a).

Concerning the small-spored isolates, sequence analyses of the *EF-a* (Fig. 3.7) and the *Alt a 1* gene (Fig. 3.8) were performed. The consensus tree of the *Alt a 1* gene and the *EF-a* (Fig. 3.2b) revealed that the small-spored isolates could be divided into two groups, namely the *Alternaria alternata* isolates and isolates belonging to the *Alternaria arborescens* species complex (*AASC*). Within the *AASC* group, different sub clusters can be distinguished. Most isolates cluster together with the reference isolates. However, some isolates were rather loosely associated with the reference isolates of the *AASC*.

Additionally, a sequence analysis of the histone h3 gene was performed (Fig. 3.9). In the consensus tree, based on the histone h3, *Alt a 1* and *EF-a* sequences (Fig. 3.2c), different sub clusters are present. One cluster contains the *A. alternata* isolates, while the isolates belonging to the *AASC* according to the *Alt a 1* and *EF-a* sequences, could be further subdivided into two groups according to their histone h3 sequence. These groups are indicated as the *A. arborescens* group A and the *A. arborescens* group B in figure 3.2c. Group A contained isolates with a histone h3 sequence of 440 bp, whereas in the histone h3 sequence of the isolates belonging the group B an intron of 106 bp was present, resulting in a histone h3 sequence of 546 bp. Some isolates could not be assigned

to one of these three groups. Additionally, isolates 14.17 and 12.20.2 form a separated group within the *A. arborescens* group A. In figure 3.10 the consensus tree for the small-spored species, based on the six different genomic regions under study, is given. Since no subdivision based on the *ITS* region and *Gpd* gene within the small-spored isolates could be made, this tree shows a similar topology compared to the tree in figure 3.2c. However, some subtle differences can be seen.

Microscopic identification

Based on the phylogenetic analyses, it was concluded that there was a subdivision within the small-spored isolates based on the histone h3, *Alt a 1* and *EF-a* sequences. Therefore, the isolates were screened for the presence or absence of arborescent to semi-arborescent conidiophores in order to find correlations between phylogenetic species groups and their catenulation structure. Figure 3.11 parts a and b show the conidial branching of two isolates (14.27 and 14.42) that were identified as *Alternaria alternata*. Parts c and d show the conidial morphology of two isolates (14.79 and 14.259) belonging to the *Alternaria arborescens* group B. In parts e and f of figure 3.11 the conidial morphology of two isolates (13.147 and 14.264) of the *A. arborescens* group A can be seen. The isolates that were identified as *A. alternata* (Fig 3.11a, b) had mostly unbranched chains of conidia, whereas the other isolates showed clusters of branching conidial chains of an arborescent appearance. However, since the differences between isolates were very subtle, we could not classify all isolates according to the results of the sequence analyses.

Table 3.2. Summary statistics with the genetic information of the seven investigated genomic regions for the small-spored and large-spored *Alternaria* species. Small: only small-spored isolates are taken into account (n = 72), Large: only large-spored isolates are taken into account (n = 11), Both: all isolates are taken into account (n = 83).

	ITS			***Gpd***			**Histone h3**			***Alt a 1***	***EF-a***	***RPB2***	**Calmodulin**
	Small	Large	Both	Small	Large	Both	Small	Large	Both	Small	Small	Large	Large
Number of isolates	72	11	83	72	11	83	72	11	83	72	72	11	11
Number of nucleotides	474	474	474	451	451	451	458	458	458	486	201	419	722
Mean genetic distance (Hamming)	0.09	0.54	1.33	1.034	1.08	0.53	1.98	0.52	2.87	0.70	0.76	0.09	0.48
Standard deviation Hamming distance	0.21	0.98	2.25	1.30	0.70	0.98	1.45	0.42	2.11	0.86	0.81	0.19	0.59
Constant characters	444	458	400	360	416	329	359	452	338	428	195	413	713
Variable characters (polymorphic sites)	30	16	74	104	35	122	99	6	120	58	6	6	9
Percentage variable characters	6.33	3.38	15.61	20.18	7.76	27.05	21.62	1.31	26.20	11.93	2.91	1.43	1.25
Parsimony informative characters	0	0	26	27	3	59	28	6	53	15	4	4	9
Percentage informative characters	0	0	5.49	5.98	0.67	10.91	6.11	1.31	11.57	3.09	1.95	0.95	1.25

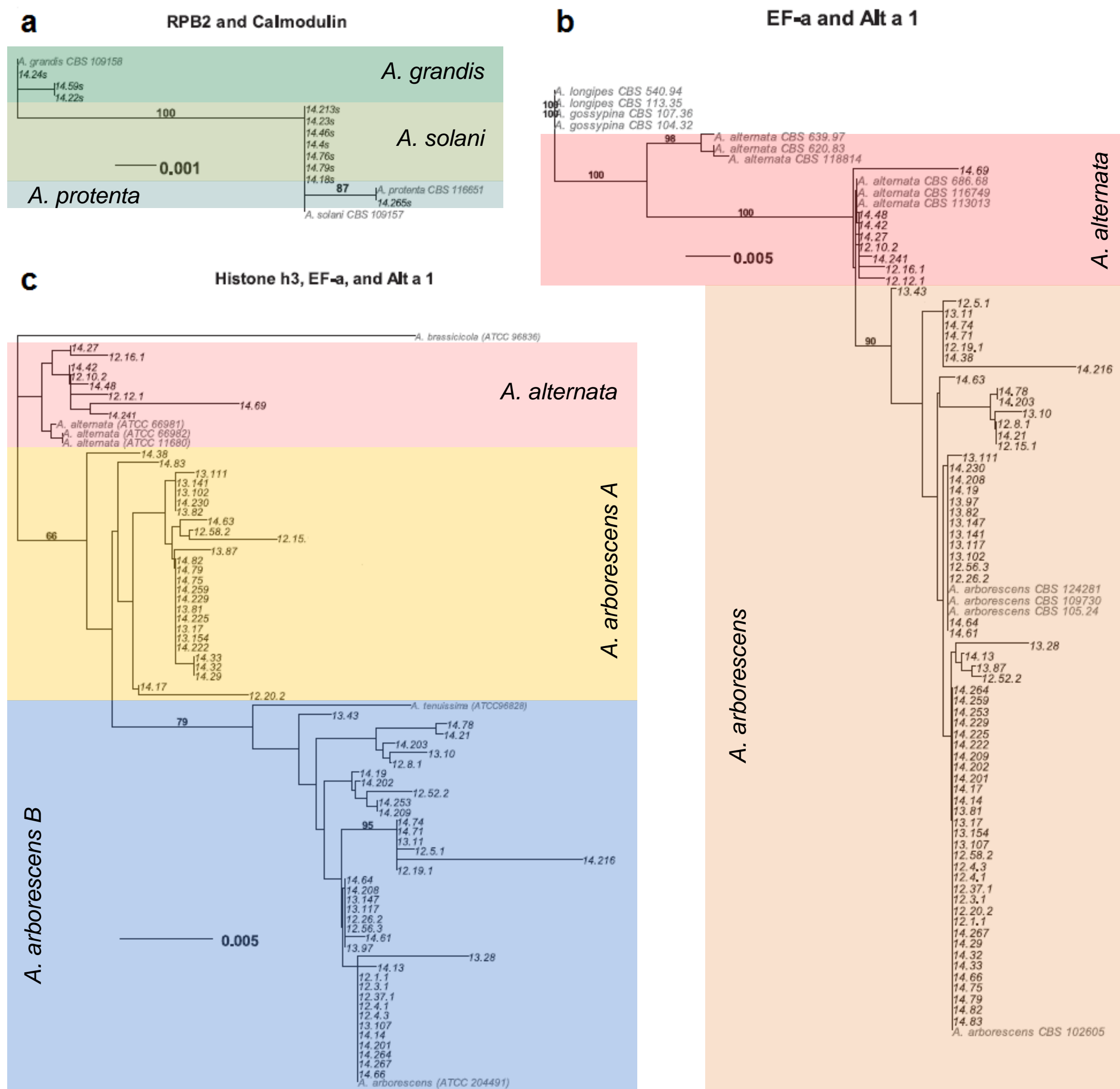


Figure 3.2. The consensus tree based on the calmodulin gene and RNA polymerase second largest subunit (*RPB2*) for the 11 large-spored species and reference isolates from Genbank (a). The consensus tree based on the *Alternaria* major allergen gene (*Alt a 1*) gene and the elongation factor-a (*EF-a*) (b). The consensus tree based on the histone h3 gene, the *Alt a 1* gene and the *EF-a* for the 72 small-spored *Alternaria* species together with reference sequences from the *Alternaria* Genome Database (*http://alternaria.vbi.vt.edu/index.html*) (c). Consensus trees are constructed by pasting the sequences from the indicated genomic regions to each other and constructing a maximum likelihood tree based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Bootstrap values above 75 % are shown in the figures. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

ITS

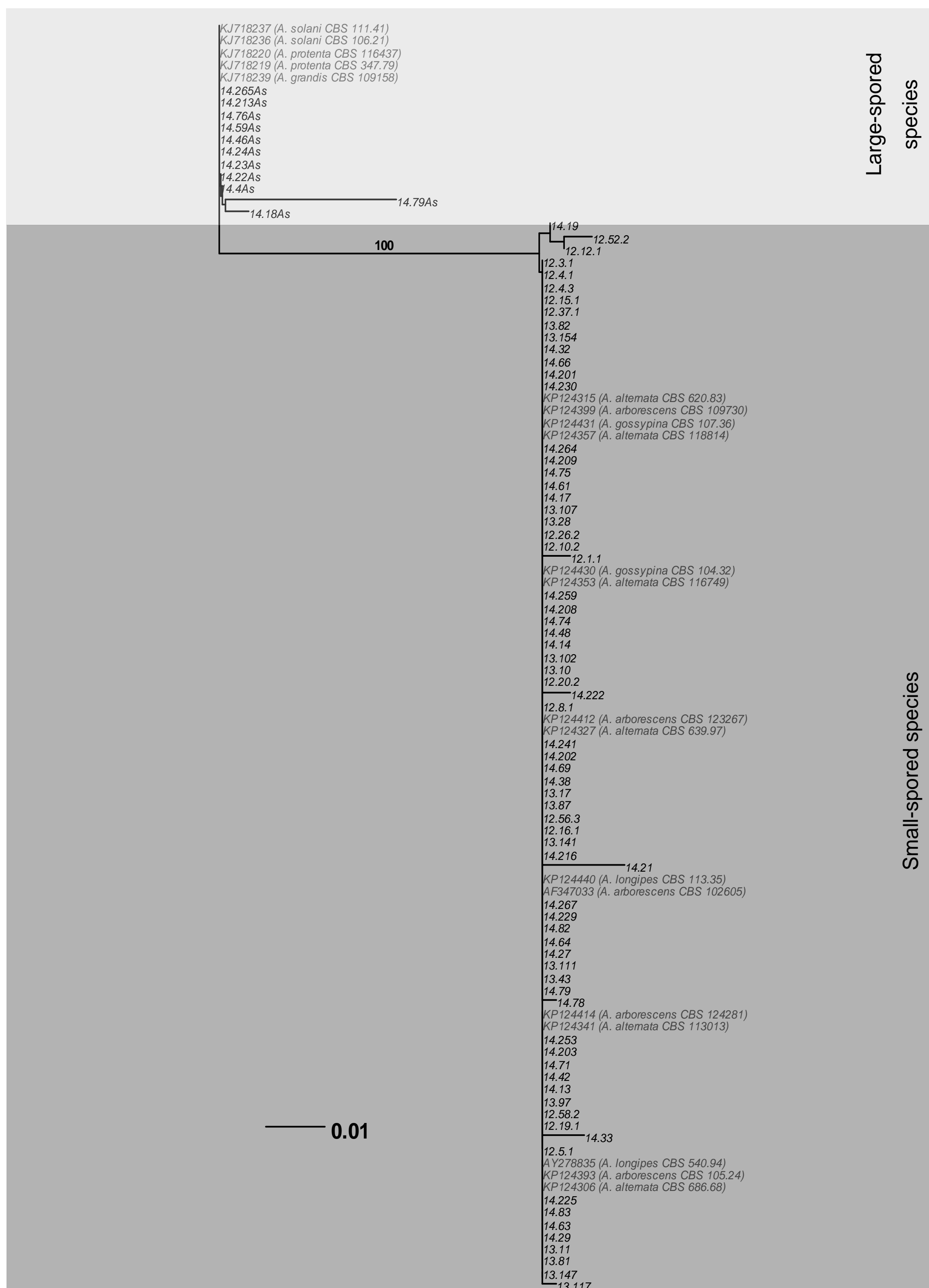


Figure 3.3. Phylogenetic tree based on the internal transcribed spacer (*ITS*) region. Tree construction was performed using a maximum likelihood method based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

Gpd

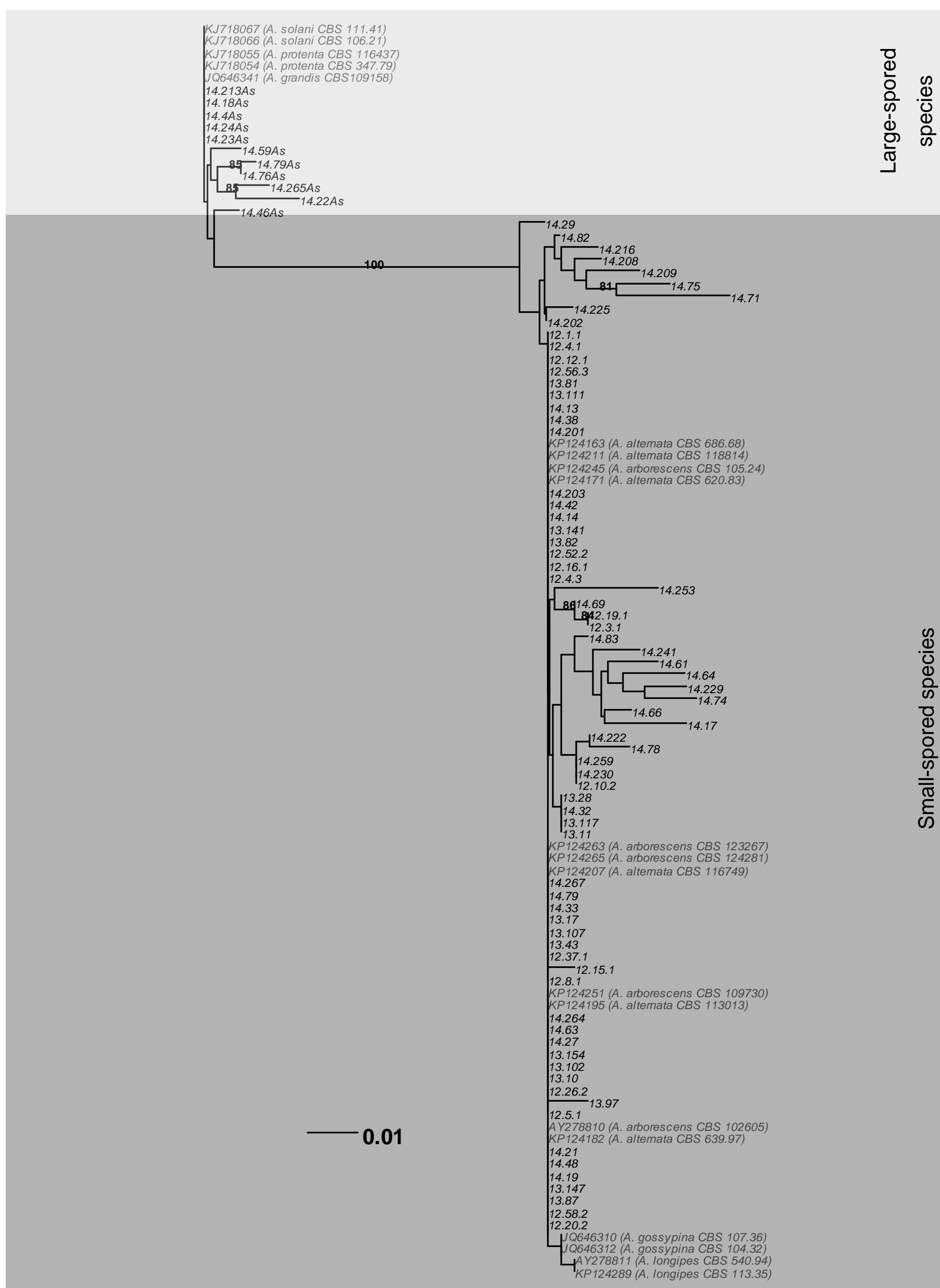


Figure 3.4. Phylogenetic tree based on the glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) gene. Tree construction was performed using a maximum likelihood method based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

Calmodulin

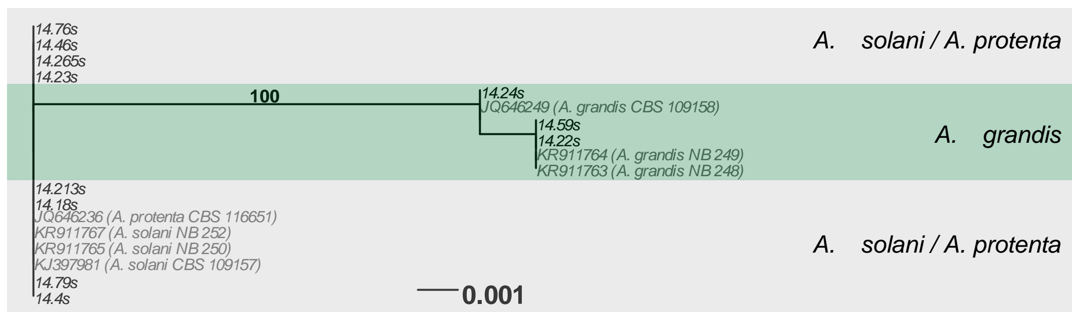


Figure 3.5. Phylogenetic tree based on the calmodulin gene. Tree construction was performed using a maximum likelihood method based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

RPB2

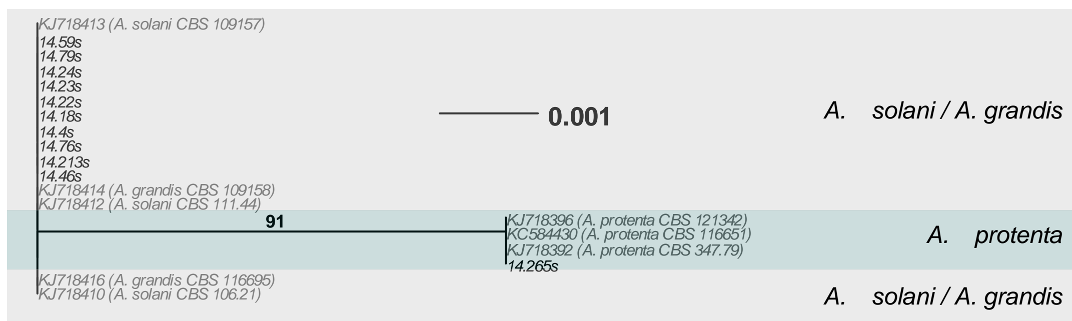


Figure 3.6. Phylogenetic tree based on the RNA polymerase second largest subunit (*RPB2*) gene. Tree construction was performed using a maximum likelihood method based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

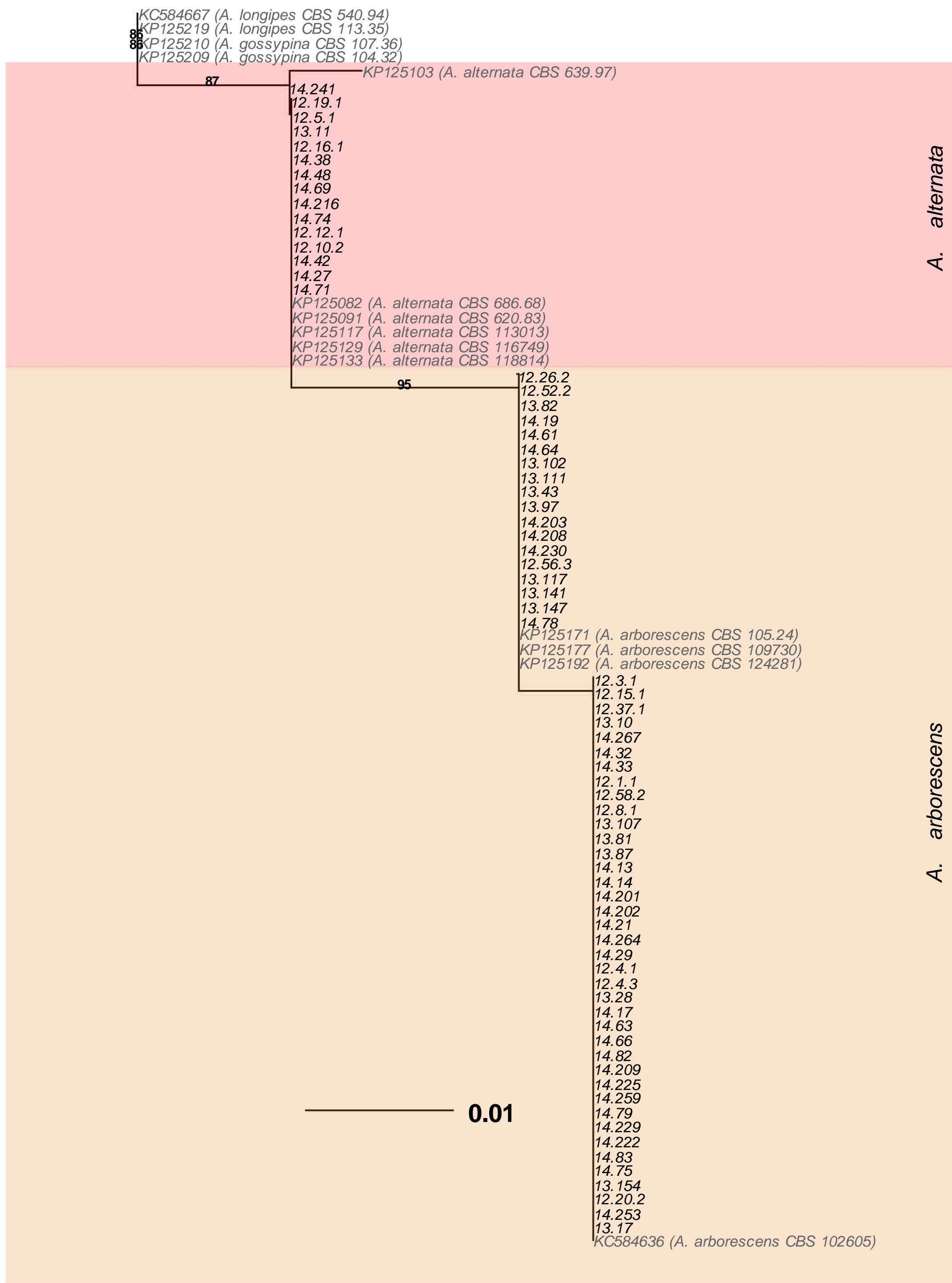


Figure 3.7. Phylogenetic tree based on the elongation factor-a (*EF-a*) gene. Tree construction was performed using a maximum likelihood method based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

Alt a 1

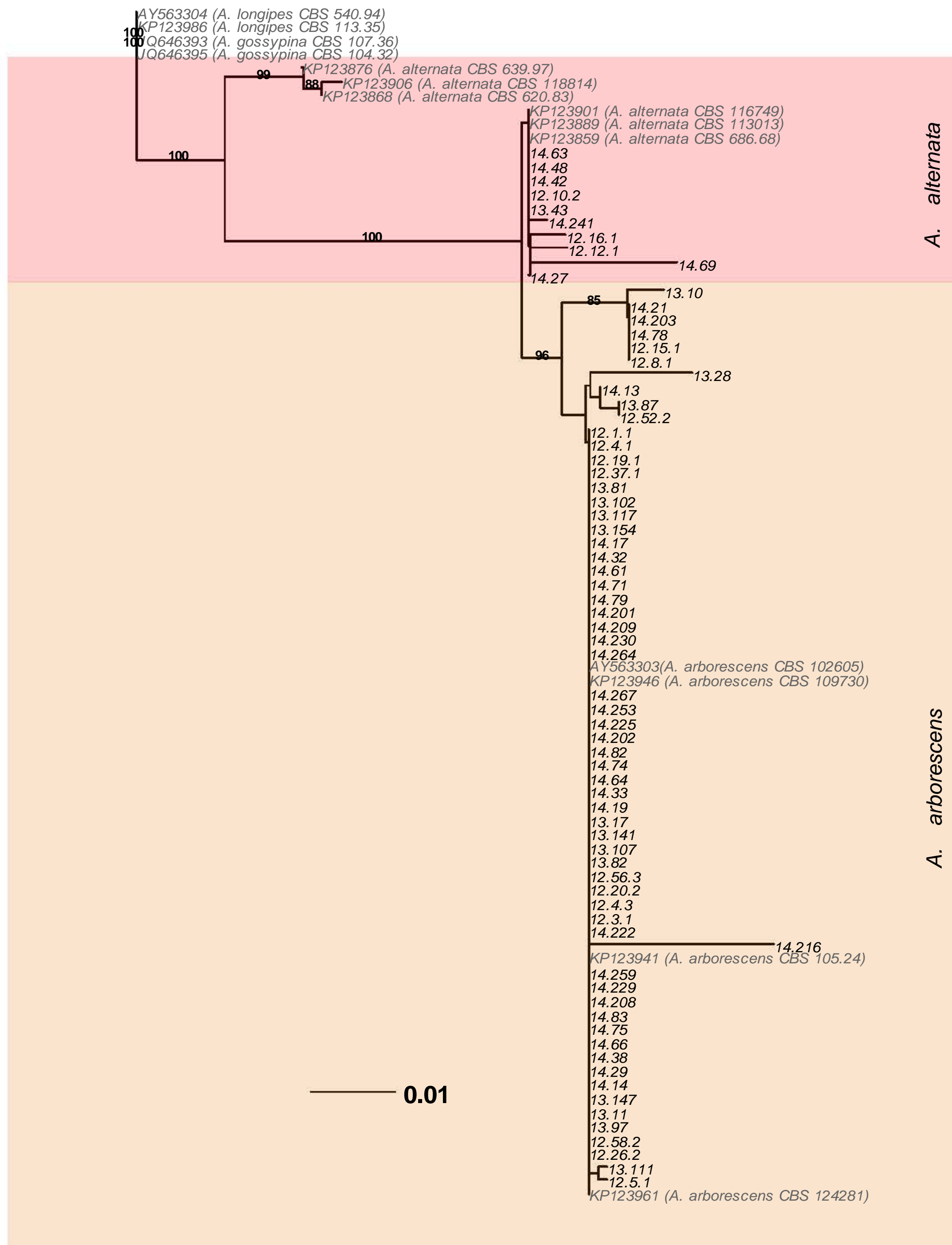


Figure 3.8. Phylogenetic tree based on the *Alternaria* major allergen a 1 (*Alt a 1*) gene. Tree construction was performed using a maximum likelihood method based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

Histone h3

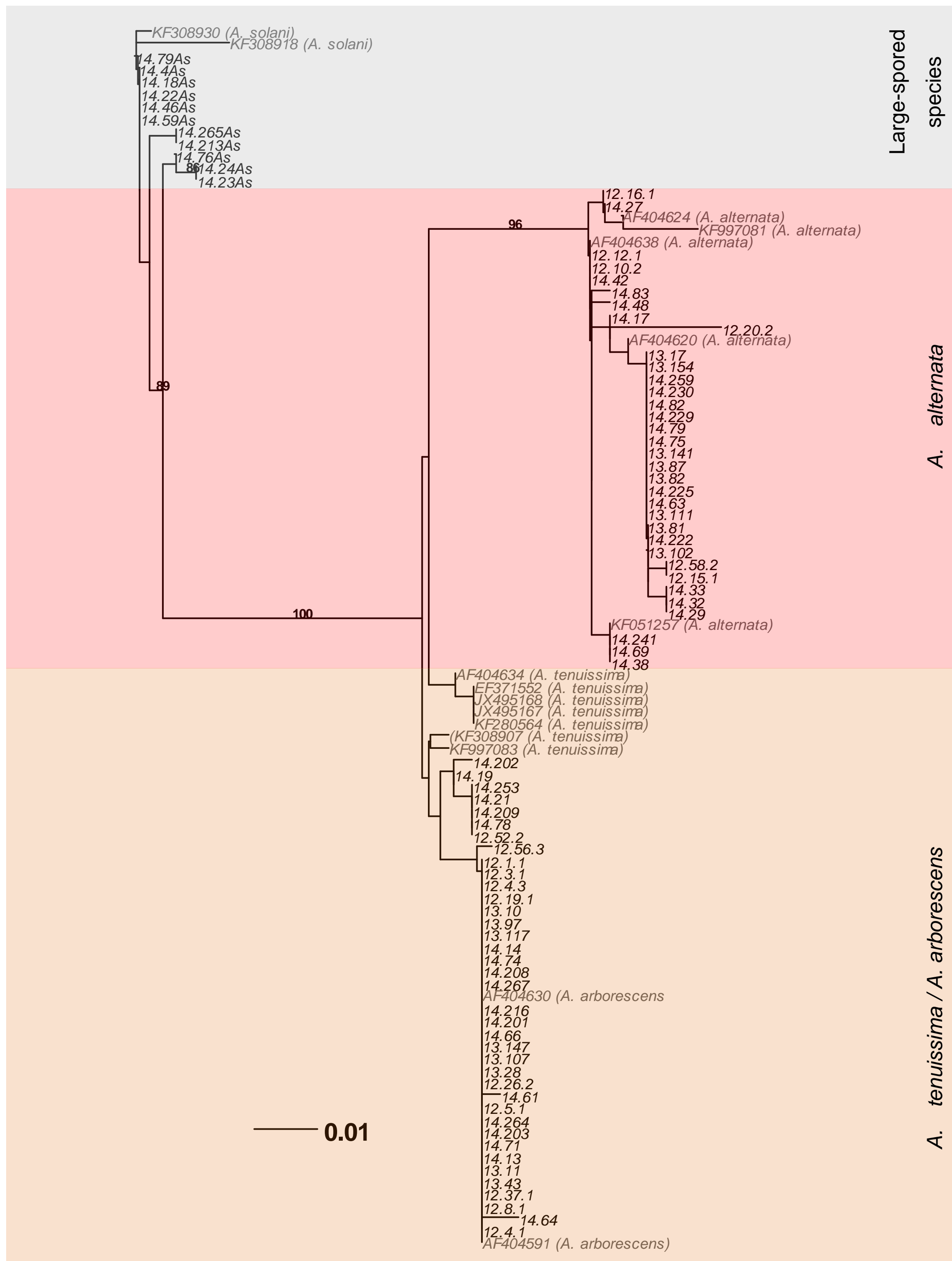


Figure 3.9. Phylogenetic tree based on the histone h3 gene. Tree construction was performed using a maximum likelihood method based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

ITS, GPD, EF-a, Histone h 3 and Alt a 1

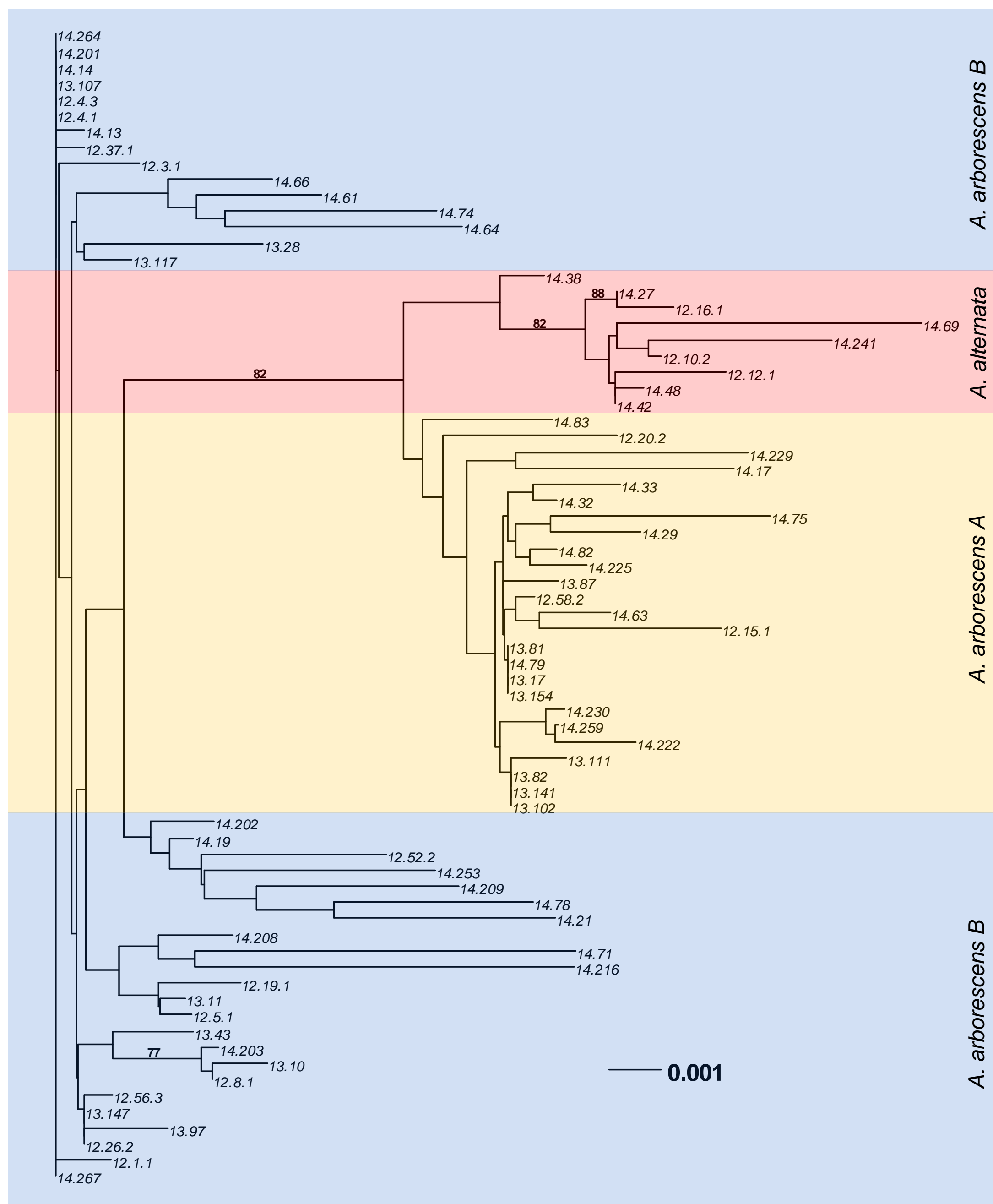


Figure 3.10. Consensus phylogenetic tree based on the sequences of the internal transcribed spacer (*ITS*) region, the glyceraldehyde-3-phosphate dehydrogenase (*Gpd*) gene, the elongation factor-a (*EF-a*) gene, the histone h3 gene and the *Alternaria* major allergen a 1 (*Alt a 1*) gene. Consensus tree was constructed by pasting all sequences from the five genomic regions to each other and constructing a maximum likelihood tree based on K2P distances (Kimura 2-Parameter distance). Branch support of the groupings obtained in the trees was assessed by bootstrapping with 1000 replicates. Bootstrap values above 75 % are shown in the figures. Clusters supported by bootstrap values over 75 % are highlighted by a colored box.

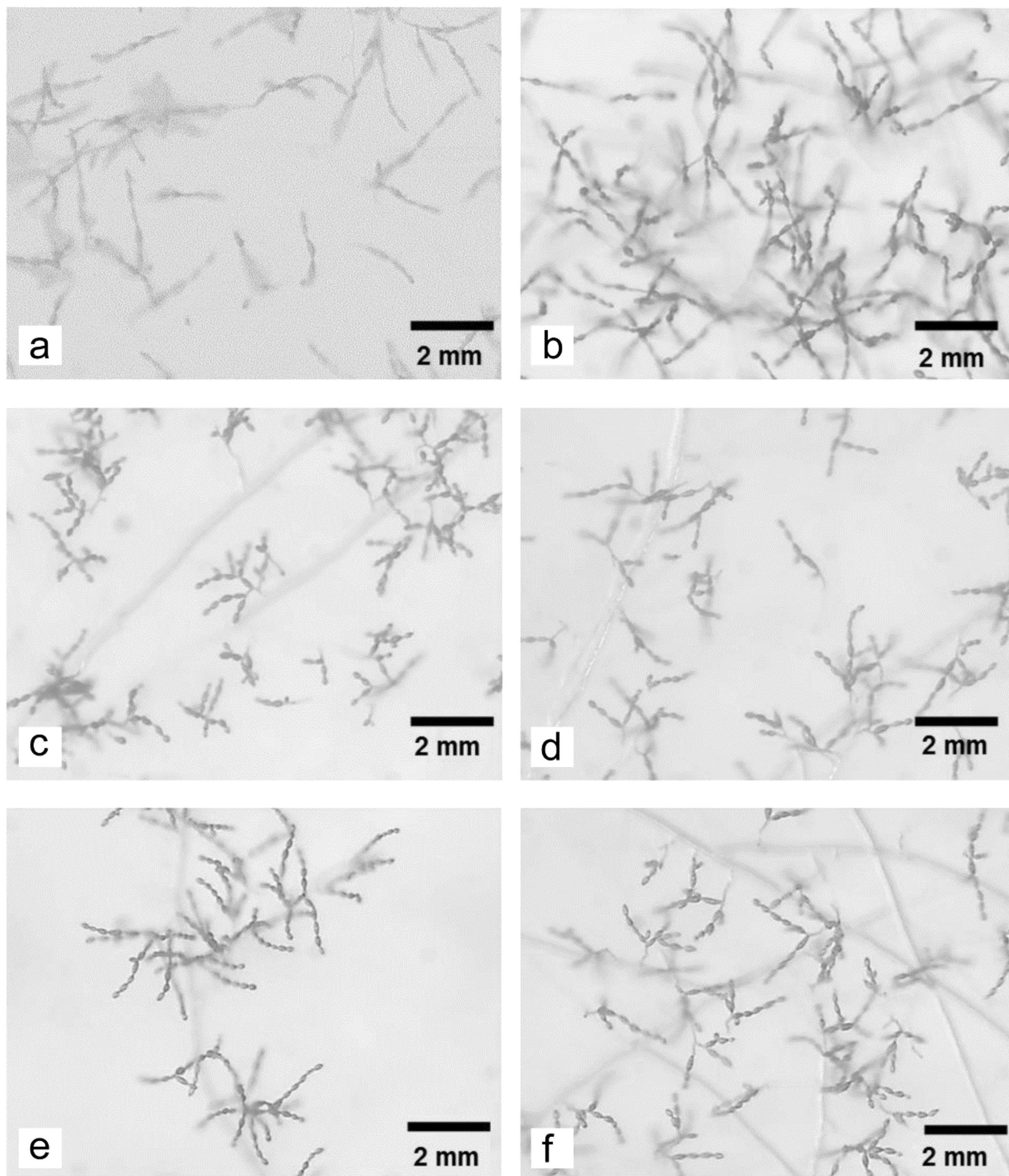


Figure 3.11. Conidia of *A. alternata* isolates (14.27 and 14.42) (a, b). Conidia of isolates belonging to the *A. arborescens* group B (14.79 and 14.259) (c, d) and conidia of isolates belonging to the *A. arborescens* group A (13.147 and 14.264) (e, f). Images were taken from 1-week-old pure cultures on SNA medium using an Olympus SZ61 stereo microscope coupled to a XC50 camera and visualized using the Cell^{F} imaging software (Olympus Soft Imaging Solutions GmbH).

DISCUSSION

To gain insight into the *Alternaria* population causing EB/BS disease on potatoes, the genetic diversity of an extensive collection of 72 small-spored and 11 large-spored *Alternaria* isolates obtained during 2012 - 2014 in Flanders (Belgium) was investigated. A genetic analysis of *Alternaria* species is important to gain further insight into this variability and into the future evolutionary potential of these pathogens. Furthermore, assessing the amount and distribution of the genetic variation in populations over time gives an idea of the pathogen’s potential to adapt to different management methods and environments (Morris et al., 2000; Aradhya et al., 2001; Leiminger et al., 2010). Therefore, in this research, a sequence analysis of seven different genes of an extended collection of 83 *Alternaria* isolates obtained during three years was performed. Based on the *ITS* region and *Gpd* gene the small-spored and large-spored *Alternaria* isolates could be separated. Similar as Woudenberg et al. (2015), this study confirmed that the discriminative power of the *ITS* region is too low to gain further insight into the variability within these two groups. The resolution of the sequences of the *Gpd* gene was greater compared to the *ITS* region. For this region, it was seen that the percentage informative characters within the small-spored isolates is clearly higher than for the large-spored isolates. However, phylogenetic analysis of this region did not result in significant sub clusters within the small-spored or large-spored *Alternaria* cluster. Other researchers have indeed reported that differentiation of the *Alternaria* isolates based on the *ITS* region is difficult due to lack of variation in this genomic region (Elansky et al., 2012; Lengi et al., 2014; Woudenberg et al., 2015; Zheng et al., 2015).

To gain further insight into the large-spored *Alternaria* population, sequence analyses of the *calmodulin* gene and *RPB2* were performed. These analyses revealed that *Alternaria solani* was not the only large-spored species on potato leaves, but also *Alternaria grandis* and *Alternaria protenta* were present. This is not the first study to report *A. grandis* on potato foliage. Indeed, this species has also been reported to be the cause of EB/BS disease in Brazil (Rodrigues et al., 2010). To our knowledge, *A. protenta* has not been reported previously as a causal agent of EB/BS disease. Concerning the small-spored species, Woudenberg et al. (2015) reported that the *AASC* can be distinguished from *Alternaria alternata* by the sequence of the *Alt a 1* gene and a particular region of the *EF-a* sequence. A sequence analysis of both regions revealed that part of our isolates belonged to the *AASC* and another part was identified as *A. alternata*. An additional sequence analysis of the *histone h3* gene revealed that the isolates belonging to the *AASC* could be subdivided into two groups. These isolates can be the result of genetic recombination. Indeed, the finding of random associations among Simple Sequence Repeat (SSR) markers strongly indicates

that most of the *A. alternata* populations sampled from potatoes in China underwent regular recombination (Meng et al., 2015). Based on this available evidence, Meng et al. (2015) concluded that *A. alternata* may adopt an epidemic mode of reproduction by combining many cycles of asexual propagation with fewer cycles of sexual reproduction. Furthermore, a study by Arie et al. (2000) revealed that the investigated *A. alternata* strains possess functional mating type loci (MAT) necessary for sexual reproduction. However, the reason as to why no sexual stage (either hidden or non-existent) has been observed in this species remains elusive (Arie et al., 2000).
In addition to the diversity of the small-spored species, the microscopic analysis revealed that there were no clear differences in conidial branching between the small-spored isolates. According to Woudenberg et al. (2013), variation in spore catenulation (conidial branching structure) is the only morphological distinction between *A. alternata* and *A. arborescens* species. Therefore, we wondered if a similar distinction could be observed in our isolate collection. Isolates that were identified as *Alternaria arborescens* (group A or B) showed more clusters of branched conidial chains of an arborescent appearance compared to the isolates identified as *A. alternata*. However, the difference was subtle and not consistent for all isolates. It is indeed known that morphological characteristics are fictile and are not only affected by intrinsic factors, but also by environmental conditions, subsequent sub-culturing, cultivation medium, etc. (Simmons, 1992; Thomma, 2003) and thus have little value to differentiate between small-spored *Alternaria* isolates.

In conclusion, this study revealed that the *Alternaria* population present on naturally infected potato leaves in Flanders is more complex than previously anticipated. At the microscopic and genetic level, the small- and large-spored isolates are easy to distinguish. Based on the *RPB2* and *calmodulin* sequence, it was concluded that besides *A. solani*, also *A. grandis* and *A. protenta* are members of the *Alternaria* population of Flemish potato fields. The sequence analyses of the *Alt a 1* gene and the *EF-a* showed that both *A. alternata* isolates and isolates belonging to the *AASC* were present in the small-spored *Alternaria* population. Based on the *histone h3* gene the isolates belonging to the *AASC* could be subdivided into two groups. This study highlights the need for molecular means to study the genetic diversity among *Alternaria* species since morphological assessment, like conidial branching, does not offer adequate resolution to delineate species within groups of small- or large spored species. With a representative collection of the *Alternaria* population identified, the population composition on different time points during the season was investigated in order to reveal potential mechanisms behind the sudden shift in disease incidence observed from mid-August (as discussed in Chapter 2). This will be discussed in the following chapter and will be supplemented with an *in vitro* virulence assay and artificially inoculated field trials using the *Alternaria* isolates collected in the studied growing seasons.

SUPPORTING INFORMATION

Table S3.1. List of *Alternaria* isolates examined in this study. The code of the isolate, date of isolation, microscopic identification, the city, coordinates (latitude and longitude) from the locations where the isolates were collected and the identification according the consensus tree of histone h3, *Alt a 1* and *EF-a* (small-spored isolates) and according to the calmodulin gene and *RPB2* (large-spored isolates).

Code	Isolation	Spore-type	City	LAT	LON	Species
12.1.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group A*
12.10.2	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. alternata*
12.12.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. alternata*
12.15.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group B*
12.16.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. alternata*
12.19.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group A*
12.20.2	2012-08-31	small-spored	Vladslo	51.046	2.916	*Undefined*
12.26.2	2012-09-05	small-spored	Kruishoutem	50.928	3.537	*A. arborescens group A*
12.3.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group A*
12.37.1	2012-08-15	small-spored	Bottelare	50.967	3.750	*A. arborescens group A*
12.4.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group A*
12.4.3	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group A*
12.5.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group A*
12.52.2	2012-08-31	small-spored	Wetteren	51.003	3.890	*A. arborescens group A*
12.56.3	2012-08-31	small-spored	Wetteren	51.003	3.890	*A. arborescens group A*
12.58.2	2012-08-15	small-spored	Oosterzele	50.947	3.816	*A. arborescens group B*
12.8.1	2012-08-31	small-spored	Vladslo	51.046	2.916	*A. arborescens group A*
13.10	2013-07-09	small-spored	Bottelare	50.967	3.750	*A. arborescens group A*
13.102	2013-08-22	small-spored	Hoeleden	50.868	4.987	*A. arborescens group B*
13.107	2013-08-25	small-spored	Bottelare	50.967	3.750	*A. arborescens group A*
13.11	2013-08-26	small-spored	Roeselare	50.950	3.129	*A. arborescens group A*
13.111	2013-08-27	small-spored	Zonnebeke	50.873	2.987	*A. arborescens group B*
13.117	2013-08-27	small-spored	Poperinge	50.845	2.721	*A. arborescens group A*
13.141	2013-09-02	small-spored	Leupegem	50.833	3.600	*A. arborescens group B*
13.147	2013-09-03	small-spored	Outgaarden	50.765	4.918	*A. arborescens group A*
13.154	2013-09-05	small-spored	Kruishoutem	50.928	3.537	*A. arborescens group B*
13.17	2013-09-23	small-spored	Kruishoutem	50.928	3.537	*A. arborescens group B*
13.28	2013-07-24	small-spored	Deftinge	50.787	3.840	*A. arborescens group A*
13.43	2013-07-29	small-spored	Leefdaal	50.850	4.583	*A. arborescens group A*
13.81	2013-08-19	small-spored	Horebeke	50.837	3.687	*A. arborescens group B*
13.82	2013-08-19	small-spored	Bevere	50.852	3.588	*A. arborescens group B*
13.87	2013-08-20	small-spored	St.-M.-Bodegem	50.862	4.213	*A. arborescens group B*
13.97	2013-08-22	small-spored	Leefdaal	50.850	4.583	*A. arborescens group A*
14.13	2014-07-31	small-spored	Outgaarden	50.765	4.918	*A. arborescens group A*
14.14	2014-08-07	small-spored	Outgaarden	50.765	4.918	*A. arborescens group A*

(Table S3.1 continued)

Code	Isolation	Spore-type	City	LAT	LON	Species
14.17	2014-07-24	small-spored	Assent	50.952	5.014	*Undefined*
14.19	2014-08-07	small-spored	Assent	50.952	5.014	*A. arborescens group A*
14.201	2014-08-18	small-spored	Wulpen	51.101	2.699	*A. arborescens group A*
14.202	2014-08-18	small-spored	Wulpen	51.101	2.699	*A. arborescens group A*
14.203	2014-08-18	small-spored	Veurne	51.073	2.668	*A. arborescens group A*
14.208	2014-08-06	small-spored	Roeselare	50.950	3.129	*A. arborescens group A*
14.209	2014-07-17	small-spored	Roeselare	50.950	3.129	*A. arborescens group A*
14.21	2014-08-29	small-spored	Leefdaal	50.850	4.583	*A. arborescens group A*
14.216	2014-08-20	small-spored	Zonnebeke	50.873	2.987	*A. arborescens group A*
14.222	2014-08-19	small-spored	Zwevegem	50.813	3.408	*A. arborescens group B*
14.225	2014-08-19	small-spored	Helkijn	50.732	3.381	*A. arborescens group B*
14.229	2014-07-31	small-spored	Bellegem	50.777	3.278	*A. arborescens group B*
14.230	2014-07-31	small-spored	Leefdaal	50.850	4.583	*A. arborescens group B*
14.241	2014-08-08	small-spored	Kasterlee	51.241	4.967	*A. alternata*
14.253	2014-08-22	small-spored	Mol	51.191	5.117	*A. arborescens group A*
14.259	2014-08-01	small-spored	Mol	51.191	5.117	*A. arborescens group B*
14.264	2014-08-14	small-spored	Mol	51.191	5.117	*A. arborescens group A*
14.267	2014-08-08	small-spored	Weelde	51.400	4.983	*A. arborescens group A*
14.27	2014-08-07	small-spored	Watervliet	51.283	3.633	*A. alternata*
14.29	2014-08-07	small-spored	Wat.-Oudeman	51.289	3.590	*A. arborescens group B*
14.32	2014-08-29	small-spored	Wat.-Oudeman	51.289	3.590	*A. arborescens group B*
14.33	2014-07-28	small-spored	Wort.-Petegem	50.850	3.500	*A. arborescens group B*
14.38	2014-09-04	small-spored	Zottegem	50.870	3.811	*Undefinded*
14.42	2014-08-13	small-spored	Kruishoutem	50.928	3.537	*A .alternata*
14.48	2014-07-24	small-spored	Kruishoutem	50.928	3.537	*A. alternata*
14.61	2014-07-14	small-spored	Outgaarden	50.765	4.918	*A. arborescens group A*
14.63	2014-07-31	small-spored	Outgaarden	50.765	4.918	*A. arborescens group B*
14.64	2014-08-07	small-spored	Outgaarden	50.765	4.918	*A. arborescens group A*
14.66	2014-07-23	small-spored	Lozer	50.919	3.569	*A. arborescens group A*
14.69	2014-07-15	small-spored	Lozer	50.919	3.569	*A .alternata*
14.71	2014-07-23	small-spored	Lozer	50.919	3.569	*A. arborescens group A*
14.74	2014-08-25	small-spored	Wort.-Petegem	50.850	3.500	*A. arborescens group A*
14.75	2014-08-18	small-spored	Oudenaarde	50.830	3.629	*A. arborescens group B*
14.78	2014-08-11	small-spored	Edelare	50.830	3.629	*A. arborescens group A*
14.79	2014-08-11	small-spored	Edelare	50.830	3.629	*A. arborescens group B*
14.82	2014-07-09	small-spored	Maarkedal	50.799	3.644	*A. arborescens group B*
14.83	2014-07-24	small-spored	Goeferdinge	50.787	3.840	*Undefined*
14.18s	2014-07-31	large-spored	Assent	50.952	5.014	*A. solani*
14.213s	2014-08-29	large-spored	Roeselare	50.950	3.129	*A. solani*
14.22s	2014-09-01	large-spored	Leefdaal	50.850	4.583	*A. grandis*
14.23s	2014-09-02	large-spored	Leefdaal	50.850	4.583	*A. solani*

(Table S3.1 continued)

Code	Isolation	Spore-type	City	LAT	LON	Species
14.265s	2014-08-29	large-spored	Mol	51.191	5.117	*A. protenta*
14.46s	2014-07-30	large-spored	Kruishoutem	50.928	3.537	*A. solani*
14.4s	2014-07-27	large-spored	Herne	50.723	4.054	*A. solani*
14.59s	2014-07-31	large-spored	Leefdaal	50.850	4.583	*A. grandis*
14.76s	2014-07-29	large-spored	Oudenaarde	50.830	3.629	*A. solani*
14.79s	2014-08-07	large-spored	Edelare	50.830	3.629	*A. solani*

CHAPTER 4

SPECIES PREVALENCE AND DISEASE PROGRESSION STUDIES DEMONSTRATE A SEASONAL SHIFT IN THE FLEMISH *ALTERNARIA* POPULATION COMPOSITION ON POTATO

Adapted from:

Vandecasteele M, Landschoot S, Carrette J, Verwaeren J, Höfte M, Audenaert K and Haesaert G (2018). Species prevalence and disease progression studies demonstrate a seasonal shift in the *Alternaria* population composition on potato. Plant Pathology 67, 327-36.

ABSTRACT

During the course of the growing season 2014, the species composition in leaves with EB/BS symptoms was assessed using real-time PCR. Results demonstrate that small-spored *Alternaria* species, such as *A. alternata* and *A. arborescens*, rather than large spored species like *A. solani* were the predominant species on potato leaves throughout the growing season, especially in July. As the disease progressed, the proportion of large-spored species increased. A fitness test of the isolates under study showed that large-spored species grew significantly slower on culturing media than small-spored species, which could introduce a bias if species quantification was performed after isolation from lesions alone. These findings highlight the need for molecular means, such as real-time PCR to conduct population composition analyses.

In view of the observed shift in population composition at the end of the season, the virulence of a collected set of *Alternaria* isolates was assessed using *in vitro* assays. Virulence profiles of the investigated isolates could substantiate that *A. solani* was more aggressive towards potato leaves than the small-spored species *A. alternata* and two phylogenetic subgroups of *A. arborescens*. Differences in aggressiveness were found within each of the small-spored species. However, no significant variation in virulence could be discerned between the small-spored species. These results were complemented with data from artificially inoculated field trials. These showed that potato plants inoculated or co-inoculated with *A. solani*, a large-spored isolate, exhibited more disease symptoms than plants that were inoculated with only small-spored species.

Since it was observed that the proportion of large-spored species increases when the disease escalates and that large-spored species such as *A. solani* display higher virulence both *in vitro* and *in vivo*, we hypothesize that the increase in disease incidence is due to this shift in population composition.

INTRODUCTION

In recent years, the virulence of *Alternaria* species found on potato leaves, has been a matter of much ambiguity. In general, *A. alternata* is considered a weak pathogen compared to its aggressive counterpart *A. solani* (Droby et al., 1984). However, several authors report conflicting findings regarding this statement. Turkensteen et al. (2010) and Spoelder et al. (2013) observed that only *A. solani* is virulent and that *A. alternata* is unable to infect potato leaflets and cause necrotic lesions. In contrast, Zheng et al. (2015) established that virulence of different *A. alternata* and *A. solani* isolates did not differ significantly between species. Kapsa and Osowski (2004) determined that *A. alternata* is the most virulent species on potato leaves while *A. solani* was more virulent towards potato tubers. Determining the primary causal agents of EB/BS disease and elucidating the mechanism behind the disease progression is therefore a matter of much discussion. An imperative factor in resolving this issue is the quantification of species proportions within the population during a specific time frame. Indeed, detection of the disease's causal agent is impossible using visual analyses alone. Additionally, when isolating species from an infected leaf, one species might grow faster on culture media than another if multiple species are present on the same leaf, introducing a bias in determining the causal agent. In an attempt to overcome these impediments, Latorse et al. (2010) optimized a real-time PCR technique using primers based on the cytochrome bc1 (*Cyt bc1*) sequences to reliably distinguish *A. solani* from *A. alternata*. Detection of other small- or large-spored *Alternaria* species on potato was not reported. They established that the technique could clearly detect both *A. alternata* and *A. solani* on field samples showing EB/BS symptoms, whereas biological analysis only identified one of the two pathogens. Leiminger et al. (2015) used a similar approach to assess the *Alternaria* species composition in German potato fields during the growing seasons of 2003 - 2007 by performing real-time PCR analyses based on internal transcribed spacer (*ITS*) sequences. In 2003 and 2004, *A. alternata* DNA was found from the beginning of the season, whereas *A. solani* DNA was only detected for the last two sampling dates (end of July). Moreover, at the end of the 2003 growing season, *A. alternata* DNA levels were twice as high as those from *A. solani*. For the remaining seasons that were studied, *A. solani* appeared to be the predominant species present in infected leaves.

However, the results from the previous chapter as well as other recent reports show that the *Alternaria* population on potato is much more elaborate than a two-species disease complex. Indeed, next to the addition of *A. arborescens*, several research groups reported other small-spored species residing on potato leaves, such as *A. tenuissima* and *A. infectoria* (Ardestani et al., 2010; Zheng and Wu, 2013; Tymon et al., 2015). Concerning large-spored species, Rodrigues et al. (2010) observed the species *A. grandis* and *A. tomatophila* on potato leaves in Brazil. The addition

of several small- or large-spored species to the *Alternaria* population on potato fields may be the reason why many authors report conflicting findings concerning the pathogenicity of *A. alternata* and *A. solani*, since the ability to cause disease may be different in other small- or large-spored species.

This investigation was undertaken because the virulence of *A. solani* and *A. alternata* isolates is still a matter of much debate. Also, the virulence of species such as *A. arborescens* on potato leaves is not well-established. Thus, this study aimed to shed light on the primary causal agents of EB/BS disease on potato in relation to the disease progression observed throughout the growing season of 2014 (see previous chapter). This was established using real-time PCR techniques to characterize the local *Alternaria* species composition. Additionally, an *in vitro* virulence assay and artificially inoculated field trials were conducted to respectively trace potential variation in virulence between field isolates of *A. alternata*, *A. arborescens* and *A. solani* and to pinpoint the causal agent of EB/BS disease pressure.

MATERIALS AND METHODS

Real-time PCR

For the preparation of fungal genomic DNA, 12 leaves showing necrotic lesions were harvested every week (three leaves per subplot) from the monitored fields during the growing seasons of 2014 and 2015 (Fig. 2.1., Chapter 2). Leaves collected per subplot were pooled, frozen under liquid nitrogen and crushed using a mortar and pestle. Genomic DNA extraction was carried out using the Invisorb Spin Plant Mini kit (Stratec Biomedical) according to the manufacturer's instructions. Afterwards, DNA concentrations were measured using a Quantus fluorometer (Promega) and adjusted to 5 ng μL^{-1} DNA. Real-time PCR primers based on sequences of *Cyt bc1* were selected from Latorse et al. (2010): the *A. solani* forward primer used in the reaction mix was SP-sol-1658F (5'-GTAGAGTATGTTGAATACTCTAACCAGACAA-3') at a concentration of 900 nM and reverse primer was SP-sol-1759R (5'-ATGTTAAGAATTTGTCCTGAACAGTTT-3') at 50 nM. For *A. alternata* the forward primer was SP-alt-153F (5'-CTTATGAGTGCTATACCTTGAGTAGGTCA-3') at 300 nM and the reverse primer was SP-alt-376R (5'-TCTCCGTCTATCAATCCTGCTAAA-3') at 50 nM. The binding of the selected primers to the *Cyt bc1* gene from *A. arborescens* subgroups A and B was tested and confirmed by running the real-time PCR on DNA extracted from pure cultures grown on PDB (as described in the Materials and methods section of the previous chapter). It was also confirmed that the primer pair SP-sol-1658F / SP-sol-1759R could amplify the *Cyt bc1* gene from *A. protenta* and *A. grandis*, indicating that both primer pairs could generate *Cyt bc1* amplicons from all *Alternaria* species under study. The qPCR mix contained 6.25 µL GoTaq qPCR Master Mix (Promega), the corresponding primers, 1 µL of diluted DNA (5 ng μL^{-1}), 0.208 µL CXR reference dye and water to 12 µL. All reactions were performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems) under the program described by Latorse et al. (2010): 2 min at 50 °C, 10 min at 95 °C; 40 cycles of 15 sec at 95 °C and 1 min at 60 °C; and finally 15 sec at 60 °C and 15 sec at 95 °C. All samples subjected to qPCR analysis were tested in four technical repeats.

Identification of field isolates

During the growing season of 2014, an effort was made to sample potato leaflets, showing EB/BS disease symptoms every week from the 22 locations under study (Fig. 2.1, Chapter 2). From each field, five leaves were collected every time. From each leaf, one necrotic lesion was excised, surface-sterilized with 5 % NaOCl for about 1 min, washed in sterile distilled water and subsequently placed on synthetic nutrient agar (SNA) medium (1 g L^{-1} KH_2PO_4, 1 g L^{-1} KNO_3, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.5 g L^{-1} KCl, 0.2 g L^{-1} glucose, 0.2 g L^{-1} saccharose, 1 M NaOH and 20 g L^{-1}

agar) at 21 °C. One week later, outgrowing mycelium was transferred to new Petri dishes and incubated at 21 °C. Pure cultures were made by additional transfers. To induce sporulation, plates were incubated at 25 °C for 2 weeks under near-UV light and with a 12 h photoperiod. To obtain single spore colonies, one spore of each plate was transferred to new SNA medium. Identification of species was performed as described in the previous chapter, based on sequence analyses of the *Alternaria* major allergen *Alt a 1*, the elongation factor-a (*EF-a*), the histone h3, calmodulin and the RNA polymerase second largest subunit (*RPB2*) genes.

Fitness test

The verify whether growth rates on culturing media of different *Alternaria* isolates differ, which could introduce a bias in the quantification of species at a specific time point during the growing season, the isolates under study were subjected to a fitness test. Therefore, the growth rate on SNA medium was assessed by inoculating petri dishes with a 5 mm mycelium plug and incubating them in the dark at 21 °C. Three, seven, ten, and 14 days after inoculation the mycelium diameter was measured and the growth rate (mm day^{-1}) was calculated. Results are displayed in box-plots which graphically depict the data by the minimum (lowest line), the lower quartile, the median (band inside the box), the upper quartile and the maximum (highest line). Outliers (> Q3 + 1.5 IQR distance) are displayed as small dots above or below the box.

Leaf disk virulence assay

Leaves from potato cultivar Bintje, grown in the phytotron for 1 month (21 °C, 16 h photoperiod), were removed from the middle level of the plants (terminal leaflets at growth stage 3 of plant development) and were surface-sterilized in a 5 % NaOCl solution for about 1 min and subsequently washed in sterile water. Next, leaf disks were cut using a 15-mm sterile cork borer and placed into 1 mL sterile water in each well of a 24-well plate. Spore suspensions were prepared by harvesting spores from colonies grown from each identified field isolate in 0.01 % Tween80® solution using a sterile inoculation loop. The suspension concentrations were quantified using a Bürker counting chamber and adjusted to 5.10^4 spores mL^{-1}. Subsequently, leaf disks were inoculated with 10 µL of a spore suspension for each isolate. All isolates were tested at 21 °C in four biological replications and the complete assay was repeated once more. Disease severity was evaluated every 3 days over the duration of 2 weeks by quantifying the necrotic area using ASSESS v. 2.0 (APS) image analysis software for plant disease quantification. Next, *rAUDPC* values were calculated as described in chapter 2. Results are displayed in box-plots which graphically depict the data by the minimum (lowest line), the lower quartile, the median (band inside the box), the

upper quartile and the maximum (highest line). Outliers (> Q3 + 1.5 IQR distance) are displayed as small dots above or below the box.

Artificially inoculated field trial

To further determine the causal agent of EB/BS disease on Flemish potato fields, field trials were set up at the experimental farm in Bottelare, Belgium, during the growing seasons of 2014 and 2015. For each season, potato seedlings of the cultivar Bintje were planted at the end of April and managed according to common agricultural practice, i.e. plants were treated weekly against *Phytophthora infestans* using the compound cyazofamid (which is not known to act against *Alternaria*). At the end of June, plants were spray-inoculated. Therefore, spore suspensions of 10^4 spores ml^{-1} were prepared of a small-spored isolate (14.79, identified as *A. arborescens* group B) and a large-spored isolate (14.4s, identified as *A. solani*). Additionally, mixed spore suspensions of both isolates with ratio's 50 / 50 and 90 / 10 (small-spored / large-spored) were prepared. For each treatment, four replications were performed. The experimental field was set up as a randomized block design with 20 blocks of approximately 50 plants to account for the five treatments (4 different inoculations and 1 control) and four replications. Disease symptoms were weekly evaluated for 2 months based on the scoring table that was used for the field survey (Table 2.1) and *rAUDPC* values were calculated as described in chapter 2.

Statistical analyses

The R v. 2.15.3 software package (R Core Team, 2014) was used for the *in vitro* virulence assay results (to test differences in virulence between species) and the field trial results (to test differences in disease severity between treatments). Statistical significance between groups of data was inferred at a significance level of $\alpha = 0.05$. Because normality assumptions of parametric tests were not met for all assays, differences between groups of data were tested for significance using a non-parametric Kruskal–Wallis test. If significant differences between the groups were found, a Dunn post hoc test at $\alpha = 0.05 / n$ with n the number of pairwise comparisons was performed.

RESULTS

Population composition study

To unravel the mechanisms behind the increased disease incidence observed from mid-August in our field survey, the composition of the *Alternaria* population of each monitored field was investigated using real-time PCR (Fig. 4.1c,d). In general, in both 2014 and 2015, small-spored *Alternaria* species (*A. alternata*, *A. arborescens* groups A and B) were dominantly present throughout the entire growing season. However, near the end of August, the large-spored species (*A. solani*, *A. grandis* and *A. protenta*) became more prominent, indicating a population shift that started around the second half of the season, in accordance with the increase of leaf necrosis described in chapter 2 (Fig 2.3, Fig. 4.1a, b).

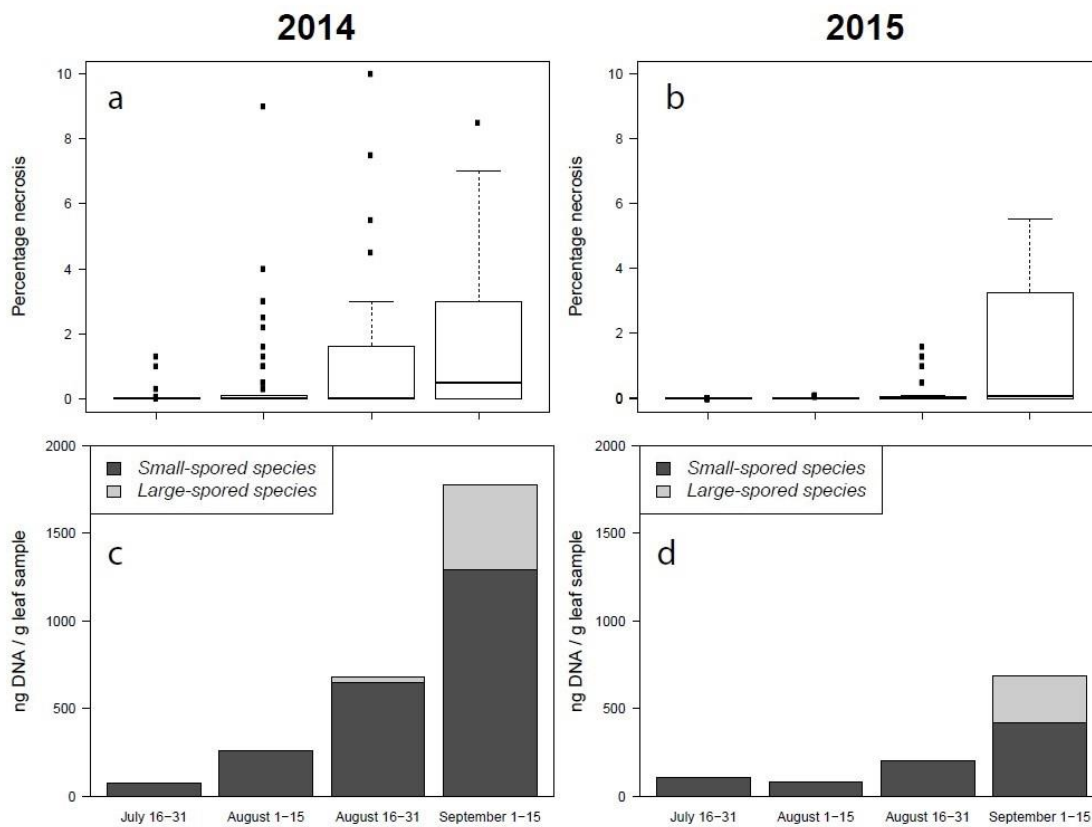


Figure 4.1. (a, b) Percentage leaf necrosis due to EB/BS disease on all monitored fields during four periods of the growing seasons 2014 and 2015 (as was shown in figure 2.3, chapter 2). (c, d) Proportion of small-spored *Alternaria* species (*A. alternata* and *A. arborescens* groups A and B) and large spored species (*A. solani*, *A. grandis* and *A. protenta*) in the population of the monitored fields during the same four periods of the growing seasons 2014 and 2015. Species proportions were analyzed using real-time PCR using the primers and conditions described by Latorse et al. (2010).

Fitness test

The growth rates on SNA media of all studied isolates was assessed to corroborate whether a bias is introduced when species are quantified and identified after isolation from necrotic lesions. Results of the test demonstrated that the growth rate of large-spored isolates was significantly lower than that of small-spored isolates (Fig. 4.2), whereas between the small-spored species (*A. alternata*, *A. arborescens* groups A and B), no significant difference in growth rate could be discerned.

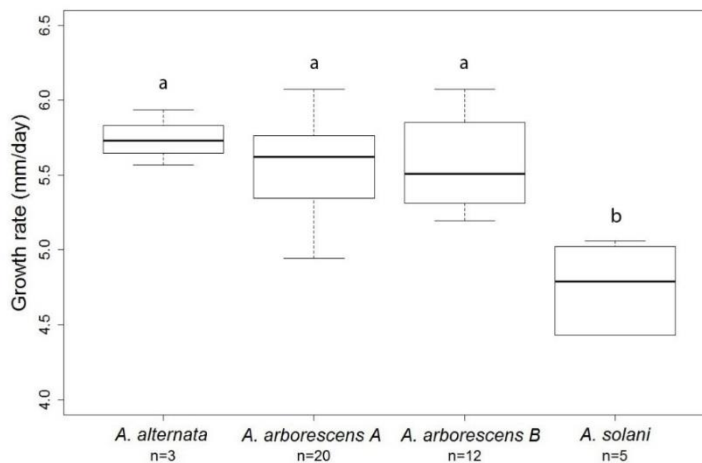


Figure 4.2. Distribution of the growth rate of the different small-spored species (*A. alternata*, *A. arborescens* group A and B) and *A. solani*. Different letters above the boxes refer to significant differences between species. The number of analyzed isolates is represented by n.

Leaf disk virulence assay

With the aim of explaining the increase in disease incidence that was in accordance with the detection of large-spored species, the growing season of 2014 was chosen as a case-study to examine the virulence of field isolates. After all, in that growing season the disease pressure was higher and the increase in disease incidence observed from mid-August was more pronounced.

Leaf samples with symptoms were harvested and isolated species were identified as described in chapter 3. All undefined species were eliminated from the study as well as those that had difficulties growing out on SNA medium, resulting in a total of 40 monosporic *Alternaria* cultures that were used for this study. The isolates were identified as three *A. alternata* isolates, 20 *A. arborescens* group A isolates, 12 *A. arborescens* group B isolates and five *A. solani* isolates (Table 4.1). The isolated cultures were consequently subjected to an *in vitro* assay to look for potential variations in virulence. To easily classify isolates according to their respective virulence profile on potato leaf discs, pathogenic responses were classified into types 0 – 6 (type 0 being an isolate that does not affect plant health, type 6 being an isolate that affects plant health the most compared to other isolates; Fig. 4.3). Clear variations in virulence within the tested species were observed (Fig. 4.4). *A. solani* isolates showed a significantly higher virulence profile than their small-spored

counterparts (Fig. 4.4b). Indeed, 100 % of *A. alternata* and *A. arborescens* subgroup B isolates and 90 % of *A. arborescens* subgroup A isolates showed response types 0 to 3, whereas 100 % of *A. solani* isolates showed response types 4 to 6 (Table 4.1). Additionally, *rAUDPC* values of all small-spored isolates never exceeded 2 % (Fig. 4.4a), whereas the values of *A. solani* isolates were up to 37 % (Fig. 4.4b). When mutually comparing virulence profiles of the three small-spored species, no significant variations in virulence profiles were found. However, significant differences in pathogenic response could be found if isolates within all tested small-spored species were compared (*A. alternata*: $P = 0.003$, *A. arborescens* subgroup A: $P < 0.001$, *A. arborescens* subgroup B: $P < 0.001$; Kruskal–Wallis test). No significant correlations were found between virulence profiles of isolates from different locations or isolates originating from different varieties.

As can be seen in figure 4.5, all small-spored isolates were clearly able to cause lesions at a concentration of 5.10^4 spores mL^{-1}, although no apparent disease progression was observed. In contrast, for *A. solani*, there was a distinct progression in leaf necrosis although the variation in pathogenic response between the different tested isolates was high (Fig. 4.4, Fig. 4.5).

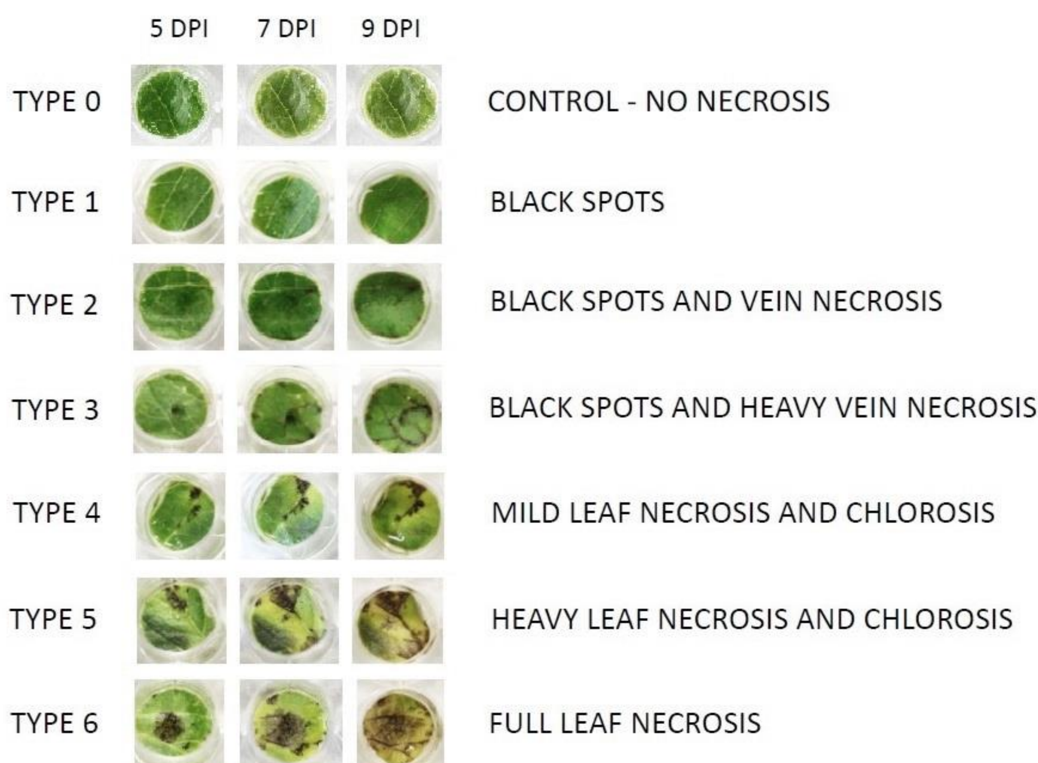


Figure 4.3. Response types of an *in vitro* virulence assay of field isolates of *Alternaria* from potato plants. Leaf disks from the cultivar Bintje were inoculated with 10 µL of a spore suspension of 5.10^4 spores mL^{-1}.

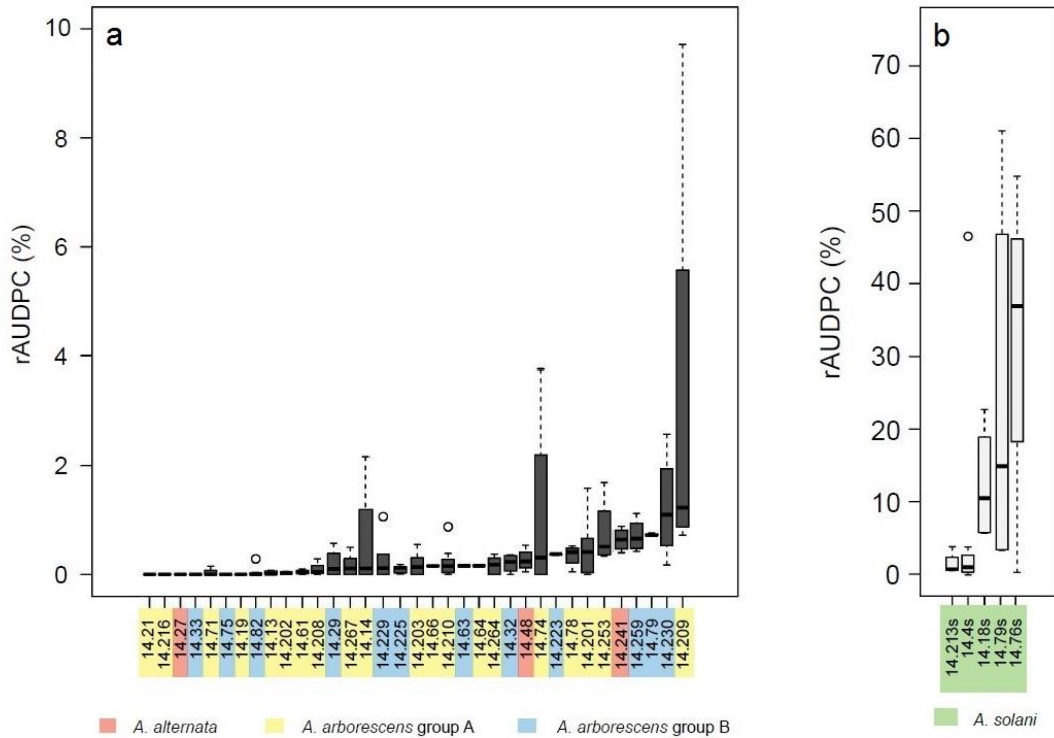


Figure 4.4. Values of relative area under the disease progress curve (*rAUDPC*) of small-spored *Alternaria* isolates (a) and *A. solani* isolates (b) (different Y-axis) in an *in vitro* virulence assay using potato leaf disks from the cultivar Bintje. Isolates are ordered from least to most virulent. For each isolate, four biological repeats were analyzed for each of the two repeats of the experiment.

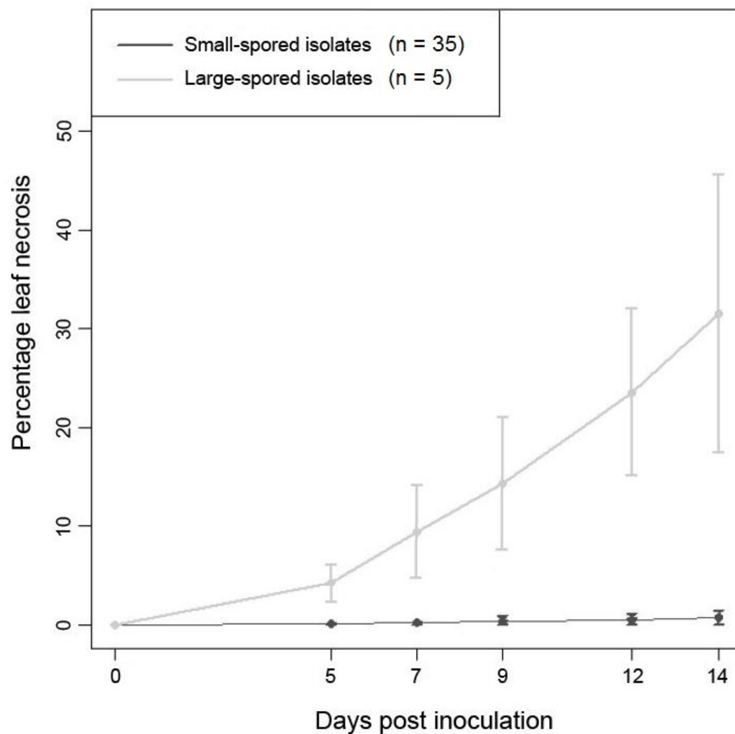


Figure 4.5. Disease progression of small-spored *Alternaria* isolates versus large-spored isolates in an *in vitro* virulence assay using potato leaf disks from the cultivar Bintje. For each isolate, four biological repeats were analyzed for each of the two repeats of the experiment. Number of analyzed isolates is represented by n. Error bars represent standard error.

Table 4.1. Information on all isolates that were subjected to the *in vitro* virulence assay. The type code represents the class by which potato leaf discs were affected after inoculation with the isolate (type 0 being not affected, type 6 being the most affected). SD: standard deviation.

Isolate number	Species	*rAUDPC* (%)	SD (%)	type code
14.13	*A. arborescens A*	0,02703	0,03447	2
14.14	*A. arborescens A*	0,59688	1,04913	3
14.19	*A. arborescens A*	0	0	3
14.21	*A. arborescens A*	0	0	1
14.27	*A. alternata*	0,00313	0,00443	1
14.29	*A. arborescens B*	0,19375	0,27109	3
14.32	*A. arborescens B*	0,20625	0,17396	3
14.33	*A. arborescens B*	0	0	3
14.48	*A. alternata*	0,26875	0,20425	3
14.61	*A. arborescens A*	0,04625	0,04424	2
14.63	*A. arborescens B*	0,1625	0	2
14.64	*A. arborescens A*	0,1625	0	2
14.66	*A. arborescens A*	0,15625	0,00722	3
14.71	*A. arborescens A*	0,05	0,0866	2
14.74	*A. arborescens A*	1,09688	1,80134	3
14.75	*A. arborescens B*	0	0	2
14.78	*A. arborescens A*	0,35	0,20917	3
14.79	*A. arborescens B*	0,72813	0,02577	3
14.82	*A. arborescens B*	0,04313	0,09956	1
14.201	*A. arborescens A*	0,47719	0,538	4
14.202	*A. arborescens A*	0,02781	0,02705	2
14.203	*A. arborescens A*	0,16979	0,18429	3
14.208	*A. arborescens A*	0,09328	0,10151	1
14.209	*A. arborescens A*	3,22188	4,33657	3
14.210	*A. arborescens A*	0,20802	0,2444	3
14.216	*A. arborescens A*	0,00469	0,00938	1
14.223	*A. arborescens B*	0,36875	0,0125	1
14.225	*A. arborescens B*	0,09859	0,06621	1
14.229	*A. arborescens B*	0,25563	0,36343	3
14.230	*A. arborescens B*	1,2375	1,00763	1
14.241	*A. alternata*	0,64281	0,21455	1
14.253	*A. arborescens A*	0,7625	0,63048	4
14.259	*A. arborescens B*	0,71438	0,26394	3
14.264	*A. arborescens A*	0,16734	0,16054	3
14.267	*A. arborescens A*	0,16375	0,19374	3

(Table 4.1 continued)

Isolate number	Species	*rAUDPC* (%)	SD (%)	type code
14.4s	*A. solani*	6,88828	16,10175	4
14.18s	*A. solani*	12,4125	8,19045	5
14.76s	*A. solani*	32,42844	22,92098	5
14.79s	*A. solani*	24,50469	24,15036	6
14.213s	*A. solani*	1,5375	1,58137	4

Artificially inoculated field trial

To test if the virulence profiles observed *in vitro* can also be discerned in the field and to verify if the increase in disease severity is due to the shift in population composition, a field trial was set up during the growing seasons of 2014 and 2015 in which inoculations or co-inoculations of both small- and large-spored isolates were applied. Figure 4.6 shows the *rAUDPC* values of the different treatments on potato plants for both field trials. For 2014, it can be observed that plants inoculated with a spore suspension that included the large-spored isolate 14.4s (either for 100, 50 or 10 %) were significantly more affected than plants that were solely inoculated with a small-spored isolate 14.79. Conversely, there was no significant difference in disease severity between the plants that were inoculated with only the small-spored isolate and the control plants, indicating that, under the applied circumstances, the small-spored isolate did not increase disease pressure. The more of the large-spored isolate was included in the inoculation suspension, the higher the mean *rAUDPC* value was, however, the differences between the treatments that included the large-spored isolate 14.4s (either for 100, 50 or 10 %) were never significant.

For the 2015 field trial (Fig. 4.7), the plants that were treated with the small-spored isolate, again did not show significantly more disease symptoms than the mock-treated plants. The plants that were inoculated or co-inoculated with a large-spored isolate were also significantly more affected than the control plants and plants inoculated with the small-spored isolate. In this trial however, there were clear differences between the plants that were treated with a large-spored isolate. Those that were solely inoculated with the large-spored *A. solani* showed significantly more disease severity than those with only 10 % of the large-spored isolate added in the inoculum. The plants inoculated with a 50 / 50 ratio of small- and large-spored isolates, showed a mean *rAUDPC* value that was in between the values for the plants treated with 100 and 10 % *A. solani*.

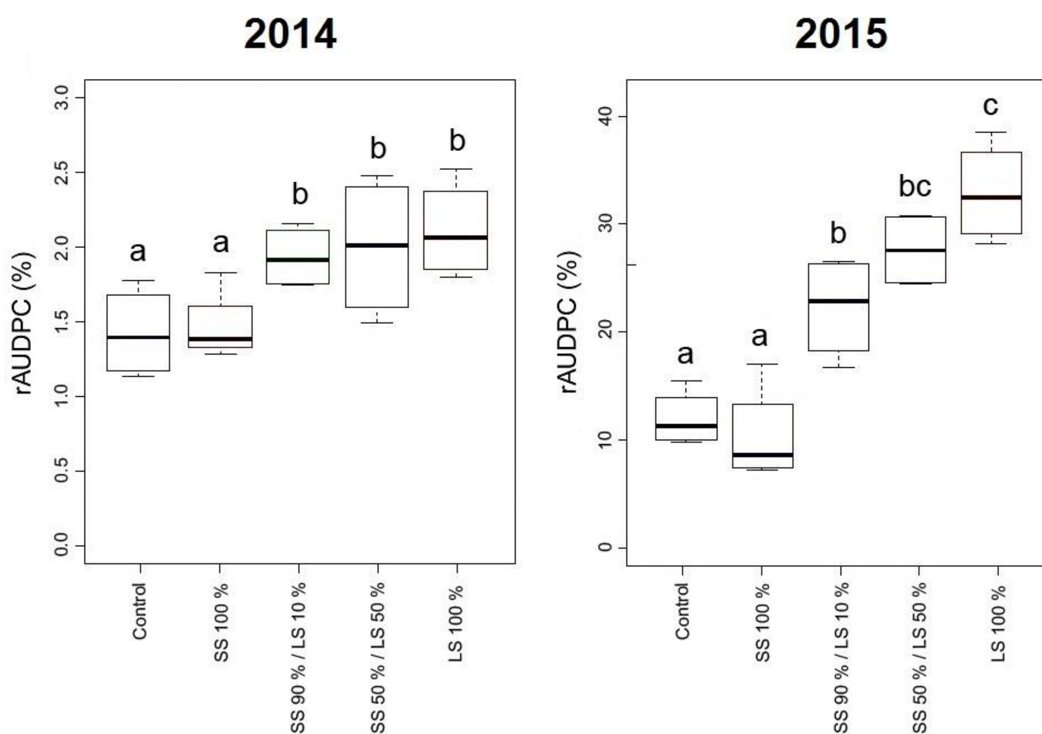


Figure 4.6. *rAUDPC* values for the different treatments in the artificially inoculated field trial (using the cultivar Bintje) during the growing seasons of 2014 and 2015. SS: small-spored inoculum, LS: large-spored inoculum. For each treatment, four biological replications were monitored weekly during two months (7 data points). Letters above the plots indicate significant differences between treatments.

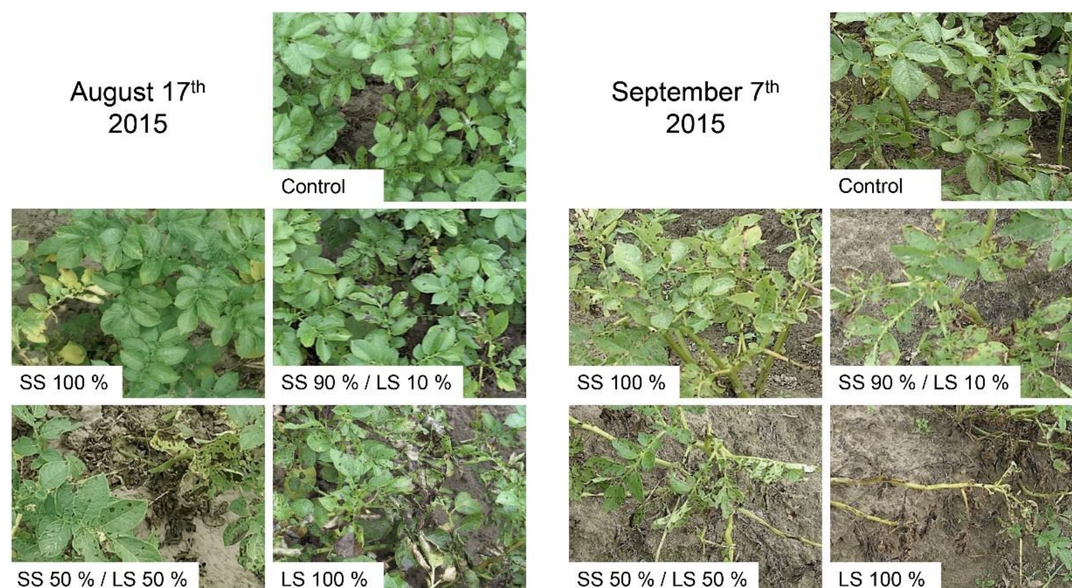


Figure 4.7. Pictures of the different treatments of the inoculated field trial (using the cultivar Bintje) on August 17th (left) and on September 7th of 2015 (right). SS: small-spored inoculum, LS: large-spored inoculum.

DISCUSSION

In order to reveal potential mechanisms behind the sudden increase in disease incidence observed from mid-August (chapter 2), the population composition at different time points during the season was investigated.

As described in chapter 2, a recurring pattern of disease progression emerged. During the first half of the growing season, leaf necrosis levels were low to negligible. From mid-August, a sharp increase in leaf necrosis was observed for all monitored fields. We hypothesize that this specific disease curve can be attributed to the *Alternaria* population composition. The real-time PCR results showed that from the beginning of the growing season, small-spored *Alternaria* species were predominantly found on potato leaves. DNA from *A. solani* was detected only from mid-August and this pathogen proliferated even more in September. It should be noted that both primer pairs that were used could detect small-spored species (*A. arborescens* groups A and B) and large-spored species (*A. solani*, *A. grandis* and *A. protenta*), but could not distinguish between the species in each group. Similar findings were made by Leiminger et al. (2015) in Germany; they also observed a latent start in disease progression, but the increase in infection began somewhat earlier (mid-July). Similarly, in 2004, they established that *A. solani* DNA could only be detected from mid-July. The observation that *A. solani* species can be detected when disease symptoms are escalating, was supported in the present study by our *in vitro* virulence assay and field trial. These demonstrated firstly that, although the small-spored species *A. alternata* and *A. arborescens* groups A and B were able to initiate leaf necrosis, disease progression was not apparent. In contrast, *A. solani* isolates were able to confer disease (up to 37 % after 14 dpi). Secondly, when potato plants were artificially inoculated with solely a small-spored isolate, no disease progression could be observed, whereas in plants solely inoculated or co-inoculated with a large-spored isolate, disease severity was significantly higher. Our results are backed up by the findings of Spoelder et al. (2013) and Stammler et al. (2013). The former research group also observed that *A. alternata* was unable to confer disease progression in an *in vitro* virulence assay. The latter researchers similarly performed an artificially inoculated field trial using different mixtures of *A. alternata* and *A. solani* in which they observed that treatments with *A. solani* exhibited much necrosis, whereas for the treatments with solely *A. alternata*, no increase in disease symptoms was detected.

Together with these reports, our findings designate the large-spored species as the causal agent of EB/BS disease in Flemish potato fields and indicate that the increase in disease pressure observed from mid-August is due to the advance of large-spored *Alternaria* species in the field.

To answer the question as to why small-spored species are predominantly detected in the beginning of the season during in what seems to be a latent stage of infection, it may be speculated that this is due to the fact that small-spored *Alternaria* species are abundantly present in the air, especially in July and August (AirAllergy.be, 2017), which could result in an abundance of spores landing on potato leaves. The latent stage of the infection process might be due to the fact that small-spored species do not pose a threat to the plant and may act as endophytes. If this is so, then *A. solani* species may take advantage of the already formed infection sites, due to wounding or wet environments, to colonize and kill the host plant more quickly. Further research, such as investigating fitness on plant leaves (by monitoring spore germination and hyphal growth), complemented with sequential inoculations where small-spored or large-spored isolates are inoculated before the other, could help to resolve this issue.

The *in vitro* assay also revealed significant variation within the small-spored *Alternaria* species, but not between them. Although no correlation was found between virulence and the variety from which the isolates originated, it has been reported previously that a number of *A. alternata* isolates from different regions in Russia exhibited varying virulence towards certain potato varieties (Kokaeva et al., 2015). Furthermore, it has been shown that *A. alternata* species can gain pathogenicity through horizontal gene flow (Hatta et al., 2002; Thomma, 2003; Akagi et al., 2009). This could also be a crucial factor, giving rise to varying *Alternaria* virulence profiles.

Unexpectedly, the majority of small-spored species isolated from the field in the present study were identified as *A. arborescens* group A or B, whereas only 3 out of 40 isolates were identified as *A. alternata*. This indicates that the majority of small-spored species on Flemish potato fields is *A. arborescens*, rather than *A. alternata*, which was previously assumed. The fact that our fitness assay revealed that there was no significant difference in growth rate between small-spored species confirms that no bias was introduced in the isolation and identification of the small-spored isolates. However, the growth rate of large-spored species was significantly lower than the small-spored counterparts. These findings highlight once again the need for identifying species at the molecular level when conducting a population composition analysis. Indeed, by using visual analyses alone, it is impossible to discriminate between different small- or large-spored species. Also, when isolating species from infected leaves, a bias could arise because small-spored species grow faster on culturing media than large-spored species, resulting in a different representation than would be shown by real-time PCR.

This study provides a clear view of EB/BS disease progression, elucidating a shift in population composition as the underlying mechanism for the increase in disease incidence. Additionally, it was confirmed that the virulence of large-spored *A. solani* isolates towards potato is higher than small-spored *Alternaria* isolates. However, no correlations were found between virulence profiles and variety or region of origin.

In the next chapter, molecular mechanisms behind the difference in virulence profiles will be further delved into. Instead of solely focusing on toxin production, the interaction between *Alternaria* and the plants hormone ethylene will also be investigated.

ACKNOWLEDGEMENTS

We would like to thank ILVO (Merelbeke, Belgium) for providing potato seedlings.

ADDENDUM – IN VITRO VIRULENCE ASSAY OPTIMIZATION

Introduction

Before arriving at the *in vitro* virulence assay setup that was used in the study described above, a number of methods was tested in order to optimize an assay that yielded reliable and reproducible results. In the paragraphs below, the different trials that were evaluated and their respective results will be briefly discussed.

Methods and results

Trial 1

Fully grown leaves (terminal leaflets at growth stage 3 of plant development) from the potato variety Bintje and Désirée, were harvested 10 days after surfacing from the lowest, middle and highest level of the plant. They were sterilized in a 5 % NaOCl solution and subsequently washed in sterile water. All leaves were put on water agar (18 g L^{-1} plant agar) (Gold Bio, St Louis, MO) and on half of the leaves, a small surface wound was made on either side of the mid vein. The other half of the leaves had no surface wound. Leaves were inoculated with a 10 µL spore drop (10^4 spores mL^{-1}) on either side of the mid vein. Two small-spored isolates and two large-spored *Alternaria* isolates were used for this setup. All infections were repeated three times. Lesion diameters were measured every two days using a digital caliper.

Lesions were hard to measure since the leaves withered and died too quickly (leaves turned brown already 4 or 5 dpi) (Fig. A4.1). Leaves with surface wounds turned brown even faster (2 or 3 dpi). No variations in necrosis between leaves from different levels or from different varieties could be discerned.

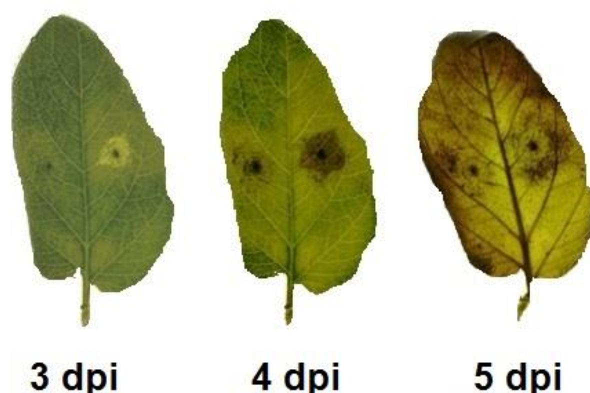


Figure A4.1. Browning of detached leaves without a surface wound and that were inoculated with a spore drop (10^4 spores mL^{-1}) of a large-spored *Alternaria* isolate.

Trial 2

A second setup was evaluated in which potato plants were grown and leaves were harvested and washed as described above. This time, no surface wounds were made. Leaves were put on water agar (18 g/L plant agar) (Gold Bio, St Louis, MO) and were inoculated with a mycelium plug of the isolates used in the previous method. Infections were repeated three times. An agar plug was used for inoculation in an attempt to increase disease pressure. Lesion diameters were measured every two days using a digital caliper.
In this trial, the leaves did not turn brown before 6 dpi. However, the lesions had not progressed enough to distinguish variation in necrosis between varieties or between leaves from different levels. Also, a lot of air mycelium was produced which might be the result of the fungus obtaining its nutrients from the agar plug instead of the plant.

Trial 3

In an attempt to make the potato leaves last longer, a new setup was approached. Leaves from the varieties Bintje and Désirée were grown, harvested and washed as described above. Instead of water agar, wetted filter paper was put in petri dishes and used to install the leaves. Half of the leaves was inoculated with a 10 µL spore drop on either side of the mid vein, the other half of the leaves was spray-inoculated over the full leaf surface in order to induce disease more evenly. Spore suspensions were of the same concentration as before. Only one small- and one large-spored isolate was used in this setup. To check if incubation temperature had an influence on the lifespan of the leaves and disease pressure, the inoculated leaflets were incubated at 15, 21 and 21 °C. All infections were performed in three-fold. Lesion diameters were measured every two days using a digital caliper.
The usage of filter paper resulted in a longer lifespan of the leaves. Based on the preliminary results that could be obtained, the variety Bintje seemed more susceptible than Désirée. At 25 °C, leaves were turning brown faster (after 4 dpi) than at 15 and 21 °C. At 15 °C, infections progressed slower than at 21 °C. The spray-inoculation resulted in low to negligible levels of necrosis especially for the large-spored isolate, presumably because spores were too large to be dispersed through the filter.

Trial 4

The following method was carried out by growing, harvesting and washing leaflets as described above. Next to wet filter paper, slants of ½ MS and 1 MS (Murashige and Skoog basal medium, Sigma-Aldrich®, Overijse, Belgium) medium were used to install the leaves in an attempt to further increase their lifespan. Based on the results of the previous trial, leaves were inoculated by applying

a 10 µL spore drop (10^4 spores mL^{-1}) on either side of the mid vein and were incubated at 21 °C. The same two isolates were used as before. All infections were tested in three biological repeats. This time, percentage necrosis was measured using the ASSESS v. 2.0 (APS) image analysis software for plant disease quantification.

The usage of MS slants did not increase the lifespan of the leaves compared to leaves on wet filter paper. Necrosis levels could be measured in more detail using the image analysis software which resulted in the observation that older leaves (from the lower level of the plant) are more susceptible to the disease than younger leaves originating from the middle or highest level of the plant. The observation that the variety Bintje is more susceptible than Désirée could be confirmed in this setup (Fig. A4.2).

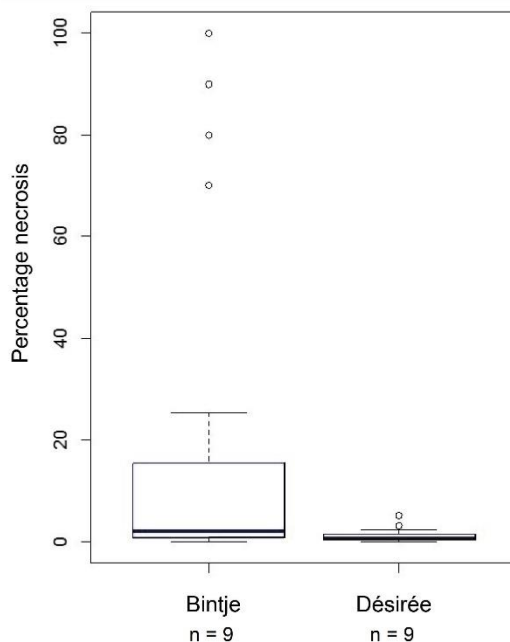


Figure A4.2. Percentage necrosis of inoculated leaves originating from the varieties Bintje and Désirée. Leaves were inoculated with one small-spored isolate or one large-spored isolate. For each isolate, three biological repeats were performed. Leaves were scored every three days during two weeks (5 time points). Number of leaves that were scored is represented by n.

Trial 5

Finally, a new setup was approached. After growing, harvesting and sterilizing leaves from the variety Bintje as described above, leaf discs were cut with a cork borer and placed on sterile water or on wet filter paper in a 24-well plate. Leaf discs were inoculated in the middle of the disc with a spore drop of either 10^4 spores mL^{-1} or 5.10^4 spores mL^{-1} and all infections were repeated four times. The same two isolates as in the previous setups were used. Necrosis levels were measured using the ASSESS v. 2.0 (APS) image analysis software for plant disease quantification.

The infections on sterile water yielded reproducible results and the leaf discs did not turn brown before full colonization (Fig. A4.3). In contrast, leaf discs on wet filter paper dried out and curled after 8 dpi, complicating necrosis measurements. Disease pressure was higher for the leaf discs

that were inoculated with spore suspensions of 5.10^4 spores mL^{-1}. Especially in the case for the large-spored isolate, many of the leaf discs were fully colonized within two weeks (Fig. A4.3).

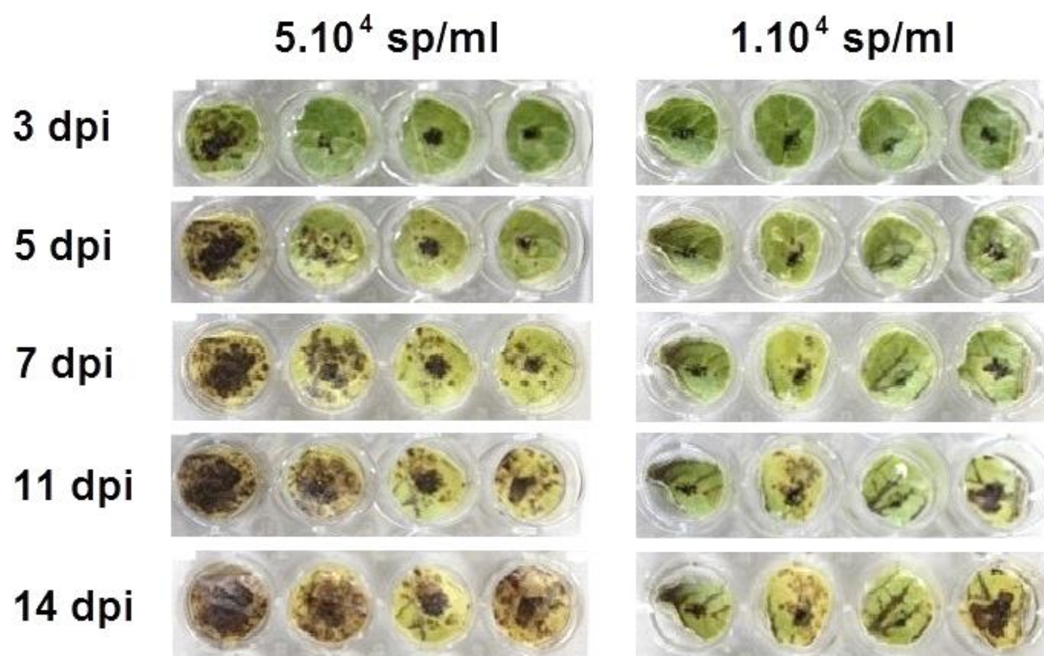


Figure A4.3. Disease progression of leaf discs inoculated with a different concentration of spore suspension. Spores were harvested from a large-spored *Alternaria* isolate. Leaf discs were cut from 10-day old leaflets from the variety Bintje. For each spore concentration, four biological repeats were performed.

Conclusions

Based on the results of the five methods described above, the method for the assay that was used in all further studies was a 24-well plate assay in which potato leaf discs of the cultivar Bintje were placed on sterile water and inoculated with a 10 µL spore drop (5.10^4 spores mL^{-1}). By spreading the spore drop across the leaf surface, chances to incorrectly score high spore densities as necrotic spots were eliminated. The assay in 24-well plates provided a fast, elegant and reproducible *in vitro* test in which treatments could easily be compared due to the small scale of the experiment. Additionally, due to this small scale, environmental factors like temperature or lighting could easily be controlled and equated. The placement on sterile water and sealing of the 24-well plates (using parafilm) also provided the necessary humidity for optimal spore germination. Due to the higher susceptibility and because it is highly cultivated in Flanders, the potato variety Bintje was chosen as the host plant. Only leaves from the middle part of the plant and at growth stage 3 of plant development were harvested in order to minimize the age factor and because leaves from the lower levels were prone to early browning and early death.

CHAPTER 5

ETHYLENE PRODUCTION AND ITS ROLE DURING *ALTERNARIA* INFECTIONS ON POTATO

This work is in preparation for publication.

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ABSTRACT

Alternaria species are known to produce a plethora of toxins, many of which are found on multiple host plants (non-host specific toxins), while others are only produced on specific hosts (host-specific toxins). Nevertheless, the involvement of toxins in the development of EB/BS disease has never been investigated on potato. In tomato, another solanaceous species, it has been previously illustrated that programmed cell death is induced by the *Alternaria* AAL-toxin and that the plant hormone ethylene (ET) is an important factor in the signaling pathway of AAL toxin-induced cell death. In view of this observation, we investigated the capacity of both small- and large-spored species for their ability to produce host- and non-host specific toxins *in vitro* and *in vivo*, and assessed if ET was involved in an infection on potato plants.

First, the time frame was determined in which spores of small- and large-spored isolates germinate on potato leaves. The assay revealed that within the first five hours post inoculation, all small and large spores had germinated and that there was no significant difference in germination rate between small- and large-spored isolates. Next, the capacity for ET production was assessed *in vitro* and *in vivo* for the isolates under study. The *in vitro* ET production assay indicated that the large-spored isolates produced more ET *in vitro* than the small-spored isolates in the presence of 1 mM of the precursor KMBA. To a much lesser extent, one large-spored isolate also produced ET in the presence of 1 mM ACC. ET production was also monitored on potato leaf discs inoculated with the same *Alternaria* isolates. After 3 days post inoculation, leaf discs inoculated with small-spored isolates released more ET than leaf discs inoculated with large-spored isolates after correcting the data according to fungal DNA content on the leaf. These results, together with the observation that symptoms start to appear at 3 days post inoculation, may suggest a role for ET during disease progression instead of disease intitiation. These data were complemented with results from an experiment where ET biosynthesis in the plant was inhibited using 2-aminoethoxyvinyl glycine (AVG). Leaf discs treated with the inhibitor exhibited a strong reduction in necrosis after inoculation with large-spored isolates. In contrast, leaf discs inoculated with small-spored isolates exhibited an increase in necrosis after treatment with the inhibitor. Finally, although isolates had the capacity to produce many host- and non-host specific toxins, none of them were produced during the first three days of infection.

Based on these observations and the data from the previous chapters, we hypothesize that on potato leaves, small-spored isolates might boost the plant ET biosynthesis, which is consequentially employed by large-spored isolates to confer necrosis and faster colonization of leaf tissue. In addition, the involvement of ET in the infection could not be linked to the production of known host- or non-host specific toxins in both small- or large-spored species.

INTRODUCTION

Although much has been revealed about bacterial pathogens and the arsenal of effectors they secrete in host cells (Kamoun, 2007; Gohre and Robatzek, 2008), information on fungal effectors and their mechanisms of pathogenicity was not readily available. Previously, our knowledge on this subject was limited to the development of specialized infection structures called 'haustoria' and the secretion of catalytic enzymes and host-specific toxins. However, in the last decade, many reports have shed new light on these virulence mechanisms and revealed that pathogens have evolved to manipulate host cell metabolism and plant immunity by secreting effectors or employing compounds such as phytohormones produced by the host plant or by the pathogen to benefit the infection process.
In this chapter, both fungal effectors and host compounds employed by pathogens will be summarized as virulence factors.

Generally, virulence factors of necrotrophic fungi can be divided into two main categories: the first category of metabolites serves to attach and colonize the host more quickly, the second set of virulence factors act on damaging host tissue (Choquer et al., 2007). Extracellular enzymes are the most common form of metabolites that help to colonize the plant tissue. These enzymes include pectin lyases, cellulases, oxydases and proteases to respectively break down pectin, carbohydrates, lignin and proteins that make up the plant cell wall (Mendgen et al., 1996). Besides these enzymes, plant colonization is helped by hydrophobic interactions that facilitate attachment of the spores to the cell wall, the induction of an oxidative burst and the formation of pressurized swollen tip-like structures called 'appressoria'. These structures can 'push' through the physical barrier of the plant by using turgor pressure (Choquer et al., 2007). Once inside the cell, the fungal pathogen will secrete virulence factors that help damage the tissue. Among these are host- or non-host specific phytotoxins and reactive oxygen species (ROS) (vonTiedemann, 1997; Deighton et al., 1999; Shinogi et al., 2003). Although much is known about the toxic effect of *Alternaria* toxins in mammalian cells, little information is available of the phytotoxic effects of these metabolites. Demuner et al. (2013) and Chen et al. (2014) were able to demonstrate that the non-host specific toxins alternariol (AOH), alternariol monomethyl ether (AME) and tenuazoic acid (TeA) inhibit the electron transport flow in the plants' chloroplasts, thereby hampering photosynthesis. In contrast, the host-specific AAL-toxin acts on inhibiting ceramide biosynthesis, which results in the accumulation of sphingosines in the plant cells, which are known to act as signaling molecules in various processes, such as the induction of programmed cell death (PCD) (Stockmann-Juvala and Savolainen, 2008). Besides being produced by the pathogen as a virulence factor, ROS are also

produced by the host plant itself upon pathogen attack as a defense mechanism that induces a hypersensitive reaction (HR), which is a type of PCD that should limit the pathogen's access to water and nutrients (Govrin and Levine, 2000). However, Gechev et al. (2004) and Prasad and Upadhyay (2010) demonstrated that exogenous application of AAL-toxin, a host-specific toxin produced by *A. alternata* f. sp. *lycopersici*, triggers H_2O_2 production in *Arabidopsis* and tomato respectively. These observations indicate that *Alternaria* pathogens can exploit the hosts' induction of HR by producing toxins that trigger ROS production, leading to faster necrosis development. Moreover, next to ROS production, application of AAL-toxin triggered ET production while application of antioxidants or ET biosynthesis inhibitors significantly reduced necrosis development (Prasad and Upadhyay, 2010). These results indicate that both ROS and ET act as virulence factors in the *Alternaria*-tomato pathosystem.

The hypothesis of ET being a virulence factor of *Alternaria* is supported by the observation that AAL toxin-triggered cell death is dependent on mitogen-activated protein kinase (MAPK) cascades and downstream ET biosynthesis (Mase et al., 2012). Additionally, ET treatment of *Nicotiana umbratica* mutants deficient in MAPK cascade signaling re-established PCD, demonstrating that toxin-triggered cell death is dependent on the action of ET. The fact that some pathogens are able to tap into the hormonal defense network to re-direct the signaling towards a more susceptible environment, has been demonstrated in several other reports. Indeed, the production of gibberellin by *Fusarium fujikuroi* was shown to be important in developing abnormal elongation of rice plants (Ou, 1987). Additionally, virulence of *Botrytis cinerea* on tomato is dependent on the stimulation of plant ABA biosynthesis, rendering the host more susceptible to colonization (Kettner and Dorffling, 1995; Audenaert et al., 2002). Finally, De Bruyne (2015) demonstrated that rice plant compromised in ET biosynthesis displayed strong resistance to brown spot disease caused by *Cochliobolus miyabeanus*.

Some pathogens have evolved to alter the plant defense network by producing phytohormones or mimics thereof by themselves (Tudzynski and Sharon, 2002; Chanclud and Morel, 2016). Because ET is involved in many defense responses, mainly as a modulator of defense pathways regulated by JA or SA (Broekgaarden et al., 2015), ET is often a target to be exploited by numerous pathogens by producing this phytohormone themselves in order to hijack defense signaling and suppress immunity responses (Table 1.4). Three distinct ET biosynthesis pathways can be discerned in fungi (Chagué, 2010) (Fig. 1.10). The first pathway has been originally characterized in higher plants and synthesizes ET through the action of three enzymes which convert L-methionine (MET) to S-adenosylmethionine (SAM), to 1-aminocyclopropane-1-carboxylic acid (ACC) and ultimately to ET (Johnson and Ecker, 1998). A second pathway also starts from MET

and relies on an unknown transaminase to produce KMBA, which is then non-enzymatically oxidized in the presence of hydroxyl radicals or photo-oxidized under light to release ET (Primrose and Dilworth, 1976; Fukuda et al., 1989). In the third ET biosynthesis pathway, ET is synthesized from 2-oxoglutarate, an intermediate of the citric acid cycle, through the action of an ethylene forming enzyme (EFE) that requires arginine or lysine, oxygen and Fe^{2+} as additional cofactors (Fukuda et al., 1992; Chagué, 2010). In fact, EFE catalyzes two simultaneous reactions where, next to ethylene, succinate, guanidine and 1-pyroline carboxylate are formed. According to Chagué (2010), the widespread presence of EFE homologues suggests that this pathway may be used by many other microorganisms to produce ethylene. In contrast, the KMBA and especially the ACC pathway are less common in microorganisms.

The role of fungal or microbial ET is not well known. However, a few examples exist where it is demonstrated that ET produced by microorganisms contribute to rendering the host more susceptible to colonization. *Pseudomonas syringae* pvs. *glycinea* and *phaseolicola* strains deficient in EFE action were significantly compromised in colonizing bean and soybean plants (Weingart et al., 2001). Additionally, Valls et al. (2006) showed that ET produced by the microbial pathogen *Ralstonia solanacearum* has an effect on the expression of host defense genes and indirectly affects *Arabidopsis* plant defense signaling. Finally, ET produced by the fungal necrotroph *C. miyabeanus* seems to be a clear susceptibility enhancer since external ET application exaggerated disease symptoms while application of ET signaling inhibitors reduced disease development (De Vleesschauwer et al., 2010). Moreover, it was shown that *C. miyabeanus* produced ET *in planta* and that ET biosynthesis genes were upregulated in rice after inoculation (De Bruyne, 2015; Van Bockhaven et al., 2015). These findings suggest that *C. miyabeanus* taps into the ET signaling pathway of rice and induces plant ET biosynthesis to induce susceptibility. Whether this induction is triggered by the fungal ET is not clear.

In this chapter, we investigate the molecular mechanisms behind the virulence profiles of small- and large-spored *Alternaria* species by focusing on two types of virulence factors. Firstly, the role of ET during infection will be investigated by monitoring ET production in potato leaves after inoculation with different *Alternaria* isolates, while also determining whether ET can be self-produced by the fungus. Furthermore, the involvement of ET during infection will be investigated by applying inhibitors that block plant ET biosynthesis. Secondly, *in vitro* and *in planta* toxin production will be profiled for both small- and large spored species to find a potential link between toxin and ET production.

MATERIALS AND METHODS

Determining spore germination time frames and variability during infection

To reveal crucial events during potato leaf infection that might be in synchronization with ET production spikes, the time frame in which spores from *Alternaria* isolates germinate was first determined. In that regard, also the spore germination variability of the different isolates was studied. Four isolates were chosen (Table 5.1) from the isolate collection that was used for the virulence study described in the previous chapter (Table 4.1). Two isolates identified as *A. arborescens* group A (14.21 and 14.209) were picked based on their extremities in virulence profile within the observed virulence spectrum (Fig. 4.3). The same was done for the large-spored *A. solani* isolates (14.4s and 14.79s). No isolates from other small-spored species, such as *A. alternata* or *A. arborescens* group B were investigated since no significant differences in virulence were observed between them (chapter 4). Table 5.1 gives an overview of the studied isolates.

To determine spore germination time frames, a similar approach to the *in vitro* virulence assay (described in the previous chapter) was applied: 10-day old leaves of the cultivar Bintje were harvested, surface-sterilized in 5 % NaOCl solution and subsequently washed in sterile water. Next, leaf discs were cut using a 15-mm cork borer and were put randomly in a 24-well plate on 1 mL of sterile water. Leaf discs were inoculated with 10 µL of a spore suspension of 5.10^4 spores mL^{-1} and incubated at 21 °C. Spore suspensions were prepared for the four isolates as described above. For each isolate, four technical repeats were performed and the complete assay was set up in 10-fold to account for the different time points after which the leaf discs were fixated and stained. After 1, 2, 3, 4, 6, 8, 10, 14, 16 and 24 hours post inoculation, the leaf discs were fixated and removed of chlorophyll by putting them in an acetic acid:ethanol solution (1:3) for 24 hours and under continuous shaking (110 rpm). Subsequently, leaf discs were transferred to an acetic acid:ethanol:glycerol solution (1:4:1) for 12 hours under continuous shaking (110 rpm), after which they were stained by placing them in a bath of lactic acid:demineralized water (1:1) with 0.01 % trypan blue stain (VWR, Leuven, Belgium) for 12 hours and under continuous shaking (110 rpm). Finally, excess staining solution was washed away using a 60 % glycerol solution. The stained leaf discs were microscopically analyzed by counting the number of germinated spores and calculating the mean percentages for each isolate at each time point. Additionally, all leaf discs were scanned for penetrated cells at each time point.

Table 5.1. Information on the investigated isolates.

Isolate	Species	Virulence type
14.21	*A. arborescens A* (small-spored)	Type 1 (least virulent)
14.209	*A. arborescens A* (small-spored)	Type 3 (most virulent)
14.4s	*A. solani* (large-spored)	Type 4 (least virulent)
14.79s	*A. solani* (large-spored)	Type 6 (most virulent)

In vitro ethylene production

To test if *Alternaria* isolates can produce ET *in vitro*, the same set of isolates that was studied in the spore germination assay was grown on SNA medium to induce sporulation and spore suspensions were prepared similarly as before. Next, 50 µL of each spore suspension was pipetted in sterile glass injection vials (10 mL) (VWR, Leuven, Belgium) that were filled with 3 mL of SNA medium. The growth medium was supplemented with one of the three direct precursors for ethylene production: 1 mM of either ACC or KMBA, or a combination of 5 mM 2-oxoglutarate and 1,25 mM arginine. Vials were capped air tight to ensure volatile build-up within the vials' headspace. For each isolate, three biological repeats were prepared. Total accumulated ET in the vials' headspace was measured after 24 h using laser-based photoacoustic spectroscopy (LPAS) (Sensor Sense, ETD-300) in stop and flow modus and re-calculated as nL h^{-1}. In a second experiment, 5 mM of KMBA, 5 mM of L-MET, or no substrate was administered. Two days post inoculation, samples were put in the dark in order to discern a light-dependent conversion. Total accumulated ET in the vials' headspace after 24 h, between 24 h and 2 dpi, and between 2 dpi and 5 dpi was measured using laser-based photoacoustic spectroscopy (LPAS) (Sensor Sense, ETD-300) in stop and flow modus and re-calculated as nL h^{-1}.

In planta ethylene production

To reveal potential ET spikes during an infection of *Alternaria* on potato leaves (which can either be produced by the plant, by the fungus, or by both), ET accumulation was measured at 5 hpi, 1 dpi and 3 dpi. Therefore, 10-day old leaf discs of the cultivar Bintje were prepared as described above, after which they were left on the bench for 8 hours to get rid of residual wound ethylene. Inoculation was performed by pipetting 10 µL of a spore suspension of 5.10^4 spores mL^{-1} on the leaf disc. Spore suspensions were prepared for the four isolates similarly as before. The inside of glass injection vials (10 mL) (VWR, Leuven, Belgium) was covered with wet filter paper in order to 'stick' the inoculated leaf discs on the sides within the glass vials. One leaf disc was put on the

bottom of each glass vial and four leaf discs were put on the side, so that a total of five inoculated leaf discs were placed in one vial. The vials were capped air tight to ensure volatile build-up within the vials' headspace. For each isolate, three biological repeats (three vials, a total of 15 inoculated leaf discs) were prepared. Five hours prior to each time point, the present ET was flushed from the vial and the amount of ET that accumulates in the following five hours was measured using laser-based photoacoustic spectroscopy (Sensor Sense, ETD-300) in stop and flow modus and re-calculated as nL h^{-1}. Measured ET quantities were standardized with the *Alternaria* DNA quantity detected on the leaf. Therefore, immediately after ET measurement, leaf discs were frozen under liquid nitrogen and crushed using a mortar and pestle. Genomic DNA extraction and subsequent detection by real-time PCR was then carried out as described in the '*Materials and methods*' section of the previous chapter. All ET measurements were calibrated against the mock-treated samples.

Pharmacological assays

In order to see if the ET production by the pathogens plays a role during infection, the ET biosynthesis inhibitor 2-aminoethoxyvinyl glycine (AVG) was dissolved at a concentration of 1 mM and was applied by placing 10-day old leaves from the cultivar Bintje in the solution for 24 hours and under continuous shaking (110 rpm) prior to inoculation. This compound blocks the action of ACC synthase, a key component in plant ET biosynthesis (and also the fungal ACC pathway to produce ET). The other half of the leaf discs was similarly treated with water instead of AVG. Leaf discs were prepared as described above and were inoculated either by 10 µL of a spore suspension (5.10^4 spores mL^{-1}), or by 10 µL of a spore suspension supplemented with 1 mM of KMBA or ACC in order to discern the re-establishment of necrosis by fungal ET production (via the KMBA pathway) or by the plant and / or fungal ET (via the ACC pathway) respectively. Next, leaf discs were placed in a 24-well plate on 1 mL of the inhibitor solution or 1 ml of sterile water and incubated for 10 days at room temperature before determining necrotic area sizes. These were obtained using spectral data acquired by the Pathoviewer phenotyping platform and which were analyzed by the CropReporter software that was developed by Phenovation B.V.

In vitro toxin analysis

To determine the *in vitro* toxin production profile, the four studied isolates were grown on malt extract agar (MEA) for 7 days at room temperature and in the dark. The complete MEA medium with fungal culture was cut in to small pieces (ca. 1 cm^2) and transferred to glass test tubes. Next, 40 mL of extraction solvent (methanol:dichloromethane:ethyl acetate (1:2:3), supplemented with 1 % formic acid) was added, followed by one hour of extensive shaking on an overhead shaker (Agitelec, J. Toulemonde & Cie, Paris, France). Then, samples were subjected to 5 min of

centrifugation (3291 g) after which 5 mL of the resulting supernatant was transferred to a new test tube in four-fold and evaporated under nitrogen flow in an thermostatic hot water bath at 40 °C (Grant Instruments Ltd, Cambridge, United Kingdom). Immediately before analysis, samples were resolved in 200 µL injection solvent (ultra-pure water:acetonitrile:methanol, (4:3:3)) and thoroughly mixed on a vortex mixer. Finally, samples were subjected to ultracentrifugation for 10 min at 10000 g (Ultrafree®-MC centrifugation filter; Millipore, Bedford, MA, USA).

Liquid chromatography coupled with tandem mass-spectrometry (LC-MS/MS) was used as multi-mycotoxin determination methodology. Table 5.2 displays all *Alternaria* toxins that were screened for. For this study, toxins were separated using Ultra Performance Liquid Chromatography (UPLC) coupled with the SYNAPT G2-Si High Definition Mass Spectrometer (Waters Belgium, Zellik) which operated in MS^E continuous mode (records product ion data for every detectable molecular ion). A sample volume of 5 µL was injected in an HSS T3 column (1.8 µm, 2.1 x 100 mm) (Agilent Technologies, Santa Clara, USA) which was applied with a gradient elution program using the following mobile phase: (A: [ultrapure water:methanol (95:5) + 0,1 % formic acid], B: [methanol:ultrapure water (95:5) + 0,1 % formic acid]) and a flow rate of 0,4 mL min^{-1}. The gradient program was as follows: 95 % A and 5 % B for 0.5 min followed by an increase of solvent B to 95 % during 15.5 min. 95 % B was held for 1 min after which 95 % A was again applied in 0.1 min. These starting conditions were then maintained for 3 min. Ionization occurred through ESI in negative mode. Reference standards of alternariol (AOH), alternariol mono methyl ether (AME), altenuene (ALT) and tenuazonic acid (TeA) were available in the lab and working solutions (10 µg mL^{-1}) were prepared in methanol. Subsequently, the standards were stored at -20 ° C until analysis.

***In planta* toxin analysis**

Mycotoxins produced during an *Alternaria* infection on potato leaves were investigated by first growing potato plants from the cultivar Bintje in the phytotron for 1 month (21 °C, 16 h photoperiod) and harvesting leaves from the middle level of the plants (terminal leaflets at growth stage 3 of plant development). Leaves were surface-sterilized in a 5 % NaOCl solution for about 1 min and subsequently washed in sterile water. Next, leaf disks were cut using a 15-mm sterile cork borer and placed into 1 mL sterile water in each well of a 24-well plate. For preparing spore suspensions, the four isolates under study were grown on synthetic nutrient agar (SNA) medium (1 g L^{-1} KH_2PO_4, 1 g L^{-1} KNO_3, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.5 g L^{-1} KCl, 0.2 g L^{-1} glucose, 0.2 g L^{-1} saccharose, 1 M NaOH and 20 g L^{-1} agar) and incubated at 25 °C for 2 weeks under near-UV light and with a 12 h photoperiod to induce sporulation. Spores were harvested in a 0.01 % Tween80® solution with a sterile inoculation loop and were adjusted to 5.10^4 spores mL^{-1} using a Bürker counting chamber. Subsequently, leaf disks were inoculated with 10 µL of a spore suspension for each isolate and

incubated at room temperature under a 16 h photoperiod cycle for 3 days. All isolates were tested in four biological replications. Toxins were extracted by crushing leaf discs under liquid nitrogen with a mortar and pestle. The resulting plant powder was transferred to a test tube. Next, 15 mL of extraction solvent (methanol:dichloromethane:ethyl acetate (1:2:3), supplemented with 1 % formic acid) was added and the suspension was thoroughly shaken on an overhead shaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 30 min. Four milliliters of the supernatant was transferred to a new test tube in three-fold and evaporated by nitrogen in a thermostatic hot water bath at 40 °C (Grant Instruments Ltd, Cambridge, United Kingdom). Sample preparation and analysis were performed as described above.

Table 5.2. *Alternaria* toxins (non-host specific and host-specific) that were investigated.

	Mycotoxin	Chemical formula	Molecular weight (g mol^{-1})
Non-host specific toxins	Alternariol (AOH)	$C_{14}H_{10}O_5$	258.22620
	Alternariol monomethyl ether (AME)	$C_{15}H_{12}O_5$	272.25278
	Altenuene (ALT)	$C_{15}H_{16}O_6$	292.28394
	Tentoxin (TEN)	$C_{22}H_{30}N_4O_4$	414.49800
	Tenuazonic acid (TeA)	$C_{10}H_{15}NO_3$	197.23100
	Altertoxin-I (ATX-I)	$C_{20}H_{16}O_6$	352.33744
	Altertoxin-II (ATX-II)	$C_{20}H_{14}O_6$	350.32156
	Altertoxin-III (ATX-III)	$C_{20}H_{12}O_6$	348.30568
	Altenusin	$C_{15}H_{14}O_6$	290.26806
	Altenuisol	$C_{14}H_{10}O_6$	274.22560
	Alternaric acid	$C_{21}H_{30}O_8$	410.45810
Host-specific toxins	AAL-TA1/TA2	$C_{25}H_{47}NO_{10}$	521.64138
	AAL-TB1/TB2	$C_{25}H_{47}NO_9$	505.64198
	AK-toxin II	$C_{22}H_{25}NO_6$	399.43700
	AK-toxin I	$C_{23}H_{27}NO_6$	413.46358
	AF-toxin II	$C_{17}H_{24}O_6$	324.36886
	AF-toxin I	$C_{22}H_{32}O_9$	440.48408
	ACT-toxin II	$C_{26}H_{39}NO_9$	509.58916
	ACT-toxin I	$C_{26}H_{39}NO_{10}$	525.58856
	AM-toxin I	$C_{26}H_{37}N_3O_6$	445.50874
	ACR-toxin I	$C_{19}H_{30}O_6$	354.43790

Statistical analyses

The R v. 2.15.3 software package (R Core Team, 2014) was used for the pharmacological assay results (to test differences in necrotic area size between treatments) and the ET production results (to test differences in ET production between treatments). Statistical significance between groups of data was inferred at a significance level of $\alpha = 0.05$. Because normality assumptions of parametric tests were met for all assays, differences between groups of data were tested for significance using a one-way ANOVA test. If significant differences between the groups were found, a Tukey post hoc test at $\alpha = 0.05 / n$ with n the number of pairwise comparisons was performed.

RESULTS

Variability of spore germination time frames on inoculated potato leaves

In order to delineate the time frame of spore germination, inoculated leaf discs were microscopically analyzed at different time points during infection. In the meantime, the variability of spore germination rate of the different isolates was also assessed. Figure 5.1 displays the percentage of germinated spores of the four investigated isolates over time. In this graph, it can be observed that spore germination on potato leaves happens within hours after application of the spores on the leaves. Indeed, almost all spores have germinated within 5 to 6 hours post inoculation. Additionally, spore germination rate seems to be equal for all of the isolates (all p-values >> 0.05, one-way ANOVA test), however, there seems to be a longer lag time for the small-spored isolate 14.21, which is the least virulent of the two small-spored isolates.

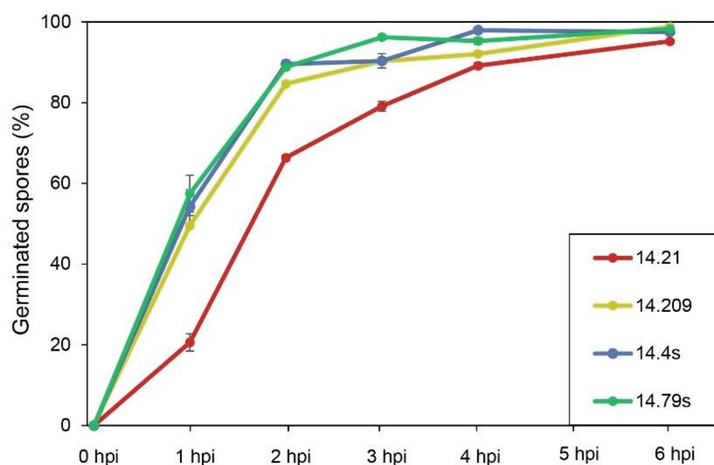

Figure 5.1. Spore germination rate of the investigated *Alternaria* isolates. Error bars represent standard deviations. For each time point, 50 leaf discs were analyzed per isolate. Small-spored isolates: 14.21 and 14.209. Large-spored isolates: 14.4s and 14.79s.

In figure 5.2, some microscope images can be seen of spores residing on cleared potato leaf discs. Here, it can also be observed that spore germination starts within the first hour after inoculation (Fig. 5.6a, d). Four hours after inoculation, spores from both small- and large-spored isolates already show germ tubes of considerable length (Fig. 5.2e, f). After 24 hours, hyphae start to proliferate and tangle (Fig. 5.2h). In figure 5.6g, some germ tubes that have penetrated and that are migrating underneath the cuticle are also shown.

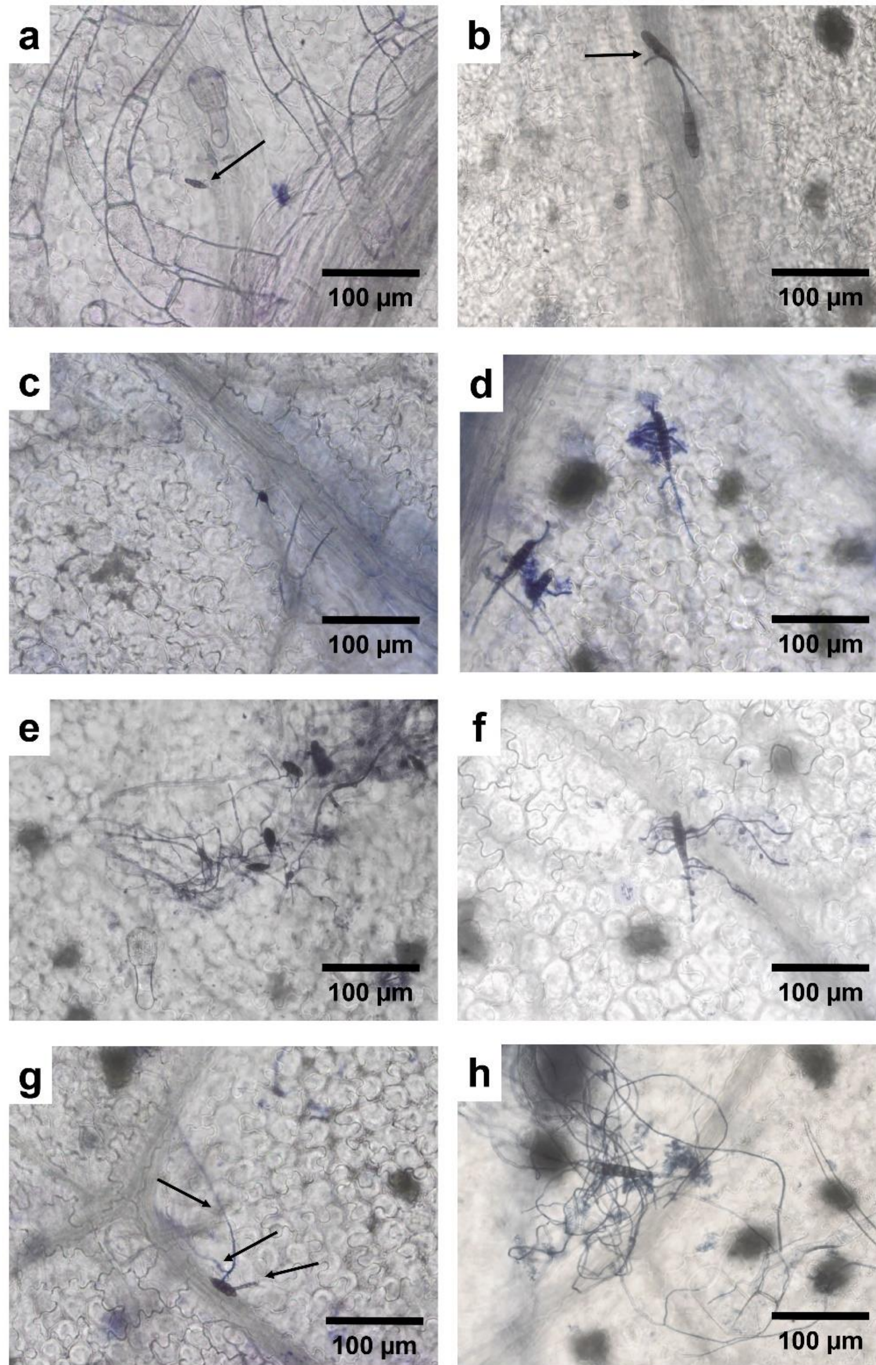

Figure 5.2. Microscope images of *Alternaria* spores residing on potato leaves. (a) Non-germinated *A. arborescens* (isolate 14.21) spore (indicated by the arrow). (b) Non-germinated *A. solani* (isolate 14.4s) spores (arrow indicates germ tube formation). (c) *A. arborescens* (isolate 14.209) spore at 1 hpi. (d) *A. solani* (isolate 14.4s) spores at 1 hpi. (e) *A. arborescens* (isolate 14.209) spores at 4 hpi. (f) *A. solani* (isolate 14.79s) spore at 4 hpi. (g) *A. arborescens* (isolate 14.21) spore at 12 hpi (arrows indicate penetration sites). (h) *A. solani* (isolate 14.79s) spore at 24 hpi. Germ tubes were stained with trypan blue. Black bars represent scale bars of 100 µm. Images were taken using an Olympus SZ61 stereo microscope coupled to a XC50 camera and were visualized using the Cell^F imaging software (Olympus Soft Imaging Solutions GmbH).

In vitro ethylene production

Based on the germination time frame that was established in the previous section, we assessed the production of ET as this plant hormone ET is increasingly reported as being produced by microorganisms, among which are several fungal species. In that regard, the potential of our isolates to produce ET *in vitro* was assessed. Therefore, the direct precursors of the three putative ET biosynthesis pathways in microorganisms (ACC, KMBA or 2-oxoglutarate) were administered in the growth medium and ET production was determined after 24 hours.

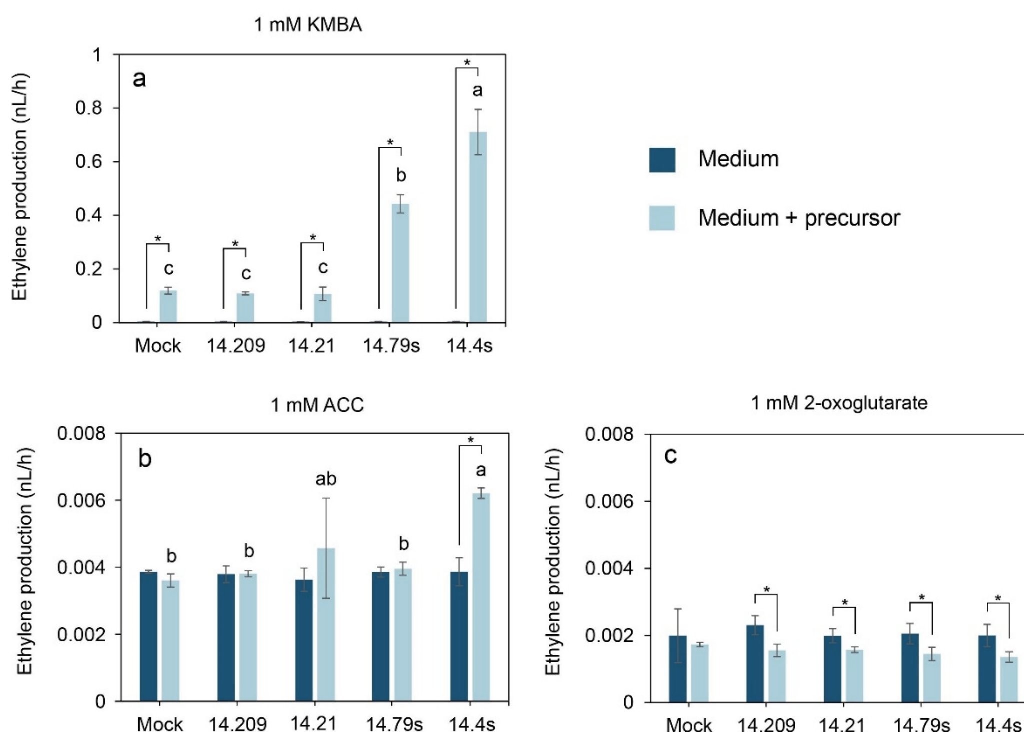

Figure 5.3. *In vitro* ET production (nL h^{-1}) for the investigated *Alternaria* isolates after application of (a) 1 mM KMBA, (b) 1 mM ACC or (c) 1 mM 2-oxoglutarate in the growth medium after 24 h of accumulation. Error bars represent standard deviations. For each treatment, three biological repeats were performed. Letters above bars represent significant differences between isolates for the treatment with precursor (differences between isolates without precursor were never significant). Asterisks above bars represent significant difference between samples with just the medium and samples with medium and the respective precursor. Mock: medium (± precursor) without fungal strain, KMBA: α-keto methylthiobutyric acid, ACC: 1-aminocyclopropane-1-carboxylic acid.

Figure 5.3 shows the results of the assay. It can be observed that, if 1 mM KMBA is administered, the large-spored isolates 14.79s and 14.4s display high ET production levels (Fig. 5.3a). In contrast, ET production for the small-spored isolates 14.209 and 14.21 was not higher compared to the mock-inoculated sample. These results indicate that the large-spored *Alternaria* species, and not

the small-spored species, might be able to use the KMBA pathway to produce ET during spore germination and vegetative growth. Conversely, for the samples that had 1 mM 2-oxoglutarate administered (Fig. 5.3c), ET production was lower than the samples without the substrate. In addition, no significant differences could be discerned between isolates. Concerning the samples that had 1 mM of ACC administered (Fig. 5.3b), only one isolate, 14.4s, displayed higher ET production than the mock-inoculated sample. For the small-spored isolate 14.21, there was also an increase of ET production, albeit the difference was not significant (p-value = 0.462, one-way ANOVA test). This result suggests that the ACC pathway might also be present within some *Alternaria* species, however, it does not seem to be the preferred pathway *in vitro*. Indeed, it should be noted that the average amount of ET production when ACC or 2-oxoglutarate was applied, was much lower than for the samples that had KMBA administered. When no precursor was provided, there were never significant differences in ET production between isolates.

To test whether the increased ET levels are due to spontaneous photo-oxidation from KMBA or enzymatic oxidation, a similar experiment was performed. This time, L-MET was provided as a substrate next to KMBA and samples were kept in the dark after 2 dpi.

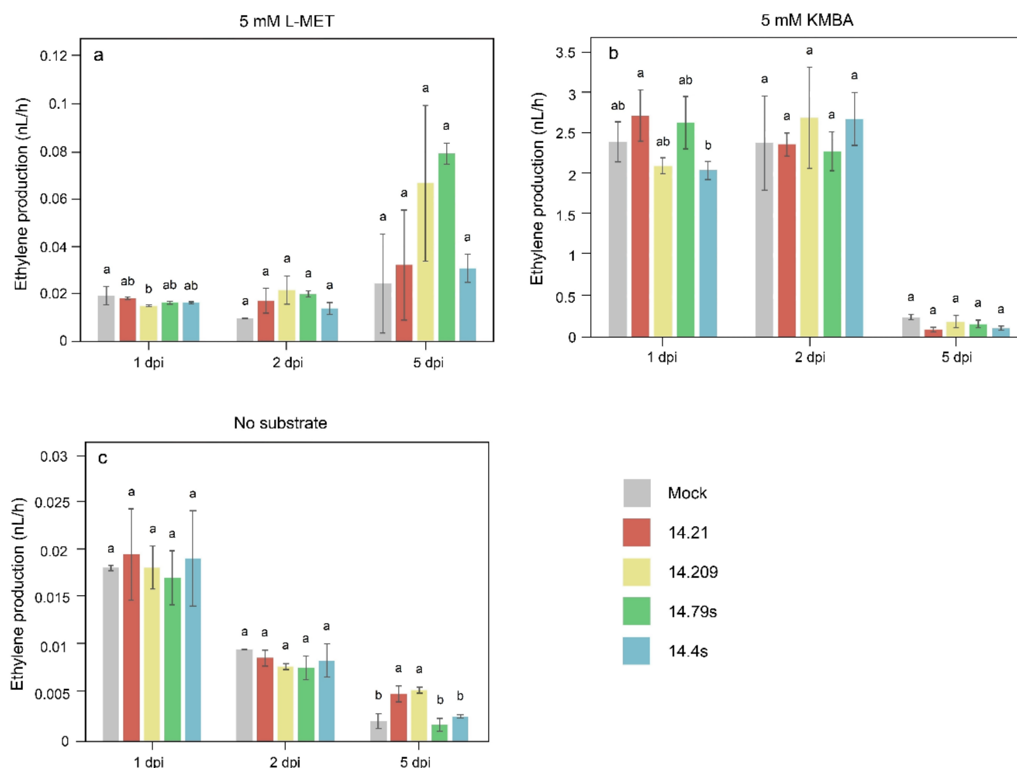

Figure 5.4. *In vitro* ET production (nL h^{-1}) for the investigated *Alternaria* isolates after application of (a) 5 mM L-MET, (b) 5 mM KMBA or (c) no substrate in the growth medium. Samples were put in the dark after 2 dpi. Error bars represent standard deviations. For each treatment, three biological repeats were performed. Letters above bars represent significant differences between isolates. Mock: medium (± precursor) without fungal strain, KMBA: α-keto methylthiobutyric acid, L-MET: L-methionine.

Figure 5.4b shows that, at 5 dpi, ET production is strongly reduced when samples are put in the dark, indicating that the administered KMBA was spontaneously converted to ET in the presence of light. When L-MET was applied as a substrate, ET levels were increasing after 5 days (Fig. 5.4a), illustrating that ET production by *Alternaria* species occurs through a methionine-dependent pathway *in vitro*, that some of the studied isolates have the capability of producing ET independently of the presence of light, and that ET production is associated with vegetative growth, rather than spore germination. Figure 5.4a also reveals increased ET levels for the small-spored isolate 14. 209 (the more virulent of the two small-spored isolates) at 5 dpi. However, the difference with the mock-treated sample and the sample inoculated with isolate 14.21 was not significant (p-values = 0.241 and 0.312 respectively, one-way ANOVA test). In contrast to the previous experiment where the least virulent large-spored isolate 14.4s displayed the highest ET levels when 1 mM of KMBA was administered (Fig. 5.3a), ET levels were not higher than the mock-treated sample when L-MET was applied as substrate. Instead, the more virulent large-spored isolate 14.79s now showed the highest production level.

Ethylene production during *Alternaria* infections on potato leaves

Since it was confirmed that *Alternaria* species can produce ET *in vitro*, the ET emission (by the plant and / or fungus) during an infection on potato leaves was investigated in order to reveal a potential role for the phytohormone during the onset of infection or during disease progression. According to the results of the previous experiment, ET emission levels were measured at 5 hpi to look for a potential role during spore germination. Additionally, ET was measured at 1 dpi and 3 dpi (before and during symptom progression).

Figure 5.5a shows that for all four isolates, ET levels were higher than those for the mock-treated samples at 3 dpi, hinting at a role for ET during symptom progression. Before 3 dpi, ET levels remained at normalized level (mock level) for all isolates. Since variation in ET production between repeats was high for most isolates, the data were also standardized with the fungal DNA content (Fig. 5.5b). It can be observed that at 3 dpi, the least virulent small-spored isolate 14.21 displays the highest ethylene levels, followed by the other small-spored isolate 14.209. The large-spored isolates showed the lowest amount of ET. Especially the ET levels on leaves inoculated with the large-spored isolate 14.4s were significantly lower than with the small-spored isolate 14.21. Before 3 dpi, no clear differences could be discerned between isolates.

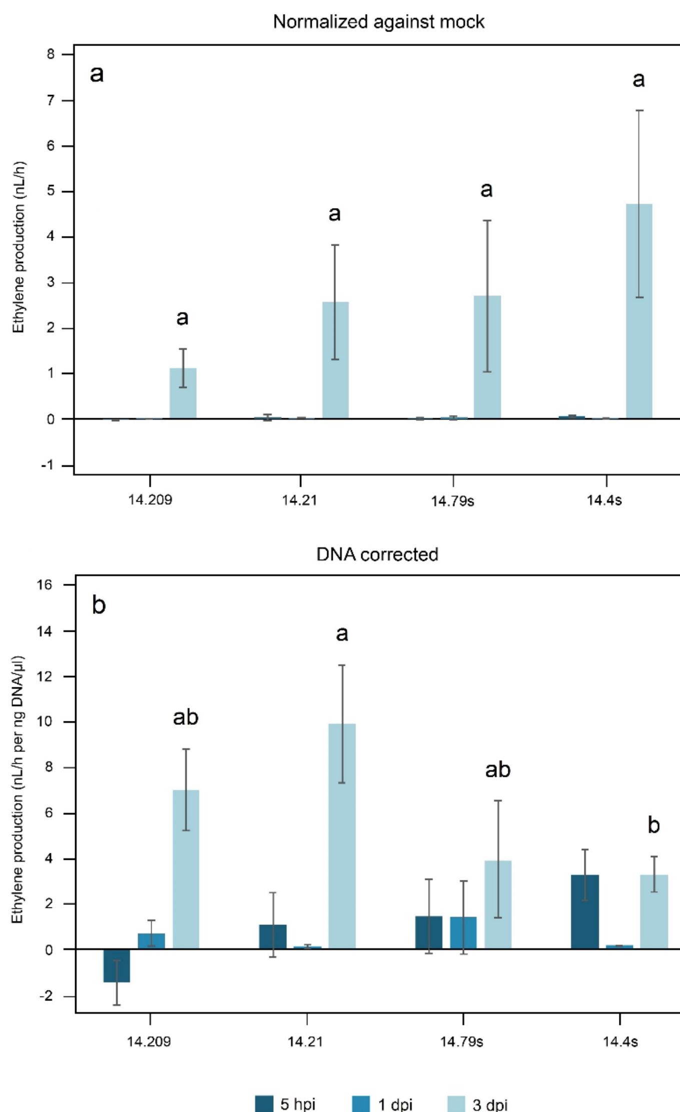

Figure 5.5. *In planta* ET emission for the investigated *Alternaria* isolates 5 hpi, 1 dpi and 3 dpi. (a) Data is shown which is normalized against mock-treated samples. (b) Data is shown which is normalized against the mock-treated samples and standardized with the fungal DNA content. Error bars represent standard deviations. For each treatment, three biological repeats were performed. Letters above bars represent significant differences between isolates at 3 dpi.

Ethylene biosynthesis inhibition assay

In order to confirm a role for ET during infections of *Alternaria* on potato, the ET biosynthesis inhibitor 2-aminoethoxyvinyl glycine (AVG) was used. This inhibitor blocks the action of ACC synthase, a key enzyme involved in plant ET biosynthesis (and the fungal ACC pathway to produce ET). It can be seen from figure 5.6a that AVG treatment strongly reduced symptoms of leaf discs inoculated with large-spored isolates compared to the non-treated control samples. When the spore suspension was supplemented with the ET precursors KMBA or ACC (AVG + KMBA or AVG + ACC), symptoms were increased, respectively indicating that fungal ET (via the KMBA pathway) and plant and / or fungal ET (via the ACC pathway) might partially re-establish necrosis after inhibitor treatment. However, the differences with the AVG treatment without precursor were never significant. Leaf discs that were not treated with the inhibitor, but were inoculated with a spore

suspension with added KMBA or ACC, exhibited more necrosis compared to the control, although the differences were also not significant.

In regard to the leaf discs inoculated with the small-spored isolates (Fig. 5.6b), AVG treatment surprisingly increased symptoms compared to the control. Adding KMBA to the spore suspension did not increase symptoms, although a slight increase of symptoms could be observed for leaf discs that were inoculated with a spore suspension with added ACC. However, the difference was not significant. Similarly as for the large-spored isolates, ACC treatment without inhibition by AVG resulted in more necrosis compared to the control. The KMBA treatment resulted in much variation in necrosis, so no clear-cut observations could be made.

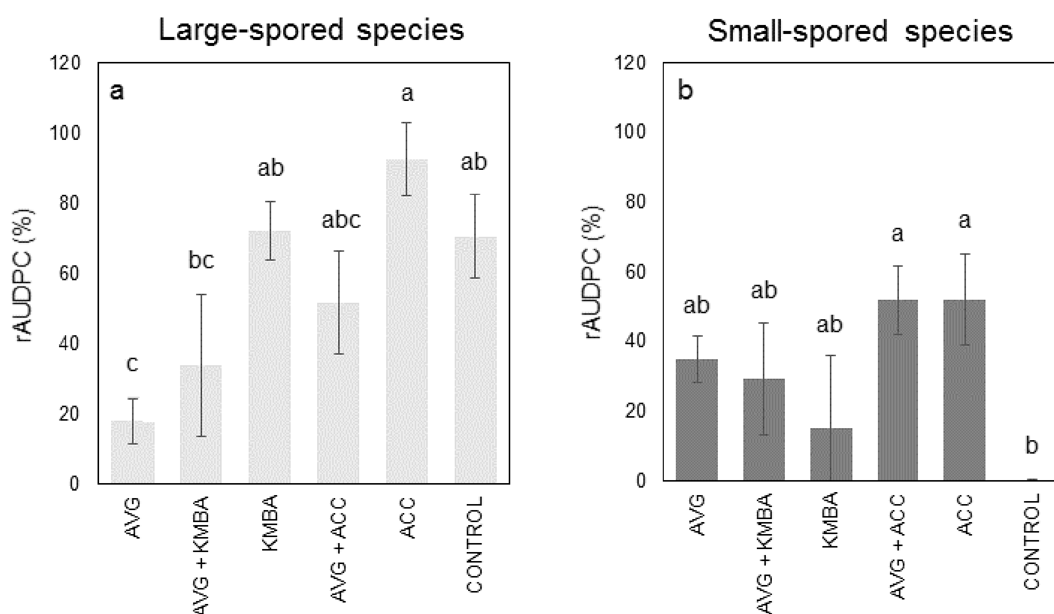

Figure 5.6. Relative *AUDPC* values of leaf discs inoculated with (a) large-spored *Alternaria* isolates (n = 2) or (b) small-spored *Alternaria* isolates (n = 2) after treatment with AVG (1 mM), KMBA (1 mM), ACC (1 mM) or a combination. Letters above bars represent significant differences between treatments. For each treatment, three biological repeats were performed. Error bars represent standard deviations. AVG: 2-aminoethoxyvinyl glycine, KMBA: α-keto-γ-methyl-thiobutyric acid, ACC: 1-aminocyclopropane-1- carboxylic acid.

In vitro toxin analysis

To determine whether a discrepancy could be observed in the toxin production profiles between small- and large-spored *Alternaria* isolates that could explain the variation in aggressiveness, their capability in *in vitro* toxin production was assessed. Results of the assay are displayed in table 5.3. It should be noted that no host-specific toxins (HST) could be detected for any of the four isolates that were investigated. For this reason, these toxins are not displayed in the table. In contrast, many

of the non-host specific toxins were found to be produced by the small-spored isolates 14.21 and 14.209. Some attention should be given to the fact that, when a specific toxin within a chemical group is produced, it does not automatically mean all toxins within this group are produced. Indeed, it can be seen in the table that ALT was not detected for any of the isolates while the other toxins within the group of benzopyrones (AOH, AME and altenuisol) could be picked up. The results of the assay demonstrate some variability in the amount that each toxin is produced, however, tenuazonic acid seemed to be highly produced by the two small-spored isolates, whereas it was not produced by any of the two large-spored species, 14.4s and 14.79s. Similarly, the biphenyl toxin altenusin and the benzopyrone altenuisol were detected for the small-spored isolates and not for the large-spored isolates. Only the toxins ATX-I and ATX-II were observed for both large-spored isolates. The benzopyrone toxins AOH and AME were caught for the large-spored isolate 14.4s, but not for the isolate 14.36s.

In planta toxin analysis

Since none of the HSTs could be detected in the *in vitro* analysis and no discernable toxin profile was observed that could explain the difference in aggressiveness between small- and large-spored species, the *in planta* toxin production profiles were assessed for the four isolates under study. Surprisingly, after repeating the assay once more, none of the toxins (non-host- or host-specific) that were investigated could be detected in infected potato leaf discs.

Table 5.3. *In vitro* toxin production profiles of the *Alternaria* isolates under study. Only non-host specific toxins are displayed because no host-specific toxins were detected in the analysis. Toxin production was analyzed using UPLC-MS/MS in MS^E continuous mode. Ionization occurred through ESI in negative mode. Numbers represent the amount of times the metabolite was detected in the analysis. If a metabolite was detected, a color code was allocated according to the scale below the table. AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenusin, TEN: tentoxin, TeA: tenuazonic acid, ATX: altertoxin.

	Benzopyrones				Cyclic tetrapeptides	Tetramic acid derivatives	Perylenequinones			Biphenyls
	AOH	**AME**	**ALT**	**Altenuisol**	**TEN**	**TeA**	**ATX-I**	**ATX-II**	**ATX-III**	**Altenusin**
14.21	826966	144839		3922		2595250	18595	91610		29442
14.209	123836	387947		75		2199978	1543	9665		1134351
14.4s	166752	133671					59649	3358		
14.79s							98671	39537		

Color code (# counts)	Not detected	< 1,00E+04	> 1,00E+04	> 5,00E+04	> 1,00E+05	> 5,00E+05	> 1,00E+06	> 5,00E+06	> 1,00E+07	> 2,50E+07	> 5,00E+07	> 1,00E+08	> 2,50E+08

DISCUSSION

This study was performed in order to reveal some of the molecular mechanisms behind the different virulence profiles of *Alternaria* isolates described in the previous chapter. First, we focused on the plant hormone ET as a virulence factor, either self-produced by the fungus or synthesized by the plant and exploited by the pathogen. The phytohormone ET has received considerable attention as a virulence factor recently and has been repeatedly reported to be produced by multiple microorganisms (Weingart et al., 2001; Valls et al., 2006; Chagué, 2010; De Vleesschauwer et al., 2010; De Bruyne, 2015; Van Bockhaven et al., 2015). Also, it has been previously observed that ET is involved in *Alternaria* spore germination (Kepczynska, 1994).

With that in mind, we first delineated the time frame in which spore germination occurred on potato leaves. For this assay, two small-spored (*A. arborescens* group A) and two large-spored isolates (*A. solani*) were studied. For both species, the most and least virulent isolates (described in chapter 4: Fig. 4.4, Table 4.1) were chosen. It could be established that the time frame in which all of the spores germinated, was within 5 hours post inoculation. Additionally, no apparent differences in germination rate between the studied isolates were observed.

Next, we investigated if our *Alternaria* isolates were able to produce ET *in vitro* if they were supplied with the direct precursor in three possible pathways (ACC, KMBA or 2-oxoglutarate) (Chagué, 2010). The results indicated that only the large-spored species were able to produce ET through the KMBA pathway and that to a much lesser extent, one large-spored isolate was able to produce ET through the ACC pathway. Our small-spored species were seemingly not able to produce ET under the applied circumstances. Although ET production levels were low, especially for the ACC and 2-oxoglutarate treatments, our data were out of the noise range since the range of detection of the ET sensor lies between 0 - 5 ppmv (parts per million by volume) and our lowest measured values were around 2.4 ppmv. Additionally, a low amount of ET was detected in the mock samples, which is most likely due to a basal level of ET present in the air. When L-MET was applied as a precursor (next to KMBA) and samples were incubated in the dark after 2 dpi, it could be observed that all of the isolates could produce ET starting from L-MET and that the conversion of KMBA to ET happened through photo-oxidation. The fact that one of the small-spored isolates could produce ET when 5 mM of L-MET was provided and that it could not when 1 mM KMBA was administered, suggests that this isolate may possess the ability to produce ET through a methionine-dependent pathway, and that its ability thereof may be concentration-dependent. ET measurements with increasing concentrations of L-MET could clarify this issue. The assay also revealed that, in the presence of 5 mM L-MET, ET production was increased only at 5 dpi, which indicated that ET production occurs during *in vitro* vegetative growth instead of spore germination. Indeed, in a side

experiment, a small sample of spores that were incubated under the same conditions as in the assay, revealed that at 2 dpi, all spores were germinated and vegetative growth had started. The observation that ET production was only observed at 5 dpi is in direct contrast with the publication from Kepczynska (1994). This report stated that spore germination was compromised after treatment with an ET binding inhibitor and that the inhibition was reduced when ACC was provided, indicating that endogenous ET is required for spore germination *in vitro*. Our data show that ET is not produced within the time frame of spore germination and by consequence, is not required for spore germination but rather during vegetative growth of the fungus. The reason why these contrasting results are observed remains elusive and requires additional experiments to make clear-cut conclusions. It should be noted that all of the *in vitro* ET production assays should be repeated to obtain statistically significant data. Our conclusions are based on only one iteration of both experiments. Therefore, these data should be interpreted with caution.

In view of the results from the *in vitro* assay, we wondered if a species-specific ET emission pattern could be observed when the studied isolates were inoculated on potato leaves. Based on the spore germination data and visual assessment of symptoms (described in chapter 4), we opted to measure ET emission at 5 hpi (during spore germination), at 1 dpi (at the onset of infection) and at 3 dpi (during disease progression). The assay revealed that, compared to the mock-treated samples, ET emission at 3 dpi was higher for leaves inoculated with an *Alternaria* isolate, again hinting at a role for ET during disease progression or *in planta* propagation and not during spore germination. The fact that variation in ET levels was high among replications, made us wonder whether ET emission is dependent on fungal growth *in planta*. For this reason, we corrected the data according to fungal DNA content. Contrary to our expectations, it could be observed that leaf discs inoculated with the small-spored isolates exhibited higher ET levels than leaf discs inoculated with large-spored isolates, indicating higher ET emission levels are not specifically correlated with more virulent species. In contrast, the publication by Zhu et al. (2017) showed that ET emission levels from grape berries inoculated with different strains of *A. alternata* are correlated with lesion diameters. It should be noted though, that Zhu et al. (2017) measured ET levels at 7 dpi, whereas in our experiment, ET levels were measured at 3 dpi. Therefore, the development of symptoms (after 3 dpi) might also contribute to the total ET emission levels. Additional ET measurements after 3 dpi might confirm this hypothesis.

The role of ET during an infection on potato was further explored by an assay in which plant ET biosynthesis is blocked using the inhibitor 2-aminoethoxyvinyl glycine (AVG). This compound blocks the action of ACC synthase (ACS), a key enzyme in the production of ET by the plant. Since

the ACC pathway might also be present in our isolates, AVG could also block fungal ET biosynthesis produced via this pathway. Indeed, it should be noted that the selectivity of AVG towards plant / fungal isoforms of ACS is not clearly defined. For this reason, no distinction between the effect of AVG on plant ET biosynthesis and on fungal ET biosynthesis could be made by treating leaf discs with this inhibitor. After treatment of the leaf discs, symptom development was strongly reduced when leaf discs were inoculated with the large-spored isolates. In contrast, symptoms were enhanced in the case of small-spored isolates. This result, together with the facts that inoculation of potato leaf discs with small-spored isolates induces a strong ET emission response and did not result in disease progression, hints at an antagonistic role for ET during infection of potato leaves by small- versus large-spored *Alternaria* species. More specifically, the ET defense response by the plant seemingly compromizes propagation of small-spored species, but increases susceptibility towards large-spored species. Therefore, it seems that only the large-spored species are able to use plant (and / or fungal) ET as a virulence factor during symptom development. Additionally, after supplying 1 mM of KMBA or ACC in the inoculation suspension, symptoms were slightly, but not significantly enhanced on AVG-treated leaf discs inoculated with large-spored isolates. These results indicate that the plant ET is predominantly employed by this pathogen to develop symptoms, and that this may be partially complemented with self-produced ET, which is synthesized either by the KMBA or the ACC pathway (due to our *in vitro* ET measurements, the former is more likely). To distinguish fungal ET from plant ET, a mutant strain of *Alternaria*, deficient in ET production through the KMBA pathway might be of use. However, the key transaminase of the KMBA pathway is currently unknown. It could also be that the KMBA present in the spore suspension is automatically converted to ET in the presence of light or that the added ACC is converted to ET by the plant and that this extra amount of ET resulted in more symptoms. Indeed, leaf discs that were not treated with AVG, but were inoculated with a spore suspension of a large-spored isolate supplemented with KMBA or ACC, also showed increased symptoms compared to the control. In contrast, ACC treatment of leaf discs inoculated with small-spored isolates resulted in higher necrosis levels, which is unexpected since ET seems to increase resistance against small-spored *Alternaria* species. In that regard, the phytotoxicity of the compound ACC, as well as that of KMBA and AVG was tested on leaf discs that were not inoculated with *Alternaria* strains. It could be observed that none of the compounds were phytotoxic on potato leaf discs, since no necrosis was observed for the duration of two weeks. Therefore, the reason for the increased necrosis observed for the ACC treatment of leaf discs inoculated with small-spored isolates remains elusive. It should be noted that this experiment has only been performed once, which warrants caution in interpreting the results. Repetitions of the assay are mandatory in order to arrive at significant conclusions. Additionally, although AVG is exceedingly used as an inhibitor of ET biosynthesis, it

is known that AVG blocks the action of other PLP-dependent enzymes (Fig. 1.10, chapter 1), two of which are involved in auxin biosynthesis. Since auxins are also linked to resistance against necrotrophs, the involvement of these phytohormones in the interaction between potato and *Alternaria* should not be neglected. In that regard, the usage of a more specific compound, such as the ET perception inhibitor 1-methylcyclopropene, could be a useful alternative.

In a previous report, Mase et al. (2012) highlighted that ET was involved in AAL-toxin-triggered cell death in the interaction between *Alternaria* and tomato. With the eye on revealing a similar mechanism in the interaction between *Alternaria* and potato, the toxin production profiles of the isolates under study was assessed. The assay revealed that on MEA medium, the small-spored isolates could produce many of the non-host specific toxins, such as AOH, AME, TeA, altenuisol and altenusin. None of the host-specific toxins (HST) were found on this artificial growth medium. This is to be expected, since it is believed that HSTs are exclusively produced on one specific host plant (Tsuge et al., 2013). It should be noted that most of the non-host specific toxins are not produced by the large-spored isolates, except for ATX-I and ATX-II and that only the least virulent large-spored isolate 14.4s was able to produce the toxins AOH and AME. Due to the fact that our small-spored isolates could not confer any disease symptoms on potato leaves and that the most virulent isolate produced the least amount of the investigated toxins, it seems that virulence on potato leaves is not achieved by the ability to produce an arsenal of non-host specific toxins. The roles of non-host specific toxins are largely unexplored, however, it is believed that these metabolites have a contributive function in disease development and might help the pathogen propagate *in planta* (Tsuge et al., 2013). In view of these reports, it may be that virulence is accomplished by the ability to produce some HST(s), which may or may not be exclusively produced on potato. Therefore, the toxin production of the same set of isolates after inoculation on potato leaves was assessed. Surprisingly, none of the investigated toxins could be detected in infected potato leaves, even after an independent repeat of the experiment. The reason for this observation might be that the non-host specific toxins are not produced before 3 dpi and by consequence, are not deployed as virulence factors that initiate necrosis, but rather act as additional metabolites that help to colonize the plant like previously stated. Additional toxin measurements after symptom development should help to resolve this issue. The fact that none of the HSTs were detected may be simply explained by the fact that the known HSTs are all being produced in different hosts and that a toxin, exclusively produced in potato, is yet to be discovered. Considering the results of the *in planta* toxin measurements, the involvement of ET in the infection could not be linked to the production of known host- or non-host specific toxins in both small- or large-spored species.

In conclusion, through a combination of observations in which (1) an increase in disease incidence in the field co-occured with the detection of both small- and large-spored species, in which (2) large-spored species were found to be more virulent than small-spored species, in which (3) it was found that potato leaves inoculated with the small-spored isolates exhibited enhanced ET emission levels and that large-spored isolates were able to produce ET *in vitro* predominantly through the KMBA pathway, and in which (4) ET biosynthesis inhibition resulted in strongly reduced symptoms in case of the large-spored isolates but not in case of the small-spored isolates, we hypothesize that small-spored species are able to boost the hosts' ET biosynthesis (either by indirectly stimulating biosynthesis by the plant and / or by self-production of ET), which is consequently employed by the large-spored species as a virulence factor to colonize the host and to develop symptoms faster. This colonization mechanism of large-spored species could then be further aided by their own production of ET. It should be made clear that our results only provide an indication towards this hypothesis. Repetitions of the ET measurements (*in vitro* and *in planta*), combined with sequential inoculation experiments should help to confirm or deny the proposed interaction between small-spored and large-spored *Alternaria* species on potato.

ACKNOWLEDGEMENTS

We would like to thank ILVO (Merelbeke, Belgium) for providing potato seedlings. Our thanks also go to Olivier Leroux (UGent, Faculty of Sciences, Department of Biology) for kindly providing the protocol for the trypan blue staining method, and to Jeroen Walravens for the UPLC-MS/MS method.

ADDENDUM 1 – OPTIMIZATION OF THE IN PLANTA INFECTION TECHNIQUE FOR MEASURING ETHYLENE EMISSION

Introduction

Before arriving at the infection assay that was used in the study described above, a few methods were tried out in order to minimize variation in ET production between repeats and consequently develop a more reproducible assay.

Methods and results

Infection assay 1

Leaf discs from potato plants from the cultivar Bintje were grown and prepared similarly as described in the Materials and methods sections of this chapter. Also, spore suspensions of each isolate that was investigated, were prepared as described in that section. Leaf discs were inoculated by a spore drop of one of the isolates which was spread across the leaf surface using a sterile inoculation loop. One inoculated leaf discs was placed at the bottom of a 20 mL glass vial filled with 4 mL of sterile water. Additionally, one vial without a leaf disc was included to account for background ET detection. The vials were capped air tight to ensure volatile build-up within the vials' headspace. For each isolate, three biological repeats were prepared. Total accumulated ET in the vials' headspace was measured using laser-based photoacoustic spectroscopy (Sensor Sense, ETD-300) in stop and flow modus and re-calculated as nL h^{-1}.

Results from this assay were not reproducible and showed much variation among repeats (Fig. A5.1). There was also high background signal detected in the vial with just water. Between inoculated leaf discs and the mock treatment, not much differences could be observed.

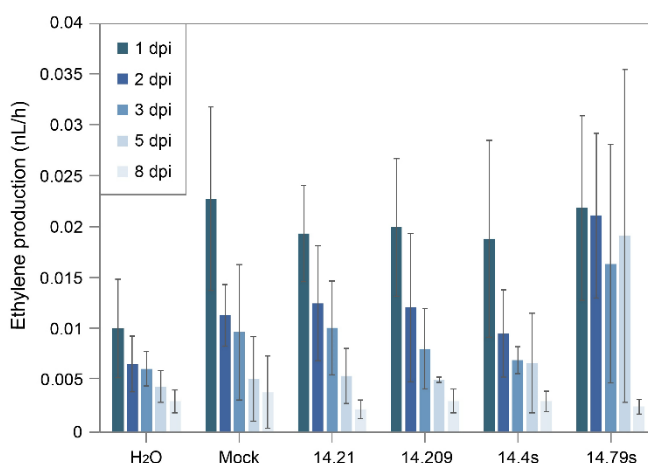

Figure A5.1. Ethylene emission levels of leaf discs inoculated with one of the isolates under study. For each treatment, three biological repeats were performed. Error bars represent standard deviations.

Infection assay 2

In an attempt to minimize variation or equalize infections among repeats, the same setup was applied as above, but instead of sterile water, leaf discs were placed on wet filter paper at the bottom of the 20 mL vial. Vials were capped air tight and ethylene production was measured as described in the previous assay.

Although the background ET levels that were detected in the previous assay could now be eliminated, the resulting ET levels were again too variable for a robust assay.

Infection assay 3

For this assay, only one of the small-spored and one of the large-spored isolates were tested in order to reduce the experiment size and to save time. The same setup as before was applied. However, in each vial, the number of inoculated leaf discs was increased to five discs per vial, again in an attempt to reduce variation among repeats. Additionally, the experiment was set up three times in parallel so that, for each of three measurements, DNA content could be determined as a way of correcting the data. Discs were again placed on wet filter paper either on the bottom or on the side of each vial. Three technical repeats were performed for each isolate. Vials were capped air tight and DNA content was determined by pooling the leaf discs from one vial and proceeding as described in the Materials and methods section of chapter 4.

Figure A5.2 shows that DNA content measurements of the three repeats were reproducible for each the three time points. This was the case for both isolates that were investigated, except at 3 dpi, some variation among repeats could be observed for the large-spored isolate.

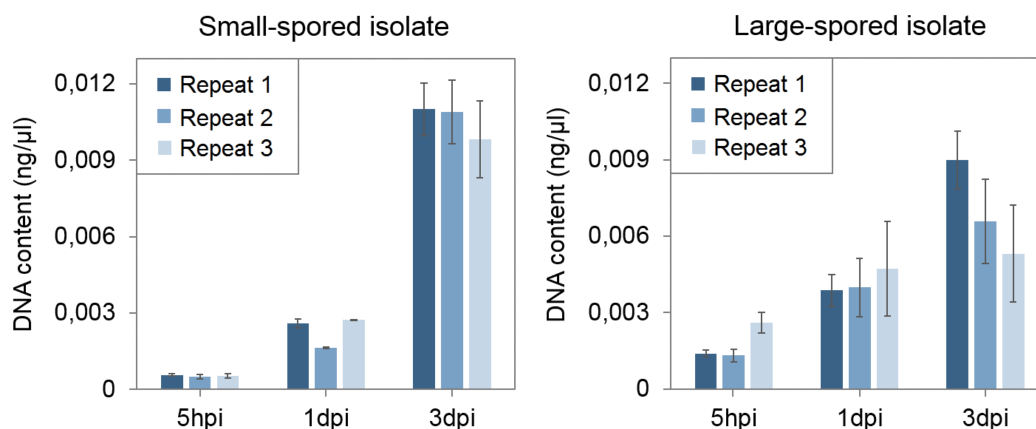

Figure A5.2. DNA contents for a small-and large spored *Alternaria* isolate (n = 1) on potato leaf discs. DNA quantities were determined by real-time PCR using primers based on *Cyt bc1* sequences (chapter 4, Materials and methods). For each repeat, three technical repeats were performed. Error bars represent standard deviations.

Conclusion

Since water could potentially dissolve some of the produced ET and because of the risk of the leaf discs becoming submerged, we opted to continue working with wet filter paper to place the leaf discs in the vials. Increasing the number of inoculated leaf discs could reduce some of the variation in ET production between repeats. However, we observed that the variation in ET levels might be due to differences in necrotic area size. A small difference in lesion size, could result in large differences in ET production. For this reason, we chose to correct the data according to fungal DNA content present on the leaf discs. As can be seen from figure A5.2, the DNA measurements were reproducible and could reliably be used to correct the data. Since we were interested in the molecular events before symptom development (before 3 to 4 dpi) and because we could confirm that spore germination was completed before 5 hpi, we chose to measure at three time points: 5 hpi, 1 dpi and 3 dpi. Due to the fact that DNA measurements are destructive methods, the experiment had to be setup in threefold (one for each measurement). The final assay that was used in the study is described in the Materials and methods sections of this chapter.

ADDENDUM 2 – OPTIMIZATION OF A STAINING METHOD FOR MICROSCOPY ANALYSIS OF INFECTED LEAF DISCS

Introduction

To determine the time frames in which our studied *Alternaria* isolates germinated and penetrated leaf cells, we attempted to optimize a paraffin embedding protocol in order to cut transverse sections of infected leaf tissue before turning to the trypan blue staining method that was used in the study.

Methods and results

Paraffin embedding protocol 1

Leaves of the cultivar Bintje were harvested, surface-sterilized, cut in to 15-mm leaf discs and inoculated as described in the Materials and methods section of this study. After 1, 2, 3, 4, 5, 7, 10 and 12 hours post inoculation, the infection was arrested by freezing the leaf discs at -20 °C. Next, an attempt to embed the leaf discs with paraffin was performed in order to cut slices for microscopy analysis. Therefore, leaf discs were fixated by placing them overnight in a 10 % neutral buffered formalin solution at 4 °C and under continuous shaking (110 rpm). Next, leaf discs were dehydrated by placing them sequentially in an increasing concentration of absolute ethanol for 15 minutes each (20, 50, 70, 80, 90 and 100 %). Subsequently, 100 % ethanol was replaced by 100 % xylene under the fume hood. Finally, leaf discs were transferred to liquid paraffin at 60 °C for 1 hour. Microscopy slices were made by pouring liquid paraffin in a paraffin steel mold (22 x 22 x 12 mm) (Thermo Fisher Scientific, Hampton, NH, USA) while holding the leaf discs in a perpendicular way to the base of the mold and subsequently cutting 7 µm microscopy slices using a microtome (Micron HM360, Microm-Walldorf, Germany). Finally, the slices were put on a glass slide and placed on a heating plate at 60 °C to liquefy and remove the paraffin.

At this point, cell structure was assessed under the microscope before attempting any staining procedure. However, cell structure was barely visible and mostly disintegrated in successfully made slices. Indeed, most attempts to cut slices were unsuccessful due to the fact that the microtome could not cut leaf tissue resulting in slices without leaf tissue, which indicated an incomplete embedding of the cells with paraffin.

Paraffin embedding protocol 2

In an attempt to optimize the previously described method, a few changes were made to the protocol. The preparation and inoculation of leaf discs was similar as before. Also, leaf discs were

frozen at the same time points during infection and fixated as described above. Dehydration was now performed by placing the leaf discs in an increasing ethanol concentration for longer periods of time (1 hour instead of 30 min). Replacing ethanol by xylene now also happened in an step-by-step manner, where ethanol:xylene mixtures were made of 25, 50 and 75 % xylene where the leaf discs were sequentially placed in before being transferred to 100 % xylene. For the paraffin embedding, xylene was now gradually replaced by paraffin by pouring small amounts of liquid paraffin (60 °C) to the xylene that was holding the leaf discs. By using this method, the paraffin solidified at first, but gradually dissolved in xylene when the suspension was placed at 38 °C. When no solid paraffin was visible, new paraffin was added to the solution until a small layer of solid paraffin remained, indicating that the solvent was saturated at that temperature. The same approach was applied at 50 and at 60 °C, so that eventually, the xylene fraction in the solvent was minimal. Finally, the leaf discs were placed in a 100 % paraffin solution at 60 °C. The preparation of the microscopy slices was similar as before.
Before any staining procedure was attempted, the cell structure was assessed under the microscope. This time, the microtome was able to cut slices holding leaf tissue more easily, however, removing the paraffin from the glass slice was troublesome and resulted in damaging the leaf tissue more times than not. Also, due to the longer incubation periods at high temperatures (60 °C), leaf discs started to curl, which made it more difficult to make paraffin blocks holding the leaf discs.

Trypan blue staining method

Due to the above mentioned complications and shortage of time, we focused on spore germination instead of hyphal cell penetration and approached a staining method that did not involve tissue sectioning. Therefore, a trypan blue staining method (see Materials and methods section of this study) was used that was developed by Olivier Leroux (UGent, Faculty of Sciences, Department of Biology) and that could stain germ tubes and dead cells and did not stain individual spores.

Conclusion

Using the trypan blue staining method, the number of germinating spores on the plant leaf surface could easily be determined, while an indication of beginning cell necrosis could also be observed.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Authors:

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GENERAL DISCUSSION

Introduction

As mentioned in the problem statement and dissertation outline, the main objectives of this project were to gain insight in (1) the *Alternaria* population on Flemish potato fields with regard to the composition at different time points during the season, its phylogenetic and toxin production profiles, and (2) the influence of environmental factors on disease incidence and development, while also focusing on the role of the plant hormone ethylene during an infection on potato. Resulting from these objectives, five research questions were posed upon which the data presented throughout this manuscript provide an answer:

1. How much EB/BS disease incidence is there on Flemish potato fields?
2. How complex is the *Alternaria* population on Flemish potato fields?
3. What is the role of *A. alternata* and *A. solani* in the infection process? Is *A. alternata* merely a saprophytic species or does it have a virulent nature?
4. What are the toxin profiles of *Alternaria* isolates encountered in Flanders? Are these related to the aggressiveness of the isolates?
5. Are abiotic stress factors triggers for *Alternaria* disease development? What is the role of the plant hormone ethylene during an *Alternaria* infection on potato?

In this chapter, these five research questions will be answered and discussed in separate paragraphs, supplemented with some thoughts on future research challenges and perspectives.

How much EB/BS disease incidence is there in Flanders?

The field survey demonstrated low disease incidence in two growing seasons

In chapter 2, a field survey was presented in which the incidence of *Alternaria*-caused diseases (which are summarized as EB/BS disease in this manuscript) on Flemish potato fields was assessed. Therefore, potato fields at 22 locations across Flanders were monitored every week for EB/BS symptoms. This survey was performed during the growing seasons of 2014 and 2015. It was established that during these growing seasons, disease incidence was low, especially in the first half of the season (Fig. 2.3, Fig. S2.1). It was only from mid-August onwards, that disease symptoms started to escalate. However, disease incidence remained at manageable levels (maximum 10 % foliage necrosis) until harvest. These data indicate that the applied control measures are sufficient to keep EB/BS disease at controllable levels. The potato growers were asked to apply their conventional spray method, which implied a weekly treatment against late

blight (*Phytophthora infestans*) using mainly a combination of cymoxanil and mancozeb, in rotation with specific compounds against *Alternaria* (such as strobilurins). Although the compound cymoxanil is primarily targeting *P. infestans*, it seems that the collateral effects of mancozeb, in combination with specific compounds against *Alternaria* species are sufficient to control the disease. A few years earlier, in 2003, 2004 and 2005, Hausladen (2006) reported necrosis levels of up to 100% in Germany. Since no specific active ingredients were mentioned, the influence of the applied fungicides on the disease incidence is difficult to discuss. Although no information is available on the effect of cymoxanil against *Alternaria*, our research group has shown that the use of fungicides has an impact on the *Alternaria* population on the field. Indeed, later in the growing season, more large-spored isolates were found that were resistant to strobilurin fungicides (azoxystrobin, pyraclostrobin) due to mutations in the *cytochrome b* gene. This was to be expected since these strains have been exposed to numerous applications of strobilurins, resulting in the survival of only the resistant strains (Landschoot et al., 2017c). It could therefore be argued that the *Alternaria* field population in the survey by Hausladen (2006) consisted of a majority of *Alternaria* strains that were resistant to these fungicides. The same research group has published a report in which they assessed the resistance to strobilurin fungicides of a large set of isolates collected between 2005 and 2014 (which was after their field survey) (Hausladen et al., 2015). There, it could indeed be read that almost 30 % of the collected large-spored isolates was resistant to strobilurins, while in our case (Landschoot et al., 2017c), only 6 % was found to possess the mutation for resistance, which may be the reason for the much lower disease incidences observed in our field survey.

How complex is the *Alternaria* population on Flemish potato fields?

The local Alternaria population is more elaborate than a two-species complex

To obtain insight into the local *Alternaria* population, symptomatic leaflets were collected from the monitored fields during the growing seasons of 2012 until 2015, fungal species were extracted and molecularly identified. Genetic variation and phylogenetic relations were subsequently inferred on the sequence data of the obtained isolates.

Firstly, after the phylogenetic analysis using the sequences of 7 different genetic loci, it could be established that the Flemish *Alternaria* population comprises of three phylogenetically distinct clusters of small-spored isolates and three distinct clusters of large-spored isolates. Based on the reference sequences obtained from Genbank, these isolates could be identified as *A. alternata*, *A. arborescens*, *A. solani*, *A. grandis* and *A. protenta* species. The isolates that were identified as *A. arborescens* could be further subdivided in two groups, group A and group B. These observations sparked our interest, since it was previously believed that EB/BS disease was caused by two

species, namely *A. alternata* and *A. solani*. Only recently, other publications have reported additional species on potato plants. Rodrigues et al. (2010) observed *A. grandis* and *A. tomatophila* on potatoes in Brazil, while Tymon et al. (2016) reported *A. solani*, *A. arbusti* and *A. arborescens* on potatoes in Columbia, USA. Additionally, other *Alternaria* species, such as *A. tenuissima*, *A. linariae* and *A. longipes* have been detected on potato foliage in China, Algeria and Pakistan respectively (Zheng and Wu, 2013; Shoaib et al., 2014; Ayad et al., 2018). The observation of multiple species residing on potato plants could be explained by regular events of genetic recombination. Indeed, the finding of random variations within simple sequence repeat (SSR) markers, strongly indicates that *Alternaria* species underwent regular genetic recombination events (Meng et al. 2015) and that the fungus predominantly reproduces asexually, which might give rise to regular genetic insertions or deletions. Although the genetic tools for sexual propagation have been identified in *A. alternata* (Arie et al. 2000), to date, a sexual stage has never been observed in *Alternaria*. The reason for this is still elusive, however, it is believed that asexual propagation has more short-term benefits for populations that need to grow and reproduce rapidly in a stable environment, which is the case for *Alternaria* species or other pathogens. Conversely, sexual reproduction seems to be more beneficial for species that grow in a rapidly changing environment such as plants or animals (Dawson, 1995).

The multilocus sequence analysis demonstrates considerable inter- and intraspecific genetic variation

Secondly, the results of the assay showed considerable genetic variation among isolates. In table 3.2, it could be seen that, if all isolates were taken into account, 15 % of variable characters were found for the *ITS* region and around 27 % in the *Gpd* and *histone h3* sequences. Within the small-spored isolates, the latter sequences showed 20 % and 21.6 % variable characters respectively, while the *Alt a 1* sequences showed almost 12 % variable characters. These data show that there exists considerable intraspecific genetic variation, which might also have arisen from the regular events of genetic recombination. Many studies have been performed to assess genetic variation between strains of *Alternaria*. Although the genetic variation between strains of *A. solani* and *A. alternata* has been reported several times (Petrunak and Christ, 1992; Weir et al., 1998; Pryor and Gilbertson, 2000; Peever et al., 2002), information on genetic variation within small-spored species such as *A. alternata* is scarce. However, for large-spored species (*A. solani*), van der Waals et al. (2004) demonstrated 27 % of genetic diversity between isolates, while Weir et al. (1998) and Weber and Halterman (2012) reported considerable genetic variation within strains of this species as well. These results, together with the data from our experiments indicate that *Alternaria* species, both small- and large-spored, are sensitive to genetic changes which could result in rapid resistance

development to specific fungicides, which is also reflected in our publication on fungicide sensitivity discussed above (Landschoot et al., 2017c).

What is the role of *A. alternata* and *A. solani* in the infection process?

Large-spored isolates are more virulent than small-spored isolates

In chapter 3, it was shown that more species are present in the *Alternaria* population on Flemish potatoes than originally anticipated and that there exists considerable genetic variability among small-spored species (Table 3.2). Despite this observation, we did not observe much differences in virulence between *A. alternata*, *A. arborescens* group A and *A. arborescens* group B (Fig. 4.4). On the other hand, a clear discrepancy could be observed between the aggressiveness of small- and large-spored species, where the large-spored species *A. solani* proved to be the more virulent species in an *in vitro* virulence assay described in chapter 4 (Fig. 4.5). In that assay, it could also be observed that the small-spored species could cause small lesions, although no disease development could be asserted.

For some time now, the virulence of small-spored and large-spored species, or in a narrower sense of *A. alternata* and *A. solani*, has been a matter of much discussion. As previously cited, most authors agree on the higher virulence of *A. solani* (Droby et al., 1984; Hausladen and Leiminger, 2007; Turkensteen et al., 2010; Spoelder et al., 2013; Stammler et al., 2013), but the ability of *A. alternata* to cause lesions is ambiguous. On the other hand, Zheng et al. (2015) did not observe a significant difference in virulence between *A. alternata* and *A. solani*, while Kapsa and Osowski (2012) have reported that *A. alternata* is the most virulent species on potato leaves. The results from our virulence assay and field trials also demonstrate that *A. solani* is more virulent than *A. alternata* (and other small-spores species, such as *A. arborescens* group A and B). Our observation that *A. solani* is the more virulent species is in agreement with publications from Hausladen and Leiminger (2007), Turkensteen et al. (2010), Stammler et al. (2013) and Spoelder et al. (2013). However, Turkensteen et al. (2010) and Spoelder et al. (2013) mentioned that *A. alternata* was unable to infect potato leaves and cause necrosis, while from our assays, we concluded that *A. alternata* (and the other small-spored species *A. arborescens* group A and B) were able to infect leaves and cause small lesions, but that no disease progression could be observed. The reason why *A. alternata* is sometimes found to be less, similarly or more virulent than *A. solani* could be attributed to horizontal gene transfer (HGT) and by consequence, their ability to make rapid changes in pathogenicity. It has indeed been previously reported that conditionally dispensable chromosomes carry genes for toxin production and that they are easily transferred to non-pathogenic species through HGT, which could give rise to new pathogenic strains (Hatta et al., 2002; Akamatsu et al., 2003; Hatta et al., 2006). This inevitably means that the conditionally

dispensable chromosomes can also be lost, resulting in the loss of pathogenicity (Johnson et al., 2001).

The detection of large-spored species in the field is in co-occurrence with an increase in disease incidence

Additionally, a population composition analysis on symptomatic leaves harvested from the monitored potato fields was described in chapter 4. The analysis revealed that in the beginning of the season, when disease incidence was low, only small-spored isolates could be detected. Around mid-August, when disease symptoms were escalating, large-spored species were picked up by the real-time PCR analysis. These results indicated that the population composition shifts during the growing season and that this co-occurs with an increase in disease incidence. Similar observations were made by Leiminger et al. (2015), who observed a latent start in disease progression, followed by an increase of symptoms that co-occurred with the detection of large-spored species in the field. In this study however, the increase in disease incidence started a month earlier (mid-July).

Together with the data from the *in vitro* virulence assay, the observations from the population composition analysis suggest that the increase in disease symptoms around mid-August is due to the propagation of large-spored species on potato leaves. To confirm this hypothesis, an artificially inoculated field trial was set up at the experimental farm in Bottelare (Belgium) in the growing seasons of 2014 and 2015 in which different ratios of small- and large-spored inocula were applied. Results from the trials demonstrated that, when only small-spored species were used as inoculum, no increase in disease symptoms could be observed. When large-spored species were added in the inoculum suspension, lesions increased. Especially in 2015, the effect was clear, showing an apparent boost in disease incidence when the portion of large-spored species increased. The incidence was found to be highest when only large-spored species were applied. Based on these results, we concluded that, although small-spored species can infect potato leaves and do not merely act as saprophytes, EB/BS disease in Flanders is in fact caused by large-spored *Alternaria* species, and not by a disease complex of small- and large-spored species. This could also be discerned in the studies performed by Stammler et al. (2013). Like our field trial, an artificial infection trial on potato plants performed by these authors similarly demonstrated that the higher the concentration of *A. solani* was in the inoculation suspension, the more necrosis could be observed.

It should be noted that, when our leaf samples were brought to the lab for isolating *Alternaria* strains, the presence of small or large spores was first visually assessed. Surprisingly, on non-symptomatic leaves or on leaves with slight symptoms that were harvested in the beginning of the season, both small and large ungerminated *Alternaria* spores were detected (Fig. 6.1). When the

season progressed, the proportion of large *Alternaria* spores increased gradually. The reason why large spores are present on the leaves but remain dormant in the first half of the season remains elusive, although it could be speculated that these species might await the optimal wheather conditions and plant growth stage to initiate infection. It is indeed known that temperature and relative humidity have an influence on spore germination and that maturing leaves are more susceptible to infection (Rotem, 1994, van der Waals et al., 2001, Wharton and Kirk, 2007).

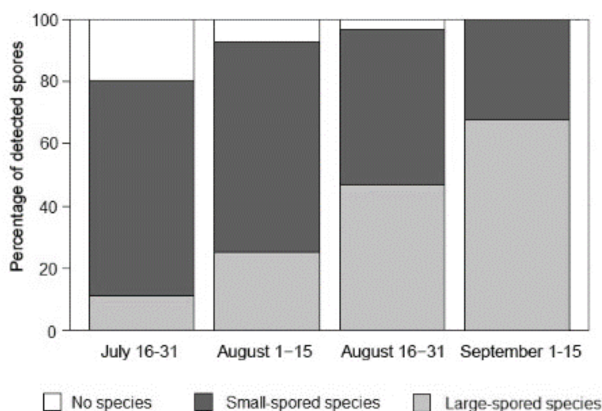

Figure 6.1. Percentage of small and large *Alternaria* spores detected on leaves harvested from 30 agricultural potato fields in Flanders during the growing season of 2014. For each time frame, 100 leaves were analyzed.

The fact that in the first half of the season, ungerminated small-spored species can be found abundantly on potato leaves and that only few symptoms develop, might indicate that a majority of the small-spored *Alternaria* strains are saprophytic strains that will only germinate after maturation and dessiciation of the foliage. This hypothesis is in direct contrast to our observation that small-spored species can cause small lesions but do not confer disease progression. However, if these ungerminated spores are indeed saprophytic strains, the isolation on artificial medium of small-spored species from lesions might only have selected the virulent small-spored strains, which would have resulted in only virulent strains in our collection while all saprophytic strains were overgrown by the more aggressive strains. By performing real-time PCR analysis, all *Alternaria* strains present on leaf samples are picked up, including the putative saprophytic strains and dormant large-spored strains. Since no DNA from large-spored species was detected during that stage, while they could be visually detected on the leaf surface, small-spored species are seemingly omnipresent compared to large-spored species during the first half of the growing season.

In that regard, a report by Turkensteen et al. (2010) stated that, next to the observation that *A. alternata* is not pathogenic, 28 % of all leaves that were harvested were void of both *A. alternata* and *A. solani*. Based on these observations and additional tests with ozone toxicity, they hypothesized that most lesions detected during the first half of the growing seasons are caused by ozone damage instead of pathogenic *Alternaria* species. The data presented in figure 6.2 also indicate that on 20 % of all symptomatic leaves harvested between July 15th and 31st , no *Alternaria*

spores could be detected. In view of this, the hypothesis of lesions caused by ozone damage seems likely. However, during the later stages of the growing season, the percentage of leaves out of which a small-spored *Alternaria* strain could be isolated, increased up to 100 %. This observation, together with the results from our *in vitro* virulence assay, which showed clear lesions caused by small-spored *Alternaria* species, suggests that these lesions are due to an infection with *Alternaria* and that the total sum of lesions observed during the first half of the season is most likely caused by a combination of ozone damage and small-spored *Alternaria* strains.

Our results regarding virulence of small- and large-spored species, combined with the population composition during the growing season could be of use for the decision-support-system (DSS) developer. Indeed, as described above, our reseach group published a study regarding sensitivity of isolates collected in the field towards fungicides with diverse modes of action (Landschoot et al., 2017c). Since the isolate collection from that paper was the same as in the experiments described in this dissertation, a putative timetable of fungicide sensitivity coupled to strain virulence during the course of the growing season could be drawn, which could be helpful to the DSS developer. This way, the usage of fungicides could be made more efficient and economical. However, to obtain a reliable DSS, our results should be supplemented with population compositions and fungicide sensitivity data from additional growing seasons.

Alternaria species are able to produce ET in vitro via the KMBA pathway

To look deeper into the role of small- versus large-spored species during an infection on potato, the molecular mechanisms of virulence were investigated. Since recent publications have reported the importance of the plant hormone ethylene (ET) as a virulence factor of several pathogenic microorganisms such as *C. miyabeanus* (De Bruyne, 2015; Van Bockhaven et al., 2015), *Botrytis cinerea* (Kettner and Dorffling, 1995; Audenaert et al., 2002) and *Pseudomonas syringae* pvs. *glycinea* (Weingart et al., 2001), we chose to focus our research on ET as a virulence factor of *Alternaria*. Our data showed that both small- and large-spored *Alternaria* species could produce ET *in vitro*, but that this is dependent on the precursor and the concentration in which it is supplied. When 1 mM KMBA was provided, only the large-spored isolates could produce ET within 24 hours post infection. When 1 mM ACC was supplied, only one large-spored isolate produced ET levels higher than the mock but to a much lesser extent. In the presence of 5 mM L-MET, the small-spored isolates also showed increased ET levels. Additionally, ET levels produced in the presence of 5 mM KMBA dropped rapidly when samples were incubated in the dark. These results suggested that the studied *Alternaria* isolates could produce ET *in vitro* via the light-dependent KMBA pathway, and to some extent, via the ACC pathway. Confirmation for these results could be found

in a publication by Zhu et al. (2017) in which it was demonstrated that strains of *A. alternata* could also produce ET via the light-dependent KMBA pathway in the presence of 10 mM of L-MET. As stated above, one isolate was seemingly able to produce ET via the ACC pathway in our assay. However, the ET levels were much lower than those detected when KMBA was supplied. Concerning this observation, it could be that the molecular tools to produce ET via the ACC pathway are present in some *Alternaria* strains, but that this pathway is not active under the applied circumstances and may be triggered during infection of potato plants. Two-oxoglutarate did not seem to be a substrate for ET production and by consequence, we concluded that our *Alternaria* isolates could not produce ET through an ethylene-forming enzyme (EFE) *in vitro*. Similarly as for the ACC pathway though, it could be that this pathway is only activated *in planta*. It should be noted that these experiments should be repeated and supplemented with experiments using extra isolates from our collection to make hard statements on the ability of *Alternaria* strains to produce ET *in vitro* via the ACC, the KMBA or the EFE pathway.

Infections with small-spored isolates cause higher ET emission levels than with large-spored isolates

Since it was established that our *Alternaria* isolates produce ET *in vitro*, we investigated wether ET plays a role during infection on potato leaves. Therefore, we measured ET emission during an infection of both small- and large-spored species on potato leaves. Data show that only after 3 days post inoculation, ethylene levels could be discerned that were higher than the mock-inoculated sample. However, no significant differences between small- and large-spored species were observed. At 3 dpi, symptoms have just started to progress and no apparent differences in necrotic sizes could be seen. For this reason, we chose to correct the data according to the fungal DNA content detected on the leaves. After correction of the data, it could be observed that ET emission levels of leaves inoculated with small-spored species were higher compared to large-spored species. Especially for the least virulent small-spored isolate, ET emission levels were highest. The fact that ET emission was only discernable after 3 days can be related to our spore germination assay (chapter 5). Results from this assay revealed that the time frame in which small and large spores germinated was within 5 hours post inoculation. This result together with the *in planta* ET emission data indicate that ET is involved in disease progression, rather than disease initiation. Although it was previously reported that endogenous ET is involved in *Alternaria* spore germination (Kepczynska, 1994), our hypothesis is supported by our *in vitro* ET production data. These equally showed that ET production for small-spored species could only be observed after 2 to 5 dpi. In the presence of 1 mM KMBA, large-spored species produced ET within 1 dpi. Both these time points have surpassed the 5 hpi mark, indicating that vegetative propagation was ongoing. However, it is

important to note that these *in planta* experiments do not distinguish between fungal or plant ET. Therefore, from these results, we could not make any conclusions on whether the detected ET was produced as a defense response by the plant, whether it was solely produced by the fungus, or whether it was a combination of both.

Small-spored species boost the plant ET biosynthesis, which is used by large-spored species to colonize the host faster

In our pursuit to reveal the role of ET during infection, an ET biosynthesis inhibition assay was also performed using 2-aminoethoxyvinyl glycine (AVG). This component blocks the action of ACC synthase, an enzyme in the plant's ET biosynthesis pathway. Since this pathway may also be present in our Alternaria isolates, AVG should also block fungal ET produced via this pathway. Leaves that were inoculated with large-spored isolates and that were pre-treated with AVG exhibited a significant reduction in symptoms, indicating that ET plays a crucial role in disease development of large-spored *Alternaria* species. In contrast, AVG-treated leaves inoculated with small-spored isolates did not show diminished symptoms. Instead, an increase in necrotic area could be seen. This observation, combined with the facts that inoculations with small-spored *Alternaria* species exhibit stronger ET emissions and do not confer disease, strongly indicates that in this case, the detected ET level is the result of a defense response by the plant and that plant ET has an antagonistic role during infection of small- versus large-spored *Alternaria* species on potato leaves. However, leaves that were not treated with AVG and that had 1 mM ACC supplied in the spore suspension, displayed an increase in lesion size, which could mean that ACC is differently used by small-spored species. The manner in which ACC is then used by these species remains elusive. To obtain statistically significant data and to make strong conclusions regarding this matter, these experiments should be repeated first.

In summary, we have established that (1) large-spored species such as *A. solani* are more virulent than small-spored species (*A. alternata* and *A. arborescens* groups A and B) that (2) small-spored species appear first on potato leaves, followed by the large-spored species in the second half of the season, that (3) an increase in disease incidence in the field is due to this advance of large-spored species, that (4) both small- and large-spored species can produce ET *in vitro*, predominantly via the light-dependent KMBA pathway, that (5) small-spored species produce more ET *in planta* than large-spored species and that (6) ET plays a important role in disease development by large-spored species on potato. In view of these results, we hypothesized that small-spored species are able to boost the host plants' ET biosynthesis (either by indirectly stimulating biosynthesis by the plant as a defense response and / or by self-production of ET),

which is consequently employed by the large-spored species as a virulence factor to colonize the host and to develop symptoms more rapidly. If this hypothesis could be confirmed, it may also be coupled to the fact that senescing plants are more susceptible to EB/BS disease (Rotem, 1994). Indeed, since maturing plants have higher levels of endogenous ET (Burg, 1968), and since large-spored *Alternaria* species would use this as a virulence factor, this could be the reason for the higher susceptibility of senescing plants. Additionally, if the hypothesis would prove to be true, this information would be valuable for the DSS developer, especially since applying fungicides to control small-spored species would reduce the *in planta* ET production, and consequently delay maturing and colonization by the more virulent large-spored species.

What are the toxin profiles of *Alternaria* isolates encountered in Flanders?

Small-spored Alternaria species display different toxin production profiles but these could not be linked with virulence or ET production

In a publication by Mase et al. (2012), it was demonstrated that ET was involved in AAL-toxin-induced cell death in the interaction between *Alternaria* and tomato. Due to this report, we investigated if the involvement of ET during the infection could be linked to toxin production and wether the virulence profiles of our isolates could be linked to their respective toxin production profiles. Firstly, an *in vitro* toxin production assay indicated that no host-specific toxins were produced by any of the small- or large-spored isolates, which was expected since these toxins are believed to be produced only in their respective host plant. Secondly, it revealed that the two small-spored isolates (*A. arborescens* group A) under study produced many of the known non-host specific toxins, whereas this could not be observed for the large-spored isolates (*A. solani*). Not much research has been done on the toxins produced by *A. solani*, however, one publication by Maiero et al. (1991) mentioned that this species mainly produced alternaric acid and zinniol. Although the production of zinniol was not determined in our assay, we did not detect alternaric acid in our *A. solani* samples. Logrieco et al. (2009) also included alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TeA) to the list. Only in case of our least virulent large-spored isolate, AOH and AME were picked up in the analysis. On the other hand, both of the large-spored isolates produced the toxins altertoxin-I (ATX-I) and altertoxin–II (ATX-II). To our knowledge, this is the first report of these toxins being produced by *A. solani*. Concerning the toxin production by the small-spored isolates, it could be observed that all toxins detected were produced by both our isolates. However, some differences could be discerned in the amount of the toxins produced. In that regard, AOH and TeA were found to be produced the most by the least-virulent small-spored isolate, whereas for the toxin altenusin, it was the other way round. Most of these toxins (AOH, AME, TeA and ATX-I) were also found to be produced by *A. arborescens* in the study

of Andersen et al. (2002). Since considerable variation in toxin production could be found between four *Alternaria* isolates, it cannot be excluded that other isolates in our collection might exhibit another toxin production profile. Indeed, since it has been proven that genes for toxin production reside on conditionally dispensable chromosomes (Johnson et al., 2001; Hatta et al., 2002; Akamatsu et al., 2003: Akagi et al., 2009), it seems likely that strains might quickly gain or lose the ability to produce certain toxins and that consequently, there might exist a considerable diversity in toxin production profiles between isolates in the same population. It should also be noted that our assay was performed on malt extract agar (MEA), which also has an influence on toxin production. A preliminary toxin production test (data not shown) using different artificial growth media such as synthetic nutrient agar (SNA), potato dextrose agar (PDA), V8 medium and MEA, indicated that the amount of different non-host specific toxins that were detected varied strongly depending on the artificial medium on which the fungi growed. Indeed, on a poor nutrient medium such as SNA, only the toxins TeA, ATX-I and ATX-II were produced by our isolates. The richer the medium was in nutrients, the more toxins were detected (both in number and in concentration). On MEA, most of the investigated toxins were produced and also in the highest quantities. For this reason, we chose to continue the experiment using MEA medium as we were primarily interested in the arsenal of toxins our isolates were able to produce.

Next to the *in vitro* toxin measurements, also the *in planta* toxin production was determined for the isolates under study. Surprisingly, none of the investigated toxins (both host- and non-host specific toxins) could be detected. Except for two unidentified compounds (Matern et al., 1978), no information is available to date on the toxin production of *Alternaria* strains in potato leaves. Since our small-spored isolates were not a threat to the plant but produced many non-host specific toxins, and because the large-spored isolates could cause disease but did not produce many known toxins, we uncouple pathogenicity on potato from the ability to produce any of the investigated known toxins. However, it should be mentioned that an unknown host-specific toxin might still be involved. In our assay, toxins were extracted after 3 days post inoculation. In this stage, first symptoms start to appear. Since no toxins were detected in that time frame, it can be concluded that toxin production is not involved in disease initiation by small- or large-spored *Alternaria* species on potato. However, we did not determine toxin production during disease progression (after 3 dpi). Therefore, we cannot make any hard statements about the investigated toxins not being involved during the later stages of infection. Additionally, with regard to the *in planta* ET production, it was demonstrated that high ET emission levels could be observed at 3 dpi. For this reason, the involvement of ET during infection on potato by small- or large-spored species could not be linked with the production of known toxins *in planta*. It should be noted that also in this signaling mechanism, an unknown toxin might be involved.

Are abiotic stress factors triggers for *Alternaria* disease development?

Sandy soils seem to be more prone to EB/BS disease

With the aim of a thorough characterization of the *Alternaria* population and EB/BS disease in Flanders, the environmental factors influencing the disease were also investigated. For this objective, an environmental analysis in which the effect of local weather factors and soil type on the relative Area Under the Disease Progress Curve (*rAUDPC*) values of the monitored fields was performed. From the analysis, we have concluded that in 2014, crops grown in sandy soils were more prone to EB/BS disease. In figure 2.5a, it could indeed be observed that the monitored fields in the east of Flanders and that had been typefied as a sandy soil type, only exhibited *rAUDPC* values that were classified as high (5 - 10 %) or medium (1 – 5 %). In contrast, all fields that were positioned in clay or loamy soils exhibited *rAUDPC* values in the lowest class (0 - 1 %). A similar observation was made by Shtienberg (2014), who also observed a positive correlation with disease incidence and sandy soils. In contrast to clay or loamy soils, sandy soils are known to be 'loose' and do not hold water and nutrients very well. This might explain why potatoes grown in this type of soil are more prone to the disease, since it has been previously shown that nutrient stress is positively correlated with many potato diseases, among which was EB/BS disease (Mackenzie, 1981; Kumar et al., 1983; van der Waals et al., 2001; Lambert et al., 2005). Mackenzie (1981) demonstrated that, as the amount of nitrogen (N) increased over the range of 0 to 200 kg ha^{-1}, early blight incidence decreased linearly. In view of this, we have performed a preliminary nitrogen fertilization field trial which equally showed that plots that were fertilized with 200 kg N ha^{-1} instead of the standard 160 kg N ha^{-1}, were more tolerant to EB/BS disease and had a longer 'latent' period of infection, resulting in an overall lower disease incidence (Fig. 6.2). The observation that crops with lower amounts of N-fertilizer are more prone to the disease might again be explained by the fact that high N levels prolong vegetative growth and delay the ripening stage, consequently reducing the amount of endogenous ET in the plant and slowing down the colonization by large-spored *Alternaria* species.

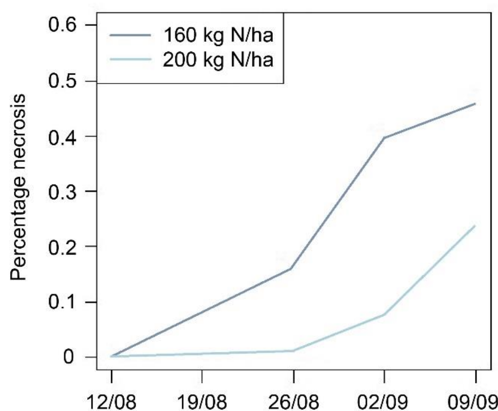

Figure 6.2. EB/BS disease progression of artificially inoculated potatoes on the experimental farm (Bottelare, Belgium) that were fertilized with either 160 or 200 kg N ha^{-1} (using NH_4NO_3). Inoculation was performed by spray inoculation with a spore suspension of *A. solani* (5.10^4 sp ml^{-1}). For each treatment, three biological repeats were performed.

Next, it is known that long 'wet-leaf' periods have a positive effect on disease incidence. However, not much information is available on the effect of drought stress in potatoes on disease pressure. Based on the positive correlation between drought stress and disease incidence of other plant-pathogen interactions such as the tomato-*Botrytis* pathosystem (Achuo et al., 2006), it is believed that drought stress would also have a stimulating effect on EB/BS disease of potatoes. However, a preliminary greenhouse trial (data not shown) surprisingly suggested that drought stress rendered the plants more tolerant to infection with *A. solani.* This could be explained as follows: under dry circumstances, the stomatal openings of potato leaves will be closed to prevent further dehydration. Since penetration of leaf tissue often occurs indirectly through these stomata, further colonization might be compromised in that way. In addition, because ET is generally believed to be positively involved in drought stress, one could argue that, based on our observation, a higher level of ET in the plant results in fewer symptoms. However, in direct contrast to this, our ET biosynthesis inhibition assay demonstrated that in case of infection with large-spored *Alternaria* species (which have been found to be the causal agents of EB/BS disease), ET inhibition resulted in less symptoms. It is important to remark that drought stress responses are regulated in a complex manner and are very much dependent on the severity of water deficit (Morgan and Drew, 1997). Indeed, as it has been previously reported that rapid dessication of detached leaves promotes ET biosynthesis, it has also been observed that slow soil drying resulted in a decrease in ET biosynthesis (Morgan, 1990). In that regard, if our drought stress experiment resulted in slow dessication, the resulting decrease in ET biosynthesis could explain the lower amount of symptoms, since large-spored species seem to use ET as a virulence factor. Due to the fact that it was difficult to determine when the plant was 'under stress' and that the repeats were highly variable, we did not include this trial in the manuscript and care should be taken when interpreting the results.
Either way, the facts that sandy soils do not hold nutrients well and that nutrient stress stimulates disease incidence, might explain our observation that sandy soils have higher incidence values than clay or loamy soils. Due to the low disease pressure in 2015, this observation could not be discerned for that growing season.

Precipitation and relative humidity accounted for the differences in disease incidence between two growing seasons

In regard to the difference in disease incidence between the two studied growing seasons, we could conclude that in 2014 but not in 2015, wet-related factors such as precipitation and relative humidity were positively influencing disease pressure, whereas temperature was negatively correlated. In 2015, disease pressure was probably too low to discern any correlations between environmental factors and disease pressure. Although it is known that heavy precipitation and long periods of

relative humidity have a positive effect on *Alternaria* spore germination and plant colonization (Bashi and Rotem, 1975; Holley et al., 1985; van der Waals et al., 2001), the negative influence of temperature is unexpected. Indeed, Ganie et al. (2015) reported a significant positive correlation between increasing temperatures and disease progression, while Hooker (1981) mentioned that temperatures around 23 °C were optimal for conidia formation and that increasing temperatures up to 28 °C stimulated hyphal growth of *A. solani* strains. It should be noted that EB/BS disease pressure is dependent on the combination of multiple environmental factors. The negative correlation between temperature and disease pressure might in that sense be explained by the fact that higher temperatures shorten the duration of leaf wetness, thereby restraining disease progression.

It was stated in the discussion section of chapter 2 that temperature also has an impact on the spore concentrations in the air. Indeed, Escuredo et al. (2011) reported that for the three years of study, the concentration of *A. solani* spores in the air was highest in the latest stages of the growing seasons. Especially in 2007, spore concentration was highest in September, after the mean temperature increased from 11 to 16 °C. In view of this, our population composition assay (chapter 4) might be linked to our temperature data (Supporting information, chapter 2). Since the mean temperatures around mid-August and the beginning of September were fluctuating around the mark of 14 - 16 °C, and since *A. solani* DNA was detected on the leaves around that time, it might be speculated that the shift in population observed in our study (and consequently, the escalated disease incidence) is due to an increase of spores from large-spored species in the air, which is induced at a mean temperature of around 14 – 16 °C. In turn, this might explain the negative correlation between temperature and disease incidence (as observed in our study), since the increase of disease incidence coincided with the period of the season that had the lowest mean temperature.

In view of the fact that temperature was negatively correlated with disease pressure in the field, we performed a preliminary *in vitro* growth rate assay and infection test were the isolates or inoculated leaf discs were incubated either at 16 °C or 26 °C (data not shown). The experiment revealed that at 16 °C, both small- and large-spored isolates exhibited slower growth rates on artificial medium compared to those at 26 °C. In terms of virulence, no significant differences could be observed between incubation temperatures for both small- and large-spored isolates. Although these data cannot be directly implemented on the situation in the field, they suggest that temperature does not have a negative influence on fitness or aggressiveness of *Alternaria* species, which is in direct constrast to what was observed in the field. However, it should be noted that *in vitro* versus field circumstances can vary greatly, since temperature and relative humidity can easily be controlled *in vitro*, these factors definitely influence one another in the field.

FUTURE PERSPECTIVES

Potato yield in relation to control measures

In the first paragraph of the general discussion, it was stated that the applied control measures in Flanders are adequate to control EB/BS disease. In that regard, it might be interesting to investigate potato yields of fields that are controlled with the conventional spray method and fields that had additional applications of fungicides that are specific against *Alternaria* species such as Narita (difenoconazole) or Terminett (pyraclostrobin and boscalid). Preliminary field trials performed by our research group in 2014 indicated that there were no significant differences in potato yield between fields treated against *Phytophthora* and fields that had additional treatments against *Alternaria*. However, to support and confirm the hypothesis, data from extra growing seasons should be added.

Additionally, since our results demonstrate a shift in *Alternaria* population composition during the growing season, different timings of fungicide application based on our results might provide information that could be useful in the development of more efficient and economical control strategies.

Sensitivity of the isolate collection to HGT

Since we have optimized a technique to molecularly identify the local *Alternaria* population in a season, it might be of value to collect leaf samples during extra growing seasons, to extract the fungi, and to molecularly identify them in order to obtain information about the evolution of the *Alternaria* population in the field. This way, we could assess whether the different *Alternaria* species detected in a growing season remain consistent and whether genetic evolution of species is a fast process or not. If the collected isolates would be subjected to virulence assays, it could provide an idea of the occurrence of HGT in a field. These data could be complemented with an assay in which we co-cultivate a non-virulent isolate with a virulent isolate of the same species or of a different species and determine the virulence of both strains after co-cultivation. This is a similar experiment as was performed by Ma et al. (2010) where they co-cultivated a virulent *F. oxysporum* isolate with a non-virulent isolate of the same species and observed that the non-virulent strain had become virulent on tomato. By performing an experiment as such, we could also determine whether *Alternaria* strains gain or lose virulence in single HGT events between strains of the same species or of different species and whether small-spored species are more prone to HGT than large-spored species (or vice versa). All of these data combined could provide us with an idea of the rate in which an *Alternaria* population evolves in terms of the species that are present and in terms of virulence.

In silico and PCR analysis for ET biosynthesis enzymes and development of mutants

The *in vitro* ET biosynthesis assay indicated that the isolates under study predominantly use the KMBA pathway to produce ET *in vitro*. However, when ACC was supplied as a precursor, one isolate was seemingly capable of producing ET, although to a much lesser extent. Due to this observation, it cannot be excluded that more isolates in our collection would possess the molecular machinery to produce ET via the ACC pathway. To substantiate this, an *in silico* analysis of putative ACC synthase homologues present in the genome of *Alternaria* could be performed. Consequently, primers could be developed in order to screen our isolate collection for the presence of an ACC synthase homologue by PCR. These isolates that were found positive for the presence of such a gene could then be subjected to the *in vitro* ET production assay with ACC as precursor to determine whether the ACC pathway is active *in vitro* or not.

These results would only provide information of the capability of producing ET *in vitro*. It should be noted that if a certain pathway does not seem to be active *in vitro*, it might still be triggered *in planta*. Therefore, it could also be interesting to search for an EFE homologue *in silico*, since this pathway did not seem to be active *in vitro*. To confirm if these pathways are either active, inactive or non-existent in our isolates, it might be possible to develop mutants of the EFE or ACC synthase genes using CRISPR/Cas9 technology and subject these mutants to an infection assay. Indeed, the methodology of CRISPR/Cas9 has been succesfully employed in *A. alternata* to develop mutants in the melanin biosynthesis pathway (Wenderoth et al., 2017). The development of a mutant that is deficient in converting KMBA to ET would prove equally useful since the involvement of fungal ET during infection is unclear. However, this gene is currently unknown, which impedes the development of KMBA mutants.

In vitro and *in vivo* toxin production assay

In our *in vitro* toxin production assay, only four isolates were tested in their capability of producing host- and non-host specific toxins. However, considerable variation in toxin production could be observed for these isolates. This hints at a complex regulation and possibly a role for toxins during the fungus' life cycle. However, the data from our experiments point at a life cycle where no toxins are involved. In view of this, a complete *in vitro* and *in vivo* screening for toxin production of all our isolates would provide additional information on variation in toxin production profiles, as well as an opportunity to find other non-host specific toxins produced by large-spored species such as *A. solani*.

Next, since no toxins could be picked up in our *in vivo* assay, the possibility of an unknown host-specific toxin being produced by our isolates should be investigated. Therefore, cultivation extracts

could be used to first inoculate potato leaves to corroborate whether symptoms develop. This might provide an indication whether compounds produced by the pathogen are involved in disease development. UPLC-MS/MS analysis of such an extract, combined with the available data from our untargeted analysis, could be used to identify new toxic compounds by comparing the chromatograms with a library of known or similar phytotoxic molecules. If ultimately the biosynthesis pathway of this host-specific toxin is unraveled, a mutant strain could be developed that could be used to investigate the involvement of such a toxin during infection and a putative interaction with ET. In regard to toxin production being linked to ET during infection, it might also be of interest to investigate the time frame in which toxins are produced *in planta*. In our experiments, toxin production was checked at 3 days post inoculation. Since ET production was registered at 3 dpi, we hypothesized that ET is involved during disease progression rather than disease initiation. If the production of specific toxins would be involved in this mechanism, it would make sense to also investigate the toxin production *in planta* after 3 dpi. Indeed, a publication by Kheder et al. (2012) indicated that the distribution of the host-specific AAL-toxin was widespread in tomato after 7 dpi.

Further exploration of the role of small- versus large-spored *Alternaria* species during an infection on potato leaves

In chapter 5, it was hypothesized that small-spored *Alternaria* species boost the plant's ET biosynthesis (either by indirectly stimulating production by the plant and / or by self-production of ET), which is consequently employed by the large-spored species as a virulence factor to colonize the host faster. To confirm or reject this hypothesis, sequential inoculations could be useful to discern whether a faster disease progression can be observed if small-spored species are inoculated before the large-spored species. Additionally, our *in vitro* and *in planta* experiments, as well as our inhibitor experiments should first be repeated to obtain statistically significant data.

Influence of nitrogen and drought stress on disease pressure in relation to sandy soils being more prone to EB/BS disease

In chapter 2, we established that in 2014, sandy soils were more prone to EB/BS disease. In view of this observation, it was suggested that the permeability of sandy soils and the resulting decrease in nutrients and water availability might be the reasons behind the observed discrepancy. Moreover, extreme weather events such as the long periods of drought during the summer months in the last few years are generally attributed to climate change, which possibly has had more effect on sandy soils than other types of soil. In view of this, it might be interesting to further investigate this by extending the field survey with extra growing seasons to confirm wether the increased sensitivity of sandy soils for EB/BS disease can be discerned.

Effect of temperature and relative humidity on EB/BS disease pressure

Since in the previous section, it was established that factors such as relative humidity and temperature are heavily influencing disease pressure but that the effect of temperature is dependent on the amount of relative humidity and *vice versa*, an artificially inoculated greenhouse trial could be set up in which inoculated potato plants could be grown under different temperatures and different levels of relative humidity. This way, the *in vivo* effect of temperature on EB/BS disease could be more strictly defined, as well as the correlations with relative humidity.

Correlation between temperature and concentration of small and large *Alternaria* spores in the air

In view of the publication of Escuredo et al. (2011), in which it was shown that the highest concentrations of *A. solani* spores in the air were detected at temperatures of 14-16 °C, we wondered if there might be a correlation between the mean temperature of a certain period during the growing season and the amount of spores (both small- and large-spore species) in the air. Since *Alternaria* spores are important allergens, spore traps have been placed in numerous locations to monitor spore concentrations in the air. By gathering data from such spore traps, determining the amount of small and large spores at different time points and by coupling this information to temperature data (which is readily available online), a putative correlation might be found. If such a relation is observed, it might explain the *Alternaria* population composition during the season and consequently, the sudden increase in disease incidence in mid-August. Moreover, correlations such as these could be used in EB/BS disease forecasting systems.

Implement the data in existing forecasting models

Although several models exist to predict *Alternaria* epidemics (FAST, P-Days, IWP, PLANT-Plus) (Madden et al., 1978; Raatjes et al., 2004; Sands and Regel, 1983; van der Waals et al., 2003), only few can reliably predict a disease outbreak in multiple regions. This is due to the fact that EB/BS disease is dependent on many factors and in that regard, environmental factors can vary greatly between regions. Moreover, most models are either plant-based or pathogen-based, which only take into account plant-related factors (such as growth stage) or pathogen-related factors (such as optimal sporulation conditions) respectively. The drawback of these prediction models is that they do not take into account the life cycle of the pathogen (in case of plant-based models) or the susceptibility of the host (in case of pathogen-based models). However, Raatjes et al. (2004) developed a plant-pathogen-based model (PLANT-Plus) that takes into account the life cycle of the fungus, the growth stage of the host plant and the degree of protection of the leaves by previous

fungicide applications. This model was found to hold potential in South Africa, although some re-calibration of threshold conditions was needed (van der Waals et al., 2003).

In a parallel project performed by our research group, a new model is being developed that is adjusted to Belgian conditions. In a previous publication, we concluded that none of the existing models were able to accurately predict the date of the first lesions (Landschoot et al., 2017b). This can be attributed to the fact that these models were developed and validated for different regions. Indeed, since Belgium is characterized by varying weather conditions between growing seasons, disease pressure will fluctuate accordingly and the existing models are adjusted to regions with more stable weather conditions. Because in this dissertation we have established useful information such as the onset of disease incidence (Mid-August), the influence of environmental factors (temperature, soil type, precipitation and relative humidity) on disease pressure, the species composition at different time points, the virulence of different species and the germination time frame on potato leaves, these data could be used in the development of a plant-pathogen-based prediction model, which would be adjusted to Belgian conditions and that could be used as a decision support system to help potato growers optimally apply fungicide treatments.

SUMMARY

In the last decade, disease symptoms caused by fungal *Alternaria* species are increasingly reported by potato cultivators in Flanders. Although the exact cause of this increase remains elusive, in professional literature a few causes are mentioned such as climate change, the restricted use of the fungicide mancozeb, shifts in the cultivation of specific potato varieties that may be more susceptible to *Alternaria* infections, and a more economical fertilization. Generally, it is believed that *Alternaria* symptoms on potato are due to a disease complex caused by two species, *A. alternata* and *A. solani*. The former species was considered to be the causal agent of potato brown spot (BS), while the latter causes potato early blight (EB). However, both species are commonly found together on an infected leaf. Moreover, symptoms of both diseases are hard to discriminate without microscope analyses. For these reasons, *Alternaria*-caused diseases on potato are synonymized as EB/BS disease in this dissertation.
Despite the increasing incidence, not much is known about the local *Alternaria* population found on potato fields, the factors contributing to the disease and its control. Additionally, little information is available on the molecular mechanisms involved in disease development. Due to this lack of knowledge, the current control strategies mostly imply the application of preventive fungicides. A better understanding of the disease progression, the population composition and genetic diversity and the effect of abiotic factors on disease development could ultimately lead to more integrated and sustainable disease management strategies. This research project therefore aimed to obtain insight in (1): the genetic diversity, phylogenetic relations, toxin profiles and population structure of the *Alternaria* population on Flemish potato fields, and (2): the influence of environmental factors with respect to disease development and the role of the plant hormone ethylene during *Alternaria* infections on potato.

Firstly, by conducting a field survey of 22 potato fields across Flanders during the growing seasons of 2014 and 2015, we could establish that the overall disease incidence was low during both seasons of study. Disease incidence in 2014 was higher than in 2015. However, in both growing seasons, after a latent stage of the disease, a sharp increase in symptoms could be observed around mid-August. To explain the differences in disease incidence between locations and between growing seasons, an environmental analysis was performed. Based on the results, we hypothesized that sandy soils are more prone to EB/BS disease than clay or loamy soils and that precipitation and relative humidity were important factors contributing to the disease in 2014, whereas temperature was not. In 2015, similar correlations could not be discerned and extending

the survey with data from additional growing seasons is mandatory to substantiate these hypotheses.

Next, the local *Alternaria* population was molecularly identified using a multi-locus sequencing analysis, while also inferring phylogenetic relations. Based on the sequence analyses of the *internal transcribed spacer region* (*ITS*) and the *glyceraldehyde-3-phosphate dehydrogenase* gene, the small-spored and large-spored *Alternaria* isolates could be separated from each other. However, the resolution of the analysis was not sufficient to reveal phylogenetic clusters within these two groups. Sequence analyses of the *calmodulin* gene and the RNA polymerase second largest subunit showed that besides *A. solani*, also *A. grandis* and *A. protenta* were present in the Flemish large-spored *Alternaria* population. Sequences of the *Alternaria* major allergen gene *Alt a 1* and the *elongation factor-a* revealed that both *A. alternata* and species belonging to the *Alternaria arborescens* species complex were present in the small-spored *Alternaria* population. Finally, according to the *histone h3* sequences, the members of the *A. arborescens* species complex could be subdivided into two distinct phylogenetic groups (A and B). These results indicate that the *Alternaria* population present on naturally infected potato leaves in Flanders is more complex than previously anticipated.

In a third part of this dissertation, the mechanism behind the sudden increase in disease incidence during the two growing seasons was investigated. Real-time PCR analyses demonstrated that small-spored *Alternaria* species, such as *A. alternata* and *A. arborescens* were the predominant species on potato leaves throughout the growing season. As the season progressed, the proportion of large-spored species (such as *A. solani*) increased. Based on a virulence assay and an artificially inoculated field trial, we established that large-spored species are more virulent than small-spored species. Also, no significant differences were found within the small- or large-spored species. Based on these observations, we hypothesized that the increase in disease incidence observed in the field is due to the simultaneous advance of large-spored species. Additionally, a fitness assay confirmed the need for molecular means to perform population assays since small-spored species grow out faster on culturing media than large-spored species which could introduce a bias if the population was determined by visual analyses alone.

The experiments were continued by a study in which the molecular mechanisms behind the difference in virulence between small- and large-spored species were investigated. Therefore, since the plant hormone ethylene (ET) is increasingly reported as a virulence factor of plant pathogens, our research efforts were focused on this compound. An *in vitro* ET production assay in which one of three possible precursor for ET biosynthesis was administered, indicated that the large-spored isolates produced more ET *in vitro* than the small-spored isolates in the presence of the precursor KMBA (α-keto-γ-methylthiobutyric acid). This experiment was followed by an *in planta*

ET production assay that showed that after 3 days post inoculation, leaf discs inoculated with small-spored isolates emitted more ET than leaf discs inoculated with large-spored isolates after correcting the data according to fungal DNA content on the leaf. These data were complemented with results from an experiment where ET biosynthesis in the plant was inhibited and that showed that blocking ET biosynthesis in the plant strongly compromised symptom development in leaf discs inoculated with large-spored isolates. In contrast, for small-spored isolates, inhibition of ET biosynthesis did not result in a reduction of the symptoms. These results, combined with an experiment that showed that spore germination on potato leaves was completed at 5 hours post inoculation for both small- and large-spored species, point to a differential role and production profile of ET during disease progression (rather than disease initiation) on potato leaves by respectively small- and large-spored isolates.
Finally, in the interaction between *Alternaria* and tomato, the role of ET has been previously linked with toxin production. In view of this report, using UPLC-MS/MS, a set of small-spored and large-spored isolates was screened *in vitro* for their capacity to produce a broad spectrum of host-specific and non-host specific toxins. Results showed that most of the small-spored isolates produced the non-host specific toxins alternariol, alternariol monomethyl ether, tenuazonic acid, altenusin and altenuisol. In contrast, none of these toxins were found to be produced by large-spored species *in vitro*. Both groups of isolates produced the toxins altertoxin-I and altertoxin-II. In order to assess the physiological relevance of these toxins, an inoculation test was carried out assessing the toxin production *in planta*. Remarkably, none of the investigated toxins were detected during the infection process.

Through the combination of observations in which (1) an increase in disease incidence in the field co-occured with the detection of large-spored species, in which (2) large-spored species were found to be more virulent than small-spored species, in which (3) it was found that potato leaves inoculated with the small-spored isolates exhibited enhanced ET emission levels and that large-spored isolates were able to produce ET *in vitro* predominantly through the KMBA pathway, and in which (4) ET biosynthesis inhibition resulted in strongly reduced symptoms in case of the large-spored isolates, we hypothesized that small-spored species are able to boost the hosts' ET biosynthesis (either by indirectly stimulating biosynthesis by the plant and / or by self-production of ET), which is consequently employed by the large-spored species as a virulence factor to colonize the host and to develop symptoms faster. In addition, the involvement of ET during inititation of the infection could not be linked to the production of known host- or non-host specific toxins in both small- or large-spored species.

In summary, this dissertation provides a thorough characterization of the *Alternaria* population on Flemish potato fields. The results from the experiments widen the scope on how EB/BS disease is perceived by providing new information on the population composition, genetic diversity and toxin production, while broadening the knowledge on the influence of environmental factors on disease incidence. Furthermore, a glimpse of the molecular machinery behind the difference in virulence between small- and large-spored *Alternaria* species is given by showing that ET has a differential role during infection of potato leaves by respectively small- and large-spored species. In conclusion, the data presented in this manuscript could contribute in the development of *Alternaria*-resistant potato cultivars, prediction models and ultimately, in more sustainable, integrated disease management strategies.

SAMENVATTING

In de laatste 10 jaar worden door aardappeltelers in Vlaanderen steeds meer ziektesymptomen gerapporteerd die veroorzaakt worden door *Alternaria* schimmelsoorten. Hoewel de precieze oorzaak van deze toename niet gekend is, worden in professionale literatuur een aantal oorzaken voorgesteld zoals de klimaatopwarming, de beperking op het gebruik van het fungicide mancozeb, verschuivingen in de teelt van aardappelvariëteiten die gevoeliger kunnen zijn voor infecties door *Alternaria*, en een economischere bemesting. Algemeen wordt aangenomen dat de symptomen op aardappel voortkomen uit een ziektecomplex veroorzaakt door twee soorten, namelijk *A. alternata* en *A. solani*. De eerstgenoemde werd beschouwd als de veroorzaker van brown spot (BS), terwijl de laatstgenoemde de veroorzaker zou zijn van de ziekte early blight (EB). Nochtans worden de twee soorten dikwijls samen teruggevonden op eenzelfde blad. Daarnaast zijn de symptomen van beide ziektes moeilijk te onderscheiden van elkaar zonder microscoopanalyses. Om deze redenen worden beide ziektes in dit proefschrift samengebracht onder de naam 'EB/BS ziekte'.
Hoewel er een stijging werd waargenomen in ziekte-incidentie is er weinig gekend over de lokale *Alternaria*populatie op aardappelvelden, over de factoren die bijdragen tot de ziekte, en over het controleren van de ziekte. Daarnaast is er weinig informatie beschikbaar over de moleculaire mechanismen die betrokken zijn tijdens symptoomontwikkeling. Door dit gebrek aan kennis worden de huidige controlemaatregelen voornamelijk gericht op het gebruik van preventieve bespuitingen. Het beter begrijpen van de ziekteprogressie, de populatiesamenstelling, de genetische diversiteit en de invloed van abiotische factoren op de ziekteontwikkeling, zou uiteindelijk kunnen leiden tot betere, geïntegreerde en duurzamere controlestrategieën. Daarom beoogden we met dit onderzoeksproject inzicht te verkrijgen in (1): de genetische diversiteit, de fylogenetische relaties, de toxineprofielen, en de populatiestructuur van *Alternaria* op Vlaamse aardappelvelden, en (2): de invloed van omgevingsfactoren gerelateerd aan de ziekteontwikkeling en de rol van het plantenhormoon ethyleen tijdens een infectie van *Alternaria* op aardappel.

Ten eerste, door een veldonderzoek uit te voeren tijdens de groeiseizoenen van 2014 en 2015 waarin 22 aardappelvelden in Vlaanderen opgevolg werden, kon er vastgesteld worden dat de ziekte-incidentie tijdens beide groeiseizoenen laag was. De ziekte-incidentie in 2014 bleek hoger te zijn dan deze in 2015. Nochtans werd in beide seizoenen opgemerkt dat, na een latente periode van infectie, de symptomen snel stegen rond midden augustus. Om de verschillen in ziekte-incidentie tussen de locaties en tussen de groeiseizoenen te verklaren, werd een omgevingsanalyse uitgevoerd. Gebaseerd op de resultaten werd verondersteld dat zandbodems

gevoeliger zijn aan EB/BS ziekte dan klei- of leembodems, en dat neerslag en relatieve vochtigheid, maar niet de temperatuur, belangrijke factoren zijn die bijdroegen aan de ziekte-incidentie van 2014. Dezelfde correlaties konden niet onderscheiden worden in 2015, wat de nood onderstreept om data van meerdere groeiseizoenen aan het onderzoek toe te voegen om deze hypotheses te ondersteunen.

Vervolgens werd de lokale *Alternaria*populatie op moleculair niveau geïdentificeerd door een multilocus-sequentieanalyse uit te voeren en de fylogenetische relaties af te leiden. Gebaseerd op de sequenties van de *internal transcribed spacer* (*ITS*) regionen en het *glyceraldehyde-3-fosfaat-dehydrogenase*gen konden de klein- en grootsporige isolaten van elkaar onderscheiden worden. Deze sequenties boden weliswaar niet genoeg resolutie om fylogenetische clusters binnen deze groepen waar te nemen. De sequentieanalyses van het *calmoduline*gen en de *tweede grootste subunit van het RNA polymerase*gen toonden aan dat naast *A. solani*, ook *A. grandis* en *A. protenta* aanwezig waren in de Vlaamse grootsporige *Alternaria*populatie. De sequenties van een belangrijk *Alternaria* allergeen *Alt a 1*, en de *elongatiefactor-a* onthulden dan weer dat zowel stammen van de soort *A. alternata* als van de soort *A. arborescens* aanwezig waren in de kleinsporige populatie. Tenslotte konden de *A. arborescens* isolaten via de *histon h3*-sequenties opgedeeld worden in twee fylogenetisch verschillende groepen (A en B). Samen duiden deze resultaten aan dat de *Alternaria*populatie die aanwezig is op natuurlijk geïnfecteerde aardapelbladeren in Vlaanderen, complexer is dan oorspronkelijk gedacht.

In een derde deel van dit proefschrift werd het mechanisme achter de plotse stijging in ziekte-incidentie op het veld onderzocht. *Real-time PCR*-analyses toonden aan dat de kleinsporige species (*A. alternata* en *A. arborescens* groepen A en B) de voornaamste soorten waren doorheen het groeiseizoen. Pas als de ziekte-incidentie toenam, verhoogde het aandeel van grootsporige soorten (zoals *A. solani*). Gebaseerd op de resultaten van een virulentietest en een artificieel geïnoculeerde veldproef, werd aangetoond dat grootsporige soorten virulenter zijn dan kleinsporige soorten en dat er geen significante verschillen in virulentie te vinden zijn binnen deze twee groepen. Door deze observaties werd voorgesteld dat de toename in ziekte-incidentie tijdens het groeiseizoen rond midden-augustus veroorzaakt werd door de gelijktijdige opkomst van grootsporige soorten in het veld. Daarnaast kon het belang om moleculaire technieken te gebruiken bij het uitvoeren van populatie-analyses bevestigd worden doordat kleinsporige soorten sneller uitgroeien op groeimedium dan grootsporige soorten en dit zou kunnen leiden tot een verkeerd beeld van de populatiesamenstelling als er enkel uitgegaan zou worden van visuele analyses.

Deze experimenten werden gevolgd door een studie waarbij de moleculaire mechanismen van virulentie die achter het verschil in aggressiviteit tussen klein- en grootsporige isolaten schuilen, uit te voeren. Omdat het plantenhormoon ethyleen (ET) steeds meer als virulentiefactor van

plantpathogenen wordt gerapporteerd, werd het onderzoek gericht op deze molecule. *In vitro* ET biosynthese-experimenten waarbij één van drie mogelijke precursoren werd toegediend, toonden aan dat de grootsporige isolaten *in vitro* meer ET konden produceren dan kleinsporigen in de aanwezigheid van de precursor KMBA (*α-keto-γ-methylthiobutyric acid*). In navolging van dit experiment werd de *in planta* ET emissie nagegaan, wat onthulde dat, na drie dagen na inoculatie, bladeren die geïnoculeerd werden met kleinsporige isolaten meer ET uitstootten dan deze die geïnoculeerd werden met grootsporige isolaten nadat de data gecorrigeerd werden volgens DNA hoeveelheid van de schimmel. Deze data werden aangevuld door resultaten van een experiment waarbij de ET biosynthese in de plant geblokkeerd werd en die wees op een sterke vermindering van symptomen op bladeren geïnoculeerd met grootsporige isolaten na het blokkeren van de ET biosynthese. Daartegenover kon geobserveerd worden dat de inhibitie van de plant ET biosynthese niet resulteerde in een vermindering van symptomen op bladeren die geïnoculeerd waren met kleinsporige isolaten. Deze resultaten, gecombineerd met een proef die aantoonde dat sporenkieming van zowel klein- als grootsporige isolaten op aardappelbladeren binnen 5 uur na inoculatie gebeurde, wijzen op een differentiële rol en productieprofiel van ET tijdens ziekteprogressie (en niet ziekte-initiatie) op aardappelbladeren door klein- en grootsporige *Alternaria*soorten.

Tenslotte, door een eerdere publicatie waarbij ET gekoppeld werd aan toxineproductie in de interactie tussen tomaat en *Alternaria*, werd via UPLC-MS/MS een set van klein- en grootsporige isolaten *in vitro* gescreend op hun mogelijkheid tot het produceren van een breed spectrum aan gastheer- en niet-gastheerspecifieke toxines. De resultaten demonstreerden dat de kleinsporige isolaten de toxines alternariol, alternariol monomethyl ether, tenuazonzuur, altenusin en altenuisol konden produceren *in vitro*, terwijl dit niet het geval was voor de grootsporige isolaten. Beide groepen produceerden wel de toxines altertoxine-I en altertoxine-II. Om de fysiologische relevantie van deze toxines na te gaan, werd een inoculatieproef uitgevoerd om de *in planta* toxines te kunnen detecteren, maar geen van de onderzochte toxines kon gedetecteerd worden binnen de eerste drie dagen na inoculatie.

Door de combinatie van observaties waarbij (1) een stijging in ziekte-incidentie gelijktijdig gebeurde met de detectie van grootsporige *Alternaria*soorten in het veld, waarbij (2) grootsporige isolaten virulenter waren dan kleinsporige isolaten, waarbij (3) aardappelbladeren geïnoculeerd met kleinsporige isolaten verhoogde ET emissieniveaus vertoonden en de isolaten *in vitro* ET konden produceren via de KMBA-pathway, en waarbij (4) inhibitie van de ET biosyntheseweg in de plant resulteerde in een sterke vermindering van symptomen in het geval van de grootsporige isolaten, werd volgende hypothese voorgesteld: kleinsporige *Alternaria*soorten kunnen de ET biosynthese

in de plant verhogen (door indirect de synthese te stimuleren en / of door zelf bij te dragen aan de productie), wat vervolgens als virulentiefactor kan gebruikt worden door grootsporige isolaten om een snellere kolonisatie van de aardappelplant te bekomen en om sneller symptomen te ontwikkelen. Daarnaast kon de betrokkenheid van ET tijdens de start van infectie niet gekoppeld worden aan de productie van gekende gastheer- of niet-gastheerspecifieke toxines voor zowel klein- als grootsporige soorten.

Samengevat beschrijft dit proefschrift een grondige karakterisatie van de *Alternaria*populatie op Vlaamse aardappelvelden. De resultaten die voortkomen uit de experimenten verbreden onze blik op de manier waarop de EB/BS ziekte wordt waargenomen door nieuwe informatie te brengen over de populatiesamenstelling, de genetische diversiteit en toxineproductie, alsook door de kennis te verruimen over de invloed van omgevingsfactoren op de ziektedruk. Daarnaast wordt een eerste kijk gegeven in de moleculaire machine achter het verschil in virulentie tussen klein- en grootsporige soorten door aan te tonen dat ET op een verschillende manier gebruikt wordt door beide groepen van *Alternaria*soorten. Tot slot kan gesteld worden dat de data die gepresenteerd worden in dit manuscript kunnen bijdragen tot het ontwikkelen van *Alternaria*-resistente aardappelvariëteiten, voorspellingsmodellen en uiteindelijk, tot duurzamere, geïntegreerde ziektebestrijdingsstrategieën.

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CURRICULUM VITAE

PERSONAL INFORMATION

Full name: Michiel M. R. Vandecasteele

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EDUCATION

2001-2007: General secondary education (science-maths)
Immaculata Instituut, De Panne, Belgium

2007-2010: Bachelor in Biochemistry and Biotechnology
Faculty of Sciences
Ghent University, Ghent, Belgium

2010-2012: Master in Biochemistry and Biotechnology
(Major: Plant Biotechnology, Minor: Structural Biotechnology)
(Thesis: Study of strigolactone-responsive genes in *Arabidopsis thaliana*)
Faculty of Sciences
Ghent University, Ghent, Belgium

ADDITIONAL TRAINING

From PhD to Job Market Workshop, September 2017, ArcelorMittal Gent, Wachtebeke, Belgium.

Presentation Techniques Module 1 (by theater group ‘Klein Barnum’), February 2018, Ghent, Belgium.

PROFESSIONAL RECORD

2013-2016 PhD candidate

(Project title: *Alternaria* species on potato: genetic characterization, chemotyping and disease development in relation to abiotic stress.)

Department of Applied Biological Sciences

Faculty of Bioscience-engineering

Ghent University, Ghent, Belgium

2016-2019 Assistant

(Project title: *Alternaria* species on potato: genetic characterization, chemotyping and disease development in relation to abiotic stress.)

Department of Plants and Crops

Faculty of Bioscience-engineering

Ghent University, Ghent, Belgium

PUBLICATIONS

Landschoot S, Vandecasteele M, De Baets B, Höfte M, Audenaert K, Haesaert G, 2017. Identification of *A. arborescens*, *A. grandis*, and *A. protenta* as new members of the European *Alternaria* population on potato. *Fungal Biology* **121**, 172-188.

Landschoot S, Vandecasteele M, Carrette J, *et al.*, 2017. Assessing the Belgian potato *Alternaria* population of sensitivity to fungicides with diverse modes of action. *European Journal of Plant Pathology* **148**, 657-672.

Landschoot S, Carrette J, Vandecasteele M, *et al.*, 2017. Boscalid-resistance in *Alternaria alternata* and *Alternaria solani* populations: An emerging problem in Europe. *Crop Protection* **92**, 49-59.

Vandecasteele M, Landschoot S, Carrette J, *et al.*, 2017. Species prevalence and disease progression studies demonstrate a seasonal shift in the *Alternaria* population composition on potato. *Plant Pathology* **67**, 327-336.

TEACHING & TUTORSHIP

Practical courses: Cell Biology (2016-2017, 2017-2018, 2018-2019)
Agro-biotechnology (2018-2019)
Ecology (2016-2018)

Theoretical courses: Biochemical and molecular analyses (guest lectures in 2017 & 2018)
Crop Protection (guest lectures in 2017, 2018 & 2019)

Individual students: Bachelor students 2012-2013

Cedric Lefebvre, Elias van de Vijver, Jonas Van Damme *(Pathogeniciteit van Alternaria spp. op aardappelen)*

François Bouche, Eline Braet, Sofie Venneman *(Invloed van fungicidenbehandeling op Alternaria bij aardappelen)*

Bachelor students 2013-2014

Sander Bauwens, Thierry Heyman, M. Vansteenkiste *(Pathogeniciteitscreening van Alternaria-isolaten)*

Hanne De Vos, Emily Galmart, Amber Tilley *(Invloed van fungiciden op de pathogeniciteit van Alternaria isolaten)*

Master students 2013-2014

Eline Braet (*Invloed van abiotische stressfactoren op de ontwikkeling van Alternaria bij aardappelen*)

Astrid Van haecht (*Het voorkomen en beheersen van Alternaria bij aardappel in Vlaanderen*)

Master students 2014-2015

Iris Jiang (*Invloed van abiotische factoren op de ontwikkeling en pathogeniciteit van Alternaria solani en Alternaria alternata en fylogenetisch onderzoek op Vlaamse isolaten aan de hand van de ITS-regio)*

Master students 2015-2016

Martina M. Sasia *(University of Parma)* (*Studying the Flemish Alternaria population and the relationship between Alternaria toxins and the plant hormone ethylene*)

Master students 2017-2018

Jolien Van Poucke (*Studie van ethyleenproductie tijdens de verschillende fasen van een Alternaria infectie op aardappel*)

External teaching: STEM course (Koninklijk Atheneum Zottegem) (2017-2018, 2018-2019) *(Analyse van de microbiële detoxificatie van deoxynivalenol (DON) via biotransformatie)*

PARTICIPATION AT CONFERENCES AND SYMPOSIA

Vandecasteele M, Audenaert K, Höfte M, Haesaert G. *Alternaria* species on Flemish potato. Annual Mytox Happening, March 2014, Ghent, Belgium. Oral presentation.

Landschoot S, Vandecasteele M, Heremans B, *et al*. Towards an integrated control and prediction of *Alternaria spp*. in the Flemish potato cultivation. 14th Euroblight subgroup Workshop, München, Germany. Oral presentation by Landschoot S.

Vandecasteele M, Audenaert K, Höfte M, Haesaert G. *Alternaria* species on Flemish potato. 66th International Symposium on Crop Protection, May 2014, Ghent, Belgium. Oral presentation.

Landschoot S, Vandecasteele M, Heremans B, *et al*. Towards an integrated control and prediction of *Alternaria spp*. in the Flemish potato cultivation. 19th triennial Conference of European Association for Potato Research, July 2014, Brussels, Belgium. Poster presentation by Landschoot S.

Vandecasteele M, Landschoot S, Höfte M, De Saeger S, Audenaert K, Haesaert G. Characterization of *Alternaria* species on potato. 5th International Mytox Symposium, May 2016, Ghent, Belgium. Poster presentation.

Vandecasteele M, Landschoot S, Audenaert K, Höfte M, De Saeger S, Haesaert G. Identification and characterization of *Alternaria* species causing early blight on potato in Belgium. 68th International Symposium on Crop Protection, May 2016, Ghent, Belgium. Oral presentation.

Vandecasteele M, Landschoot S, Höfte M, De Saeger S, Audenaert K, Haesaert G. Characterization of *Alternaria* species on potato. 9th World Mycotoxin Forum & 14th International Symposium on Mycotoxins (WMF meets IUPAC), June 2016, Winnipeg (MB), Canada. Poster presentation.

Vandecasteele M, Landschoot S, Verwaeren J, Höfte M, Haesaert G, Audenaert K. Investigating the *Alternaria* disease progression, genetic diversity and species composition on potato in Belgium. 12th European Foundation for Plant Pathology (EFPP) & 10th French Society for Plant Pathology (FSP) conference, May 2017, Dunkirk, France. Poster presentation.

Vandecasteele M, Landschoot S, Verwaeren J, Höfte M, Haesaert G, Audenaert K. Investigating the *Alternaria* disease progression, genetic diversity and species composition on potato in Belgium. 1st MYCOKEY International Conference, September 2017, Ghent, Belgium. Oral presentation.

CONFERENCES AND SYMPOSIA WITHOUT PARTICIPATION

Annual Mytox Happening, March 2013, Ghent, Belgium.

Annual Euroblight Subgroup Meeting, February 2014, BASF Limburgerhof, Germany.

69th International Symposium on Crop Protection, May 2017, Ghent, Belgium.

Digital PCR Mini-symposium, May 2018, Ghent, Belgium.

AWARDS

Best Speaker Award at Annual Mytox Happening, March 2014, Ghent, Belgium.

Best Poster Award at 12th European Foundation for Plant Pathology (EFPP) & 10th French Society for Plant Pathology (FSP) conference, May 2017, Dunkirk, France.

DANKWOORD

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Michiel_