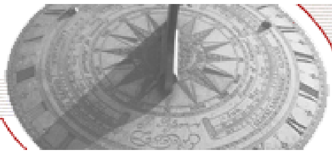
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"Distribution of the airborne inoculum of wheat leaf rust and septoria tritici blotch : impact on epidemics in wheat fields and implications for integrated pest management"

Duvivier, Maxime

Abstract

Like pollen, spores of pathogenic fungi can take to the air in order to disperse. We have hypothesized that a quantitative analysis of the airborne inoculum of wheat pathogens would lead, first, to a better understanding of the epidemiology of wheat diseases and, second, to an improvement in short-term predictions, as a guide to farmers. Two major foliar diseases of wheat in Belgium were chosen: septoria tritici blotch (STB) and wheat leaf rust (WLR), which differ in propagation, development and survival mechanisms. STB occurs early in the wheat development, mainly through airborne contamination by ascospores, produced by the sexual cycle. Its spread to the upper leaves during wheat development is known to be mainly due to asexual pycnidiospores dispersed by rain splashes. However, airborne inoculum analysis indicated that STB inoculum was present throughout the growing season, with a seasonal pattern. Using mechanistic models, the study showed that contamination of the upper leaves ...

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Faculté des bioingénieurs

Earth and Life Institute – Applied Microbiology – Phytopathology

Distribution of the airborne inoculum of wheat leaf rust and septoria tritici blotch: impact on epidemics in wheat fields and implications for integrated pest management

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Novembre 2015

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Abbreviations and definitions

AI load – Total quantity of airborne inoculum detected in a trial field from the emergence of a given leaf layer to PIasc-max

Avr – Avirulence gene

CT – Cycle Threshold

D0 – Date of last observation without symptoms

DD – Degree days base temperature 0°C

DDsep – Degree days above a base temperature of -2.37°C

DMI – Demethylation inhibitors fungicides

Dsymp – Date of first symptoms visible

EC – European Commission

GS – Zadoks growth stage

GS11 – Emergence

GS30 – Beginning of stem elongation

GS31 – First node stage (L3 emerging)

GS32 – Second node stage

GS39 – Flag leaf stage

GS55 – Heading stage

GS61 – Beginning of flowering

GS69 – End of flowering

GS75 – Stage medium milk (grain development)

GS85 – Stage soft dough (grain maturation)

Healthy-time – Cumulative DDsep between emergence of a leaf and the Dsymp

IPM – Integrated Pest Management

L – Leaf (L1 = flag leaf; L2= leaf under the flag leaf, …)

Lr – Race specific WLR resistance gene

MBC – Methyl benzimidazole carbamtes fungicides

PCR – Polymerase Chain Reaction

QTL – Quantitative trait loci

PIasc – Period of infection calculated for ascospores and delimited by PIasc-min and PIasc-max

PIpyc – Period of infection calculated for pycnidiospores and delimited by PIpyc-min and PIpyc-max

R – Resistance gene

RH – Relative Humidity

Source-distance – Number of leaf stage between a given leaf layer and the higher position of the disease on the plants at PIpyc-max

SDHI – Succinate dehydrogenase inhibitor fungicides

SNK – Student-Newman-Keuls

STB – Septoria tritici blotch

WLR – Wheat leaf rust

Chapter 1
Introduction and objectives

1.1. Wheat and diseases

Wheat (*Triticum* spp.) is one of the most widely grown crops, providing one-fifth of the total calories consumed by the world's population (Food and Agricultural Organization of the United Nations, 2010). Currently (2015), wheat is still by far the most popular cereal grown in the European Union (EU) in terms of quantity (139.5 Mio t) and area (24.3 Mio ha), accounting for nearly half the total production of cereals (301.7 Mio t and 57.9 Mio ha) (http://ec.europa.eu/agriculture/cereals/balance-sheets/). In 2014, Wallonia (the southern region of Belgium), of the 193,106 ha sown with cereals, more than 130,587 ha were devoted to winter wheat, representing more than 30% of the total arable crop (http://statbel.fgov.be,2014). In this region, winter wheat is cultivated intensively, reaching a mean yield of more than 8.5 t/ha.

This level of productivity has been achieved through the adoption of Green Revolution technologies, including high-yielding varieties and synthetic fertilizers, pesticides and grow regulators (Evenson & Gollin 2003). The cultivation of high potential cultivars, however, has considerably widened the gap between potential yield and yield obtained without the use of pesticides and fertilizers (Austin 1999). Although potential losses due to weeds are significant in wheat production worldwide, the incidence and impact of pathogens, especially *Mycosphaerella graminicola* (anamorph *Septoria tritici*) and rust fungi, increase with the intensification of crop cultivation. In Western Europe, potential yield loss due to pathogens is considered as important the loss due to weeds (Oerke 2006).

Wheat disease management in Western Europe is achieved mainly through the use of fungicides and disease-resistant cultivars (Eyal 1987; Roelfs *et al.* 1992; Bai & Shaner 2004). In Wallonia, wheat diseases responsible for important yield losses include wheat leaf rust (WLR) (*Puccinia triticina*), stripe rust (*P. striiformis*), *M. graminicola, Phaeosphaeria nodorum* and the complex of pathogens responsible for Fusarium head blight. Farmers in this region usually apply between one and three fungicide treatments to the winter wheat crop, with a mean amount of 1 kg of active substances/ha/year (Lievens *et al.* 2015). Knowledge of the pests and diseases likely to affect the health and quality of wheat plants is critical to maintaining high productivity with minimum impact on the environment.

Detailed knowledge of the mechanisms of infection, dispersion, survival and other aspects of pathogen epidemics is a basic prerequisite for the development of efficient prediction tools for diseases in a targeted area.

Integrated pest management (IPM) is an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimize the use of pesticides. This approach is currently promoted as the preferred approach to crop protection because it is a pillar of both sustainable intensification of crop production and pesticide risk reduction. Indeed, the continuing rise in food demand poses huge challenges for the sustainability of both food production systems and terrestrial and aquatic ecosystems. It reinforces the urgent need to establish policies for ensuring the sustainability of agriculture and ecosystems (Tilman et al. 2002; Duveiller et al. 2007). Policy examples include the European Commission (EC) regulation 1107/2009/EC concerning the introduction of plant protection products into the market and the European Parliament directive (2009/128/EC) establishing a framework for EU actions on the sustainable use of pesticides. Directive 2009/128/EC concerns in particular tools for monitoring pesticide applications and thresholds for guaranteeing optimal and timely applications.

In the case of wheat production, integrated control of cryptogammic pathogens should rely on at least four different and complementary strategies:

- the use of resistant cultivars,
- the adapted agronomical practices,
- the knowledge of the pathogens populations occurring in the fields,
- the use of efficient fungicides with adapted doses and timing.

This dissertation will mainly focus on the third evocated strategy, in trying to better understand the epidemics of wheat pathogens using spore traps and developing tools or models to predict their evolutions.

Two main different ways can be used to develop models able to predict the development of plant diseases: the empirical or the mechanistic approach. Empirical models are based on direct observation, measurement and extensive data records. In the context of plant disease epidemiology, the objectives of this approach will be to find strong correlations between certain conditions (e.g. environmental, meteorological, biological…) and the occurrence of the disease. These relationships, if validated, can be used to predict disease in the future. However, it does not mean that the processes

causing the diseases have been perfectly understood. This kind of approach has already been used with success to simulate the infection of WLR in wheat fields (El Jarroudi *et al.*, 2014a,b) or for early-warning of STB (te Beest *et al.*, 2009). The mechanistic approach is based on the knowledge of different processes ruling the disease (e.g. infection conditions, latency period, spore dispersal…). It assumes that a system can be divided in individual parts and that the manner in which these parts are coupled can be understood. A typical example for this kind of model is the 'Proculture' forecasting system used in Wallonia to predict the development of *Septoria tritici* on the upper parts of the wheat plants (Moreau & Maraite 2000). This model is able to simulate the development of the plant, the infection of the different leaf layers and the evolution of the disease severity on these leaves. Conditions conducive to the infection of leaves and the duration of the latency period of the pathogen were determined and finely tuned using past knowledge and experiments. The equation used for the evolution of symptoms on the leaves was determined using an empirical approach. All these processes (e.g. spore dispersal, infection, latency period, evolution of disease severity) were combined to simulate the progression of disease severity on the five last leaf layers in fields based on hourly data of meteorological conditions and cultural practices. .

The 'Proculture' forecasting system adapted to conditions in Wallonia is suitable only for predicting the evolution of septoria tritici blotch (STB) symptoms on plants, the most problematic disease in the region. Advice on fungicide applications in the field is therefore based on weekly observations of the evolution of disease symptoms in a network of trials covering Wallonia. Weekly bulletins for farmers are published carrying information on the evolution of epidemics in various sub-areas and informing them if the intervention threshold has been exceeded. Although this approach has been validated in recent years (Duvivier 2014; Duvivier & Bataille 2015), there could be an overall improvement in the sustainable use of pesticides if prediction tools were available and validated for all the main diseases occurring in Wallonia. Some models have been constructed for predicting infection and showing risk before the symptoms appear. Depending on the availability of the data needed to run these models, they can predict the evolution of infection on a smaller scale than a survey network. Models for predicting WLR caused by *P. triticina* (El Jarroudi *et al.* 2014a) and Fusarium head blight (Prandini *et al.*, 2009) have been developed for the border areas of Wallonia, but have never been used or validated for the whole region.

In addition, most of the proposed disease prediction systems for wheat are based on the occurrence of weather conditions conducive to the production and release of pathogen inoculum or host infection (e.g.,

Moreau & Maraite 2000; Robert & Bancal 2004; El Jarroudi *et al.* 2014b; Savary *et al.* 2015), assuming the inoculum needed for infection is always present. Disease epidemics, however, only result from the combination of a favorable environment, host susceptibility and **the presence of inoculum** (Campbell & Madden, 1990).

1.2. Importance of airborne inoculum in wheat diseases

In plant pathology, inoculum can be defined as the particles that cause infection in a host under favorable conditions. With regard to the inoculum of a cryptogrammic pathogen, the term 'inoculum' is used to refer to a specific part of the cycle of a pathogen (e.g., ascospores, pycnidiospores, conidia, basidiospores, urediniospores). Often, this is further qualified by the dispersal pattern of the inoculum, such as splashborne inoculum (Shaw, 1987; Walklate *et al.*, 1989; Shaw & Royle, 1993), seedborne inoculum (Milus & Chalkley, 1997; Bennett & Milgroom, 2007), soilborne inoculum (Bockus & Shroyer, 1998; Saari, 1998) and airborne inoculum (Roelfs *et al.*, 1992; Shaw & Royle, 1993; Del Ponte, 2003). A specific example of airborne inoculum is the wet deposition by rain of spores from the atmosphere. This type of dispersal is known to be involved in rust epidemics (Barnes *et al.* 2008; Li *et al.* 2009).

The epidemics of the main foliar diseases of wheat that occur in Wallonia (*M. graminicola*, *P. triticina* and *P. striiformis*) are not governed by the same dispersal mechanisms. Most Walloon farmers use a crop rotation system based on one wheat crop on the same land every 2 or 3 years, and in such a system soilborne contamination by crop residues is unlikely to be involved in primary infection (Krupinsky & Bailey 2002). In monocultural systems in Wallonia, ploughing before the wheat is sown is common practice, reducing crop residue impact (Bailey & Lazarovits, 2003). Seedborne infections are not a dispersal mechanism for *M. graminicola* (Suffert *et al.*, 2011) and *Puccinia triticina*. Different agent-specific dispersal mechanisms might be involved in secondary infections by fungal pathogens. In STB epidemics in wheat fields, the main spore dispersed are thought to be splashborne pycnidiospores (Shaw, 1987, 1999; Fitt, 1989; Shaw & Royle, 1993). In the case of rust epidemics, however, the main way of dispersion are airborne inoculum (Roelfs, 1989; Singh & Saari, 1992a).

Teleomorph and anamorph spores can use different dispersal mechanisms and are usually involved at different phases of the pathogen cycle. In some diseases, however, spores involved in the sexual/asexual stages can co-exist and simultaneously participate in the spread of the epidemic. For example, *M. graminicola* can develop on the plants of a previous wheat crop, with

the primary infection of the plantlets originating from either splashborne pycnidiospores or airborne ascospores (Shaw & Royle, 1989a; Suffert *et al.*, 2011). The subsequent development of the disease on the upper leaves is generally assumed to be caused by splashborne pycnidiospores, but it is also likely that airborne ascospores could affect the disease dynamics during the final stage of growth (Kema *et al.*, 1996b; Zhan *et al.*, 1998; Hunter *et al.*, 1999a; Selim & Roisin-Fichter, 2014).

1.3. Quantification of airborne inoculum

Since the rebuttal of Pasteur's proposal of spontaneous generation (Pasteur *et al.*, 1878), many methods have been developed for collecting, studying and quantifying airborne spores (Lacey & West, 2007). Wheat leaf pathogens can be quantified using trap plants (Shaw & Royle, 1989a; Sache *et al.*, 2000) or spore traps (Park & Felsenstein, 1998; Hunter *et al.* 1999;). An example of a spore trap adapted for airborne inoculum is the volumetric Burkard 7-day spore trap (Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK; Figure 1A). It has been used successfully for various purposes in many studies of winter wheat pathogens (Hunter *et al.*, 1999a; Fernando & Miller, 2000; Fraaije *et al.*, 2005a). The Burkard device traps air samples at 10 L/min and the particles (usually more than 1 um diameter) adhere to tape that rotates 2 mm/h, providing a continuous record of the airborne inoculum (Lacey & West, 2007). It has the advantage of being volumetric and providing continuous sampling, making it easy to calculate the time the spores are collected, and it also needs only one manipulation every 7 days. It has been used successfully with polymerase chain reaction (PCR) for identifying and quantifying arable crop pathogens (Calderon *et al.*, 2002b; Fraaije *et al.*, 2005a; Rogers *et al.*, 2009; Selim & Roisin-Fichter, 2014). Conventional methods for identifying and/or quantifying airborne inoculum were based on microscopy or on counting colonies after the culture of organisms on adapted growth substrates (West *et al.*, 2008). These methods were extremely time-consuming and require considerable expertise in order to identify the organisms accurately. Recent advances in molecular technologies have made quantifying the airborne inoculum of pathogens much easier and less expensive. Real-time PCR is based on classic PCR, but enables a targeted DNA sequence to be simultaneously detected and quantified. During each cycle of amplification, the amount of total DNA ('amplicon') is measured using a fluorescent label. Recording the complete kinetics of the polymerization reaction enables the initial amount of targeted DNA to be quantified, which was very difficult to achieve with classic PCR. A fluorescence threshold is set in the PCR software. The number of cycles at which the fluorescence exceeds this

threshold is called the cycle threshold (CT). With this value, the DNA can be quantified using an equation designed with known quantities. Currently, four technologies are used for applying this technique in plant pathology (Schena *et al.*, 2004). They can be separated into amplicon sequence non-specific (SYBR Green I) and sequence specific (TaqMan, Molecular beacons, and Scorpion-PCR) methods (Mackay *et al.*, 2002). In addition, real-time PCR technologies allow all the post-amplification steps needed to visualize the amplicon to be eliminated, reducing the time and labor required for the analysis. This makes real-time PCR particularly suitable for large-scale analysis.

1.4. Using airborne inoculum quantification in disease prediction

The existence of airborne inoculum near a wheat crop depends on many factors, including the presence of a source and the occurrence of climatic conditions conducive to spore release. The ejection of spores from contaminated hosts, survival structures or residues is often conditioned by climatic factors such as humidity (rain, dew and relative humidity [RH]), temperature and light. The distance between a susceptible crop and a source of airborne inoculum is also a key factor. The dispersal of airborne inoculum from a source and its subsequent deposition on a crop are complex processes influenced by wind direction, turbulence and many other parameters that cannot always be quantified (McCartney & Fitt, 1998; Aylor, 1999, 2003; McCartney & West, 2007), making it difficult to accurately estimate the spore density likely to infect a crop. Although it is possible for weather-based models to predict airborne inoculum release and density, accurate large-scale datasets are needed for the construction and validation of these models.

Combining molecular diagnostics with strategic sampling of airborne inoculum enables data on the temporal distribution of airborne inoculum in a field to be collected. This type of information could be used to predict epidemics more accurately where disease severity is influenced by timing or amount of inoculum (West *et al.*, 2008). As noted earlier, models for predicting wheat foliar pathogens often take account **only of the climatic conditions involved in the propagule infection process** when recommending the best time for fungicide applications to control the disease (e.g., Moreau & Maraite 2000; Robert & Bancal 2004; El Jarroudi *et al.* 2014b; Savary *et al.* 2015). Most of these models do not consider the presence of initial or secondary inoculum as a limiting factor, although it

has been shown that including information on airborne inoculum density can provide more accurate predictions of the risk of severe epidemics.

In Canada, Botrytis leaf blight, caused by *Botrytis squamosa*, is a common and often damaging disease of onion crops, causing variable losses from year to year. Studies on the relationship between airborne conidium concentration and lesion development led to the determination of a spore density threshold that should be reached before fungicide was applied. Using this approach, it was shown that the number of fungicide applications could be reduced by 75% without causing a significant yield reduction (Carisse *et al.*, 2005).

In Poland, stem canker of crucifers caused by the ascomycete fungi *Leptosphaeria maculans* and *Leptosphaeria biglobosa* is a serious disease of oilseed rape. Information on the concentration of *Leptosphaeria* spp. in air samples is important for controlling the disease. The period of ascospore release differs greatly from year to year and region to region. A network of Burkard 7-day spore traps was set up to help predict the occurrence of these diseases more accurately (Jedryczka & Kaczmarek, 2008).

Many other studies have clearly demonstrated the potential of using spore trapping to predict the risk of epidemics in crops (Harrison *et al.* 1965; Guyot *et al.* 2014; Klosterman & Anchieta 2014; Mahaffee 2014). Studies that combined observations of epidemics in the field with data on climatic conditions and spore detection have made an important contribution, **directly or indirectly**, to the construction of disease prediction systems.

This approach could be used to improve the understanding of wheat disease epidemics caused by fungal pathogens in Wallonia and to develop an accurate prediction system. For example, data on airborne inoculum in relation to the disease dynamics could be helpful in understanding the amount of inoculum needed for epidemics to take hold. The use of weather data and/or environmental conditions could also help in the development of inoculum density prediction models.

1.5. Network of spore traps in Wallonia

Within the context of research project D1300 (‘Spatio-temporal evolution of the airborne inoculum of cryptogamic diseases of winter wheat in relation with climate and the dynamic of epidemics’), subsidized by the Walloon Region, a network of Burkard 7-day spore traps was set up throughout the region in order to sample the air (Figures 1.1A and 1.1B).

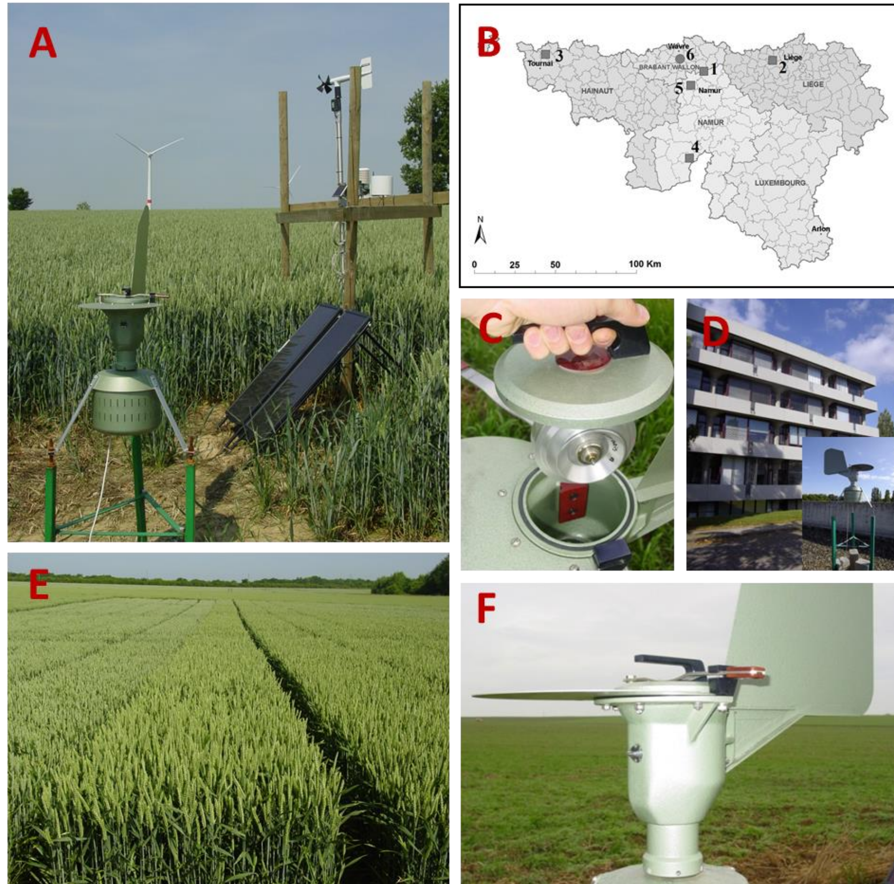

Figure 1.1. A) Imetos meteorological station (Pessl Instruments, Austria) and Burkard 7-day spore traps in a trial field. Both devices are solar-powered. B) Location of the main trial fields and spore traps in Wallonia from 2009 to 2013. C) Wax-coated Melinex tape on a drum that is revolved once every 7 days. D) Building in Louvain-la-Neuve on which a spore trap was placed from 2009 to 2013. E) Fungicide field trial sown with selected wheat cultivar close to the spore traps. F) Burkard spore trap opening, always facing the wind.

There was one trap per field at four or five sites (depending on the year), operating continuously between 2009 and 2013. An additional trap was placed on the roof of a building 20 m high and 2 km from cultivated fields (Figure 1.1D). The tapes from the spore traps were collected weekly (Figure 1.1F) and total DNA was extracted from daily fragments of tapes with the aim of creating a DNA bank collection. Specific real-time PCR assays for *M. graminicola*, *P. triticina* and *P. striiformis* were developed in order to quantify the airborne inoculum trapped in the trial fields. All the spore traps, apart from the one on the roof, were in fields in which cultivars susceptible to and resistant of the main foliar diseases of wheat were

grown and where fungicide trials had been conducted (Figure 1E). In each growing season from March-April to July, the dynamics of fungal disease epidemics were assessed in all the fields. Data on the main climatic parameters known to play a role in the epidemics were collected in or near the fields (Figure 1A). By allowing comparisons between sites and years, this set-up proved a powerful tool for studying the effect of airborne pathogen inoculum on the development of epidemics, as well as the potential of using of airborne inoculum data to improve prediction systems.

1.6. Two contrasting case studies

The causal agents of STB and WLR (*M. graminicola* and *P. triticina*, respectively) were chosen to illustrate the benefit of collecting data on airborne inoculum in order to better understand and predict epidemics. These two diseases are among the most damaging diseases of wheat in Wallonia and the rest of Europe (King *et al.* 1983; Eyal 1987, 1999; Huerta-Espino *et al.* 2011). The survival and dispersal modes of both fungal agents differ greatly and airborne inoculum probably plays a major role in the progression of epidemics. An improvement in the understanding of the epidemics of both diseases in Belgium is needed in order to develop more effective integrated pest management (IPM) strategies. The following section discusses the epidemiology and management of epidemics of these pathogens in Wallonia, and the issues that need to be addressed.

1.6.1. *Mycosphaerella graminicola*

1.6.1.1. Overview

Mycosphaerella graminicola (anamorph, *Septoria tritici*) is the causal agent of STB, an important wheat disease that occurs worldwide (King *et al.* 1983; Eyal 1987; Daamen & Stol 1992; Halama 1996; Jørgensen 1999; Hardwick et *al.* 2001; Jones & Harrison 2004). The introduction of shorter and more susceptible high-yielding cultivars and the increase in nitrogen use (Bayles 1991) has increased the economic impact of this disease on wheat production. Under favorable environmental conditions, epidemics can reduce yield by 30-40% (Eyal 1987). *Mycosphaerella graminicola* is an heterothallic ascomycete with a bipolar mating system (Kema *et al.* 1996c). In Belgium, this pathogen is endemic, but the dynamics and severity of epidemics vary greatly from year to year (Moreau & Maraite 2000; Duvivier *et al.* 2013, 2014, 2015). Serious yield losses occur when the three upper leaves of plants become severely infected (Shaw & Royle 1989b; Thomas *et al.* 1989). Controlling the disease on these leaves is generally achieved by

planting resistant cultivars and applying fungicides (Palmer & Skinner 2002). The ability of this pathogen to overcome cultivar resistance (Kema *et al.* 1996a; Cowger *et al.* 2000) and develop resistance to fungicides (Amand *et al.* 2002; Stergiopoulos 2003; Fraaije *et al.* 2005b; Leroux *et al.* 2007; Torriani & Brunner, 2009), however, has made STB the most serious wheat disease in Europe.

1.6.1.2. Epidemiology

Epidemics of STB in wheat fields involve two types of propagule dispersal: airborne ascospores formed during sexual reproduction in the pseudothecia; and pycnidiospores produced in the pycnidia by asexual reproduction (Figure 1.2.) (Palmer & Skinner 2002). The fungal agent can survive from one wheat cropping season to the next one as mycelia, pycnidia or pseudothecia on crop residues and volunteer hosts (Suffert *et al.* 2013). Rotation and full tillage are the current practice in Wallonia and therefore ascospores forming on pseudothecia on crop residues are the main source of primary infection in young plants (Shaw & Royle 1989a). Under moist conditions, airborne ascospores are released from fruiting bodies (Sanderson & Hampton 1978) and can travel long distances, contaminating newly sown fields (Zhan *et al.* 1998; Linde *et al.* 2002). Where wheat was the previous crop, pycnidiospores from residues can contaminate young wheat plants (Suffert & Sache 2011). In both cases, infection by spores occurs almost entirely through leaf stomata (Cohen & Eyal 1993; Duncan & Howard 2000). The pathogen colonizes the mesophyll tissue in an intercellular way without producing feeding structures, such as haustoria. Under optimal infection conditions, the mesophyll collapses after 10 days, resulting in the initial chlorosis and necrosis of the host leaf (Kema *et al.* 1996c), with brown or black globose pycnidia appearing in the lesions after 14-21 days (Eyal 1987). Asexual spores exudate from the pycnidia when the leaf surface is wet (Eyal 1971; Gough & Lee 1985). Thereafter, the pycnidiospores can be dispersed by rain splash, contact or runoff and infect other leaves (Shaw 1987; Shaw & Royle 1993; Lovell *et al.* 1997, 2004b). It is thought that STB progression from the lower part of plant to the upper leaves is achieved mainly through rain splash carrying pycnidiospores. Infection by pycnidiospores requires moist conditions and temperatures above 3-4°C, with optimal temperatures of 17-19°C (Shaw, 1990; Lovell *et al.*, 2004a; Bernard *et al.*, 2013). The time needed to complete the asexual cycle depends largely on temperature (Shaw 1990; Lovell *et al.* 2004a). In Belgium, it generally lasts from 17 to 21 days during spring and early summer.

Severe economic losses caused by STB occur only if important surfaces of the three upper leaves are contaminated (i.e., when infection occurs when these leaves have just emerged and one or two asexual cycles have been completed prior to senescence) (Shaw & Royle 1993). Some recent studies have questioned the importance of the effect of airborne inoculum from the sexual reproduction on vertical STB dispersal on wheat plants (Kema *et al.* 1996b; Zhan *et al.* 1998; Hunter *et al.* 1999a; Linde *et al.* 2002; Fraaije *et al.* 2005a; Sameh *et al.* 2011).

1.6.1.3. Role of asexual and sexual reproduction in STB dispersal

Vertical STB dispersal refers to the progression of the pathogen from the lower part of the plant to the upper part, whereas horizontal STB refers to dispersal from plant to plant at the same height. Pycnidiospores can be dispersed by a single rain splash within a radius of 1 m, but the number of spores dispersed rapidly decreases over a half-distance of 10 cm (Shaw 1999). The concept of half-distance relates to the distance needed to reduce by two the number of spores transported. Studies of the population genetics have corroborated this observation, with the dispersion of clones in a crop generally being limited to a range of about 1 m^2 (McDonald *et al.* 1999). Despite this low dispersal capacity, a crop can often be rapidly and homogeneously contaminated by STB, given the enormous potential production of pycnidiospores from a single infection (50,000 and 500,000 spores) (Eyal 1971) and the relatively lax conditions for infection (Shaw 1990; Shaw & Royle 1993). After one or two asexual cycles, symptoms can generally be observed throughout a crop, even where the primary infection is moderate. This is why primary infection by ascospores is hardly considered as a limiting factor. The severity of epidemics is determined more by leaf development and environmental conditions (weather and cultivar) than by primary inoculation (Shaw 1999). The vertical dispersal of STB needs to be taken into account in the development of a prediction model aimed at protecting the three upper leaves. For *M. graminicola*, the model would be determined by leaf development and the capacity of the pathogens to be dispersed on symptom-free upper leaves. Vertical dispersion is thought to be driven mainly by splashborne pycnidiospores (Shaw & Royle 1993; Moreau & Maraite, 2000). It has been shown, however, that the vertical movements by droplets are more limited than horizontal movements with an half-distances approximated to 5 cm (Shaw, 1987).

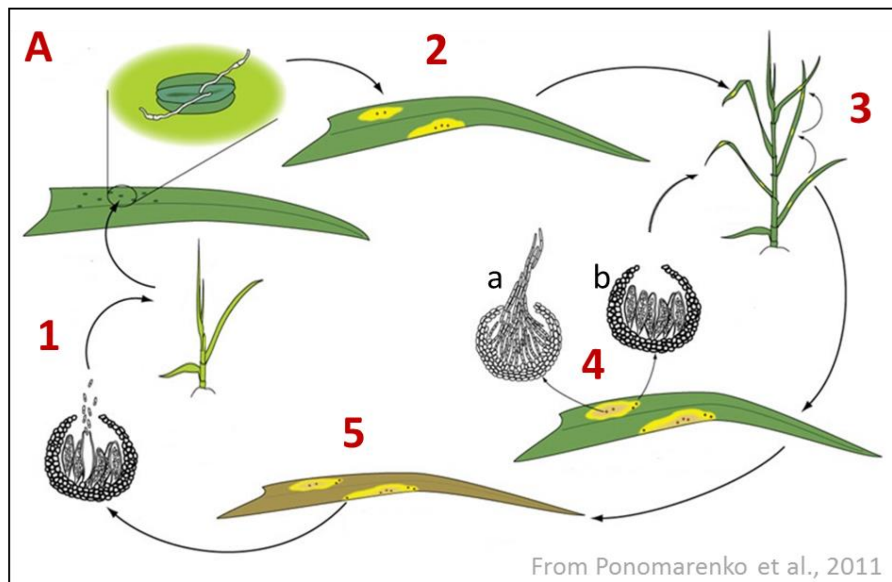

Figure 1.2. A) Disease cycle of STB caused by *Mycosphaerella graminicola*. A1) In a crop rotation system, the primary infection of plants is caused mainly by airborne ascospores produced on residues of previous wheat crops. Ascospores contaminate young plants through leaf stomata. A2) First lesions on young plants develop pycnidia. A3) Secondary spread of pycnidiospores (vertical and horizontal dispersion) caused by contact and rain dispersal. A4a) Pycnidia that have developed on lesions produce great quantities of pycnidiospores throughout the growing season. A4b) Pseudothecia develop on lesions, releasing mobile airborne ascospores that can contaminate wheat plants. 5) Pathogens overwinter as mycelia, pycnidia, pseudothecia on crop debris and on autumn-sown crops and volunteer hosts. B & C) Typical STB symptoms on an upper leaf of winter wheat

The efficiency of this dispersal mechanism could be significantly decreased, if the crop growth rate is rapid and the disease is low in the canopy. Depending on the cultivar, the architecture of a wheat plant can also greatly affect the vertical dispersal of pathogens. In addition, during emergence a leaf might be at a similar height or even lower than an

infected older leaf. A young leaf can therefore be infected by droplet runoff or simply by contact, without the need for high intensity rainfall (Lovell *et al.* 1997, 2004b).

Another way of vertical disease progression could involve the ascospores, which are the spores produced by sexual reproduction. Several sexual cycles can occur in a growing season (Kema *et al.* 1996b). Hunter *et al.* (1999a) reported that ascospores were trapped in fields throughout the growing season, with a seasonal pattern. In addition, pseudothecia are regularly observed on wheat leaves from March onwards, with production at the leaf scale increasing with leaf age (Eriksen & Munk 2003). It has also been shown that the DNA content of STB on the upper leaves could greatly increase after airborne inoculum peaks (Selim *et al.* 2011; Selim & Roisin-Fichter 2014). Work based on genetic analyses assumed that sexual recombination often occurs during the season and that strains found in the field are sometimes immigrant or recombined strains responsible for the inoculation of the fields (Zhan *et al.* 1998). The considerable genetic diversity at the field scale or even the leaf scale suggests a constant mixing of populations, indicating that ascospores could provide secondary as well as primary inoculum (Boeger *et al.* 1993; McDonald *et al.* 1999; Linde *et al.* 2002). The great adaptation ability of *M. graminicola* suggests that airborne ascospores play an important role throughout the growing season. This was illustrated in a study measuring the selection of resistant strains after the application of strobilurin fungicides (Fraaije *et al.* 2005a). Monitoring using real-time PCR showed that fungicide treatment rapidly selected isolates carrying resistant (R) alleles within field populations. A rapid increase in R-allele frequencies (35-80%) was also measured in airborne ascospores populations sampled in wheat plots immediately after the first treatment at the 'second node' growth stage (GS32). After the second strobilurin application, most of the R-allele frequencies measured for *M. graminicola* populations in the leaves and airborne inoculum sampled from the treated plots exceeded 90%. The spatial sampling and testing of *M. graminicola* flag leaf populations derived from ascospores in the surrounding crop showed that ascospores carrying R alleles could spread easily within the crop across distances up to 85 m.

The findings of all these studies suggest that airborne ascospores could also be involved in the vertical progression of STB. These mobile airborne spores might therefore be responsible for some primary infection on the upper leaves. The asexual cycle could thereafter take the lead and be responsible for the horizontal spread and subsequent increase in severity in a leaf layer. Given this possibility, it would be useful to include data on airborne sexual spores in a prediction model. It has been suggested that once there are a

few sporulation lesions on a reasonable proportion of the upper leaves in wheat crops, there is potential for multiplication, leading to widespread infection and premature leaf death, except in very dry weather (Shaw 1999).

In an infected STB-sensitive crop, the occurrence of ascospore production when the upper leaves are emerging is usually less important than pycnidiospore production due to the longer latency period of the pseudothecia (Kema *et al.* 1996b; Hunter *et al.* 1999a; Eriksen & Munk 2003). Pseudothecia are produced long after the first pycnidia are observed in a crop and the sexual fruiting bodies produce fewer spores (Eyal 1971; Gough & Lee 1985). Airborne ascopores, however, have high mobility (Shaw & Royle 1989a; Fraaije *et al.* 2005a) and can be produced on residues for a very long time (Suffert & Sache, 2011; Suffert *et al.*, 2011). It would be necessary to measure the exact density of spores above a crop before concluding whether or not this dispersal mechanism plays a significant role in the contamination of upper wheat leaves.

1.6.1.4. Control and prediction system in Wallonia

In Wallonia, STB control is achieved through an integrated approach that combines partially resistant cultivars, cultural practices, crop rotation and fungicide applications. The main cultivars available are tested annually for STB resistance and these resistance levels are released in September, (Livre Blanc Céréales, Gembloux) allowing farmers to make the appropriate choice. With regard to host resistance, there are two concepts in pathogenicity involved: **virulence** of the pathogen, which refers to the ability of the parasite to attack a host; and **aggressiveness** of the pathogen, which refers to the extent of the disease that could be induced by a pathogen. The specific or qualitative resistance in a host prevents the infection (virulence) and corresponds to a gene-for-gene interaction between the fungus and its host. It is based on a recognition between the plant and the pathogens that triggers a cascade of defense reactions and prevents the infection. This resistance is a single-gene carrier and its expression prevents infection completely if the resistance gene (R) corresponds to the avirulence gene (Avr) of the pathogen. The quantitative or non-specific resistance is related to the aggressiveness of the pathogen. In most cases, it is governed by several genes whose effects are cumulative (Schiff *et al.*, 2001) and which are described as quantitative trait loci (QTL), each QTL being assumed to contain a gene determining a particular effect on the development of the pathogen (Lindhout, 2002). In the case of *M. graminicola*, both types of resistance can be found in a wheat cultivar. Analyses of the genetics of the host and the pathogen have shown clear

interactions between a resistance gene in the host and the corresponding virulence in the pathogen, confirming the existence of gene-for-gene interactions at least for some cultivar-isolate pairs. Much of the resistance against *M. graminicola* in wheat, however, is clearly quantitative and possibly non-specific. Both mechanisms appear to be important in conferring full/durable resistance in the field (Arraiano & Brown, 2006). Given the great diversity of the population and its ability to adapt rapidly, however, it is likely that quantitative resistance has greater potential for the durable control of STB. Cultivar resistance against STB offers the greatest potential for reducing dependence on fungicides in integrated control strategies. The use of resistant cultivar also allows wheat growers to gain in flexibility for the choice of the dose and the timing of spraying.

In addition to using resistant cultivars, tillage and crop rotation are also common practices in Wallonia aimed at reducing the potential of primary inoculum, although it is known that primary inoculum is not one of the main factors in severe epidemics Effective cultural practices implemented to reduce STB risk include late seeding and limited fertilization (Eyal, 1987, 1999; Thomas *et al.*, 1989; Simón *et al.*, 2003).

Most farmers in Wallonia avoid STB-related yield losses by using fungicides, because there is no cultivar with total resistance. Methyl benzimidazole carbamates fungicides ([MBC], MBC inhibits tubulin formation in mitosis) and demethylation inhibitors fungicides (DMI) such as triazols (DMI inhibits the 14-alpha demethylase enzyme) have been used against STB since the 1970s. A new type of fungicide, Qo inhibitors (strobilurin), has been widely used since the 1990s. Strobilurins used to be almost completely effective again STB, but resistance to benzimidazoles and Qo inhibitors spread rapidly after 1984 and 2002, respectively, because these fungicides were only single-site inhibitors. Resistance to DMIs has also occurred over time, resulting in decreased sensitivity to their mode of action. The succinate dehydrogenase inhibitor fungicides (SDHI) are a new type of fungicides that are proving to be effective against STB and, so far, no concrete example of resistance has been reported. Chemical companies are now developing mixtures of active substances from distinct families (with distinct modes of action) in order to prevent the selection of resistant strains. It is known that the best way to prevent selection is to use a mix of active substances, alternate fungicide modes of action and efficient doses of products. STB is prone to developing resistance to fungicides and it is therefore important that fungicide strategies are based on reducing the likelihood of resistance development.

The timing of fungicide applications is critical for achieving adequate disease control. When pressure is high early in the season, an initial

treatment is often applied at about the time of the second node stage (Zadoks growth stage 32, GS32). This slows down the vertical progression of the disease by the asexual mode. With an early application, another treatment should be applied a maximum of 3-4 weeks later to protect the flag leaf, the most important organ for yield. If disease pressure is low in early spring (e.g., because a resistant cultivar is used or when meteorological conditions are not conducive to STB), fungicide applications can be postponed to the flag leaf stage (GS39) or even the heading stage (GS55). In spring each year, advice on STB control is given to farmers based on observations of disease pressure in a network of untreated wheat plots established annually throughout Wallonia and on the predictions obtained with Proculture, a model that predicts the evolution of symptoms on the upper leaves (Moreau & Maraite 1999; Lemaire *et al.* 2003). This model focuses on the phenological development of wheat, the identification of infection periods and the estimation of disease severity on the five upper leaves. It takes account of the progression of the disease only by splash-dispersed pycnidiospores and **does not consider airborne inoculum from sexual reproduction**. This type of dispersal could play a significant role in the contamination of upper leaves, particularly when the asexual progression of the disease begins to slow down (e.g., resistant cultivar or treatment GS32).

1.6.2. *Puccinia triticina*

1.6.2.1. Overview

Puccinia triticina, the causal agent of WLR, is the most widespread and common of the three wheat rusts worldwide (Saari & Prescott 1985; Roelfs *et al.* 1992; Kolmer 2005a). At the field scale, damage due to WLR is usually less important than that from stem or stripe rust, but the frequency of epidemics, linked to wide distribution, result in greater annual losses overall (Huerta-Espino *et al.* 2011). In Europe, yield losses in susceptible cultivars can reach 30% (Hartleb *et al.*, 1995; El Jarroudi *et al.*, 2014b). In Wallonia, significant symptoms are generally observed from spring and early summer, although they can appear in autumn on early seedlings. The development of WLR is particularly noticeable when conditions are moist and the temperature is 15-25°C (Vallavieille-Pope *et al.* 1995). The severity of WLR epidemics in Wallonia varies greatly from year to year (El Jarroudi *et al.* 2014a). The greatest losses occur when early contamination of susceptible cultivars is followed by climatic conditions conducive to the disease (Roelfs *et al.* 1992). Controlling WLR is usually achieved by using adapted cultural practices, resistant cultivars and applying fungicides. Modern fungicides are

particularly effective for controlling the pathogens in wheat crops (Duvivier *et al.* 2013, 2014). Fungicide use could be considerably reduced in Wallonia, however, if the variability in the dynamics and severity of the epidemics was better understood.

1.6.2.2. Epidemiology

Puccinia triticina is a heteroecious fungus, requiring an alternative host, *Thalicitrum speciosissimum* or *Isopyrum fumaroides*, to complete its full life cycle. These plant species are grown mainly as ornamental plants in Wallonia and it is therefore likely that only the uredinial stage (asexual cycle) of the fungus contributes to WLR epidemics. This fungus is a biotrophic pathogen of wheat, developing mainly on leaves and requiring living green plant material in order to survive and multiply (Bolton *et al.* 2008). In Belgium, successive wheat crops do not overlap; mature plants are usually harvested 2 months before newly sown plants emerge. It is thought that WLR survives during this period by infecting volunteers (self-sown) of wheat, which provide a 'green bridge' for the pathogen's survival across seasons (Figure 1.3). A newly sown crop can be infected from the time the young plants emerge, thanks to this bridge. This phenomenon has been reported in several areas, including the southern Great Plains of the United States (Eversmeyer & Kramer 1998), Australia (Beard 2005) and Europe (Casulli 1988). WLR in winter wheat can over-winter as mycelia or urediniospores if temperatures are suitable (Roelfs 1989) and can survive in the same environmental conditions in which wheat survives (Roelfs *et al.* 1992). From spring, the inoculum that is already present in the field initiates epidemics, leading to infection of the upper leaves. Independently of this green bridge, the first symptoms observed in a field can also result from infection by urediniospores carried upwind by air masses from distant infected fields (Kolmer 2005; Nagarajan & Singh 1990; Roelfs *et al.* 1992).

The asexual cycle of *P. triticina* comprises three main steps: infection of leaves, pathogen growth in leaf tissue and spore production. Urediniospores germinate in the presence of free water with temperatures of 15-25°C (Vallavieille-Pope *et al.* 1995). A temperature of 15-20°C, combined with a dew period of at least 3-4 h, is generally considered as optimal (Eversmeyer *et al.* 1988). The spores elongate until they meet leaf stomata (Dickinson, 1970) and they then propagate in the intercellular spaces, forming haustoria structures for the plant's nutrient uptake. About 7-10 days after infection, in optimal conditions, the mycelia produce uredinia (pustules), which release urediniospores after rupturing the leaf epidermis (Allen 1926). The latency period is strongly correlated with temperature; a period of 8-20 days was observed for air temperatures

ranging from 10 to 20°C (Eversmeyer *et al.* 1988). More than 3,000 urediniospores/day for about 20 days can be produced from a single pustule (Chester, 1946), which explains the explosive nature of the disease. The spores are spread mainly by wind. Rain is also able to wash the atmosphere from spores and facilitate their deposition on crops in high moisture conditions (Barnes *et al.* 2009; Li *et al.* 2009). Although most rainfall will help disperse spores through splash, heavy rain can also induce the leaching of spores deposited on leaves and can totally deplete the pustules of their urediniospores (Sache 2000). Severe epidemics result from a succession of four to five cycles of asexual reproduction in the season, when environmental conditions are favorable (Zadoks & Bouwman 1985). In essence, excellent conditions for rapid disease development are a combination of cool spring-summer nights with high moisture conditions that favor the infection process and warm (up to 25°C) windy days that accelerate dispersal and reduce the latency period. Because of climate change, mild winters and warm springs are likely to become more frequent in Wallonia, and therefore WLR could become a greater problem earlier in the season (Coakley 1999).

1.6.2.3. Inoculum survival and long distance transport

Because of the biotrophic character of the pathogens, disease survival is a key factor in the WLR epidemics in Wallonia. It is thought that the green bridge phenomenon is important in the survival of the inoculum, but there are no data yet to confirm this. Warm and wet climatic conditions in late summer and autumn influence the green bridge phenomenon by promoting infection on volunteer plants and, later, on newly sown wheat. In contrast, a heavy winter can partially destroy WLR on young plants (Daamen *et al.* 1992; Eversmeyer & Kramer 1998). The survival rate of WLR until the following spring could influence the inoculum and spore density detected when upper leaves are emerging. A lack of inoculum when the first conducive climatic conditions occur for upper leaf infection could delay epidemics and considerably reduce the risk of severe loss. Urediniospores, however, can travel long distances, in keeping with their viability (Hirst & Hurst 1967). As a result, the infection of a field could also derive from contamination by immigrant spores.

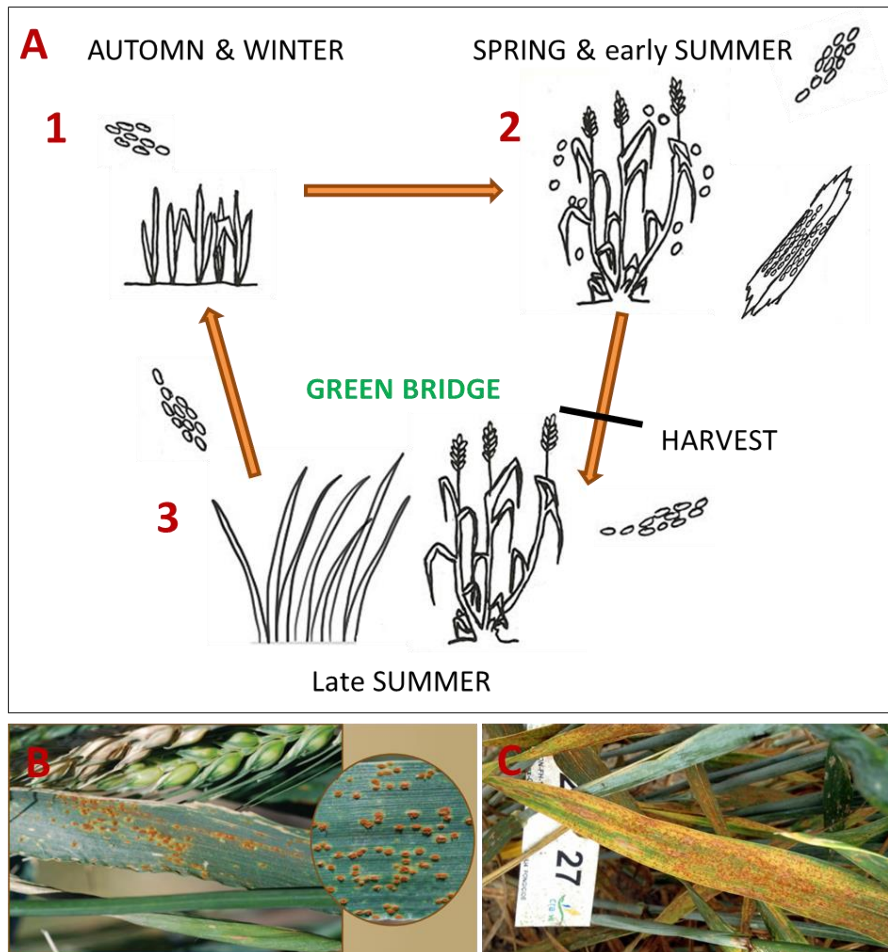

Figure 1.3. A) Asexual cycle of WLR caused by *Puccinia triticina*. A1) In autumn, urediniospores contaminate young plants in a new crop from volunteer wheat plants and the pathogen then overwinters on these plants. A2) Secondary cycles of infection occur under favorable conditions. Depending on the temperature, a full asexual cycle can be completed in 8-12 days. Infection spread within a crop occurs rapidly by wind and contact, and can also occur with spores coming from distant infected fields. A3) After harvesting, urediniospores are produced on volunteer, leading to the infection of wheat crops in the following season. This phenomenon is called the 'green bridge'. B&C) Typical WLR symptoms on the upper leaves of wheat. The fungus forms small reddish-orange pustules (uredinia), which rupture the upper surface of the leaf blade as the spores mature. Individual pustules are circular to oval, with a diameter of 1.5 mm.

In Europe, there is an air current that carries WLR urediniospores from Morocco (where they survive throughout the year) to Scandinavia (Nagarajan & Singh 1990). It is known that leaf rust uredinia developing in

the spring from infections that occurred in the autumn or winter (endogenous inoculum) are usually limited in the canopy, with the oldest infections on the lowest leaves. WLR developing from airborne (exogenous) inoculum usually occurs higher in the canopy, with the upper leaves being infected (Roelfs *et al.* 1992).

1.6.2.4. Control and prediction system in Wallonia

Yield losses depend greatly on the susceptibility of the wheat cultivar, the weather conditions, the rate of disease development and the crop stage when the disease first appeared (Eversmeyer & Kramer 2000). Biotrophic fungi such as *P. triticina* affect the plant by exporting assimilates to produce tissues and fungal spores, reducing the photosynthetic area and accelerating leaf senescence, which results in yield losses (Robert & Bancal 2004; Robert *et al.* 2005; Bancal *et al.* 2007). The use of resistant cultivars is clearly the most effective and inexpensive way to reduce disease risk. Genetic studies have shown that most WLR resistance genes confer effective resistance on a specific race of *P. triticina* (Bolton *et al.* 2008). These race-specific resistance genes in the host plant have been described (McIntosh *et al.* 1995) and most of them follow the 'gene-for-gene' concept described earlier (Section 1.6.1.4). As the WLR population is enormous, it is likely that random mutations in the fungal genome lead to the development of virulent races. As a result, there is a great diversity of races among *P. triticina* populations worldwide (Goyeau *et al.* 2007; Kolmer *et al.* 2007). It is thought that virulence probably exists for most known race-specific WLR resistance genes (Lr). New races with virulence to resistance genes can be introduced from distant sources into commonly wheat grown cultivars (Kolmer 2001). Planting resistant cultivars can exert selective pressure that could lead to the development of new races containing genes virulent to these cultivars (Singh & Huerta-Espino, 2000). New WLR races do arise, and therefore monitoring new races and using prediction systems remain very important even where most of the plants are resistant. The development of wheat cultivars with non-race-specific resistance should lead to the development of effective and durable resistance. This resistance is additive, and combining 3-5 minor genes results in plants that are highly resistant (Bolton *et al.* 2008).

Adapting agriculture practices can also help to reduce yield losses due to WLR. Working the soil after a cereal harvest is a good way of reducing the risk of severe epidemics by breaking the green-bridge phenomenon (Eversmeyer & Kramer 2000). In Wallonia, farmers often destroy debris, crop residues and volunteer plants after a harvest. Delaying seed sowing can also reduce the risk of early crop contamination, although it has been

observed that late maturity can expose a crop to significant losses due to WLR (Roelfs *et al.*, 1992; Beard, 2005).

With regard to the chemical control of WLR, many fungicides from distinct families are effective against this disease and are registered in Belgium (yellow sheets of the Livre Blanc Céréales, Gembloux). Walloon farmers sometimes used susceptible cultivars with high yield potential. Specific fungicide applications against WLR are normally recommended only in the case of susceptible cultivars if the disease starts early, risking severely infected flag leaves. The choice of fungicides for controlling *P. triticina* is less important than the timing and rate. In Wallonia, this choice is influenced by opportunities to control other diseases, such as STB and Fusarium head blight, because most of the products available are highly effective against WLR (Duvivier *et al.* 2013, 2014). The decision to spray should be based on the disease severity on the three upper leaves, which is strongly correlated with potential yield loss (Seck *et al.* 1991; Bancal *et al.* 2007). As with most of pathogens of wheat, fungicides should be applied before WLR is present on the upper leaves if they are to be completely effective (El Jarroudi *et al.* 2014b). Assessing disease infection is therefore a key variable in optimizing fungicide spraying time (Moreau & Maraite 2000). A WLR prediction system has not yet been validated for Wallonia.

Improved knowledge of the factors influencing the survival of the inoculum until spring should help in WLR prediction/control. A model that enables the level of inoculum survival in the spring to be identified would be useful because the earlier in a season that a rust epidemic starts the greater the potential yield losses. In addition, a model for predicting infection needs to be validated for Wallonia, as timing is critical for the effective control of rust diseases with fungicides in the case of susceptible cultivars. A prediction system adapted to Walloon conditions needs to be developed in order to prevent the risk of epidemics. Inoculum is sometimes thought to be a limiting factor in Wallonia and therefore combining a prediction model with spore trap detection could be an effective solution.

1.7. Objectives

Airborne inoculum is thought to play an important role in all the main foliar diseases of wheat that occur in Wallonia, and in Belgium in general, and could therefore be useful in predicting the risk of severe epidemics. Before this study was undertaken, information on the airborne inoculum of wheat pathogens in Belgium was limited or non-existent. Precise data on the temporal distribution of airborne inoculum above fields would therefore be

very valuable both for gaining a better understanding of epidemics and for predicting them more accurately.

The main objective of this dissertation was to show how a study of the temporal distribution of airborne inoculum in fields could contribute to:

- **improving the understanding of the epidemiology of wheat pathogens in Wallonia**
- **developing IPM strategies for controlling the main wheat fungal diseases in Wallonia**

It used two case studies for this work: *M. graminicola*, the causal agent of STB, and *P. triticina,* the causal agent of WLR. The following two chapters focus on *M. graminicola*. Chapter 2 describes the development and validation of a method based on using real-time PCR and Burkard 7-day spore traps to quantify *M. graminicola* airborne inoculum. The method was used to investigate the occurrence of airborne inoculum in Wallonia during the wheat growing season. The temporal distribution of *M. graminicola* airborne inoculum was then studied at four sites over 5 years. The relationship between airborne inoculum distribution, STB prevalence in fields and meteorological conditions was studied in order to evaluate the factors influencing airborne inoculum density.

Based on this dataset and on monitoring the dynamics of the epidemics in field, Chapter 3 focuses on the importance of airborne inoculum in STB dispersal. Various disease propagation scenarios were simulated in order to explain the initial contamination of the three upper leaves observed in fields, using climatic factors, plant development and airborne inoculum data.

Chapter 4 discusses the development and testing of a rapid method of quantifying *P. triticina* airborne inoculum combining the use of a spore trap network with specific real-time PCR. The temporal distribution of *P. triticina* airborne inoculum was then studied in Belgium over five growing seasons in a network of trial fields. Evidence of the existence of the green bridge phenomenon in Wallonia was researched and conditions influencing the inoculum availability required to start epidemics were identified. The final chapter (Chapter 5) describes the integration of the data on the quantification of WLR airborne inoculum into a model for predicting WLR infection. The objective was to provide a clear example of how airborne spore monitoring could lead to economic and environmental improvement if the risk of epidemics could be predicted more accurately.

The conclusion to this dissertation summarizes and discusses the main findings and proposes possible avenues for improving the management and control of STB and WLR.

Chapter 2

Real-time PCR quantification and spatio-temporal distribution of airborne inoculum of *Mycosphaerella graminicola* in Belgium

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Comments

A paper on this topic was accepted and published in July 2013 by the *European Journal of Plant Pathology*:

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This paper constitutes the first part of this chapter. It includes data covering only the first two growing seasons (2009-10 and 2010-11) of the five seasons covered in the study. A compilation of the main results, including data from the 2011-12 to 2013-14 seasons, was therefore prepared and presented as an addendum (Section 2.5) in support of the main conclusions presented in this chapter.

Abstract

Two kinds of propagules play a role in *Mycosphaerella graminicola* dissemination: splash-dispersed pycnidiospores and airborne sexual ascospores. A method based on real-time polymerase chain reaction (PCR) assay and using Burkard spore traps was developed to quantify *M. graminicola* airborne inoculum. The method was tested for its reliability and applied in a spore trap network over a 2-year period in order to investigate the spatio-temporal distribution of airborne inoculum in Belgium. At four experimental sites, airborne inoculum was detected in both years. A seasonal distribution was observed, with the highest mean daily quantities (up to 351.0 cDNA) trapped in July and with clusters detected from September to April. The first year of trapping, a mean daily quantity of 15.7 cDNA of *M. graminicola* airborne inoculum was also detected in the air above a building in a city where the spatio-temporal distribution showed a similar pattern to that in the field. Mean daily quantities of up to 60.7 cDNA of airborne inoculum were measured during the cereal stem elongation and flowering stages, suggesting that it contributes to the infection of upper leaves later in the season. Most detection, however, tended to occur between flowering and harvest, suggesting significant production of pseudothecia during that period. Variations in mean daily quantities from 1.0 to 48.2 cDNA were observed between sites and between years in the patterns of airborne inoculum. After stem elongation, the quantities detected at a site were positively correlated with the disease pressure in the field. Quantities trapped at beginning of the growing season were also well correlated with the disease level the previous year. Multiple regressions revealed that some factors partly explain the daily variations of airborne inoculum.

2.1. Introduction

Mycosphaerella graminicola (anamorph, *Septoria tritici*) is the causative agent of septoria tritici blotch (STB) in winter wheat. The disease is endemic in Belgium, although STB pressure in fields varies from year to year (Moreau & Maraite, 1999). STB can cause yield losses of up to 30-40% when the upper leaves are severely affected (King *et al.*, 1983; Eyal, 1987).

Two kinds of propagules play a role in the dissemination of the pathogen: splash-dispersed pycnidiospores and airborne sexual ascospores (Eyal, 1999; Palmer & Skinner, 2002). Airborne ascospores originating in stubble or trash are now considered to be the most important source of primary infection occurring in autumn and winter (Scott *et al.*, 1988; Shaw & Royle, 1989a). The subsequent development of STB occurs through the infection of the upper leaves by splash-dispersed pycnidiospores produced on the lower leaves under favorable weather conditions (Shaw & Royle, 1993). Several sexual cycles and ascospore generations can be produced by *M. graminicola* during the growing season (Kema *et al.*, 1996b), however, the role played by ascospores in the progress of STB epidemics, especially after stem elongation in wheat plants, has been addressed in several studies (Zhan *et al.*, 1998; Hunter *et al.*, 1999; Fraaije *et al.*, 2005; Selim *et al.*, 2011). Hunter *et al.* (1999) studied the patterns of detection of airborne inoculum of *M. graminicola* using a Burkard volumetric trap sited next to winter wheat field trials and confirmed that ascospores can be released throughout the year. Although the patterns of ascospores captured differed over the 3 years studied, a seasonal gradation was observed, with two periods of important detection: in autumn and early winter and between April and July. These results suggested that the maturation of pseudothecia and the production of ascospores might have occurred at different periods of the year, depending on weather conditions and the sources of pseudothecia. These sources include wheat crop debris, currently infected wheat crops and, to a lesser extent, wheat volunteer plants or other host plants (Suffert *et al.*, 2011).

In many countries, the major release of ascospores from crop residues occurs during the early growth stage of young plants and falls to a low level after winter (Brown *et al.*, 1978; Scott *et al.*, 1988; Shaw & Royle, 1989a; Bathgate & Loughman, 2001; Suffert & Sache, 2011). Great quantities of ascospores, similar to those trapped during the early growth stage of wheat crops, have also been trapped in the spring, between April and June (Hunter *et al.*, 1999; Fraaije *et al.*, 2005; Selim *et al.*, 2011). From field observations in the UK, France, Denmark and Belgium (Hunter *et al.*, 1999;

Eriksen & Munk, 2003; Clinckemaillie *et al.*, 2010), pseudothecia were reported on infected wheat plants but only later in the growing season, generally after stem elongation. In these studies, mature pseudothecia were visible first on the lower leaves of plants, from March-April, and then progressively on the upper leaves. Their number rose during the growing season with the senescence of each leaf layer. It is thought, therefore, that in the northern hemisphere the major source of airborne inoculum changes in about March-April from pseudothecia developing on wheat debris to pseudothecia developing on the infected leaves of wheat crops.

Very little is known about the factors influencing the density of ascospores of *M. graminicola* because most studies focusing on airborne *M. graminicola* ascospores were performed only at one site. The density of ascospores of *M. graminicola* trapped at the end of the growing season in Argentina was positively correlated with temperature, relative humidity and rainfall (Cordo *et al.*, 1999). Other studies on Ascomycetes showed that rainfall, dew point, wind speed and wind direction positively affected the daily density of ascospores (Trout & Levetin, 2001; Burch & Levetin, 2002). Hasnain (1993) showed significant correlations between the emission of ascospores and relative humidity, rainfall the day of recording and rainfall the day before. Radiation, minimum humidity, changes in humidity and minimum wind velocity were also correlated with ascospore release (Lyon *et al.*, 1984).

Hirst-type air samplers, combined with inoculum detection using PCR assays or microscopy observations, have been used to study the density of airborne inoculum of various plant pathogens (Calderon *et al.*, 2002a; Holb & Heijne, 2004; Luo *et al.*, 2007; Rogers *et al.*, 2009; Fountaine *et al.*, 2010), including *M. graminicola* (Fraaije *et al.*, 2005; Selim *et al.*, 2011); Fraaije used a specific real-time PCR assay to study the role of ascospores in the spread of QoI resistance strains in *M. graminicola*. Conventional methods of monitoring airborne inoculum using microscopy are time-consuming and require experienced operators. The accuracy and sensitivity of the real-time PCR method compared with microscopy observation and the potential of spore traps, coupled with real-time PCR to investigate the development of epidemics in crops, had already been demonstrated (Fraaije *et al.*, 2005b; Luo *et al.*, 2007; Rogers *et al.*, 2009; Fountaine *et al.*, 2010).

A rapid and reliable method of quantifying *M. graminicola* airborne inoculum combining the use of a spore trap network with a specific assay targeting the nucleic DNA of *M. graminicola* was first developed and tested for its reliability. The spatio-temporal distribution of *M. graminicola* airborne inoculum was then studied in Belgium over a 2-year period and factors influencing the daily density of ascospores were identified.

2.2. Materials and methods

2.2.1. Burkard 7-day recording spore traps.

Airborne inoculum was collected using Burkard 7-day recording spore traps (Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK). For all the experiments, the spore trap openings were always placed 1 m above ground level in a wheat-free plot measuring 9 m^2, in order to avoid capturing the splash-dispersed conidia of *M. graminicola*. The throughput of each spore trap was regulated at 10 liter per minute, corresponding to 14.4 m^3 every 24 h. The traps collected airborne particles on wax-coated Melinex tape (Burkard Manufacturing Co. Ltd) (345 mm x 20 mm) attached to a drum that completed one rotation over a 7-day period. The spore trap tapes were covered with a thin film of Vaseline and were replaced weekly. After exposure, each tape was cut into seven daily segments of 48 x 20 mm.

2.2.2. Spore disruption and DNA extraction.

Each daily tape segment was placed in a 2 ml microtube for total DNA extraction, using the method described by Lee and Taylor (1990) and modified by Williams *et al.* (2001) and Calderon *et al.* (Calderon *et al.*, 2002b). A volume of 220 µl of Nonidet P40 0.1% and 0.2 g of 0.5 mm Zirocnia/Silica Beads (BioSpec Products, Inc., Bartlesville, Oklahoma, US) was added to the microtube. The tubes were shaken in a FastPrep® machine (ThermoSavant, Carlsbad, California) for two periods of 40 s, at 6 m s^{-1}, with 5 min cooling in ice between these periods. DNA was extracted from the resulting suspensions. The samples were mixed with lysis buffer (containing Tris 50 mM, EDTA 50 mM, SDS 3% and β-mercaptoethanol 0.1%), incubated at 65°C for 1 h, and the DNA was extracted from each sample with phenol:chloroform (1:1) precipitated with isopropanol and 20 µg of glycogen (Roche Diagnostics Ltd, Lewes, UK) and washed with ethanol. The DNA pellet was dissolved in 50 µl of molecular biology grade water (5 PRIME, Inc., Gaithersburg, Maryland, US).

2.2.3. Specific and quantitative detection of *M. graminicola* using real-time PCR.

A set of primers and Taqman probe specific to *M. graminicola* was designed (MG-probe: acg act cgc ggc ttt cac cca acg, labeled with FAM and containing BHQ-1 as quencher; MG-For: att ggc gag agg gat gaa gg; MG-Rev: ttc gtg tcc cag tgc gtg ta) using the Beacon Designer 3.0 program (Biosoft International, Corina Way, California, US). The target region was a part (a

fragment of 101bp) of the DNA lyase coding gene of *M. graminicola* (GenBank accession number: AF440399 and AF440398). Real-time PCR protocol was optimized by varying product quantities, times and temperatures applied in the PCR reaction. The mixture for the real-time PCR assay was prepared with 12.5 µl of 2x qPCR MasterMix (Eurogentec, Seraing, Belgium), forward and reverse primers at 500 nM, probe at 500 nM and 2.5 µl of DNA extract in a total volume of 25 µl. The amplification reaction was conducted on the Bio-Rad iCycler (Bio-Rad and software ICycler IQ version 3.0, Hercules, California) as follows: an initial denaturation at 95°C for 10 min, and 40 cycles each at 95°C for 15 s, 60°C for 20 s and 72°C for 40 s. The increase in fluorescence from the probe was recorded at 72°C during every cycle. Each sample was run in two replicates.

The specificity of the primers and probe was assessed using the Blastn algorithm (Altschul *et al.*, 1990). The specificity of the method was also tested by including DNA extracts from reference isolates of *M. graminicola* (MUCL references 45550, 45549), *Staganospora nodorum* (MUCL references 30165, 44704 and 44707), *Fusarium graminearum* (MUCL references 43802, 43803 and 46388), *F. culmorum* (MUCL references 43796, 43797 and 43798), *F. poae* (MUCL references 42824, 42836 and 42842), *Oculimacula yallundae* (MUCL references 40386 and 40387), *O. acuformis* (MUCL references 40388, 40389 and 40637), *Sclerotinia sclerotiorum* (MUCL references 11553 and 30163) and *Microdochium nivale* (MUCL references 15949 and 31963).

The amplified fragment of 101 bp obtained after PCR using DNA extracted from *Mycosphaerella graminicola* strain MUCL reference 45550 was cloned into a plasmid using the pGEM®-T Vector System (Promega, Madison, Wisconsin) for standardizing the PCR reaction. Four tenfold serial dilutions of plasmid (from 1.86E+06 to 1.86E+03 DNA copies (cDNA)) were used as a template for each real-time PCR run. The construction of the standard curve was based on the relationship of CT values (CT value is defined by the cycle at which an increase of fluorescence exceeds the background) and known DNA copies (cDNA). The CT values were plotted against the logarithm of starting quantity of the standard for each dilution. Amplification efficiency was calculated from the slope of the standard curve by the software ICycler IQ version 3.0 (BioRad, Hercules, California, US).

In order to determine the detection threshold of the real-time PCR and the relationship between cDNA values and number of ascospores trapped, a conidia suspension was prepared from the *M. graminicola* strain MUCL reference 45550 using 0.1% Nonidet P40. The concentration of conidia in suspension was estimated using a Thoma counting chamber. The suspension was adjusted to 2 x 10^7 conidia/ml and a tenfold serial dilution

up to 200 conidia/ml was prepared. A volume of 220 µl of each conidia suspension, corresponding to 4.4 x 10^6 conidia to 44 conidia on Melinex tapes, was placed in a 2 ml microtube with a piece of 48 x 20 mm clean Melinex tape covered with a thin layer of Vaseline, and DNA was extracted and quantified by real-time PCR as described above. The test was replicated twice. Pycnidiospores contain between four and eight nuclei and ascospores are binucleated cells. The same level of cDNA response should be obtained with a given number of pycnidiospores and 3 times the number of ascospores, providing a similar extraction yield for both types of particles.

2.2.4. Reliability of the quantification method.

In order to assess the relevance of airborne inoculum information emanating from trapping, the data from distinct traps placed either side by side or at distinct locations were compared. Two Burkard 7-day spore-recording traps (spore traps A and B) were placed 1 m apart in a wheat field at Perwez, Belgium from 17 November 2009 to 2 December 2010. Three other Burkard 7-day spore-recording traps were placed at Perwez, Belgium from 8 July to 10 October 2009. Two of them (spore traps 1 and 2) were set up in a wheat field, 100 m apart. The third trap (spore trap 3) was placed 70 m away from the two other traps in a sugar beet field adjacent to the wheat field (Figure 2.1). In both these experiments, one of the traps (at Perwez) was part of the network described below. Both experiments were set up under normal cropping conditions for the Walloon region, Belgium, on previous crops known not to be involved in *M. graminicola* primary inoculum or the development of STB epidemics (Table 2.1). Between-trap variations in the airborne inoculum data were assessed using descriptive statistics and the Kruskal-Wallis test for non-parametric data (Minitab 13; Minitab Inc., State College, US). Data corresponding to a day without detection for all traps and data from the month of August corresponding to the harvest period were excluded.

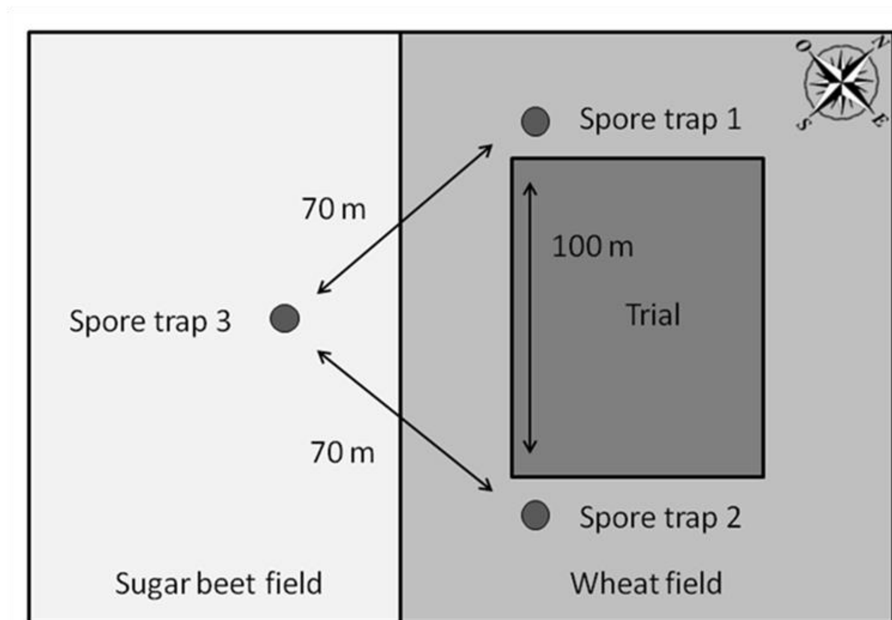

Figure 2. 1. Location of the three spore traps in a wheat field and a sugar beet field at Perwez, Belgium

2.2.5. Spore trap network.

A network of five Burkard 7-day spore-recording traps was set up in the Belgian Walloon region and run continuously from 15 April 2009 to 14 April 2011 (Figure 2.2). Four traps were in wheat fields at Perwez, Voroux-Goreux, Tournai and Niverlée. The spore trap openings were always placed 1 m above ground level. These sites provided a good representation of Walloon's wheat-growing regions. In mid-October 2009 and mid-October 2010, the spore traps were moved several kilometers away to newly sown wheat fields. The fifth spore trap was placed on the roof of a four-storey building (30 m high) in Louvain-la-Neuve, 14 km from the Perwez site, in order to study airborne inoculum in a higher air layer. Between-site and between-year variations in the airborne inoculum data were assessed, using descriptive statistics and the Kruskal-Wallis test. Data from the month of August corresponding to the harvest period were excluded from the analysis.

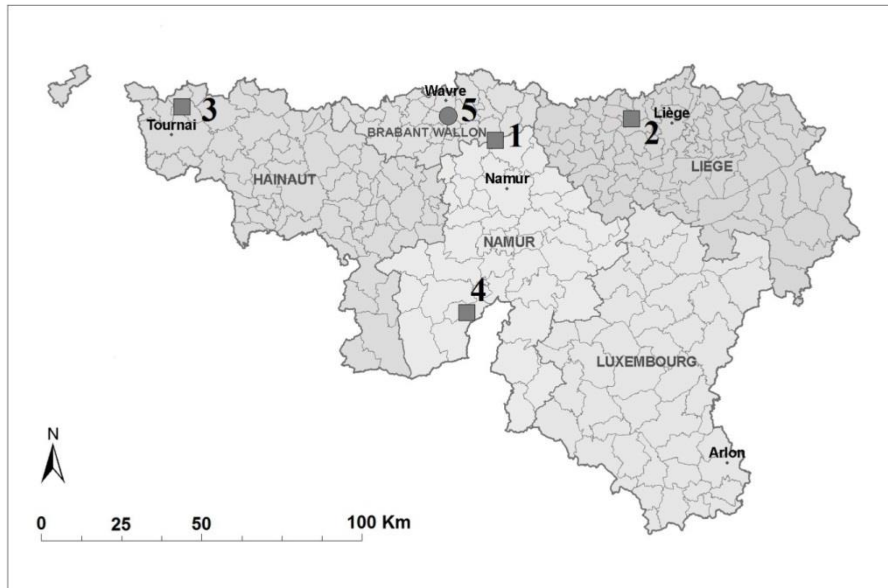

Figure 2. 2. Location of the spore traps in the Walloon region, Belgium (1: Perwez; 2: Voroux-Goreux; 3: Tournai; 4: Niverlée; 5: Louvain-la-Neuve). The squares represent spore traps in a wheat field and the circle represents the spore trap set up on the roof of a building

2.2.6. Factors influencing detection of airborne inoculum.

Meteorological data were obtained from iMetos stations (Pessl Instruments, Austria) set up in trial fields or 5-10 km away for measuring temperature, rainfall quantity, relative humidity wind speed and wind direction. Data from two national networks of meteorological stations, Pameseb (Asbl Pameseb, Libramont) and Institut royal météorologique de Belgique (IRM, Brussels), were used to add missing data. Daily detections from all traps placed in fields were first correlated with daily rainfall in order to highlight the possible contamination by splash-dispersed pycnidiospores on the Melinex tapes. The analysis was made using data (omitting 0 data) from all field sites for the period between 15 April and 31 July when STB is at its height in Belgian fields.

The four spore traps in the fields were all placed next to a fungicide trial (Table 2.1). Each fungicide trial field contained, in addition to other treatments, four untreated plots (1.5 * 10 m) of Istabraq, a cultivar known for its susceptibility to STB. The winter wheat trials were established in fields cropped by the farmers themselves, using good agricultural practices typical of the region. In the fungicide trial area, farmers applied all the treatments except the fungicide ones. In all the trial fields, the main Zadoks

growth stages were recorded and the developments of the three last leaf layers were assessed weekly.

Table 2. 1. Agronomic conditions in the wheat field trials, sown close to each spore trap and in which the disease was assessed.

Season	Site	Seedling date	Harvest date	Previous crop
2008-09	Perwez	17/11/2008	4/08/2009	Sugar beet
	Voroux-Goreux	4/11/2008	10/08/2009	Sugar beet
	Tournai	7/11/2008	5/08/2009	Sugar beet
	Niverlée	19/11/2008	17/08/2009	Sugar beet
2009-10	Perwez	18/10/2009	18/08/2010	Carrot
	Voroux-Goreux	15/10/2009	2/08/2010	Pea
	Tournai	26/10/2009	10/08/2010	Potato
	Niverlée	20/10/2009	12/08/2010	Sugar beet

In all the trial fields, disease severity (leaf area % covered by *M. graminicola* symptoms) was assessed from emergence to senescence by observing symptoms from leaf 3 to leaf 1 (flag leaf). Every week from the end of April to mid-July, 15 Istabraq plants were randomly observed in each of the four untreated plots. For each assessed leaf layer, the area under the disease progress curve (AUDPC) was calculated as follows:

$$\text{AUDPC} = \sum_{i=1}^{n} [(Y_{i+1} + Y_i) \div 2] \times [X_{i+1} + X_i]$$

in which Y_i = disease severity (%) at the i*th* observation, X_i = time (days) at the i*th* observation, and n = total number of observations.

The AUDPC was calculated from the emergence of the leaf until a sum of Celsius degree day (DDc) specific to each leaf layer (L3 = 800 DDc; L2=850 DDc and L1=900 DDc). The AUDPC values obtained for the three last leaf layers were added up to obtain a unique set of data (disease level) reflecting the disease pressure in a field late in the growing season. The log-transformed disease-level values were subjected to an analysis of variance (ANOVA) followed by a multiple comparison of means test with α = 0.05 (Tukey's test).

As the main sources of airborne inoculum varied during the growing season, analyses were performed separately for both the following periods: the first period ran from stem elongation (15 April) to the beginning of the harvest (31 July); the second period started after the harvest (1 September) and

ended at stem elongation (14 April). The measurements made in the eight fields were considered as independent.

A Pearson coefficient correlation was computed between the disease level values and the mean daily quantity of airborne inoculum. Multiple linear regression was used to assess the effect of field, rainfall, temperature, relative humidity, wind speed and wind direction on the log-transformed intensity of daily detection. All statistical analyses were performed using Jump 8 (SAS institute Inc., US).

2.3. Results

2.3.1. Assessment of the specificity of real-time PCR assay for detecting *M. graminicola*.

The amplification of plasmids prepared for quantifying *M. graminicola* for two replicates of four serial dilutions of plasmid (from 1.86E+06 to 1.86E+03 DNA copies (cDNA)) gave amplification curves with following parameters: R^2 : 0.999 and y=3.347x+41.148. The kinetics of the fluorescence curves for four tenfold dilutions of the standard had an efficiency of 99%. The equation used for calculating the cDNA quantities in each DNA extract was: $y = 10^{(41.148-x)/3.347}$ (y = cDNA quantity and x = CT).

The primers and probe designed for *M. graminicola* tested by Blastn showed high specificity. No amplification of the DNA extracts was obtained using the real-time PCR assay with DNA extracts from all the tested reference isolates of *Staganospora nodorum*, *Fusarium graminearum*, *F. culmorum*, *F. poae*, *Oculimacula yallundae*, *O. acuformis*, *Sclerotinia sclerotiorum* and *Microdochium nivale*.

The detection threshold of the PCR assay was estimated using DNA extracted from known quantities of pycnidiospores. The DNA of *Mycosphaerella graminicola* that had been extracted from 44 conidia on the daily segment (48 x 20 mm) of Melinex tape was detected in two out of the four tested replications (spore disruption and DNA extraction were replicated twice; real-time PCR was also replicated twice), corresponding to 6.237 cDNA in the real-time PCR, equivalent to 9 ascospores /m^3. The reproducibility of the method decreased when the CT was higher than 36 (Table 2.2).

Table 2. 2. Amplification of DNA extracts from 4.4 x 106 conidia to 0 conidia of *M. graminicola* on a daily segment (48 x 20 mm) of Melinex tape by real-time PCR. DNA extraction and real-time PCR were replicated twice. A volume of 2.5µl of DNA extract was used in each reaction. The threshold position was determined at a value of 30 CF RFU.

No. of conidia on tape	No. of conidia in real-time PCR	Mean CT	Standard deviation	cDNA in real-time PCR	Estimated no. of ascospores/ m^3
4,400,000	220,000	21.94	0.34	560,000	916,666
440,000	22,000	25.29	0.60	58,400	91,666
44,000	2,200	28.69	0.28	5,360	9,166
4,400	220	32.44	0.56	421	916
440	22	36.17	1.22	40	92
44	2	37.48*	NA	6	9
0	0	NA	0.00	0	0

*1 DNA extract detected on 2 tested

2.3.2. Reliability of the detection method.

At Perwez, from November 2009 to November 2010, over a 350-day period, the mean daily quantity of *M. graminicola* airborne inoculum, trapped with spore traps A and B placed 1 m apart in a wheat field, was estimated to be 12.6 and 11.9 cDNA, respectively. *M. graminicola* airborne inoculum was detected for 125 and 126 days in traps A and B, respectively. The profiles of daily quantities of airborne inoculum trapped in A and B were similar (Figure 2.3). The Kruskal-Wallis test performed on the data showed no significant differences for the two spore traps (H=0.06; DL=1; N global=328 and p-value=0.807).

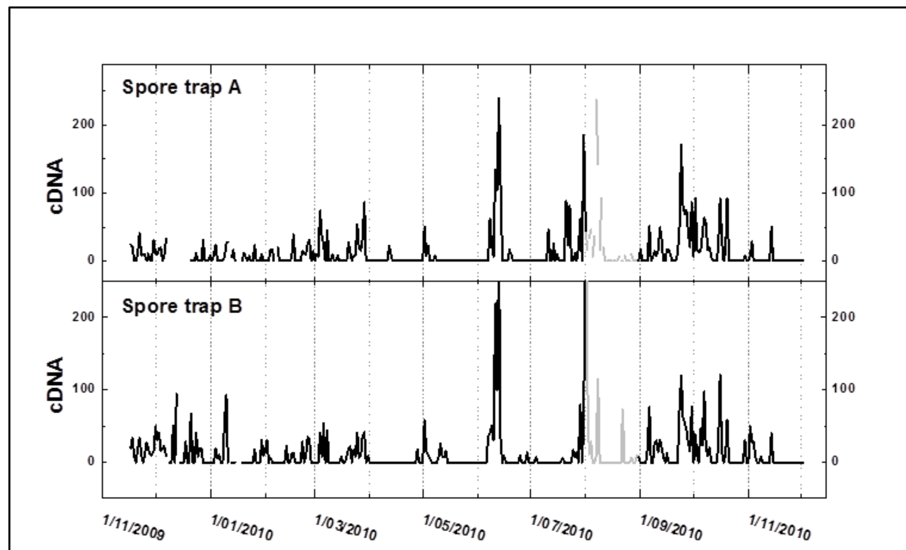

Figure 2. 3. Daily quantities of *M. graminicola* airborne inoculum trapped by two spore traps 1 m apart in a wheat field at Perwez, Belgium from 17 November 2009 to 2 December 2010. Data are expressed in cDNA in the real-time PCR (see Table 2.2). Data in grey correspond to the harvest period in Belgium.

The mean daily quantity of *M. graminicola* airborne inoculum obtained from three spore traps over 66 days in or near the wheat field at Perwez was estimated to be 97.8 and 36.4 for traps 1 and 2 in the wheat field, respectively, and 54.6 cDNA for trap 3 in the adjacent sugar beet field. Detections of *M. graminicola* occurred for 24, 22 and 21 days of the 66 tested days for traps 1, 2 and 3, respectively. The patterns of airborne inoculum trapped by the three spore traps in or near a wheat field were similar (Figure 2.4). No significant differences were observed between the profiles of the *M. graminicola* daily concentrations collected by the three spore traps (H=1.43; DL= 2; N global = 90 and p-value=0.490).

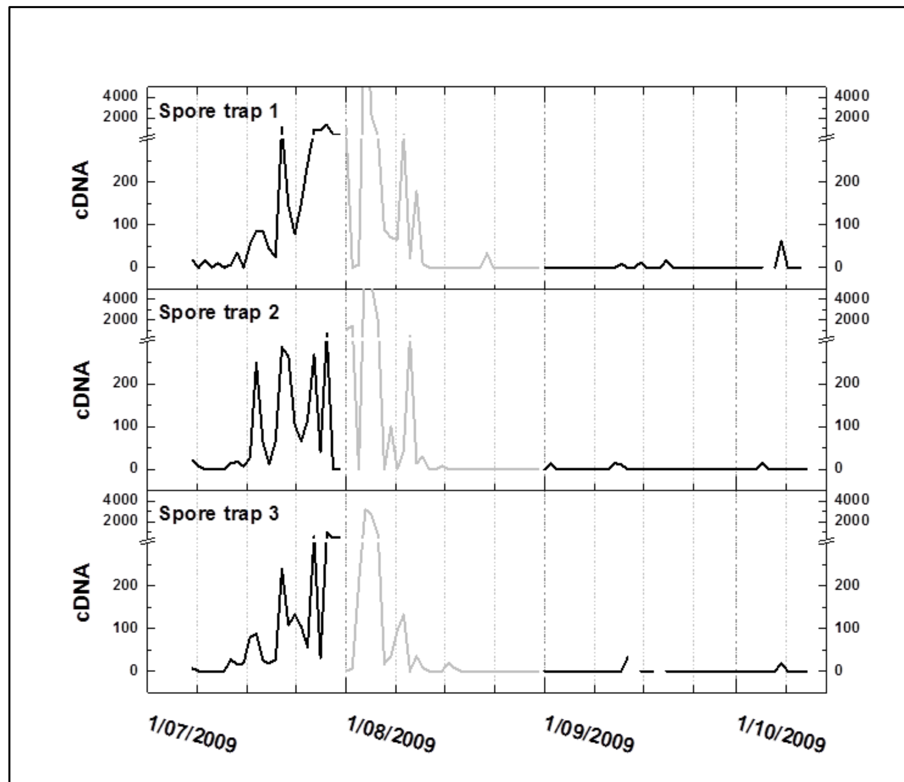

Figure 2. 4. Daily quantities of *M. graminicola* airborne inoculum trapped by three spore traps in a wheat field (1 and 2) and in a sugar beet field (3) at Thorembais, Belgium from 8 July to 12 October 2009. Data are expressed in cDNA in the real-time PCR (see table 2.2). Data in grey correspond to the harvesting period in Belgium.

2.3.3. Seasonal pattern.

The data (Table 2.3 and Figures 2.5 and 2.6) on the spatio-temporal distribution of *M. graminicola* airborne inoculum are presented in two sets covering 1 year, from 15 April in one year to 14 April in the following year, for the four spore traps in the fields and the one on the roof of a building. During the first year of trapping, from 15 April 2009 to 14 April 2010, airborne inoculum of STB was detected throughout the four seasons at all the sites (Figure 2.5). Detections occurred at all the sites from stem elongation (mid-April) to flowering. Detections of less than 100 cDNA occurred when the upper leaves were emerging. Detection frequency and peak levels were higher after flag leaf senescence. The highest detections occurred at the beginning of July, with some detections exceeding 400

cDNA. From October to mid-November 2009, groups of airborne inoculum peaks were clearly distinguishable in all the fields, but the daily quantity rarely exceeded 100 cDNA at any of the sites. Lower quantities of airborne inoculum were trapped in the coldest months of the year, December 2009 and January 2010.

Table 2. 3. Mean daily quantities and percentages of days with detection in the spore trap network for both years of trapping for different periods.

Year of trapping	Site	Mean daily quantities (cDNA) and percentages of days with detection							
		15 Apr – 30 June		1 July – 31 July		1 Sept – 14 Apr		15 Apr – 14 Apr	
1st year	Building roof Louvain-la-Neuve	no data		106,2	66,7%	6,0	30,8%	15,7	31,0%
	Perwez	60,7	61,1%	202,4	74,2%	14,1	50,3%	42,1	51,3%
	Voroux-Goreux	5,8	27,5%	111,6	80,0%	8,7	31,8%	17,2	33,0%
	Tournai	13,7	41,7%	351,0	96,7%	20,8	50,0%	48,2	48,8%
	Niverlée	7,2	16,2%	120,0	80,6%	no data		22,8	27,6%
2nd year	Building roof Louvain-la-Neuve	0,5	16,9%	5,4	18,5%	1,3	10,7%	1,3	8,7%
	Perwez	10,4	2,6%	17,8	32,3%	8,9	25,4%	9,4	22,1%
	Voroux-Goreux	0,6	5,2%	4,1	22,6%	0,7	5,4%	1,0	6,0%
	Tournai	1,4	26,0%	3,0	16,1%	1,8	14,8%	1,7	11,5%
	Niverlée	8,6	3,9%	5,3	22,6%	1,7	6,4%	3,6	12,3%

Some detections of airborne inoculum occurred over successive days between February and April 2009 at Perwez, Voroux-Goreux and especially at Tournai. In the second year of trapping, from the 15 April 2010 to 14 April 2011, airborne inoculum of STB was also detected throughout the year at all the sites. Between mid-April and the end of June, a cluster (50-100 cDNA) of peaks was detected at Perwez and Niverlée after the flag leaf had fully emerged. Only very small quantities were detected sporadically at Voroux-Goreux and Tournai during this period. At all the sites, detections occurred in July. At Perwez in 2010, clustered peaks were detected in October and November, but there were only sporadic detections at the other sites. These results reveal between-site and between-year variations. Nevertheless, in both years of trapping, a clear seasonal pattern was observed, with the highest quantities trapped between June and July and with clustered detections occurring from September to April. The detections between September and April generally peaked first in October-

November and then again in February-April, after the coldest month of the year.

2.3.4. Spatio-temporal distribution.

Important between-site variations were observed in the first year of trapping. The highest mean daily quantity was at Tournai and the smallest was at Voroux-Goreux (Table 2.3). The percentages of day with detections were the most important in Perwez. The variation between sites for this first year of trapping was significant (H=29.45, df= 3, N global = 1154 and P-value <0.001).

The quantities trapped in the second year were considerably lower (by more than 15 times) and less frequent than those trapped in the previous year. The Perwez site showed a pattern of airborne inoculum being detected more frequently and in larger quantities. The variation between sites in the second year of trapping was also considered as significant (H=38.2, df= 3, N global = 505 and P-value <0.001).

2.3.5. Airborne inoculum trapped on the roof site.

On the roof of the building in Louvain-la-Neuve, an average of 15.7 cDNA was trapped between 15 April 2009 and 14 April 2010. Detection occurred on 31% of the days in the first year of sampling. These values were slightly lower than the inoculum trapped in the field sites. The temporal distribution of the inoculum trapped on the roof was similar to that trapped in the fields. In the first year, there were small quantities detected (<50 cDNA) in June. High peaks were then detected in July. After the wheat harvest in 2009, peaks of less than 100 cDNA were regularly recorded, showing a similar pattern to that in the field. In the second trapping year, the mean daily quantity trapped as much lower and fairly insignificant compared with the quantities

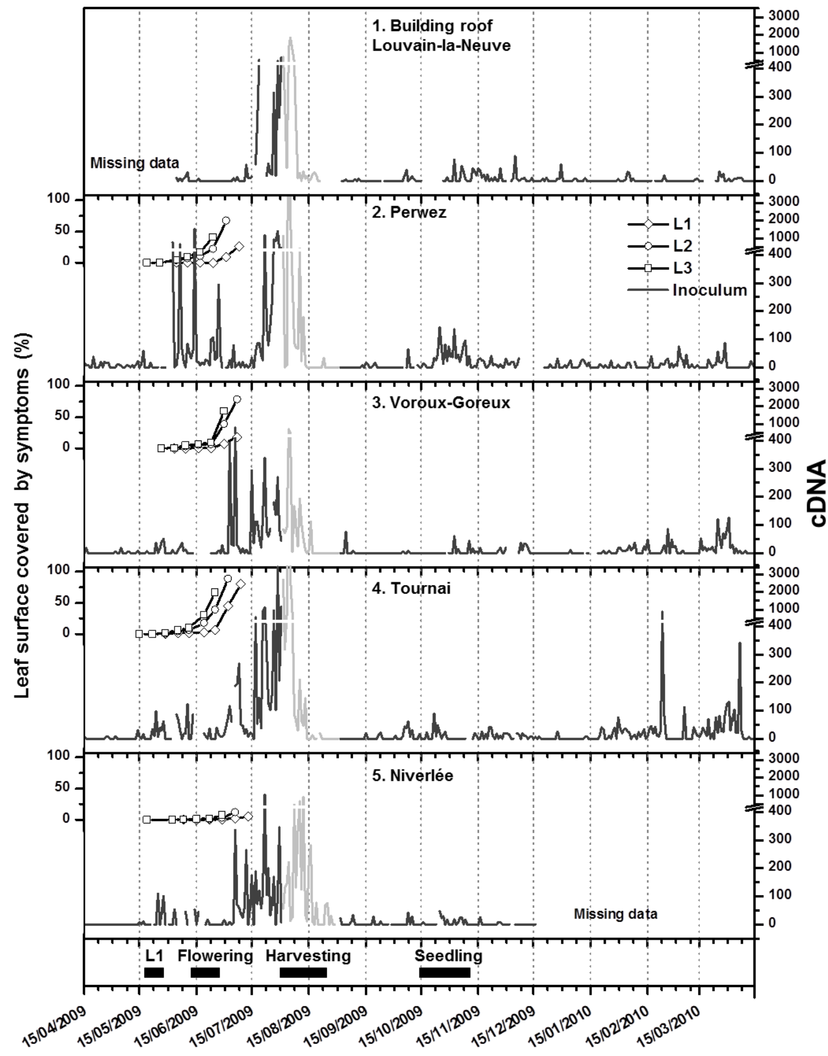

Figure 2. 5. Severity of septoria tritici blotch (STB) disease on the three last leaf layers of an unsprayed susceptible cultivar, and daily quantities of *M. graminicola* airborne inoculum trapped at five Walloon sites between 15 April 2009 and 14 April 2010. Data in grey correspond to the harvesting period in Belgium.

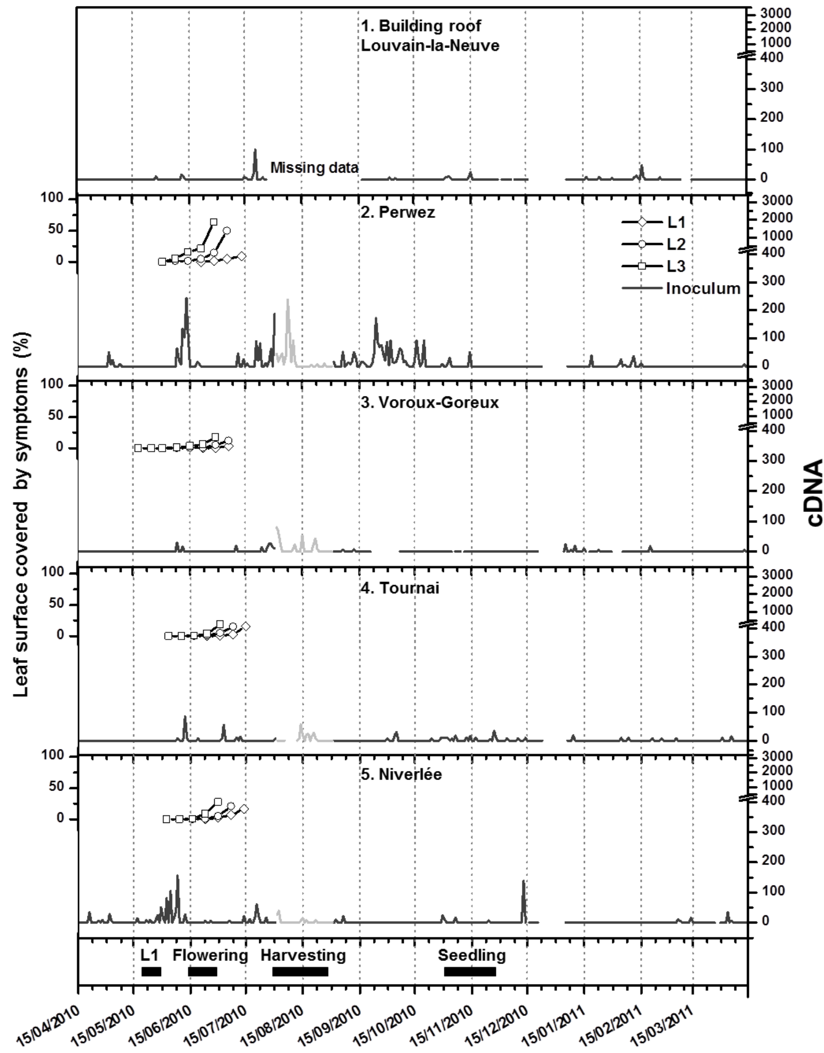

Figure 2. 6. Severity of septoria tritici blotch (STB) disease on the three last leaf layers of an unsprayed susceptible cultivar, and daily quantities of *M. graminicola* airborne inoculum trapped at five Walloon sites between 15 April 2010 and 14 April 2011. Data in grey correspond to the harvesting period in Belgium

2.3.6. Factors influencing presence of airborne inoculum

No correlation was found between the quantity of airborne inoculum trapped late in the growing season and the rainfall quantity measured on the same day (n=859 Pearson coef. =0.010, p-value=0.869).

In the trial fields, significant differences in disease pressure occurred between trial fields according to the ANOVA (F=124.3, df = 7, P-value <0.001) (Table 2.4). The disease pressure in 2009 was higher, overall, than in 2010. The disease was the highest in the field of Tournai and Perwez in 2009. The disease was the lowest in the field of Tournai, in 2010 and in Niverlée, the first year of trapping. The difference between the disease levels of these two low-level-disease fields was not significantly different (P-value =0.967).

Table 2. 4. STB disease level in eight trial fields over two growing seasons and results of a multiple comparison of means test with α = 0.05 (Tukey's test) applied on the log transformed disease level values.

Field denomination and disease level (into brackets)			**1st year 2009**				**2nd year 2010**		
			Perwez	Voroux-Goreux	Tournai	Niverlée	Perwez	Voroux-Goreux	Tournai
			(1217)	(532)	(1616)	(114)	(758)	(174)	(99)
1st year 2009	Voroux-Goreux	(532)	**<0.001**						
	Tournai	(1616)	0.435	**<0.001**					
	Niverlée	(114)	**<0.001**	**<0.001**	**<0.001**				
2nd year 2010	Perwez	(758)	**0.031**	0.225	**<0.001**	**<0.001**			
	Voroux-Goreux	(174)	**<0.001**	**<0.001**	**<0.001**	0.071	**<0.001**		
	Tournai	(99)	**<0.001**	**<0.001**	**<0.001**	0.967	**<0.001**	**0.007**	
	Niverlée	(327)	**<0.001**	**0.016**	**<0.001**	**<0.001**	**<0.001**	**0.003**	**<0.001**

The mean daily quantities of airborne inoculum trapped between plant stem elongation (15 April) and 31 July were positively correlated with the disease pressure at a site (Figure 2.7A, Pearson coef. = 0.87, P-value=0.005). From harvest to the stem elongation stage of the next crop, the mean daily quantities trapped in a field at a given location showed a close relationship with the disease pressure that had been observed at that location in the previous year (Figure 2.7B, Pearson coef. = 0.98, P-value <0.001).

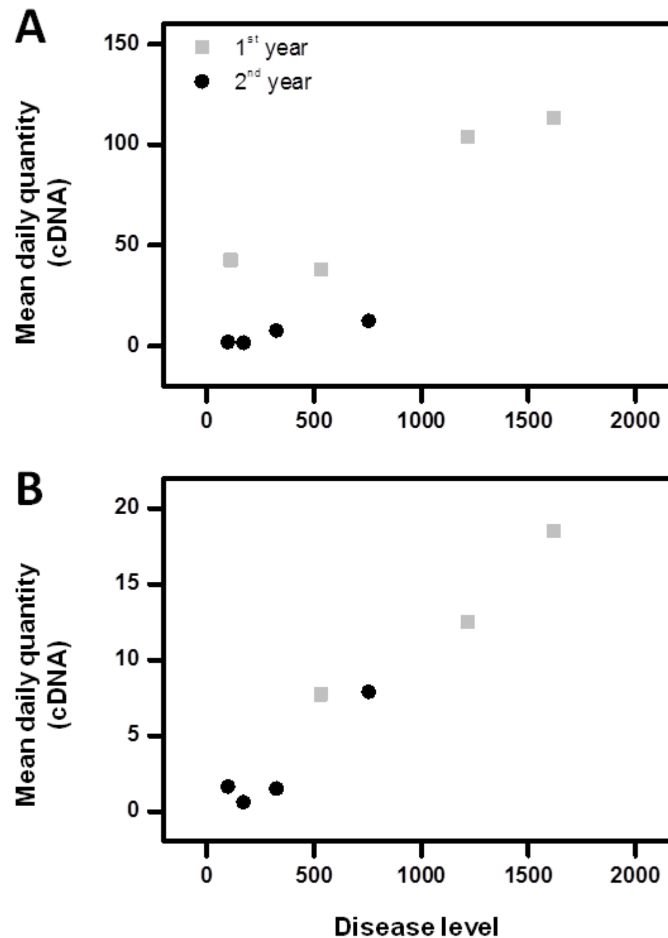

Figure 2. 7. Disease level values for untreated plants of a given crop in relation to the mean daily airborne inoculum quantities trapped over two periods: A, from stem elongation (mid-April) to the beginning of harvest (31 July); and B, from 1 September to stem elongation of the next crop. Grey squares represent the data for the first year of trapping and black circles represent data for the second year of trapping. On the figure B, one point was removed because the spore trap was moved to a new field 15 km away in October 2011, without any knowledge of the STB disease level in the previous wheat crop in this new field.

The coefficients of the multiple linear regression models built on data from the first and second period appear in Tables 2.5 and 2.6, respectively. For the period between 15 April and 31 July, 29.2% of the variability of the log-transformed daily airborne inoculum quantity trapped was explained by the variables in the model. The analysis of variance of this multiple linear model

showed a global significant effect for the field variable. In this model, the field is a categorical variable with eight distinct levels. The Perwez site, the 2nd year, was taken as the reference level and the regression coefficient of each particular field corresponds therefore to the difference of the log-transformed daily airborne inoculum quantity between this field and the reference level. Significant positive regression coefficients were observed for the field of Perwez, Voroux-Goreux and Tournai, the 1st year showing that log-transformed daily airborne inoculum quantities for these three fields were significantly higher than the reference level. It is worth noting that these three fields were also characterized by a high disease level value (table 2.4). By contrast, significant negative coefficients were observed for the field of Voroux-Goreux, Tournai and Niverlée, the 2nd year of trapping meaning that log-transformed daily airborne inoculum quantities for these three fields were significantly lower than the reference level. These fields were characterized by a particularly low disease level value. Temperature, precipitation and wind direction significantly affected detection intensities. In this model, wind direction is also a categorical variable with four distinct levels. Northwesterly winds are the reference level. The model indicates that southwesterly winds had a positive effect.

For the period between 1 September and 14 April, only 11.8% of the daily trapped Log-transformed quantity was explained by the variables in the model. Similarly in this second model, the field variable and the wind direction variable were categorical variable with respectively eight and four levels. As observed in the first model, the field variable is still a significant predictor, with the Perwez and Tournai fields in the 1st year showing a positive effect compared with the reference level (Perwez field, 2nd year) (Table 2.6). These fields had been characterized by high disease level the previous growing season (table 2.4). By contrast, significant negative coefficients were observed for the field of Tournai and Niverlée, the 2nd year of trapping meaning that log-transformed daily airborne inoculum quantities for these two fields were significantly lower than the reference level. These fields had been characterized by a low disease level value the previous growing season. The model also included temperature, relative humidity and wind speed as significant positive predictors.

Table 2. 5. Results from a multiple linear regression assessing the effect of field, rainfall, temperature, relative humidity, wind speed and wind direction on the log-transformed intensity of daily detection between 15 April and 31 July. All meteorological parameters are expressed as a daily average.

Variable	Coefficient	95% Confidence interval	P-value
Constant	-0.57	(-1.20, 0.07)	0.080
Field			
Perwez 1st year	0.45	(0.32, 0.58)	**<0.001**
Voroux-Goreux 1st year	0.21	(0.05, 0.37)	**0.009**
Tournai 1st year	0.41	(0.27, 0.55)	**<0.001**
Niverlée 1st year	0.04	(-0.09, 0.18)	0.521
Perwez 2nd year	Ref	NA	NA
Voroux-Goreux 2nd year	-0.35	(-0.51, -0.19)	**<0.001**
Tournai 2nd year	-0.41	(-0.55, -0.26)	**<0.001**
Niverlée 2nd year	-0.17	(-0.33, -0.01)	**0.035**
Temperature	0.05	(0.04, 0.06)	**<0.001**
Rainfall	0.03	(0.01, 0.04)	**<0.001**
Relative humidity	0.00	(0.00, 0.01)	0.530
Wind speed	0.06	(-0.21, 0.03)	0.072
Wind direction			
0-90°	-0.09	(-0.21, 0.03)	0.135
90-180°	-0.05	(-0.14, 0.05)	0.361
180-270°	0.16	(0.07, 0.25)	**<0.001**
270°-360°	Ref	NA	NA

Table 2. 6. Results from a multiple linear regression assessing the effect of field, rainfall, temperature, relative humidity, wind speed and wind direction on the log-transformed intensity of daily detection between 1 September and 14 April. All meteorological parameters are expressed as a daily average.

Variable	Coefficient	95% Confidence interval	P-value
Constant	-0.59	(-1.03, -0.15)	0.009
Field			
Perwez 1st year	0.28	(0.20, 0.36)	**<0.001**
Voroux-Goreux 1st year	0.00	(-0.08, 0.09)	0.909
Tournai 1st year	0.33	(0.25, 0.41)	**<0.001**
Niverlée 1st year	-0.10	(-0.21, -0.00)	0.054
Perwez 2nd year	Ref	NA	NA
Voroux-Goreux 2nd year*			
Tournai 2nd year	-0.18	(-0.26, -0.1)	**<0.001**
Niverlée 2nd year	-0.31	(-0.44, -0.18)	**<0.001**
Temperature	0.01	(0.01, 0.02)	**<0.001**
Rainfall	0.00	(-0.00, 0.01)	0.145
Relative humidity	0.01	(0.00, 0.01)	**<0.001**
Wind speed	0.03	(0.01, 0.06)	**0.010**
Wind direction			
0-90°	-0.04	(-0.12, 0.04)	0.321
90-180°	0.03	(-0.03, 0.09)	0.386
180-270°	-0.01	(-0.06, 0.05)	0.826
270°-360°	Ref	NA	NA

* Wind direction information not available for Voroux-Goreux in the second year.

2.4. Discussion

In this study, a method to quantify the airborne inoculum of *M. graminicola* at the field scale from the nuclear DNA of this fungus was developed and validated. DNA from *M. graminicola* airborne inoculum trapped by Burkard 7-day spore traps was amplified by real-time PCR in a specific and accurate way. The primers and probe designed for *M. graminicola* were highly specific and no amplification of DNA extracts from reference isolates of other species was observed. The detection threshold was estimated at 44 conidia on a daily Melinex tape (14.4 m^3 of air). Assuming an ascospore contains about a quart to a half of the nuclear DNA of a conidium, the detection threshold would correspond to about 9 ascospores.m^{-3}. This is of the same order of magnitude as the detection threshold of the method developed by Fraaije *et al.* (2005), which targets mitochondrial DNA.

The profiles of the daily quantities of *M. graminicola* airborne inoculum trapped in the two spore traps placed 1 m apart in the wheat field at Perwez were very similar. This result reveals the reproducibility of the whole quantification method using two different spore traps. The trapping profiles obtained with the two traps placed 100 m apart in a wheat field at Perwez and the one of the spore trap in the adjacent sugar beet field were all related. The number of days with detections hardly differed. Nevertheless, the mean daily quantities trapped by the three spore traps did differ because of two or three high detections, as the Kruskal-Wallis test based on rank seems confirm. Using the methodology described in this paper, trapping at a location in or near a field seemed to be representative of that field.

Although the spores deposited on the tapes were not observed microscopically, several factors indicate that the airborne inoculum trapped in the spore traps was composed mainly of ascospores. The orifices of all the spore traps were placed 1 m above ground level in a wheat-free 9 m^2 plot in order to prevent contamination by splash-dispersed conidia. Very few droplets would have been able to reach the orifice under these conditions (Fitt, 1989). This was confirmed by the total lack of correlation between the quantity of cDNA measured and the quantity of precipitation. In addition, in the first year of trapping, when frequent detection occurred, the pattern obtained on the roof of the building in Louvain-la-Neuve was similar to that obtained in the field at Perwez, 14 km away. Finally, detections made during the harvest were not taken into account at all in the study. These detections were probably influenced by contamination of the tapes by airborne plant particles or dust containing *M. graminicola* DNA.

In the four experimental fields, *M. graminicola* airborne inoculum was detected throughout the 2 years of the experiment at the four locations. Important differences in detection frequency and quantities trapped between site and between years were observed, and a seasonal pattern was apparent, with the highest quantities generally trapped between June and July and with clustered detections occurring from September to April. A similar seasonal release of ascospores was also shown in the UK (Hunter *et al.*, 1999a), with important quantities trapped in late autumn (October-December) and a second period of detections at the end of the growing season (June-July). As observed in other studies (Cordo & Simon, 1999; Hunter *et al.*, 1999a), the ascospore density in the air was highest at the end of the growing season.

This suggests that ascospores could have an impact not only as primary inoculum in autumn and winter (Shaw & Royle, 1989a), but also as secondary inoculum at the end of spring and in summer. This airborne inoculum could help to colonize upper leaf without needing splash-dispersed pycnidiospores or could exacerbate the damage caused by STB because of the presence of an addition potential inoculum. The observation by Selim *et al.* (2011), obtained with a real-time PCR assay and Burkard spore traps, that late ascospore arrival increases the amount of *M. graminicola* DNA in the upper leaf layers supports this hypothesis.

Our study also showed a positive relationship between STB on the disease pressure measured at a site and the amount of inoculum trapped in the period between stem elongation and harvest. Ascospores trapped during this period would come mainly from pseudothecia developing on the infected leaves of the current wheat crop. The significant detections in spring and summer could not entirely be the result of ascospores release from pseudothecia on the wheat residues of the previous crop, given that various authors have shown that ascospore production falls to low levels after winter (Scott *et al.*, 1988; Suffert *et al.*, 2011). The release of mature ascospores from pseudothecia on the upper leaves of plants in the growing season in Belgium, observed in 2009, confirmed that the sexual stage of *M. graminicola* can be completed on growing plants during the growing season (Clinckemaillie *et al.*, 2010).

The multiple regression showed that the field variable is a significant predictor in explaining daily detections after stem elongation. This effect seems linked to the disease pressure measured in the field. Temperature and rainfall are positive predictors in spore dispersal of *M. graminicola*, as also observed by Cordo *et al.* (1999). Southwesterly winds, coming from France (where crops are more advanced chronologically), would positively influence the quantity trapped at the end of the growing season.

The records of annual patterns of airborne inoculum and STB severity in fields highlights the positive relationship between the inoculum trapped after harvest until mid-April and the STB pressure measured at the site in the previous wheat-growing season. This relationship suggests that airborne inoculum trapped after harvest until mid-April would mainly be the result of ascospores produced by pseudothecia on plant residues. The multiple regression also confirmed that fields near a site where high disease pressure had been measured in the previous season had a positive effect on the daily detection of airborne inoculum.

This is consistent with observations reported in various other studies. In the UK, mature pseudothecia were observed on the wheat debris of the five upper leaves from August to March and on volunteer plants from November to January (Hunter *et al.*, 1999a). A great number of mature pseudothecia were still being observed in March on wheat debris. In France, Suffert *et al.* (2011) showed that ascospore release from wheat debris occurred from September to March. Scott *et al.* (1988) showed that the number of pseudothecia on wheat debris peaked in December-January and declined to a low level thereafter.

In the air above a city building, the spatio-temporal distribution of *M. graminicola* airborne inoculum showed a similar pattern to that in the field. For a given period, the quantities trapped on the building roof were generally lower than those trapped in the fields. The quantities trapped on the roof differed in both years, probably because of the differences in disease pressure observed in Walloon region in this period. Inoculum detection on the roof indicated that long transport distances of *M. graminicola* airborne inoculum is frequent, which could contribute to an increase in infection over the seasons. This type of long-distance dispersion probably explains the high amount of *M. graminicola* airborne inoculum trapped at Niverlée between 10 and 31 July 2009, although, unlike the other experimental fields, STB severity was quite low in the field near the spore trap.

The two multiple regressions correlating the log-transformed daily quantity of airborne inoculum, the field and meteorological parameters only partly explain the daily variations in detection intensity. An improvement could be made by adding parameters on the dynamic of the source (availability of pseudothecia on current crop and/or on residues), parameters on the long distance contribution and other climatic parameters such as leaf humidity and turbulence in the canopy. Ascospores can be trapped in a wheat field under various environmental and meteorological conditions. The peaks detected in Belgium were spasmodic and did not appear at the same time at the different locations. Their occurrence and the quantities trapped

therefore remain difficult to integrate into risk-based advice. However, the exploration of the relationship between disease pressure, airborne inoculum detection and meteorological conditions would provide valuable information for integrated crop protection.

Data on the spatial-temporal dispersion of ascospores will be used in a further paper focusing on the role of airborne inoculum in STB epidemics and its control.

2.5. Addendum

2.5.1. The 3 following growing seasons

In this section, the results presented relate to the entire dataset covering five growing seasons, in support of the main conclusions given in Chapter 2.

The data on the spatio-temporal distribution of *M. graminicola* airborne inoculum for the three last growing seasons (2011-12 to 2013-14) are presented in Figures 2.8, 2.9 and 2.10 and Table 2.7 in sets covering 12 months (from 15 April to 14 April in the following year) for the four spore traps in the fields and the one on the roof of a building.

In spring and early summer 2011, there was little detection of airborne *M. graminicola* inoculum except at Niverlée, where a cluster of detections occurred during the flowering period. With the dry conditions, the STB epidemics in this growing season were not severe, especially at Voroux-Goreux and Tournai. In Niverlée and Perwez, disease severity was higher. During the harvest period, there were more detections at both sites. From September 2011 to 15 April 2012, there was little detection at Perwez, Voroux-Goreux and Niverlée. Most detections were less than 100 cDNA. The Tournai site was the exception, with higher detection frequencies and quantities being trapped. The spore trap was moved to a new field 15 km away in October 2011, however, without any knowledge of the STB disease level in the previous wheat crop in this new field.

From 15 April 2012 to 14 April 2013, there were very high detection frequencies and quantities at all the sites, even higher than for the first year of trapping. From stem elongation (mid-April) to flowering (early June), many detections had peaks of more than 200 cDNA. At Perwez, Voroux-Goreux and Tournai, epidemics in the fields were intense and developed rapidly. At Niverlée, where the frequency and quantity of airborne inoculum trapped during spring were not significant, the STB epidemic was moderate. During harvesting, significant quantities were trapped at all sites. The following autumn and winter were also characterized by frequent detections and high quantities trapped.

In spring 2013, once again there were many detections (with peaks of up to 200 cDNA) at all the sites, but fewer at Voroux-Goreux and Niverlée. STB disease severity was also lower at these sites than at Tournai and Perwez.

These results of the three last growing seasons of the study confirmed the existence of between-site and between-year variations in airborne inoculum patterns. A clear seasonal pattern of airborne inoculum detection

was observed, with the highest quantities trapped between June and August and with clustered detections occurring from September to April. The important detections in spring 2012 and 2013, when the upper leaves were emerging, suggest that airborne inoculum could have an impact on the contamination of upper leaves.

The results for these seasons showed that the temporal distribution of the inoculum trapped on the roof was similar to that trapped in the fields. For a given period, detection quantity and frequency were generally less significant on the roof than in the fields. The detections on the roof (3 km from any field) showed a similar pattern to that in the fields, indicating that airborne STB inoculum is very mobile. Airborne inoculum from a given source could contaminate a distant field.

In the trial fields, there were important differences in disease levels between years as well as between sites. Using all the data available (16 fields), the mean daily quantities of airborne inoculum trapped at a site between stem elongation (15 April) and 14 July were positively correlated with the disease pressure at this site (Figure 2.7A, Pearson coef. = 0.85, P-value<0.001). The data for the fifth growing season were not included because sampling stopped in this season on 30 June. This positive correlation again indicates that ascospores trapped during this period came mainly from pseudothecia developing on the infected leaves of the current wheat crop. From harvest to the stem elongation stage of the following crop, the mean daily quantities trapped in a field at a given location showed a close relationship with the disease pressure observed at that location in the previous year (Figure 2.7B, Pearson coef. = 0.80, P-value <0.001). These observations support the hypothesis that airborne inoculum trapped after harvest until mid-April would result mainly from ascospores produced by pseudothecia on plant residues. Taken together, these two relationships indicate that in Wallonia the major source of airborne inoculum changes in about April from pseudothecia developing on wheat debris to pseudothecia developing on the infected leaves of wheat crops.

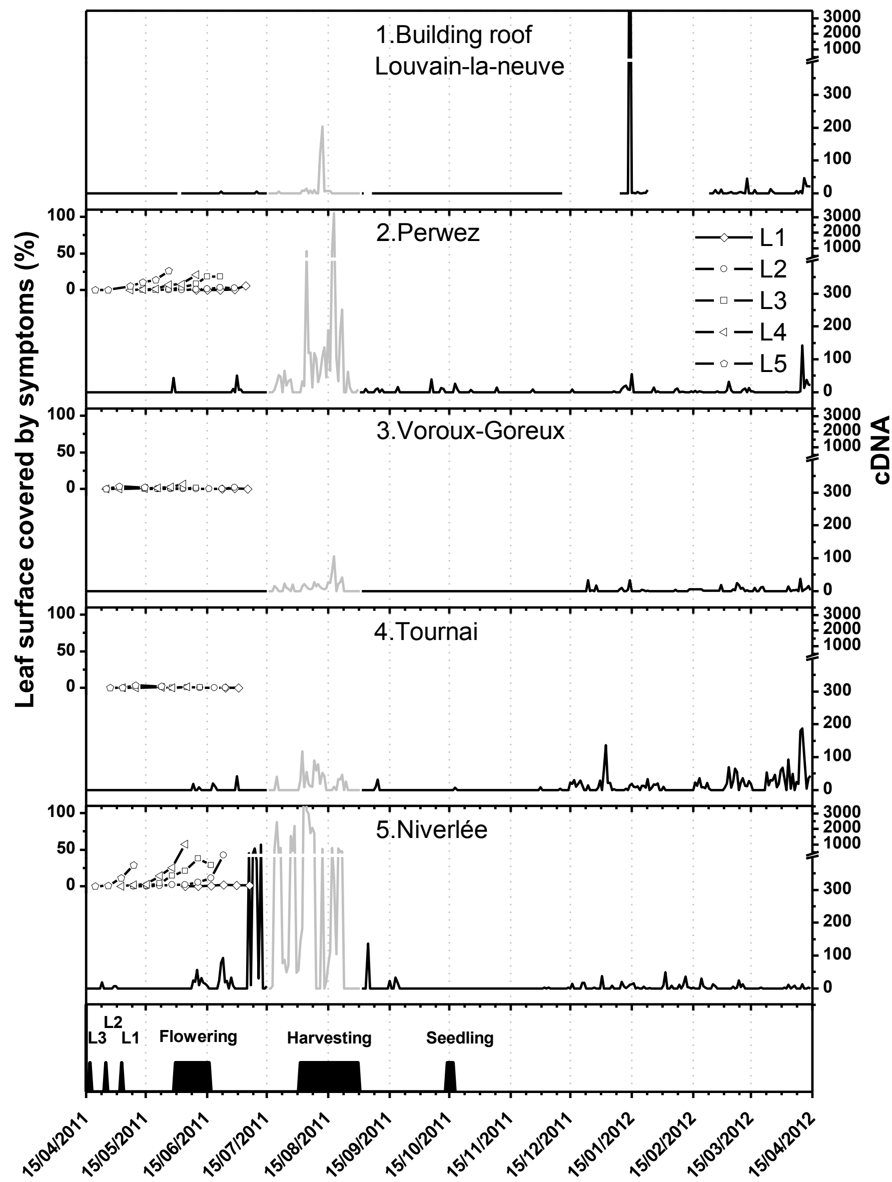

Figure 2.8. Severity of septoria tritici blotch (STB) disease on the fifth last leaf layer of an unsprayed susceptible cultivar of wheat, and daily quantities of *M. graminicola* airborne inoculum trapped at five sites in Wallonia between 15 April 2011 and 14 April 2012. Data in grey correspond to the harvest period in Belgium.

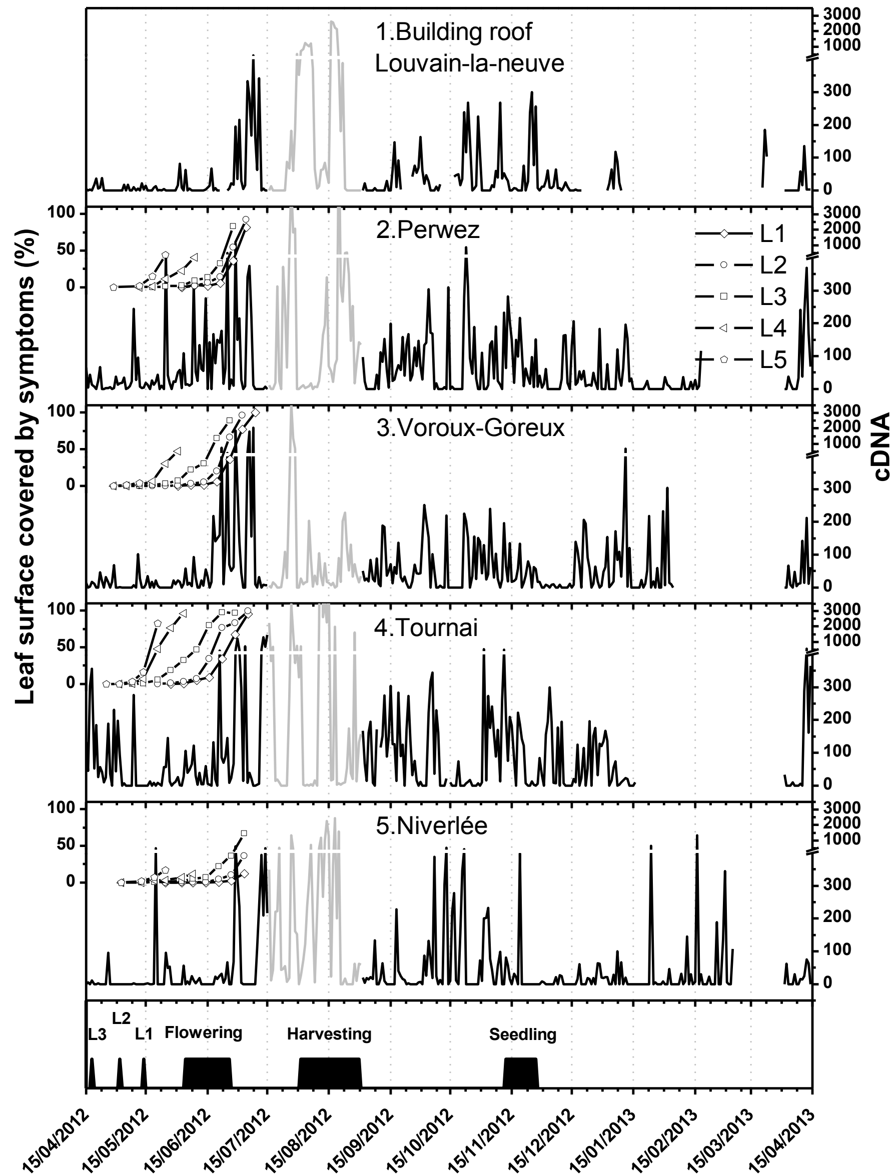

Figure 2.9. Severity of septoria tritici blotch (STB) disease on the fifth last leaf layer of an unsprayed susceptible cultivar of wheat, and daily quantities of *M. graminicola* airborne inoculum trapped at five sites in Wallonia between 15 April 2012 and 14 April 2013. Data in grey correspond to the harvest period in Belgium.

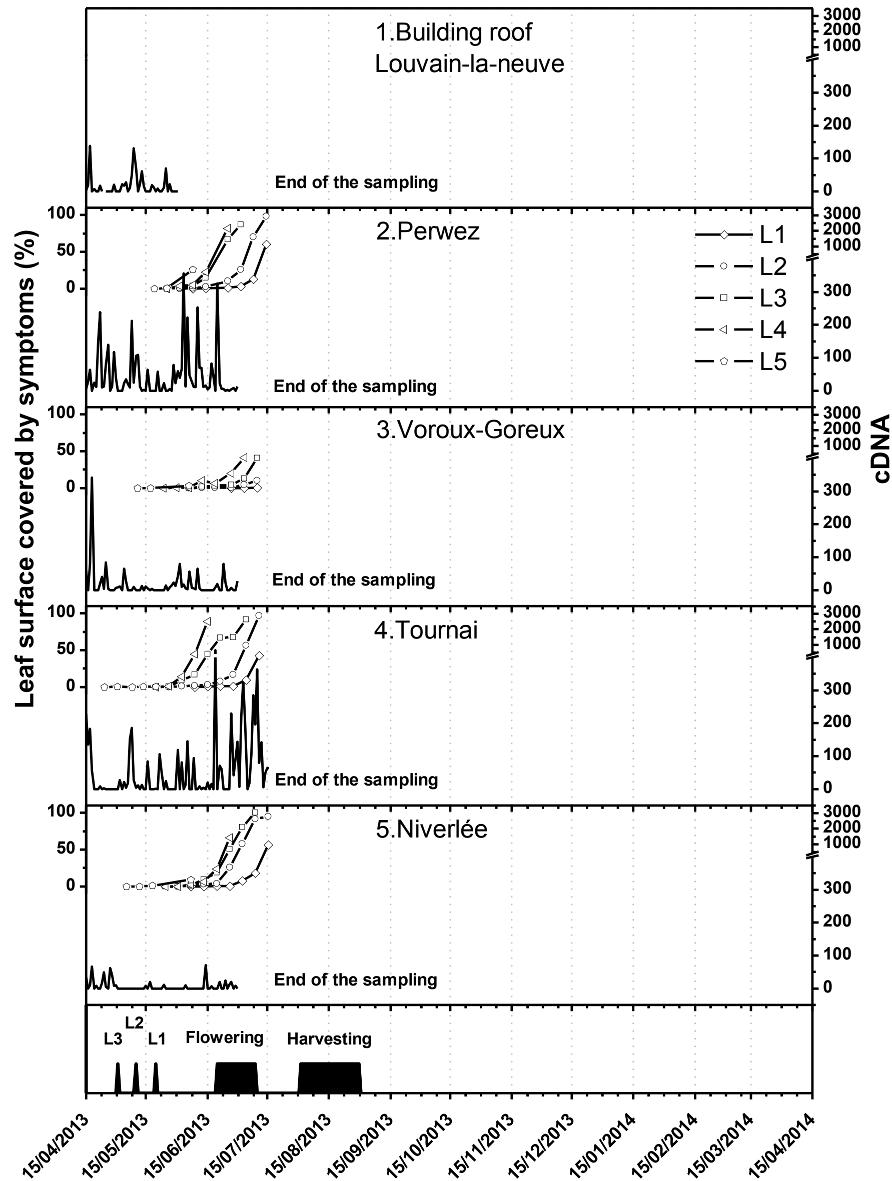

Figure 2.10. Severity of septoria tritici blotch (STB) disease on the fifth last leaf layer of an unsprayed susceptible cultivar of wheat, and daily quantities of *M. graminicola* airborne inoculum trapped at five sites in Wallonia between 15 April 2013 and 30 June 2013.

Table 2.7. Mean daily quantity and frequency of days of detection via the spore trap network over 5 years of trapping at different periods. Data for August (harvest period) have been retained in these statistics. Some of the statistics presented in this table may slightly differ with table 2.3 because many suspicious data (e.g., presence of insects on the bands, possible contaminations…) were removed for the publication in European Plant Pathology, it was not the case for this table presenting row data.

Year of trapping	Site	Mean daily quantity (cDNA) and frequency of days of detection							
		15 Apr – 14 July		15 July – 31 Aug		1 Sept – 14 Apr		15 Apr – 14 Apr	
1st year	Building roof LLN	4.4	0.27	188.1	0.83	5.4	0.28	35.0	0.37
	Perwez	50.6	0.62	517.7	0.60	12.7	0.48	88.8	0.53
	Voroux-Goreux	20.8	0.38	88.5	0.67	8.1	0.31	21.8	0.38
	Tournai	24.4	0.54	573.6	0.67	18.5	0.45	93.6	0.50
	Niverlée	17.2	0.33	141.3	0.77	4.1	0.21	35.6	0.36
	Mean (field)	**28.3**	**0.47**	**330.3**	**0.68**	**10.9**	**0.36**	**59.9**	**0.44**
2nd year	Building roof LLN	0.4	0.03	11.2	0.38	1.1	0.09	1.3	0.09
	Perwez	9.6	0.16	24.9	0.46	7.9	0.23	10.6	0.24
	Voroux-Goreux	0.7	0.03	9.3	0.31	0.6	0.05	1.9	0.08
	Tournai	2.2	0.09	3.8	0.19	1.6	0.13	2.1	0.13
	Niverlée	7.6	0.24	5.1	0.23	1.5	0.06	3.6	0.13
	Mean (field)	**4.1**	**0.11**	**10.9**	**0.31**	**2.5**	**0.11**	**3.9**	**0.13**
3nd year	Building roof LLN	0.1	0.02	8.4	0.25	83.8	0.15	47.1	0.13
	Perwez	1.3	0.05	144.4	0.67	3.0	0.21	21.1	0.23
	Voroux-Goreux	0.0	0.00	11.5	0.54	1.7	0.21	2.6	0.20
	Tournai	1.1	0.05	16.2	0.40	10.1	0.34	8.6	0.28
	Niverlée	38.1	0.27	692.5	0.69	3.0	0.22	102.1	0.29
	Mean (field)	**10.1**	**0.10**	**216.2**	**0.57**	**4.4**	**0.24**	**33.6**	**0.25**
4nd year	Building roof LLN	32.2	0.51	393.1	0.65	39.7	0.63	102.1	0.59
	Perwez	72.5	0.80	491.0	0.85	60.3	0.63	127.6	0.71
	Voroux-Goreux	108.5	0.64	164.6	0.88	56.4	0.73	88.4	0.73
	Tournai	110.0	0.76	1021.9	0.83	88.3	0.71	250.2	0.74
	Niverlée	41.8	0.40	355.3	0.85	46.3	0.47	88.7	0.50
	Mean (field)	**83.2**	**0.65**	**508.2**	**0.85**	**62.8**	**0.63**	**138.7**	**0.67**
Year of trapping	**Site**	**15 Apr – 30 June**							
5nd year	Building roof LLN	17.0	0.48	NA		NA		NA	
	Perwez	45.7	0.74						
	Voroux-Goreux	17.2	0.53						
	Tournai	56.4	0.55						
	Niverlée	6.8	0.29						
	Mean (field)	**31.5**	**0.53**						

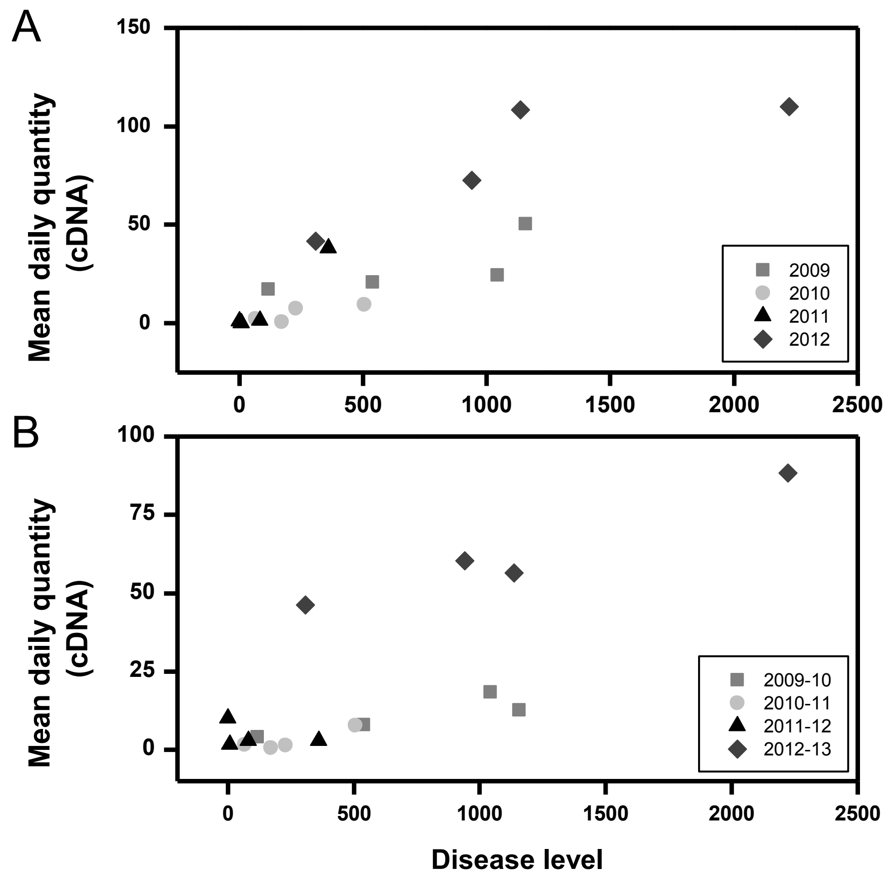

Figure 2.11. Disease level values for untreated plants in relation to the mean daily airborne inoculum quantities trapped over two periods: A, from stem elongation (mid-April) to the beginning of the harvest period (fixed for 14 July); and B, from 1 September to stem elongation of the next crop. Data from four experimental fields over four growing seasons.

2.5.2. Occurrence of higher incidence of STB on upper leaves

An incidence curve is defined here as the evolution of the percentage of leaves showing symptoms of *M. graminicola* on a leaf layer. During this five years study, STB was assessed each year on 4 sites and two cultivars. Disease incidence of STB measured on a given leaf layer was sometimes higher than on a lower leaf layer (Table 2.8). These observations do not support a vertical progression of the disease from below, associated to the dispersion of the disease by splash-borne pycnidiospores. Such an higher incidence on upper leaves was observed 17 times during the study (Table

2.8). This happened, in all the cases, in fields where high quantities of airborne inoculum were trapped.

Table 2.8. Listing of the moment when "abnormal disease gradient" of STB where observed during the study. For example, case N°1 means that on 11/07/2011 in Perwez, a higher disease incidence was observed on L1 than on L2 on the cultivar Istabraq.

N°	Cultivar	Site	year	l1-l2	l2-l3	l3-l4	l4-l5
1	Istabraq	Perwez	2011	11/07/2011			
2			2012	13/06/2012			
3		Voroux-Goreux	2009		3/06/2009 16/06/2009		
4			2012			11/06/2012	
5		Tournai	2009		5/06/2009 11/06/2009		
6			2012				16/05/2012
7		Niverlée	2013			11/06/2013 18/06/2013	
8	Lexus	Tournai	2009	5/06/2009 11/06/2009 19/06/2009		22/05/2009	
9			2011				19/05/2011
10	Julius	Tournai	2012	14/06/2012	21/06/2012		

A good example is given by the development of STB on the sensitive cultivar Istabraq in the field of Perwez in 2012 (Figure 2.12). Here, a higher disease incidence was observed on L1 than on L2 in the beginning of June when leaves were primary contaminated. In that field, significant airborne inoculum was trapped during the emergence of these two upper leaf layers. These observations do not quantify or prove the impact of the airborne inoculum on a STB epidemic on the upper leaves, but they reinforce the hypothesis that airborne inoculum play a role in the epidemic phase of STB.

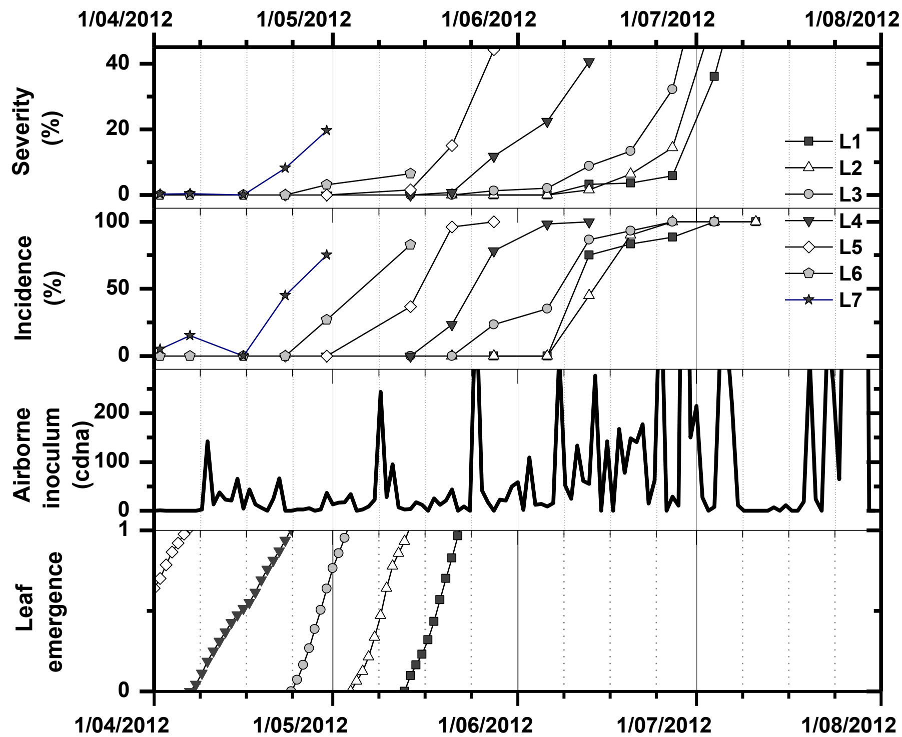

Figure 2.12. A) Severity and B) incidence of septoria tritici blotch (STB) disease on the seventh last leaf layers of an unsprayed susceptible cultivar of wheat, and C) daily quantities of *M. graminicola* airborne inoculum trapped in Perwez between 1 April 2012 and 1 Augustus 2012. D) Curves representing the leaf development of the fifth last leaf layers.

Chapter 3

A mechanistic approach for assessing the role of splashborne vs airborne inoculum in septoria tritici blotch infections of the three upper leaves of wheat

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Comments

This chapter is the basis of a paper being prepared for publication in *Plant Pathology*.

Abstract

Mycosphaerella graminicola (anamorph *Septoria tritici*) is the causal agent of septoria tritici blotch (STB), a major wheat disease that occurs worldwide. Its vertical dispersal on plants can be achieved by splashborne pycnidiospores or airborne ascospores. A third possible dispersal mechanism that has been reported is the overlapping between an infected leaf and an emerging one, leading to a transfer of pycnidiospores without the need for high-energy droplets. It is now recognized that airborne ascospores can be produced throughout the year, but the importance of airborne ascospore contamination on the upper leaf layers remains unknown. In the present study, a network of four Burkard 7-day volumetric spore traps, set up in trial fields throughout Wallonia in Belgium, operated continuously between March and June over five growing seasons (2009-2013) with the aim of quantifying airborne STB inoculum above the canopy. In most cases, airborne STB inoculum was detected between the emergence of the three last leaf layers in the wheat plants and the end of their calculated period of infection, with the inoculum load sometimes exceeding 1,000 cDNA (load of ±2,200 ascospores m^3day^{-1}). In an attempt to quantify the relative importance of each dispersal mechanism in the initial contamination observed on the upper leaf layers, three models were developed using a mechanistic approach based on knowledge of STB epidemiology and the data collected over the five growing seasons, including information on the phenology of the plants in the fields, on the disease severity and on climatic conditions. When each dispersal mechanism was considered separately, the fitted models, obtained by maximizing the probability of detection and keeping the false alarm ratio below 30%, could partly explain the contamination of the three upper leaf layers: 50-58% by splashborne dispersal, 42-46% by airborne dispersal and 12% by leaf overlapping. When the three models were combined, a more accurate model was obtained that explained 68-78% of the contamination observed in the fields. The compilation model was particularly well suited to predicting contamination on the flag leaves of plants treated with fungicides at GS32 (96-92%). Our mechanistic approach showed that airborne STB particles could possibly play an important role in the primary contamination of the upper leaves of wheat when upward dispersal by asexual spores is slowed down (e.g., by early fungicide treatment or the use of a resistant cultivar).

3.1. Introduction

Mycosphaerella graminicola (anamorph, *Septoria tritici*) is the causal agent of septoria tritici blotch (STB), an important cause of a wheat disease that occurs worldwide (King *et al.* 1983; Eyal 1987; Daamen & Stol 1992; Halama 1996; Jørgensen 1999; Hardwick *et al.* 2001; Jones & Harrison 2004). Under favorable environment conditions, severe epidemics can reduce yields by up to 30-40% (Eyal 1987). In Belgium, this pathogen occurs every year, although the dynamics and severity of epidemics vary greatly from year to year (Moreau & Maraite 2000; Duvivier 2013). The most serious yield losses occur when the three upper leaves of plants become severely infected (Shaw & Royle 1989; Thomas *et al.* 1989). The control of STB on these leaves is usually achieved by planting resistant cultivars and applying fungicides (Palmer & Skinner 2002). The ability of the pathogen to rapidly overcome cultivar resistance (Kema *et al.* 1996a; Cowger *et al.* 2000), however, and to develop resistance to fungicides (Amand *et al.* 2002; Stergiopoulos, 2003; Fraaije *et al.* 2005; Leroux *et al.* 2007; Torriani & Brunner, 2009) have made it the most problematic wheat disease in Europe. Within this context, a model for predicting the evolution of infection on the upper leaves could be helpful in adapting fungicide treatments (quantity and timing) according to disease development in the field.

The dissemination of STB involves two types of spores: splashborne asexual pycnidiospores and airborne sexual ascospores (Eyal 1987; Palmer & Skinner 2002). STB survives from one wheat cropping season to the next one as mycelia, pycnidia or pseudothecia on crop residues and volunteer hosts (Suffert *et al.* 2013). After moist conditions, airborne ascospores are released from pseudothecia (Sanderson & Hampton 1978) and can travel long distances, contaminating newly sown fields (Zhan *et al.* 1998; Linde *et al.* 2002). Where wheat was the previous crop, pycnidiospores produced on residues are able to contaminate new young wheat plants (Suffert & Sache 2011). After landing on a host leaf, both types of spores infect foliar tissue through the stomata (Eyal 1987; Kema *et al.* 1996c). About 10 days after infection under optimal conditions, the pathogen colonizes the mesophyll, resulting in the initial chlorosis and necrosis (Kema *et al.* 1996c). Brown-black globose pycnidia appeared within the lesions after 14-21 days (Eyal 1987). The time needed to complete the asexual cycle depends largely on the mean temperature (Shaw 1990; Lovell *et al.* 2004a). Thereafter, pycnidiospores can be dispersed by splash, contact or simple runoff, and infect other leaves (Shaw 1987; Shaw & Royle 1993; Lovell *et al.* 1997, 2004b). The ascomycete *M. graminicola* has a heterothallic, bipolar mating system (Kema *et al.* 1996c). An even distribution of both mating types has

been observed, even at a very small scale, as different pycnidia in the same lesion (Zhan *et al.* 2002), meaning that sexual reproduction is probably always possible. Sexual reproduction of *M. graminicola* is thought to be conditioned by infection density, since the two mating types have to meet for the formation of pseudothecia (Cowger *et al.* 2002). On a leaf, however, pseudothecia are usually observed long after pycnidia (46-76 days) (Suffert *et al.* 2011). The pseudothecia/pycnidia ratio rises during the growing season with the senescence of the leaf layer (Eriksen & Munk 2003).

The environmental factors that affect infection and disease development have been well studied, and the results show that the success of an infection depends largely on air temperature and high humidity during the process and after inoculation (Holmes & Colhoun 1974; Hess & Shaner 1985, 1987; Shaw 1990; Magboul *et al.* 1992; Chungu *et al.* 2001). In the field, a 15 h period of leaf wetness was found to be the minimum requirement for infection (Renfro & Young 1956). Cardinal temperatures reported for the germination of *M. graminicola* conidia were a minimum of 2-3°C and a maximum of 33-37°C, with an optimum of 20-25°C (Eyal 1987). The conditions conducive to infection are not really a limiting factor because the pathogen tolerates extended breaks in humidity during the infection process (Shaw 1991; Shaw & Royle 1993). Cultivar resistance and inoculum concentration are two other factors known to influence infection success (Ahmed & Mundt 1996; Chungu *et al.* 2001).

The latent period *of M. graminicola* depends largely on temperature (Shaw 1990; Henze *et al.* 2007). This period is also influenced by other factors, such as cultivar resistance, pathogenicity of the strains and environmental conditions during and after infection (Shaw 1990; Chungu & Gilbert 1996) (Viljanen-Rollinson & Marroni 2005; Suffert *et al.* 2013). In a study conducted in outdoor conditions with two cultivars with different levels of resistance to *M. graminicola*, the time between inoculation and the appearance of the first lesions was estimated to be 250 and 257 degree-days (DD) above the estimated base temperature of -2.37°C for the susceptible and resistant cultivars, respectively (Lovell *et al.* 2004a). In spring in Belgium, the latent period should last between 14 and 21 days, as is the case in other countries with comparable environmental conditions (Eyal 1987; Shaw, 1990; Lovell *et al.* 2004a). A recent study comparing the pathogenicity of ascospores and pycnidiospores concluded that the latent period was significantly longer (60 DD, above 0°C) after contamination with ascospores rather than with pycnidiospores (Morais *et al.* 2015). The lesion size, the appearance of symptoms and the density of pycnidia measured in the lesions were found to be similar, regardless of the type of spores.

In many models for predicting *M. graminicola,* including the one currently used in Belgium **(ProCulture,** Moreau & Maraite, 2000), vertical dispersal is assumed to occur only via rainfall splash, allowing the disease to progress from lower leaves to upper leaves under favorable weather conditions. Splashborne dispersal is often considered to be the limiting factor of the vertical progression of *M. graminicola*. In a well-conducted study on factors determining the severity of epidemics, Shaw & Royle (1993) argued that serious damage occurred when splashborne pycnidiospores from the lower part of the canopy infected the two newly emerged upper leaves. The timing of infection and, to a lesser extent, the amount of initial inoculum deposited on an upper leaf were found to be the most critical factors determining the impact of the disease on the crop. The conditions of infection were rarely found to be a limiting factor, with infection occurring even in exceptionally dry and hot years. It has been shown that *M. graminicola* retains an infectious potential after long and repeated interruptions of humid periods (Shaw 1991). Upward dispersal by splashing is a complex mechanism that depends on many parameters (McCartney & Fitt 1998) and is not very efficient, mainly because of the exponential decay of droplets above a height of 5-10 cm (Shaw 1987). It would be unusual for a rain splash to cause more than a few lesions on an upper leaf. One or (rarely) two cycles of asexual multiplication following the infection are therefore more likely to be responsible for an increase in disease severity on the leaves (Shaw & Royle 1993). Given this vertical dispersal, an initial fungicide treatment at Zadoks growth stage 32 (GS32) is recommended in order to slow down the progression of STB and restrict it to the lower part of plants (Cook *et al.* 1995, 1999).

More recent field studies on the contamination of the upper leaves by M. graminicola, however, have shown that vertical dispersal could have two other origins involving mechanisms other than the splash dispersal mode. Lovell et al. (1997, 2004b) showed that during the development of wheat plants, for a short period new leaves can be lower than or as high as developed leaves, with sporulating lesions of M. graminicola. The greatest degree of ‘overlap’ could bridge three successive leaves, with an emergent leaf overlapping the three more recently developed ones. The newly emerged upper leaves could be infected without any need for high energy rain splash simply by contact between a sporulating older leaf and an emerging leaf during a wet period or by the runoff of dew or droplets (after non-splashy rain) carrying pycnidiospores.

Since Kema *et al.* (1996b) showed that several sexual stages of *M. graminicola* could occur during the growing season, the role played by ascospores in the progress of STB epidemics has been addressed in several

studies, especially after stem elongation in wheat plants (Selim *et al.*; Zhan *et al.* 1998; Hunter *et al.* 1999; Moschini & Pérez 1999; Fraaije *et al.* 2005; Sameh *et al.* 2011). All these studies support the finding, based on a genetics approach or spore trap devices, that ascospores play a role in epidemics late in the growing season, as well as in the primary infection of the crop in autumn and winter. A recent study (Duvivier *et al.* 2013) showed that, in Belgium, ascospores are present in the air above the canopy when the upper leaves are emerging. This airborne inoculum could be involved in the infection of the upper leaves in spring and summer, accelerating the upward progression of the disease on the plants.

In the current study, the temporal distribution of airborne *M. graminicola* inoculum was quantified in spring over five seasons via a network of 20 trial fields (four fields each year) using Burkard 7-day spore traps and qPCR assays (Duvivier *et al.* 2013). In order to gain a better understanding of STB epidemiology and the role of the three dispersal mechanisms, this distribution was analyzed in parallel with the dynamics of the progression of STB on the upper leaves of two types of cultivars (susceptible to and resistant to STB) on untreated plants and on plants treated at GS32. The parameters of a mechanistic model involving the three known mechanisms of vertical propagation (splashborne inoculum, leaf overlapping and airborne inoculum) were determined with the aim of finding the best way to explain the first symptom occurrence on the three upper leaves. Using this original approach, the importance of the three mechanisms was investigated.

3.2. Materials and Methods

3.2.1. Experimental field network

Field trials were established over five growing seasons, from 2009 to 2013, at four sites at Niverlée, Tournai, Perwez and Voroux-Goreux in the wheat-growing region in Wallonia (Figure 3.1). For each site, field trials were conducted in five fields, varying from one year to the next in order to avoid a situation where winter wheat was the preceding crop.

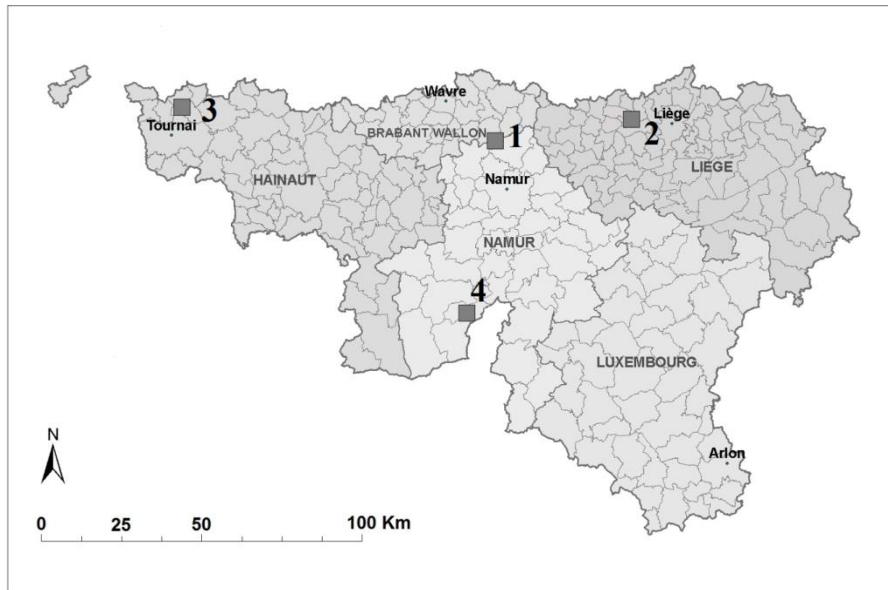

Figure 3.1. Location of the field trials in Wallonia, Belgium (1: Perwez, N 50°37'43.507'', E4°48'27.259''; 2: Voroux-Goreux, N50°39'58.414'', E5°25'40.108''; 3: Tournai N50°32'55.392'', E3°32'44.656''; 4: Niverlée, N50°7'3.716'', E4°42'35.996'').

In each field, two cultivars of winter wheat (Triticum aestivum) known to differ in their level of quantitative resistance to M. graminicola were used. Istabraq (rating 41 in 2008) was chosen as the reference for highly susceptible cultivars for the whole study. As moderately resistant cultivar, Lexus (rating[1] 7.5 in 2008), was chosen for the first three growing seasons and Julius (rating 7.51 in 2011) for the last two seasons (Livre Blanc Céréales, Gembloux). The plots (1.5-2 × 10-13 m) were organized in four

[1] Resistance evaluations were based on a rating scale of 1 to 9, where 1 was the lowest and 9 the highest rating.

randomized complete blocks and included untreated plots and plots treated with a fungicide at GS32, when L2 starts to emerge. For this fungicide treatment, 1.5 L/ha of Opus team (125 g epoxiconazole and 375 g fenpropimorph), 1 L/ha of Mirage (450g prochloraz) and 0.5 L/ha of Comet (125g pyraclostrobine) were sprayed together at a rate of 200 L/ha. This mixture is known to be effective against all the main wheat diseases in Wallonia, including STB. The winter wheat trials were established in fields cropped by the farmers themselves, using the good agricultural practices common in the region. In the trial area, the farmers applied all the treatments except the fungicide ones. Data on cultural practices and plant phenology in each field are given in Table 3.1.

Table 3.1. Agronomic parameters of the five experimental sites set up each year in the 2009-13 growing seasons. Zadoks growth stages (GS) are given for the cultivar Istabraq. GS32: second node visible; GS39: flag leaf fully emerged; GS61: end of flowering.

Year	Site	Seed density (seed/m²)	Previous crop	Sowing date	GS32	GS39	GS69	Nitrogen (kg N ha^{-1})
2008-09	Perwez	400	sugar beet	17/11/2008	7/05/2009	29/05/2009	26/06/2009	184
	Voroux-Goreux	310	sugar beet	4/11/2008	7/05/2009	25/05/2009	23/06/2009	178
	Tournai	310	sugar beet	7/11/2008	6/05/2009	22/05/2009	19/06/2009	194
	Niverlée	400	sugar beet	19/11/2008	4/05/2009	29/05/2009	26/06/2009	195
2009-10	Perwez	250	carrot	18/10/2009	10/05/2010	25/05/2010	28/06/2010	190
	Voroux-Goreux	250	pea	15/10/2009	11/05/2010	25/05/2010	29/06/2010	175
	Tournai	250	potato	26/10/2009	13/05/2010	27/05/2010	27/06/2010	227
	Niverlée	250	sugar beet	20/10/2009	15/05/2010	2/06/2010	30/06/2010	168
2010-11	Perwez	250	sugar beet	13/10/2010	3/05/2011	16/05/2011	13/06/2011	175
	Voroux-Goreux	250	sugar beet	15/10/2010	25/04/2011	10/05/2014	14/06/2011	180
	Tournai	370	chicory	20/11/2010	2/05/2011	12/05/2011	16/06/2011	205
	Niverlée	250	sugar beet	13/10/2010	4/05/2011	11/05/2011	14/06/2011	197
2011-12	Perwez	250	flax	16/10/2011	7/05/2012	21/05/2012	26/06/2012	180
	Voroux-Goreux	250	sugar beet	15/10/2011	30/04/2012	21/05/2012	25/06/2012	175
	Tournai	250	sugar beet	14/10/2011	3/05/2012	24/05/2012	20/06/2012	170
	Niverlée	250	oilseed rape	17/10/2011	3/05/2012	23/05/2012	24/06/2012	175
2012-13	Perwez	250	potato	22/10/2012	16/05/2013	3/06/2013	8/07/2013	190
	Voroux-Goreux	300	sugar beet	14/11/2012	20/05/2013	5/06/2012	6/07/2013	178
	Tournai	250	sugar beet	25/10/2012	13/05/2013	30/05/2013	4/07/2013	165
	Niverlée	250	sugar beet	27/10/2012	14/05/2013	4/06/2013	9/07/2013	180

3.2.2. Crop growth monitoring

From April to May, the development of the leaves of 10 marked plants in two untreated plots was monitored once a week at each site. The development of each plant over a marked point was observed using a percentage scale. For example, 250 corresponded to the development of two leaves and one half-emerged leaf above the mark. These datasets allowed the phyllotherm of the three cultivars to be estimated using the method of least squares. The phyllotherm was defined as the number of accumulated degree-days (DD) above a base temperature of 0°C (Gallagher 1979; Baker 1980) counted from the beginning of emergence to the full development of a leaf. This adapted phyllotherm was used to model the foliar development of the cultivar leaves as described by Moreau and Maraite (2000). The leaves were numbered relative to the flag leaf (e.g., L2 was the leaf immediately below L1, and L3 was immediately below L2, and so on). Plant growth stages (GS) were also assessed according to a decimal scale (Zadoks *et al.* 1974).

3.2.3. Disease monitoring

In all the fields, disease severity (% of total leaf area covered by STB symptoms) in untreated plants was assessed from emergence to senescence from L4 to L1. From GS30 to the end of the senescence of L1, 15 plants were randomly picked each week from each untreated plot and were examined for disease severity. Before GS30, observations were also made of the lower leaves (L7 to L5) on 15 plants once every 2 weeks. Three weeks after the fungicide treatment, disease severity in each treated plot was assessed from emergence to senescence for L4 to L1. It is worth noting that over the study period, other fungal diseases were also observed (i.e., wheat leaf rust and wheat powdery mildew).

Disease levels at the different sites on the susceptible and resistant cultivars were compared in each cropping season using an analysis of variance (ANOVA), followed by a Student-Newman-Keuls (SNK) test ($\alpha=0.05$), in order to demonstrate the diversity of disease pressure in Wallonia each year. This comparison was made for summed disease severity on L5 and L6 when L3 were emerging (GS31) and for summed disease severity on L1 and L2 at GS75. Similar analyses were used to compare the overall mean disease severity in the five growing seasons at GS31 and GS75. The arcsine square root transformations of the disease severity data for each leaf, recommended for proportional data, were calculated before performing the ANOVA (McDonald 2009).

3.2.4. Measurement of airborne inoculum

Airborne inoculum was collected using Burkard 7-day spore traps (Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK) in all the trial fields between 1 September and 30 June each year. The position of the spore traps in the fields, the treatment of the tapes and DNA extraction were similar to that described by Duvivier *et al.* (2013). The quantification of *M. graminicola* was done by real-time PCR using the method described by Duvivier *et al.* (2013). The results were presented as numbers of cDNA on the PCR (cDNA).

3.2.5. Weather data

Hourly meteorological data were captured by iMetos weather stations (Pessl Instruments, Austria) set up in most of the trial fields. Fields without a station were at a maximum distance of 10 km from a weather station. Temperature, rainfall and relative humidity (RH) were recorded at a height of 2 m above the ground. Data from the national networks of meteorological stations, Pameseb (Asbl Pameseb, Libramont) and the Institut Royal Météorologique de Belgique (IRM, Belgium) were used to fill in the missing data.

3.2.6. Assessment of periods of STB infection of L3 to L1

For the three upper leaves, the actual date of symptom occurrence was between the last date on which symptoms were not yet visible (**D0**) and the first date on which symptoms were visible (**Dsymp**). The boundaries of this time period, reduced by a period of latency, enabled the likely period of infection to be calculated. In order to estimate the period of infection by pycnidiospores **(PIpyc)**, the latency periods were determined using the equations devised by Lovell *et al.* (2004a). This was 250 DD and 257 DD above a base temperature of -2.37°C (**DDsep**) for the susceptible and resistant cultivars, respectively. In order to estimate the period of infection by ascospores (**PIasc**), 69 DDsep were subtracted from **PIpyc** (**PIpyc-min** and **PIpyc-max**), in line with a recent study by Morais *et al.* (2015). Because the period of latency is also influenced by many other factors, such as plant cultivar, fungal isolate and overall environmental conditions during and after infection, an extended estimation of the periods of infections for pycnidiospores (**extPIpyc**) was obtained by removing 21 days from **D0** and 14 days from **Dsymp**. These values were chosen according the maximum and minimum days post-inoculation needed for the appearance of symptoms reported in various outdoor studies (Eyal 1987; Shaw 1990). In

order to obtain an extended estimation of the periods of infections for ascospores (**extPIasc**), 4 days were removed from **extPIpyc-min** and **extPIpyc-max** (Morais *et al.* 2015). A graphic example is given in Figure 3.2.

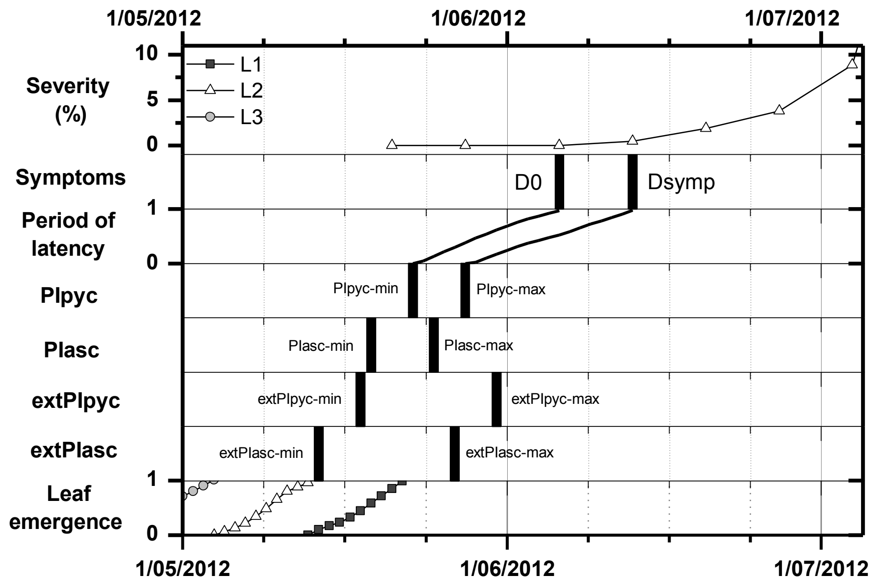

Figure 3.2. Example of the assessment of periods and extended periods of infection by pycnidiospores or ascospores for the leaf layer L2 of the resistant cultivar at Perwez in 2012. Dsymp: date when first symptoms visible; D0: last date when symptoms not yet visible; PIpyc: period of infection calculated for pycnidiospores and delimited by PIpyc-min and PIpyc-max. PIasc: period of infection calculated for ascospores and delimited by PIasc-min and PIasc-max. ExtPIpyc and extPIasc: extended period of infection calculated for pycnidiospores and ascospores, respectively.

3.2.7. Inoculum availability and infection

The **healthy-time** for a given leaf layer was taken as the total DDsep counted between leaf emergence and the occurrence of the first symptoms on this leaf. A scatter plot between healthy-time and the maximum disease severity observed on the upper leaves (L3 to L1) was computed. The airborne inoculum load (**AI-load**) for a leaf layer was defined as the total quantity of inoculum detected between the leaf emergence and PIasc-max. For each leaf layer from L3 to L1, an estimation of the number of leaf layers between the given leaf layer (L1 to L3) and the higher position of the disease on the plants at the estimated time of infection of the leaf (PIpyc-max) was calculated and called **source-distance.** For example, '1' meant

that STB symptoms were present on the leaf directly beneath at **Plpyc-max**. These three parameters – healthy-time, AI-load and source-distance – were calculated for all the leaf layers in our study (untreated plants = 5 seasons * 4 fields * 2 cultivars * 3 leaf layers = 120; treated plants = 4 seasons * 4 fields * 2 cultivars * 1 leaf layer =32).

For the untreated plants, the effect of the field, leaf layer and cultivar resistance to STB on healthy-time and AI-load were tested in an analysis of variance (ANOVA). The effect of the field, leaf layer and cultivar type on source-distance were also tested using a generalized linear model with a Poisson distribution. Similarly, ANOVA was used to test the effect of cultivar resistance to STB, fungicide treatments at GS32 and the field on the healthy-time and AI-load measured for the flag leaf (L1). A generalized linear model with a Poisson distribution was used to test the effect of the cultivar resistance to STB, the treatments and the field on source-distance measured for the flag leaf.

3.2.8. Simulation of infection by mechanistic models

Mechanistic models were developed to simulate STB dispersal and infection on the three upper leaves of wheat plants. The models used only parameters from the datasets described earlier (i.e., meteorological data, plant development, disease severity and quantification of airborne inoculum in the fields). They identified an infection event only when the transport of spores by one of three mechanisms (splash dispersal, leaf overlapping or airborne inoculum) was fulfilled and was followed by a period where conditions were conducive to infection. Similar conditions were considered for both types of spores (ascospores and pycnidiospores), in line with previous studies conducted in Belgium and Luxembourg (Lemaire *et al.* 2003; Jarroudi & Delfosse 2009): a period of RH higher than 60% over 16 h with a temperature remaining above 4°C for 24 h.

The conditions for the transport of the inoculum on given leaves (L3-L1) by splash dispersion were met if there was heavy rain when a lower leaf was infected. Rain splash was simulated by an amount of rainfall occurring in 1 h (**Rainfall.splash**). Humectation, which could be needed for pycnidiospore extrusion, was simulated by a minimum amount of rainfall (0.1 mm) in the preceding hour (**Humectation.event**). The maximum height (**Height.splash**) that a rain splash could cover for the infection of a healthy leaf was expressed in the number of leaf layers between the targeted leaf layer (L1 to L3) and the higher position of the disease. For example, an increment of '2' meant that pycnidiospores could move from L4 up to L2, or from L5 up to L3. Assuming that an emerging leaf could be at the same height as the

leaf immediately below it (Lovell *et al.* 2004b), the Height.splash increment could be raised to 1 during leaf emergence (**Emerging.leaf)**. The quantity of inoculum needed on a 'source' leaf in order for splashes to be loaded with pycnidiospores was represented by disease severity (**Source.availibility.splash**).

The conditions for the transport of the inoculum on given leaves (L3-L1) by leaf overlapping were met if a lower leaf was infected during the emergence of one of the upper leaves. Various increments of overlapping were tested (**Overlap**). For example, an increment of '2' meant that an emerging leaf could be contaminated by the two leaf layers just below it. The minimum quantity of inoculum needed on a developed leaf in order for the inoculum to be dispersed on the emerging leaf was represented by disease severity (**Source.overlap**).

The conditions for the transport of spores on given leaves (L3-L1) by airborne inoculum were met if a given quantity of airborne inoculum was detected once a leaf layer was present. Various cumulative amounts of airborne inoculum were tested (**Load**) using a range of thresholds in order to take detection into account (**Threshold.detection**).

3.2.9. Evaluation of the parameters used in the model

A wide range of values was tested for each parameter (Table 3.2) in order to find the best combination for each of the three dispersal mechanisms. The model was assessed using the data collected from all the trial fields from the beginning of L3 emergence in the five growing seasons. Optimal values of each parameter were determined separately for the three mechanisms of inoculum dispersal. An infection event predicted by the model was assumed if it was reached during the period of PIpyc or PIasc, depending on the dispersal mechanism involved. Infection events predicted between leaf emergence and the beginning of the period of infection were considered to be false alarms.

The best values of the parameters for each of the three dispersal mechanisms were determined using the probability of detection (POD), false alarm ratio (FAR) and critical success index (CSI) of the STB infection. These statistical scores were calculated as follows (Wilks 1995):

POD = a/(a+c)

FAR = b/(a+b)

CSI = a/(a+b+c)

where:

a = observed and predicted infections

b = predicted infections, but not observed (**false alarm**)

c = observed infections, but not predicted

The values for the three scores (POD, FAR and CSI) ranged from 0 to 1. A perfect score for the POD and CSI was 1, and for the FAR it was 0.

Contingency tables were produced to calculate statistical scores (POD, FAR and CSI) for all the combinations of the parameters of each dispersal mechanism. The most interesting scenarios (combination of parameter values) among those tested were chosen, based on this rule: maximize the POD and keep the FAR at <0.3. The limit of 0.3 was chosen arbitrarily. The statistical scores calculated with the extended estimations of the periods of infection for pycnidiospores and ascospores (extPIpyc and extPIasc) were also presented. The models using the best scenarios of the three dispersal mechanisms were assessed using the dataset for leaf layers of untreated plants (L1, L2 and L3) and for the flag leaf of fungicide-treated plants (at GS32).

Algorithms and statistical analyses were performed using R Software R 2.15.0 (www.r-project.org). Graphics were designed using OriginPro 8 (OriginLab, Northampton).

Table 3.2. Values tested for the parameters involved in each of the three dispersal mechanisms.

Mechanism of dispersal	Parameters and units	Values tested
Splash dispersion	Rainfall.splash (mm/h)	0.3 - 0.4 - 0.5 - 0.6 - 0.7- 0.8 - 1 - 1.2 - 1.5 - 2
	Humectation.event	0 - 1
	Inoculum.position.splash (leaf layer)	1 - 2 - 3
	Emerging.leaf	0 - 1
	Source.availibility.splash (%)	0.01 - 0.5 - 1 - 2 - 5 - 10
Leaf overlapping	Inoculum.position.overlap (leaf layer)	1 - 2 - 3
	Source.availibility.overlap (%)	0.01 - 0.5 - 1 - 2 - 5 - 10
Airborne inoculum	Load (cDNA)	5 - 10 - 20 - 30 - 40 - 50 - 60 - 70 - 80 - 90 - 100 - 120 - 150 - 200 - 400
	Threshold.detection (cDNA)	0 - 1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 - 9 - 10 - 20 - 40 - 50 - 60 - 70 - 80

3.3. Results

3.3.1. Leaf development

For each cultivar used in the study, an adapted phyllotherm was calculated in order to interpolate precisely the development of the three upper leaves in all the trial fields and to compare the dates when a given stage was reached. Using the square roots method, the phyllotherm that best fitted the development of each of the three upper leaves observed in the fields was the sum of the temperatures (115.1, 118.2 and 111.8 DD) for the Istabraq, Lexus and Julius cultivars, respectively.

The emergence of the three upper leaves at all the sites occurred close together in the same growing season, but there were significant variations between the growing seasons (Table 3.3). Flag leaf emergence in 2009, 2010 and 2012 occurred during the same period, between 10 and 22 May. The growing season in 2011 was characterized by the early emergence of the three upper leaves. In contrast, the long and cold winter of 2013 considerably stunted the development of wheat, resulting in the late emergence of the flag leaf, at the end of May.

Table 3.3. Time interval, including all the simulated dates for leaf emergence (L1-L3), calculated for all the trial fields in a given growing season (2009-2013). The emergence of the upper leaves was interpolated with the phyllotherm of the cultivar and a reference stage of development observed in the trial fields.

Year	L3	L2	L1
2009	17/04 - 27/04	29/04 - 08/05	10/05 - 17/05
2010	19/04 - 29/04	30/04 - 10/05	16/05 - 22/05
2011	12/04 - 21/04	23/04 - 28/04	02/05 - 07/05
2012	18/04 - 27/04	30/04 - 07/05	10/05 - 17/05
2013	29/04 - 03/05	09/05 - 13/05	20/05 - 26/05

3.3.2. Disease pressure

In all the trial fields, whatever the growing season, STB was present on L7 of both types of cultivars when L3 was emerging (GS31) (Figure 3.3). At this point, the disease was present on L6 and L5 of the resistant cultivar in 80% and 15% of the fields, respectively, and on L6 and L5 of the susceptible cultivar in 95% and 35% of the fields, respectively. STB inoculum was present in the lower part of the plants in all the trial fields, and this was therefore not a limiting factor. In all the fields, disease severity measured at GS31 was lower on the resistant cultivar (Lexus or Julius) than on the susceptible one (Istabraq). Disease pressure at this growth stage differed in

all the trial fields in every growing season except for 2009. In terms of the annual mean of disease severity on L5 and L6, 2010 showed higher overall pressure in the early spring compared with the other growing seasons.

The levels of STB severity on the two upper leaves during grain development and maturation also differed between fields in the same growing season (Figure 3.4). In most cases, the symptoms observed at a site were at least half as severe on the resistant cultivar as on the susceptible one. In 2009 and 2012, there were severe epidemics in all the fields except at Niverlée, where the pressure had remained low. For these two growing seasons, 20% of the flag leaf area of the susceptible cultivar was covered by symptoms at some sites. The pressure in 2013 was a little lower than in 2009. The 2010 and 2011 seasons were characterized by slow progression of the disease. The dry climatic conditions at end of the 2011 growing season resulted in the rapid drying of the upper leaves, making it difficult to detect and measure STB symptoms in some fields. The STB pressure in 2011 was very low anyway, with an epidemic outbreak observed only in Perwez. The layout of the trial fields and the monitoring of disease progression on the same susceptible cultivar over 5 years at four sites allowed the significant variations in STB between sites and years, even in as small an area as Wallonia, to be revealed.

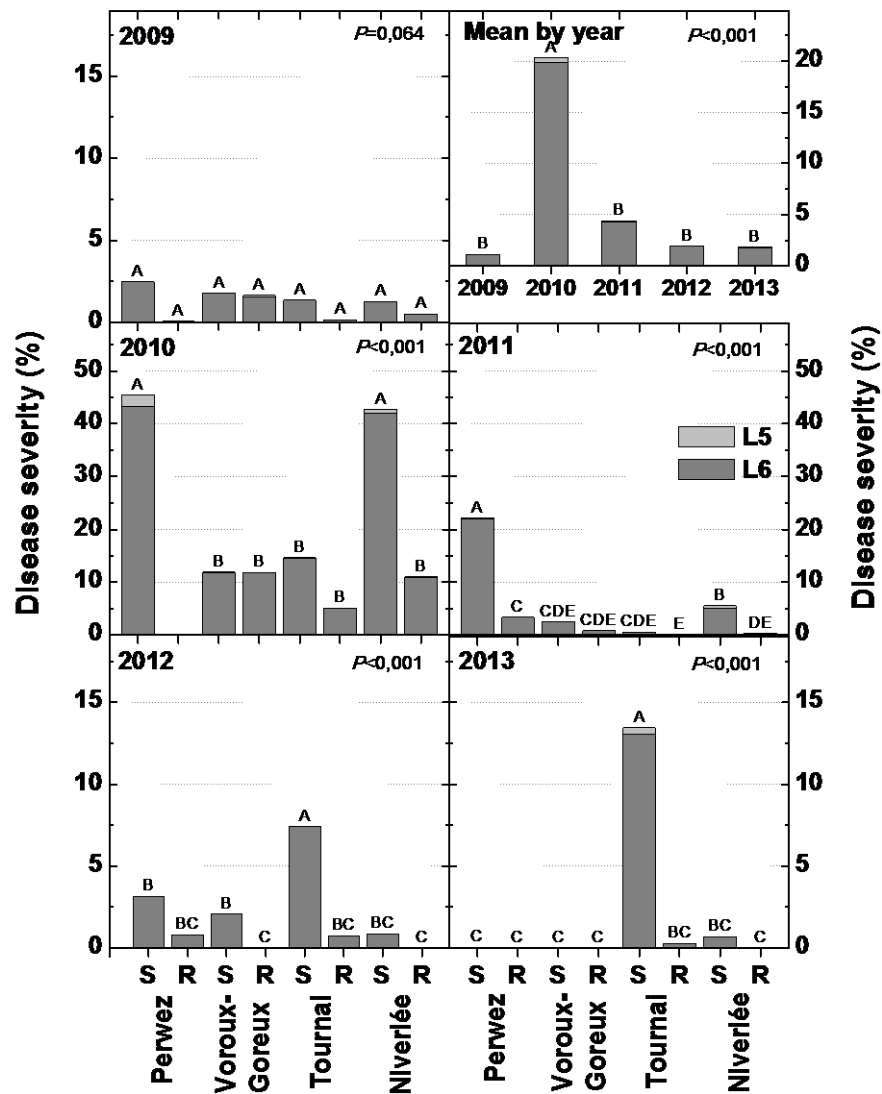

Figure 3.3. STB severity (%) on leaves L5 and L6 estimated when L3 was emerging (GS31) in the 2009-2013 growing seasons. Observations were made of two cultivars that differed in their resistance to STB (S = susceptible, R = resistant) from the end of April up to the first 10 days of June, depending on the year. Comparisons between sites were made for each growing season using ANOVA, followed by a Student-Newman-Keuls test (α=0.05). Significant differences between sites are indicated by different letters. A comparison between the mean of each year was made using the same method.

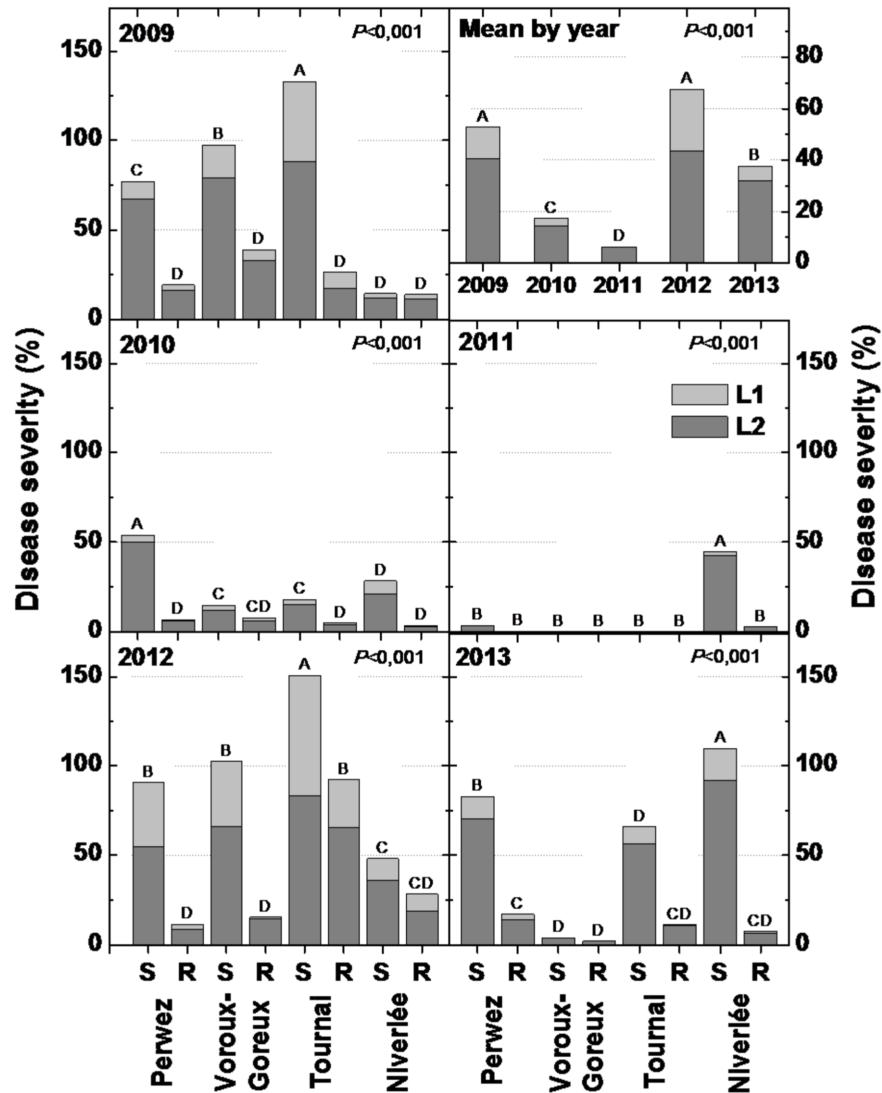

Figure 3.4. STB severity (%) on the upper leaves (L1 and L2) between GS75 and GS85 in the 2009-2013 growing seasons. Observations were made of two cultivars that differed in their resistance to STB (S = susceptible, R = resistant) from the end of June up to mid-July, depending on the year. Comparisons between sites were made for each growing season using ANOVA, followed by a Student-Newman-Keuls test (α=0.05). Significant differences between sites are indicated by different letters. A comparison between the mean of each year was made using the same method.

3.3.3. Contamination of upper leaves in untreated plants

The time between the emergence of a leaf and the occurrence of STB symptoms (Dsymp) on this leaf was determined for each of the three upper leaves in all the trial fields (healthy-time) (Figure 3.2). It ranged between 21 and 64 days, corresponding to between 303 and 1,092 DDsep (113 leaves). When healthy-time was above 550 DDsep, there was a lower risk of high disease severity on a leaf (>20%). Contamination of a leaf soon after emergence often resulted in severe symptoms (Figure 3.5).

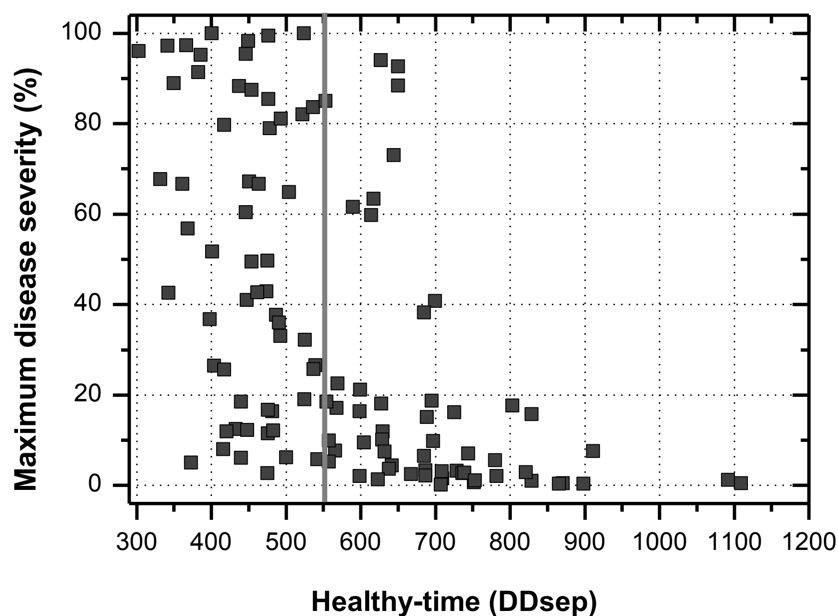

Figure 3.5. The relationship between maximum disease severity observed on a leaf (L1-L3) and the healthy-time. Observations of STB-susceptible and STB-resistant cultivars were made between 2009 and 2013 at four sites. In 2011, seven leaves layers dried prematurely because of drought and were not taken into account here (ntot =113).

The ANOVA revealed that the healthy-time calculated for a dataset of 102 untreated leaf layers depended on the field concerned, the type of cultivar and the leaf layer (Table 3.4). Three leaf layers in four fields every season were used in this analysis, except for 2011 when only the Niverlée site was considered because some leaves dried prematurely at the other sites. The field concerned had the greatest effect on the healthy-time, with a mean between 449.7 DDsep (Voroux-Goreux 2012) and 678.8 DDsep (Voroux-Goreux 2013). The upper leaves of the susceptible cultivar were usually

infected more rapidly (511.5 DDsep) than those of the resistant cultivars (592.7 DDsep). The leaf L1 was significantly less rapidly infected (606.9 DDsep) than L2 and L3 (519.4 DDsep and 530 DDsep, respectively).

Table 3.4. Effects of the 'field', 'leaf layer' and 'cultivar resistance to STB' factors on the healthy-time. For the five growing seasons from 2009 to 2013, the healthy-time was assessed for the three upper leaves of an STB-susceptible and an STB-resistant cultivar at four sites each year. In 2011, only the Niverlée site was retained because some leaves dried prematurely at the other sites (ntot=102).

Healthy-time	df	Mean square	F	P
Field	16	890134	4.96	<0.0001
Leaf layer	2	155000	6.91	0.0017
Cultivar resistance to STB	1	168207	14.99	0.0002

The **AI-load** for a leaf layer was defined as the total quantity of inoculum detected between the emergence of this leaf layer and PIasc-max (Figure 3.1). The presence of airborne inoculum in a field was detected between the emergence of a leaf layer and the end of the estimated PIasc in 81.5% of cases (n=113). The quantities of airborne inoculum varied greatly, however, ranging between 1 cDNA and 1,862.7 cDNA. The AI-load for 102 untreated leaf layers was significantly affected by the field concerned, the type of cultivar and the leaf layer (Table 3.5).

Table 3.5. Effects of the 'field', 'leaf layer' and 'cultivar resistance to STB' factors on the AI-load. For the five growing seasons from 2009 to 2013, the AI-load was calculated for the three upper leaves of an STB-susceptible and an STB-resistant cultivar at four sites each year. In 2011, only the Niverlée site was retained because some leaf layers dried prematurely at the other sites (n=102).

AI-load	df	Mean square	F	P
Field	16	48.8	9.37	<0.0001
Leaf layer	2	2.4	3.63	0.0309
Cultivar resistance to STB	1	2.5	7.78	0.0066

The three fields that suffered from drought in 2011 were discarded when the ANOVA was conducted. The 'field' factor had the strongest effect on AI-load. The mean for a given field ranged from 7.6 cDNA (Niverlée 2009) to 733.9 cDNA (Perwez 2012). The mean AI-load for the susceptible cultivar (176.3 cDNA) was significantly smaller than that for the resistant one (253.4 cDNA) (Figure 3.6A). Significantly higher AI-loads (267.4 cDNA) were calculated for the L1 layer than for the L2 and L3 layers (168.3 cdna and 209.0 cdna, respectively) (Figure 3.6B).

Source-distance is an estimation of the number of layers between a given leaf layer (L1 to L3) and a higher position of the disease on the plants at the estimated time of infection of the leaf (PIpyc-max) (Figure 3.1). It was calculated for all leaf layers. Values between 1 and 4 were observed for both types of cultivar. This parameter was not significantly related to the 'field' or 'cultivar resistance to STB' factors, but it was to the 'leaf layer' factor (Table 3.6 and Figure 3.6C), with the values observed for the L1 layers being significantly lower than those observed for L2 and L3 (Figure 3.6D).

Table 3.6. Effects of the 'field', 'leaf layer' and 'cultivar resistance to STB' factors on source-distance. For the five growing seasons from 2009 to 2013, source-distance was calculated for the three upper leaves of an STB-susceptible and an STB-resistant cultivar at four sites each year. In 2011, only the Niverlée site was retained because some leaves dried prematurely at the other sites (n=102).

Source-distance	df	Likelihood ratio	P
Field	16	3.5	0.9995
Leaf layer	2	26.6	<0.0001
Cultivar resistance to STB	1	0.2	0.6632

Figure 3.6. Comparison between a susceptible and resistant cultivar of AI-load (A) and source-distance (C). Comparisons of AI-load (B) and source-distance (D) were also made between the three upper leaves, mixing data from both types of cultivar. For the five growing seasons, parameters were assessed for the three upper three leaves of the untreated plants (two cultivars) at four sites. In 2011, only the Niverlée site was retained (ntot=102).

3.3.4. Contamination of flag leaf by STB in treated plants (GS32) vs untreated plants

In the following analyses, all the data from the 2011 growing season were excluded because the flag leaf layers of treated plants dried before the appearance of symptoms. The period between leaf emergence and the occurrence of STB symptoms on this leaf (healthy-time) was also evaluated for the flag leaf of plants treated at GS32 in all the fields and compared with healthy-time measured for the flag leaf of untreated plants. For the 2010, 2012 and 2013 growing seasons, the healthy-time for the flag leaf was

significantly linked with the field concerned and the type of cultivar. The mean values of healthy-time for L1 ranged between 555.2 DDsep (Niverlée 2012) and 786.61 DDsep (Voroux-Goreux 2013) (Table 3.7). The susceptible cultivar had a mean of 625 DDsep and the resistant cultivar had one of 703.2. There was no statistical difference between treated and untreated plants, which had an average of 681.6 and 646.4 DDsep, respectively (Figure 3.7A).

Table 3.7. Effects of the 'field', 'fungicide treatment at GS32' and 'cultivar resistance to STB' factors on the healthy-time of flag leaves. For the 2009-10, 2011-12 and 2012-13 growing seasons, the healthy-time of L1 in treated and untreated plants was assessed for both types of cultivar at four sites (ntot=48).

Healthy-time	df	Mean square	F	P
Field	11	531,141	4.06	0.0008
Treatment	1	14,882	1.25	0.2712
Cultivar resistance to STB	1	69,942	5.88	0.0208

The AI-load for the flag leaf was linked with the field (Table 3.8). The means by site ranged between 47.0 cDNA (Voroux-Goreux 2010) and 1,213.0 cDNA (Perwez 2013). A slightly higher AI-load mean was observed for the flag leaf in the treated plants (480.9 cDNA) than in the untreated ones (361.3 cDNA) (Figure 3.7B). Similarly, the AI-load mean for the resistant cultivar (501.79 cDNA) was higher than that for the susceptible cultivar (340.5 cDNA). The effect of fungicide treatment and cultivar resistance to STB on the AI-load of flag leaf, however, was not statically significant.

Table 3.8. Effects of 'field', 'fungicide treatment at GS32' and 'cultivar resistance to STB' factors on the AI-load calculated for the flag leaves. For the 2009-10, 2011-12 and 2012-13 growing seasons, the healthy-time of L1 in treated and untreated plants was assessed for both types of cultivar at four sites (ntot=48).

AI-load	df	Mean square	F	P
Field	11	12.2	4.33	0.0005
Treatment	1	0.2	0.76	0.3898
Cultivar resistance to STB	1	0.2	0.77	0.3861

The 'treatment' factor had an effect on source-distance, with the values observed for the flag leaf of treated plants being significantly higher than those for untreated plants (Figure 6C). The 'field' and 'cultivar resistance to STB' factors were not significantly related to source-distance (Table 3.9 and Figure 7C).

Table 3.9. Effects of 'field', 'fungicide treatment at GS32' and 'cultivar resistance to STB' factors on source-distance evaluated for the flag leaves. For the 2009-10, 2011-12 and 2012-13 growing seasons, the healthy-time of L1 in treated and untreated plants was assessed for both types of cultivar at four sites (ntot=48).

Source-distance	df	Likelihood ratio	P
Field	11	12.8	0.3084
Treatment	1	7.3	0.0070
Cultivar resistance to STB	1	0.2	0.6393

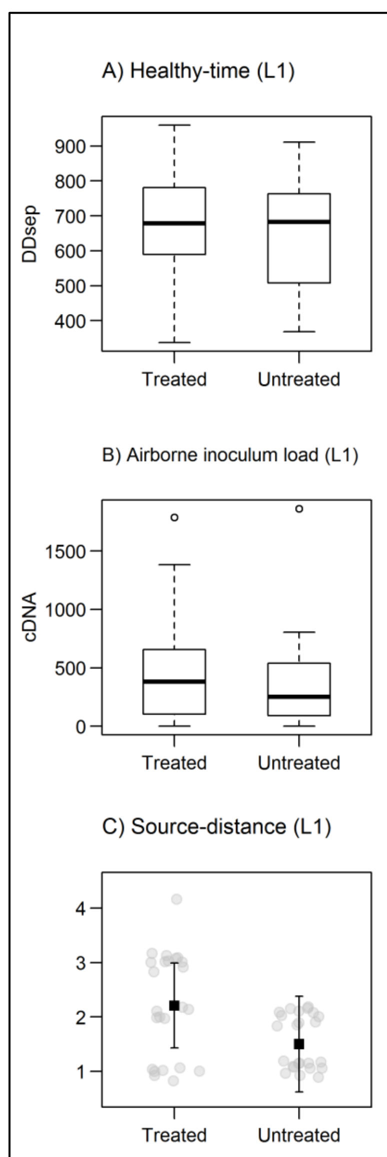

Figure 3.7. Comparisons between fungicide-treated plants (GS32) and untreated plants for healthy-time (A), AI-load (B) and source-distance (C) measured for the flag leaf (L1). The measurements were made on an STB-susceptible and an STB-resistant cultivar, grown in four fields each year in the 2009-10, 2010-12 and 2012-13 growing seasons (ntot=48).

3.3.5. Modeling the contamination by STB of the upper leaf of untreated plants

Three mechanisms of dispersal affecting the upper leaves were considered: splash dispersal, leaf overlapping and airborne inoculum. Each one was simulated by mechanistic models using parameters described in detail in 'Materials and Methods'. For each parameter included in the three models, a wide range of values was tested in order to explain the contamination observed on the upper leaf of untreated plants (n_{tot}=113).

For the three dispersal mechanisms, the POD, FAR and CSI of the STB infection were calculated for all combinations of the parameter values (Table 3.2). The best combinations for each of the three mechanisms (SD1, LO1 and AI1) are underlined in grey in Table 3.10.

With regard to infection due to splashborne inoculum, and keeping the FAR under 0.3, the values of parameters maximizing the POD assumed rainfall of at least 0.6 mm/h was needed to disperse pycnidiospores to an upper leaf from a source one or two leaves below. Using these parameters for splash dispersal, half the contamination on the upper leaf can be explained. Some scenarios similar to this optimal combination are presented in Table 3.10. An additional increment of one leaf layer when the upper leaves were emerging (emerging.leaf=1) increased the POD, but led to unacceptable FAR values (e.g., SD3, SD5 and SD6 in Table 3.10). An increase in the threshold value for the quantity of inoculum required for the infection (source.splash) reduced the POD considerably (e.g., SD8 in Table 3.10). Taking account of a preliminary humectation before a splash event did not provide a better prediction of infection (e.g., SD4 and SD6 in Table 3.10). With regard to an extended period of infection, a better prediction of infection by splashborne inoculum was obtained in all cases, with an increase in the POD and CSI and a decrease in the FAR.

With regard to dispersal due to leaf overlapping, less infection was predicted compared with dispersal by splashborne inoculum. The optimal combination of parameters (LO1, Table 3.10) was based on a leaf layer with symptoms infecting an emerging leaf immediately above it. Little contamination was predicted with this scenario (<10%), but the FAR was only 0.08%. An increase in the degree of overlap leaf layers in the model considerably increased the POD for this dispersal mechanism, but resulted in a drastic and unacceptable increase in the FAR (e.g., LO3, LO4 and LO5 in Table 3.10). An increase in the threshold value for the inoculum needed for contamination reduced the FAR slightly, but also had the effect of reducing the POD (e.g., LO2 and LO5 in Table 3.10).

Two specific parameters were considered in the mechanistic model for infection due to airborne inoculum: the cumulative amounts of airborne inoculum required for infection; and the minimal quantity of cDNA required for detection. Cumulative values of at least 80 cDNA of inoculum reduced the POD, compared with 10 or 40 cDNA, and also reduced the FAR, especially when a threshold of 8 cDNA was applied when taking an infection into account (Table 3.10). The parameters allowed more than 40% of the contamination to be explained. Lower AI-load values without a change in the threshold value increased the FAR without a significant increase in the POD (e.g., AI3 in Table 3.10). A higher threshold value (e.g., AI4 in Table 3.10) considerably reduced the POD without any significant change in the FAR (e.g., AI4 in Table 3.10). An AI-load of 10 cDNA with a threshold of 5 cDNA explained much of the contamination, but the FAR was over the limit (e.g., AI2 in Table 3.10).

The parameters chosen for each of the three dispersal mechanisms were used in a compilation model (scenario CM in Table 3.10). More than 68% of the initial contamination was explained for the three upper leaves and up to 78% if extended periods of infection were taken into account. Under the same conditions (FAR<0.3), the splashborne mechanism could not explain more than 50% of the initial contamination, reaching 58% if extended periods of infection were taken into account.

The compilation model (CM) allowed very similar contamination percentages for both types of cultivars with the same FAR range (Table 3.12) to be simulated. Using this model, infections due to dispersal by airborne inoculum alone or in combination with either or both the other dispersal mechanisms (splash and leaf overlapping) were less frequent for the susceptible cultivar (33%) than for the resistant one (50%) during a normal period of infection (PI). In contrast, infections due to splashborne dispersal were more frequent for the susceptible cultivar (54%) than for the resistant one (43%). The leaf overlapping dispersal mechanism played a significant role only for the susceptible cultivar (13%).

3.3.6. Testing retained model on the flag leaf of treated (GS32) plants

The compilation model was tested to assess the prediction of STB infection based on data collected from trial fields treated with a fungicide at GS32. This model was able to predict 96% of the STB contamination of the flag leaves, with a FAR of only 17% (Table 3.11). For extended periods of infection, the FAR was reduced to 11% and the POD reached 92%. As shown in the table for treated (GS32) plants, the proportions of flag leaf

contamination explained by the three dispersal mechanisms (using optimal parameters) were 42% for the splashborne inoculum mechanism, 0% for the leaf overlapping mechanism and 85% for the airborne inoculum mechanism.

Using the compilation model with CM parameters based on a normal period of infection, the splashborne inoculum mechanism could explain 50% of the flag leaf contamination in the susceptible treated cultivar, but only 33% in the resistant cultivar (Table 3.12). For both types of cultivar, the most important dispersal mechanism in flag leaf infection was the airborne inoculum mechanism (83-86%). In the resistant and susceptible cultivars, 25% and 36%, respectively, of the flag leaf symptoms could be explained both by the airborne and splashborne inoculum mechanisms.

Table 3.10. Prediction of STB infection on the three upper leaves of untreated plants obtained using mechanistic models simulating the three possible ways of STB dispersal and infection (SI: splashborne inoculum; LO: leaf overlapping; AI: airborne inoculum) in a normal period of infection (PI, Figure 3.2). Statistical scores (FAR, POD and CSI) are presented for both types of infection periods (PI and extended PI, Figure 3 2). Scenarios with the value of parameters maximizing the POD, with the FAR at <0.3, are underlined in grey for each mechanism. Statistical scores of other scenarios (presenting variations of parameter values around the optimal scenarios) are also given for each dispersal mechanism. Statistical scores for a compilation model (CM) using the three optimal dispersal mechanisms are presented. All the models were assessed using data from 20 trial fields (four fields in each growing season from 2008-09 to 2012-13) that always included a susceptible cultivar and a resistant one. In 2011, seven leaf layers dried prematurely and are therefore not taken into account here (ntot =113).

Code	Parameters									PI						Extended PI		
	Source splash (%)	Splash heigth (L layer)	Emerging leaf	Humectation (mm/h)	Rainfall splash (mm/h)	Source overlap (%)	Overlap (L layer)	Load (cDNA)	Threshold detection (cDNA)	Predicted before observed	Predicted and observed	Not predicted in time	FAR	POD	CSI	FAR	POD	CSI
Splash Dispersion																		
SD1	**0.01**	**2**	**0**	**0**	**0.6**					**21**	**57**	**56**	**0.27**	**0.50**	**0.43**	**0.15**	**0.58**	**0.53**
SD2	0.01	1	0	0	0.6					5	19	94	0.21	0.17	0.16	0.08	0.20	0.20
SD3	0.01	1	1	0	0.6					14	32	81	0.30	0.28	0.25	0.16	0.32	0.30
SD4	0.01	2	0	0.1	0.6					20	50	63	0.29	0.44	0.38	0.17	0.50	0.46
SD5	0.01	2	1	0	0.6					51	86	27	0.37	0.76	0.52	0.28	0.82	0.62
SD6	0.01	2	1	0.1	0.6					48	80	33	0.38	0.71	0.50	0.27	0.75	0.59
SD7	0.01	3	0	0	0.6					61	95	18	0.39	0.84	0.55	0.31	0.89	0.64
SD8	0.5	2	0	0	0.6					17	42	71	0.29	0.37	0.32	0.15	0.45	0.42
SD9	0.01	2	0	0	2					15	46	67	0.25	0.41	0.36	0.09	0.44	0.42
Leaf Overlapping																		
LO1						**0.01**	**1**			**1**	**12**	**101**	**0.08**	**0.11**	**0.11**	**0.00**	**0.12**	**0.12**
LO2						0.5	2			11	22	91	0.33	0.19	0.18	0.22	0.25	0.23
LO3						0.01	2			16	31	82	0.34	0.27	0.24	0.14	0.27	0.25
LO4						0.01	3			39	53	60	0.42	0.47	0.35	0.33	0.50	0.40
LO5						0.5	3			30	44	69	0.41	0.39	0.31	0.28	0.42	0.36
Airborne Inoculum																		
AI1								**80**	**8**	**18**	**47**	**66**	**0.28**	**0.42**	**0.36**	**0.16**	**0.46**	**0.42**
AI2								10	5	37	80	33	0.32	0.71	0.53	0.23	0.73	0.59
AI3								40	8	27	58	55	0.32	0.51	0.41	0.23	0.58	0.49
AI4								80	40	12	29	84	0.29	0.26	0.23	0.20	0.29	0.27
Compilation Model																		
CM	**0.01**	**2**	**0**	**0**	**0.6**	**0.01**	**1**	**80**	**8**	**32**	**77**	**36**	**0.29**	**0.68**	**0.53**	**0.19**	**0.78**	**0.66**

Table 3.11. Prediction of STB infection on the flag leaf of treated plants obtained using mechanistic models simulating the three possible ways of STB dispersal and infection (SI: splashborne inoculum; IO: leaf overlapping; AI: airborne inoculum) in a normal period of infection (PI, Figure 3.2). Parameters used in the three models constitute the optimal scenario obtained from the assessment of models for untreated plants (Table 3.10). Statistical scores (FAR, POD and CSI) are presented for both types of infection periods (PI and extended PI, Figure 3 2). Statistical scores for a compilation model using the three optimal dispersal mechanisms are presented (CM). All the models were assessed using data from 13 trial fields (four fields in the 2009-10, 2011-12 and 2012-13 growing seasons and one field in the 2010-11 growing season) that always included a susceptible cultivar and a resistant one. In 2011, three sites were discarded because of the dry climatic conditions (n_{tot} =26).

Code	Parameters									PI						Extended PI		
	Source splash (%)	Splash heigth (L layer)	Emerging leaf	Humectation (mm/h)	Rainfall splash (mm/h)	Source overlap (%)	Overlap (L layer)	Load (cDNA)	Threshold detection (cDNA)	Predicted before observed	Predicted and observed	Not predicted in time	FAR	POD	CSI	FAR	POD	CSI
Splash dispersion																		
SDtr	0.001	2	0	0	0.6					2	11	15	0.15	0.42	0.39	0.15	0.42	0.39
SI2tr	0.001	2	1	0	0.6					2	11	15	0.15	0.42	0.39	0.15	0.42	0.39
Leaf overlapping																		
LOtr						0.001	1			0	0	26	NA	0.00	0.00	NA	0.00	0.00
LOtr						0.5	2			0	0	26	NA	0.00	0.00	NA	0.00	0.00
Airborne inoculum																		
AItr								80	8	4	22	4	0.15	0.85	0.73	0.08	0.85	0.79
AItr								10	5	12	25	1	0.32	0.96	0.66	0.17	0.96	0.81
Compilated model																		
CMtr	0.001	2	0	0	0.6	0.001	1	80	8	5	25	1	0.17	0.96	0.81	0.11	0.92	0.83

Table 3. 12. Proportion (%) of STB contamination explained by each of three dispersal mechanisms in the compilation model using the CM scenario parameter values (Table 3.10) for the three upper leaves of untreated plants and the flag leaf of plants treated with fungicide at GS32. Percentages are given for a susceptible cultivar and a resistant one in both a normal and extended period of infection.

	Untreated (L1-L3)				**Treated (L1)**			
Type of cultivar	**Susceptible**		**Tolerant**		**Susceptible**		**Tolerant**	
Period of infection (PI)	**PI**	**extPI**	**PI**	**extPI**	**PI**	**extPI**	**PI**	**extPI**
Number of leaf layers	58		55		14		12	
Splah dispersion (SD)	0.26	0.29	0.13	0.16	0.14	0.07	0.08	0.08
Leaf overlapping (LO)	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Airborne inoculum (AI)	0.14	0.17	0.20	0.18	0.50	0.43	0.58	0.58
SD & LO	0.09	0.09	0.04	0.04	0.00	0.00	0.00	0.00
LO & AI	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
SD & AI	0.17	0.21	0.25	0.29	0.36	0.43	0.25	0.25
SD & LO & AI	0.02	0.02	0.05	0.07	0.00	0.00	0.00	0.00
Predicted	**0.69**	**0.79**	**0.67**	**0.76**	**1.00**	**0.93**	**0.92**	**0.92**
Not predicted in time	**0.31**	**0.21**	**0.33**	**0.24**	**0.00**	**0.07**	**0.08**	**0.08**
FAR	**0.27**	**0.15**	**0.31**	**0.24**	**0.18**	**0.13**	**0.15**	**0.08**

3.4. Discussion

As reported by Shaw & Royle (1993), the present study clearly showed that STB in wheat is often severe when the upper leaves are contaminated early in their life. Our study also showed that the time between leaf emergence and symptom appearance was influenced by the field concerned, the cultivar resistance to STB and the leaf layer, with the flag leaf showing symptoms later. This variability reinforced the need to develop tools that can predict infection in the upper leaves accurately enough for an effective adjustment to be made to fungicide applications in intensive cropping systems.

It is now known that airborne ascospores can be produced throughout the year (Kema *et al.* 1996a; Hunter *et al.* 1999; Fraaije *et al.* 2005; Sameh *et al.* 2011; Duvivier *et al.* 2013), although opinions differ about the importance of airborne ascospores in the contamination of upper leaves.

In wheat sown in a field where the previous crop was not wheat, primary STB infection comes mainly from ascospores (Shaw & Royle 1989; Suffert *et al.* 2011), but subsequent horizontal and vertical dispersion is still thought to be driven primarily by splashborne pycnidiospores (Shaw 1999). The impact of the spread of pycnidiospores by rain splash, however, is low, particularly because of the limited extent of upward movement (Shaw 1987; Walklate et *al.* 1989; McCartney & Fitt 1998). This limited impact might be partially compensated for by the considerable production potential of pycnidiospores from a single initial infection (50,000 to 500,000 spores) (Eyal 1971).

Like the findings reported by Lovell *et al.* (2004b), our results showed that applying fungicide treatments early in the growing season and using STB-resistant cultivars can slow down STB progression through asexual reproduction, increasing the distance between emerging leaves and the height of the disease symptoms in the canopy. Under hot and dry climatic conditions, a high leaf development rate could put the upper leaves of wheat out of reach of splashborne pycnidiospores. In this situation, the impact of airborne inoculum in the initial contamination of upper leaves is questionable. This contamination derives from the sexual reproduction of STB and could accelerate the upward progression of the disease. A subsequent asexual cycle in which a huge quantity of spores is produced could thereafter be responsible for the horizontal spread and subsequent increase in disease severity in a leaf layer.

In our previous study at four sites over two growing seasons, a reliable method was developed for studying the daily concentration of *M.*

graminicola airborne inoculum above the canopy (Duvivier *et al.* 2013). In the present study, a network of four Burkard 7-day spore traps was set up in trial fields throughout Wallonia and operated continuously between March and June over five growing seasons (2009-2013). In most cases, airborne inoculum was detected between the emergence of the three upper three leaves and the end of the calculated period of infection, giving an AI-load that sometimes exceeded 1,000 cDNA (load of ±2,200 ascospores $m^3 day^{-1}$). In experiments measuring ascospore density at the time of STB primary infection in the fields, daily detections of ascospores of about 100 ascospores m^3day-1 were observed and were related to the appearance of symptoms in the fields (Bathgate & Loughman 2001; Morais *et al.* 2015). This indicated that the airborne inoculum that was trapped could have been responsible for at least some infection of the upper leaves under favorable conditions. Our study also showed that during the periods of infection for a given leaf layer, the height of the disease in the canopy was sometimes three or four leaf layers beneath the leaf layer concerned (Figures 3.6C, 3.6D and 3.7D). The number of splash carrying pycnidiospores travelling such a distance (20-30 cm) will be considerably decreased, given the half-distance[1] of 5 cm for this mechanism (Shaw, 1987, 1999).

In addition to infection due to the dispersal by splashborne and airborne inoculum, Lovell *et al.* (1997, 2004b) showed that the STB infection of a young leaflet can also occur (even without rain splash) by inoculum transfer because of the overlap between emergent and established leaves containing sporulating lesions of *M. graminicola*.

In an attempt to quantify the relative importance of each dispersal mechanism in the initial contamination observed on upper leaves, mechanistic models were developed based on knowledge of STB epidemiology and data collected over the five growing seasons. The models assumed that the transport of the inoculum had to be followed by favorable conditions for STB infection in order to explain the appearance of symptoms on leaves. One of the main issues was to determine the likely period of infection (PI) of each infected leaf layer in order to fit the model. The equations developed by Lovell *et al.* (2004a) and based on the accumulation of thermal time were used to estimate the period of latency. Since these equations were designed for optimal conditions of infection, which does not necessarily reflect field conditions, and since many other factors (e.g., infection conditions and pathogenicity of the strains) could influence the length of the latent period in a field (Shaw 1990; Chungu & Gilbert, 1996 ; Viljanen-Rollinson & Marroni 2005; Suffert *et al.* 2013), we also included extended periods of infection (extended PI) in the modeling. This was done in order to avoid under-estimation, or even over-estimation,

of the latent period using a simple temperature-based relationship. The calculation of the extended PI was based on the number of days (14-21 days) observed to be the latent period in spring reported in studies conducted in areas bordering Belgium (Eyal 1987; Shaw 1990).

For each parameter included in the modeling of the three dispersal mechanisms, the optimal values were selected from the large dataset on infected leaf layers collected from untreated plots over five growing seasons at four sites on two cultivars that differed in their resistance to STB, using the maximization of the POD, with the FAR not exceeding 0.3, as the criteria. The three mechanisms were initially considered independently.

The research on the best value of the five parameters included in the model of propagation by splash-dispersed pycnidiospores led to a model that was fairly similar to the one developed in Wallonia by Lemaire *et al.* (2003). The best model obtained for the vertical propagation by splash-dispersed pycnidiospores assumed that rainfall of at least 0.6 mm/h generates splashes that can contaminate a leaf that is one or two leaf layers above the infected leaf layer. Rainfall has not been seen as a required condition for pycnidiospore extrusion. The release of *Septoria* spp. pycnidiospores usually occurs rapidly (30 min) when pycnidia are wet, and it is likely that the same rainfall event (over 1 hour) allows both the extrusion of pycnidiospores and their subsequent transport (Brennan & Fitt 1986). Using this model for dispersion/infection by splashborne inoculum, it was possible to explain only 50-58% (PI or extended PI) of the infection observed on the three upper three leaves of untreated plants. Efforts to explain a higher proportion of the infection using this model (e.g., increasing splash.height or decreasing rainfall.splash) resulted in many false alarms, indicating that the model fitted (SP1) was not suitable or, more probably, that other dispersal mechanisms were involved in the infection of the upper leaves.

In the attempt to simulate inoculum dispersal by transfer due to leaf overlapping, the fitted model assumed that an emerging leaf layer would probably be contaminated if the leaf just beneath it showed symptoms. This explained only 11-12% of the infection, however. This situation is probably unusual in Wallonia, especially for STB-resistant cultivars and for plants treated with fungicides early in the growing season. Increasing the degree of overlapping resulted in many false alarms. Although it is possible that an infected leaf could overlap and contaminate leaf layers two or three layers above it (Lovell *et al.* 1997, 2004b), this does not seem to occur systematically. It would depend on many factors, such as the size and architecture of the plants and the position of symptoms on the leaf. These factors are difficult to quantify and therefore difficult to integrate into a simple mechanistic model.

For airborne inoculum dispersal, our fitted model assumed that a load of 80 cDNA with a detection threshold of 8 cDNA, followed by favorable conditions, is required for infection. This detection threshold accords with the one fixed in our previous study (Duvivier et *al.* 2013). The 80 cDNA load corresponds with the range of values measured for primary STB infection in wheat in other studies (Bathgate & Loughman 2001; Suffert et *al.* 2011; Morais et *al.* 2015). According to our model, this dispersal mechanism could account for 42-46% (PI or extended PI) of the first contamination observed, which is fairly important.

When the three separate models were combined it was possible to explain 68-78% (PI or extended PI) of the initial contamination of the three upper leaves. This suggests that integrating the three dispersal mechanisms produces a model that is better able to explain contamination than models focusing on each mechanism independently.

These results, especially the importance of airborne inoculum in the initial contamination of upper leaves, indicate that the sexual cycle of *M. graminicola* plays a major role in STB epidemics in crop growing seasons. Previous studies on the genetic structure of *M. graminicola* populations, particularly the high level of genetic variability at field or even leaf scale and the high degree of recombination within and among populations indicating a constant mixing of strains, have provided indirect evidence that sexual reproduction occurs at high frequency throughout the growing season (Boeger et *al.* 1993; McDonald et *al.* 1999; Linde et *al.* 2002). The importance of sexual reproduction in leaf contamination was studied by Zhan *et al.* (1998), who, using a genetic approach, estimated that by the end of a growing season 34% of the isolates resulted from sexual reproduction (immigrants or recombinants of the 10 inoculated isolates in the field) and 66% were asexual progeny from the inoculated isolates. Although our compilation model could not explain all the contamination, an approximation could be made by keeping the same ratio for non-explained contamination and dividing by two the amount of contamination that both types of reproduction could explain. Our results, based on a mechanistic approach, revealed that airborne inoculum would have been responsible for 43%-42% (PI and extended PI) of the initial contaminations observed and that 57%-58% could be attributed to the asexual cycle (splash dispersion and leaf overlapping). These proportions, obtained using a completely different approach, are comparable with those obtained by Zhan *et al.* (1998). This argues for dispersion by airborne inoculum being included in STB prediction models.

So far as we know, airborne contamination has not been included in any STB prediction system. Since mature pseudothecia are produced long after

the appearance of pycnidia in the field and are less productive, it was thought that ascospores did not significantly influence STB epidemics (Eyal 1971; Gough & Lee 1985; Kema *et al.* 1996b; Hunter *et al.* 1999; Eriksen & Munk 2003). Their great mobility (Shaw & Royle 1989; Fraaije *et al.* 2005), however, and the fact that they can be produced on residues for a long time after the harvest (Suffert & Sache, 2011; Suffert *et al.* 2011) are two other factors to take into account in the evaluation of ascospore concentrations above fields when upper leaves are emerging. Our previous study led to the conclusion that airborne inoculum trapped in the field when the three upper leaves are emerging (March-May) should derive from two sources: the residues of a previous wheat crop and the pseudothecia developing in the wheat canopy (Duvivier et *al.* 2013). It was therefore necessary to measure the exact density of spores above a crop in many situations before concluding that this dispersal mechanism did not play a significant role in the contamination of the newly emerged upper leaves of wheat.

Pycnidiospores infections of the upper leaves probably occur at the same time as contamination by ascospores, especially with STB-susceptible cultivars. In some cases, the effect of contamination by airborne inoculum could be completely overshadowed by asexual reproduction. The estimation of infection attributable to airborne vs splashborne inoculum obtained with our fitted model seemed to confirm this result for untreated plots. For example, on an STB-susceptible cultivar, the results indicate that of the 33%-40% (PI and extended PI) of contamination possibly due to airborne inoculum, 19%-23% could also be explained by asexual reproduction. The fitted model was therefore tested on the flag leaf using an efficient fungicide treatment at GS32 to halt asexual reproduction. Our fitted model was then able to explain 96-92% (PI extended PI) of the contamination observed. This slowing down of the asexual propagation of STB resulted in a longer distance between the height of the disease in the canopy and the flag leaf than observed in untreated plants. There was no significant difference between these two groups, however, with regard to the appearance of symptoms. Vertical dispersal by pycnidiospores could explain only about 40% of the contamination, whereas more than 80% of the contamination could have involved airborne inoculum, depending on the cultivar or period of infection considered. Inoculum dispersion by leaf overlapping never seemed to be involved in the contamination of the flag leaves of treated plants. These results indicate that airborne inoculum plays a major role in the infection of the flag leaves of treated plants, especially in the case of STB-resistant cultivars.

The selection of *M. graminicola* populations with a high level of fungicide resistance combined with their sexual reproduction in the same growing season explains why STB adapts so rapidly to fungicides. Our results suggest that a significant part of the infection of the upper leaves of plants treated with fungicides at GS32 is caused by airborne spores. These spores probably come from a recombination of strains that have survived fungicide treatment. Zhan *et al.* (1998) showed that sexual recombination in a field population of *M. graminicola* increased over the growing season, whereas the proportion of immigrants in a field decreased between mid and late season. This suggests that, during the growing season, there is an initial mixing of strains receiving treatments that might give the progeny even higher resistance levels than the parents. These progeny could then infect the upper leaves and reproduce in high quantities, thanks to the asexual cycle. The selective pressure due to a second or even a third fungicide treatment on the upper leaves could reinforce the process. This again shows how important it is to mix substance actives in single spray program and/or to alternate the use of active substances in fungicide treatment programs. The same findings could explain why resistant cultivars are rapidly overcome by STB, with sexual contamination often apparently involved in the contamination of the upper leaves of resistant cultivars.

In the case of susceptible cultivars, asexual reproduction is often dangerous in mid-season (GS31 -GS32) because STB is already high in the canopy and could reach the flag leaf through splash dispersion. It is often necessary to slow down disease propagation by asexual reproduction. The case for resistant cultivars is different. Asexual dispersal does not necessarily constitute a risk of contamination of the upper leaves at GS31-GS32, as noted earlier. It would therefore be interesting to postpone fungicide treatment where possible and directly target the flag leaf. A simple strategy based on slowing down the disease at the bottom of the plants is no longer valid. Airborne inoculum above the canopy could directly infect the upper leaves in a field where dispersal by asexual reproduction does not appear to be a threat.

In conclusion, the assessment of the role of airborne inoculum in STB epidemics in fields using a mechanistic approach and datasets from successive growing seasons confirmed that this inoculum is involved in the infection of the upper three leaves. Complementary data, such as the measurement of splash height using specific devices (Shaw 1987; Lovell & Parker 2002) or data on the canopy architecture (Lovell *et al.* 1997, 2004b) and the viability of airborne inoculum (Hunter *et al.* 1999; Jackson & Bayliss 2011), would have improved the accuracy of the findings. Given the possible effects of infection by airborne inoculum on yield and on the

genetic diversity of *M. graminicola* populations, further research is needed to develop an accurate model for predicting STB infection after GS30, taking account of the three dispersal mechanisms and the STB resistance of wheat cultivars. It would be useful to define the conditions conducive to high airborne inoculum concentrations at the critical period in order to predict of the risk of airborne inoculum infection more easily.

Chapter 4

Real-time PCR quantification and spatio-temporal distribution of airborne inoculum of Puccinia triticina in Belgium

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Abstract

In order to better understand the epidemiology of *Puccinia triticina* and the relationship between airborne inoculum and disease severity, a method for quantifying airborne inoculum was developed using volumetric Burkard 7-day spore traps and real-time PCR. The method was applied using a spore trap network from 1 March to 30 June over a 5-year period. At one site, the inoculum was quantified continuously over 3 years, during which it showed a seasonal distribution, with the highest quantities and detection frequencies occurring between May and June. High mean daily quantities (65.8-121.2 spores/day) and detection frequencies (±20% of days) were also reported after harvest from September to December. In the coldest months of the year, almost no detection was recorded (1-6% of days). The study results indicate that the absence of inoculum in the air when upper leaves are emerging could be a limiting factor for the risk of epidemics. Mean daily quantities of airborne inoculum from 0 spore/day to 131.4 spores/day were measured from the beginning of stem elongation (GS30) to the flag leaf stage (GS39). These values were well correlated with the disease severity levels measured during grain development. A multiple regression analysis showed that total rainfall in late summer and autumn and mean minimum temperature in winter positively influence spore density between GS30 and GS39 in the following spring (R^2=0.73). This relationship and the patterns of airborne inoculum observed in fields strongly suggest that fields are contaminated early in the growing season possibly by the existence of a 'green bridge' phenomenon in Belgium. Our study also showed that the quantification of airborne inoculum or its estimation using a weather-based predictive model could be useful for interpreting disease severity models and avoiding over-estimates of disease risk.

4.1. Introduction

Wheat leaf rust (WLR), caused by *Puccinia triticina* Eriks., is the most widespread of the three rust diseases affecting winter wheat and causing significant losses worldwide (Bolton *et al.* 2008; Kolmer 2005; Roelfs 1989; Saari & Prescott 1985). *Puccinia triticina* is an obligate biotrophic and macrocyclic fungal pathogen with five spore stages. It requires an alternate host, *Thalictrum speciosissimum* or *Isopyrum fumaroides*, to complete its life cycle (Bolton *et al.* 2008). In Belgium, only the uredinial stage (urediniospores) is thought to contribute epidemiologically to the disease because the alternate hosts are uncommon ornamental plants. Successive wheat crops do not overlap in Belgium, with mature plants being harvested at least 2 months before newly sown plants emerge. The survival of the pathogen during this period therefore depends on the presence of volunteer plants. At the end of the growing season, large numbers of urediniospores can be produced and blown away from contaminated fields. Although most urediniospores are deposited near their source (Roelfs and Martell 1984), some can be dispersed over considerable distances by the wind (Hirst & Hurst 1967). As observed in the southern Great Plains of the United States, it is likely that infections of volunteer wheat plants in Belgium could serve as a bridge leading to the infection of newly sown wheat (the 'green bridge' phenomenon) (Eversmeyer *et al.* 1988a). Leaf rust on winter wheat can over-winter as mycelial or uredinial infections in areas with favorable temperature conditions (Roelfs 1989). It is known that *P. triticina* can survive in the same environmental conditions as wheat leaves, provided that infection, but not sporulation, has occurred (Roelfs *et al.* 1992). A warm and wet late summer and autumn is conducive to the infection of volunteer plants and therefore the green bridge phenomenon is more likely to occur under these climatic conditions, whereas a very cold winter can decrease the rate of survival of *P. triticina* on young plants (Daamen *et al.* 1992; Eversmeyer & Kramer 1998). Independently of the green bridge, the first symptoms of WLR in a field could also be the result of infection by urediniospores carried upwind by air masses from distant infected fields in warmer areas (Kolmer 2005; Nagarajan & Singh 1990; Roelfs *et al.* 1992).

From these two sources of primary infection, the pathogen can spread rapidly in fields given suitable environmental conditions in the spring and summer. Average disease severity of WLR estimated on the upper three leaves of wheat plants is highly correlated with reduced grain weight (Seck *et al.* 1991). The severity of rust epidemics on these leaves depends on the precocity of the infection by primary inoculum, host resistance and climatic conditions (Eversmeyer & Kramer 2000; Moschini & Pérez 1999). Several

predictive models of WLR infection have been developed, based only on climatic factors (El Jarroudi *et al.* 2014; Eversmeyer & Kramer 1998; Rao *et al.* 1990). Some models include other environmental factors, such as cultivar resistance or inoculum pressure (Audsley *et al.* 2005; Eversmeyer & Burleigh 1970; Moschini & Pérez 1999). On WLR-susceptible cultivars, moisture and temperature have been reported as the most important meteorological parameters influencing the disease (de Vallavieille-Pope *et al.* 2002). Urediniospores come into contact with winter wheat leaves through the action of wind or rain (de Vallavieille-Pope *et al.* 2002; Barnes *et al.* 2008; Li *et al.* 2009). In addition to wet deposition (spores deposited by rain), rainfall can also be a factor in the dispersal of uredinispores through the transfer of motion energy to the leaf or through splashing (Sache 2000). The success of an infection depends mainly on the duration of the wet period, which varies as a function of temperature (Huber & Gillespie 1992). The period of latency is strongly correlated with temperature; a latency period of 8-20 days was observed for air temperatures ranging from 10 to 20 °C (Eversmeyer *et al.* 1988b). Severe epidemics result from the succession of four to five cycles of asexual reproduction in the season, when environmental conditions are favorable (Zadoks & Bouwman 1985). Therefore, high disease pressure causing subsequent yield losses is usually reflected in the infection of newly emerged upper leaves (Roelfs *et al.* 1992).

The dispersal of airborne inoculum from the source and after deposition on a crop is a complex process influenced by wind direction, turbulence and many other parameters that are difficult to quantify (Aylor 2003, 1999; McCartney & West 2007; McCartney & Fitt 1998). Recent developments in molecular technology, however, have made it easier to estimate spore concentration above the canopy of wheat fields. This type of information could help in predicting epidemics more accurately where disease severity is influenced by timing or amount of inoculum (West *et al.* 2008). Spore traps, combined with inoculum detection and real-time PCR assays, are being increasingly used to quantify the airborne inoculum of plant pathogens and to improve precision in disease risk management (Luo *et al.*, 2007; Rogers *et al.*, 2009; Dedeurwaerder *et al.*, 2011; Wieczorek & Jørgensen, 2013; Duvivier *et al.*, 2013a; Chandelier *et al.*, 2014; Almquist & Wallenhammar, 2015). Accurate data on the spatio-temporal distribution of airborne *P. triticina* inoculum, trapped above wheat field canopy, would contribute to a better understanding of the epidemiology of WLR. This information could also be used to establish the relationship between airborne inoculum load and the risk of severe epidemics and therefore help in the prediction of the disease.

In the study reported here, a rapid method for quantifying airborne *P. triticina* inoculum combining the use of spore trap with a specific real-time PCR assay was developed and tested for reliability. The method was used in a network of trial fields in Belgium to study the temporal distribution of airborne *P. triticina* inoculum over five seasons. The relationships between airborne inoculum, disease pressure in the field and meteorological factors were analyzed. The study focused on their role in WLR prediction.

4.2. Materials and methods

4.2.1. Quantification of *P. triticina* using real-time PCR and spore traps

Airborne inoculum was collected using Burkard 7-day spore traps (Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK). The traps collected airborne particles on wax-coated Melinex tape (Burkard Manufacturing Co. Ltd; 345 x 20 mm). The tape was covered with Vaseline and replaced weekly. The throughput of each spore trap in the study was regulated at 10 l/min, corresponding to 14.4 m^3 every 24 h. After exposure, the tape was cut into seven daily segments of 48 x 20 mm. Each segment was placed in a 2 ml microtube for total DNA extraction, using the method described by Duvivier *et al.* (2013).

A set of primers and Taqman probe specific to *P. triticina* was designed (Ptriti-probe: Texas-Red-CTCTCGGTGTACTCTACTGATCGC-BHQ 2, labeled with Texas Red and containing BHQ-2 as quencher; Ptriti_btub_For: CGCAGATGACTATCCCTTTA; Ptriti_btub_Rev: GTGCGATGTCACAATGAG) using the Beacon Designer 3.0 program (Biosoft International, Corina Way, California, US). The target region was part (110 bp) of the β-tubuline coding gene of *P. triticina* (GenBank accession number HQ317599), because fungal β-tubuline gene sequences are known to be well conserved within this species (Fraaije *et al.*, 1999). The specificity of the primers and probe was then assessed using the Blastn algorithm (Altschul *et al.*, 1990). The mixture for the real-time PCR assay was prepared with 12.5 µl of iTaq™ Universal Probes Supermix (Biorad, Hercules, California, US), forward and reverse primers at 500 nM, probe at 50 nM and 2.5 µl of DNA extract in a total volume of 25 µl. The amplification reaction was conducted using the Bio-Rad CFX96™ Real-Time System (Bio-Rad and software Bio-Rad CFX Manager 3.0, Hercules, California, US) as follows: initial denaturation at 95°C for 3 min and 40 cycles each; denaturation at 95°C for 5 s; and hybridization and elongation at 60°C for 30 s. The increase in fluorescence from the probe was recorded at 60°C during each cycle. The real-time PCR protocol was optimized by varying product quantities, times and temperatures applied in the PCR reaction.

The specificity of the method was tested by including, in the qPCR-assay, DNA extracts from several species: *P. triticina* spores collected from WLR-infected wheat leaves; *P. striiformis* spores collected from wheat leaves (cultivar Toisondor) infected by wheat stripe rust; *in vitro* culture of reference isolates of *Mycosphaerella graminicola* (MUCL references 45550, 45549), *Staganospora nodorum* (MUCL references 30165, 44704 and

44707), *Fusarium graminearum* (MUCL references 43802, 43803 and 46388), *F. culmorum* (MUCL references 43796, 43797 and 43798), *F. poae* (MUCL references 42824, 42836 and 42842), *Oculimacula yallundae* (MUCL references 40386 and 40387), *O. acuformis* (MUCL references 40388, 40389 and 40637), *Sclerotinia sclerotiorum* (MUCL references 11553 and 30163) and *Microdochium nivale* (MUCL references 15949 and 31963).

Four tenfold serial dilutions of a *P. triticina* DNA extract were used as a template for each real-time PCR run. The cycle threshold (CT) values were plotted against the logarithm of the starting quantity of the DNA extract for each dilution. Amplification efficiency was calculated from the slope of the standard curve using ICycler IQ software, version 3.0 (BioRad, Hercules, California, US).

In order to determine the detection threshold of the real-time PCR and the relationship between CT values and number of trapped spores, a spore suspension was prepared from *P. triticina* spores collected from the field using 0.1% Nonidet P40. The spore concentration in suspension was estimated using a Thoma counting chamber. The suspension was adjusted to 2 x 10^7 spores/ml and a tenfold serial dilution of up to 200 spores/ml was prepared. A volume of 50 µl of each spore suspension (10^6 spores to 10 spores) was placed in a 2 ml microtube with a piece of clean Melinex tape (48 x 20 mm) covered with Vaseline. DNA was extracted and quantified using real-time PCR, as described earlier. The test was replicated twice. The CT values were plotted against the logarithm of the starting quantity of each spore suspension in order to create a calibration curve and to present the results as numbers of spores on the tape each day (spores/day). During the development and for routine use, each sample was run in two replicates.

The specificity of the qPCR for detecting *P. triticina* in the DNA extracts from the spore trap tapes was confirmed by sequencing PCR products from 10 positive samples (DNA extracts with a positive detection using the qPCR assay), originated from randomly chosen spore trap tapes from the 5-year study, and comparing these sequences with the expected sequence.

4.2.2. Trial fields and spore trap network

A network of five Burkard 7-day spore traps was set up in the Walloon region of Belgium and run from March 2009 to July 2013 (Figure 4.1). The traps were placed in wheat fields at Perwez, Voroux-Goreux, Tournai, Niverlée and Gembloux; the GPS coordinates are given in Figure 4.1 for the 2012 growing season. The trap openings were placed 1 m above ground

level in a wheat-free plot measuring 9 m². The sites provided a good representation of Walloon's wheat-growing regions. Each year, in mid-October, the spore traps were moved to newly sown wheat fields a maximum of 10 km away.

Each year, the daily quantities of *P. triticina* inoculum trapped between 1 March and 30 June in the five spore traps were assessed. The spore trap at Gembloux (5) operated in only 2011 and 2012. At Voroux-Goreux, the analyses were completed by assessing the quantities of inoculum from September to February in the 2009-2010, 2010-2011 and 2011-2012 growing seasons. The data were always expressed in terms of the number of spores on a daily segment of tape (spores/day).

The five spore traps in the trial fields were all placed next to a fungicide trial. Each fungicide trial field contained, in addition to other treatments, four untreated plots (1.5 × 10 m) of Lion, a cultivar known for its susceptibility to WLR. The winter wheat trials were established in fields cultivated by the farmers themselves, using the good agricultural practices typical of the region. In the trial area, farmers applied all the treatments apart from the fungicide ones. Trial field management data are given in Table 4.1.

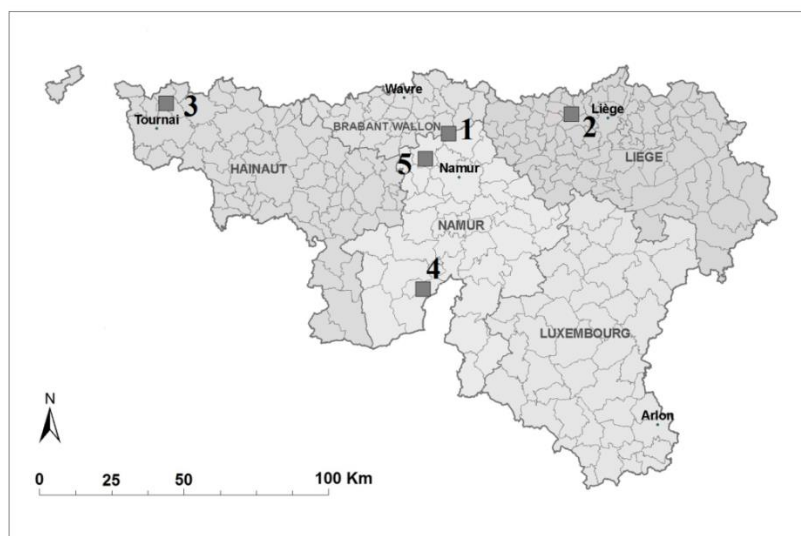


Figure 4.1. Location of the spore traps for the growing season 2012 in the Walloon region, Belgium (1: Perwez, N 50°37'43.507'', E4°48'27.259''; 2: Voroux-Goreux, N50°39'58.414'', E5°25'40.108''; 3: Tournai N50°32'55.392'', E3°32'44.656''; 4: Niverlée, N50°7'3.716'', E4°42'35.996''; 5: Gembloux N50°33'42.124'', E4°42'36.345'').

4.2.3. Weather data

Hourly meteorological data were obtained from iMetos stations (Pessl Instruments, Austria) set up in the trial fields or a maximum of 10 km away for measuring temperature, rainfall quantity and RH. Data from the national network of meteorological stations, Pameseb (Asbl Pameseb, Libramont, Belgium) and the Institut Royal Météorologique de Belgique (IRM, Belgium) were used to supply the missing data. Air temperature, rainfall quantity and RH were measured at a height of 2 m above the ground.

4.2.4. Relationship between airborne inoculum and disease level

The leaves were numbered relative to the uppermost leaf (L1, the flag leaf), with the leaf immediately below L1 designated L2, followed by L3, and so on (Shaner & Buechley 1995). Plant growth stages (GS) were also assessed according to a decimal scale. The main Zadoks growth stages of wheat in all the trial fields were recorded (Zadoks et *al.* 1974) and the development of the upper three leaves was assessed weekly. The modified Cobb scale was used to measure the percentage of leaf tissue affected by WLR (Peterson 1948). Data on disease severity on 15 untreated plants of the LION cultivar (cv susceptible to *P. triticina*), randomly collected from each of the four plots per field, were assessed weekly, from emergence to senescence, by observing symptoms from L3 to L1. After performing the arcsine square root transformations recommended for proportional data, the mean disease severity on the two uppermost leaves measured during grain development or maturation (between GS75 and GS85) were used to indicate **the disease level** of a site. For the five growing seasons, the disease level of each site was compared using ANOVA, followed by a Student-Newman-Keuls test (α=0.05). Similar analyses were used to compare the overall mean disease level of the five growing seasons. It is worth noting that, over the study period, other fungal diseases also occurred, including *Septoria triciti* blotch (STB) and wheat powdery mildew. Simple linear regression was used to study the relationship between disease pressure in a field and spore concentration above the canopy. The response variable was disease level, the explanatory variable being the log-transformed daily mean detection of airborne *P. triticina* inoculum for three periods: from the beginning of stem elongation (GS30) to the flag leaf stage (GS39); from GS30 to the beginning of flowering (GS61); and from 1 March to GS39.

4.2.5. Factors influencing airborne inoculum concentration

A regression equation explaining the variability of the log-transformed daily mean detection of airborne inoculum (GS30 to GS39) was used, with weather conditions and environmental factors as potential explanatory variables. The meteorological parameters tested were the daily mean temperature, mean minimum temperature, mean maximum temperature, total rainfall and number of days with >70% RH but no rainfall, in line with the findings reported by Moschini *et al.* (1999). The mean minimum/maximum temperatures for a given period were computed as the average of hourly minimum/maximum temperatures observed each day. For these meteorological parameters, the highest and most significant Pearson coefficient correlations were first identified for all the periods between August and April (minimum step: 1 month). The significance threshold of the coefficients was adjusted for multiple testing using Bonferroni correction, as recommended by Vittinghoff *et al.* (2005). The environmental factor tested was the disease level observed in adjacent fields in the previous growing season. A multiple linear regression was computed using the highest correlated parameters to explain spore density variability between GS30 and GS39 in the fields. A scatter plot between the observed and predicted values was computed. The accuracy of the model was assessed using fivefold cross validation. This cross validation was performed using data from all the trial fields in a growing season as the validation set and the remaining observations as the training set. The relationship between predicted value and disease level was checked using simple linear regression.

Statistical analyses were performed using R Software R 2.15.0 (www.r-project.org). Graphics were created using OriginPro 8 (OriginLab, Northampton, UK).

Table 4. 1. Agronomic data for winter wheat fields at the experimental sites in the Walloon region in the 2009-2013 growing seasons. GS30: beginning of stem elongation, GS39: flag leaf fully emerged, GS61: beginning of flowering.

Year	Site	Seed density (seed/m2)	Previous crop	Sowing date	GS30	GS39	GS69	Nitrogen (kg N ha^{-1})
2009	Perwez	400	sugar beet	17/11/08	15/04/09	29/05/09	12/06/09	184
	Voroux-Goreux	310	sugar beet	4/11/08	15/04/09	25/05/09	9/06/09	178
	Tournai	310	beetroot	7/11/08	15/04/09	22/05/09	5/06/09	194
	Niverlée	400	sugar beet	19/11/08	14/04/09	29/05/09	12/06/09	195
2010	Perwez	250	carrot	18/10/09	20/04/10	25/05/10	14/06/10	190
	Voroux-Goreux	250	pea	15/10/09	20/04/10	25/05/10	15/06/10	175
	Tournai	250	potato	26/10/09	22/04/10	27/05/10	13/06/10	227
	Niverlée	250	sugar beet	20/10/09	21/04/10	2/06/10	16/06/10	168
2011	Perwez	250	sugar beet	13/10/10	13/04/11	14/05/11	30/05/11	175
	Voroux-Goreux	250	sugar beet	15/10/10	5/04/11	10/05/11	31/05/11	180
	Tournai	370	chicory	20/11/10	14/04/11	12/05/11	2/06/11	205
	Niverlée	250	sugar beet	13/10/10	17/04/11	11/05/11	1/06/11	197
	Gembloux	250	beetroot	25/10/10	27/04/11	16/05/11	5/06/11	175
2012	Perwez	250	flax	16/10/11	16/04/12	21/05/12	8/06/12	180
	Voroux-Goreux	250	sugar beet	15/10/11	17/04/12	24/05/12	4/06/12	175
	Tournai	250	sugar beet	14/10/11	12/04/12	24/05/12	4/06/12	170
	Niverlée	250	oil rape	17/10/11	10/04/12	23/05/12	6/06/12	175
	Gembloux	250	sugar beet	16/10/11	15/04/12	22/05/12	8/06/12	165
2013	Perwez	250	potato	22/10/12	3/05/13	3/06/13	24/06/13	190
	Voroux-Goreux	300	sugar beet	14/11/12	6/05/13	5/06/13	23/06/13	178
	Tournai	250	sugar beet	25/10/12	30/04/13	30/05/13	22/06/13	165
	Niverlée	250	sugar beet	27/10/12	30/04/13	4/06/13	20/06/13	180

4.3. Results

4.3.1. Assessment of the specificity of real-time PCR assay for detecting *P. triticina*

The primers and probe designed in specific regions of the β-tubuline coding gene of *P. triticina* tested by Blastn showed high specificity for this species. No amplification was obtained with DNA extracts from the other tested fungal species (*S. nodorum*, *F. graminearum*, *F. culmorum*, *F. poae*, *O. yallundae*, *O. acuformis*, *S. sclerotiorum, M. nivale* and *P. striiformis*). The amplification of four serial dilutions of *P. triticina* DNA extract gave amplification curves with 90-110% efficiency and an R^2 of 0.99.

The detection threshold of the PCR assay was estimated using DNA extracted from known quantities of urediniospores deposited daily on segments (48 x 20 mm) of Melinex tape (Table 4.2). Mean CT values for DNA from 1,000,000 to 100 spores ranged from 24.97 to 38.02. The PCR assay allowed up to 10 urediniospores to be detected daily on the tape. In this case, however, the DNA of *P. triticina* that had been extracted from 10 spores was detected in only two of the four tested replications (spore disruption and DNA extraction were replicated twice, as was real-time PCR). The reproducibility of the method therefore decreased when the CT value was higher than 38 (Table 4.2). The relationship between the CT values and spore quantities was estimated from these results. The equation used for calculating spore quantities (Y) in each DNA extract was:

$$Y = 10^{12}.e^{(-0,682.CT)}$$

The PCR assay was applied for the specific quantification of airborne *P. triticina* inoculum from all the samples using the Burkard 7-day spore trap network (see later; e.g., Figure 4.3). The sequences obtained from the sequenced PCR products for the 10 positive samples collected over the five growing seasons all matched the expected sequence of *P. triticina*.

Table 4.2. Amplification of DNA extracts from 10^6 to 10 spores of *P. triticina* on a daily segment (48 x 20 mm) of Melinex tape using real-time PCR. DNA extraction and real-time PCR were replicated twice. A volume of 2.5µl of DNA extract was used in each reaction.

No. of spores on tape	No. of spores in real-time PCR tube	Mean CT	Standard deviation
1,000,000	50,000	24.97	0.18
100,000	5,000	27.65	0.02
10,000	500	31.19	0.16
1,000	50	35.16	0.22
100	5	38.02	2.21
10	0.5	39.32*	NA
0	0	NA	0.00

***One detection out of two tested DNA extracts**

4.3.2. Wheat and disease development

The 2008-2009, 2009-2010 and 2011-2012 growing seasons were similar in terms of plant development, with the flag leaf usually emerging (GS39) in the last 10 days of May. The 2010-2011 growing season was characterized by a more rapid development of plants. In contrast, the long and severe winter of 2012-2013 considerably delayed wheat development.

The date on which WLR symptoms appeared in the fields varied considerably between years. Although initial symptom detection sometimes occurred on young plants in the winter, as in 2011-2012, the symptoms then disappeared with cold temperatures and heavy rain. In our study, the initial WLR detection on the upper leaves (L3-L1) always occurred after the flag leaf had fully emerged (GS39) (Tables 4.1 and 4.3). The disease level (between GS75 and GS85) varied greatly over the 5 years (Figure 4.2). In 2009, 2010 and 2012, mean disease severity on the two uppermost leaves was above 10% most of the time. In 2011, there was an outbreak of WLR (>10%), with the highest severity observed at Niverlée and Gembloux. In contrast, the 2013 growing season was characterized by particularly low WLR severity, with no symptoms observed apart from at Voroux-Goreux, where there was late development of the disease. Wallonia covers a small area and yet every year the variations in WLR occurrence were significant, according to the ANOVA.

Table 4.3. Detection of WLR in the field: date and leaf layer of the first detection and date of the first detection on the three upper leaves in each field. L1 corresponds to the flag leaf, L2 the leaf immediately below it, and so on. (ND: not detected).

Year	Site	First detection in the field		First detection on three upper leaves		
		Date	Organ	L3	L2	L1
2009	Perwez	17/06/09	L3-L2-L1	17/06/09	17/06/09	17/06/09
	Voroux-Goreux	03/06/09	L4	16/06/09	16/06/09	23/06/09
	Tournai	29/05/09	L4	11/06/09	11/06/09	11/06/09
	Niverlée	22/06/09	L3-L2	22/06/09	22/06/09	29/06/09
2010	Perwez	24/06/10	L4-L3-L2-L1	24/06/10	24/06/10	24/06/10
	Voroux-Goreux	22/06/10	L3-L2	22/06/10	22/06/10	29/06/10
	Tournai	24/06/10	L4-L3-L2-L1	24/06/10	24/06/10	24/06/10
	Niverlée	23/06/10	L4-L3-L2-L1	23/06/10	23/06/10	23/06/10
2011	Perwez	16/05/11	L5-L4	23/05/11	30/05/11	30/05/11
	Voroux-Goreux	31/05/11	L4-L3-L2	31/05/11	31/05/11	06/06/11
	Tournai	19/05/11	L4-L3-L2	19/05/11	19/05/11	01/06/11
	Niverlée	27/04/11	Lower leaves	25/05/11	25/05/11	25/05/11
	Gembloux	16/05/11	L4-L3	16/05/11	16/05/11	01/06/11
2012	Perwez	January	Lower leaves	20/06/12	20/06/12	20/06/12
	Voroux-Goreux	March	Lower leaves	21/05/12	21/05/12	04/06/12
	Tournai	January	Lower leaves	31/05/12	31/05/12	31/05/12
	Niverlée	January	Lower leaves	28/05/12	28/05/12	19/06/12
	Gembloux	March	Lower leaves	01/06/12	01/06/12	15/06/12
2013	Perwez	ND	/	ND	ND	ND
	Voroux-Goreux	10/07/13	L3-L2-L1	10/07/13	10/07/13	10/07/13
	Tournai	ND	/	ND	ND	ND
	Niverlée	ND	/	ND	ND	ND

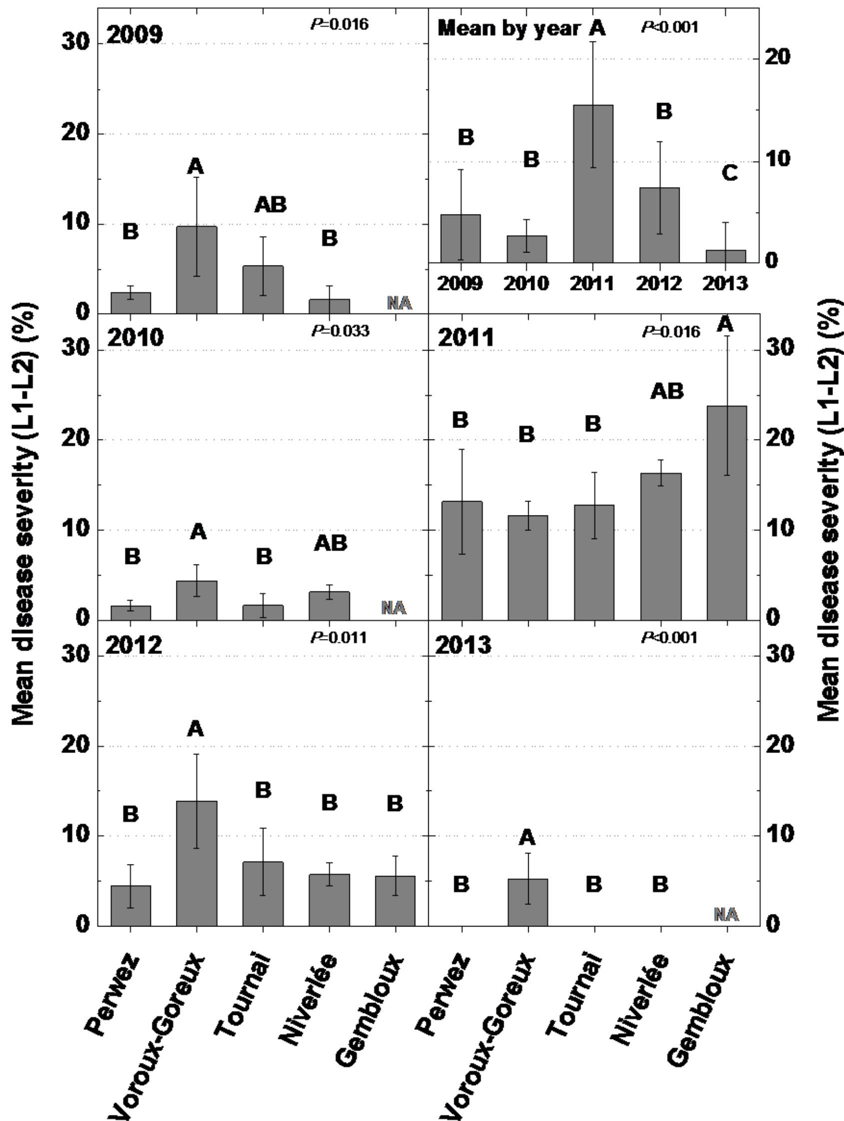


Figure 4.2. Mean wheat leaf rust (WLR) severity (%) and standard deviation on the two upper leaves (L1 and L2) measured during grain development or maturation (between GS75 and GS85) in the 2009-2013 growing seasons. Observations were made in four untreated plots per field from the end of June until mid-July, depending on the year. Comparisons between sites were made for each growing season using ANOVA, followed by a Student-Newman-Keuls test (α=0.05). Significant difference among sites are indicated by different letters. Comparison between the mean of each year was made using the same methodology. (NA: not available).

4.3.3. Temporal distribution of airborne inoculum

The data on the temporal distribution of airborne *P. triticina* inoculum at Voroux-Goreux (Figure 4.3) are presented in three 1-year datasets covering the 1 April to 31 March period. In April and May, there were occasional detections of less than 300 spores/day. This period corresponds to the time when the three upper leaves were emerging. Each year, detection frequency intensified alongside an increase in the quantity of spores trapped. This did not occur at the same time each year, however. In 2009 and 2011 the intensification occurred in mid-May, but in 2010 it occurred almost 1 month later, in mid-June. Similarly, the first detection of symptoms on the upper leaves occurred later that season. The highest quantities and detection frequencies occurred in June when the disease was well established in the field. At that time, detections sometimes exceeded 1,000 spores/day. The mean daily detection for this period was 202.7, 337.2 and 37.9 spores/day for 2009, 2010 and 2011, respectively. The frequency of detection was about 20% for 2010 and 2011, but up to 32% in 2009.

The September-December period was characterized by sporadic clusters of detection, which sometimes exceeded 1,000 spores/day. The mean daily detection for this period was 114.5, 65.8 and 121.2 spores/day in 2009, 2010 and 2011, respectively, and the frequency of detection was about 20%. This period corresponds to the emergence of volunteer plants and newly sown wheat plants.

In the coldest months, there was very little detection and the quantities trapped rarely exceeded 100 spores/day. The mean daily detection for this period was 2.7 spores/day in 2010 and 1.2 spores/day, with a detection frequency of 1% and 3%, respectively. Greater quantities were trapped between the beginning of January and the end of March 2012 at Voroux-Goreux (mean daily detection = 7.3 spores/day, frequency of detection = 6%). It should be noted that WLR symptoms were present at that site on the lower leaves of the plants from March.

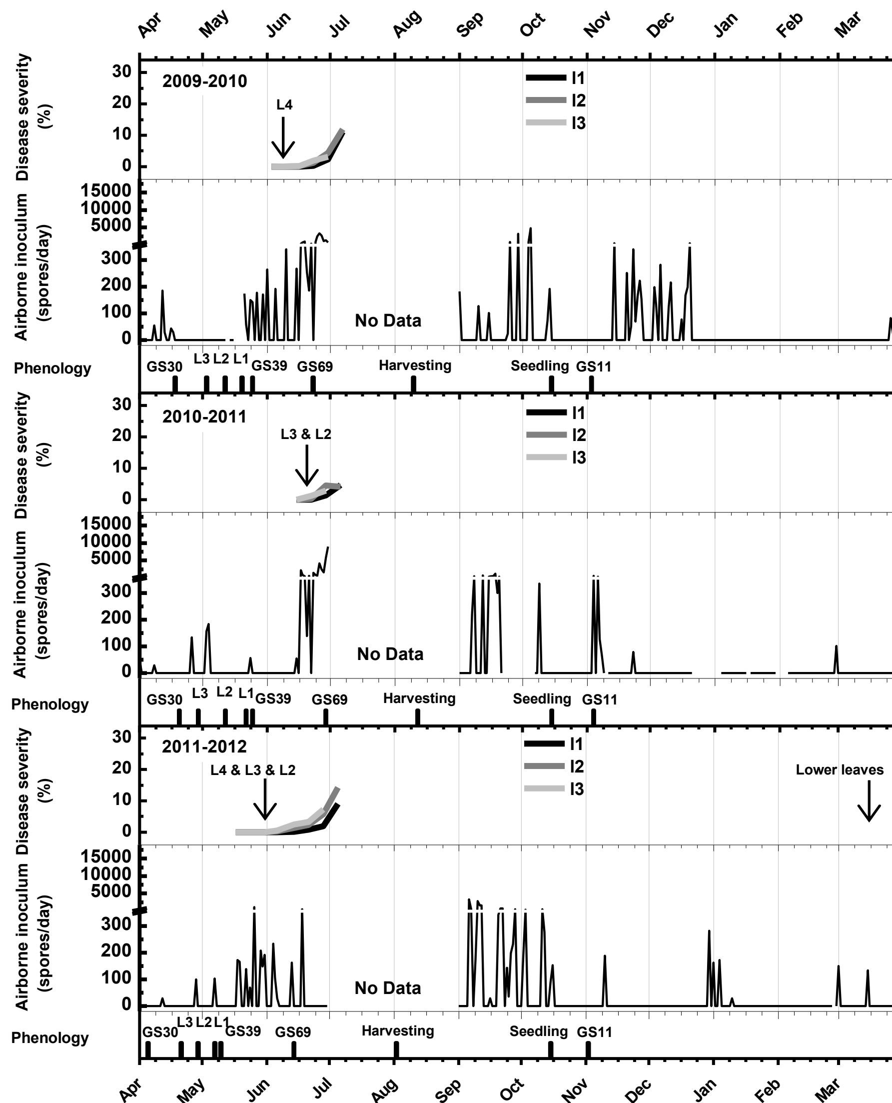


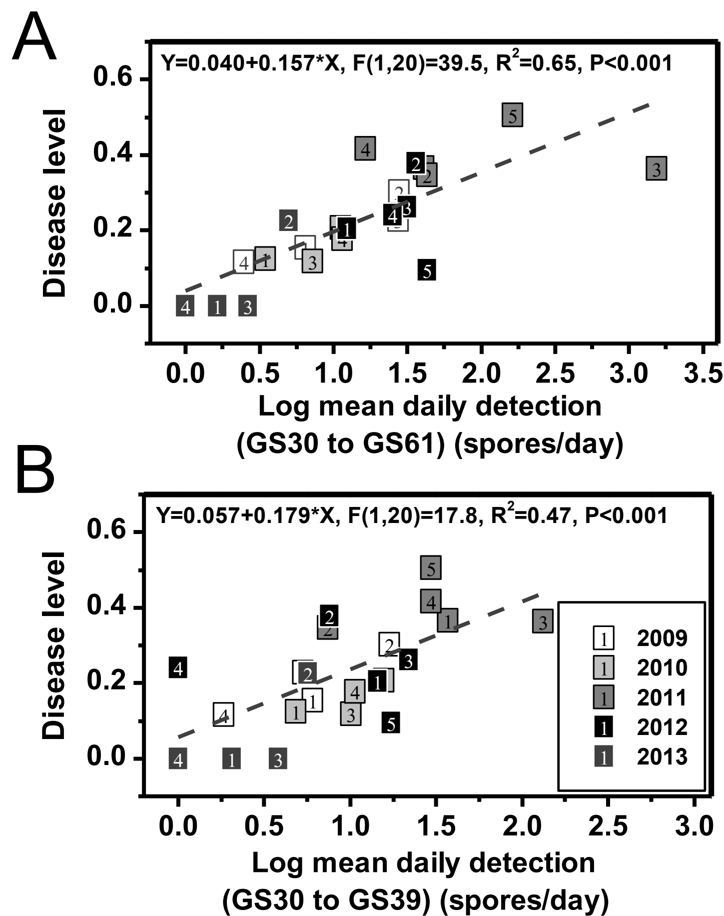
Figure 4.3. Daily quantities of airborne *P. triticina* inoculum trapped at Voroux-Goreux from April 2009 to March 2013. The evolution of wheat leaf rust (WLR) severity on the three upper leaves observed on a susceptible cultivar (Lion) near the spore traps is represented for each growing season. The arrows show the time of the first detection of WLR in the field. Plant phenology, including the appearance of the three upper leaves (L1-L3), is shown at the bottom of each figure. GS11: emergence, GS30: beginning of stem elongation, GS39: flag leaf fully emerged, GS61: beginning of flowering.

4.3.4. Relationship between airborne inoculum and disease epidemics

From the quantities of airborne inoculum collected at four to five sites from 2009 to 2013, between GS30 and GS39, it is clear that airborne *P. triticina* inoculum was detected at most of the sites apart from Niverlée in 2012 and 2013. Detections during this period were sporadic and rarely exceeded 300 spores/day. The mean daily detections for this period showed significant between-site and between-year variations (Figure 4.4B.) After GS39, usually corresponding to the time when the first WLR symptoms were detected on the upper leaves, detection frequency increased greatly, with daily quantities of more than 10,000 spores/day trapped in some fields (Figure 4.4A). In 2013, the quantities and detection frequencies of airborne inoculum were very low throughout the period sampled.

The relationship between disease pressure and mean daily quantities trapped in a field for a given period was studied. The airborne *P. triticina* inoculum trapped between GS30 and GS61 was positively and significantly correlated with the disease severity observed in the fields, explaining 65% of the variability in this parameter (Figure 4.4A). This period covered the time when the upper leaves had not yet been infected to the time when they first showed WLR symptoms. The quantity of airborne *P. triticina* inoculum trapped between GS30 and GS39 was also positively and significantly correlated with disease levels (Figure 4.4B). The inoculum was trapped before the appearance of symptoms on the upper leaves (L3-L1) in all the fields. This measure explained 47% of the disease pressure, late in the growing season. When the mean daily detection for this period was less than 5 spores/day, there was a low risk of high disease pressure during grain development. It is worth noting that measurements taken before GS30 did not help to explain the disease severity on the upper leaves late in the growing season. For example, the model computed with mean daily detection values from 1 March to GS39 explained only 32% of the variability in disease severity.

Figure 4.4. The relationship between the disease level measured in untreated susceptible wheat plants and the log-transformed mean daily detection of airborne inoculum of *Puccinia triticina* measured A) between GS30 and GS61 and B) between GS30 and GS39. Dashed line represents the regression equations. Data were collected over five growing seasons from 2009 to 2013 from a network of trial fields. The number refers to the position of the trial fields in the network set up each year in Wallonia (Figure 4.1).



4.3.5. Factors influencing airborne inoculum concentration in fields (GS30 to GS39)

Knowing the density of airborne inoculum between GS30 and GS39 could help in the prediction of final disease severity on the upper leaves. In order to build a weather-based model that explained the variability in this measurement, we looked for the highest correlation coefficients between

spore density (from GS30 to GS39) and climatic data over all time periods from 1 August to 31 April (minimum step: 1 month) (Table 4.4). The mean minimum temperature from 1 January to 31 March was highly and significantly correlated with the log-transformed mean daily detection from GS30 to GS39. Minimum temperature was better correlated than mean temperature or maximum mean temperature. A significant relationship was also found with total rainfall from 1 August to 31 December. Spore density was not significantly linked either with the number of days with high humidity and no precipitation, or with the disease level in the previous year.

Table 4.4. Pearson correlation coefficients between the log-transformed mean daily inoculum trapped (GS30 to GS39) and the climatic and environmental factors. The coefficients shown for each climatic parameter are the three highest values for all the periods from August to April (minimum step: 1 month). The significance threshold of the coefficients was adjusted for multiple testing using Bonferroni correction. Significant P –value (α=0.05) in bold.

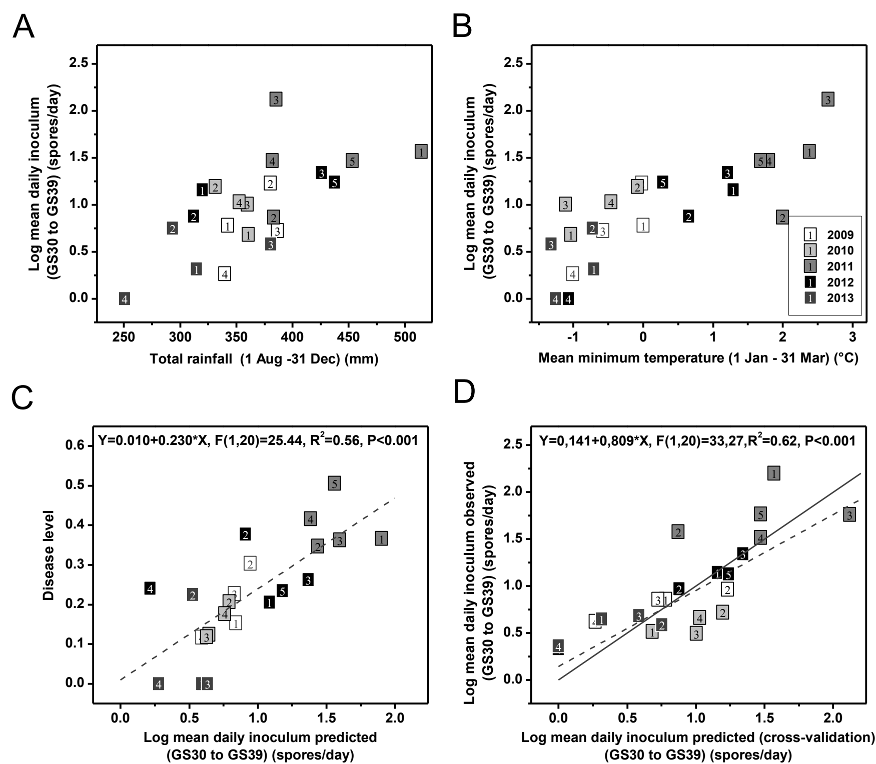
Variables	From	To	R	P-value	Corrected P-value
Mean minimum temperature	1 Jan	31 Mar	0.80	0.000	**0.001**
	1 Feb	31 Mar	0.79	0.000	**0.001**
	1 Jan	30 Apr	0.75	0.000	**0.005**
Mean maximum temperature	1 Jan	30 Apr	0.67	0.001	**0.048**
	1 Feb	31 Mar	0.67	0.001	0.053
	1 Feb	30 Apr	0.67	0.001	0.055
Mean temperature	1 Feb	31 Mar	0.77	0.000	**0.002**
	1 Jan	30 Apr	0.73	0.000	**0.009**
	1 Jan	31 Mar	0.72	0.000	**0.012**
Total rainfall	1 Aug	31 Dec	0.71	0.000	**0.018**
	1 Aug	30 Nov	0.66	0.001	0.062
	1 Aug	31 Jan	0.66	0.001	0.063
Humidity (>70%, 0 mm)	1 Oct	30 Apr	0.46	0.031	1.000
	1 Sep	30 Apr	0.46	0.033	1.000
	1 Sep	31 Mar	0.46	0.033	1.000
Disease level			-0.41	0.088	

A multiple linear regression model was computed to explain the log-transformed mean daily inoculum trapped from GS30 to GS39 (Y). The chosen variables were total rainfall from 1 August to 31 December (X_1) and mean minimum temperature from 1 January to 31 March (X_2) (Figures 4.5A and 4.5B). The regression equation obtained using all the data (n=22) was:

$$Y= -0.1363+0.0029*X_1+0.2386*X_2.$$

This fitted model enabled 73% of the variability in the log-transformed spore density to be explained ($F(2,19)=25.15$, $P<0.001$). Both parameters were positively and significantly correlated ($\beta_1=0.0029$, 95% CI = [0.0005, 0.0701], $P=0.025$, $\beta_2= 0,2386$, 95% CI = [0.1202,0.7202], $P<0.001$). The predicted values obtained with this equation were also in accord with the disease level (Figure 4.5C). The model was validated using fivefold cross validation. For each site in a given growing season, a predicted value was calculated using the model computed with all the data from the other growing seasons and using the same variables. The observed data corresponded well with the values obtained with the fitted models (Figure 4.5D). A correct and significant goodness of fit was obtained between the predicted value of the cross validation and the observed value.

Figure 4.5. The relationship between the log-transformed mean daily detection of airborne *Puccinia triticina* inoculum measured in wheat fields and A) the total rainfall calculated from 1 August to 31 December and B) the mean minimum temperature calculated from 1 January to 31 March. C) The relationship between the disease levels measured on untreated susceptible wheat plants and the predicted log-transformed mean daily detection between GS30 and GS39. Dashed line represents the regression equation. Predicted values were obtained by generating a multiple linear regression model using as explanatory factors the total rainfall calculated from 1 August to 31 December (X_1) and the mean minimum temperature calculated from 1 January to 31 March (X_2), the equation of the model being $Y=-0.1363+0.0029*X_1+0.2386*X_2$. D) The relationship between the values observed and predicted with a fivefold cross validation. The predicted values of a given growing season were obtained with a model computed with all the data from the other growing seasons. Dashed line is the regression equation. Grey line is the identity line. For each figure, the number refers to the position of the trial fields in the network set up each year in Wallonia (Figure 4.1).



4.4. Discussion

Recent studies combining real-time PCR and air sampling show that these tools can be useful in gaining a better understanding of the epidemiology of arable crop pathogens (Carisse *et al*. 2005; Duvivier *et al*. 2013; Fraaije *et al*. 2005; Wieczorek and Jørgensen 2013). The biological cycle of obligatory biotrophic fungi involved in polycyclic epidemics such as *P. triticina*, the causal agent of WLR, is particularly influenced by the dispersal of airborne inoculum (West *et al*. 2008), and tools that combine spore traps with modern quantitative PCR could be helpful in WLR prediction.

In this study, an original method combining specific real-time PCR with the use of Burkard 7-day spore traps was developed initially to quantify airborne *P. triticina* inoculum. The primers and probe designed for the study were highly specific to *P. triticina* and the assay allowed very small quantities of spores to be detected: up to 10 spores on the daily spore trap tape could be detected, although repeatability was not high at this level. All the data used in the analysis were log-transformed and averaged, considerably reducing the weight of these small quantities. This method was applied in Wallonia using a spore trap network from 2009 to 2013, with two main objectives: to learn more about the epidemiology of WLR and the spatio-temporal distribution of the airborne inoculum; and to explore the potential of using such measurements to prevent the risk of serious epidemics.

At Voroux-Goreux, a spore trap was run continuously for 3 years. Airborne *P. triticina* inoculum was detected throughout the period and showed a clear seasonal pattern. In spring, spore detection always occurred before symptom occurrence on the three upper leaves of wheat plants. As expected, due to the production and dispersal of secondary *P. triticina* urediniospores, the importance and frequency of detections increased significantly once the disease was established in the field. The airborne inoculum was not quantified in July and August, this period corresponding with plant maturation and harvest, when new infections probably have less impact on yield. In addition, detection during this period might have been influenced by contamination of the tapes by airborne plant particles or dust containing *P. triticina*. Unexpectedly, from September to December the airborne inoculum densities detected in all 3 years was high. This probably contributed to the infection of volunteer wheat plants and newly sown crops, which in turn ensured the survival of WLR from harvest through to the spring emergence of the next crop (Eversmeyer *et al.* 1988a). This constitutes a critical part of the life cycle of WLR because in Belgium successive wheat crops do not usually overlap and the alternate hosts are

uncommon ornamental plants. This green bridge phenomenon has been reported in various areas, including the southern Great Plains of the United States (Eversmeyer & Kramer 1998), Australia (Beard 2005) and Europe (Casulli 1988). After harvest, a high density of airborne inoculum is needed to compensate for the weak density of congenial hosts present at this time. The detection of airborne inoculum in autumn suggested that in Belgium *P. triticina* over-winters as mycelia without producing urediniospores, given the low quantities trapped from December to March. Preliminary studies on the quantification of *P. triticina* DNA in young plants collected from fields in Belgium in March show that it is possible to detect the pathogen at this time, even in the absence of symptoms on plants (data not shown). The sporulation of WLR observed on young wheat plants early in the season in 2012, after a mild winter, suggested that the wheat plantlets had been infected in the autumn. The relatively high temperatures in the winter of 2011-2012 were probably conducive to the production of urediniospores.

Disease severity on the two uppermost leaves is the main factor responsible for yield loss caused by WLR (Seck *et al.* 1991). The symptoms on these leaves rarely appear before GS39 in Belgium. The quantity of airborne inoculum measured in our study from GS30 to GS39 was well correlated with the mean severity of the disease observed at the end of the growing season on these two leaves. It has been reported that most severe WLR epidemics occur when uredinia and/or latent infections survive on wheat crops during winter above a particular threshold or when the crops have been affected by exogenous inoculum at an early stage before heading (Roelfs *et al.* 1992). In contrast, a lack of inoculum when climatic conditions are conducive to secondary infection could delay the epidemics and considerably reduce the risk of severe losses. The inoculum measured from GS30 to GS39 in our study, however, was not enough to explain the severity observed. The deposition of spores on leaves should be followed by favorable weather conditions in order for the WLR cycle to be completed and the first symptoms to appear and thereafter increase in severity. Also, an absence of spores detected above the canopy does not necessarily mean that infection and dispersion cannot occur because spores are likely to be deposited within a small distance of the source lesions (Mundt,2009). Epidemiologically important spores can also come from wet deposition (spores carried and dropped by rain), which is an important mechanism in crop contamination by rusts (Barnes *et al.* 2008; Li *et al.* 2009). A final source of bias in the relationship was that our method, based on using real-time PCR, did not distinguish viable spores.

Nevertheless, the relationship obtained between airborne inoculum quantity between GS30 and GS39 and disease severity indicated that spore

concentration measured when the upper leaves are emerging could be used as an initial indicator of the risk of an epidemic affecting the upper leaves. The method requires specific skills, however, and is still expensive. For these reasons, we looked at the possibility of predicting inoculum densities between GS30 and GS39 based on our data. In Wallonia, the spore concentration detected at this time should depend mainly on the survival of the disease, which is determined by weather conditions from the end of the summer until the following spring (Daamen *et al*. 1992; Eversmeyer & Kramer 1998). In order to build an easy-to-use weather-based model that could predict spore density levels, the correlations using weather statistics from different time periods between 1 August (before seedling) and 30 April were investigated. We observed a clear and significant relationship between spore density from GS30 to GS39 and total rainfall from the beginning of the previous August until the end of December. Rainfall occurring during this period influences the green bridge in two ways. First, rainfall in late summer promotes the germination of volunteer plants. It is known that a dry summer and autumn can prevent the establishment of volunteer wheat plants after harvest, which reduces the survival of *P. triticina* inoculum from the previous crop (Chester 1946). Second, rainfall promotes inoculum dispersal and WLR infection of volunteer plants and, later, newly sown wheat plants. Most rainfall events result in spore dispersal (Sache 2000), and wet deposition can bring spores at a time when the conditions are ideal for infection (Barnes *et al.* 2008; Li *et al.* 2009). There was also a significant and positive correlation with mean minimum temperatures measured from the beginning of January until the end of March. In Belgium, January and February are the coldest months (IRM, Belgium). Prolonged sub-freezing temperatures can affect the survival of inoculum present in field soils, autumn-planted wheat or volunteer plants (Eversmeyer & Kramer 1994). Spore density between GS30 to GS39 could also be influenced by urediniospores carried upwind by air masses from distant infected fields. In Europe, there is an air current that carries WLR urediniospores from Morocco, where they survive throughout the year, to Scandinavia (Nagarajan & Singh 1990).

The severity of epidemics that occurred during the previous growing season is also thought to influence the green bridge phenomenon by promoting the infection of volunteer plants and, later, newly sown wheat. No correlation was found, however, between spore density from GS30 to GS39 and the disease pressure measured in the previous year. When we combined rainfall from 1 August to 31 December and mean minimum temperatures from 1 January to 31 March in a multiple regression model, this explained 73% of the spore density from GS30 to GS39. The fivefold

cross validation showed that the model was robust, although it is worth keeping all the information to compute the equation. The model confirmed that, in Belgium, WLR survives from year to year because of a green bridge. This accords with results reported by Eversmeyer (1998), which showed that the winter survival of inoculum on volunteer wheat plants or autumn-planted crops depends largely on the presence of sufficient moisture for infection and the absence of sudden drops to sub-freezing temperatures.

An airborne inoculum survey conducted when the upper leaves are emerging, or an evaluation of spore concentration at this time with a weather-based model, could be used to complete the disease severity model for predicting time of infection. This parallel information could be useful in avoiding over-estimations of disease risk. In Belgium, for example, exceeding a given threshold of spore density from GS30 to GS39 should be the first factor to consider in a disease prediction model such as the one described by El Jarroudi (2014a) in Luxembourg. The results of this study also highlighted the need to manage green material surviving as volunteer plants before the next crop is sown in order to reduce the impact of WLR during the following cropping season.

Chapter 5

Spore trapping: a tool to improve the prediction of Puccinia triticina infection

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Comments

Few modifications have still to be made before the submission in Plant Pathology. The determination of the best load of spore needed for the infection and the threshold to consider detections will be looked after using a cross validation methodology.

Abstract

Wheat leaf rust (WLR), caused by *Puccinia triticina*, is one of the most important wheat diseases causing important losses worldwide. In the Grand Duchy of Luxembourg, a model for forecasting WLR infection using night-time climatic conditions has recently been developed. In the study described here, this model was assessed in Belgium and upgraded with data collected over five growing seasons from a network of trial fields and spore traps. The original model correctly predicts disease infection on the three uppermost leaves of the canopy in certain cases, but there were large underestimates and overestimates for 2011 and 2013, respectively. In our previous study, it was shown that the presence of spores could be a limiting factor of severe epidemics. For this reason, the data on airborne inoculum obtained using spore traps and real-time PCR were integrated into the forecasting model. The use of spore detections to simulate primary infection on each of the three upper leaf layers increases the overall mean of the probability of infection ratio from 0.73 to 0.86. It also reduces the overall mean of the false alarm ratio from 0.19 to 0.13. In 2013, consideration of airborne inoculum led to a correct prediction of no risk of disease. This provides a clear example of how airborne spore monitoring can have economic and environmental benefits. The combination of molecular diagnostics with the quantification of airborne inoculum could be exploited in order to predict the risk of epidemics in wheat agro-ecosystems more accurately.

5.1. Introduction

Wheat (*Triticum aestivum L.*) is the third most important cereal crop globally, with more than 600 million tons harvested annually (Shewry, 2009). In 2013, the total world harvest was about 710 million tons, compared with 1,015 million tons of maize and 745 million tons of rice (http:// faostat.fao.org/).

Stripe, stem and leaf rusts, caused by *Puccinia striiformis*, *P. graminis* and *P. triticina*, respectively, are important diseases of wheat (Marasas *et al.*, 2004). Wheat leaf rust (WLR), the most common of these rust diseases, is widely distributed and causes yield losses in wheat production worldwide (Saari & Prescott 1985; Roelfs 1989; Roelfs *et al.* 1992; Kolmer 2005; Bancal *et al.* 2007). In Belgium, the disease occurs almost every year, but the onset and severity of the epidemics vary considerably from year to year (Duvivier *et al.* 2015, chapter 4).

Since the alternate hosts (*Thalictrum speciosissimum* or *Isopyrum fumaroides*) are almost absent from Belgium, survival depends on the biotrophic causal agent of WLR, *P. triticina,* which is ensured only by infection of wheat plants and dissemination (long-distance dispersion and dispersion in fields) by the urediniospores. This pathogen can survive between two successive crops by infecting volunteer wheat plants that serve as a bridge in the contamination of the following wheat crop (Eversmeyer *et al.* 1988a). Evidence of this phenomenon, known as a 'green bridge', has been reported in various areas worldwide (Casulli 1988; Eversmeyer & Kramer 1998; Beard 2005) and more recently in Belgium (Duvivier *et al.* 2015, chapter 4). Primary infection in fields could also be the result of the infection of plants by airborne inoculum provided by a distant source (long-distance transport) (Nagarajan & Singh 1990; Roelfs *et al.* 1992; Kolmer 2005).

When urediniospores are in contact with the leaves of a susceptible cultivar, infection success needs a minimal duration of wetness, which varies as a function of temperature (Huber & Gillespie 1992). De Vallavieille-Pope *et al.* (1995) showed that optimum temperatures for urediniospore germination ranged from 12 to 15°C and that the germination process ceased above 35°C. The presence of free water on the leaf surface is also essential for urediniospore germination. In an earlier study, Eversmeyer *et al.* (1988b) proposed an optimum temperature of 16°C for the completion of the infection process by urediniospores. Infection needs a dew period of at least 3-4 hours. In the same study, it was shown that the latent period was about 8-20 days for air temperatures ranging from 10 to 20°C; this can be approximated with a linear relationship

using the daily mean air temperature. It has also been shown that the germination of *P. triticina* urediniospores could be delayed or inhibited by light intensity (Chang 1973; Eversmeyer *et al.* 1988b). This is why infections tend to occur at night. The genetic resistance of wheat cultivars is also one of the most important factors in determining the impact of the disease (Moschini & Pérez 1999; Marasas *et al.* 2004). Urediniospores are deposited by wind or rain on both sides of wheat leaves. Turbulence on the leaf surface enables the urediniospores to disperse (Roelfs *et al.* 1992; Eversmeyer & Kramer, 2000; Bolton *et al.*, 2008). Wet deposition (spores carried and deposited by rain) is also considered to be an important cause of crop contamination by rusts (Barnes *et al.* 2009; Li *et al.* 2009). Although most rainfall events promote spore dispersal produced in the field, heavy rain can also induce the leaching of spores deposited on leaves and can completely deplete the pustules of the urediniospores (Sache 2000).

Against this background, an empirical approach for simulating WLR infection on susceptible cultivars and its progress on the three uppermost leaf layers has been proposed and validated for the Grand Duchy of Luxembourg (El Jarroudi *et al.* 2014a). This model based on statistical correlations uses only night-time weather data (from 8 pm to 5 am), on the assumption that spore germination is inhibited by light. Each infection event requires a period of at least 12 consecutive hours on at least two nights, with air temperatures ranging between 8 and 16°C and a relative humidity higher than 60 %. The hourly rainfall during these 12 hours should be less than 1 mm to prevent the spores from being leached from the leaves. Primary infection in a field also requires light rain (0.1-1.0 mm) in the first hour of an infection event, on the assumption that this rainfall facilitates the first deposition of the inoculum in the field. This model has led to a decision-support system that enables farmers to optimize the timing of fungicide spraying for controlling the development of WLR in fields in the Grand Duchy of Luxembourg (El Jarroudi *et al.* 2014b).

In this model, airborne inoculum is considered to be present continuously. A recent study on the spatio-temporal distribution of airborne inoculum in fields, however, revealed that spore densities vary depending on the season and the field (Duvivier *et al.* 2015, chapter 4). This parameter could therefore be a limiting factor in the infection of newly emerged leaves. In our 5-year study (2009-13), airborne inoculum was detected every year in the fields from March to May, but there were important between-site and between-year variations in the spore distribution patterns. The severity of the WLR epidemics during grain development was strongly influenced by spore density in the fields before the first symptoms appeared on the three uppermost leaves. The most severe epidemics occurred when uredinia

and/or latent infections had not been completely destroyed by a harsh winter or if airborne inoculum from distant sources infected the crop before the heading stage (Roelfs *et al.* 1992).

The presence of spores in the canopy when the upper leaves are developing is not easy to predict because it depends of numerous factors, such as spore deposition from distant infected fields, uredinia production, the depletion of urediniospores in the field and turbulence in the canopy, which are all difficult to quantify with simple tools (Legg & Wall 1983; McCartney 1994; Aylor 1999; Sache 2000; Aylor & Flesch 2001). Indications of the presence of wheat pathogen spores such as *Mycosphaerella graminicola, P. striiformis* and *P. triticina* in the upper part of the canopy can now be easily obtained with spore traps combined with molecular detection methods such as quantitative PCR (Dedeurwaerder *et al.* 2011, Duvivier *et al.* 2013, Duviver, chapitre IV). It has been demonstrated that the onset of epidemics of various diseases, including *Botrytis squamosa* on onion (Carisse *et al.* 2005, 2008), phoma stem canker (*Leptosphaeria maculans*) on oilseed rape (Kaczmarek *et al.* 2011; West *et al.* 2002) and soybean rust (*Phakopsora pachyrhizi*) (Isard *et al.* 2011), can be better forecasted using spore trapping.

The quantification of airborne inoculum in the field or the interpolation of spore concentration in the early lives of upper leaves layers could be useful in optimizing a model for predicting *P. triticina* infection. The aim of our study was twofold: to assess the ability of the empirical model developed in the Grand Duchy of Luxembourg to simulate WLR infection using data collected in Belgium and to evaluate the usefulness of the data on airborne inoculum in predicting the disease. Field trials were performed in the 2009-2013 growing seasons in order to quantify WLR progress, meteorological conditions and airborne inoculum. All these data were integrated into the model in order to test its validity and improve its efficiency.

5.2. Materials and methods

5.2.1. Field trials

Field trials were established for five growing seasons from 2009 to 2013 in four to five sites each year (Figure 4.1, chapter 4, page 118). For each site, trials were established in different fields for agronomic reasons. These sites provided a good representation of Walloon's wheat-growing region. The winter wheat variety Lion was chosen for its high susceptibility to WLR. In each field, four untreated plots (1.5 × 10-13 m) were delimited in an experimental plan devised to test the efficacy of fungicide treatments. The winter wheat trials were established in fields cropped by the farmers themselves, using good agricultural practices common in the region. In the trial area, farmers applied all the treatments apart from the fungicide ones. Data on the cultural practices and phenology of plants in each field are given in Table 4.1 (Chapter 4, page 121).

5.2.2. Crop growth monitoring

The leaves were numbered relative to the flag leaf (L1). L2 was the leaf immediately below L1, and below L2 was L3, and so on. From April to May, the development of the leaves of 20 marked plants in each plot was monitored once a week at each site. These datasets enabled the phyllotherm of the cultivar Lion to be estimated using the least squares method and a base temperature equal to 0. The phyllotherm was defined here as the number of accumulated degree days above a base temperature of 0°C (**DD**) (Gallagher, 1979; Baker, 1980) counted from beginning of emergence to the full development of a leaf. This adapted phyllotherm was then used to model the foliar development of the cultivar as described by Moreau and Maraite (2000). Plant growth stages (GS) were also assessed according to a decimal scale (Zadoks *et al.* 1974).

5.2.3. Disease monitoring

In all the fields, the modified Cobb scale was used to measure the percentage of diseased leaf tissue (Peterson 1948). Disease severity (% of total leaf area covered by WLR symptoms) in untreated plants was assessed from emergence to senescence by observing symptoms from L3 to L1. Each week from the end of April to mid-July, 15 plants were randomly picked from each of the four untreated plots and were examined. It is worth noting

that over the study period, other fungal diseases were also observed (i.e., *Septoria* leaf blotch and wheat powdery mildew).

5.2.4. Measurement of airborne inoculum

Airborne inoculum was collected using Burkard 7-day spore-recording traps (Burkard Manufacturing Co. Ltd, Rickmansworth, Hertfordshire, UK) in all the trial fields between 1 March and 7 July. The position of the spore traps, the treatment of the tapes and DNA extraction were as described by Duvivier *et al.* (2013) (Chapter 2, page 39). The quantification of *P. triticina* was done by real-time PCR using the method described in the chapter 4 (page 116). The results were presented as numbers of spores trapped per day (spores/day).

5.2.5. Weather data

Hourly meteorological data were measured by iMetos weather stations (Pessl Instruments, Austria) set up in the trial fields. Fields without a station were at a maximum distance of 10 km from a weather station. Temperature, rainfall quantity (RQ) and relative humidity (RH) were recorded at a height of 2 m above the ground surface. Data from the national network of meteorological stations, Pameseb (Asbl Pameseb, Libramont) and the Institut Royal Météorologique de Belgique (IRM, Brussels, Belgium) were used to supply missing data.

5.2.6. Determination of the periods of infection

In line with the study by El Jarroudi *et al.* (2014a), only data from 8 pm to 5 am were considered in the determination of infection events. A period of at least 12 consecutive hours, with air temperatures ranging from 8 to 16°C (optimum values 12-16°C) and an RH greater than 60 %, is needed. Hourly rainfall during this 12-hour period should not exceed 1 mm. Primary infection (the first infection event in the fields) needs a supplementary condition. There should be light rain (0.1-1mm) in the first hour of the 12-hour period in order to facilitate the wet deposition of WLR spores ('rain condition'). This light rain is not compulsory once primary infection has occurred.

In order to improve the prediction capacity of the model, data on airborne inoculum were used, given that the presence of inoculum is necessary to initiate infection on each leaf. Each infection event predicted is considered after spore deposition on a given leaf layer. For each leaf layer, when the

leaf is at least 50% emerged, 100 spores/day (or lower quantities of airborne inoculum in the field detected at least twice) must be recorded before primary infection of the leaf occur ('inoculum condition'). These conditions were chosen in accordance with the detection threshold of the method of quantification. Once this condition had been met, the infection events were determined as described below.

5.2.7. Assessment of the models

After performing the arcsine square root transformations recommended for proportional data, the mean disease severity on the two uppermost leaf layers measured during grain development or maturation (between GS75 and GS85) were used to indicate **the disease level** of a site. For the five growing seasons, the disease level of each site was compared using ANOVA, followed by a Student-Newman-Keuls test ($\alpha=0.05$) in order to show the diversity of disease pressure in the fields used in this study. Similar analyses were used to compare the global mean disease level of the 5 growing seasons.

The efficiency of the model under the conditions described for the original model by El Jarroudi *et al.* (2014a), as well as under these conditions combined with the detection of airborne inoculum (**model+**), was assessed. The ability of the model to predict the occurrence of new symptoms on the three upper leaf layers was tested using a contingency scores method described by El Jarroudi et al (2014a). The assessment was conducted using data collected in all the fields from the beginning of L3 emergence (GS31) to dough development (GS85) in the 2009-2013 growing seasons. This method was used to see if the infection events predicted by the model resulted in a significant increase of symptoms on a leaf layer after the latency period. The period of latency was determined using the equation devised by Eversmeyer *et al.* (1988a):

$P=1/(0.00741*T)$, where P refers to the latent period and T is the mean air temperature (°C).

The validation method used contingency scores for the probability of detection (POD), false alarm ratio (FAR) and critical success index (CSI) of WLR infection. These statistical scores were calculated as follows (Wilks 1995):

$$POD = a/(a+c)$$

$$FAR = b/(a+b)$$

$$CSI = a/(a+b+c)$$

$$BIAS = (a+b)/(a+c)$$

where:

a = observed and predicted infections

b = predicted infections, but not observed

c = observed infections, but not predicted

The values for the three scores (POD, FAR and CSI) can range from 0 to 1. A perfect score for the POD and CSI is 1. The BIAS is the ratio between the simulated infection events and observed infection events in a given field. It is a measure of over- or under-prediction. The best score for BIAS is also 1, while 0 is the best score for FAR.

An infection forecasted by the model was assumed if, after the latency period, WLR appeared for the first time on a leaf layer or if there was a significant increase in disease severity between two observations (comparison t-test with a threshold of $\alpha=0.05$). Given the likely variability between the replicates, an infection was also assumed if disease severity increased by more than 1% between two observations when the severity level was low (0-10% of severity). When the disease severity level exceeded 10%, an increase higher than 10% meant infection could be assumed. It is important to note that between two field observations, two or three infection events could be detected. The statistical scores allowed the original model and the model+ to be compared. When no WLR symptoms were observed on the upper leaves in the fields, the numbers of infection events calculated using both models were compared.

Statistical analyses were performed using R Software R 2.15.0 (www.r-project.org). Graphics were created using OriginPro 8 (OriginLab, Northampton, UK).

5.3. Results

5.3.1. Plant and leaf development

Using the square root method, the phyllotherm that best fitted the development of each of the three upper leaves observed in fields was equal to a sum of temperature 109.3 degree days (base 0). This allowed for leaf development to be assessed and compared, with the aim of reaching a same given stage in all the fields (Table 5.1). The between-site variation in the emergences of the three upper leaves never exceeded 5 days, although between-year variation sometimes exceeded 20 days. Plant development observed in 2009, 2010 and 2012 was similar, with flag leaf emergence occurring between 14 and 25 May. The 2011 growing season was characterized by the early emergence of the three upper leaves. In contrast, the long, cold winter of 2012-2013 stunted crop development considerably, with the flag leaf appearing only at the end of May in 2013.

Table 5. 1.Comparison of the dates of half-emergence (50% deployed) for the three last leaf layers in all the experimental fields.

Year	Sites	L3	L2	L1
2009	Perwez	04/05	13/05	21/05
	Voroux-Goreux	03/05	12/05	20/05
	Tournai	28/04	08/05	17/05
	Niverlée	04/05	13/05	21/05
2010	Perwez	28/04	10/05	22/05
	Voroux-Goreux	29/04	12/05	22/05
	Tournai	29/04	14/05	23/05
	Niverlée	05/05	18/05	25/05
2011	Perwez	23/04	30/04	08/05
	Voroux-Goreux	21/04	29/04	07/05
	Tournai	21/04	29/04	07/05
	Niverlée	21/04	29/04	08/05
	Gembloux	22/04	30/04	08/05
2012	Perwez	29/04	08/05	18/05
	Voroux-Goreux	27/04	06/05	14/05
	Tournai	26/04	06/05	16/05
	Niverlée	01/05	10/05	20/05
	Gembloux	26/04	05/05	14/05
2013	Perwez	09/05	18/05	30/05
	Voroux-Goreux	08/05	18/05	30/05
	Tournai	05/05	15/05	28/05
	Niverlée	08/05	18/05	31/05

5.3.2. Detection of wheat leaf rust and disease pressure

The date of first detection of WLR in the fields varied greatly between years (table 4.3, chapter 4, page 124). Between-site variations were lower. WLR detection often occurred after the flag leaf had emerged, apart from in 2012 and in Niverlée in 2011, where the pathogen was detected earlier on the lower leaves. When the pathogen was not detected early in the season, it generally appeared simultaneously in several upper leaf layers.

During grain development and maturation in 2009, 2010 and 2012, the mean severity on the two uppermost leaf layers was less than 10%, except in Voroux-Goreux in 2012 (Figure 4.2, chapter 4, page 125). In 2011, severe epidemics occurred in all the fields, with the highest disease severity observed in Niverlée and Gembloux. No symptoms were observed in 2013, apart from at Voroux-Goreux where the late development of the disease was observed. The experimental layout, including cultivating the same susceptible cultivar for 5 successive years at four or five sites each year, enabled us to show that, even in a small area such as Wallonia, there were significant between-site variations in WLR each year.

5.3.3. Evaluation of the original model in Belgium

The model devised by El Jarroudi *et al*. (2014 a) was assessed for use in Belgium in 21 sample situations. The trial field at Tournai in 2010 was excluded because of invalidity of the meteorological data on RH for that site.

For 13 out of 18 fields where the POD could be calculated, this score was higher than 0.8, indicating a satisfactory adjustment between simulations and field observations. It should be noted that statistical scores could not be calculated in fields where no WLR symptoms were observed. In the five other fields, the rain conditions needed to simulate primary infection were not reached or were reached too late, resulting in a low POD value. For example, in 2011, the POD calculated was between 0 and 0.35 in three fields, whereas disease pressure that season was the highest of the study. For all the cropping seasons, the number of infection events forecasted but not expressed was fairly high, especially for the 2013 growing season, where the model simulated many infection events, but no disease was observed in three out of the four fields studied (Table 5.2).

The model gave an overall mean of 0.73 for the POD. The FAR ranged from 0.00 to 0.46, depending on the year, with an overall mean of 0.19. The overall mean for CSI was 0.63, with a BIAS slightly lower than 1, but with large variations (from 0 to 1.83).

5.3.4. Improvement in spore detections (model+)

The inoculum detections recorded from March to July in the same fields were used : infection events were considered only if detections had occurred previously, once a given leaf layer had emerged. This model using spore detection was then assessed and the scores were compared with those obtained with the original model. The statistical POD, FAR, CSI and BIAS scores and the number of infection events on the three upper leaves were calculated using the data presented in Table 5.3.

The use of spore detection to simulate primary infection on each of the three upper leaf layers increased the overall POD mean from 0.73 to 0.86. For five fields, the POD value increased, but at two it fell slightly. With the model+, the number of detections forecasted, but not observed, decreased considerably in most of the fields tested. The FAR was between 0.00 and 0.36, depending on the year, with an overall mean of only 0.13, whereas in the original model the FAR was between 0.00 and 0.46 with a mean of 0.19. The use of inoculum detection in the model reduced the total number of infection events simulated on the three upper leaf layers at almost every site where disease pressure was low. Because of the inoculum detection condition, no disease was predicted at any site in 2013 where no symptoms occurred subsequently. In 2012, the number of infection events was similar using both models, but in 2011 where the disease pressure was high the number of infection events increased when inoculum detection was taken into account.

The CSI also improved in 11 out of the 18 fields where this score could be compared. The BIAS was also closer to 1.00 overall.

The improvement in the model’s accuracy by using airborne inoculum detection was illustrated by three situations observed in Perwez in 2009, 2011 and 2013 (Figure 5.1). In 2009 (Figure 5.1A), using the original rain condition to simulate primary infection, the disease was predicted on L3 almost 1 month before the appearance of symptoms in the field. When the model was used after the detection of airborne inoculum, the predictions corresponded to the period when the disease was observed for the first time in the field.

In 2011 (Figure 5B), the original model did not predict an infection event at any time during the growing season. In contrast, the inoculum detection condition enabled a primary infection event to be predicted first for L3 and then for L2. The observed disease progress on the three leaf layers corresponded closely with the predicted infection. A delay was also

observed between the detection of first symptoms on L3 and their detection on L2.

In 2013 (Figure 5 C), the condition needed for the original model was reached early in the season, but no inoculum was detected after the emergence of the upper leaf layers. On this basis, 35 infection events were forecast for this field, but the disease was never observed.

Table 5. 2. Comparison between the forecasted values of WLR infections and the observed data using the original model (El jarroudi *et al.*, 2014) and the model using airborne inoculum data. Results given by the model using the inoculum detections condition (model+) are underlined in grey. The disease development was measured on leaves L3 to L1 at 4 sites in the Walloon area during the cropping seasons 2009-2013. For a safe of clarity, data for Gembloux are not shown. (NA=No Available).

Year	Leaf	Perwez						Voroux-Goreux						Tournai						Niverlée					
		Forecasted and expressed		Forecasted but not expressed		Expressed but not forecasted		Forecasted and expressed		Forecasted but not expressed		Expressed but not forecasted		Forecasted and expressed		Forecasted but not expressed		Expressed but not forecasted		Forecasted and expressed		Forecasted but not expressed		Expressed but not forecasted	
2009	L1	4	4	1	1	0	0	1	1	2	2	2	2	7	4	0	0	0	2	1	1	2	2	1	1
	L2	2	2	2	1	0	0	3	3	2	0	2	2	6	4	1	0	2	2	3	3	1	0	0	0
	L3	5	5	2	0	1	0	3	3	2	0	1	1	4	2	2	0	0	1	2	2	1	0	0	0
	Total	**11**	**11**	**5**	**2**	**1**	**0**	**7**	**7**	**6**	**2**	**5**	**5**	**17**	**10**	**3**	**0**	**2**	**5**	**6**	**6**	**4**	**2**	**1**	**1**
2010	L1	1	4	0	0	2	1	4	4	4	0	1	1	NA	NA	NA	NA	NA	NA	5	5	0	0	2	2
	L2	1	4	0	0	1	0	4	4	4	0	1	1	NA	NA	NA	NA	NA	NA	5	5	0	0	0	0
	L3	0	2	0	0	1	0	6	6	4	4	1	1	NA	NA	NA	NA	NA	NA	8	8	0	0	0	0
	Total	**2**	**10**	**0**	**0**	**4**	**1**	**14**	**14**	**12**	**4**	**3**	**3**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**18**	**18**	**0**	**0**	**2**	**2**
2011	L1	0	7	0	2	5	1	10	10	0	0	0	0	5	7	0	0	1	0	0	3	0	2	6	4
	L2	0	7	0	0	5	1	10	10	1	0	1	1	5	6	0	2	1	0	0	5	0	0	5	2
	L3	0	2	0	3	4	1	6	6	2	0	1	1	3	5	0	2	1	0	0	4	0	0	3	1
	Total	**0**	**16**	**0**	**5**	**14**	**3**	**26**	**26**	**3**	**0**	**2**	**2**	**13**	**18**	**0**	**4**	**3**	**0**	**0**	**12**	**0**	**2**	**14**	**7**
2012	L1	9	9	1	0	0	0	7	6	0	0	0	1	10	10	0	0	0	0	3	3	4	4	2	2
	L2	9	9	0	2	0	0	6	5	1	0	2	2	10	10	0	0	0	0	7	7	0	0	1	1
	L3	9	9	0	6	0	0	4	2	2	0	0	1	10	10	2	2	0	0	7	7	0	0	1	1
	Total	**27**	**27**	**1**	**8**	**0**	**0**	**17**	**13**	**3**	**0**	**2**	**4**	**30**	**30**	**2**	**2**	**0**	**0**	**17**	**17**	**4**	**4**	**4**	**4**
2013	L1	0	0	9	0	0	0	2	5	0	0	0	0	0	0	8	0	0	0	0	0	8	0	0	0
	L2	0	0	11	0	0	0	2	5	0	0	0	0	0	0	8	0	0	0	0	0	13	0	0	0
	L3	0	0	15	0	0	0	2	5	0	0	0	0	0	0	8	0	0	0	0	0	15	0	0	0
	Total	**0**	**0**	**35**	**0**	**0**	**0**	**6**	**15**	**0**	**0**	**0**	**0**	**0**	**0**	**24**	**0**	**0**	**0**	**0**	**0**	**36**	**0**	**0**	**0**

Table 5. 3. Statistical scores of the original model using the "rain condition" to simulate the primary infection compared with scores obtained with the model+ using the inoculum detections conditions (underlined in grey).

Year	Site	POD		FAR		CSI		BIAS		Infection events (L1+L2+L3)	
2009	Perwez	0.92	1.00	0.31	0.15	0.65	0.85	1.33	1.18	16	13
	Voroux-Goreux	0.58	0.58	0.46	0.22	0.39	0.50	1.08	0.75	13	9
	Tournai	0.89	0.67	0.15	0.00	0.77	0.67	1.05	0.67	20	10
	Niverlée	0.86	0.86	0.40	0.25	0.55	0.67	1.43	1.14	10	8
	Mean	**0.81**	**0.78**	**0.33**	**0.16**	**0.59**	**0.67**	**1.22**	**0.94**	**15**	**10**
2010	Perwez	0.33	0.91	0.00	0.00	0.33	0.91	0.33	0.91	2	10
	Voroux-Goreux	0.82	0.82	0.46	0.22	0.48	0.67	1.53	1.06	26	18
	Tournai	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Niverlée	0.90	0.90	0.00	0.00	0.90	0.90	0.90	0.90	18	18
	Mean	**0.69**	**0.88**	**0.15**	**0.07**	**0.57**	**0.83**	**0.92**	**0.96**	**15**	**15**
2011	Perwez	0.00	0.84	NA	0.18	0.00	0.67	0.00	1.11	0	21
	Voroux-Goreux	0.93	0.93	0.10	0.00	0.84	0.93	1.04	0.93	29	26
	Tournai	0.81	1.00	0.00	0.18	0.81	0.82	0.81	1.22	13	22
	Niverlée	0.00	0.63	NA	0.14	0.00	0.57	0.00	0.74	0	14
	Gembloux	0.30	0.77	0.00	0.00	0.30	0.77	0.30	0.77	3	10
	Mean	**0.41**	**0.83**	**0.03**	**0.10**	**0.39**	**0.75**	**0.43**	**0.95**	**9**	**19**
2012	Perwez	1.00	1.00	0.04	0.23	0.96	0.77	1.04	1.30	28	35
	Voroux-Goreux	0.89	0.76	0.15	0.00	0.77	0.76	1.00	1.00	20	13
	Tournai	1.00	1.00	0.06	0.06	0.94	0.94	1.07	1.07	32	32
	Niverlée	0.81	0.81	0.40	0.36	0.68	0.68	1.00	1.00	21	21
	Gembloux	1.00	1.00	0.45	0.36	0.55	0.64	1.83	1.57	11	11
	Mean	**0.94**	**0.91**	**0.22**	**0.20**	**0.78**	**0.76**	**1.19**	**1.19**	**22**	**22**
2013	Perwez	**No disease detected**								35	0
	Voroux-Goreux	1.00	1.00	0.00	0.00	1.00	1.00	1.00	1.00	6	15
	Tournai	**No disease detected**								24	0
	Niverlée	**No disease detected**								36	0
	Mean	**1.00**	**1.00**	**0.00**	**0.00**	**1.00**	**1.00**	**1.00**	**1.00**	**25**	**4**
Global mean		**0.73**	**0.86**	**0.19**	**0.13**	**0.61**	**0.76**	**0.93**	**1.02**	**17**	**15**

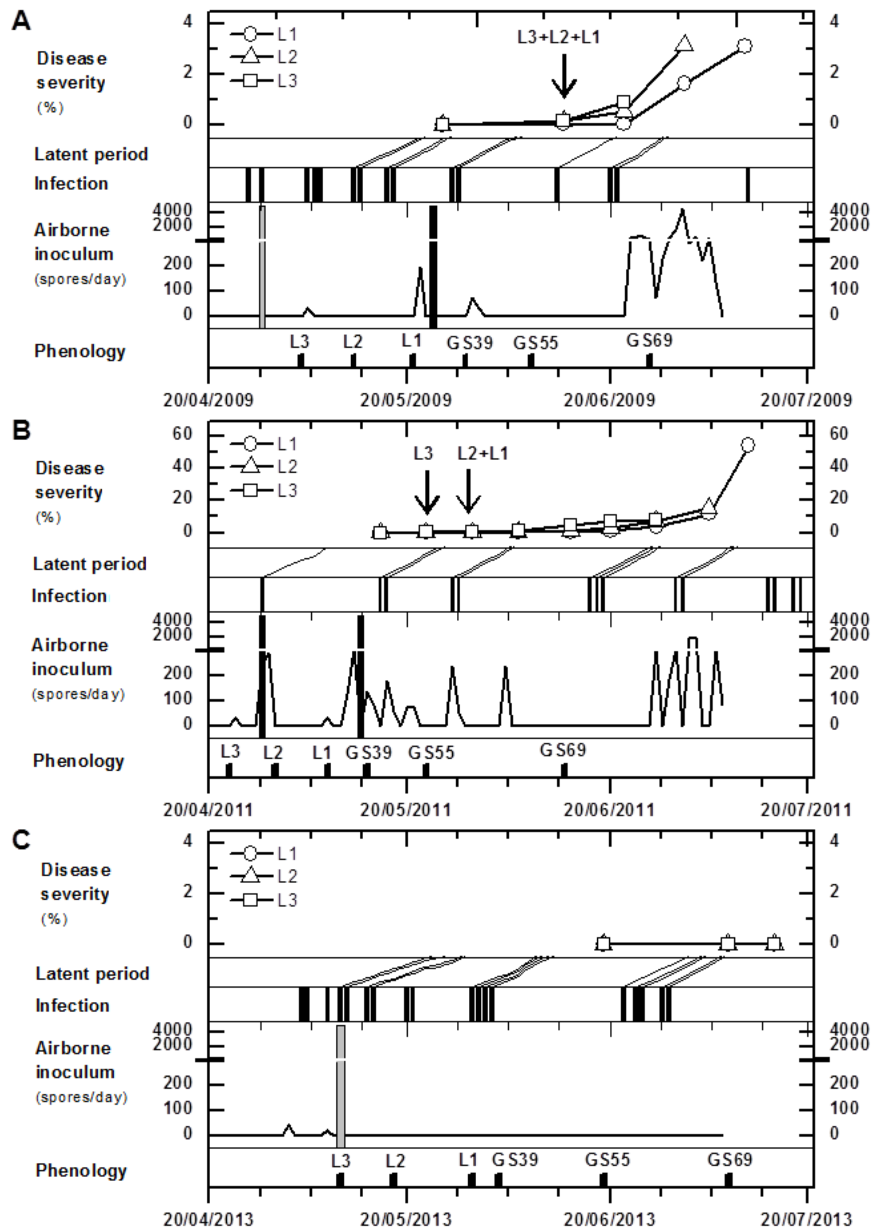

Figure 5. 1. Wheat leaf rust severity on the three upper leaves ; WLR infection and latent periods determined on the basis of favorable night weather conditions at Perwez in 2009 (A), 2011 (B) and 2013 (C). The arrows show the time of the first disease observation in the field.Phenology of the plants including the apparition of the 3 top leaf layers is represented in the bottom of each figure. The airborne inoculum trapped in the field allows determining when the "inoculum condition" was reached (black bars). The grey bars symbolize the moment when the "rain conditions" of the original model were reached.

5.4. Discussion

In Belgium, WLR occurs almost every year, although the time the first WLR symptoms appear and the severity of the disease vary greatly from year to year. For this reason, fungicide protection programs can be difficult to devise. Predicting the appearance of the first symptoms and the severity of epidemics enables appropriate control measures to be optimized, bringing economic and environmental benefits.

Epidemics of WLR have been successfully predicted using mechanistic and empirical approaches (Eversmeyer & Burleigh 1970; Benizri & Projetti 1992; Burleigh *et al.* 1972a; Moschini & Pérez 1999). In the Grand Duchy of Luxembourg, the correlations between disease progress on the three upper leaf layers and the day- or night-time weather data have been studied (El Jarroudi *et al.* 2014a). This works has clearly shown that predicting WLR based on night-time weather conditions greatly improves model performance. The best correlation was obtained using period of at least 12 consecutive hours (two nights involved) under the following conditions: air temperatures between 8 and 16°C, RH greater than 60 %, and rainfall lower than 1 mm. This approach was used in a model to predict infection events. A light rainfall event (≤ 1 mm) was considered necessary for laying down spores on leaves and initiating infection. The model was validated and is now currently used in the Grand Duchy of Luxembourg through a decision-support system for the application of fungicides against WLR (El Jarroudi *et al.* 2014b). This model was tested with data collected during a 5-year study in Wallonia that involved the highly susceptible cultivar Lion. The statistical scores obtained through the validation study were not always satisfactory, due mainly to an inaccurate condition for using the model, the 'rain condition'. This often led to an overestimate of the number of possible infection events. Detection probability was high in only 60% of the fields. Two main problems were identified. When disease pressure was low (as in 2009 and 2013), the model was used too early, leading to a significant FAR about the conditions in Wallonia. In 2013, many infection events were predicted by the model in the Grand Duchy of Luxembourg, but no disease was observed in the experimental fields (El Jarroudi 2014, pers. comm.). In contrast, in 2011 the original model did not predict any infection events, but disease pressure that year was very high. This led to a dramatic decrease in the probability of detection.

Various studies have reported that wet deposition is an effective way of removing rust spores from the air and depositing them on a crop (Barnes and Szabo 2007; Barnes *et al.* 2008; Li *et al.* 2009; Sache 2000). In recent research in fields in Belgium, airborne inoculum of WLR was not detected

every day during the emergence of the three upper leaf layers (Duvivier *et al.,* 2015, chapter 4). This indicated that inoculum could be a limiting factor in the contamination of upper leaves, even when conditions favorable to wet deposition and infection by spores prevail. Seasonal differences in weather patterns can affect the timing of pathogen spore release. Many forecasting strategies are based on the occurrence of weather parameters that favor the production and release of pathogen inoculum or the infection of the host, assuming inoculum is present. The use of spore samplers that measure the amounts of airborne pathogen inoculum directly could provide more accurate forecasts of the risks of severe epidemics. Such forecasts could be helpful in predicting changes in the timing and severity of epidemics and guide farmers in decisions about treatments (West *et al.* 2008, 2009). Against this background, we proposed a model for predicting WLR infection based on the detection of inoculum using spore traps. No previous study has included airborne inoculum data in a WLR prediction model. The specific method developed in our earlier study for detecting and quantifying *P. triticina* airborne inoculum using real-time PCR was aimed at detecting the presence of spores when the upper leaves had emerged. Using airborne inoculum data to predict the first infection on leaf layers would limit false alerts in most of the studied situations. In 2011, we were also able to use the model without the constraint of the original model where there is a requirement for light rain to follow the period of infection. A similar case of underestimation was reported in 2007, when *P. triticina* caused severe epidemics in wheat crops throughout northern Europe, including the UK, but the severity of these epidemics had not been predicted by the available model (West *et al.* 2009). During the 2013 growing season, no spores were detected after the emergence of the upper leaves in most of the fields studied. As a result, the model using the 'inoculum condition' did not take into account the infection events simulated in these fields. The environmental conditions prevalent during this growing season offered a clear example of how airborne spore monitoring can have economic and environmental benefits.

The use of the model combined with spore detection gave good predictions of the infection of susceptible cultivars. Disease occurrence was accurately predicted for most of the situations, with POD and BIAS close to 1, and FAR close to 0. Nevertheless, the overall mean POD and CSI were lower than the scores provided by the original model for the 2004-2006 growing seasons in the Grand Duchy of Luxembourg. This could be explained by the use of Lion, a more susceptible cultivar, in our study. The use of this cultivar resulted in the number of unforecasted infection events increasing in most of the

fields, thus explaining the lower overall mean POD and CSI values than those reported by El Jarroudi *et al.* (2014a).

The combination of molecular diagnostics with airborne inoculum sampling could be exploited to predict the risk of epidemics in wheat agro-ecosystems more accurately. As reported by Lacey and West (2007), however, a potential limitation to using spore traps combined with molecular analysis for disease forecasting is that it is impossible to discriminate living spores from dead ones. The ability of rust spores, including WLR, to survive after long-distance transport by air, however, has been widely reported (Brown & Hovmøller 2002; Nagarajan & Singh 1990). High quantities of airborne inoculum detected during the growing seasons are likely to contain a significant amount of viable spores.

The density of *P. triticina* spores could therefore be very useful in the prediction of WLR. In terms of cost, it is worth noting that the price of the equipment (spore traps) is falling rapidly. The main constraint in using molecular techniques remains the time needed to obtain results. When Burkard 7-days spore-recording traps are used, it can take about 10 days before information related to the first day of trapping (autonomy of the traps, cutting band operation, DNA extraction and real-time PCR analysis) is available. The improvement and development of tools for this type of monitoring should aim at accelerating this process. In Belgium, the first symptoms of brown rust in fields in different regions are generally observed in the same time, with the most significant variations observed between successive growing seasons (Table 3). This suggests that a reliable WLR prediction model might not need more than a couple of well-placed spore traps. The prediction of spore density offers another way of increasing the efficiency of such a model. Multiple regression analysis revealed that rain during late summer and autumn and mean minimum temperature during winter could explain more than 70% of the variations in spore density between GS30 and GS39 (Duvivier *et al.* 2015, chapter 4). Weather conditions favoring the growth of volunteer plants, the infection and the survival of WLR promote the ‘green bridge’ phenomenon, which results in high spore density when the upper leaves of wheat are emerging. This inoculum predictive model could be used in a decision-support system in addition to infection event calculations. In Belgium, WLR management is achieved mainly through the use of fungicides and resistant cultivars. According to IPM guidelines, pesticide applications should be linked to tools for monitoring and to thresholds that guarantee optimal application timing (Directive 2009/128/EC 2009). If spore density exceeds a threshold during the critical period (GS30-GS39), and an infection event occurs before GS39, this would indicate the need for early fungicide treatment. In contrast,

weak spore density would indicate that fungicide treatment against this disease could be postponed or even cancelled.

Our study has shown that measuring spore density could considerably increase the efficiency of predicting infection by WLR and its development in fields. Thus, a forecasting approach based on detecting airborne inoculum or interpolating spore density, in addition to predicting infection events, could be set up and used to protect wheat from losses due to this disease.

Chapter 6

Conclusions and perspectives

The objective of protecting wheat against foliar diseases is simple: the upper leaves should remain healthy and functional for as long as possible because crop yield is greatly affected by any disruption in the proper functioning of these vital organs (Seck *et al.*, 1991; Bancal *et al.*, 2007). In the crucial weeks from stem elongation to flowering, everything happens rapidly and the difficulty lies in choosing protection strategies that come closest to achieving the economic optimum. In the context of integrated pest management, reduction of pesticide use and resistance of fungal pathogens towards fungicides, knowledge on the epidemiology of main foliar pathogens, on their natural pressure, on their interactions with cultivars and on their diversity should help in the development of useful decision support systems.

When this study on the two commonest and most damaging wheat diseases in Wallonia began, it was accepted that septoria tritici blotch (STB) occurred early in wheat development and that its progression to the upper leaves during grain development was linked to splashborne dispersal. It was thought that it spread fairly early and aggressively to the upper leaves, depending on plant growth kinetics and rainfall occurrence. In contrast, wheat leaf rust (WLR) was considered to be an exogenous disease, with the spores probably originating some distance away and reaching the wheat fields in Wallonia fairly early in the season and with varying incidence, depending on the weather conditions being conducive to the winter survival of *Puccinia triticina* and to its release, transport and deposition onto leaves. In both cases, advice on protecting wheat against these diseases was based on the earliness and intensity of symptoms observed in the field and collected through a monitoring network. With regard to STB, a 'ProCulture' prediction model simulating *Septoria tritici* infection and development (anamorph phase) and based on weather conditions was used to supplement the information from the monitoring network.

This study was based on the hypothesis that a quantitative analysis of the airborne inoculum of wheat pathogens would lead, first, to a better understand the epidemiology of wheat diseases and, second, to an improvement in the short-term prediction of these diseases, as a guide to farmers.

The study produced two major findings. The first one was that STB primary infection of the upper leaves could derive from airborne inoculum, which in some cases seemed to be the major source of primary infection of these leaves. The second was that WLR inoculum is not continuously present, but varies greatly from one year to another, although in the same season patterns can be fairly similar between sites.

These two findings have a direct use for cereal farmers. Currently, farmers protect the upper leaves of wheat against STB mainly by applying the initial fungicide treatment at growth stage 32 (GS32) and a second one at GS55. The first treatment slows the progression of infection due to splashborne pycnidiospores transported from the lower to the upper leaves, whereas the second treatment aims at protecting both the last leaf and the ear. The possibility of the upper leaves be infected by airborne inoculum, as demonstrated by the study, suggests that the 'GS32 + GS55' strategy is flawed because it means that the last leaf (GS39) receives no fungicidal protection until heading and is therefore exposed to infection by airborne inoculum for 10-18 days. The study shows that, if the STB pressure is low at GS32 (particularly in resistant varieties), it is best to delay the first treatment and to apply it instead at GS39. This would provide better protection of the last leaf against STB and of the whole crop until flowering. At this stage, it would then be possible to determine whether or not the risk of *Fusarium* head blight and late wheat leaf rust justifies any treatment. If so, this treatment could take place at the stage that it is most likely to be effective (early flowering); if not, there would be no need for the second fungicide treatment.

As for WLR, the study showed that an analysis of airborne inoculum data improves the prediction of infections using the model developed by El Jaroudi (2014a) and removes most of the false alarms inherent in the model. This is also very useful information for cereal farmers.

What future and what role for spore trapping of STB and WLR?

The airborne inoculum concentration measurements showed that there was a link with diseases observed in neighboring fields. The extent to which these measurements are representative, however, remains unclear. It might be possible in the future to determine fairly accurately the concentration of spores of certain pathogens anywhere in the country by interpolation. This could be the case for WLR, for example. Future research might lead to the development of a weather-based model that could predict spore density and improve the prediction of infection periods. A prediction that the spore density threshold would be exceeded during the critical period (GS30-GS39) and that an infection would occur before GS39

would indicate the need for early fungicide treatment against the disease on sensitive cultivar, whereas a low spore density prediction would point to the opportunity to postpone or cancel fungicide treatment. The development of such a model is far less likely with STB, however, because the proximity effect of source fields causes greater irregularity in the spatial distribution of the airborne inoculum.

Extending spore trap networks to other wheat pathogens and to other crops

Spores traps are not selective. They collect the airborne inoculum of various crop pathogens indiscriminately. They could be used, however, to simultaneously collect the airborne inoculum of several important crop pathogens in Wallonia, in addition to STB and WLR, provided that they operate at appropriate times and sites.

For example, an approach similar to that used in this study could provide a better understanding of the factors influencing the presence of stripe rust (*Puccinia striiformis*) inoculum early in the season (February-March). This would help identify risk years when increased monitoring of plots would be necessary in order to decide on a possible early treatment against the disease. Studies on the detection of spores of *Fusarium* head blight pathogens could also provide a better understanding of the conditions favoring the presence of these spores at flowering.

Research conducted in Denmark showed that the early, pre-symptomatic detection of the airborne inoculum of *Ramularia beticola* and *Cercospora beticola*, which are causal agents of sugar beet diseases, could be used more effectively to assess treatment requirements (Wieczorek & Jørgensen, 2013). In rapeseed, *Sclerotinia sclerotiorum* is harmful only when the weather during flowering favors the dispersal of inoculum and the spread of infection. Preventive fungicide treatments are applied almost systematically, and yet, in seven out of the past 10 years, the damage has been negligible. If the absence of inoculum could be determined, this would negate the need for the systematic use of preventive fungicide treatments to protect plants against this disease and help in reducing the use of fungicides when they are not necessary.

The role of airborne inoculum sampling in integrated pest management

The quantification of airborne inoculum using molecular technologies is a powerful tool for studying the conditions of disease infection. The inclusion of airborne inoculum profiles, as well as weather conditions, in the analysis of disease development enables the conditions required for infections and

epidemics to occur to be identified. **This approach would make it possible to target the critical periods when it would most useful to quantify pathogen inoculum.** As shown in this study, the airborne inoculum of diseases can sometimes be a limiting factor in crop infection. Seasonal climatic differences influence the way spores are produced and it is therefore important to identify these critical periods and the inoculum loads necessary to trigger severe epidemics.

With regard to integrated pest management (IPM), there are two possible approaches: to develop models for predicting inoculum concentrations; or to monitor the airborne inoculum of the diseases in fields in real time during these critical periods (Figure 6.1). The first option would be based on past scientific work, whereas the second requires real-time monitoring. Using both options simultaneously would probably produce more reliable results. This additional information on the level of airborne inoculum would enable the prediction models of epidemics to be refined. In the perspective of an integrated approach, the prediction of epidemics should also integrated other factors such as the agricultural practices (rotation, sowing date, cultivar, fertilization, …) and other control strategies that play a role in epidemics.

Interest in the use of spore traps in IPM has increased in recent years due to various technological advances, including PCR quantification. Some traps have even been developed exclusively for molecular or immunological diagnostic purposes in order to save time in the preparation of analytical samples (miniature cyclone, Micro Titre Immuno Spore Trap Ionic Spore Trap) (West *et al.*, 2008, 2009). The ability to combine air sampling with molecular tools for quantification can provide rapid, reliable and repeatable results. Furthermore, these methods give the opportunity to detect the presence of pathogens pre-symptomatically. In order to meet biosafety requirements, however, the developed methodologies should be effective and enable pathogens to be quantified rapidly. Within this context, great progress still needs to be made, particularly with regard to distinguishing viable particles from those that no are longer viable. A final important point relates to the location of the traps. More work is needed to determine the spatial variability of spore production at different scales.

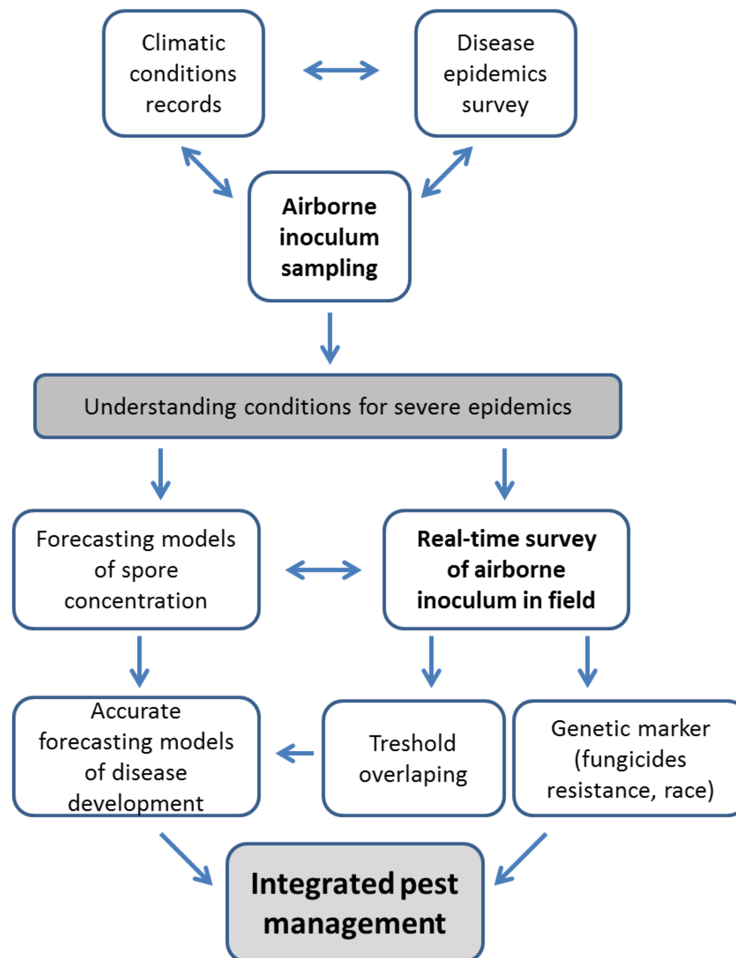

Figure 6. 1. Role of the quantification of airborne inoculum in the development of disease prediction models and IPM.

Airborne inoculum quantification tools can also be combined with an analysis of genetic markers in order to gather information about the specific traits of a pathogenic population occurring during a growing season (e.g., fungicide resistance, ability to produce mycotoxins or race type). In the future, IPM is likely to be based on this type of information, which cannot be obtained using visual microscopy.

From modeling disease development to modeling the best protection

Clearly, disease development modeling constitutes progress in that it enables agronomists to anticipate risks and farmers to choose effective protection strategies. In the case of wheat, however, several diseases evolve together, including wheat leaf rust (WLR), septoria tritici blotch

(STB), stripe rust and *Fusarium* head blight. Even if the development of each disease was correctly modeled, aggregating all the data would be difficult and would not necessarily provide a clear answer about which protection strategy to adopt for maximum economic benefit.

To this end, another type of modeling should be developed, based on the **probability of the best economic choice**. Such a model would use the observational data from the field (symptoms) as well as model predictions, including airborne inoculum occurrence. In addition – and this is where its originality would lie – it would use, as data, the performance results of a large number of trials comparing various fungicide protection strategies. For any situation described (e.g., variety, sowing date, fertilization, previous crop, pathogen development prediction), the model would search the database for situations sowing the greatest similarity and identify protection strategies that usually proved to be the most effective. Such a model would give farmers the practical answer to the question: 'What to do in order to do what is best?' In Wallonia, and particularly at CRA-W, fungicide trial data are abundant and could be suitable for such 'data mining'.

The range of wheat varieties and fungicides evolves continuously. The virulence of pathogens and their resistance to fungicides are changing too. Prices are so unpredictable that they are often described as 'volatile', but it is nevertheless important to deal with these evolving data in order to get closer to the optimum. The obvious major constraint in all this is the need to update data continuously if they are to remain relevant, but this relates to agronomy in general.

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