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Accuracy of Real-Time PCR to Study *Mycosphaerella graminicola* Epidemic in Wheat: From Spore Arrival to Fungicide Efficiency

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1. Introduction

Mycosphaerella graminicola (Fuckel) J. Schroeter in Cohn (anamorph *Septoria tritici* Roberge in Desmaz.) is one of the most important pathogens on winter wheat in northern Europe (Leroux et al., 2007; Stammler et al., 2008b). This fungus is responsible for *Septoria* leaf blotch and causes 30–40% yield reduction under extreme conditions (Palmer & Skinner, 2002). The primary inoculum is commonly described by the arrival of *M. graminicola* ascospores (Shaw & Royle, 1989). At high humidity, spores germinate and germ tubes penetrate 12 hours post inoculation into the leaves, exclusively through the stomata. The internal colonization is still intercellular between mesophyll cells until 10–12 days post inoculation. Then, the host cells die in response to the necrotrophic mode of *S. tritici,* with the development of visual chlorosis and necrotic symptoms. The formation of brown/black pycnidia in the substomatal cavities of the necrotic spots results from asexual reproduction, which appears 14–21 days after inoculation (Kema et al., 1996). Pycnidiospores are the secondary inoculum and are responsible for the repetition of the infection in the upper foliar layers by the rain effect on the vertical spore transfer, called "splashing" (Shaw & Royle, 1993).

The application of fungicides is the most common method used for controlling this pathogen. Several families of fungicides exist and are used against *M. graminicola*, but their efficiencies differ and decline with use (Fraaije & Cools, 2008). The azole class of sterol demethylation inhibitors (DMIs), which includes triazoles, is able to control *S. tritici* targeting of the *CYP51* enzyme (14α-demethylase), and it has been used frequently over the past 25 years (Leroux et al., 2007; Stammler et al., 2008b). Over the past 20 years, however, significant changes in the sensitivity of *M. graminicola* strains toward DMI fungicides have been widely reported (Leroux et al., 2007), especially over the past five years in the case of triazoles.

Resistance to DMIs has been described by various mechanisms, one of which is point mutations in the *CYP51* gene, which reduces the affinity between DMIs and the 14α-demethylase enzyme (Leroux et al., 2007; Stammler et al., 2008b). To date, 17 DMI-resistant genotypes (R-types) characterized by amino acid alterations have been reported in the *M. graminicola CYP51* gene: L50S, V136A/C, A379G, S188N (Leroux et al., 2007); G510C, N513K, Y137F (Cools et al., 2005; Leroux et al., 2007); I381V (Cools et al., 2005; Leroux et al., 2007);

and Y459D/S/N/C, G460D, Y461H/S, ΔY459/G460 (Cools et al., 2005; Leroux et al., 2007). Each *CYP51* variant is selected differently by triazole fungicides, and there are two main categories: strains that contain the isoleucine amino acid at the position 381 of the *CYP51* protein sequence (I381) and strains with the point mutation (V381), where isoleucine is replaced by valine (Leroux et al., 2007; Stammler et al., 2008b).

As this disease has a long latent period, presymptomatic detection and quantification of *M. graminicola* and genotypes that are related to different DMI resistance levels are very important for the effective control of strains that are highly resistant to DMIs. This allows better timing, choice and dose of fungicide applications. However, it is not possible to achieve such detection by conventional methods, and hence fungicides cannot be applied until visible symptoms appear.

Molecular detection of disease in plants was developed using the polymerase chain reaction (PCR) technique, which offers a rapid and sensitive diagnostic method (Henson and French 1993). The association of specific chemistries and a fluorescent reporter molecule (TaqMan chemistry) with PCR has permitted the development of real-time PCR (Schena et al., 2004). This technique is an accurate and reliable tool for many phytopathological studies (Guo et al., 2006 & 2007; Fraaije et al., 1999; Schena et al., 2004). Real-time PCR analysis, also referred to as quantitative PCR (qPCR), can provide a deeper insight into host–pathogen interactions by detecting the primary inoculum, general infection level and genotypes, depending on the specific target gene. For *M. graminicola*, qPCR based on the specific and stable gene β-tubulin has been described as a diagnostic tool by (Bearchell et al., 2005) and on the *CYP51* gene for 14α-demethylase mutations by (Selim, 2009).

In the present study, we used qPCR to study the epidemiological context of *M. graminicola,* taking into account the effect of many factors, including external contamination by ascospores, cultivars resistance, leaf colonization stages and fungicide efficiency. The correlation between results of molecular qPCR analysis and visual symptoms observations were investigated.

2. Materials and methods

2.1 In vivo studies of *Septoria* leaf blotch using qPCR

2.1.1 Trapping of spores

The spore trap assay is described by (Fraaije et al., 2005). A spore trap was installed in the field, at a distance of 3 m from the wheat trial in order to avoid the capture of pycnidiospores that move vertically as a result of splashing raindrops. Spores were collected from a plastic film, and then they were ground using an MM 300 Mixer Mill (Qiagen, USA) in a 2-ml Eppendorf® Safe-lock micro test tube containing 250 mg white quartz (Sigma® S-9887) and one 5-mm stainless steel bead (Cat. No. 69989, Qiagen, France).

2.1.2 Evolution of *Septoria* leaf blotch and resistant wheat cultivars

The field sites were located at the Beauvais Agricultural Research Station of the Institut Polytechnique LaSalle-Beauvais, Beauvais, France. Based on the susceptibility to *M. graminicola*, one resistant cultivar (Maxwell) and four susceptible cultivars (Dinosor, Alixan, Trémie and Maxyl) were selected (Table 1). Rainfall, temperature and leaf wetness in the trial field were monitored daily (Figure 1a). Trials were performed during the 2008–2009 growing season using a completely randomized block design with three replicate plots.

Fertilization was according to plant requirements and protection against other diseases was carried out. The plot size was 2 × 12 m. Twenty leaves were randomly sampled from one foliar layer of the main stem. The foliar layer number (Fn) was determined by counting the position of the leaf from the flag leaf (F1). Disease evolution was determined by assessing visual symptoms and by qPCR analysis. The necrotic area related to Septoria blotch was recorded and then the leaves were stored at –80 °C until lyophilization.

Cultivar	MAXWELL	DINOSOR	ALIXAN	TREMIE	MAXYL
Producer	Saaten Union	Unisigma	Nickerson	Serasem	Momont
Year	2008	2005	2005	1992	2005
Susceptibility rating*	7	4	4	4	5

* Susceptibility rating (1–9): 1 (susceptible) to 9 (resistant).

Table 1. Wheat cultivars used in the resistance study against *Mycosphaerella graminicola*

2.1.3 *S. tritici* DMI-resistant genotypes

S. tritici DMI-resistant genotypes were determined for wheat leaf samples collected from the five wheat cultivars (listed above) grown under field conditions. Intentional mismatch primers (Table 2) and allele-specific qPCR were used as described by (Selim, 2009), to quantify the mutation proportions of *CYP51* in DNA samples.

Primers	Sequence (5′–3′)	Mutation	GenBank accession number	Amplicon size	T_m
SYBER Green					
MG for	CCTCGCCGAACTTACGATCT		EF418622		58
MG rev	CGCGGACTTCCTTTCCTTG			60	59
R6&7- for	CCCTTCGTATTCACGCTCgA**G**	I381V	EF418630		60
R6 rev	GCTTACCAGGCCGTAGCCA		EF418628	276	60
R7- rev	CCTTGCTTAC**C**AGGCCGT**t**//GT	ΔY459/G460	EF418630	273	60
R7+ for	CAAAGAAACCCTTCGTATTCAtG**G**	A379G			60
R7+ rev	AATGGAGGCAGTCGGGAAA			167	59
TaqMan					
MG for	GCCTTCCTACCCCACCATGT		AY547264	63	60
MG rev	CCTGAATCGCGCATCGTTA				60
MG P	FAM-TTACGCCAAGACATTC-MGB				69
W for	AGTCGTCAAAGAAACCCTTCGTA		EF418622	106	58
W rev	GGTGGTTGGAATGACGTATGC				59
W P	Vic-CGCTCCAATCCACTC-MGB				68

Lower case nucleotide indicates the intentional mismatch, nucleotides in bold are located at the site of single nucleotide polymorphism (SNP), (for) and (rev) indicate forward and reverse orientation, (W) indicates wild-type specific primer, (T_m) indicates primer melt temperature, (//) and Δ**Y**459/**G**460 indicate double deletion of tyrosine (Y) and glycine (G) at positions 459 and 460 of the amino acid sequence, respectively.

Table 2. Sequences of primers and probes used to determine moderate resistant (R6, R7– or R7+) or general (MG) genotypes of *M. graminicola*

2.2 *In vitro* studies of *Septoria* leaf blotch using qPCR

2.2.1 Plant material

Susceptible and resistant winter wheat cultivars Dinosor and Maxwell, respectively, were used. Grains were disinfected by incubation with 1% sodium hypochlorite for 5 minutes, with shaking, and then washed three times in autoclaved distilled water. Grain germination was realized in a 0.5% water–agar medium. After incubation in darkness; 24 hours at 20 °C, 48 hours at 4 °C and then 24 hours at 20 °C (Arraiano et al. 2001), grains were transferred into 500 ml pots containing an autoclaved soil mixture of horticultural compost, sand and silt–loam soil (1:1:2 v/v/v). Pots were incubated at 15 °C, for a 16-hour photoperiod, with 150 µmol of photon.$m^{-2}.s^{-1}$. Plantlets were watered twice a week with 50 ml distilled water per pot, and once a week with 50 ml water containing 25% Murashige and Skoog basal medium (Sigma® M 5519).

2.2.2 Inoculum preparation

M. graminicola isolates T0248, T0254 and T0256 were obtained from *S. tritici* collection strains held in the authors' laboratory. Sporidia (yeast-like cells) stored at –80 °C were activated by transfer to fresh potato dextrose agar medium (39 $g.l^{-1}$, Sigma, USA). After 10 days of incubation at 18 °C, with a 12-hour photoperiod, mycelia and spores were scraped off the surface and grown in a liquid yeast–sucrose medium (yeast extract 10 $g.l^{-1}$, sucrose 10 $g.l^{-1}$; Sigma, USA) for 7 days at 18 °C with permanent light (100 µmol of photon.$m^{-2}.s^{-1}$) and shaking (150 rpm). Spores were collected by centrifugation at 1500 rpm for 5 minutes at 15 °C, washed twice with sterile distilled water, and then suspended in 10 mM $MgSO_4$ (Sigma® M-9397) containing 0.1% Tween 20 surfactant. The concentration was adjusted to 10^5 spores.ml^{-1}.

2.2.3 Plantlet inoculation and leaf sampling

Twenty-one-day-old plantlets were inoculated either with 10 µl (one drop) of *M. graminicola* inoculum (10^5 spores.ml^{-1}) at the bottom part of the second leaf for microscopic observations, or by spraying a 3 ml covering over the whole plantlet to assess fungicide efficiency. The plants were enveloped in a transparent polyethylene bag for 3 days. The bag provided an atmosphere of saturated humidity around the inoculated leaves. Disease development was followed for 1 month after inoculation by sampling inoculated leaves at 0, 1, 2, 3, 5, 7, 12, 14, 16, 19, 23, 26, 28 and 30 days post inoculation (dpi). Six leaves per leaf layer were collected each time, three for the microscopic observations and three for qPCR analyses.

2.2.4 Fungicide treatments

JOAO (prothioconazole 250 g a.i. l^{-1}, Bayer CropSciences, Langenfeld, Germany) in its recommended dose (0.8 $l.ha^{-1}$) was tested for its efficiency in containing the development of *Septoria* disease under controlled conditions. Depending on the day of inoculation (d0), four modalities of fungicide application were tested: one preventive modality at day one before inoculation (d–1) and three curative modalities at 3, 7 and 10 days after inoculation (d+3,

d+7 and d+10, respectively). Each plant was covered once with 3 ml of the fungicide solution, supplemented with one drop of Tween 20, using a TLC sprayer (Grace®, USA).

2.2.5 Microscopic observations

Microscopic observations were carried out according to (Shetty et al., 2003), with modifications. Leaves were cut and cleared by placing them overnight between two filter papers saturated with glacial acetic acid and absolute ethanol (1:3 v/v). They were then washed three times with distilled water and stored between two filter papers in a solution of lactoglycerol (lactic acid/glycerol/distilled water, 1:1:1, v/v/v) until observation. Coloration was carried out by incubating the leaf parts in 0.1% trypan blue in lactoglycerol for 1 hour at 50 °C.

Stained slides were microscopically assessed using a Leica DM 4500P research microscope (Leica Microsystems, Bensheim, Germany). Further magnification was achieved by analyzing the surface of cryofractured nonstained leaf fragments with an electron microscope (TM-1000 Tabletop Microscope; HITACHI High-Technology Corporation, Japan).

2.2.6 DNA extraction

All plant leaves analyzed by qPCR had less than 40% necrotic surface. Collected leaf samples were placed directly in liquid nitrogen and then lyophilized in a Virtis 12 XL lyophilizer for 48 hours. The dried samples were then ground using an MM 300 Mixer Mill (Qiagen, USA). DNA was extracted using the DNeasy 96 Plant kit (Qiagen, USA) according to the manufacturer's protocol. DNA was quantified by measurement of UV absorption at 260 nm (Cary 50 UV–Vis spectrophotometer; Varian, France).

2.2.7 Quantification of *S. tritici* using qPCR analysis

To quantify infection levels of *S. tritici*, primers and TaqMan minor groove binder probes were used to target a 63-bp fragment of the β-tubulin gene (GenBank accession no. AY547264), as described in (Bearchell et al., 2005). A TaqMan assay was carried out in 25 µl of a reaction mixture that contained the following: 12.5 µl Universal TaqMan PCR Master Mix (Applied Biosystems, USA), 0.3 µM of each primer, 0.2 µM probe, 200 ng DNA and water up to a volume of 25 µl. The conditions of qPCR determination were the following: 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. All qPCR experiments were carried out using an ABI PRISM 7300 sequence detection system (Applied Biosystems, USA).

qPCR analysis of the *M. graminicola* β-tubulin gene was calibrated from 10^2 to 10^7 copies by serial dilution of the appropriate cloned target sequence.

3. Results

3.1 External contamination by ascospores

Data in figure 1a show that there was dry weather during the experimental season (2008–2009) and low temperatures of around 10 °C until the end of April. Results of spore

capture showed a low level of external ascospore contamination (Figure 1b). Three periods of contamination were observed. The first period was during April, with two main peaks that represented 30 and 3000 ascospores per day. The second was the main period, which represented a continuous arrival of ascospores during May, with a stable number of about 100 spores per day. The third period was a classical period of ascospore production, which was at the end of the wheat growing season during July (about 30 spores per day).

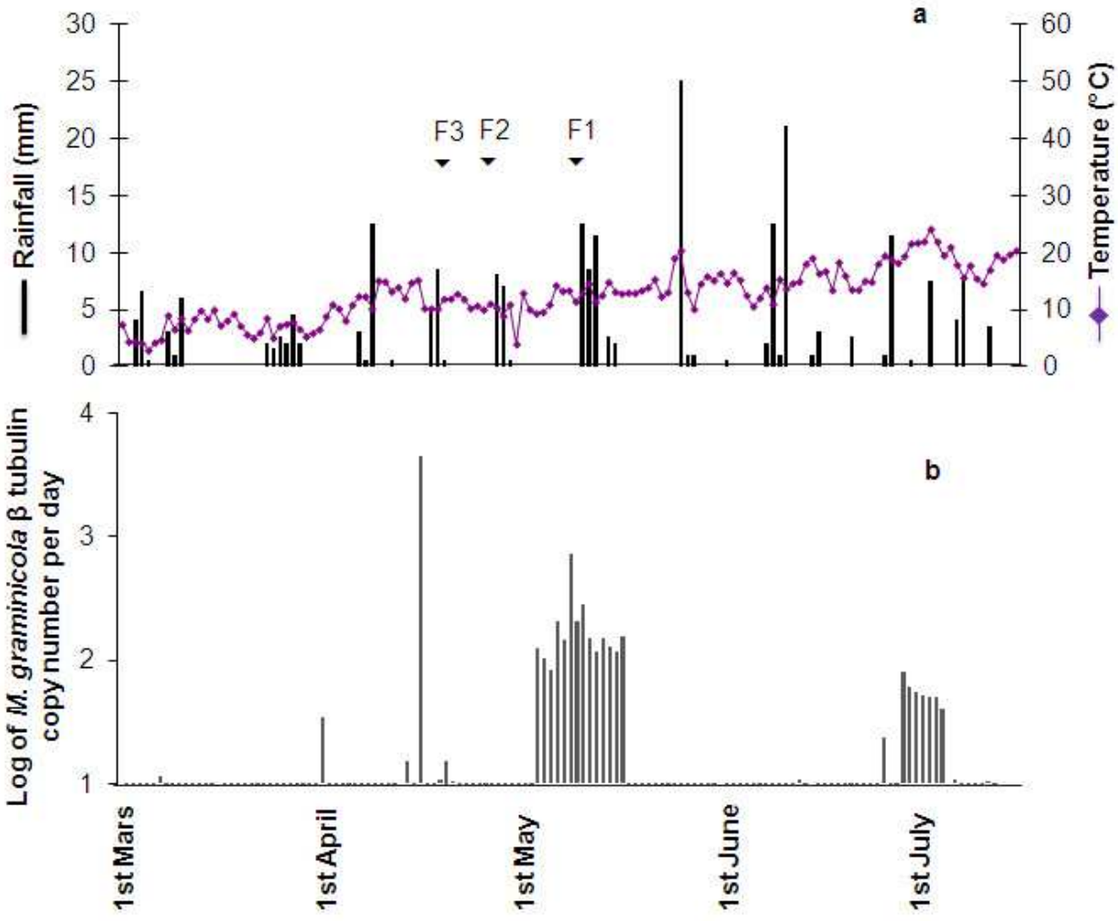

Fig. 1. (a) Weather conditions and (b) temporal dispersal of ascospores in *M. graminicola* DNA presented as the amount per day.

3.2 Evolution of fungal biomass

Evolution of *S. tritici* was characterized by simultaneously using visual symptoms and *M. graminicola* β-tubulin gene qPCR (Figure 2). Observation of necrotic areas on the top three leaf layers of the two cultivars Maxwell and Dinosor showed late disease development

during June, with a "croissant"-like gradient from the top of the plant (flag leaf) to the bottom. The epidemic began in the susceptible cultivar Dinosor 15 days earlier than in the resistant cultivar Maxwell. By 16 June (Zadok's growth stage (GS) 85), Dinosor's leaf necrotic surface was 5, 22 and 25% for F1, F2 and F3, respectively, whereas Maxwell had not shown any symptoms. Two weeks later, all three leaf layers of Dinosor had more than 60% necrotic surface, whereas the values for Maxwell were 11.6, 23.5 and 67.5%, respectively.

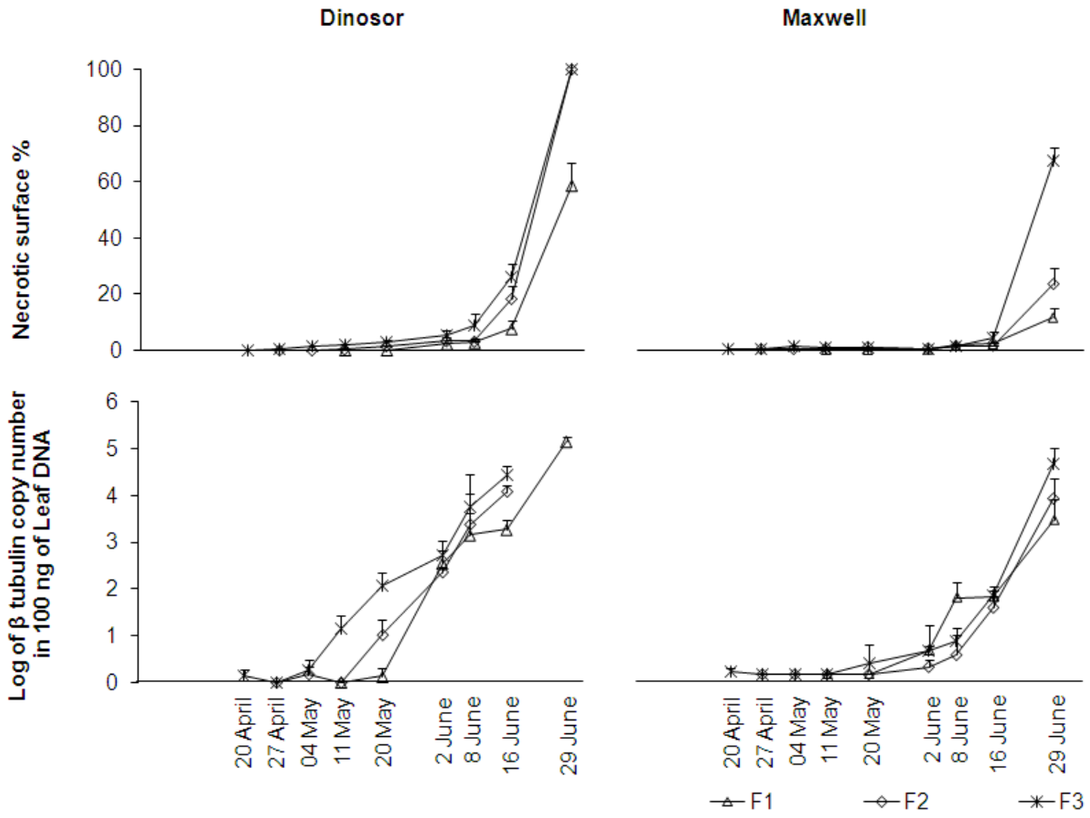

Fig. 2. Septoria blotch disease progression on Dinosor and Maxwell cultivars measured by necrotic surface observation and by qPCR.

The development of epidemics of *M. graminicola* was detected earlier with qPCR analysis (Figure 2) than when the same samples were observed visually. qPCR was not used for samples with > 40% of the surface area necrotic because of the negative effect observed on the accuracy of analysis. The *M. graminicola* β-tubulin gene on F3 was observed 2–4 weeks before symptoms appeared. There were five copies of the β-tubulin gene per 100 ng leaf DNA (CBT_{100ng}). The detection of *M. graminicola* DNA was two weeks later in the resistant cultivar than in the susceptible cultivar. The croissant gradient of the disease from the top of the plant to the bottom was also observed by qPCR analysis on all analysis dates except 2 and 8 June (GS 61 and 65 for Maxwell and GS 64 and 70 for Dinosor, respectively), when F1 qPCR values were higher than F2 and higher than or equal to F3. This increase was 14 days

after the second period of the external contamination as determined by spore capture. This was characterized by the continuous arrival of ascospores (Figure 1b), combined with disease-favorable conditions, a high rate of precipitation (25 mm) and a temperature of 20–25 °C (Figure 1a).

3.3 Wheat resistance to Septoria leaf blotch

The resistance of wheat cultivars to *M. graminicola* was evaluated using visual symptoms assessment and qPCR analysis. Leaf samples were collected from the two leaf layers below the flag leaf, from the F3 leaf layer on 2 June at GS 61, 64, 65, 68 and 67, and from the F2 leaf layer on 16 June at GS 72, 71, 73, 73 and 73, of Maxwell, Dinosor, Alixan, Tremie and Maxyl, respectively. Results obtained for the *M. graminicola* DNA amount (Figure 3b), using either the F3 or F2 leaf layer, correlated well with the susceptibility rating given by the Arvalis Institut du Végétal (Arvalis Institut du Végétal 2008). Where two statistical groups were obtained, one presented the four susceptible cultivars (Dinosor, Alixan, Trémie and Maxyl) and the second presented the resistant cultivar (Maxwell). A close correlation was obtained between the results of the amount of DNA and the percentages of leaf necrotic area, especially when using the F2 leaf layer, where the discrimination between the two categories was clearer than with the F3 leaf layer (Figure 3a).

3.4 Distribution of R-types of *M. graminicola* to DMI

Quantification of the R-types of *M. graminicola* was not possible in the case of the Maxwell cultivar because of its low level of contamination. Therefore, the quantification of R-types (R6, R7–, R7+ and I381) was realized only for the susceptible cultivars Dinosor, Alixan, Trémie and Maxyl. Analyses of R-types were carried out on two dates and using two leaf layers: F3 leaf samples were used on 2 June and F2 leaf samples on 16 June. For F3, the averages of CBT_{100ng} were 1492, 2284, 1378 and 3606, and for F2 were they 3174, 5194, 3591 and 3642 for Dinosor, Alixan, Trémie and Maxyl, respectively. No significant differences were observed between R-types over the four cultivars used and over the two dates of analyses (Figure 3c). Regardless of the wheat cultivar, a high proportion of V381-strains (≥ 94%) was found, with an average of 74.2, 0.1 and 19.7% for R6, R7– and R7+ genotypes, respectively, whereas, I381-strains represent only 6% of all *M. graminicola* populations.

3.5 Fungicide efficiency

The effect of fungicide application on the development of Septoria blotch disease was investigated in an *in planta* experiment over 21 days. Within this period, the nongreen leaf area reached 15% on nontreated-inoculated control plants. Between 3 to 15 dpi, disease dynamics on control plants was slow (CBT_{100ng} was 113–237 for Dinosor and 67–191 for Maxwell) but accelerated between 15 to 21 dpi to reach 1922 and 764 for Dinosor and Maxwell, respectively. The area under the disease progression curve (AUDPC) was calculated using qPCR analysis (CBT_{100ng}) for the period from 3 to 21 dpi. For control plants, AUDPC was 8633 and 4614 for Dinosor and Maxwell, respectively. The AUDPC for all fungicide treatments was lower than in the control. Figure 4 shows a significant negative effect of all fungicide treatments on the development of fungal DNA regardless of the plant

cultivar. The most effective application occurred with the preventive treatment (d–1), where fungal DNA was strongly decreased on all dates of observations: protection levels of 79% and 85% were achieved for Dinosor and Maxwell, respectively. Microscopic observations showed that spores failed to germinate when fungicide was applied one day before inoculation (Figure 5). Curative treatment (d+3) was similar to preventive treatment; the level of protection for Dinosor and Maxwell was 73 and 71%, respectively. Less fungicidal impact was observed when the fungicide was applied seven or ten days after inoculation (d+7 and d+10); the level of protection was 45–60%.

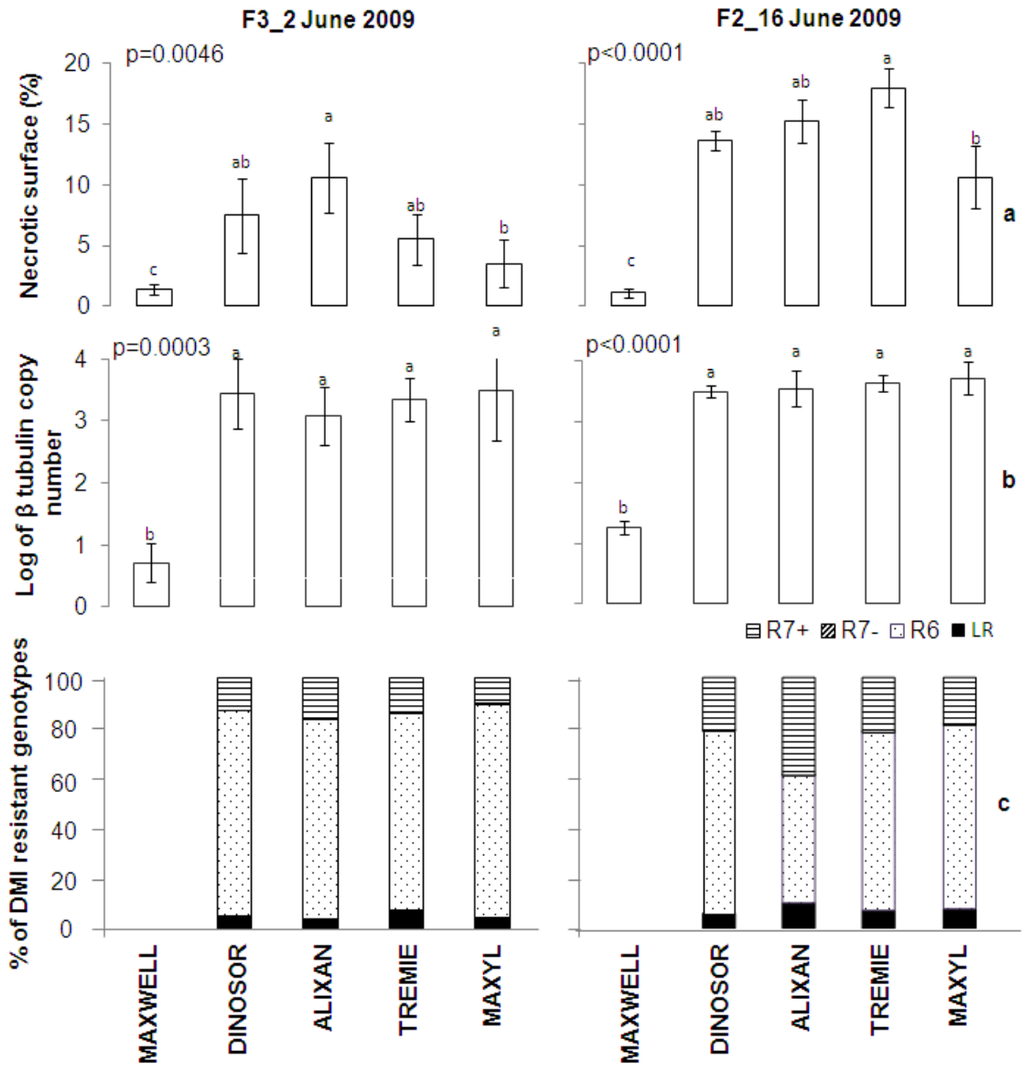

Fig. 3. (a and b) Evaluation of cultivar resistance using visual symptoms observations and qPCR. (c) Frequency of DMI-resistant genotypes of *M. graminicola*; low resistant (I381) and moderate resistant (R6, R7– and R7+). (Leaf samples were collected from leaf layers F3 and F2 on 2 and 16 June 2009, respectively.)

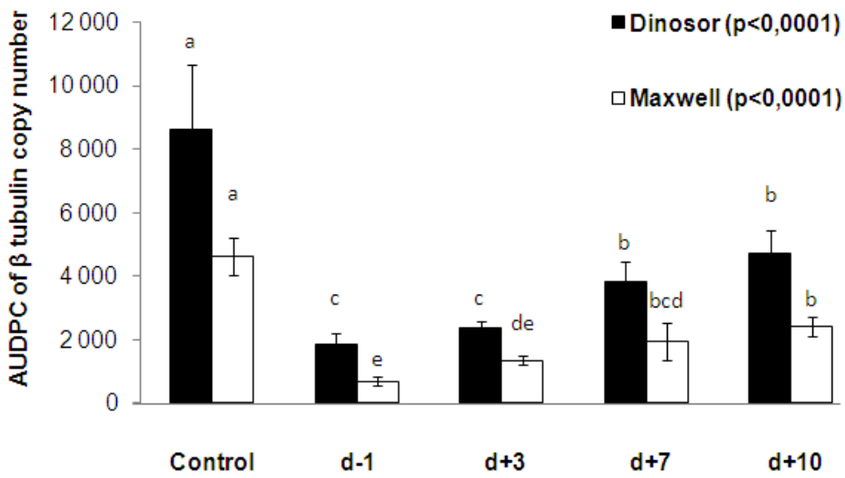

Fig. 4. Prothioconazole efficiency against Septoria leaf blotch on Maxwell and Dinosor: d–1, d+3, d+7 and d+10 are the fungicide treatments used one day before inoculation, and 3, 7 and 10 days after inoculation, respectively.

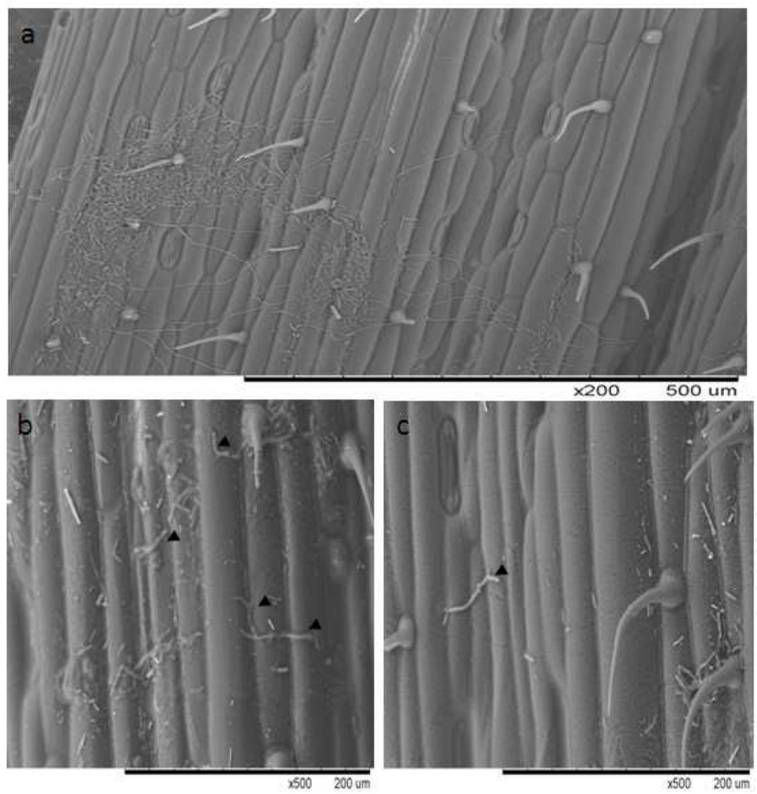

Fig. 5. Electron microscopic exposures of Dinosor leaves sampled at 15 dpi by a 10 µl-drop of *M. graminicola* conidiospores $10^5.ml^{-1}$: (a) nontreated control, where almost all spores germinated and stomata near from the point of inoculation were penetrated; and (b and c) wheat leaf treated with prothioconazole one day before inoculation (d–1), where spores failed to germinate and almost all spores degraded.

3.6 Validation of qPCR analyses

3.6.1 Relationship between qPCR analyses and *S. tritici* necrotic symptoms

The second leaf layer (F2) of the susceptible cultivar Maxyl was used to study the correlation between DNA amounts measured by qPCR for leaf samples with different levels of necrotic surfaces, from 0 to 100% (Figure 6). *M. graminicola* DNA was detected in samples without symptoms and the DNA amount increased proportionally with an increase in leaf necrotic surface. A high level of correlation (CF = 0.95) was observed up to 40% leaf necrotic surface. The amount of *M. graminicola* DNA decreased beyond 50% leaf necrotic surface and remained stable.

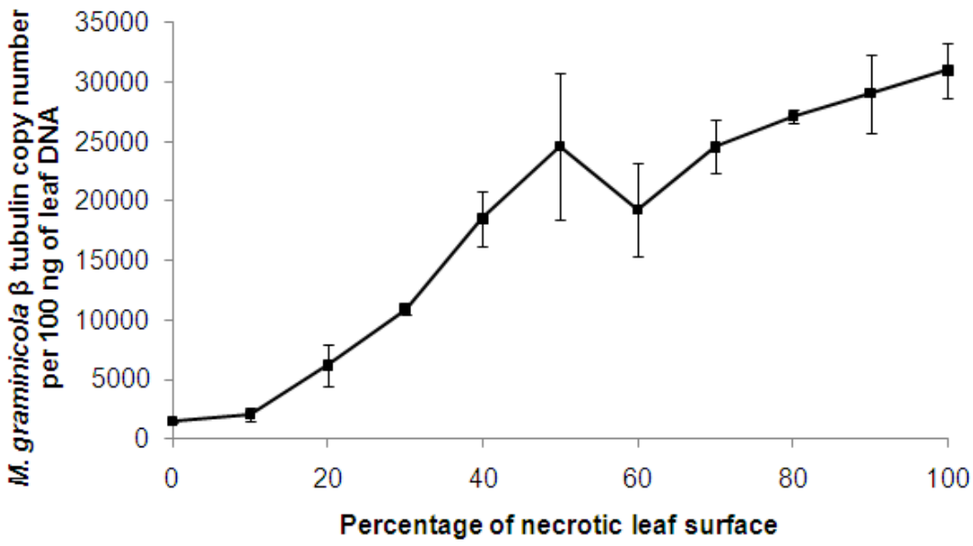

Fig. 6. Linear relationship between necrotic symptoms and qPCR analysis for cultivar Maxyl (F2 leaf layer)

3.6.2 Relationship between qPCR analysis and fungal colonization stages

To understand the relationship between the amounts of *M. graminicola* DNA measured by qPCR and leaf colonization stage, a *M. graminicola*–wheat pathosystem with one point of inoculation was developed. The base part of the second leaf of 21-day-old plantlets (GS 13) was inoculated using 10 µl (one drop) of *M. graminicola* inoculum (10^5 spores.ml^{-1}). Fungal colonization stages and the development of the amount of DNA are tabulated in Table 3.

One day after inoculation, 70% of spores had germinated and almost all germ tubes growing on the leaf surface were oriented toward the stomata (Figure 7a and b). Stomata located near the inoculation point were penetrated (Figure 7c and d). Appressorium-like structures (swellings of germ tube tips) were observed in contact with the ridges found at the guard cell lips (Figure 8a and b) and also sometimes over anticlinal walls, within the depressions

between epidermal cells (Figure 8c, d and e). Electron microscopic observations of cryofractured leaf samples showed a direct penetration of leaf tissue in positions far from the stomata (Figure 8f). Intercellular colonization by mycelia growth was observed 5 and 7 dpi (Figure 7e). It was characterized by a progression from the inoculation point toward the top part of the leaf. This progression was fast at the beginning and became slower until 9 dpi. At 12 dpi, 55% of stomata were colored blue and cell death reached 20% of the leaf surface (Figure 7f). Disease development remained stable at this level until the appearance of symptoms at 26 dpi. This started from the leaf tip and progressed downward. Under the conditions used in this experiment, no pycnidium formation was observed by visual or microscopic observations.

Day post inoculation	Colonization state	**CBT_g***
0	Inoculation	327
1	Germination	367
3	Penetration	443
5–7	Internal colonization	533–564
12	Cells degradation	683
26	Symptoms (2%)	1785

*Copy of *M. graminicola* β-tubulin gene per gram of fresh leaf.

Table 3. Relationship between *M. graminicola* colonization stages and results of qPCR analysis

The qPCR assay was performed for samples collected on the same dates as for samples used for the microscopic observations. *M. graminicola* DNA evolution was characterized by an increase in the number of β-tubulin gene copies per gram (CBT_g) of fresh leaf weight from 327 at the day of inoculation to 683 copies at 12 dpi. The amount of DNA then remained stable over 10 days. At 26 dpi, during the appearance of symptoms, an increase in the amount of DNA becomes important.

4. Discussion

Septoria leaf blotch in wheat has a long latent period (Verreet et al., 2000). Prompt information about epidemic diseases, external contamination (arrival of ascospores) and fungicide-resistant alleles can be used to modify disease management strategies, based on the optimal use of host resistance, chemical control and cultural practices (Fraaije et al., 2005). In the present study, we highlight the importance of the use of qPCR in the study of Septoria leaf blotch epidemics.

Ascospores of *M. graminicola* present the primary disease inoculation. These could be produced all year round under specific conditions, such as certain weather conditions (Cordo et al., 1999), and by mature pseudothecia (Kema et al., 1996). Results of spore traps have indicated that external contamination by ascospores affects the *Septoria* leaf blotch epidemic by increasing the level of contamination in the upper leaf layers (F1 and F2).

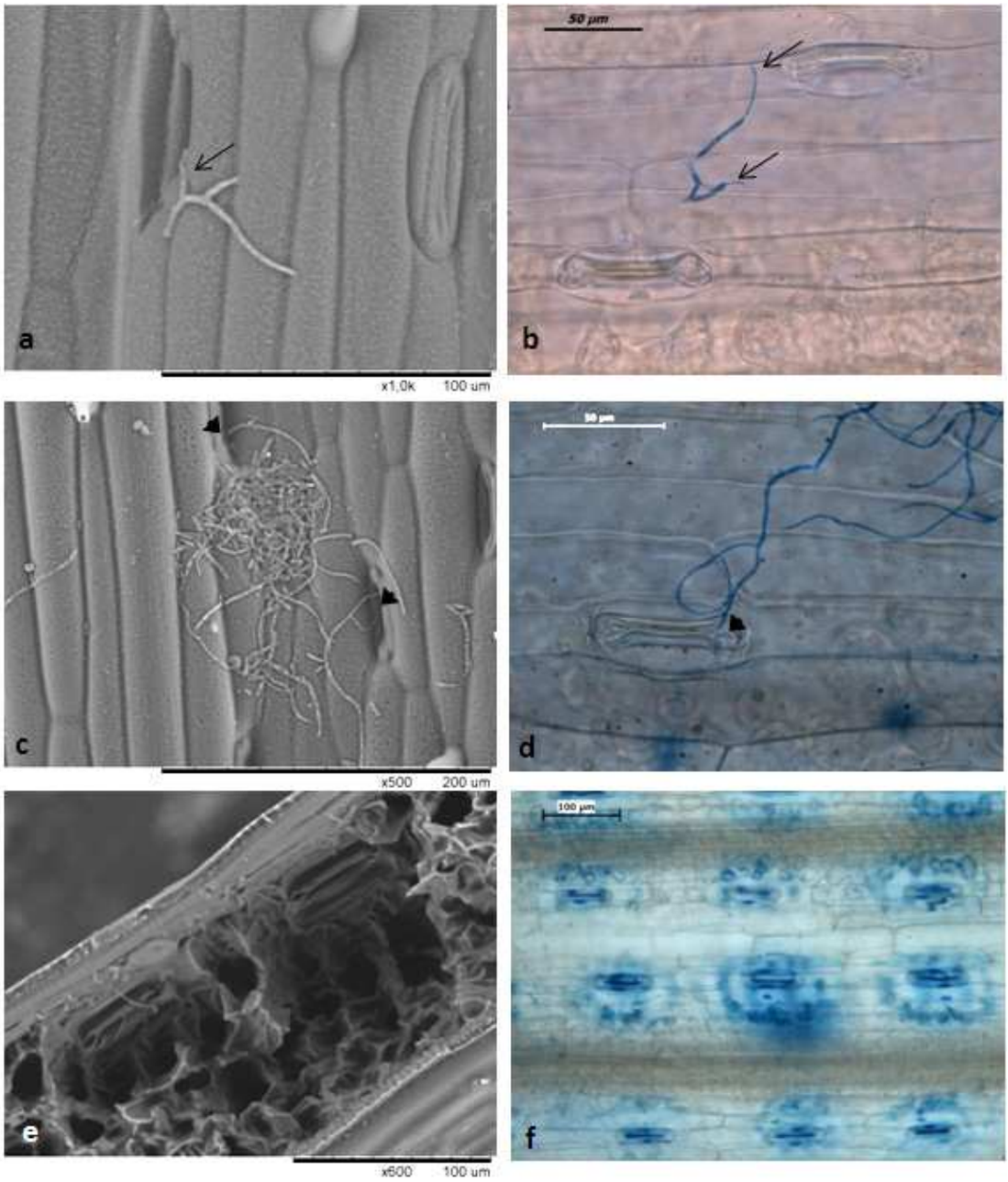

Fig. 7. Electron microscopic (a, c and e) and light microscopic (b, d and f) exposures of the colonization stages of Dinosor leaves inoculated by a 10 µl-drop of *M. graminicola* conidiospores (10^5.ml^{-1}): (a and b) spore germination at 1 dpi, 70% of spores germinated and almost of germ tubes grew on the leaf surface oriented toward the stomata; (c and d) at 3 dpi, stomata located near the inoculation point were penetrated; (e) at 5 and 7 dpi, the stomata were penetrated by passing through the stomatal guard cells and then intercellular colonization by mycelia growth; (f) at 12 dpi, 55% of the stomata were colored blue and cell death had reached 20% of the leaf surface. (Note the colonization of adjacent substomatal chambers and the surrounding tissue of the substomatal chamber.)

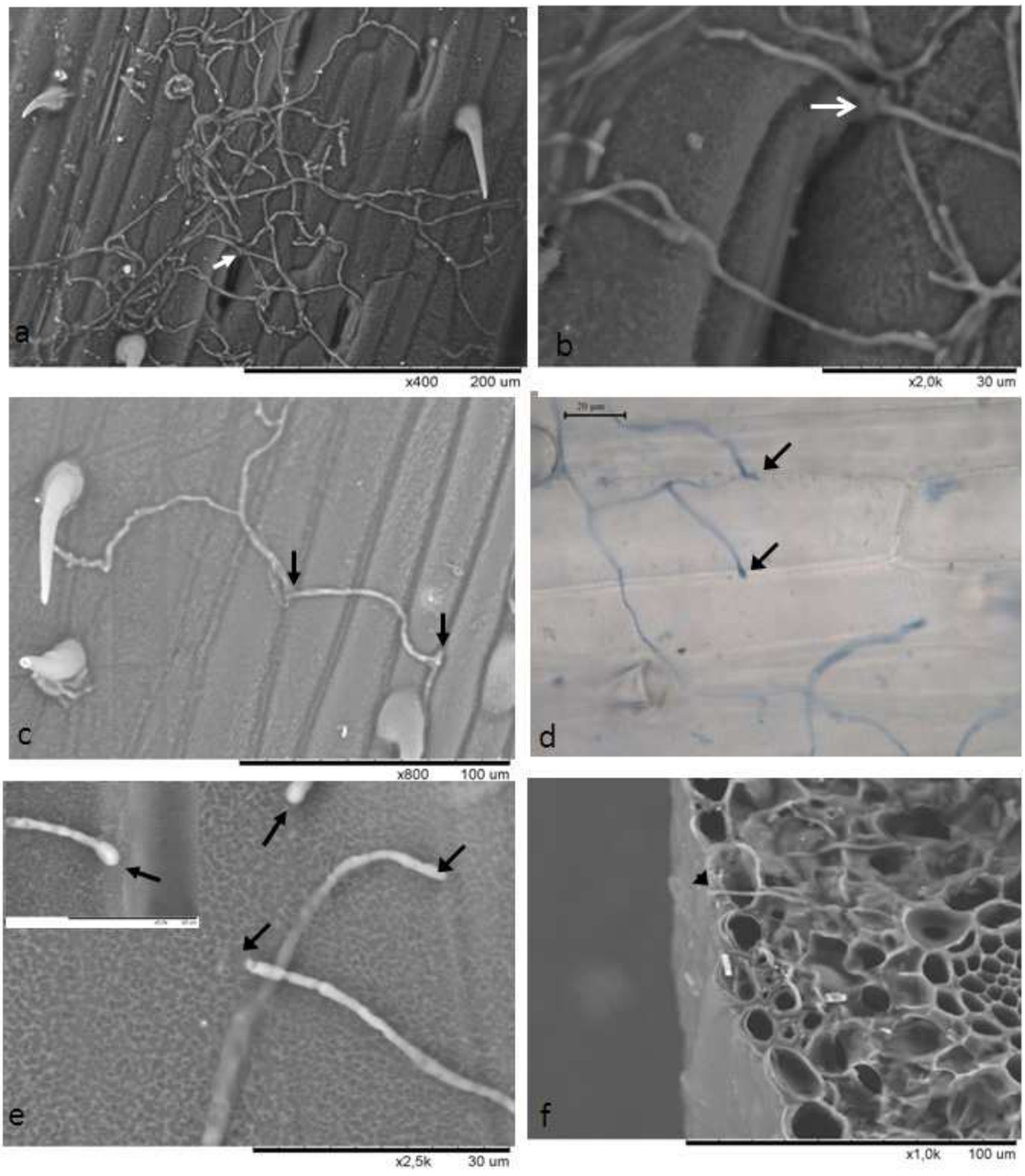

Fig. 8. Electron microscopic (a, b, c, e and f) and light microscopic (d) exposures of Dinosor leaves inoculated by a 10-µl drop of *M. graminicola* conidiospores ($10^5.ml^{-1}$) sampled at 15 dpi: (a) stomata penetration by germ tubes; (b) two germ tubes with an appressorium-like structure penetrate a single stomata (arrows); (c, d and e) germ tube apical differentiations, which are similar to those seen over stomatal openings but here are associated with a leaf surface depression over an epidermal cell anticlinal wall (arrows); (f) cryofracture of *M. graminicola* colonized wheat leaf. Note that the site of penetration (arrowhead) is not per stomata, which proves the direct penetration by this fungus.

The position of these leaf layers allows them to capture more air spores than the leaf layers at the bottom of the plant. This external contamination could affect fungicide efficiency by presenting a potential reinfection process, which perhaps coming out of the potential period of fungicide. Therefore, the detection of this reinfection source provides an actual interpretation of the efficiency of chemical treatment. In ascospore traps, the limitation of qPCR is the absence of specific gene markers that can discriminate between conidiospores and ascospores. However, *M. graminicola* conidia have a limited spread of only a few meters (Boeger et al., 1993), whereas ascospores spread over longer distances (Fraaije et al., 2005). Therefore, the limitation of qPCR was now successfully eliminated by removing all plants within a 3 m diameter of the traps in order to avoid the capture of rain-splash-dispersed conidia, as described in (Fraaije et al., 2005).

The accuracy of the use of qPCR to evaluate the resistance level of wheat cultivars to *M. graminicola* was investigated. Generally, results of qPCR analysis have a high correlation with the results of visual disease assessment (0.95–0.99). Detection of *M. graminicola* using qPCR was achieved 2–4 weeks before symptoms appeared and was 2 weeks earlier in the susceptible cultivars than in the resistant one. However, the *M. graminicola* epidemiology was well characterized for the resistant cultivar by a low level of contamination and a longer period of incubation than the susceptible cultivars. Generally, results of qPCR analyses of F3 or F2 DNA samples correlated well with the susceptibility rating of cultivars that were previously identified by the (Arvalis Institut du Végétal, 2008). A close correlation was obtained between qPCR results and the percentages of leaf necrotic area, especially with the F2 leaf layer. However, under natural infection conditions, visual assessment is suitable for assessing the combined resistance to all pathogens involved, but it is not suitable for assessing resistance to individual pathogens in a disease complex (Loughman et al., 1996). Visual assessment lacks accuracy and specificity and may be confused with other diseases, stress-related symptoms or even normal plant development (Hollomon et al., 1999; Parker et al., 1995; Shimin, 2005). This problem was eliminated with qPCR, which has a high level of specificity of *M. graminicola* β-tubulin and other microorganisms on the leaf surface that interfere with *M. graminicola* DNA are avoided (Fraaije et al., 1999). Furthermore, the using of multiplex qPCR (Fraaije et al., 2001), permits the quantification of other wheat pathogens in the same DNA sample.

The effect of wheat genotypes on the distribution of DMI-resistant R-types of *M. graminicola* was also studied. As observed previously by (Selim, 2009), no significant effect of wheat cultivar was observed and the population structure was stable over all the analysis dates in the same season. The frequency of V381-genotypes was > 90%, which agrees with the frequency of > 50% reported in previous surveys carried out in 2005, 2006, 2007 and 2008 (Leroux et al., 2007; Stammler et al., 2008b), and > 70% in 2009 (Selim, 2009). However, the presymptomatic detection and quantification of *M. graminicola* R-types is very important for the effective control of strains that are highly resistant to DMIs, as it allows better timing, choice and dose of fungicide applications. For example, genotypes R6, R7– and R7+ are less sensitive to tebuconazole but are sensitive to prochloraz, whereas genotypes carrying substitution V136A (R5) are most resistant to prochloraz (Fraaije et al., 2007; Leroux et al., 2007; Stammler et al., 2008a).

In the absence of total wheat cultivar resistance to *Septoria* leaf blotch, DMIs remain the key fungicide agents against *M. graminicola* (Leroux et al., 2007; Mavrolidi & Shaw, 2005).

Their effects on *M. graminicola* are mostly attributed to the systemic action and inhibition of spore germination (Godwin et al., 1999). In the present study, qPCR analysis was used to evaluate the preventive and curative efficiency of DMI fungicides to control the more frequent genotypes of *M. graminicola* (R6, R7– and R7+). Results showed that prothioconazole treatments significantly decreased the amount of *M. graminicola* DNA by 80% in preventive treatment, and they were still 70% efficient when applied curatively at 3 dpi. Electron microscope observations showed that, after the preventive fungicide treatment (d–1), the spores failed to germinate. Although all fungicide treatments resulted in a significant DNA reduction, compared with a nontreated control, later applications of prothioconazole, at 7 to 10 dpi, resulted in a high loss of efficiency (> 50%). These results are in agreement with those reported by (Godwin et al., 1999) and (Guo et al., 2007), namely that prothioconazole has a significant inhibitive effect against spore germination and postgermination, and could have a good curative effect against *M. graminicola* when applied at up to 20% of the latent period. However, most fungicides work best when applied early in the infection cycle, prior to visual symptom expression (O'Reilly et al., 1988). Thus, a qPCR monitoring process could help determine when fungicide application would be needed, and hence it could be determined when spraying would be most economical (Guo et al., 2006).

For a good understanding of the *M. graminicola* epidemic, cytological investigations are very important; however, under field conditions they are difficult to carry out (Kema et al., 1996). Therefore, we studied the relationship between qPCR analysis and the *M. graminicola* infection processes under controlled conditions and by using a one-drop inoculation method. This method is efficient because it controls the number of spores at the beginning of infection, it reduces points of penetration that delay disease development and it eliminates the limitation of the qPCR method, which arises from the DNA quantification of dead and nongerminated spores (Allmann et al., 1995; Josephson et al., 1993). However, penetration of leaves occurred mostly through the stomata, which is in agreement with previous reports (Cohen & Eyal 1993; Duncan & Howard, 2000; Kema et al., 1996; Shetty et al., 2003). Where, appressorium-like swellings were produced over the stomata as well as periclinally and anticlinally, and all stomatal penetration took place from germ tubes with swellings. Direct penetrations have been demonstrated previously (Cohen & Eyal, 1993; Dancer et al., 1999; Rohel et al., 2001; Shetty et al., 2003) and was also in rare cases. It has been suggested that it was due to a secondary mechanism of invasion of the host (Cohen & Eyal, 1993); however, its trigger factors are not known. The time course of the wheat infection processes of *M. graminicola* has been described by (Kema et al., 1996) and (Duncan & Howard, 2000). A strong correlation between the results of qPCR and microscopic observations was found, and disease development determined using qPCR had a similar pattern to that previously revealed by ELISA (Kema et al., 1996) or by PCR/PicoGreen assay (Fraaije et al., 1999). Until the formation of necrotic lesions, the biomass increased only slightly (or even decreased), but then increased rapidly during necrosis and the formation of pycnidia.

Our data confirm that qPCR is an accurate and specific method for studying *Septoria* leaf blotch epidemics in wheat, and for evaluating cultivar resistance, fungicide efficiency and the appearance of fungicide-resistant genotypes. Further research is required to improve our understanding of *M. graminicola* epidemics, taking in account wheat genotype resistance and the effects of fungicide application.

5. Conclusion

Close correlations were obtained between qPCR analysis and leaf colonization stages as well as with visual observations when the leaf had less than 40% necrotic area.

Real-time PCR results showed that late ascospore arrival increase the amount of *M. graminicola* DNA in the upper leaf layers (F1 and F2) and affect the real evaluation of fungicide efficiency. Distribution of DMI-resistant populations of *M. graminicola* was not affected by wheat cultivars. Cultivar resistance determined by qPCR correlated well with the susceptibility rating given by disease symptoms evaluation. Direct penetration of leaf tissue was confirmed by electron microscopy and, coupled with qPCR results, prothioconazole showed a significant inhibitive effect against spore germination and postgermination. We concluded that qPCR is an accurate and specific quantitative method for detecting and quantifying *M. graminicola* leaf blotch, in wheat, spore arrival, fungicide efficiency and fungicide-resistant genotypes, and for assessing the resistance of cultivars.

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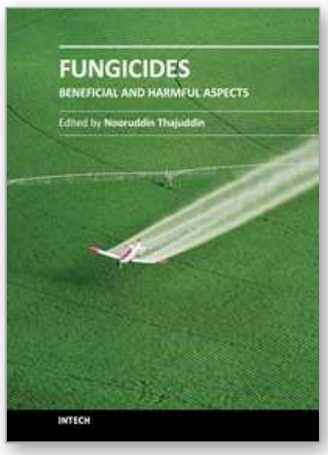

Fungicides - Beneficial and Harmful Aspects
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Fungicides are a class of pesticides used for killing or inhibiting the growth of fungus. They are extensively used in pharmaceutical industry, agriculture, in protection of seed during storage and in preventing the growth of fungi that produce toxins. Hence, fungicides production is constantly increasing as a result of their great importance to agriculture. Some fungicides affect humans and beneficial microorganisms including insects, birds and fish thus public concern about their effects is increasing day by day. In order to enrich the knowledge on beneficial and adverse effects of fungicides this book encompasses various aspects of the fungicides including fungicide resistance, mode of action, management fungal pathogens and defense mechanisms, ill effects of fungicides interfering the endocrine system, combined application of various fungicides and the need of GRAS (generally recognized as safe) fungicides. This volume will be useful source of information on fungicides for post graduate students, researchers, agriculturists, environmentalists and decision makers.

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