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Impact of fungicide mode of action and application timing on the control of *Mycosphaerella graminicola* and the physiology and yield of wheat

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List of abbreviations

A

ABA	Abscisic Acid
ACC	1-Aminocyclopropane-1-Carboxylic Acid
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate

B

BABA	β-Amino-Butyric Acid
BRs	Brassinosteroids
BTH	Benzothiadiazole S-Methylester

C

cDNA	Complementary DNA
CK	Cytokinins
cRNA	Complementary RNA

D

DAMPS	Damage-Associated Molecular Patterns
DAPA	Days After the Preventative Application
DMIs	De-Methylation-Inhibitors
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Phosphate
dpi	Days Post Inoculation
dt/ha	Decitonnes per Hectare
DTT	Dithiothreitol

E

EPPO	European and Mediterranean Plant Protection Organization
ESTs	Expressed Sequence Tags
ET	Ethylene
ERF	Ethylene Responsive Factor
ETS	Effector-Triggered Susceptibility

F

FAD	Flavin-Adenine Dinucleotide
FAO	Food and Agriculture Organization of the United Nations
FRAC	Fungicide Resistance Action Committee

G

g ai/ha	Grams Active Ingredient per Hectare
GA	Gibberellin
GA20-ox	Gibberellin 20 Oxidase
GO	Gene Ontology
GS	Growth Stage of Wheat

H

HR	Hypersensitive Response
HSP	Heat Shock Protein

I

IAA	Indole-3-Acetic Acid
ISP	Rieske Iron-Sulfur Protein
ISR	Induced Systemic Resistance

J

JA	Jasmonate
JA-Ile	Jasmonoyl-Isoleucine
JAMe	Jasmonate Methyl Ester
JAZ	Jasmonate Zim Domain

L

L/ha	Litters per Hectare
LSD	Least Significant Difference

M

MAMP	Microbe-Associated Molecular Pattern
MAPK	Mitogen-Activated Protein Kinase
MAS	Marker Assisted Selection
MBC	Methyl Benzimidazole Carbamates
MYA	Malt-Yeast Extract Agar

N

NADH	Nicotinamide Adenine Dinucleotide
NPR1	Non-Expressor of PR Genes1

P

PAMP	Pathogen-Associated Molecular Pattern
PCD	Program Cell Death
PCR	Polymerase Chain Reaction
PLS	Physiological Leaf Spot Disorder
PP	PhenylPyrroles
PR	Pathogenesis-Related Protein
PRR	Pattern Recognition Receptors
PTI	PAMP-Triggered Immunity

Q

Qi	Quinone binding site inside of the inner mitochondrial membrane
Qo	Quinone binding site outside of the inner mitochondrial membrane
QoI	Quinone Outside Inhibitor
qPCR	Quantitative Real Time PCR

R

RIN	RNA Integrity Number
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription PCR

S

SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SBIs	Sterol Biosynthesis Inhibitors
SDHIs	Succinate Dehydrogenase Inhibitors
SEM	Scanning Electron Microscope
SEPTTR	EPPO code system for the pathogen *M. graminicola* (*Zymoseptoria tritici*)
SLB	Septoria Leaf Blotch

T

TCA	Tricarboxylic Acid
TKW	Thousand Kernel Weight
TMV	Tobacco Mosaic Virus

U

UQ	Ubiquinone
USA	United States of America

1. Introduction

The demand for global food production is expected to increase due to the constantly rising world population which is estimated to reach 9.1 billion inhabitants by the year 2050. In order to feed the foreseen population, FAO estimates that global agricultural production would have to increase by 60% from the 2005-2007 levels (FAO, 2012b). Furthermore, a reduction in available land is expected to occur due to environmentally extreme situations such as drought and flooding; as well as through increased urbanization (Goklany, 1999). Nonetheless, technological improvements in the areas of plant breeding, fertilization and crop protection, together with optimized farming practices, have had a positive impact on agricultural productivity (Goklany, 1999; Oerke & Dehne, 2004). Furthermore, the income per capita is foreseen to increase 1.8 fold by 2050 (FAO, 2012b). With a wealthier world population, changes in dietary habits in developing countries towards more milk, meat and grain products are expected to occur, leading to an increase in the cultivation of monocultures (FAO, 2012b; Oerke & Dehne, 2004). According to the FAO (2013a), more than half of the world harvested area is destined to cereals such as rice, maize and wheat.

Even though high yielding cereal varieties have had a positive impact on the worldwide production of food, they may be more prone to pests and biological diseases leading to significant amount of yield losses (Oerke & Dehne, 2004). According to Oerke and Dehne (2004), taking into account the most cultivated crops, the estimated loss potential of yield due to biological stress factors such as weeds, fungal and bacterial pathogens, insects and viral diseases can reach up to 50%, 75% and 66% for wheat, rice and maize respectively, when no crop protection strategies are performed. The integration of crop protection practices can reduce the already mentioned losses to 29%, 37% and 20% in the respective crops. For this reason, safeguarding agricultural production either by chemical, biological or mechanical means is of great importance (Oerke & Dehne, 2004). However, in order to obtain the most benefits from applying agrochemicals, understanding the plant-pathogen-crop protection product interaction is of great importance in not only providing the best crop protection but also in obtaining the highest possible yield with the lowest fungicidal input. As that wheat is the most important cereal in Europe (FAO, 2012a), and the Septoria leaf blotch disease is considered to be the most economically damaging fungal disease in wheat growing areas

around the world (Ponomarenko *et al.*, 2011); this work focuses on the effects that fungicides with varying modes of action have on the control of this disease and their subsequent influence on yield at both molecular and physiological levels.

1.1 Wheat (*Triticum aestivum*)

According to the (FAO, 2012a; 2013a), 2.3 billion tons of cereals were produced worldwide in 2010, from which 43% were food products for human consumption, 33% were used as animal feed and the remaining 22% were destined for industrial purposes, seed for the next season or waste. Cereals are considered as the main source of food supply, with rice as the most important cereal followed by wheat and maize, whereas maize is mainly used as animal feed.

Triticum aestivum is a hexaploid wheat belonging to the grass family Poaceae, which represents one of the largest families of flowering plants (Winfield, n.d.). It is believed to have originated in south western Asia, in the region known as the Fertile Crescent and the Nile Delta, through repeated hybridizations of *Triticum* spp. with wild grasses members of the genus *Aegilops*. Upon each hybridization event, the chromosomes were doubled in the new hybrid (Winfield). The location of the Fertile Crescent between coastal, desert and mountainous terrain makes it one of the most diversified regions in the world; with a temperate Mediterranean climate in the west, a continental climate in the east, a desert climate along the Saharo-Arabian region in the south and the Euro-Siberian climate in the north(Bonjean & Angus, 2001).

The most ancient domesticated species of wheat is the Einkorn (*Triticum monococcum*) which is a diploid plant (genome AA); a subsequent hybridization between a wild grass and a Triticum plant gave rise to tetraploid wheats (genome AABB) such as *T. durum.* With its low levels in gluten, which make it suitable for macaroni products, *T. durum* is the most common tetraploid wheat currently used. Approximately 10.000 years ago, the hybridization between cultivated tetraploid emmer wheat (*T.* dicoccum) and a wild diploid grass gave rise to the hexaploid (genome AABBDD) wheat *T. aestivum*, also known as bread wheat (Bonjean & Angus, 2001; Oz *et al.*, 2001).

Wheat has been considered a key plant to the emergence of city-based societies throughout history due to its ability to i) grow in a wide range of environments, ii) its

large-scale production capabilities and iii) the possibility of its long-term storage as a food product (FAO, 2012a).

Among all cereals produced, wheat occupies third place after maize and rice with a world production volume of approximately 704 million tons in 2011, of which 65% is destined for food consumption, 17%for animal feed and 12% for industrial applications and biofuels. With an annual production volume of 226 million tons per year, wheat is the most important cereal in Europe; the largest share produced in France, with 38 million tons, followed by Germany with 23 million tons in 2011 (FAO, 2012a; 2013a).

Winter wheat is the most common type of wheat cultivated in Germany, where sowing starts in mid-September and lasts until late November, with October being the most common sowing month. Early sowing in the autumn can lead to foot rot diseases and dense crop population, which leads to higher foliar diseases risk in the vegetative growth period. Harvesting takes place from late July in the warmer south until the end of August in the north (Bonjean & Angus, 2001; Proplanta, 2006-2013).

As with all plants, wheat is subject to different kinds of stress that eventually have an effect on the yield and quality of the final product. Fungal diseases such as septoria leaf blotch (SLB), powdery mildew and brown leaf rust cause a reduction in the green leaf area of the top two leaves available for photosynthesis. Since these leaves significantly affect crop yield during the grain-filling period, the hindrance of their photosynthetic ability by fungal disease results in significant economic losses worldwide (Duveiller *et al.*, 2007; Wegulo *et al.*, 2009). Fungicide spraying either before or during anthesis has been the most important tool in controlling fungal diseases and increasing crop yield (Gooding *et al.*, 2000; Wiik, 2009; Wiik & Rosenqvist, 2010). However, Wegulo *et al.* (2011) demonstrated that the profitability of fungicide spraying depends on several factors such as weather conditions, cultivar resistance, wheat price, and disease severity.

1.2 Septoria Leaf Blotch

Septoria leaf Blotch (SLB) is a fungal disease produced by the hemibiotrophic fungus *Mycosphaerella graminicola* (Fuckel) J. Schröt in Cohn (anamorph *Septoria tritici* Roberge in Desmaz, recently renamed *Zymoseptoria tritici* (Desm.) Quaedvlieg & Crous), which, due to an increase in wheat cultivation area, lack of cultivar resistance in newly

introduced semi-dwarf varieties, early sowing, increased nitrogen fertilization, high summer rainfall and less management of crop residues due to low tillage programs, has experienced a significant shift in economic importance since the 1950s (Eyal, 1987; 1999; Lucas *et al.*, 1999). Since the top, or flag, leaf of wheat is considered to be the most photosynthetically active leaf, and to provide almost half of the carbohydrates to the developing grain (Gooding *et al.*, 2000), SLB epidemics can lead to low quality grains and to a yield reduction between 30 and 40% if not combatted (Eyal, 1987). Annual losses are estimated to be $400 million and $275 million in Europe and the United States, respectively. SLB is thus considered to be the most important economically damaging fungal disease in Europe and the second most important, after wheat rust, in the wheat growing regions of the United States (Ponomarenko *et al.*, 2011).

1.2.1 Disease cycle

The ascomycete fungus *M. graminicola* is the sexual stage of the pathogen (asexual stage: *Zymoseptoria tritici*), and belongs to the Mycosphaerellaceae family, which includes several other plant pathogenic fungi such as the banana black Sigakota *Mycosphaerella fijiensis*. Fungal propagation can be achieved either by sexual ascospores dispersed by wind over long distances or by asexual pycnidiospores disseminated over short distances by rain splash (R.-S. Chen & McDonald, 1996).

Disease epidemics take place in two phases; the so-called winter and summer epidemics (**Figure 1.1**). The main source of inoculum for the first phase originates predominately from air-blown ascospores present in plant debris from the previous season, which, depending on the environmental conditions in autumn and winter; cause and influence the severity of the primary infection for the following summer. *M. graminicola* infections generate lesions bearing pycnidiospores, which are repeatedly dispersed from lower to upper leaves within the same plant or to neighboring ones by rainfall splash, thus starting the secondary (summer) epidemic. Moreover, infection by air-borne ascospores from *M. graminicola* can complement the secondary epidemic caused by pycnidiospores from *Z. tritici*. Throughout the growing season, the fungus can have many sexual and asexual reproduction events that allow epidemics to develop rapidly. Nonetheless, disease severity depends on rainfall amounts and frequency as well as canopy structure (R.-S. Chen & McDonald, 1996; Eyal, 1999; Lucas *et al.*, 1999; Ponomarenko *et al.*, 2011).

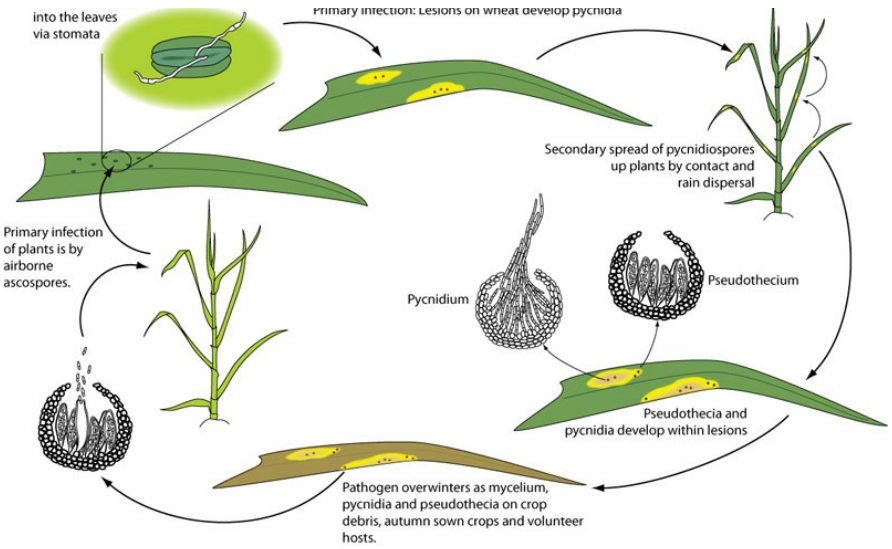


Figure 1.1. SLB disease cycle

Representation of Septoria Leaf Blotch on wheat produced by the fungus *Mycosphaerella graminicola*. Primary infection starts in autumn sown seedlings by airborne ascospores or rain-splashed pycnidiospores present in crop debris from the previous season. Secondary infections occur mainly from pycnidiospores, which are dispersed upwards and to neighboring plants during rainfall events. Source: Ponomarenko et al (2011)

1.2.2 Infection biology

Disease severity is higher in temperate climates with high rainfall. Disease symptoms are characterized by necrotic blotches that contain asexual reproduction structures, known as pycnidia, scattered within the lesion area (Kema *et al.*, 1996).

The *M. graminicola* infection starting from a germinating pycnidiospores is associated with a latent period that can extend several weeks before symptoms appear. Cytological analysis of the *M. graminicola* infection biology (**Figure 1.2**) have revealed that, upon inoculation with pycnidiospores, germination usually starts within two hours as germ tubes start branching and growing towards the stomatal apertures where penetration mainly occurs. Nonetheless, it has also been reported that fungal penetration can take place through the cuticle and even appressoria-like structures have been observed above stomatal apertures or at the junction of two epidermal cells. Unlike other fungal pathogens, *M. graminicola* does not require appressorium for successful plant penetration (Cohen & Eyal, 1993). Once the fungus has entered the host, colonization of the substomatal cavity and the neighboring regions begins. Fungal growth remains intercellular without causing any damage to the plant cell for a period of approximately 7 days. At this point in time, initial disease symptoms are visible as chlorotic spots on

the leaf surface (Duncan & Howard, 2000). Afterwards, the pathogen switches to a necrotrophic lifestyle characterized by rapid mycelial proliferation in the intercellular spaces of the epidermal and mesophyll cells, followed by the development of asexual sporulation structures in substomatal cavities. The pycnidia appear as black dots within the necrotic lesion (Duncan & Howard, 2000). This period is associated with the collapse of the host tissue and massive host cell death, providing the fungus with the nutrients needed for further colonization and pycnidium formation (Cohen & Eyal, 1993; Kema *et al.*, 1996; Keon *et al.*, 2007). Under ideal climate conditions for fungal infection, as mentioned above, fully developed disease symptoms in susceptible wheat cultivars are visible within 14 and 21 days after inoculation (Eyal, 1987; 1999; Kema *et al.*, 1996). In climates with high humidity, pycnidiospores are released from the pycnidium in an extracellular matrix known as the cirrus. Between five and ten thousand pycnidiospores are exuded from a pycnidium; with the majority being liberated during the first wetting (Duncan & Howard, 2000; Eyal, 1987; 1999).

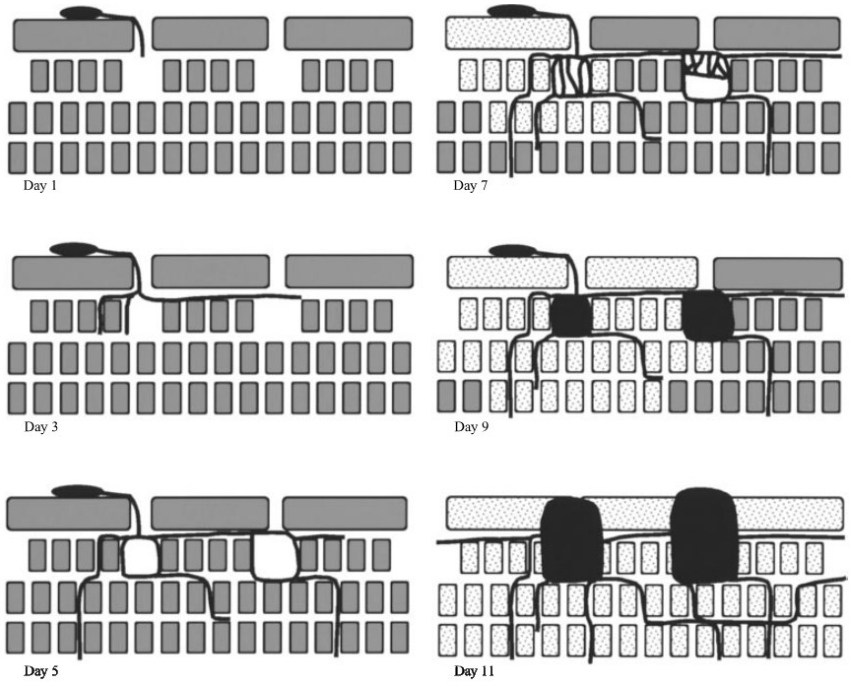


Figure 1.2. *M. graminicola* infection process

Microscopic, cross-sectional view of a wheat leaf representing the *M. graminicola* infection process in wheat. In each leaf, cells of the epidermis, mesophyll, stomatal openings and substomatal chambers are illustrated as filled rectangles. (Day 1): Pycnidiospore germination; germ tube formation and host cell penetration through stomatal opening. (Day 3): Hyphal colonization of subsomatal cavity and substomatal neighboring cells. (Day 5): Intercellular growth and colonization of adjacent substomatal cavities. (Day 7): Massive mycelial proliferation around substomatal cavities leading to an onset of macroscopic symptoms visible as chlorotic

spots on the leaf surface. (Day 9): Development of pycnidium in substomatal cavities as well as increase in cell chlorosis. (Day 11): Fully mature pycnidia visible as black dots within the necrotic lesion. Fungal hyphae continue to spread further. Source: Duncan and Howard, (2000)

Unlike *S. nodorum,* which is known to produce phytotoxic compounds upon plant cell death (Friesen *et al.*, 2006; 2007; Z. H. Liu *et al.*, 2004), known biologically active pathogen-produced toxins in the *M. graminicola*-wheat interaction have not yet been found, even though enough evidence for the involvement of a toxic compound exists (Eyal, 1999; Keon *et al.*, 2007; Ponomarenko *et al.*, 2011). Nonetheless, three *Cladosporium fulvum* effector homologs for the gene Ecp2 have been identified in the *M. graminicola* and *M. fijiensis* genome (Stergiopoulos *et al.*, 2010). Even though the function of the Ecp2 gene is unknown, treatment of the *M. fijiensis* Ecp2 homolog in tomato plants induced necrosis (Stergiopoulos *et al.*, 2010). In addition to these results, mutant *C. fulvum* without the Ecp2 gene showed reduced fungal infection; both findings indicate that Ecp2 is a virulence factor (Laugé *et al.*, 1997).

1.2.3 Control measures

Understanding the biology of the plant-pathogen interaction plays a vital role in the continual development of effective control strategies (Lucas *et al.*, 1999). Based on this knowledge, SLB disease control currently relies on resistance breeding, intensive fungicide use, crop rotation measures, residue management and disease forecasting (Eyal, 1999; Lucas *et al.*, 1999; Wegulo *et al.*, 2009)

1.2.3.1 Resistance breeding

Due to the increased use of fungicides in controlling Septoria Leaf Blotch (SLB) epidemics, concern over rising prices for agrochemicals and their environmental impact along with growing signs of fungicide resistance; breeding crops for higher resistance against SLB has gained interest in both the agricultural and scientific world (Arraiano *et al.*, 2001; 2009). SLB resistance can be divided into two categories; specific and quantitative. Specific resistance is monogenic and follows the gene for gene model which, however, is only effective against certain *M. graminicola* isolates (Brading *et al.*, 2002). Quantitative resistance is polygenic; it is isolate non-specific and is considered to be more durable than specific resistance. Although is not as complete as specific resistance (Brown, 2002; Chartrain *et al.*, 2004a).

Since their first discovery in the mid-1960s, several genes conferring resistance to *M.* graminicola have been identified and mapped to different regions and chromosomes of wheat (**Table 1.1**). Of these, *Stb 1* has displayed the most durable resistance since the 1970s in wheat growing areas in the United States (Adhikari *et al.*, 2003; 2004a). *Stb 4* was extensively used in California since 1975 and in Oregon in the 1990s, but in the past ten years it has been losing its effectiveness in the field (Adhikari *et al.*, 2004b). Only after three years of its introduction in the Willamette Valley in Oregon, *M. graminicola* isolates started to become virulent to *Stb 4* (Cowger et al 2000). Nowadays however, wheat cultivars containing the *Stb4* gene are considered susceptible (Goodwin, 2007). *Stb 6*, which follows in combination with isolate IPO323 of *M. graminicola* the gene-for-gene model (Brading *et al.*, 2002), has been widely used worldwide in breeding programs (Chartrain et al 2004b) and is considered the most common type of SLB resistance in wheat germplasm in the world (Chartrain *et al.*, 2005b). However, the possibility that *Stb 6* resistance can be overcome by *M. graminicola* due to selection pressure is very high (Brading *et al.*, 2002; Cowger *et al.*, 2000; Orton *et al.*, 2011).

Although resistant cultivars against SLB exist and are used in modern agriculture, the mechanisms behind their durability are unknown (Chartrain *et al.*, 2004b). The possibility that this pathogen will overcome current resistant varieties is very high due to its rapid evolution upon selection pressure and its genetic diversity. Therefore, the adoption of *Stb* resistance genes in current breeding programs is scarce; hence the control of *M. graminicola* still relies heavily on fungicide use (Cools *et al.*, 2007; Thygesen *et al.*, 2008).

1.2.3.2 Chemical control

Seed treatments are currently used in order to control seed-borne infections and to protect wheat seedlings from air-borne ascospores during autumn. Nonetheless, they cannot prevent severe epidemics during the vegetative growth period in the spring (Lucas *et al.*, 1999).

Table 1.1: Resistance genes in wheat to septoria leaf blotch

Gene	Source variety	Chromosomal location[a]	Molecular markers[b]	Reference
Stb 1	Bulgaria 88	5BL	Xbarc74 (2.8)	Adhikari *et al.* (2004a); Rillo and Caldwell (1966)
Stb 2	Veranopolis	1BS	Xwmc40 (6) Xbarc008 (5)	Y. Liu et al. (2012); Wilson (1985)
Stb 3	Israel 493	7AS	*Xwmc83* (2.1)	Goodwin *et al.* (2007); Wilson (1985)
Stb 4	Tadinia	7DS	Xgwm111 (0.7)	Adhikari *et al.* (2004b); Somasco *et al.* (1996)
Stb 5	SHW 'Synthetic 6x'	7DS	Xgwm44 (7.0)	Arraiano *et al.* (2001)
Stb 6	Flame	3AS	Xgwm369 (2.0)	Brading *et al.* (2002)
Stb 7	Estanzuela Federal	4AL	Wmc313	McCartney *et al.* (2003)
Stb 8	SHW 'W7984'	7BL	Xgwm146 (3.5) Xgwm577 (5.3)	Adhikari *et al.* (2003)
Stb 9	Courtot	2BL	Xfbb226 (3.6) XksuF1b (9)	Chartrain *et al.* (2009)
Stb 10	Kavkaz-K4500 L.6.A.4 (KK)	1Dc	Xgwm848 (8)	Chartrain *et al.* (2005c)
Stb 11	TE 9111	1BS	Xbarc008	Chartrain *et al.* (2005a)
Stb 12	Kavkaz-K4500 L.6.A.4 (KK)	4AL	Xwmc219 (13) Xwmc313 (16)	Chartrain *et al.* (2005c)
Stb 15	Arina	6AS	Xpsr904 (14)	Arraiano *et al.* (2007)
Stb 16	SHW 'M3'	3DL	Xgwm494	Ghaffary *et al.* (2012)
Stb 17	SHW 'M3'	5AL	Xhbg247	Ghaffary *et al.* (2012)
Stb 18	Balance	6DS	Xgpw3087 Xgpw5176	Ghaffary *et al.* (2011)

[a] S: short arm of the chromosome, L: long arm of the chromosome, SHW: synthetic hexaploid wheat
[b] Closest molecular markers reported with their genetic distances in centiMorgans (cM)

In adult plants, foliar fungicides are applied in order to prevent green leaf area reduction related to fungal diseases and avoid the subsequent loss in yield (Cook *et al.*, 1999). From the total estimated volume of agrochemicals used in Europe to control fungal diseases on cereals, approximately 70% is employed for the control of SLB (Goodwin, 2007; Ponomarenko *et al.*, 2011). The use of fungicides has not only improved the control of *SLB*, it has also helped to increase yields of cereals in Europe by protecting and extending the green leaf area duration of the flag leaf. Up to 42% yield loss could be avoided by applying foliar fungicides under moderate to severe disease levels (Wegulo *et al.*, 2009; 2011).

The period with the highest risk of yield-damaging epidemics is considered to be from the start of the emergence of the second leaf from the top until ripening. It is therefore of great importance to protect the plant throughout this period (Lucas *et al.*, 1999).

Several spraying experiments have demonstrated that appropriate timing and type of fungicide active ingredient used play a major role in the control of SLB; where a single fungicide application between wheat growth stages (GS) 37-39 (**Appendix 1**) provided the best disease control and the most significant yield benefit (Cook & Thomas, 1990; Hardwick *et al.*, 2001; Lucas *et al.*, 1999; Wiik, 2009). Furthermore, in order to achieve a yield increase in north European countries, where the ripening process tends to take longer, a secondary spray at GS 59 is recommended (Cook & Thomas, 1990). Cook *et al.* (1999) demonstrated that an average yield loss of 17 kg per hectare occurs by each day that a fungicide treatment is delayed beyond growth stage 37 (**Appendix 1**).

Among the available fungicides, the triazoles group are not only very successful in managing fungal diseases like SLB, brown rust and Fusarium head blight, but also in delaying flag leaf senescence and thus increasing grain protein content and yield (Blandino & Reyneri, 2009; Watson *et al.*, 2010). However, even the most effective triazole fungicide is limited to hyphal colonization. Once pycnidia appear, there is no available fungicide that is able to control the disease, thus flag leaf protection has to be performed before disease symptoms appear (Lucas *et al.*, 1999).

1.2.3.3 Integrated disease management or cultural practices

Changes in agricultural practices have had an effect on the development of wheat fungal diseases which were not considered economically significant in the past. Some of the most predominant include; i) the introduction of shorter early-maturing wheat varieties in order to enhance yield and avoid lodging lead to an increase in the occurrence of fungal diseases sprayed by rain splash like SLB and tan spot as observed by the higher susceptibility of these wheat plants towards the mentioned diseases (Eyal, 1987; McIntosh, 1998). ii) The switch towards low tillage programs, on the other hand, where crop debris is left on the soil surface, has had an impact on the development of SLB and tan spot epidemics and the subsequent increase in fungicide use (Bockus & Claassen, 1992; McIntosh, 1998; Wegulo *et al.*, 2009; 2011).

Other common practices that can help to decrease the primary infection levels and thus, lowered spring epidemics include crop rotation with non-host plants, plowing of crop debris and late planting of winter wheat (Ponomarenko *et al.*, 2011). Moreover, the use of resistant semi-dwarf varieties can help to avoid or alleviate the susceptibility to SLB while maintaining their high yield properties (Adhikari *et al.*, 2004a). Additionally, by using marker assisted selection (MAS) in wheat breeding programs, stacking of isolate-specific genes associated with SLB resistance may help to increase their durability and hence, decrease fungicidal input (Chartrain *et al.*, 2004b; 2005a; 2005c; Ghaffary *et al.*, 2011; 2012)

Conventional agriculture decisions including fungicide application are based on the growth stage of the plant rather than on pathogen epidemiology (Burke & Dunne, 2008). Targeted fungicide spraying programs based on infection events rather than standard spraying regimes can not only help to reduce fungicidal use while still protecting the net yield, they can also help to avoid the development of fungicide resistance (Beyer *et al.*, 2012; Burke & Dunne, 2008). However, due to the long latent period of SLB development, infection events may be difficult to identify and when the first disease symptoms become visible, fungicide application would already be ineffective. Therefore, several disease models have been developed (Henze *et al.*, 2007; Verreet *et al.*, 2000) with the goal of forecasting *M. graminicola* infection events based on meteorological data and thus give guidance on the timing of fungicide application.

1.3 Fungicides

Even though compounds like sulfur, copper and lime have been used to control diseases in plants since the beginning of agriculture, active research on new chemistries, activity spectra and application techniques did not start until the beginning of the 20th century, albeit limited to chemical compounds with protectant activities but low residual activity. In the mid- to late-1900s, the first systemic products that could either be applied to the leaves or the roots to enter the plants through their vascular system, entered the market (Russell, 2005). Due to this advantage, systemic fungicides became part of a routine-based crop protection method in cereal production (Dunne, 2002). Between the 1960s and 1980s, the first fungicides with a single mode of action, the most predominant being the carboxamides and triazoles, due to their enhanced disease control and lower rates,

entered the market and soon became the standard plant protection products against various cereal diseases (Kuck *et al.*, 2012a; PhillipsMcDougall, 2012). In 1997, a new family of fungicides reached the market; the Quinone outside inhibitors (QoI), also known as strobilurins, not only provided control against a broad spectrum of pathogens but also displayed physiological properties related to increased yields (PhillipsMcDougall, 2012; Russell, 2005). Although single target fungicides proved to be more effective in controlling fungal pathogens, they presented a new problem; resistance development (Kuck *et al.*, 2012a). The widespread use of the strobilurins since their market introduction led to rapid resistance development in certain pathogen populations like *M. graminicola* and *Blumeria graminis* (Earley et al., 2012). At present, QoI fungicides are only used as mixture partners with a restricted number of applications per season, as suggested by the Fungicide Resistance Action Committee (FRAC) (PhillipsMcDougall, 2012; Russell, 2005). Research on new chemistries that required lower dosages, and are safer not only to the environment but also to humans has continued, with the most recent market introduction of a new generation of carboxamide fungicides in 2011 and 2012 (PhillipsMcDougall, 2012).

In 2011, European countries dominated the global fungicide market with approximately 70% of the total market share, 50% of which attributable to France, Germany and the United Kingdom (PhillipsMcDougall, 2012). According to the FRAC, more than forty fungicide modes of action are currently available, though not all are accessible in every country due to regulatory differences or market size (Kuck *et al.*, 2012a). Over the last years, two fungicide families have dominated the agrochemical business in cereals, the QoI and DMI fungicides (**Table 1.2**), with 24 and 27% market share, respectively (Kuck *et al.*, 2012a; PhillipsMcDougall, 2011; Thygesen *et al.*, 2008). Nonetheless, with the successful introduction of a new generation of carboxamide fungicides belonging to the succinate dehydrogenase inhibitors (SDHI) group, the distribution of the fungicide market is expected to change as this group of chemicals gains market share (Kuck *et al.*, 2012a; topagrar, 2011; 2013).

Table 1.2: Percentage of fungicide market sales by chemical sector in 2010

Fungicide group[a]	Mode of action[a]	Target site[a]	%
DMI	Sterol biosynthesis in membranes	C14-demethylase	27.5
QoI	Cellular respiration	Complex III: cytochrome bc1 complex	24.1
Dithiocarbamates	Multi-site contact activity		7
Inorganics	Multi-site contact activity		5.8
Phthalimides	Multi-site contact activity		3.9
Other multisite fungicides			2.8
MBC	Mitosis and cell division	β-tubuline	3.8
SDHI	Cellular respiration	Complex II: succinate-dehydrogenase	3.7
Phenylamides	Nucleic acid synthesis	RNA polymerase I	3.1
Morpholines	Sterol biosynthesis in membranes	Δ^{14}-reductase $\Delta 8 \rightarrow \Delta 7$ isomerase	2.7
Anilino-pyrimidines	amino acid and protein biosynthesis	Methionine biosynthesis	1.9
Dicarboxamides	-	-	1.5
Other fungicides			12.2
Total fungicide sales in 2010: $ 11,475 million			

Adapted from: (PhillipsMcDougall, 2011)
[a]According to the Fungicide Resistance Action Committee (FRAC)
Abbr. DMI: DeMethylation inhibitors, QoI: Quinone outside inhibitors, MBC: Methyl-benzimidazole-carbamates, SDHI: Succinate dehydrogenase inhibitors

1.1.1 Sterol biosynthesis inhibitors (SBIs)

Sterols are organic molecules with great importance in eukaryotes, where they are essential in the organization and structure of membranes, determine membrane fluidity and permeability, and serve as precursors for hormones essential for regulation, growth and development (Dupont *et al.*, 2012; Lepesheva *et al.*, 2008). There are three main types of sterols that vary depending on the organism; cholesterol is predominant in vertebrates, phytosterol in plants and ergosterol in fungi (Dupont *et al.*, 2012). The synthesis of sterols in vertebrates, plants and fungi shares a common pathway, starting at acetyl CoA up to squalene (Dupont *et al.*, 2012; Mercer, 1984). In most of the fungi belonging to the Ascomycota and Basidiomycota families, ergosterol is the most common type of sterol present in their membranes. Based on this along with the fact that the post-squalene segment, from lanosterol to ergosterol, are specific for fungi, has led this part of the pathway to be of great importance in research and development of new targets for antifungal compounds in the agrochemical and pharmaceutical industries (Kuck *et al.*, 2012b; Mercer, 1984; 1991)

The post-squalene segment of sterol synthesis in fungi includes eleven enzymes that catalyze the main steps from squalene to ergosterol (Kuck *et al.*, 2012b). Even though the reaction sequence is not the same for all fungi, the pathway displayed in **Figure 1.3** can be considered as representative (Kuck *et al.*, 2012b; Mercer, 1984; 1991). Different agrochemical compounds within the SBI family inhibit different steps of the pathway (**Figure 1.3**). The inhibition of the 14-demethylation step represents the most important group of fungicides over the last 30 years based on their rising market share and the number of compounds developed since their introduction (Kuck *et al.*, 2012b; Mercer, 1991). By inhibiting the synthesis of ergosterols, SBIs hinder the *de novo* synthesis and the deterioration of existing fungal membranes (Kuck *et al.*, 2012b).

1.3.1.1 De-Methylation inhibitors (DMI)

De-Methylation-Inhibitors (DMIs) target the haeme-iron part of C-14 demethylase, a P450 enzyme in the sterol biosynthesis pathway (**Figure 1.3**). By inhibiting the activity of this vital enzyme, no final sterols can be synthesized and an accumulation of 14α-methyl sterols is observed (Burden *et al.*, 1989; Kwok & Loeffler, 1993) leading to membrane destabilization, abnormal fungal growth such as incomplete septum formation, irregular cell-wall thickening, excessive branching and cell necrosis eventually leading to fungal death (Burden *et al.*, 1989; Han *et al.*, 2006; Kuck *et al.*, 2012b). These agrochemicals have no outstanding preventative activity because they do not hinder the pathogens from obtaining enough ergosterol from reserves stemming from the fungal spores and thus, have no effect on spore germination (Bartlett *et al.*, 2002; Pontzen & Scheinpflug, 1989).

With more than 30 agricultural compounds belonging to this fungicide family, and due to their broad spectrum of action, the DMI class of sterol inhibitors are considered the most important SBI fungicides with high curative and eradicative activity (Kuck *et al.*, 2012b). Within the DMI group of fungicides, one particular chemical group, the triazoles, dominates the market (Kuck *et al.*, 2012b). In 2010, 20% of total fungicide sales were attributed to the triazoles group. Furthermore, in 2010, five out of the fifteen leading fungicides in the global market (**Table 1.3**) belonged to this group (PhillipsMcDougall, 2011).

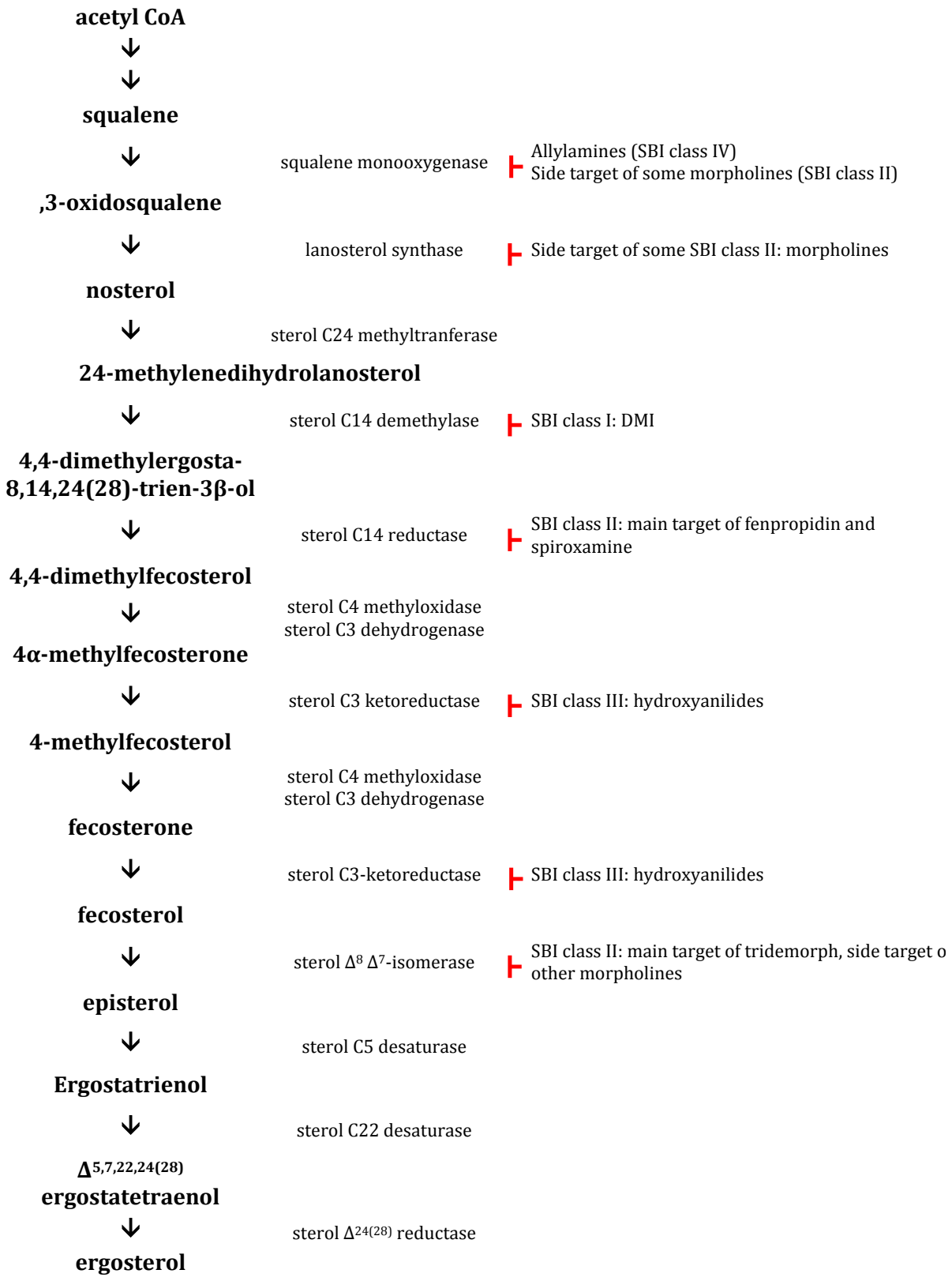


Figure 1.3: Post-squalene segment of the ergosterol biosynthesis in Ascomycota and Basidiomycota

Pathway of ergosterol biosynthesis in fungi of the ascomycetes and basidiomycetes. Products of each reaction are displayed in bold. The central column displays the name of each enzyme, followed by the sites of inhibition by the different SBI fungicides classes on the right as indicated by (⊢). Abbr.: SBI: sterol biosynthesis inhibitors, DMI: De-Methylation inhibitors. Adapted from: Kuck *et al.* (2012b)

The dominance of the triazoles in the cereal market can be attributed to ever-present spreading of the Septoria Leaf Blotch disease, the effectiveness of these agrochemicals against this pathogen and the moderate development of resistance that occurs according to FRAC estimates (Kuck *et al.*, 2012b). However, over the years, a gradual reduction in their effectiveness, known as shifting, has been observed. Reasons for the decline in the fungicidal performance of triazoles include, among others: i) mutations in the gene CYP51, which encodes the 14α-demethylase enzyme, causing a reduction in fungicide binding capacity. ii) Changes in the expression profile of genes encoding efflux pumps like membrane-bound transporters that could release the chemicals from the cells. iii) Overexpression of the gene encoding the 14α-demethylase enzyme, which has been observed in both human and plant pathogens with azole resistance phenotypes (Cools & Fraaije, 2008; 2013; Kuck *et al.*, 2012a; 2012b; Thygesen *et al.*, 2008). In spite of this, triazoles are still the most important chemical control against *M. graminicola* (Cools & Fraaije, 2008; Thygesen *et al.*, 2008). Nonetheless, constant tracking of changes in azole sensitivity in field isolates is essential in maintaining the effectiveness of this class of fungicides against important fungal diseases like SLB in the future (Cools & Fraaije, 2013).

It has been reported that SBI fungicides also have a physiological effect on plants; after application of products belonging to this class, shorter nodes and internodes as well as smaller dark green leaves have been observed, which had led to the suggestion that SBIs can act as regulators of plant growth (Kuck *et al.*, 2012b). Activity of DMI fungicides on cytochrome P450 monooxygenases in plants, such as the 14α-demethylase in sterol biosynthesis and the kaurene oxidase in the gibberellin synthesis pathway, has been suggested as the mode of action in which triazoles retard plant growth (Benton & Cobb, 1995; Burden *et al.*, 1989; Rozhon *et al.*, 2013). Furthermore, evidence of the possible effect of triazoles on the brassinosteroid-dependent sterol synthesis in plants was demonstrated by Rozhon *et al.* (2013) in an experiment where monocotyledonous and dicotyledonous plants were treated with the pharmaceutical fungicide Voriconazole. The treatment led to phenotypic changes characteristic of those associated with brassinosteroid deficiency, such as reduced size, shortened hypocotyls and internode elongation, reduced root growth and dark green leaves. Additionally, Rozhon *et al.*

(2013) were able to demonstrate that the observed phenotype could be rescued by exogenous application of the phytohormone brassinosteroid.

Table 1.3: Leading fungicides in the global cereal market in 2011

Product	Chemical group[a]	Fungicide group[a]	Sales ($m)
Azoxystrobin	Methoxy-acrylates (Strobilurin)	QoI	1,065
Pyraclostrobin	Methoxy-carbamates (Strobilurin)	QoI	700
Mancozeb	Dithio-carbamates	Multi-sites Dithiocarbamates	570
Trifloxystrobin	Methoxy-acrylates (Strobilurin)	QoI	535
Copper fungicides	Inorganic	Multi-sites Inorganic	450
Epoxiconazole	Triazole	DMI (SBI)	415
Tebuconazole	Triazole	DMI (SBI)	395
Prothioconazole	Triazolinthiones	DMI (SBI)	385
Metalaxyl	Acylalanines	Phenylamides	305
Boscalid	Pyridine- carboxamides	SDHI	295
Chlorothalonil	Chloronitriles	Multi-sites Chloronitriles	285
Cyproconazole	Triazole	DMI (SBI)	250
Propiconazole	Triazole	DMI (SBI)	250
Difenoconazole	Triazole	DMI (SBI)	225
Thiophanate	Thiophanates	MBC	190
Carbendazim	Benzimidazoles	MBC	190
Fludioxonil	Phenylpyrroles	PP	190

Adapted from: PhillipsMcDougall (2011)
[a] According to the Fungicide Resistance Action Committee (FRAC)
Abbr. , QoI: Quinone outside inhibitors ; DMI: De-Methylation inhibitors, SBI: Sterol biosynthesis inhibitors, SDHI: Succinate dehydrogenase inhibitors; MBC: Methyl Benzimidazole Carbamates; PP: PhenylPyrroles

Besides the physiological effects of triazole treatments on plants, Siefert *et al.* (1996) suggested that the triazole fungicide Epoxiconazole has an influence on the plant´s immune system. According to their findings, Epoxiconazole treatment of wheat plants triggers a *de novo* synthesis of the fungal hydrolases chitinase and β-1,3-glucanase in the shoots. Based on this reaction, they suggest that the effectiveness of Epoxiconazole as a fungicide is due to its effect on both the fungus as well as its enhancement of the plant's immune system.

1.3.2 Cellular respiration inhibitors

Carbohydrates and fatty acids are the main sources of energy for the majority of cellular processes. The breaking-down of these substances takes place through cellular

respiration, with the corresponding release of energy due to a series of oxidation reactions. The first step of the cellular respiration takes place in the cytoplasm, where glucose degradation leads to pyruvate. Subsequently, pyruvate enters the mitochondrion where it is oxidized in order to produce carbon dioxide, water and energy in the form of ATP. The process that leads to ATP production is known as oxidative phosphorylation (Agrios, 2005; Earley *et al.*, 2012). Once the substrates for the oxidation are inside the mitochondrion, a series of redox reactions with the transfer of electrons from NADH and/or FADH to oxygen takes place through protein complexes located in the inner mitochondrial membrane, a process known as the electron transport chain. Together with the transfer of electrons to the protein complexes, the release of hydrogen ions outside the inner mitochondrial membrane space ensues. Every time a proton is pumped out of the inner mitochondrial membrane, an electrochemical gradient is built, leading to the synthesis of ATP by the enzyme ATP synthase (**Figure 1.4-A**) (Earley *et al.*, 2012; Saraste, 1999)

Between the protein complexes, electrons are transported by carriers such as Ubiquinone and cytochrome c. Initially, two electrons are transferred from the reduced nicotinamide adenine dinucleotide (NADH) to the complex I and four protons are pumped out. Subsequently, the two electrons are transferred to ubiquinone (UQ), which is in charge of passing them to the complex III. In this complex, one electron is used for the generation of a proton via the Q cycle mechanism (**Figure 1.4-C**). This mechanism involves using one electron for the oxidation and deprotonation of UQ at a site outside of the inner mitochondrial membrane (Q_o) to reduce UQ at a site inside of the inner mitochondrial membrane (Q_i). The other electron stemming from ubiquinone is transferred to the cytochrome c via the Rieske iron-sulfur protein (ISP). Finally, cytochrome c transfers one electron at a time to the fourth complex, which, once it has received four electrons, catalyzes the reduction of oxygen together with the release of hydrogen ions outside of the inner mitochondrial membrane.

In addition to complex I, the electron transfer chain can also start in the succinate dehydrogenase, otherwise known as the complex II. Unlike complex I, where the oxidation reaction is catalyzed by NAD^+, in complex II the electron from the oxidation of succinate to fumarate is generated by flavin-adenine dinucleotide (FAD). FAD then, via the iron cluster located in the subunit B of the complex II, transfers the electrons to

ubiquinone, which binds where domains B, C and D converge (**Figure 1.4-B**). Once UQ has been reduced, it can continue the electron transfer to complex III. The succinate dehydrogenase protein complex is the only one in the electron transport chain that does not release protons, but it is also the only protein complex in the mitochondrial respiratory chain that also participates in the tricarboxylic acid (TCA) cycle (Earley *et al.*, 2012; Saraste, 1999; Scalliet *et al.*, 2011).

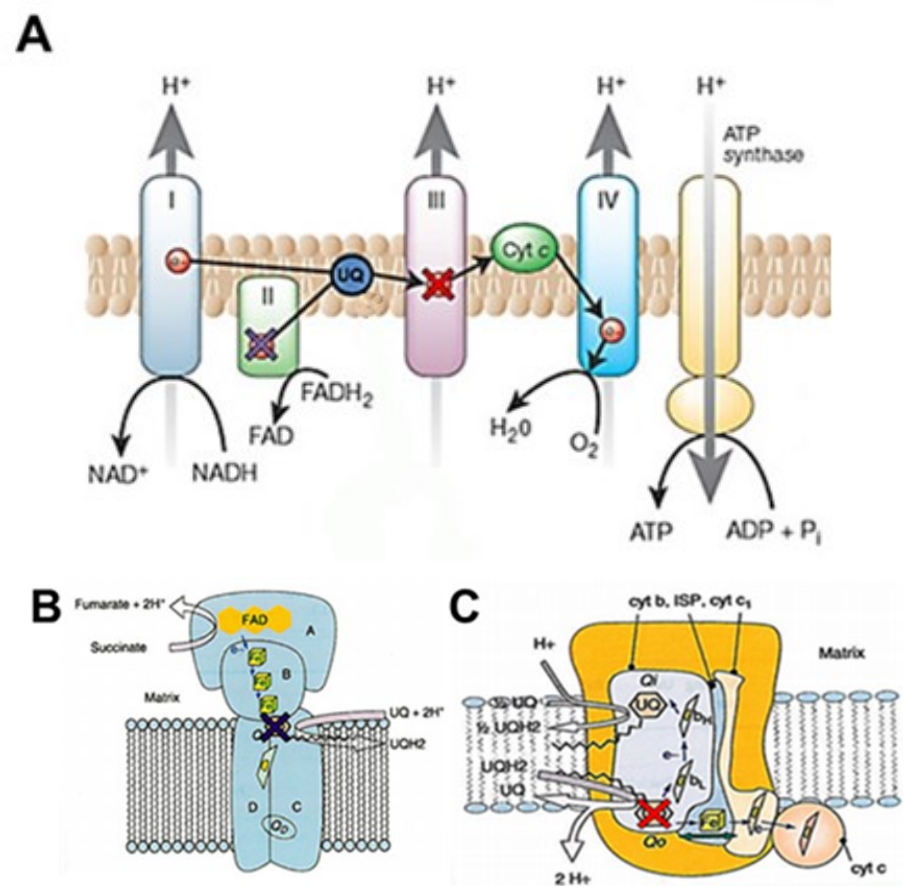


Figure 1.4: Mitochondrial electron transport chain

Diagram representing the oxidative phosphorylation and the active site of fungicides that inhibit the process. **A** Electrons originating from the oxidation of nutrients such as glucose or succinate are transferred from the reduced cofactors NADH and $FADH_2$ to oxygen through a series of protein complexes located on the inner mitochondrial membrane. When electron transfer to one of the protein complexes occurs, hydrogen ions are pumped to the outside of the inner mitochondrial membrane. The electrochemical gradient generated through this process is the driving force for ATP synthesis by ATP synthase. By obstructing the oxidative phosphorylation process (purple and red crosses), fungicides are able to block the energy production capability of fungi and inhibit their growth **B**. Detailed look at the succinate dehydrogenase or complex II. Carboxamide fungicides block the UQ binding site at complex II of the electron transport chain (purple cross) and, thus, obstruct the electron transfer from succinate to UQ via FAD and via the iron clusters located in the complex domains A and B. **C.** Detailed view of the complex III, also known as the cytochrome c reductase. Strobilurin fungicides block the oxidation of UQ at the site outside of the inner mitochondrial membrane (red cross), preventing the transfer of electrons to the Qi site and to the cytochrome c. For this reason, strobilurins are also known as Qo inhibitors.

Abbr. H^+: hydrogen ions, NAD+: Nicotinamide adenine dinucleotide, NADH: reduced NAD+, FAD: Flavin adenine dinucleotide, $FADH_2$: reduced FAD, O_2: oxygen, H_2O: water, ATP: Adenosine triphosphate, ADP: Adenosine diphosphate, Pi: Phosphate, UQ: ubiquinone, UQH_2: reduced ubiquinone, Qo: Quinone binding outside of the inner mitochondrial membrane, Qi: Quinone binding inside of the inner mitochondrial membrane, cyt: cytochrome, ISP: Rieske iron-sulfure protein. Adapted from: Brownlee (2001); Earley *et al.* (2012)

As that oxidative phosphorylation is an indispensable process for every obligate aerobic organism, it has been a leading target in the development of agrochemicals to control fungal diseases and pests (Earley *et al.*, 2012). Among the major available fungicides on the global market, the Qo, or complex III, inhibitors together with the succinate dehydrogenase, or complex II, inhibitors represent a quarter of the global market (**Table 1.2**).

1.3.2.1 Complex III inhibitors

Strobilurins belong to the QoI, or complex III, inhibitors that bind to the Q_o site of the cytochrome b in the inner mitochondrial membrane of fungi (**Figure 1.4-C**), causing a disruption in the electron transport chain that eventually obstructs ATP production (Bartlett *et al.*, 2002; Earley *et al.*, 2012). Spore germination and early germ tube growth are highly energy-demanding processes in fungi. By inhibiting this process, fungicides acting on the oxidative phosphorylation are considered to display strong preventative activity, hindering pathogen growth before it can penetrate the host plant and cause damage. (Bartlett *et al.*, 2002; Earley *et al.*, 2012). Additionally, fungal growth inhibition has also been reported in pathogens after infection has occurred but before the development of disease symptoms, evidence of the curative activity of these agrochemicals. Nonetheless, in order to provide optimum disease control, Qo inhibitors are recommended as preventative products (Bartlett *et al.*, 2002).

The introduction of the strobilurin group of fungicides in 1996 provided additional, highly effective opportunities against *M. graminicola* due to the group's preventative and curative action, low application rates and, ultimately, broad-spectrum activity that encompassing all classes of pathogenic fungi, such as ascomyscetes, basidiomycetes and oomycetes (Bartlett *et al.*, 2002; Earley *et al.*, 2012). Strobilurins have been considered as the most important group of fungicides after the introduction of the DMIs (Russell, 2005). Within four years of its market introduction, it represented 10% of the global fungicide sales, reaching USD 620 million (Bartlett *et al.*, 2002). Sales have increased

continuously since then, reaching approximately USD 2,800 million in 2010 with almost one quarter of global fungicide market share (PhillipsMcDougall, 2011).

Along with remarkable antimycotic performance, a boost on grain yield associated with greener leaves, delayed senescence and increased drought resistance has been reported after strobilurin treatment (Grossmann & Retzlaff, 1997; Grossmann *et al.*, 1999), especially after Pyraclostrobin application (Earley *et al.*, 2012). The observed physiological effects are attributed to the inhibition of the complex III in the plant electron transport chain along with changes in the plant phytohormonal levels. According to Gerhard *et al.* (1998); Grossmann and Retzlaff (1997); and Koehle *et al.* (2002), inhibition of mitochondrial respiration in plants leads to higher biomass production due to increased CO_2 uptake and enhanced nitrogen assimilation rates, which are caused by the activation of the alternative oxidase pathway and increased nitrate reductase levels. In addition to the above mentioned effects on the plant electron transport chain, reduced ethylene levels in plants submitted to drought stress have also been reported. Reduction in the ethylene biosynthetic process can have a positive impact on the grain-filling period and yield production by prolonging the photosynthetic capacity of the leaves through a slower ripening process (Gerhard *et al.*, 1998; Grossmann *et al.*, 1999; Koehle *et al.*, 2002).

Because these physiological effects were observed both under disease and low disease pressure, new market opportunities have been found besides the protection of crops from fungal diseases. Nowadays, products like Pyraclostrobin are offered as plant physiology enhancers in maize and soybean cultures (Earley *et al.*, 2012; PhillipsMcDougall, 2011).

Nonetheless, due to the intensive use of this class of fungicides resistant strains of important plant pathogenic fungi like *M. graminicola* and *B. graminis*, were identified soon after its market introduction, and have dispersed rapidly across the United Kingdom and Ireland (Fraaije *et al.*, 2005). In the meantime, in northern Europe, *M. graminicola* populations are completely resistant to QoI fungicides (Stammler *et al.*, 2012). Unlike to the polygenic resistance observed with the DMI fungicides, resistance to the Qo inhibitors is caused due to a single point mutation at the position 143 in the cytochrome b gene of fungi that changes the amino acid glycine to alanine (G143A

mutation). This single-point mutation at the target site of Qo inhibitors, leads to complete loss of function of the fungicide by modifying its binding site (Earley *et al.*, 2012; Gisi *et al.*, 2002), and to low or no fitness penalty for the pathogen (Fraaije *et al.*, 2005; Gisi *et al.*, 2002; Kuck *et al.*, 2012a). Other resistance mechanisms with minor agronomic impacts have been identified in other fungal pathogens such as the phenylalanine to leucine mutation (F129L) of the cytochrome b gene (Bartlett *et al.*, 2002; Earley *et al.*, 2012; Gisi *et al.*, 2002; Lucas & Fraaije, 2008). Even though strobilurins are no longer active against resistant *M. graminicola* populations, they are still a recommended active ingredient in modern fungicide products, due to their yield enhancement effects (Kildea *et al.*, 2010; McCartney *et al.*, 2007), their broad spectrum activity and the lack of resistance development on important pathogen populations, especially those belonging to the rust diseases (PhillipsMcDougall, 2011).

Nowadays, agrochemical products for the cereal market are preferably used in mixtures with active ingredients with different modes of action in order to avoid resistance development (PhillipsMcDougall, 2011). In the German cereal market for example, Pyraclostrobin is recommended as a co-formulation with Epoxiconazole and Fenpropinorph, as the product with the trade name Diamant® (BASF) for the control of the major fungal diseases like powdery mildew, net blotch, leaf spot, yellow and brown leaf rust, and the Septoria diseases (topagrar, 2011; 2013). Nonetheless, more than two applications of a strobilurins-based product during a growing season is not recommended (FRAC, 2012a). Additionally, constant monitoring of resistance development is of great importance not only to analyze the distribution of resistant isolates, but also to identify the outbreak of new resistant pathogens.

1.3.2.2 Complex II inhibitors

Complex II inhibitors comprise a wide range of chemicals responsible for the inhibition of the ubiquinone binding site in the succinate dehydrogenase (SDH) enzyme of the electron transport chain (**Figure 1.4-B**). In this way, fungal growth is significantly constrained due to the lack of energy production (Earley *et al.*, 2012). Even though this class of chemical crop protection products has existed for more than forty years, a revival of the SDHI fungicides started with the introduction of Boscalid by BASF in 2003. This product broadened both their activity spectrum and market share, including the

fruit, vegetables, cereals and oilseed segments (Earley *et al.*, 2012; Glättli *et al.*, 2011). With sales reaching USD 295 million in 2010, Boscalid is now considered to be the most important active ingredient of the SDHI family (PhillipsMcDougall, 2011). Nonetheless, Boscalid-resistant strains of the fungal pathogens *Botrytis cinerea* in grapes and strawberries (Stammler *et al.*, 2008), and *Alternaria alternata* in pistachio leaves (Avenot *et al.*, 2009) have already been reported in various field locations (Scalliet *et al.*, 2012). *B. cinerea* resistance resulted from a single point mutation (P225L/T) in the SDH b subunit, at the UQ binding site (Stammler *et al.*, 2008), whereas Boscalid resistance in *A. alternata* resulted from amino acid substitutions at the SDH c and d subunits, namely H134R and H133 R, respectively (Avenot *et al.*, 2009). These amino acid substitutions may drive changes in the binding affinity of carboxamides, causing reduced effectiveness (Avenot *et al.*, 2009; Stammler *et al.*, 2008).

In the last couple of years, the major agrochemical companies introduced a new generation of SDHI products belonging to the carboxamide chemical class (Semar *et al.*, 2011; Suty-Heinze *et al.*, 2011). These products are not only active against basidiomycetes, as the products introduced in the late 1900s already were, they are also effective against ascomycetes as seed treatments, foliar applications and paddy fields for the cereals, fruits and vegetables markets (Earley *et al.*, 2012; Glättli *et al.*, 2011). Furthermore, they provide a long-lasting efficacy as preventative and curative treatments at lower dosages than their predecessor Boscalid (Semar *et al.*, 2011; Walter, 2011).

Currently, *M. graminicola* control relies mostly on the triazoles fungicide family (Fraaije *et al.*, 2012; McCartney *et al.*, 2007). Due to the lack of alternative active ingredients to control this pathogen, if this class of chemicals were to be removed from the European market through, for example the introduction of new plant protection product legislation (Directive 91/414/EEC), the European cereal production would be in jeopardy (Clarke *et al.*, 2009). For this reason, and the fact that the new generation of carboxamide fungicides provide strong activity against *M. graminicola* (Semar et al., 2011; Suty-Heinze et al., 2011), new control strategies besides the triazoles fungicides have arisen to control this pathogen (Earley *et al.*, 2012). Moreover, because the SDHI are single site fungicides, the FRAC has assigned them to the group with moderate to high-resistance risk. Despite the fact that resistant cereal pathogen populations have not

yet been identified in the field, laboratory mutants carrying amino acid substitutions in the b, c and d complexes of SDH near the ubiquinone binding site have shown reduced sensitivity to modern SDHIs (Fraaije *et al.*, 2012; Scalliet *et al.*, 2012). As a consequence, if proper measures are not carried out, there is a high probability that field isolates of *M. graminicola* will be able to develop resistance mechanisms to adapt to this class of chemicals (Fraaije *et al.*, 2012). In order to avoid resistance development, these products are mainly offered as mixed formulations in combination with triazoles (Semar *et al.*, 2011; Suty-Heinze *et al.*, 2011; Walter, 2011), with a maximum of two applications per season recommended (FRAC, 2012b). Furthermore, constant monitoring of the development of resistance mechanisms in field pathogen populations is of great importance in developing up-to-date anti-resistance strategies (Fraaije *et al.*, 2012).

As with the QoI products, physiological effects including delayed leaf senescence, increased flag leaf size and drought stress tolerance have also been reported for the new generation of carboxamide fungicides under disease-free conditions (Berdugo *et al.*, 2011; Suty-Heinze *et al.*, 2011), effects which result in higher yields compared to untreated plants (Berdugo *et al.*, 2011; Berdugo *et al.*, 2012)

Even though sales data including the new generation of carboxamides was not available when this thesis was written, a significant revenue potential awaits this chemical class in seed and foliar application treatments (PhillipsMcDougall, 2011)

1.4 Plant defense responses

Plants have to cope with a great number of diseases and pests including among others, fungi, bacteria, viruses, oomycetes, insects and nematodes. Since they are sessile organisms and lack the somatic immune system of mammals, plants rely on both; i) pre-formed barriers, such as the waxy cuticle and secondary metabolites with antimicrobial properties like saponins (Buchanan *et al.*, 2000; Dangl & Jones, 2001), and ii) a very robust induced immunity system, which is able to recognize foreign molecules or internal signals and activate the corresponding response to stop the invading organism (Buchanan *et al.*, 2000; Jones & Dangl, 2006; Pieterse *et al.*, 2012).

1.4.1 Induced defense responses

Jones and Dangl (2006) introduced the four phased zig-zag model (**Figure 1.5**) to describe the co-evolutionary plant-microbe interaction. In the first phase, plants recognize specific molecules present in microorganisms such as chitin, flagellin, glycoproteins and polysaccharides, or endogenous signals that are activated by cells damaged by pathogens or insects. The first set of molecules is known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), while the second set is known as damage-associated molecular patterns (DAMPs). PAMPs, MAMPs or DAMPs are recognized by pattern recognition receptors (PRR) in the plant, which in turn activate the first set of defense responses known as PAMP-triggered immunity (PTI).

In the course of evolution, pathogens have developed virulence effector proteins in order to adapt to, or to inhibit, PTI activation by the host (J. Zhang & Zhou, 2010). According to this, phase two of the zig-zag model occurs when pathogens are able to either suppress PTI, or elude plant detection of their PAMPs by using effectors, and thus, continue colonization of the host. This phase is known as effector-triggered susceptibility (ETS). Nonetheless, plants have also developed mechanisms to stop disease by developing resistance (R)-proteins that detect a specific effector from the pathogen and trigger an enhanced defense response, kwon as effector triggered immunity (ETI), previously called R-gene-based resistance (Boller & Felix, 2009). ETI is phase three of the zig-zag model and, in some cases; can end in hypersensitive cell death response at the site of infection. Through evolution, microbes have also developed means of avoiding or suppressing ETI, leading back to ETS. (Jones & Dangl, 2006; Pieterse *et al.*, 2012).

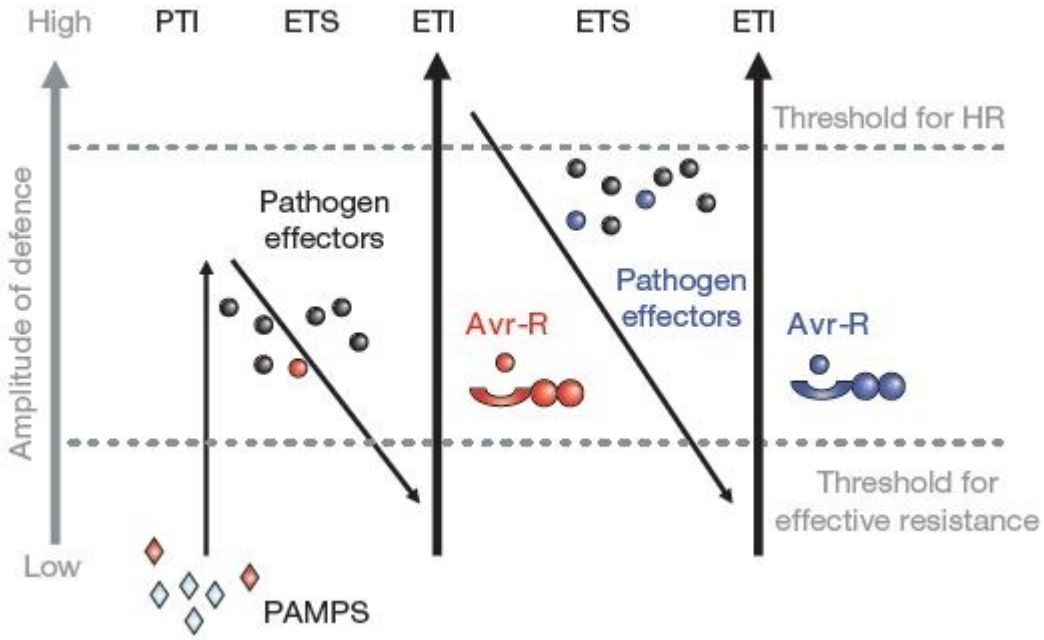


Figure 1.5: Zig-Zag model illustrating the plant-microbe interaction

Representation of the plant immune system as a four phased zig-zag model. Phase 1: Detection of PAMPs or DAMPs by pattern recognition receptors leads to PAMP-triggered immunity (PTI) to halt pathogen colonization. Phase 2: Interference of PTI by pathogen effectors, which allow further colonization of the plant, resulting in effector triggered susceptibility (ETS). Phase 3: A resistance (R)-protein from the plants is able to recognize an effector from the pathogen and triggers an enhanced defense response, known as effector-triggered immunity (ETI). If ETI passes the HR threshold, this response can end in programmed cell death. Phase 4: Natural selection has led to pathogens that have either lost the effector that a plant could recognize or have developed new effectors that lead to ETI suppression. Nonetheless, plants can also develop new R-proteins that recognize the new effector and respond once again with ETI. Abbr. PAMPs, pathogen acquired molecular patterns; PTI, PAMP-triggered immunity; ETS, effector triggered susceptibility; ETI, effector-triggered immunity; Avr; avirulence; R, resistance; HR, hypersensitive response. Source: Jones and Dangl (2006).

1.4.1.1 PAMP-triggered immunity

Activation of PTI occurs within minutes of pathogen detection (Boller & Felix, 2009). Initially, changes in cell potential occur due to the simultaneous in- and efflux of ions across the plasma membrane, which serve as messengers for the activation of downstream signals. The downstream signaling events activated include, the induction of mitogen-activated protein kinase (MAPK) signaling cascades, WRKY transcription factors and the production of reactive oxygen species with antimicrobial properties, which serve as secondary messengers for the activation of subsequent defense responses (Boller & Felix, 2009). Activation of early signaling events leads to i) Callose depositions at penetration sites in order to block pathogen invasion; ii) Synthesis of the

stress hormones ethylene, jasmonate or salicylic acid; iii) Induction of defense-related genes, such as hydrolytic enzymes that cause the degradation of microbe cell wall polysaccharides, and; iv) the synthesis of secondary metabolites with antimicrobial properties (Boller & Felix, 2009; Buchanan *et al.*, 2000)

1.4.1.2 Effector-triggered immunity

As mentioned by Jones and Dangl (2006), during the evolution, pathogens have found a way of avoiding or suppressing PAMP-triggered immunity by using effectors that, unlike MAMPs, are pathogenicity factors specific to each microorganism (Boller & Felix, 2009).

At the same time, plants have evolved improved surveillance systems for detecting effectors by using specific recognition proteins, a pattern that follows the gene-for gene model, where pathogens carrying a specific avirulence genes (*avr*-gene*)* are recognized by plants containing its corresponding resistance gene (R-gene) and trigger a set of defense responses known effector-triggered immunity (ETI) (Glazebrook, 2001; Jones & Dangl, 2006). Most of the resistance genes are nucleotide-binding leucine rich repeat (NB-LRR) proteins (Boller & Felix, 2009; Dangl & Jones, 2001; Jones & Dangl, 2006), which upon direct or indirect activation, lead to local and systemic defense responses that can end up in a programmed cell death response. Nonetheless, ETI is a highly regulated process, which depending on the type of microorganism that is invading the cell, the corresponding response is activated. Counteraction against biotrophic pathogens is mediated by salicylic acid, whereas against necrotrophs and chewing insects, jasmonate and ethylene are the main signaling components (Jones & Dangl, 2006).

1.4.2 Roles of phytohormones in plant innate immunity

PTI and ETI cause the activation of synthesis and response events that include several hormones such as salicylic acid, ethylene, jasmonate, abscisic acid, auxin, gibberellin and brassinosteroids. This phytohormones are not only known for their functions in regulating plant growth and development, but also in their pivotal role as signaling molecules in defense responses (Bari & Jones, 2009; Pieterse *et al.*, 2012). Pathogen infection results in changes in plant hormonal levels that trigger a defense response to stop pathogen invasion and colonization (Bari & Jones, 2009). Depending on the type of

hormone, its activation and further effect can determine whether a plant is susceptible or not against a certain kind of disease (Pieterse *et al.*, 2012)

1.4.2.1 Salicylic acid, ethylene and jasmonate

Microbial pathogens have different lifestyles that vary from those feeding on living cells to those who live by getting their nutrients from dying cells. The first group of pathogens are known as biotrophs, whereas the second ones are called necrotrophs; in between there is a group of microorganisms known as hemibiotrophs, which at the beginning carry a biotrophic lifestyle and then they switch to a necrotrophic one (Browse, 2009; Buchanan *et al.*, 2000) It is already known that salicylic acid is the main signaling molecule in regulating defense responses against biotrophic or hemibiotrophic microorganisms, while both jasmonate and ethylene mediate resistance against necrotrophic pathogens and herbivorous insects. Evidence of hormone crosstalk that modulate not only the magnitude of the response but also its specificity towards several pathogens exists. Nonetheless, the molecular mechanisms involved in the highly controlled hormonal regulation of plants upon pathogen attack are not well understood (Bari & Jones, 2009)

Upon pathogen recognition, salicylic acid (SA) levels in the plant cell increase, and this leads to a series of events involved in the activation of defense responses in local tissues. Furthermore, this response can subsequently be triggered in distant parts of the plants in order to protect undamaged tissues, a process that is known as the systemic acquired resistance (SAR). SAR is associated with increased SA levels and the activation of gene products with antimicrobial properties (Bari & Jones, 2009; Grant & Lamb, 2006; Pieterse *et al.*, 2009; Pieterse *et al.*, 2012). Evidence on the specificity of salicylic acid towards biotrophic and hemibiotrophic pathogens was achieved by using mutant plants impaired in either the synthesis or downstream signaling of this hormone. The results of the experiments revealed that SA-impaired mutants showed enhanced susceptibility only towards the microorganisms mentioned (Bari & Jones, 2009)

Salicylic acid accumulation due to pathogen recognition causes the activation and translocation of a regulatory protein named non-expressor of PR genes1 (NPR1) from the cytosol to the nucleus. In the nucleus, NPR1 monomers interact with TGA transcription factors and stimulate the transcription of pathogenesis-related (PR)

proteins. (Grant & Lamb, 2006; Pieterse *et al.*, 2012). Besides the activation of several PR proteins upon salicylic acid synthesis, many WRKY transcription factors are also induced, which not only have a function as SA-mediated response inducers but they also act as feedback inhibitors (Pieterse *et al.*, 2012; D. Wang *et al.*, 2006). Because the activation of the systemic resistance is quite costly for the plant, where the expression profiles of more than 1000 genes change; plants have developed ways of controlling not only the activation of SAR but also its timespan. D. Wang *et al.* (2006) identified two WRKY transcription factors acting downstream of NPR1 to control SA accumulation when the plant has been able to halt pathogen attack. Although, most of the research in SA-mediated defense response has been done in dicot plants, evidence of similar mechanisms and analog genes have been identified in certain monocots as well such as rice, barley and wheat (Colebrook *et al.*, 2012; Kogel & Langen, 2005; Vlot *et al.*, 2009). Nonetheless, whether biologically induced SAR in monocots and dicots are conserved needs further investigation (Balmer *et al.*, 2013; Colebrook *et al.*, 2012).

Jasmonate (JA) and its methyl ester (JAMe) act as regulators of several processes related to plant development, gene expression and response to external stimuli such as wounding, pathogen attack or water stress. Jasmonate is involved in leaf abscission, fruit ripening and seed germination. Under biotic stress conditions, jasmonate is known for inducing the synthesis of several enzymes involved in the production of secondary metabolites such as flavonoids and sesquiterpenoids, induction of antifungal compounds and protease inhibitor proteins (Creelman & Mullet, 1995; Gundlach *et al.*, 1992).

Upon necrotrophic pathogen or insect attack, jasmonic acid or its metabolites are synthesized via the oxylipin biosynthesis pathway. JA synthesis starts when linoleic acid is either released from the plasma or plastid membranes by phospholipases, and is then converted to jasmonic acid by the action of lipooxigenases, allene oxide synthase, allene oxide cyclase and 12-oxo-DPA reductase. (Browse, 2009; Creelman & Mullet, 1995; Vick & Zimmerman, 1984). Many jasmonate derivatives are subsequently produced which have different roles in plant biology. As for instance, methylation of jasmonate leads to the volatile molecule JAMe that is the active signal transduction molecule upon pathogen attack, which activates defense responses in neighboring plants. Another important jasmonate derivative is the Jasmonoyl-Isoleucine (JA-Ile), which is believed to be an important down-stream signaling molecule (Browse, 2009).

The role of this plant hormone in disease resistance was achieved by using mutants impaired in JA synthesis or signaling, which were more susceptible to insect and necrotrophic pathogen attacks (Browse, 2009; Howe *et al.*, 1996). Furthermore, exogenous applications of jasmonate or JAMe to leaf tissues have resulted in the local induction of JA-related defense genes (Bari & Jones, 2009). After damage of the plant cell wall either by wounding or by pathogen elicitors, JA transiently accumulates both at the lesion site and in distant plant tissues by its distribution through the phloem, this process leads to an increased disease resistance (Browse, 2009; Truman *et al.*, 2007). Jasmonate synthesis leads to the activation of COI1, an F-box protein belonging to the SCF^{COI1} ubiquitinase complex that is in charge of regulating the proteasomal degradation of the Jasmonate Zim Domain (JAZ) proteins. JAZ proteins are negative regulators of jasmonate responses, and upon their degradation, down-stream signaling mechanisms that lead to the induction of defense responses are activated (Browse, 2009; Wasternack, 2007; 2013).

Because jasmonate is involved in the defense responses against both, pathogen attack and wounding by insects or mechanical action, plants have evolved a way of prioritizing the corresponding response based on the type of stress (Lorenzo *et al.*, 2004). In *Arabidopsis thaliana*, two branches of JA signaling have been identified, the MYC and the ERF branches. The first one is regulated by the MYC-type transcription factor and includes the vegetative storage protein 2 (VSP2), which is a marker for JA-mediated response, and is associated to wound responses and defense against insects (Lorenzo *et al.*, 2004; Memelink, 2009; Pieterse *et al.*, 2012). The second branch of JA signaling is controlled by ethylene responsive factor 1 (ERF1) and includes the marker gene plant defensin 1.2 (PDF1.2). The ERF branch is a downstream component of jasmonate and ethylene signaling and it involves the activation of defense responses due to attack by necrotrophic microorganisms (Browse, 2009; Lorenzo *et al.*, 2003; Memelink, 2009; Pieterse *et al.*, 2012). In order to activate MYC-mediated pathway only jasmonate is required, whereas the ERF-mediated signaling branch involves the synergistic action of JA and ethylene (Lorenzo *et al.*, 2003; 2004; Memelink, 2009).

Besides being involved in physiological aspects of plant development such as leaf abscission, fruit ripening and senescence, ethylene (ET) is also in charge of the regulation of defense responses to biotic and abiotic stress factors (Broekaert *et al.*,

2006; Y.-F. Chen *et al.*, 2005). The successful recognition of PAMPs by the plant surveillance systems leads to the activation of the ethylene synthesis pathway, which converts methionine into ethylene by a series of reactions involving the intermediates *S*-adenosyl-methionine (AdoMet) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The synthesis of ACC by the enzyme ACC synthase is considered to be the rate-limiting step in ethylene synthesis, and it is therefore a major focus in scientific research (Broekaert *et al.*, 2006). Upon ethylene synthesis, this hormone is recognized by ETR1/2 and ERS1/2 receptors localized in the endoplasmic reticulum that lead to the inactivation of the ethylene signaling repressor protein CTR1. Once the ethylene signaling is restored, the EIN2 gene is in charge of the transduction of down-stream signaling events into the nucleus, where the activation of ERF transcription factors and the subsequent induction of defense-related genes take place (Broekaert *et al.*, 2006; Y.-F. Chen *et al.*, 2005). Evidence of the pivotal role that ERF transcription factors play in plant-pathogen interactions was provided by using transgenic *Arabidopsis* plants with constitutive expression of ERF1. These plants were highly resistant to several necrotrophic fungi such as *B. cinerea* and *P. cucumerina* (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003)

As mentioned before, evidence of the synergistic interaction between jasmonate and ethylene have been reported in plant defense responses towards necrotrophic fungi. Furthermore, these responses are mediated by ERF1 as a common downstream signaling molecule of both pathways (Lorenzo *et al.*, 2003). However, when it comes to the activation of insect- or wounding-induced responses, these two phytohormones act antagonistically (Lorenzo & Solano, 2005). Upon necrotrophic pathogen detection ERF1 induces the JA/ET-mediated activation of pathogen-specific resistance genes, while repressing the MYC branch of the jasmonate pathway that is restricted to insect/wounding-triggered responses (Lorenzo *et al.*, 2003; 2004). Based on that, it has been concluded that ethylene specifies which jasmonate-signaling pathway is activated depending on the type of stimuli, cell damage by insects or necrotrophic microorganisms (Lorenzo *et al.*, 2003; 2004; Memelink, 2009)

Program cell death (PCD) is a highly regulated induced response to control biotrophic pathogens that feed on living cells. Nonetheless, PCD would benefit necrotrophic microorganisms that obtained their nutrients from dying cells (Broekaert *et al.*, 2006).

For this reason, plants have developed highly regulatory systems that involve hormonal cross-talk in order to respond properly against pathogens with different kinds of lifestyles. For instance, upon infection of SAR-inducing pathogens, antagonistic interactions between SA and JA have been observed indicating that plants are able to prioritize between SA-dependent defense responses over JA-dependent ones (Spoel *et al.*, 2003). Furthermore, the SA-mediated suppression of JA/ET-responsive genes during SAR is a highly regulated process that involves cytosolic NPR1 (Spoel *et al.*, 2003), WRKY transcription factors, MAP kinases and glutaredoxins (Bari & Jones, 2009). Similarly, the repression of SA-mediated responses during necrotrophic plant-pathogen interactions is regulated by MAP kinase 4 and ERF1, which are responsible for the induction of JA/ET-responsive genes (Lorenzo & Solano, 2005) (**Figure 1.6**). Even though it is widely known that salicylic acid and jasmonate antagonize each other to prioritize defense reactions, Mur *et al.* (2006) reported a transient synergistic interaction when *Arabidopsis* and tobacco plants were treated with low concentrations of these two hormones. This report led to the conclusion, that the outcome of this interaction is dependent of both hormonal concentration and timing (Mur *et al.*, 2006). Additionally, on a study regarding the molecular mechanisms of acquired resistance in rice, Schweizer *et al.* (1997) reported a synergistic interaction between JA and the SA-analog 2,6-dichloroisonicotinic acid (DCINA) in rice plants challenged with the fungus *Magnaporthe oryzae*. According to these authors, in order to activate acquired resistance in rice, SA or its analogs are necessary, whereas JA act as an enhancer of the induced resistance (Colebrook *et al.*, 2012; Schweizer *et al.*, 1997). In a co-expression analysis using publicly available stress-related microarray experiments in *Arabidopsis,* van Verk *et al.* (2011) were able to demonstrate how already known and new potential regulatory elements during stress responses like transcription factors and MAP kinase signaling cascades are interconnected in order to fine-tune hormonal-mediated defense responses.

Due to the important role that phytohormones play in regulating defense responses to a given microorganism, pathogens have also evolve ways of hijacking the plant immunity system for their own benefit (Wasternack & Hause, 2013). In order to successfully infect tomato plants, the necrotrophic fungus *Botrytis cinerea* produces the exopolysaccharide β-(1,3)-(1,6)-glucan that activates the SA-mediated response and hence, the JA/ET-

mediated activation of resistance genes will be inhibited due to the antagonistic role that SA has over JA/ET-induced responses (El Oirdi *et al.*, 2011). On the other hand, the hemibiotrophic pathogen *Pseudomonas syringae* injects a toxin into the plant cell known as coronatine, which is a molecular mimic of JAIle. By doing so, the plant will activate the JA/ET signaling pathway and repress the SA-mediated responses to (hemi)biotrophic organisms, which leads to successful bacterial growth (Pieterse *et al.*, 2012; Wasternack & Hause, 2013).

1.4.2.2 Other phytohormones

Although the main players in regulating defense responses to biotic stress responses are salicylic acid, jasmonate and ethylene, other phytohormones such as abscisic acid, auxin, gibberellins and brassinosteroids also help to modulate plant immunity by interacting with SA, JA or ET in an antagonistic or synergistic manner (Pieterse *et al.*, 2012). A representation of the highly interconnected network of hormonal interaction under biotic stress conditions is represented in **Figure 1.6**

Abscisic acid (ABA) is a phytohormone that plays an important function in providing pants tolerance to drought and high salinity conditions (Ton *et al.*, 2009). Nonetheless, the role that ABA plays in plant responses to biotic stress factors have been reported to be both negative and positive depending on the type of the plant-pathogen interaction that comes into play (Bari & Jones, 2009). Among the positive effects that this hormone has on plant immunity are i) the ABA-mediated closure of the stomata that upon PAMP detection inhibits the penetration of microorganisms through plant openings (Bari & Jones, 2009; Ton *et al.*, 2009); ii) upon wounding or herbivore attack, ABA induces the JA-mediated MYC branch, while suppressing the JA/ET-responsive ERF pathway. By doing so plants are able to prioritize between responses to herbivorous insects or necrotrophic pathogens (Pieterse *et al.*, 2012; Wasternack & Hause, 2013).

On the other hand, once a bacterial pathogen has successfully entered the plant by means of its effectors, ABA inhibits the callose depositions that the plant induces in order to halt microbial invasion (Ton *et al.*, 2009). Moreover, Yasuda *et al.* (2008) demonstrated that in *Arabidopsis* ABA plays a negative role in the local and systemic activation of SA-mediated defense responses, where upon ABA treatment, plants were not able to activate the systemic acquire resistance (SAR).

The plant growth hormone auxin and its most common member, indole-3-acetic acid (IAA), not only have physiological effects in plants by stimulating cell division, differentiation and growth (Mano & Nemoto, 2012; Zhao, 2010) but they also have an effect in plant innate immunity (Bari & Jones, 2009; Z. Zhang *et al.*, 2007). Auxin treatment has led to increased susceptibility levels in plants, and it has been reported that some pathogen effectors like the AvrRpt2 from *P. syringae* induce auxin synthesis in order to achieve successful pathogen invasion (Bari & Jones, 2009; Z. Chen *et al.*, 2007). Based on transcriptome analyses of the responses of *Arabidopsis* plants to the SA analog, benzothiadiazole S-methylester (BTH), D. Wang *et al.* (2007) suggested that plants inhibit IAA signaling- and synthesis-related genes in local and distant tissues during salicylic acid-induced SAR in order to attenuate the negative effects that auxins have on plant immunity and thus, increase plant´s resistance.

Giberellins (GA) are plant growth promoting hormones produced not only by plants but also by fungi and bacteria (Bari & Jones, 2009; MacMillan, 2001). Upon GA synthesis, this hormone is recognized by the GID1 receptor, which activates downstream signaling by modulating the degradation of the negative regulators of plant growth known as DELLA proteins. This leads to changes in plant physiology and defense (Schäfer *et al.*, 2009). The role of GA in SA- and JA/ET-mediated defense responses was first demonstrated by Navarro *et al.* (2008) using *Arabidopsis* DELLA mutants impaired in GA signaling. Mutants with stabilized DELLAs revealed high susceptibility to the bacterial pathogen *P.syringae* DC3000, while being more resistant to the necrotrophic pathogens *B. cinearea* and *A. brassicola*. Based on their results, the authors suggested that DELLAs alter SA and JA/ET homeostasis in order to promote colonization of biotroph whereas necrotrophic pathogen infection was hindered. Nonetheless, upon gibberellin synthesis, DELLAs are degraded leading to the activation of SA-induced defense responses and the repression of JA/ET-mediated signaling pathway due to the antagonistic interaction of SA and JA (Bari & Jones, 2009; Navarro *et al.*, 2008). Due to the role that GA plays in plant-pathogen interactions, it has been suggested that this hormones acts as a virulence factor in plant-pathogen interactions (Bari & Jones, 2009). The necrotrophic fungus *Gibberella fujikuroi* in rice is able to achieve successful plant colonization by secreting gibberellin and hence, repress the activation of JA/ET-mediated defense responses (Navarro *et al.*, 2008)

Besides the antagonistic interaction that gibberellin and jasmonate play in defense responses towards necrotrophic pathogens, crosstalk between these two hormones leads to an antagonistic effect between GA-mediated growth and JA-responsive resistance genes due to the competitive interaction between MYC2 and DELLAs in JAZ binding. Upon gibberellin synthesis, DELLA proteins will be degraded, which leads to the suppression of JA-responsive genes due to the inhibition of MYC2 activity by the JAZ proteins. Furthermore, the activation of the MYC pathway upon biotic stress detection leads to interference with the GA-mediated DELLA degradation and thus, it is believed that jasmonate is in charge on prioritizing defense responses over growth by affecting gibberellin downstream signaling events in monocots and dicots (Wasternack & Hause, 2013; Yang *et al.*, 2012).

Cytokinins (CKs) are widely known as plant growth promoting enzymes that have also been reported to accumulate under drought stress responses (Bari & Jones, 2009; Choi *et al.*, 2011). However, not until recently, the roles that these hormones play in plant immunity have started to be more understood. It has been reported that some biotrophic pathogens modify CK metabolism in plants either by secreting CK analogs into the plant cells or by inducing CK synthesis in the plant in order to divert nutrients from the host into infection sites. This phenomenon leads to a reduced photosynthetic metabolism, delayed cell death and the formation of "green-islands" along the leaf (Choi *et al.*, 2011; Walters & McRoberts, 2006). Based on this, one would think that CKs play negative roles in plant-pathogen interactions. Nonetheless, it has been reported that transgenic plants with elevated resistance to several viruses displayed high cytokinins levels, which leads to the conclusion that these hormones induce resistance to viral infections, and that this resistance involves salicylic acid accumulation (Choi *et al.*, 2011). Additionally, it has also been suggested that plant-derived CKs stimulate resistance of *Arabidopsis* plants to the hemibiotrophic pathogen *P. syringae* pv. tomato DC3000 by positively interacting with SA-induced resistance transcripts (Choi *et al.*, 2010; 2011)

Brassinosteroids (BRs) are steroid hormones synthesized from a plant sterol known as campesterol. BRs have similar chemical structure to the steroid hormones in mammals and ecdysteroids in insects, and regulate growth and development in plants by controlling cell elongation, division and differentiation (Bari & Jones, 2009; Rozhon *et*

al., 2013). Nakashita *et al.* (2003) reported that the bioactive BR, Bassinolide is able to induce systemic resistance in rice and tobacco plants subjected to different pathogens. Furthermore, this resistance did not involve SAR markers such as SA synthesis and the expression of PR, nor the synthesis of JA-induced resistance; and no evidence of cross-talk between these responses was reported. Nonetheless, the molecular mechanisms involved in BR-induced resistance are still poorly understood (Choudhary *et al.*, 2012). The brassinosteroid insensitive 1 (BRI1)-associated kinase 1 (BAK1) and the BR-signaling kinase1 (BSK1) are key molecules in the downstream signaling cascade of BRs, which have also been reported to be involved in the activation of PAMP-triggered immunity (Bari & Jones, 2009; Shi *et al.*, 2013). Additionally, upon BR synthesis, downstream signaling events are activated that lead to the repression of PTI independently of BAK1, which suggests an antagonistic interaction between BR-mediated plant growth and PTI (Albrecht *et al.*, 2012; Shi *et al.*, 2013)

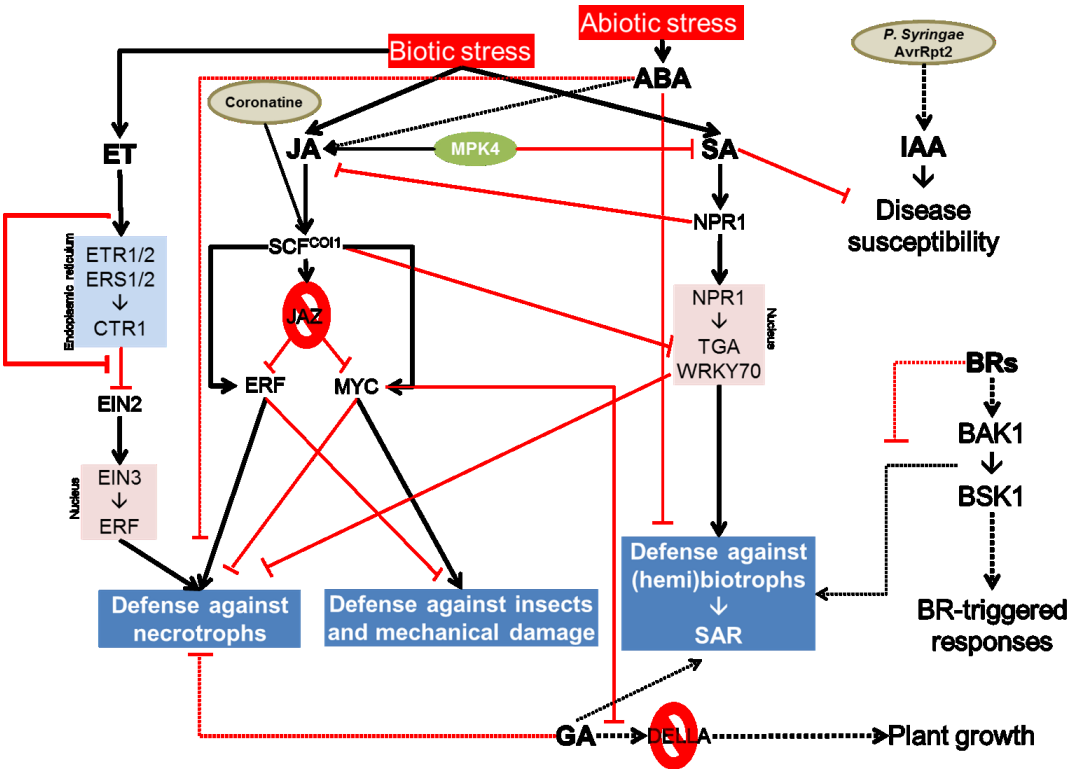


Figure 1.6: Roles of phytohormones in biotic stress responses

Phytohormones play a pivotal role in downstream signaling mechanisms that lead to the activation of defense responses upon pathogen or insect detection. Displayed is the highly interconnected network of hormonal crosstalk that not only specifies which defense responses are activated depending on the nature of the stimulus but it also regulates between hormonal-triggered growth responses and defense reactions. Due to the fact that phytohormones play such an important role in regulating plant innate immunity, microorganisms have found a way of

achieving successful colonization by injecting effectors (tan-filled ovals) into the plant cell that are able to hijack hormonal signaling pathways for their own benefit. Solid lines represent the backbone of plant defense signaling regulated by SA, JA and ET. Dashed lines represent secondary signaling pathways regulated by other hormones. Black arrows represent positive effects whereas red-truncated arrows indicate antagonistic interactions. No symbol represents degradation steps. Abbr. ET: ethylene; JA: jasmonate; SA: salicylic acid; ABA: abscisic acid; IAA: auxin; BRs: brassinosteroids; GA: gibberellin; MPK4: mitogen-activated protein kinase 4; NPR1: non-expressor of PR genes 1; SCF^{COI1} Skp1/Cullin/F-box-Coronatine-insensitive 1 complex; JAZ: Jasmonate Zim domain; ERF: ethylene responsive factor; ETR1/2; ethylene receptor 1 and/or 2; ERS1/2: ethylene response sensor 1 ad/or 2; EIN: ethylene insensitive. Adapted from: Lorenzo *et al.* (2004;2005); Pieterse *et al.* (2009)

1.4.3 Responses of wheat against SLB

Fungal infection of *M. graminicola* on wheat leaves causes clear disease symptoms observed as chlorotic and necrotic lesions starting at the point of pathogen penetration, which will then extent throughout the leaf surface. As the disease advances further, the asexual fruiting bodies of the fungus are produced within the lesions, and under high humidity conditions spores are released from the pycnidium within the cirri (Eyal, 1987). However, depending on the way plants react to *M. graminicola* infection, the severity of the disease can vary. The number of pycnidiospores being produce by the fungus is highly dependent on the host response to the pathogen, where resistant varieties are able to hinder pathogen growth and hence, the necrotic lesions and pycnidia are significantly reduced (Cohen & Eyal, 1993; Eyal, 1987; Kema *et al.*, 1996; Palmer & Skinner, 2002; Shetty *et al.*, 2003).

Adhikari *et al.* (2007) suggested that there are two peaks of gene induction during a wheat-*M. graminicola* incompatible interaction that may be related to the hemibiotrophic lifestyle of the fungus. The particular biphasic lifestyle of *M. graminicola* allows resistant plants to respond to fungal colonization events twice during the infection process. The first set of defense responses observed in the wheat resistant cultivars Tadinia and W7984, carrying the resistance genes *Stb4* and *Stb8* respectively, are activated at pre- and early post-penetration stages. These plants are able to detect PAMPs from *M. graminicola* such as β-1,3-glucan, a polysaccharide from the fungal cell wall, and trigger a series of defense responses that help to minimize pathogen penetration and colonization (Shetty *et al.*, 2009). These responses are related to i) the induction of defense-related genes like phenylalanine ammonia lyase (PAL), and PR proteins such as chitinases, β-1,3-endoglucanases, PR1 and PR5 (Adhikari *et al.*, 2007; Ray *et al.*, 2003; Shetty *et al.*, 2009), ii) the appearance of auto-fluorescent material and

callose deposition at penetration sites (Cohen & Eyal, 1993; Shetty *et al.*, 2003; 2009), and iii) the production of ROS that are toxic to the fungus after the penetration but not during the necrotrophic stage (Shetty *et al.*, 2003). The fact that no or significantly less defense responses have been detected in susceptible cultivars upon pathogen penetration have been attributed to effector molecules released by the fungus in order to avoid its detection and the activation of PTI by the plant (R. Marshall *et al.*, 2011; Shetty *et al.*, 2003). After the publication of the *M. graminicola* genome (Goodwin *et al.*, 2011), a pathogen effector (Mg3LysM) that binds to chitin fragments and prevents the activation of early defense responses was identified (R. Marshall *et al.*, 2011). According to these authors, *M. graminicola* suppresses PTI in order to achieve successful host colonization. Subsequently, when a given fungal biomass concentration in reached, host program cell death is induced, leading to rapid fungal growth and sporulation (Kema *et al.*, 1996; R. Marshall *et al.*, 2011)

The late peaks of gene induction that was observed in the resistant cultivars Tadinia and W7984, and not in the susceptible varieties, are related to the switch of the pathogen lifestyle from a biotroph to a necrotroph. (Adhikari *et al.*, 2007). Apparently, resistant plants are able to detect a change in the pathogen and activate a series of responses that lead to pathogen sequestration and the inhibition of the cell necrosis that is normally observed during a compatible interaction. Among the responses found to be induced during this time point of the infection process in resistant cultivars, those belonging to signaling transduction, energy metabolism, response to stress probably due to the action of a fungal toxin, and the synthesis and post-translational modification of proteins were reported (Adhikari *et al.*, 2007).

Significant changes in gene expression have also been described to occur during a compatible interaction at this time point, which correlates with an exponential growth in fungal biomass and the appearance of disease symptoms (Keon *et al.*, 2005b; 2007; Rudd *et al.*, 2008; Shetty *et al.*, 2009). Keon *et al.* (2007) demonstrated that wheat responses at the time of symptom development were similar to a hypersensitive response, which is normally induced after biotrophic pathogen detection and leads to host PCD. At the onset of massive fungal growth, the leakage of cytochrome c from mytochondria into the cytoplasm of infected plants; and the host DNA fragmentation, seen as DNA laddering on gel electrophoresis, led to conclude that the development of

disease symptoms are related to loss in host cell membrane integrity at penetration sites and in neighboring cells (Keon *et al.*, 2007; Rudd *et al.*, 2008). Cell lysis causes the release of electrolytes into the apoplast, which would provide the fungus with the necessary nutrients to drive the massive host colonization and the sporulation process later on (Keon *et al.*, 2007). Additionally, a sudden and exponential increase in the expression of the MAP kinase gene TaMPK3 and the PR2 (β-1,3-glucanase) protein, together with a rise in the concentration of ROS were recorded just before the period of massive fungal colonization and the appearance of necrotic lesions in the leaf surface (Rudd *et al.*, 2008; Shetty *et al.*, 2003; 2009). Furthermore, ROS levels reached a peak toward the end of the infection process when fungus starts to sporulate (Shetty *et al.*, 2003; 2007). However, it is unclear whether H_2O_2 production is produced by the plant or by the fungus (Keon *et al.*, 2005a; 2007; Orton *et al.*, 2011; Shetty *et al.*, 2003). Based on the fact that the rapid fungal growth is preceded by host responses similar to the ones triggered during an incompatible interaction that leads to program cell death, it has been suggested that an effector molecule like a toxin or a necrosis-inducing peptide is secreted by the pathogen when a given fungal growth stage is reached in order to hijack plant innate immunity and thus, induce PCD in the host (Kema *et al.*, 1996; Keon *et al.*, 2007; Motteram *et al.*, 2009; Rudd *et al.*, 2008).

1.5 Objectives

Plants being sessile organisms are not able to escape stresses situations such as changes in the environmental conditions and attack by microbial organisms or herbivorous insects that threatened their biological capacity. For this reason, they must rely on a solid innate immunity system in order to survive biological stress responses. Upon detection of pathogens, insects or mechanical damage, plants need to reprogram their genetic profile in order to translocate the resources they would normally use for growth into defense. This trade-off between growth and defense will affect their fitness that will be reflected in reduced yields (Bilgin *et al.*, 2010; Walters & Heil, 2007)

The introduction of DNA microarrays enabled researches to study the response of thousands of genes to a given stimulus in parallel. For this reason it has become a widely used technology not only in clinical and animal research but also in plant science. From the publically available microarray experiments, the most relevant to this project

include; i) the *in vivo* and *in vitro* evaluation of *M. graminicola* infection strategy (Keon *et al.*, 2005b; 2007), ii) the evaluation of Epoxiconazole resistance development in *M. graminicola* field isolates with varying resistance levels (Cools *et al.*, 2007); and iii) the effect of fungicide application on plant physiology under disease-free conditions (Pasquer *et al.*, 2005).Nonetheless, the effect that fungicide application timing and mode of action have on the wheat transcriptome in the presence of a fungal disease like SLB has not, to the author´s knowledge, been evaluated. For this reason, a microarray-based experiment was carried out in order to investigate the impact that application timing of different fungicides and their modes of action have on the control of *M. graminicola* and the physiology of wheat. When a given agrochemical with protective activity is sprayed to the plant before the infection has occurred, the activation of defense reactions will be avoided and no trade-off between immunity and growth will take place. Furthermore, when an infection has taken place, the application of a fungicide will hinder pathogen growth and avoid yield losses due to disease damage. For this purpose, the aim of this study was to determine that not only 15% yield increase will be obtained by applying an agrochemical to hinder pathogen growth but also to identify, at the molecular level, differences between the active ingredients related to their efficacy and the beneficial effects they provide to the plant to boost its yield. Three different active ingredients (Epoxiconazole, Pyraclostrobin and Fluxapyroxad) with varying modes of action, namely the inhibition of sterol biosynthesis and the complex III and II in the cellular respiration process respectively were employed. Subsequently, their effect on disease control and plant physiology was evaluated at the molecular level in wheat seedlings grown under natural glasshouse conditions. Furthermore, in order to identify whether the effects observed at the genetic levels can be transferred to nature, yield trials under semi-natural and field conditions were carried out.

2. Materials and methods

2.1 Employed organisms

Plants for molecular biology and microscopy experiments were grown in glasshouse chambers under controlled conditions. For this purpose, seedlings from the winter wheat variety Riband were used. For the yield trials in the field and in the micro-plots, two winter wheat varieties were used; Akteur and Riband (**Table 2.1**). Both varieties are known to be highly susceptible to *M. graminicola* (*Beschreibende Sortenliste*, 2012; Chartrain *et al.*, 2004a), and are therefore preferred for a fungicide screening test.

Glasshouse and micro-plot experiments were inoculated with pycnidiospores from a mixture of four *M. graminicola* isolates collected in 1999 from field trials in Germany, Holland and Belgium. The four isolates were chosen because of their high sensitivity towards SDHI, QoIs and DMIs fungicide classes (Simone Miessner, Laboratory of resistance research, Agricultural Center, BASF, Limburgerhof, Germany; personal communication). For the field trials, wheat straw infected with *M. graminicola* was collected from the previous season and use as the source of inoculum (**Table 2.1.**)

Table 2.1: Organisms used and their corresponding/correlating experiments

Organism	Variety/Isolate	Experiment
Triticum aestivum	Akteur	Micro-plots 2011 Field 2011
	Riband	Micro-plots 2011, 2012 Field 2012 Glasshouse
Mycosphaerella graminicola	2806 (Germany)	Glasshouse and micro-plots
	2101 (Holland)	Glasshouse and micro-plots
	2107 (Belgium)	Glasshouse and micro-plots
	2108 (Belgium)	Glasshouse and micro-plots
	Wild	Field

The winter wheat varieties Riband and Akteur were obtained from the agricultural research station (BASF, limburgerhof, Germany). The mixture of the four different *M. graminicola* isolates was obtained from Dr. Gerd Stammler (Laboratory of resistance research, Agricultural Center, BASF, Limburgerhof, Germany)

2.2 Cultivation, growth conditions and trial design

2.2.1 Wheat (Triticum aestivum)

2.2.1.1 Glasshouse experiments

Ten seeds of winter wheat variety Riband were planted in a 0.23 litter pot with Universal soil substrate (Floragard, Oldenburg, Germany) and placed in a glasshouse chamber at 22/17 °C day/night temperature with a 16 hour light period at 70% relative humidity for 11 days. After inoculation with pycnidiospores from *M. graminicola* (**see 2.3.2**), the plants were enclosed in a plastic cover for four days in order to achieve the 100% relative humidity needed for fungal infection. Afterwards, the plants remained at 16/22 °C day/night temperature with a 15 hour light period and 88% relative humidity for approximately 25 days or until the severity of the infection reached approximately 100% in the untreated plants. Each pot was treated only once, with each treatment containing three biological repetitions (i.e. 3 different pots) with a complete randomization arranged within each wagon (**see 2.3.1**).

Fertilization took place three times a week with 0,3% Kamasol® Brilliant Blau (Compo GmbH & Co.K.G., Münster, Germany)

Kamasol® Brillant Blau

8%	Total nitrogen
8%	Water soluble phosphate (P_2O_5)
6%	Water soluble potassium oxide (K_2O)
0,01%	Water soluble Boron (B)
0,002%	Water soluble copper (Cu)
0,02%	Water soluble iron(Fe)
0,01%	Water soluble manganese(Mn)
0,001%	Water soluble molybdenum(Mo)
0,002%	Water soluble zinc (Zn)

2.2.1.2 Micro-plot experiments

Before planting, all seeds were treated with Rubin TT (BASF, Ludwigshafen, Germany), a mixture of different fungicide active ingredients in order to protect the plants from seed- and soil-borne fungal diseases. In the first week of October, 35 wheat seeds were planted in 0.12 cm^2 plastic boxes with local soil substrate (4:1; compost soil: peat). After seeding was completed, the plants were placed outside to grow under natural conditions. However, in order to protect them from adverse weather conditions and animals, the plants were placed under a netted tunnel and remained there until harvest.

Each plastic box was considered a treatment (treated once), and trials were laid out in a randomized complete block design (7 treatments, 8 repetitions per treatment).

Fertilization with 0,8% Kamasol® Brillant Grün (Compo GmbH & Co.K.G., Münster, Germany) was done only when a yellowish leaf colorization was observed. In case any fungal disease, apart from SLB or an insect pest, was observed, control measurements were taken according to common agricultural practices. The entire plot was harvested with yield and thousand kernel weight (TKW) values reported at 15% water content. Weight of grains per box was estimated to determine yield.

Rubin TT (200 mL/100 Kg seeds)

25 gai/L	Triticonazole
42 gai/L	Prochloraz copper chloride complex
42 gai/L	Pyrimethanil

Kamasol® Brillant Grün

10%	Total nitrogen
4%	Water soluble phosphate (P_2O_5)
7%	Water soluble potassium oxide (K_2O)
0,01%	Water soluble Boron (B)
0,002%	Water soluble copper (Cu)
0,02%	Water soluble iron(Fe)
0,01%	Water soluble manganese(Mn)
0,001%	Water soluble molybdenum(Mo)
0,002%	Water soluble zinc (Zn)

2.2.1.3 Field trials

Rubin TT was applied as a seed treatment before sowing. Winter wheat seeds were planted in autumn at three different locations in the Rhineland-Palatinate region in Germany (**Table 2.2**). In each location, wheat was the previous crop. For the 2010/2011 growing season, the winter wheat variety Akteur was used, whereas for the 2011/2012 season Riband was the chosen variety. Within each location, a randomized complete block design was chosen (5 treatments and 6 repetitions per treatment), with a plot size of 6x2 m and a harvested area of 10.2 m^2 per plot. For all trials, seeding, fertilization, weeding, disease and pest control were carried out according to common agricultural practices. Yield and TKW values were reported at 15% water content. Yield data was reported in decitonnes per hectare (dt/ha).

Table 2.2: Field trials seeding information

Season	Wheat variety	Place	Seeding date
2010/2011	Akteur	Ruchheim	September 16, 2010
		Limburgerhof	September 16, 2010
		Gronau	September 16, 2010
2011/2012	Riband	Böhl	October 11, 2011
		Limburgerhof	October 6, 2011
		Ruchheim	October 6, 2011

2.2.2 *Mycosphaerella graminicola*

Mycelium from each isolate was sub-cultured on a new agar plate containing malt-yeast extract agar (MYA)-growing medium. Two isolates per plate were grown together, each one in one half of the agar plate. After 9-12 days of incubation at 20°C and a daily 12 hour light period, pycnidiospores were collected for inoculation.

MYA medium
10 g Malt extract
4 g Yeast extract
4 g Glucose
20 g Agar
in Millipore water to a final volume of 1 litter and autoclave

To prepare the spore suspension, 1% tween-water and a Drigalski spatula were used to harvest the spores from the MYA medium. After being filtered through miracloth to remove hyphae and agar, the suspension was collected in an Erlenmeyer flask. The spores were counted with a Fuchs-Rosenthal counting chamber and then adjusted to obtain a final concentration of $2x10^6$ spores/mL.

For the micro-plot yield trials, the spore suspension was prepared by washing the spores from the MYA-medium with 2% malt extract and then filtering them through miracloth. After counting of the spores was completed, they were frozen at -20°C until further use. At the day of inoculation, depending on the spore concentration in the malt extract, dilution with tween-water was carried-out in order to achieve a final concentration of $2x10^6$ spores/mL

2.3 Treatments

2.3.1 Fungicide application

For the glasshouse and micro-plots trials, three different fungicides were used with alternative modes of action: Pyraclostrobin (Comet®, BASF, Ludwigshafen, Germany),

Epoxiconazole (Opus®, BASF, Ludwigshafen, Germany) and Fluxapyroxad (Imtrex®, BASF, Ludwigshafen, Germany). In the field trials, Pyraclostrobin was not used because the *M. graminicola* isolates present in the area contain the G143A mutation and are thus resistant to this class of chemicals. In all experiment setups, one fungicide application took place at the corresponding application timing; depending on preventative or curative treatment.

2.3.1.1 Glasshouse experiments

Based on application timing, plants were divided into the following treatment groups: preventative, curative, application under disease-free conditions, untreated control and untreated-uninfected control (**Table 2.3**). Untreated and untreated-uninfected controls were sprayed with water as a mock treatment

Table 2.3: Group of plants according to their treatment

Treatment Group	Innoculation	Common name	Trade name	Application time
Preventative	*M. graminicola* pycnidiospores	Pyraclostrobin Epoxiconazole Fluxapyroxad	Comet® Opus® Xemium®	-1 dpi
Curative	*M. graminicola* pycnidiospores	Pyraclostrobin Epoxiconazole Fluxapyroxad	Comet® Opus® Xemium®	7 dpi
Disease-free	Tween-water	Pyraclostrobin Epoxiconazole Fluxapyroxad	Comet® Opus® Xemium®	-1 dpi
Untreated control	*M. graminicola* pycnidiospores		Water	Each time water
Untreated-uninfected control	Tween-water		Water	Each time water

Once the first true leaf of wheat seedlings was fully developed (approximately 10 days after sowing), the first fungicide application took place. In order to prepare the plants for the fungicide treatments, they were placed in different glasshouse wagons based on their treatment group (**Table 2.3**) in order to avoid any cross-contamination later on. Preventative and disease-free application of fungicides took place one day before inoculation (-1 dpi), whereas the curative application was performed seven days post-inoculation (7 dpi). In order to have the same stress conditions in all plants at each application date, all plants were sprayed either with the chemical product or mock-treated with water based on their corresponding treatment. For example, when the first

application took place (-1 dpi), plants belonging to the curative group were sprayed with water, while the preventative and disease-free groups were sprayed with chemicals; on 7 dpi vice versa.

Fungicide application was performed within the in-house facility using twin-fluid flat fan nozzles (Lechler GmbH, Metzingen, Germany). Chemicals were sprayed at a rate of 32 grams of active ingredient per hectare (gai/ha), based on pre-experiment results.

2.3.1.2 Micro-plot experiments

Table 2.4: Fungicide treatments in the micro-plots and field trials

Treatment	Product name	Trade name	Concentration	Application timing
1	Untreated control		-	-
2 3	Pyraclostrobin	Comet®*	200 g ai/ha	Preventative Curative
4 5	Epoxiconazole	Opus®	125 g ai/ha	Preventative Curative
6 7	Fluxapyroxad	Xemium®	125 g ai/ha	Preventative Curative

*In the field trials Comet® was not used due to *M. graminicola* resistance development

Because the flag leaf is the most photosynthetically active leaf of a wheat plant, as well as the main source of carbohydrates for developing grain, protection of this leaf is of great importance. By protecting the flag leaf from a foliar damaging disease like SLB, higher yields and better quality grains can be obtained. With this in mind, for this trial, preventative fungicide application started once the flag leaf was fully developed (Growth stage (GS) 39; **Appendix 1**) while curative treatment was conducted after infection occurred. Due to the size of the plots and technical reasons, only the plants belonging to the corresponding application timing were sprayed at the corresponding date, no water treatment was done in-between as in the glasshouse.

Fungicide application was carried-out at a rate based on the manufacturer´s recommendations (**Table 2.4**) at the in-house facility using flat spray nozzles (Teejet, Wheaton, USA) with an application pressure of 3.2 bar and volume of 400 liters of water per hectare. After fungicidal treatment, plants were left to dry before bringing them back to the netted tunnels. Due to technical reasons, untreated controls were not sprayed at all.

2.3.1.3 Field trials

As in the micro-plot experiments, the preventative fungicide application for the field trials was conducted at GS 39 in order to protect the flag leaf. Since these experiments did not include artificial inoculation of the fungus, the timing of curative application was approximately two weeks after preventative treatment. In the meantime, sprinklers were used to simulate rain splash, which, as mentioned in the introduction, promotes the development of SLB disease to the upper leaf layers.

Fungicide application was performed only to the corresponding plots using a high-pressure plot sprayer configured to 300 L/ha spray volume. Like in the micro-plot experiments, fungicide rates were chosen according to the manufacturer´s recommendations (**Table 2.4**). Untreated plots were not sprayed due to technical reasons.

2.3.2 Inoculation of wheat leaves with pycnidiospores from *M. graminicola*

The inoculation methods selected and/or the timing of inoculation, related to the growth stage of the plants, varied based on the experiment locations as mentioned above.

In the glasshouse trials, first true leaves were inoculated with a spore suspension (**see 2.2.2**) one day after the first fungicide application. Inoculation was done using twin fluid flat fan nozzles (Lechler GmbH, Metzingen, Germany) at 2 bar. After inoculation, the wagons were enclosed in plastic covers for four days in order to achieve the 100% relative humidity the fungus needs for leaf infection. Following this period, during disease development, the plants were kept in 80% relative humidity and 16/22 °C day/night temperature with a 15 hour light period. Disease assessment was carried out when 100% disease severity was observed in the untreated plants (approximately 25 dpi).

In the micro-plot experiments, flag leaves were inoculated at night over a 3 day period with a spore suspension (**see 2.2.2**) and pycnidia-bearing straw from the micro-plot trials from the previous season. Infected straw from the field trials were not used in these experiments in order to avoid contamination with *M. graminicola* resistant isolates. When pycnidia-bearing straw from micro-plot trials was not available, infected leaves from fungicide-screening glasshouse tests were used because they contain the

same *M. graminicola* isolate mixtures as the one used for these experimental setup. Contrary to the glasshouse experiments, inoculation took place one week after the first application using a sprayer. In the first year, a RTS back pack sprayer (Baumann Saatzuchtbedarf, Waldenburg, Germany) was used with flat fan nozzles (Agrotop GmbH, Obertraubling, Germany) set at 2 bar, whereas in the second year a moveable one-wheel plot sprayer (Baumann Saatzuchtbedarf, Waldenburg, Germany) was used with flat fat nozzles (Agrotop GmbH, Obertraubling, Germany), and based on the manufacturer´s instructions, the nozzles pressure was set at 4.8 bar. Immediately after inoculation was achieved, plots were sprinkler-irrigated every 10 minutes for 10 seconds in order to keep leaf wetness at 100%, and thus, improve disease development. Weather conditions (temperature, relative humidity, leaf wetness and rainfall) were constantly monitored with a micro station (WatchDog 1000 series; Spectrum, Plainfield, USA). Approximately two weeks after preventative application was performed, curative application took place.

In the field trial experiments, pycnidia-bearing straw from field experiments from the previous season was laid in the plots in autumn in order to settle enough inoculum for natural disease development in spring and summer. In order to simulate rainfall and, therefore, promote disease development in the upper leaf layers, plots were sprinkler-irrigated once spring began. After conditions for SLB infection were determined to be favorable by weather monitoring with a micro stration (WatchDog 1000 series; Spectrum, Plainfield, USA), curative application to the corresponding plots was initiated.

2.4 Assessments

2.4.1 Disease assessment

The assessment of the disease severity was performed by estimating the percentage of infected leaf area according to (Bleiholder, 1997).

For the glasshouse experiments, the first true leaves of each pot were evaluated at approximately 25 dpi to report the percentage of infection per pot. In the micro-plot experiments, five flag leaves located at the center of each box were assessed to determine the average disease severity per box. In the field trials, at that plot length was 6 m, disease assessment was carried out in three sub-plots by randomly selecting ten

flag leaves and evaluating their disease severity. The averaged percentage of infected leaves for each plot was then reported.

2.4.2 Chlorophyll content and flag leaf area

As mentioned in **1.3**, after the application of some fungicides, higher yields have been obtained, which have been related to higher chlorophyll levels and delayed leaf senescence. To determine if higher yields are related to higher chlorophyll content or due to better disease control (by chemical application), the chlorophyll content in the flag leaf was measured. An SPAD-502Plus instrument (Konica Minolta Optics Inc, Tokyo, Japan) was used for this purpose. The SPAD meter is non-invasive equipment that measures the light absorbance in the red and near-infrared regions of a given leaf resulting in a value related to the amount of chlorophyll present. Besides the chlorophyll content measurements, the area of the flag leaf was also measured in order to see whether a fungicide treatment causes any change to this parameter and if this change is related to enhanced or lower yield levels. For this purpose, the noninvasive Ci-203 handheld laser area meter (CID Bio-Science Inc, Camas, USA) was used.

The same number of leaves was used as in the disease assessments (**see 2.4.1**) in order to report the averaged chlorophyll content and flag leaf area of wheat plants in a plot.

2.4.3 Yield and thousand kernel weight (TKW)

In the micro-plots, all plants within a box where harvested by cutting the ears and threshing them with a laboratory thresher (Haldrup, Ishofen, Germany). The weight of all grains per plot was measured and yield and thousand grain weight were reported at 15% water content.

For the field trial, the dual plot combine Wintersteiger Split (Wintersteiger AG, Ried im Innkreis, Austria), was used for harvesting the experiments. Because the borders of each plot were not included, yield and TKW data was calculated based on a 10.2 m^2 plot size and a water content of 15%.

2.4.4 Data analysis

The R statistical program (R-Core-Team, 2012) was used to analyze the data using ANOVA. To see whether the data needed any transformation due to normality

distribution and homogeneity of variance violation, the R-diagnostic plots within the ANOVA model were used. To compare differences between treatments, the agricolae package (de Mendiburu, 2012) within the R statistical program was used to calculate the Least significant difference (LSD) based on an alpha value of 0.05.

2.5 Molecular biology methods

In order to analyze the effect that different fungicide modes of action and application timing have on the plant at the molecular level, a gene expression analysis using wheat microarrays was done at two different time points during the symptomless phase of *M. graminicola* infection and under controlled growing conditions in the glasshouse. Additionally, in order to see whether there is any difference in the disease control efficiency within the different treatments, DNA fungal quantification was performed during the symptomless phase on wheat seedlings. This phase was chosen because even though all chemicals are successful controlling this fungus and during a screening test no or few visible symptoms are observed upon fungicide treatment, one would think that there is no difference in the type of fungicide use or in the application timing. However, due to the long symptomless phase of this fungus, differences in the control of this disease at this point could help plants saving costly stress response activation that could be expressed in lower yields. For all the molecular biology experiments 10 days old seedlings from the winter wheat variety Riband were used. Growth conditions, fungicide treatments and *M. graminicola* inoculation were performed according to **2.2.1.1, 2.3.1.1** and **2.3.2** respectively.

2.5.1 First true leaf harvesting

For the microarrays and fungal colonization analysis, ten first true leaves per pot were harvested at 3, 5, 7, 10 and 17 days post inoculation (dpi). In the case of the fungicide treatment under disease-free conditions only 3 dpi was the chosen time point, because after this period the probability that the products were already metabolized by the plant were higher. The first true leaves were cut at the bottom and the leaf tip was removed before freezing them in liquid nitrogen. Frozen leaves were then ground in liquid nitrogen and samples were distributed in Eppendorf tubes, each one containing 100-200 mg of the powder. If samples were not immediately analyzed, they were stored at -80°C.

2.5.2 *M. graminicola* quantification via qPCR

2.5.2.1 DNA extraction

Fungal genomic DNA extraction from wheat leaves was performed using the NucleoSpin® plant II extraction kit (Macherey-Nagel GmbH &Co. KG, Düren, Germany) according to manufacturer´s instruction in order to get an elution volume of 70 µl. Subsequently, DNA concentration was measured with the nano-drop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) at 260/280 nm.

2.5.2.2 Primer selection

The chosen endogenous control gene for *M. graminicola* was cytochrome-b (Stammler *et al.*, 2012) and for wheat was Actin. The sequences and conditions of the used primers can be seen in **Appendix 2**. The primers for the gene Actin were designed using Primer-BLAST (Ye *et al.*, 2012). For each oligonucleotide pair, a standard PCR approach was used to proof their binding conditions and amplified product using the sample that contain the highest amount of fungal DNA, being the untreated infected plant at 17 dpi.

2.5.2.3 Standard PCR

Before the PCR process started, primers and template DNA were diluted with 5mM tris-HCL to a final concentration of 500nM and 5 ng/µl respectively. The amplification process was carried out using the 2x Phusion® Flash high-fidelity PCR master mix (Finnzymes, Espoo, Finland) that contains a proofreading polymerase and thus, subsequent sequencing of the PCR product would be more accurate. A standard PCR amplification consists of the following components:

12.5 µl	2x Phusion® Flash PCR master mix
2.5 µl	Forward and reverse primers
7.5 µl	Nuclease-free water (Thermo Fisher Scientific Inc., Waltham, USA)
2.5 µl	Template DNA

For the amplification process, a DNA engine Dyad Peltier thermal cycler (MJ Research™ Inc, Copenhagen, Denmark) was used with the specifications provided by the master mix chosen (**Table 2.5**). Subsequently, the size of the amplified product was checked by agarose gel electrophoresis (90V for 50 minutes). If the size of the product was the same as the theoretical one, it was cleaned from any gel or master mix residues with the NucleoSpin® gel and PCR cleanup kit (Macherey-Nagel GmbH &Co. KG, Düren, Germany)

and sequenced. Sequencing was performed at the DNA laboratory from BASF. The analysis of the sequence was performed with the SeqMan Pro software (DNASTAR Inc, Madison, USA), and if both, the sequence from BLAST and the one from the PCR product were the same, the chosen primer pairs could be used for further studies.

Table 2.5: PCR program for primer check

Temperature (°C)	Time	Repetitions
98	20 sec.	
98	10 sec.	
60	5 sec.	35 times
72	1 min	
72	1 min.	

Agarose gel
2 g. Agarose
200 mL 1 x TAE buffer

50x TAE buffer
242 g. Tris base
57.1 mL Glacial acetic acid
100 mL 0.5M EDTA (PH 8.0)
Bring to a final volume of 1L with double distillated water

2.5.2.4 Quantitative real time PCR (qPCR)

In order to evaluate if there was any difference in the in the control of *M. graminicola* when using different types of fungicides with different application timings, the fungal biomass in infected first true leaves from wheat seedlings was quantified via qPCR. The reaction mix (25µl) for the quantitative amplification consisted of the following components:

12.5 µl	MESA FAST qPCR MasterMix plus dTTP for SYBR® assay no ROX (Eurogentec, Seraing, Belgium)
1 µl	Forward and reverse primers (200 nM)
9 µl	Nuclease-free water (Thermo Fisher Scientific Inc., Waltham, USA)
2.5 µl	Template DNA

In the first place and in order to evaluate the efficiency of the primers, a standard curve was built for the two primer pairs using eight 1:5 serial dilutions of the untreated infected sample at 17 dpi as template DNA. The primer efficiency calculation based on the standard curve from the serial dilutions was made using the Rotor-gene Q series software (Qiagen, Hilden, Germany), and if the calculated efficiency was between 0.9 and 1.1, fungal quantification of all the samples via qPCR was performed. The amplifications

for both the standard curve and the fungal quantification were done with the Rotor-gene Q cycler (Qiagen, Hilden, Germany).

The PCR program consisted of an initial denaturing step at 95°C for five minutes followed by 40 amplification cycles, where each one of them consisted on the following steps: i) denaturing step at 95°C for 15 seconds, ii) primer annealing at 60°C for 30 seconds, and iii) elongation step at 72°C for 30 seconds. After each cycle the emitted fluorescence on the green channel was measured and the cycle threshold (CT) values for each sample were calculated according to the Rotor-gene Q series software (Qiagen, Hilden, Germany). In order to identify if any primer dimers were formed that could lead to miscalculated CT values due to sample contamination, after every PCR program a melting curve analysis was performed. For this purpose, a 1°C step-wise temperature increase in the range of 72-95°C was done and after each step the fluorescence was measured. For each PCR made samples were loaded in duplicate for the non-template control and the DNA samples. Besides, four samples from the standard curve were also loaded every time.

The fungus vs. plant DNA ratio ($2^{-\Delta CT}$) was calculated according to (Livak & Schmittgen, 2001) using the wheat constitutive expressed gene Actin as reference.

2.5.2.5 Data analysis

In order to evaluate differences in the fungal growth in wheat seedling upon fungicide treatment ANOVA and the LSD test were used as mentioned in **2.4.4**.

2.5.3 Gene expression analysis

2.5.3.1 RNA extraction and quality assessment

The isolation of total plant RNA for gene expression analysis was done using the NucleoSpin® RNA Plant isolation kit (Macherey-Nagel GmbH &Co. KG, Düren, Germany) according to manufacturer´s instructions. Once the total RNA from wheat plants was isolated, samples remained on ice if immediately used or were stored at -80°C until needed.

RNA concentration was measured with the nano-drop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) at 260/280 nm. In order to evaluate if the quality of

the isolated RNA was good enough for microarray hybridization, the Agilent RNA 6000 Nano kit (Agilent, Santa Clara, USA) was used according to manufacturer´s instructions in order to prepare RNA for analysis with the Bioanalyzer 2100 (Agilent, Santa Clara, USA). This equipment needs only 1 µl of RNA from each sample in order to perform an electrophoretic separation of the sample and provide an electropherogram for visual assessment of the RNA quality. Furthermore, in order to remove the human-based visual interpretation, the plant RNA Nano assay within the 2100 Expert software (version B.02.07; Agilent, Santa Clara, USA) uses its own algorithm in order to calculate the RNA integrity number (RIN) based on the electrophoretic separation of the samples. According to the software instructions, bellow a RIN value of 5, the RNA from the samples is partially degraded, while a RIN value of 10 represents an intact RNA. The chosen RIN threshold for downstream experiments was 7.

2.5.3.2 Microarray hybridization and data analysis

For the microarray experiments three biological replicas were used, where each replica was a pot that contained 10 wheat seedlings. Leaf harvesting and RNA extraction were done as explained in **2.5.1** and **2.5.3.1** respectively. Samples were hybridized to the 8x66K wheat gene expression microarray from Agilent, where the design of the 44K array from Agilent was taken and the rest 22K were filled up with technical replicas from probes within the chip. One glass slide contained 8 high-definition 66K arrays. All steps that need to be followed in order to obtain the expression data of the genes within the array were done according to the One-color microarray-based gene expression analysis. Low input quick amp labeling protocol (version 6.5, May 2010; Agilent, Santa Clara, USA).

Every microarray experiment contains positive controls that are used to monitor the sample amplification and labeling processes as well as the image analysis independently of the quality of the starting material. These positive controls are called spike-in probe sets which were designed in such a way that will not hybridize to the plant samples.

Before synthesizing labeled complementary RNA (cRNA), the spike-in positive controls had to be added to the samples. For this purpose, four serial dilutions of the spike mix were done, and 2 µl of the fourth dilution were added to 90 ng of total wheat RNA. In order to isolate messenger RNA from the samples, 1.8 µl of the T7 promoter primer mix

were added to the total RNA and incubated at 65°C for 10 minutes. Subsequently the complementary strand DNA (cDNA) was synthesized by mixing 4.7 µl of the cDNA master mix with the messenger RNA. Probes were incubated for two hours at 40°C, and in order to inactivate the AffinityScript RNase, samples were incubated for 15 minutes at 70°C. Finally, labeled cRNA was synthesized by adding 6 µl of the transcription master mix and incubating the samples for two hours at 40°C. The transcription master mix contains the T7 RNA polymerase, which not only amplifies the template material but it also incorporates the labeling dye simultaneously.

T7 promoter mix

0.8 µl	T7 promoter primer
1 µl	Nuclease free water

cDNA master mix

2 µl	5x first strand buffer
1 µl	0.1M DTT
0.5 µl	10mM dNTP mix
1.2 µl	AffinityScript RNase block mix

Transcription master mix

0.75 µl	Nuclease-free water
3.2 µl	5x transcription buffer
0.6 µl	0.1M DTT
1 µl	NTP mix
0.21 µl	T7 RNA polymerase blend
0.24 µl	Cyanine 3-CTP

Purification of the amplified cRNA samples was done with the RNeasy mini kit (Qiagen, Hilden, Germany). cRNA concentration and cyanine 3 dye integration were evaluated using the microarray measurement function of the Nano-drop software. If the recommended values for cRNA yield and dye integration were accomplished, samples could then be hybridized to the microarrays.

Fragmentation of the cRNA was done by adding 25 µl of the fragmentation mix and incubating the samples at 60°C for 30 minutes. Subsequently, 25µl of 2x GEx hybridization buffer HI-RPM were added to the fragmented cRNA in order to stop the fragmentation reaction. Finally, taking care not to introduce bubbles into the samples, they were pipetted onto the array, the Agilent SureHyb chamber was assembled and the hybridization of the complementary labeled RNA to their corresponding sequence in the microarray took place in the hybridization oven by continuously rotating the slides at 10

rpm and 65°C for 17 hours. The disassembly of the hybridization chamber and the first washing step took place in the GE wash buffer 1 and the second washing step was done at 37°C with the pre-warmed GE wash buffer 2. Slides were shortly dried with a delicate wipe and scanned with the Agilent high-resolution C scanner. Gene expression values were obtained using the Feature extraction software (version A.8.4.1; Agilent, Santa Clara, USA) with the protocol GE1-107-Sep09

Fragmentation mix

600 ng	Cyanine 3-labeled, linearly amplified cRNA
5 µl	10x blocking agent
1 µl	25x fragmentation buffer

Bring to a final volume of 24 µl with nuclease free water

The microarrays were analyzed by Dr. Birgit Samans from the Institute of Biometry and Population Genetics from the Justus Liebig University of Gießen using limma (Smyth, 2005), a Bioconductor package within the R statistical programming (http://www.bioconductor.org/). The data were first background corrected applying the *normexp* option, and then log2 transformed and normalized with the *quantile* method. The intensities of oligonucleotides that were spotted more than once on the microarray were averaged. For each comparison, the log fold change and the moderate t-statistic was calculated. The selection of differentially expressed genes was done based on a fold change of 2 (log2 = 1), a moderate t-statistic of 1.96 and an average spot intensity (A) of 5. In the cases where we had replicate samples, the intensities for each gene were averaged.

Subsequently, the gene sequences were annotated using Blast2GO. Blast2GO provides gene annotation based on sequence similarity, gene ontology terms, Interpro IDs and the assignment to KEGG pathways (Conesa et al., 2005; Conesa & Götz, 2008; Götz et al., 2008; 2011). A cluster analysis of the treatments was done within the R environment using the k-mean clustering method. The hyper geometric test from the GOStats package in Bioconductor was used in order to find significant enriched GO terms for the genes within each cluster.

2.5.4 Microarray validation via qPCR

Based on the results obtained from the gene expression analysis, nine genes were chosen to validate the microarray experiments. The sequences and the running

conditions of the primers designed during this experiment can be seen in Appendix 2 (**Appendix 2**). For this purpose, RNA was extracted from the same samples used for the microarray experiment according to **2.5.3.1**. Subsequently the RNA was reverse transcribed in order to synthesize cDNA, from which the gene expression analysis was carried out by means of qPCR.

2.5.4.1 cDNA synthesis

For the cDNA synthesis the Maxima® First Strand cDNA Synthesis kit for the reverse transcription PCR (RT-PCR) (Thermo Fisher Scientific Inc., Waltham, USA) was employed. For this purpose, 1µg RNA was incubated at 70°C for 2 minutes, in order to denature its secondary structures, and samples were immediately placed on ice. The denatured RNA was mixed with the cDNA master mix and the reverse transcription took place in the PCR cycler according to the following program:

25°C 10 min.
50°C 30 min.
85°C 5 min.

cDNA master mix

4 µl	5X Reaction Mix
2 µl	Maxima® Enzyme Mix
1 µg	RNA

Bring to a final volume of 20 µl with nuclease free water

Once the cDNA was synthesized it was diluted with 80 µl of milli-Q water in order to get a final concentration of 10 ng/µl cDNA. For every sample a reverse transcriptase negative control was included in order to check if there was any genomic DNA contamination.

In order to prove the quality of the cDNA synthesis, a PCR check (**Table 2.6**) was carried out with the primer pair for the endogenous control gene Actin, where the expression of this gene should be the same across samples. The PCR reaction for the cDNA check was performed with the DCSPol DNA polymerase (DNA Cloning Service e.K., Hamburg, Germany), which consists on the following components:

2.5 µl	10X BD Buffer
2.5 µl	2mM DNTPs
0.6 µl	Actin forward primer
0.6 µl	Actin reverse primer
0.2 µl	DCS Taq polymerase
2 µl	Template cDNA (10 ng/µl)

Table 2.6: PCR program for cDNA check

Temperature (°C)	Time	Repetitions
98	2 min.	
98	20 sec.	27x
60	20 sec.	
72	20 sec.	
72	5 min.	

Once the amplification was accomplished, the PCR products of every sample were analyzed with agarose gel electrophoresis (90V for 45 minutes) before proceeding with the quantitative PCR.

Agarose gel (1.5%)

3 g	Agarose
200 µl	1X TBE buffer
20 µl	Ethidium bromide solution 1%

10X TBE buffer

900 mM	Tris
900 mM	Boric acid
25 mM	EDTA pH 8

2.5.4.2 Quantitative real time PCR (qPCR)

For the qPCR experiments two internal controls were used. The first one was as in **2.5.2.4** the wheat gene Actin. The second reference gene was obtained using the RefGenes tool from the Genevestigator platform (Hruz *et al.*, 2008; 2011). Among the genes obtained from RefGenes two probes with GeneBank accession numbers AK334339.1 and DQ681104.1were selected. However, during the qPCR experiments the expression of the gene DQ681104.1 was not stable. For this reason, the two internal control genes employed for the microarray validation were Actin and AK334339.1.

Because the microarray experiments were performed in the Institute of Phytopathology and Applied Zoology (IPAZ) from the Justus Liebig University of Gießen, the validation of the experiment by means of qPCR was also performed in the IPAZ facilities using the Applied Biosystem 7500 Fast real time PCR system (Life Technologies GmbH, Darmstadt, Germany). For this purpose, the SYBR® Green JumpStart™ Taq Ready Mix™ (Sigma-Aldrich, Munich, Germany) was used according to the following volumes:

10 µl	2x SYBR Green JumpSart Taq Ready Mix
1 µl	Forward and reverse primers (200 mM)
7 µl	Milli-Q water
20 ng	cDNA

The PCR program consisted in an initial Taq polymerase activation step at 95°C for 5; followed by 40 cycles, each consisting on a denaturing step at 95°C for 15 seconds, 60°C for 30 seconds for primer annealing and a final extension step at 72°C for 30 seconds. After each cycle the emitted fluorescence was measured and the cycle threshold (Ct) was calculated according to the 7500 Software (version 2.01; Life Technologies, Darmstadt, Germany). Once the amplification was completed, a melting curve analysis was carried out in order to identify any primer dimer formation that can lead to misleading Ct values. The melting curve analysis consisted on a 1°C step-wise temperature increase in the range of 68-95°C, where the fluorescence measured took place after each step. For each reaction, cDNA samples and non-template control were loaded in triplicate. The fold change in gene expression was calculated according to (Livak & Schmittgen, 2001) for the different experimental setups as follows: i) fungicide treated plants under disease-free conditions vs. uninfected, untreated plants; and ii) fungicide treated wheat seedlings under disease pressure relative to Septoria infected untreated plants. For each of the experiments, the two previously mentioned genes Actin and AK334339.1 were employed as internal controls.

2.6 Microscopy of *M. graminicola* infected leaves

Microscopical images of the *M. graminicola* infection process were carried out with the Hitachi analytical tabletop electron microscope TM3000 (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) and the research stereo zoom microscope SZX16 (Olympus Deutschland GmbH, Hamburg, Germany).

In order to get better quality pictures in the scanning electron microscope, sample preparation was done by coating leaves with gold using the Leica EM SCD005 table-top spotter coater (Leica Microsystems GmbH, Wetzlar, Germany) under 10^{-1} mbar vacuum pressure and 60 mA fixed voltage, chamber was flushed with argon. Gold sputtering took place for 30 seconds under vacuum conditions. The Scanning electron microscope works under low vacuum (0.1 mbar) and images were detected with the analysis mode.

3. Results

The aim of this project was to evaluate the effect of different fungicides and timing of application on the physiology and yield of wheat in relation to the development of the fungal disease caused by *Mycosphaerella graminicola*. For this purpose, different experimental setups were analyzed: i) under controlled conditions, the effects of the fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad were evaluated at the molecular level using microarrays from wheat. To study the purely physiological effects of the fungicides on the plant, the experiments were carried out under disease-free conditions. Additionally, for the evaluation of the effect that the fungicides have on the plant-pathogen interaction, two different application time points, a preventative and curative, were carried out. Subsequently, the effects that the fungicides have on disease control and plant physiology were evaluated at the molecular level with microarrays. ii) In, order to evaluate the effect that fungicide application timing has on yield, trials under semi-controlled and completely natural conditions were carried out as an attempt to correlate the results observed at the molecular level to those obtained on the micro-plots and on the field. If fungicide application takes place preventatively or early enough in the growing season, the activation of costly defense responses will be hindered and thus, higher yields should be obtained. On the other hand, if the fungicide application first takes place after disease symptoms are visible, the yield produced should be lower because the disease has already caused enough damage to the plant.

Before the experiments with the fungicides were started, however, the infection biology of *M. graminicola* in wheat seedlings was studied as a control since that, according to Eyal (1987), the infection process can vary depending on the environmental conditions, wheat cultivar and fungal isolates used.

3.1 Infection biology of *M. graminicola* in wheat seedlings

For the analysis of the infection process of *M. graminicola* on wheat seedlings, the winter wheat cultivar Riband was chosen due to its high susceptibility to this pathogen (Chartrain *et al.*, 2004a). Wheat seedlings were inoculated with pycnidiospores from four *M. graminicola* isolates (**see 2.1**). The experiments were carried out in the glasshouse with the following environmental conditions: 16/22 °C day/night temperature, 15 hour light period and 88% relative humidity.

Under the environmental conditions present in the glasshouse, fungal penetration events of the selected plant variety and *M. graminicola* isolates were observed at 3dpi mainly via the stomata (**Figure 3.1A-B**). In some cases, when present, appressoria were observed at the penetration site (**Figure 3.1-B** arrow). While initial colonization by the fungus did not cause any visible damage to the plants (**Figure 3.1C**), visible symptoms, such as chlorotic spots on the leaf surface, were observed as the fungus switched to a necrotrophic lifestyle and started killing the plant. The first visible symptoms appeared between 15 and 17 dpi (**Figure 3.4**). Seven days later, leaves were completely necrotic with pycnidia extruding from the stomata (**Figure 3.1D)** and visible by the unaided eye as black spots scattered on the leaf surface (**Figure 3.1E**). Under high humidity conditions, conidia were released from the pycnidium within a gelatinous matrix, known as cirrus (**Figure 3.1E-F**). In natural conditions, when it rains, the gel in the cirri would dissolve and conidia would be dispersed within short distances by rain-splash.

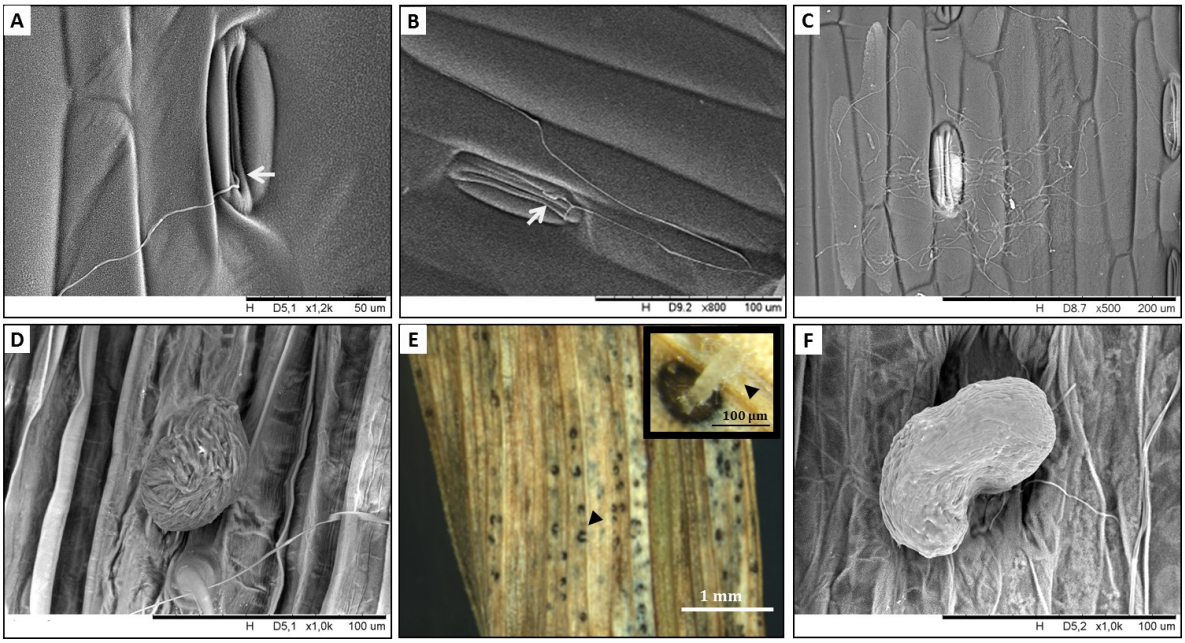


Figure 3.1: *M. graminicola* infection process in winter wheat seedlings of the susceptible variety Riband

The fungal infection process was monitored by scanning electron microscopy (SEM) at 3, 5, 17 and 25 days post inoculation (dpi). Fungal penetration through the stoma (arrows) as visualized at 3 dpi (**A**) and at 5 dpi (**B**). Fungal growth seen as a cluster of hyphae surrounding the stoma observed at 17 dpi (**C**). At 25 dpi, mature pycnidia have extruded from the stomata (**D**) and can be seen by the unaided eye (**E**) as black spots (black arrow-head) on the necrotic tissue. When leaves are incubated in a moist environment conidia extrude from the pycnidium within the cirrus (**F)**.

Along with visual assessment of the *M. graminicola* infection process, the amount of fungal DNA present in the infected plants was measured during the symptomless phase of the infection in order to evaluate whether a steady increase in fungal biomass was present, or if the absence of visible symptoms was due to the fact that the fungus may not have been actively growing during this period. According to the data obtained (**Figure 3.2**), during the first 10 days of the infection process no significant increase in fungal growth occurred. At 17dpi, there was an exponential growth in the biomass of the pathogen, which, according to the macroscopic pictures of the leaves, was also the point when visual disease symptoms appeared as chlorotic spots on the leaf surface (**Figure 3.4**). From 3 to 5 dpi a slight decrease of the fungal DNA could be observed; probably because the first leaf kept growing while the fungus did not, therefore making it appear as if there was a reduction in the biomass of the fungus.

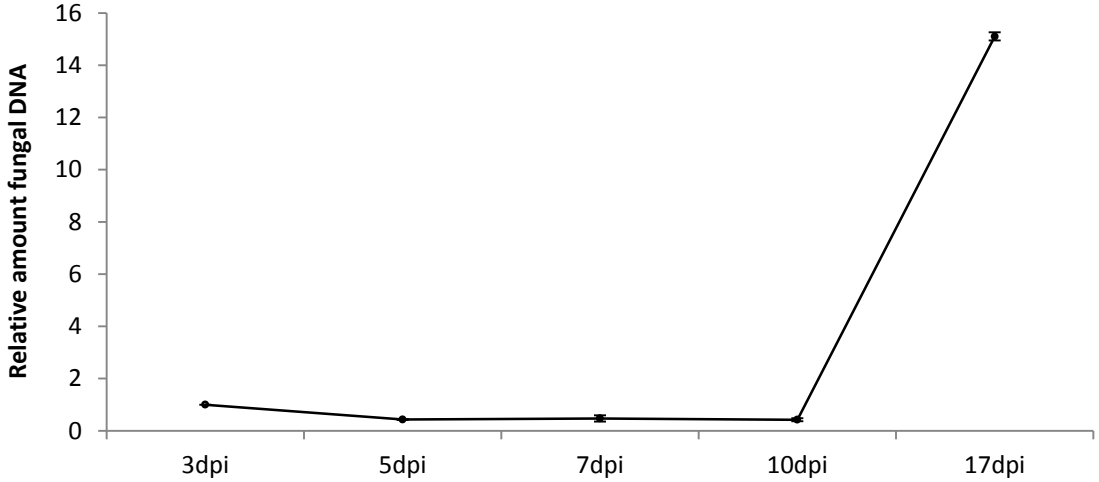


Figure 3.2: Relative amount of *M. graminicola* DNA during the symptomless phase of the infection process

qPCR was carried-out in order to quantify the fungal DNA based on the amount of *M. graminicola* cytochrome-b relative to wheat actin gene during the symptomless phase of the fungal infection. At 3, 5, 7, 10 and 17 dpi first true leaves in each pot were collected for fungal DNA quantification. Ten plants were seeded in a pot, where each pot represented an experimental unit. The experiment was carried out with three biological replicas (i.e. 3 different pots). Displayed above are the means with standard error of the three independent experiments. Abbr: dpi: days post inoculation

3.2 Efficacy of fungicides with different modes of action on the control of septoria leaf blotch in wheat seedlings

In order to see whether different fungicide active ingredients vary in their efficacy in treating to septoria leaf blotch (SLB), three products were employed with spore

germination, fungal growth and disease symptom development evaluated under curative and preventative spraying regimes. As mentioned in the introduction, Epoxiconazole and Pyraclostrobin represent the most important chemical classes in the global fungicide market, the triazoles (DMI) and the strobilurins (QoI), respectively. Fluxapyroxad belongs to the new generation of carboxamide fungicides (SDHI) recently introduced to the global market.

By inhibiting different steps in the electron transport chain, Pyraclostrobin and Fluxapyroxad disrupt fungal energy production. Therefore, these fungicides display very strong preventative activity by hindering the spore germination and preventing an infection to occur in the first place (Bartlett *et al.*, 2002; Semar *et al.*, 2011). Scanning electron microscope images taken at 3 days post inoculation confirmed that both cellular respiration inhibitors are able to hinder spore germination and germ tube formation (**Figure 3.3-A and C**). In contrast, the sterol biosynthesis inhibitor, Epoxiconazole was not able to do so; allowing fungal spores to penetrate the plant through stomatal openings or directly at the junction of two epidermal cells (**Figure 3.3B**).

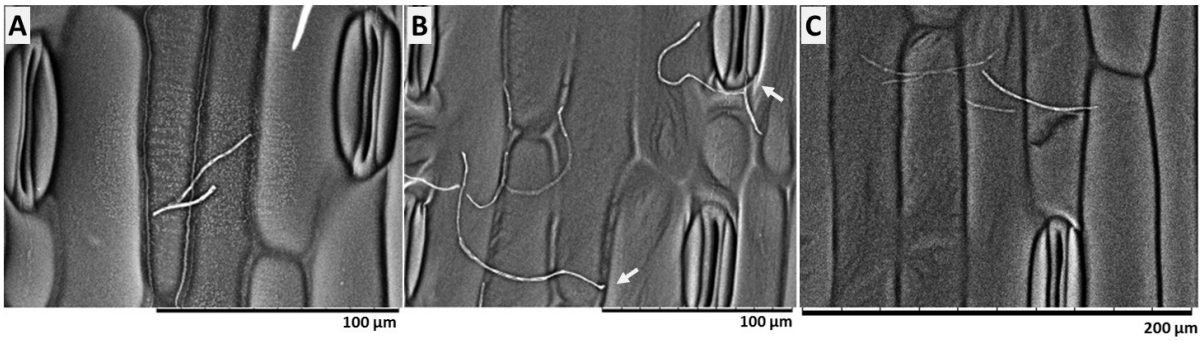


Figure 3.3: Effect of fungicide treatment on *M. graminicola* spore germination

The fungicides Pyraclostrobin (A), Epoxiconazole (B) and Fluxapyroxad (C) were sprayed on wheat seedlings one day before inoculation with pycnidiospores from *M. graminicola*. Three days after the inoculation the first leaf was cut and the spore germination, germ tube elongation and penetration events (arrows) were observed under a scanning electron microscope (SEM).

Unlike in Pyraclostrobin- and Fluxapyroxad-treated plants; *M. graminicola* growth was not completely hindered when Epoxiconazole was applied preventatively, with the infected plants developing disease symptoms at 17 dpi, albeit less aggressive in comparison to untreated plants (**Figure 3.4**). When fungicide application took place after the infection had already been established, all three products were able to hinder

pathogen growth and retard the appearance of symptoms. After 25 dpi, however, when the untreated plants were heavily diseased, a clear difference between the sterol biosynthesis inhibitor and the two respiration inhibitors could be observed. During this experiment, Epoxiconazole-treated plants showed evident necrotic lesions while Fluxapyroxad- and Pyraclostrobin-treated wheat seedlings did not. Although the plants that were treated curatively with Epoxiconazole still developed some disease symptoms, no pycnidia were observed. In plants treated with the same active ingredient preventatively, on the other hand, mature pycnidia were still detected as black spots in the lesion area. When compared to untreated plants, nonetheless, the control of disease under Epoxiconazole treatment was still very effective regardless of the application timing.

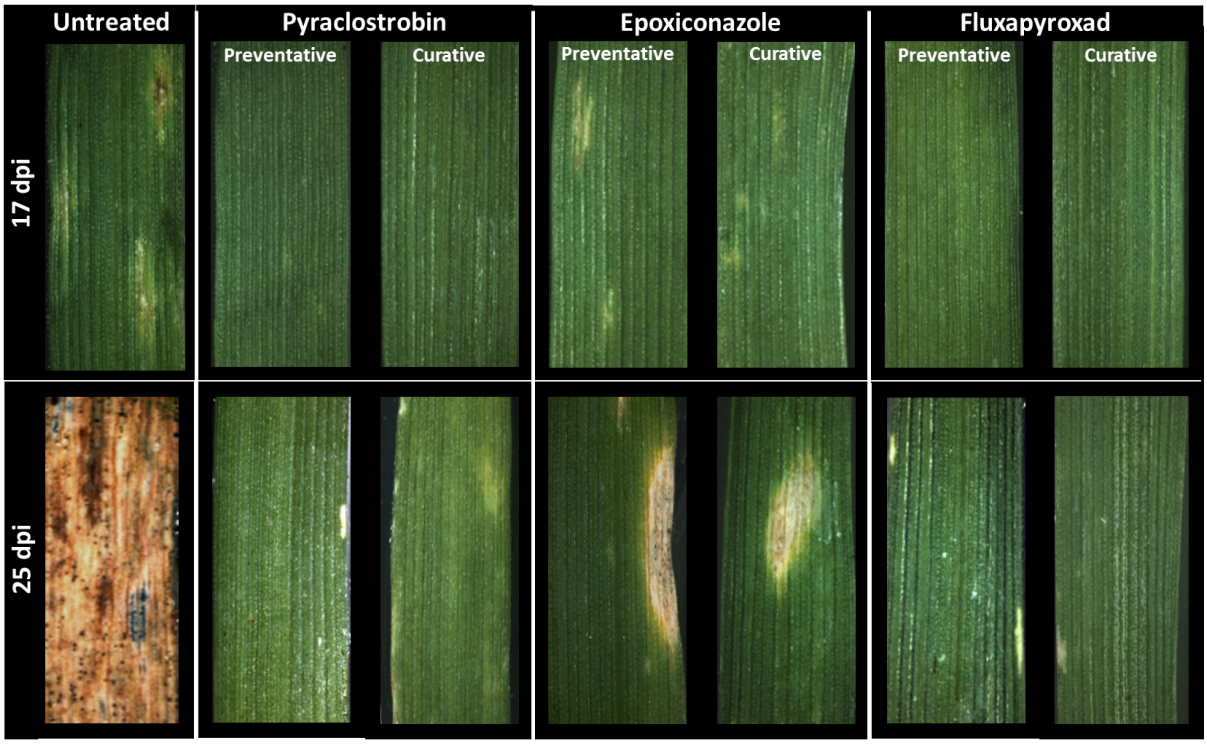


Figure 3.4: Effect of fungicide treatment and application timing on septoria leaf blotch symptom development

The fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad were applied either preventatively, one day before inoculation, or curatively, seven days post inoculation. Displayed above are macroscopic pictures of disease symptoms in fungicide-treated and untreated wheat leaves at the onset of lesion appearance (17 dpi), and when untreated plants reached 100% disease infection (25 dpi). At 25 dpi mature pycnidia could be seen as black dots along the leaf surface. Abbr.: dpi; days post inoculation.

Alternatively, in plants treated with Fluxapyroxad, no visible difference in symptom development between preventative and the curative applications were observed (**Figure 3.4**). Plants treated curatively with Pyraclostrobin, on the other hand, displayed minor chlorotic lesions. These observations could lead to the conclusion that QoI fungicides are less effective in controlling the disease once it has established than the SDHI products.

The results obtained previously relied on macroscopic observations of the disease development and can be influenced by the evaluator's perception and expertise. Furthermore, assessments can only be performed after disease symptoms have developed (Guo *et al.*, 2007). In order to estimate the effect that different fungicide modes of action have on controlling septoria leaf blotch, either as preventative or as curative treatments, the fungal biomass was quantified. Fungal DNA quantification took place before the development of disease symptoms via qPCR at 3, 5, 7, 10 and 17 dpi. Subsequently, the efficacy of each fungicide was calculated as the percentage of the reduction in fungal DNA relative to the untreated control.

A single ANOVA was performed to test for differences among the distinct modes of action and application timings in the control of *M. graminicola* in wheat seedlings. The efficacy of the fungicides differed significantly at the different measuring time points (**Table 3.1**).

Table 3.1: ANOVA summary table of the % reduction in fungal DNA relative to the untreated control at different time points after inoculation with pycnidiospores from *M. graminicola*

	Fungicide treatment
Response variable	F-value; P
% reduction in DNA at 3dpi	$F_{(2,6)}$= 70.7; ***
% reduction in DNA at 5dpi	$F_{(2,6)}$= 12.9; **
% reduction in DNA at 7dpi	$F_{(2,6)}$=4.9; *
% reduction in DNA at 10dpi[a]	$F_{(5,12)}$=15.6; ***
% reduction in DNA at 17dpi[a]	$F_{(5,12)}$=7.1; **

Significance codes F-test(DF treatment; DF error): P < 0.001 ***, P < 0.01 **, P < 0.05 *, P > 0.05 not significant (NS). Abbr.: DF; degrees of freedom

[a] For the statistical analysis of the data a logit transformation was performed.

The post-hoc least significant difference comparison indicates a clear difference between the preventative application of Epoxiconazole and both cellular respiration inhibitors (**Figure 3.5**). The efficacy of Pyraclostrobin and Fluxapyroxad exceeded 80% along the entire infection process. At 5 dpi, it seems that the effectiveness decreased, likely attributable, as mentioned earlier, to the reduction in fungal biomass observed in the untreated control (**Figure 3.2**). Even though the preventative application of Epoxiconazole reached almost 100% efficacy at 17 dpi, there was a clear difference between it and the other fungicides earlier on in the process, especially at 3 dpi.

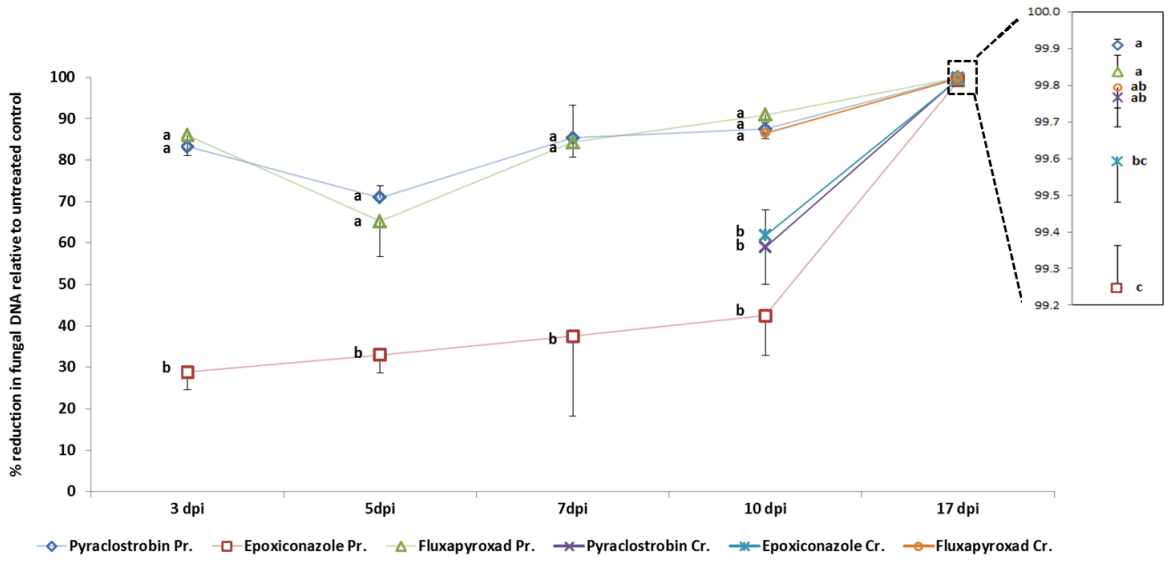


Figure 3.5: Fungicide efficacy during the symptomless phase of the *M. graminicola* infection process.

The fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad were sprayed on wheat seedlings either as a preventative treatment, one day before the *M.* graminicola infection, or as a curative treatment 7 days after the inoculation. At 3, 5, 7, 10 and 17 dpi, first true leaves in each pot were collected for fungal DNA quantification by qPCR. The amount of fungal cytochrome-b in fungicide-treated plants relative to the untreated control was determined using the wheat actin gene as the endogenous control. Based on the data obtained, the efficacy of each fungicide was calculated as the percentage of fungal DNA reduction relative to the untreated control. Ten plants were seeded in a pot, with each pot represented an experimental unit. The experiment was carried out with three biological replicas (i.e. 3 different pots). The means of three replicates are displayed above; bars represent the standard error of the experiment. Means with the same letter within a time point are not significantly different (Least significant difference at P=0.05). Abbr. dpi: Days post inoculation; Pr.: Preventative treatment one day before the inoculation; Cr.: Curative treatment 7 days after the inoculation; LSD: Least significant difference

Among the curative treatments, Fluxapyroxad was the most effective fungicide along the entire infection process (**Figure 3.5**). Furthermore, the effectiveness of the curative

application of this fungicide even achieved the same level as the preventative application of Pyraclostrobin and Fluxapyroxad. Based on these results, the observations from SEM images on the effect of different modes of action on spore germination could be confirmed. Even though the efficacy of all tested products reached approximately 100% by the end of the assessment, a clear distinction between the active ingredients could be identified before the point at which chlorotic lesions appeared (17 dpi). Differences in fungicidal efficacy along the infection process may have an impact on the physiology of the plant.

3.3 Transcriptional analysis of wheat seedlings upon fungicide treatment

The previous results demonstrated that efficacy of the fungicides Pyraclostrobin (QoI), Epoxiconazole (DMI) and Fluxapyroxad (SDHI) in controlling septoria leaf blotch in wheat seedlings varies according to the mode of action and application timing used. For this reason, a transcriptional analysis of wheat seedlings via microarrays was performed in order to evaluate whether: i) the fungicides mentioned above have a positive influence on the plant when applied under disease-free conditions, or ii) the higher yields reported in the literature are mainly due to better disease control strategies as suggested by Bertelsen *et al.* (2001) and Swoboda and Pedersen (2009).

In order to evaluate the physiological effects that Pyraclostrobin, Epoxiconazole and Fluxapyroxad have on wheat at the molecular level, the products were sprayed on wheat seedlings under disease-free conditions. The effects of the fungicides on wheat relative to a mock-treated plant were evaluated four days after fungicide application (4 dpa) using an Agilent microarray containing 44 thousand wheat probes.

Additionally, to evaluate the effect that the application timing of the selected fungicides has on controlling *M. graminicola* and the physiology of wheat, these products were sprayed either preventatively, one day before the inoculation with pycnidiospores from *M. graminicola*, or seven days after the inoculation as a curative application. Subsequently, a genome-wide transcriptional analysis of the treated wheat seedlings relative to the infected un-treated controls was carried out using the 44K Agilent microarray at 3 and 10 dpi. The selected time points for this analysis were based on a microarray pre-experiment (**see 3.3.2.1**) of the responses of wheat seedlings to *M. graminicola* during the symptomless phase of the infection.

A total of 54 microarrays were hybridized, including one microarray from each time point from the pre-experiment samples (**Appendix 3**).The microarray experiments were analyzed by Dr. Birgit Samans from the Institute of Biometry and Population Genetics from the Justus Liebig University of Gießen using limma (Smyth, 2005), a Bioconductor package within the R statistical programming (http://www.bioconductor.org). The data were first background corrected applying the *normexp* option, and then log2 transformed and normalized with the *quantile* method. After pre-processing of the raw data, 40644 genes were left for further analysis. The selection of differentially expressed genes was performed based on a fold change of 2 (log2 = 1), a moderate t-statistic of 1.96 and an average spot intensity (A) of 5. According to this, a total of 2577 differentially expressed genes (6% of the analyzed genes) resulted from the application of either treatment under disease-free conditions at 4 dpa. The preventative application of any of the fungicides resulted in 1057 (almost 3% of the probes) differentially expressed genes at 3dpi and 2634 (more than 6%) genes at 10 dpi. The curative treatment led to 1557 genes being differentially expressed at 10 dpi, representing almost 4% of the analyzed genes. Subsequently, for each of the significantly up- and down-regulated transcripts obtained, an enrichment analysis of Gene Ontology (GO) terms was performed in November 2012 to identify the biological processes that were modified in the plants after a given fungicide treatment or after the infection with *M. graminicola*. The top 50 GO terms for each comparison can be found in **Appendix 4**.

3.3.1 Microarray experiments under disease-free conditions

With the purpose of evaluating whether there is any difference in the effect that fungicides with varying modes of action have in the plant at the transcriptome level, three products (Pyraclostrobin, Epoxiconazole and Fluxapyroxad) were applied to wheat seedlings under controlled, disease-free conditions. The responses of the plants four days after the application (4dpa) were then evaluated versus water-treated plants using the Agilent microarray containing 44 thousand probes from wheat. Epoxiconazole belongs to the fungicide group inhibiting the C-14 demethylase enzyme in the sterol biosynthesis pathway in fungi, and are thus, known as sterol biosynthesis inhibitors (Burden *et al.*, 1989; Kwok & Loeffler, 1993). Fluxapyroxad and Pyraclostrobin, on the other hand, block the energy production of fungi by inhibiting the electron transfer chain at the complexes II and III in the inner mitochondrial membrane, respectively. For

this reason these fungicides are also known as cellular respiration inhibitors (Bartlett *et al.*, 2002; Earley *et al.*, 2012)

Pyraclostrobin, Epoxiconazole and Fluxapyroxad treatment under disease-free conditions resulted in 1878, 1646 and 1569 differentially expressed genes respectively. Regardless of the fungicide treatment, approximately 60-67% of the genes were up-regulated compared to the mock-treated control. From the 2577 differentially expressed genes obtain after fungicide application, 971 (38%) overlapped between the three fungicides, from which more than 70% were induced (**Figure 3.6**). Additionally, 465 (18%), 353 (14%) and 214 (8%) gene transcripts resulted to be differentially expressed specifically to Pyraclostrobin, Epoxiconazole and Fluxapyroxad respectively. Among the genes that responded exclusively to a given fungicide, 50% were induced after Pyraclostrobin application, 47% after the treatment with Epoxiconazole and 32% were up-regulated only in Fluxapyroxad-treated plants.

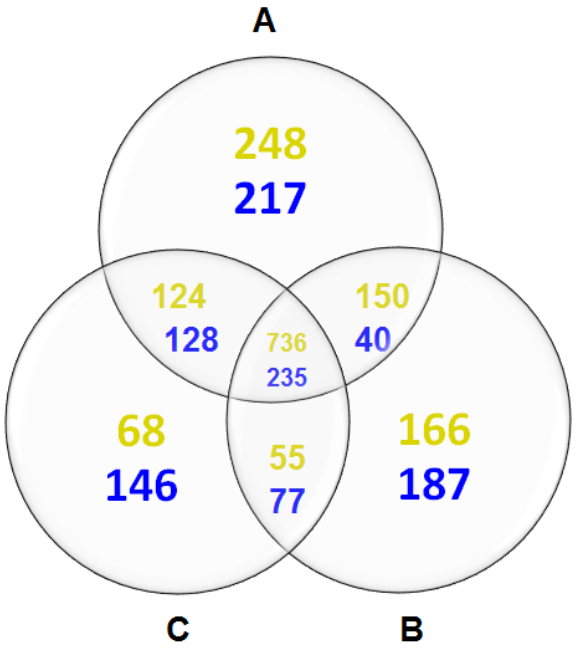


Figure 3.6: Up- and down-regulated transcripts in fungicide treated wheat seedlings under disease-free conditions at 4dpa

The fungicides Pyraclostrobin (**A**), Epoxiconazole (**B**) and Fluxapyroxad (**C**) were sprayed on wheat seedlings under disease-free conditions. The untreated control was sprayed with water instead. Ten seedlings were planted per pot, with each pot representing an experimental unit. The experiment was carried out with three biological replicas (i.e. 3 different pots). Four days after the fungicide application (4dpa) the first true leaf in each pot was collected for transcriptional analysis using an Agilent microarray from wheat. The Venn diagram in the picture displays the number and overlap of the transcripts found to be differentially expressed

in fungicide-treated plants versus the untreated controls. Up-regulated genes are displayed in yellow, whereas down-regulated genes are represented in blue.

After BLAST2GO annotation (http://www.blast2go.com; Retrieved December 2013), genes could be classified in 13 categories as follows (**Figure 3.7**): stress response, transport, gene expression, response to hormone stimulus, lipid metabolic process, signal transduction, hormone-mediated signaling pathway, cell wall organization or biogenesis, photosynthesis, electron transport chain, secondary metabolic process and protein degradation. Between 35-40% of the differentially expressed genes did not have any annotation, thus their functions are still unknown. Among the annotated genes, one can infer that stress responses represent the biggest group with approximately 30% of the genes belonging to this category in each fungicide-treated plant; followed by transport, gene expression and response to hormone stimulus each with 15%.

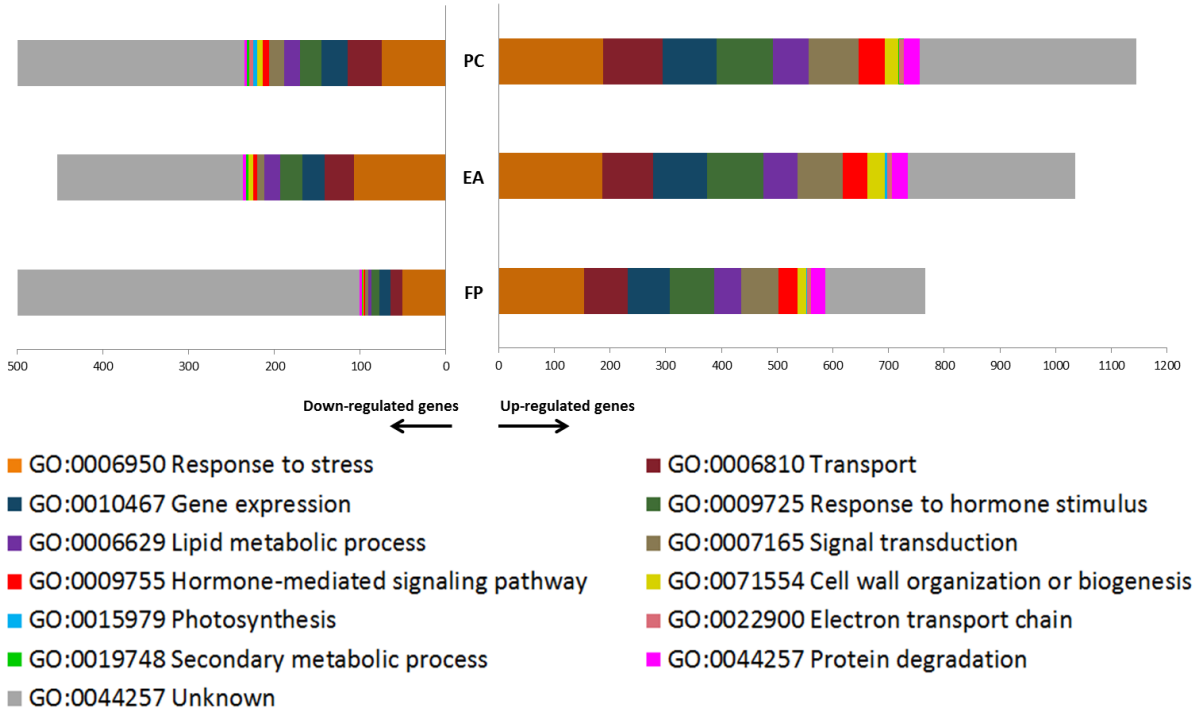


Figure 3.7: Categories of differentially expressed genes upon fungicide treatment under disease-free at 4dpa

Ten seedlings were planted per pot; each pot represented an experimental unit and the experiment was carried out with three biological replicas (i.e. 3 different pots) under disease-free conditions. At 4dpa the first true leaf in each pot was collected for transcriptional analysis using an Agilent microarray from wheat. The selection of differentially expressed genes was done based on a fold change of 2 (log2 = 1), a moderate t-statistic of 1.96 and an average spot intensity (A) of 5. Differentially expressed genes were annotated with BLAST2GO and categorized according to their predicted function. Stacked bars represent the number and categorization of the differentially expressed genes after fungicide application in comparison to an untreated plant. The left part of the graphic corresponds to down-regulated genes,

whereas the right side represents up-regulated genes. Different colors describe the corresponding category present in the legend. Abbr: FP, Fluxapyroxad; EA, Epoxiconazole; PC, Pyraclostrobin; 4dpa, four days after the fungicide application.

Subsequently, mapman (http://mapman.gabipd.org; Retrieved January 2013) was employed to identify differences in the regulation of biotic stress-related genes after Pyraclostrobin, Epoxiconazole and Fluxapyroxad treatment (**Figure 3.8**). According to these results obtained, it is evident that at 4dpa, the three products induced significant changes in the hormonal balance, the components of the cell wall, signaling-related genes and transcription factors involved in defense response activation to biotic stresses. All three products induced the expression of auxin- and abscisic acid-responsive genes. Ethylene biosynthesis at the ACC synthase and ACC oxidase levels was induced in plants treated with any of the fungicides. On the other hand, it could be observed that some ethylene-responsive transcripts were down-regulated in the plants after fungicide application. Unlike ethylene, the expression of jasmonate synthesis-related genes was repressed, while the jasmonate-responsive genes were up-regulated with the three active ingredients. Nonetheless, more down-regulated bins were observed in Pyraclostrobin-treated wheat seedlings. Interestingly, none of the fungicides had an effect on the salicylic acid-dependent synthesis or signaling gene transcripts on wheat.

Up-regulation of genes involved in cell redox homeostasis processes can be observed upon fungicide treatments regardless of their mode of action. Interestingly, genes involved in the hydrogen peroxide metabolism are mainly induced in plants treated with Pyraclostrobin and Fluxapyroxad.

Additionally it is worth noting that all three products down-regulate the transcription of heat shock proteins, with the strongest response observed in Fluxapyroxad-treated plants (**Figure 3.8**). Heat shock proteins (HSPs) are not only known for their role on protein folding, assembly and degradation, but they are also crucial in re-establishing the cellular homeostasis after abiotic stress circumstances (W. Wang *et al.*, 2004). Therefore, the data obtained here suggest that fungicides may not only have an effect as antimycotic agents but also as stress scavengers in wheat seedlings.

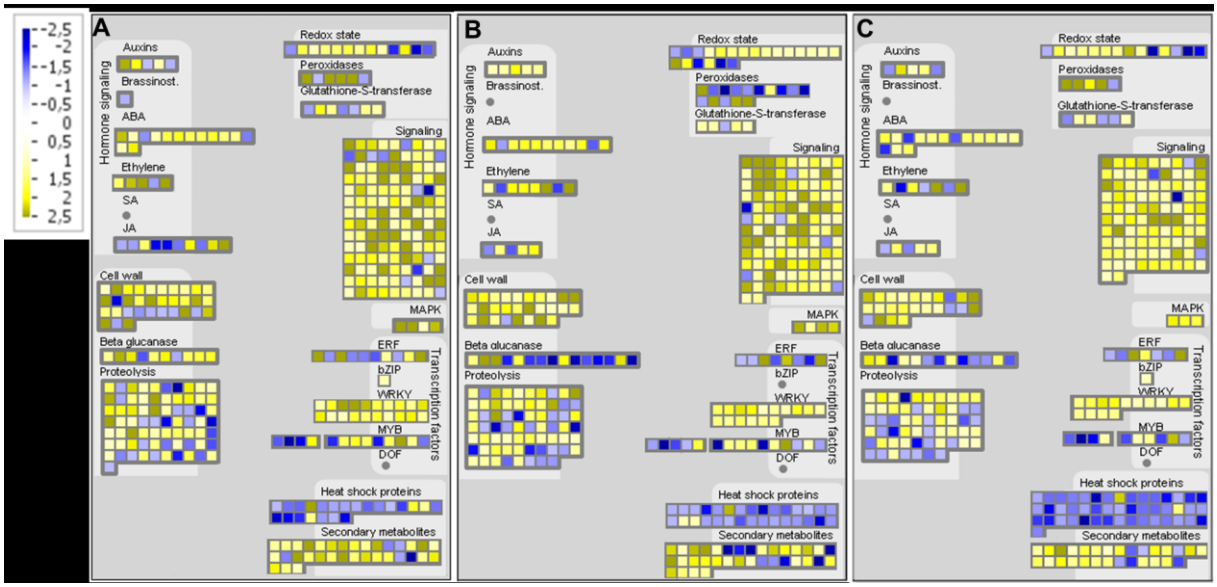


Figure 3.8: Putative involvement of fungicide treatment in biotic stress responses under disease-free conditions four days after the application

The fungicides Pyraclostrobin (**A**), Epoxiconazole (**B**) and Fluxapyroxad (**C**) were sprayed onto wheat seedlings under disease-free conditions. Untreated control plants were sprayed with water. At 4dpa first true leaves in each pot were collected for transcriptional analysis using an Agilent microarray from wheat. Mapman was employed to display the differentially regulated genes obtained from the gene expression analysis (44K wheat chip) onto the biotic stress responses diagram. Blue colors denote down-regulation, whereas yellow symbolizes up-regulation versus the untreated control. Ten seedlings were planted per pot; each pot represented an experimental unit and the experiment was carried out with three biological replicas (i.e. 3 different pots) under disease-free conditions. Abbr.: ABA, abscisic acid; SA, salicylic acid; JA, jasmonate; MAPK; mitogen-activated protein kinase.

Even though the expression profiles of defense-related genes in plants was modified upon fungicide application under disease-free conditions, it can be inferred from **Figure 3.8** that the expression pattern obtained did not differ between the three products albeit their different modes of action. For this reason, a cluster and gene ontology enrichment analysis was performed in order to assess whether there was any difference or similarity in the expression pattern of fungicide-treated and *M. graminicola*-infected plants. Furthermore, this analysis was performed including all genes obtained after data quality control (40644 genes). By doing so, minor gene expression changes could be detected that may have a stronger impact on plant physiology. According to their expression profile, six clusters were selected from the fifteen clusters obtained. A picture of all the clusters with their corresponding gene set enrichment analysis can be found in **Appendix 5**. The first cluster corresponds to gene transcripts that were induced in *M. graminicola*-infected plants but were mostly down-regulated in fungicide-

treated seedlings relative to the untreated control (**Figure 3.9-I**). The gene set enrichment analysis indicated that these genes are involved in the degradation of phenyl-alanine, such as phenyl-alanine ammonia-lyase. Furthermore, transcripts related to cell wall organization were also identified in this cluster. The second cluster represents genes involved in auxin biosynthesis and in post-translational protein modification processes. The gene probes belonging to this cluster were up-regulated in plants treated with the fungicides and the induction levels were even higher than in the infected seedlings. Furthermore, a stronger up-regulation of genes could be observed upon Epoxiconazole treatment (**Figure 3.9-II**). The third cluster corresponds to probes implicated in abiotic stress responses after temperature change and hyperosmotic conditions. Interestingly, the genes in this cluster responded similarly after Pyraclostrobin and Fluxapyroxad treatment but not after Epoxiconazole application (**Figure 3.9-III**). The opposite expression pattern was observed in *M. graminicola*-infected plants. The genes obtained in cluster number four were, according to the GO terms, related to stress responses such as heat shock proteins (HSP), WRKY transcription factors and PR proteins. The expression of the above mentioned genes was mostly induced in *M. graminicola*-infected plants, whereas in fungicide-treated wheat seedlings these transcripts were down-regulated by more than two fold relative to the untreated control (**Figure 3.9-IV**). Treatment with the fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad resulted in the induction of photosynthesis-related transcripts and genes involved in membrane lipid metabolism. The expression of these transcripts was, on the other hand, down-regulated in *M. graminicola*-infected wheat seedlings (**Figure 3.9-V**), probably due to the induction of defense-related genes (**Figure 3.9-IV**). In addition, as observed in the mapman analysis, genes involved in ethylene and auxin metabolism were up-regulated in fungicide-treated and in *M. graminicola*-infected plants. The induction levels observed in the plants that were sprayed with the fungicides was however even stronger (**Figure 3.9-VI**). Additionally, among the fungicides tested, Pyraclostrobin treatment resulted in a stronger induction of the genes present in cluster VI.

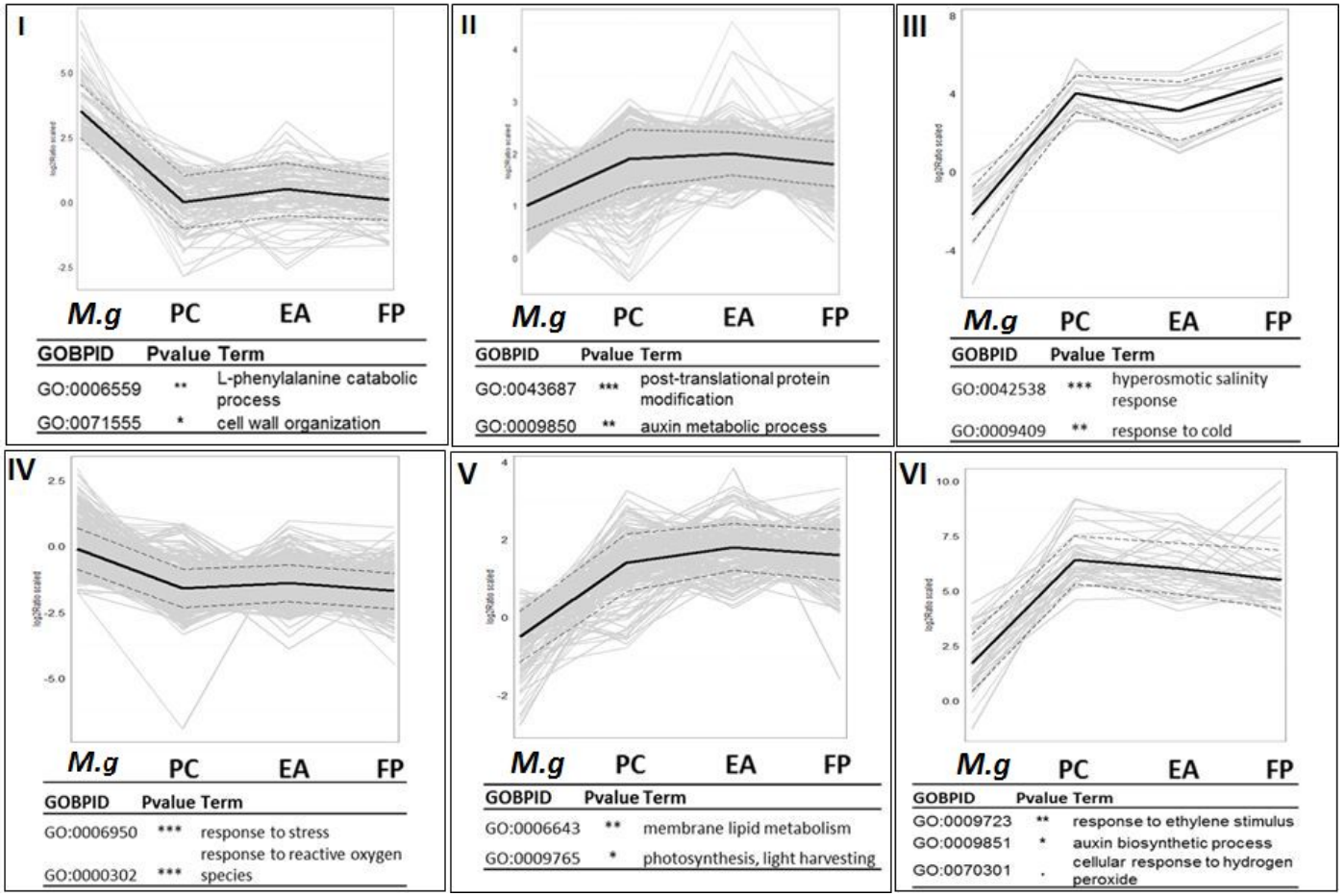


Figure 3.9: Cluster analysis of infected control and treated plants four days after the fungicide application

Microarrays containing 44 thousand *T. aestivum* probes were hybridized with samples isolated from first true leaves that were either infected with pycnidiospores from *M. graminicola* or were treated with fungicides under disease-free conditions. Fungicides sampling time for the evaluation of the gene expression was carried out 4 days after fungicide application. Cluster analysis was performed using the k-mean method. For each cluster a gene ontology enrichment analysis for the biological processes was carried out. *Mg.* represents the relative fold change in gene expression of infected-untreated plants versus the untreated uninfected control. PC, EA and FP represent the fold change of fungicide treated in comparison to untreated healthy plants. The individual expression profiles of every gene in a specific cluster are displayed as grey lines, the average profile is displayed as a black line; the dash lines represent the mean +/- the standard deviation of the expression pattern. Abbr.: *M.g*, *Mycosphaerella graminicola*; PC, Pyraclostrobin; EA, Epoxiconazole; FP, Fluxapyroxad; GOBPID, gene ontology identification number of biological process. Significance codes: P < 0.0001 ***; P < 0.001 **; P < 0.01 *; P < 0.05 ·0.001 **; P < 0.01 *; P < 0.05.

According to the gene cluster analysis, the three fungicide tested induced a similar response in wheat seedlings, which differed from the gene expression profile observed in plants inoculated with pycnidiospores from *M. graminicola*. Nonetheless, the expression pattern and the magnitude of the responses obtained varied within the three active ingredients, probably due to their different chemistry and mode of action.

3.3.2 Microarray experiments of fungicide-treated plants infected with pycnidiospores from *M. graminicola*

The beneficial effects that fungicides like strobilurins have in plants are quite controversial. On the one hand, there is the suggestion that this class of chemicals alters the mitochondrial respiration of the plant, thereby modifying the plant metabolism resulting in higher yields (Grossmann & Retzlaff, 1997; Grossmann *et al.*, 1999; Koehle *et al.*, 2002). On the other hand, opponents of this theory imply that the delayed senescence and the subsequent yield increase related to strobilurin spraying is due to its outstanding fungicidal effect and its preventative activity (Bartlett *et al.*, 2002; Earley *et al.*, 2012).

By inhibiting the spore germination and germ tube elongation, fungi will not be able to penetrate plant cells and thus, is no necessarily for the plants to activate the high energy-demanding process of defense that lead to premature senescence (Bertelsen *et al.*, 2001). For this reason, a genome-wide expression analysis was carried out on wheat seedlings subjected to fungicide spraying. The fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad were sprayed either as a preventative treatment one day before the inoculation with pycnidiospores from *M. graminicola*, or as a curative treatment seven days after the infection. In this way, differences in the activation of defense responses by the plant can be identified that may be related to the mode of action of the three products. Nonetheless, in order to adequately select the time points of leaf sampling, a microarray pre-experiment was carried out to evaluate the responses of wheat seedlings to *M. graminicola* during the symptomless phase of the infection process.

3.3.2.1 Microarray pre-experiment

The microarray pre-experiment was focused on the activation of defense responses and the effect on photosynthesis- and chlorophyll-related genes in wheat seedlings inoculated with pycnidiospores from *M. graminicola.* With this purpose, first true leaves of plants infected with *M. graminicola* pycnidiospores, and plants infected with water

were collected at 3, 5, 7, 10 and 17 days post inoculation (dpi). Genome-wide expression analysis on the Agilent wheat chip containing 44 thousand wheat probes was then carried out.

To observe whether there is a specific expression pattern of the defense- and photosynthesis-related genes during the latent period of fugal growth, a k-mean cluster analysis was performed using GOStats, within the bioconductor package in the R statistical programing (http://www.bioconductor.org/). Subsequently, for each cluster a gene ontology enrichment analysis was carried out. From the defense responses enriched in cluster I (**Figure 3.10-I**) it can be observed that the plant is able to detect pathogen penetration and slightly activates the expression of defense-related genes at 3dpi. With the use of effectors, the pathogen is able to inhibit PAMP-triggered immunity and proceed with the plant colonization. However, as the fungus grows further and switches to a necrotrophic lifestyle (10 dpi) the plant is able to detect the pathogen again and activates effector-triggered immunity to try to hinder fungal growth. At the onset of disease symptoms (17 dpi) defense responses in the plant have exponentially increased. Gene Ontology (GO) terms for the plant-type hypersensitive response as well as the synthesis and response to reactive oxygen species such as hydrogen peroxide (H_2O_2) are associated to this time point (**Figure 3.10-II**). Besides the induction of responses related to defense and reactive oxygen species reactions, the down-regulation of photosynthesis-related genes, and transcripts involved in chlorophyll and carbohydrate metabolism was observed (**Figure 3.10-III**). Furthermore, the exponential increase observed in genes involved in defense responses and oxidative stress correlates with the rapid fungal growth observed in the qPCR experiment (**Figure 3.2**). Based on the genetic profile obtained from the pre-experiment, 3 and 10 dpi were the chosen time points for further analysis in fungicide-treated plants.

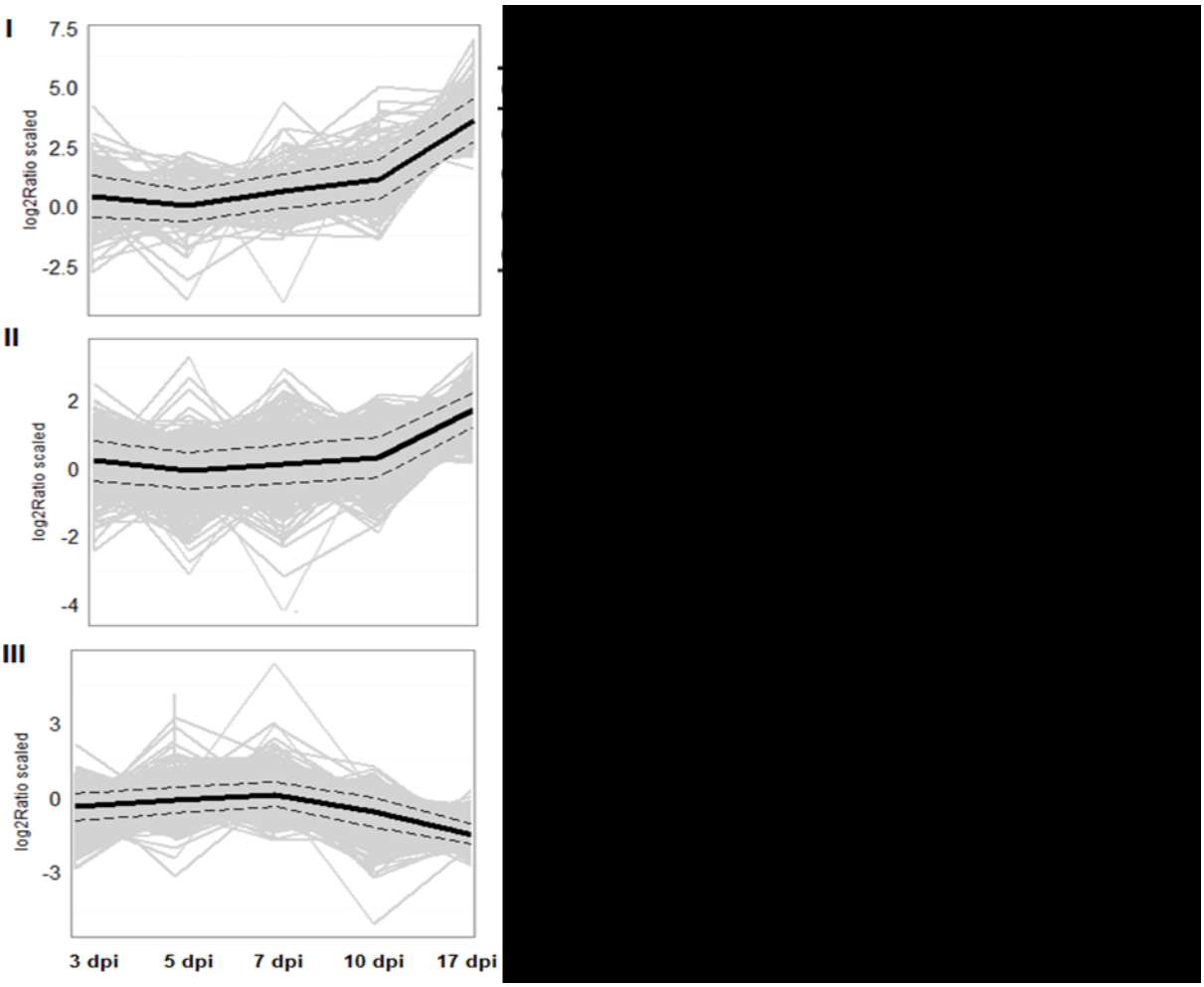


Figure 3.10: Expression profile of the responses of wheat seedlings to the fungus *M. graminicola* with emphasis on defense and photosynthesis

As a pre-experiment, the responses of wheat seedlings to the fungus *M. graminicola* were evaluated during the symptomless phase on the infection process using an Agilent microarray. The fold change (log2 ratio) gene expression of the infected plants relative to the un-infected control was calculated. Displayed are the scaled log2 ratio of the defense- (**I-II**) and the photosynthesis-related responses (**III**) obtained after k-mean cluster and gene ontology enrichment analyses. The individual expression profile of every gene in a specific cluster are displayed as grey lines, the average profile is displayed as a black line; the dash lines represent the mean +/- the standard deviation of the expression pattern. Abbr.: dpi, days post inoculation; GOBPID, gene ontology identification number of biological process. Significance codes: P < 0.0001 ***; P < 0.001 **; P< 0.01 *; P < 0.05 ·0.001 **; P < 0.01 *; P < 0.05.

3.3.2.2 Transcriptional analysis with fungicides with different modes of action and application timing under disease pressure

To evaluate the effect of fungicide mode on wheat seedlings under *M. graminicola* disease pressure, three different products (Pyraclostrobin, Epoxiconazole and Fluxapyroxad) and two spraying regimes (preventative and curative) were assessed using the Agilent wheat microarray. Changes in gene expression of fungicide-treated

plants were calculated relative to an infected mock-treated plant. As explained in **3.3**, the raw data obtained from the microarray experiment was first log2 transformed and normalized according to the *quantile* method, leaving 40644 genes for further analysis. From the 40644 genes, only those with a minimum fold change of 2, a moderate t-statistic of 1.96 and an average spot intensity of 5 were considered to be differentially expressed.

The preventative application of the Pyraclostrobin, Epoxiconazole and Fluxapyroxad resulted in 158, 590 and 629 differentially expressed genes at 3 dpi (**Figure 3.11-I.**) and 2008, 1176 and 978 at 10 dpi respectively (**Figure 3.11-II.**). The curative treatment of the same products brought about 1024, 431 and 715 differentially expressed genes at 10dpi (**Figure 3.11-III**). At each time point (3 and 10 dpi) and spraying timing (preventative or curative), between 70 and 80% percent of the differentially expressed genes after Pyraclostrobin treatment were less-expressed than *M. graminicola*-infected wheat seedlings. A similar scenario resulted for Epoxiconazole application; where between 67 and 77% of the genes were displayed lower expression than the untreated control at 3 and 10 dpi. Fluxapyroxad treatment, however, displayed a different expression profile. At 3 dpi, the preventative application of this fungicide resulted in 67% of the genes being higher expressed than untreated-infected plants. At 10 dpi, on the other hand, 80% of the differentially expressed genes were less expressed after the preventative treatment and 70% after the curative application.

According to BLAST2GO annotation (http://www.blast2go.com; Retrieved December 2013), the differentially expressed genes after fungicide application under disease pressure could be categorized in 13 groups (**Figure 3.12**). Among the annotated genes that were de-regulated or lower expressed than the infected control, approximately 20% were classified as stress-related responses at 3 dpi and 15% at 10 dpi. Epoxiconazole preventative treatment at 3dpi resulted in the highest number of stress-related genes being less induced than an untreated-infected plant. The preventative application of Fluxapyroxad, however, led to the highest number of stress genes being higher expressed than the infected control at 3 dpi.

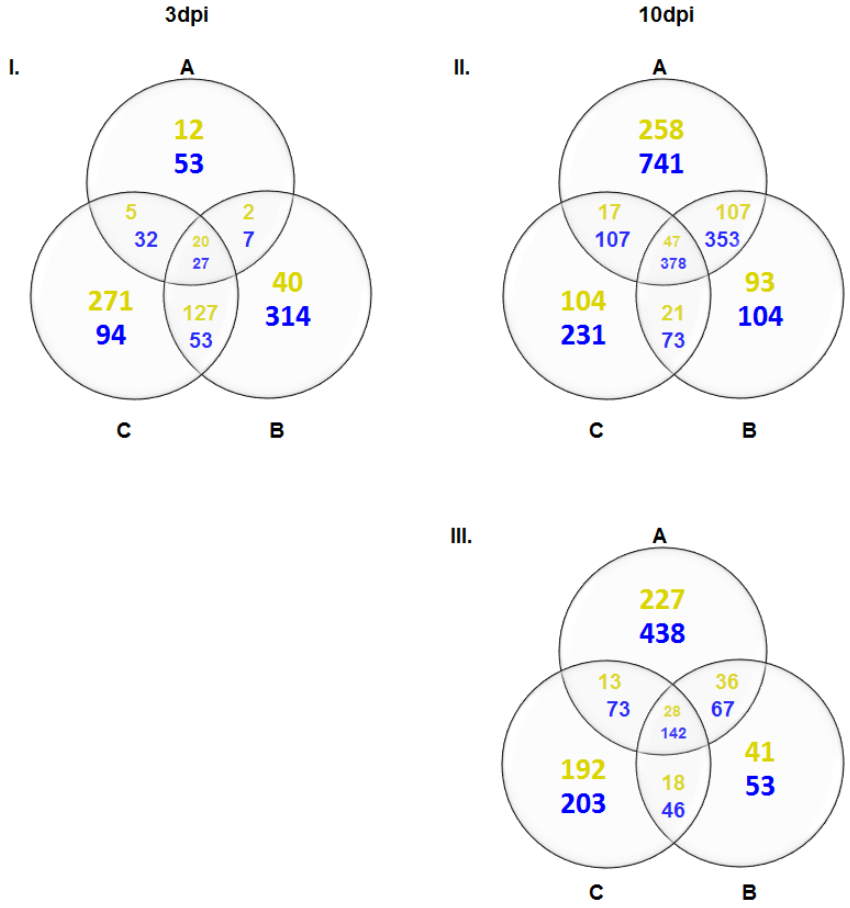


Figure 3.11: Number of differentially expressed genes in fungicide-treated plants relative to the infected-untreated controls

The fungicides Pyraclostrobin (**A**), Epoxiconazole (**B**) and Fluxapyroxad (C) were applied either preventatively, one day before inoculation, or curatively, seven days post inoculation (dpi). The infected-untreated controls were sprayed with water instead. Ten seedlings were planted per pot, with each pot representing an experimental unit. The experiment was carried out with three biological replicas (i.e. 3 different pots). First true leaves in each pot were collected at 3 and 10 dpi for transcriptional analysis using an Agilent microarray from wheat. Venn diagrams in the picture display the number and overlap of the transcripts found to be differentially expressed after the preventative application of the above mentioned fungicides at 3 dpi (**I.**) and at 10 dpi (**II.**). Number and overlap of the differentially expressed genes at 10 dpi after the curative application of the Pyraclostrobin, Epoxiconazole and Fluxapyroxad are displayed in **III.** Genes with higher expression than the infected-untreated controls are shown in yellow, whereas de-regulated genes are represented in blue.

A clear difference between the application timings can be observed at 10 dpi regardless of the active ingredient. The preventative application of any of the fungicides resulted in more stress-related genes with reduced expression compared to the infected control, than in plants where a curative application was performed. Furthermore, treatment with the QoI, Pyraclostrobin, displayed the highest amount of stress responses with a relative fold change lower than the infected control (**Figure 3.12-10**). Among the genes found to

be higher expressed in Pyraclostrobin- and Epoxiconazole-treated plants at 10 dpi, BLAST2GO annotation included them in the gene expression category, whereas in Fluxapyroxad-treated plants, these genes belonged to stress responses.

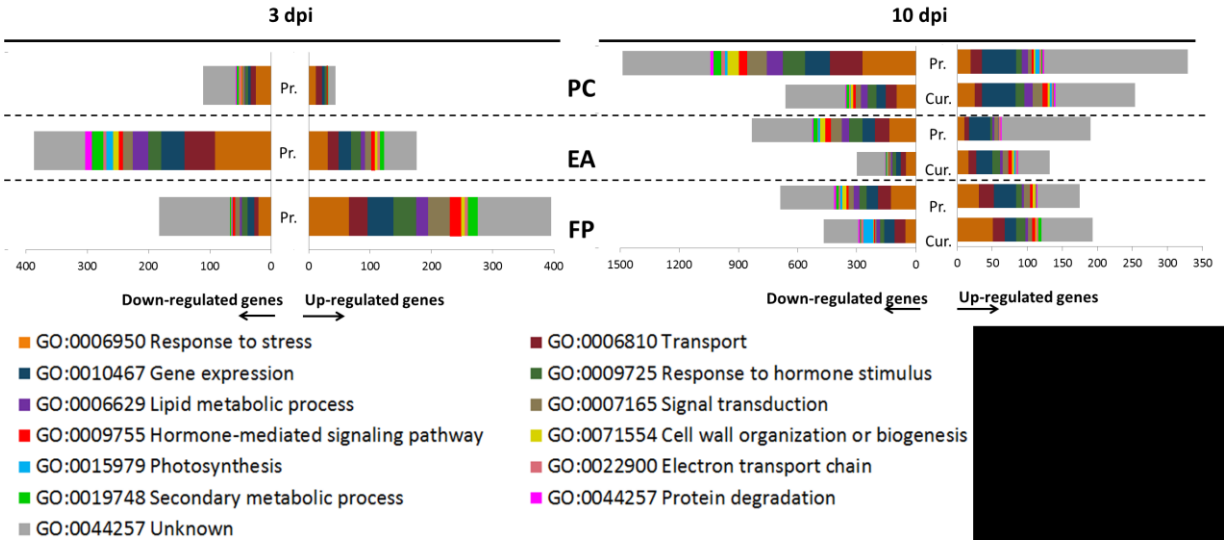


Figure 3.12: Categories of differentially expressed genes upon fungicide treatment under disease pressure at 3 and 10 days after *M. graminicola* inoculation

The fungicides Pyraclostrobin (PC), epoxiconazole (EA) and fluxapyroxad (FP) were sprayed on wheat seedlings either as a preventative treatment, one day before the *M. graminicola* infections, or as a curative treatment 7 days after the inoculation was performed. The first leaves of each pot where collected for microarray analysis at 3 and 10 days post inoculation (dpi). Ten seedlings were planted per pot; each pot represented an experimental unit and the experiment was carried out with three biological replicas (i.e. 3 different pots). After data analysis the differentially expressed genes were annotated using BLAST2GO, and genes were categorized according to their function. The stacked bars represent the number and categories of the differentially expressed genes after fungicide application in comparison to infected, un-treated plants. The left part of each graphic corresponds to genes being less expressed than the infected untreated plants, whereas the right side represents genes whose expression level was higher than *M. graminicola*-infected wheat seedlings. Different colors describe the corresponding category present in the legend. Abbr.: Pr. Preventative treatment; Cur. Curative treatment.

Because a great part of the wheat responses upon fungicide treatment is related to stress responses, defense related transcript were analyze in more detail with mapman (http://mapman.gabipd.org; Retrieved January 2013). According to mapman annotation of biotic stress responses, Fluxapyroxad treatment resulted in the greatest number of transcripts being higher expressed at 3 dpi, whereas Pyraclostrobin treatment did not cause many difference related to an infected-untreated plant (**Figure 3.13**). Among the genes related to phytohormones whose expression, relative to an infected-untreated plant, significantly changed at 3 dpi where related to auxin, abscisic acid, ethylene and

jasmonate. Ethylene synthesis was only lower expressed with Pyraclostrobin treatment. Auxin- and ABA-related signaling and responsive genes were stronger expressed upon Fluxapyroxad and Epoxiconazole treatment. Jasmonate synthesis-related genes were less expressed in Epoxiconazole treated plants and jasmonate signaling transcripts were higher regulated with Fluxapyroxad treatment.

At 3 dpi, the preventative application of Fluxapyroxad resulted in a higher expression of genes related to cell wall synthesis and modification, as well as transcripts involved in ROS detoxification or synthesis such as peroxidases. Genes implicated in cellular recognition and downstream signaling were also highly expressed. The results obtained during the early stages of pathogen infection suggested that the fungicide Fluxapyroxad not only has a fungistatic effect but it also activates plant innate immunity, perhaps through the JA/ET related signaling pathways.

At 10 dpi there is a clear difference between the two application timings (**Figure 3.13**), suggesting that the preventative application of any of the fungicides evaluated reduced the activation of defense responses observed in an infected-untreated plant. Even though the curative application of fungicides resulted in successful fungal control (**Figure 3.5-A**), the plant was still able to detect the pathogen and activate its immune system to control fungal spread, which can be observed as the lower amount of differentially expressed transcripts being de-regulated in comparison to the preventative treatment. Besides, signaling-related transcripts are even more expressed than the infected control after the curative treatment.

Even though the fungicide efficacy of the cellular respiration inhibitors is the same when they are sprayed as a preventative treatment (**Figure 3.5-B**), there is a clear difference in the effect that these two fungicides cause on the transcriptome of wheat seedlings under disease pressure. Pyraclostrobin treatment resulted in higher number of transcripts being less expressed than Fluxapyroxad and Epoxiconazole. For this reason, a cluster analysis was carried out in order to compare the expression pattern of fungicide-treated plants relative to an infected untreated plant.

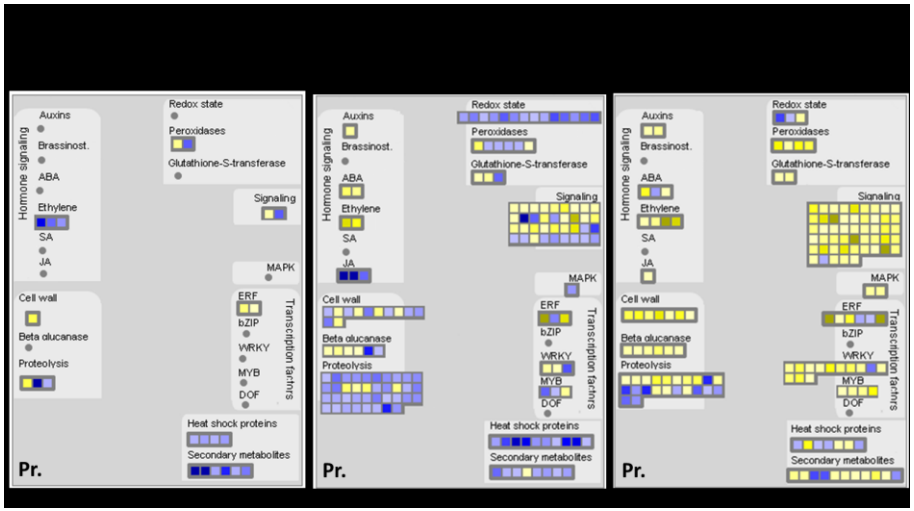


Figure 3.13 Putative involvement of fungicide treatment in biotic stress responses under disease pressure

The fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad were sprayed onto wheat seedlings either one day before inoculation with *M. graminicola* pycnidiospores or seven days after the infection took place as preventative and curative treatments, respectively. Untreated infected control plants were sprayed with water. At 3 and 10 dpi, the first leaves were used for RNA extraction and Microarray hybridization. Ten seedlings were planted per pot; each pot represented an experimental unit and the experiment was repeated three times. Mapman analysis was employed to display the differentially regulated genes obtained from the gene expression analysis (44K wheat chip) onto the biotic stress responses diagram. Blue colors denote down-regulation, whereas yellow symbolizes up-regulation versus the untreated infected control. Abbr.: ABA, abscisic acid; SA, salicylic acid; JA, jasmonate; MAPK, mitogen-activated protein kinase; Pr. Preventative treatment; Cr. Curative treatment; dpi, days post inoculation.

According to the k-mean cluster analysis, the genes obtained from the microarray experiment can be divided into 15 clusters (**Appendix 6**). Among these clusters, six of them were related with genes involved in photosynthesis processes, post-translational protein modifications, phytohormone metabolism, cell wall biosynthesis, defense responses and responses to oxidative stress. As observed in the microarray pre-experiment, infection with the fungus *M. graminicola* resulted in the down-regulation of photosynthesis-related genes at 3 and 10 dpi in infected plants relative to the un-infected control (**Figure 3.14-I**). Genes involved in defense response to fungus, synthesis of cell wall components, post-translational protein modification and oxidative stress responses were induced in *M. graminicola*-infected plants at 3 and 10 dpi (**Figure 3.14-II, IV, V, VI**). On the contrary, the application of Pyraclostrobin, Epoxiconazole and Fluxapyroxad resulted a higher expression of genes involved in photosynthesis and energy at 3 dpi, with higher expression peaks observed in plants treated the cellular respiration inhibitors (**Figure 3.14-I**). Genes implicated in protein modification processes and in downstream pathways related to phenyl alanine were highly expressed in the infected control at 10 dpi as well as in plants curatively treated with the fungicides (**Figure 3.14-II**). Soon after fungicides were sprayed preventatively a transient induction of genes involve in auxin, abscisic acid and gibberellin metabolism was observed in the three products and even more in Fluxapyroxad-treated plants. Nonetheless, four days later the expression levels of the above mentioned genes was de-regulated to control levels (**Figure 3.14-III**). Transcripts related to cell wall synthesis were highly induced in infected-untreated plants at 10dpi, probably due to the switch of *M. graminicola* to its necrotrophic life style (**Figure 3.14-IV**). As expected, the preventative application of the fungicides de-regulated these responses. When plants were curatively sprayed, genes enriched in cluster four were higher expressed than in preventatively-treated plants. Defense responses related to fungal infection were up-regulated in infected plants at 10 dpi, and these transcripts were less expressed upon preventative fungicide treatment and to a lesser extent in plants treated with a fungicide curatively (**Figure 3.14-V**). Interestingly the curative application of Fluxapyroxad resulted in a stronger expression of defense responses than the infected control (**Figure 3.14-V**). Responses to reactive oxygen species were only de-regulated in Pyraclostrobin and Fluxapyroxad-treated plants at 3dpi (**Figure 3.14-VI**).

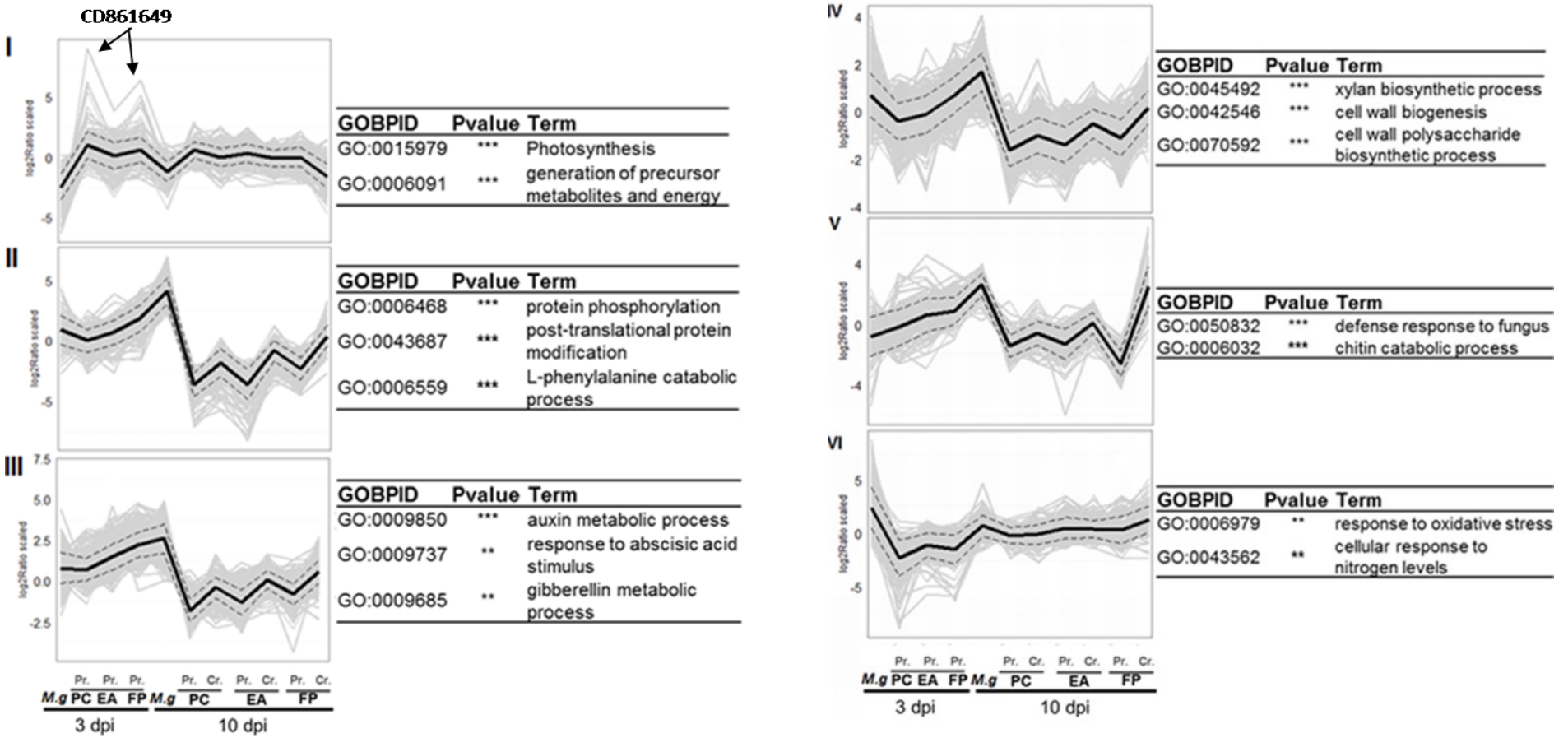


Figure 3.14: Cluster analysis of infected control and fungicide treated plants at 3 and 10 dpi.

Differentially expressed genes from the microarray hybridization were clustered and a gene ontology enrichment analysis for the biological processes was performed. A picture describing the expression profiles of the genes from six clusters (**I-VI**) and their corresponding enrichment analysis are displayed. *Mg.* represents the relative fold change in gene expression of infected-untreated plants versus the untreated uninfected control. PC, EA and FP represent the fold change of fungicide treated vs. untreated infected plants. The individual expression profiles of every gene in a specific cluster are displayed as grey lines, the average profile is displayed as a black line; the dash lines represent the mean +/- the standard deviation of the expression pattern. In Cluster I, the GeneBank accession number of the gene transcript corresponding to the highly expressed peaks is reported. Abbr.: Pr. Preventative fungicide application; Cr. Curative fungicide treatment; *M.g*, *Mycosphaerella graminicola*; PC., Pyraclostrobin; EA., Epoxiconazole; FP., Fluxapyroxad; dpi, days post inoculation; GOBPID, gene ontology identification number of biological process. Significance codes: P < 0.0001 ***; P < 0.001 **; P < 0.01 *; P < 0.05 ·0.001 **; P < 0.01 *; P < 0.05.

According to the above mentioned results, it may be suggested that treatment with a complex II inhibitor such as Fluxapyroxad may have a transient effect on the plant transcriptome (**Figure 3.13-3dpi**) perhaps by modifying the expression of genes involved in the activation of defense responses of wheat seedlings to *M. graminicola* (**Figure 3.14-V**), without having any penalty in the expression of transcripts involved in energy production and photosynthesis (**Figure 3.14-I**). Additionally, it could be proven than when a fungicide is applied early enough to hinder pathogen penetration the activation of costly defense responses can be avoided (**Figure 3.13-10dpi-Pr.**). Interestingly, fungicides with similar mode of action resulted in similar results. The preventative application of the cellular respiration inhibitors, Fluxapyroxad and Pyraclostrobin, not only resulted in better disease control along the entire infection process (**Figure 3.5**), but a stronger effect on genes involved in oxidative stress (**Figure 3.14-VI**) and photosynthesis (**Figure 3.14-I**) was also observed

3.3.3 Validation of transcriptome analysis of selected genes via qPCR

In order to proof the results of the microarray experiments, nine genes were selected and their expression values were evaluated by means of qPCR using the same RNA material as for the microarray experiments. Gene Bank accession number and primer information of the selected transcripts can be found in **Appendix 2.**

From the experiments under disease-free conditions, gene transcripts involved in regulation of defense responses, hormone metabolism and carbon fixation were chosen based on their expression profile observed upon fungicide treatment (**Figure 3.15**). Additionally, from cluster III (**Figure 3.9-III**)one gene transcript with unknown function (AZO1; Accession number CD861649) was selected as a marker for cellular respiration inhibitors because of its specific activation (**Figure 3.15-E**). According to Genevestigator (Hruz *et al.*, 2008), an internet-based gene expression profiling tool, this gene was found to be induced under abiotic stress responses such as temperature, light and high salinity conditions. Generally, the results between the microarray and the qPCR data were comparable, meaning that the transcriptome analysis could be validated by means of qPCR. The only exception was the transcript corresponding to the Rubisco alpha subunit (**Figure 3.15-F**). According to the microarray results, this transcript was induced by 2 fold only in the plants treated with Pyraclostrobin and Fluxapyroxad.

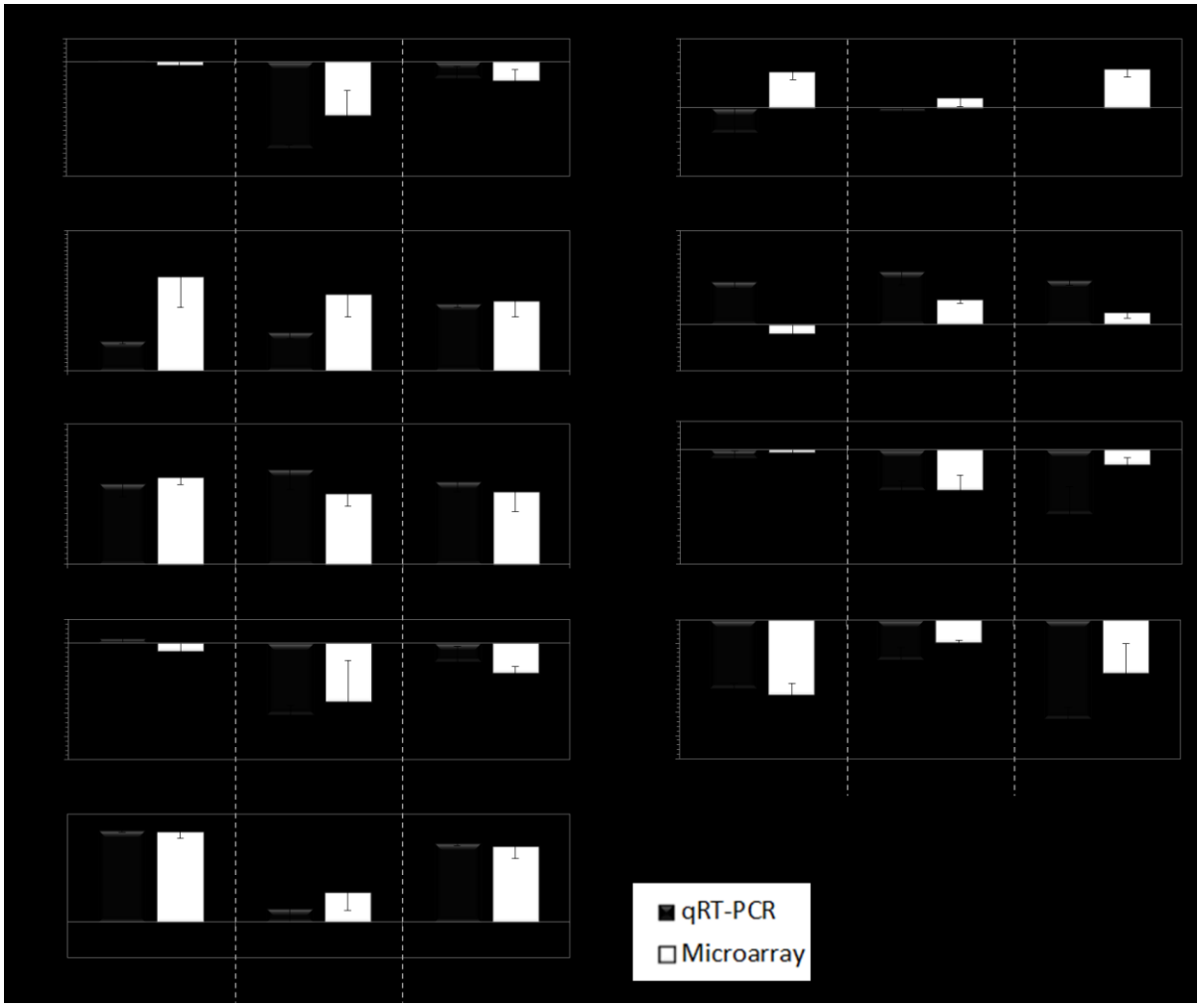


Figure 3.15: Validation of the microarray experiments under disease-free conditions by means of quantitative real time PCR (qPCR)

Comparison of the gene expression profiles between the microarray experiment (white) and the qPCR (black) of wheat seedlings 4 days after the application of the fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad. Displayed are the relative expression (log fold change) of the genes PR5 (**A**), ACC synthase (**B**), ACC oxidase (**C**), Chitinase (**D**), AZO1 (**E**), Rubisco alpha subunit (**F**),Lipid transfer protein (**G**), Root peroxidase (**H**) and Gibberellin 20 oxidase (**I**) relative to an uninfected-untreated control. Actin and AK334339.1 were employed as the reference genes. The fold change of the qPCR samples was calculated according to Livak and Schmittgen (2001) using the $2^{-\Delta\Delta CT}$ method. The average expression of the two reference genes was used for normalization. Columns represent the average of three independent experiments, while the bars displayed the standard error.

For the validation of the experiments carried out under disease pressure, five gene transcripts that were differentially expressed in at least one time point of the infection process were chosen (**Figure 3.16**). According to their gene annotation, the chosen transcripts belong to the pathogenesis-related proteins PR3 (chitinase) and PR5 (thaumatin-like), as well as the ethylene synthesis pathway (ACC synthase and ACC oxidase). As explained above, AZO1 does not have any annotation yet, but could be used

as a marker for the cellular respiration inhibitors, as this gene was transiently induced in wheat seedlings only after the application of these products (**Figure 3.14-I**), and after abiotic stress conditions as found on Genevestigator.

The expression profiles of the microarray experiments and the qPCR are similar (**Figure 3.16**). Based on these results, it can be concluded that the microarray experiments could be verified using qPCR, which demonstrates the robustness of the data.

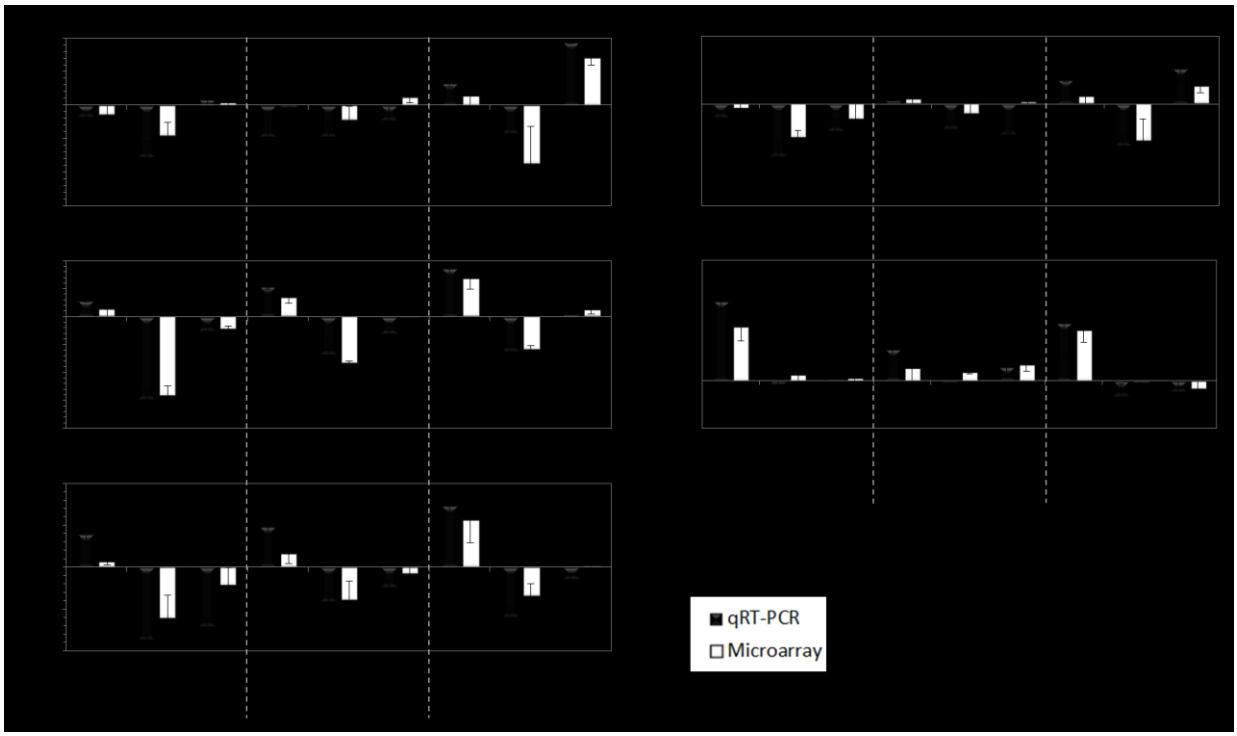


Figure 3.16: Validation of the microarray experiments of fungicide-treated plants under disease pressure by means of quantitative real time PCR (qPCR)

The fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad were sprayed either preventatively, one day before the inoculation with pycnidiospores from *M. graminicola,* or curatively, seven days after the inoculation. Infected control plants were sprayed with water as a mock treatment. RNA extraction from the first true leaves took place at three days and seven days post inoculation (dpi). Gene expression changes of fungicide-treated plants vs infected controls were analyzed using cDNA microarrays. Validation of the microarray experiments was carried out by means of qPCR according to Livak and Schmittgen (2001) using the $2^{-\Delta\Delta CT}$ method. The average expression of the two reference genes was used for normalization. Displayed are the comparison between microarray (white) and qPCR (black) expression profiles of the genes PR5 (**A**), ACC synthase (**B**), ACC oxidase (**C**), Chitinase (**D**) and AZO1 (**E**). Three independent experiments were performed. Columns represent the average expression of fungicide-treated plants relative to an infected-untreated control using Actin and AK334339.1 as the reference genes. Bars displayed the standard error. Abbr.: Pr.: Preventative fungicide application; Cr.: Curative fungicide application.

Based on the obtained results, yield trials under semi-natural and natural conditions were carried out to confirm whether higher yields could be obtained by applying a

fungicide preventatively instead of waiting until the infection has settled down. Additionally, differences in yield levels related to the efficacy profiling carried out (**3.2**) were also expected to occur.

3.4 Effect of fungicide treatment on controlling septoria leaf blotch and the subsequent development of yield

The previous results demonstrated that, due to their outstanding preventative action in comparison to Epoxiconazole, the preventative application of Fluxapyroxad or Pyraclostrobin has a significant impact on controlling of septoria leaf blotch. Moreover, when the products were sprayed curatively, Fluxapyroxad displayed a significantly higher efficacy, followed by Pyraclostrobin and Epoxiconazole (**Figure 3.5**). Visually, differences in disease symptom development between the two respiration inhibitors and Epoxiconazole-treated plants were observed (**Figure 3.4**). Furthermore, according to the transcriptome analysis of wheat seedlings after fungicide application under disease pressure, the activation of plant defense reactions and photosynthesis-related genes also varied based on the timing of application and the different modes of action (**Figure 3.14**). For this reason, yield trials were carried out under semi-natural and natural conditions in a two year period in order to assess whether higher yields can be obtained when the flag leaf, the most photosynthetically active part of wheat, can be protected from fungal diseases. As with the previous experiments, two different spraying timings, preventative and curative, were utilized along with untreated controls to estimate the severity of the disease and the related yield loss.

3.4.1 Yield trials under semi-natural conditions

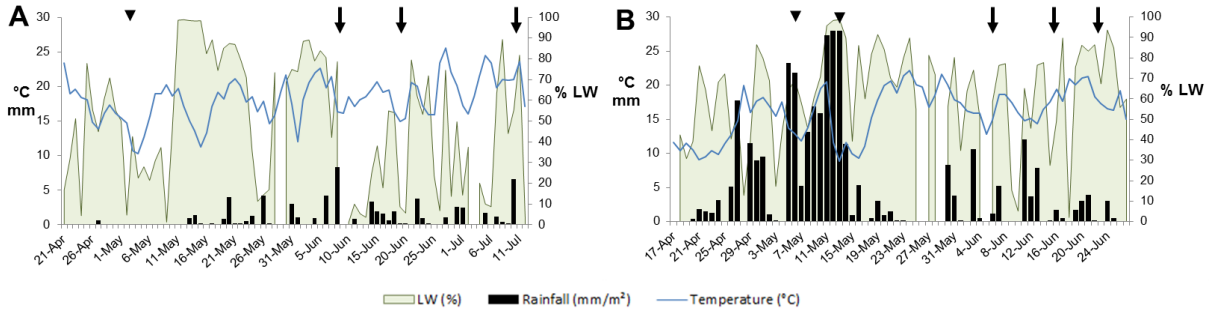
Winter wheat seedlings were planted in Autumn on micro-plots using plastic boxes (0.12 cm^2) as containers, and grown under natural environmental conditions. To protect plants from adverse weather conditions and birds, the plastic boxes were placed under a netted tunnel until harvest. Once the vegetative period started (early spring), environmental conditions were constantly monitored with a micro station (WatchDog 1000 series; Spectrum, Plainfield, USA). Each micro-plot was considered an experimental unit and was sprayed with the fungicide only once at the corresponding time point, preventatively or curatively. Each year two independent trials were carried out, in each of them, the micro-plots were laid out in a randomized complete block

design (7 treatments, 8 repetitions per treatment). Fungicide application took place at growth stage 39 (**Appendix 1**) as a preventative treatment; or after *M. graminicola* infection had occurred as a curative treatment (**see 2.3.1.2**). Untreated-infected controls were not sprayed at all. Artificial inoculation with a spore suspension and pycnidia-bearing straw was performed one week after the preventative application had taken place.

Based on the model developed by Verreet *et al.* (2000), *M. graminicola* infection events can be predicted by the environmental conditions during the growing season. According to these authors, dispersal and infection events of this pathogen take place when; i) there is a more than two-day long period of leaf wetness at more than 98%, ii) total precipitation within a three-day period is higher than 10mm; and iii) the temperature is above 7°C for more than two days. The optimum temperature for a *M. graminicola* infection is between 16 and 21°C.

For the experiments conducted in 2011, the winter wheat varieties Akteur and Riband where used. The Akteur variety is a relatively modern cultivar, which according to the German variety bureau, Bundessortenamt (BSA), is considered an elite wheat variety due to its outstanding backing quality but, even so, is relatively susceptible to the septoria diseases such as septoria leaf blotch. The Riband wheat variety, on the other hand, is a long-standing winter wheat cultivar with a high susceptibility to septoria diseases and, therefore, the favored variety for fungicide screening programs (Dr. Jurith Montag, Advanced testing fungicides, Agricultural Center, BASF, Limburgerhof, Germany; personal communication).

The year 2011 was marked by relatively dry spring with raining events that started late in the summer (**Figure 3.17A**). Under these weather conditions, the development of the disease was retarded; visible symptoms appeared first 43 days after the first application and reached their maximum seven days later when the plant was at the ripening stage. Even though a high level of leaf wetness could be maintained by an irrigation system, the fungus still relied on rain to be dispersed within the plant and to neighboring ones.



C

Year	2011				2012	
Wheat variety	Akteur		Riband		Riband	
Trial number	001	002	001	002	001	002
Seeding date	6.Oct.10	6.Oct.10	6.Oct.10	6.Oct.10	20.Sept.11	5.Oct.11
Preventative application (GS 39)	3.May.11	3.May.11	3.May.11	3.May.11	4.May.12	8.May.12
Artificial inoculation	10.May.11	10.May.11	10.May.11	10.May.11	9.May.12	14.May.12
Curative application	18.May.11	18.May.11	18.May.11	18.May.11	16.May.12	28.May.12
Harvesting	29.Jul.11	29.Jul.11	29.Jul.11	29.Jul.11	24.Jul.12	27.Jul.12

Figure 3.17: Weather data and summary information from the micro-plot trials during the 2011-2012 periods

Weather data during the micro-plot trials was constantly monitored as soon as the main growing period began in order to be able to predict, according to environmental conditions, when natural infection events, (arrows) apart from the artificial inoculation (arrow heads), would occur. **A.** Weather data from the 2011 growing season. **B.** Weather data from the 2012 yield trials. **C.** Table summarizing the main events during the seasons. Each micro-plot was considered an experimental unit and was sprayed with the fungicide only once at the corresponding time point, preventatively or curatively. In 2011 two trials, each with 8 replicas per treatment were carried out. In 2012, however, due to the harsh winter, one trial consisted on 5 repetitions and the second trial on 4 replicates. Abbr. LW (%): percentage leaf wetness; GS39; growth stadium of wheat plants.

The two-way analysis of variance (ANOVA) did not reveal an interaction effect between the different fungicide treatments and the two trials, meaning, the treatments behaved in the same manner in both experiments, just the magnitude of the evaluated variable differed. Therefore, the model was simplified and the interaction effect was removed (**Table 3.2**). According to the new ANOVA model, in the winter wheat variety Riband the two trials did not significantly differ from one another in terms of flag leaf area, chlorophyll content, disease severity and yield. In the case of the Akteur variety, the two trials differed significantly when taking the latest disease infection assessment and the thousand kernel weight (TKW) into consideration. Fungicide treatments only displayed a significant effect in regard to fungal growth inhibition; no significant differences were observed when assessing physiological parameters like flag leaf area, chlorophyll

content, thousand kernel weight and yield (**Table 3.2-2011**). Since flag leaf area measurement is very time consuming and significant leaf size differences were not observed, this factor was removed in the experiments of the 2012 season. Additionally, the chlorophyll content measurements were reduced to only one measurement, carried out seven days after the first application.

The prolongation of the disease development not only reduced the infection levels in the untreated plants, where 50% disease severity was observed for Riband and 33% for Akteur, it also had an effect on the fungicide application timing. In 2011, the flag leaf was fully developed at the beginning of May; thus the preventative application was carried out in the same period. The curative application took place two weeks later; during the week in-between artificial inoculation was performed (**Figure 3.17C**). Due to the slow fungal growth, however, the preventative-treated plants displayed stronger disease severity than the curative-treated examples (**Table 3.3-2011-Disease severity**). An explanation for these results could be that the first fungicide application was too far apart from the actual infection that occurred later in the summer, and the second application, which was supposed to have a curative effect, became indeed more of a preventative treatment.

The Least Significant Difference comparison of the effect of the fungicide treatments in terms of disease control revealed, that for both varieties, Pyraclostrobin provided the most significant disease control, followed by the curative application of Fluxapyroxad and Epoxiconazole (**Table 3.3-2011**). Although significant differences among the treatments regarding pathogen control were observed, significant differences in the yield of the Riband variety did not occur. A tendency for lower yields in untreated plants was noticed, as well as a tendency for higher yields in Pyraclostrobin-treated plants. In the Akteur variety, on the other hand, the preventative treatment of Fluxapyroxad resulted in significantly reduced yield levels even though the disease levels were significantly lower than in the untreated plants. This result could possibly be explained by eating damage caused by mice that built a nest inside the containers where the plants were cultivated and ate the ears of some plants from this experiment.

Table 3.2: Two-way ANOVA summary table for the micro-plot trials of the 2011 and 2012 seasons

	Leaf area		Chlorophyll content			Disease severity				TKW	Net Yield
	3 DAPA GS 39-45	13 DAPA GS 47-52	7 DAPA GS 37-41	13 DAPA GS 47-52	23 DAPA GS 59-61	27 DAPA GS 61-69	42 DAPA GS 71-75	43 DAPA GS 71-77	50 DAPA GS 83-85	-	-
Factor	F-value; P										
Riband 2011											
Trial	$F_{(1,104)}$=1.4; NS	$F_{(1,104)}$=2.7; NS	N.A.	$F_{(1,104)}$=0.3; NS	$F_{(1,104)}$=0.6; NS	N.A.	N.A.	$F_{(1,104)}$=1.1; NS [a]	$F_{(1,104)}$=0.1; NS [a]	$F_{(1,104)}$=7.4; **	$F_{(1,104)}$=2.6; NS
Treatment	$F_{(6,104)}$=0.07; NS	$F_{(6,104)}$=0.4; NS	N.A.	$F_{(6,104)}$=0.8; NS	$F_{(6,104)}$=0.4; NS	N.A.	N.A.	$F_{(6,104)}$=11.1; *** [a]	$F_{(6,104)}$=11.9; *** [a]	$F_{(6,104)}$=1.3; NS	$F_{(6,104)}$=0.5; NS
Trial x Treatment	/	/	N.A.	/	/	N.A.	N.A.	/	/	/	/
Akteur 2011											
Trial	$F_{(1,104)}$=0.6; NS	$F_{(1,104)}$=0.5; NS	N.A.	$F_{(1,104)}$=0.1; NS	$F_{(1,104)}$=3.6; NS	N.A.	N.A.	$F_{(1,104)}$=1.1; NS [a]	$F_{(1,100)}$=23.1; *** [a]	$F_{(1,100)}$=19.7; ***	$F_{(1,100)}$=1.2; NS
Treatment	$F_{(6,104)}$=0.1; NS	$F_{(6,104)}$=0.003; NS	N.A.	$F_{(6,104)}$=0.6; NS	$F_{(6,104)}$=0.8; NS	N.A.	N.A.	$F_{(6,104)}$=12.3; *** [a]	$F_{(6,100)}$=8.5; *** [a]	$F_{(6,100)}$=2.1; NS	$F_{(6,100)}$=1.8; NS
Trial x Treatment	/	/	N.A.	/	/	N.A.	N.A.	/	/	/	/
Riband 2012											
Trial	N.A.	N.A.	$F_{(1,54)}$=17; ***	N.A.	N.A.	$F_{(1,55)}$=5.6; * [a]	$F_{(1,55)}$=33.4; ***	N.A.	N.A.	$F_{(1,53)}$=14.1; ***	$F_{(1,53)}$=107.9; ***
Treatment	N.A.	N.A.	$F_{(6,54)}$=0.7; NS	N.A.	N.A.	$F_{(6,55)}$=6.1; *** [a]	$F_{(6,55)}$=17.2; ***	N.A.	N.A.	$F_{(6,53)}$=2.4; *	$F_{(6,55)}$=2.6; *
Trial x Treatment	N.A.	N.A.	/	N.A.	N.A.	/	/	N.A.	N.A.	/	/

Significance codes (F-test): P < 0.001 ***, P < 0.01 **, P < 0.05 *, P > 0.05 not significant (NS). ANOVA analysis was based on 16 repetitions (2 trials x 8 replicas) for the 2011 seasons and 9 replications (1 trial x 5 repetition + 1 trial x 4 repetitions) for the 2012 season

/: Due to not significant interaction between the trials and treatments, the model was simplified and the interaction effect was removed.

[a] For the statistical analysis, a logarithmic transformation on the data was performed.

Abbr. TKW: Thousand kernel weight; DAPA: Days after preventative application; GS: Growth stage according to the BBCH code for cereals; N.A: Not applicable; NS: not significant

Table 3.3: Effect of different fungicide treatments on *M. graminicola* disease severity and wheat physiology under semi-natural conditions during the 2011 and 2012 growing seasons

	2011								2012				
	Leaf area (cm^2)		Chlorophyll content		Disease severity (%)		TKW ($g\ box^{-1}$)	Net Yield ($g\ box^{-1}$)	Chlorophyll content	Disease severity (%)		TKW ($g\ box^{-1}$)	Net Yield ($g\ box^{-1}$)
	3 DAPA GS 39-45	13 DAPA GS 47-52	13 DAPA GS 47-52	23 DAPA GS 59-61	43 DAPA[a] GS 71-77	50 DAPA[a] GS 83-85	-	-	7 DAPA GS 37-41	27 DAPA[a] GS 61-69	42 DAPA GS 71-75	-	-
Riband													
Treatment													
Untreated Control	17.5 a	17.7 a	46.3 a	48.0 a	6.9 a	50.2 a	42.1 b	130.7 a	42.8 a	18.2 a	73.6 a	35.9 b	68.6 c
Pyraclostrobin Pr.	16.7 a	16.9 a	46.4 a	47.1 a	15.0 cd	11.2 de	45.8 a	144.2 a	43.1 a	2.8 d	21.9 c	41.1 a	84.1 ab
Pyraclostrobin Cr.	17.4 a	18.1 a	46.3 a	48.0 a	8.8 d	7.5 e	45.4 ab	135.7 a	41.9 a	8.2 bc	25.6 c	40.8 a	72.0 bc
Epoxiconazole Pr.	17.1 a	17.4 a	46.0 a	47.7 a	13.3 b	27.5 bc	43.9 ab	142.8 a	41.0 a	5.9 bcd	39.4 b	39.0 ab	71.5 bc
Epoxiconazole Cr.	17.3 a	16.2 a	46.9 a	47.9 a	3.8 bc	15.5 d	45.7 a	143.6 a	41.8 a	10.5 b	38.4 b	41.5 a	86.9 a
Fluxapyroxad Pr.	16.7 a	16.5 a	45.7 a	47.1 a	5.7 b	27.5 b	42.9 ab	133.1 a	41.2 a	3.7 cd	33.3 bc	40.8 a	87.0 a
Fluxapyroxad Cr.	17.2 a	18.1 a	45.3 a	47.6 a	24.8 cd	16.3 cd	44.9 ab	136.5 a	40.8 a	7.8 b	32.8 bc	42.0 a	83.9 ab
Akteur													
Treatment													
Untreated Control	17.2 a	16.4 a	49.1 a	52.2 a	7.0 a	32.8 a	50.1 abc	135.0 a					
Pyraclostrobin Pr.	17.0 a	16.5 a	49.5 a	51.3 a	1.2 c	14.8 bc	50.9 a	134.4 a					
Pyraclostrobin Cr.	17.4 a	16.9 a	49.6 a	52.3 a	0.9 c	8.5 d	50.8 a	131.6 ab					
Epoxiconazole Pr.	15.9 a	16.1 a	49.8 a	52.2 a	3.3 b	20.1 bc	49.8 abc	140.7 a					
Epoxiconazole Cr.	17.9 a	18.0 a	50.6 a	52.5 a	4.2 ab	18.2 bc	49.2 c	124.1 ab					
Fluxapyroxad Pr.	18.3 a	17.8 a	49.4 a	51.4 a	4.0 b	22.6 b	49.4 bc	112.3 b					
Fluxapyroxad Cr.	16.0 a	17.0 a	49.5 a	52.3 a	1.6 c	12.6 c	50.7 ab	124.8 ab					

Means with the same letter are not significantly different (Least significant difference at P=0.05). Displayed values are based on 16 repetitions (2 trials x 8 replicas) for the 2011 seasons and 9 replications (1 trial x 5 repetition + 1 trial x 4 repetitions) for the 2012 season.

[a] For the statistical analysis, a logarithmic transformation on the data was performed. Values represent the mean in the original scale.

Abbr. DAPA: days after preventative treatment; GS: growth stage according to the BBCH code for cereals; TKW: Thousand kernel weight; $g\ box^{-1}$: grams per box.

Although the eating damage was distributed along the entire experiment, most of the damage occurred to the plants treated with this fungicide. Therefore, due to the disturbance created by the mice, the data obtained cannot be used to come to the conclusion that Fluxapyroxad causes reduced yield levels.

In an attempt to overcome the weather effect, some changes were made to the micro-plot trials for the 2012 growing season: i) the Akteur winter wheat variety was removed from the experiments, since, even though it is considered susceptible to septoria diseases by the Bundessortenamt, the disease levels obtained with this variety in the untreated controls were significantly lower in comparison to Riband. In order to obtain clearer conclusions as to the effect of the fungal disease on wheat yield, stronger disease severity levels are needed. ii) The irrigation nozzles were changed in order to attain thicker water drops and thus simulate rain events that would enhance disease infection no matter what the weather conditions actually were. Last but not least, iii) to avoid the problem that the curative application ended up becoming the preventative, weather conditions were monitored closely and the second application was only carried out when, according to the weather station, an infection would take place based on the septoria disease model from Verreet *et al.* (2000) (more than two-days period of long leaf wetness, more than 10mm precipitation in three days and a temperature range of 16-21°C). Some plants, however, were severely affected during the extreme freezing temperatures that occurred in February 2012, and had to be disposed of. Therefore, unlike the 2011 season, during which two trials with eight repetitions each were carried out, the 2012 experiments were performed for a total of nine repetitions, with one trial containing five and another four replicas.

Based on the changes mentioned above, the preventative application started again at the beginning of May, when the flag leaf reached full development, and the curative application occurred, depending on the trial, between 15 and 20 days after the initial application (**Figure 3.17C**). As can be seen in the weather data in **Figure 3.17B**, during artificial inoculation the environmental conditions were ideal for fungal development and infection to occur. Furthermore, the disease levels in the untreated plants were notably higher and developed faster than in the previous year, with more than 70% disease severity levels was obtained 42 days after the first application, when the plants were in the fruit development growth stadium (**Table 3.3**).

As with the 2011 experiments, no significant interaction between the different experiments and the treatments was observed in the 2012 season, allowing for interaction effect in the two-way ANOVA to be removed. According to the ANOVA model, no significant effect on the chlorophyll content of wheat leaves upon fungicide application was detected, though significant differences in disease control, thousand kernel weight and yield were achieved (**Table 3.2**).

At the beginning of the developments of symptoms, when disease levels in the untreated control were at approximately 18%, the post-hoc comparison of the disease levels upon fungicide application suggested that the preventative treatment of the two respiration inhibitors was more successful in controlling septoria leaf blotch than the curative treatment (**Table 3.3**). In plants treated with the sterol biosynthesis inhibitor, a difference between the two application timings was not detected. Moreover, the infection levels of the plants treated with this product were the same as the curative treatments of Pyraclostrobin and Fluxapyroxad, suggesting that the preventative application of fungicides that inhibit fungal sporulation, such as the two respiration inhibitors, was more effective in hampering the development of *M. graminicola* in wheat plants.

As the infection developed further, however, the differences in the efficacy of the fungicides applied changed. When disease severity reached 73% in the untreated plants, there was no longer a significant distinction between the preventative and the curative application. The type of fungicide used did make a difference, however, as the application of Pyraclostrobin resulted in lower infection levels than the other products; significantly different even from the plants treated with Epoxiconazole. Additionally, even though the difference is not considered to be significant, a tendency for better efficacy and higher yield levels was observed in the preventative application of Pyraclostrobin.

Regarding the thousand kernel weight and yield, fungicide application not only resulted in significantly reduced disease levels in comparison to the untreated plants, it also resulted in significantly heavier grains and higher yields, the only exception being the preventative application of Epoxiconazole. Nonetheless, though this product is not that effective as a protectant fungicide, its application after the pathogen has been

established is still recommended due to its strong curative activity. In conclusion, by applying fungicides under strong *M. graminicola* epidemics, approximately 18% of the yield could be saved. Furthermore, if the proper active ingredients are used at the recommended application timing, more than 24% of the yield that otherwise would have been lost to a devastating disease like septoria leaf blotch could be preserved.

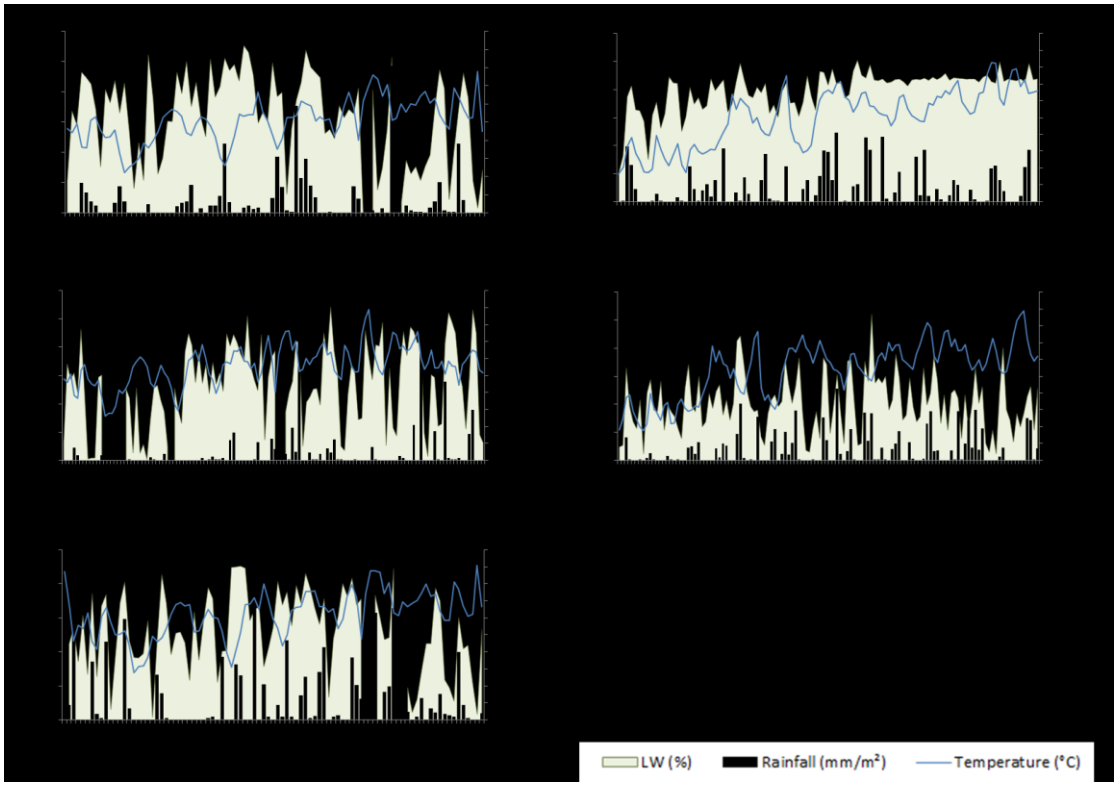
3.4.2 Yield trials under natural conditions

The field trials in the 2010/2011 season consisted of three experiments located in three different locations in the Rhineland-Palatinate region in Germany. Due to space limitations, only one variety was used for the field trials. As that Akteur is a cultivar that is still employed by growers in Germany, this was the chosen variety for the yield field trials. As with the micro-plot trials, the lack of rainfall during the spring hindered the development of the disease in the upper leaf layers. As explained in **3.4.1**, the fungus needs specific environmental conditions in order to induce the spores to germinate and cause an infection. Even though in some of the locations there was enough rainfall to cause an infection, the necessary leaf wetness duration was not reached, hindering fungal growth (**Figure 3.18A-C**).

Two-way ANOVA was used to analyze whether the different fungicide treatments and application timings had an effect on controlling *M. graminicola* and the subsequent effect on yield development. Additionally, the fact that certain products may induce physiological changes in the crop cannot be excluded. To test these effects, the area and the amount of chlorophyll on the flag leaf were determined. According to the two-way ANOVA, no interaction between the different trial locations and treatments in the variables measured (**Table 3.4**) occurred; hence the interaction effect was removed to strengthen the statistical model. Significant difference ($p<0.001$) between the three locations was obtained for all of the variables measured, possibly indicating a relation to environmental conditions or seeding dates. Since no interaction effect was obtained, however, these differences corresponded to the magnitude of the variables measured and not to the effect of the fungicide treatments. With this in mind, the results of the three trials were analyzed together. According to the two-way ANOVA, the fungicide treatments demonstrated a significant effect on disease severity ($p<0.001$) and yield ($p<0.05$).

The preventative application of fungicides took place at the beginning of May, when the flag leaf reached full development, with the curative application performed approximately two weeks later (**Figure 3.18F**). During 2011, the last disease assessment was conducted fifty days after the first fungicide application, before the conclusion of the ripening process, which was necessary to avoid misinterpretations of the disease symptoms due to the normal senescing process of the plants. The post-hoc least significant difference comparison revealed that the disease severity in the untreated plants was significantly higher than fungicide-treated plants with an average infection level of 54% (**Table 3.5**). Furthermore, the disease severity of the plants treated preventatively with Fluxapyroxad was significantly higher than in those treated curatively. In the Epoxiconazole-treated plants, on the contrary, although it seems that the efficacy of the preventative application is lower, the difference is not statistically significant, despite this class of fungicide being known for its higher curative performance.

Even though, no significant difference in the application timing of the fungicides on the control of septoria leaf blotch in wheat grown under natural conditions in 2011 was obtained, whether the plants were treated with fungicides or not did have a significant impact on plant yield (**Table 3.5**). Despite the fact that the environmental conditions were not optimal for the early development of the disease, 4% yield loss in untreated plants was still possible. As with the yield trials performed under semi-natural conditions, in 2012 the wheat variety was switched to Riband. This cultivar is more susceptible to septoria leaf blotch than Akteur, allowing a clearer analysis of the fungicide treatments on yield under high disease pressure. February 2012 was marked by strong freezing temperatures that caused severe damages to the plants and an entire trial had to be discarded. Therefore, in 2012 only two trials were carried out. In the spring of 2012 more rainfall events were detected than in the previous year, providing optimal conditions for infections to occur (**Figure 3.18D-E**). The year was marked, however, by a mixed infection of *M. graminicola* with other fungal diseases.



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Year	2011			2012	
Wheat variety	Akteur			Riband	
Location	Gronau	Ruchheim	Limburgerhof	Böhl	Limburgerhof
Seeding date	16.Sep.10	16.Sep.10	16.Sep.10	11.Oct.11	6.Oct.11
Inoculation with infected straw	Oct.10	Oct.10	Oct.10	Oct.11	Oct.11
Preventative application (GS 39)	12.May.11	10.May.11	4.May.11	14.May.12	9.May.12
Curative application	23.May.11	23.May.11	18.May.11	24.May.12	22.May.12
Harvesting	2.Aug.11	4.Aug.11	11.Aug.11	24.Jul.12	3.Aug.12

Figure 3.18: Weather data and summary from the field trials during the 2011 and 2012 periods

Field trial weather data was constantly monitored as the main growing period started in order to be able to predict when natural infection events (arrows) took place, based on the *Septoria* model developed by Verreet *et al.* (2000). **A-C.** Weather data from the 2011 growing season in Gronau, Ruchheim and Limburgerhof. **D-E.** Weather data from the 2012 season in Böhl and Limburgerhof. **F**. Table summarizing main events during the seasons. Abbr. LW (%): percentage leaf wetness; GS: growth stadium of wheat plants. Due to lack of weather stations in 2012, environmental conditions for the Limburgerhof location were obtained from (Agrarmeteorologie-Rheinland-Pfalz, 2012)

Table 3.4: Two-way ANOVA summary table for the field trials of the 2011 and 2012 seasons

	Leaf area	Chlorophyll content				Disease severity				Net Yield
	9 DAPA GS 49-56	1 DBPA GS 37-47	7-11 DAPA GS 47-56	21 DAPA GS 59-61	35 DAPA GS 65-71	22-27 DAPA GS 61-65	36-41 DAPA GS 69-75	43-44 DAPA GS 75-77	50 DAPA GS 77-85	-
Factor	F-value; P									
Akteur 2011										
Location	$F_{(2,83)}$=57.5; ***	$F_{(2,83)}$=44.9; ***	$F_{(2,83)}$=111.6; ***	$F_{(2,83)}$=67.4; ***	$F_{(2,83)}$=48; ***	N.A.	$F_{(2,83)}$=74.4; ***[a]	N.A.	$F_{(2,83)}$=124.7; ***	$F_{(2,83)}$=49.9; ***
Treatment	$F_{(4,83)}$=0.4; NS	$F_{(4,83)}$=0.1; NS	$F_{(4,83)}$=1.9; NS	$F_{(4,83)}$=1.3; NS	$F_{(4,83)}$=0.5; NS	N.A.	$F_{(4,83)}$=16.2; ***[a]	N.A.	$F_{(4,83)}$=7.8, ***	$F_{(4,83)}$=0.01, *
Location x Treatment	/	/	/	/	/	N.A.	/	N.A.	/	/
Riband 2012										
Location	N.A.	$F_{(1,54)}$=86.1; ***	$F_{(1,54)}$=44.6; ***	N.A.	N.A.	$F_{(1,54)}$=50.4; ***[a]	$F_{(1,54)}$=59.2; ***	$F_{(1,54)}$=22.6; ***	N.A.	$F_{(1,54)}$=0.3; NS
Treatment	N.A.	$F_{(4,54)}$=0.1; NS	$F_{(4,54)}$=1.6; NS	N.A.	N.A.	$F_{(4,54)}$=15.5; ***[a]	$F_{(4,54)}$=26; ***	$F_{(4,54)}$=25; ***	N.A.	$F_{(4,54)}$=10.5; ***
Location x Treatment	N.A.	/	/	N.A.	N.A.	/	/	/	N.A.	/

Significance codes (F-test): P < 0.001 ***, P < 0.01 **, P < 0.05 *, P > 0.05 not significant (NS)

/: Due to not significant interaction between the trials and treatments, the model was simplified and the interaction effect was removed.

[a] For the statistical analysis, a logarithmic transformation on the data was performed.

Abbr. DAPA: Days after preventative application; DBPA: Days before the preventative application; GS: Growth stage according to the BBCH code for cereals; N.A: Not applicable; NS: not significant

Table 3.5: Effect of different fungicide treatments on *M. graminicola* disease severity and wheat physiology under natural conditions during the 2011 and 2012 growing seasons

	2011							2012					
	Leaf area (cm^2)	Chlorophyll content			Disease severity (%)		Net Yield ($dt\ ha^{-1}$)	Chlorophyll content		Disease severity (%)			Net Yield ($dt\ ha^{-1}$)
	7-9 DAPA	1 DBPA	7-9 DAPA	21 DAPA	41 DAPA[a]	50 DAPA	-	1 DBPA	7 DAPA	22-27 DAPA[a]	36-39 DAPA	43-44 DAPA	-
	GS 49-56	GS 37-47	GS 49-56	GS 59-61	GS 69-73	GS 77-85		GS 37-47	GS 37-41	GS 61-65	GS 71-75	GS 75-77	
	Akteur							Riband					
Treatment													
Untreated Control	19.2 a	47.5 a	54.8 ab	52.9 a	10.5 a	54.0 a	79.7 a	47.5 a	50.1 a	9.9 a	45.3 a	75.9 a	56.2 c
Epoxiconazole Pr.	18.7 a	47.7 a	54.5 ab	53.3 a	5.1 bc	40.1 bc	83.1 b	47.7 a	49.5 ab	2.8 b	23.3 b	51.7 bc	69.6 a
Epoxiconazole Cr.	19.3 a	47.7 a	54.4 ab	53.6 a	3.8 c	36.3 c	82.1 b	47.7 a	49.4 ab	3.8 b	22.8 b	49.6 c	67.7 ab
Fluxapyroxad Pr.	19.4 a	47.6 a	54.3 b	53.4 a	5.7 b	45.8 b	83.6 b	47.6 a	48.7 b	3.8 b	27.8 b	57.6 b	63.2 b
Fluxapyroxad Cr.	18.9 a	47.5 a	55.0 a	53.5 a	4.5 bc	35.8 c	82.8 b	47.5 a	49.4 ab	3.7 b	23.3 b	47.3 c	68.0 a

Means with the same letter are not significantly different (Least significant difference at P=0.05). Displayed values are based on 18 repetitions (3 trials x 6 replicas) for the 2011 seasons and on 12 replications (2 trial x 6 repetitions) for the 2012 season.

[a] For the statistical analysis, a logarithmic transformation on the data was performed. Values represent the mean in the original scale.

Abbr. DAPA: days after preventative treatment; DBPA: days before preventative application; GS: growth stage according to the BBCH code for cereals; cm^2: square centimeters; g: grams; $dt\ ha^{-1}$: decitons per hectare.

Based on the results obtained in the previous year regarding the physiological effects that the fungicides had on the plants, for 2012, the chlorophyll measurements were reduced to two assessments, one carried out before the first application took place and a second assessment, seven days after the preventative fungicide treatment. Leaf area measurements were not performed as they are highly time consuming and brought little value since fungicide treatment did not appear to have a significant effect on flag leaf area in the 2011 field and micro-plot trials, as well as the 2012 micro-plot experiments.

During the 2012 experiments, fungal growth progressed faster than in the previous year due to improved weather conditions for the disease and the use of a more susceptible variety, Riband. Infection levels reached an average of over 70% in untreated plants 40 days after the first fungicide application, as the plants where still developing the kernels. The two way analysis of variance ANOVA, did not indicate an interaction effect between the treatments and the two trial locations. In light of this, the model was simplified by removing the interaction effect and analyzing both trials together (**Table 3.4**). As in the previous year, there was no physiological effect on the plants from the fungicide application. Nonetheless, the products displayed a significant influence ($p<0.001$) on the disease control and crop yield.

Least significant difference analysis of the fungicide effects indicates that during the early development of the infection, the products did not differ from one another (**Table 3.4**). At 44 days, however, after the first application, a significantly reduced effect on the disease control was observed in the plants that were treated preventatively with Fluxapyroxad. Moreover, the yield obtained in these plants was significantly lower than in the plants sprayed with the same active ingredient as a curative treatment. One reason for this result may be attributable to the mixed infection. As in the previous year, no difference between the timing of the applications of Epoxiconazole in regards to disease control and yield was identified.

Furthermore, the fact that higher yields can be obtained when a fungicide is used to control fungal diseases was proven once again. Under similar conditions to those present in the 2012 field trials, the lack of fungicide treatment can lead to a 20% yield loss caused by fungal diseases, with the subsequent significant impact on cereal production during the corresponding growing season.

4. Discussion

Wheat is considered the second most important source of food supply after rice (FAO, 2012a). In 2011, with a global harvested area of approximately 220 million hectares, the global net wheat production reached 704 million tons (FAO, 2012a). As with any other plant, however, wheat is exposed to many biotic and abiotic stress factors that directly, or indirectly, threaten the yield and the final quality of its grains. Among the biological pressures that negatively affect the global wheat production, the septoria leaf blotch disease (SLB), caused by the fungus *Mycosphaerella graminicola,* has the greatest impact, followed by brown rust and powdery mildew, caused by the fungi *Puccinia triticina* and *Blumeria graminis* f.sp. *tritici*, respectively (FAO, 2013a; 2013b; Jahn *et al.*, 2012; Wegulo *et al.*, 2011; Wiik, 2009). The loss of photosynthetic tissue caused by this pathogen is related to significant yield and quality losses that, under severe epidemics, can range between 40 and 60% (Eyal, 1987) and generate serious economic losses (Ponomarenko *et al.*, 2011). Therefore, successful control of this, as well as other fungi, is of great importance in protecting global wheat production. Resistant wheat varieties do exist, though, with low durability due to the rapid evolutionary rate of *M. graminicola* upon selection pressure. Therefore, the control of SLB is still heavily reliant on fungicidal use in order to protect the upper leaf layers of the plant, which play a vital role in determining crop yield (Cools *et al.*, 2007; Thygesen *et al.*, 2008).

The use of agrochemicals is an old and traditional way of combating plant diseases in order to secure yield (Russell, 2005). Furthermore, evidence of yield increase due to fungicide treatment under low and high disease pressure has been reported for several crops such as wheat, barley (Berdugo *et al.*, 2011; 2012; McCartney *et al.*, 2007; Suty-Heinze *et al.*, 2011), soybean (Mueller *et al.*, 2009), rice (Bartlett *et al.*, 2002; Harrison & Tedford, 1999) and potato (Bartlett *et al.*, 2002; Stevenson *et al.*, 1999). Nonetheless, there is still the discussion if the achieved yield increase is related more to physiological effects or due to successful pathogen control (Bertelsen *et al.*, 2001; Swoboda & Pedersen, 2009). The last hypothesis takes into consideration that by controlling the disease before it enters the plant, the activation of plant innate immunity system will be avoided and thus there will be no yield penalty (Bertelsen *et al.*, 2001).

In the present study, the effects of three fungicides with different modes of action were evaluated regarding their impact on the wheat physiology, *M. graminicola* disease

control and yield. For this reason several experimental setups were assessed in order to evaluate the effects of the fungicides under different experimental conditions from glasshouse to natural field environments. In the first part of the project, the biology of the *M. graminicola*-wheat interaction was studied using molecular and cytological tools, to optimize the experimental conditions in the glasshouse, as it has been reported that many factors including plant variety, fungal isolates and environment are involved in the development of the disease (Eyal, 1987; Lovell *et al.*, 2004). Based on the results obtained from the infection biology study, critical time points during the symptomless infection process could be identified for further studies. Additionally, by making use of molecular biology techniques and microscopical observations, the efficacy profile of the fungicides Pyraclostrobin, Epoxiconazole and Fluxapyroxad in controlling septoria leaf blotch on wheat seedlings was analyzed to identify differences in the mode of action that could explain a specific respond from the plant.

With the development of DNA microarray chips at the end of the 1990s, the possibility of evaluating the response of thousands of genes in parallel to a given stimulus became possible. Since then, this technology has improved considerably and has been used extensively in different areas of plant research biology including, among others, disease resistance, abiotic stress responses and plant developmental processes (Reymond, 2001; Zhu, 2003). With this in mind, a microarray-based approach was employed to identify, at the molecular level, the effects that fungicides have on the transcriptome of wheat seedlings grown in glasshouse environmental conditions. Although RNA Seq represents an improved technique for quantifying an organism´s transcriptome (Mortazavi *et al.*, 2008), it was decided not to implement it during the experiments due to its high experimental cost and less developed analytical capabilities in comparison to the standardized microarray analytical tools. The high complexity of the hexaploid wheat genome has caused delays in the genome sequencing projects current underway and, therefore, there is currently not a complete reference genome and gene annotation for this organism. Based on this, mapping the transcriptome profile obtained from RNA Seq to a reference genome would not be possible, which would limit the advantages of this new technology.

In order to differentiate between the physiological from fungicidal effects on the plants, two different approaches were employed. In the first approach, agrochemicals were

sprayed under disease-free conditions with the reactions of wheat seedlings to the fungicides evaluated at the transcription level with microarray chips. The second approach consisted of estimating the influence of different fungicide application time points, preventative or curative, on the wheat transcriptome when plants are infected with pycnidiospores from *M. graminicola*. With this in mind, the agrochemicals were sprayed either one day before, or seven days after, inoculation. The responses of wheat seedlings at the molecular level were evaluated at three and ten days after inoculation. To the author´s knowledge, this is the first study reporting the effects of the interaction between fungicide application timing and the *M. graminicola* infection on wheat at the transcriptome level. Microarray studies have been performed on *M. graminicola* grown *in vitro* and *in vivo* under different nutrient levels in order to understand the pathogen´s infection strategy (Keon *et al.*, 2005b; 2007). Transcriptional analysis of two *M. graminicola* isolates, each exhibiting a different levels of resistance to Epoxiconazole, have also been carried out with the purpose of identifying the pathways responsible for fungicide resistance development (Cools *et al.*, 2007). Pasquer *et al.* (2005) evaluated the transcriptome responses of wheat leaves to the Fenpropinorph and Azoxystrobin fungicides, as well as to the chemical resistance inducer benzo(1,2,3) thiadiazole-7-carbothioic acid S-methylester (BTH). Nonetheless, during that study, barley, instead of wheat, microarrays were chosen and the experiments were carried out in a glasshouse under disease-free conditions. During the field trials, Pasquer *et al.* (2005) did not report the influence of naturally occurring fungal diseases.

Yield trials under semi-controlled and completely natural conditions were also carried out to evaluate whether the results obtained at the molecular level, in terms of physiological effects, could also be validated in the field. As with the glasshouse experiments, the differences between the preventative and the curative application of fungicides with varying modes of action on controlling *M. graminicola,* along with the subsequent effect on yield, were evaluated.

4.1 *M. graminicola* infection biology

Wheat septoria leaf blotch, caused by the *Mycosphaerella graminicola* fungus (anamorph *Zymoseptoria tritici*), is a common disease prevalent in wheat growing areas and characterized by a long latent period followed by the sudden appearance of necrotic lesions in the leaf. The disease gained economic importance due to the switch to early-

maturing semi-dwarf wheat varieties highly susceptible to this fungus as well as changes in agronomical practices (Eyal, 1987; 1999; Lucas *et al.*, 1999). The fungus requires high humidity conditions during all stages of its infection process. Approximately 12 hours after inoculation, spores germinate; 24 hours later, leaf penetration occurs via stomatal openings or directly through the cell wall. After 14 to 21 days, disease symptoms appear as black spots scattered within the necrotic lesions (Eyal, 1987). However, depending on the environmental conditions and which wheat cultivars and fungal isolates are present, symptom appearance under glasshouse conditions can vary between 8 to 16 days after inoculation (Cohen & Eyal, 1993; Guo *et al.*, 2005; 2006; Siah *et al.*, 2010). In the field, this period can extend up to 42 days (Henze *et al.*, 2007; Lovell *et al.*, 2004). Under ideal glasshouse environmental conditions for fungal growth, the spore germination and plant penetration via stomatal openings of a mixture of four *M. graminicola* isolates (2806, 2101, 2107 and 2108) in the Riband winter wheat variety was visualized three days after the inoculation (**Figure 3.1-A**). In some cases, appressoria-like structures at the penetration sites near guard cells were also observed (**Figure 3.1-B**). Such events have also been reported by other authors (Cohen & Eyal, 1993; Duncan & Howard, 2000; El Chartouni *et al.*, 2012; Kema *et al.*, 1996; Shetty *et al.*, 2003; 2007). Based on cytological analysis of the *M. graminicola* infection process, Kema *et al.* (1996) concluded that, contrary to rust fungi, *M. graminicola* uses appressoria-like structures to attach itself to the plant surface rather than to achieve successful penetration. First visible disease symptoms were observed between 15 and 17 days post-inoculation (dpi) as chlorotic regions along the leaf surface and, in certain cases, necrotic lesions were already present within the chlorotic area (**Figure 3.4**). Microscopic observations at this time point also revealed significant mycelial growth on the leaf surface (**Figure 3.1-C**). Advanced disease symptoms were detected at 25 dpi, as infected leaves became completely necrotic and pycnidia were visible as black dots linearly distributed along the leaf (**Figure 3.1-D-F**).

Since evidence of fungal growth and leaf damage could not be visualized during the first 15 days of infection, quantitative real time PCR (qPCR) was employed to quantify fungal development during this period. For this purpose, the DNA of infected plant material was extracted at 3, 5, 7, 10, and 17 dpi to calculate the amount of fungal DNA relative to the plant actin gene. The obtained results support the conclusion that no disease

symptoms are detectable in the plants due to the limited growth of the fungus. A sudden, rapid fungal growth stage was measured after 10 dpi due to a "switch" in the lifestyle of the pathogen that leads to aggressive host colonization. At 17 dpi, a 16-fold increase in fungal DNA was detected (**Figure 3.2**), revealing massive fungal colonization at this point in the infection process. Moreover, the exponential increase in fungal biomass, detected between 10 and 17 dpi, coincided with the appearance of the first visible disease symptoms (**Figure 3.4**). Measurements of *M. graminicola* biomass in wheat plants using qPCR also established a narrow fungal growth during the symptomless phase of infection, followed by a sudden and rapid increase between 8 and 16 dpi (Guo *et al.*, 2006; Keon *et al.*, 2007; Shetty *et al.*, 2007). Kema *et al.* (1996) performed an enzyme-linked immunoabsorbent assay (ELISA) in order to quantify mycelial growth of *M. graminicola* isolate IPO87016 in the susceptible wheat cultivar Shafir during the infection process. The authors also observed that the antigen level did not significantly increase until 6 dpi, when the first chlorotic lesions were visualized. The reported differences in the point in time at which exponential fungal growth is detected are believed to depend on the plant-pathogen interaction as along with the environmental conditions present during the experiment (Guo *et al.*, 2006; Lovell *et al.*, 2004).

4.1.1 Gene expression analysis during the wheat-*M. graminicola* interaction

In addition to the quantification of the fungal biomass, a microarray pre-experiment to evaluate the global responses of Riband winter wheat seedlings to *M. graminicola* during the symptomless phase of the infection process was conducted. The purpose of this part of the project was to identify the time points during the infection at which the plant is able to detect the fungus and initiate a defense response to hinder fungal colonization. Based on the results obtained from this pre-experiment, time points in a second microarray experiment could be selected to analyze, the effect of the fungicides on controlling the disease and their subsequent effect on wheat stress response. It is important to keep in mind that Riband is a highly susceptible SLB cultivar (Chartrain *et al.*, 2004a) and thus, the time and the magnitude in the defense response reported in this paper may vary to that of a resistant variety.

Once the fungus has penetrated its host, either through stomatal openings or directly through the epidermis, and colonization has started, the plant is able to recognize the pathogen invasion and slightly increases the expression of defense-related genes. The

defense related genes activated include, among others, chitinase and beta-glucanase, as well as transcripts involved in jasmonate and ethylene responses. The plant, however, down-regulates the expression of these genes at 5 dpi (**Figure 3.10-I**), suggesting the fungus is, somehow, able to suppress PAMP-triggered immunity (PTI) responses and/or hide from the plant´s surveillance system while establishes itself inside its host. The recent publication of the finished *M. graminicola* genome (Goodwin *et al.*, 2011) has already provided further insight into the pathogenicity of this fungus. A fungal effector protein (Mg3LysM), for instance, that binds to chitin fragments from the microorganism and prevents the activation of PTI by the plant, has been identified (R. Marshall *et al.*, 2011). According to the authors mentioned, the pathogen is able to hide from the plant´s surveillance system and proceed with the symptomless phase of the disease infection (do Amaral *et al.*, 2012; R. Marshall *et al.*, 2011).

Between 10 and 17 dpi, an exponential rise in the expression of plant defense-related genes along with the activation of transcripts involved in oxidative stress and the hypersensitive response (HR) was observed (**Figure 3.10-I-II**). These responses were not induced at the earlier time points. It has been reported, that differences in the activation of defense responses during a compatible and incompatible interaction arise shortly after pathogen penetration has taken place, and are crucial for the development of the disease (Cohen & Eyal, 1993; Kema *et al.*, 1996; Shetty *et al.*, 2003). ROS, such as hydrogen peroxide (H_2O_2), plays a dual role in *M. graminicola* growth and further colonization. Resistant wheat cultivars are able to hinder pathogen colonization and disease development by accumulating large quantities of ROS around the penetration site. The effectiveness of ROS in halting M. graminicola suggests that, upon host penetration, as well as during its biotrophic phase, the fungus is sensitive to ROS. On the other hand, susceptible cultivars probably because are not able to detect the pathogen, only start to accumulate ROS at the onset of disease symptom development during the necrotrophic stage of the fungus, with elevated ROS levels first detected upon pycnidium formation. The high ROS levels observed at the onset of visual disease symptoms have been attributed to host cell collapse and massive fungal colonization (Shetty *et al.*, 2003; 2007). Nonetheless, whether, at this point, ROS are derived from the pathogen or induced by the plant is still unclear (Keon *et al.*, 2005b; 2007; Shetty *et al.*, 2003). According to Shetty *et al.* (2007), at the onset of symptom development, large quantities

of carbohydrates like sucrose, fructose and glucose are released in the apoplastic tissue. The accumulation of these sugars not only provides the fungus with nutrients to drive massive host colonization and sporulation, it also may trigger host tissue necrosis and ROS synthesis. The sudden accumulation of H_2O_2, therefore, induces a change in the lifestyle of the fungus, from biotroph to necrotroph, to cope with the newly existing toxic environment in the host (Shetty *et al.*, 2007). Global gene expression analysis of *M. graminicola* responses during host colonization revealed an increase in the expression levels of genes related to ROS detoxification after the onset of disease symptom development. The genes involved included, among others, peroxidases, catalases and superoxide dimutases; genes which are considered to help the fungus tolerate the harsh environment present during host program cell death (Keon *et al.*, 2007).

The strong induction of genes involved in oxidative stress and hypersensitive response, as observed in **Figure 3.10-I and II,** between 10 and 17 dpi during the experiment, correlate to a sudden increase in the amount of fungal DNA as measured by qPCR (**Figure 3.2**) along with the appearance of visible necrotic spots on the leaf surface (**Figure 3.4**). According to these results, it can be concluded that the genetic profile observed in the Riband winter wheat variety after *M. graminicola* colonization is similar to the defense responses observed during compatible interactions (Adhikari *et al.*, 2007; Hammond-Kosack & Rudd, 2008; Rudd *et al.*, 2008; Shetty *et al.*, 2003; 2009).

The sudden increase in fungal DNA, as well as the exponential rise in the expression profile of the plant-type hypersensitive response and oxidative stress-related genes, suggests that the fungus may induce a programmed cell death (PCD) response in the host for its own benefit. Other studies reached similar conclusions, suggesting the involvement of a fungal toxin in the unexpected induction of necrosis and cell collapse, a toxin which is only produced when a critical amount of fungal biomass has been attained (Kema *et al.*, 1996; Keon *et al.*, 2007). The fact that fungal toxins are able to induce PCD is a method known to be used by other necrotrophic pathogens such as *Pyrenophora tritici-repentis* (Manning & Ciuffetti, 2005), *Botrytis cinerea* (Govrin & Levine, 2000) and *Botrytis elliptica* (Van Baarlen *et al.*, 2004) for a successful plant infection (Keon *et al.*, 2007). A toxin acting as elicitor of PCD in the *M. graminicola*-wheat interaction, however, has not yet been identified (Keon *et al.*, 2007; Ponomarenko *et al.*, 2011; Rudd *et al.*, 2008). Motteram *et al.* (2009) reported that the necrosis and ethylene-inducing

peptide 1 (Nep1)-like protein from the fungus *M. graminicola* (MgNLP) is only significantly induced *in planta* during the symptomless phase of a compatible infection process, with an expression peak shortly before the exponential fungal growth and the development of disease symptoms. Even though infiltration of MgNLP in *Arabidopsis* leaves triggers plant cell death and the induction of defense-related genes, treatment of wheat leaves with this protein did not induce any of the above mentioned responses. Additionally, gene deletion mutants were still able to infect the plants and sporulate at a later time. According to these results, Motteram *et al.* (2009) determined that, although this protein is only expressed during compatible interactions, it is not a pathogenicity or virulence factor, and its function in the *M. graminicola*- wheat interaction is still unknown.

*M. graminicola m*icroarray experiments have been conducted to evaluate the infection process of the fungus with the hope to gain more information regarding its pathogenicity. Fungal transcripts activated during the symptomless phase of the infection have strong similarities to fungal genes up-regulated under nutrient-limited conditions. Genes associated to high-nutrient status and oxidative stress responses, on the other hand, were induced during the period of exponential fungal growth (Keon *et al.*, 2005b; 2007). Additionally, at the onset of symptom development, increased ion leakage of infected plant cells as well as higher metabolite and sugar levels in the plant apoplast were reported to precede cell lysis, necrosis and death (Keon *et al.*, 2007; Shetty *et al.*, 2007). The conclusions obtained from these studies is that the fungus barely survives (feeds) during the asymptomatic phase, with sudden exponential growth triggered by a programmed cell death-like response in the host. PCD is not only incapable of controlling the spread of the pathogen, it even provides the fungus with an environment rich in nutrients for further growth and sporulation (Kema *et al.*, 1996; Keon *et al.*, 2005b; 2007). Studies covering the *M. graminicola* infection process have led Keon *et al.* (2007) to suggest that this pathogen should not be categorized as a hemibiotroph but, rather, as a necrotroph, as that no evidence of it using plant nutrients to grow or pursue intracellular colonization during the biotrophic phase exists. This conclusion is also supported by R. Marshall *et al.* (2011), who described the fungus as a necrotroph that needs a long symptomless period of host association. During this project, the first set of defense responses (3dpi) activated by the plant were not related

to salicylic acid-mediated synthesis or signaling, but rather to jasmonate- and ethylene-related responses, a fact that supports this theory. Salicylic acid is a plant hormone involved in the regulation of defense responses to biotrophic and hemibiotrophic pathogens (Bari & Jones, 2009), whereas ethylene and jasmonate act synergistically to mediate responses to necrotrophs (Browse, 2009; Lorenzo *et al.*, 2003; 2004; Memelink, 2009).

The activation of defense and oxidative stress responses revealed a simultaneous down-regulation of transcripts involved in photosynthesis, chlorophyll and carbohydrate metabolism (**Figure 3.10-III**); suggesting that defense is a costly process for a plant in terms of growth and development. Similar results have been obtained by Bilgin *et al.* (2010) during a meta-analysis of publically available microarray experiments involving the responses of assorted plants to various pathogens and pests.

Based on the results mentioned above, a microarray-based experiment was carried out to investigate the influence of fungicides on the plant transcriptome under disease pressure, with spraying occurring before, or after, infection had taken place. If, by applying a fungicide before infection, the activation of costly plant defense responses can be avoided and higher yields could be obtained. Nonetheless, before evaluating the effects of the fungicide products on the plant transcriptome, the efficacy profile of each fungicide in controlling *M. graminicola* was evaluated under preventative and curative application regimes to determine the impact that fungicide modes of action have on controlling septoria leaf blotch.

4.2 Efficacy of fungicides on the early control of *M. graminicola* depends on their mode of action

Application timing plays a very important role in controlling septoria leaf blotch. If chemical application takes place after disease symptoms appear, it can be ineffective since the fungus has already established itself in the plant (Beyer *et al.*, 2012; Lucas *et al.*, 1999). For this reason, the chemical control effectiveness of different application timings was determined using fungicides that vary not only in their manner of hindering pathogen growth, but also in their chemistry. Pyraclostrobin belongs to the group of the strobilurins, Fluxapyroxad to the pyrazole carboxamides and Epoxiconazole to the triazoles fungicides (Kuck *et al.*, 2012a). Pyraclostrobin and Fluxapyroxad bind to

protein complexes III and II of the electron transport chain, respectively, and, thus are able to block fungi energy demand and hamper its growth (Bartlett *et al.*, 2002; Earley *et al.*, 2012). The agrochemical Epoxiconazole, on the other hand, obstructs fungal growth by inhibiting the C-14 demethylase enzyme in the fungal sterol synthesis pathway, leading to membrane destabilization (Burden *et al.*, 1989; Kuck *et al.*, 2012b; Kwok & Loeffler, 1993)

The fact that Pyraclostrobin (PC) and Fluxapyroxad (FP) hinder the energy production of the fungus, while Epoxiconazole (EA) does not, has an influence on the disease development. Scanning electron microscopy observations revealed that, when the products were sprayed preventatively, the PC and FP fungicides were able to hinder spore germination and germ tube formation, while the EA fungicide was not (**Figure 3.3**). When the disease development was evaluated, minor disease symptoms, including pycnidia, were even observed in the plants treated preventatively with Epoxiconazole but not, however, in the plants treated with Pyraclostrobin and Fluxapyroxad (**Figure 3.4**). In a study carried out to determine the activity of Epoxiconazole, Schöfl and Zinkernagel (1997) demonstrated that when this product was sprayed preventatively, leaf damage due to necrotic lesions could still be observed, albeit at a much lesser extent compared to an untreated plant. Furthermore, when Epoxiconazole was applied in a mixture with a strobilurin fungicide, Kresoxim-methyl, for example, the disease symptoms displayed were significantly lower due to the spore inhibiting properties of the latter fungicide, which is absent in the azoles.

Fungal biomass quantification via qPCR revealed a clear difference between the efficacy of the triazole fungicide and the two fungal respiration inhibitors in controlling septoria leaf blotch in wheat seedlings. Pyraclostrobin and Fluxapyroxad preventative application hindered fungal development by more than 80% from the onset of the infection process. The preventative treatment of Epoxiconazole, on the other hand, did not achieve over 40% efficacy until 10 dpi and, though it reached more than 90% efficacy at 17 dpi, it was not able to attain the efficacy levels of the other two products (**Figure 3.5**). The results proved the outstanding preventative control of the cellular respiration inhibitors in the management of fungal diseases, such as septoria leaf blotch on wheat. Reports of the strong preventative activity of strobilurins (Bartlett *et al.*, 2002; Inoue *et al.*, 2012) and carboxamide fungicides (Stammler & Speakman, 2006; Suty-

Heinze *et al.*, 2011) on fungal spore germination inhibition have already been published. Moreover, it has been suggested that fungicides inhibiting sterol synthesis do not provide strong preventative activity as the fungi are able to obtain enough ergosterol from reserves stowed in their spores. For this reason, these fungicides are not able to hinder pathogen penetration (Bartlett *et al.*, 2002; Pontzen & Scheinpflug, 1989). Nevertheless, fungal colonization is significantly affected as the efficacy of this product stood at more than 90% at 17 dpi (**Figure 3.5**).

When the plants where sprayed with Fluxapyroxad and Pyraclostrobin seven days after inoculation to simulate a curative application, minor differences in disease symptom development not visualized when the products were sprayed preventatively were observed. Pyraclostrobin-treated plants developed slight chlorotic lesions, indicating some level of disease development, whereas the application of the new generation of SDHI fungicide, Fluxapyroxad, resulted in healthy plants without any evidence of septoria leaf blotch disease (**Figure 3.4**). The quantification of fungal DNA indicated that even though at 17 dpi all three fungicides were equally effective, a clear difference among them at 10 dpi was observed. At this time point, the SDHI product provided significantly higher disease control than the strobilurin and triazole fungicides (**Figure 3.5-curative treatments**). These results suggest that when Fluxapyroxad is sprayed shortly after pathogen infection, the fungal control level is the same as when the product is sprayed preventatively, a demonstration of exceptional curative activity. Regarding the triazole fungicide Epoxiconazole, a significant increase in the efficacy of this product was observed when sprayed curatively, reaching similar efficacy levels as the curative application of Pyraclostrobin (**Figure 3.5**). Furthermore, the disease symptoms under application of this fungicide were not only more moderate in comparison to its preventative counterpart; the fungus was not even able to develop pycnidia (**Figure 3.4**), demonstrating its curative effect in hindering fungal growth and reproduction. According to these results, it can be concluded that a resistance management strategy involving a fungicide with a strong preventative activity, such as Pyraclostrobin or Fluxapyroxad, in an Epoxiconazole spraying program would provide the highest protection level without risking fungal resistance development (due to the different fungicide action modes). Therefore, major agrochemical producers currently include tank mixtures of triazole fungicides with either pyrazole carboxamides, or strobilurins,

in their product portfolio, leading to outstanding results in the cereal fungicide market (topagrar, 2013)

The results provided above demonstrate that differences in the efficacy of fungicides with varying modes of action in controlling septoria leaf blotch can be detected before the appearance of disease symptom by including molecular biology techniques, such as qPCR, in fungicide screening programs. Besides enabling more accuracy, this technique is not biased by the evaluators experience in disease assessment methods. Though qPCR is costly and requires automation, its employment is highly beneficial in the early evaluation of the efficacy profiles of various agrochemicals. These benefits are not limited to the control of septoria leaf blotch in wheat, but can be extended to other diseases and crops as well; leading to the possible conclusion that the advantages of including such assessment methods in a fungicide screening program may outweigh the costs. The successful use of qPCR for the early detection of *M. graminicola* infection events in the field, along with the evaluation of fungicide efficacy, has already been concluded (Guo *et al.*, 2006; 2007). Nonetheless, an evaluation of the effects of different application timing and fungicide mode of action on disease control by using this technique has not yet been published. Additionally, the fact that adequate application timing and selection of the proper active ingredient are of great importance in disease management decisions was observed and demonstrated, once more, by this project.

4.3 Analysis of the fungicidal effect on the wheat transcriptome

Previously, it was demonstrated that application timing and fungicide mode of action play a very important role in the control of septoria leaf blotch in wheat seedlings. For further analysis, a microarray experiment was pursued in order to evaluate whether the observed differences in fungicide efficacy can be attributed to their influence on the plant transcriptome and the corresponding physiological effects or activation of plant defense responses. In order to be able to distinguish between purely physiological and fungicidal effects on the plant, the microarray experiments were carried out either under disease-free conditions or under disease pressure. Finally, to evaluate whether the effects observed on transcriptome level correlate to yield levels in the field, yield trials under semi-natural and natural conditions were conducted. No genome-wide expression analysis was performed in the field since, under natural conditions, plants are constantly triggered by so many environmental factors beyond pathogens and pests,

making it impossible to differentiate between the purely fungicide and environmental effects on the plants. After analyzing the gene expression profile of wheat plants subjected to chemical treatments in the field and glasshouse, Pasquer *et al.* (2005) found no correlation between the results obtained in the two experimental setups. The authors also measured the gene expression levels of mock-treated plants in the field, and found that the stress-related genes highly expressed in fungicide-treated plants in the glasshouse were also highly induced in the field. According to their results, they concluded that stress-related genes of the plants grown under natural conditions were constitutively expressed and, thus, the fungicide effects on the plant transcripts in the field could not be detected. Choosing the proper environmental conditions when testing the effects of plant protection products on plant physiology is, therefore, of great importance (Pasquer *et al.*, 2005).

4.3.1 Effect of fungicides on the wheat transcriptome under disease-free conditions

Beneficial aspects of Pyraclostrobin treatment considered to increase the net yield at a given season, regardless of the disease pressure, have been observed (Koehle *et al.*, 2002). These physiological benefits are attributed to delayed senescence, reduced water consumption due to lower stomatal aperture, increased tolerance to oxidative stress and improved CO_2 assimilation (Beck, 2005; Koehle *et al.*, 2002). Based on these results, it has been suggested that Qo inhibtors not only have an effect on the mitochondrial respiration process of fungi but also on that of the treated plants (Gerhard *et al.*, 1998; Grossmann & Retzlaff, 1997; Koehle *et al.*, 2002). Berdugo *et al.* (2012) reported that treatment with the new carboxamide fungicide Bixafen under glasshouse, disease-free conditions resulted in even higher yields than in plants treated with the azole and strobilurin products, Prothioconazole and Fluoxastrobin, respectively. According to their results, which were based on digital infrared images, they suggested that the physiological benefits of Bixafen resulted from the product-driven lower tissue temperature in the treated plants, which positively impacts plant vitality and photosynthetic activity. These authors also observed delayed senescence in leaves and ears treated with the Fluoxastrobin, Prothioconazole and Bixafen fungicides and the corresponding improvement in yield during the grain-filling period. The authors, therefore, concluded that higher yields in treated, disease-free plants were obtained.

Besides being an important signaling molecule in plant defense response, growth and development (Apel & Hirt, 2004; Foyer & Shigeoka, 2011), reactive oxygen species (ROS) promote leaf senescence (Leshem, 1988; Thompson *et al.*, 1987; Wu & von Tiedemann, 2001). Upon fungicide treatment under disease-free conditions, gene transcripts related to plant peroxidases were highly induced in the wheat seedlings, regardless of the active ingredient being applied (**Figure 3.8**). Furthermore, according to the cluster and gene enrichment analyses, all three products caused the down-regulation of transcripts involved in oxidative stress responses (**Figure 3.9-IV**). Depending on their catalytic cycle, peroxidases have dual functions in the plant; either as scavenging enzymes that reduce H_2O_2, or as generators of ROS through the hydroxylic cycle (Passardi *et al.*, 2005). The fact that the gene expression of peroxidases was induced after fungicide treatment, along with the repression of oxidative stress-related genes, leads to the suggestion that the agrochemicals Pyraclostrobin, Epoxiconazole and Fluxapyroxad are not only effective in controlling fungal infections, they may also have an effect on leaf senescence by modifying the ROS metabolism. Other groups have reported the positive effect of strobilurins and triazole fungicides on ROS scavenging enzymes, such as the superoxide dismutase (SOD) and peroxidases (POX) on wheat and barley, and the subsequent effect on retarding leaf senescence (Jabs *et al.*, 2002; Koehle *et al.*, 2002; Wu & von Tiedemann, 2001; 2002). Application of Epoxiconazole and the strobilurin fungicide Azoxystrobin led to higher SOD and POX enzymatic activity in treated plants and thus, were able to i) delay the naturally occurring leaf senescence in wheat beyond the grain filling period, and ii) prevent ozone injury in barley caused by the production of ROS such as peroxide (Koehle *et al.*, 2002; Wu & von Tiedemann, 2001; 2002). Jabs *et al.* (2002) also demonstrated that, by inhibiting the activation ROS, Pyraclostrobin and Epoxiconazole prevent the development of the non-parasitic physiological leaf spot disorder (PLS) on barley that involves severe necrosis, loss of chlorophyll content and reduced yields. By delaying leaf senescence, the grain-filling period will be extended; hence higher yields are obtained (Berdugo *et al.*, 2012; Gerhard *et al.*, 1998; Jabs *et al.*, 2002). Koehle *et al.* (2002) reported more than two-fold increase in the peroxidase levels in Pyraclostrobin-treated barley in the field. This response lasted more than four weeks and led to the conclusion that this fungicide helps cereals to cope with oxidative stress induced either by biotic or abiotic stress conditions.

It has been suggested that the elevated biomass levels observed in plants upon application of a strobilurin fungicide are not only caused by higher chlorophyll levels and delayed leaf senescence but also as a result of enhanced nitrogen assimilation. Treatment of wheat leaves with Pyraclostrobin (Koehle *et al.*, 2002) and spinach leaf discs with Kresoxim-methyl (Glaab & Kaiser, 1999), led to higher nitrate uptake levels due to the activation of the enzyme nitrate-reductase at night. Furthermore, the improved nitrate assimilation in the wheat leaves observed after application of Pyraclostrobin correlated to 20% higher biomass production (Koehle *et al.*, 2002). Although the microarray experiments performed during this study did not provide any evidence of nitrate reductase activation after Pyraclostrobin, Epoxiconazole or Fluxapyroxad treatment, the effect that these fungicides have on nitrate assimilation cannot be discarded. According to Koehle *et al.* (2002), nitrite and ammonia only accumulated on Pyraclostrobin-treated leaves at night. Nitrate reductase is usually inactive in leaves during the night and re-activated by light (Glaab & Kaiser, 1999). It has therefore, been suggested that strobilurin fungicides inhibit the darkness-driven inactivation of nitrate reductase, enabling improved nitrate assimilation in treated plants (Glaab & Kaiser, 1999; Koehle *et al.*, 2002). These considerations may help to explain why similar results in nitrate reductase levels were not noted in the leaves, which were harvested at day time, used in the microarray analysis of this study. In order to confirm the effect that strobilurins, or any other fungicides, may have on nitrate assimilation rates, the expression levels and enzymatic activity of nitrate reductase upon fungicide treatment should also be evaluated at night.

4.3.2 Effect of fungicides on hormone homeostasis in wheat seedlings

According to the mapman and gene enrichment analysis conducted in this study, wheat seedlings treated with Pyraclostrobin, Epoxiconazole and Fluxapyroxad either under disease pressure or under disease-free conditions, revealed changes in the expression levels of genes involved in phytohormone synthesis and signaling. The main hormone genes found to be induced or repressed after fungicide treatment were, among others, ethylene, jasmonate, auxin, gibberellin and abscisic acid.

Ethylene synthesis starts with the conversion of methionine to *S*-adenosyl-methionine, which is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the ACC synthase enzyme. Subsequently, ACC is oxidized by ACC oxidase to form ethylene.

Several studies suggest that the first pivotal step in regulating the synthesis of this phytohormone is mediated by ACC synthase (Barry *et al.*, 2000; Broekaert *et al.*, 2006; Peck & Kende, 1998). Nonetheless, various external stimuli such as pathogen attack, wounding or abiotic stress, cause a change in the expression of ACC oxidase genes, indicating that ethylene synthesis is not only regulated by ACC synthases but, also, to a lesser extent, by ACC oxidases (Barry *et al.*, 2000; Broekaert *et al.*, 2006). Moreover, strobilurin fungicides have been reported to inhibit the enzyme ACC synthase in the ethylene biosynthetic pathway and induce endogenous levels of phytohormones auxin and abscisic acid (Grossmann *et al.*, 1999; Koehle *et al.*, 2002). These phytohormonal changes are considered to play an important role in the physiology of plants; delaying leaf senescence and improving their adaptation to drought conditions (Grossmann *et al.*, 1999; Koehle *et al.*, 2002). According to the results obtained from the microarray experiments, all three fungicides appear to induce ethylene synthesis at the ACC synthase and at the ACC oxidase transcriptional levels under disease-free conditions (**Figure 3.8; Figure 3.15-B-C**). The discrepancy between these findings to those of the authors mentioned above, may be attributed to the fact that the results of Grossmann *et al.* (1999) and Koehle *et al.* (2002) were based on measurements of the enzymatic level in plants subjected to drought stress, and not of plants under stress-free conditions, as with the experiments of this study.

To evaluate whether or not ethylene synthesis is inhibited by the selected fungicides in plants subject to stress conditions other than water deprivation, ACC synthase and ACC oxidase levels were examined in *M. graminicola*-infected plants during this study. Three days after inoculation, the expression of ethylene synthesis genes increased in the fungicide-treated plants in comparison to the infected, untreated specimens. Nonetheless, seven days later, genes involved in ethylene and jasmonate synthesis and response were strongly de-regulated in the fungicide-treated plants (**Figure 3.13**; **Figure 3.16-B-C**). Jasmonate and ethylene are known to act synergistically in the activation of disease resistance to necrotrophic pathogens (Bari & Jones, 2009; Lorenzo *et al.*, 2003; 2004; Memelink, 2009). Due to its long latent period of fungal colonization followed by a sudden appearance of necrotic leaf lesions, *M. graminicola* was initially considered to be a hemibiotrophic fungus. Genetic studies of its infection process have revealed new insights into *M. graminicola´s* pathogenicity, however, leading to the

suggestion that the pathogen should not be classified as a hemibiotroph, but as a necrotroph with a long period of symptomless association (Keon *et al.*, 2007; R. Marshall *et al.*, 2011). Ethylene, though, not only has a role in mediating defense responses towards necrotrophic microorganisms, the phytohormone is also involved in the induction of leaf abscission, fruit ripening and senescence in plants (Barry *et al.*, 2000; Y.-F. Chen *et al.*, 2005; Grossmann *et al.*, 1999). The fact that, at 10 dpi, the fungicide-treated plants displayed lower levels of the ethylene synthesis genes ACC synthase and ACC oxidase than the untreated-infected controls, may indicate a positive influence of the fungicides in reducing biotic stress and delaying leaf senescence. Though all fungicides inhibited the gene transcripts involved in ethylene biosynthesis, the reduction levels measured in Pyraclostrobin-treated leaves were higher than in those treated with the other two products (**Figure 3.13**; **Figure 3.16-B-C**). The negative effect that strobilurin fungicides like Pyraclostrobin and Kresoxim-methyl have on ethylene synthesis under stress conditions have been previously reported (Grossmann *et al.*, 1999; Koehle *et al.*, 2002). It has also been suggested that the so called "greening-effect" observed after strobilurin application is caused by the inhibition of ethylene synthesis (Grossmann *et al.*, 1999; Koehle *et al.*, 2002). The transient induction of ACC synthase and ACC oxidase, as observed upon fungicide treatment vs. untreated-infected plants is inconsistent with the hypothesis mentioned above and requires further study. Measurement of the ethylene levels in infected plants pre-treated with these fungicides may help to determine their effect on ethylene at the transcriptional and post-translational levels in *M. graminicola*-infected wheat plants.

Auxin metabolism was found to be induced at 3 dpi in fungicide-treated plants under disease-free conditions (**Figure 3.9-II**) as well as in *M. graminicola*-infected wheat seedlings by more than 3-fold (**Figure 3.14-III**). Grossmann and Retzlaff (1997) reported that the strobilurin fungicide Kresoxim-methyl displays auxin-like characteristics in plants; demonstrated by the triggering of shoot and root formation in tobacco stem explants and germinating wheat seeds treated with Kresoxim-methyl (Grossmann *et al.*, 1999). Koehle *et al.* (2002) also measured elevated indole-3-acetic acid levels in Pyraclostrobin-treated wheat exposed to drought stress, suggesting that the observed induced hormonal change may explain the greening effect exhibited by plants treated with this fungicide.

The plant growth regulation effect displayed by plants after the application of triazole fungicides has been attributed to the inhibition of the Cytochrome P450-depenent enzymes in the gibberellin synthesis pathway (Benton & Cobb, 1995). Even though noted evidence of the inhibitory effect of Epoxiconazole on the transcription level of the enzyme *ent*-kaurene oxidase was not observed, the expression of gibberellin 20 oxidase (GA20-ox) was repressed by two fold in Epoxiconazole-treated wheat seedlings under disease-free conditions. GA20-ox was, however, suppressed to an even greater extent in Pyraclostrobin- and Fluxapyroxad-treated plants in comparison to the untreated mock-inoculated control (**Figure 3.15-I**). Nonetheless, no evidence of plant growth inhibition was observed in the wheat seedlings after the application of any of the evaluated fungicides. Gibberellin 20 oxidase (GA20-ox) belongs to the family of the 2-oxoglutarate-dependent dioxygenases (2ODDs) and, together with gibberellin 3 oxidase, catalyzes the synthesis of bioactive gibberellins (Yamaguchi, 2008). Besides a study from Grossmann and Retzlaff (1997), which accounts of a slight induction in the bioactive gibberellin GA_1 levels in wheat after Kresoxim-methyl application, no further evidence concerning the hormonal effects of cellular respiration inhibitors has been found. For this reason, further studies would need to be performed in order to understand the impact the fungicides examined in this study have on gibberellin at the transcriptional level.

Interestingly, an effect of the Epoxiconazole treatment on plant brassinosteroids was not observed even though other triazoles, such as the pharmaceutical fungicides Fluconazole and Voriconazole, are known for their inhibitory action of the brassinosteroid synthesis in monocotyledonous and dicotyledonous plants. Plants treated with these compounds revealed phenotype-like characteristics of BR deficiency, including among others reduced growth (Rozhon *et al.*, 2013).

4.3.3 Effect of fungicides on induced resistance in plants under disease-free conditions or infected with *M. graminicola*

Besides the physiological effects that fungicides have on treated plants, it has been suggested that they may also enhance plant defense responses against pathogens. Studies focusing on the induced defense responses in plants after fungicide application have noted the induction of cell wall appositions and the production of lignin and PR proteins like chitinases and β-1,3-glucanases after the following events: i) Epoxiconazole treatment of wheat seedlings roots and sunflower cell suspension cultures under

disease-free conditions (Siefert *et al.*, 1996); ii) the foliar application of Tebuconazole to wheat leaves infected with the stripe rust fungus *P. striiformis* f. sp. t*ritici* (Han *et al.*, 2006). Strobilurin fungicides including Pyraclostrobin also have an effect on plant defense responses. Pre-treatment of the Xhanti nc tobacco cultivar with Pyraclostrobin revealed an enhanced more rapid activation of the pathogenesis-related protein PR1, conferring increased resistance against the tobacco mosaic virus (TMV) and pathogenic bacteria *Pseudomonas syringae* pv *tabaci* (Herms *et al.*, 2002). Moreover, the activation of PR1 was only observed when tobacco leaves were challenged with TMV and not on intact uninfected plant tissue. Therefore, Koehle *et al.* (2002) and Herms *et al.* (2002) suggest that Pyraclostrobin primes tobacco plants for a faster, more resilient defense response against viral and bacterial pathogens.

According to the microarray analysis and validation experiments via qPCR, the Fluxapyroxad and Pyraclostrobin fungicides did not induce the expression of pathogenesis-related proteins under disease-free conditions (**Figure 3.8; Figure 3.15-A-D**). Nonetheless, when the plants were infected with pycnidiospores from *M. graminicola* after first being sprayed with Fluxapyroxad and Epoxiconazole, a slight induction of genes involved in defense responses against fungi was observed at 3 dpi, albeit to a lesser extent in the Epoxiconazole-treated wheat seedlings. Furthermore, when evaluated seven days later, the plant response expression profile had been repressed by more than two fold (**Figure 3.16-A-D**). Of the various treatment examined, the curative application of Fluxapyroxad resulted in a significantly higher increase in the defense response of the wheat seedling to *M. graminicola*. The results obtained here suggest that, in addition to displaying outstanding fungicidal efficacy, Fluxapyroxad transiently induces plant immunity responses to hinder pathogen growth. Furthermore, according to the cluster and gene enrichment analysis at 3 dpi, photosynthesis-related genes and transcripts involved in energy and metabolites production where induced in plants pre-treated with Fluxapyroxad (**Figure 3.14-I**). This observation leads to the suggestion that the induction of defense-related genes, as observed at 3 dpi does not compromise plant growth.

The enhanced more rapid activation of plant defense responses after a given biotic or abiotic stimulus is known as priming (Conrath *et al.*, 2006; Kogel & Langen, 2005). Since during priming, disease resistance is only induced after a pathogen or abiotic challenge,

trade-off between defense and growth during priming has been reported not to exist (Conrath *et al.*, 2006; Stein *et al.*, 2008; van Hulten *et al.*, 2006). The priming state has been observed in plants pre-treated with low dosages of salicylic acid or any of its synthetic analogs (Katz *et al.*, 1998; 2002; Thulke & Conrath, 1998), as well as after the application of the non-protein amino acid β-amino-butyric acid (BABA) in *Arabidopsis* (Ton & Mauch-Mani, 2004). Additionally, non-pathogenic microorganisms and mutualistic fungi may also induce resistance to fungal or insect attack. Such a response can be seen for example, when the colonization of *Arabidopsis* roots by the rhizobacteria *Pseudomonas fluorescens* strain WCS417r activates the expression of the MYC branch of the Jasmonate-responsive pathway, leading to an induced systemic resistance (ISR) against insect attack (Pozo *et al.*, 2008). In another instance, the mutualistic plant root-colonizing fungus *Piriformospora indica* provides cereals with enhanced resistance against fungal pathogens in their roots and leaves by means of the jasmonate-mediated pathway (Kogel & Langen, 2005; Stein *et al.*, 2008; Waller *et al.*, 2005). Moreover, *P. indica* primes plants for abiotic stress conditions, such as salt stress, enhances the anti-oxidative capacity of cereals, and stimulates the plant growth and net yield of barley (Waller *et al.*, 2005). To the author´s knowledge, there has not been any publication so far that deals with the priming effects on plants treated with this or any other pyrazole carboxamide fungicide. Nonetheless, based on the results obtained during this study, it cannot be concluded that Fluxapyroxad primes wheat seedlings for subsequent fungal attacks. To properly test this hypothesis, further experiments on induced defense response and its effect on plant growth after Fluxapyroxad treatment still need to be conducted.

Interestingly, the preventative application of Pyraclostrobin resulted in the lowest amount of differentially expressed genes at 3 dpi (**Figure 3.13**). It has been reported that strobilurin fungicides not only inhibit the cytochrome b complex of the electron transport chain of fungi (Bartlett *et al.*, 2002), they also partially inhibit the mitochondrial respiration process in treated plants (Gerhard *et al.*, 1998; Grossmann & Retzlaff, 1997; Grossmann *et al.*, 1999; Koehle *et al.*, 2002). According to Herms *et al.* (2002) and Koehle *et al.* (2002), the inhibition of the electron transport chain in plants by Pyraclostrobin leads to the activation of the alternative oxidase pathway, which, at the same time, causes a re-programing of cellular functions such as elevated cytosolic H^+

levels, improved nitrate assimilation and Nitrogen oxide (NO) production, promotes pathogen recognition and changes the phytohormonal balance of the plants. Nonetheless, it must be noted that these responses were observed under stress conditions generated by water deprivation or pathogen challenge.

As shown in diagram **4.1.1**, the infection of wheat seedlings with pycnidiospores of the pathogenic fungus *M. graminicola* is considered by the plant as a stress situation that leads it to focus its resources on defense instead of growth. For this reason, it was expected that the responses obtained when Pyraclostrobin is sprayed preventatively to be similar to that of when sprayed on plants under stress conditions, such as the inhibition of ethylene synthesis and the rapid activation of defense-related genes that prime plants to subsequent pathogen attack (Beckers & Conrath, 2007; Herms *et al.*, 2002; Koehle *et al.*, 2002). During this study, though it could not be proved that Pyraclostrobin primes wheat seedlings to *M. graminicola* attack. Induced tolerance levels against viral (Koehle *et al.*, 2006) and bacterial (Koehle *et al.*, 2009) diseases have been observed, on the other hand, in a wide variety of plants after Strobilurin treatment, but not, however, against fungal organisms. These pathogens have lifestyles and colonization strategies that differ starkly from *M. graminicola*; induced disease resistance to this fungus may therefore, rely on other mechanisms. As a consequence, though, the fact that no priming effect was observed in wheat seedlings treated with Pyraclostrobin and subsequently challenged by *M. graminicola* during this study does not imply that reporting this specific active ingredient as a defense inducer on other plant-pathogen interactions is not valid.

4.3.3.1 Preventative application of fungicides down-regulates genes involved in biotic stress responses

Plant defense activation is a costly process for plants that requires cell reprogramming in order to balance resources. By analyzing several publically available microarray experiments, Bilgin *et al.* (2010) were able to confirm that, upon pathogen or insect attack, plants are able to relocate their resources to defense activities by down-regulating the transcription of genes involved in photosynthesis reactions, while, at the same time up-regulating defense- and detoxification-related genes. The switch from growth to disease resistance manifests itself as a loss of fitness that, consequently leads to lower yields (Bilgin *et al.*, 2010; Heil & Baldwin, 2002; Walters & Heil, 2007).

One of the objectives of this project was to assess, at the molecular level, the responses of susceptible wheat seedlings to *M. graminicola* and how they change when a fungicide is applied either before, or after, the infection has taken place. Furthermore, an attempt to correlate the effects that fungicides have on the wheat transcriptome under disease pressure with yield responses in adult plants grown under controlled, semi-control and natural conditions was pursued.

Through the molecular biology experiments performed during this study, it could be proven that the preventative application of fungicides hinders the development of fungal diseases due to either the prevention of spore germination and plant penetration, or the inhibition of fungal growth shortly after host colonization. Furthermore, at 10 dpi, the time point at which *M. graminicola* starts massive host invasion, wheat transcripts involved in biotic stress responses were highly repressed in the plants pre-treated with any of the evaluated fungicides (**Figure 3.14-V**). The activation of plant defense responses was also repressed after the curative applications, most likely due to the outstanding curative actions of the fungicides. Nonetheless, the magnitude of the repression was not as strong as that of the preventative application. Furthermore when considering differences between the three active ingredients, Pyraclostrobin-treated plants revealed stronger down-regulation of genes involved in biotic stress responses such as signaling regulation, MAP-kinase signaling cascade, WRKY transcription factors and the production of secondary metabolites (**Figure 3.13 10dpi-Pr.**). The fact that treatment with the strobilurin fungicide repressed more defense-related genes than the triazole product can be attributed to the lower preventative efficacy of Epoxiconazole. Nonetheless, according to the fungal biomass quantification by qPCR as well as disease symptom development, the two cellular respiration inhibitors provided comparable fungal control levels when sprayed as a preventative treatment (**Figure 3.5**). Differences in the regulation of defense related genes should, therefore, not be observed in Pyraclostrobin- and Fluxapyroxad-treated wheat seedlings. These results suggest that, in addition to the anti-mycotic effect of Pyraclostrobin, the fungicide may assist plants in coping with stress conditions. During a two-year field trial carried out to evaluate the effects that strobilurin and triazole fungicides have on plant vitality and yield under disease-free conditions, Gerhard *et al.* (1998) observed that strobilurin-treated plants were able to respond better to the dry season, which transpired in 1997, and, in

addition, provided higher biomass levels than Epoxiconazole-treated plants and untreated controls. Based on these results, the authors suggested that strobilurin fungicides such as Azoxystrobin and Kresoxim-methyl support plants in compensated for unfavorable conditions.

Xylanases produced by fungi are able to degrade xylan, the main polymer found in the cell wall of cereals, and are, therefore considered to play a major role in plant pathogen interactions (Beliën *et al.*, 2006) including, among others, *M. graminicola* in wheat (Siah *et al.*, 2010). At the onset of disease symptom development, Siah *et al.* (2010) reported an increase in xylanase activity only during compatible interactions. According to these authors, the ability of *M. graminicola* to induce cell wall degrading enzymes during programmed cell death can determine the outcome of the disease. The gene cluster enrichment analysis carried out during this project revealed that, at 10 dpi, gene transcripts involved in xylan biosynthesis and the cell wall polysaccharide biosynthetic process were strongly induced in *M. graminicola*-infected seedlings (**Figure 3.14-IV**); confirming the results reported by Siah *et al.* (2010). This response is likely related to an activation of fungal xylanase activity with the goal of disrupting the plant cell wall and releasing plant nutrients into the plant apoplast. As expected, a down-regulation of xylan and cell wall polysaccharide biosynthetic genes was observed in the fungicide-treated plants (**Figure 3.14-IV**). Furthermore, inhibited fungal growth caused by pre-treatment with the fungicides led to a stronger down-regulation of the genes belonging to this cluster. Even though the curative application of fungicides is still highly effective in hampering fungal growth, no significant change in comparison to the infected untreated control in the expression of genes involved in cell wall synthesis, defense responses to fungi or oxidative stress response was observed (**Figure 3.14-IV-VI**). Based on these results, it can be concluded that, a fungicide sprayed before an infection has taken place, not only leads to significantly improved disease control; costly stress responses which reduce yields later on can also be avoided.

In conclusion, in the current study it could be proven that fungicides are not only successful in hindering fungal infection regardless of their mode of action, they also have an effect on the plant transcriptome; under disease-free conditions as well as under disease pressure. The expression of defense-related genes, changes in the phytohormonal balance of plants, gene transcripts involved in cell wall synthesis, post-

translational modifications and photosynthesis-related reactions were all responses found to be significantly altered by fungicide treatment. All transcriptional changes may have positive impact on plant physiology by assisting the plant in coping better with biotic or abiotic stress conditions. Although all products were able to hinder disease development, clear differences in their mode of action and application timing were observed. For this reason, yield trials were carried out under semi-natural and completely natural conditions in order to evaluate whether the observed effects on wheat seedlings in the glasshouse could be transferred to the field in terms of yield. Based on the results obtained previously, it was hypothesized that higher yield should be obtained in fungicide-treated plants than in plants in which no agrochemical treatment takes place. Furthermore, by avoiding the activation of defense responses, by applying fungicides, no trade-offs between growth and defense will take place. Higher yields should also, therefore, be obtained when a fungicide is sprayed preventatively in comparison to when the application first takes place after an infection has already begun.

4.4 Fungicide effects on yield

Environmental conditions play a very important role in *M. graminicola* development and growth; the fungus needs at least 10mm of rainfall and 98% leaf wetness, within a three-day period, to be able release spores and cause infection (Eyal, 1987; Verreet *et al.*, 2000). For this reason, the severity of the disease can vary between years and locations, with rainfall events in May and June considered crucial for the development of summer epidemics (Beyer *et al.*, 2012; Hardwick *et al.*, 2001; Wiik & Ewaldz, 2009). In 2011 these conditions were rarely met (**Figure 3.17-A; Figure 3.18-A-C**) resulting in slower fungal growth, which in turn, notably influenced fungicide application timing effectiveness (**Table 3.3; Table 3.5**). The fungicide application that, according to the septoria model developed by Verreet *et al.* (2000), occurred at the curative time point, really had a preventative nature due to its proximity to the beginning of the infection. Moreover, the application which should have been the preventative treatment did not have a significant effect on controlling the disease since it was conducted too far in advance of the actual infection and the active ingredient may have already been metabolized by the plant. Nonetheless, in 2012, the environmental conditions were optimal for septoria leaf blotch infection (**Figure 3.17-A; Figure 3.18-A-C**), with an

average disease severity of 73.6% and 76% achieved in the untreated controls at the ripening stage in the micro-plot and field trials respectively. However, due to the high variation of the data, no significant differences in controlling *M. graminicola* infection between the application timings in the micro-plot experiments were obtained (**Figure 4.1-A**). Nonetheless, a clear difference between the Pyraclostrobin and Epoxiconazole fungicide applications was measured, with a tendency for higher efficacy levels after the preventative treatment of Pyraclostrobin; proving its outstanding fungicide action when susceptible isolates are present under natural conditions. The new generation of carboxamide fungicides, Fluxapyroxad provided the same level of effectiveness as Pyraclostrobin. An effect that could not, however, be transferred to the field trials due to the mixed fungal infections that occurred later on in the growing season (**Figure 4.1-B**).

The fact that *M. graminicola* has a very long latent period in its infection process, during which no visual disease symptoms are detectable complicates disease control strategies. For this reason, appropriate timing of fungicide application is of great importance. If chemical treatment first takes place after the appearance of disease symptoms, the fungus will have already established itself in the plant and started to cause enough damage to account for yield losses (Beyer *et al.*, 2012; Lucas *et al.*, 1999). If fungicide application is conducted early enough before infection, however, fungal infection can be prevented with no significant damages brought to the plant, maintaining crop yields. Verreet *et al.* (2000) reached similar conclusions, where, according to their study, if a fungicide is sprayed during the epidemiologically sensitive phase of the disease, US $ 200/ha net economical increase can be obtained. Additionally, Mueller *et al.* (2009) demonstrated that early fungicide application against the soybean pathogen *Phakopsora pachyrhizi,* the causal agent of Asian soybean rust, provided both the greatest disease control and the highest yield in comparison to when an agrochemical was applied after disease symptoms detection.

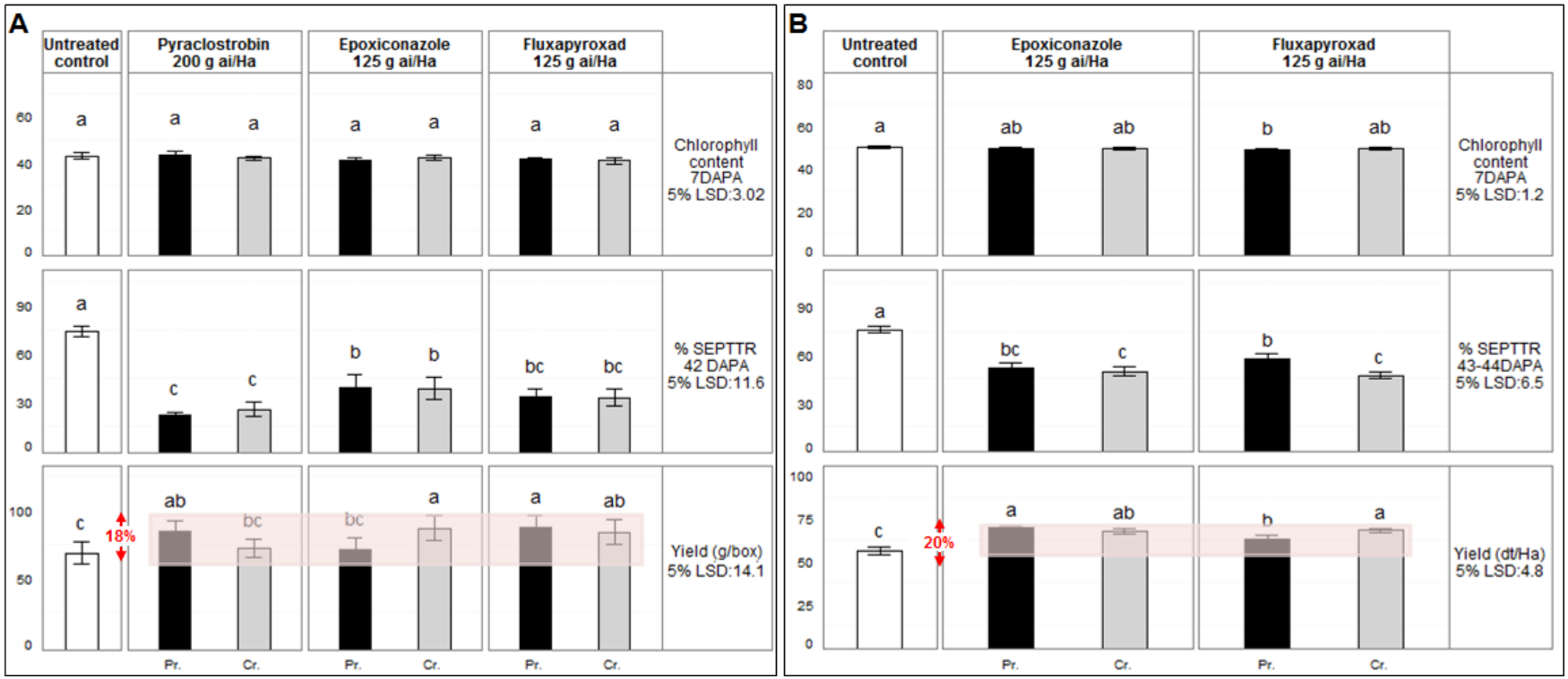


Figure 4.1: Summary of yield trial results under semi-natural and natural conditions in 2012

Wheat plants grown either under semi-natural conditions in micro-plots and covered with a netted tunnel, or in field trials carried out under natural conditions were sprayed with fungicides either as a preventative treatment once the flag leaf was fully developed, or as a curative treatment, approximately two weeks later. Three different products were evaluated in the micro-plot trials, namely Pyraclostrobin, Fluxapyroxad and Epoxiconazole. In the field trials, Pyraclostrobin was not utilized due to resistance development in *M. graminicola* wild isolates. Displayed are the measured variables in the micro-plot (**A**) and field (**B**) trials. Columns represent mean values of the two independent experiments with a total of nine repetitions in the micro-plot, and 12 replicas in the field trials. Bars display the standard error. Means with the same letter are not significantly different (Least significant difference at P=0.05). The light red area represents the average yield increase obtained in fungicide-treated plants as compared to the corresponding untreated control. Abbr.: g ai/ha, grams active ingredient per hectare; DAPA, days after the preventative application; SEPTTR, EPPO code system for the pathogen *M. graminicola* (*Zymoseptoria tritici*); LSD, least significant difference; dt/ha, decitons per hectare; Pr. Preventative treatment; Cr. Curative treatment.

No difference in yield between fungicide-treated and untreated plants could be observed in 2011 micro-plot trials (**Table 3.3**); most likely due to feeding damage caused by mice or slower disease development due to unfavorable weather conditions. Swoboda and Pedersen (2009) also reported that fungicide application to soybean fields under low disease severity did not result in significant higher yields than in untreated plants. Due to the favorable weather conditions present in the spring of 2012, on the other hand, the results obtained during this period confirmed the hypothesis that higher yields can be obtained by applying a fungicide treatment to protect plants from fungal diseases, regardless of treatment timing (**Figure 4.1**). Other studies have demonstrated the profitability of fungicide application and its dependency on i) environmental conditions for disease development, ii) the fungal disease present and its severity, iii) the efficacy of the product(s), as well as iv) the susceptibility of the cultivars (Paul *et al.*, 2011; Ruske *et al.*, 2003; Wegulo *et al.*, 2011).

In the micro-plot trials in 2012, an average yield increase of 18% was obtained in fungicide-treated plants, whereas in the field trials the yield increase was 20% (**Figure 4.1**). Wegulo *et al.* (2009) reported that fungicide application prevented yield losses ranging from 27 to 42% by controlling wheat foliar diseases such as tan spot, spot blotch, septoria leaf blotch, leaf rust and powdery mildew. Additionally, the authors also reported that disease epidemics during flowering are more related to yield losses than epidemics during ripening. In a 5 year yield trial study on the effect of fungicide application on controlling septoria leaf blotch and yield levels in Ireland, Burke and Dunne (2008) detected higher yields in fungicide-treated plants in every season. The magnitude of the increase was, however, related to disease severity; under strong epidemics, 40% yield increase was measured in fungicide-treated plants, while 16% yield lost was detected in untreated plants under low disease conditions. Meta-analysis reports on the effect of fungicide application on the yield of hybrid corn in the United States under low and strong disease pressure have also found a correlation between yield increase after fungicide application and the level of disease severity (Paul *et al.*, 2011).

During this study, 20% yield increase under strong septoria leaf blotch epidemics was measured, which may not seem very significant when compared to the previously reported results. Nonetheless, it is worth mentioning that these trials were carried out

with only one fungicidal application taking place either at GS 39 as a preventative treatment, or as a curative treatment later on; with only one active ingredient. Under common wheat agricultural practices, two fungicide applications are usually used to control major diseases like powdery mildew and septoria leaf blotch (Hardwick *et al.*, 2001; topagrar, 2011; 2013). Furthermore, in regions with strong *fusarium* spp. risk, a third application is generally planned at GS 65. Additionally, the agrochemicals are often sprayed as mixtures of fungicides with different modes of action in order to avoid resistance development and enable the widening of the disease control spectra (topagrar, 2011; 2013). With this in mind, the fact that even only a single application of one product provided 20% yield increase during severe epidemics demonstrates the importance of fungicidal use in the controlling septoria leaf blotch and secondary fungal diseases. In an 8-year study evaluating the efficacy differences of application timings on fungal disease control in wheat using different fungicide active ingredients, Cook and Thomas (1990) reported an average yield increase of 4.3% of one application at GS 31, 9.1% after a single application of at GS 39, and a 11.6 and 16.2% yield increase after two and three applications at GS 31+39 and 31+39+59 respectively.

Even though a distinction in the efficacy of septoria leaf blotch control between Pyraclostrobin and Epoxiconazole treatment under semi-natural conditions exists, no significant difference in the yield achieved between these two products could be observed. The application of Fluxapyroxad to wheat plants provided higher yields than in untreated plants regardless of application timing. The yield increase was not, however, significantly different than in plants treated with the other two products (**Figure 4.1-A**). Blandino and Reyneri (2009) reported that the addition of a strobilurin fungicide to a triazole program did not delay leaf senescence or increase yield when compared to a triazole only program. In a study of the physiological effects of fungicides on wheat under disease-free conditions, no differences in the yield obtained after Fluxastrobin (QoI) and Prothioconazole (DMI) treatment were found (Berdugo *et al.*, 2012). Similar results were obtained by Bertelsen *et al.* (2001) using Azoxystrobin and Epoxiconazole. These results though, are not in concordance to reports that relate strobilurin (Beck, 2005; Gerhard *et al.*, 1998; Gooding *et al.*, 2000) and carboxamide (Berdugo *et al.*, 2011; 2012) treatments to longer green leaf area, leading in turn, to yield increase, and improved photosynthesis and carbohydrate assimilation activity.

Regarding the effect of fungicide application timing on yield, based on the high variation of the data, no significant differences in Pyraclostrobin- and Fluxapyroxad-treated plants could be observed. A tendency to higher yield levels in the plants sprayed preventatively, however, could still be perceived (**Figure 4.1-A**). The fact that Epoxiconazole possess better curative performance, as often ascertained, could not be confirmed in this study due to the high variability of the data. The preventative and curative application of this active ingredient did not provide any significant distinction in terms of septoria leaf blotch control. Nonetheless, the curative application of this active ingredient resulted in significantly higher yield levels than in the preventative application. This result could not be transferred to the field trials though, where no significant difference in the yield levels between the two application timings was noted; perhaps due to other factors besides the *M. graminicola* infection.

The results of the genome-wide gene expression analysis carried out on wheat seedlings under controlled conditions suggest that the preventative application of fungicides avoids the activation of costly plant defense responses. Based on the fact that the trade-off between defense activation and growth leads to fitness penalty (Bilgin *et al.*, 2010; Heil & Baldwin, 2002; 2007), one of the aims of this study was to prove that higher yields should be obtained when a chemical application is carried out early enough in the growing season. Nonetheless, the hypothesis of whether a routine-based application of a fungicide at GS 39, aimed at protecting the flag leaf, provides higher yield than an application based purely on weather monitoring could not be demonstrated due to the high variability of the data. In a study carried out in four different locations in Nebraska, USA on the effect of fungicide application timing on tan spot/spot blotch disease severity and yield, due to the high variability of the results, Wegulo *et al.* (2009) could not reach a conclusion on the most appropriate timing for fungicide application. Cook and Thomas (1990), on the other hand, suggest that an application with timing centering on protecting the flag leaf (GS39), is the most important treatment in a routine fungicide spraying program; with a 50% yield benefit over that of a three-spray program.

Elevated yields and thousand kernel weight after strobilurin and carboxamide fungicide application have been reported to correlate to delayed leaf senescence (Beck, 2005; Berdugo *et al.*, 2011; Berdugo *et al.*, 2012; Gerhard *et al.*, 1998). During the two years of micro-plot and field experiments, no effect on yield and leaf senescence related to higher

chlorophyll levels was observed; neither after Pyraclostrobin application nor after Epoxiconazole or Fluxapyroxad treatment (**Table 3.3; Table 3.5**). Furthermore, no significant fungicide effects on leaf area during the two year experiments in the micro-plot trials and the 2011 field trials were noted. In an attempt to clarify the effect strobilurins have on flag leaf area, Gerhard *et al.* (1998) measured the effect of this fungicide on flag leaf area of different wheat varieties grown in the field over a three-year period. In two out of the three years, they observed positive results in one of the varieties, which led to the conclusion that higher flag leaf areas could be obtained through fungicide treatment, with the level of effect depending on the wheat variety used and the environmental conditions during the growing season. According to the results attained, one can conclude that the yield increase observed after fungicide application in trials under natural and semi-natural conditions was not related to physiological effects but rather, as suggested by Bertelsen *et al.* (2001), due to the control of fungal diseases. Nonetheless, it is worth mentioning that the chlorophyll assessments were carried out with an SPAD meter, which measures only one section of the plant and thus, is highly dependent on the point where measurement takes place. Infrared (IR) thermography is used to evaluate changes in plant physiology related to fungicide application in glasshouses (Berdugo *et al.*, 2012). This technique, which measures the entire plant, has proven to be a reliable and sensitive method that is not influenced by an evaluator´s experience. Therefore, more modern techniques with the capability to evaluate plant physiology in plants grown in the field may provide further insights into the additional effects that fungicides have on plants in natural conditions.

5. Summary / Zusammenfassung.

5.1 Summary

Septoria leaf blotch is a devastating wheat fungal disease caused by the fungus *Mycosphaerella graminicola* that, under severe epidemics, can cause up to 60% yield loss. Even though resistant varieties do exist, the possibility that *M. graminicola* will overcome resistant varieties is very high due to its genetic diversity and rapid evolution upon selection pressure. *M. graminicola* control is therefore, heavily dependent on fungicide treatment. Modern agrochemicals compounds with varying modes of action are successful in controlling a wide variety of fungal diseases that threaten crop yield potential. Nonetheless, in order to provide the most appropriate protection and, hence, secure crop productivity, appropriate application timing is of great importance. For this reason, the present study aimed at evaluating the effects of application timing and mode of action of the Pyraclostrobin, Epoxiconazole and Fluxapyroxad fungicides on controlling septoria leaf blotch and the corresponding development of wheat yield.

Three different experimental setups were tested, ranging from controlled conditions in a glasshouse, semi-control conditions in micro-plots to natural growth environments in the field. Firstly, by employing molecular biology techniques such as quantitative real time PCR (qPCR) and microarray DNA chips, it could be demonstrated that preventative fungicide application not only provides the most appropriate disease control, it also avoids the activation of costly defense responses that would have a negative impact on wheat yield. Furthermore, changes in plant hormone metabolism, which may have additional impacts on plant growth and development, after the application of Pyraclostrobin, Epoxiconazole and Fluxapyroxad under disease pressure and disease-free conditions, were observed. Additionally, a transient induction of photosynthesis- and energy-related genes after the preventative application of the cellular respiration inhibitors Pyraclostrobin and Fluxapyroxad was also observed.

Based on these results, yield trials under semi-controlled and natural conditions were carried out in order to evaluate if higher yields would be obtained when a product is sprayed preventatively, thereby avoiding the activation of plant defense responses, than when applied after the infection has already occurred. Even though no significant differences between the application timings could be identified due to the high variability of the data, a tendency for higher yields were detected in the micro-plot trials

under semi-natural conditions after the preventative application of Pyraclostrobin and Fluxapyroxad. These results could not be transferred to the field, however, most likely due to the fact that in nature deciding the point of time at which a preventative application is needed is more complicated as *M. graminicola* infection occurs throughout the growing season. Nonetheless, it could be demonstrated that an approximately 20% yield increase can be obtained by performing a single fungicide application to treat the septoria leaf blotch disease in wheat.

5.2 Zusammenfassung

Septoria-Blattdürre gehört zu einer der bedeutendsten Pilzkrankheiten im Weizenbau Nord-West-Europas. Die Krankheit wird durch den Pilz *Mycosphaerella graminicola* verursacht. Bei für den Pilz günstigen Umweltbedingungen können Ertragsverluste von bis zu 60% entstehen. Obwohl Weizensorten mit Resistenzen gegen Septoria-Blattdürre im Markt weit verbreitet sind, ist chemischer Pflanzenschutz zur Kontrolle der Krankheit unverzichtbar. Moderne Fungizide unterschiedlicher Wirkmechanismen können die Pilzkrankheit sehr erfolgreich bekämpfen und Erträge sichern. Zielsetzung dieser Arbeit war es zu untersuchen, welchen Einfluss der Zeitpunkt der Applikation (protektiv oder kurativ) sowie die unterschiedlichen Wirkmechanismen der drei fungiziden Wirkstoffe Pyraclostrobin, Epoxiconazole und Fluxapyroxad auf die Entwicklung von Septoria-Blattdürre und letzten Endes den Ertrag im Weizen haben.

Die Fragestellung wurde anhand drei verschiedener Versuchseinstellungen, die von kontrollierten- über semi-kontrollierte- bis hin zu Freilandbedingungen reichten. Unter kontrollierten Versuchsbedingungen im voll klimatisierten Gewächshaus wurden Proben genommen und mit Hilfe von quantitativer real-time PCR (qPCR) und Microarray Technologien untersucht. Anhand dieser Versuchseinstellungen und Messmethoden konnte gezeigt werden, dass die protektive Applikation eines die Sporenkeimung hemmenden Fungizids die Pflanze nicht nur effektiv vor einer Infektion mit *M. graminicola* schützt, sondern auch die Aktivierung der pflanzlichen Abwehr verhindert. Desweiteren konnten Veränderungen im Phytohormonhaushalt nach Applikation der Fungizide sowohl nach Infektion des Erregers als auch unter befallsfreien Bedingungen festgestellt werden. Bei Pflanzen, die präventiv mit Pyraclostrobin und Fluxapyroxad behandelt wurden, konnte darüber hinaus eine vorübergehende Hochregulation von Genen der Photosynthese und des

Energiehaushaltes gezeigt werden. Mögliche positive und negative Effekte der gefunden Veränderungen der phytohormon-, phytosynthese- sowie energiebezogenen Gene werden im Kontext des aktuellen Stand des Wissens diskutiert.

In Versuchen semi-kontrollierten sowie unter Freilandbedingungen sollte geprüft werden, ob sich die unter kontrollierten Bedingungen gefunden Effekte im Ertrag widerspiegeln. Aufgrund der hohen biologischen Varianz gelang es nicht, statistisch signifikante Unterschiede nachzuweisen. Lediglich bis zu 20 % höhere Erträge gegenüber den unbehandelten Kontrollen konnten bei mit *M. graminicola* infiziertem Weizen statistisch abgesichert werden.

6. References

Adhikari, T. B., Anderson, J. M., & Goodwin, S. B. (2003). Identification and Molecular Mapping of a Gene in Wheat Conferring Resistance to Mycosphaerella Graminicola. *Phytopathology, 93*(9), 1158-1164. doi: 10.1094/phyto.2003.93.9.1158

Adhikari, T. B., Yang, X., Cavaletto, J. R., Hu, X., Buechley, G., Ohm, H. W., . . . Goodwin, S. B. (2004a). Molecular Mapping of *Stb1* , a Potentially Durable Gene for Resistance to Septoria Tritici Blotch in Wheat. *TAG Theoretical and Applied Genetics, 109*(5), 944-953. doi: 10.1007/s00122-004-1709-6

Adhikari, T. B., Cavaletto, J. R., Dubcovsky, J., Gieco, J. O., Schlatter, A. R., & Goodwin, S. B. (2004b). Molecular Mapping of the Stb4 Gene for Resistance to Septoria Tritici Blotch in Wheat. *Phytopathology, 94*(11), 1198-1206. doi: 10.1094/phyto.2004.94.11.1198

Adhikari, T. B., Balaji, B., Breeden, J., & Goodwin, S. B. (2007). Resistance of Wheat to Mycosphaerella Graminicola Involves Early and Late Peaks of Gene Expression. *Physiological and Molecular Plant Pathology, 71*(1-3), 55-68. doi: 10.1016/j.pmpp.2007.10.004

Agrarmeteorologie-Rheinland-Pfalz. (2012). Wetterstation Schifferstadt - Vorhersage, from http://www.dlr-rheinpfalz.rlp.de/Internet/AM/NotesAM.nsf/meteoweb/aea26ec6eb6672cbc1257171002e8a2f?OpenDocument&TableRow=2.3#2.

Agrios, G.N. (2005). *Plant Pathology* (Fifth ed.): Elsevier Academic Press.

Albrecht, Catherine, Boutrot, Freddy, Segonzac, Cécile, Schwessinger, Benjamin, Gimenez-Ibanez, Selena, Chinchilla, Delphine, . . . Zipfel, Cyril. (2012). Brassinosteroids Inhibit Pathogen-Associated Molecular Pattern–Triggered Immune Signaling Independent of the Receptor Kinase Bak1. *Proceedings of the National Academy of Sciences, 109*(1), 303-308. doi: 10.1073/pnas.1109921108

Apel, K., & Hirt, H. (2004). Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction. *Annual review of plant biology, 55*, 373-399. doi: 10.1146/annurev.arplant.55.031903.141701

Arraiano, L. S., Worland, A. J., Ellerbrook, C., & Brown, J. K. M. (2001). Chromosomal Location of a Gene for Resistance to Septoria Tritici Blotch (Mycosphaerella Graminicola) in the Hexaploid Wheat 'Synthetic 6x'. *Theoretical and Applied Genetics, 103*(5), 758-764. doi: 10.1007/s001220100668

Arraiano, L. S., Chartrain, L., Bossolini, E., Slatter, H. N., Keller, B., & Brown, J. K. M. (2007). A Gene in European Wheat Cultivars for Resistance to an African Isolate of Mycosphaerella Graminicola. *Plant Pathology, 56*(1), 73-78.

Arraiano, L. S., Balaam, N., Fenwick, P. M., Chapman, C., Feuerhelm, D., Howell, P., . . . Brown, J. K. M. (2009). Contributions of Disease Resistance and Escape to the Control of Septoria Tritici Blotch of Wheat. *Plant Pathology, 58*(5), 910-922. doi: 10.1111/j.1365-3059.2009.02118.x

Avenot, H., Sellam, A., & Michailides, T. (2009). Characterization of Mutations in the Membrane-Anchored Subunits Aasdhc and Aasdhd of Succinate Dehydrogenase from Alternaria Alternata Isolates Conferring Field Resistance to the Fungicide Boscalid. *Plant Pathology, 58*(6), 1134-1143.

Balmer, Dirk, Planchamp, Chantal, & Mauch-Mani, Brigitte. (2013). On the Move: Induced Resistance in Monocots. *J Exp Bot, 64*(5), 1249-1261. doi: 10.1093/jxb/ers248

Bari, R., & Jones, J. D. (2009). Role of Plant Hormones in Plant Defence Responses. *Plant Mol Biol, 69*(4), 473-488. doi: 10.1007/s11103-008-9435-0

Barry, C. S., Llop-Tous, M. I., & Grierson, D. (2000). The Regulation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase Gene Expression During the Transition from System-1 to System-2 Ethylene Synthesis in Tomato. *Plant Physiol, 123*(3), 979-986. doi: 10.1104/pp.123.3.979

Bartlett, Dave W., Clough, Jon M., Godwin, Jeremy R., Hall, Alison A., Hamer, Mick, & Parr-Dobrzanski, Bob. (2002). The Strobilurin Fungicides. *Pest Manag Sci, 58*, 649-662. doi: 10.1002/ps.520

Beck, C. L. (2005). *Einfluss Von Fungiziden Auf Die Ertragsphysiologie Von Weizen.* Doktor der Agrarwissenschaften Doctorate thesis, Rheinischen Friedrich-Wilhelms Universität zu Bonn, Bonn.

Beckers, G. J., & Conrath, U. (2007). Priming for Stress Resistance: From the Lab to the Field. *Current Opinion in Plant Biology, 10*(4), 425-431. doi: 10.1016/j.pbi.2007.06.002

Beliën, Tim, Van Campenhout, Steven, Robben, Johan, & Volckaert, Guido. (2006). Microbial Endoxylanases: Effective Weapons to Breach the Plant Cell-Wall Barrier or, Rather, Triggers of Plant Defense Systems? *Molecular Plant-Microbe Interactions, 19*(10), 1072-1081. doi: 10.1094/mpmi-19-1072

Benton, J. M., & Cobb, A. H. (1995). The Plant Growth Regulator Activity of the Fungicide, Epoxiconazole, on *Galium Aparine* L. (Cleavers). *Plant Grwoth Regulation, 17*, 149-155.

Berdugo, C. A., Steiner, U., Oerke, E. C., & Dehne, H. W. (2011). *Effects of the New Sdhi-Fungicide Bixafen on the Physiology and Yield of Wheat Plants*. Paper presented at the Modern Fungicides and Antifungal Compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

Berdugo, C. A., Steiner, U., Dehne, H. W., & Oerke, E. C. (2012). Effect of Bixafen on Senescence and Yield Formation of Wheat. *Pesticide Biochemistry and Physiology, 104*(3), 171-177.

Berrocal-Lobo, Marta, Molina, Antonio, & Solano, Roberto. (2002). Constitutive Expression of Ethylene-Response-Factor1 in Arabidopsis Confers Resistance to Several Necrotrophic Fungi. *The Plant Journal, 29*(1), 23-32.

Bertelsen, J. R., de Neergaard, E., & Smedegaard-Petersen, V. (2001). Fungicidal Effects of Azoxystrobin and Epoxiconazole on Phyllosphere Fungi, Senescence and Yield of Winter Wheat. *Plant Pathology, 50*(2), 190-205. doi: 10.1046/j.1365-3059.2001.00545.x

Beschreibende Sortenliste. (2012). Hannover, Germany: Bundessortenamt.

Beyer, Marco, El Jarroudi, Moussa, Junk, Jürgen, Pogoda, Friederike, Dubos, Tiphaine, Görgen, Klaus, & Hoffmann, Lucien. (2012). Spring Air Temperature Accounts for the Bimodal Temporal Distribution of Septoria Tritici Epidemics in the Winter Wheat Stands of Luxembourg. *Crop Protection, 42*(0), 250-255. doi: 10.1016/j.cropro.2012.07.015

Bilgin, D. D., Zavala, J. A., Zhu, J., Clough, S. J., Ort, D. R., & DeLucia, E. H. (2010). Biotic Stress Globally Downregulates Photosynthesis Genes. *Plant Cell Environ, 33*(10), 1597-1613. doi: 10.1111/j.1365-3040.2010.02167.x

Blandino, M., & Reyneri, A. (2009). Effect of Fungicide and Foliar Fertilizer Application to Winter Wheat at Anthesis on Flag Leaf Senescence, Grain Yield, Flour Bread-Making Quality and Don Contamination. *European Journal of Agronomy, 30*(4), 275-282. doi: 10.1016/j.eja.2008.12.005

Bleiholder, Hermann. (1997). *Methods for the Layout and Evaluation of Plant Protection Field Trials*. Germany.

Bockus, W.W., & Claassen, M.M. (1992). Effects of Crop Rotation and Residue Management Practices on Severity of Tan Spot of Winter Wheat. *Plant Disease, 76*(6), 633-636.

Boller, Thomas, & Felix, Georg. (2009). A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual review of plant biology, 60*(1), 379-406. doi: doi:10.1146/annurev.arplant.57.032905.105346

Bonjean, A.P., & Angus, W.J. (2001). *The World Wheat Book: A History of Wheat Breeding*: Intercept.

Brading, Penny A., Verstappen, Els C. P., Kema, Gert H. J., & Brown, James K. M. (2002). A Gene-for-Gene Relationship between Wheat and *Mycosphaerella Graminicola*, the Septoria Tritici Blotch Pathogen. *Phytopathology, 92*(4), 439-445.

Broekaert, W. F., Delaure, S. L., De Bolle, M. F. C., & Cammue, B. P. A. (2006). The Role of Ethylene in Host-Pathoven Interactions. *Annu Rev Phytopathol, 44*, 393-416. doi: 10.1146/annurev.phyto.44.070505.143440

Brown, J. K. M. (2002). Yield Penalties of Disease Resistance in Crops. *Current Opinion in Plant Biology, 5*(4), 339-344. doi: 10.1016/s1369-5266(02)00270-4

Brownlee, Michael. (2001). Biochemistry and Molecular Cell Biology of Diabetic Complications. *Nature, 414*(6865), 813-820.

Browse, J. (2009). Jasmonate Passes Muster: A Receptor and Targets for the Defense Hormone. *Annual review of plant biology, 60*, 183-205. doi: 10.1146/annurev.arplant.043008.092007

Buchanan, B.B., Gruissem, W., & Jones, R.L. (2000). *Biochemistry & Molecular Biology of Plants*: American Society of Plant Physiologists Waldorf^ eMD MD.

Burden, Raymond S., Cooke, David T., & Carter, Geoffrey A. (1989). Inhibitors of Sterol Biosynthesis and Growth in Plants and Fungi. *Phytochemistry, 28*(7), 1791-1804. doi: http://dx.doi.org/10.1016/S0031-9422(00)97862-2

Burke, J. J., & Dunne, B. (2008). Investigating the Effectiveness of the Thies Clima "Septoria Timer" to Schedule Fungicide Applications to Control Mycosphaerella Graminicola on Winter Wheat in Ireland. *Crop Protection, 27*(3-5), 710-718. doi: 10.1016/j.cropro.2007.10.007

Chartrain, L., Brading, P. A., Widdowson, J. P., & Brown, J. K. M. (2004a). Partial Resistance to Septoria Tritici Blotch (Mycosphaerella Graminicola) in Wheat Cultivars Arina and Riband. *Phytopathology, 94*(5), 497-504. doi: 10.1094/phyto.2004.94.5.497

Chartrain, L., Brading, P. A., Makepeace, J. C., & Brown, J. K. M. (2004b). Sources of Resistance to Septoria Tritici Blotch and Implications for Wheat Breeding. *Plant Pathology, 53*(4), 454-460. doi: 10.1111/j.1365-3059.2004.01052.x

Chartrain, L., Joaquim, P., Berry, S. T., Arraiano, L. S., Azanza, F., & Brown, J. K. M. (2005a). Genetics of Resistance to Septoria Tritici Blotch in the Portuguese Wheat Breeding Line Te 9111. *Theoretical and Applied Genetics, 110*(6), 1138-1144. doi: 10.1007/s00122-005-1945-4

Chartrain, L., Brading, P. A., & Brown, J. K. M. (2005b). Presence of the Stb6 Gene for Resistance to Septoria Tritici Blotch (Mycosphaerella Graminicola) in Cultivars Used in Wheat-Breeding Programmes Worldwide. *Plant Pathology, 54*(2), 134-143. doi: 10.1111/j.1365-3059.2005.01164.x

Chartrain, L., Berry, S. T., & Brown, J. K. M. (2005c). Resistance of Wheat Line Kavkaz-K4500 L.6.A.4 to Septoria Tritici Blotch Controlled by Isolate-Specific Resistance Genes. *Phytopathology, 95*(6), 664-671. doi: 10.1094/phyto-95-0664

Chartrain, L., Sourdille, P., Bernard, M., & Brown, J. K. M. (2009). Identification and Location of Stb9, a Gene for Resistance to Septoria Tritici Blotch in Wheat Cultivars Courtot and Tonic. *Plant Pathology, 58*(3), 547-555.

Chen, Ruey-Shyang, & McDonald, Bruce A. (1996). Sexual Reproduction Plays a Major Role in the Genetic Structure of Populations of the Fungus Mycosphaerella Graminicola. *Genetics, 142*(4), 1119-1127.

Chen, Yi-Feng, Etheridge, Naomi, & Schaller, G Eric. (2005). Ethylene Signal Transduction. *Annals of Botany, 95*(6), 901-915.

Chen, Zhongying, Agnew, Jennifer L., Cohen, Jerry D., He, Ping, Shan, Libo, Sheen, Jen, & Kunkel, Barbara N. (2007). Pseudomonas Syringae Type Iii Effector Avrrpt2 Alters Arabidopsis Thaliana Auxin Physiology. *Proceedings of the National Academy of Sciences, 104*(50), 20131-20136. doi: 10.1073/pnas.0704901104

Choi, J., Huh, S. U., Kojima, M., Sakakibara, H., Paek, K. H., & Hwang, I. (2010). The Cytokinin-Activated Transcription Factor Arr2 Promotes Plant Immunity Via Tga3/Npr1-Dependent Salicylic Acid Signaling in Arabidopsis. *Developmental Cell, 19*(2), 284-295.

Choi, J., Choi, D., Lee, S., Ryu, C. M., & Hwang, I. (2011). Cytokinins and Plant Immunity: Old Foes or New Friends? *Trends Plant Sci, 16*(7), 388-394. doi: 10.1016/j.tplants.2011.03.003

Choudhary, S. P., Yu, J. Q., Yamaguchi-Shinozaki, K., Shinozaki, K., & Tran, L. S. P. (2012). Benefits of Brassinosteroid Crosstalk. *Trends Plant Sci, 17*(10), 594-605.

Clarke, J., Wynn, S., Twining, S., Berry, P., Cook, S., Ellis, S., & Gladders, P. (2009). Research Review 70. Pesticide Availability for Cereals and Oilseeds Following Revision of Directive 91/414/Eec; Effect of Losses and New Research Priorities, 2013, from http://www.hgca.com

Cohen, L., & Eyal, Z. (1993). The Histology of Processes Associated with the Infection of Resistant and Susceptible Wheat Cultivars with *Septoria Tritici*. *Plant Pathology, 42*, 737-743.

Colebrook, E. H., Creissen, G., McGrann, G. R. D., Dreos, R., Lamb, C., & Boyd, L. A. (2012). Broad-Spectrum Acquired Resistance in Barley Induced by the Pseudomonas Pathosystem Shares Transcriptional Components with Arabidopsis Systemic Acquired Resistance. *Molecular Plant-Microbe Interactions, 25*(5), 658-667. doi: 10.1094/mpmi-09-11-0246

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2go: A Universal Tool for Annotation, Visualization and Analysis in Functional Genomics Research. *Bioinformatics, 21*(18), 3674-3676. doi: 10.1093/bioinformatics/bti610

Conesa, A., & Götz, S. (2008). Blast2go: A Comprehensive Suite for Functional Analysis in Plant Genomics. *Int J Plant Genomics, 2008*. doi: 10.1155/2008/619832

Conrath, Uwe, Beckers, Gerold J. M., Flors, Victor, García-Agustín, Pilar, Jakab, Gábor, Mauch, Felix, . . . Mauch-Mani, Brigitte. (2006). Priming: Getting Ready for Battle. *Molecular Plant-Microbe Interactions, 19*(10), 1062-1071. doi: 10.1094/mpmi-19-1062

Cook, R. J., & Thomas, M. R. (1990). Influence of Site Factors on Yield Response of Winter Wheat to Fungicide Programmes in England and Wales, 1979–1987. *Plant Pathology, 39*(3), 548-557. doi: 10.1111/j.1365-3059.1990.tb02532.x

Cook, R. J., Hims, M. J., & Vaughan, T. B. (1999). Effects of Fungicide Spray Timing on Winter Wheat Disease Control. *Plant Pathology, 48*, 33-50.

Cools, H. J., Fraaije, B. A., Bean, T. P., Antoniw, J., & Lucas, J. A. (2007). Transcriptome Profiling of the Response of Mycosphaerella Graminicola Isolates to an Azole Fungicide Using Cdna Microarrays. *Mol Plant Pathol, 8*(5), 639-651. doi: 10.1111/j.1364-3703.2007.00411.x

Cools, H. J., & Fraaije, B. A. (2008). Are Azole Fungicides Losing Ground against Septoria Wheat Disease? Resistance Mechanisms in Mycosphaerella Graminicola. *Pest Manag Sci, 64*(7), 681-684. doi: 10.1002/ps.1568

Cools, H. J., & Fraaije, Bart A. (2013). Update on Mechanisms of Azole Resistance in Mycosphaerella Graminicola and Implications for Future Control. *Pest Manag Sci, 69*(2), 150-155. doi: 10.1002/ps.3348

Cowger, C., Hoffer, M. E., & Mundt, C. C. (2000). Specific Adaptation by Mycosphaerella Graminicola to a Resistant Wheat Cultivar. *Plant Pathology, 49*(4), 445-451. doi: 10.1046/j.1365-3059.2000.00472.x

Creelman, R.A., & Mullet, J.E. (1995). Jasmonic Acid Distribution and Action in Plants: Regulation During Development and Response to Biotic and Abiotic Stress. *Proceedings of the National Academy of Sciences, 92*(10), 4114-4119.

Dangl, J. L., & Jones, J. D. G. (2001). Plant Pathogens and Integrated Defence Responses to Infection. *Nature, 411*(6839), 826-833. doi: 10.1038/35081161

de Mendiburu, Felipe. (2012). Agricolae: Statistical Proceedures for Agricultural Research (Version R package version 1.1-3). Retrieved from http://tarwi.lamolina.edu.pe/~fmendiburu

do Amaral, A. M., Antoniw, J., Rudd, J. J., & Hammond-Kosack, K. E. (2012). Defining the Predicted Protein Secretome of the Fungal Wheat Leaf Pathogen Mycosphaerella Graminicola. *Plos One, 7*(12). doi: 10.1371/journal.pone.0049904

Duncan, Keith E., & Howard, Richard J. (2000). Cytological Analysis of Wheat Infection by the Leaf Blotch Pathogen Mycosphaerella Graminicola. *Mycological Research, 104*(9), 1074-1082. doi: 10.1017/s0953756299002294

Dunne, B. (2002). *New Fungicides and Their Role in Disease Control Programmes.* Paper presented at the National tillage crop conference, Crops Research Centre, Oak Park, Carlow, Ireland.

Dupont, Sebastien, Lemetais, Guillaume, Ferreira, Thierry, Cayot, Philippe, Gervais, Patrick, & Beney, Laurent. (2012). Ergosterol Biosynthesis: A Fungal Pathway for Life on Land? *Evolution, 66*(9), 2961-2968. doi: 10.1111/j.1558-5646.2012.01667.x

Duveiller, Etienne, Singh, Ravi P., & Nicol, Julie M. (2007). The Challenges of Maintaining Wheat Productivity: Pests, Diseases, and Potential Epidemics. *Euphytica, 157*(3), 417-430. doi: 10.1007/s10681-007-9380-z

Earley, Fergus, Sauter, Hubert, Rheinheimer, Joachim, Rieck, Heiko, Coqueron, Pierre-Yves, Whittingham, William G., & Walter, Harald. (2012). Fungicides Acting on Oxidative Phosphorylation. In W. Krämer, U. Schirmer, P. Jeschke & M. Witschel (Eds.), *Modern Crop Protection Compounds* (pp. 559-691): Wiley-VCH Verlag GmbH & Co. KGaA.

El Chartouni, L., Randoux, B., Duyme, F., Renard-Merlier, D., Tisserant, B., Bourdon, N., . . . Halama, P. (2012). Correlation of Cytological and Biochemical Parameters with Resistance and Tolerance to Mycosphaerella Graminicola in Wheat. *Plant Biol (Stuttg), 14 Suppl 1*, 11-21. doi: 10.1111/j.1438-8677.2011.00443.x

El Oirdi, Mohamed, El Rahman, Taha Abd, Rigano, Luciano, El Hadrami, Abdelbasset, Rodriguez, María Cecilia, Daayf, Fouad, . . . Bouarab, Kamal. (2011). Botrytis Cinerea Manipulates the Antagonistic Effects between Immune Pathways to Promote Disease Development in Tomato. *The Plant Cell Online, 23*(6), 2405-2421. doi: 10.1105/tpc.111.083394

EPPO. (2013). Eppo Plant Protection Thesaurus, from http://eppt.eppo.org

Eyal, Z. (1987). *The Septoria Diseases of Wheat: Concepts and Methods of Disease Management*: Cimmyt.

Eyal, Z. (1999). The Septoria Tritici and Stagnospora Nodorum Blotch Diseases of Wheat. *European Journal of Plant Pathology, 105*, 629-641.

FAO. (2012a). Fao Statistical Yearbook. World Food and Agriculture. Part 3: Trends in the Crop Sector. Retrieved Date| Accessed from Food and Agriculture Organization of the United Nations website: http://www.fao.org

FAO. (2012b). World Agriculture: Towards 2030/2050. The 2012 Revision. Retrieved Date| Accessed from Food and Agriculture Organization of the United Nations website: http://www.fao.org

FAO. (2013a). Fao Statstical Yearbook. World Food and Agriculture. Part 3: Feeding the World. Retrieved Date| Accessed from Food and Agriculture Organization of the United Nations website: http://www.fao.org

FAO. (2013b). Faostat. Retrieved Date| Accessed from Food and Agriculture Organization of the United Nations website: http://faostat.fao.org

Foyer, C. H., & Shigeoka, S. (2011). Understanding Oxidative Stress and Antioxidant Functions to Enhance Photosynthesis. *Plant Physiol, 155*(1), 93-100. doi: 10.1104/pp.110.166181

Fraaije, B. A., Cools, H. J., Fountaine, J., Lovell, D. J., Motteram, J., West, J. S., & Lucas, J. A. (2005). Role of Ascospores in Further Spread of Qoi-Resistant Cytochrome B Alleles (G143a) in Field Populations of Mycosphaerella Graminicola. *Phytopathology, 95*(8), 933-941. doi: 10.1094/phyto-95-0933

Fraaije, B. A., Bayon, C., Atkins, S., Cools, H. J., Lucas, J. A., & Fraaije, M. W. (2012). Risk Assessment Studies on Succinate Dehydrogenase Inhibitors, the New Weapons in the Battle to Control Septoria Leaf Blotch in Wheat. *Mol Plant Pathol, 13*(3), 263-275. doi: 10.1111/j.1364-3703.2011.00746.x

FRAC. (2012a). Qoi Working Group. Minutes of 2012 Meeting Recommendations for 2013, from http://frac.info/

FRAC. (2012b). Succinate Dehydrogenase Inhibitor (Sdhi) Working Group. Protocol of the Discussions and Use Recommendations of the Sdhi Working Group of the Fungicide Resistance Action Committee (Frac), from http://frac.info/

Friesen, Timothy L., Stukenbrock, Eva H., Liu, Zhaohui, Meinhardt, Steven, Ling, Hua, Faris, Justin D., . . . Oliver, Richard P. (2006). Emergence of a New Disease as a Result of Interspecific Virulence Gene Transfer. *Nat Genet, 38*(8), 953-956. doi: http://www.nature.com/ng/journal/v38/n8/suppinfo/ng1839_S1.html

Friesen, Timothy L., Meinhardt, Steven W., & Faris, Justin D. (2007). The Stagonospora Nodorum-Wheat Pathosystem Involves Multiple Proteinaceous Host-Selective Toxins and Corresponding Host Sensitivity Genes That Interact in an Inverse Gene-for-Gene Manner. *The Plant Journal, 51*(4), 681-692. doi: 10.1111/j.1365-313X.2007.03166.x

Gerhard, M., Habermeyer, J., & Zinkernagel, V. (1998). *The Impact of Strobilurins on Plant Vitality on Winter Wheat under Field Conditions*. Paper presented at the Modern fungicides and antifungal compounds II: 12th International Reinhardsbrunn Symposium Berg Hotel, Friedrichroda, Thuringia, Germany.

Ghaffary, Seyed Mahmod Tabib, Robert, Olivier, Laurent, Valerie, Lonnet, Philippe, Margale, Eric, van der Lee, Theo A. J., . . . Kema, Gert H. J. (2011). Genetic Analysis of Resistance to Septoria Tritici Blotch in the French Winter Wheat Cultivars Balance and Apache. *Theoretical and Applied Genetics, 123*(5), 741-754. doi: 10.1007/s00122-011-1623-7

Ghaffary, Seyed Mahmod Tabib, Faris, Justin, Friesen, Timothy, Visser, Richard, van der Lee, Theo, Robert, Olivier, & Kema, Gert. (2012). New Broad-Spectrum Resistance to Septoria Tritici Blotch Derived from Synthetic Hexaploid Wheat. *TAG Theoretical and Applied Genetics, 124*(1), 125-142. doi: 10.1007/s00122-011-1692-7

Gisi, Ulrich, Sierotzki, Helge, Cook, Alison, & McCaffery, Alan. (2002). Mechanisms Influencing the Evolution of Resistance to Qo Inhibitor Fungicides. *Pest Manag Sci, 58*(9), 859-867. doi: 10.1002/ps.565

Glaab, Johanna, & Kaiser, Werner M. (1999). Increased Nitrate Reductase Activity in Leaf Tissue after Application of the Fungicide Kresoxim-Methyl. *Planta, 207*(3), 442-448. doi: 10.1007/s004250050503

Glättli, A., Grote, T., & Stammler, G. (2011). *Sdh-Inhibitors: History, Biological Performance and Molecular Mode of Action*. Paper presented at the Modern Fungicides and Antifungal Compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

Glazebrook, J. (2001). Genes Controlling Expression of Defense Responses in Arabidopsis - 2001 Status. *Current Opinion in Plant Biology, 4*(4), 301-308. doi: 10.1016/s1369-5266(00)00177-1

Goklany, I. M. (1999). Meeting Global Food Needs: The Environmental Trade-Offs between Increasing Land Conversion and Land Productivity. *Technology, 6*, 107-130.

Gooding, M. J., Dimmock, Jpre, France, J., & Jones, S. A. (2000). Green Leaf Area Decline of Wheat Flag Leaves: The Influence of Fungicides and Relationships with Mean Grain Weight and Grain Yield. *Annals of Applied Biology, 136*(1), 77-84. doi: 10.1111/j.1744-7348.2000.tb00011.x

Goodwin, S. B. (2007). Back to Basics and Beyond: Increasing the Level of Resistance to Septoria Tritici Blotch in Wheat. *Australasian Plant Pathology, 36*(6), 532-538. doi: 10.1071/ap07068

Goodwin, S. B., Cavaletto, J. R., Lowe, I, Thompson, I., Xu, S.X., Adhikari, T. B., & Dubcovsky, J. (2007). *Validation of a New Map Location for the Stb3 Gene for Resistance to Septoria Tritici Blotch in Wheat*. Paper presented at the 7th International Mycosphaerella and Stagnospora symposium, , Ascona, Switzerland.

Goodwin, S. B., M'Barek S, B., Dhillon, B., Wittenberg, A. H., Crane, C. F., Hane, J. K., . . . Kema, G. H. (2011). Finished Genome of the Fungal Wheat Pathogen Mycosphaerella Graminicola Reveals Dispensome Structure, Chromosome Plasticity, and Stealth Pathogenesis. *PLoS Genet, 7*(6), e1002070. doi: 10.1371/journal.pgen.1002070

Götz, S., Garcia-Gomez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., . . . Conesa, A. (2008). High-Throughput Functional Annotation and Data Mining with the Blast2go Suite. *Nucleic Acids Research, 36*(10), 3420-3435. doi: 10.1093/nar/gkn176

Götz, S., Arnold, R., Sebastián-León, P., Martín-Rodríguez, S., Tischler, P., Jehl, M.A., . . . Conesa, A. (2011). B2g-Far, a Species-Centered Go Annotation Repository. *Bioinformatics, 27*(7), 919-924.

Govrin, E. M., & Levine, A. (2000). The Hypersensitive Response Facilitates Plant Infection by the Necrotrophic Pathogen Botrytis Cinerea. *Current Biology, 10*(13), 751-757. doi: 10.1016/s0960-9822(00)00560-1

Grant, Murray, & Lamb, Chris. (2006). Systemic Immunity. *Current Opinion in Plant Biology, 9*(4), 414-420. doi: http://dx.doi.org/10.1016/j.pbi.2006.05.013

Grossmann, K., & Retzlaff, G. (1997). Bioregulatory Effects of the Fungicidal Strobilurin Kresoxim-Methyl in Wheat (Triticum Aestivum). *Pesticide Science, 50*(1), 11-20.

Grossmann, K., Kwiatkowski, J., & Casper, G. (1999). Regulation of Phytohormone Levels, Leaf Senescence and Transpiration by the Strobilurin Kresoxim-Methyl in Wheat (Triticum Aestivum). *J Plant Physiol, 154*(5-6), 805-808.

Gundlach, H, Müller, M J, Kutchan, T M, & Zenk, M H. (1992). Jasmonic Acid Is a Signal Transducer in Elicitor-Induced Plant Cell Cultures. *Proceedings of the National Academy of Sciences, 89*(6), 2389-2393.

Guo, J. R., Schneider, F., Beyer, M., & Verreet, J. A. (2005). Rapid Detection of *Mycosphaerella Graminicola* in Wheat Using Reverse Transcription-Pcr Assay. *Journal of Phytopathology, 153*, 674-679.

Guo, J. R., Schnieder, F., & Verreet, J. A. (2006). Presymptomatic and Quantitative Detection of Mycosphaerella Graminicola Development in Wheat Using a Real-Time Pcr Assay. *FEMS Microbiol Lett, 262*(2), 223-229. doi: 10.1111/j.1574-6968.2006.00393.x

Guo, J. R., Schnieder, F., & Verreet, J. A. (2007). A Real-Time Pcr Assay for Quantitative and Accurate Assessment of Fungicide Effects on Mycosphaerella Graminicola Leaf Blotch. *Journal of Phytopathology, 155*(7-8), 482-487. doi: 10.1111/j.1439-0434.2007.01261.x

Hammond-Kosack, K. E., & Rudd, J. J. (2008). Plant Resistance Signalling Hijacked by a Necrotrophic Fungal Pathogen. *Plant Signalling and Behavior, 3*(11), 993-995.

Han, QM, Kang, ZS, Buchenauer, H, Huang, LL, & Zhao, J. (2006). Cytological and Immunocytochemical Studies on the Effects of the Fungicide Tebuconazole on the Interaction of Wheat with Stripe Rust. *Journal of Plant Pathology, 88*(3), 263-271.

Hardwick, N. V., Jones, D. R., & Slough, J. E. (2001). Factors Affecting Diseases of Winter Wheat in England and Wales, 1989–98. *Plant Pathology, 50*(4), 453-462. doi: 10.1046/j.1365-3059.2001.00596.x

Harrison, S., & Tedford, E. (1999). *Quadris™: A Novel Fungicide for Disease Control in Rice.* Paper presented at the Second Temperate Rice Conference, Sacramento, California.

Heil, M., & Baldwin, I. T. (2002). Fitness Costs of Induced Resistance: Emerging Experimental Support for a Slippery Concept. *Trends Plant Sci, 7*(2), 61-67.

Henze, M., Beyer, M., Klink, H., & Verreet, J. A. (2007). Characterizing Meteorological Scenarios Favorable for Septoria Tritici Infections in Wheat and Estimation of Latent Periods. *Plant Disease, 91*(11), 1445-1449. doi: 10.1094/pdis-91-11-1445

Herms, S., Seehaus, K., Koehle, H., & Conrath, U. (2002). A Strobilurin Fungicide Enhances the Resistance of Tobacco against Tobacco Mosaic Virus and Pseudomonas Syringae Pv Tabaci. *Plant Physiol, 130*(1), 120-127. doi: 10.1104/pp.004432

Howe, Gregg A, Lightner, Jonathan, & Ryan, CA. (1996). An Octadecanoid Pathway Mutant (Jl5) of Tomato Is Compromised in Signaling for Defense against Insect Attack. *The Plant Cell Online, 8*(11), 2067-2077.

Hruz, Tomas, Laule, Oliver, Szabo, Gabor, Wessendorp, Frans, Bleuler, Stefan, Oertle, Lukas, . . . Zimmermann, Philip. (2008). Genevestigator V3: A Reference Expression Database for the Meta-Analysis of Transcriptomes. *Advances in Bioinformatics, 2008*. doi: 10.1155/2008/420747

Hruz, Tomas, Wyss, Markus, Docquier, Mylene, Pfaffl, Michael, Masanetz, Sabine, Borghi, Lorenzo, . . . Zimmermann, Philip. (2011). Refgenes: Identification of Reliable and Condition Specific Reference Genes for Rt-Qpcr Data Normalization. *BMC Genomics, 12*(1), 156.

Inoue, K., Tsurumi, T., Ishii, H., Park, P., & Ikeda, K. (2012). Cytological Evaluation of the Effect of Azoxystrobin and Alternative Oxidase Inhibitors in Botrytis Cinerea. *FEMS Microbiol Lett, 326*(1), 83-90. doi: 10.1111/j.1574-6968.2011.02438.x

Jabs, T., Pfirrmann, J., Schäfer, S., Wu, X. Y., & Tiedemann, A. (2002). *Anti-Oxidative and Anti-Senescence Effects of Strobilurin Pyraclostrobin in Plants: A New Strategy to Cope with Environmental Stress in Cereals.* Paper presented at the The BCPC Conference - Pest and diseases 2002, Brighton Hilton Metropole Hotel, UK.

Jahn, Marga, Wagner, Christina, & Sellmann, Jörg. (2012). Ertragsverluste Durch Wichtige Pilzkrankheiten in Winterweizen Im Zeitraum 2003 Bis 2008 - Versuchsergebnisse Aus 12 Deutschen Bundesländern. *Journal für Kulturpflanzen, 64*(8), 273-285.

Jones, J. D., & Dangl, J. L. (2006). The Plant Immune System. *Nature, 444*(7117), 323-329. doi: 10.1038/nature05286

Katz, Vera, Thulke, Oliver, & Conrath, Uwe. (1998). A Benzothiadiazole Primes Parsley Cells for Augmented Elicitation of Defense Responses. *Plant Physiol, 117*(4), 1333-1339.

Katz, Vera, Fuchs, Annette, & Conrath, Uwe. (2002). Pretreatment with Salicylic Acid Primes Parsley Cells for Enhanced Ion Transport Following Elicitation. *FEBS Letters, 520*(1–3), 53-57. doi: http://dx.doi.org/10.1016/S0014-5793(02)02759-X

Kema, Gert H. J., Yu, DaZhao, Rijkenberg, Frits H. J., Shaw, Michael W., & Baayen, Robert P. (1996). Histology of the Pathogenesis of *Mycosphaerella Graminicola* in Wheat. *Phytopathology, 86*(7), 777-786.

Keon, J., Antoniw, J., Rudd, J., Skinner, W., Hargreaves, J., & Hammond-Kosack, K. (2005a). Analysis of Expressed Sequence Tags from the Wheat Leaf Blotch Pathogen Mycosphaerella Graminicola (Anamorph Septoria Tritici). *Fungal Genet Biol, 42*(5), 376-389. doi: 10.1016/j.fgb.2004.12.005

Keon, J., Rudd, J. J., Antoniw, J., Skinner, W., Hargreaves, J., & Hammond-Kosack, K. (2005b). Metabolic and Stress Adaptation by Mycosphaerella Graminicola During Sporulation in Its Host Revealed through Microarray Transcription Profiling. *Mol Plant Pathol, 6*(5), 527-540. doi: 10.1111/j.1364-3703.2005.00304.x

Keon, J., Antoniw, J., Carzaniga, R., Deller, S., Ward, J. L., Baker, J. M., . . . Rudd, J. J. (2007). Transcriptional Adaptation of Mycosphaerella Graminicola to Programmed Cell Death (Pcd) of Its Susceptible Wheat Host. *Molecular Plant-Microbe Interactions, 20*(2), 178-193. doi: 10.1094/mpmi-20-2-0178

Kildea, S., Dunne, B., Mullins, E., Cooke, L. R., Mercer, P. C., & O´Sullivan, E. (2010). Pyraclostrobin Reduces Tube Growth Og Qoi-Resistant *Mycosphaerella Graminicola* Pycnidiospores and the Severity of Septoria Tritici Blotch on Winter Wheat. *Plant Pathology, 29*, 1091-1098. doi: Doi: 10.1111/j.1365-3059.2010.02348.x

Koehle, H., Grossman, Klaus, Jabs, T., Gerhard, M., Kaiser, W., Glaab, J., . . . Herms, S. (2002). *Physiological Effects of the Strobilurin Fungicide F500 on Plants*. Paper presented at the Modern Fungicides and Antifungal Compounds III.13th international Reinhardsbrunn symposium, Friedriechroda, Germany.

Koehle, H., Conrath, U., Sechaus, K., Niedenbrock, M., Tavares-Rodrigues, M. A., Sanchez, W., . . . Oliveira, C. (2006). Method of Inducing Virus Tolerance of Plants. US 2006/0172887 A1. BASF-AG.

Koehle, H., Bardinelli, T. R., & Tavares-Rodrigues, M. A. (2009). Method of Inducing Tolerance of Plants against Bacterioses. US 20090011935A1. BASF-SE.

Kogel, K. H., & Langen, G. (2005). Induced Disease Resistance and Gene Expression in Cereals. *Cellular Microbiology, 7*(11), 1555-1564. doi: 10.1111/j.1462-5822.2005.00592.x

Kuck, Karl-Heinz, Leadbeater, Andy, & Gisi, Ulrich. (2012a). Frac Mode of Action Classification and Resistance Risk of Fungicides. In W. Krämer, U. Schirmer, P. Jeschke & M. Witschel (Eds.), *Modern Crop Protection Compounds* (pp. 539-557): Wiley-VCH Verlag GmbH & Co. KGaA.

Kuck, Karl-Heinz, Stenzel, Klaus, & Vors, Jean-Pierre. (2012b). Sterol Biosynthesis Inhibitors *Modern Crop Protection Compounds* (pp. 761-805): Wiley-VCH Verlag GmbH & Co. KGaA.

Kwok, Iris M-Y, & Loeffler, R Thomas. (1993). The Biochemical Mode of Action of Some Newer Azole Fungicides. *Pesticide Science, 39*(1), 1-11.

Lancashire, P.D., Bleiholder, H., Langelüddecke, P., Stauss, T., Van den Boom, T., Weber, E., & Witzenberger, A. (1991). An Uniform Decimal Code for Growth Stages of Crops and Weeds. *Annals of Applied Biology, 119*, 561-601.

Laugé, Richard, Joosten, Matthieu H. A. J., Van den Ackerveken, Guido F. J. M., Van den Broek, Henk W. J., & De Wit, Pierre J. G. M. (1997). The in Planta-Produced Extracellular Proteins Ecp1 and Ecp2 of Cladosporium Fulvum Are Virulence Factors. *Molecular Plant-Microbe Interactions, 10*(6), 725-734. doi: 10.1094/mpmi.1997.10.6.725

Lepesheva, GalinaI, Hargrove, TatyanaY, Kleshchenko, Yuliya, Nes, W. David, Villalta, Fernando, & Waterman, MichaelR. (2008). Cyp51: A Major Drug Target in the Cytochrome P450 Superfamily. *Lipids, 43*(12), 1117-1125. doi: 10.1007/s11745-008-3225-y

Leshem, Y. Y. (1988). Plant Senescence Processes and Free-Radicals. *Free Radical Biology and Medicine, 5*(1), 39-49. doi: 10.1016/0891-5849(88)90060-3

Liu, Yanyan, Zhang, Lingli, Thompson, Ian, Goodwin, Stephen, & Ohm, Herbert. (2012). Molecular Mapping Re-Locates the *Stb2* Gene for Resistance to Septoria Tritici Blotch Derived from Cultivar Veranopolis on Wheat Chromosome 1bs. *Euphytica*, 1-12. doi: 10.1007/s10681-012-0796-8

Liu, Z. H., Faris, J. D., Meinhardt, S. W., Ali, S., Rasmussen, J. B., & Friesen, T. L. (2004). Genetic and Physical Mapping of a Gene Conditioning Sensitivity in Wheat to a Partially Purified Host-Selective Toxin Produced by Stagonospora Nodorum. *Phytopathology, 94*(10), 1056-1060. doi: 10.1094/phyto.2004.94.10.1056

Livak, Kenneth J., & Schmittgen, Thomas D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative Pcr and the 2−Δδct Method. *Methods, 25*(4), 402-408. doi: http://dx.doi.org/10.1006/meth.2001.1262

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J. J., & Solano, R. (2003). Ethylene Response Factor1 Integrates Signals from Ethylene and Jasmonate Pathways in Plant Defense. *Plant Cell, 15*(1), 165-178. doi: 10.1105/tpc.007468

Lorenzo, O., Chico, J. M., Sanchez-Serrano, J. J., & Solano, R. (2004). Jasmonate-Insensitive1 Encodes a Myc Transcription Factor Essential to Discriminate between Different Jasmonate-Regulated Defense Responses in Arabidopsis. *Plant Cell, 16*(7), 1938-1950. doi: 10.1105/tpc.022319

Lorenzo, O., & Solano, R. (2005). Molecular Players Regulating the Jasmonate Signalling Network. *Current Opinion in Plant Biology, 8*(5), 532-540. doi: http://dx.doi.org/10.1016/j.pbi.2005.07.003

Lovell, D. J, Hunter, T., Powers, S. J, Parker, S. R, & Van den Bosch, F. (2004). Effect of Temperature on Latent Period of Septoria Leaf Blotch on Winter Wheat under Outdoor Conditions. *Plant Pathology, 53*(2), 170-181. doi: 10.1111/j.0032-0862.2004.00983.x

Lucas, J. A., Bowyer, P., & Anderson, H. M. (1999). *Septoria on Cereals: A Study of Pathosystems*: CABI Pub.

Lucas, J. A., & Fraaije, B. (2008). *Qoi Resistance in Mycosphaerella Graminicola: What Have We Learned So Far?* Paper presented at the Modern Fungicides and Antifungal Compounds V. 15th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

MacMillan, Jake. (2001). Occurrence of Gibberellins in Vascular Plants, Fungi, and Bacteria. *Journal of Plant Growth Regulation, 20*(4), 387-442. doi: 10.1007/s003440010038

Manning, V. A., & Ciuffetti, L. M. (2005). Localization of Ptr Toxa Produced by Pyrenophora Tritici-Repentis Reveals Protein Import into Wheat Mesophyll Cells. *Plant Cell, 17*(11), 3203-3212. doi: 10.1105/tpc.105.035063

Mano, Y., & Nemoto, K. (2012). The Pathway of Auxin Biosynthesis in Plants. *J Exp Bot, 63*(8), 2853-2872.

Marshall, Owen J. (2004). Perlprimer: Cross-Platform, Graphical Primer Design for Standard, Bisulphite and Real-Time Pcr. *Bioinformatics, 20*(15), 2471-2472. doi: 10.1093/bioinformatics/bth254

Marshall, Rosalind, Kombrink, Anja, Motteram, Juliet, Loza-Reyes, Elisa, Lucas, John, Hammond-Kosack, Kim E., . . . Rudd, Jason J. (2011). Analysis of Two in Planta Expressed Lysm Effector Homologs from the Fungus Mycosphaerella Graminicola Reveals Novel Functional Properties and Varying Contributions to Virulence on Wheat. *Plant Physiol, 156*(2), 756-769. doi: 10.1104/pp.111.176347

McCartney, C. A., Brûlé-Babel, A. L., Lamari, L., & Somers, D. J. (2003). Chromosomal Location of a Race-Specific Resistance Gene to Mycosphaerella Graminicola in the Spring Wheat St6. *Theoretical and Applied Genetics, 107*(7), 1181-1186. doi: 10.1007/s00122-003-1359-0

McCartney, C. A., Mercer, P. C., Cooke, L. R., & Fraaije, B. A. (2007). Effects of a Strobilurin-Based Spray Programme on Disease Control, Green Leaf Area, Yield and Development of Fungicide-Resistance in Mycosphaerella Graminicola in Northern Ireland. *Crop Protection, 26*(8), 1272-1280. doi: 10.1016/j.cropro.2006.10.027

McIntosh, R.A. (1998). Breeding Wheat for Resistance to Biotic Stresses. *Euphytica, 100*, 19-34.

Memelink, J. (2009). Regulation of Gene Expression by Jasmonate Hormones. *Phytochemistry, 70*(13-14), 1560-1570.

Mercer, E. I. (1984). The Biosynthesis of Ergosterol. *Pestic. Sci., 15*(133-155).

Mercer, E. I. (1991). Sterol Biosynthesis Inhibitors: Their Current Status and Modes of Action. *Lipids, 26*(8), 584-597. doi: 10.1007/bf02536422

Mortazavi, Ali, Williams, Brian A., McCue, Kenneth, Schaeffer, Lorian, & Wold, Barbara. (2008). Mapping and Quantifying Mammalian Transcriptomes by Rna-Seq. *Nat Meth, 5*(7), 621-628. doi: http://www.nature.com/nmeth/journal/v5/n7/suppinfo/nmeth.1226_S1.html

Motteram, Juliet, Küfner, Isabell, Deller, Siân, Brunner, Frédéric, Hammond-Kosack, Kim E., Nürnberger, Thorsten, & Rudd, Jason J. (2009). Molecular Characterization and Functional Analysis of Mgnlp, the Sole Npp1 Domain–Containing Protein, from the Fungal Wheat Leaf Pathogen Mycosphaerella Graminicola. *Molecular Plant-Microbe Interactions, 22*(7), 790-799. doi: 10.1094/mpmi-22-7-0790

Mueller, T. A., Miles, M. R., Morel, W., Marois, J. J., Wright, D. L., Kemerait, R. C., . . . Hartman, G. L. (2009). Effect of Fungicide and Timing of Application on Soybean Rust Severity and Yield. *Plant Disease, 93*(3), 243-248. doi: 10.1094/pdis-93-3-0243

Mur, L. A. J., Kenton, P., Atzorn, R., Miersch, O., & Wasternack, C. (2006). The Outcomes of Concentration-Specific Interactions between Salicylate and Jasmonate Signaling Include Synergy, Antagonism, and Oxidative Stress Leading to Cell Death. *Plant Physiol, 140*(1), 249-262.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., . . . Yoshida, S. (2003). Brassinosteroid Functions in a Broad Range of Disease Resistance in Tobacco and Rice. *Plant Journal, 33*(5), 887-898. doi: 10.1046/j.1365-313X.2003.01675.x

Navarro, L., Bari, R., Achard, P., Lison, P., Nemri, A., Harberd, N. P., & Jones, J. D. G. (2008). Dellas Control Plant Immune Responses by Modulating the Balance and Salicylic Acid Signaling. *Current Biology, 18*(9), 650-655. doi: 10.1016/j.cub.2008.03.060

Oerke, E. C., & Dehne, H. W. (2004). Safeguarding Production - Losses in Major Crops and the Role of Crop Protection. *Crop Protection, 23*(4), 275-285. doi: 10.1016/j.cropro.2003.10.001

Orton, E. S., Deller, S., & Brown, J. K. (2011). Mycosphaerella Graminicola: From Genomics to Disease Control. *Mol Plant Pathol, 12*(5), 413-424. doi: 10.1111/j.1364-3703.2010.00688.x

Oz, Pickersgill, David, & Wroot, Sarah. (2001). History of Wheat. *Farm-Direct*, from http://www.farm-direct.co.uk/farming/stockcrop/wheat/wheathist.html

Palmer, Claire-Louise, & Skinner, Wendy. (2002). Mycosphaerella Graminicola: Latent Infection, Crop Devastation and Genomics. *Mol Plant Pathol, 3*(2), 63-70. doi: 10.1046/j.1464-6722.2002.00100.x

Pasquer, F., Isidore, E., Zarn, J., & Keller, B. (2005). Specific Patterns of Changes in Wheat Gene Expression after Treatment with Three Antifungal Compounds. *Plant Mol Biol, 57*(5), 693-707. doi: 10.1007/s11103-005-1728-y

Passardi, F., Cosio, C., Penel, C., & Dunand, C. (2005). Peroxidases Have More Functions Than a Swiss Army Knife. *Plant Cell Reports, 24*(5), 255-265.

Paul, P. A., Madden, L. V., Bradley, C. A., Robertson, A. E., Munkvold, G. P., Shaner, G., . . . Esker, P. (2011). Meta-Analysis of Yield Response of Hybrid Field Corn to Foliar Fungicides in the Us Corn Belt. *Phytopathology, 101*(9), 1122-1132. doi: 10.1094/phyto-03-11-0091

Peck, S. C., & Kende, H. (1998). Differential Regulation of Genes Encoding 1-Aminocyclopropane-Carboxylate (Acc) Synthase in Etiolated Pea Seedlings: Effects of Indole-3-Acetic Acid, Wounding, and Ethylene. *Plant Mol Biol, 38*(6), 977-982. doi: 10.1023/a:1006033030081

PhillipsMcDougall. (2011). Products Section – 2010 Market Retrieved April 2013, from http://www.phillipsmcdougall.com

PhillipsMcDougall. (2012). Crops Section – 2011 Market Retrieved April 2013, from http://www.phillipsmcdougall.com

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., & Van Wees, S. C. M. (2009). Networking by Small-Molecule Hormones in Plant Immunity. *Nature Chemical Biology, 5*(5), 308-316.

Pieterse, C. M. J., Van der Does, Dieuwertje, Zamioudis, Christos, Leon-Reyes, Antonio, & Van Wees, Saskia C.M. (2012). Hormonal Modulation of Plant Immunity. *Annual Review of Cell and Developmental Biology, 28*(1), 489-521. doi: doi:10.1146/annurev-cellbio-092910-154055

Ponomarenko, A., Gooding, S. B., & Kema, G. J. (2011). Septoria Tritici Blotch (Stb). *Plant Health Instructor*, from http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/Septoria.aspx

Pontzen, R., & Scheinpflug, H. (1989). Effects of Triazole Fungicides on Sterol Biosynthesis During Spore Germination of Botrytis Cinerea, Venturia Inaequalis and Puccinia Graminis F. Sp. Tritici. *Netherlands Journal of Plant Pathology, 95*(1), 151-160. doi: 10.1007/bf01974294

Pozo, Maria J., Van Der Ent, Sjoerd, Van Loon, L. C., & Pieterse, Corné M. J. (2008). Transcription Factor Myc2 Is Involved in Priming for Enhanced Defense During Rhizobacteria-Induced Systemic Resistance in Arabidopsis Thaliana. *New Phytologist, 180*(2), 511-523. doi: 10.1111/j.1469-8137.2008.02578.x

Proplanta. (2006-2013). Infothek - Weizen Retrieved December 2012, from http://www.proplanta.de

R-Core-Team. (2012). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Ray, S., Anderson, J. M., Urmeev, F. I., & Goodwin, S. B. (2003). Rapid Induction of a Protein Disulfide Isomerase and Defense-Related Genes in Wheat in Response to the Hemibiotrophic Fungal Pathogen Mycosphaerella Graminicola. *Plant Mol Biol, 53*(5), 741-754. doi: 10.1023/b:plan.0000019120.74610.52

Reymond, Philippe. (2001). DNA Microarrays and Plant Defence. *Plant Physiology and Biochemistry, 39*(3–4), 313-321. doi: http://dx.doi.org/10.1016/S0981-9428(00)01235-3

Rillo, AO, & Caldwell, RM. (1966). Inheritance of Resistance to Septoria Tritici in Triticum Aestivum Subsp. Vulgare 'Bulgaria 88'. *Phytopathology, 56*, 897.

Rozhon, Wilfried, Husar, Sigrid, Kalaivanan, Florian, Khan, Mamoona, Idlhammer, Markus, Shumilina, Daria, . . . Fujioka, Shozo. (2013). Genetic Variation in Plant Cyp51s Confers Resistance against Voriconazole, a Novel Inhibitor of Brassinosteroid-Dependent Sterol Biosynthesis. *Plos One, 8*(1), e53650.

Rudd, J. J., Keon, J., & Hammond-Kosack, K. E. (2008). The Wheat Mitogen-Activated Protein Kinases Tampk3 and Tampk6 Are Differentially Regulated at Multiple Levels During Compatible Disease Interactions with Mycosphaerella Graminicola. *Plant Physiol, 147*(2), 802-815. doi: 10.1104/pp.108.119511

Ruske, R. E., Gooding, M. J., & Jones, S. A. (2003). The Effects of Adding Picoxystrobin, Azoxystrobin and Nitrogen to a Triazole Programme on Disease Control, Flag Leaf Senescence, Yield and Grain Quality of Winter Wheat. *Crop Protection, 22*(7), 975-987. doi: http://dx.doi.org/10.1016/S0261-2194(03)00113-3

Russell, P. E. (2005). A Century of Fungicide Evolution. *Journal of Agricultural Science, 143*, 11-25. doi: 10.1017/s0021859605004971

Saraste, Matti. (1999). Oxidative Phosphorylation at the Fin De Siècle. *Science, 283*(5407), 1488-1493. doi: 10.1126/science.283.5407.1488

Scalliet, G., Boehler, M., Bowler, J., Green, P. S., Kilby, P. M., & Fonne-Pfister, R. (2011). *Sdhis and the Fungal Succinate Dehydrogenase*. Paper presented at the Modern Fungicides and Antifungal Compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

Scalliet, G., Bowler, J., Luksch, T., Kirchhofer-Allan, L., Steinhauer, D., Ward, K., . . . Fonne-Pfister, R. (2012). Mutagenesis and Functional Studies with Succinate Dehydrogenase Inhibitors in the Wheat Pathogen Mycosphaerella Graminicola. *Plos One, 7*(4). doi: 10.1371/journal.pone.0035429

Schäfer, Patrick, Pfiffi, Stefanie, Voll, Lars M., Zajic, Doreen, Chandler, Peter M., Waller, Frank, . . . Kogel, Karl-Heinz. (2009). Phytohormones in Plant Root-*Piriformospora Indica* Mutualism. *Plant Signaling & Behavior, 4*(7), 669-671.

Schöfl, U. A., & Zinkernagel, V. (1997). A Test Method Based on Microscopic Assessments to Determine Curative and Protectant Fungicide Properties against Septoria Tritici. *Plant Pathology, 46*(4), 545-556. doi: 10.1046/j.1365-3059.1997.d01-41.x

Schweizer, Patrick, Buchala, Antony, & Métraux, Jean-Pierre. (1997). Gene-Expression Patterns and Levels of Jasmonic Acid in Rice Treated with the Resistance Inducer 2, 6-Dichloroisonicotinic Acid. *Plant Physiol, 115*(1), 61-70.

Semar, M., Strobel, D., Strathmann, S., & Groeger, U. (2011). *Xemium® - the Basf Fungicide Innovation*. Paper presented at the Modern Fungicides and Antifungal Compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

Shetty, N. P., Kristensen, B. K., Newman, M. A., Møller, K., Gregersen, P. L., & Jørgensen, H. J. L. (2003). Association of Hydrogen Peroxide with Restriction of Septoria Tritici in Resistant Wheat. *Physiological and Molecular Plant Pathology, 62*(6), 333-346. doi: 10.1016/s0885-5765(03)00079-1

Shetty, N. P., Mehrabi, R., Lutken, H., Haldrup, A., Kema, G. H., Collinge, D. B., & Jorgensen, H. J. (2007). Role of Hydrogen Peroxide During the Interaction between the Hemibiotrophic Fungal Pathogen Septoria Tritici and Wheat. *New Phytol, 174*(3), 637-647. doi: 10.1111/j.1469-8137.2007.02026.x

Shetty, N. P., Jensen, J. D., Knudsen, A., Finnie, C., Geshi, N., Blennow, A., . . . Jorgensen, H. J. (2009). Effects of Beta-1,3-Glucan from Septoria Tritici on Structural Defence Responses in Wheat. *J Exp Bot, 60*(15), 4287-4300. doi: 10.1093/jxb/erp269

Shi, H., Shen, Q. J., Qi, Y. P., Yan, H. J., Nie, H. Z., Chen, Y. F., . . . Tang, D. Z. (2013). Br-Signaling Kinase1 Physically Associates with Flagellin Sensing2 and Regulates Plant Innate Immunity in Arabidopsis. *Plant Cell, 25*(3), 1143-1157.

Siah, A., Deweer, C., Duyme, F., Sanssené, J., Durand, R., Halama, P., & Reignault, P. (2010). Correlation of *in Planta* Endo-Beta-1,4-Xylanase Activita with the Necrotrophic Phase of the Hemibiotrophic Fungus *Mycosphaerella Graminicola*. *Plant Pathology, 59*, 661-670.

Siefert, F., Thalmair, M., Langebartels, C., Sandermann, H., Jr., & Grossmann, K. (1996). Epoxiconazole-Induced Stimulation of the Antifungal Hydrolases Chitinase and B-1,3-Glucanase in Wheat. *Plant Growth Regulation, 20*(3), 279-286. doi: 10.1007/bf00043319

Smyth, G. K. (2005). Limma: Linear Models for Microarray Data. In R. Gentleman, V. Carey, S. Dudoit, R. Irizarry & W. Huber (Eds.), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor* (pp. 397-420): Springer, New York.

Somasco, O. A., Qualset, C. O., & Gilchrist, D. G. (1996). Single-Gene Resistance to Septoria Tritici Blotch in the Spring Wheat Cultivar 'Tadinia'. *Plant Breeding, 115*(4), 261-267. doi: 10.1111/j.1439-0523.1996.tb00914.x

Spoel, Steven H, Koornneef, Annemart, Claessens, Susanne MC, Korzelius, Jerôme P, Van Pelt, Johan A, Mueller, Martin J, . . . Kazan, Kemal. (2003). Npr1 Modulates Cross-Talk between Salicylate-and Jasmonate-Dependent Defense Pathways through a Novel Function in the Cytosol. *The Plant Cell Online, 15*(3), 760-770.

Stammler, G., & Speakman, J. (2006). Microtiter Method to Test the Sensitivity of Botrytis Cinerea to Boscalid. *Journal of Phytopathology, 154*(7-8), 508-510. doi: 10.1111/j.1439-0434.2006.01139.x

Stammler, G., Brix, H. D., Nave, B., Gold, R., & Schoefl, U. (2008). *Studies on the Biological Performance of Boscalid and Its Mode of Action*. Paper presented at the Modern Fungicides and Antifungal Compounds V. 15th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

Stammler, G., Taher, K., Koch, A., Haber, J., Liebmann, B., Bouagila, A., . . . Nasraoui, B. (2012). Sensitivity of Mycosphaerella Graminicola Isolates from Tunisia to Epoxiconazole and Pyraclostrobin. *Crop Protection, 34*, 32-36. doi: 10.1016/j.cropro.2011.11.007

Stein, E., Molitor, A., Kogel, K. H., & Waller, F. (2008). Systemic Resistance in Arabidopsis Conferred by the Mycorrhizal Fungus Piriformospora Indica Requires Jasmonic Acid Signaling and the Cytoplasmic Function of Npr1. *Plant and Cell Physiology, 49*(11), 1747-1751. doi: 10.1093/pcp/pcn147

Stergiopoulos, Ioannis, van den Burg, Harrold A., Ökmen, Bilal, Beenen, Henriek G., van Liere, Sabine, Kema, Gert H. J., & de Wit, Pierre J. G. M. (2010). Tomato Cf Resistance Proteins Mediate Recognition of Cognate Homologous Effectors from Fungi Pathogenic on Dicots and Monocots. *Proceedings of the National Academy of Sciences, 107*(16), 7610-7615. doi: 10.1073/pnas.1002910107

Stevenson, W. R., James, R. V., Rand, R. E., & Flak, L. A. (1999). Wisonsin´S Fiel Experience with Azoxystrobin Fungicide Control of Early Blight on Potato. *Phytopathology, 89*(6s), S75. doi: 10.1094/phyto.1999.89.6.s1

Suty-Heinze, A., Dunkel, R., Krieg, U., & Rieck, H. (2011). *Bixafen - the New Cereal Fungicide with Yield Boosting Effects*. Paper presented at the Modern Fungicides and Antifungal Compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

Swoboda, Catherine, & Pedersen, Palle. (2009). Effect of Fungicide on Soybean Growth and Yield All Rights Reserved. No Part of This Periodical May Be Reproduced or Transmitted in Any Form or by Any Means, Electronic or Mechanical, Including Photocopying, Recording, or Any Information Storage and Retrieval System, without Permission in Writing from the Publisher. *Agron. J., 101*(2), 352-356. doi: 10.2134/agronj2008.0150

Thompson, J. E., Legge, R. L., & Barber, R. F. (1987). The Role of Free Radicals in Senescence and Wounding. *New Phytologist, 105*(3), 317-344. doi: 10.1111/j.1469-8137.1987.tb00871.x

Thulke, Oliver, & Conrath, Uwe. (1998). Salicylic Acid Has a Dual Role in the Activation of Defence-Related Genes in Parsley. *The Plant Journal, 14*(1), 35-42. doi: 10.1046/j.1365-313X.1998.00093.x

Thygesen, Karin, Jørgensen, Lise Nistrup, Jensen, Kim Skov, & Munk, Lisa. (2008). Spatial and Temporal Impact of Fungicide Spray Strategies on Fungicide Sensitivity of Mycosphaerella Graminicola in Winter Wheat. *European Journal of Plant Pathology, 123*(4), 435-447. doi: 10.1007/s10658-008-9381-3

Ton, Jurriaan, & Mauch-Mani, Brigitte. (2004). B-Amino-Butyric Acid-Induced Resistance against Necrotrophic Pathogens Is Based on Aba-Dependent Priming for Callose. *The Plant Journal, 38*(1), 119-130. doi: 10.1111/j.1365-313X.2004.02028.x

Ton, Jurriaan, Flors, Victor, & Mauch-Mani, Brigitte. (2009). The Multifaceted Role of Aba in Disease Resistance. *Trends Plant Sci, 14*(6), 310-317.

topagrar. (2011). Neue Fungizidklasse - Ihre Stärken Und Schwächen. *top agrar,* 58-61.

topagrar. (2013). Getreide-Fungizide: Das Ist Neu! *top agrar, 1,* 56-61.

Truman, W., Bennettt, M. H., Kubigsteltig, I., Turnbull, C., & Grant, M. (2007). Arabidopsis Systemic Immunity Uses Conserved Defense Signaling Pathways and Is Mediated by Jasmonates. *Proc Natl Acad Sci U S A, 104*(3), 1075-1080. doi: 10.1073/pnas.0605423104

Van Baarlen, P., Staats, M., & Van Kan, J. A. L. (2004). Induction of Programmed Cell Death in Lily by the Fungal Pathogen Botrytis Elliptica. *Mol Plant Pathol, 5*(6), 559-574. doi: 10.1111/j.1364-3703.2004.00253.x

van Hulten, M., Pelser, M., van Loon, L. C., Pieterse, C. M. J., & Ton, J. (2006). Costs and Benefits of Priming for Defense in Arabidopsis. *Proc Natl Acad Sci U S A, 103*(14), 5602-5607. doi: 10.1073/pnas.0510213103

van Verk, Marcel, Bol, John, & Linthorst, Huub. (2011). Prospecting for Genes Involved in Transcriptional Regulation of Plant Defenses, a Bioinformatics Approach. *BMC Plant Biology, 11*(1), 88.

Verreet, J. A., Klink, H., & Hoffmann, G. M. (2000). Regional Monitoring for Disease Prediction and Optimization of Plant Protection Measures: The Ipm Wheat Model. *Plant Disease, 84*(8), 816-826.

Vick, Brady A., & Zimmerman, Don C. (1984). Biosynthesis of Jasmonic Acid by Several Plant Species. *Plant Physiol, 75*(2), 458-461.

Vlot, A. C., Dempsey, D. A., & Klessig, D. F. (2009). Salicylic Acid, a Multifaceted Hormone to Combat Disease. *Annu Rev Phytopathol, 47*, 177-206. doi: 10.1146/annurev.phyto.050908.135202

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., . . . Kogel, K. H. (2005). The Endophytic Fungus Piriformospora Indica Reprograms Barley to Salt-Stress Tolerance, Disease Resistance, and Higher Yield. *Proc Natl Acad Sci U S A, 102*(38), 13386-13391. doi: 10.1073/pnas.0504423102

Walter, H. (2011). *New Fungicides and New Modes of Action*. Paper presented at the Modern Fungicides and Antifungal Compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany.

Walters, D. R., & McRoberts, N. (2006). Plants and Biotrophs: A Pivotal Role for Cytokinins? *Trends Plant Sci, 11*(12), 581-586. doi: 10.1016/j.tplants.2006.10.003

Walters, D. R., & Heil, M. (2007). Costs and Trade-Offs Associated with Induced Resistance. *Physiological and Molecular Plant Pathology, 71*(1–3), 3-17. doi: http://dx.doi.org/10.1016/j.pmpp.2007.09.008

Wang, Dong, Amornsiripanitch, Nita, & Dong, Xinnian. (2006). A Genomic Approach to Identify Regulatory Nodes in the Transcriptional Network of Systemic Acquired Resistance in Plants. *PLoS Pathog, 2*(11), e123. doi: 10.1371/journal.ppat.0020123

Wang, Dong, Pajerowska-Mukhtar, Karolina, Culler, Angela Hendrickson, & Dong, Xinnian. (2007). Salicylic Acid Inhibits Pathogen Growth in Plants through Repression of the Auxin Signaling Pathway. *Curr Biol, 17*(20), 1784-1790.

Wang, W., Vinocur, B., Shoseyov, O., & Altman, A. (2004). Role of Plant Heat-Shock Proteins and Molecular Chaperones in the Abiotic Stress Response. *Trends Plant Sci, 9*(5), 244-252.

Wasternack, C. (2007). Jasmonates: An Update on Biosynthesis, Signal Transduction and Action in Plant Stress Response, Growth and Development. *Annals of Botany, 100*(4), 681-697.

Wasternack, C., & Hause, B. (2013). Jasmonates: Biosynthesis, Perception, Signal Transduction and Action in Plant Stress Response, Growth and Development. An Update to the 2007 Review in Annals of Botany. *Annals of Botany, 111*(6), 1021-1058. doi: 10.1093/aob/mct067

Watson, Andrew M., Hare, Martin C., Kettlewell, Peter S., Brosnan, James M., & Agu, Reginald C. (2010). Relationships between Disease Control, Green Leaf Duration, Grain Quality and the Production of Alcohol from Winter Wheat. *Journal of the Science of Food and Agriculture, 90*(15), 2602-2607. doi: 10.1002/jsfa.4128

Wegulo, Stephen N., Breathnach, Julie A., & Baenziger, P. Stephen. (2009). Effect of Growth Stage on the Relationship between Tan Spot and Spot Blotch Severity and Yield in Winter Wheat. *Crop Protection, 28*(8), 696-702.

Wegulo, Stephen N., Zwingman, Michael V., Breathnach, Julie A., & Baenziger, P. Stephen. (2011). Economic Returns from Fungicide Application to Control Foliar Fungal Diseases in Winter Wheat. *Crop Protection, 30*(6), 685-692. doi: 10.1016/j.cropro.2011.02.002

Wiik, L., & Ewaldz, T. (2009). Impact of Temperature and Precipitation on Yield and Plant Diseases of Winter Wheat in Southern Sweden 1983–2007. *Crop Protection, 28*(11), 952-962. doi: http://dx.doi.org/10.1016/j.cropro.2009.05.002

Wiik, L. (2009). Yield and Disease Control in Winter Wheat in Southern Sweden During 1977-2005. *Crop Protection, 28*(1), 82-89. doi: 10.1016/j.cropro.2008.09.002

Wiik, L., & Rosenqvist, H. (2010). The Economics of Fungicide Use in Winter Wheat in Southern Sweden. *Crop Protection, 29*(1), 11-19.

Wilson, R.E. (1985). *Inheritance of Resistance to Septoria Tritici in Wheat.* Paper presented at the Septoria of cereals: proceedings of the workshop held August 2-4, 1983, at Montana State University, Bozeman, Montana, Montana State University--Bozeman. http://books.google.de/books?id=wFfQHCZX6hwC

Winfield, M. Wheat: The Big Picture Retrieved December 2012, from http://www.wheatbp.net/WheatBP/Documents/DOC_WheatBP.php

Winfield, M. (n.d.). Evolution of Wheat Retrieved December 2012, from http://www.wheatbp.net/WheatBP/Documents/DOC_Evolution.php

Witzenberger, A., Hack, H., & van den Boom, T. (1989). Erläuterungen Zum Bbch-Dezimal-Code Für Die Entwicklungsstadien Des Getreides - Mit Abbildungen. *Gesunde Pflanzen, 41*, 384-388.

Wu, Yue-Xuan, & von Tiedemann, Andreas. (2001). Physiological Effects of Azoxystrobin and Epoxiconazole on Senescence and the Oxidative Status of Wheat. *Pesticide Biochemistry and Physiology, 71*(1), 1-10. doi: http://dx.doi.org/10.1006/pest.2001.2561

Wu, Yue-xuan, & von Tiedemann, Andreas. (2002). Impact of Fungicides on Active Oxygen Species and Antioxidant Enzymes in Spring Barley *(Hordeum Vulgare* L.) Exposed to Ozone. *Environmental Pollution, 116*(1), 37-47.

Yamaguchi, Shinjiro. (2008). Gibberellin Metabolism and Its Regulation. *Annu. Rev. Plant Biol., 59*, 225-251.

Yang, Dong-Lei, Yao, Jian, Mei, Chuan-Sheng, Tong, Xiao-Hong, Zeng, Long-Jun, Li, Qun, . . . He, Sheng Yang. (2012). Plant Hormone Jasmonate Prioritizes Defense over Growth by Interfering with Gibberellin Signaling Cascade. *Proceedings of the National Academy of Sciences, 109*(19), E1192–E1200. doi: 10.1073/pnas.1201616109

Yasuda, Michiko, Ishikawa, Atsushi, Jikumaru, Yusuke, Seki, Motoaki, Umezawa, Taishi, Asami, Tadao, . . . Nakashita, Hideo. (2008). Antagonistic Interaction between Systemic Acquired Resistance and the Abscisic Acid–Mediated Abiotic Stress Response in Arabidopsis. *The Plant Cell Online, 20*(6), 1678-1692. doi: 10.1105/tpc.107.054296

Ye, Jian, Coulouris, George, Zaretskaya, Irena, Cutcutache, Ioana, Rozen, Steve, & Madden, ThomasL. (2012). Primer-Blast: A Tool to Design Target-Specific Primers for Polymerase Chain Reaction. *BMC bioinformatics, 13*(1), 1-11. doi: 10.1186/1471-2105-13-134

Zhang, Jie, & Zhou, Jian-Min. (2010). Plant Immunity Triggered by Microbial Molecular Signatures. *Molecular Plant, 3*(5), 783-793. doi: 10.1093/mp/ssq035

Zhang, Zhongqin, Li, Qun, Li, Zhimiao, Staswick, Paul E, Wang, Muyang, Zhu, Ying, & He, Zuhua. (2007). Dual Regulation Role of Gh3. 5 in Salicylic Acid and Auxin Signaling During Arabidopsis-Pseudomonas Syringae Interaction. *Plant Physiol, 145*(2), 450-464.

Zhao, Yunde. (2010). Auxin Biosynthesis and Its Role in Plant Development. *Annual review of plant biology, 61*(1), 49-64. doi: doi:10.1146/annurev-arplant-042809-112308

Zhu, Tong. (2003). Global Analysis of Gene Expression Using Genechip Microarrays. *Current Opinion in Plant Biology, 6*(5), 418-425. doi: 10.1016/s1369-5266(03)00083-9

7. Appendix

Appendix 1. Phenological growth stages and BBCH code of cereals

(Triticum aestivum, Hordeum vulgare L., Avena sativa and Secale sereale) (Lancashire *et al.*, 1991; Witzenberger *et al.*, 1989)

Code	Description
Growth stage 0: Germination	
00	Dry seed
01	Beginning of seed imbibition
03	Seed imbibition complete
05	Radicle emerged from caryopsis
06	Radicle elongated, root hairs and/or side roots visible
07	Coleoptile emerged from caryopsis
09	Emergence: coleoptile penetrates soil surface (cracking stage)
Growth stage 1: Leaf development	
10	First leaf through coleoptile
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
1.	Stages continue until…
19	9 or more leaves unfolded
Growth stage 2: Tillering	
20	No tillers
21	Beginning of tillering: first tiller detectable
22	2 tillers detectable
23	3 tillers detectable
2.	Stages continue until…
29	End of tillering. Maximum numbers of tillers detectable
Growth stage 3: Stem elongation	
30	Beginning of stem elongation: pseudostem and tillers erect, first internode begins to elongate, top of inflorescence at least 1 cm above tillering node
31	First node at least 1cm above tillering node
32	Node 2 at least 2 cm above node 1
33	Node 3 at least 2 cm above node 2
3.	Stages continue until…
37	Flag leaf just visible, still rolled
39	Flag leaf stage: flag leaf fully unrolled, ligule just visible
Growth stage 4: Booting	
41	Early boot stage: flag leaf sheath extending
43	Mid boot stage: flag leaf sheath just visibly swollen
45	Late boot stage: flag leaf sheath swollen
47	Flag leaf sheath opening
49	First awns visible

Code	Description
Growth stage 5: Inflorescence emergence, heading	
51	Beginning of heading: tip of inflorescence emerged from sheath, first spikelet just visible
52	20% of inflorescence emerged
53	30% of inflorescence emerged
54	40% of inflorescence emerged
55	Middle of heading: half of inflorescence emerged
56	60% of inflorescence emerged
57	70% of inflorescence emerged
58	80% of inflorescence emerged
59	End of heading: inflorescence fully emerged
Growth stage 6: Flowering, anthesis	
61	Beginning of flowering: first anthers visible
65	Full flowering: 50% of anthers mature
69	End of flowering: all spikelets have completed flowering but some dehydrated anthers may remain
Growth stage 7: Development of fruit	
71	Watery ripe: first grains have reached half their final size
73	Early milk
75	Medium milk: grain content milky, grain reached final size, still green
77	Late milk
Growth stage 8: Ripening	
83	Early dough
85	Soft dough: grain content soft but dry. Fingernail impression not held
87	Hard dough: grain content solid. Fingernail impression held
89	Fully ripe: grain hard, difficult to divide with thumbnail
Growth stage 9: Senescence	
92	Over-ripe: grain very hard, cannot be dented by thumbnail
93	Grains loosening in daytime
97	Plant dead and collapsing
99	Harvested product

Appendix 2. Oligonucleotide information

List of the oligonucleotides used during the projects. Primers were design with Primer-BLAST (Ye *et al.*, 2012) or PerlPrimer version 1.1.21 (O. J. Marshall, 2004) and synthesized in the DNA laboratory from BASF SE. nucleotide sequences are displayed in 5´-end to 3´-end direction. Abbr: Tm: primer melting temperature; bp: base pairs; PR: pathogenesis-related protein.

Organism	Gene	GeneBank Accession Nr./ TA number	Primer type	Primer name	Sequence (5´- 3´)	Tm (°C)	Product size (bp)
M. graminicola	Cytochrome b	AY247413	Target	Mg-Cytb-Fwd	CCCTAGAACATTAACATGAACAATCG	57	130
				Mg-Cytb-Rev	CAATAAGTTAGTTATAACTGTTGCCC		
T. aestivum	Actin	AB181991	Reference	Ta-Actin-Fwd	TCCTGTGTTGCTGACTGAGG	60	134
				Ta-Actin-Rev	CGCTGGCATACAAGGACAGA		
T. aestivum	Clone WT009_N13	AK334339.1	Reference	Ta-WT009-Fwd	ATTGCCTTTGCCTTTACCTT	60	155
				Ta-WT009-Rev	ACCATTTCATCCTTCTTATCTTGT		
T. aestivum	Clone AZO1003C02	CD861649	Target	Ta-AZO1-Fwd	CTAGCATTAGCATCACCAACAC	60	135
				Ta-AZO1-Rev	GATGTGGCTCATGATCTTCGAC		
T. aestivum	PR5	TC428016	Target	Ta-PR5-Fwd	CCTTGCCATGGACTTCTCGT	60	91
				Ta-PR5-Rev	GTTGGGGTGTTGGTAGGCTT		
T. aestivum	Chitinase 1	TA53899_4565	Target	Ta-Chitinase-Fwd	CATGTGGTTCTGGATGACGG	60	269
				Ta-Chitinase-Rev	CTAGCGAAGTTCCTCTGGGT		
T. aestivum	Root peroxidase	AY506509	Target	Ta-RootPerox-Fwd	CCACGACTGCTTTGTCCAAG	60	146
				Ta- RootPerox-Rev	ACGGTCTGCTTGCATATACTC		
T. aestivum	Gibberellin 20 oxidase	Y14007	Target	Ta-GA20ox-Fwd	CTCTGCTCCTCCCACCATTA	60	164
				Ta- GA20ox-Rev	GAAATCCTGCCATCCATCCA		
T. aestivum	Rubisco alpha subunit	X07851	Target	Ta-Rubisco-Fwd	CACCTGTCTACCTATGTCCC	60	181
				Ta-Rubisco-Rev	TTGTAACCCATCTCCCACTC		
T. aestivum	Lipid transfer protein	TA61087_4565	Target	Ta-LTP-Fwd	CTCTAAGATTCGCTGATCAACCA	60	236
				Ta-LTP-Rev	ACAATAAAGTCTCGGTTCTCCT		
T. aestivum	ACC synthase	U42336	Target	Ta-ACS2-Fwd	TGTGTGACCACGCATGGTATT	60	167
				Ta-ACS2-Rev	AACTCATCACTCCTGCGGTA		
T. aestivum	ACC oxidase	TA56545_4565	Target	Ta-ACCox-Fwd	AAGATTCGAGGCCGTCAAGG	60	129
				Ta-ACCox-Rev	AATCGCCCAAATCGCCACTA		

Appendix 3. Microarray and sample information

Displayed are the microarray chips that were employed for the analysis of the responses of wheat seedlings to fungicide treatment. The first column represents the number of the microarray, followed by the barcode identification number of the microarray slide provided by Agilent. SampleName denotes the corresponding treatment, sample time point and biological replica. Group represents the corresponding treatment and sampling point. Experimental conditions describe whether the plant was infected or mock infected with tween water. Abbr.: PC: Pyraclostrobin, FP: Fluxapyroxad, EA: Epoxiconazole, *Mg*: *Mycosphaerella graminicola,* Control: mock-treated and mock-infected plant, Pre: Preventative treatment one day before the inoculation, Cur: Curative treatment seven days after the inoculation, dpi: days post inoculation, dpa: days post fungicide application.

Nr.	Agilent Barcode	SampleName	Group	Experimental conditions
1	US09433729_253302810001	PC_Prev-3dpiR2	PC_Prev_3dpi	*Mg* infected
2	US09433729_253302810001	*Mg*-3dpiR1 (Pre-experiment)	*Mg*_3dpi	*Mg* infected
3	US09433729_253302810001	Control-3dpiR1 (Pre-experiment)	Control_3dpi	Disease-free
4	US09433729_253302810001	*Mg*-10dpiR1 (Pre-experiment)	*Mg*_10dpi	*Mg* infected
5	US09433729_253302810001	Control-10dpiR1 (Pre-experiment)	Control_10dpi	Disease-free
6	US09433729_253302810001	FP-4dpaR1	FP_4dpa	Disease-free
7	US09433729_253302810005	Control-3dpiR1	Control_3dpi	Disease-free
8	US09433729_253302810005	Control-10dpiR1	Control_10dpi	Disease-free
9	US09433729_253302810005	*Mg*-3dpiR1	*Mg*_3dpi	*Mg* infected
10	US09433729_253302810005	*Mg*-10dpiR1	*Mg*_10dpi	*Mg* infected
11	US09433729_253302810006	Control-10dpiR2	Control_10dpi	Disease-free
12	US09433729_253302810006	*Mg*-3dpiR2	*Mg*_3dpi	*Mg* infected
13	US09433729_253302810007	Control-3dpiR2	Control_3dpi	Disease-free
14	US09433729_253302810007	*Mg*-10dpiR2	*Mg*_10dpi	*Mg* infected
15	US09433729_253302810008	EA_Prev-10dpiR3	EA_Prev_10dpi	*Mg* infected
16	US09433729_253302810008	*Mg*-3dpiR3	*Mg*_3dpi	*Mg* infected
17	US09433729_253302810008	PC_Prev-3dpiR1	PC_Prev_3dpi	*Mg* infected
18	US09433729_253302810008	EA_Prev-3dpiR1	EA_Prev_3dpi	*Mg* infected
19	US09433729_253302810008	FP_Prev-3dpiR1	FP_Prev_3dpi	*Mg* infected
20	US09433729_253302810008	PC_Prev-10dpiR2	PC_Prev_10dpi	*Mg* infected
21	US09433729_253302810008	EA-4dpaR1	EA_4dpa	Disease-free
22	US09433729_253302810008	FP_Cur-10dpiR3	FP_Cur_10dpi	*Mg* infected
23	US09433729_253302810009	PC_Cur-10dpiR2	PC_Cur_10dpi	*Mg* infected
24	US09433729_253302810009	FP_Prev-3dpiR2	FP_Prev_3dpi	*Mg* infected
25	US09433729_253302810009	PC-4dpaR2	PC_4dpa	Disease-free
26	US09433729_253302810009	Control-10dpiR3	Control_10dpi	Disease-free
27	US09433729_253302810009	FP_Prev-10dpiR3	FP_Prev_10dpi	*Mg* infected
28	US09433729_253302810009	FP-4dpaR2	FP_4dpa	Disease-free
29	US09433729_253302810009	EA-4dpaR2	EA_4dpa	Disease-free
30	US09433729_253302810010	PC_Prev-3dpiR3	PC_Prev_3dpi	*Mg* infected
31	US09433729_253302810010	FP_Cur-10dpiR2	FP_Cur_10dpi	*Mg* infected
32	US09433729_253302810010	FP_Prev-3dpiR3	FP_Prev_3dpi	*Mg* infected
33	US09433729_253302810010	PC-4dpaR3	PC_4dpa	Disease-free
34	US09433729_253302810010	EA_Cur-10dpiR2	EA_Cur_10dpi	*Mg* infected
35	US09433729_253302810010	FP-4dpaR3	FP_4dpa	Disease-free
36	US09433729_253302810010	PC_Prev-10dpiR1	PC_Prev_10dpi	*Mg* infected

Nr.	Agilent Barcode	SampleName	Group	Experimental conditions
37	US09433729_253302810010	PC_Cur-10dpiR1	PC_Cur_10dpi	*Mg* infected
38	US09433729_253302810011	Control-3dpiR3	Control_3dpi	Disease-free
39	US09433729_253302810011	EA_Prev-3dpiR3	EA_Prev_3dpi	*Mg* infected
40	US09433729_253302810011	*Mg*-10dpiR3	*Mg*10dpi	*Mg* infected
41	US09433729_253302810011	EA_Prev-10dpiR2	EA_Prev_10dpi	*Mg* infected
42	US09433729_253302810011	EA_Cur-10dpiR3	EA_Cur_10dpi	*Mg* infected
43	US09433729_253302810011	FP_Prev-10dpiR2	FP_Prev_10dpi	*Mg* infected
44	US09433729_253302810011	EA-4dpaR3	EA_4dpa	Disease-free
45	US09433729_253302810012	EA_Prev-3dpiR2	EA_Prev_3dpi	*Mg* infected
46	US09433729_253302810012	PC_Prev-10dpiR3	PC_Prev_10dpi	*Mg* infected
47	US09433729_253302810012	PC_Cur-10dpiR3	PC_Cur_10dpi	*Mg* infected
48	US09433729_253302810012	EA_Prev-10dpiR1	EA_Prev_10dpi	*Mg* infected
49	US09433729_253302810012	EA_Cur-10dpiR1	EA_Cur_10dpi	*Mg* infected
50	US09433729_253302810012	FP_Cur-10dpiR1	FP_Cur_10dpi	*Mg* infected
51	US09433729_253302810012	PC-4dpaR1	PC_4dpa	Disease-free
52	US09433729_253302810013	Control-10dpiR1	Control_10dpi	Disease-free
53	US09433729_253302810013	FP-4dpaR1	FP_4dpa	Disease-free
54	US09433729_253302810013	FP_Prev-10dpiR1	FP_Prev_10dpi	*Mg* infected

Appendix 4. Enrichment analysis for Gene Ontology (GO) terms of the up- and down-regulated genes after a given treatment

Differentially expressed genes for each comparison were selected based on a fold change of 2, a moderate t statistic of 1.96 and an average spot intensity of 5. Based on the obtained lists of differentially expressed genes, an enrichment analysis for Gene Ontology (GO) terms of biological processes was performed in November 2012 for the up- and down-regulated transcripts within each comparison. Displayed are the GO terms for each treatment. The first column represents the GO identification number of biological process, followed by the significance value and the odds ratio of the result. The ExpCount column represents the expected numbers of genes that would have been found to belong to this GO term if no enrichment had been done. Count represents the number of genes being annotated to the corresponding GO term, and Size is the number of genes belonging to that category that are present on the microarray. Significance codes: P < 0.0001 ***; P < 0.001 **; P < 0.01 *; P<0.05 ·.

Up-regulated genes: Pyraclostrobin-treated plants vs. untreated-uninfected plants at 4dpa

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006468	***	4.0	27.3	85	979	protein phosphorylation
GO:0016310	***	3.5	30.8	85	1105	phosphorylation
GO:0006793	***	3.3	33.8	89	1212	phosphorus metabolic process
GO:0006796	***	3.3	33.8	89	1212	phosphate-containing compound metabolic process
GO:0043687	***	3.0	38.3	92	1376	post-translational protein modification
GO:0006464	***	2.8	41.7	94	1496	protein modification process
GO:0043412	***	2.7	43.1	94	1546	macromolecule modification
GO:0010817	***	2.7	41.0	89	1471	regulation of hormone levels
GO:0009850	***	2.6	40.1	86	1438	auxin metabolic process
GO:0042445	***	2.6	40.3	86	1447	hormone metabolic process
GO:0009851	***	2.6	39.7	85	1424	auxin biosynthetic process
GO:0042446	***	2.6	39.9	85	1432	hormone biosynthetic process
GO:0065008	***	2.2	48.7	91	1746	regulation of biological quality
GO:0065007	***	1.9	87.0	135	3121	biological regulation
GO:0006558	***	16.6	0.6	7	22	L-phenylalanine metabolic process
GO:0006559	***	19.3	0.5	6	17	L-phenylalanine catabolic process
GO:0009074	***	17.7	0.5	6	18	aromatic amino acid family catabolic process
GO:0042538	***	12.4	0.8	7	27	hyperosmotic salinity response
GO:0009685	***	22.1	0.4	5	13	gibberellin metabolic process
GO:0006467	***	Inf	0.1	3	3	protein thiol-disulfide exchange
GO:0016101	***	13.3	0.6	6	22	diterpenoid metabolic process
GO:0006972	***	9.6	0.9	7	33	hyperosmotic response
GO:0009961	***	105.5	0.1	3	4	response to 1-aminocyclopropane-1-carboxylic acid
GO:0051703	***	105.5	0.1	3	4	intraspecies interaction between organisms
GO:0010483	***	105.5	0.1	3	4	pollen tube reception
GO:0009310	**	5.4	1.9	9	68	amine catabolic process
GO:0019439	**	9.2	0.8	6	29	aromatic compound catabolic process
GO:0009409	**	3.2	5.1	15	182	response to cold
GO:0010107	**	52.8	0.1	3	5	potassium ion import
GO:0009063	**	5.3	1.7	8	62	cellular amino acid catabolic process
GO:0009719	**	2.3	10.1	22	362	response to endogenous stimulus
GO:0006952	**	2.2	11.6	24	415	defense response
GO:0019748	**	2.6	6.9	17	249	secondary metabolic process
GO:0009725	**	2.3	9.6	21	344	response to hormone stimulus

GO:0009832	**	8.4	0.7	5	26	plant-type cell wall biogenesis
GO:0044249	**	1.4	123.0	152	4413	cellular biosynthetic process
GO:0009058	**	1.4	126.2	155	4530	biosynthetic process
GO:0009737	**	3.6	3.0	10	109	response to abscisic acid stimulus
GO:0010033	*	2.1	12.7	25	457	response to organic substance
GO:0048574	*	21.1	0.2	3	8	long-day photoperiodism, flowering
GO:0071669	*	5.7	1.2	6	43	plant-type cell wall organization or biogenesis
GO:0009612	*	17.6	0.3	3	9	response to mechanical stimulus
GO:0048571	*	15.1	0.3	3	10	long-day photoperiodism
GO:0016265	*	2.2	8.5	18	306	death
GO:0008219	*	2.2	8.5	18	306	cell death
GO:0009072	*	3.6	2.4	8	86	aromatic amino acid family metabolic process
GO:0007584	*	13.2	0.3	3	11	response to nutrient
GO:0009698	*	3.5	2.5	8	88	phenylpropanoid metabolic process
GO:0009739	*	7.4	0.6	4	23	response to gibberellin stimulus
GO:0019219	*	1.7	21.7	35	780	regulation of nucleobase-containing compound metabolic process

Down-regulated genes: Pyraclostrobin-treated plants vs. untreated-uninfected plants at 4dpa

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006869	***	7.4	1.1	7	114	lipid transport
GO:0010876	***	7.4	1.1	7	114	lipid localization
GO:0031408	**	13.1	0.4	4	38	oxylipin biosynthetic process
GO:0031407	**	12.7	0.4	4	39	oxylipin metabolic process
GO:0006633	**	5.5	1.4	7	150	fatty acid biosynthetic process
GO:0009269	*	54.7	0.1	2	6	response to desiccation
GO:0006631	*	4.3	1.8	7	191	fatty acid metabolic process
GO:0071368	*	31.2	0.1	2	9	cellular response to cytokinin stimulus
GO:0009736	*	31.2	0.1	2	9	cytokinin mediated signaling pathway
GO:0006950	*	2.0	14.9	26	1615	response to stress
GO:0009735	*	9.7	0.3	3	37	response to cytokinin stimulus
GO:0009414	*	5.6	0.8	4	83	response to water deprivation
GO:0006694	*	8.2	0.4	3	43	steroid biosynthetic process
GO:0008202	*	8.0	0.4	3	44	steroid metabolic process
GO:0009415	*	5.5	0.8	4	85	response to water
GO:0019295	*	Inf	0.0	1	1	coenzyme M biosynthetic process
GO:0019296	*	Inf	0.0	1	1	coenzyme M metabolic process
GO:0050896	.	1.7	21.6	32	2345	response to stimulus
GO:0008610	.	2.8	3.1	8	333	lipid biosynthetic process
GO:0048832	.	108.4	0.0	1	2	specification of organ number
GO:0048833	.	108.4	0.0	1	2	specification of floral organ number
GO:0048657	.	108.4	0.0	1	2	tapetal cell differentiation
GO:0009786	.	108.4	0.0	1	2	regulation of asymmetric cell division
GO:0010268	.	108.4	0.0	1	2	brassinosteroid homeostasis
GO:0016126	.	10.4	0.2	2	23	sterol biosynthetic process
GO:0016125	.	9.9	0.2	2	24	sterol metabolic process
GO:0006528	.	54.2	0.0	1	3	asparagine metabolic process
GO:0006529	.	54.2	0.0	1	3	asparagine biosynthetic process
GO:0000255	.	54.2	0.0	1	3	allantoin metabolic process
GO:0000256	.	54.2	0.0	1	3	allantoin catabolic process
GO:0009955	.	54.2	0.0	1	3	adaxial/abaxial pattern specification

GO:0009943	.	54.2	0.0	1	3	adaxial/abaxial axis specification
GO:0009944	.	54.2	0.0	1	3	polarity specification of adaxial/abaxial axis
GO:0065001	.	54.2	0.0	1	3	specification of axis polarity
GO:0043605	.	54.2	0.0	1	3	cellular amide catabolic process
GO:0008356	.	54.2	0.0	1	3	asymmetric cell division
GO:0032787	.	2.5	3.0	7	323	monocarboxylic acid metabolic process
GO:0016053	.	2.3	3.7	8	403	organic acid biosynthetic process
GO:0046394	.	2.3	3.7	8	403	carboxylic acid biosynthetic process
GO:0006521	.	36.1	0.0	1	4	regulation of cellular amino acid metabolic process
GO:0048656	.	36.1	0.0	1	4	tapetal layer formation
GO:0033238	.	36.1	0.0	1	4	regulation of cellular amine metabolic process
GO:0009828	.	36.1	0.0	1	4	plant-type cell wall loosening
GO:0009725	.	2.3	3.2	7	344	response to hormone stimulus
GO:0003002	.	6.4	0.3	2	36	regionalization
GO:0009886	.	6.4	0.3	2	36	post-embryonic morphogenesis
GO:0048654	.	27.1	0.0	1	5	anther morphogenesis
GO:0048655	.	27.1	0.0	1	5	tapetal layer morphogenesis
GO:0010311	.	27.1	0.0	1	5	lateral root formation
GO:0048455	.	27.1	0.0	1	5	stamen formation

Up-regulated genes: Pyraclostrobin preventative treatment vs. Untreated *M. graminicola*-infected plants at 4dpa

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006869	***	108.2	0.1	8	114	lipid transport
GO:0010876	***	108.2	0.1	8	114	lipid localization
GO:0033036	***	19.5	0.8	8	575	macromolecule localization
GO:0050896	***	8.4	3.1	11	2345	response to stimulus
GO:0006950	***	8.0	2.1	9	1615	response to stress
GO:0006810	**	5.9	2.2	8	1706	transport
GO:0051234	**	5.9	2.2	8	1709	establishment of localization
GO:0051179	**	5.8	2.3	8	1739	localization
GO:0070887	*	11.7	0.3	3	236	cellular response to chemical stimulus
GO:0071365	*	25.0	0.1	2	71	cellular response to auxin stimulus
GO:0009734	*	25.0	0.1	2	71	auxin mediated signaling pathway
GO:0006417	*	22.7	0.1	2	78	regulation of translation
GO:0032268	*	17.9	0.1	2	98	regulation of cellular protein metabolic process
GO:0001708	*	135.4	0.0	1	7	cell fate specification
GO:0009960	.	116.1	0.0	1	8	endosperm development
GO:0051246	.	14.2	0.2	2	123	regulation of protein metabolic process
GO:0009755	.	13.3	0.2	2	131	hormone-mediated signaling pathway
GO:0010608	.	13.2	0.2	2	132	posttranscriptional regulation of gene expression
GO:0032870	.	13.2	0.2	2	132	cellular response to hormone stimulus
GO:0071495	.	12.3	0.2	2	142	cellular response to endogenous stimulus
GO:0009733	.	11.6	0.2	2	150	response to auxin stimulus
GO:0045165	.	50.8	0.0	1	17	cell fate commitment
GO:0071310	.	9.3	0.2	2	185	cellular response to organic substance
GO:0051716	.	4.9	0.7	3	550	cellular response to stimulus
GO:0042743	.	22.5	0.0	1	37	hydrogen peroxide metabolic process

GO:0042744	.	22.5	0.0	1	37	hydrogen peroxide catabolic process
GO:0070301	.	22.5	0.0	1	37	cellular response to hydrogen peroxide

Down-regulated genes: Pyraclostrobin preventative treatment vs. Untreated *M. graminicola*-infected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0055114	*	3.9	2.7	8	1352	oxidation-reduction process
GO:0015695	*	260.0	0.0	1	3	organic cation transport
GO:0015696	*	260.0	0.0	1	3	ammonium transport
GO:0006950	.	3.2	3.2	8	1615	response to stress
GO:0006621	.	104.0	0.0	1	6	protein retention in ER lumen
GO:0009835	.	104.0	0.0	1	6	ripening
GO:0009399	.	65.0	0.0	1	9	nitrogen fixation
GO:0051651	.	57.7	0.0	1	10	maintenance of location in cell
GO:0032507	.	57.7	0.0	1	10	maintenance of protein location in cell
GO:0045185	.	57.7	0.0	1	10	maintenance of protein location
GO:0051235	.	43.3	0.0	1	13	maintenance of location
GO:0006542	.	37.1	0.0	1	15	glutamine biosynthetic process
GO:0050896	.	2.4	4.7	9	2345	response to stimulus
GO:0071215	.	30.5	0.0	1	18	cellular response to abscisic acid stimulus
GO:0009738	.	30.5	0.0	1	18	abscisic acid mediated signaling pathway

Up-regulated genes: Pyraclostrobin preventative treatment vs. Untreated *M. graminicola*-infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0019219	***	5.7	5.5	24	780	regulation of nucleobase-containing compound metabolic process
GO:0051171	***	5.6	5.6	24	793	regulation of nitrogen compound metabolic process
GO:0010556	***	5.3	5.8	24	825	regulation of macromolecule biosynthetic process
GO:0031326	***	5.2	6.0	24	844	regulation of cellular biosynthetic process
GO:0009889	***	5.2	6.0	24	844	regulation of biosynthetic process
GO:0010468	***	5.0	6.2	24	876	regulation of gene expression
GO:0080090	***	4.9	6.3	24	897	regulation of primary metabolic process
GO:0031323	***	4.8	6.4	24	911	regulation of cellular metabolic process
GO:0060255	***	4.7	6.6	24	932	regulation of macromolecule metabolic process
GO:0019222	***	4.3	7.0	24	998	regulation of metabolic process
GO:0050794	***	2.9	10.5	25	1486	regulation of cellular process
GO:0006139	***	2.7	14.8	31	2099	nucleobase-containing compound metabolic process
GO:0006807	***	2.5	18.9	36	2670	nitrogen compound metabolic process
GO:0050789	***	2.8	11.5	26	1634	regulation of biological process
GO:0065007	**	2.2	22.0	38	3121	biological regulation
GO:0006516	**	95.8	0.0	2	5	glycoprotein catabolic process
GO:0010467	*	2.2	15.0	27	2118	gene expression
GO:0006694	*	10.9	0.3	3	43	steroid biosynthetic process
GO:0009058	*	1.8	32.0	45	4530	biosynthetic process
GO:0008202	*	10.6	0.3	3	44	steroid metabolic process

GO:0034645	*	2.0	15.4	26	2176	cellular macromolecule biosynthetic process
GO:0009059	*	2.0	15.4	26	2181	macromolecule biosynthetic process
GO:0044249	*	1.7	31.2	43	4413	cellular biosynthetic process
GO:0006825	*	17.9	0.1	2	18	copper ion transport
GO:0071466	*	Inf	0.0	1	1	cellular response to xenobiotic stimulus
GO:0009410	*	Inf	0.0	1	1	response to xenobiotic stimulus
GO:0006805	*	Inf	0.0	1	1	xenobiotic metabolic process
GO:0044260	*	1.7	32.1	44	4550	cellular macromolecule metabolic process
GO:0042891	.	142.1	0.0	1	2	antibiotic transport
GO:0048657	.	142.1	0.0	1	2	tapetal cell differentiation
GO:0051504	.	142.1	0.0	1	2	diterpene phytoalexin precursor biosynthetic process pathway
GO:0010268	.	142.1	0.0	1	2	brassinosteroid homeostasis
GO:0033542	.	142.1	0.0	1	2	fatty acid beta-oxidation, unsaturated, even number
GO:0015904	.	142.1	0.0	1	2	tetracycline transport
GO:0015893	.	9.9	0.2	2	31	drug transport
GO:0042493	.	9.9	0.2	2	31	response to drug
GO:0044238	.	1.6	46.7	57	6619	primary metabolic process
GO:0009955	.	71.1	0.0	1	3	adaxial/abaxial pattern specification
GO:0009943	.	71.1	0.0	1	3	adaxial/abaxial axis specification
GO:0009944	.	71.1	0.0	1	3	polarity specification of adaxial/abaxial axis
GO:0046677	.	71.1	0.0	1	3	response to antibiotic
GO:0065001	.	71.1	0.0	1	3	specification of axis polarity
GO:0006982	.	71.1	0.0	1	3	response to lipid hydroperoxide
GO:0010044	.	71.1	0.0	1	3	response to aluminum ion
GO:0033194	.	71.1	0.0	1	3	response to hydroperoxide
GO:0015850	.	71.1	0.0	1	3	organic alcohol transport
GO:0010335	.	71.1	0.0	1	3	response to non-ionic osmotic stress
GO:0009086	.	9.6	0.2	2	32	methionine biosynthetic process
GO:0010224	.	9.6	0.2	2	32	response to UV-B
GO:0043170	.	1.5	34.6	44	4893	macromolecule metabolic process

Down-regulated genes: Pyraclostrobin preventative treatment vs. Untreated *M. graminicola*-infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006468	***	3.2	36.2	93	979	protein phosphorylation
GO:0006793	***	2.7	44.8	101	1212	phosphorus metabolic process
GO:0006796	***	2.7	44.8	101	1212	phosphate-containing compound metabolic process
GO:0016310	***	2.8	40.8	95	1105	phosphorylation
GO:0010817	***	2.5	54.3	112	1471	regulation of hormone levels
GO:0043687	***	2.5	50.8	107	1376	post-translational protein modification
GO:0009850	***	2.4	53.1	108	1438	auxin metabolic process
GO:0009851	***	2.4	52.6	107	1424	auxin biosynthetic process
GO:0042445	***	2.4	53.4	108	1447	hormone metabolic process
GO:0042446	***	2.4	52.9	107	1432	hormone biosynthetic process
GO:0006464	***	2.4	55.2	110	1496	protein modification process
GO:0065008	***	2.2	64.5	120	1746	regulation of biological quality
GO:0043412	***	2.3	57.1	110	1546	macromolecule modification

GO:0065007	***	1.8	115.2	173	3121	biological regulation
GO:0006558	***	22.2	0.8	10	22	L-phenylalanine metabolic process
GO:0019439	***	14.0	1.1	10	29	aromatic compound catabolic process
GO:0006559	***	23.6	0.6	8	17	L-phenylalanine catabolic process
GO:0009074	***	21.2	0.7	8	18	aromatic amino acid family catabolic process
GO:0044238	***	1.6	244.4	296	6619	primary metabolic process
GO:0009072	***	5.2	3.2	14	86	aromatic amino acid family metabolic process
GO:0009310	***	5.7	2.5	12	68	amine catabolic process
GO:0044267	***	1.6	109.2	150	2956	cellular protein metabolic process
GO:0042546	***	8.0	1.4	9	39	cell wall biogenesis
GO:0008152	***	1.6	317.0	360	8585	metabolic process
GO:0006952	***	2.4	15.3	34	415	defense response
GO:0019538	***	1.5	119.8	160	3243	protein metabolic process
GO:0009698	***	4.6	3.2	13	88	phenylpropanoid metabolic process
GO:0010413	***	52.6	0.2	4	6	glucuronoxylan metabolic process
GO:0010417	***	52.6	0.2	4	6	glucuronoxylan biosynthetic process
GO:0045492	***	52.6	0.2	4	6	xylan biosynthetic process
GO:0009832	***	9.7	1.0	7	26	plant-type cell wall biogenesis
GO:0006467	***	Inf	0.1	3	3	protein thiol-disulfide exchange
GO:0019748	***	2.7	9.2	23	249	secondary metabolic process
GO:0006725	***	2.7	9.3	23	252	cellular aromatic compound metabolic process
GO:0009685	***	16.5	0.5	5	13	gibberellin metabolic process
GO:0070592	**	26.3	0.3	4	8	cell wall polysaccharide biosynthetic process
GO:0071669	**	6.0	1.6	8	43	plant-type cell wall organization or biogenesis
GO:0051703	**	78.7	0.1	3	4	intraspecies interaction between organisms
GO:0010483	**	78.7	0.1	3	4	pollen tube reception
GO:0006575	**	2.7	8.2	20	222	cellular modified amino acid metabolic process
GO:0070882	**	4.0	3.1	11	84	cellular cell wall organization or biogenesis
GO:0044249	**	1.4	163.0	199	4413	cellular biosynthetic process
GO:0009225	**	7.9	1.0	6	26	nucleotide-sugar metabolic process
GO:0044237	**	1.4	260.6	297	7058	cellular metabolic process
GO:0009063	**	4.5	2.3	9	62	cellular amino acid catabolic process
GO:0044260	**	1.4	168.0	203	4550	cellular macromolecule metabolic process
GO:0043170	**	1.4	180.7	216	4893	macromolecule metabolic process
GO:0010107	**	39.4	0.2	3	5	potassium ion import
GO:0006950	**	1.5	59.6	84	1615	response to stress
GO:0010383	**	8.8	0.7	5	20	cell wall polysaccharide metabolic process

Up-regulated genes: Pyraclostrobin curative treatment vs. Untreated *M. graminicola*-infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0060255	***	5.5	6.1	25	932	regulation of macromolecule metabolic process
GO:0010468	***	5.6	5.7	24	876	regulation of gene expression
GO:0019222	***	5.1	6.5	25	998	regulation of metabolic process

GO:0019219	***	5.6	5.1	22	780	regulation of nucleobase-containing compound metabolic process
GO:0051171	***	5.5	5.2	22	793	regulation of nitrogen compound metabolic process
GO:0006139	***	3.9	13.7	36	2099	nucleobase-containing compound metabolic process
GO:0080090	***	5.1	5.9	23	897	regulation of primary metabolic process
GO:0010556	***	5.3	5.4	22	825	regulation of macromolecule biosynthetic process
GO:0031326	***	5.1	5.5	22	844	regulation of cellular biosynthetic process
GO:0009889	***	5.1	5.5	22	844	regulation of biosynthetic process
GO:0031323	***	4.7	5.9	22	911	regulation of cellular metabolic process
GO:0006807	***	3.3	17.4	39	2670	nitrogen compound metabolic process
GO:0050789	***	3.7	10.7	29	1634	regulation of biological process
GO:0010467	***	3.1	13.8	32	2118	gene expression
GO:0044260	***	2.8	29.7	51	4550	cellular macromolecule metabolic process
GO:0034645	***	3.0	14.2	32	2176	cellular macromolecule biosynthetic process
GO:0009059	***	3.0	14.2	32	2181	macromolecule biosynthetic process
GO:0050794	***	3.3	9.7	25	1486	regulation of cellular process
GO:0043170	***	2.6	31.9	52	4893	macromolecule metabolic process
GO:0065007	***	2.6	20.4	38	3121	biological regulation
GO:0044238	***	2.5	43.2	61	6619	primary metabolic process
GO:0044237	**	2.4	46.1	63	7058	cellular metabolic process
GO:0009058	**	2.2	29.6	46	4530	biosynthetic process
GO:0044249	**	2.2	28.8	45	4413	cellular biosynthetic process
GO:0008152	*	2.2	56.0	69	8585	metabolic process
GO:0016458	*	10.5	0.3	3	48	gene silencing
GO:0010587	*	Inf	0.0	1	1	miRNA catabolic process
GO:0006398	*	Inf	0.0	1	1	histone mRNA 3'-end processing
GO:0008334	*	Inf	0.0	1	1	histone mRNA metabolic process
GO:0040019	*	Inf	0.0	1	1	positive regulation of embryonic development
GO:0040029	*	8.1	0.4	3	61	regulation of gene expression, epigenetic
GO:0010629	.	7.1	0.5	3	69	negative regulation of gene expression
GO:0019362	.	6.7	0.5	3	73	pyridine nucleotide metabolic process
GO:0051131	.	154.0	0.0	1	2	chaperone-mediated protein complex assembly
GO:0034661	.	154.0	0.0	1	2	ncRNA catabolic process
GO:0009987	.	1.8	57.2	67	8765	cellular process
GO:0006733	.	6.4	0.5	3	76	oxidoreduction coenzyme metabolic process
GO:0010586	.	77.0	0.0	1	3	miRNA metabolic process
GO:0051495	.	77.0	0.0	1	3	positive regulation of cytoskeleton organization
GO:0009955	.	77.0	0.0	1	3	adaxial/abaxial pattern specification
GO:0009943	.	77.0	0.0	1	3	adaxial/abaxial axis specification
GO:0009944	.	77.0	0.0	1	3	polarity specification of adaxial/abaxial axis
GO:0065001	.	77.0	0.0	1	3	specification of axis polarity
GO:0031334	.	77.0	0.0	1	3	positive regulation of protein complex assembly

GO:0032197	.	77.0	0.0	1	3	transposition, RNA-mediated
GO:0031112	.	77.0	0.0	1	3	positive regulation of microtubule polymerization or depolymerization
GO:0031116	.	77.0	0.0	1	3	positive regulation of microtubule polymerization
GO:0032273	.	77.0	0.0	1	3	positive regulation of protein polymerization
GO:0035194	.	9.1	0.2	2	36	posttranscriptional gene silencing by RNA
GO:0016441	.	8.6	0.2	2	38	posttranscriptional gene silencing

Down-regulated genes: Pyraclostrobin curative treatment vs. Untreated *M. graminicola* - infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0042219	***	20.2	0.3	5	23	cellular modified amino acid catabolic process
GO:0006598	***	72.1	0.1	3	6	polyamine catabolic process
GO:0042402	***	54.1	0.1	3	7	cellular biogenic amine catabolic process
GO:0006595	*	16.6	0.2	3	16	polyamine metabolic process
GO:0009310	*	5.8	0.9	5	68	amine catabolic process
GO:0009850	*	1.8	20.0	33	1438	auxin metabolic process
GO:0042445	*	1.8	20.1	33	1447	hormone metabolic process
GO:0010817	*	1.8	20.4	33	1471	regulation of hormone levels
GO:0046271	*	28.7	0.1	2	7	phenylpropanoid catabolic process
GO:0046274	*	28.7	0.1	2	7	lignin catabolic process
GO:0009851	*	1.8	19.8	32	1424	auxin biosynthetic process
GO:0042446	*	1.8	19.9	32	1432	hormone biosynthetic process
GO:0006468	*	1.9	13.6	24	979	protein phosphorylation
GO:0006793	*	1.8	16.8	28	1212	phosphorus metabolic process
GO:0006796	*	1.8	16.8	28	1212	phosphate-containing compound metabolic process
GO:0019439	*	8.3	0.4	3	29	aromatic compound catabolic process
GO:0043687	*	1.7	19.1	30	1376	post-translational protein modification
GO:0065007	.	1.5	43.4	57	3121	biological regulation
GO:0006952	.	2.2	5.8	12	415	defense response
GO:0051241	.	Inf	0.0	1	1	negative regulation of multicellular organismal process
GO:0010906	.	Inf	0.0	1	1	regulation of glucose metabolic process
GO:0030420	.	Inf	0.0	1	1	establishment of competence for transformation
GO:0010453	.	Inf	0.0	1	1	regulation of cell fate commitment
GO:0010455	.	Inf	0.0	1	1	positive regulation of cell fate commitment
GO:0010187	.	Inf	0.0	1	1	negative regulation of seed germination
GO:0006110	.	Inf	0.0	1	1	regulation of glycolysis
GO:0043470	.	Inf	0.0	1	1	regulation of carbohydrate catabolic process
GO:0043471	.	Inf	0.0	1	1	regulation of cellular carbohydrate catabolic process
GO:0065008	.	1.6	24.3	35	1746	regulation of biological quality
GO:0031408	.	6.2	0.5	3	38	oxylipin biosynthetic process
GO:0031407	.	6.0	0.5	3	39	oxylipin metabolic process
GO:0016310	.	1.7	15.4	24	1105	phosphorylation
GO:0006464	.	1.5	20.8	30	1496	protein modification process

GO:0071215	.	9.0	0.3	2	18	cellular response to abscisic acid stimulus
GO:0009738	.	9.0	0.3	2	18	abscisic acid mediated signaling pathway
GO:0010033	.	2.0	6.4	12	457	response to organic substance
GO:0006567	.	71.4	0.0	1	2	threonine catabolic process
GO:0015700	.	71.4	0.0	1	2	arsenite transport
GO:0015915	.	71.4	0.0	1	2	fatty-acyl group transport
GO:0015916	.	71.4	0.0	1	2	fatty-acyl-CoA transport
GO:0043412	.	1.5	21.5	30	1546	macromolecule modification
GO:0009405	.	6.8	0.3	2	23	pathogenesis
GO:0016567	.	3.2	1.3	4	93	protein ubiquitination
GO:0031589	.	35.7	0.0	1	3	cell-substrate adhesion
GO:0007160	.	35.7	0.0	1	3	cell-matrix adhesion
GO:0044282	.	2.0	4.6	9	333	small molecule catabolic process

Up-regulated genes: Epoxiconazole-treated plants vs. untreated-uninfected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006468	***	3.5	25.7	72	979	protein phosphorylation
GO:0006793	***	3.0	31.8	78	1212	phosphorus metabolic process
GO:0006796	***	3.0	31.8	78	1212	phosphate-containing compound metabolic process
GO:0043687	***	2.9	36.1	84	1376	post-translational protein modification
GO:0016310	***	3.1	29.0	73	1105	phosphorylation
GO:0006464	***	2.7	39.3	86	1496	protein modification process
GO:0043412	***	2.6	40.6	86	1546	macromolecule modification
GO:0010817	***	2.4	38.6	77	1471	regulation of hormone levels
GO:0009850	***	2.3	37.8	74	1438	auxin metabolic process
GO:0042445	***	2.3	38.0	74	1447	hormone metabolic process
GO:0009851	***	2.3	37.4	73	1424	auxin biosynthetic process
GO:0042446	***	2.3	37.6	73	1432	hormone biosynthetic process
GO:0065007	***	1.9	81.9	125	3121	biological regulation
GO:0042538	***	16.0	0.7	8	27	hyperosmotic salinity response
GO:0065008	***	2.0	45.8	79	1746	regulation of biological quality
GO:0006972	***	12.1	0.9	8	33	hyperosmotic response
GO:0006467	***	Inf	0.1	3	3	protein thiol-disulfide exchange
GO:0009409	***	3.7	4.8	16	182	response to cold
GO:0048574	***	37.5	0.2	4	8	long-day photoperiodism, flowering
GO:0009961	***	112.2	0.1	3	4	response to 1-aminocyclopropane-1-carboxylic acid
GO:0051703	***	112.2	0.1	3	4	intraspecies interaction between organisms
GO:0010483	***	112.2	0.1	3	4	pollen tube reception
GO:0009719	***	2.6	9.5	23	362	response to endogenous stimulus
GO:0048571	***	25.0	0.3	4	10	long-day photoperiodism
GO:0009725	**	2.6	9.0	22	344	response to hormone stimulus
GO:0050794	**	1.8	39.0	62	1486	regulation of cellular process
GO:0007165	**	2.3	12.5	27	477	signal transduction
GO:0019219	**	2.0	20.5	38	780	regulation of nucleobase-containing compound metabolic process
GO:0010107	**	56.1	0.1	3	5	potassium ion import
GO:0051171	**	2.0	20.8	38	793	regulation of nitrogen compound metabolic process
GO:0010556	**	1.9	21.7	39	825	regulation of macromolecule biosynthetic process
GO:0009685	**	16.7	0.3	4	13	gibberellin metabolic process
GO:0009414	**	4.6	2.2	9	83	response to water deprivation
GO:0009415	**	4.5	2.2	9	85	response to water
GO:0031326	**	1.9	22.2	39	844	regulation of cellular biosynthetic process
GO:0009889	**	1.9	22.2	39	844	regulation of biosynthetic process
GO:0010033	**	2.2	12.0	25	457	response to organic substance
GO:0071365	**	4.8	1.9	8	71	cellular response to auxin stimulus
GO:0009734	**	4.8	1.9	8	71	auxin mediated signaling pathway
GO:0023052	**	2.0	18.0	33	685	signaling
GO:0009733	**	3.3	3.9	12	150	response to auxin stimulus
GO:0019322	**	Inf	0.1	2	2	pentose biosynthetic process
GO:0019567	**	Inf	0.1	2	2	arabinose biosynthetic process
GO:0010468	**	1.8	23.0	39	876	regulation of gene expression
GO:0006559	**	11.5	0.4	4	17	L-phenylalanine catabolic process

GO:0050789	*	1.6	42.9	63	1634	regulation of biological process
GO:0009074	*	10.7	0.5	4	18	aromatic amino acid family catabolic process
GO:0009266	*	2.5	7.4	17	280	response to temperature stimulus
GO:0080090	*	1.8	23.6	39	897	regulation of primary metabolic process
GO:0009612	*	18.7	0.2	3	9	response to mechanical stimulus

Down-regulated genes: Epoxiconazole-treated plants vs. untreated-uninfected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006952	***	8.2	3.8	24	415	defense response
GO:0006950	***	3.8	14.6	41	1615	response to stress
GO:0031640	***	33.5	0.3	8	36	killing of cells of other organism
GO:0001906	***	30.2	0.4	8	39	cell killing
GO:0050832	***	15.4	0.7	9	78	defense response to fungus
GO:0016998	***	27.1	0.3	7	37	cell wall macromolecule catabolic process
GO:0009620	***	12.8	0.8	9	92	response to fungus
GO:0050896	***	2.6	21.2	43	2345	response to stimulus
GO:0044036	***	14.5	0.6	7	63	cell wall macromolecule metabolic process
GO:0009607	***	4.9	3.0	13	332	response to biotic stimulus
GO:0051707	***	5.0	2.7	12	296	response to other organism
GO:0042743	***	17.8	0.3	5	37	hydrogen peroxide metabolic process
GO:0042744	***	17.8	0.3	5	37	hydrogen peroxide catabolic process
GO:0070301	***	17.8	0.3	5	37	cellular response to hydrogen peroxide
GO:0034599	***	16.3	0.4	5	40	cellular response to oxidative stress
GO:0034614	***	16.3	0.4	5	40	cellular response to reactive oxygen species
GO:0042886	***	Inf	0.0	2	2	amide transport
GO:0015840	***	Inf	0.0	2	2	urea transport
GO:0042044	***	Inf	0.0	2	2	fluid transport
GO:0006833	***	Inf	0.0	2	2	water transport
GO:0000272	**	11.9	0.5	5	53	polysaccharide catabolic process
GO:0042542	**	11.2	0.5	5	56	response to hydrogen peroxide
GO:0006026	**	30.6	0.1	3	14	aminoglycan catabolic process
GO:0006032	**	30.6	0.1	3	14	chitin catabolic process
GO:0000302	**	8.9	0.6	5	69	response to reactive oxygen species
GO:0051704	**	3.4	3.9	12	433	multi-organism process
GO:0071554	**	5.2	1.5	7	161	cell wall organization or biogenesis
GO:0051302	**	17.7	0.2	3	22	regulation of cell division
GO:0051781	*	55.6	0.1	2	6	positive regulation of cell division
GO:0034050	*	16.0	0.2	3	24	host programmed cell death induced by symbiont
GO:0009626	*	16.0	0.2	3	24	plant-type hypersensitive response
GO:0006030	*	14.0	0.2	3	27	chitin metabolic process
GO:0051301	*	5.0	1.3	6	144	cell division
GO:0006022	*	11.6	0.3	3	32	aminoglycan metabolic process
GO:0033554	*	3.2	3.1	9	338	cellular response to stress
GO:0006979	.	3.1	2.4	7	264	response to oxidative stress
GO:0046505	.	110.3	0.0	1	2	sulfolipid metabolic process
GO:0046506	.	110.3	0.0	1	2	sulfolipid biosynthetic process
GO:0009786	.	110.3	0.0	1	2	regulation of asymmetric cell division
GO:0015915	.	110.3	0.0	1	2	fatty-acyl group transport

GO:0015916	.	110.3	0.0	1	2	fatty-acyl-CoA transport
GO:0005975	.	1.9	8.7	15	956	carbohydrate metabolic process
GO:0010044	.	55.2	0.0	1	3	response to aluminum ion
GO:0008356	.	55.2	0.0	1	3	asymmetric cell division
GO:0055085	.	2.1	5.0	10	551	transmembrane transport
GO:0006631	.	3.0	1.7	5	191	fatty acid metabolic process
GO:0006521	.	36.8	0.0	1	4	regulation of cellular amino acid metabolic process
GO:0033238	.	36.8	0.0	1	4	regulation of cellular amine metabolic process
GO:0009056	.	1.8	8.4	14	924	catabolic process
GO:0010311	.	27.6	0.0	1	5	lateral root formation

Up-regulated genes: Epoxiconazole preventative treatment vs. Untreated *M. graminicola* - infected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006468	***	5.3	4.3	17	979	protein phosphorylation
GO:0016310	***	4.6	4.8	17	1105	phosphorylation
GO:0006793	***	4.2	5.3	17	1212	phosphorus metabolic process
GO:0006796	***	4.2	5.3	17	1212	phosphate-containing compound metabolic process
GO:0006464	***	3.6	6.5	18	1496	protein modification process
GO:0043687	***	3.6	6.0	17	1376	post-translational protein modification
GO:0043412	***	3.5	6.8	18	1546	macromolecule modification
GO:0051703	**	235.8	0.0	2	4	intraspecies interaction between organisms
GO:0010483	**	235.8	0.0	2	4	pollen tube reception
GO:0006869	**	11.3	0.5	5	114	lipid transport
GO:0010876	**	11.3	0.5	5	114	lipid localization
GO:0009725	**	5.2	1.5	7	344	response to hormone stimulus
GO:0065007	**	2.5	13.7	25	3121	biological regulation
GO:0009851	**	2.9	6.2	15	1424	auxin biosynthetic process
GO:0009719	**	5.0	1.6	7	362	response to endogenous stimulus
GO:0042446	**	2.9	6.3	15	1432	hormone biosynthetic process
GO:0009850	*	2.9	6.3	15	1438	auxin metabolic process
GO:0042445	*	2.9	6.3	15	1447	hormone metabolic process
GO:0046777	*	47.1	0.1	2	12	protein autophosphorylation
GO:0010817	*	2.8	6.4	15	1471	regulation of hormone levels
GO:0065008	*	2.5	7.6	16	1746	regulation of biological quality
GO:0010033	*	3.9	2.0	7	457	response to organic substance
GO:0071365	*	10.5	0.3	3	71	cellular response to auxin stimulus
GO:0009734	*	10.5	0.3	3	71	auxin mediated signaling pathway
GO:0010500	*	Inf	0.0	1	1	transmitting tissue development
GO:0080125	*	Inf	0.0	1	1	multicellular structure septum development
GO:0080126	*	Inf	0.0	1	1	ovary septum development
GO:0048462	*	Inf	0.0	1	1	carpel formation
GO:0048445	*	Inf	0.0	1	1	carpel morphogenesis
GO:0042475	*	Inf	0.0	1	1	odontogenesis of dentin-containing tooth
GO:0042476	*	Inf	0.0	1	1	odontogenesis
GO:0006417	*	9.6	0.3	3	78	regulation of translation
GO:0044267	*	2.1	12.9	22	2956	cellular protein metabolic process

GO:0019219	*	3.0	3.4	9	780	regulation of nucleobase-containing compound metabolic process
GO:0009888	*	5.7	0.8	4	174	tissue development
GO:0051171	*	2.9	3.5	9	793	regulation of nitrogen compound metabolic process
GO:0032268	*	7.5	0.4	3	98	regulation of cellular protein metabolic process
GO:0010556	*	2.8	3.6	9	825	regulation of macromolecule biosynthetic process
GO:0050896	*	2.1	10.3	18	2345	response to stimulus
GO:0044260	*	1.9	19.9	29	4550	cellular macromolecule metabolic process
GO:0031326	.	2.7	3.7	9	844	regulation of cellular biosynthetic process
GO:0009889	.	2.7	3.7	9	844	regulation of biosynthetic process
GO:0009723	.	13.4	0.2	2	37	response to ethylene stimulus
GO:0009735	.	13.4	0.2	2	37	response to cytokinin stimulus
GO:0010468	.	2.6	3.8	9	876	regulation of gene expression
GO:0080090	.	2.6	3.9	9	897	regulation of primary metabolic process
GO:0019538	.	1.9	14.2	22	3243	protein metabolic process
GO:0051246	.	5.9	0.5	3	123	regulation of protein metabolic process
GO:0031323	.	2.5	4.0	9	911	regulation of cellular metabolic process
GO:0031214	.	77.2	0.0	1	4	biomineral tissue development

Down-regulated genes: Epoxiconazole preventative treatment vs. Untreated *M. graminicola* -infected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0009628	***	3.5	9.3	28	759	response to abiotic stimulus
GO:0071258	***	247.4	0.0	3	4	cellular response to gravity
GO:0050896	***	2.2	28.6	51	2345	response to stimulus
GO:0010038	***	4.4	3.5	14	290	response to metal ion
GO:0010035	***	3.9	4.5	16	372	response to inorganic substance
GO:0046686	***	4.8	2.8	12	226	response to cadmium ion
GO:0051258	***	11.2	0.6	6	51	protein polymerization
GO:0044281	***	2.2	21.2	40	1740	small molecule metabolic process
GO:0006520	***	3.2	5.8	17	475	cellular amino acid metabolic process
GO:0044106	***	3.1	6.5	18	529	cellular amine metabolic process
GO:0009266	**	3.8	3.4	12	280	response to temperature stimulus
GO:0006082	**	2.5	10.1	23	829	organic acid metabolic process
GO:0019752	**	2.5	10.1	23	829	carboxylic acid metabolic process
GO:0043436	**	2.5	10.1	23	829	oxoacid metabolic process
GO:0042180	**	2.5	10.2	23	834	cellular ketone metabolic process
GO:0009308	**	2.8	7.1	18	582	amine metabolic process
GO:0006950	**	2.0	19.7	35	1615	response to stress
GO:0007017	**	5.4	1.4	7	115	microtubule-based process
GO:0042221	**	2.2	13.1	26	1073	response to chemical stimulus
GO:0071214	**	11.4	0.4	4	33	cellular response to abiotic stimulus
GO:0009651	**	3.7	2.9	10	240	response to salt stress
GO:0043623	**	5.0	1.5	7	124	cellular protein complex assembly
GO:0009063	**	7.3	0.8	5	62	cellular amino acid catabolic process
GO:0016054	*	5.6	1.2	6	95	organic acid catabolic process
GO:0046395	*	5.6	1.2	6	95	carboxylic acid catabolic process
GO:0051716	*	2.6	6.7	16	550	cellular response to stimulus

GO:0006970	*	3.4	3.2	10	260	response to osmotic stress
GO:0009310	*	6.6	0.8	5	68	amine catabolic process
GO:0046688	*	54.6	0.1	2	5	response to copper ion
GO:0019464	*	15.4	0.2	3	19	glycine decarboxylation via glycine cleavage system
GO:0009629	*	14.5	0.2	3	20	response to gravity
GO:0009409	*	3.9	2.2	8	182	response to cold
GO:0034641	*	2.3	9.1	19	748	cellular nitrogen compound metabolic process
GO:0006546	*	13.7	0.3	3	21	glycine catabolic process
GO:0009071	*	13.7	0.3	3	21	serine family amino acid catabolic process
GO:0042176	*	41.0	0.1	2	6	regulation of protein catabolic process
GO:0019253	*	12.4	0.3	3	23	reductive pentose-phosphate cycle
GO:0006547	*	32.8	0.1	2	7	histidine metabolic process
GO:0000105	*	32.8	0.1	2	7	histidine biosynthetic process
GO:0009075	*	32.8	0.1	2	7	histidine family amino acid metabolic process
GO:0009076	*	32.8	0.1	2	7	histidine family amino acid biosynthetic process
GO:0034050	*	11.8	0.3	3	24	host programmed cell death induced by symbiont
GO:0019685	*	11.8	0.3	3	24	photosynthesis, dark reaction
GO:0009626	*	11.8	0.3	3	24	plant-type hypersensitive response
GO:0008152	*	1.7	104.8	121	8585	metabolic process
GO:0006544	*	11.2	0.3	3	25	glycine metabolic process
GO:0009987	*	1.7	107.0	123	8765	cellular process
GO:0071704	*	10.7	0.3	3	26	organic substance metabolic process
GO:0010073	*	10.7	0.3	3	26	meristem maintenance
GO:0015977	*	10.7	0.3	3	26	carbon fixation

Up-regulated genes: Epoxiconazole preventative treatment vs. Untreated *M. graminicola* - infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0019219	***	8.0	3.4	19	780	regulation of nucleobase-containing compound metabolic process
GO:0051171	***	7.9	3.5	19	793	regulation of nitrogen compound metabolic process
GO:0010556	***	7.5	3.6	19	825	regulation of macromolecule biosynthetic process
GO:0031326	***	7.4	3.7	19	844	regulation of cellular biosynthetic process
GO:0009889	***	7.4	3.7	19	844	regulation of biosynthetic process
GO:0010468	***	7.1	3.8	19	876	regulation of gene expression
GO:0080090	***	6.9	3.9	19	897	regulation of primary metabolic process
GO:0031323	***	6.8	4.0	19	911	regulation of cellular metabolic process
GO:0060255	***	6.6	4.1	19	932	regulation of macromolecule metabolic process
GO:0019222	***	6.1	4.4	19	998	regulation of metabolic process
GO:0050794	***	4.6	6.5	21	1486	regulation of cellular process
GO:0050789	***	4.4	7.2	22	1634	regulation of biological process
GO:0065007	***	2.9	13.7	27	3121	biological regulation
GO:0006139	*	2.6	9.2	19	2099	nucleobase-containing compound metabolic process

GO:0010467	*	2.6	9.3	19	2118	gene expression
GO:0034645	*	2.5	9.5	19	2176	cellular macromolecule biosynthetic process
GO:0009059	*	2.5	9.5	19	2181	macromolecule biosynthetic process
GO:0071466	*	Inf	0.0	1	1	cellular response to xenobiotic stimulus
GO:0009410	*	Inf	0.0	1	1	response to xenobiotic stimulus
GO:0006805	*	Inf	0.0	1	1	xenobiotic metabolic process
GO:0006807	*	2.1	11.7	20	2670	nitrogen compound metabolic process
GO:0048657	*	231.6	0.0	1	2	tapetal cell differentiation
GO:0010268	*	231.6	0.0	1	2	brassinosteroid homeostasis
GO:0006694	.	11.5	0.2	2	43	steroid biosynthetic process
GO:0008202	.	11.2	0.2	2	44	steroid metabolic process
GO:0048656	.	77.2	0.0	1	4	tapetal layer formation
GO:0043171	.	57.9	0.0	1	5	peptide catabolic process
GO:0006516	.	57.9	0.0	1	5	glycoprotein catabolic process
GO:0048654	.	57.9	0.0	1	5	anther morphogenesis
GO:0048655	.	57.9	0.0	1	5	tapetal layer morphogenesis
GO:0048455	.	57.9	0.0	1	5	stamen formation
GO:0006751	.	57.9	0.0	1	5	glutathione catabolic process
GO:0010584	.	46.3	0.0	1	6	pollen exine formation
GO:0048448	.	46.3	0.0	1	6	stamen morphogenesis
GO:0016128	.	38.6	0.0	1	7	phytosteroid metabolic process
GO:0016129	.	38.6	0.0	1	7	phytosteroid biosynthetic process
GO:0016131	.	38.6	0.0	1	7	brassinosteroid metabolic process
GO:0016132	.	38.6	0.0	1	7	brassinosteroid biosynthetic process
GO:0009058	.	1.7	19.8	27	4530	biosynthetic process
GO:0044273	.	33.1	0.0	1	8	sulfur compound catabolic process
GO:0044260	.	1.7	19.9	27	4550	cellular macromolecule metabolic process
GO:0048658	.	25.7	0.0	1	10	tapetal layer development
GO:0010208	.	23.1	0.0	1	11	pollen wall assembly

Down-regulated genes: Epoxiconazole preventative treatment vs. Untreated *M. graminicola* -infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006468	***	3.1	18.6	48	979	protein phosphorylation
GO:0010817	***	2.6	28.0	60	1471	regulation of hormone levels
GO:0043687	***	2.6	26.2	57	1376	post-translational protein modification
GO:0006793	***	2.6	23.1	51	1212	phosphorus metabolic process
GO:0006796	***	2.6	23.1	51	1212	phosphate-containing compound metabolic process
GO:0065008	***	2.3	33.2	65	1746	regulation of biological quality
GO:0016310	***	2.7	21.0	48	1105	phosphorylation
GO:0009850	***	2.5	27.4	57	1438	auxin metabolic process
GO:0042445	***	2.4	27.5	57	1447	hormone metabolic process
GO:0006464	***	2.4	28.5	58	1496	protein modification process
GO:0009851	***	2.4	27.1	56	1424	auxin biosynthetic process
GO:0042446	***	2.4	27.3	56	1432	hormone biosynthetic process
GO:0043412	***	2.3	29.4	58	1546	macromolecule modification
GO:0019439	***	16.8	0.6	7	29	aromatic compound catabolic process
GO:0065007	***	1.9	59.4	93	3121	biological regulation
GO:0006559	***	21.9	0.3	5	17	L-phenylalanine catabolic process
GO:0009074	***	20.2	0.3	5	18	aromatic amino acid family catabolic

						process
GO:0051703	***	156.5	0.1	3	4	intraspecies interaction between organisms
GO:0010483	***	156.5	0.1	3	4	pollen tube reception
GO:0009310	***	7.1	1.3	8	68	amine catabolic process
GO:0006558	***	15.4	0.4	5	22	L-phenylalanine metabolic process
GO:0042219	***	14.6	0.4	5	23	cellular modified amino acid catabolic process
GO:0009832	**	12.5	0.5	5	26	plant-type cell wall biogenesis
GO:0006598	**	52.1	0.1	3	6	polyamine catabolic process
GO:0071669	**	8.5	0.8	6	43	plant-type cell wall organization or biogenesis
GO:0042402	**	39.1	0.1	3	7	cellular biogenic amine catabolic process
GO:0009698	**	5.3	1.7	8	88	phenylpropanoid metabolic process
GO:0015712	**	Inf	0.0	2	2	hexose phosphate transport
GO:0042546	**	7.7	0.7	5	39	cell wall biogenesis
GO:0006879	*	19.5	0.2	3	11	cellular iron ion homeostasis
GO:0008645	*	103.9	0.1	2	3	hexose transport
GO:0015749	*	103.9	0.1	2	3	monosaccharide transport
GO:0006575	*	3.0	4.2	12	222	cellular modified amino acid metabolic process
GO:0008152	*	1.6	163.4	186	8585	metabolic process
GO:0009072	*	4.7	1.6	7	86	aromatic amino acid family metabolic process
GO:0044238	*	1.5	126.0	150	6619	primary metabolic process
GO:0046777	*	17.4	0.2	3	12	protein autophosphorylation
GO:0055072	*	17.4	0.2	3	12	iron ion homeostasis
GO:0009225	*	9.5	0.5	4	26	nucleotide-sugar metabolic process
GO:0019300	*	51.9	0.1	2	4	rhamnose biosynthetic process
GO:0019299	*	51.9	0.1	2	4	rhamnose metabolic process
GO:0006826	*	8.0	0.6	4	30	iron ion transport
GO:0030243	*	5.8	1.0	5	50	cellulose metabolic process
GO:0010033	*	2.2	8.7	18	457	response to organic substance
GO:0006595	*	12.0	0.3	3	16	polyamine metabolic process
GO:0006725	*	2.7	4.8	12	252	cellular aromatic compound metabolic process
GO:0009226	*	26.0	0.1	2	6	nucleotide-sugar biosynthetic process
GO:0070882	*	4.0	1.6	6	84	cellular cell wall organization or biogenesis
GO:0009063	*	4.6	1.2	5	62	cellular amino acid catabolic process
GO:0044249	*	1.4	84.0	103	4413	cellular biosynthetic process

Up-regulated genes: Epoxiconazole curative treatment vs. Untreated *M. graminicola* - infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0019219	***	10.1	1.9	12	780	regulation of nucleobase-containing compound metabolic process
GO:0051171	***	9.9	1.9	12	793	regulation of nitrogen compound metabolic process
GO:0010556	***	9.5	2.0	12	825	regulation of macromolecule biosynthetic process
GO:0031326	***	9.2	2.0	12	844	regulation of cellular biosynthetic process
GO:0009889	***	9.2	2.0	12	844	regulation of biosynthetic process

GO:0010468	***	8.9	2.1	12	876	regulation of gene expression
GO:0080090	***	8.6	2.1	12	897	regulation of primary metabolic process
GO:0031323	***	8.5	2.2	12	911	regulation of cellular metabolic process
GO:0060255	***	8.3	2.2	12	932	regulation of macromolecule metabolic process
GO:0019222	***	7.7	2.4	12	998	regulation of metabolic process
GO:0050794	***	4.9	3.5	12	1486	regulation of cellular process
GO:0006139	**	4.3	5.0	14	2099	nucleobase-containing compound metabolic process
GO:0006869	**	17.4	0.3	4	114	lipid transport
GO:0010876	**	17.4	0.3	4	114	lipid localization
GO:0034645	**	4.1	5.2	14	2176	cellular macromolecule biosynthetic process
GO:0009059	**	4.1	5.2	14	2181	macromolecule biosynthetic process
GO:0050789	**	4.4	3.9	12	1634	regulation of biological process
GO:0006807	**	3.7	6.4	15	2670	nitrogen compound metabolic process
GO:0010467	**	3.7	5.0	13	2118	gene expression
GO:0010500	*	Inf	0.0	1	1	transmitting tissue development
GO:0080125	*	Inf	0.0	1	1	multicellular structure septum development
GO:0080126	*	Inf	0.0	1	1	ovary septum development
GO:0048462	*	Inf	0.0	1	1	carpel formation
GO:0048445	*	Inf	0.0	1	1	carpel morphogenesis
GO:0044249	.	2.4	10.5	17	4413	cellular biosynthetic process
GO:0043170	.	2.3	11.6	18	4893	macromolecule metabolic process
GO:0009058	.	2.3	10.8	17	4530	biosynthetic process
GO:0065007	.	2.3	7.4	13	3121	biological regulation
GO:0008614	.	54.1	0.0	1	9	pyridoxine metabolic process
GO:0008615	.	54.1	0.0	1	9	pyridoxine biosynthetic process
GO:0042816	.	54.1	0.0	1	9	vitamin B6 metabolic process
GO:0042819	.	54.1	0.0	1	9	vitamin B6 biosynthetic process
GO:0048449	.	39.3	0.0	1	12	floral organ formation
GO:0048645	.	33.3	0.0	1	14	organ formation
GO:0048563	.	30.9	0.0	1	15	post-embryonic organ morphogenesis
GO:0048444	.	30.9	0.0	1	15	floral organ morphogenesis
GO:0044260	.	2.0	10.8	16	4550	cellular macromolecule metabolic process
GO:0033036	.	3.2	1.4	4	575	macromolecule localization

Down-regulated genes: Epoxiconazole curative treatment vs. Untreated *M. graminicola* - infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0042219	***	43.9	0.1	4	23	cellular modified amino acid catabolic process
GO:0006598	**	101.2	0.0	2	6	polyamine catabolic process
GO:0046271	**	81.0	0.0	2	7	phenylpropanoid catabolic process
GO:0046274	**	81.0	0.0	2	7	lignin catabolic process
GO:0042402	**	81.0	0.0	2	7	cellular biogenic amine catabolic process
GO:0010817	*	2.5	7.5	16	1471	regulation of hormone levels
GO:0006595	*	28.9	0.1	2	16	polyamine metabolic process
GO:0009142	*	6.8	0.6	4	126	nucleoside triphosphate biosynthetic process
GO:0009851	*	2.4	7.2	15	1424	auxin biosynthetic process

GO:0009141	*	6.4	0.7	4	133	nucleoside triphosphate metabolic process
GO:0042446	*	2.4	7.3	15	1432	hormone biosynthetic process
GO:0009850	*	2.4	7.3	15	1438	auxin metabolic process
GO:0042445	*	2.4	7.3	15	1447	hormone metabolic process
GO:0031055	*	Inf	0.0	1	1	chromatin remodeling at centromere
GO:0031508	*	Inf	0.0	1	1	centromeric heterochromatin assembly
GO:0019439	*	15.0	0.1	2	29	aromatic compound catabolic process
GO:0009061	.	199.4	0.0	1	2	anaerobic respiration
GO:0034508	.	199.4	0.0	1	2	centromere complex assembly
GO:0015975	.	199.4	0.0	1	2	energy derivation by oxidation of reduced inorganic compounds
GO:0015947	.	199.4	0.0	1	2	methane metabolic process
GO:0015948	.	199.4	0.0	1	2	methanogenesis
GO:0043446	.	199.4	0.0	1	2	cellular alkane metabolic process
GO:0043447	.	199.4	0.0	1	2	alkane biosynthetic process
GO:0065008	.	2.1	8.8	16	1746	regulation of biological quality
GO:0031086	.	99.7	0.0	1	3	nuclear-transcribed mRNA catabolic process, deadenylation-independent decay
GO:0031087	.	99.7	0.0	1	3	deadenylation-independent decapping of nuclear-transcribed mRNA
GO:0009808	.	11.2	0.2	2	38	lignin metabolic process
GO:0009165	.	4.3	1.0	4	197	nucleotide biosynthetic process
GO:0006754	.	5.6	0.6	3	113	ATP biosynthetic process
GO:0032776	.	66.4	0.0	1	4	DNA methylation on cytosine
GO:0006333	.	5.5	0.6	3	115	chromatin assembly or disassembly
GO:0046034	.	5.4	0.6	3	117	ATP metabolic process
GO:0009145	.	5.3	0.6	3	119	purine nucleoside triphosphate biosynthetic process
GO:0009201	.	5.3	0.6	3	119	ribonucleoside triphosphate biosynthetic process
GO:0009206	.	5.3	0.6	3	119	purine ribonucleoside triphosphate biosynthetic process
GO:0009199	.	5.1	0.6	3	123	ribonucleoside triphosphate metabolic process
GO:0009144	.	5.1	0.6	3	123	purine nucleoside triphosphate metabolic process
GO:0009205	.	5.1	0.6	3	123	purine ribonucleoside triphosphate metabolic process
GO:0070828	.	49.8	0.0	1	5	heterochromatin organization
GO:0031507	.	49.8	0.0	1	5	heterochromatin assembly
GO:0080121	.	49.8	0.0	1	5	AMP transport
GO:0006575	.	3.8	1.1	4	222	cellular modified amino acid metabolic process
GO:0044237	.	1.7	35.8	44	7058	cellular metabolic process
GO:0009987	.	1.8	44.4	52	8765	cellular process
GO:0009152	.	4.7	0.7	3	133	purine ribonucleotide biosynthetic process
GO:0000956	.	39.9	0.0	1	6	nuclear-transcribed mRNA catabolic process
GO:0065007	.	1.7	15.8	23	3121	biological regulation
GO:0006164	.	4.6	0.7	3	137	purine nucleotide biosynthetic process
GO:0015866	.	33.2	0.0	1	7	ADP transport
GO:0015867	.	33.2	0.0	1	7	ATP transport

Up-regulated genes: Fluxapyroxad-treated plants vs. untreated-uninfected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006468	***	4.2	22.6	73	979	protein phosphorylation
GO:0016310	***	3.6	25.5	73	1105	phosphorylation
GO:0006793	***	3.3	28.0	74	1212	phosphorus metabolic process
GO:0006796	***	3.3	28.0	74	1212	phosphate-containing compound metabolic process
GO:0010817	***	2.9	34.0	80	1471	regulation of hormone levels
GO:0043687	***	3.0	31.8	76	1376	post-translational protein modification
GO:0009850	***	2.9	33.2	78	1438	auxin metabolic process
GO:0042445	***	2.9	33.4	78	1447	hormone metabolic process
GO:0009851	***	2.9	32.9	77	1424	auxin biosynthetic process
GO:0042446	***	2.9	33.1	77	1432	hormone biosynthetic process
GO:0006464	***	2.8	34.6	78	1496	protein modification process
GO:0043412	***	2.7	35.7	78	1546	macromolecule modification
GO:0065008	***	2.5	40.3	82	1746	regulation of biological quality
GO:0065007	***	2.0	72.1	116	3121	biological regulation
GO:0042538	***	15.1	0.6	7	27	hyperosmotic salinity response
GO:0009409	***	4.2	4.2	16	182	response to cold
GO:0006558	***	16.2	0.5	6	22	L-phenylalanine metabolic process
GO:0006972	***	11.6	0.8	7	33	hyperosmotic response
GO:0048574	***	42.8	0.2	4	8	long-day photoperiodism, flowering
GO:0006559	***	17.9	0.4	5	17	L-phenylalanine catabolic process
GO:0009074	***	16.5	0.4	5	18	aromatic amino acid family catabolic process
GO:0009961	***	128.1	0.1	3	4	response to 1-aminocyclopropane-1-carboxylic acid
GO:0051703	***	128.1	0.1	3	4	intraspecies interaction between organisms
GO:0010483	***	128.1	0.1	3	4	pollen tube reception
GO:0048571	***	28.5	0.2	4	10	long-day photoperiodism
GO:0010107	**	64.0	0.1	3	5	potassium ion import
GO:0016101	**	12.6	0.5	5	22	diterpenoid metabolic process
GO:0009310	**	5.8	1.6	8	68	amine catabolic process
GO:0009685	**	19.0	0.3	4	13	gibberellin metabolic process
GO:0044249	**	1.5	102.0	131	4413	cellular biosynthetic process
GO:0009719	**	2.6	8.4	20	362	response to endogenous stimulus
GO:0009058	**	1.5	104.7	133	4530	biosynthetic process
GO:0019439	**	8.9	0.7	5	29	aromatic compound catabolic process
GO:0009063	**	5.5	1.4	7	62	cellular amino acid catabolic process
GO:0019748	**	2.8	5.8	15	249	secondary metabolic process
GO:0007165	**	2.2	11.0	23	477	signal transduction
GO:0009266	**	2.6	6.5	16	280	response to temperature stimulus
GO:0009612	**	21.3	0.2	3	9	response to mechanical stimulus
GO:0009737	**	3.9	2.5	9	109	response to abscisic acid stimulus
GO:0009725	*	2.4	7.9	18	344	response to hormone stimulus
GO:0006950	*	1.6	37.3	56	1615	response to stress
GO:0006467	*	85.1	0.1	2	3	protein thiol-disulfide exchange
GO:0006952	*	2.2	9.6	20	415	defense response
GO:0007584	*	16.0	0.3	3	11	response to nutrient
GO:0010033	*	2.1	10.6	21	457	response to organic substance
GO:0046777	*	14.2	0.3	3	12	protein autophosphorylation
GO:0048573	*	8.1	0.6	4	25	photoperiodism, flowering
GO:0023052	*	1.9	15.8	28	685	signaling

GO:0006970	*	2.5	6.0	14	260	response to osmotic stress
GO:0009414	*	4.0	1.9	7	83	response to water deprivation

Down-regulated genes: Fluxapyroxad-treated plants vs. untreated-uninfected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006950	***	2.8	14.5	33	1615	response to stress
GO:0050896	**	2.0	21.1	36	2345	response to stimulus
GO:0034050	*	16.2	0.2	3	24	host programmed cell death induced by symbiont
GO:0009626	*	16.2	0.2	3	24	plant-type hypersensitive response
GO:0031640	*	10.3	0.3	3	36	killing of cells of other organism
GO:0006952	*	2.9	3.7	10	415	defense response
GO:0016998	*	10.0	0.3	3	37	cell wall macromolecule catabolic process
GO:0001906	*	9.4	0.4	3	39	cell killing
GO:0050832	*	6.1	0.7	4	78	defense response to fungus
GO:0051241	*	Inf	0.0	1	1	negative regulation of multicellular organismal process
GO:0019295	*	Inf	0.0	1	1	coenzyme M biosynthetic process
GO:0019296	*	Inf	0.0	1	1	coenzyme M metabolic process
GO:0010187	*	Inf	0.0	1	1	negative regulation of seed germination
GO:0009620	*	5.2	0.8	4	92	response to fungus
GO:0071554	.	3.6	1.4	5	161	cell wall organization or biogenesis
GO:0009786	.	111.3	0.0	1	2	regulation of asymmetric cell division
GO:0044036	.	5.6	0.6	3	63	cell wall macromolecule metabolic process
GO:0009651	.	2.9	2.2	6	240	response to salt stress
GO:0008356	.	55.6	0.0	1	3	asymmetric cell division
GO:0006970	.	2.7	2.3	6	260	response to osmotic stress
GO:0006521	.	37.1	0.0	1	4	regulation of cellular amino acid metabolic process
GO:0033238	.	37.1	0.0	1	4	regulation of cellular amine metabolic process
GO:0055046	.	37.1	0.0	1	4	microgametogenesis
GO:0009828	.	37.1	0.0	1	4	plant-type cell wall loosening
GO:0009886	.	6.6	0.3	2	36	post-embryonic morphogenesis
GO:0048654	.	27.8	0.0	1	5	anther morphogenesis
GO:0048655	.	27.8	0.0	1	5	tapetal layer morphogenesis
GO:0010311	.	27.8	0.0	1	5	lateral root formation
GO:0031408	.	6.2	0.3	2	38	oxylipin biosynthetic process
GO:0006355	.	2.7	1.9	5	217	regulation of transcription, DNA-dependent
GO:0006633	.	3.1	1.3	4	150	fatty acid biosynthetic process
GO:0031407	.	6.1	0.4	2	39	oxylipin metabolic process
GO:0051252	.	2.6	2.0	5	220	regulation of RNA metabolic process
GO:0045087	.	3.8	0.8	3	92	innate immune response

Up-regulated genes: Fluxapyroxad preventative treatment vs. Untreated *M. graminicola* - infected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006468	***	6.4	9.9	44	979	protein phosphorylation
GO:0016310	***	5.6	11.2	44	1105	phosphorylation
GO:0006793	***	5.0	12.3	44	1212	phosphorus metabolic process

GO:0006796	***	5.0	12.3	44	1212	phosphate-containing compound metabolic process
GO:0065007	***	3.8	31.6	71	3121	biological regulation
GO:0010817	***	4.5	14.9	47	1471	regulation of hormone levels
GO:0043687	***	4.3	13.9	44	1376	post-translational protein modification
GO:0006464	***	4.2	15.2	46	1496	protein modification process
GO:0009850	***	4.3	14.6	45	1438	auxin metabolic process
GO:0042445	***	4.2	14.7	45	1447	hormone metabolic process
GO:0043412	***	4.1	15.7	46	1546	macromolecule modification
GO:0009851	***	4.2	14.4	44	1424	auxin biosynthetic process
GO:0042446	***	4.1	14.5	44	1432	hormone biosynthetic process
GO:0065008	***	3.8	17.7	48	1746	regulation of biological quality
GO:0006559	***	42.3	0.2	5	17	L-phenylalanine catabolic process
GO:0009074	***	39.0	0.2	5	18	aromatic amino acid family catabolic process
GO:0044249	***	2.3	44.7	71	4413	cellular biosynthetic process
GO:0006558	***	29.8	0.2	5	22	L-phenylalanine metabolic process
GO:0044260	***	2.3	46.1	72	4550	cellular macromolecule metabolic process
GO:0051703	***	299.8	0.0	3	4	intraspecies interaction between organisms
GO:0010483	***	299.8	0.0	3	4	pollen tube reception
GO:0009058	***	2.2	45.9	71	4530	biosynthetic process
GO:0009310	***	11.8	0.7	7	68	amine catabolic process
GO:0019439	***	21.1	0.3	5	29	aromatic compound catabolic process
GO:0043170	***	2.1	49.6	73	4893	macromolecule metabolic process
GO:0010556	***	3.0	8.4	22	825	regulation of macromolecule biosynthetic process
GO:0019219	***	3.0	7.9	21	780	regulation of nucleobase-containing compound metabolic process
GO:0031326	***	2.9	8.6	22	844	regulation of cellular biosynthetic process
GO:0009889	***	2.9	8.6	22	844	regulation of biosynthetic process
GO:0009063	***	10.9	0.6	6	62	cellular amino acid catabolic process
GO:0051171	***	3.0	8.0	21	793	regulation of nitrogen compound metabolic process
GO:0044267	***	2.1	30.0	50	2956	cellular protein metabolic process
GO:0010468	***	2.8	8.9	22	876	regulation of gene expression
GO:0080090	***	2.7	9.1	22	897	regulation of primary metabolic process
GO:0031323	**	2.7	9.2	22	911	regulation of cellular metabolic process
GO:0050794	**	2.3	15.1	30	1486	regulation of cellular process
GO:0060255	**	2.6	9.4	22	932	regulation of macromolecule metabolic process
GO:0046777	**	33.3	0.1	3	12	protein autophosphorylation
GO:0009072	**	7.6	0.9	6	86	aromatic amino acid family metabolic process
GO:0044238	**	1.9	67.1	87	6619	primary metabolic process
GO:0050789	**	2.2	16.6	31	1634	regulation of biological process
GO:0044237	**	1.9	71.5	91	7058	cellular metabolic process
GO:0008152	**	2.0	87.0	105	8585	metabolic process
GO:0016054	**	6.9	1.0	6	95	organic acid catabolic process
GO:0046395	**	6.9	1.0	6	95	carboxylic acid catabolic process
GO:0019222	**	2.4	10.1	22	998	regulation of metabolic process
GO:0019538	**	1.9	32.9	50	3243	protein metabolic process

GO:0009856	*	7.0	0.8	5	77	pollination
GO:0009698	*	6.1	0.9	5	88	phenylpropanoid metabolic process
GO:0006108	*	13.0	0.3	3	26	malate metabolic process

Down-regulated genes: Fluxapyroxad preventative treatment vs. Untreated *M. graminicola* -infected plants at 3dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0071215	**	67.6	0.0	2	18	cellular response to abscisic acid stimulus
GO:0009738	**	67.6	0.0	2	18	abscisic acid mediated signaling pathway
GO:0009786	*	520.0	0.0	1	2	regulation of asymmetric cell division
GO:0008356	*	260.0	0.0	1	3	asymmetric cell division
GO:0006521	*	173.3	0.0	1	4	regulation of cellular amino acid metabolic process
GO:0033238	*	173.3	0.0	1	4	regulation of cellular amine metabolic process
GO:0010311	*	130.0	0.0	1	5	lateral root formation
GO:0010101	.	104.0	0.0	1	6	post-embryonic root morphogenesis
GO:0010102	.	104.0	0.0	1	6	lateral root morphogenesis
GO:0016567	.	11.8	0.2	2	93	protein ubiquitination
GO:0032446	.	10.2	0.2	2	107	protein modification by small protein conjugation
GO:0009737	.	10.0	0.2	2	109	response to abscisic acid stimulus
GO:0050794	.	2.9	3.0	7	1486	regulation of cellular process
GO:0048829	.	47.2	0.0	1	12	root cap development
GO:0010565	.	40.0	0.0	1	14	regulation of cellular ketone metabolic process
GO:0009755	.	8.3	0.3	2	131	hormone-mediated signaling pathway
GO:0032870	.	8.3	0.3	2	132	cellular response to hormone stimulus
GO:0065007	.	2.3	6.2	11	3121	biological regulation
GO:0009725	.	4.8	0.7	3	344	response to hormone stimulus
GO:0048589	.	7.7	0.3	2	141	developmental growth
GO:0071495	.	7.7	0.3	2	142	cellular response to endogenous stimulus
GO:0009719	.	4.6	0.7	3	362	response to endogenous stimulus
GO:0070647	.	7.3	0.3	2	148	protein modification by small protein conjugation or removal
GO:0048527	.	30.5	0.0	1	18	lateral root development
GO:0006825	.	30.5	0.0	1	18	copper ion transport
GO:0050789	.	2.6	3.3	7	1634	regulation of biological process
GO:0051302	.	24.7	0.0	1	22	regulation of cell division

Up-regulated genes: Fluxapyroxad preventative treatment vs. Untreated *M. graminicola* -infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0019219	***	7.6	3.7	20	780	regulation of nucleobase-containing compound metabolic process
GO:0051171	***	7.5	3.8	20	793	regulation of nitrogen compound metabolic process
GO:0010556	***	7.2	3.9	20	825	regulation of macromolecule biosynthetic process
GO:0031326	***	7.0	4.0	20	844	regulation of cellular biosynthetic process

GO:0009889	***	7.0	4.0	20	844	regulation of biosynthetic process
GO:0010468	***	6.7	4.2	20	876	regulation of gene expression
GO:0080090	***	6.6	4.3	20	897	regulation of primary metabolic process
GO:0031323	***	6.5	4.3	20	911	regulation of cellular metabolic process
GO:0060255	***	6.3	4.4	20	932	regulation of macromolecule metabolic process
GO:0019222	***	5.8	4.8	20	998	regulation of metabolic process
GO:0006869	***	18.0	0.5	8	114	lipid transport
GO:0010876	***	18.0	0.5	8	114	lipid localization
GO:0050794	***	4.3	7.1	22	1486	regulation of cellular process
GO:0050789	***	4.2	7.8	23	1634	regulation of biological process
GO:0010467	***	3.1	10.1	23	2118	gene expression
GO:0010628	***	19.0	0.2	4	51	positive regulation of gene expression
GO:0051173	**	17.5	0.3	4	55	positive regulation of nitrogen compound metabolic process
GO:0045935	**	17.5	0.3	4	55	positive regulation of nucleobase-containing compound metabolic process
GO:0034645	**	2.8	10.4	22	2176	cellular macromolecule biosynthetic process
GO:0009059	**	2.8	10.4	22	2181	macromolecule biosynthetic process
GO:0010557	**	13.5	0.3	4	70	positive regulation of macromolecule biosynthetic process
GO:0031328	**	13.3	0.3	4	71	positive regulation of cellular biosynthetic process
GO:0009891	**	13.3	0.3	4	71	positive regulation of biosynthetic process
GO:0010604	**	12.7	0.4	4	74	positive regulation of macromolecule metabolic process
GO:0006139	**	2.7	10.0	21	2099	nucleobase-containing compound metabolic process
GO:0031325	**	12.3	0.4	4	76	positive regulation of cellular metabolic process
GO:0009893	**	12.3	0.4	4	76	positive regulation of metabolic process
GO:0006094	*	48.0	0.1	2	11	gluconeogenesis
GO:0033036	*	3.7	2.7	9	575	macromolecule localization
GO:0048522	*	8.4	0.5	4	109	positive regulation of cellular process
GO:0019319	*	24.0	0.1	2	20	hexose biosynthetic process
GO:0006807	*	2.1	12.7	22	2670	nitrogen compound metabolic process
GO:0048518	*	6.2	0.7	4	146	positive regulation of biological process
GO:0006090	*	18.8	0.1	2	25	pyruvate metabolic process
GO:0046364	*	18.0	0.1	2	26	monosaccharide biosynthetic process
GO:0065007	*	2.0	14.9	24	3121	biological regulation
GO:0051568	*	212.5	0.0	1	2	histone H3-K4 methylation
GO:0046165	.	9.8	0.2	2	46	alcohol biosynthetic process
GO:0006810	.	1.9	8.1	14	1706	transport
GO:0051234	.	1.9	8.1	14	1709	establishment of localization
GO:0051179	.	1.9	8.3	14	1739	localization
GO:0006817	.	35.4	0.0	1	7	phosphate ion transport
GO:0034968	.	26.5	0.0	1	9	histone lysine methylation

Down-regulated genes: Fluxapyroxad preventative treatment vs. Untreated *M. graminicola* -infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006952	***	4.1	7.0	25	415	defense response
GO:0031640	***	14.5	0.6	7	36	killing of cells of other organism
GO:0001906	***	13.2	0.7	7	39	cell killing
GO:0006468	***	2.5	16.5	36	979	protein phosphorylation
GO:0016998	***	11.6	0.6	6	37	cell wall macromolecule catabolic process
GO:0042219	***	16.6	0.4	5	23	cellular modified amino acid catabolic process
GO:0016310	***	2.2	18.6	37	1105	phosphorylation
GO:0043687	***	2.1	23.1	43	1376	post-translational protein modification
GO:0009851	***	2.1	23.9	44	1424	auxin biosynthetic process
GO:0042446	***	2.1	24.1	44	1432	hormone biosynthetic process
GO:0006793	***	2.1	20.4	39	1212	phosphorus metabolic process
GO:0006796	***	2.1	20.4	39	1212	phosphate-containing compound metabolic process
GO:0009850	***	2.1	24.2	44	1438	auxin metabolic process
GO:0042445	***	2.0	24.3	44	1447	hormone metabolic process
GO:0006464	***	2.0	25.2	45	1496	protein modification process
GO:0043412	***	2.0	26.0	46	1546	macromolecule modification
GO:0006598	***	59.3	0.1	3	6	polyamine catabolic process
GO:0008152	***	1.8	144.3	170	8585	metabolic process
GO:0010817	***	2.0	24.7	44	1471	regulation of hormone levels
GO:0051704	**	2.8	7.3	19	433	multi-organism process
GO:0051707	**	3.3	5.0	15	296	response to other organism
GO:0042402	**	44.5	0.1	3	7	cellular biogenic amine catabolic process
GO:0009607	**	3.1	5.6	16	332	response to biotic stimulus
GO:0015712	**	Inf	0.0	2	2	hexose phosphate transport
GO:0050832	**	5.9	1.3	7	78	defense response to fungus
GO:0009808	**	9.0	0.6	5	38	lignin metabolic process
GO:0044036	**	6.3	1.1	6	63	cell wall macromolecule metabolic process
GO:0044238	**	1.6	111.3	135	6619	primary metabolic process
GO:0008645	**	118.0	0.1	2	3	hexose transport
GO:0015749	**	118.0	0.1	2	3	monosaccharide transport
GO:0009620	**	4.9	1.5	7	92	response to fungus
GO:0009744	*	16.2	0.2	3	14	response to sucrose stimulus
GO:0051703	*	59.0	0.1	2	4	intraspecies interaction between organisms
GO:0010483	*	59.0	0.1	2	4	pollen tube reception
GO:0006595	*	13.7	0.3	3	16	polyamine metabolic process
GO:0005975	*	1.9	16.1	28	956	carbohydrate metabolic process
GO:0050896	*	1.6	39.4	56	2345	response to stimulus
GO:0034285	*	11.8	0.3	3	18	response to disaccharide stimulus
GO:0065008	*	1.6	29.4	44	1746	regulation of biological quality
GO:0009698	*	4.4	1.5	6	88	phenylpropanoid metabolic process
GO:0006575	*	2.8	3.7	10	222	cellular modified amino acid metabolic process
GO:0009142	*	3.5	2.1	7	126	nucleoside triphosphate biosynthetic process
GO:0046271	*	23.6	0.1	2	7	phenylpropanoid catabolic process
GO:0046274	*	23.6	0.1	2	7	lignin catabolic process

GO:0009141	*	3.3	2.2	7	133	nucleoside triphosphate metabolic process
GO:0006816	*	16.9	0.2	2	9	calcium ion transport
GO:0000272	.	4.8	0.9	4	53	polysaccharide catabolic process
GO:0006754	.	3.3	1.9	6	113	ATP biosynthetic process
GO:0006950	.	1.5	27.2	39	1615	response to stress
GO:0019439	.	6.8	0.5	3	29	aromatic compound catabolic process

Up-regulated genes: Fluxapyroxad curative treatment vs. Untreated *M. graminicola* - infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006952	***	7.7	2.3	14	415	defense response
GO:0031640	***	31.6	0.2	5	36	killing of cells of other organism
GO:0050896	***	3.4	12.8	30	2345	response to stimulus
GO:0001906	***	28.8	0.2	5	39	cell killing
GO:0006950	***	3.6	8.8	24	1615	response to stress
GO:0005975	***	3.7	5.2	16	956	carbohydrate metabolic process
GO:0050832	***	13.4	0.4	5	78	defense response to fungus
GO:0009620	**	11.2	0.5	5	92	response to fungus
GO:0006030	**	23.8	0.1	3	27	chitin metabolic process
GO:0009269	**	93.8	0.0	2	6	response to desiccation
GO:0006022	**	19.7	0.2	3	32	aminoglycan metabolic process
GO:0043562	*	41.7	0.1	2	11	cellular response to nitrogen levels
GO:0009607	*	4.3	1.8	7	332	response to biotic stimulus
GO:0006026	*	31.3	0.1	2	14	aminoglycan catabolic process
GO:0006032	*	31.3	0.1	2	14	chitin catabolic process
GO:0051707	*	4.0	1.6	6	296	response to other organism
GO:0010500	*	Inf	0.0	1	1	transmitting tissue development
GO:0019482	*	Inf	0.0	1	1	beta-alanine metabolic process
GO:0019484	*	Inf	0.0	1	1	beta-alanine catabolic process
GO:0019295	*	Inf	0.0	1	1	coenzyme M biosynthetic process
GO:0019296	*	Inf	0.0	1	1	coenzyme M metabolic process
GO:0009865	*	Inf	0.0	1	1	pollen tube adhesion
GO:0080125	*	Inf	0.0	1	1	multicellular structure septum development
GO:0080126	*	Inf	0.0	1	1	ovary septum development
GO:0048462	*	Inf	0.0	1	1	carpel formation
GO:0048445	*	Inf	0.0	1	1	carpel morphogenesis
GO:0005976	*	4.4	1.2	5	225	polysaccharide metabolic process
GO:0051704	*	3.2	2.4	7	433	multi-organism process
GO:0006572	.	185.1	0.0	1	2	tyrosine catabolic process
GO:0006979	.	3.7	1.4	5	264	response to oxidative stress
GO:0006570	.	92.5	0.0	1	3	tyrosine metabolic process
GO:0006540	.	92.5	0.0	1	3	glutamate decarboxylation to succinate
GO:0006538	.	92.5	0.0	1	3	glutamate catabolic process
GO:0009450	.	92.5	0.0	1	3	gamma-aminobutyric acid catabolic process
GO:0009448	.	92.5	0.0	1	3	gamma-aminobutyric acid metabolic process
GO:0010236	.	92.5	0.0	1	3	plastoquinone biosynthetic process
GO:0016998	.	10.7	0.2	2	37	cell wall macromolecule catabolic process
GO:0006105	.	61.7	0.0	1	4	succinate metabolic process
GO:0009605	.	3.8	1.1	4	206	response to external stimulus

GO:0046520	.	46.3	0.0	1	5	sphingoid biosynthetic process
GO:0000272	.	7.3	0.3	2	53	polysaccharide catabolic process
GO:0007154	.	4.4	0.7	3	132	cell communication
GO:0006081	.	6.9	0.3	2	56	cellular aldehyde metabolic process
GO:0051260	.	30.8	0.0	1	7	protein homooligomerization
GO:0016337	.	30.8	0.0	1	7	cell-cell adhesion
GO:0030148	.	30.8	0.0	1	7	sphingolipid biosynthetic process
GO:0010189	.	30.8	0.0	1	7	vitamin E biosynthetic process
GO:0042360	.	30.8	0.0	1	7	vitamin E metabolic process
GO:0051259	.	26.4	0.0	1	8	protein oligomerization
GO:0045426	.	26.4	0.0	1	8	quinone cofactor biosynthetic process

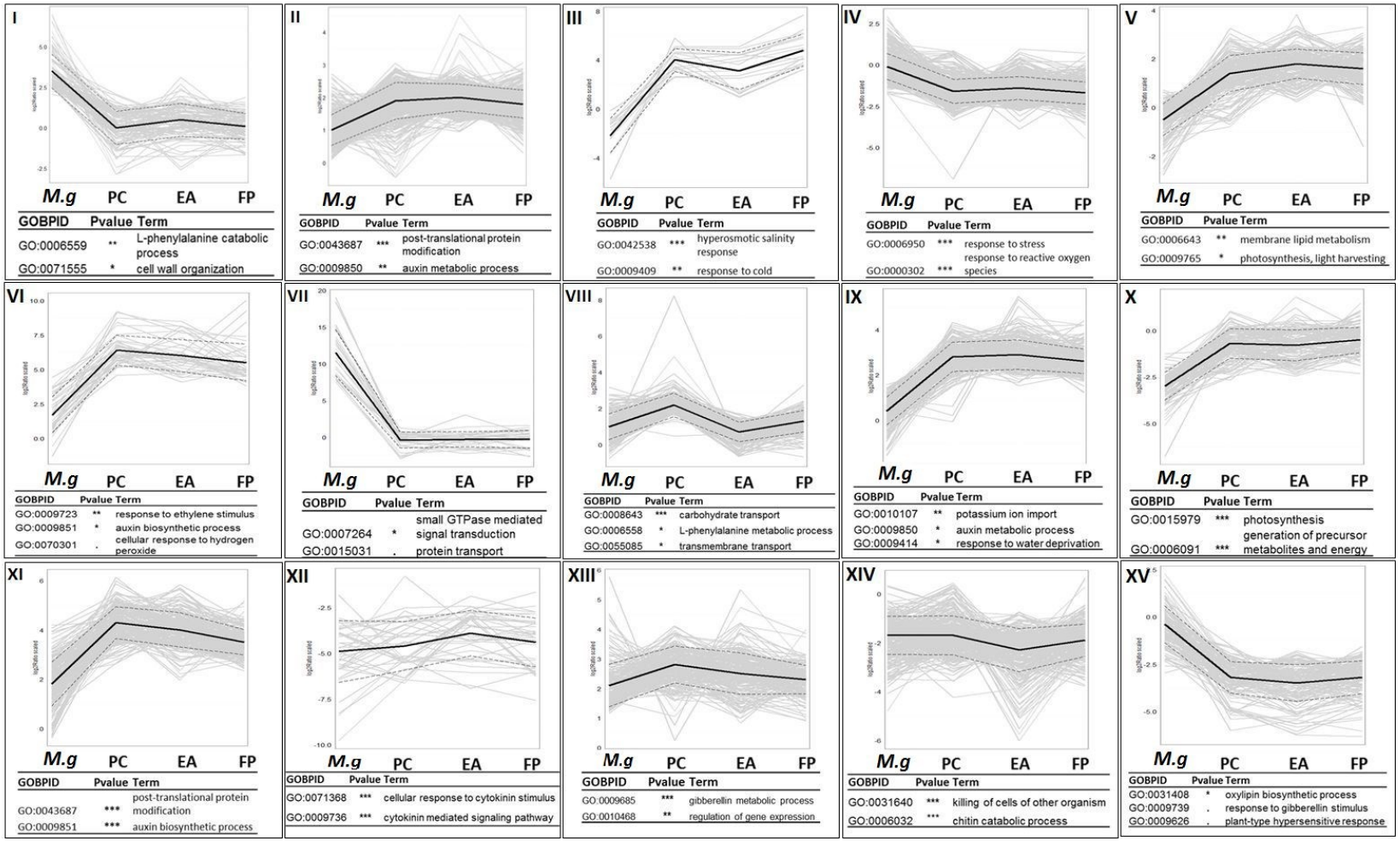
Down-regulated genes: Fluxapyroxad curative treatment vs. Untreated *M. graminicola* - infected plants at 10dpi

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0009765	***	63.8	0.9	29	83	photosynthesis, light harvesting
GO:0015979	***	23.5	2.9	41	271	photosynthesis
GO:0019684	***	35.7	1.5	32	141	photosynthesis, light reaction
GO:0018298	***	73.3	0.5	20	50	protein-chromophore linkage
GO:0006091	***	9.2	7.1	44	673	generation of precursor metabolites and energy
GO:0010196	***	67.6	0.3	10	25	nonphotochemical quenching
GO:0010114	***	33.8	0.4	10	40	response to red light
GO:0010218	***	33.8	0.4	10	40	response to far red light
GO:0009637	***	27.4	0.5	10	47	response to blue light
GO:0009639	***	10.8	1.1	10	103	response to red or far red light
GO:0009416	***	5.8	3.0	15	284	response to light stimulus
GO:0009314	***	5.5	3.1	15	299	response to radiation
GO:0044237	***	2.4	74.2	101	7058	cellular metabolic process
GO:0009768	***	43.0	0.1	4	13	photosynthesis, light harvesting in photosystem I
GO:0042550	***	96.2	0.1	3	6	photosystem I stabilization
GO:0009780	***	96.2	0.1	3	6	photosynthetic NADP+ reduction
GO:0009987	***	2.3	92.2	113	8765	cellular process
GO:0008152	***	2.2	90.3	111	8585	metabolic process
GO:0043687	**	2.3	14.5	29	1376	post-translational protein modification
GO:0050821	**	32.0	0.1	3	12	protein stabilization
GO:0031086	**	190.9	0.0	2	3	nuclear-transcribed mRNA catabolic process, deadenylation-independent decay
GO:0031087	**	190.9	0.0	2	3	deadenylation-independent decapping of nuclear-transcribed mRNA
GO:0006464	**	2.2	15.7	30	1496	protein modification process
GO:0042548	**	22.2	0.2	3	16	regulation of photosynthesis, light reaction
GO:0031647	**	22.2	0.2	3	16	regulation of protein stability
GO:0043412	**	2.1	16.3	30	1546	macromolecule modification
GO:0043467	**	20.6	0.2	3	17	regulation of generation of precursor metabolites and energy
GO:0044282	**	3.4	3.5	11	333	small molecule catabolic process
GO:0010109	**	19.2	0.2	3	18	regulation of photosynthesis
GO:0006096	*	4.5	1.7	7	159	glycolysis
GO:0000956	*	47.7	0.1	2	6	nuclear-transcribed mRNA catabolic process

GO:0009773	*	14.4	0.2	3	23	photosynthetic electron transport in photosystem I
GO:0046271	*	38.2	0.1	2	7	phenylpropanoid catabolic process
GO:0046274	*	38.2	0.1	2	7	lignin catabolic process
GO:0009628	*	2.3	8.0	17	759	response to abiotic stimulus
GO:0010608	*	4.6	1.4	6	132	posttranscriptional regulation of gene expression
GO:0006402	*	31.8	0.1	2	8	mRNA catabolic process
GO:0046496	*	6.2	0.7	4	66	nicotinamide nucleotide metabolic process
GO:0006769	*	6.2	0.7	4	66	nicotinamide metabolic process
GO:0044238	*	1.6	69.6	85	6619	primary metabolic process
GO:0006007	*	3.5	2.1	7	200	glucose catabolic process
GO:0019320	*	3.5	2.1	7	201	hexose catabolic process
GO:0046365	*	3.5	2.1	7	201	monosaccharide catabolic process
GO:0009820	*	5.8	0.7	4	70	alkaloid metabolic process
GO:0046164	*	3.4	2.2	7	210	alcohol catabolic process
GO:0006401	*	19.1	0.1	2	12	RNA catabolic process
GO:0019362	*	5.6	0.8	4	73	pyridine nucleotide metabolic process
GO:0043603	*	5.5	0.8	4	74	cellular amide metabolic process
GO:0016052	*	3.0	2.9	8	273	carbohydrate catabolic process
GO:0006733	*	5.4	0.8	4	76	oxidoreduction coenzyme metabolic process

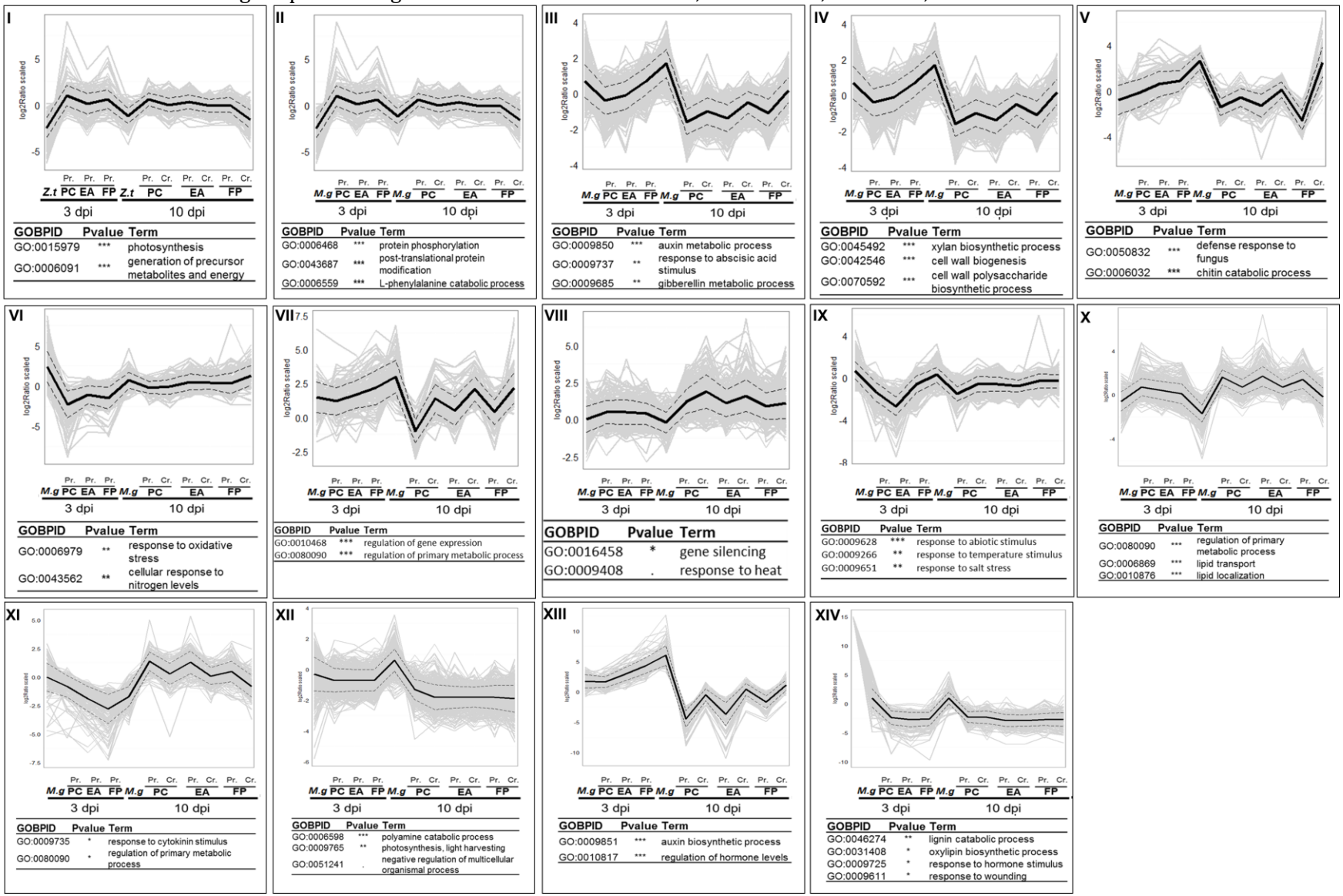
Appendix 5. Expression profile via k-mean clustering of fungicide treated plants under disease-free conditions and infected untreated plants at 3 dpi.

Displayed are the log2 scaled ratio of the gene expression analysis of i) fungicide-treated plants relative to untreated plants, and ii) the infected untreated plants relative to untreated uninfected plants. The individual expression profiles of every gene in a specific cluster are displayed as grey lines, the average profile is displayed as a black line; the dash lines represent the mean +/- the standard deviation of the expression pattern. Abbr.: *M.g*, *Mycosphaerella graminicola*; PC. Pyraclostrobin; EA., Epoxiconazole; FP., Fluxapyroxad; dpi, days post inoculation; GOBPID, gene ontology identification number of biological process. Significance codes: P < 0.0001 ***; P < 0.001 **; P < 0.01 *; P < 0.05 ·0.001 **; P < 0.01 *; P < 0.05.



Appendix 6. Cluster and gene ontology enrichment analysis of fungicide-treated plants under disease pressure and infected untreated control at 3 and 10 dpi.

Displayed are the log2 scaled ratio of the gene expression analysis of i) fungicide-treated plants relative to infected untreated plants, and ii) the infected untreated control relative to an untreated uninfected plant. The individual expression profiles of every gene in a specific cluster are displayed as grey lines, the average profile is displayed as a black line; the dash lines represent the mean +/- the standard deviation of the expression pattern. Abbr.: Pr. Preventative fungicide application; Cr. Curative fungicide treatment; *M.g*, *Mycosphaerella graminicola*; PC., Pyraclostrobin; EA., Epoxiconazole; FP., Fluxapyroxad; dpi, days post inoculation; GOBPID, gene ontology identification number of biological process. Significance codes: P < 0.0001 ***; P < 0.001 **; P < 0.01 *; P < 0.05 ·.



Erklärung

Ich erkläre:

Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Gießen, April 2014

Maria Angelica Quintero Palomar

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