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der Christian-Albrechts-Universität zu Kiel

**Rapid Detection and Quantification of *Mycosphaerella graminicola*  
in Wheat Using PCR and RT-PCR Assays**

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**Abbreviations**

µg	microgram
AFLP	amplified fragment length polymorphism
AP	alkaline phosphatase
APS	ammoniumpersulfate
ATP	adenosintriphosphate
bp	base pair
BPB	bromophenol blue
cDNA	complemental deoxyribonucleic acid
CTAB	cetyltrimethylammonium bromide
d	day(s)
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	1,4-dithiothreitol
EDTA	ethylenedinitrilotetra acid
ELISA	enzyme linked immunoabsorbent assay
EST	expressed sequence tag
h	hour(s)
ITS	internal transcribe spacer
kb	kilo bases
LB medium	luria bertani medium
Mb	mega bases
min	minute
MOPS	4-morpholinepropanesulfonic acid, sodium salt
mRNA	messenger ribonucleic acid
MYA	malt yeast agar
MYB	malt-yeast broth
PCI	phenol/chloroform/isoamylalcohol
PCR	polymerase chain reaction
PDA	potato dextrose broth agar
PDB	potato dextrose broth

pg	picogram
ng	nanogram
RAPD	random amplified polymorphic DNA
PVP	polyvinylpyrrolidone
PVPP	polyvinyl polypyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
RFLP	restriction fragment length polymorphism
RL-buffer	restriction-ligation-buffer
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
s	second
SDS	sodium dodecylsulfate
TAE	Tris-acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TDFs	transcript derived fragments
TEMED	N,N,N',N'-tetramethylethylenediamin
Tris	Tris(hydroxymethyl)-aminowethan
<i>T<sub>m</sub></i>	melting temperature

## 1 Introduction

### 1.1 Biology and epidemics of *Mycosphaerella graminicola* leaf blotch

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops worldwide and foods processed from it are the basic nutrition sources in many regions. In 2003, wheat was harvested at a total area of 208,765,010 ha and had a total production of 556,348,627 million tons in the world. In Germany, wheat holds a dominant position among arable crops, with a harvest area of 2,967,379 ha which covers about 30% of the cereals and 19,296,100 million tons in 2003 (FAO data). Increasing intensity of wheat cultivation has been achieved by shorter rotations, high plant density and high nitrogen fertilization. These agricultural practices not only bring substantial grain yield and quality, but also increase disease pressure at the same time. Disease management has become a prominent part of the wheat production (Verreet *et al.* 2000).

*Mycosphaerella graminicola* (Fuckel) Schroeter (anamorph *Septoria tritici* Roberge in Desmaz), the cause of wheat leaf blotch disease, is a widespread and economically important pathogen of bread wheat and durum wheat in the major wheat production regions of the world, especially in regions with a humid, temperate climate (Eyal 1999). In Germany, this disease is currently one of the most important foliar diseases in wheat (Verreet *et al.* 2000). Its infection and epidemics can result in serious yield losses ranging from 30-40% (Caldwell and Narvaez 1960, Eyal *et al.* 1987).

Wheat leaf blotch was described for the first time in the 1840s and its asexual state was described as *Septoria tritici* by Desmazières in 1842 (Zadoks 2003). The sexual state *M. graminicola* was first described and named by Sanderson (1972) in New Zealand and later reported in many other countries. In Germany, Verreet *et al.* (1990) gave the first proof of the sexual state *M. graminicola* and observed the complete life cycle on different medium. *M. graminicola* can infect seedlings and adult plants. The pathogen generally infects wheat leaves but can also cause low levels of glume blotch (Wainshilbaum and Lipps 1991). Its initial symptoms are yellowish and chlorotic flecks usually found on the lower leaves, especially on those in contact with the soil. These flecks enlarge into irregular lesions that are brown to reddish-brown in colour. As the lesions age, the centres become somewhat bleached with gray or ash-white colour. During this time, small, dark brown to black pycnidia appear in the centre. The presence of those small, black pycnidia in the lesions is the most reliable character for identifying the disease (Gair *et al.* 1987).

The primary inoculum sources within the disease cycle are airborne ascospores, rainsplashed pycnidioconidia from infected plant debris or grass hosts and possibly seed-borne infections (Eyal 1999). The asexual pycnidioconidia play an important role in the vertical and horizontal spread and the disease epidemics (Shaner and Finney 1976, Verreet *et al.* 2000). Its production and development is greatly influenced by moisture and temperature (Shaw 1990). Vertical and horizontal spread of the pathogen takes place usually through the kinetic energy of striking raindrops. Spread of conidia is thought to occur mainly during heavy rain when large raindrops splash the fungal spores from the lesions on the lower leaves to the upper leaves (Eyal 1999). Some horizontal spread can also occur from the combined effects of leaf wetness and wind, or via leaf contact, but it is proved to be very limited (McDonald *et al.* 1999).

In the field, pseudothecia develop on dead tissues within the confines of the old lesions (Sanderson 1972), and ascospores are released from mature pseudothecia throughout the year (Hunter *et al.* 1999). Currently, the perfect stage is considered important in the epidemiology and may be associated with the widespread infections (Shaw *et al.* 1989, Hunter *et al.* 1999). A principal role for airborne ascospores is the onset and probably the perpetuation of epidemics. The environmental factors associated with the ascospore discharge and the resulting infection intensity on the wheat genotype have been used in formulating models for disease forecasting and disease control (Royle 1994). Uniform spatial distribution of infection by *M. graminicola* found throughout the growing season may be indicative of a distant airborne inoculum source. A continuous within-crop supply of ascospores from lower to upper leaves is also operative (Hunter *et al.* 1999).

A high level of genetic variation has been reported within *M. graminicola* populations (McDonald 1999, Linde *et al.* 2002, Zhan *et al.* 2004). In Germany, Schnieder *et al.* (1998, 2001) analysed the population structure and genotypic diversity of 203 *M. graminicola* isolates from six fields using amplified fragment length polymorphism (AFLP) and randomly amplified polymorphism (RAPD) markers. A high degree of genotypic diversity was observed in Germany and a high amount of pathogen variation could be found even on a single host plant.

Host specialization has been confirmed in the last 30 years, but the results have been inconsistent. Van Ginkel and Scharen (1988) did not find significant host-pathogen

interactions in the wheat-*M. graminicola* pathosystem. They found that isolates of *M. graminicola* varied for aggressiveness rather than virulence and concluded that clear gene-for-gene relations were absent in this pathosystem. Other studies suggested that a gene-for-gene interaction exists between host and pathogen due to the fact that avirulence in *M. graminicola* can be controlled by one gene and many wheat cultivars can carry single genes (Kema *et al.* 1996b, 2000). It is now shown that gene-for-gene interactions between wheat and *M. graminicola* do exist (Brading *et al.* 2002). However, the changes in the frequencies of marked genomes and cultivars differing in resistance indicate that host-pathogen reaction could be complex in the wheat-*M. graminicola* pathosystem (Zhan *et al.* 2002).

Lately, some progress has been made regarding the genetics and genomics of *M. graminicola*. The size of the *M. graminicola* genome was estimated 31 Mb (McDonald and Martinez 1991). An ascomycete fungus possesses averagely between 8,000 and 9,000 genes (Kupfer *et al.* 1997). A group of expressed sequence tags (ESTs) from *M. graminicola* were generated (Skinner *et al.* 2001). There are currently 2,926 unisequences for *M. graminicola* which are publicly available at the Phytopathogenic Fungi EST Database (<http://cogeme.ex.ac.uk/>). A detailed genetic linkage map of *M. graminicola* is constructed with RAPD and AFLP markers (Kema *et al.* 2002). Maarten de Waad and his group have performed functional analysis of ABC-transporters of *M. graminicola*. At least five related genes have been cloned and characterized (Zwiers *et al.* 2000, 2003). Hamer *et al.* (2001) performed genome-sized mutagenesis experiments in *M. graminicola* using Paradigm's TAG-KO™ technology and reported the mutagenesis of 70% of the total genes. The understanding of genetics and genomics will facilitate the development of resistant cultivars, novel fungicides and new control measurements (Palmer *et al.* 2002).

## **1.2 The integrated pest management (IPM) of wheat leaf blotch**

Some cultural practices have been thought to be efficient to reduce the disease. In management systems where wheat is planted in short consecutive yearly intervals, discarding infected stubble by ploughing or burning can drastically reduce the primary inoculum (Eyal 1999). Jordan and Hutcheon (1999) reported that minimizing the risk of

disease could be achieved by multi-function crop rotation, combined with resistance and manipulation of the crop structure.

The application of resistance is regarded as an ideal measure in the disease management. A lot of resistance genes and sources have been discovered so far. Jlibene *et al.* (1992) found that resistance to *M. graminicola* in the highly resistant spring wheat accessions Bobwhite 'S' (BOW) and KK is controlled by several genes with predominantly additive effects. Up to now, eight dominant genes (*Stb1* to *Stb8*) conferring resistance to *M. graminicola* have been described and mapped using different types of molecular markers (Adhikari *et al.* 2003). Resistance to *M. graminicola* by a single dominant gene was assigned to the winter wheat Bulgaria 88 (Rillo and Caldwell 1996). Several quantitative loci (QTL) were further identified (Simón *et al.* 2004, Chartrain *et al.* 2004). However, the irregular epidemics, diverse genotypes and virulence patterns, complicated wheat-pathogen relationship and the lack of knowledge on pathogenicity make it hard to use resistant varieties to control this pathogen. So far, there are very few examples where resistance controlled by major genes was recognized and incorporated into a specifically designated *Septoria tritici* blotch breeding scheme, and none is sufficiently resistant to give a satisfactory control (Eyal 1999).

Currently, *M. graminicola* management relies mainly on the application of fungicides (Palmer and Skinner 2002). Over the last decade, a number of efficient fungicides have been developed to control *M. graminicola*, including a variety of 14-demethylase inhibitors of sterol biosynthesis (such as cyproconazole and epoxiconazole) and the systemic fungicides famoxadone and strobilurins (Jordan and Hutcheon 1999, Godwin *et al.* 1999). Chemical control provides a simple, convenient and efficient tool for plant protection, but it also causes some negative impact on the environment and results in the resistance of pathogens. Rational use of fungicides is an important prerequisite for chemical control to ensure optimal and cost-efficient control and to minimize the problems mentioned (Knight *et al.* 1997). The choice of suitable fungicides and spray timing depends on the precise prediction of disease risk and epidemics (Verreet *et al.* 2000). Several prediction models of *M. graminicola* epidemics have been formulated and used for instructing the use of fungicides (Royle 1994, Parker *et al.* 1999). Verreet *et al.* (2000) developed a threshold-orientated integrated plant protection system (IPM wheat model) for *S. tritici* leaf blotch. According to over ten-year data of epidemiological studies, weather and yield, a flexible

threshold value (disease incidence > 50% at F6 leaf and leaf wetness > 98% for at least 48 h) was determined. However, efficient application of these models strongly depends on the accurate diagnosis and assessment of disease severity.

### 1.3 Early and precise diagnosis — a key to disease prognosis and control

Disease diagnosis is an important prerequisite to optimise fungicide input, to practice integrated pest management (IPM) and to work towards sustainable agriculture (Leonard and Fry 1986). Accurate identification of the pathogens and assessment of population densities is a basis for precise prognosis of disease and an essential for precise timing in fungicide applications to get the best control effects (Fraaije *et al.* 1999).

Microscopic diagnosis and cultural methods are traditional approaches to isolate and identify potential pathogens. But these methods are either inaccurate, or time-consuming, and often require skilled taxonomists to identify the pathogen. They are sometimes difficult to carry out due to a number of factors, namely, ambiguities in morphological characters, the specific nutrient requirements, growth conditions of certain pathogens grown *in vitro* as well as the time constraint imposed by slow-growing pathogens. Furthermore, both methods can only be used for qualitative diagnosis of diseases (Leonard and Fry 1986). Visual diagnosis and assessment is a routine approach for disease assessment in the field. This approach is solely based on browning or necrosis characterizing disease intensity and often causes unreal conclusions because the symptoms are often confused with those caused by other pathogens (Verreet *et al.* 2000). The occurrence of wheat leaf blotch is usually accompanied by other foliar diseases in the field. *Stagonospora nodorum* is a pathogen which gives rise to leaf spots similar to *M. graminicola*. Other pathogens, such as *Pseudocercospora herpotrichoides*, *Drechslera tritici*, *Botrytis cinerea*, *Alternaria alternata*, *Didymella exitialis* and some *Fusarium* spp., can also cause leaf spots and lesions in wheat (Gair *et al.* 1987). These pathogens usually co-occur with *M. graminicola* in the field, which makes it hard to identify this disease accurately by conventional visual assessment, especially in the early stages of disease.

The latent period of *M. graminicola* is quite long in winter wheat before its epidemic period. Based on an epidemiological field study of the pathogen development cycle,

Vereet *et al.* (2000) concluded that *M. graminicola* has a latent period of at least 28 days before visual infection in winter wheat in Schleswig-Holstein. Lovell *et al.* (2004) reported that the latent period of *M. graminicola* is greatly influenced by temperature. The latent period until the first visible symptoms ranged from 11 to 42 days with mean batch temperatures of 2.9-20.2°C. A fungicide treatment based alone on visual diagnosis only leads to partial success in terms of disease control in that the causative infection already started nearly four weeks ago. Therefore, early prediction is of high importance to the management of *M. graminicola* leaf blotch.

#### **1.4 Molecular diagnostics of plant diseases**

In recent years, the development of molecular diagnostic techniques has provided a valuable approach for the early and precise detection and quantification of plant pathogens (Miller and Martin 1988). Currently, two principle molecular diagnostic techniques, immunologic techniques and polymerase chain reaction (PCR)-based assays, have been successfully applied for the qualitative or quantitative detection of various pathogens including viruses, bacteria and fungi (Clark 1981, Henson and French 1993). In comparison to traditional visual and microscopic diagnosis, these methods have a higher specificity and sensitivity and therefore may be useful to detect the infection presymptomatically and identify the target diseases without time-consuming isolation and culturing of pathogens (Martin *et al.* 2000).

##### **1.4.1 Immunologic techniques**

Immunologic techniques have been applied to the detection of plant viruses since the 1960s. They employ specific antibodies for target pathogens. Most applications were directed at virus detection with little emphasis on fungal and bacterial pathogens prior to the development of enzyme linked immunosorbent assays (ELISA) and monoclonal antibody technologies (Martin *et al.* 2000). Presently, ELISA has been widely applied to identify viral and bacterial pathogens selectively as a routine diagnostic assay. Lately, this approach has been employed for the identification of several cereal pathogens including *M. graminicola* (McCartney *et al.* 2003). An immunology-based “On-site” technology (Stewart *et al.* 1996) which can be directly performed in the field within minutes was developed. Kema *et al.* (1996a) used ELISA to detect and quantify *M. graminicola* during the infection of wheat leaves under controlled conditions in the greenhouse. Furthermore,



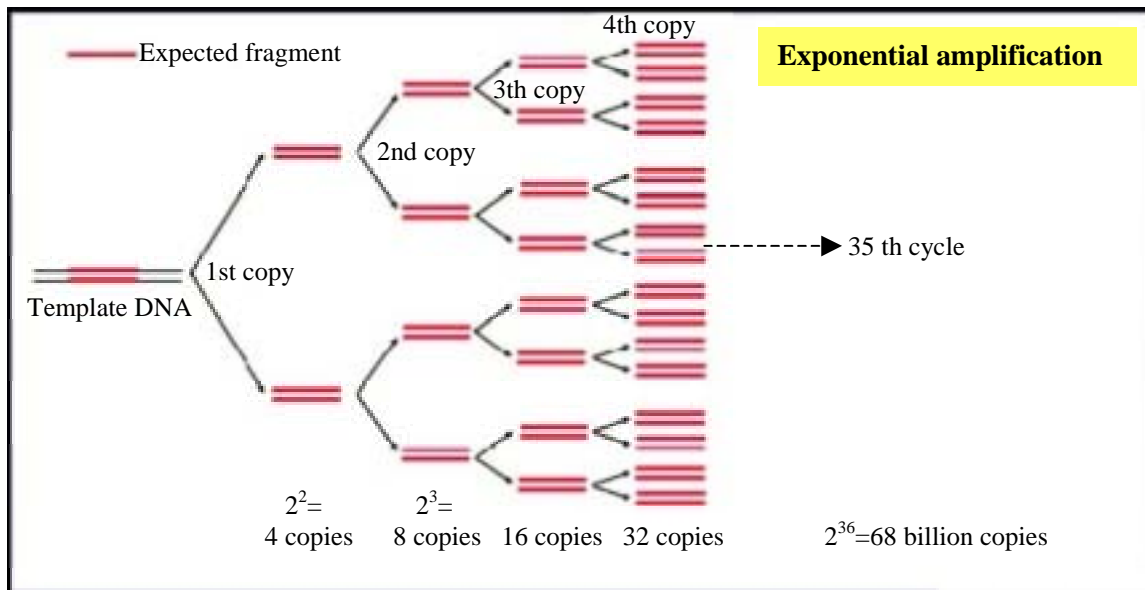
Kendall *et al.* (1998) applied ELISA for an accurate pre-symptomatic detection and quantification of *M. graminicola*. These results improved disease control effect through better timing as well as choice and dose-rate of fungicides. Tian *et al.* (2002, 2004) developed a specific and quantitative biotin/avidin format ELISA (BA-ELISA) to detect *S. tritici* and *S. nodorum* and successfully applied it to assess fungicide effect and resistance level of varieties in the field. The results detected by BA-ELISA showed significant correlation with the visual assessment data. However, some shortages of immunologic diagnostic techniques block their wide application. First, the production and purification of pathogen-specific antibody is expensive and time consuming. Second, it is also difficult to produce a quality-stable, highly specific antibody for pathogens, especially for fungi (Clark 1981). In addition, false positive reactions can be encountered with immunologic assays due to cross-reactivity of antibodies with antigens derived possibly from present plants or other microbes (Beck and Ligion 1995).

#### 1.4.2 PCR-based methods

PCR is a powerful tool in molecular biology to exponentially amplify specific DNA sequences by DNA synthesis *in vitro* (Fig. 1). It has been the most common technique for DNA analysis in molecular biology since its invention by Kary Mullis in 1983 (Saiki *et al.* 1985, the first paper describing PCR). The method includes three essential steps: melting of target DNA (denaturing), annealing of two oligonucleotide primers to the denatured DNA strands, and primer extension by a thermostable DNA polymerase. Newly synthesized DNA strands serve as targets for subsequent DNA synthesis in following cycles (Fig. 1, McPherson and Møller 2000). By this method, large numbers of target DNA sequences can be amplified from tiny amounts of DNA.

Although PCR had been successfully used to detect pathogens in infected animal tissues by biomedical researchers for some time, it had not been applied to detect plant pathogens until 1990 (Rollo *et al.* 1990). In the last decade, this method has been greatly developed and serves currently as an usual molecular technique for the diagnosis of various plant pathogens including virial, bacterial and fungal pathogens (Annamalai *et al.* 1995). By now it has been successfully applied to the identification or quantification of at least 144 phytopathogenic fungal species of 77 genera (<http://www.sppadbase.com/>). Table 1 lists the successful applications of PCR in qualitative or quantitative detection of common fungal pathogens in wheat using specific primers. In addition, this method is of great

significance to the epidemiological studies and the management of plant diseases since it is able to detect pathogens within infected tissues pre-symptomatically and quantitatively (McCartney *et al.* 2003).



**Fig. 1** Scheme of exponential amplification of DNA by PCR (Vierstraete 1999)

PCR offers several advantages compared with traditional diagnostic methods: (1) organisms do not need to be cultured prior to their detection as for ELISA; (2) the technique possesses high sensitivity, with the potential to detect a single target molecule theoretically without using radioactive probes; (3) it allows a rapid and simple identification of pathogens without knowledge of complex taxonomy; (4) by this method several different pathogens can be detected in one tube using multiplex PCR; (5) due to its high sensitivity, pathogens can be detected presymptomatically. Compared to immunological techniques, PCR has a higher specificity and a relatively lower cost (Henson and French 1993).

In recent years, several PCR-based methods such as multiplex PCR (Claustres *et al.* 1989), immuno-capture PCR (Wetzel *et al.* 1992), competitive PCR (Diviacco *et al.* 1992) and real-time PCR (Higuchi *et al.* 1992, 1993) have been developed. These methods greatly facilitate the application of PCR in molecular diagnosis of pathogens. The development of quantitative PCR provides the possibility to quantify fungal biomass in infected plant tissues by PCR assay. Before the application of real-time PCR, the PCR quantification of fungal biomass had been generally performed by competitive PCR or by quantification of

**Table 1** Applications of PCR to common fungal pathogens of wheat using specific primers

Pathogens	Target sequences	Purpose	References
<i>Fusarium</i> spp.	ITS region, <i>tri5</i> gene,	Conventional PCR	Doohan <i>et al.</i> 1998, Waalwijk
	RAPD fragments and	Real-time PCR	<i>et al.</i> 2004, Mishra <i>et al.</i>
	Fot1 transposon		2003, Schnerr <i>et al.</i> 2001
<i>Gaeumannomyces graminis</i>	ITS region	Conventional PCR	Bryan <i>et al.</i> 1995, Goodwin
	Mitochondrial DNA	Conventional PCR	<i>et al.</i> 1995
	18S rDNA	Conventional PCR	Schesser <i>et al.</i> 1991, Ward
	Avenacinase gene	Conventional PCR	1995
<i>Microdochium nivale</i>	RAPD fragment	Conventional PCR	Fouly and Wilkinson 2000
	genomic DNA	Multiplex PCR	Rachdawong <i>et al.</i> 2002
		Real-time PCR	Nicholson <i>et al.</i> 1996a
<i>Mycosphaerella graminicola</i>	ITS region	Conventional PCR	Waalwijk <i>et al.</i> 2004
	$\beta$ -tubulin gene	PCR/PicoGreen assay	Beck and Ligon 1995
	$\beta$ -tubulin gene	Multiplex PCR	Fraaije <i>et al.</i> 1999
<i>Pseudocercospora herpotrichoides</i>		Real-time PCR	Fraaije <i>et al.</i> 2001
	RAPD fragment	Conventional PCR	Nicholson <i>et al.</i> 1997
	ITS region	Conventional PCR	Nicholson <i>et al.</i> 1997
<i>Rhizoctonia solani</i>	ITS region	Conventional PCR	Poupard <i>et al.</i> 1993
<i>Stagonospora nodorum</i>	ITS region	Conventional PCR	Nicholson and Parry 1996b
	$\beta$ -tubulin gene	Multiplex PCR	Beck & Ligon 1995
		Real-time PCR	Fraaije <i>et al.</i> 2001
<i>Puccinia recondita</i>	tubulin gene	Multiplex PCR	Fraaije <i>et al.</i> 2001
<i>Puccinia striiformis</i>		Real-time PCR	Fraaije <i>et al.</i> 2001
	tubulin gene	Multiplex PCR	Fraaije <i>et al.</i> 2001
<i>Puccinia striiformis</i>	tubulin gene	Real-time PCR	Fraaije <i>et al.</i> 2001
<i>Tilletia indica</i>	mitochondrial DNA	Conventional PCR	Smith <i>et al.</i> 1996
	mitochondrial DNA	Real Time PCR	Frederick <i>et al.</i> 2000
<i>Botrytis cinerea</i>	$\beta$ -tubulin gene	Conventional PCR	Luck and Gillings 1995
	RAPD fragment	Conventional PCR	Rigotti <i>et al.</i> 2002

the PCR end-products. At that time, competitive PCR was one of the most facile quantitative methods which include known amounts of reporter or internal control sequences in PCR reactions (Diviacco *et al.* 1992, Hu *et al.* 1993). However, such quantitative data is not directly derived from target DNA but from the ratio between target and competitor DNA. Hence, the result is affected when the amplification efficiency of the target sequence is different from the competitor sequence (Hensen and French 1993). In addition, post-PCR confirmation steps such as running gels and image processing are needed.

#### **1.4.2.1 Real-time PCR**

Real-time PCR is a quantitative PCR method which allows the cycle-to-cycle monitoring of the amplified DNA (Higuchi *et al.* 1992, 1993). It quantifies the initial amount of the template DNA with high specificity and sensitivity. This method is a preferable alternative to other forms of quantitative PCR that detect the amount of final amplified product at the endpoint (Raeymaekers, 2000). Compared with conventional PCR and other quantitative PCR methods, real-time PCR can monitor the whole process of PCR reaction in real-time, eliminates time-consuming post-PCR processing of PCR products, and therefore avoids the use of hazardous dyes like ethidium bromide (Abd-Elsalam 2003, Schena *et al.* 2004).

The real-time PCR system is based on the detection and quantification of a fluorescent reporter (Lee 1993, Livak *et al.* 1995). This signal increases in proportion to the amount of PCR product present in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase. A fixed fluorescence threshold is set significantly above the baseline and can be altered by the operator. The parameter threshold cycle (Ct) is defined as the cycle number at which the fluorescence emission exceeds the set threshold. The detection system records this specific cycle number which is inversely proportional to the log of the initial DNA template. In contrast to those quantitative PCR methods with quantification at endpoint, real-time PCR is more accurate since it quantifies the initial DNA based on the Ct value measured at an early exponential stage of the reaction (Qi and Yang 2002).

There are two main fluorescence-monitoring systems for real-time PCR based on used chemistries: amplicon sequence nonspecific and sequence specific methods (Wittwer *et al.* 1997, Schena *et al.* 2004). The formers are based on certain dsDNA intercalating dyes such

as SBRY Green I. SYBR Green I is the most utilised DNA-binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison *et al.* 1998). SYBR Green-based real-time PCR is a simple and reliable low-cost method for monitoring PCR amplicons and for quantifying template DNA. Disadvantages of this method are that it requires extensive optimisation and follow-up assays (melting point curve or dissociation analysis) for amplicon identification because SYBR Green I can not discriminate the target amplicon from other nonspecific amplicons and primer dimers (Ririe *et al.* 1997). The amplicon sequence specific methods include TaqMan probes (Livak *et al.* 1995), molecular beacons (Tyagi and Kramer 1996) and scorpions (Whitcombe *et al.* 1999). Compared with the amplicon nonspecific methods, they are more specific for target sequence, and can detect two or more distinct sequences in a single PCR reaction without melting curve analysis, but are also more expensive (Skena *et al.* 2004).

As the fungal DNA amount is proportional to fungal biomass, real-time PCR can quantify fungal biomass by measuring the fungal DNA amount in infected plants. During the infection, the amount of fungal biomass increases in infected tissues with time. Consequently, the proportion of fungal DNA in the DNA of infected plants also increases with time. Thus, real-time PCR can be used not only to assess disease severity, but also to monitor infection development and disease progression in infected plants. Currently, successful application of real-time PCR to fungal plant pathogens includes the following main aspects: (1) detection and quantification of pathogen biomass in host tissues (Schnerr *et al.* 2001, Schaad *et al.* 2002); (2) monitoring disease development of pathogens and assessing disease severity in the field (Qi and Yang 2002, Gachon and Saindrenan 2004); (3) assessment of pathogen virulence and varietal resistance (Fraaije *et al.* 2001, Hietala *et al.* 2003, Vandemark *et al.* 2003); (4) analysis of fungicide resistance and efficacy (Nascimento *et al.* 2003); and (5) detection of gene expression level (McMaugh and Lyon 2003, Ray *et al.* 2003, Schenk *et al.* 2003).

#### **1.4.2.2 RT-PCR**

Reverse transcription (RT)-PCR (Lee *et al.* 1989) is another PCR-based method for the detection of target mRNA. It is composed of two initial steps: (1) reverse transcription of target mRNA to cDNA; and (2) PCR amplification of cDNA (McPherson and Møller

2000). This technique realises the indirect amplification of RNA by PCR, and provides another possibility to qualitatively and quantitatively detect pathogens in crops.

DNA, the PCR template, is relatively stable and can also be found in dead organisms. It was demonstrated that PCR products could be amplified from DNA of cells killed by heat or other treatments several days after cell death (Josephson *et al.* 1993, Allmann *et al.* 1995). Hartung *et al.* (1993) detected plasmid DNA sequences from *Xanthomonas campestris* pv. *citri* in 7-month-old dried lesions on inoculated grapefruit leaves, but viable organisms were not recovered. Hence, the results obtained by PCR do not necessarily reflect viable and infectious pathogen structures. In contrast to DNA, mRNA is rapidly turned over in dead cells, and most mRNA species have half-lives measured in minutes (Ingle and Kushner 1996). Therefore, detection of mRNA by RT-PCR, as opposed to DNA-based methods, is considered a better indicator of cell viability (Sheridan *et al.* 1998).

In the past years, RT-PCR has been broadly applied to detect and quantify viral pathogens (Saade *et al.* 2000, Bertolini *et al.* 2003, Moravec *et al.* 2003). For fungal pathogens, RT-PCR has been used to detect and quantify the expression of some pathogenesis- or resistance-related genes (Ray *et al.* 2003, McMaugh and Lyon 2003, Schenk *et al.* 2003). Bleve *et al.* (2003) successfully applied real-time RT-PCR to monitor contaminations of yeasts and molds in yogurts and pasteurized food products by quantifying an actin mRNA target. Maher *et al.* (2000) applied the RT-PCR assay to detect *Pneumocystis carinii* in AIDS patients using a mRNA-specific primer set. No application in the diagnosis and epidemics of fungal plant pathogens has been reported by now.

#### **1.4.2.3 Applications of PCR based methods in *M. graminicola* diagnosis**

Beck and Ligon (1995) applied the PCR assay to distinguish *M. graminicola* from *S. nodorum* using a specific primer set (ITS1/JB446) developed from the ITS regions. However, this primer set was confirmed to amplify a second fragment of about 280 bp from healthy wheat plants (Fraaije *et al.* 1999). Therefore, It is not appropriate for *M. graminicola* quantification in infected plants. Fraaije *et al.* (1999) developed a specific primer set E1/STSP2R from a sequence of  $\beta$ -tubulin genes, and successfully applied a PCR/PicoGreen assay to study the colonization, infection and disease development of *M. graminicola* in wheat (Fraaije *et al.* 1999). However, this technique has some

disadvantages compared with real-time PCR. It has an even lower sensitivity (10 pg genomic DNA) than conventional PCR with detection on an agarose gel (2 pg genomic DNA). Furthermore, it is difficult to distinguish between similar template DNA inputs like other quantitative PCR by quantifying PCR products at endpoint. In 2001, a multiplex PCR assay was developed by Fraaije *et al.* (2001) to detect and quantify *M. graminicola* and three other foliar pathogens on wheat. In this work, a specific primer set (STIF2/BAF4ST) which amplified a single fragment of 555-561 bp was designed from the fifth intron of  $\beta$ -tubulin genes. This primer set has been used for the quantification of *M. graminicola* infection levels in the flag leaf of 19 cultivars by real-time-PCR. The results which showed a good correlation with those by visual assessment were used to evaluate the resistance of the wheat variety. A quantitative PCR was successfully applied to monitor the effect of curative and preventative applications of azoxystrobin for the control of *M. graminicola* leaf blotch on wheat, compared with visual assessment (Rohel *et al.* 2002).

### **1.5 Overall background and objectives of this research**

*M. graminicola* is an economically important pathogen in wheat. Currently, its control relies mainly on the use of fungicides. Early and precise diagnosis is a key to an optimal control effect of this pathogen with fungicides. PCR-based methods provide beneficial approaches to presymptomatically and quantitatively detect *M. graminicola* in infected wheat plants due to their high specificity and sensitivity. Despite some successful applications reported (see section 1.4.2.3), research is still limited in this field. Although three specific primer sets were developed, they either can not be used for quantitative PCR or can only distinguish *M. graminicola* from several other pathogens. New and more specific primer sets which can be used not only for qualitative but also for quantitative detection need to be developed. It also needs to be further studied how these methods can be applied to monitor disease epidemics in the field.

Although PCR-based methods have been successfully applied to detect and quantify many plant pathogens, they are not yet routinely used for fungal pathogens due to their relatively high cost and some technical problems. It is necessary to develop a rapid, easy-to-use and cheaper procedure. A simple and efficient protocol of DNA or RNA extraction suitable for various fungi and infected plants is needed for practical application of these techniques. Optimal reaction conditions should be designed for these techniques because their

specificity and sensitivity is usually affected by different conditions. Furthermore, contamination is always a concern regarding PCR since it is extremely sensitive. The development of more specific primers, careful performance and rational sampling may be an effective way to minimize the incidence of false positives.

The objective of this research was to develop a rapid and efficient PCR-based method to detect and quantify *M. graminicola* epidemics in wheat. For this purpose, we attempted to develop several highly specific primer sets, and to detect *M. graminicola* in pure culture and in infected plants by conventional PCR or real-time PCR. Besides, RT-PCR, a RNA-based method, was used to identify *M. graminicola*. Summarily, the following were the main aims in this study:

1. Development of simple and efficient methods for DNA and RNA extraction suitable for different fungi as well as for infected wheat plants
2. Development of specific primers
3. Optimisation of PCR conditions
4. Detection and identification of *M. graminicola* in inoculated plants and field samples by conventional PCR and RT-PCR
5. Monitoring of disease development in artificially inoculated plants and in naturally infected plants by real-time PCR
6. Assessment of fungicide efficacy by real-time PCR.



## 2 Materials and Methods

### 2.1 Plant material

#### 2.1.1 Preparation of inoculated wheat plants

The susceptible winter wheat cultivar Ritmo was used for the artificial inoculation. Seeds were first placed in humid sand on a Petri dish covered with aluminium foil and vernalized for about six weeks at 4°C. After germination, the seedlings were transplanted to 11-cm plastic pots, one plant per pot, in a potting mix of peat moss/composted steam-disinfested Wooster silt loam soil (1:5, v/v) and grown to about four to five leaves for about one month in a temperature- and light-regulated room at 20°C.

An isolate of *M. graminicola* newly isolated from infected wheat leaves was used for the inoculation. Conidia were released from pycnidia by immersing a leaf in 5 ml of sterile distilled water, and the resulting conidial suspension was streaked onto a malt-yeast agar (MYA) plate. Culture was grown at 22°C. After five days, one creamy, pink-coloured colony which was confirmed to be *M. graminicola* by microscopy was picked up into one ml sterile distilled water and mixed well. The conidial suspension was pipetted onto the MYA plates and cultured for five days at 22°C. The conidial suspension was prepared for inoculation by flooding Petri dish cultures with distilled water and scraping the agar surface. The conidia in the suspension were counted using a hemacytometer (Fuchs-Rosenthal) under a light microscope. The suspension was adjusted to  $1 \times 10^7$  conidia/ml with deionised H<sub>2</sub>O containing 0.3% Tween-80.

Thirty-day-old seedlings were inoculated by spraying the conidial suspension until the leaves surface was completely humid but before runoff. After inoculation, the seedlings were immediately placed in a humidity-saturated plastic box for 72 h, and then grown in a growth chamber (Heraeus/Vötsch HPS 2000) at 70% relative humidity, 22°C during the day, 17°C during the night, and 12 h photoperiod. To differentiate old inoculated leaves from uninoculated leaves grown after inoculation, inoculated leaves were marked with yellow paint. The inoculated leaves (only marked leaves) were sampled on 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 d after inoculation (each time three pots, 21-34 leaves).

The disease severity of all sampled leaves was assessed visually by a disease classification. The leaves were classified according to the following grading scales:

Scale 0: no infection;

Scale 1: 1-10% lesion area of total leaf area;

Scale 2: 11-20% lesion area of total leaf area;

Scale 3: 21-30% lesion area of total leaf area;

Scale 4: 31-40% lesion area of total leaf area;

Scale 5: over 40% lesion area of total leaf area.

The disease index was calculated according to the following equation:

$$\text{Disease index} = \frac{\sum (\text{leaf number of each scale} \times \text{scale number})}{\text{total leaf number} \times 5} \times 100$$

After that, the leaves were stored at  $-80^{\circ}\text{C}$  until being used for DNA and RNA extraction. To remove the adhering spores from leaf surfaces, collected leaves were cleaned with distilled water before DNA and RNA extraction.

### 2.1.2 Design of fungicide treatment plots and collection of field samples

Field experiments were performed with winter wheat variety Ritmo in Gettorf, Schleswig-Holstein, Germany, where *M. graminicola* epidemics were frequently observed in the past. Three plots (two fungicide treatment and one untreated control (CK), each plots 30 m<sup>2</sup>) were parallel designed with an interval area of 15 m<sup>2</sup> between them. Two fungicide treatments (1) Fandango (100 EC prothioconazole and 100 EC fluoxastrobin) + Prosaro (125 EC prothioconazole and 125 EC tebuconazole) and (2) Input (160 EC prothioconazole and 300 EC spiroxamine) + Prosaro (Bayer CropScience, Germany) were tested in this experiment. The wheat plants in the plots were sprayed for three times using a sprayer (System2, Schachtner, Germany) according to the design listed in Table 2.

**Table 2** Fungicide spraying date and dose used in the field

Treatment	April 22, 2004	May 19, 2004	June 10, 2004
Fandango	Fandango 0.750 l/ha	Fandango 0.625 l/ha	Prosaro 0.500 l/ha
Input	Input 1.250 l/ha	Input 1.250 l/ha	Prosaro 1.000 l/ha
CK (control)	untreated	untreated	untreated

To monitor the disease development during the *M. graminicola* epidemic period, thirty wheat plants in fungicide-untreated control plots were randomly collected at weekly intervals during

the epidemic period from May 3 to June 21, 2004. The disease severity of the three youngest leaves (F2 = third leaf from top to F = flag leaf) was investigated by disease classification as well as by pycnidia-counting method. The investigation by disease classification was performed as described in section 2.1.1. Meanwhile, the number of pycnidia on each leaf was counted under a light microscope. The severity was assessed by the average number of pycnidia of all investigated leaves. After investigation, the leaves were put into a plastic bag and stored at  $-70^{\circ}\text{C}$  for DNA, RNA extraction and real-time PCR.

In fungicide-treated plots, the efficacies of fungicide treatments were assessed for two times on May 17 and June 14, 2004. At each time, 30 leaves at different leaf positions (F4 = fifth leaf from top to flag leaf) were sampled and their disease severity was assessed by disease classification and by counting pycnidial number (see above). After that, these leaves were stored at  $-70^{\circ}\text{C}$  for later DNA extraction and fungal DNA quantification by real-time PCR (see section 2.8). The efficacy of fungicide treatment was calculated based on the following equation:

$$\text{Fungicide efficacy} = \frac{\text{CK} - \text{fungicide treatment}}{\text{CK}} \times 100\%$$

The work of fungicide treatment and sampling was performed by Dr. F. Schnieder, and all the field samples were kindly provided by him.

## **2.2 Fungal isolates and species**

### Collection and storage of fungal species and isolates

The fungal species and isolates used in the study are listed in Table 3. Six species were kindly provided by Dr. K. Abd-Elsalam in Egypt, and the other species were collected by Dr. F. Schnieder of the Institute of Phytopathology, University of Kiel.

For a long-term preservation, the isolates were stored at  $-70^{\circ}\text{C}$  in Skim milk. *M. graminicola* isolates were cultured on MYA plates for 5 d at  $22^{\circ}\text{C}$ , and other fungal species were grown on potato dextrose agar (PDA) plates until the mycelium covered most of the surface of plates. Five sterile 1.5-ml Eppendorf tube (five replicates) were prepared for each isolate. The initial inoculum mycelium was first removed from the Petri dishes with a sterile wire loop. After that, 7-10 ml autoclaved 10% skim milk was added. The mycelium and spores were harvested

**Table 3** Fungal species and isolates used in this research

Fungal species	Isolate	Resource	Origin
<i>Mycosphaerella graminicola</i>	Hor-116	wheat	Heideberg, Germany
	Gor-86	wheat	Göttingen, Germany
	Kor-68	wheat	Kiel, Germany
	Kor-92	wheat	Kiel, Germany
	Kor-11	wheat	Kiel, Germany
	K-Bc-105	wheat	Kiel, Germany
	K-Bc-121	wheat	Kiel, Germany
	Gor-9	wheat	Göttingen, Germany
	OK-109	wheat	Austria
	OK-102	wheat	Austria
	OK-120	wheat	Austria
	FN2	wheat	France
	FN4	wheat	France
	FC2	wheat	France
	GBW1	wheat	England
	GBE2	wheat	England
	GBE4	wheat	England
	CH2	wheat	Switzerland
	CH4	wheat	Switzerland
<i>Stagonospora nodorum</i> GB1		wheat	Germany
<i>Microdochium nivale</i> Hul4		wheat	Germany
<i>Erysiphe graminis</i> f. sp. <i>tritici</i>		wheat	Germany
<i>Macrophomina phaseolina</i> *		cotton	Egypt
<i>Pseudocercospora anguioides</i> 22b3998		wheat	Germany
<i>Pseudocercospora herpotrichoides</i> var. <i>acuformis</i>		wheat	Germany
<i>Cercospora beticola</i>		sugarbeet	Germany
<i>Phytophthora infestans</i>		potato	Germany
<i>Aspergillus</i> spp.		wheat	Germany
<i>Trichoderma harzianum</i> *		cotton	Egypt
<i>Verticillium dahliae</i> *		cotton	Egypt
<i>Septoria petroselini</i>		celery	Germany
<i>Rhizoctonia solani</i> *		cotton	Egypt
<i>Fusarium tricinctum</i>		wheat	Germany
<i>Fusarium oxysporum</i> f.sp. <i>vasinfectum</i> *		cotton	Egypt
<i>Fusarium avenaceum</i> Rg 7/5		wheat	Germany
<i>Fusarium culmorum</i> 7-LBP		wheat	Germany
<i>Fusarium graminearum</i> G4.1		wheat	Germany
<i>Penicillium</i> spp.		wheat	Germany
<i>Sclerotium</i> spp.		wheat	Germany
<i>Botrytis</i> spp.		wheat	Germany
<i>Cladosporium</i> spp.		wheat	Germany
<i>Mucor</i> spp.		wheat	Germany
<i>Alternaria alternata</i>		wheat	Germany
<i>Rhizopus</i> spp.		cotton	Egypt

\*These isolates were kindly provided by Dr. K.A. Abd-Elsalam in Egypt.

by scraping gently with a stainless steel stalk and mixed well with skim milk. One ml of mycelium mixture was transferred to each Eppendorf tube. The tubes were closed and placed in liquid nitrogen in an Air-Liquide Nicoool LM-10 freezing machine (AIR LIQUIDE GmbH, Düsseldorf, Germany) for 15 min until they were completely frozen. Finally, isolates were preserved in a cryo-refrigerator at  $-70^{\circ}\text{C}$ .

For a short-term storage of three to six months, the fungi were cultured on PDA plates (*M. graminicola* on MYA plates) until well grown. The plates were sealed up with parafilm and stored at  $4^{\circ}\text{C}$ .

### Culture of fungal species and isolates

The isolates preserved at  $-70^{\circ}\text{C}$  were taken out and first grown on PDA plates at  $22^{\circ}\text{C}$  for 5-7 d. Then several pieces of mycelium and spores were carefully scraped up into 30 ml autoclaved liquid medium with a sterile steel loop. *M. graminicola* was cultured in malt-yeast broth (MYB) liquid medium and the other species in potato dextrose broth (PDB) liquid medium on a shaker at  $22^{\circ}\text{C}$  and 120 rpm. *M. graminicola* was harvested after 5 d by centrifuging at 8,000 *g* for 2 min and then washed once with sterile distilled water. Other species were harvested after 5-15 d by filtering and finally removing residual water with filter papers and stored at  $-20^{\circ}\text{C}$ .

## **2.3 DNA and RNA extraction**

### **2.3.1 DNA extraction from fungal species**

#### DNA extraction using DNAzol reagent

DNA extraction from different fungi using DNAzol reagent (Molecular Research Center, Cincinnati, OH, USA) was performed as described by Guo *et al.* (2005). Fifty mg fresh or 5 mg freeze-dried mycelium ground in liquid nitrogen were put into a pre-cooled 2-ml Eppendorf tube, resuspended and lysed in 1 ml DNAzol reagent by inverting 5-6 times. The mixture was incubated for 10 min at room temperature and shaken every 3 min. To eliminate more efficiently tissue debris and traces of phenol, proteins and lipids, an additional elution step was performed by adding 500  $\mu\text{l}$  chloroform. After a 10-min centrifugation at 12,000 *g* and  $4^{\circ}\text{C}$ , the supernatant was transferred carefully into a new 1.5-ml Eppendorf tube. Then 0.5 ml 100% ethanol per ml lysate was added, mixed by inverting 5-6 times and stored for 3 min at room temperature. To increase DNA yield, the mixture was centrifuged for 5 min at

a higher speed of 15,000 *g*. The pellet was first washed with 500  $\mu$ l of 70% (v/v) DNAzol reagent and 30% (v/v) ethanol and then with 500  $\mu$ l 70% (v/v) ethanol. The remaining supernatant was discarded completely with a pipette after an additional 1-min centrifugation. The resulting pellet was subsequently exposed to ambient air for 10 min and dissolved in 100  $\mu$ l sterile water. The DNA yield was measured by Hoeffer<sup>®</sup> DyNA Quant<sup>®</sup> 200 Fluorometer (Amersham Pharmacia Biothech, Uppsala, Sweden) according to the manufacturer's protocol. The purity was evaluated by  $A_{260}/A_{280}$  tested with a Unikon-942 spectrophotometer (Kontron Instrument Inc., Montigny le Bretonneux, France) and by electrophoresis on a 1% (w/v) agarose gel.

#### Fungal DNA extraction using Cenis (1992) method

The extraction was carried out as described by Cenis (1992) with a slight modification. Fifty mg mycelium homogenized with liquid nitrogen was put into a 1.5-ml sterile Eppendorf tube. Four hundred  $\mu$ l DNA extraction buffer 1 (see section 8.1.2, the same below) and 6  $\mu$ l 10  $\mu$ g/ $\mu$ l RNase A were added and mixed well by shaking vigorously. The mixture was incubated for 10 min at 65°C in a water bath. After that, 130  $\mu$ l 3 M sodium acetate (pH 5.2) were added and tubes were placed at -20°C for about 10 min and then centrifuged for 5 min at 15,000 *g*. The upper aqueous phase was transferred into a fresh centrifuge tube and an equal volume of isopropanol was added. The microtube was stored at room temperature for 5 min. The precipitated DNA was pelleted by centrifugation for 10 min at 15,000 *g*. The pellet was washed twice with 500  $\mu$ l 70% (v/v) ethanol, vacuum dried for 15 min, and resolved in 100  $\mu$ l TE buffer.

#### A simple modified CTAB-PVP method

For a miniprep DNA extraction, 100 mg mycelium-PVP mixture homogenized as described in section 2.3.2 were transferred into a 2-ml microtube. Five hundred  $\mu$ l heated DNA extraction buffer 2 (see section 8.1.2, 80°C) were added and mixed well by shaking vigorously. Subsequently, 500  $\mu$ l PCI (phenol (pH 7.9):chloroform:isoamyl alcohol 25:24:1) were added, strongly mixed and then centrifuged for 10 min at 15,000 *g*. The aqueous phase was transferred to a new microtube and an equal volume of isopropanol was added. The mixture was well mixed by inverting several times and centrifuged for 10 min at 15,000 *g*. The DNA pellet was washed twice with 70% (v/v) ethanol, vacuum dried and resolved in 100  $\mu$ l TE buffer containing 40 ng/ $\mu$ l RNase A.

DNA extraction from large amount of fresh mycelium and spores was also performed using the same protocol as described in section 2.3.2.

### **2.3.2 DNA extraction from infected wheat leaves**

2.5-3 g healthy or infected wheat leaves were first ground to powder in liquid nitrogen, and then mixed with 0.6 g insoluble polyvinylpyrrolidone (PVP) (molecular weight 36,000, Sigma) and 1 g NaCl. The sample was further ground with liquid nitrogen and transferred to a pre-chilled 50 ml-centrifuge tube. 10 ml pre-warmed DNA extraction buffer 2 (80°C) were added and mixed well by vortexing. 5 ml PCI were added, stirred strongly and vortexed until a complete emulsion was formed, and then centrifuged for 10 min at 10,000 g, 4°C. Eight ml of aqueous phase were transferred to a new 10 ml tube (an additional elution with 5 ml PCI is necessary for dried or severely diseased leaves). Subsequently, an equal volume of isopropanol was added, mixed well by inverting 5-6 times, and centrifuged for 15 min at 10,000 g, 4°C. The pellet was washed with 1 ml 70% ethanol and transferred into a 1.5-ml Eppendorf tube, and centrifuged for 5 min at 15,000 g. The pellet was washed with 500 µl 70% ethanol and centrifuged for 5 min at 15,000 g. The pellet was dried for 20 min in vacuum and resolved in 200 µl TE buffer containing 40 ng/µl RNase A. The DNA yield was measured with a Hoeffer<sup>®</sup> DyNA Quant<sup>®</sup> 200 Fluorometer, and the purity was roughly assessed by electrophoresis on a 1% agarose gel.

The agarose gel was used for the assessment of DNA or PCR products. 1-2% agarose gel was made depending on the size of tested fragments and different use requirements. A proper amount of 100% agarose was weighed, put in 1x TAE buffer and melted completely by heating in a microwave. After the melted agarose was cooled down to about 60°C, 5 µl/100 ml etidium bromide (10 mg/ml) were added, mixed well and poured in the gel casting tray with a comb. The gel was cooled and solidified at room temperature for about 20 min. The apparatus was then filled with 1x TAE buffer until it covered completely the gel and an appropriate amount of the samples were loaded onto the gel. The samples were first mixed with 1/6 volume of DNA loading buffer and added into the gel well. The electrophoresis was run in 1x TAE buffer for 60-90 min at 60-100 volt (depending on the gel size and length). After that, the gel was photographed using the GelSystem Flexi 4,000 (Biostep, Jahnsdorf, Germany). The images were processed for optimal contrast and size cutting with Corel Photo-Paint software (Corel Corporation, Unterschleissheim, Germany).

### 2.3.3 RNA extraction

#### Extraction of total RNA from fungi and wheat leaves

Total RNA was extracted as described by Guo *et al.* (2003). Briefly, two grams of fresh mycelium or wheat leaves were first homogenized into powder in liquid nitrogen, then 2.5 ml homogenization buffer (4 M guanidium isothiocyanate, 100 mM Tris-HCl (pH 7.0)), 0.9 g NaCl and 0.6 g insoluble PVP-36 were added. The sample was further ground with liquid nitrogen and then transferred to a prechilled 50 ml centrifuge tube. Subsequently, 5 ml prewarmed extraction buffer (2% CTAB, 100 mM Tris-HCl (pH 7.0), 25 mM EDTA and 2M NaCl) were added and well vortexed. The mixture was eluted 2-3 times with 5 ml acidic phenol (pH 4.5-5):chloroform:isoamylalcohol (25:24:1). The supernatant was precipitated with ¼ volumes 10 M LiCl overnight at 4°C and harvested by centrifugation at 15,000 g and 4°C for 30 min. The RNA pellet was first washed with 1 ml 2 M LiCl and then washed two times with 70% ethanol, dried in vacuum and resolved in 100 µl DEPC-treated water. The quantity and purity of the RNA was spectrophotometrically tested at wavelengths of 210, 230, 260, 280 nm using a Unikon-942 spectrophotometer and electrophoresed on 1% agarose gel in 1x TBE buffer.

For electrophoresis, the gel was prepared as described in section 2.3.2 except that 1x TBE buffer was used instead of 1x TAE buffer. The RNA samples were denatured before loaded on the gel. Five µl RNA samples (2.5 µg) (for RNA ladder: 3 µl RNA ladder, 2 µl DEPC-treated H<sub>2</sub>O) were mixed with 5 µl RNA loading buffer 1, denatured for 10 min at 65°C in a water bath, and cooled immediately on ice. The denatured sample was loaded and run on the gel for 60-90 min in 1x TBE buffer at 70 volt. The gel was photographed as described in section 2.3.2.

#### mRNA extraction from total RNA

mRNA was extracted from 500 µg total RNA using an Oligotex<sup>TM</sup> mRNA Kit (Qiagen, Hilden, Germany). Briefly, total RNA was mixed with 500 µl DEPC-treated water, 500 µl 2x Binder Buffer and 30 µl Oligotex suspension. The mixture was incubated for 3 min at 65°C, then stayed for 10 min at room temperature and centrifuged for 2 min at 15,000 g. The pellet was resuspended in 650 µl Wash Buffer OW<sub>2</sub>, pipetted into a spin column and centrifuged for 15,000 g for 30 s. The pellet was further washed with 650 µl Wash Buffer OW<sub>2</sub> as described above. mRNA was finally eluted with 30 µl preheated (70°C) 5 mM Tris-HCl (pH 7.5) and stored at -70°C.



## **2.4 Isolation of *M. graminicola*-specific sequences and design of specific primers**

### **2.4.1 Search of *M. graminicola*-specific sequences in public gene banks**

A 574-bp rDNA sequence (AJ300330) and a 768-bp Act1 gene sequence (AJ300310) of *M. graminicola* were first identified in the NCBI-GenBank (<http://www.ncbi.nlm.nih.gov/>). After that, a BLAST search was further performed in this gene bank with the two target sequences. According to the search results, the *M. graminicola*-specific subsequences in the target sequence were identified and used for the design of specific primers. Primers were designed using the internet programme Primer3 ([http://frodo.wi.mit.edu/cgi-in/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-in/primer3/primer3_www.cgi)).

### **2.4.2 Isolation of *M. graminicola*-specific fragments by RAPD-PCR**

#### **2.4.2.1 RAPD-PCR**

First, a primary screening was performed by PCR using 80 random primers listed in Table A1 with genomic DNA of three *M. graminicola* isolates GBW1, CH2, FN2, and *S. nodorum*. Second, those primers which gave rise to clear fingerprints and amplified differential DNA fragments from *M. graminicola* were selected for a further test by RAPD-PCR with *M. graminicola* GBE2 and Gor-92, *S. nodorum*, *F. culmorum*, *M. phaseolina*, *F. poae*, *M. nivale* and *P. anguoides*. The 25 µl PCR reaction mixture was composed of 10 ng purified DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton<sup>®</sup> X-100 and 2 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 1U *Taq* DNA polymerase and 0.6 µM primer. The PCR was carried out in a Primus 96 Plus thermocycler (MWG Biotech, Esbersberg, Germany) under the following conditions: initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 1.5 min with a final extension at 72°C for 10 min. PCR products were analyzed on a 2% (w/v) agarose gel. Those *M. graminicola*-specific DNA fragments were cut out from the gel, isolated, cloned and finally sequenced.

#### **2.4.2.2 Isolation of DNA fragments from agarose gel**

DNA fragments from agarose gel were isolated using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The DNA fragment was excised from the agarose gel using a clean, sharp scalpel, with minimized gel slice by removing extra agarose. The gel slice was weighed in a colourless tube. Three volumes of Buffer QG were added to 1 volume of gel (100 mg ~ 100 µl). The mixture was incubated at 50°C for 10 min (or until the gel slice has completely dissolved) and mixed by vortexing the tube every 2–3 min during the incubation. After the gel slice has dissolved completely, one equal volume of isopropanol was

subsequently added to the sample and mixed. To bind DNA, the sample was transferred into the QIAquick column placed in a 2 ml collection tube, and centrifuged for 1 min. The liquid was discarded and the QIAquick column was placed back in the same collection tube. To remove all traces of agarose, 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 min. 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for 1 min. The liquid was discarded and centrifuged for an additional minute. After that, the QIAquick column was placed into a clean 1.5-ml microcentrifuge tube. The DNA was eluted with 50  $\mu$ l of Buffer EB (10 mM Tris·Cl, pH 8.5) and centrifuged for 1 min. To check the purity and quantity, 5  $\mu$ l of the purified PCR-product were run on a 1% (v/v) agarose gel. The quantity was roughly assessed by comparing with 50 ng and 100 ng standard  $\lambda$ -DNA (Promega, Madison, USA) on the gel. The isolated DNA fragment was stored at -20°C.

#### **2.4.2.3 Purification of PCR products**

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol before cloning and sequencing. Five volumes of Buffer PB were added to one volume of the PCR products and mixed by inverting several times. To bind DNA, the mixture was applied to a QIAquick column in a 2-ml collection tube and centrifuged for 1 min. The liquid was discarded and the QIAquick column was placed back into the same tube. The DNA on the membrane was washed with 0.75 ml Buffer PE and centrifuged for 1 min. The following steps were the same as those described in section 2.4.2.2.

#### **2.4.2.4 Cloning of DNA fragments**

The cloning was carried out using the pGEM<sup>®</sup>-T Vector Systems I (Promega, Madison, WI, USA) according to the manufacturer's protocol.

#### Preparation of competent cells

The competent cells were prepared according to the protocol described by Sambrook *et al.* (1989). The strain (*Escherichia coli* JM 109, Promega) was stored at -70°C grown on a LB agar plate overnight at 37°C. A single colony was picked with a steel loop, transferred into a 1.5-ml sterile culture tube containing 5 ml LB liquid medium and cultured overnight in a shaker incubator at 37°C, 225 rpm. One ml of the culture was transferred into a pre-warmed 50-ml Falcon tube (37°C) containing 40 ml LB liquid medium, and incubated at 37°C, 150 rpm up to OD<sub>600</sub> = 0.6 (about 9 h). After that, the culture was cooled down to -4°C,

divided to 20 ml culture per Falcon tube and centrifuged for 5 min at 3 000 g, 4°C. The pellet was resuspended with 12 ml of sterile, cold 100 mM CaCl<sub>2</sub> (4°C) and incubated on ice for 45 min. The mixture was then centrifuged for 5 min at 2,000 g, 4°C and the pellet was gently resuspended 1 ml cold and sterile Store Solution (20% (v/v) glycerol, 75 mM CaCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.0, filter sterilized, 4°C). Finally, 500 µl competent cells were added to each 1.5-ml Eppendorf tube, quickly frozen in liquid nitrogen, and stored at -70°C.

#### Ligation of insert DNA with the vector

The cloned DNA fragment (purified PCR products) was ligated with the pGEM<sup>®</sup>-T Vector. The 10µl ligation mixture contained the following components: 50 ng pGEM<sup>®</sup>-T Vector, 25-30 ng insert DNA (purified PCR product), 3 U T4 DNA ligase, 30 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM ATP, 5% (w/v) polyethylene glycol and H<sub>2</sub>O. One negative control without insert DNA was simultaneously set up. The mixture was incubated overnight at 4°C.

#### Transformation

The ligated plasmid was transformed into competent cells of *E. coli* JM109 strain. First, 5 µl ligation mixture was added to a sterile 1.5-ml microcentrifuge tube on ice. Meanwhile, another tube was set up on ice with 0.1 ng uncut plasmid for determination of the transformation efficiency of the competent cells. 60 µl of competent cells melted in an ice bath were carefully transferred into the tube containing ligation mixture, mixed by flicking gently the tube and placed on ice for 20 min. The tubes were immediately replace into ice for 2 min after heat-shocked for 45–50 s in a water bath at exactly 42°C (without shaking). 450 µl liquid SOC medium was added to the tubes and incubated for 2 h at 37°C and 150 rpm. The transformation culture was plated onto three selective LB/ampicillin/IPTG/X-Gal plates (each LB/ampicillin medium plate plated with 20 µl 50 mg/ml X-Gal and 100 µl 0.1 M IPTG). The plates were incubated overnight (16–24 hours) at 37°C. The transformed colonies were identified by white/blue colour selection. Generally, white colonies contain inserts. Four to six white colonies were further tested by PCR with the M13-uni and M13-rev primers. The clones which were confirmed to contain expected DNA inserts were further cultured overnight in 5 ml liquid LB/ampicillin medium for the extraction of plasmids. One part of culture was mixed with an equal volume of sterile glycerol and stored at -70°C.

#### 2.4.2.5 Plasmid isolation

The selected clones were incubated in 5ml liquid LB medium and cultured on a shaker overnight at 37°C and 150 rpm. Plasmid was isolated using the Silica Spin Disc Plasmid DNA Minprep Kit (Biometra, Göttingen, Germany). The cells were harvested by centrifugation at 7,500 *g* for 1 min and the pellet was resuspended in 200 µl P1 buffer. Subsequently, 200 µl P2 buffer was added and mixed gently by inverting 4-6 times. 200 µl P3 buffer was added, mixed immediately by gently inverting 4-6 times and centrifuged for 5 min at room temperature. The supernatant was transferred into a spin filter in a 1.5-ml Eppendorf tube and centrifuged 1 min to remove the liquid. The DNA pellet on the filter was first washed with Buffer W1 and further with Buffer W2. After 1-min additional centrifugation, the spin filter was placed in a clean 1.5-ml Eppendorf tube. The plasmid DNA was eluted with 100 µl TE buffer (pH8.0) by centrifugation for 30 s, and stored at -20°C.

#### 2.4.2.6 Sequencing of DNA fragments and primer design

The cloned DNA fragments were reamplified from the relevant plasmid by PCR with M13-uni and M13-rev primers. The 25 µl reaction mixture contained 1 µl purified plasmid DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton<sup>®</sup> X-100 and 2 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 1U *Taq* DNA polymerase, 0.4 µM of M13-uni and M13-rev primer. The PCR was carried out under the following conditions: initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min with a final extension at 72°C for 5 min. PCR products were loaded on a 1 % (w/v) agarose gel. The target fragment was cut out of the agarose gel and was purified according to the method described in section 2.4.2.3, and then sent to the Institute of Biology and Genetics, Humboldt University of Berlin for sequencing. The sequencing data were analysed using DNASTAR software (DNASTAR, Inc., Madison, WI, USA). First, a consensus sequence was obtained based on the original forward and reverse sequences, and then the plasmid sequences were eliminated from this consensus sequence. Finally, two positions of the random primer were identified. The analysed sequences were further used for Primer design which was performed with the help of an Internet programme Primer3 ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)).

### 2.4.3 cDNA-AFLP and AFLP

#### 2.4.3.1 cDNA synthesis

100 µg total RNA was treated with 5 U RQ1 RNase-free DNase (Promega) to remove DNA contamination before cDNA synthesis according to the manufacturer's instructions. The efficiency of DNase treatment was tested by PCR with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and the RNA quantity was remeasured spectrophotometrically at 260 nm.

First-strand of cDNA was synthesized using the M-MLV reverse transcriptase (Promega). 30 µg DNase-treated total RNA or 2 µg mRNA was mixed with 1.5 µg of the oligo (dT)<sub>15</sub> primer in a total volume of 28 µl. After denaturation at 70°C for 5 min, the following components were added: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 500 µM of each dNTP, 25 U RNasin<sup>®</sup> ribonuclease inhibitor (Promega) and 300 U M-MLV RT in a final volume of 50 µl. The samples were gently mixed and incubated for 60 min at 42°C.

The synthesis of second-strand was performed according to the standard protocol (Sambrook *et al.* 1989) with a slight modification. The following components were added to the first-strand solution: 50 mM Tris-HCl (pH 7.2), 10 mM MgSO<sub>4</sub>, 100 µM DTT, 25 U DNA polymerase I (Promega), 10 U RNase H, 150 µM of each dNTP and H<sub>2</sub>O in a final volume of 150 µl. The components were gently mixed and incubated at 14°C for 3 h. Then 10 µg ATP, 6 U T4 DNA ligase and 5U T4 polynucleotide kinase were added and incubated at RT for 15 min. Finally, 5 µl 0.5 M EDTA (pH 8.0) were added to terminate the reaction.

To extract the double-stranded cDNA, an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol was added, mixed thoroughly, and centrifuged for 5 min at 15,000 g, 4°C. The aqueous phase was transferred to a new tube, 0.1 volume of 3 M sodium acetate (pH5.2) and two volumes of cold 99.9% ethanol were added and mixed well by inverting several times. The mixture was placed at -70°C for 30 min and then centrifuged for 5 min at 15,000 g, 4°C. The pellet was washed with 70% (v/v) ethanol, vacuum dried and dissolved in 30 µl sterile Milli-Q water. The cDNA concentration was evaluated by a Hoeffler<sup>®</sup> DyNA Quant<sup>®</sup> 200 Fluorometer.

### 2.4.3.2 AFLP

The AFLP procedure was performed as described by Vos *et al.* (1996). Fingerprint patterns were visualized using silver staining.

#### Restriction

About 300 ng extracted cDNA or DNA was used for the restriction reaction with 5 U *EcoRI* and 3 U *MseI*. The restriction reaction mix was composed of 8 µl 5x RL-buffer, 300 ng extracted DNA or cDNA, 5 U *EcoRI* and 3 U *MseI* in a final volume of 40 µl, and incubated at 37°C for 2 h in a thermocycler.

#### Ligation

The *EcoRI*- and *MseI*-adapters were first denatured at 95°C for 5 min before added to the ligation reaction. The ligation reaction mixture which was composed of 2 µl of 5x RL-buffer, 1U T4-DNA Ligase, 1 mM ATP (always fresh prepared), 5 pmol of denatured *EcoRI*-adapter and 50 pmol of denatured *MseI*-adapter in a total volume of 10 µl was put into the restriction mixture and incubated for 3 h at 37°C in a thermocycler. The reaction mixture was then diluted with 150 µl 0.1x TE-buffer before pre-amplification.

#### Pre-amplification

Pre-amplification was performed using *EcoRI*-0/*MseI*-0 (for cDNA) or *EcoRI*-A/*MseI*-A (for genomic DNA). The PCR reaction was composed of 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCl, 2 mM MgSO<sub>4</sub>, 0.1% (v/v) Triton X-100, pH 8.8, 5 µl of diluted restriction/ligation mixture, 200 µM of each dNTP, 0.3 µM of each primer and 1 U *Taq* polymerase (Biolabs, Beverly, MA, USA) in a total volume of 20 µl. The conditions were 20 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 90 s.

#### Selective amplification

The pre-amplification products were diluted 2.5-10 folds with 0.1x TE buffer before selective amplification. The primer pairs used are listed in Table A2. The reaction mixture contained 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCl, 2 mM MgSO<sub>4</sub>, 0.1% (v/v) Triton X-100, pH 8.8, 5µl of diluted pre-amplification product, 200 µM of each dNTP, 0.3 µM of each primer and 1 U *Taq* polymerase (Biolabs) in a total volume of 20 µl. The reaction conditions were 30 s of denaturation at 94°C; 9 cycles of 30 s at 94°C, 30 s at 65°C (annealing temperature was subsequently reduced each cycle by 1°C) and 60 s at 72°C; 25 cycles of

30 s at 94°C, 30 s at 56°C and 60 s at 72°C; 15 min at 72°C. The PCR products were analysed by electrophoresis on a 5% denaturing polyacrylamide gel.

The gel was prepared as follows. First, a glass plate sandwich was constructed. The glass plates and spacers were cleaned with 99.9% ethanol. The long glass was first placed on the bench, then the spacers (0.4 mm) were positioned along the edge of glass plate. Subsequently, the long glass plate was placed onto the spacers. The sandwich was then fixed with clamps. A 5% polyacrylamide mix (70 ml Sequence Verdünner buffer, 20 ml Sequencing Konzentrate, 10 ml Sequence buffer) was prepared. To avoid the gel to flow out from the glass plates, a rapid-solidifying mix (10 ml 5% (w/v) polyacrylamide mix, 500 µl APS, 25 µl TEMED) was first prepared and immediately poured into the glass plate sandwich. After it was solidified, the rest 5% (w/v) polyacrylamide mix was mixed with 500 µl 10% (w/v) APS and 40-45 µl TEMED, and poured into the sandwich. The comb was then placed into the gel. The gel was solidified for 1-1.5 h at room temperature.

The DNA samples were denatured with deionised formamide before loading on the gel. 5 µl PCR products were mixed with 5 µl loading dye (98 ml formamide, 2 ml 0.5 M EDTA (pH 8.0), 0.025% orange G, 0.025% (w/v) bromophenolblue), denatured for 3 min at 95°C in a thermocycler and immediately cooled on ice. The denatured PCR products were analysed by electrophoresis on a 5% denaturing polyacrylamide gel and 1x TBE buffer at 50 watt for ~3 h. The gel was stained with silver nitrate as described by Creste *et al* (2001).

## **2.5 Specificity and sensitivity test of designed primer sets**

### **2.5.1 Conventional PCR**

The specificity of designed primers was tested by PCR with three screening steps. First, the primer pairs were tested with genomic DNA of *M. graminicola* and healthy wheat plants. Second, those primer pairs which were not amplified from healthy plant DNA but only from *M. graminicola* DNA were further tested with genomic DNA of *M. graminicola* OK-102, and seven other fungal pathogens (*S. nodorum*, *M. nivale*, *F. culmorum*, *F. graminearum*, *M. phaseolina*, *P. herpotrichoides*, *R. solani*). Finally, the specificity of those primer pairs selected from the second screening was further tested with 14 *M. graminicola* isolates collected from different regions, 22 other fungal pathogens (Table 3), *M. graminicola*-inoculated plants and healthy plants. The sensitivity of specific primer sets

was tested with 100 ng, 10ng, 1ng, 100 pg, 50pg, 20 pg, 10 pg, 5 pg, 2 pg, 1pg, 0.5 pg, 0.2 pg and 0.1 pg of *M. graminicola* genomic DNA.

The PCR reaction consisted of 5 ng genomic DNA (plant DNA, 25 ng), 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCl, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, pH 8.8, 200 μM of each dNTP, 0.4 μM of each primer and 1 U *Taq* polymerase (Biolabs) in a total volume of 25 μl. For the primer set E1/STS2R and STIF2/BAF4ST, the PCR was performed in a PTC-200 Peltier thermal cycler (MJ Research, Inc., Waltham, MA, USA) with the PCR programme as described by Fraaije *et al.* (1999). For the other tested primer sets, the PCR was performed under the following conditions: 2 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C. Twenty μl of PCR products were analysed on a 2% ethidium bromide-stained agarose gel.

### 2.5.2 RT-PCR

The specificity of developed primer sets was tested by one-step RT-PCR using a commercial kit of “Acess RT-PCR Introductory System” (Promega). The sensitivity was tested by one-tube RT-PCR as well as by two-step RT-PCR. The reaction mixture of one-tube RT-PCR was composed of 500 pg to 2 μg total RNA, 10 mM Tris-HCl (pH 8.8), 3 mM MgCl<sub>2</sub>, 0.3% (v/v) Triton X-100, 30 pmol of each primer, 200 μM of each dNTP, 5 U AMV reverse transcriptase and 5 U *Tfl* DNA polymerase in a final volume of 50 μl. The reaction conditions were 45 min at 48°C, 2 min at 94°C, 40 cycles of 30 s at 94°C, 1 min at 60°C and 2 min at 68°C, followed by a final step of 7 min at 68°C. Twenty μl RT-PCR products were analysed on a 2% agarose gel with ethidium bromide staining.

For two-step RT-PCR, the first strand of cDNA was synthesized according to the following reaction: 2 μg total RNA of *M. graminicola*, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 500μM dNTP, 25 U rRNasin<sup>®</sup> Ribonuclease inhibitor, 0.6 μM of each specific primer, 100 U M-MLV reverse transcriptase (Promega) in a final volume of 25 μl. The reaction was incubated for 60 min at 42°C. The cDNA solution was first diluted in concentration series equal to 100 ng, 10 ng, 1 ng, 100 pg, 50 pg, 10 pg, 5 pg and 1 pg total RNA respectively and added to the 25-μl PCR reactions. The PCR was performed as described in the section 2.5.1. Twenty μl PCR products were analysed on a 2% (w/v) agarose gel using ethidium bromide staining.



## 2.6 Optimization of PCR conditions of specific primer sets

### 2.6.1 Magnesium concentration

To test the influence of magnesium concentration on the PCR reaction of specific primer sets, eight different concentrations of MgCl<sub>2</sub> (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM) were used. The PCR mixture was composed of 100 pg genomic DNA of *M. graminicola*, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton<sup>®</sup> X-100, 0.5-4.0 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.4 µM of each primer and 1U *Taq* DNA polymerase (Promega) in a total volume of 25 µl. The PCR reaction was run in a PTC-200 Peltier thermal cycler under the following conditions: 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 60°C and 45 s at 72°C. Twenty µl of PCR product were loaded on a 2% (w/v) agarose gel.

### 2.6.2 Different annealing temperatures

To get the optimum annealing temperature of each specific primer set, a PCR was performed with 12 different annealing temperatures of 50.1°C, 50.5°C, 51.3°C, 52.7°C, 54.4°C, 56.4°C, 58.3°C, 60.3°C, 62.6°C, 64.0°C, 64.7°C and 65.2°C in a Px2 gradient PCR thermal cycler (Thermo Hybaid, Ashford, United Kingdom). 200 pg genomic DNA was used and the other conditions were the same as those in section 2.5.1.

### 2.6.3 Different PCR programmes

To obtain a simple and efficient PCR programme for each developed primer set, eight different PCR programmes were tested in a PTC-200 Peltier thermal cycler (Table 4). The PCR components and reaction conditions are the same as those in section 2.6.1.

**Table 4** Eight different PCR programmes tested and their duration

Programme No.	Programme	Duration
1	2 min at 94°C, 40 cycles of 15 s at 94°C, 15 s at 60°C and 30 s at 72°C	1 h 10 min
2	2 min at 94°C, 40 cycles of 15 s at 94°C, 15 s at 60°C and 45 s at 72°C	1 h 20 min
3	2 min at 94°C, 40 cycles of 15 s at 94°C, 15 s at 60°C and 60 s at 72°C	1 h 32 min
4	2 min at 94°C, 40 cycles of 15 s at 94°C, 30 s at 60°C and 30 s at 72°C	1 h 20min
5	2 min at 94°C, 40 cycles of 15 s at 94°C, 30 s at 60°C and 45 s at 72°C	1 h 34 min
6	2 min at 94°C, 40 cycles of 15 s at 94°C, 30 s at 60°C and 60 s at 72°C	1 h 43 min
7	2 min at 94°C, 30 cycles of 15 s at 94°C, 15 s at 60°C and 45 s at 72°C	1 h 6 min
8	2 min at 94°C, 30 cycles of 15 s at 94°C, 30 s at 60°C and 45 s at 72°C	1 h 12 min

## 2.7 Non-radioactive Northern hybridization

To test the expression level of the  $\beta$ -tubulin, actin and rRNA genes corresponding to three specific primers in *M. graminicola* tissues with different culture time, a non-radioactive DIG Northern hybridization was performed using total RNA and digoxigenin-labelled DNA probes.

### 2.7.1 Culture of *M. graminicola* and total RNA extraction

*M. graminicola* Kor-102 was cultured with seven different culture time of 3, 5, 7, 9, 12, 13 and 15 days. The isolate was first grown on a MYA plate for five days at 22°C. Some conidial culture was scraped with a sterile steel loop, put into 1 ml sterile water and mixed thoroughly. 200  $\mu$ l of the conidial suspension were added to 30 ml autoclaved liquid MYB medium and cultured on a shaker at 22°C, 120 rpm. *M. graminicola* was harvested after cultivation by centrifugation for 2 min at 8,000 g and then washed once with sterile distilled water. The fresh cultured cells were immediately used for total RNA extraction. The extraction was performed as described in section 2.3.3.

### 2.7.2 Preparation of the digoxigenin-labelled probes by PCR

The digoxigenin-labelled probes were prepared by PCR according to the manufacturer's instruction with a slight modification. The PCR was performed using genomic DNA of *M. graminicola* as template, and three primer sets E1/STS2R (for  $\beta$ -tubulin gene), St-act F/R (for actin gene) and St-rRNA F/R (for rRNA gene). The PCR solution contained 10 ng genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton<sup>®</sup> X-100, 1.5 mM MgCl<sub>2</sub>, 5  $\mu$ l of DIG DNA Labelling Mix (Roche Molecular Biochemicals, Mannheim, Germany) (for control reaction with 200  $\mu$ M of each dNTP), 0.4  $\mu$ M of each primer and 1 U *Taq* DNA polymerase (Promega) in a total volume of 25  $\mu$ l. The PCR was run in a PTC-200 Peltier Thermal Cycler (MJ Research) under the following conditions: 5 min at 95°C, 40 cycles of 30 s at 95°C, 60 s at 56°C and 120 s at 72°C. To assess the concentration and quality of synthesized probes, 3  $\mu$ l of each PCR product were loaded on a 1% (w/v) agarose gel by ethidium bromide staining and run for ca. 60 min at 70 volt. The concentration of probes was roughly assessed by comparing the band intensity with the 500 bp band of the 100 bp DNA ladder (MBI Fermentas, St. Leon-Rot, Germany).

### **2.7.3 Northern hybridization**

Northern hybridization was performed according to the manufacturer's protocol with a slight modification. It was composed of four major steps: electrophoresis of total RNA, Northern transfer, hybridization with DIG EasyHyb and chemiluminescent detection.

#### Preparation of experimental instruments and chemical solutions

In order to obtain satisfactory results, it is necessary that all the processes of Northern hybridization are carefully carried out under a RNase-free condition. Therefore, all glassware was baked in an oven at 230°C for 6 h. The plasticware such as the bottle tops, conical tubes and tips were soaked in 0.1% (v/v) DEPC for at least 4 h at 37°C and autoclaved for 30 min. All electrophoresis mode, comb, chamber and lid were soaked in 0.1% (w/v) SDS solution for 2 h and then cleaned with DEPC-treated water before use. All the chemical solutions were prepared with the DEPC-treated water and then treated with DEPC whenever possible.

#### Electrophoresis of total RNA

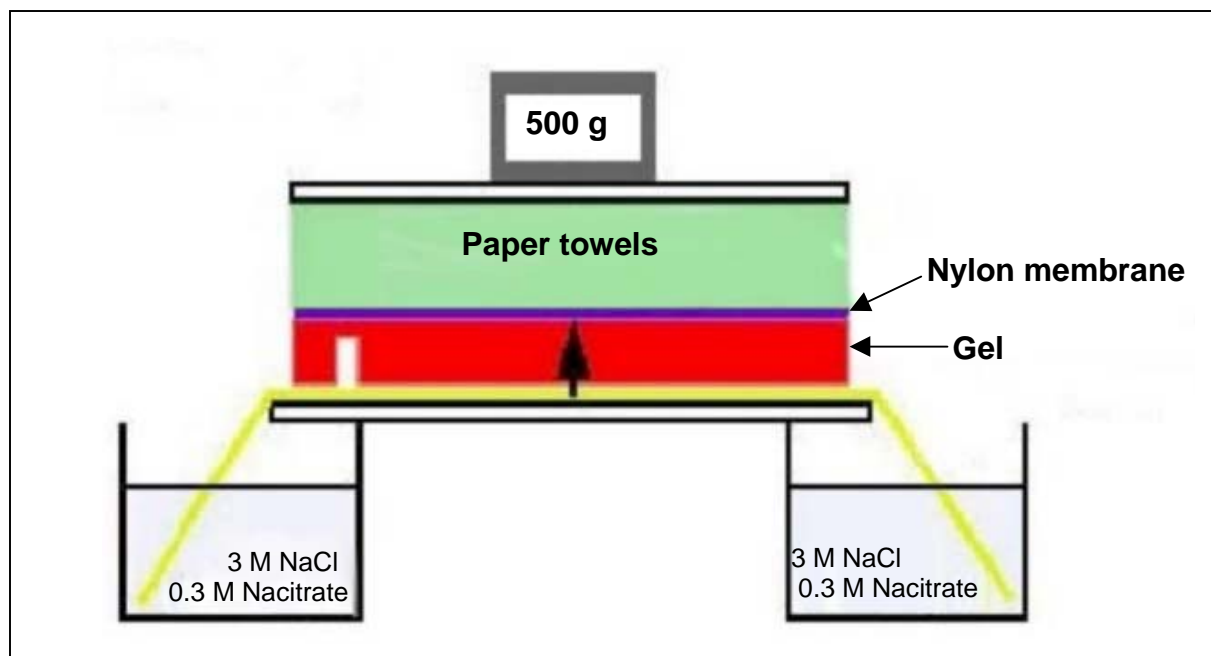
First, a 1% (w/v) formaldehyde-denatured agarose gel was prepared as follows: 1 g of agarose and 94.5 ml 1 x MOPS buffer was poured into a RNase-free bottle and melted in a microwave. The gel was cooled down to about 60°C, 5.5 ml of prewarmed 37% formaldehyde were added in the fume hood, and poured into the gel casting tray with the comb. The gel was cooled and solidified at room temperature about 30 min.

The RNA samples were diluted to 1 µg/µl with DEPC-treated H<sub>2</sub>O. 6 µl (3-6 µg) total RNA (for RNA ladder 3 µl +3 µl H<sub>2</sub>O) was mixed with 12 µl RNA loading buffer 2 and denatured for 10 min at 65°C in a water bath, cooled immediately on ice, and centrifuged for a short time. The samples were loaded into the gel wells, and 1x MOPS buffer was put into the chamber (without covering the gel). The gel was first run at 80 V for 8 min or until RNA sample ran in gel, then 1x MOPS buffer was added over the gel, and went on running the gel at 35 volt for ca. 4.5 h until the tracking dye migrated about 2/3 of the gel. The gel was photographed and used for the following Northern transfer step.

#### Northern transfer

The gel was soaked twice (2x 15 min) in 20x SSC to remove formaldehyde before the transfer was set up. Six sheets of 3 MM paper (the size was a little bigger than the gel) and one piece of positively charged Nylon membrane (Roche, Mannheim, Germany) cut to the size of the

gel were prepared. A blot transfer was set up as follows (Fig. 2): a 20 x 40 cm Whatman 3 MM paper soaked with 20x SSC was placed on a “bridge” and its two edges were put in a shallow reservoir of 20x SSC. Then the gel was put on the Whatman 3 MM paper. All air bubbles between the gel and paper were removed by rolling with a sterile glass tube. Subsequently, a Nylon membrane was placed carefully on the gel. The air bubbles were eliminated as above. The blot assembly was completed by press with two dry sheets of Whatman 3 MM paper and a stack of paper towels cut to the size of gel, a glass plate, and a 500-g weight. After the overnight transfer, the position and orientation of the wells was marked on the membrane and it was rinsed briefly in 2x SSC. The membrane was wrapped between two sheets of Whatmann 3 MM paper, baked for 30 min at 120°C and then used for pre-hybridization and hybridization.



**Fig. 2** A schematic presentation of Northern transfer blot construction

#### Hybridization using DIG EasyHyb

The membrane was put into a new sterile glass roller and 20 ml pre-warmed DIG Easy Hyb was added. First, a pre-hybridization was performed. The membrane was evenly soaked by inversion in the hybridization oven for 1 h at 50°C. After that, the pre-hybridization solution was poured out and 10 ml EasyHyb solution with 250–500 ng denatured DIG-labeled DNA probe were added. The membrane was hybridized overnight at 50°C, then washed twice for

5 min with 20 ml 2x SSC, 0.1% (w/v) SDS at 50°C and twice for 15 min with 20 ml 0.1x SSC, 0.1% (w/v) SDS at 50°C.

#### Chemiluminescence detection

The membrane was first rinsed in Tween washing buffer for 1 min at RT, then incubated in blocking buffer for 30 min at RT and finally incubated in 10 ml anti-DIG-AP solution for 30 min at RT. Subsequently, the membrane was washed 2x 15 min with washing buffer at RT, equilibrated in detection buffer for 5 min and finally carefully transferred onto a new plastic foil. After CSPD buffer was dropped onto the membrane (20-30 droplets/100 cm<sup>2</sup> membrane), the membrane was covered with this foil without air bubbles and incubated for 5 min at RT. The excess CSPD buffer was eliminated. The membrane was immediately covered with another clean plastic foil (without the membrane drying), and incubated at 37°C for 15 min. To detect the chemiluminescent signal, the membrane was exposed to Lumi-Film (Roche). The exposure time was from 5 min to several hours, depending on the signal strength.

## **2.8 Real-time PCR**

### **2.8.1 Preparation of DNA samples**

The samples of inoculated plants were prepared as described in section 2.1.1 and the field samples were collected as described in section 2.1.2. *M. graminicola* DNA was extracted according to the method described in section 2.3.1. DNA of healthy and infected leaves was prepared according to the protocol described in section 2.3.2. The plant DNA was adjusted to 25 ng/μl before being used for quantification of real-time PCR.

### **2.8.2 Real-time PCR using the LightCycler system**

Real-time PCR was performed using the QuantiTect™ SYBR® Green PCR Kit (Qiagen) and the LightCycler system (Roche) according to the manufacturer's instructions. The QuantiTect™ SYBR® Green PCR Kit is a ready-to-use kit. A 2x QuantiTect SYBR Green PCR Master mix contains HotStarTaq DNA polymerase, buffer (Tris-HCl, KCl, (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, 5mM MgCl<sub>2</sub>, pH 8.7 (20°C)), dNTP mix and SYBR Green I.

First, all relevant parameters in the LightCycler system were defined before preparation of PCR reactions. All the functions including the machine operation, PCR reaction process,

signal acquisition as well as data analysis were controlled by a computer using the LightCycler Software version 3.5. To start a run, the LightCycler Software was opened and the run module was selected after the computer and the LightCycler machine were switched on. The machine started to run and automatically checked the system. The fluorimeter gains were automatically adapted for the fluorescence channel F1/1.

To perform a LightCycler run, a cycle programme suitable for certain primer pairs which include an initial denaturation, PCR amplification with repeated cycles of denaturation, annealing and elongation, melting curve and cooling step was first filled in the Programming Screen of the LightCycler Run Software. The cycle programmes for four different primer sets used in this research are outlined in Table A3. In order to suppress fluorescence readings caused by primer-dimers or non-specific products, an additional data acquisition step was always added to the cycle programme. The acquisition temperature was determined according to the results of a melting curve analysis. It was above the melting temperature ( $T_m$ ) of primer-dimers or non-specific products but at least 3°C below the  $T_m$  of the target PCR product. The defined programme could be saved in the computer for next use. Subsequently, the sample information such as sample carousel position, name, type, replicates and concentration was entered in the Loading Screen window of sample data before a run was started.

A 18 µl PCR master mix consisting of 10 µl 2x QuantiTect SYBR Green PCR Master Mix, 0.5 µM of each primer and H<sub>2</sub>O was prepared according to the manufacturer's protocol. The mixture was thoroughly mixed and put into PCR capillaries placed in the adapters of the cooling block. Subsequently, 2 µl template DNA were added. The PCR capillaries were sealed with a plastic stopper and the mixture was descended to the bottom by a short centrifugation (3-5 s, up to 4,000 rpm). The capillaries were placed into a LightCycler sample carousel and put into the LightCycler machine. Finally, the cycle programme was started to run. The machine ran automatically according to the designed cycle programme, acquired and accumulated the fluorescent signal emitted by SYBR Green I. The reaction data were saved for later analysis.

For the test of all plant samples, real-time PCR was performed using 50 ng genomic DNA with three replicates. To calibrate the possible errors resulting from various experimental conditions such as pipetting, difference of PCR solutions and machine detection system, a

positive control was always parallel set with 1 ng genomic DNA of *M. graminicola* in each run. To control the results of real-time PCR, the amplified real-time PCR products were again visualised by electrophoresis on an 2% (w/v) agarose gel with ethidium bromide staining.

The real-time PCR data were first analysed using the LighCycler Analysis Software. The reaction curve and the cycle number of each sample was obtained by a quantification analysis. A melting curve analysis was used for the identification of target PCR product and non-specific products such as primer dimers, determination of fluorescence acquisition temperature. Furthermore, the melting curve was used to analyse the specificity and availability of the primer set used and the potential contaminations.

### **2.8.3 Generation of standard curves**

To calculate the fungal DNA amount in tested DNA of infected plants, a standard curve of real-time PCR was first constructed for each tested specific primer set using a serial tenfold dilution of purified *M. graminicola* genomic DNA (0, 0.1, 1, 10, 100, 1000 pg) with three replicates. The standard curve and the regression equation was drawn and calculated using Sigma Plot 2000 (SPSS Science Software GmbH, Erkrath, Germany).

First, the variation between the cycle number of 1 ng standard DNA of *M. graminicola* in each run and that in the standard curve was calculated. The cycle numbers of plant samples obtained in each run were first calibrated with the average variation value. Fungal DNA amount in tested samples was calculated according to the calibrated cycle number of each reaction using the regression equation of standard curve.

### **2.8.4 Evaluation of fungicide efficacy**

Wheat leaves at different positions were sampled two times from the fungicide-treated plots and fungicide-untreated plots on May 17 and June 14, 2004, respectively (see section 2.1.2). The disease severity on these leaves (F, F1, F2, F3 and F4 leaf on May 17 and F, F1, F2, F3 leaf on June 14) was assessed visually and by real-time PCR. Disease visual assessment was carried out as described in section 2.1.2. Real-time PCR was performed as described in section 2.8.2. The fungicide efficacy by real-time PCR was calculated according to the equation in section 2.1.2.

## **2.9 Processing of photographs and statistic analyses**

The photographs in this research were processed using Corel Photo Paint Software (Corel Corporation, Unterschleissheim, Germany). The statistic analyses of the results were carried out using MS-Office 97 and SigmaPlot 2000 (SPSS Science Software). MS-Office 97 was used for calculation of initial data including the mean and standard deviation (SD) of replicates, and for generation of disease progression curves. SigmaPlot 2000 was used for generation of standard curve, regression and correlation analysis. The significance level was described with “\*”: \* = significant at  $P < 0.05$ , \*\* = significant at  $P < 0.01$ , and \*\*\* = significant at  $P < 0.001$ .



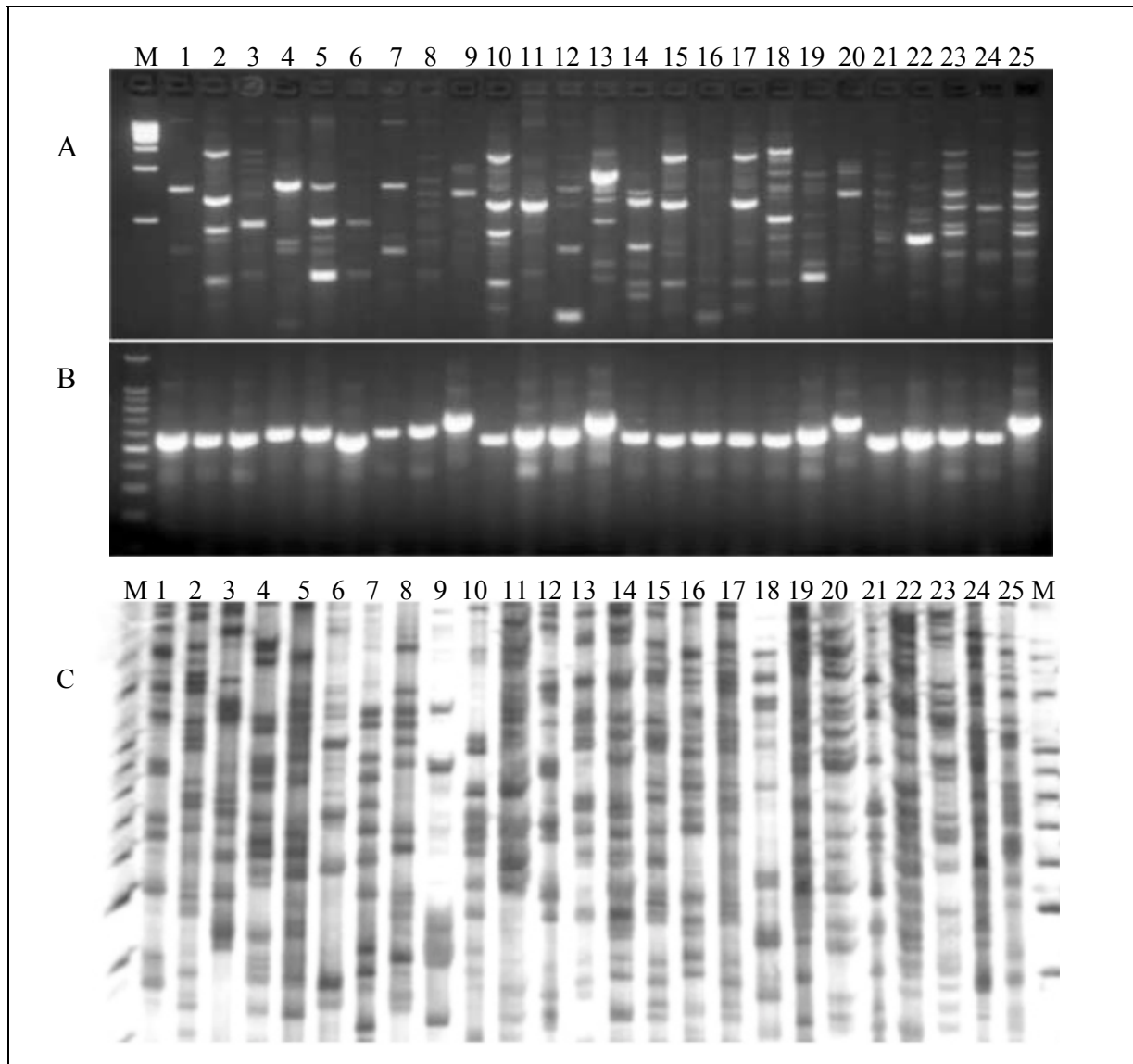
### 3. Results

#### 3.1 DNA and RNA extraction

Several different materials such as various fungal species, healthy plants and infected wheat plants had to be studied in the present thesis to achieve the objectives of the project. Therefore, a simple method of DNA and RNA extraction suitable for various fungal species or plants was needed. Furthermore, it was also needed for the practical application of PCR-based methods. The objectives of this initial part of the thesis were (1) to develop a simple procedure DNA extraction suitable for various plant pathogenic fungi; (2) to improve a simple method of DNA extraction from severely diseased wheat leaves; (3) to integrate a simple procedure of RNA extraction suitable for fungi, healthy and infected plants.

DNAzol reagent was successfully used to extract genomic DNA from *M. graminicola* isolates and 24 other fungal species tested, compared with a commonly used method described by Cenis (1992). The results reported in this section were accepted for publication by the journal "Biotechnology Letters" (Guo *et al.* 2005). To improve the adaptability of DNAzol reagent to various phytopathogenic fungi, Several modifications were made to the manufacturer's protocol. They included an additional elution step with chloroform, a higher centrifugation speed for DNA precipitation, an additional washing step with 500 µl of 70% DNAzol reagent and 30% ethanol instead of 70% ethanol, and a 10-min incubation time of the DNA pellet to remove the ethanol residue. For *M. graminicola*, the homogenization in liquid nitrogen was not necessary since DNAzol reagent lysed conidia effectively.

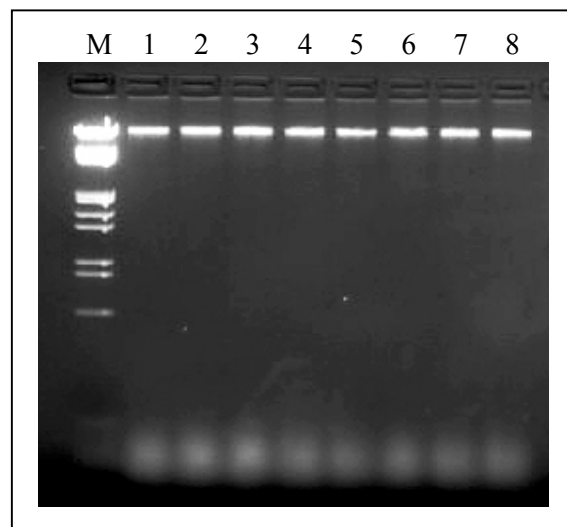
The DNA samples of 25 species were tested by RAPD-PCR, PCR amplification of ITS regions and AFLP. Clear band patterns were generated from the DNAs of 25 fungi using the three PCR-based methods (Fig. 3), indicating that potential PCR inhibitors were removed from the final DNA extracts. It could be demonstrated that the DNA purity is high enough for RAPD and AFLP analyses as well as for the amplification of ITS regions.



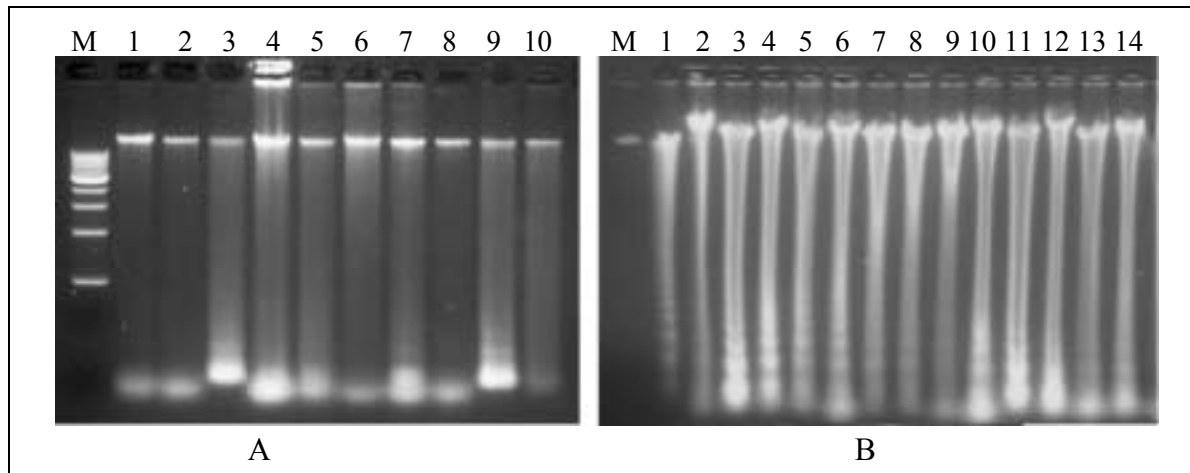
**Fig. 3** PCR amplification with genomic DNAs of 25 fungal species extracted using a modified procedure with DNAzol reagent. (A) RAPD-PCR with the random primer Roth-A 20; (B) PCR amplification of ITS region with the primer set ITS1/ITS4; and (C) AFLP fingerprint generated using the primer combination *EcoRI*-AA/*MseI*-AA for the selective amplification and silver staining. Lanes 1-25 are *Mycosphaerella graminicola*, *Microdochium nivale*, *Macrophomina phaseolina*, *Pseudocercospora anguioides*, *P. herpotrichoides*, *Cercospora beticola*, *Penicillium infestans*, *Aspergillus* spp., *Trichoderma harzianum*, *Verticillium dahliae*, *Septoria petroselini*, *Stagonospora nodorum*, *Rhizotonia solani*, *Fusarium tricinctum*, *F. oxysporum*, *F. avenaceum*, *F. culmorum*, *F. graminearum*, *Penicillium* spp., *Sclerotium* spp., *Botrytis* spp., *Cladosporium* spp., *Mucor* spp., *Alternaria alternata* and *Rhizopus* spp., respectively. M in A 1 kb ladder, and in B and C is the 100 bp DNA ladder (Promega).

The *M. graminicola* DNA was extracted using four different procedures with DNAzol reagent: (1) without homogenization, the conidia were directly mixed by inverting several times, but with chloroform elution, (2) without homogenization and chloroform elution step, (3) with homogenization and chloroform elution, the conidia were first ground in liquid nitrogen and then mixed with DNAzol reagent. (4) with homogenization but without chloroform elution. Homogenization and chloroform elution did not enhance DNA purity and yield (Fig. 4). It was demonstrated that the DNAzol reagent effectively lysed the conidia of *M. graminicola*. The homogenization and chloroform steps were dispensable when this procedure was used for the extraction of *M. graminicola* isolates.

**Fig. 4** Genomic DNA of *Mycosphaerella graminicola* extracted using DNAzol reagent. 10  $\mu$ l DNA solution were run on a 1% agarose gel. Lanes 1-4: without homogenization step, 50 mg conidia were directly mixed in 1 ml DNAzol reagent. Lanes 5-8: 50 mg conidia ground in liquid nitrogen were mixed with 1 ml DNAzol. Lanes 1-2 and Lanes 5-6: with chloroform elution step. Lanes 3-4 and lanes 7-8: without chloroform elution step. M is 1 kb DNA ladder (Promega).



Meanwhile, a simple method of DNA extraction suitable for minipreps and large-amount of tissues from fungi and infected plants was developed. The procedure was described in the sections 2.3.1.3. and 2.3.2. In this procedure, CTAB, insoluble PVP, SDS and chloroform elution were combined into one step such that the procedure was greatly simplified. The whole process can be finished within 40 min. This procedure was tested with 10 fungal species and used for DNA extraction from healthy and infected wheat leaves. Figure 5 shows DNA of 10 fungi (A) and 14 field samples (B). DNA of high purity was obtained from severely infected leaves or dried leaves infected using this procedure. The purity of the DNA is an evidence that the CTAB-PVP method effectively removed proteins, polysaccharides and polyphenols in infected leaves. The DNA purity was high enough for conventional PCR as well as for real-time PCR (see section 3.4).



**Fig. 5** Genomic DNA of 10 fungal species (A) and 14 plant samples (B) extracted using a CTAB-PVP method. Five  $\mu$ l DNA solution were run on a 1% agarose gel. Lanes 1 to 10 in A are *Mycosphaerella graminicola*, *Stagonospora nodorum*, *Pseudocercospora anguioides*, *P. herpotrichoides*, *Fusarium culmorum*, *F. graminearum*, *Microdochium nivale*, *Septoria petroselini*, *Rhizotonia solani*, *Macrophomina phaseolina*, respectively. Lanes 1 to 6 in B are flag leaves sampled on May 17 and 24 and June 1, 7, 14 and 21, 2004, and lanes 7 to 14 are F1 leaves sampled on May 3, 10, 17 and 24 and June 1, 7, 14 and 21, 2004. M in A is 1 kb DNA ladder, and M in B is 50 ng  $\lambda$  standard DNA.

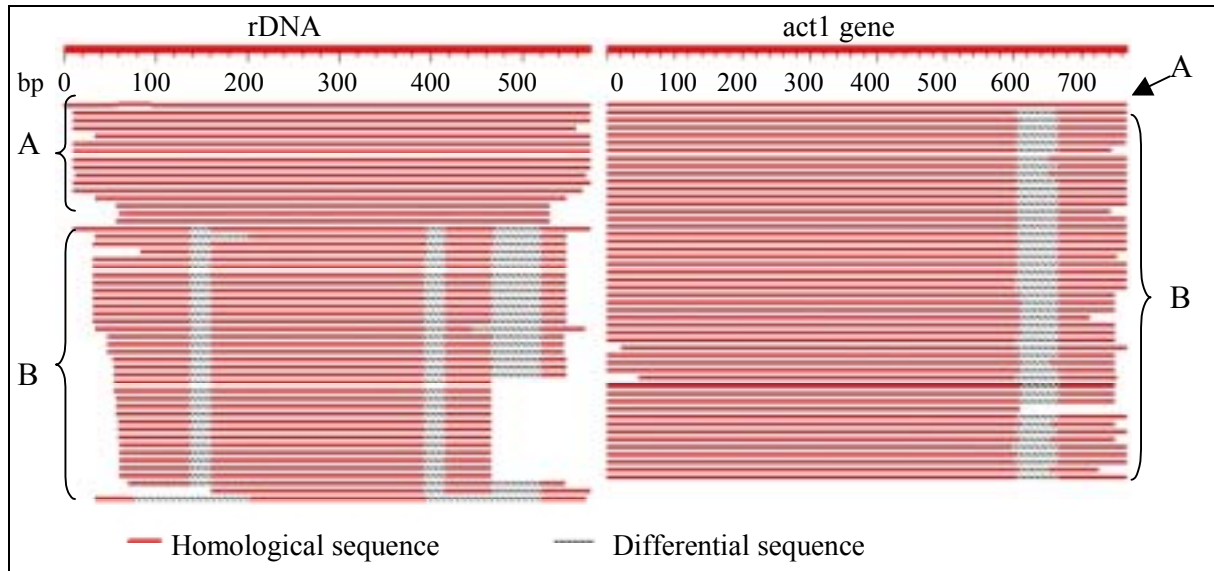
In addition, a simple procedure of RNA extraction was improved, which was published in the journal “Journal of Rapid Methods and Automation in Microbiology” (Guo *et al.* 2003). In this procedure, guanidinium isothiocyanate, CTAB and insoluble PVP were combined in one step and simplified the extraction process. It was successfully used for the extraction of total RNA from *M. graminicola* (Fig. 34), other fungal pathogens as well as *M. graminicola*-infected plant leaves, and resulted in high yield of total RNA. The total RNA extracted using this procedure was used for Northern hybridization (section 3.5.1), RT-PCR (section 3.5.2), as well as cDNA synthesis (section 3.2.3).

## 3.2 Isolation of specific sequences and development of specific primers

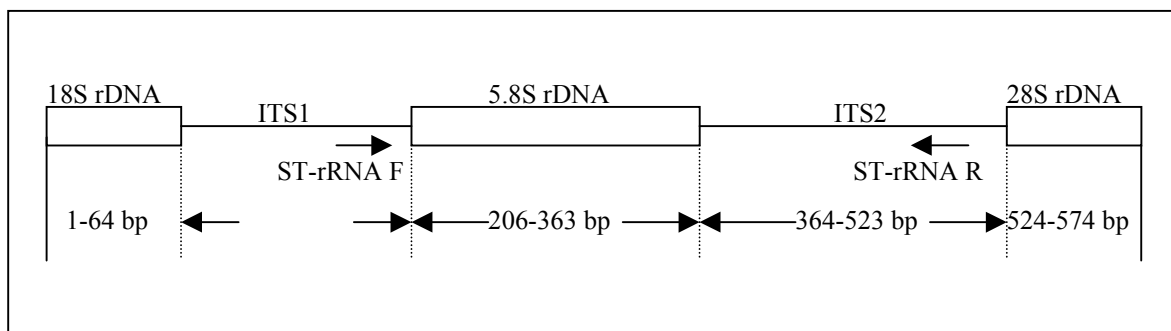
### 3.2.1 Search of *M. graminicola*-specific sequences by BLAST search

A 574-bp sequence of rDNA (AJ300330) and a 768-bp sequence of act1 gene (AJ300310) of *M. graminicola* were selected as target from the NCBI-GenBank (<http://www.ncbi.nlm.nih.gov/>) because they are highly conserved and have high copy numbers in the genome. The sequence AJ300330 contains the 18S rRNA gene (partially), internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, internal transcribed spacer 2 (ITS2)

and the 28S rRNA gene (partially) (Fig. 7). The selected sequence of the act1 gene is the exons 1-2 of the act1 gene. Both sequences contain no intron, hence they can be completely transcribed to RNA. Therefore, the primers designed based on both sequences may be used for PCR as well as RT-PCR.



**Fig. 6** BLAST search in the NCBI-GenBank using one rDNA sequence and one partial sequence of the act1 gene of *Mycosphaerella graminicola* as target sequences. (A) Sequence of *M. graminicola* isolates; (B) Sequences of other organisms.



**Fig. 7** A scheme of the structure and components of the AJ300330 sequence of *Mycosphaerella graminicola* and the location of the primer set ST-rRNA F/R.

**A sequence of rDNA (574 bp)**

ttcatttagaggaagtaaaagtcgtaacaaggtctccgtaggtgaacctgcggagggatcattaccgagcgagggcctccgggtc  
gacctccaacctttgtgaacacatcccgttgcttcgggggcgacctgccgggcgccccggaggaccacaaaaaacactgca  
tctctgcgctcgaggttacgagtaaatcgaacaaaaacttcaacaacggatctcttggtctggcatcgatgaagaacgcagcgaat  
gcgataagtaatgtgaattgcagaattcagtgatcatcgaatctttgaacgcacattgcgccccctggattccggggggcatgcc  
gttcgagcgtcattacaccactccagcctcgtgggtattgggcgtctttcgcgggggatcactccccgcgcgctcaaagtctcc  
ggctgagcgggtctctcccagcgttggtgcatcacgtctcgcgaggttcacgagccctcacggcgttaaatcacacctcag  
gttgacctcgatcggttaggataaccgctgaacttaagcatatcaataa

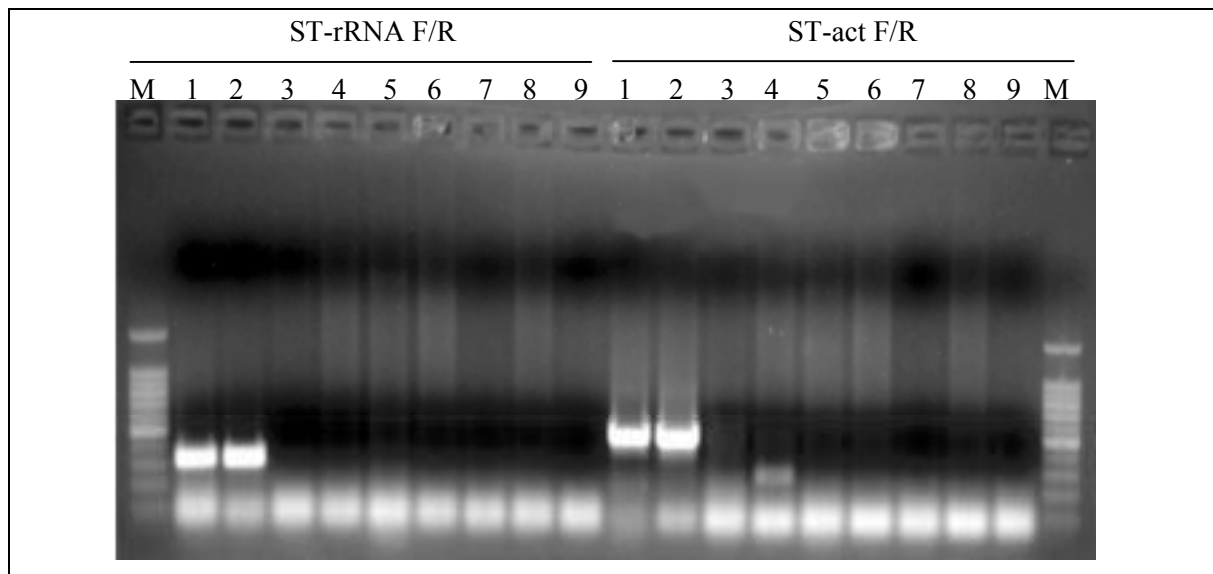
**A sequence of act1 gene (768 bp)**

cgaggagcaccctctctcaccgagggtcccatcaacccaagtccaaccgtgagaagatgaccagatcgtcttcgagacctcaacgc  
cccagccttctactctccatccaggtgtgctctccctctacgctcc ggctgtaccaccggtattgtctcgact ctggtgacggtgacccac  
ggtgtccaatctacgagggtttcccttccccacgccatctctcgtgacatggctggtgattgaccgactacctgaagatcttggc  
cgagcgcggatacgtctcagcaccaccgccgagcgtgaaatgctcgtgacatcaaggagaagctctgctacgtcgcctcgactttgagcag  
gagatccaaaccgccaccagctcttccctcgagaagctctacgaactccccgatgggcaagtcaccatcggaacgagcgttccgtg  
ctccagaggtctctccagcctccgctcctcggctcgaatccggcggcatccacgtcaccacctcaactccatcatgaagtgtgatgctgatg  
cgcaaggatctctacggaacatcgtcatggtgagtgtgacatactgctgactccatccgatcgagtaaacgatattcccagtcggcgac  
caccatgtaccaggtatctccgaccgatgcagaaggaaatcaccgcctcgcctccatccagcatgaaggtcaagatcgcgccaccaga  
gcgcaa

**Fig. 8** The sequences of rDNA and act1 gene of *Mycosphaerella graminicola* selected for the BLAST search. The differential subsequences are shaded and the sequences of designed primers are underlined.

To identify potential specific subsequences in both target sequences, a BLAST search was performed. Three differential subsequences were identified in the ITS sequence which were located between 140-160 bp (21 bp), 385-420 bp (36 bp) and 467-521 bp (55 bp), respectively (Fig.6, Fig. 8). Another differential subsequence was identified in the act1 gene sequence (606-668 bp, Figs. 6 and 8). These differential subsequences might be used as specific sequences for *M. graminicola*. Two primer sets (ST-rRNA F/R and ST-act F/R) were designed, using the Internet programme Primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>). The primers ST-rRNA F and St-act F were located within the homological sequences, and ST-rRNA R and ST-act R were involved in the differential subsequences (Fig. 8).

The specificity of the two developed primer sets ST-rRNA F/R and ST-act F/R was primarily tested with two isolates of *M. graminicola* and seven other fungal phytopathogens by conventional PCR. Both primer sets gave rise to a single fragment from two isolates of *M. graminicola*, but not from seven other fungal pathogens (Fig. 9). It is suggested that both primer sets were specific for *M. graminicola* within the tested range of isolates and species.



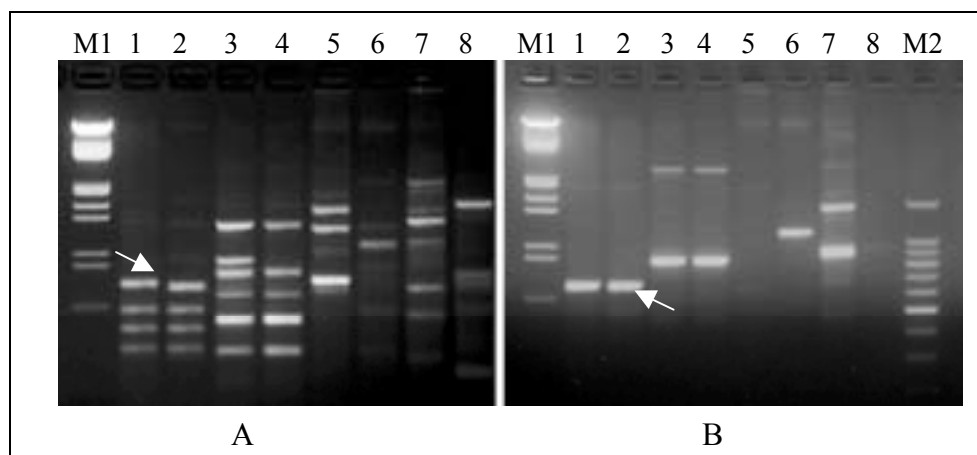
**Fig. 9** Specificity test of the two primer sets ST-rRNA F/R and ST-act F/R with two isolates of *Mycopaerella graminicola* and seven other fungal phytopathogens. Lanes 1 to 9 are *M. graminicola* FC2, Kor-92, *Stagonospora nodorum*, *Microdochium nivale*, *Pseudocercospora herpotrichoides*, *Fusarium culmorum*, *Rhizotonia solani*, *F. graminearum* and *Macrophomina phaseolina*. M is the 100 bp DNA ladder (Promega).

### 3.2.2 Isolation of specific fragments by RAPD-PCR

To isolate some *M. graminicola*-specific fragments, a primary screening was performed by RAPD-PCR using 80 random primers (Table A1) and genomic DNA of three *M. graminicola* isolates and *S. nodorum*. Out of the 80 primers, twelve were selected and further tested with two isolates and six other fungal pathogens. Figure 10 shows the band patterns of RAPD-PCR using the random primers Roth-A 19 and Roth-A 20. Nine specific DNA fragments, R1072, R2996, R3005, R3006, R3009, R5861, R5869, R5870-1 and R5870-2, were cut out and purified from the agarose gel. The size of these fragments ranged from 520 to 1700 bp, respectively (Table 5). These fragments were further cloned in the vector of *E. coli*.

Four fragments R3005, R3006, R3009 and R5870-2 were sequenced. The sequences are shown in Fig. A2. In order to check the homologues and specificity of these sequences, they were compared with the sequence data by BLAST search in the NCBI GenBank and the Co-Geme EST Database (a phytopathogenic fungi and oomycete EST database, <http://cogeme.ex.ac.uk/index.html>). No complete homologue to the four fragments, but only some short homologous sequences ranging from 20-28 bp were identified in the two database

(Table 6). Among the four fragments, R5870-2 had the least BLAST hits, indicating that this fragment might be more specific than the other three fragments.



**Fig. 10** Isolation of *Mycosphaerella graminicola*-specific fragments by RAPD-PCR using random primers Roth-A 20 (A) and Roth-A-19 (B). The arrow in A shows the position of the specific fragment R5870-2 and the arrow in B shows that of R3009. Lanes 1-8 are *M. graminicola* GBE2 and Gor-92, *Microdochium nivale*, *Fusarium culmorum*, *Stagonospora nodorum*, *Macrophomina phaseolina*, *F. poae* and *Pseudocercospora anguioides*. M1 is the 1 kb DNA ladder (Promega) and M2 is the 100 bp DNA ladder (Promega).

**Table 5** The *Mycosphaerella graminicola*-specific fragments isolated by RAPD-PCR

Fragments	Approximate size (bp)	Used random primers	Clone number <sup>a</sup>
R1072	1500	Roth-A 14	P1072
R2996	900	Roth-A 06	P2996
R3005	520	Roth-A 15	P3005
R3006	636	Roth-A 16	P3006
R3009	649	Roth-A 19	P3009
R5861	1700	Roth-A 11	P5861
R5869	700	Roth-A 19	P5869
R5870-1	1700	Roth-A 20	P5870-1
R5870-2	709	Roth-A 20	P5870-2

<sup>a</sup> The relevant clones of these fragments were deposited at  $-80^{\circ}\text{C}$  at the Institute of Phytopathology, University of Kiel.



**Table 6** Results of the BLAST search of four RAPD fragments in two public DNA databases

Database	Fragment	Number of BLAST hits	Length of alignments (base)	Alignment scores (bits) <sup>a</sup>	E-value <sup>b</sup>
NCBI	R5870-2	7	20-24	40-44	0.45-7.0
	R3009	22	20-24	40	6.3
	R3005	31	20-28	40-48	0.021-5.1
	R3006	28	20-28	40-48	0.026-6.3
Co-Geme	R5870-2	7		32-34	0.89-3.5
	R3009	9		32-34	0.81-3.2
	R3005	14		32-38	0.042-2.6
	R3006	11		32-34	0.8-3.2

<sup>a</sup> The alignment score is a measure for the sequence similarity. The higher the alignment score, the better the alignment.

<sup>b</sup> The E-value is a parameter to describe how many matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990).

Altogether 13 forward and 10 reverse primers were designed based on these sequences, in which one forward and one reverse primer from R3005, two forward and two reverse primers from R3006, six forward and four reverse primers from R3009 as well as four forward and three reverse primers from R5870-2 were designed (Table A2). To find several potential primer sets specific to *M. graminicola*, the specificity of 31 primer sets was tested with DNA of *M. graminicola*, healthy plant and other fungi (Table 7). The primer set R3005-F/R designed from R3005 was unspecific since it amplified the same fragment of *M. graminicola* from seven other fungal pathogens and healthy plants (Table 7). Four primer sets from R3006 were tested. R3006-F/R3006-R amplified multiple bands from all samples. Although R3006-F/R3006-1R, 3006-1F/R3006-R and R3006-1F/R3006-1R amplified a single fragment, they amplified unspecific fragments from other fungi as well as healthy plants. All primer sets from R3005 and R3006 have no specificity for *M. graminicola* (Table 7). Therefore, R3005 and R3006 can not be used as target sequences for the detection of *M. graminicola*. Fourteen primer sets from R3009 amplified no product from other fungi tested, but a product of the same size was amplified from healthy plants, indicating that R3009 was a good marker to discriminate *M. graminicola* from other fungal pathogens, but

**Table 7** Specificity test of primer pairs developed from four RAPD fragments by PCR with DNA of healthy wheat plants and seven other pathogens

RAPD fragment	Primer (forward)	Primer (reverse)	Product size (bp)	Band(s)	Specificity <sup>a</sup>		
					Healthy plant	Other fungi	
R3005	R3005-F	R3005-R	517	single	No	No	
R3006	R3006-F	R3006-R	631	mutiple	No	No	
	R3006-1F	R3006-1R	260	single	No	No	
	R3006-F	R3006-1R	277	single	No	No	
	R3006-1F	R3006-R	615	single	No	No	
R3009	R3009-F	R3009-R	643	single	No	Yes	
	R3009-1F	R3009-R	448	single	No	Yes	
	R3009-2F	R3009-R	492	single	No	Yes	
	R3009-3F	R3009-R	566	single	No	Yes	
	R3009-4F	R3009-R	492	single	No	Yes	
	R3009-5F	R3009-R	278	single	No	Yes	
	R3009-1F	R3009-1R	337	single	No	Yes	
	R3009-2F	R3009-1R	381	single	No	Yes	
	R3009-3F	R3009-1R	455	single	No	Yes	
	R3009-4F	R3009-1R	381	single	No	Yes	
	R3009-5F	R3009-1R	168	single	No	Yes	
	R3009-1F	R3009-4R	418	single	No	Yes	
	R3009-2F	R3009-4R	462	single	No	Yes	
	R3009-3F	R3009-4R	535	single	No	Yes	
	R5870	R5870-F	R5870-R	665	single	Yes	No
		R5870-F	R5870-1R	588	single	Yes	
R5870-F		R5870-2R	485	single	No		
R5870-1F		R5870-R	503	single	Yes		
R5870-1F		R5870-1R	426	single	Yes	Yes	
R5870-1F		R5870-2R	323	single	Yes	Yes	
R5870-2F		R5870-R	376	single	Yes		
R5870-2F		R5870-1R	299	single	No		
R5870-2F		R5870-2R	196	single	No		
R5870-3F		R5870-R	631	single	No		
R5870-3F		R5870-1R	549	single	Yes		
R5870-3F		R5870-2R	451	single	No		

<sup>a</sup>“Yes” means “specific”; “No” means “unspecific”.

not from healthy plants. Hence, the primer sets designed from this fragment could not be used to detect *M. graminicola* in infected plants. For the fragment R5870-2, six out of 11 primer sets tested amplified no band from healthy plants (Table 7). Two of the six primer sets R5870-1F/R5870-1R and R5870-1F/5870-2R were selected since both primer sets amplify products of suitable size for real-time PCR. They were further tested with *M. graminicola*-infected plants and 22 other fungal pathogens (see the section 3.3).

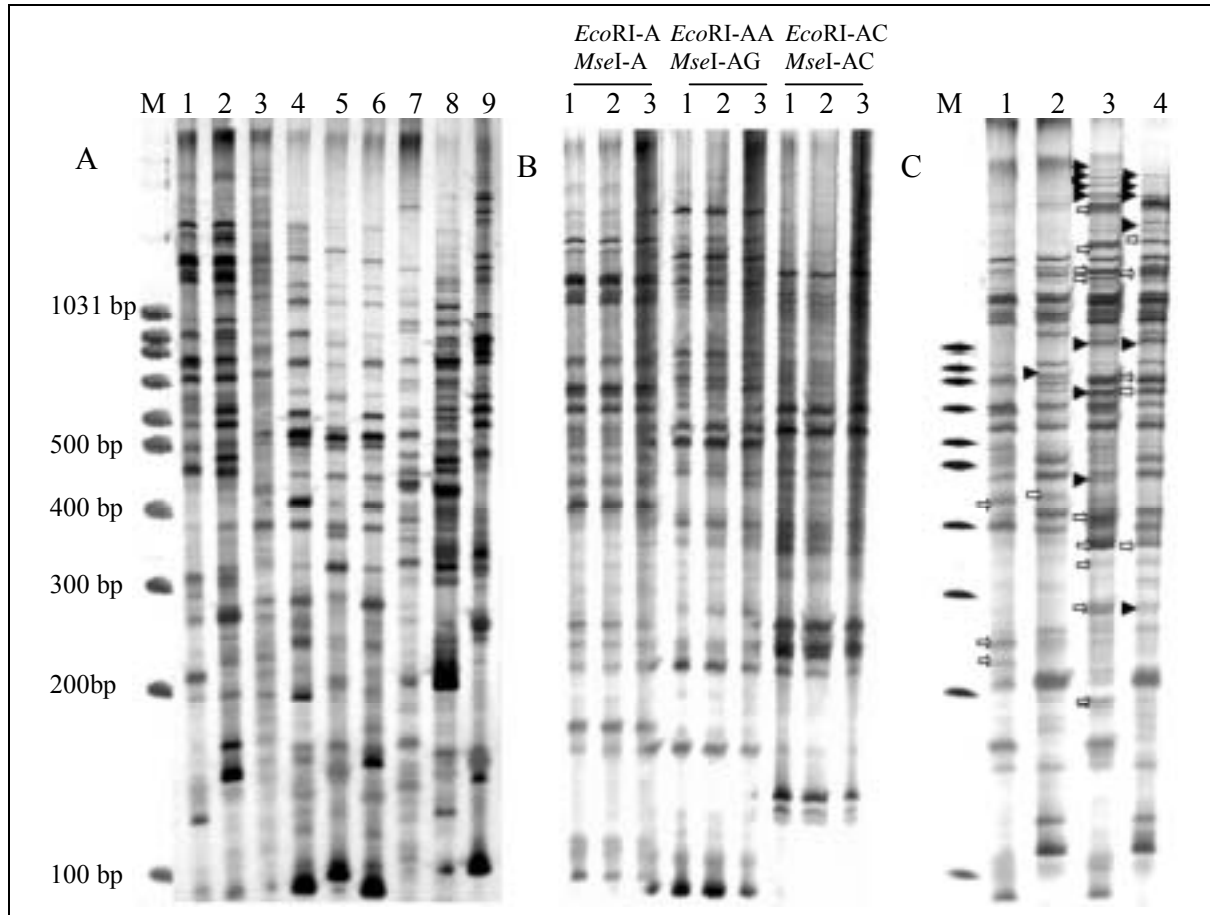
### 3.2.3 Establishing a cDNA-AFLP procedure

A cDNA-AFLP procedure suitable for *M. graminicola* and other fungal pathogens was first established in order to isolate some transcripts specific for *M. graminicola*. The procedure outlined by Bachem *et al.* (1998) is a basic procedure, but it is laborious and requires radioactive labeling. Therefore, a slight modification was made to the standard protocol. First-strand was synthesized with M-MLV RT instead of AMV RT since M-MLV RT is more suitable for long mRNA templates. In second-strand synthesis, the commercial buffer of DNA polymerase I was used as it is optimal for the activity of DNA polymerase I. For the display of transcript-derived fragments (TDFs), a silver staining system was used.

To test the suitability of using total RNA as starting template for the cDNA synthesis, cDNA-AFLP was carried out using 30 µg total RNA of nine different fungal pathogens and the primer combination *EcoRI*-A/*MseI*-A. Clear cDNA-AFLP fingerprints were generated from two isolates of *M. graminicola* and seven other fungal pathogens (Fig. 11A). Forty-two to 55 TDFs were detected in the fingerprints. The variation of TDFs was observed not only between different fungal species, but also between two isolates of *M. graminicola* (Fig. 11A).

To compare the effect of total RNA with that of mRNA, cDNA-AFLP was performed with 30 µg total RNA or 2 µg mRNA of the two *M. graminicola* isolates FN4 and OK-120. The fingerprints generated from total RNA were obviously different from those from mRNA, especially in TDFs with high molecular mass (Fig. 11C). For FN4, the fingerprints generated from mRNA showed seven additional TDFs, nine TDFs with increased intensity and three TDFs with decreased intensity in comparison with that from total RNA. For OK-120, the fingerprint from mRNA showed six additional TDFs, five TDFs with increased intensity and, and the fingerprint from total RNA showed one additional TDF and one TDF with increased intensity (Fig. 11C). Different input of total RNA for cDNA synthesis and different input of

cDNA for AFLP were tested, but these trials produced similar fingerprints (results not shown).



**Fig. 11** cDNA-AFLP fingerprints on a 5% denaturing polyacrylamide gel with silver staining. (A) cDNA-AFLP fingerprints generated from total RNA of nine different fungal species. Lanes 1 to 9 in A are *Mycosphaerella graminicola* FN4, OK-120, *Pseudocercospora herpotrichoides*, *Microdochium nivale*, *Fusarium graminearum*, *F. culmorum*, *Macrophomina phaseolina*, *Rhizotonia solani* and *Stagonospora nodorum*, respectively. (B) Comparison of cDNA-AFLP fingerprints generated with three different volumes of preamplification products and three different primer combinations. Lanes 1 to 3 in B are 10, 5 and 2-fold dilutions of preamplification products. (C) Comparison of cDNA-AFLP fingerprints using total RNA of two *M. graminicola* isolates with those using the respective mRNA. Lanes 1 to 4 are total RNA of FN4 isolate, total RNA of OK-120 isolate, mRNA of FN4 isolate and mRNA of OK-120 isolate, respectively. “▶” shows bands with higher intensity, and “⇨” shows increased bands. M in A and C is the 100 bp DNA ladder (MBI Fermentas).

The effects with a series of 2, 5, 10-fold dilutions of the secondary template (pre-amplification products) for the selective PCR were tested with three primer combinations of *EcoRI-A/MseI-A*, *EcoRI-AA/MseI-AG* and *EcoRI-AC/MseI-AC*. It was shown that the 5-fold and 10-fold dilutions generated similar fingerprints, but the fingerprints generated with the 2-fold dilution had a stronger background (Fig. 11B). Three primer combinations generated obviously different fingerprint profiles. *EcoRI-A/MseI-A* amplified the largest number of TDFs and *EcoRI-AC/MseI-AC* generated markedly less TDFs than *EcoRI-AA/MseI-AG*. The effects of three dilutions of preamplification products on the fingerprint profiles generated with the three primer combinations were different. Although the 2-fold dilution resulted in a strong background in the fingerprint profiles generated with *EcoRI-A/MseI-A* and *EcoRI-AA/MseI-AG*, the pattern of the fingerprint remained broadly similar to those with 5- and 10-fold dilutions and the TDFs could be clearly distinguished (Fig. 11B). However, the 2-fold dilution resulted in a stronger background in the fingerprint with *EcoRI-AC/MseI-AC*, and some TDFs could not be distinguished.

### **3.3 Specificity and sensitivity of the developed primer sets by conventional PCR**

#### **3.3.1 Specificity test of developed primer sets**

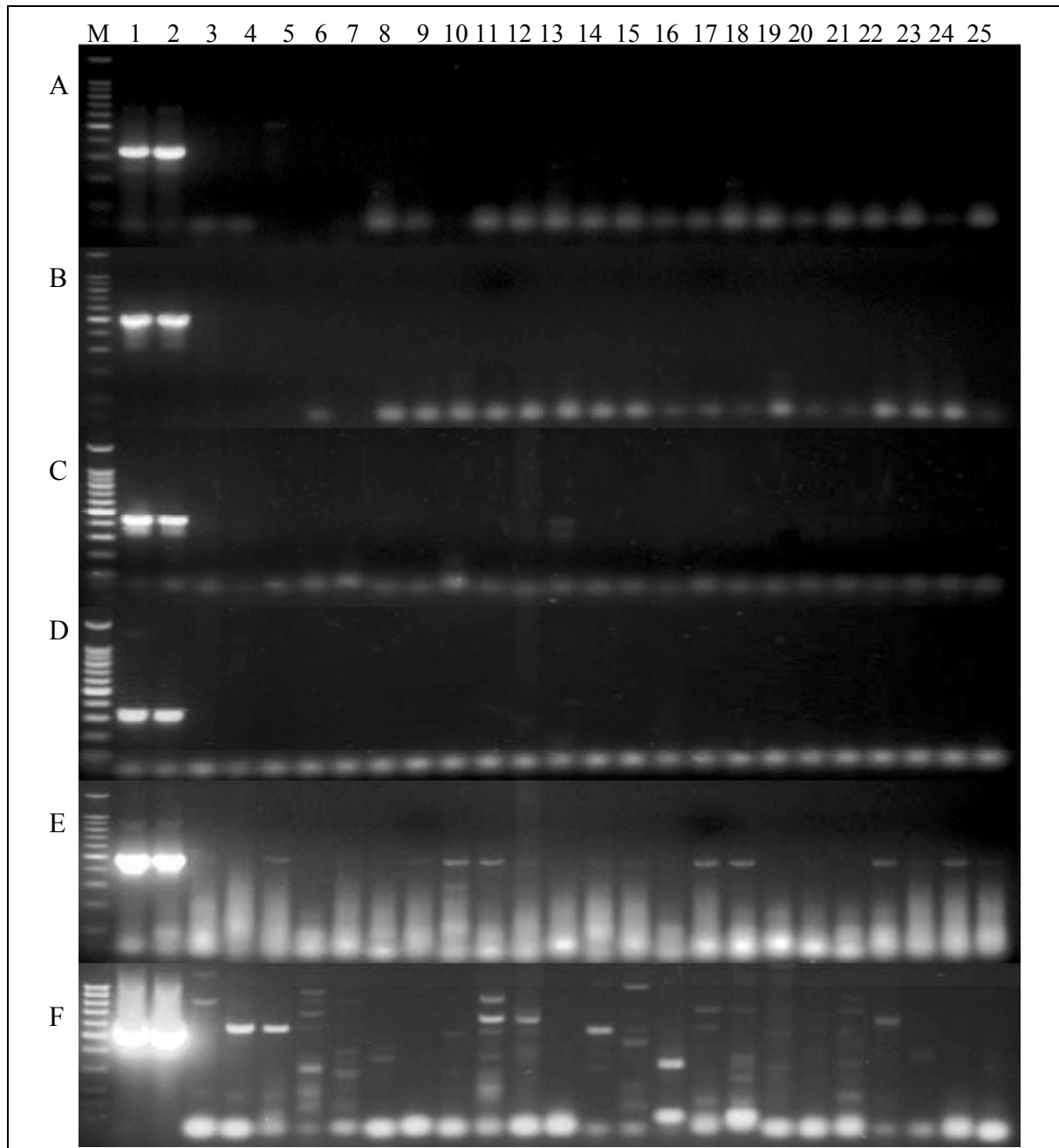
In the four developed primer sets, ST-rRNA F/R was developed from a rDNA sequence, ST-act F/R from the Act1 gene sequence, and R5870-1F/R5870-1R and R5870-1F/R5870-2R were developed from the RAPD fragment R5870-2. They were primarily proved to be specific for *M. graminicola*. These primers have different GC content, but have similar melting temperature ( $T_m$ , Table 8). Of the four primers, ST-act F and ST-act R have the highest self-complementarity score, indicating that they have the highest tendency to anneal to itself or form secondary structure. The 3' self-complementarity scores of all primers were small, showing that they have a low tendency to form a primer-dimer. The target sizes of the four primer sets ST-rRNA F/R, ST-act F/R, R5870-1F/R5870-1R and R5870-1F/R5870-2R were 328 bp, 496 bp, 426 bp and 323 bp, respectively. The GC content of these targets differed slightly.

**Table 8** Characters of the developed primers and their target products

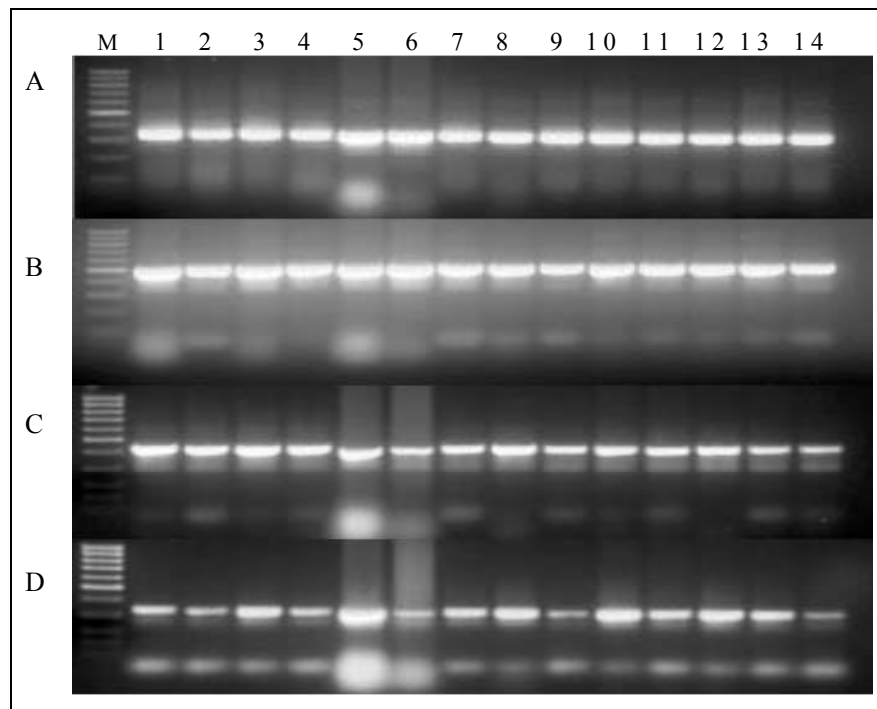
primer	Primer length	GC%	T <sub>m</sub>	Any	3'	Target size	Target GC%
ST-rRNA F	20	55.00	60.45	5.00	2.00	328 bp	55.18
ST-rRNA R	19	63.16	60.82	4.00	2.00		
ST-act F	20	55.00	59.60	8.00	0.00	496 bp	56.85
ST-act R	20	55.00	60.23	5.00	1.00		
R5870-1F	20	60.00	59.99	2.00	0.00	426 bp	54.17
R5870-1R	20	50.00	60.01	3.00	1.00		
R5870-1F	20	60.00	59.99	2.00	0.00	323 bp	54.46
R5870-2R	20	50.00	60.07	5.00	0.00		

The values of T<sub>m</sub>, Any and 3' in this table were obtained from the internet programme Primer3 when these primers were designed. Any means the self-complementarity score of the oligo or primer (taken as a measure of its tendency to anneal to itself or form secondary structure). 3' means the 3' self-complementarity of the primer or oligo (taken as a measure of its tendency to form a primer-dimer with itself).

The specificity of the four developed primer sets was further tested with genomic DNA of *M. graminicola*, the infected plants, healthy plants and 22 other phytopathogenic fungi, compared with two reported primer sets E1/STSP2R and STIF2/BAF4ST (Fraaije *et al.* 1999, 2001). The results showed that three primer sets, ST-rRNA F/R, ST-act F/R, and R5870-1F/R5870-2R, amplified one single fragment of expected size only from *M. graminicola* and its infected leaves, but not from healthy plants or 22 other fungi tested (Fig. 12A, B and D). R5870-1F/R5870-1R also showed high specificity except *P. anguioides*, where one slight band was present (Fig. 12C). However, two reported primer sets revealed low specificity. Although E1/STSP2R produced one strong single band from *M. graminicola* and its infected plants, a slight but clear band with the same fragment size was also present in 14 other pathogens (*S. petroselini*, *M. nivale*, *F. graminearum*, *F. avenaceum*, *P. infestans*, *P. herpotrichoides*, *R. solani*, *M. phaseolina*, *C. beticola*, *Alternaria* spp., *Rhizopus* spp., *Sclerotium* spp., *Botrytis* spp. and *Mucor* spp., Fig. 12E). Although STIF2/BAF4ST gave rise to a strong single band from *M. graminicola* and its inoculated plants, it produced multiple bands from healthy plants and most fungal pathogens tested (Fig. 12F). The repetition of the trial produced a similar band pattern with E1/STSP2R and STIF2/BAF4ST, indicating that both primer sets had a lower specificity than the four developed primer sets when using *Taq* polymerase in the PCR reaction.



**Fig. 12** The specificity of the six primers sets ST-rRNA-F/R (A), ST-act F/R (B), R5870-1F/R5870-1R (C), R5870-1F/R5870-2R (D), E1/STSP2R (E) and STIF2/BAF4ST (F) by PCR with genomic DNA of *Mycosphaerella graminicola*, its infected plants, healthy wheat plants and 22 fungal pathogens. The lanes 1 to 25 are *M. graminicola*, *M. graminicola*-infected plants, healthy plants, *Erysiphe graminis* f.sp. *tritici* infected plants, *Septoria petroselini*, *Stagonospora nodorum*, *Microdochium nivale*, *Fusarium culmorum*, *F. gramineum*, *F. avenaceum*, *Phytophthora infestans*, *Pseudocercospora herpotrichoides*, *P. anguioides*, *Rhizotonia solani*, *Macrophomina phaseolina*, *Verticillium dahliae*, *Cercospora beticola*, *Alternaria* spp., *Trichoderma harzianum*, *Aspergillus* spp., *Cladosporium* spp., *Rhizopus* spp., *Sclerotium* spp., *Botritis* spp. and *Mucor* spp., respectively. M in A, B, C, D and E is the 100 bp DNA ladder (Promega), and M in F is the 100 bp DNA ladder (Carl Roth, Karlsruhe, Germany).



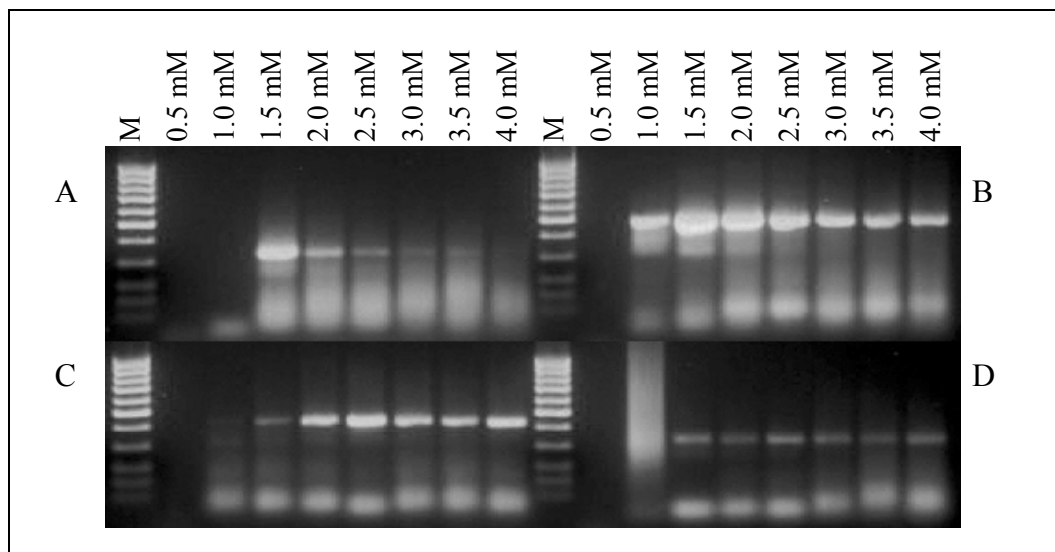
**Fig. 13** The PCR amplification with four primer sets developed in the study and genomic DNA of 14 isolates originated from different regions of Europe. Primer sets were (A) ST-rRNA F/R; (B) ST-act F/R; (C) R5870-1F/R5870-1R and (D) R5870-1F/R5870-2R. Lanes 1 to 14 are *Mycosphaerella graminicola* Kor-11, Kor-92, Gor-86, Gor-9, K-Bc-105, K-Bc-121, OK-102, OK-120, FN4, FC2, CH2, CH4, GBW1 and GBE2, respectively. M is the 100 bp DNA ladder (Carl Roth).

The genetic diversity in *M. graminicola* isolates is high (Linde *et al.* 2002, Schnieder *et al.* 2001), so that if the primer sets developed using one isolate are located in genetic diverse regions, they may fail to amplify products from other isolates with different sequences in the primer-binding position. Accordingly, the four primer sets were tested with 14 isolates of *M. graminicola* originated from different regions in Europe by conventional PCR. All four primer sets amplified an expected PCR product from 14 isolates tested (Fig. 13). Although the band intensity produced by R5870-1F/R5870-2R showed variation between isolates (Fig. 13D) and a stronger background was observed in the isolates of K-Bc-105 and K-Bc-121, the primer sets ST-rRNA F/R, ST-act F/R and R5870-1F/R5870-1R produced band pattern with similar intensity between isolates (Fig. 13A, B and C). This was probably due to these DNA samples which were extracted with a different preparation.



### 3.3.2 Optimization of the PCR conditions

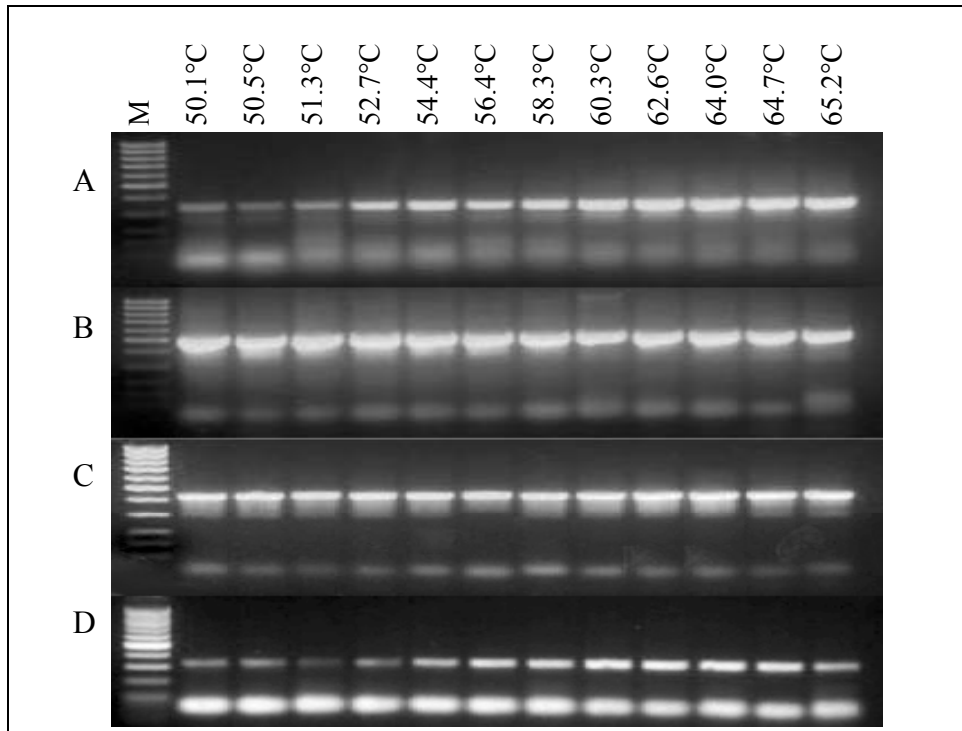
Initially, the influence of  $Mg^{2+}$  concentration on the amplification of four specific primer sets was tested. The PCR was performed using 100 pg genomic DNA of *M. graminicola* at eight different  $Mg^{2+}$  concentrations ranging from 0.5-4.0 mM. The results indicated that the effect of  $Mg^{2+}$  on the four primer sets were different. All primer sets failed to amplify products when 0.5 mM  $MgCl_2$  was used. On ST-rRNA F/R,  $Mg^{2+}$  had a clear influence (Fig. 14A). At 0.5 and 1.0 mM, no band was present. The optimal concentration was 1.5 mM. At higher concentrations, the amplification efficiency decreased. ST-act F/R was not as sensitive as ST-rRNA-F/R, and produced a band at 1.0-4.0 mM  $Mg^{2+}$  except 0.5 mM. The optimum concentration for ST-act F/R was 1.5-2.0 mM (Fig. 14B). R5870-1F/R5870-1R and R5870-1F/R5870-2R amplified no product at 0.5 and 1.0 mM (Fig. 14C and D). R5870-1F/R5870-1R produced a slight band at 1.5 mM, and a similar clear band at 2.0-4.0 mM. The optimum concentration for this primer set was 2.5 mM. R5870-1F/R5870-2R had a similar amplification efficiency at 1.5-4.0 mM (Fig. 14D).



**Fig. 14** Effect of  $Mg^{2+}$  concentration on the amplification of the four specific primer sets ST-rRNA F/R (A), ST-act F/R (B), R5870-1F/R5870-1R (C) and R5870-1F/R5870-2R (D). The values shown are the concentration of  $MgCl_2$  used in the PCR. M is the 100 bp DNA ladder (Carl Roth).

The effect of 12 annealing temperatures on the PCR amplification of the four primer sets was tested using 200 pg genomic DNA of *M. graminicola*. The effect on four primer sets was different (Fig. 15). For ST-act F/R and R5870-1F/R5870-1R, similar bands were present at all 12 temperatures tested, indicating that the PCR efficiency was hardly affected by the annealing temperature within the range of 50-65°C (Fig. 15B and C), but the PCR efficiency

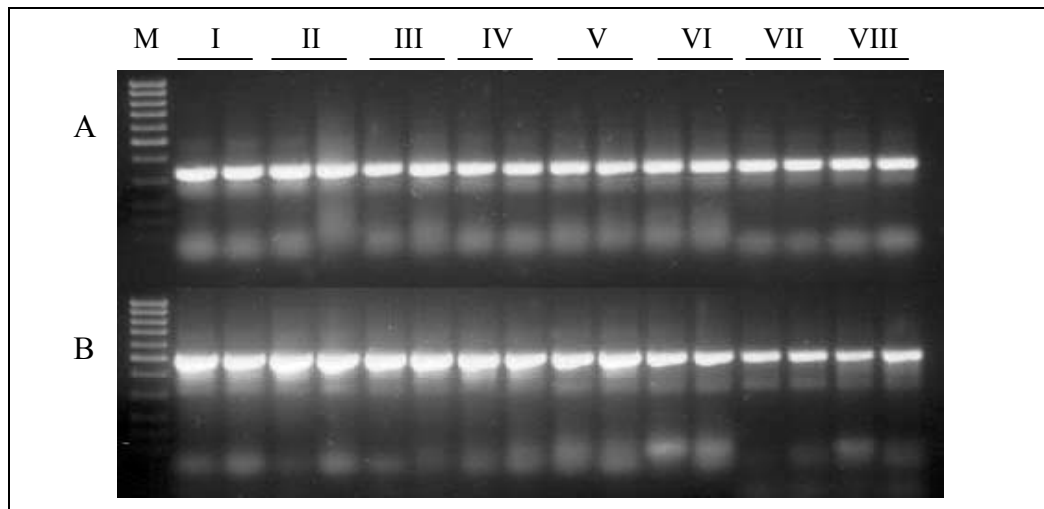
of ST-rRNA F/R and R5870-1F/R5870-2R was obviously influenced by annealing temperature (Figs. 15A and D). Both primer sets had a relatively low efficiency between 50.1 and 51.3°C. The optimum temperature for ST-rRNA F/R was 60.3-65.2°C and for R5870-1F/R5870-2R was 60.3-60.7°C.



**Fig. 15** Effect of annealing temperature on the PCR amplification efficiency of the four primer sets ST-rRNA F/R (A), ST-act F/R (B), R5870-1F/R5870-1R (C), R5870-1F/R5870-2R (D). Lanes 1 to 12 are 12 annealing temperatures tested. M is the 100 bp DNA ladder (Carl Roth).

To obtain a short and efficient PCR programme for the developed specific primer sets, PCR was performed using 200 pg genomic DNA of *M. graminicola* and run with eight different PCR programmes ranging from 1 h 6 min to 1 h 43 min in a PTC-200 Peltier thermal cycler. Two replicates were employed in each programme. The results showed that the shortest PCR programme I gave rise to a high band intensity for both primer sets (Fig. 16, I), but clear differences among the first six 40-cycle programmes were not observed (Figs. 16A and B, I-VI). For ST-act F/R, two 30-cycle programmes (Fig. 16B, VII and VIII) gave rise to a markedly lower band intensity than six 40-cycle programmes (Fig. 16B, I-VI), indicating that 40 cycles were necessary for ST-act F/R. But for ST-rRNA F/R, the 30-cycle programmes produced band patterns with lower background than the 40-cycle programmes, and the band intensity did not differ clearly from that produced by the 40-cycle programmes (Fig. 16A).

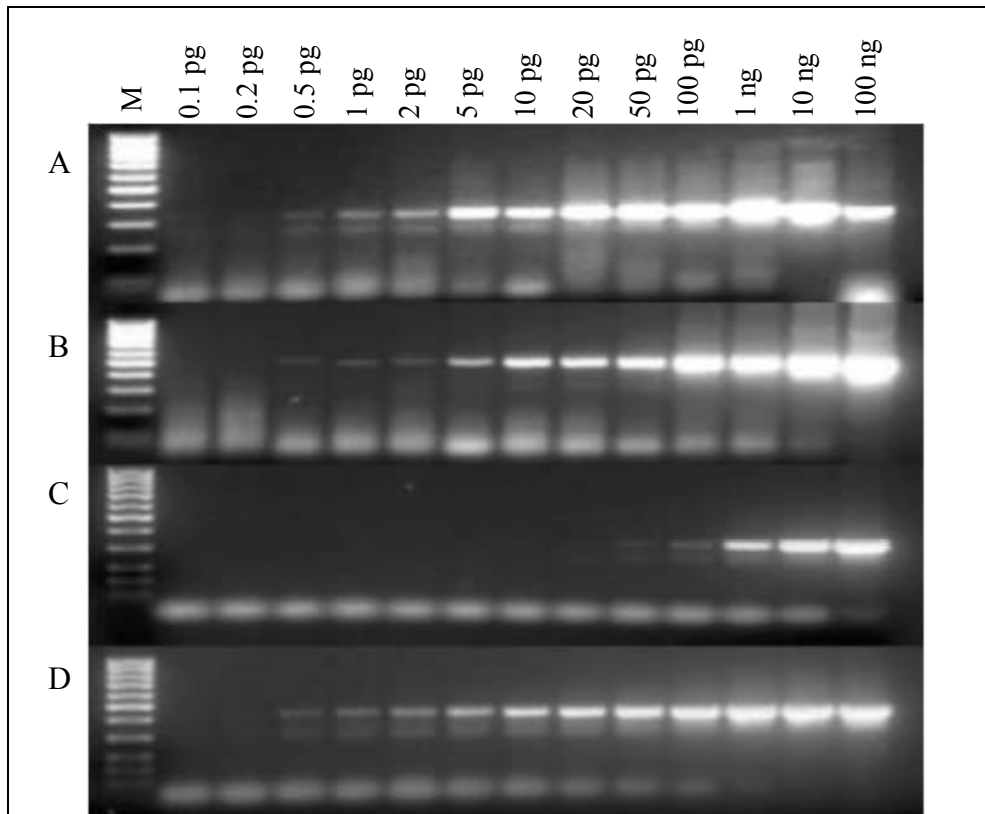
The PCR specificity and background did not differ among six 40-cycle programmes for both primer sets.



**Fig. 16** Effect of eight different PCR programmes on the PCR efficiency of the two primer sets ST-rRNA F/R (A) and ST-act F/R (B). Lanes I to VIII are eight PCR programmes tested (see Table 4). M is the 100 bp DNA ladder (Carl Roth).

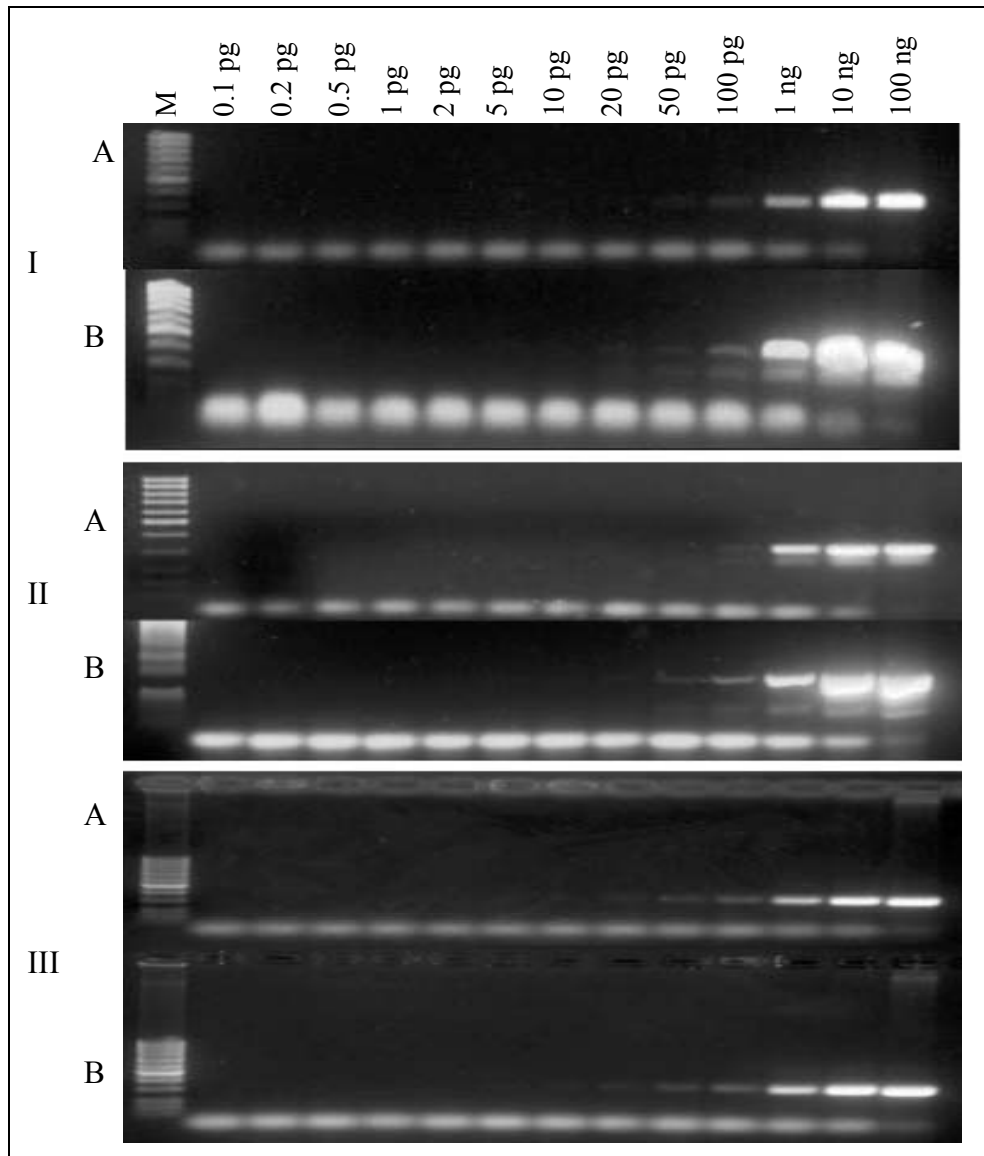
### 3.3.3 Sensitivity of four specific primer sets

The detection sensitivity is an important element for the diagnostic application of a primer set. To test the sensitivity of four developed primer sets, a conventional PCR was performed using 13 different concentrations of genomic DNA of *M. graminicola* ranging from 0.1 pg to 100 ng. Twenty  $\mu$ l of PCR products were visualized on a 2% agarose gel stained with ethidium bromide. Three primer sets ST-rRNA F/R, ST-act F/R and R5870-1F/R5870-1R could detect as little as 0.5 pg of genomic DNA (Fig. 17A, B and D), but R5870-1F/R5870-2R had much lower detection sensitivity and only up to 50 pg of genomic DNA were detectable (Fig. 17C). For ST-rRNA F/R, the band intensity with 100 ng was clearly lower than that with 10 ng and 1 ng (Fig. 17A), indicating that excessive DNA input might inhibit the PCR amplification of this primer set.



**Fig. 17** The sensitivity of the four primer sets ST-rRNA F/R (A), ST-act F/R (B), R5870-1F/R5870-2R (C) and R5870-1F/R5870-1R (D) in a conventional PCR on a 2% ethidium bromide-stained agarose gel. Lanes 1 to 13 are 20  $\mu$ l PCR product amplified using these primer sets and 13 different amounts of *Mycosphaerella graminicola* DNA. M is the 100 bp DNA ladder (Carl Roth).

SYBR Green I is a sensitive fluorescent dye available for detecting dsDNA in agarose gel. We attempted to improve the detection sensitivity of the specific primer sets on agarose gel using SYBR Green I staining. The PCR products used for the test were amplified using the primer set R5870-1F/R5870-2R and 13 different concentrations of genomic DNA of *M. graminicola*. The staining efficiency of SYBR Green I in three different staining methods compared with ethidium bromide staining: I. staining the PCR products before electrophoresis; II. adding the dye in the gel; III. staining the gel after electrophoresis. In treatment I, SYBR Green I staining obviously improved the detection sensitivity from 100 pg (Fig. 18IA) to 20 pg (Fig. 18IB), but obvious band shift was present. In treatment II, the detection sensitivity was improved from 100 pg to 50 pg (Fig. 18II). Although the band intensity on the gel stained with SYBR Green I was somewhat stronger than that stained with ethidium bromide, the detection limit has no obvious difference between both stains in treatment III (Fig. 18III).



**Fig. 18** Comparison of the detection sensitivity of ethidium bromide (EtBr) staining (A) and SYBR Green I (SYBR) staining (B). The gel was photographed under 312 nm transillumination with a EtBr filter OF03-A0105. I. Staining the PCR product before electrophoresis (EtBr 1:2000, SYBR 1:1000). II. Adding the dyes into the gel (EtBr 1:20,000, SYBR 1:10,000). III. Staining the gel after electrophoresis (EtBr 1:10,000, SYBR 1:10,000). Lanes 1 to 13 are 10  $\mu$ l PCR products amplified using R5870-1F/R5870-2R and 13 different amounts of *Mycosphaerella graminicola* DNA. M is the 100 bp DNA ladder (Carl Roth).

### 3.4 Real-time PCR

#### 3.4.1 Effects of primer degradation and DNA template input

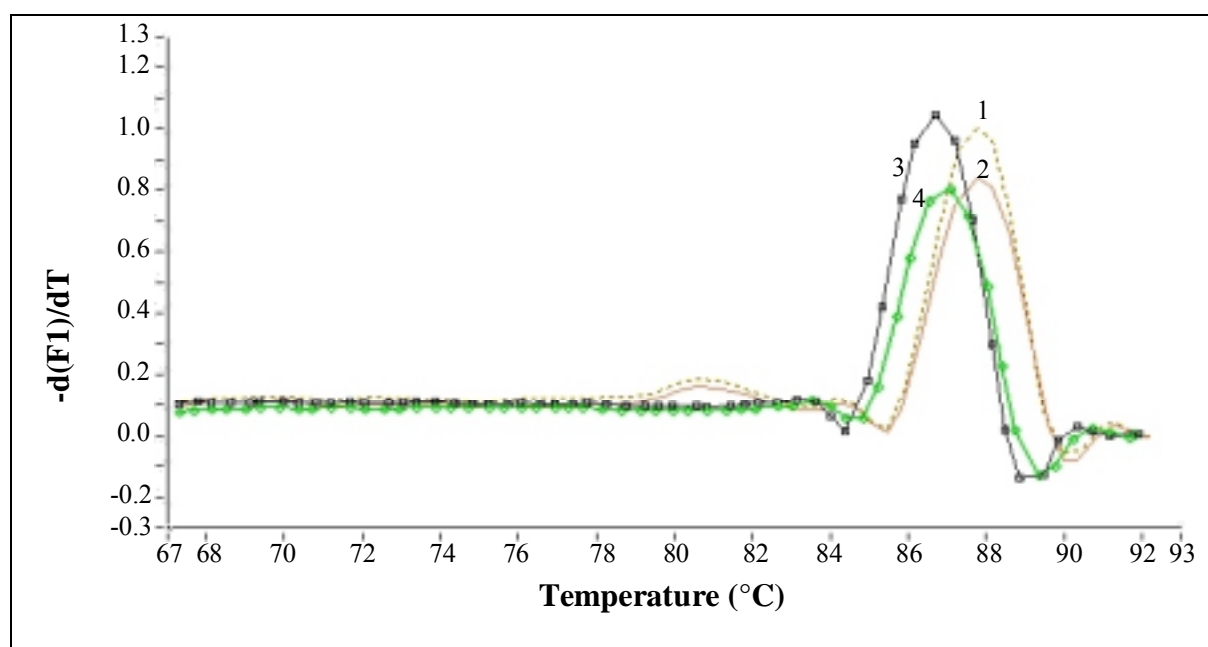
To test the influence of potential primer degradation on real-time PCR, two old primer sets ST-rRNA F/R and ST-act F/R stored for 14 months at  $-20^{\circ}\text{C}$  were tested by real-time PCR using 1 ng genomic DNA of *M. graminicola*. New primer sets served as control. 0.3  $\mu\text{M}$  of

each primer were used as recommended in the instruction of the kit. The results showed that the fluorescence decreased clearly when using the old primers in comparison with new primers, especially the old ST-act F/R primers were almost three cycles later than the new primers (Table 9). It is likely that the old primers had somewhat decomposed during storage and result in the decrease of primer concentration. It is suggested that the concentration of primers is an important factor for real-time PCR. The melting curve revealed that no unspecific products were produced in the real-time PCR using the old primers (Fig. 19), indicating that the degradation of primers did not cause the formation of unspecific products.

**Table 9** Effect of primer degradation on real-time amplification efficiency

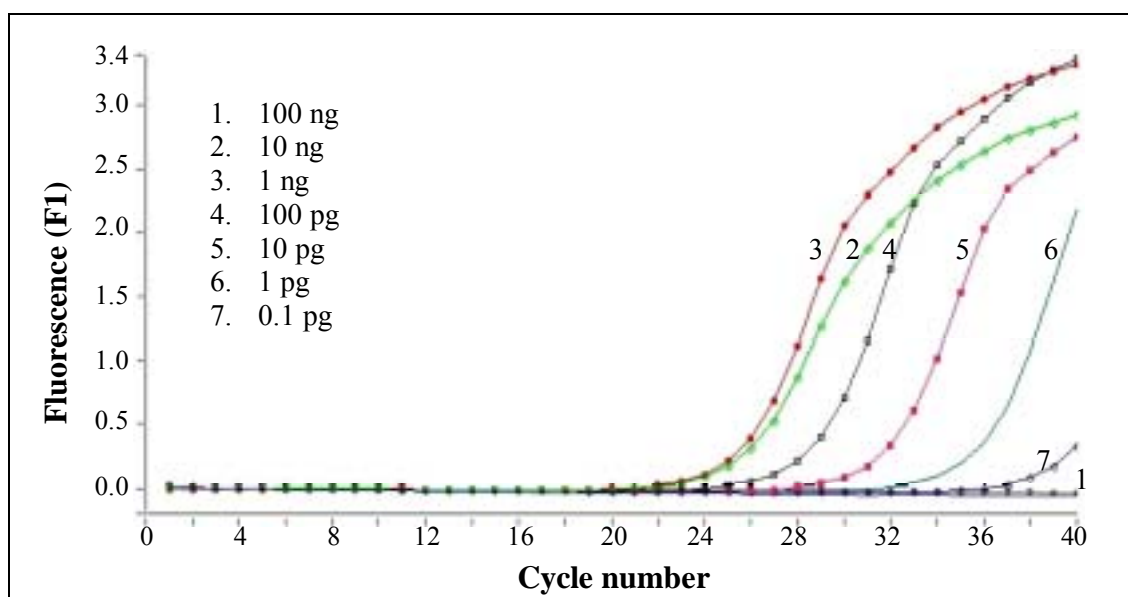
Primers	Cycle number <sup>a</sup>
Old ST-act F/R	25.69 ± 0.52
New ST-act F/R	22.89 ± 0.07
Old ST-rRNA F/R	14.84 ± 0.13
New ST-rRNA F/R	13.88 ± 0.10

<sup>a</sup> The values are the mean of two replicates ± SD.



**Fig. 19** Melting curves generated by real-time PCR using old (stored for 14 month at -20°C) and new primers. 1. New primers of ST-rRNA F/R; 2. Old primers of ST-rRNA F/R; 3. New primers of ST-act F/R; 4. Old primers of ST-act F/R.

In order to test the influence of DNA template input, real-time PCR was performed using a dilution series of *M. graminicola* DNA ranging from 0.1 pg to 100 ng with the primer sets ST-act F/R. The results show that the reaction in the 100-ng sample hardly worked and the reaction was somewhat inhibited in the 10-ng sample (Fig. 20, profiles 1 and 2). The suitable concentration range of DNA template for ST-act F/R was between 0.1 pg and 1 ng (Fig. 20). Similar results were obtained from real-time PCR using the primer sets ST-rRNA F/R and R5870-1F/R5870-2R (results not shown). It was demonstrated that an excessive input of DNA template inhibited the amplification in real-time PCR.

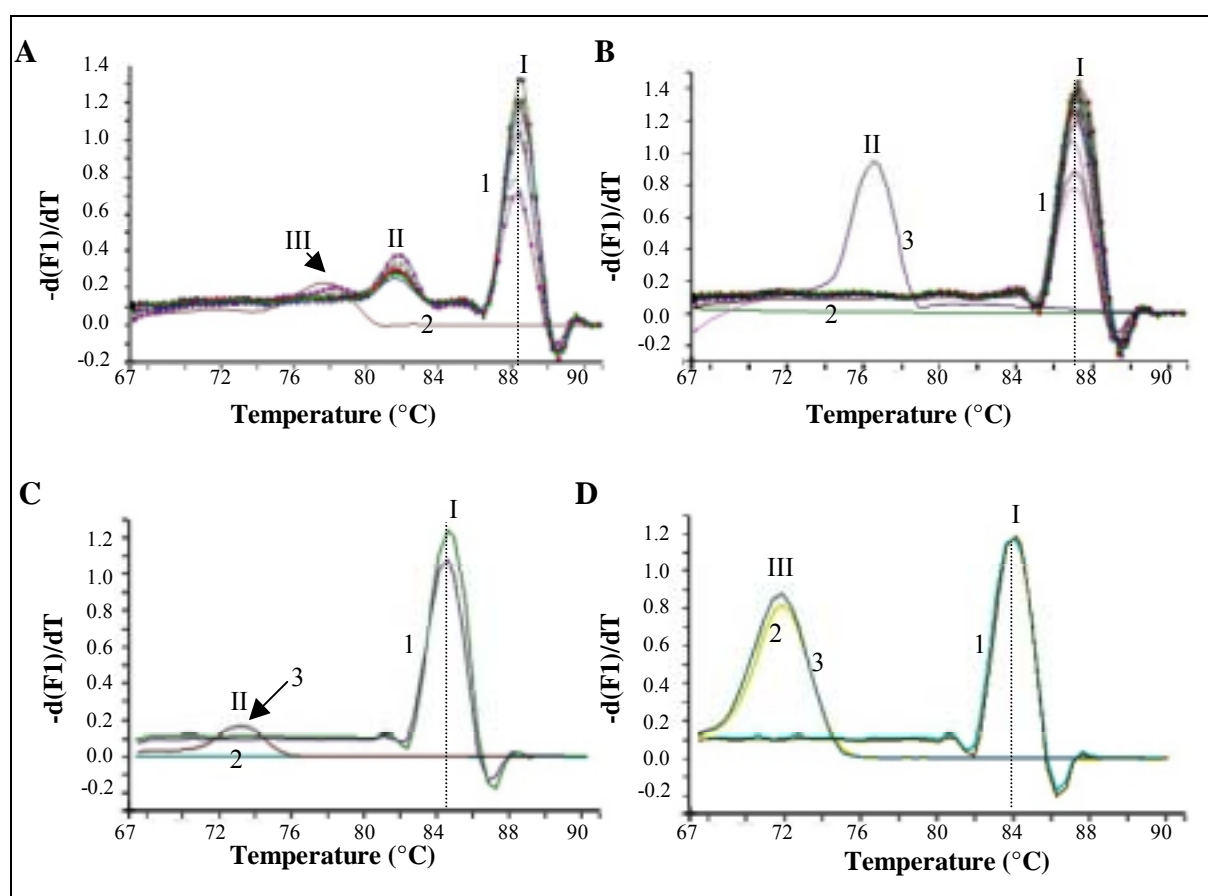


**Fig. 20** Real-time PCR profiles generated from cycle to cycle collection of fluorescence using different concentrations of *Mycosphaerella graminicola* DNA ranging from 0.1 pg to 100 ng and using ST-act F/R.

### 3.4.2 Suitability of four specific primer sets for real-time PCR

To test the specificity of four primer sets and to identify their target PCR products by real-time PCR, a melting curve analysis was performed using genomic DNA of *M. graminicola*, healthy plants and H<sub>2</sub>O (negative control). According to the melting curves, all four primer sets amplified the target product only from *M. graminicola* DNA, but not from healthy plant DNA, although primer dimers or non-specific products were present in such samples with lower template concentration or without template (Fig. 21). For ST-rRNA F/R, the melting temperature ( $T_m$ ) maximum of the target product was  $88.4 \pm 0.2^\circ\text{C}$ , a non-specific product was present at a  $T_m$  maximum of  $81.8 \pm 0.2^\circ\text{C}$ , primer dimers were formed from samples with lower template concentration and negative control at a  $T_m$  maximum of

$77.5 \pm 0.5^\circ\text{C}$  (Fig. 21A, Table 10). For ST-act F/R, the target product had a  $T_m$  maximum of  $87.2 \pm 0.2^\circ\text{C}$ , a non-specific product was produced from healthy plant DNA at a  $T_m$  maximum of  $76.5 \pm 0.1^\circ\text{C}$ , but no peak was present from the H<sub>2</sub>O negative control, indicating no primer dimer was formed (Fig. 21B, Table 10). For R5870-1F/R5870-1R, the target product has a  $T_m$  maximum of  $85.7 \pm 0.2^\circ\text{C}$ , a non-specific product was produced from healthy plant DNA at a  $T_m$  maximum of  $73.2 \pm 0.2^\circ\text{C}$ , and no primer dimer formed (Fig. 21C, Table 10). For R5870-1F/R5870-2R, the maximum  $T_m$  of target product was  $84.3 \pm 0.1^\circ\text{C}$ , clear primer dimers were formed from plant DNA and H<sub>2</sub>O control at a  $T_m$  maximum of  $71.9 \pm 0.1^\circ\text{C}$  (Fig. 21D, Table 10).



**Fig. 21** Melting curve of the four specific primer sets ST-rRNA F/R (A), ST-act F/R (B), R5870-1F/R5870-1R (C) and R5870-1F/R5870-2R (D) generated by real-time PCR using DNA of *Mycosphaerella graminicola* (1) and healthy plants (3) and H<sub>2</sub>O (2), plotted against the negative derivative of the fluorescence with respect to temperature [ $-\text{d}(\text{F}1)/\text{dT}$ ] versus temperature. The curves reveal specific target product, non-specific products or primer dimers: (I) target product, (II) nonspecific products and (III) primer dimers.



**Table 10** Melting temperature ( $T_m$ ) of target product, nonspecific products and primer dimers obtained from the melting curves shown in Fig. 21

Primer set	$T_m^a$		
	Target product	Nonspecific products	Primer dimers
ST-rRNA F/R	87.2 ± 0.2	76.5 ± 0.1	
ST-act F/R	88.4 ± 0.2	81.8 ± 0.2	77.5 ± 0.5
R5870-1F/R5870-1R	85.7 ± 0.2	73.2 ± 0.2	
R5870-1F/R5870-2R	84.3 ± 0.1	71.9 ± 0.1	

<sup>a</sup>  $T_m$  is manually defined using the manual  $T_m$  tool of the LightCycler data analysis software.

In order to suppress fluorescence readings caused by primer dimers or non-specific products, an additional data acquisition step was set up for each primer set according to the melting curve analysis. For ST-act F/R, R5870-1F/R5870-1R and R5870-1F/R5870-2R, the data acquisition was at 80°C, and for ST-rRNA F/R it was at 82°C.

**Table 11** Sensitivity comparison of four primer sets by real-time PCR and by conventional PCR

Primer set	Target fragment	Cycle number <sup>a</sup>	Detection sensitivity	
			Conventional PCR <sup>b</sup>	Real-time PCR
ST-rRNA F/R	328	20.49 ± 0.15	0.5 pg	0.1 pg
ST-act F/R	496	23.37 ± 0.11	0.5 pg	0.1 pg
R5870-1F/R5870-1R	426	27.45 ± 0.15	0.5 pg	0.1 pg
R5870-1F/R5870-2R	326	25.93 ± 0.06	50 pg	1 pg

<sup>a</sup> Cycle numbers were measured by real-time PCR using 1 ng genomic DNA of *Mycosphaerella graminicola*. The values are the means of three replicates ± SD.

<sup>b</sup> The results of detection sensitivity of conventional PCR were from section 3.3.3.

The sensitivity of four primer sets was primarily compared by real-time PCR using 1 ng *M. graminicola* DNA under the same conditions. Among the four primer sets, ST-rRNA F/R gave rise to the smallest cycle number (20.49) and R5870-1F/R5870-1R had the largest when used the same DNA template input (27.45, Table 11), indicating that ST-rRNA F/R may have the highest sensitivity and R5870-1F/R5870-1R may have the lowest. The sensitivity of three primer sets of ST-rRNA F/R, ST-act F/R and R5870-1R/R5870-2R was further tested using five 10-fold dilution series of 1 ng, 100, 10, 1 and 0.1 pg *M. graminicola* DNA. The results show that ST-rRNA F/R and ST-act F/R could detect at least 0.1 pg and R5870-1R/R5870-2R could detect 1 pg *M. graminicola* DNA (Table 11). In comparison with conventional PCR,

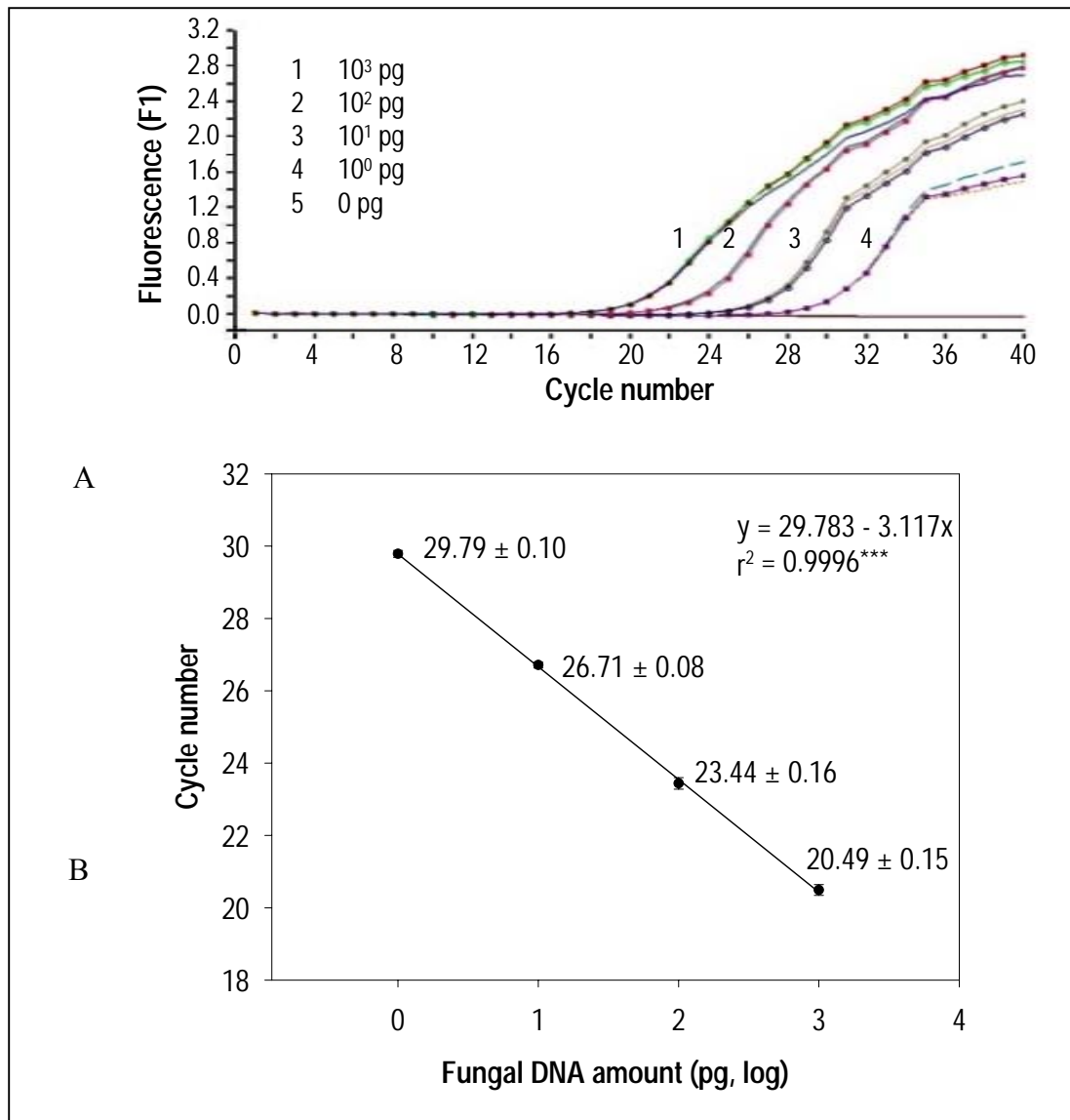
real-time PCR represented increased sensitivity for four primer sets tested, especially for R5870-1F/R5870-2R.

### 3.4.3 Generation of DNA standard curves

Three primer sets (ST-rRNA F/R, ST-act F/R and R5870-1-F/R5870-2-R) were selected for the quantification of *M. graminicola* DNA in wheat by real-time PCR. To calculate the fungal DNA amount from the cycle number measured by real-time PCR, three DNA standard curves (one for each primer set) were generated, using five delution series of *M. graminicola* genomic DNA ( $10^{-1}$  to  $10^3$  pg).

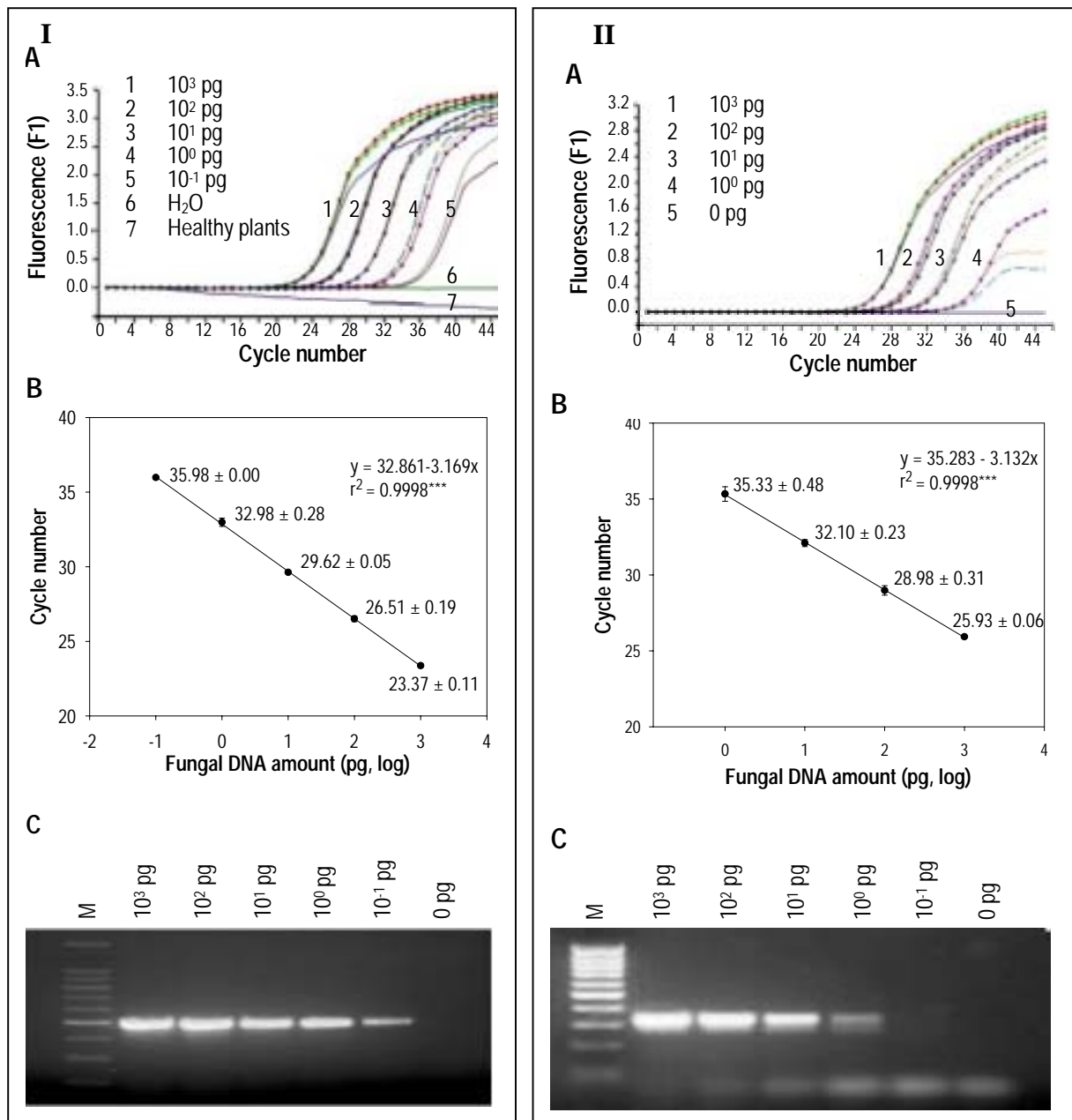
For ST-rRNA F/R, four dilutions of  $10^0$ ,  $10^1$ ,  $10^2$  and  $10^3$  pg of *M. graminicola* genomic DNA were used for the generation of the standard curve since a linear relationship was no more suitable for  $10^{-1}$  pg. Amplification profiles of the samples generated with cycle-by-cycle collection of fluorescence showed that three replicates produced almost the same amplification profiles in four different concentrations (Fig. 22A), and the standard deviation was rather small in all four DNA dilutions (Fig. 22B). This is evidence that the reproducibility of real-time PCR was high. The fluorescence value for the no-template control ( $H_2O$ ) remained at the baseline throughout 40 cycles, indicating that no fluorescent signal was detected in the  $H_2O$  samples. The cycle numbers for the samples of  $10^3$ ,  $10^2$ ,  $10^1$  and  $10^0$  pg genomic DNA were  $20.49 \pm 0.15$ ,  $23.44 \pm 0.16$ ,  $26.71 \pm 0.08$  and  $29.79 \pm 0.10$ , respectively (Fig. 22B).

A standard curve was generated by plotting the concentration of *M. graminicola* DNA versus their cycle number obtained from real-time PCR. A linear relationship was observed when the values of cycle number were plotted against the logs of the respective DNA amount (Fig. 22B). The regression equation was  $y = 29.783 - 3.117x$ . With this standard curve, a 10-fold dilution corresponded to approximately three cycles. The linearity ranged at least from  $10^0$  to  $10^3$  pg. The coefficient of determination ( $r^2$ ) for the standard curve was 0.9996\*\*\*. It indicated that the slope of the line was different from zero and hence there was a significant relationship between fungal DNA amount and cycle number.



**Fig. 22** Real-time PCR analysis and generation of DNA standard curve using ST-rRNA F/R and a dilution series of *Mycosphaerella graminicola* DNA (0 to 10<sup>3</sup> pg, each dilution with three replicates). (A) Real-time PCR profiles generated from cycle-to-cycle collection of fluorescence. (B) Standard curve for the calculation of *M. graminicola* DNA amount based on the respective cycle number obtained from the real-time PCR test.

The standard curve for ST-act F/R was generated with five dilutions ranging from 10<sup>-1</sup> to 10<sup>3</sup> pg of *M. graminicola* genomic DNA, using DNA of healthy plants and water as negative controls. The amplification profiles showed no fluorescence was accumulated in negative control throughout 45 cycles (Fig. 23IA). The logs of fungal DNA amounts were linearly related to the values of cycle number, ranging at least from 10<sup>-1</sup> to 10<sup>3</sup> pg. The regression equation was  $y = 32.861 - 3.169x$ , and the coefficient of determination was 0.9998<sup>\*\*\*</sup> (Fig. 23IB). The PCR products were visualized in a 2% agarose gel. A clear change of



**Fig. 23** Generation of standard curves for the quantification of *Mycosphaerella graminicola* DNA by real-time PCR using ST-act F/R (I) and R5870-1F/R5870-1R (II). Five dilutions of *M. graminicola* DNA ( $10^{-1}$  to  $10^3$  pg) were used, H<sub>2</sub>O and DNA of healthy plants were used as negative control. Three replicates were used for each concentration. (A) Real-time PCR profiles generated from cycle-to-cycle collection of fluorescence. (B) DNA standard curve. The logs of the initial fungal DNA amount are plotted versus the corresponding values of cycle number. Whenever no errorbars are visible, standard deviations are smaller than the plot symbols (C) Visualization of real-time PCR products by electrophoresis. Twenty  $\mu$ l of each product were loaded on a 2% agarose gel. M in IC is the 100 bp DNA ladder (Promega) and M in IIC is the 100 bp DNA ladder (Carl Roth).

The band intensity between different dilutions could be observed, and a clear band was also visualized in the  $10^{-1}$ -pg sample (Fig. 23IC).

For the primer set R5870-1F/R5870-2R, real-time PCR was performed with five dilution series from  $10^{-1}$  to  $10^3$  pg of *M. graminicola* genomic DNA. The amplification profiles showed that a good reproducibility was obtained in the  $10^3$ -,  $10^2$ - and  $10^1$ -pg samples and no fluorescence was detected in the H<sub>2</sub>O negative control throughout 45 cycles (Fig. 23IIA). Although the fluorescence collection was observed in the  $10^{-1}$ -pg sample, a linear relationship extends no more at this concentration. Therefore, the standard curve for R5870-1F/R5870-2R was constructed with the cycle number values of the  $10^3$ -,  $10^2$ -,  $10^1$ - and  $10^0$ -pg samples. A good linear relationship was observed between the logs of *M. graminicola* DNA and the values of cycle numbers obtained from real-time PCR within the concentration range of  $10^{-1}$  to  $10^3$  pg. The regression equation for the standard curve was  $y = 35.283 - 3.132x$ ,  $r^2 = 0.9998^{***}$  (Fig. 23IIB). A visualization of PCR products showed that this primer set could detect as little as 1 pg *M. graminicola* genomic DNA by real-time PCR (Fig. 23IIC).

#### 3.4.4 Monitoring of the disease development in inoculated wheat plants

By quantifying the fungal DNA amount *in planta*, real-time PCR was used to monitor the disease development of *M. graminicola* in inoculated wheat plants using the specific primer sets ST-rRNA F/R and ST-act F/R. Thirty-day-old seedlings were inoculated with an isolate newly isolated from infected plants, and then grown in a growth chamber. The infected leaves were collected every two days after inoculation and the disease severity was assessed visually by a disease classification. The first lesions were observed eight days after inoculation in 50% of the leaves, and the disease index was 12.94 (Table 12). After 12 days, 100% leaves were symptomatic, the disease index increased rapidly to 67.2, and necrotic lesions with pycnidia were present in most leaves. The rapid progression period was observed between eight and 12 days after inoculation (Table 12, Fig. 24 ).

The results obtained from real-time PCR showed that it could detect *M. graminicola* on the inoculation day, although the first visible symptoms appeared eight days after inoculation (Table 12, Fig. 24). During the first two days, the DNA amount increased slightly. Despite no symptom appeared before eight days, the fungal DNA amount in four days and in six days obviously increased. Between six and eight days, fungal DNA amount increased rapidly by more than five times. The rapid growth period of fungal DNA was observed between 6 and 16

days according to the data tested with ST-rRNA F/R and between 6 and 18 days according to that tested with ST-act F/R.

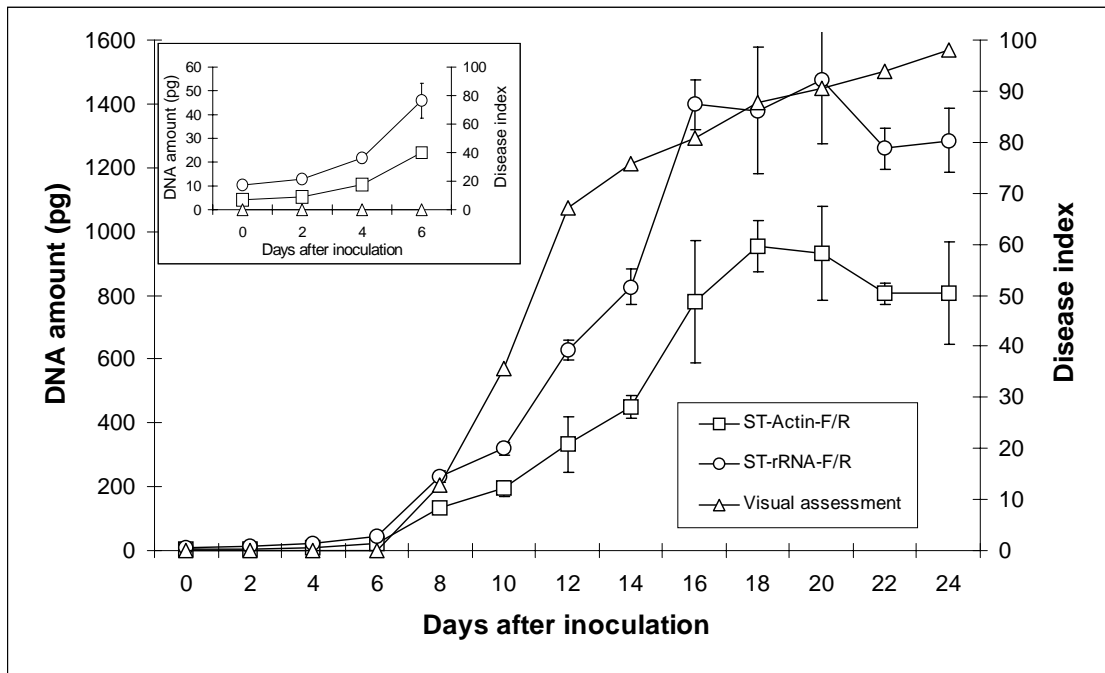
**Table 12** Quantification of *Mycosphaerella graminicola* DNA amount by real-time PCR using the primer sets ST-rRNA F/R and ST-act F/R during the disease development in inoculated plants in comparison with the disease index

Days after inoculation	Disease index <sup>a</sup>	ST-rRNA F/R <sup>b</sup>		ST-act F/R <sup>b</sup>	
		Cycle number	DNA amount (pg)	Cycle number	DNA amount (pg)
0	0.00	26.63 ± 0.20	10.32 ± 1.50	30.94 ± 0.38	4.15 ± 1.22
2	0.00	26.34 ± 0.08	12.77 ± 0.76	30.55 ± 0.10	5.37 ± 0.40
4	0.00	25.63 ± 0.14	21.63 ± 2.26	29.62 ± 0.25	10.63 ± 1.86
6	0.00	24.62 ± 0.22	45.83 ± 7.38	28.49 ± 0.10	24.00 ± 1.86
8	12.94	22.41 ± 0.10	233.00 ± 18.20	26.13 ± 0.15	133.25 ± 14.62
10	35.76	21.97 ± 0.09	320.68 ± 20.10	25.61 ± 0.19	194.82 ± 25.54
12	67.20	21.06 ± 0.07	627.75 ± 30.11	24.90 ± 0.37	332.84 ± 87.06
14	75.65	20.69 ± 0.09	825.72 ± 55.57	24.45 ± 0.11	450.85 ± 35.94
16	80.80	19.98 ± 0.08	1397.86 ± 78.35	23.73 ± 0.36	779.85 ± 192.51
18	87.83	20.01 ± 0.20	1379.06 ± 198.34	23.42 ± 0.12	953.13 ± 79.80
20	90.43	19.91 ± 0.19	1476.15 ± 203.70	23.46 ± 0.23	932.04 ± 148.18
22	94.00	20.12 ± 0.07	1260.31 ± 65.43	23.65 ± 0.06	804.99 ± 33.53
24	98.18	20.10 ± 0.11	1248.64 ± 100.55	23.67 ± 0.28	805.76 ± 160.38

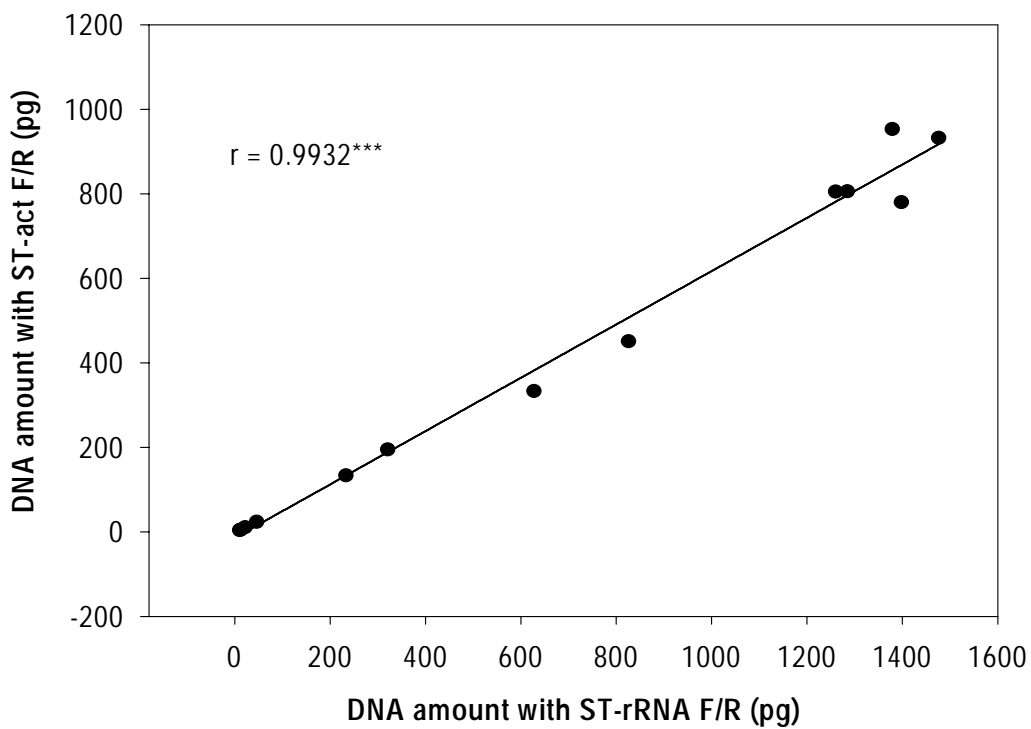
<sup>a</sup> Disease indices were assessed visually by disease classification. Each value was calculated from all leaves sampled on each date (the original data were shown in Table A4).

<sup>b</sup> Mean of three replicates ± SD, n = 3 (the original data were shown in Table A7).

The DNA amount measured using ST-rRNA F/R was 1.45 - 2.49 times higher than that using ST-act F/R (Table 12), but the shape of both fungal DNA growth curves was similar except 18 days after inoculation (Fig. 24). The correlation between the fungal DNA amounts measured using both primer sets was highly significant, and the relationship between them are linear ( $r = 0.9932^{***}$ , Fig. 25).

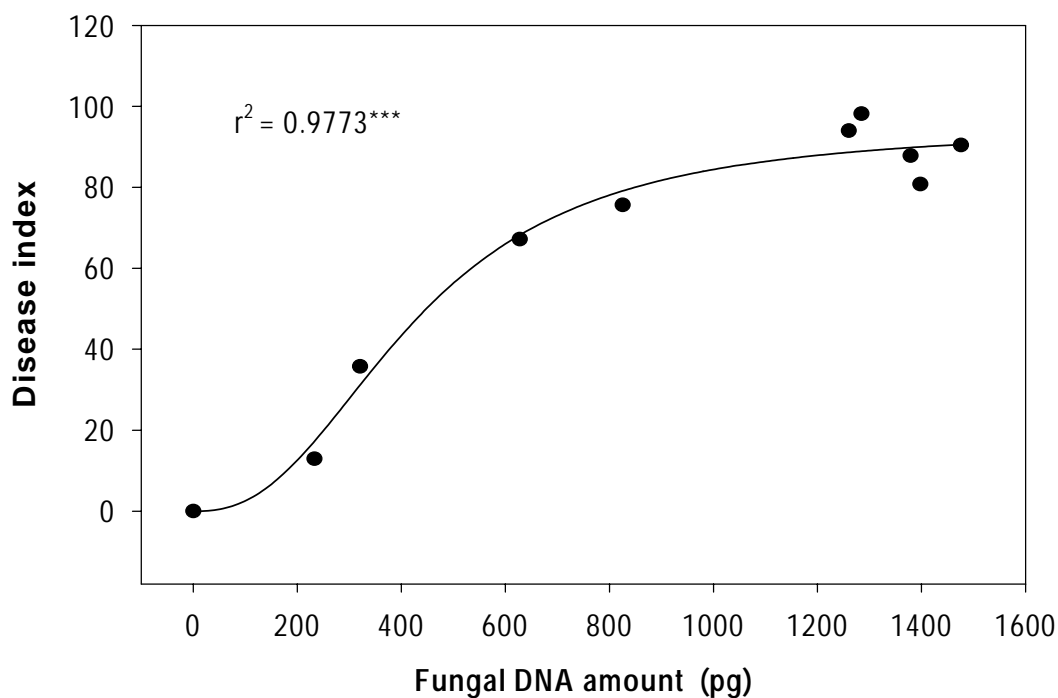


**Fig. 24** Monitoring *Mycosphaerella graminicola* growth in inoculated wheat plants as measured by the fungal DNA amount using real-time PCR with the primer sets ST-rRNA F/R and ST-act F/R, compared with the visual assessment. Each data point obtained from real-time PCR represents the average of three replicates  $\pm$  SD.



**Fig. 25** Correlation between the DNA amounts measured with ST-rRNA F/R and ST-act F/R during the disease development in inoculated plants.

The fungal DNA growth tested by real-time PCR was compared with the results visually assessed by a disease classification. Real-time PCR could detect *M. graminicola* on the inoculation day and a clear increase of fungal DNA was obtained before symptoms appeared (Table 12). However, the visual assessment could be performed only after the symptoms were visible (since eight days after inoculation). The shape of disease progression curves obtained by both methods was basically identical at most date points tested except for 18, 22 and 24 days after inoculation. The fungal DNA amount obtained from real-time PCR was essentially constant after 18 days (Fig. 24). The results obtained by real-time PCR were significantly correlated with those obtained by a conventional visual assessment ( $r = 0.9115^{***}$ ). However the relationship between fungal DNA amount and disease index was non-linear (Fig. 26). In particular, very low disease indices were represented when the fungal DNA amounts were less than 200 pg. This result was expected because fungal DNA could be detected in the absence of symptoms at the early stage of disease development. Furthermore, the fungal DNA amount still further increased at disease indices close to 100.



**Fig. 26** Relationship between the fungal DNA amount measured by real-time PCR using ST-rRNA F/R and the disease index in the inoculated plants.



### 3.4.5 Monitoring of *M. graminicola* disease progression in the field

#### 3.4.5.1 Real-time PCR using the primer sets ST-rRNA F/R and ST-act F/R

In order to monitor the disease progression of *M. graminicola* in F, F1 and F2 leaves during the epidemic period, thirty leaves were collected weekly from a fungicide-untreated plot from May 3 to June 21, 2004. The disease severity in these samples were first assessed by a disease classification and by pycnidia-counting method before DNA extraction. The results showed that the disease developed slowly before May 24 (Table 13, Fig. 27). On May 24, the disease index and the number of pycnidia per leaf (NPL) in F2 leaves were only 19.31 and 10, respectively and in most leaves only chlorotic spots appeared. But from May 24 to June 1, the disease index increased rapidly from 19.31 to 70 and the NPL from 10 to 49.3. On June 14, most F2 leaves dried up, the disease index amounted to 100 and the NPL to 394. In F1 leaves, no visible symptoms appeared before May 10. On May 17, only chlorotic spots or small lesions were present on some leaves. The rapid disease progression period was between May 24 (the disease index and NPL were 14.48 and 0.67, respectively) and June 7 (88.66 and 293.7). The first flag leaves were collected on May 17. The disease in flag leaves developed slowly during the sampling period. The visible symptoms on F leaves were observed on June 1 for the first time, and disease index and NPL on the final sampling day (June 21) were only 16.67 and 18.7. The disease progression curves showed that the disease progression obtained by counting pycnidial number was approximately one week later than that by disease classification (Figs. 27A and B). Before May 24, NPL was very low in F1 and F2 leaves, whereas the disease indices were almost 20.00 in F2 leaves on May 17 and 24, and in F1 leaves on May 24. The NPLs in F1 and F2 leaves were very similar (Fig. 27B), but the disease indices in F1 leaves were somewhat less than those in F2 leaves between May 24 and June 14 (Fig. 27A).

Meanwhile, the disease development in the F, F1 and F2 leaves was monitored by quantifying the fungal DNA amount in these leaves using real-time PCR (Table 13). Real-time PCR using ST-rRNA F/R could detect *M. graminicola* DNA in all samples tested, whereas when using ST-act F/R could not detect *M. graminicola* DNA in four F samples (May 17, 24 and June 1, 14), three F1 samples (May 3, 10, 17 and 24) and three F2 leaves (May 3, 10 and 24, Table 13). It indicates that ST-act F/R had a higher detection limit than ST-rRNA F/R when used for the detection of field samples. In addition, the DNA amount obtained from the real-time PCR data using ST-act F/R was obviously lower than that using ST-rRNA F/R (Table 13) and similar to the results obtained with the inoculated plants (Table 12).

**Table 13** Monitoring of the disease progression of *Mycosphaerella graminicola* in F, F1 and F2 leaves sampled in a fungicide-untreated plot during the epidemic period by visual assessment and by real-time PCR using the primer sets ST-rRNA F/R and ST-act F/R

Leaf	Date	Disease index <sup>a</sup>	NPL <sup>b</sup>	ST-rRNA F/R <sup>c</sup>		ST-act F/R <sup>c</sup>	
				Cycle number	DNA amount (pg)	Cycle number	DNA amount (pg)
F	May 17	0.00	0.00	30.66 ± 0.29	0.53 ± 0.12		0.00
F	May 24	0.00	0.00	30.51 ± 0.25	0.59 ± 0.11		0.00
F	June 1	5.33	6.30	30.78 ± 0.64	0.37 ± 0.04		0.00
F	June 7	6.67	10.70	27.69 ± 0.13	4.71 ± 0.48	29.17 ± 0.23	14.77 ± 2.49
F	June 14	10.67	18.70	25.00 ± 0.10	34.31 ± 2.41		0.00
F	June 21	16.67	0.00	24.60 ± 0.33	47.14 ± 10.85	28.19 ± 0.38	30.53 ± 9.07
F1	May 3	0.00	0.00	29.20 ± 0.12	1.54 ± 0.14		0.00
F1	May 10	0.00	0.00	29.65 ± 0.23	1.11 ± 0.21		0.00
F1	May 17	3.23	0.00	28.38 ± 0.20	2.46 ± 0.65		0.00
F1	May 24	14.48	0.67	27.11 ± 0.13	7.23 ± 0.66		0.00
F1	June 1	46.00	84.00	24.56 ± 0.05	48.00 ± 2.09	29.05 ± 0.24	16.06 ± 2.70
F1	June 7	88.66	293.70	22.13 ± 0.31	291.72 ± 63.00	26.96 ± 0.15	73.26 ± 7.72
F1	June 14	91.33	416.70	21.25 ± 0.22	554.29 ± 88.96	25.44 ± 0.46	227.69 ± 78.18
F1	June 21	95.33	0.00	20.84 ± 0.04	744.07 ± 20.52	24.80 ± 0.15	351.11 ± 39.07
F2	May 3	1.33	0.00	29.25 ± 0.12	1.49 ± 0.16		0.00
F2	May 10	4.13	0.30	27.32 ± 0.23	6.17 ± 0.07		0.00
F2	May 17	15.56	0.67	27.17 ± 0.20	7.00 ± 1.35		0.00
F2	May 24	19.31	10.00	27.24 ± 0.13	6.47 ± 0.94	29.91 ± 0.12	8.58 ± 0.75
F2	June 1	70.00	49.30	24.79 ± 0.05	41.10 ± 11.71	28.50 ± 0.16	23.85 ± 2.69
F2	June 7	96.00	288.30	21.06 ± 0.22	635.20 ± 76.34	25.53 ± 0.25	208.42 ± 35.59
F2	June 14	100.00	394.00	20.75 ± 0.31	800.30 ± 110.51	25.12 ± 0.14	278.73 ± 27.22
F2	June 21	100.00		20.90 ± 0.04	709.08 ± 80.71	25.29 ± 0.03	244.98 ± 5.03

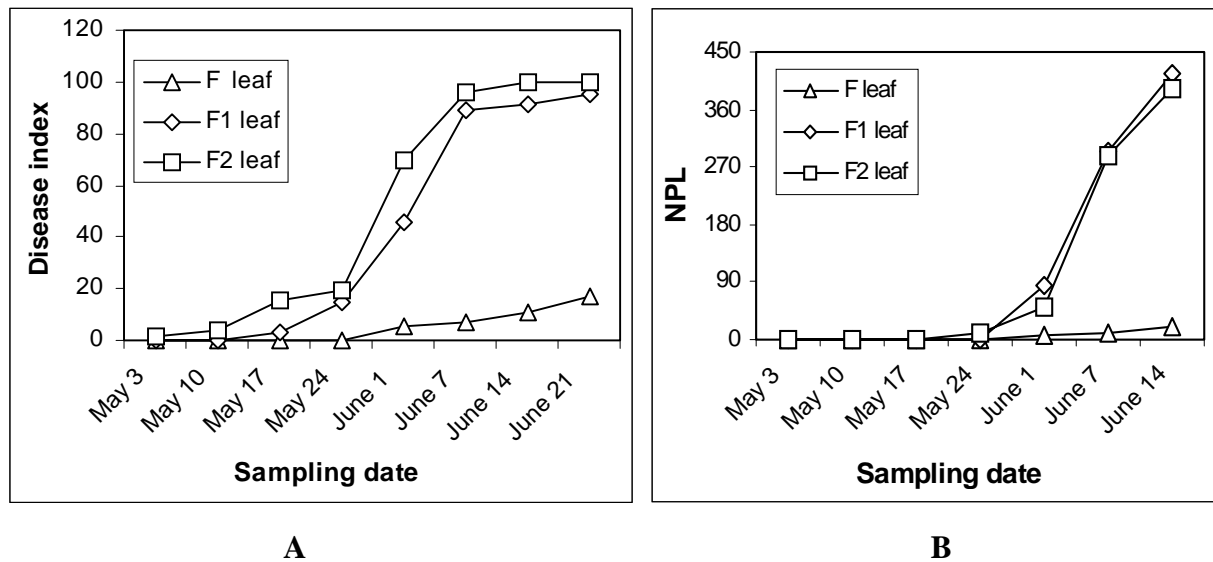
<sup>a</sup> Disease index was assessed by a disease classification. The values were calculated from the disease scale of 30 leaves randomly sampled from a fungicide-untreated plot on each sampling date (the original data were shown in Table A5).

<sup>b</sup> The data of NPL (number of pycnidia per leaf) were kindly provided by Dr. F. Schnieder. The value represents the mean of 30 leaves.

<sup>c</sup> The data obtained from real-time PCR are the mean of three replicates ± SD (the original data were shown in Table A8). The DNA amount is calculated from the cycle number according to the corresponding equations of standard curves (Fig. 22B, Fig. 23IB,)

Blank column in the table means no value obtained by such assessment.

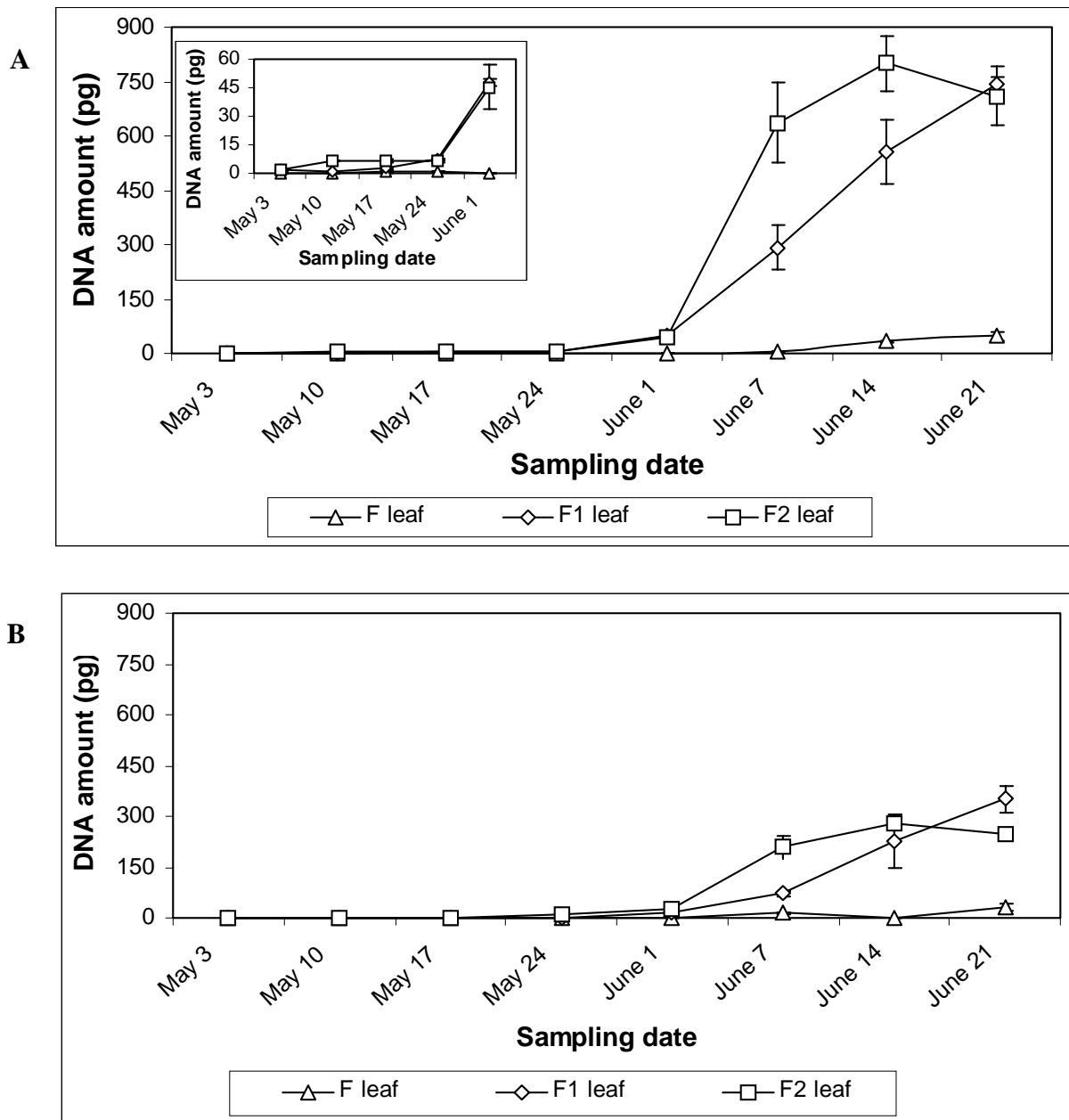
The shape of *M. graminicola* DNA growth curves in F, F1 and F2 leaves obtained from real-time PCR with both primer sets was similar between May 24 and June 21 (Fig. 28A, B), although their DNA amounts were different. In F2 leaves, the DNA amount of *M. graminicola* was low before May 24, but then it increased rapidly until June 14. After that DNA amount was essentially constant. In F1 leaves, the DNA amount increased very slowly before May 24, but increased rapidly between June 1 and June 21. In contrast to the DNA amounts in F1 and F2 leaves, *M. graminicola* DNA in F leaves was detected at a relatively low level during the whole epidemic period although it was already detected at the first sampling date (May 17, Table 13, Fig. 28A). Nevertheless, a clear growth of *M. graminicola* DNA in F leaves was observed between June 1 and June 14 (Table 13).



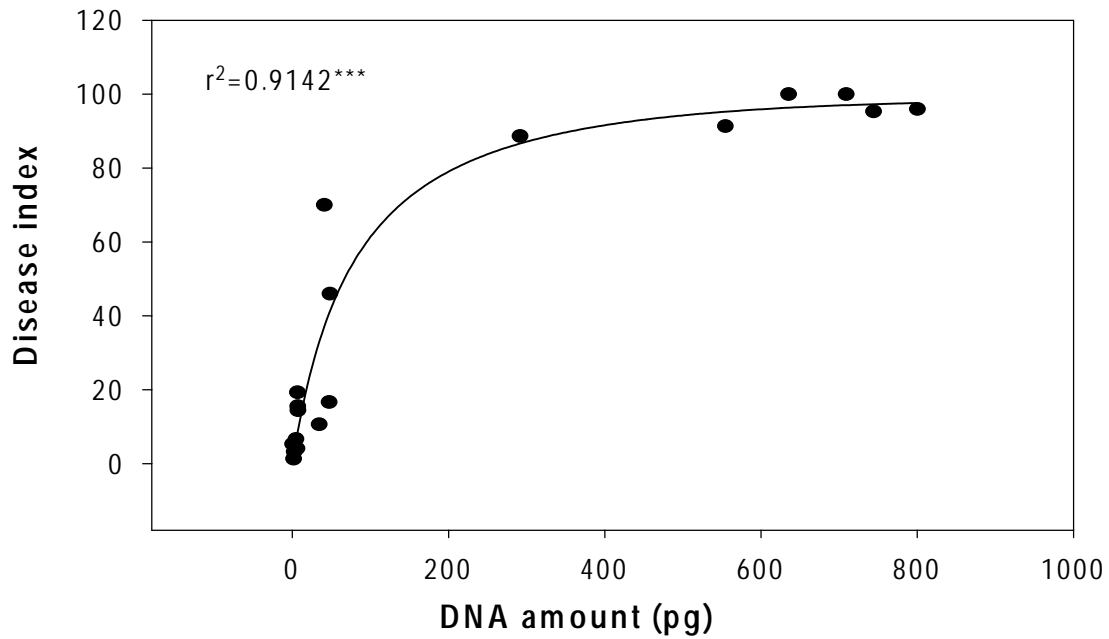
**Fig. 27** Monitoring of *Mycosphaerella graminicola* disease progression in F, F1 and F2 leaves sampled from a fungicide-untreated plot during the epidemic period as assessed by two visual methods. (A) Disease classification; (B) Counting the number of pycnidia per leaf (NPL).

The disease progression in three leaf positions obtained from real-time PCR using the primer set ST-rRNA F/R (Fig. 28A) revealed a close relationship with that obtained from pycnidia counting method (Fig. 27B). However, the rapid disease progression period observed with disease classification (Fig. 27A) was approximately one week earlier than that assessed by real-time PCR. The *M. graminicola* DNA amount measured by real-time PCR using ST-rRNA F/R was significantly correlated with the disease index ( $r = 0.8937^{**}$ ) as well as the NPL ( $r = 0.9106^{***}$ ), but their relationships were non-linear (Figs. 29, 30). The relationship between DNA amount and disease was not expected (Fig. 29). The disease index increased rapidly from 0 to 60 at low DNA amounts (range from 0 to 80 pg). Afterwards, the DNA

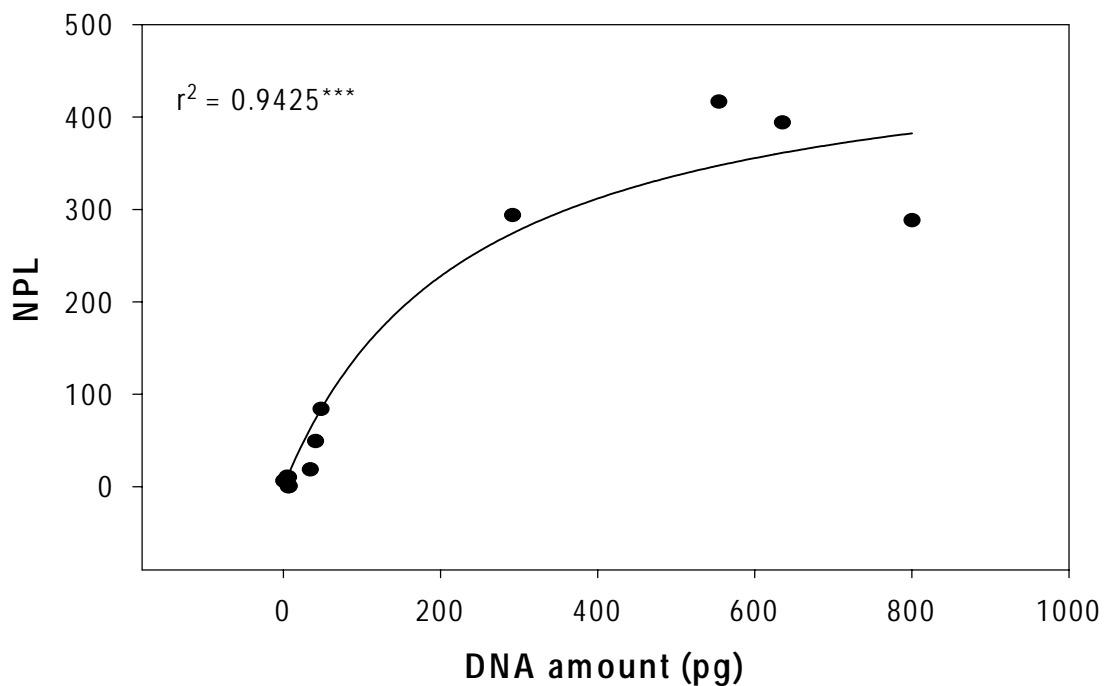
amount increased up to approximately 820 pg, but the disease index was close to its maximum level (100) at approximately 300 pg DNA amount and could not increase any more at higher DNA amounts. In contrast, the DNA amount was more closely related to the NPL (Fig. 30). When the NPL was less than 100, the DNA amount was also at a low level. The DNA amount was accordingly high when the NPL was more than 300.



**Fig. 28** Monitoring of disease progression of *Mycosphaerella graminicola* in F, F1 and F2 leaves in a fungicide-untreated plot during the epidemic period by quantifying the fungal DNA amount by real-time PCR using the primer set ST-rRNA F/R. (A) and ST-act F/R (B). Each data point represents the average of three replicates  $\pm$  SD.



**Fig. 29** Relationship between the *Mycosphaerella graminicola* DNA amount measured by real-time PCR using the primer set ST-rRNA F/R and the disease index in the F, F1 and F2 leaves sampled from a fungicide-untreated plot.



**Fig. 30** Relationship between the *Mycosphaerella graminicola* DNA amount measured by real-time PCR using the primer set ST-rRNA F/R and the number of pycnidia per leaf (NPL) in F, F1 and F2 leaves sampled from a fungicide-untreated plot.

### 3.4.5.2 Real-time PCR using the primer set R5870-1F/R5870-2R

The primer set R5870-1F/R5870-2R was lately developed from one RAPD fragment after the tests in section 3.4.5.1 had been already finished. As described before, this primer set has a target of 323 bp, and is smaller than the targets of the primer sets of ST-rRNA F/R and ST-act F/R. Therefore, it may be more suitable for real-time PCR test (for real-time PCR, small targets are generally more suitable than big targets). In a primary test, the primer set represented markedly higher sensitivity in real-time PCR than in conventional PCR (Table 11). Meanwhile, its standard curve (relationship between cycle number and log of DNA amount) was almost completely linear (Fig. 23IIB). These results indicated that this primer set might be available for the quantification of *M. graminicola* DNA *in planta*.

**Table 14** Quantification of *Mycosphaerella graminicola* DNA in F1 leaves sampled in a fungicide-untreated plot using real-time PCR and the primer set R5870-1F/R5870-2R

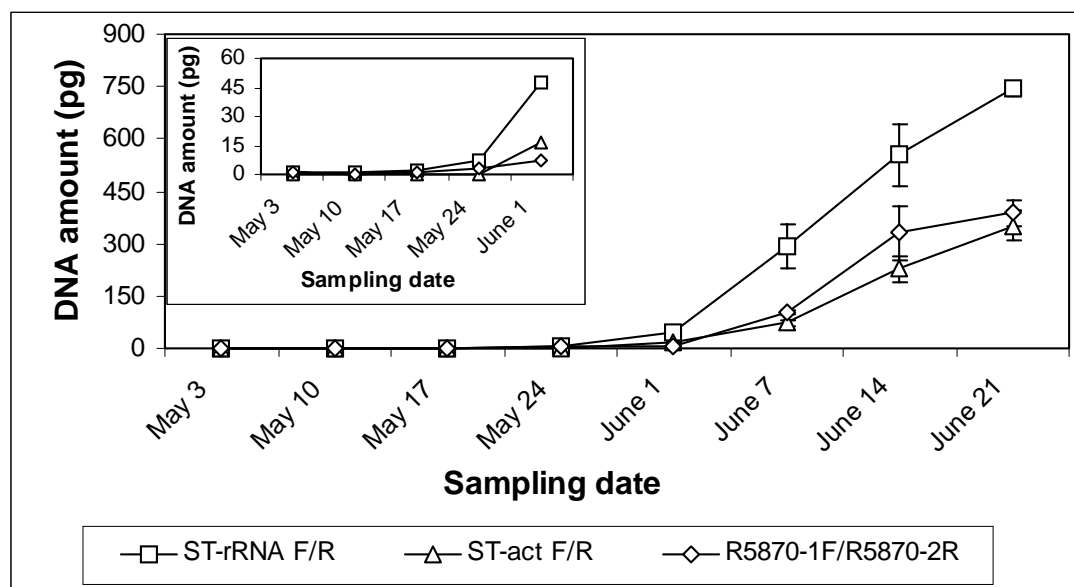
Sampling date <sup>a</sup>	Cycle number	DNA amount (pg) <sup>b</sup>
May 3	35.95 ± 0.17	0.61 ± 0.07
May 10	36.70 ± 0.45	0.37 ± 0.13
May 17	34.80 ± 0.13	1.43 ± 0.13
May 24	33.68 ± 0.19	3.26 ± 0.45
June 1	32.53 ± 0.03	7.59 ± 0.18
June 7	28.99 ± 0.13	102.46 ± 9.51
June 14	27.40 ± 0.15	330.97 ± 36.18
June 21	27.18 ± 0.16	387.78 ± 44.25

<sup>a</sup> The samples used are the same as those used for ST-rRNA F/R and ST-act F/R shown in Table 13.

<sup>b</sup> The DNA amount was calculated from the cycle number obtained from real-time PCR according to the regression equation of the standard curve for R5870-1F/R5870-2R (Fig. 23IIB). Data are presented as mean of three replicates ± SD.

In order to test the suitability of the primer set R5870-1F/R5870-2R for quantification of *M. graminicola* DNA in naturally infected plants, nine F1 leaf samples were analysed. The results showed that real-time PCR using this primer sets could detect *M. graminicola* DNA in all nine samples and as little as 0.37 pg DNA could be detected (Table 14). It indicated that the primer set R5870-1F/R5870-2R had high sensitivity like ST-rRNA F/R when used for the tests of field samples. The DNA amounts were more similar to those measured with the primer set ST-act F/R, whereas they were clearly less than those tested with the primer set ST-rRNA F/R (Table 13). Meanwhile, the shape of disease progression curve obtained with

this primer set was not as linear as that with the primer sets ST-rRNA F/R and ST-act F/R (Fig. 31). Real-time PCR with three different primer sets gave rise to different DNA amount (Fig. 31) even though the same DNA samples were used, indicating that the quantification results could be influenced by different primers.



**Fig. 31** Comparison of *Mycosphaerella graminicola* DNA amount in F1 leaves quantified by real-time PCR using the primer sets ST-rRNA F/R, ST-act F/R and R5870-1F/R5870-2R. Each data point represents the average of three replicates  $\pm$  SD.

### 3.4.6 Assessment of fungicide efficacy

The efficacy of two fungicide treatments on *M. graminicola* leaf blotch on leaves at different positions was assessed by real-time PCR using the primer sets ST-rRNA F/R on May 17 and June 14, 2004 and compared with the results obtained by disease classification and by pycnidia-counting method. Real-time PCR results showed that two fungicide treatments had high efficacy against *M. graminicola* leaf blotch (Table 15). In F2, F3, F4 leaves on May 17 and in all leaves tested on June 14, *M. graminicola* DNA amounts were much lower in the Fandango treatment than in CK. Similar results were obtained in the Input treatment. *M. graminicola* DNA amounts were much lower than those in CK in F, F2, F3, F4 leaves on May 17 and in all leaves tested on June 14. In contrast to the two visual methods, real-time PCR could detect *M. graminicola* DNA in some symptomless leaves such as in F and F1 leaves on May 17 and in dried leaves such as in F3 leaves on June 14 (Table 15). Nevertheless, on May 17, the DNA amounts in F, F1 and F2 leaves of two fungicide

treatments were similar or even higher than those in CK, although they could be clearly detected in these samples (Table 15).

**Table 15** Quantification of *Mycosphaerella graminicola* DNA amount in leaves of different positions from two fungicide-treated plots and an untreated control plot (CK) by real-time PCR<sup>a</sup>

Date	LP <sup>b</sup>	CK		Fandango		Input	
		Cycle number	DNA amount (pg)	Cycle number	DNA amount (pg)	Cycle number	DNA amount (pg)
May	F	30.66 ± 0.29	0.53 ± 0.12	30.29 ± 0.18	0.63 ± 0.18		
17	F1	28.38 ± 0.20	2.46 ± 0.65	28.40 ± 0.10	2.79 ± 0.42	27.32 ± 0.11	6.20 ± 0.52
	F2	27.17 ± 0.20	7.00 ± 1.35	28.83 ± 0.10	2.02 ± 0.15	27.48 ± 0.09	5.49 ± 0.35
	F3	22.73 ± 0.31	185.81 ± 42.02	23.99 ± 0.09	72.47 ± 6.72	25.11 ± 0.07	31.59 ± 1.65
	F4	23.44 ± 0.11	108.87 ± 8.46	23.83 ± 0.12	81.68 ± 7.29	23.70 ± 0.11	89.85 ± 7.18
June	F	25.00 ± 0.10	34.31 ± 2.41	31.42 ± 0.13	0.30 ± 0.03	30.50 ± 0.39	0.60 ± 0.17
14	F1	21.25 ± 0.22	554.29 ± 88.96	24.08 ± 0.22	68.30 ± 10.71	26.61 ± 0.13	10.43 ± 0.96
	F2	21.06 ± 0.22	635.20 ± 76.34	21.42 ± 0.02	482.00 ± 6.14	22.71 ± 0.06	185.51 ± 7.81
	F3	20.75 ± 0.30	801.61 ± 171.04	22.31 ± 0.09	249.53 ± 17.13	22.12 ± 0.12	287.38 ± 25.16

<sup>a</sup> The values shown in the table are the mean of three replicates ± SD (the original data were shown in Table A9). The DNA amount is calculated from the cycle number measured by real-time PCR according to the regression equation of the standard curve for ST-rRNA F/R (Fig. 22B). Blank column means that no efficient cycle number was obtained.

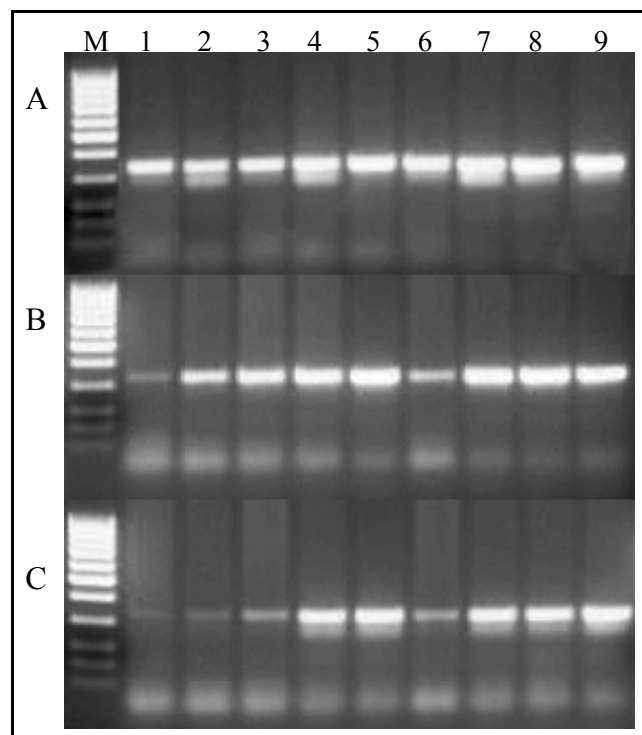
<sup>b</sup> LP = leaf position.

Meanwhile, the final PCR products were visualized in an agarose gel. In the Input treatment (Fig. 32C), the band intensity of F, F1 and F2 leaves on May 17 and F leaves on June 14 was clearly more slight than five other leaf samples in which their band intensity could not be clearly distinguished. In the Fandango treatment, clear difference of band intensity was observed only in F leaves on May 17 and June 14 (Fig. 32B). In CK, the band intensity had no obvious difference among different leaf samples (Fig. 32A). In the F leaves of the Input treatment, *M. graminicola* was not successfully detected by real-time PCR, whereas a very slight band was shown on the agarose gel.

The efficacy of two fungicide treatments were assessed by real-time PCR and compared with those obtained with two visual methods. The fungal DNA amount decreased due to both fungicide treatments (Table 16). In the Fandango treatment, the efficacy could not be assessed



in F and F1 leaves on May 17 by two visual methods because of no visible symptoms, and real-time PCR also produced no positive results in these samples since the DNA amounts in Fundango-treated plot exceed those in CK (Table 15). In F2, F3, F4 leaves on May 17, the results assessed by real-time PCR were closely related to those obtained by disease classification, whereas the pycnidia-counting method gave unsatisfactory results in such samples (Table 16). On June 14, the results assessed by real-time PCR had a close relationship with those obtained by pycnidia-counting method. In addition, real-time PCR could assess the efficacy in F3 leaves, but both visual methods could not since most F3 leaves were dried on June 14 (Table 16).



**Fig. 32** Visualization of real-time PCR products on a 2% agarose gel. (A) CK; (B) The Fandango treatment; (C) The Input treatment. Lanes 1-9 are F, F1, F2, F3 and F4 leaves collected on May 17, and F, F1, F2 and F3 leaves collected on June 14, respectively. M is the 100 bp DNA ladder (Carl Roth).

In the Input treatment on May 17, the efficacy in F and F1 leaves could not be assessed by two visual methods since the disease severity was too slight. According to the data assessed by real-time PCR (Table 16), no positive efficacy in F and F1 leaves was evident like in the Fandango treatment (Table 16). In F2 leaves, the efficacy obtained by real-time PCR was less than that obtained by the two visual methods. In F3 leaves, it was related to that obtained by pycnidia-counting method, and in F4 leaf, it was more closely related to that obtained by

disease classification. On June 14, the results assessed by real-time PCR were closely related to those assessed by pycnidia-counting method. Although most F3 leaves were dried, real-time PCR could assess the efficacy in F3 leaves (68.87%) and the two visual methods could not (Table 16).

**Table 16** Fungicide efficacy assessed by three different methods

Treatment	Date	Leaf position	Fungicide efficacy (%) <sup>a</sup>		
			Disease classification <sup>a</sup>	Pycnidia-counting method <sup>a</sup>	Real-time PCR <sup>b</sup>
Fandango	May 17	F	0.00 <sup>d</sup>	0.00 <sup>d</sup>	0.00 <sup>e</sup>
		F1	0.00 <sup>d</sup>	0.00 <sup>d</sup>	0.00 <sup>e</sup>
		F2	73.46	0.00 <sup>e</sup>	71.14 ± 2.13
		F3	42.93	13.25	61.00 ± 2.46
		F4	9.91	0.00	24.97 ± 6.70
	June 14	F	74.98	98.40	99.42 ± 0.51
		F1	29.19	80.97	87.68 ± 1.93
		F2	0.00 <sup>e</sup>	17.18	24.12 ± 0.97
INPUT	May 17	F	0.00 <sup>d</sup>	0.00 <sup>d</sup>	0.00 <sup>e</sup>
		F1	0.00 <sup>d</sup>	0.00 <sup>d</sup>	0.00 <sup>e</sup>
		F2	86.70	100.00	21.62 ± 5.06
		F3	22.41	89.69	88.67 ± 9.83
		F4	0.00	68.74	17.47 ± 6.59
	June 14	F	94.25	100.00	98.83 ± 1.07
		F1	63.00	96.47	98.12 ± 0.17
		F2	29.33	76.57	70.79 ± 1.24
		F3	13.33	0.00 <sup>d</sup>	68.87 ± 3.48

<sup>a</sup> Fungicide efficacy was calculated by comparing the disease index or NPL obtained from 30 leaves of each leaf position in fungicide-treated plot with those in CK according to the equation in section 2.1.2 (the original data were shown in Table A6).

<sup>b</sup> Fungicide efficacy assessed by real-time PCR was calculated by comparing *M. graminicola* DNA amounts in fungicide-treated samples with those in CK according to the equation in section 2.1.2. The value is the mean of three replicates ± SD (the original data were shown in Table A9).

0.00<sup>d</sup> No symptoms or pycnidia observed in those leaves.

0.00<sup>e</sup> Disease severity of such sample was assessed, but no positive efficacy was given since the disease severity or DNA amount in fungicide-treated plot was higher than that in CK.

According to the results assessed by real-time PCR, two fungicide treatments controlled *M. graminicola* leaf blotch efficiently. On June 14, the effects of the Fundango treatment in F, F1, F2 and F3 leaves were 99.42%, 87.68%, 24.12% and 65.15% respectively, and those of Input treatment were 98.83%, 98.12%, 70.79% and 68.87%, respectively (Table 16). On May 17, the results assessed by real-time PCR were not in agreement with those assessed by pycnidia-counting method. It is probably due to that the NPL on the leaves except F4 leaves on May 17 were too small so that the pycnidia-counting method could not assess the efficacy accurately (Table A6). On June 14, the results assessed by real-time PCR and by pycnidia-counting method showed that the Input treatment had obviously better efficacy in F1 and F2 leaves than the Fandango treatment, but the efficacy in F and F3 leaves was similar in both treatments (Table 16).

### **3.5 Detection of *M. graminicola* in wheat by RT-PCR**

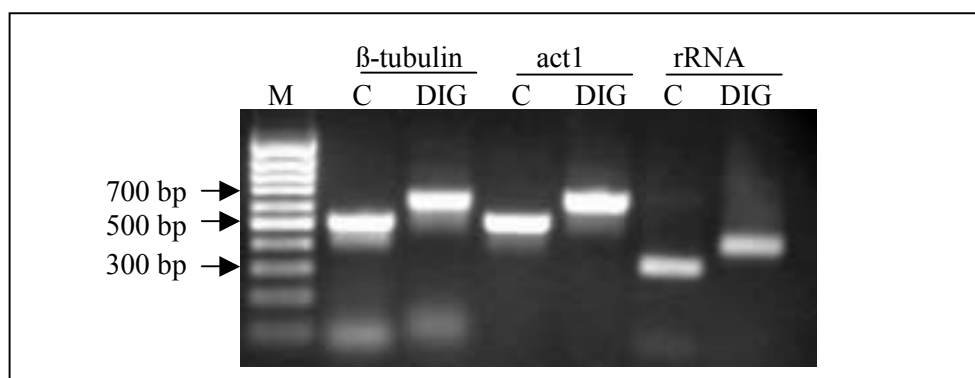
#### **3.5.1 Detection of the contents of three target RNA in *M. graminicola* cells**

The ideal RNA targets for RT-PCR molecular diagnosis should be basically stable in different structures and developmental stages of *M. graminicola*. For this purpose, the target RNA contents of the three primer sets E1/STSP2R, ST-rRNA F/R and ST-act F/R in seven *M. graminicola* cultures with different culture times ranging from 3 to 15 days were assessed by Northern hybridization and RT-PCR.

*M. graminicola* cells were cultured in MYB liquid medium and harvested by centrifugation after 3, 5, 7, 9, 11, 13 and 15 days. The colour of cultures turned from bright yellow to dark black with increasing culture time. Only conidia were observed before five days. From nine days, the mycelium was visible in the culture and the colour became grey. The culture of 15 days contained numerous mycelium.

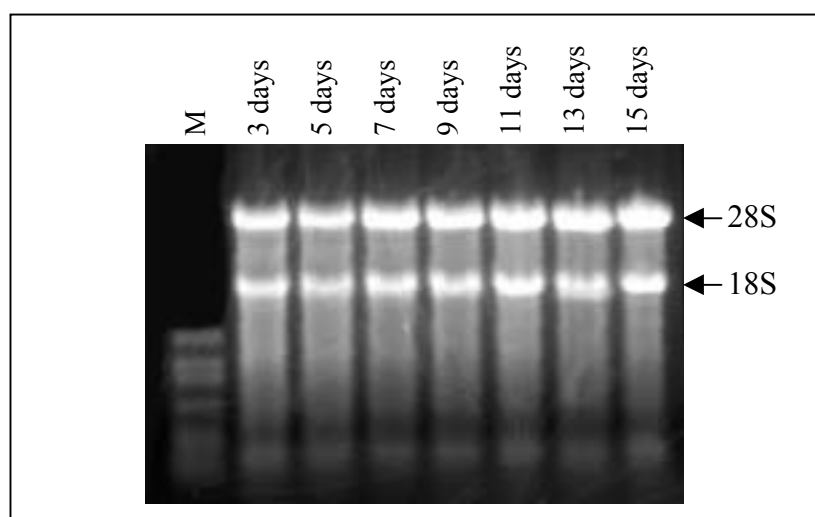
Northern hybridization was performed with a non-radioactive DIG hybridization system using DNA probes. First, three DNA probes were labelled with digoxigenin (DIG) by PCR using dUTP-DIG mix and three corresponding primer sets. To assess the quality and concentration of these probes, 3 µl PCR products labelled with DIG were visualized on a 2% agarose gel, compared with the PCR product without DIG. The concentration of three probes was roughly evaluated as 50 ng/µl ( $\beta$ -tubulin and act1) and 30 ng/µl (rRNA), by comparing with the

500 bp band of the 100 bp DNA ladder (82 ng). The DIG-labelled PCR-products had a higher molecular weight due to the insertion of dUTP-DIG (Fig. 33).



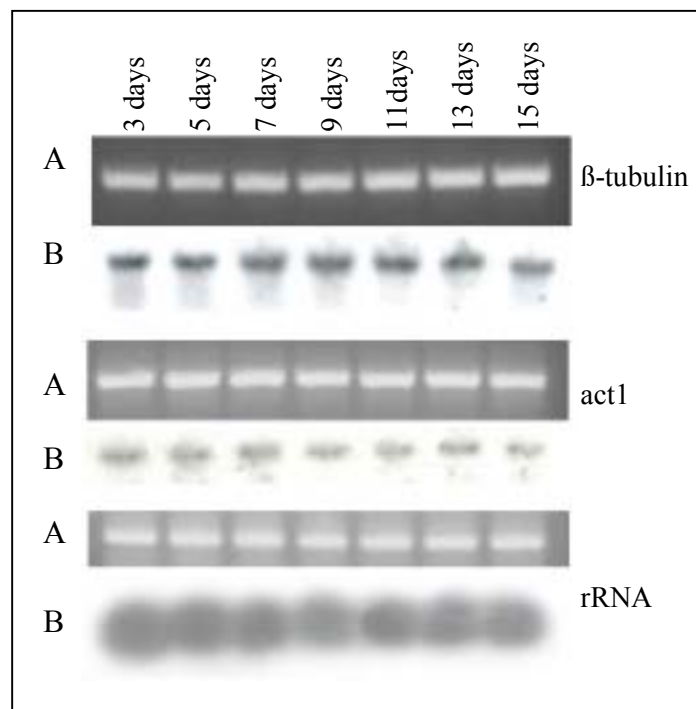
**Fig. 33** Preparation of  $\beta$ -tubulin, actin and rRNA probes labelled with digoxigenin (DIG) by PCR. 3  $\mu$ l PCR products were run on a 2% agarose gel. The DIG-labelled probes (DIG) have higher molecular weight than those PCR products with normal dNTPs (C). M is the 100 bp DNA ladder (MBI Fermentas).

Six  $\mu$ g total RNA of each culture were first separated on a 1% formaldehyde-denatured agarose gel for ca. 3 h at 45 volt. To check the degradation of RNA during the electrophoresis, the gel was photographed before Northern transfer. Two clear bands of 28S and 18S rRNA showed that the total RNA had a sufficient purity and no obvious degradation during the electrophoresis (Fig. 34).



**Fig. 34** Separation of total RNA of *Mycosphaerella graminicola* cultures with different culture time in a 1% formaldehyde-denatured agarose gel. *M. graminicola* cells were cultured for 3, 5, 7, 9, 11, 13 and 15 days before total RNA extraction. Six  $\mu$ g total RNA of each culture was loaded. M is the low range RNA ladder (MBI Fermentas).

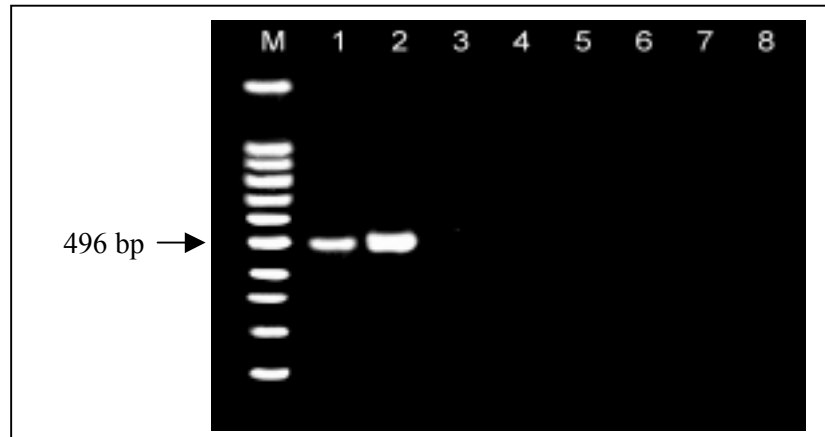
The expression level of the three genes in seven different cultures was also tested by one-step RT-PCR. The results tested by Northern hybridization and RT-PCR showed that the signal intensity of the three RNA targets  $\beta$ -tubulin and act1 RNA and rRNA had no obvious difference among the seven culture times (Fig. 35). It indicated that their contents were basically stable in different growth stages of *M. graminicola*. Therefore, the three RNA sequences may be used as target sequences for the detection and quantification of *M. graminicola* in wheat by RT-PCR assay.



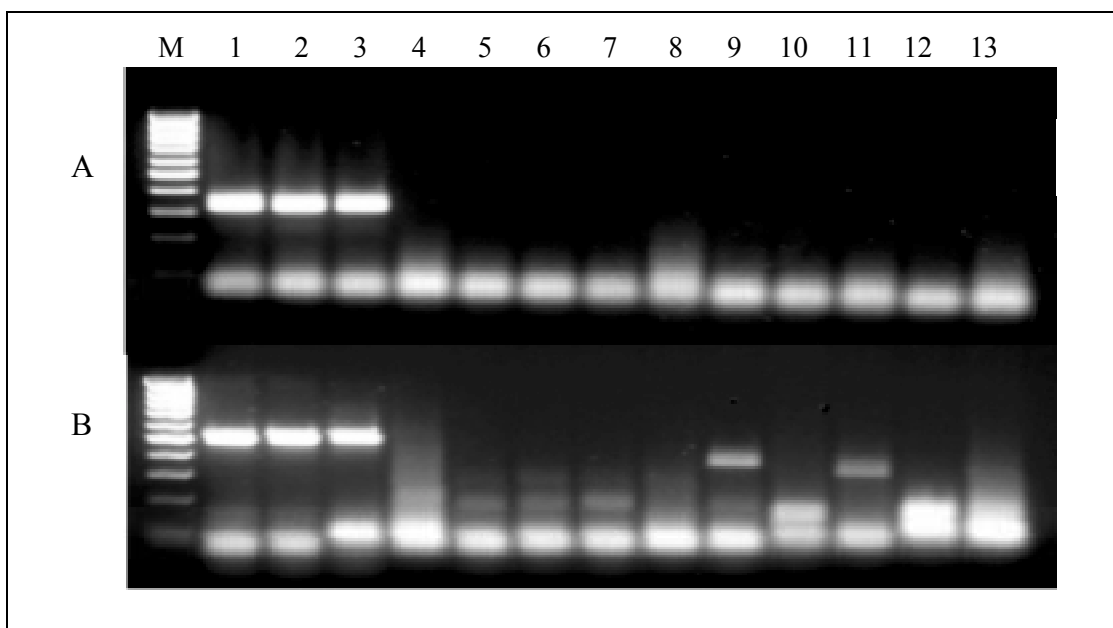
**Fig. 35** Detection of the expression level of the  $\beta$ -tubulin, actin and rRNA genes in *Mycosphaerella graminicola* cells with different culture times of 3, 5, 7, 9, 11, 13 and 15 days using RT-PCR (A) and Northern hybridization (B).

### 3.5.2 Suitability of three primer sets for the RT-PCR assay

The primer set E1/STSP2R was developed by Fraaije *et al.* (1999) from a sequence of  $\beta$ -tubulin genes and used for a PCR assay. The primer sets ST-rRNA F/R and ST-act F/R were developed from a sequence of rDNA and a partial sequence of the act1 gene. They were proved to be specific and be able to be used for the detection of *M. graminicola* in wheat by PCR (Fig. 12). In order to test the suitability of the three primer sets for RT-PCR assay, their specificity and sensitivity was tested.

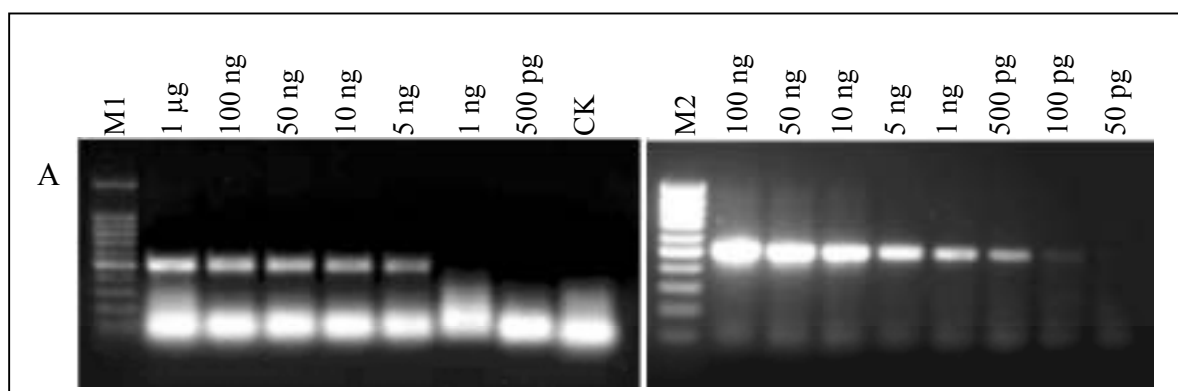


**Fig. 36** Specificity of the primers E1 and STSP2R with one-step RT-PCR. Lanes 1 to 8 are total RNA of *M. graminicola*, leaves infected with *Mycosphaerella graminicola*, *Stagonospora nodorum*, *Microdochium nivale*, *Fusarium graminearum*, *F. culmorum*, *Pseudocercospora herpotrichoides* and healthy leaves, respectively. A 496-bp single fragment was amplified from *M. graminicola* and its infect plants. Lane M is the 100-bp DNA ladder (Promega).

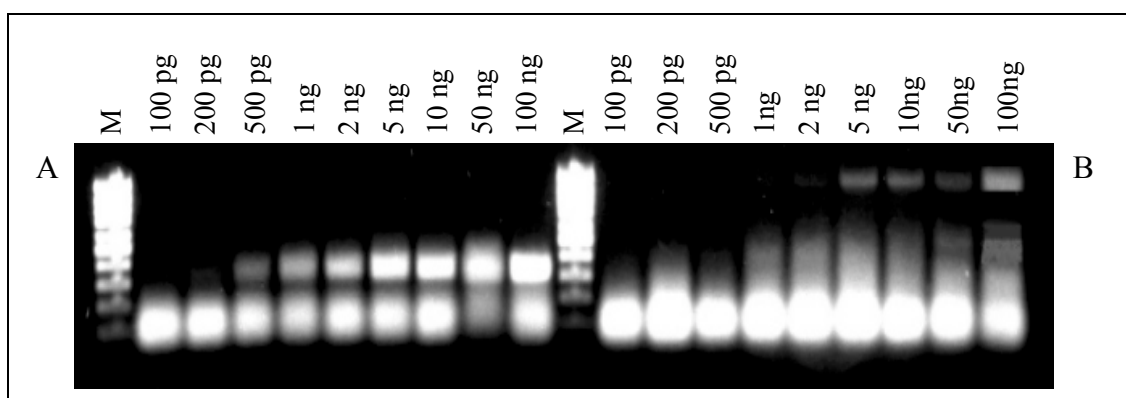


**Fig. 37** The specificity of ST-rRNA F/R (A) and ST-act F/R (B) by RT-PCR with total RNA of different fungal pathogens. Lanes 1-13 are *Mycosphaerella graminicola* Ki-Or-11, *M. graminicola* FN4, *M. graminicola* infected plants, *Stagonospora nodorum*, *Microdochium nivale*, *Fusarium Culmorum*, *F. graminearum*, *Pseudocercospora herpotrichoides*, *Macrophomina phaseolina*, *Rhizotonia solani*, *Erisphe graminis* f. sp. *Tritici*, healthy plants and *M. graminicola* without reverse transcriptase, respectively. M is the 100-bp DNA ladder (MBI Fermentas). The PCR products were treated with 2  $\mu$ l (10  $\mu$ g/ $\mu$ l) RNase at room temperature for 1 h before electrophoresis.

For the primer set E1/STSP2R, one single fragment was amplified not from that of healthy plants or five other fungal pathogens tested, but from total RNA of *M. graminicola* and from infected leaves (Fig. 36). The primer set St-rRNA F/R also amplified one single fragment only from *M. graminicola* and its infected wheat plants, but not from eight other fungal pathogens and healthy plants (Fig. 37A). No unspecific bands were present in *M. graminicola*, other fungal pathogens and healthy plants. It indicated that both primer sets were specific to *M. graminicola* by RT-PCR like PCR. For ST-act F/R, the expected band was only present in *M. graminicola* and its infected plants although several unspecific bands of different size (Fig. 37B) were present in other pathogens. Therefore, this primer set could also be useful for the qualitative detection of *M. graminicola* with RT-PCR.



**Fig. 38** Detection sensitivity of one-step RT-PCR (A) and two-step RT-PCR (B) using the primer set E1/STSP2R. Twenty  $\mu$ l of PCR products were run on a 2% agarose gel stained with ethidium bromide. M1 is the 100-bp DNA ladder (Promega) and M2 is the 100-bp DNA ladder (MBI Fermentas).



**Fig. 39** The sensitivity of one-step RT-PCR with the primer sets ST-rRNA F/R (A) and ST-act F/R (B) using nine different concentrations of *Mycosphaerella graminicola* total RNA. M is the 100-bp DNA ladder (MBI Fermentas).

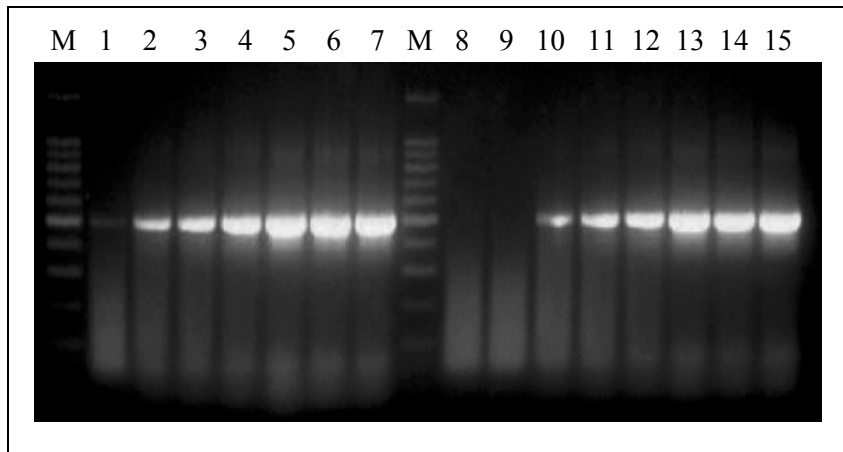
The sensitivity of three primer sets was tested by one-step PCR using a dilution series of total RNA. Among them, ST-rRNA F/R showed the highest sensitivity and could detect as little as 200 pg total RNA by one-step RT-PCR (Fig. 39A). The detection limit of E1/STSP2R was 5 ng total RNA by one-step RT-PCR (Fig. 38A). Although ST-act F/R could detect up to 2 ng total RNA, it produced a low band intensity even with 50 ng total RNA (Fig. 39B). To compare the sensitivity of one-step and two-step RT-PCR, a two-step RT-PCR was performed using E1/STSP2R and a dilution series of cDNA synthesized from total RNA. cDNA was diluted into relevant quantities equal to 100 ng, 50 ng, 10 ng, 5 ng, 1ng, 500 pg, 100 pg and 50 pg of total RNA. It showed that two-step RT-PCR with E1/STSP2R could detect up to 100 pg total RNA (Fig. 38B), and was approximately 50 times more sensitive than one-step RT-PCR (5 ng total RNA, Fig. 38A).

### **3.5.3 Detection of *M. graminicola* in infected wheat plants**

The *M. graminicola* disease development in inoculated wheat plants and in naturally infected F1 leaves sampled in a field was monitored by one-step RT-PCR assay using the primer set E1/STSP2R. Results obtained by one-step RT-PCR showed that a clear single product was amplified from the samples taken on 4, 8, 12, 16, 20 and 24 days after inoculation (Fig. 40, lanes 1-7). Even though leaves were thoroughly washed with water prior to RT-PCR, a slight band was also present in the 0-day sample taken immediately after inoculation. Although no symptoms were observed at four days after inoculation (Table 12), an obvious product was amplified (Fig. 40, lane 1) at this early stage of disease development.

In the leaves sampled from the field, the first small lesions were found in about 15% of the F1 leaves sampled on May 17, 2004, and the rapid progression period of the disease was observed between May 24 and June 7 (Table 13). The results obtained by RT-PCR showed that the expected fragment was amplified from F1 leaves collected after May 17, 2004 with a gradual increase in band intensity, which was in general agreement with the disease severity assessed by visual investigation (Fig. 40, lanes 8-15, Table 13).





**Fig. 40** Detection and diagnosis of *Mycosphaerella graminicola* in artificially inoculated wheat leaves and F1 leaves sampled in the field by one-step RT-PCR. Lanes 1 to 7 are inoculated leaves sampled at 0, 4, 8, 12, 16, 20 and 24 days after inoculation, respectively. Lanes 8 to 15 are F1 leaves sampled on May 3, May 10, May 17, May 24, Juni 1, Juni 7, Juni 14 and Juni 21, 2004, respectively. Lane M is the 100-bp DNA ladder (Promega). The PCR products were treated with 2  $\mu$ l (10  $\mu$ g/ $\mu$ l) RNase at room temperature for 1 h before electrophoresis.

#### 4. Discussion

Reliable methods for disease severity assessment are of crucial importance in the study of either disease diagnosis in the field, or plant pathogen interactions, or effect assessment of fungicide treatments. Currently, most of the assays used in fungal disease diagnosis rely on visual assessment of the symptoms, lesion diameter measurement or spore counting. However, these tests are tedious and often cannot accurately assess the disease severity, especially during the early stages or later stages (dried diseased leaves). Molecular diagnostics provides an alternative approach to accurately and presymptomatically detect pathogens.

*M. graminicola* which causes wheat leaf blotch is an economically important pathogen of wheat in most regions of the world. Early detection is of great significance for the efficient management of this pathogen as it has a long latent infection period. In the experiments described in this thesis, PCR and RT-PCR were successfully applied to detect *M. graminicola* in infected wheat plants. A simple and effective method of DNA extraction suitable for fungi and infected wheat leaves was developed. Four specific primer sets with high specificity and sensitivity were developed and efficiently used for qualitative and quantitative detection of *M. graminicola* in wheat by conventional PCR as well as by real-time PCR. Two specific primer sets were used for identification of *M. graminicola* using RT-PCR assay. Furthermore, real-time PCR was successfully used to monitor the disease development in inoculated wheat plants and in naturally infected F, F1 and F2 leaves during the epidemic period, and to assess the efficacies of two fungicide treatments compared with the conventional visual assessment.

##### 4.1 Improvement and simplification of DNA and RNA extraction procedures

DNA and RNA extraction is a prerequisite for studying biological processes on a molecular level. Simple DNA or RNA extraction methods suitable for various fungi and wheat plants are needed to achieve the objectives of this study. Although several different methods have been described (Cenis 1992, Möller *et al.* 1992, Chen *et al.* 2000), they are either time-consuming and laborious, or useful for only specific materials. In this study, two simple and effective procedures of DNA extraction and one simple procedure of RNA extraction were improved.

DNAzol reagent was successfully applied to the DNA extraction of 25 different phytopathogenic fungi. In the procedure, several modifications were made to the standard protocol of the manufacturer in order to improve the adaptability of DNAzol reagent to various phytopathogenic fungi. These modifications markedly raised the purity of DNA and the suitability of DNAzol for various fungi. The DNA obtained with this procedure was directly used for RAPD-PCR, AFLP, ITS-PCR (Fig. 3) as well as for the specificity test of primer sets (Fig. 12). In comparison to other methods, the modified procedure using DNAzol reagent is rapid, easy to use, and can yield genomic DNA of satisfactory yield and purity from a wide range of pathogenic fungi.

Although DNAzol has been used for the DNA extraction of *M. graminicola* isolates in the past (Czember and Arseniuk 1999, Zwiers *et al.* 2003), a homogenization step was used in those protocols. Homogenization is time-consuming and not suitable for DNA extraction from small quantities of tissues. The results indicated the DNAzol reagent could efficiently lyse the conidia of *M. graminicola*, and therefore the laborious homogenization step could be omitted (Fig. 4). The conidia needed only to be mixed with DNAzol by conversing several times and incubated for 2-3 min, which greatly simplified the process. It is even more time-saving when this procedure is employed for DNA extraction of numerous isolates.

Other pathogens tested were ground in liquid nitrogen. Further tests are needed to simplify the homogenization step for these pathogens. Many different homogenization methods were reported. For instance, Cenis (1992) used a conical grinder to crush the mycelium in a 1.5-ml microtube by hand. But its efficiency for various fungi with different structures needed to be further tested. A microwave-based method was used for the homogenization of natural soil samples for DNA extraction (Orsini and Romano-Spica 2001). Yeates and Grillings (1998) used a bead-beating machine to homogenize the soil samples in DNA extraction. These methods make it possible to simplify the fungal homogenization during DNA extraction.

Meanwhile, a simple CTAB-PVP method was developed for DNA extraction from fungi and infected wheat plants. Plants, particularly infected plants, contain high amounts of polyphenols, polysaccharides, proteins as well as latex which bind to DNA or are the PCR inhibitors. Therefore, efficient elimination of these substances is a key to getting sufficient DNA yield and purity. CTAB can efficiently precipitate polysaccharides in presence of high salt conditions, PVP binds to both polysaccharides and phenolic compounds and SDS inactivates proteins (Möller *et al.* 1992, Chang *et al.* 1993, Chen *et al.* 2000). Möller *et al.*

(1992) developed a simple method using CTAB, SDS/Proteinase K and  $\beta$ -mercaptoethanol for DNA extraction from filamentous fungi and infected plants. Chen *et al.* (2000) used CTAB, insoluble PVPP and chloroform-phenol elution in one step for RNA extraction from infected plants.

In the procedure improved here, CTAB, insoluble PVP, SDS and chloroform-phenol elution were combined in one step. Insoluble PVP was used instead of soluble PVP since the latter is incompatible with phenol extraction and obstructs nucleic acid precipitation (Claros and Cánovas 1998, Chen *et al.* 2000). Although CTAB was incompatible with SDS in the DNA extraction buffer, it did not influence DNA extraction when the buffer was heated to about 80°C. This protocol was successfully used to extract DNA from ten fungal species tested, healthy and infected wheat plants (Fig. 5). DNA with purity high enough for conventional PCR and real-time PCR was obtained from severely or even dried diseased leaves. In comparison to other reported methods, this method is simple and cheap, with no expensive chemicals and materials used. The whole process can be finished within 40 min. The time can be further shortened when other simpler tissue homogenization methods are used.

In addition, a simple procedure of RNA extraction was improved, in which guanidinium isothiocyanate, CTAB, insoluble PVP and chloroform were all combined in one step, which greatly simplified the extraction processes. The acidic phenol is used to promote cell disruption, RNA precipitation and removal of DNA. The acidic pH helps to remove polyphenols after PVP treatment (Claros and Cánovas 1998). LiCl precipitation was proved to be very effective (Chomczynski and Sacchi 1987, Chen *et al.* 2000). LiCl does not precipitate DNA, proteins or carbohydrates except the RNA, so most DNA and proteins can be effectively removed by it (Birboim 1992).

This procedure was effectively used for RNA extraction from infected wheat plants and more than 10 phytopathogenic fungi. Total RNA extracted with this procedure was directly employed for RT-PCR, Northern hybridization and cDNA-AFLP. This protocol needs no expensive chemicals and facilities, and offers an interesting and efficient alternative for RNA extraction from various fungi and infected wheat plants.

## **4.2 Isolation of target-specific sequences and development of specific primers**

Isolation of target-specific sequences is the key work in the development of PCR-based molecular diagnostic methods. Theoretically, any DNA or RNA sequences that are specific for a particular organism can be used for PCR detection of this organism. For fungal pathogens, the following main strategies can be utilised to identify the specific target sequences: (1) searching conserved DNA sequences of target pathogen and other closely related pathogens from existing DNA databases; (2) amplifying and sequencing conserved genes with universal primers, (3) amplifying unknown random fragments from the fungal genome with PCR-based techniques such as RAPD-PCR, and (4) isolating unknown unique genomic fragments or cDNA-fragments by AFLP or cDNA-AFLP.

### **4.2.1 Search of *M. graminicola*-specific sequences by BLAST search**

In the last decades, with the rapid development of genomics and bioinformatics, a lot of gene banks with large sequence data have been established (Soanes *et al.* 2002). Many conserved gene sequences of common fungal pathogens can be found directly in the present gene banks, making it possible to identify target-specific sequences by analysing the sequence similarity in public sequence databases using BLAST search. BLAST search is a common database similarity programme and is a very effective and powerful computational tool. With this programme, the sequence similarity and homogeneity of target sequences can be analysed rapidly and automatically in public databases (Altschul *et al.* 1990, Zhang and Madden 1997).

It has been demonstrated that searching target-specific sequences from present sequence databases is a simple and efficient way since it needs no laborious process of cloning and sequencing. Many specific primer sets have successfully been developed through this approach. For instance, Schaad *et al.* (2002) designed specific primers for the real-time PCR quantification of *Xylella fastidiosa* from a 16S rDNA and 16S-23S internal transcribed spacer (ITS) region sequences obtained from GenBank accessions. Qi and Yang (2002) developed a specific primer set from a 28S rRNA gene of *Magnaporthe grisea* for real-time PCR. Schnerr *et al.* (2001) developed a primer set from a sequence of *tri5* gene to quantify trichothecene-producing *Fusarium* spp. in wheat using real-time PCR.

The selection of suitable target sequences is a fundamental step for this approach. Generally, the chosen target sequence should be highly conserved and have high copy numbers in the

genome. It should be popular in many other organisms. For fungal species, rDNA is an attractive target sequence for molecular detection and phylogenetic studies (Henson and French 1993). It occurs in multiple copies in the genome (60-200 copies per haploid genome) and contains internal transcribed spacer (ITS) regions and non-transcribed spacer (NTS). The ITS regions consist of the 18S small subunit, the 5.8S, and the 28S large subunit genes separated by internal transcribed spacer (ITS) regions ITS1 and ITS2 (Bruns *et al* 1991, Liew *et al.* 1998). Furthermore, they are the most widely sequenced regions in fungi. The sequences of most economically important pathogens are known and can be easily found in public DNA databases (Schena *et al.* 2004). The NTS is the spacer between 18S and 28S rRNA genes, which has a greater sequence variation than the ITS regions (Henson and Feench 1993). Despite the conservation of fungal rDNA, this sequence often contains large sequence differences (Hibbett 1992). Sufficient sequence variation between different species or even different strains or isolates can exist, which allows them to be used as targets for different amplification. A lot of specific primer sets presently used for the detection of plant fungal pathogens were developed from these regions (Henson and French 1993, Schena *et al.* 2004, Table 1).

Meanwhile, some highly conserved house-keeping genes such as  $\beta$ -tubulin and actin were also used as usual target sequences for molecular diagnosis of plant diseases (Fraaije *et al.* 2001, Benito *et al.* 1998). These gene sequences of many fungal pathogens were known or partially known in the present DNA databases. Some other genes, such as ascomycete mating-type genes (Dyer *et al.* 2001, Foster *et al.* 2002), the laccase gene (Hietala *et al.* 2003), the avenacinase gene (Rachdawong *et al.* 2002) and the tri5 gene (Nissen *et al.* 2004) were also used as targets for the detection of phytopathogenic fungi. Other sequences such as mitochondrial DNA were used to develop specific primers for the fungal DNA amplification of *G. graminis* and *T. indica* in infected wheat (Ward 1995, Schesser *et al.* 1998, Frederick *et al.* 2000).

In this study, The act1 gene and rDNA sequences were selected as target sequences due to the following reasons. First, they are highly conserved and have high copy numbers in the genome. Second, both sequences of many fungi were known and can be easily found in gene banks so that the *M. graminicola*-specific subsequence loci in two gene sequences can be identified with reasonable accuracy by comparing the two sequences of *M. graminicola* with the corresponding sequences of other fungal pathogens. In addition, both sequences are

transcribed and therefore the primer developed from them may be used not only for PCR assay but also for RT-PCR assay. In this study, by comparing with the same sequence region of other organisms using BLAST search, three differential subsequences in the rDNA sequence and one specific differential subsequence in the act1 gene sequence were successfully identified (Figs. 6 and 8). Two *M. graminicola*-specific primer sets, ST-rRNA F/R and ST-act F/R, were successfully developed from these differential subsequences.

#### **4.2.2 Isolation of target-specific sequences by RAPD-PCR**

RAPD-PCR is also known as arbitrarily primed PCR (AP-PCR) and is a relatively rapid PCR-based genomic fingerprint method based on the use of synthetic 10 mer oligonucleotides as primer (Williams *et al.* 1990). This method uses only a single random primer and needs no knowledge of genomic sequences. It is very simple and requires only minimal amounts of genomic DNA. PCR-amplified fragments can be easily analysed by gel electrophoresis. Compared with other techniques like restriction fragment length polymorphism (RFLP) and AFLP, this method is technically simple, fast, cheap, and efficient for revealing DNA polymorphisms. The shortage of this technique is that the reproducibility of RAPD polymorphisms is to some extent influenced by DNA purity and reaction conditions. Currently, RAPD-PCR has been a common method of DNA analysis for different uses such as the construction of genetic maps, identification of populations and the genetic variation between different fungal species and isolates (Annamalai *et al.* 1995).

RAPD-PCR has also been a useful approach for isolation of target-specific sequences for many phytopathogenic fungi although this approach requires more time and expense compared to the amplification of conserved DNA sequences (Schena *et al.* 2004). The species- or isolate-specific fragments can be amplified randomly from the fungal genome using random primers and further purified, cloned and sequenced. Lots of primers with different levels of specificity for a large number of phytopathogenic fungi were successfully developed from target-specific sequences isolated by RAPD-PCR. For instance, Nicholson *et al.* (1996, 1997 and 1998) developed specific primer sets for *M. nivale*, *P. herpotrichoides*, *F. culmorum* and *F. graminearum* from RAPD fragments. Rigotti *et al.* (2002) developed specific primers from RAPD fragments for the identification and detection of *Botrytis*

*cinerea*. A specific primer set was developed for the quantification of *Aphyanomyces euteiches* in alfalfa with real-time PCR (Vandemark *et al.* 2002).

In this study, RAPD-PCR was employed to isolate *M. graminicola*-specific DNA fragments. By a primary screening, 12 of 80 primers which produced clear band patterns and had good reproducibility were selected and further used to isolate specific RAPD fragments. Nine reproducible RAPD fragments specific to *M. graminicola* were identified from the fingerprints amplified by RAPD-PCR (Table 5). They were purified from agarose gel and further cloned. Four of nine fragments were sequenced (Fig. A2).

In order to develop several specific primer sets from RAPD fragments, 13 forward and 11 reverse primers were designed based on four isolated specific RAPD fragment sequences (Table 7). Altogether 31 primer combinations were tested with genomic DNA of *M. graminicola*, seven other fungi and healthy plants. The specificity of primer sets varied between four RADP fragments. No valuable primer sets were obtained from fragments R3005 and R3006. For R3009, although all 14 primer sets tested amplified no expected band from other pathogens tested, they amplified the same fragment from healthy plant DNA. Hence, these primer sets are not valuable for the identification of *M. graminicola* in wheat plants. It is likely that a sequence homologous to R3009 is also present in the wheat genome. In order to confirm the hypothesis, a BLAST search was performed in the Tiger Wheat Genome Database (<http://tigrblast.tigr.org/euk-blast/index.cgi?project=tae1>) as well as the Tiger Rice Genome Database (<http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1>), but no homologue was identified in either databases. It is suggested that wheat plant may contain no complete homologue of the R3009 fragment. Six of 11 primer sets developed from the fragment R5870-2 amplified no band from healthy plants, indicating that this fragment might be utilized to develop specific primer sets. Two of the six primer sets, R5870-1F/R5870-1R and R5870-1F/5870-2R, were further tested with seven other pathogens. Both primer sets were specific for *M. graminicola* (Table 7).

Although RAPD-PCR is a very common and useful approach for the DNA fingerprint analysis, it can sometimes cause problems. In RAPD-PCR, differing from the conventional PCR, only one single primer was used, which means the fragments can be amplified only when two priming sites for the 10 mer primer occur in the genome close to each other in an inverted orientation (Annamalai *et al.* 1995). In a primary screening, about half of the



80 primers tested produced no band. Probably there are no efficient priming sites in the fungal genome for these primers presented, or the PCR conditions were not suitable for these primers. Furthermore, its annealing and extension is more greatly affected by annealing temperature, PCR conditions as well as PCR programmes since the primer sequence is short and random. Yu and Pauls (1992) confirmed that different PCR programmes including different annealing temperatures, duration of each step and cycle number have a great influence on RAPD-PCR amplification. Different thermostable polymerases may also amplify different RAPD products (Schierwater and Ender 1993).

Additionally, its reproducibility is sometimes problematic (Milcheli *et al.* 1994). During the experiments, DNA templates extracted with different procedures produced somewhat different RAPD fingerprints. In most fungal pathogens tested, more stable RAPD fingerprints with more bands were generated using DNA obtained with DNazol than using DNA obtained with Cenis (1992) method and the CTAB-PVP method (Guo *et al.* 2005). It is probable that the DNA samples extracted with the last two methods still contained inhibitors of RAPD-PCR. Micheli *et al.* (1994) tested the influence of DNA templates with different preparations on RAPD-PCR, and concluded that the DNA purity and components affect primer annealing and can be one major reason for irreproducibility of RAPD patterns. The reproducibility is also affected by different PCR conditions (Yu and Pauls 1992, Schierwater and Ender 1993). Therefore, it is necessary to use the same DNA prepared with the same procedure under constant PCR conditions in order to improve the reproducibility of RAPD-PCR.

#### **4.2.3 Construction of a cDNA-AFLP procedure**

cDNA-AFLP is an effective RNA fingerprint technique to display differentially expressed genes (Bachem *et al.* 1996). In contrast to other differential display methods, cDNA-AFLP is more stringent and reproducible. Currently, cDNA-AFLP has been broadly applied for the identification of differentially expressed genes (Aubry *et al.* 2003), the construction of EST libraries (Qin *et al.* 2001, Suarez *et al.* 2000) and analysis of gene expression profile (Breynne *et al.* 2003). It may also be used for diagnostic purposes (Bachem *et al.* 1998). We intended to isolate *M. graminicola*-specifically expressed TDFs which may be used as targets for RT-PCR diagnostic assays. However, this work was not continued after some initial trials due to the following reasons. First, two specific primer sets had been successfully developed by

BLAST search and could be suitable for RT-PCR assay. Second, in comparison with the other two methods used in this study, this method is much more laborious, expensive and requires advanced experimental techniques. Third, the expression abundance and stability of these randomly amplified cDNA fragments in pathogen cells is unknown, so extensive analyses are required to assess their specificity, stability and reproducibility.

Some primary work has been done to construct an efficient non-radioactive cDNA-AFLP procedure suitable for various fungi in our laboratory. Total RNA was attempted to use as starting material for the cDNA synthesis instead of mRNA. Although mRNA is usually used, total RNA was, however, also reportedly used for cDNA synthesis or cDNA-AFLP. For instance, Ivanova and Belyavsky (1995) used it as starting material in the cDNA synthesis for the identification of differentially expressed genes by restriction endonuclease-based gene expression fingerprinting. Dilger *et al.* (2003) identified differentially expressed genes during conidial germination in *Pyrenophora teres* using a radioactive cDNA-AFLP procedure with total RNA as starting material. In this test, the cDNA-AFLP fingerprints produced using total RNA were compared with those produced using mRNA, employing a non-radioactive procedure with silver staining. Clear fingerprints were generated from both materials (Fig. 11C). Although somewhat more TDFs in the fingerprint generated from mRNA than that from total RNA, most of these TDFs were of high molecular weight. Clear fingerprints were successfully generated using total RNA of eight fungi and more than 40 TDFs were displayed (Fig. 11A). Therefore, in consideration of cost and time, total RNA can be also alternatively available for the generation of cDNA-AFLP fingerprint.

The cDNA-AFLP fingerprint can be analysed by autoradiography, fluorescence labelling, silver staining or using a LiCor automated system. Autoradiography is a very sensitive technique which can display average 70 bands per primer combination (Bachem *et al.* 1998). Hence, it is still a commonly used method for the display of cDNA-AFLP fingerprints despite its harmfulness to human health. It is demonstrated that an AFLP display method using fluorescence-labelled primers and LiCor automated system is similarly sensitive to the autoradiography. In these experiments, a conventional silver-staining method was used. It was proved to detect 40-50 bands (Fig 11A). Although these staining method is less sensitive than radioautography, it is simple, nonradioactive and requires no expensive facilities and chemicals. Therefore, it provides an alternative to display cDNA-AFLP fingerprints for those laboratories without facilities to use autoradiography.

### **4.3 Development of specific primer sets and test of their specificity and sensitivity**

#### **4.3.1 Design of primers and their specificity test**

The quality of primers is one of the most important factors for successful PCR amplification since the specificity, sensitivity and amplification efficiency of PCR detection depend largely on the efficiency of primers (He *et al.* 1994). A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation (Dieffenbach *et al.* 1995). The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors including: a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures; b) duplex stability of mismatched nucleotides and their location; and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. The primers which are unique for the target sequence to be amplified should fulfill certain criteria like primer length, GC content, annealing and melting temperature, 5' end stability and 3' end specificity etc. (Dieffenbach *et al.* 1995, Erlich *et al.* 1991). There are a number of professional web-based resources and software packages for PCR and primer design (Abd-Elsalam 2003). Besides, many commercial biosoftwares were developed, such as Molecular Biology Insights, PREMIER Biosoft International, DNA Star and Advanced American Biotechnology and Imaging. Some algorithms and computer programmes have also been developed for primer design of various purposes (Plasterer 1997, Gorelenkov *et al.* 2001, Singh and Kumar, 2001). Among web-based primer design resources, Primer3 ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) employed for all primer design in this study is comprehensive, easy-to-use and are freely available. The user can define different elements such as product size range, maximum mispriming, maximum 3' stability, primer size,  $T_m$  and GC content according to his demands. Then the natures and sequences of several primer sets will be automatically represented and the user can select the optimal primer set from them. High-quality primers for most purposes can be achieved with this tool.

The specificity of PCR assays is an important factor affecting their practical application. As PCR-based assays are very sensitive, a low specificity of primers can increase the risk of producing false-positives (Henson and French 1993). Its specificity mainly depends on the specificity of primers for target pathogens although it is also influenced by PCR conditions as discussed in section 4.3.2. Our aim was to develop several specific primer sets which could be applied to qualitatively and quantitatively detect *M. graminicola* in the field. Therefore, it is

desired that these primers are highly specific and can distinguish *M. graminicola* from other pathogens, healthy plants or other non-pathogenic organisms.

For this purpose, 22 fungal pathogens including 17 wheat pathogens were collected and used for the specificity test of the four developed primer sets ST-rRNA F/R, ST-act F/R, R5870-1F/R5870-1R and R5870-1F/R5870-2R. Among them, only R5870-1F/R5870-1R produced one slight band from *P. anguoides*, the other three amplified no fragments from all the other 22 fungal pathogens tested. They represented dominantly higher specificity than two reported specific primers when used with the same PCR mixture (DNA, polymerase, PCR buffer). E1/STSP2R produced a visible band from 14 of the 22 pathogens tested, and STIF2/BAF4ST amplified multiple bands from most pathogens and from healthy plants (Fig. 12). The results indicate that the specificity of two reported primer sets was unstable, and varied obviously in different PCR conditions. Both primer sets were developed from a partial coding sequence of  $\beta$ -tubulin genes by Fraaije *et al.* (1999, 2001). They are proved to be specific by testing with *M. graminicola* and several other wheat fungal pathogens. However, different results were obtained in this test. Different PCR conditions (DNA polymerase and PCR buffer) are probably the main reasons resulting in the variant specificity. In this experiment, although the PCR programme was performed as described by Fraaije *et al.* (1999) for both primers, different DNA polymerase and PCR buffer were used. A commonly used *Taq* DNA polymerase and the PCR buffer (Biolabs) were employed here for six primer sets, while Fraaije *et al.* (1999, 2001) used 0.5 U Red Hot<sup>®</sup> DNA polymerase and the relevant PCR buffer (Abgene). In the manufacturer's instructions, however, it is not mentioned that the Red Hot DNA polymerase can enhance the specificity.

*M. graminicola* is a pathogen with high genetic variation and genotypic diversity (McDonald *et al.* 1999, Schnieder *et al.* 2001). The genotype of the populations is diverse from lesions to continents (Linde *et al.* 2002). The primer sets ST-rRNA F/R and ST-act F/R were developed from the highly conserved rDNA and act1 gene sequences, their location in the genome is clear. Although the act1 gene and rDNA sequences are variable between *M. graminicola* and other organisms, there is no report on the base variation within both sequences between different isolates of *M. graminicola* by now. As for two other primer sets developed from RAPD fragment, the location of their targets in the genome is still unknown. Four primer sets were tested for the suitability for different isolates with 14 *M. graminicola* isolates which originate from five different European countries and have high genotypic diversity

(Schnieder, 1998). They all amplified an expected PCR product from 14 isolates tested (Fig. 13). It is suggested that the four primer sets can be used for the detection of various *M. graminicola* isolates. The primer set R5870-1F/R5870-1R produced band patterns with variant band intensities (Fig. 13D). It is probably due to the DNA template with different preparations. DNA of three isolates (K-Bc-121, FN4 and GBE2) which gave rise to slight band intensity was provided by Dr. Schnieder. They were prepared using a CTAB method described by Möller *et al.* (1991). Other DNA samples were extracted with DNAzol procedure. As discussed before, DNA with different preparations can obviously affect the efficiency of RAPD-PCR (see section 4.2.2). Later experiments proved that R5870-1F/R5870-2R had a much lower detection sensitivity than the other three primer sets developed by conventional PCR (Fig. 17). This suggests that the amount of final PCR product amplified with R5870-1F/R5870-2R may be influenced more by the initial DNA template. Nevertheless, it is also possible that certain bases of this primer set are variant in these isolates and result in worse primer annealing and amplification efficiency. Further tests for this primer set may be needed using more isolates and DNA samples with the same preparation and concentration.

In conclusion, four primer sets developed in this study amplified target fragment only from *M. graminicola* and its infected plants, but not from healthy wheat plant and other 22 fungal species. They amplified the same target PCR products from 14 different *M. graminicola* isolates and were more specific than the two reported primer sets when used under the same DNA and PCR conditions. It is proved that the four primer sets can be used for the identification and detection of *M. graminicola* in wheat.

#### 4.3.2 Optimization of the PCR conditions

It is well-known that the reaction conditions are critical for PCR amplification as they can obviously influence not only the reaction specificity but also the detection sensitivity (Erich *et al.* 1991). Many PCR parameters such as DNA polymerase, magnesium concentration, annealing temperature, reaction buffer, cycle number and length have an obvious effect on PCR specificity and sensitivity (Erich *et al.* 1991, McPherson and Møller 2000, Cao *et al.* 2004).

DNA polymerase is important for the amplification. *Taq* DNA polymerase, for instance, increased more greatly the amplification specificity and efficiency compared with the Klenow

fragment (Saiki *et al.* 1988). Recent application of hot start DNA polymerase further enhances PCR specificity and efficiency (Kaijalainen *et al.* 1993, Kermekchiev *et al.* 2003). On the one hand, it is thermostable and inactive until it is activated by high temperature incubation (93-96°C for several minutes) and it avoids the potential nonspecific amplification before the reaction reaches 72°C. In this way, it further improved the amplification specificity. On the other hand, it is more thermostable and has longer half-lives at denaturation temperature and therefore withstands more DNA amplification cycles than *Taq* polymerase (Arnheim and Erlich 1992, Kong *et al.* 2004).

Magnesium ion concentration is another critical parameter affecting the specificity and efficiency of the reaction (Innis and Gelfand 1990). dNTPs–  $Mg^{2+}$  complexes are an actual substrate of the polymerase. The concentration of free  $Mg^{2+}$  is affected by the concentration of dNTPs, free pyrophosphate, and ion-binding agents such as EDTA.  $Mg^{2+}$  influences the activity and fidelity of *Taq* DNA polymerase, primer annealing,  $T_m$  of template, product and primer-template associations as well as product specificity. A too low concentration of  $Mg^{2+}$  may result in unsuccessful amplification or a low yield of target products, while excessive  $Mg^{2+}$  can reduce the fidelity of polymerase and lead to non-specific primer binding and increase the non-specific background of the reaction (McPherson and Møller 2000).

Besides, a suitable annealing temperature is also important for PCR amplification (Wu *et al.* 1991). In general, the higher the annealing temperature is, the more specific annealing of the primer and the less mismatches between primer and template can be tolerated (McPherson and Møller 2000). Rychlik *et al.* (1990) reported that if the annealing temperature is increased by 1°C every other cycle, specificity of amplification and yield of products < 1kb in length are both increased. Williamson and Rybicki (1991) suggested that the primer annealing may also be influenced by the GC content and many other factors. The annealing temperature is usually empirically determined based on the melting temperature  $T_m$  of the primer (McPherson and Møller 2000). However, there are different empirical formulas for the calculation of  $T_m$  (Rychlik *et al.* 1990, Wallace *et al.* 1979, Sambrook *et al.* 1989) which can give obviously different  $T_m$  values (Rochelle *et al.* 1997). Therefore, such calculations only provide a guide for the annealing temperature in the PCR. Recently, the gradient thermal cycler with which a series of annealing temperatures can be simultaneously tested has greatly simplified the optimization of annealing temperature.

The PCR programme includes different cycles with three essential steps of denaturation, primer annealing and primer extension. As different target templates and primers have different natures, the optimum PCR programme for different primers may differ. Anyway, a short and efficient PCR programme not only saves running time but also increases the catalyzation efficiency of DNA polymerase. An overlong running time may also cause nonspecific amplification or increase the background (McPherson and Møller 2000). Numerous PCR programmes with different running time or cycle number have been reportedly used for the amplification of different DNA targets. The successful application of some short PCR programmes indicates that it is possible to shorten the running time of each step. Beck and Ligon (1995), for instance, used a short PCR programme (30 cycles of 15 s at 94°C, 15s at 60°C and 45 s at 72°C) to efficiently amplify a 345-bp target product from *M. graminicola* and its infected plants. A simple PCR programme (94°C for 3 min, 30 cycles of 94°C for 30 s, 65°C for 15 s and 72°C for 30 s) was also employed efficiently for the amplification of an over 500-bp ITS region with ITS1 and ITS4 primers (Flowers *et al.* 2003).

In order to test the possible influence of  $Mg^{2+}$  concentration on four specific primer sets, a conventional PCR was performed with eight different concentrations of  $Mg^{2+}$  ranging from 0.5-4.0 mM. The results indicated that  $Mg^{2+}$  concentration had a clearly variant effect on the amplification of the four primer sets (Fig. 14). For ST-rRNA F/R and ST-Act F/R, the optimal concentration was 1.5 mM, and the amplication efficiency decreased obviously with the increase of  $Mg^{2+}$  concentration from 1.5-4.0 mM. The possible reason is that excessive  $Mg^{2+}$  either reduces the activity of *Taq* DNA polymerase, or inhibits primer annealing and product and primer-template associations. Kim and Marians (1995) concluded that a DNA-DNA and RNA-DNA annealing activity is stimulated by  $Mg^{2+}$ , and higher concentrations inhibited the reaction and increased the rate of independent DNA annealing. For the other two primer sets, the amplification efficiency did not vary so obviously between 1.5-4.0 mM (Fig. 14). It is suggested that the two primer sets had a large adaption range of  $Mg^{2+}$  concentration. Increased  $Mg^{2+}$  concentrations did not lead to amplification of non-specific products or higher background. The four primer sets tested failed to amplify products at a concentration of 0.5 mM, indicating that an overlow concentration does result in unsuccessful amplification. It is suggested that the effect of  $Mg^{2+}$  on PCR amplification may be complex and differs according to different primer sets.

The effect of 12 annealing temperatures on the PCR amplification of the four primer sets was tested in a gradient thermal cycler. Different effects were indicated on them. For ST-act F/R and R5870-1F/R5870-1R, the PCR efficiency was not clearly affected by the annealing temperature between 50-65°C, whereas the PCR efficiency for ST-rRNA F/R and R5870-1F/R5870-2R was obviously influenced by it and was lower at low temperatures (Fig. 15). It is concluded that the effect of annealing temperature differs with different primers. Meanwhile, it is also evident that empirical determination of annealing temperature is often not accurate.

Eight PCR programmes with different running time ranging from 1 h 6 min to 1 h 43 min were tested for the PCR amplification of the two primer sets ST-rRNA F/R and ST-act F/R. The increase of the running time of PCR did not improve the PCR amplification efficiency, and the the shortest PCR programme I (94°C for 2 min, 40 cycles of 94°C for 15 s, 60°C for 15 s and 72°C for 30 s) represented a high PCR efficiency for both primer sets (Fig. 16). It indicated that this programme was sufficient for the amplification of both targets. The band intensity between 30 and 40 cycles was variant in PCR with ST-act F/R but not in PCR with ST-rRNA F/R. It is suggested that the optimal cycle number differs according to primer sets.

How much time each step of a PCR programme need may depend mainly on the template length as well as the nature of template and primers. The denaturation step should be as short as possible since *Taq* polymerase has a half-life of 30 min at 95°C. An overlong denaturation time of each cycle reduces the cycle number of an efficient amplification (McPherson and Møller 2000). Normally the denaturation time is 1 min at 94°C. But it is possible for short template sequences to reduce it to 30 s or less. Innis and Gelfand (1990), for instance, recommended 96°C for 15 s. In the PCR programme described by Beck and Ligon (1995), 94°C for 15 s was used for denaturation. Our results indicated that 94°C for 15 s was adequate for the denaturation of at least 496-bp target products. It is necessary to shorten the annealing time since the polymerase must have activity at the annealing temperature. In the primer annealing step, long annealing time increases the risk of nonspecific amplification. Mcpherson and Møller (2000) reported that most primers can anneal efficiently in 30 s or less unless the actual temperature is too close to the  $T_m$  or the targets are unusually long. Our results showed 15 s was sufficient for the annealing of the two primer sets tested. The primer extension depends essentially on the length of target product. It is normally performed at 72°C for 0.5-3 min. Innis and Gelfand (1990) suggested that about 1 min is sufficient for a



reliable amplification of 2 kb sequences. In this experiment, a time of 30 s was used efficiently for primer extension of a 496-bp fragment amplified by the primer set ST-act F/R (Fig. 16).

The test results greatly shortened the PCR running time, requiring only 1 h 13 min for 40 cycles. It may be helpful for the amplification of other target PCR products with similar size. Due to different heating and cooling efficiency of thermal cyclers, the same PCR programme may require differential running time in different thermal cyclers. These differences may cause differential PCR efficiency. All PCR programmes in this experiment were run only in a PTC-200 Peltier Thermal Cycler. It may be necessary to further test it in other PCR thermal cyclers.

### 4.3.3 Detection sensitivity in a conventional PCR

The detection sensitivity of PCR is an important factor for diagnostic applications. In this study, presymptomatic detection of *M. graminicola* in the field is desired. Therefore, high sensitivity is necessary in order to detect *M. graminicola* in the latent period as early as possible.

PCR is an extremely sensitive assay in comparison with other biochemical and immunological assays. Nevertheless, its sensitivity can be affected by many factors such as the nature of target sequences and primers, and PCR conditions as discussed in section 4.3.2. In order to test the detection limit of the four primer sets, a conventional PCR was performed using 13 different dilutions of *M. graminicola* DNA. It indicated that PCR with the three primer sets ST-rRNA F/R, ST-act F/R and R5870-1R/R5870-1R had a high detection sensitivity, and could detect as little as 0.5 pg genomic DNA of *M. graminicola* (equal to about 50 spores) on an ethidium bromide-stained agarose gels (Fig. 17). This detection limit is somewhat lower than that of the two reported primer sets E1/STSP2R (2 pg, Fraaije *et al.*) and ITS1/JB446 (2.5 pg, Beck and Ligon 1995). PCR with the primer set R5870-1F/R5870-2R had a much higher detection limit (50 pg, Fig. 17C). Similar results are also shown in Fig. 14. Although R5870-1F/R5870-2R was designed from the same RAPD sequence as R5870-1F/R5870-1R, its sensitivity was much lower probably due to the difference in target sequence size. The target sequence of R5870-1F/R5870-2R (323 bp) is 103 bp smaller than that of R5870-1F/R5870-1R (426 bp). It is clear that small fragment reveals a lower band intensity than big fragment in the gel when their PCR products have the

same copy number. Another possible reason is the different nature of the two primers and their target sequences. The self-complementary score of R5870-2R is 5.0, higher than that of R5870-1R (3.0), and the 3' complementary is 0.0, lower than that of R5870-1R (1.0, Table 8). It indicates that R5870-2R has a higher tendency to form a primer-dimer with itself, but has a lower tendency to form primer dimers than R5870-1R. These differences probably cause the difference in the PCR efficiency of both primer sets.

Different staining can result in a different detection sensitivity. For agarose gel, ethidium bromide is a routine staining dye. Although this dye is cheap, efficient and easy to use, it has a high mutagenicity frequency. SYBR Green I is a novel fluorescent dye available for nucleic acid staining. Because of its strong DNA binding affinity, it has a much higher sensitivity than ethidium bromide, whereas it has a much lower mutagenicity frequency (Singer *et al.* 1999). It can be used to stain double-stranded DNA in agarose or other gels before electrophoresis, in gels during electrophoresis or after electrophoresis (Rye *et al.* 1993).

The staining efficiency of SYBR Green I for PCR products in agarose gel with three different staining procedures was compared with that of ethidium bromide. The results showed that SYBR Green I does somewhat increase the detection sensitivity and band intensity when staining has been performed by adding the dye into the gel or before electrophoresis (Fig. 18). But the increased intensity was not as high as that described in the instructions of manufacturer. The probable reason is that the SYBR Green I dye-stained gel was also excited using 312 nm transillumination. As described in the instructions, the excitation at 254 nm results in the highest sensitivity. For SYBR Green I, staining by adding into the gel represented the most efficient method since it obviously increased the detection sensitivity and band intensity without causing obvious band shift (Fig.18II). Staining before electrophoresis using SYBR Green I somewhat increases the detection sensitivity and band intensity, but caused clearly band shift (Fig. 18IB).

Although SYBR Green I represents a somewhat higher sensitivity for agarose gel staining, it is too expensive to use for it. When staining by adding into agarose gel is used, SYBR Green I needs 10  $\mu$ l/100 ml agarose solution, and ethidium bromide needs 5  $\mu$ l/100 ml. Considering their price, SYBR Green I costs about 80 times more than ethidium bromide.

#### 4.4 Real-time PCR

Real-time PCR is a quantitative PCR approach which can quantify the amount of template DNA input and which allows the cycle-to-cycle monitoring of the amplified DNA (Higuchi *et al.* 1992). Compared to conventional PCR, it has significant advantages. This technique does not require post-amplification processing steps and thus greatly reduces time and labour, largely increasing the throughput of PCR testing as an automated diagnostic system suitable for large-scale analyses (Schena *et al.* 2002, Abd-Elsalam 2003). Real-time PCR includes amplicon sequence non-specific and sequence specific methods (Schena *et al.* 2004). In this research, an amplicon sequence non-specific method was employed using SYBR Green I and the LightCycler system. SYBR Green I (Morrison *et al.* 1998) is the most frequently used intercalating dye in real-time PCR. It is also a simple and low-cost method for monitoring PCR amplicons and for quantifying template DNA in comparison with other real-time PCR methods. In solution, the unbound dye exhibits very little fluorescence, but fluorescence is enhanced upon DNA-binding. As a consequence, the fluorescence is proportional to the amount of total dsDNA in the reaction. The shortage of this method is that these dyes do not differentiate the target PCR product from other non-specific amplified products or primer dimers (Schena *et al.* 2004). But this problem may be solved when the Lightcycler system is used. Through a melting curve analysis, the target product can be distinguished from nonspecific products or primer dimers based on their different melting points. In order to suppress fluorescence caused by the primer dimers or nonspecific products, an additional data acquisition step can be added.

Currently, there are numerous real-time PCR kits produced by different companies. It is important to select a suitable PCR kit because different kits have different chemical components and may produce greatly differing results. An initial comparison of real-time PCR efficiency between the LightCycler FastStart DNA Master SYBR Green I (Roche) and the QuantiTect<sup>TM</sup> SYBR<sup>®</sup> Green PCR Kit (Qiagen), for example, revealed that the former resulted in two cycles earlier than the latter. Therefore, it is necessary to use always the same kit for the construction of standard curves and sample tests. The QuantiTect<sup>TM</sup> SYBR<sup>®</sup> Green PCR Kit (Qiagen) was selected for all the tests of real-time PCR in this research since it is effective and relatively cheap in comparison to other kits. This kit is a ready-to-use kit in that all components are optimized in a master mix. Only DNA template, primers and H<sub>2</sub>O have to be added in the reaction so that the errors or contaminations caused by pipetting can be significantly reduced. A hot start *Taq* DNA polymerase is used in the kit, allowing high PCR

specificity and accurate quantification. But the shortage of this kit is that the  $Mg^{2+}$  concentration is defined in the master mix with 2.5 mM in the PCR reaction. As tested, different primer sets have different optimal  $Mg^{2+}$  concentrations (Fig. 14). Hence, for those primer sets that have the optimal  $Mg^{2+}$  concentration less than 2.5 mM or more than 2.5 mM, such as for ST-rRNA F/R, this kit may not generate data with an optimal specificity and sensitivity.

#### 4.4.1 Effects of several factors on real-time PCR amplification

Like other PCR-based methods, real-time PCR is also affected by different factors such as DNA purity, kits used,  $Mg^{2+}$  concentration and primer amount (McPherson and Møller 2000). During the experiments, real-time PCR using an old solution of the ST-act F/R primer set showed a much lower sensitivity and failed to detect *M. graminicola* DNA from those leaves with a low infection level. A comparison of old and new solution indicated that it was due to primer degradation during the storage (Table 9, Fig. 19). It is probable that the primer degradation resulted in the decrease of efficient primer concentration in the solution and reduced the PCR efficiency. It is suggested that the primer concentration may be an important factor affecting the efficiency of real-time PCR. In the instructions of LightCycler system, it is noted that equimolar concentrations of both primers should be used to avoid asymmetric (unbalanced) reaction conditions, which have been shown to decrease PCR fidelity. The optimum concentration of primers is usually 0.1–0.6  $\mu$ M. Higher concentrations may promote mispriming and accumulation of nonspecific products, while lower primer concentrations may be exhausted before the reaction is completed, resulting in lower yields of desired products. The potential influence of primer concentration on PCR was partially discussed in section 4.3.1 and 4.3.3.2 and is also described in some publications (Gunson *et al.* 2003, McPherson and Møller 2000).

Every successful real-time PCR depends on the quality of the nucleic acid template that contains the target sequence(s). There are numerous protocols of DNA preparation suitable for real-time PCR from various biological materials. We integrated a simple CTAB-PVP method suitable for DNA extraction from large or small samples of infected wheat plants and fungi (Fig. 5). The DNAs prepared with this protocol had a purity high enough for real-time PCR test (see section 3.4). Some simple methods, such as that described by Fraaije *et al.* (1999) and that described by Qi and Yang (2002), are reportedly suitable for DNA

preparation from fungi and infected plants for real-time PCR. In the instructions of LightCycler system, it is mentioned that excessive DNA template input may inhibit real-time PCR amplification and that DNA template amount shall not exceed 50 ng genomic DNA in a 20- $\mu$ l reaction for SYBR Green assay. Our results indicated that more than 10 ng initial input of *M. graminicola* DNA indeed inhibited the real-time PCR amplification with ST-act F/R (Fig. 20). Similar results also occurred in the two other primer sets ST-rRNA F/R and R5870-1F/R5870-2R by real-time PCR. Meanwhile, a trial was done with 25 ng, 50 ng and 100 ng genomic DNA of infected wheat plants in a 20- $\mu$ l reaction. The results showed that the 100-ng genomic DNA input inhibited clearly the amplification, but the 25- and 50-ng input had no inhibitory effect (result not shown). It is likely that SYBR Green I is a nonspecific intercalating fluorochrome, which emits fluorescent signal when intercalated to double-strand DNA template. When the initial DNA template concentration is too high, a large amount of SYBR Green intercalate to these DNA templates. It results in a strong background and also indicates that the SYBR Green molecules useful for the amplicons are greatly reduced. Furthermore, excessive initial DNA template can inhibit PCR amplification (McPherson and Møller 2000). This phenomenon was observed in the sensitivity test of ST-rRNA F/R by conventional PCR, in which the band intensity of the 100-ng DNA input was clearly lower than that of the 10-ng DNA input (Fig. 17A).

#### **4.4.2 Suitability of the four specific primer sets for real-time PCR**

Although the specificity of the four primer sets has been demonstrated by conventional PCR, these primer sets may not be suitable for real-time PCR since the signal detection system of real-time PCR is completely different from conventional PCR. The real-time PCR system is based on the detection and quantification of a fluorescent reporter such as SYBR Green I during the PCR reaction. The conventional PCR, however, usually detects the final PCR products by visualizing on the agarose gel. In conventional PCR, the specificity of primers is evaluated based on the fragment size. Nonspecific PCR products or primer dimers with a size different from the target fragment do not affect the detection of targets by conventional PCR, but may affect real-time PCR if these nonspecific or primer dimers have the same or a similar melting temperature with the target product. In addition, the amplification of nonspecific fragments and the formation of primer dimers may reduce the amplification efficiency of the target fragment.

The specificity of primer sets can be assessed through a melting curve analysis. Usually, the non-specific products or primer dimers have different  $T_m$  from the target product. The results of a primary test revealed that the four primer sets amplified the target product only from the standard DNA of *M. graminicola*, but not from DNA of healthy wheat plants or H<sub>2</sub>O. Although some unspecific products or primer dimers were amplified from samples with low DNA template input or negative control, their  $T_m$  was over 3°C lower than the  $T_m$  of target product (Fig. 21, Table 10). In the LightCycler system, the fluorescent signals caused by these nonspecific products or primer dimers can be avoided by adding a fluorescence acquisition step at a temperature between the  $T_m$  of specific and nonspecific products. In this study, a SYBR Green real-time PCR using these primer sets was successfully performed for the quantification of *M. graminicola* in inoculated plants as well as in field samples (see sections 3.4.4 and 3.4.5).

The results indicate that real-time PCR was relatively more sensitive than conventional PCR for the four primer sets when the two methods were used to detect the standard DNA of *M. graminicola* (Table 11). Especially for R5870-1F/R5870-2R, real-time PCR (Fig. 23IIC) represented a much higher sensitivity (Fig. 17C). Similar results have also been reported (Cullen *et al.* 2001, Schena *et al.* 2002). Amplification of *R. solani* target DNA from soil was achieved with real-time PCR, but not with conventional PCR (Lees *et al.* 2002), which is probably due to the characteristics of real-time PCR. Real-time PCR detects the fluorescence signal during the entire reaction and defines the cycle number when the accumulated fluorescence exceeds the fluorescence threshold which depends on the start point of the PCR exponential phase, but not on the target sequence size. Hence, real-time PCR is usually more sensitive for small sequences than for large sequences. It is recommended in the LightCycler system that the suitable sequence size for SYBR Green assay is 100-1000 bp and the optimal size is 100-300 bp. It is suggested in the instructions of the QuantiTect™ SYBR® Green PCR Kit that targets should ideally be 100-150 bp in length in order to get the highest efficiency. In contrast, conventional PCR performed by visualizing in an agarose gel has a lower sensitivity for small fragment than large fragment when their amplified copy numbers are the same. Another possibility is that the LightCycler system can more efficiently and rapidly change the temperature than conventional thermal cyclers during the running, which greatly shortens the running time of the PCR programme and enhances the amplification efficiency of the *Taq* DNA polymerase.

#### 4.4.3 Construction of DNA standard curves

Real-time PCR provides an accurate, reliable and high-throughput quantification of target DNA of pathogens *in planta*. Although several methods have been developed with conventional PCR for quantitative analysis of phytopathogenic fungi (Fraaije *et al.* 1999, Mahuku and Platt 2002), they are laborious and not accurate enough because the amplification efficiency decreases during later cycles (Ginzinger 2002). As previously described, real-time PCR measures the amplicons at an early stage of the reaction when the efficiency is still constant. The cycle number is automatically generated when a fluorescent signal exceeds the cycle threshold. A linear correlation to the log of the initial DNA amount should result. Fungal DNA amounts in infected plants can be calculated from the cycle number measured by real-time PCR based on a standard curve.

It was demonstrated that the four developed primer sets could be used for the quantification of *M. graminicola* DNA. The three primer sets ST-rRNA F/R, ST-act F/R and R5870-1F/R5870-2R were selected for quantification of *M. graminicola* DNA in infected wheat plants because they were more sensitive. In order to calculate unknown fungal DNA amount from the cycle number value measured by real-time PCR, three standard curves were constructed for three corresponding primer sets using a series of 10-fold dilutions of the standard DNA of *M. graminicola*. The standard curve was constructed by plotting the initial amount of *M. graminicola* DNA versus their cycle number obtained from real-time PCR. Three standard curves revealed an almost complete linearity, and their regression coefficients ( $r^2$ ) exceeded 0.9996 and were highly significant (Figs. 22, 23). This indicates that the initial DNA amounts of *M. graminicola* were highly correlated with their cycle number obtained from the real-time PCR data using the three primer sets at least within the tested dilution range.

The accurate quantitation of the standard DNA is a prerequisite for the construction of standard DNA. Currently, there are a number of methods to quantitate DNA amounts. The most commonly used is the determination of absorbance at 260 nm since it is simple and requires no additional chemical reagents, but its major disadvantages are the large potential contribution of nucleotides and single-stranded nucleic acids to the signal, the inability to distinguish between DNA and RNA and the relative insensitivity and inaccuracy of this assay (an A260 corresponds to five  $\mu\text{g/ml}$ ). A Hoefer<sup>®</sup> DyNA Quant<sup>®</sup> 200 fluorometer was therefore used to quantitate genomic DNA of *M. graminicola* and wheat plants in this study.

This method uses the Hoechst 33528 fluorescence dye (bisbenzimidazole) which binds selectively to dsDNA, excites at 356 nm and emits fluorescence at 458 nm. The fluorescent signal is only rarely interfered with RNA because RNA does not generally bind Hoechst 33528 under high salt conditions. Therefore this method is more accurate for the quantitation of dsDNA concentration than the absorbance method and has been commonly used for DNA quantitation (Teare *et al.* 1997, Al-Samarrai and Schmid 2000). In addition, it is reported that PicoGreen dsDNA quantitation reagent is a more sensitive fluorescent dye for quantitating dsDNA in solution than Hoechst 33528 (Ahn *et al.* 1996, Singer *et al.* 1997, Fraaije *et al.* 1999).

#### 4.4.4 Monitoring of disease development in inoculated wheat plants

An accurate monitoring of disease progression is important for the study of fungal colonization, fungal infection and development *in planta*, or disease susceptibility (Leonard and Fry 1986). There are several methods for monitoring disease progression. Visual or microscopic methods are the most commonly used, but they are not suitable for the assessment at the early stage of fungal development. Biochemical tests like ergosterol or chitin quantification have also been used, but such tests are not specific for a given pathogen and are often not sufficiently sensitive (Ekblad *et al.* 1998). Indirect assessment of fungal biomass has been used, like monitoring the expression of the GUS (Pnini-Cohen *et al.* 1999) or GFP gene (Rohel *et al.* 2001) in transgenic fungal strains. However, such techniques are labour-intensive and quantification accuracy may sometimes be questioned. In contrast, real-time PCR has been proved to be an accurate and sensitive way to quantify the disease progression of phytopathogenic fungi (Schaad and Frederick 2002). Brouwer *et al.* (2003), for instance, quantified disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR and the LightCycler system. Gachon and Saindrenan (2004) also applied real-time PCR to monitor fungal development in *A. thaliana* infected by *A. brassicicola* and *B. cinerea*.

In this study, real-time PCR was successfully applied to monitor the development of *M. graminicola* in inoculated wheat plants from 0 to 24 days after inoculation using two specific primer sets. *M. graminicola* DNA, although in low amount, was detected on the inoculation day (Table 12). Although the first visible lesions appeared eight days after inoculation, a dynamic increase of fungal DNA before that time was obtained with real-time PCR (Table 12, Fig. 24). This indicates that real-time PCR is a very sensitive method for the



quantitative detection of *M. graminicola* in plants. It could presymptomatically detect *M. graminicola* eight days before visible symptoms appear. Fraaije *et al.* (1999) monitored the fungal biomass growth during the infection of *M. graminicola* with a PCR/PicoGreen assay. An exponential biomass increase is observed only after the appearance of necroses and production of pycnidia (about 14 days after inoculation). Pnini-Cohen *et al.* (1999) monitored fungal biomass progression during the infection of a GUS transformant of *M. graminicola* by measuring GUS activity. Fungal biomass is detected two days after inoculation, but a rapid increase starts 12 days after inoculation. These differences may be caused by two factors: (1) the detection limit of the latter two methods, which can not accurately detect the fungal biomass increase at the early stage, is much higher than that of real-time PCR assay; (2) different inoculation conditions such as incubation temperature and the variety susceptibility result in different development speed of fungal infection (Shaw *et al.* 1990). Regarding the fungal infection and growth speed of *M. graminicola* in infected plant, it is proved that the penetration can be completed within 24 h after inoculation, the fungal growth speed depends on the temperature and cultivar resistance, and the fungal biomass can increase rapidly at the early stage under suitable conditions (Hilu *et al.* 1957, Rohel *et al.* 2001).

The tendency of disease progression obtained from real-time PCR basically agreed with that visually assessed by a disease classification between eight and 20 days after inoculation (Fig. 24). The fungal DNA amounts were significantly correlated with the disease indices ( $r = 0.9115^{***}$ ). However, their relationship was non-linear, particularly when the fungal DNA amount felt short of 200 pg or exceeded 600 pg (Fig. 26). This result was expected because fungal DNA could be detected in the absence of symptoms at the early stage of disease development. Furthermore, although the disease indices were close to 100, the disease could further develop since the leaf was not completely diseased. Therefore, the fungal DNA could further grow until the leaf was dried.

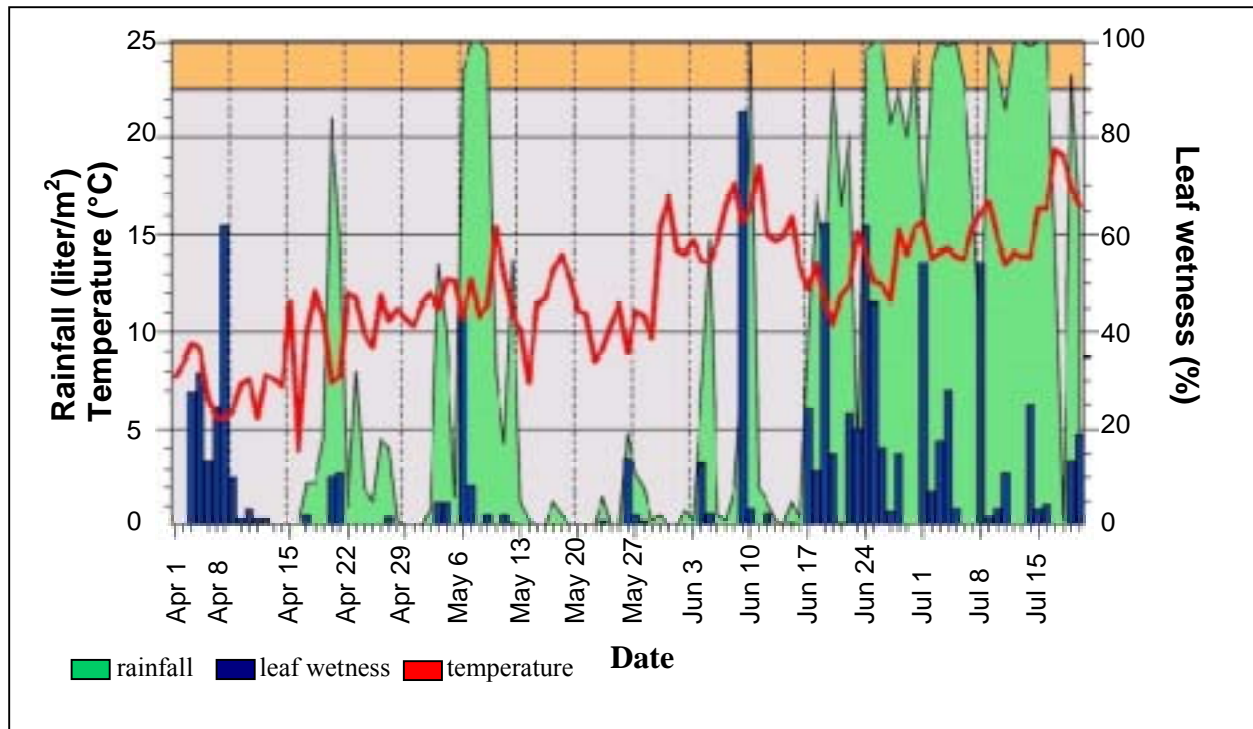
In order to compare the suitability of different primer sets for the quantification of *M. graminicola* in plants, the fungal DNA growth during disease development was measured by real-time PCR using the two primer sets ST-rRNA F/R and ST-act F/R. Theoretically, the DNA amount values obtained from both primer sets should be the same because the samples used were identical. However, the values obtained were obviously different although their relationship was almost linear (Table 12, Fig. 25). The values tested with ST-act F/R were clearly lower than those obtained with ST-rRNA F/R. A similar phenomenon also occurred

in the experiments performed for quantifying *M. graminicola* DNA in naturally infected leaves by real-time PCR with three different primer sets (Table 14, Fig. 31). This indicates that the quantification results obtained by real-time PCR can be affected by the primer sets used. Vet and Marras (2005) described that optimal design of primer sets and targets, and use of appropriate primer concentrations are the prerequisites for successful real-time PCR using SYBR Green. In the instructions of the kit, it is also remarked that different characters of primer sets and target sequences can largely influence the PCR results. As discussed in sections 4.3.1 and 4.3.2, the quality and nature of primers and the nature of target sequences can obviously influence the PCR specificity and efficiency. The three primer sets used have different values of  $T_m$ , any and 3' self complementary, their target sequences have different size and GC content (Table 8) and they may have different copy numbers in the genome. Due to these differences, the three primer sets may require different optimal PCR conditions. Their amplification efficiency and the intercalating amount of SYBR Green molecules within target sequences can be variant. Furthermore, it was demonstrated that the three primer sets had different requirements on  $Mg^{2+}$  concentration, annealing temperature and had different sensitivity (see section 3.3.2, table 11). In the experiments, the same kit was used for the three primer sets. The QuantiTect™ SYBR® Green PCR Kit is a ready-to-use kit, the parameters of PCR conditions can not be changed except primer concentration, template input and PCR programme. The amplification efficiency of the three primer sets can vary under the same PCR condition given in the kit. Another reason is probably that the three primer sets have a different potential to produce nonspecific products and primer dimers (Fig. 21). The early amplification of these products inhibited the amplification of the target product.

#### **4.4.5 Monitoring of disease progression in the field**

Accurate monitoring of *M. graminicola* disease progression in the field during the disease epidemic period plays an important role in precisely prognosing epidemics and efficiently managing this disease. The commonly used visual assessment based on the diagnosis of visible symptoms is not suitable for the assessment at the early stage of disease development and often can not precisely and specifically evaluate the disease severity. Recently, real-time PCR has been successfully used for the assessment of various plant diseases in the field (Schaad *et al.* 2002, Qi and Yang 2002). For *M. graminicola* wheat leaf blotch, Fraaije *et al.* (2001) applied real-time PCR to assess the resistance of different varieties by quantifying the fungal biomass in wheat leaves.

In this study, real-time PCR was successfully applied to follow *M. graminicola* disease progression in F, F1 and F2 leaves in the field during the epidemic period and was compared with two common visual assessment methods. The results indicated that *M. graminicola* DNA amounts measured by real-time PCR were significantly correlated with disease indices and with the NPL. Nevertheless, their relationship was nonlinear (Figs. 29 and 30). The disease progression curves showed that the disease progression obtained by real-time PCR was more closely related to that obtained by pycnidia-counting method than to that obtained by disease classification (Figs. 27, 28). A relatively high disease severity in F1 and F2 leaves before May 24, 2004 was revealed by a disease classification, compared to that obtained by counting pycnidial number and by real-time PCR (Table 13, Figs. 27, 28). The possible reason is that some lesions may be caused by other pathogens or some environmental factors were regarded as *M. graminicola* lesions during the classification of disease severity since the lesions are hard to distinguish by eye in the early stage of disease. Although the pycnidia-counting method can specifically assess *M. graminicola* disease severity, it can be performed only after pycnidia appear. The data indicate that real-time PCR using specific primer sets was very sensitive and could detect and quantify accurately and specifically the pathogen biomass. The real-time PCR assay using ST-rRNA F/R could detect as little as 0.37 pg *M. graminicola* DNA from 50 ng DNA of a field sample, and *M. graminicola* DNA was detected in all F, F1 and F2 leaf samples tested, even in those symptomless leaf samples, indicating that real-time PCR can presymptomatically detect *M. graminicola* in the field. Meanwhile, a dynamic progression of *M. graminicola* DNA in these leaves was quantitatively determined (Fig. 28A). It is demonstrated that real-time PCR can be a powerful tool to quantitatively detect *M. graminicola* and to assess the disease severity during the early stage of disease epidemics. One of its disadvantage is that, like other PCR-based methods, the sensitivity of real-time PCR can cause false positive results due to possible cross contaminations during leaf sampling or PCR performance (Schena *et al.* 2004). Therefore, during the preparation of sample and reaction, potential contaminations of target pathogens or DNA have to be avoided.



**Fig. 41** Leaf wetness, rainfall and temperature in the experimental field (Gettorf) between April 1 and July 20, 2004.

The disease progression obtained by real-time PCR can be explained by the weather conditions in the experimental field. The weather data showed that the leaf wetness exceeded 98% and the mean daily temperature was 10-15°C between May 5 and May 10 (Fig. 41). As reported, the conidia germinate from 2 to 37°C (Shipton *et al.* 1971). The suitable temperature for infection is 11-25°C (Hess and Shaner 1987). Infection takes place when total precipitation exceeds 10 mm within three days, or two days total precipitation exceeds 5 mm (Shaw 1990). According to these reported infection conditions, the temperature and precipitation was sufficient for *M. graminicola* infection. Hence, an infection occurred during that time. Between May 10 and June 17, there were no suitable infection conditions due to low leaf wetness. Therefore, the F1, F2 leaves and partial F leaves were probably infected between May 5 and 10. As the temperature was basically low (around 10°C) before May 29, the disease developed very slowly during that time. But after May 29, the temperature increased rapidly to more than 15°C until June 14, and fungal biomass and the number of pycnidia increased rapidly accordingly (Fig. 28). The data suggest that temperature may be one of the most important factors for disease development after *M. graminicola*'s penetration into the host plants.

According to the results, the latent period of *M. graminicola* in F1 and F2 leaves was assessed as about 26 days in the winter wheat variety Ritmo, which is very close to that (28 days) reported by Verreet *et al.* (2000). Shaw *et al.* (1990) regarded that the latent period of *M. graminicola* depends largely on temperature and the cultivar resistance. Brokenshire (1976) found that the latent period varied between 14 and 25 days in the field, depending on cultivar, while Arsenijević (1964) observed a latent period varying from 36 days in December to 11 days in June. By a inoculation, Lovell *et al.* (2004) reported that the latent periods ranged from 11 to 42 days with mean batch temperatures of 2.9 - 20.2°C, but the latent period can be shorter under optimal conditions. In our inoculation experiment with 22°C on day 17°C during the night, and 12 hours photoperiod, lesions were observed in the cultivar Ritmo eight days after inoculation, and the latent period in the cultivar Kanzler was about three days longer than in Ritmo. Furthermore, discoloration symptoms is observed by Fraaije *et al.* (1999) on three days after inoculation at 18°C.

Real-time PCR was performed using the primer set ST-rRNA F/R and compared with the primer set ST-act F/R. Real-time PCR using ST-act F/R did not detect *M. graminicola* DNA in 11 of 22 field samples, whereas real-time PCR using ST-rRNA F/R could detect it in all samples tested (Table 13). This indicates that the former had a much higher detection limit than the latter, but such case did not occur in inoculated samples in which as little as 4.15 pg fungal DNA amount was detected and no nonspecific products were amplified using real-time PCR with ST-act F/R (Table 12). It is probable that the specificity of ST-act F/R was not sufficient so that it produced some nonspecific products from either other pathogens or non-pathogenic microorganisms. Unlike plants inoculated with a pure pathogen, the plants in the field are usually co-infected by multiple pathogens. Furthermore, the organism populations in the field are much complex than in a climatic room. The melting curve analysis showed that non-specific products or primer dimers were amplified when there were few target DNA templates (Fig. A5). It was also shown that a nonspecific fragment was strongly amplified from the negative control of healthy plants (Fig. 21). The prior amplification of these nonspecific products inhibits the amplification of the target sequence in late cycles. Similar phenomena were also reported by Fraaije *et al.* (2001) who used real-time PCR to test field samples.

Later, the primer set R5870-1F/R5870-2R was used to detect *M. graminicola* in the nine F1 field samples in order to test its suitability for naturally infected plants. Like the primer set

ST-rRNA F/R, this primer set could detect *M. graminicola* in all nine F1 samples tested. The DNA values resembled those measured by ST-act F/R, but were less than those measured by ST-rRNA F/R (Table 13, 14). Theoretically, the DNA amounts tested with the three primer sets should be the same since the same samples used were the same for the three primer sets. The possible reasons have been discussed in section 4.4.4.

#### 4.4.6 Assessment of fungicide efficacy

Recently, several quantitative PCR methods have been applied to assess fungicide efficacy. Doohan *et al.* (1999) and Edwards *et al.* (2001) determined the efficacy of fungicide against *Fusarium* head blight of winter wheat by a competitive PCR assay. Rohel *et al.* (2002) used a PicoGreen/PCR assay to monitor the efficacy of azoxystrobin treatments on *M. graminicola* epidemics in wheat. These assays assess initial fungal DNA amount in the leaves by quantifying final PCR products, and their detection accuracy is worse than that of real-time PCR. As discussed before, real-time PCR could quantitatively and specifically detect *M. graminicola* in the field.

In this study, real-time PCR was successfully applied to assess the efficacy of two fungicide treatments at different leaf positions by quantifying fungal DNA amounts in fungicide-treated and –untreated plants. Compared with two visual methods, real-time PCR had an obvious advantage because it could assess the efficacy in different diseased leaves. Real-time PCR gave expected positive efficacy in 14 of 18 leaf samples including several severely diseased leaves. In contrast, the disease classification and the pycnidia counting method gave only 11 and 10, respectively (Table 16). The pycnidia counting method gave unsatisfactory results in Fandango treatment on May 17, and the disease classification gave no positive efficacy in F2 and F3 of Fandango treatment on June 14 since most leaves were dried diseased. The disease classification gave unsatisfactory results in the early stage and later stage of disease development (F and F1 on May 17 and F2 and F3 on June 14). In F and F1 leaves on May 17, all three methods gave no positive efficacy for two fungicide treatments since the disease was almost free even in the untreated control during that time (Table 16). Therefore, it is meaningless to assess fungicide efficacy on these leaves. On June 14, the efficacies of two treatments obtained by real-time PCR were very close to those obtained by the pycnidia counting method. This possibly suggests that real-time PCR can assess the fungicide efficacy on *M. graminicola* specifically like the pycnidia counting method.

Fandango, Input and Prosaro are three novel systemic fungicides produced by Bayer CropScience. The results obtained by real-time PCR and visual assessments indicated that two fungicide treatments controlled *M. graminicola* leaf blotch efficiently (Table 16). The efficacy of both treatments on June 14 was somewhat higher than those on May 17. One possible reason is that the first assessment (May 17) was performed 25 days after fungicide treatment and the second assessment (June 14) was performed four days after the third treatment. Another reason is probably that the disease on June 14 was much severe than that on May 17. According to the results assessed on June 14, the Input treatment was somewhat better than Fandango treatment.

#### **4.5 Detection of *M. graminicola* in wheat using an RT-PCR assay**

RT-PCR is another PCR-based method to detect pathogens *in planta*. Although most genes are expressed differentially in different structures or developmental stages of cells (Ingle and Kushner 1996), some genes, such as rRNA,  $\beta$ -tubulin and actin genes, serve as house-keeping genes and are constitutively expressed in cells. Usually, the expressed RNA contents of these genes are proportional to the fungal biomass and therefore they may be used as target to quantify fungal biomass (Benito et al. 1998, Qi and Yang 2002). Some RNA-based methods such as Northern blot analysis and RT-PCR have been used for quantifying the fungal biomass in infected plants. For instance, Qi and Yang (2002) applied Northern blot analysis to quantify *Magnaporthe grisea* biomass during the infection in rice by measuring the expression of rRNA genes. Their results show that the radioactive counts measured by Northern blot have a linear relationship with the fungal RNA amount. Maher *et al.* (2000) applied the RT-PCR assay to detect *Pneumocystis carinii* in AIDS patients using an RNA-specific primer set. Bleve *et al.* (2003) successfully applied real-time RT-PCR to monitor contaminations of yeasts and molds in yogurts and pasteurized food products by quantifying an actin mRNA target. Besides, RT-PCR has also been extensively applied to detect and quantify viral pathogens (Saade *et al.* 2000, Bertolini *et al.* 2003, Moravec *et al.* 2003).

RT-PCR is considered a better method to detect viable pathogen structures because mRNA molecules, as opposed to DNA molecules, are usually decomposed rapidly after the death of an organism. Maher *et al.* (2000) and Bleve *et al.* (2003) concluded that RT-PCR could distinguish between viable and non-viable fungi, but PCR assays could not. For

*M. graminicola*, Gough and Lee (1985) reported that high relative humidities clearly decreased the survival rate of the conidia in cirri after discharge. Survival in the field may also be affected by environmental factors like temperature and sunlight, therefore it is desirable to distinguish viable from nonviable pathogen cells in field samples.

#### 4.5.1 Suitability of three primer sets for RT-PCR assay

Although the three primer sets E1/STSP2R, ST-rRNA F/R and ST-act F/R were successfully used for PCR assays, their suitability for RT-PCR assay should be further tested since RT-PCR which uses RNA as target has some characters different from PCR. Therefore, as described before, the primer sets ST-rRNA F/R and ST-act F/R were developed from a transcribed rDNA sequence and an exon sequence of the *act1* gene, respectively, and E1/STSP2R is developed from an exon sequence of  $\beta$ -tubulin genes by Fraaije *et al.* (1999). Hence, the three primer sets can possibly be used for RT-PCR assay.

The ideal RNA targets for RT-PCR molecular diagnosis should be basically stable in different structures and ages of *M. graminicola*. Although the three genes are regarded as house-keeping genes in tissues, there are no direct reports about their expression level in *M. graminicola* cells of different structures. For this purpose, their RNA contents in seven *M. graminicola* cultures with different culture time ranging from three to 15 days were tested by Northern hybridization and RT-PCR. The results indicated that the contents of the three target gene sequences were basically stable in *M. graminicola* cells of different cultivation time (Fig. 35). Therefore, they may be used as target sequences for the detection and quantification of *M. graminicola* biomass in wheat by RT-PCR assay.

Due to their different characters, the detection specificity and sensitivity of RT-PCR assay may differ from that of PCR assay. The results indicated that ST-rRNA F/R and ST-act F/R were specific by RT-PCR like by PCR (Fig. 37). E1/STSP2R amplified one slight band from *M. nivale* and *P. herpotrichoides* by PCR, but it did not amplify by RT-PCR (Figs. 12, 36). This indicates that it had a higher specificity in RT-PCR than in PCR. There may be two possible reasons. First, the RT-PCR reaction conditions used enhance the detection specificity of RT-PCR with this primer set. Second, RT-PCR is composed of cDNA synthesis and PCR steps with two times primer annealing and extension during the reaction. During the reverse transcription, RNA is used as template, which is more critical for DNA primer annealing than



DNA template since RNA-DNA binding is not so stable as DNA-DNA binding (Sambrook *et al.* 1989).

The sensitivity is an important element of a detection method for practical diagnosis. Our results show that RT-PCR using three primer sets gave rise to different detection limit. Among them, RT-PCR using ST-rRNA F/R represented the lowest detection limit (100 pg total RNA, Fig. 39). It is likely that the three genes have different copy numbers in the genome and therefore the abundance of their mRNA is also different in tissues. rRNA is the most abundant RNA which covers more than 70% of total RNA (Qi and Yang 2002), and may be an ideal RNA target for RT-PCR assay. Meanwhile, two-step RT-PCR was much more sensitive than one-step RT-PCR (Fig. 38). It is likely that the reaction conditions in two-step RT-PCR were more suitable for the PCR amplification. Nevertheless, two-step RT-PCR was also more laborious and expensive.

#### **4.5.2 Detection of *M. graminicola* in infected wheat plants**

In this study, an RT-PCR assay was successfully applied to monitor the disease development in inoculated plants and the disease progression in the field using the primer set E1/STS2R. Its specificity and sensitivity was high enough to detect the pathogen presymptomatically in inoculated plants as well as in field samples. Although no symptoms were observed four days after inoculation (Table 12), an obvious product was amplified (Fig. 40) at this early stage of disease development, demonstrating that RT-PCR could detect *M. graminicola* at least four days before the first lesions were visible. *M. graminicola* has a long latent period in infected winter wheat. Since fungicides applied during the latent period are particularly effective, presymptomatic detection offers a valuable improvement in the management of *M. graminicola* leaf blotch.

Similar to the results obtained by real-time PCR (Table 12, Fig. A4), a slight band was also present in the 0-day sample (Fig. 40), indicating that some residual conidia may still have been attached to the leaf surface after washing. Several mechanisms may have been responsible for strong spore adhesion. First, the presence of Tween-80 in the spore suspension used for artificial infection not only improved surface wetting of the wheat leaves, but may also have improved adhesion of the spores to the leaves so that spore removal by subsequent washing may have been incomplete. Second, several fungal spores contain surface-acting

proteins (hydrophobins and integrins, Tucker and Talbot 2001) to ensure firm adhesion to host surfaces.

After symptoms were visible on the leaves, the increase of band intensity of the amplified RT-PCR products in the gel was in general agreement with the conventional visual disease assessments (Table 13, Fig. 40) and hence may have reflected to some extent the increase of *M. graminicola* biomass in infected leaves since close relationships between fungal biomass and fungal RNA amount were reported previously in other fungi (Benito *et al.* 1998).

The recent development of real-time RT-PCR has provided a technical possibility to quantify *M. graminicola* biomass in wheat plants indirectly. It has also used to quantify viral infections (Dhar *et al.* 2002; Salmon *et al.*, 2002) and biomass of certain yeasts and fungi (Bleve *et al.*, 2003). However, because of its high costs and technical requirements, real-time RT-PCR has not been used for the quantification of fungal biomass on a routine basis by now. Further study is needed for the quantification of *M. graminicola* biomass by real-time RT-PCR.

This work provides another effective and sensitive method to detect and identify fungal pathogens in plants. Like PCR, RT-PCR has a high specificity and sensitivity and can detect pathogens rapidly and pre-symptomatically in infected plants. The results can more accurately represent the situation of living pathogen populations than those obtained by PCR. The reduce of prices will probably lead to a broader application of RT-PCR for the detection of fungal pathogens.

#### **4.6 Outlook**

In this study, three PCR-based assays (conventional PCR, real-time PCR and RT-PCR) were successfully applied to detect and identify *M. graminicola* in infected plants using specific primer sets. Real-time PCR was successfully used to monitor the disease development in inoculated wheat plants and in naturally infected leaves during the epidemic period, and to assess fungicide efficacies. These assays provide a valuable alternative to presymptomatically and quantitatively detect *M. graminicola* in wheat. Compared with conventional visual assessment methods, PCR-based assays, especially real-time PCR, have incomparable advantages for a precise and specific detection and quantification of target pathogens during the early stage of disease. Real-time PCR has a high potential to address central questions in

plant pathology with a precision that was impossible a few years ago. Its sensitivity, speed, versatility and its quantitative nature will facilitate its wide applications in plant pathology. Although PCR-based methods are no yet used as routine approaches for the diagnosis of plant diseases and disease assessment in the field due to their relatively high costs and the laborious process, it is believed that with their technical improvement and cost reduction, these assays will possibly become a standard method suitable for large-scale diagnosis of pathogenic fungi in extension services.

## 5 Summary

*Mycosphaerella graminicola* leaf blotch is an economically important fungal disease in wheat worldwide. Its accurate and early diagnosis is a prerequisite for the precise prognosis of disease epidemics and optimisation of fungicide input within an integrated pest management system. The objective of this research was to develop a rapid and efficient PCR-based method to detect and quantify *M. graminicola* epidemics in wheat. In this study, two molecular diagnostic methods, polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR), were successfully applied to qualitatively and quantitatively detect *M. graminicola* in wheat.

Two different methods were tested to simplify the process of DNA extraction. A modified procedure using DNAzol reagent was employed to extract genomic DNA from 25 fungal pathogens. High yield and purity was obtained from *M. graminicola* isolates using this method without homogenization and chloroform elution steps. The DNA could be directly used for PCR, RAPD and AFLP analysis. Furthermore, a simple method of DNA extraction suitable for minipreps and large-amounts of tissues from fungi and infected plants was developed, in which CTAB, insoluble PVP, SDS and chloroform elution were combined into one step. These DNA samples were directly used for conventional PCR and real-time PCR. For RNA extraction, a simple procedure which combined guanidinium isothiocyanate, CTAB and insoluble PVP in one step was improved. This procedure was successfully used for total RNA extraction from *M. graminicola*, nine other fungal pathogens as well as from infected wheat leaves. The total RNA was sufficiently pure for Northern hybridization, RT-PCR and cDNA synthesis.

Identification of *M. graminicola*-specific sequences was performed using three different approaches. First, four specific subsequences were identified from two highly conserved rDNA and act1 gene sequences by BLAST search in a public gene bank. The primer sets ST-rRNA F/R and ST-act F/R were developed. Second, target specific sequences were further isolated by RAPD-PCR. Nine specific fragments were isolated and cloned from RAPD-PCR products. Twenty-four primer sets were designed from four sequenced fragments R3005, R3006, R3009 and R5870-2, respectively. After a primary specificity test, the primer sets R5870-1F/R5870-1R and R5870-1F/R5870-2R were selected for further test. Third, an efficient cDNA-AFLP procedure using silver staining was established to isolate differentially expressed transcripts of *M. graminicola*. The efficiency of cDNA-AFLP using total RNA as

starting material was compared with that using mRNA. The variation of transcript derived fragments was displayed between the cDNA-AFLP fingerprints of *M. graminicola* isolates and other fungal species.

Four primer sets were highly specific since they amplified one single fragment only from 14 *M. graminicola* isolates and infection plants, but not from 22 other fungal pathogens and healthy plants. The only exception was that R5870-1F/R5870-1R amplified one very faint band from *Pseudocercospora anguioides*. These primer sets were more specific than the two reported primer sets E1/STSP2R and STIF2/BAF4ST. The effects of the Mg<sup>2+</sup> concentration and annealing temperature on them differed. Eight different PCR programmes were compared using ST-rRNA F/R and ST-act F/R. The primer sets ST-rRNA F/R, ST-act F/R and R5870-1F/R5870-1R could detect as little as 0.5 pg *M. graminicola* genomic DNA, and R5870-1F/R5870-2R could detect up to 50 pg in a conventional PCR. The detection sensitivity could be somewhat improved using SYBR Green I staining.

The disease development of *M. graminicola* in inoculated wheat plants was monitored by real-time PCR using the primer sets ST-rRNA F/R and ST-act F/R. *M. graminicola* DNA was detected on the inoculation day, a clear growth tendency of fungal biomass was evaluated before visible symptoms appeared. According to the real-time PCR data, the rapid progression period was between six to 16 days after inoculation. After 16 days, the amount of *M. graminicola* was essentially constant. The correlation between the fungal DNA amounts obtained using both primer sets was highly significant ( $r = 0.9932^{***}$ ). The fungal DNA amount obtained from real-time PCR was significantly correlated with the disease index ( $r = 0.9115^{**}$ ), however their relationship was non-linear.

Real-time PCR was applied to monitor the disease progression in F, F1 and F2 leaves in the field from May 3 to June 21, 2004. The detection with the primer sets ST-rRNA F/R and ST-act F/R was compared with two visual assessment methods. *M. graminicola* amounts could be detected in all 22 leaf samples including five symptomless samples by real-time PCR with ST-rRNA F/R. Before May 24, the DNA amount increased very slowly in F1 and F2 leaves, but after that increased rapidly until June 14 in F2 leaves and until June 21 in F1 leaves. In F leaves, the DNA amount increased slowly after June 1. The amount of *M. graminicola* DNA measured by real-time PCR using ST-rRNA F/R was significantly correlated with the disease index ( $r = 0.8937^{**}$ ) and the number of pycnidia ( $r = 0.9106^{***}$ ).

Real-time PCR using ST-act F/R had a much higher detection limit than that using ST-rRNA F/R, and failed to detect fungal DNA from 11 leaf samples. The amount of *M. graminicola* DNA amount in F1 leaves was also followed by real-time PCR with R5870-1F/5870-2R. Although the DNA amounts in F1 leaves measured with three primer sets were different, the shape of their their disease progression curves during the epidemic period was similar.

The efficacy of two fungicide treatments (Input and Fandango) on *M. graminicola* leaf blotch in different leaf positions was assessed for two times using real-time PCR. The results were compared with two visual methods. Real-time PCR could detect *M. graminicola* DNA in all samples tested including some leaves without symptoms and dried leaves. the pycnidia-counting method gave no results for dried leaves or leaves without pycnidia, and the method of disease classification could not accurately assess the efficacy in the early stage of disease development. According to the results by real-time PCR, both treatments revealed remarkable efficacy against *M. graminicola* in most leaves. The Input treatment had a relatively better efficacy in F1 and F2 leaves on June 14 than the Fandango treatment.

RT-PCR assay was used to detect and identify *M. graminicola* in wheat with the three specific primer set E1/STSP2R, ST-rRNA F/R and ST-act F/R. The content of three targets  $\beta$ -tubulin, actin RNA and rRNA showed no obvious difference between seven different culture times according to the results by Northern hybridization and RT-PCR. It indicates that three RNA targets may be used for RT-PCR assay to detect *M. graminicola*. Three primer sets were specific as they amplified one single fragment from total RNA of *M. graminicola* and infected leaves, but not from those of healthy leaves or other fungal pathogens tested. RT-PCR using ST-rRNA F/R could detect as little as 100 pg total RNA and had a lower detection limit than that with E1/STSP2R (5ng) and with ST-act F/R (2 ng). Using E1/STSP2R, two-step RT-PCR could detect up to 100 pg total RNA and was approximately 50 times more sensitive than one-step RT-PCR (5 ng total RNA).

The disease development in inoculated wheat plants and in naturally infected F1 leaves during the epidemic period was monitored using RT-PCR with E1/STSP2R. This assay could detect *M. graminicola* at least four days before lesions appeared in inoculated plants. After symptoms were visible on the leaves, the band intensities of the amplified RT-PCR products were in general agreement with conventional visual disease assessments.

## 6 Zusammenfassung

Der pilzliche Erreger der Getreideblatfleckenkrankheit *Mycosphaerella graminicola* ist ein Pathogen mit weltweit großer ökonomischer Bedeutung. Eine präzise und frühzeitige Diagnose für diesen Erreger ist die Voraussetzung für eine optimale Fungizidanwendung im Rahmen des Integrierten Pflanzenschutzes. Es war das Ziel dieser Untersuchung, eine schnelle und effiziente PCR-basierte Methode zur Detektion und Quantifizierung von *M. graminicola* in Weizen zu entwickeln. In der vorliegenden Arbeit wurden zwei molekulare Diagnosemethoden, die Polymerase-Kettenreaktion und Reverse Transkription (RT-PCR) zur qualitativen und quantitativen Feststellung eines Befalls mit *M. graminicola* im Weizen erfolgreich eingesetzt.

Verschiedene Methoden wurden getestet um den Prozess der DNA-Isolierung zu vereinfachen. Es wurde eine modifizierte Methode durch die Nutzung des DNazol Reagenz angewandt zur Isolation genomischer DNA für 25 pilzliche Erreger. Für *M. graminicola* wurden hohe Ausbeuten reiner DNA ohne eine Homogenisierung und Chloroform Fällungsschritte erzielt. Die DNA konnte direkt für PCR, RAPD und AFLP verwendet werden. Gleichzeitig wurde eine einfache Methode zur DNA-Extraktion für Minipreps oder eine große Menge von Pilzen und infiziertem Pflanzengewebe entwickelt, in der CTAB, unlösliches PVP und SDS sowie Chloroform-Fällung in einem Schritt zusammengefasst wurde. Die gewonnene DNA wurde direkt in PCR und Real- Time-PCR weiterverwendet. Für die RNA-Extraktion wurde eine einfache Methode, die Guanidinium-Isothiocyanat, CTAB und unlösliches PVP in einem Schritt kombiniert, entwickelt. Die Extraktionsmethode wurde erfolgreich zur Extraktion der gesamten RNA von *M. graminicola* sowie von neun weiteren pilzlichen Pathogenen und infizierten Weizenblättern eingesetzt. Die Gesamt-RNA war hinreichend rein, um damit Northern-Hybridisierung, RT-PCR und cDNA-Synthese durchzuführen.

Mittels dreier verschiedener Ansätze wurde versucht, *M. graminicola*-spezifische Sequenzen zu identifizieren. Über einer BLAST-Analyse in öffentlich zugänglichen Genbanken wurden vier spezifische Sequenzen in zwei hoch konservierten rDNA- und Act1-Gensequenzen identifiziert. Es wurden dementsprechend die Primerpaare ST-rRNA F/R und ST-act F/R entwickelt. Des Weiteren wurden spezifische Sequenzen über RAPD-PCR ermittelt. Neun spezifische Fragmente wurden aus RAPD-PCR-Produkten isoliert und geklont. Vierundzwanzig Primerpaare wurden mittels der vier Fragmentsequenzen R3005, R3006,

R3009 und R5870-2 erstellt. Nach einem ersten Spezifitätstest wurden zwei Primerpaare, R5870-1F/R5870-1R und R5870-1F/R5870-2R für weitere Tests ausgewählt. Eine effiziente cDNA-AFLP-Methode unter Einsatz einer Silberfärbung wurde zur Isolation unterschiedlich exprimierter Transkripte von *M. graminicola* eingeführt. Die Effizienz von cDNA-AFLP unter Verwendung der gesamten RNA als Ausgangsmaterial wurde mit der Verwendung von mRNA verglichen. Die Variation von Transkripten abstammender Fragmente zeigte sich zwischen den cDNA-AFLP-Fingerabdrücken von *M. graminicola*-Isolaten und anderen pilzlichen Erregern,

Die vier Primerpaare waren hoch spezifisch, da sie lediglich für 14 verschiedene *M. graminicola*-Isolate und für infizierte Pflanzen ein PCR-Produkt zeigen, nicht jedoch für 22 andere getestete Pathogene und gesunde Pflanzen. Die einzige Ausnahme war ein schwaches Band für den Primer R5870-1F/R5870-1R bei dem Pathogen *Pseudocercospora anguioides*. Die in dieser Arbeit entwickelten Primer zeigten eine deutlich höhere Spezifität als die in der Literatur zu findenden Primer E1/STSP2R and STIF2/BAF4ST. Von den vier Primer-Paaren waren für die Primer ST-rRNA F/R, ST-act F/R und R5870-1F/R5870-1R geringe Mengen genomischer *M. graminicola*-DNA von bis zu 0,5 pg ausreichend. Für R5870-1F/R5870-2R waren bis zu 50 pg DNA in einer konventionellen PCR notwendig. Die Sensitivität konnte durch den Einsatz von SYBR-Green-Anfärbung deutlich erhöht werden.

Die Infektionsentwicklung von *M. graminicola* in künstlich infizierten Weizenpflanzen wurde durch Real-Time-PCR überprüft unter Verwendung der Primer ST-rRNA F/R und ST-act F/R. *M. graminicola*-DNA wurde bereits am Tag der Inokulation festgestellt. Es konnte zudem eine starke Zunahme pilzlicher DNA festgestellt werden, noch bevor visuelle Symptome achte Tage nach der Inokulation sichtbar wurden. Eine starke Zunahme war insbesondere zwischen sechs und 16 Tagen nach der Inokulation zu verzeichnen. Nach 16 Tagen war die *M. graminicola*-DNA im wesentlichen konstant. Die Korrelation zwischen der Menge pilzlicher DNA unter Verwendung der beiden Primerpaare war hoch signifikant ( $r = 0,9932^{***}$ ). Es bestand eine signifikante Korrelation zwischen den Ergebnissen der Real-Time-PCR und denen der visuellen Bonitur ( $r = 0,9115^{***}$ ).

Real-Time-PCR wurde eingesetzt um die Krankheitsentwicklung in den Blättern F, F1 und F2 im Feld vom 3 Mai bis zum 21 Juni 2004 zu erfassen. Die Ergebnisse der verwendeten Primer ST-rRNA F/R und ST-act F/R wurden mit zwei visuellen Diagnosemethoden verglichen. In



allen 22 Blattproben konnte *M. graminicola* über Real-Time-PCR mit den Primern ST-rRNA F/R und ST-act F/R nachgewiesen werden mit Ausnahme von fünf symptomlosen Proben. Bis zum 24. Mai kam es zu einer langsamen Zunahme der gemessenen DNA Menge. Danach nahm die DNA-Menge von *M. graminicola* schnell zu, in den F2-Blättern war dies bis zum 14. Juni und in den F1-Blättern bis zum 21. Juni zu beobachten. In den Fahnblättern hat die DNA-Menge ab 1. Juni zugenommen. Die mit dem Primerpaar ST-rRNA F/R über Real-Time PCR gemessene pilzliche DNA korrelierte signifikant mit dem über die visuelle Bonitur ermittelten Krankheitsbefall ( $r = 0.8937^{**}$ ) sowie mit der Anzahl gezählter Pyknidien ( $r = 0.9106^{***}$ ). Die PCR mit dem Primerpaar ST-act F/R zeigte eine deutlich höhere Nachweisgrenze als das Primerpaar ST-rRNA F/R und konnte für 11 Blattproben keine DNA nachweisen. Die Entwicklung der DNA-Zunahme in F1 Blättern wurde auch über Real-Time-PCR verfolgt unter Verwendung der Primer R5870-1F/5870-2R. Obwohl die gemessene DNA-Menge in den F1-Blättern für die verschiedenen Primerpaare unterschiedlich war, war die Tendenz der Zunahme für alle Primerpaare ähnlich.

Der Einfluss von zwei Fungizidbehandlungen (Input und Fandango) auf die *M. graminicola*-Blattfleckenkrankheit auf den unterschiedlichen Blattetagen wurde über Real-Time-PCR bestimmt. Die Ergebnisse wurden mit zwei visuellen Diagnosemethoden verglichen. Über Real-Time-PCR konnte *M. graminicola*-DNA in allen getesteten Proben nachgewiesen werden einschließlich symptomloser und vollständig abgestorbener Blätter. Über das Auszählen der Pyknidien konnten keine Aussagen für abgestorbene oder Blätter ohne Pyknidien gemacht werden und die Methode der Befallsstärkenbestimmung war im frühen Stadium der Erkrankung ungenau. Nach der PCR-Analyse zeigten beide Fungizidbehandlungen einen deutlichen Bekämpfungserfolg gegen *M. graminicola* auf den meisten Blattetagen. Die Input-Behandlung war dabei wirkungsvoller in den F1- und F2-Blättern als die Fandango Behandlung.

RT-PCR wurde eingesetzt, um *M. graminicola* mittels der drei Primerpaare E1/STSP2R, ST-rRNA F/R und ST-act F/R nachzuweisen und zu bestimmen. Die drei RNA-Zielsequenzen,  $\beta$ -Tubulin, Actin-RNA und rRNA, zeigten keine Unterschiede zwischen sieben Kultivierungszeiten. Dies wurde über Northern-Hybridisierung und RT-PCR nachgewiesen. Demnach sind die drei Primer geeignet, *M. graminicola*-Biomasse über RT-PCR nachzuweisen. Sie sind zudem spezifische Primer da sie ein einziges Fragment der gesamten *M. graminicola*-RNA und infizierter Blätter amplifizieren, jedoch kein Fragment

bei gesundem Blattmaterial oder aus der DNA anderer Pathogenen. RT-PCR unter Verwendung der Primer ST-rRNA F/R konnte bis zu 100 pg Gesamt-RNA nachweisen und hatte eine deutlich niedrigere Nachweisgrenze als mit den Primern E1/STSP2R (5ng) und ST-act F/R (2 ng). Mit einer Two-Step-RT-PCR und E1/STSP2R konnte 100 pg Gesamt RNA nachwiesen werden; mit einer One-Step-RT-PCR (5 ng).

Die Entwicklung von *M. graminicola* in künstlich infizierten Weizenpflanzen und in den F1-Blättern vom Feld wurde mittels RT-PCR unter Verwendung der Primer E1/STSP2R untersucht. Die Methode war in der Lage, *M. graminicola* mindestens vier Tage vor dem Ausbruch visueller Krankheitssymptome in der Pflanze nachzuweisen. Nachdem die Krankheitssymptome auf den Blättern sichtbar waren, stimmten die Intensitäten der amplifizierten RT-PCR Produkte generell mit den Ergebnissen der konventionellen visuellen Boniturmethode überein.

## 7 References

- Abd-Elsalam, K. A., Aly, I. N., Abdel-Satar, M. A., Khalil, M. S., and Verreet, J.-A. 2003. PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. *African Journal of Biotechnology* 2:82-85.
- Abd-Elsalam, K. A. 2003. Non-gel based techniques for plant pathogen genotyping. *Polish Journal of Microbiology* 52:329-41.
- Adhikari, T. B., Anderson, J. M., and Goodwin, S. B. 2003. Identification and molecular mapping of a gene in wheat conferring resistance to *Mycosphaerella graminicola*. *Phytopathology* 93:1158-1164.
- Ahn, S. J., Costa, J., and Emanuel, J. R. 1996. PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. *Nucleic Acids Research* 24:2623-2625.
- Allmann, M., Hofelein, C., Koppel, E., Luthy, J., Meyer, R., Niederhauser, C., Wegmuller, B., and Candrian, U. 1995. Polymerase chain reaction (PCR) for detection of pathogenic microorganisms in bacteriological monitoring of dairy products. *Research in Microbiology* 146:85-97.
- Al-Samarrai, T. H., and Schmid, J. 2000. A simple method for extraction of fungal genomic DNA. *Letters in Applied Microbiology* 30:53-56.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410.
- Annamalai, P., Ishii, H., Lalithakumari, D., and Revathi, R. 1995. Polymerase chain reaction and its applications in fungal disease diagnosis. *Journal of Plant Diseases and Protection* 102:91-104.
- Arnheim, N., and Erlich, H. 1992. Polymerase chain reaction strategy. *Annual Review of Biochemistry* 61:131-156.
- Arsenijević, M. 1964. *Septoria tritici* Rob. et Desm. Parazit pšenice u SR Srbiji. *Zastita Bilja* 16:5-70.
- Aubry, C., Morere-Le Paven, M.C., Chateigner-Boutin, A. L., Teulat-Merah, B., Ricoult, C., Peltier, D., Jalouzot, R., and Limami, A. M. 2003. A gene encoding a germin-like protein, identified by a cDNA-AFLP approach, is specifically expressed during germination of *Phaseolus vulgaris*. *Planta* 217:466-475.
- Bachem, C. W. B., van der Hoeven, R. S., de Bruijn, S. M., Vreugdenhil, D., Zabeau, M., and Visser, R. G. F. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant Journal* 9:745-753.
- Bachem, C. W. B., Oomen, R. J. F. J., and Visser, R. G. F. 1998. Transcript imaging cDNA-AFLP: a step-by-step protocol. *Plant Molecular Biology Reporter* 16:157-173.

- Beck, J. J., and Ligon, J. M. 1995. Polymerase chain reaction assay for the detection of *Stagonospora nodorum* and *Septoria tritici* in wheat. *Phytopathology* 85:319-324.
- Bertolini, E., Olmos, A., López, M. M., and Cambra, M. 2003. Multiplex nested reverse transcription-polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in olive trees. *Phytopathology* 93:286-292.
- Benito, E. P., Ten Have, A., Van Klooster, J. W., and Van Kan, J. A. L. 1998. Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. *European Journal of Plant Pathology* 104:207-220.
- Bertolini, E., Olmos, A., López, M. M., and Cambra, M. 2003. Multiplex nested reverse transcription-polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in olive trees. *Phytopathology* 93:286-292.
- Birnboim, H. C. 1992. Extraction of high molecular weight RNA and DNA from cultured mammalian cells. *Methods: A Companion to Methods in Enzymology* 216:154-160.
- Bleve, G., Rizzotti, L., Dellaglio, F., and Torriani, S. 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. *Applied and Environmental Microbiology* 69:4116-4122.
- Brading, P. A., Verstappen, E. C. P., Kema, G. H. J., and Brown, J. K. M. 2002. A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the *Septoria tritici* blotch pathogen. *Phytopathology* 92:439-445.
- Breyne, P., Dreesen, R., Cannoot, B., Rombaut, D., Vandepoele, K., Rombauts, S., Vanderhaeghen, R., Inze, D., and Zabeau, M. 2003. Quantitative cDNA-AFLP analysis for genome-wide expression studies. *Molecular Genetics and Genomics* 269:173-9.
- Brokenshire, T. 1976. The reaction of wheat genotypes to *Septoria tritici*. *Annals of Applied Biology* 82:415-423.
- Brouwer, M., Lievens, B., van Hemelrijck, W., van den Ackerveken, G., Cammue, B. P., and Thomma, B. P. 2003. Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *FEMS Microbiology Letters* 228:241-248.
- Bruns, T. D., White, T. J., and Taylor, J. L. 1991. Fungal molecular systematics. *Annual Review of Ecological System* 22:525-564.
- Bryan, G. T., Daniels, M. J., and Osbourn, A. E. 1995. Comparison of fungi within the *Gäumannomyces-Phialophora* complex by analysis of ribosomal DNA sequences. *Applied and Environmental Microbiology* 61:681-689.
- Caldwell, R. M., and Narvaez I. 1960. Losses to winter wheat from infection by *Septoria tritici*. *Phytopathology* 50:630.

- Cao, Y., Zheng, Y., and Fang, B. 2004. Optimization of polymerase chain reaction-amplified conditions using the uniform design method. *Journal of Chemical Technology and Biotechnology*, 79:910-913(4).
- Cenis, J. L. 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research* 20:2380.
- Chang, S., Puryear, J., and Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* 11:113-116.
- Chattrain, L., Brading, P. A., Widdowson, J. P., and Brown, J. K. M. 2004. Partial resistance to *Septoria tritici* blotch (*Mycosphaerella graminicola*) in wheat cultivars Arina and Riband. *Phytopathology* 94:497-504.
- Chen, G. Y. J., Jin, S., and Goodwin, P. H. 1999. An improved method for the isolation of total RNA from *Malva pusilla* tissues infected with *Colletotrichum gloeosporioides*. *Journal of Phytopathology* 148:57-60.
- Chomezynski, P., Mackey, K., Drews, R., and Wilfinger, W. 1997. DNAzol: a reagent for the rapid isolation of genomic DNA. *Biotechniques* 22:550-553.
- Clark, M. F. 1981. Immunosorbent assays in plant pathology. *Annual Review of Phytopathology* 19:83-106.
- Claros, M. G. and Cánovas, F. M. 1998. Rapid high quality RNA preparation from pine seedlings. *Plant Molecular Biology Reporter* 16:9-18.
- Claustres, M., Kjellberg, P., Desgeorges, M., Bellet, H., Sarda, P., Bonnet, H., and Boileau, C. 1989. Detection of deletions by the amplification of exons (multiplex PCR) in Duchenne muscular dystrophy. *Journal of Human Genetics* 37:251-257.
- Creste, S., Tulmann-Neto, A., and Figueira, A. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Molecular Biology Reporter* 19:1-8.
- Cullen, D. W., Lees, A. K., Toth, I. K., and Duncan, J. M. 2001. Conventional PCR and real-time PCR quantitative PCR detection of *Helminthosporium solani* in soil and potato tubers. *European Journal of Plant Pathology* 107:387-398.
- Czember, P.C., and Arseniuk E. 1999. Study of genetic variability among monopycnidial monopycnidiospore isolates derived from single pycnidia of *Stagonospora* spp. and *Septoria tritici* with the use of RAPD-PCR, MP-PCR and Rep-PCR techniques. *Journal of Phytopathology* 147:539-546.
- Dieffenbach, C. W., Lowe, T. M. J., and Dveksler, G. S. 1995. General concepts for PCR primer design. In: *PCR Primers, A Laboratory Manual*, Dieffenbach CW, Dveksler GS Ed., Cold Spring Harbor Laboratory Press, New York, pp133-155.
- Dilger, M., Felsenstein, F. G., and Schwarz, G. 2003. Identification and quantitative expression analysis of genes that are differentially expressed during conidial germination in *Pyrenophora teres*. *Molecular Genetics and Genomics* 270:147-55.

- Diviacco, S., Norio, P., Zentilin, L., Menzo, S., Clementi, M., Biamonti, G., Riva, S., Falaschi, A., and Giacca, M. 1992. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. *Gene* 122:313-320.
- Doohan, F. M., Parry, D. W., Jenkinson, P., and Nicholson, P. 1998. The use of species-specific PCR-based assays to analyse *Fusarium* ear blight of wheat. *Plant Pathology* 47:197-205.
- Doohan, F. M., Parry, D. W., and Nicholson, P. 1999. *Fusarium* ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control. *Plant Pathology* 48:209-217.
- Dyer, P. S., Furneaux, P. A., Douhan, G, and Murray, T. D. 2001. A multiplex PCR test for determination of mating type applied to the plant pathogens *Tapesia yallundae* and *Tapesia acuformis*. *Fungal Genetics and Biology* 33:173-180.
- Edwards, S. G., Pirgozliev, S. R., Hare, M. C., Jenkinson, P. 2001. Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against *Fusarium* head blight of winter wheat. *Applied and Environmental Microbiology* 67:1575-1580.
- Ekblad, A., Wallander, H., and Näsholm, T. 1998. Chitin and ergosterol combined to measure total and living fungal biomass in ectomycorrhizas. *New Phytology* 138:143-149.
- Erlich, H. A., Gelfand, D., and Sninsky, J. J. 1991. Recent advances in the polymerase chain reaction. *Science* 252:1643-1651.
- Eyal, Z., Scharen, A. L., Prescott, J.M., and Van Ginkel, M. 1987. The *Septoria* diseases of wheat. Concepts and methods of disease management. CIMMYT, Mexico City, DF, Mexico.
- Eyal, Z. 1999. The *Septoria tritici* and *Stagonospora nodorum* blotch diseases of wheat. *European Journal of Plant Pathology* 105:629-641.
- Flowers, J., Hartman, J., and Vaillancourt, L. 2003. Detection of latent *Sphaeropsis sapinea* infection in Austrian pine tissues using nested-polymerase chain reaction. *Phytopathology* 93:1471-1477.
- Foster, S. J., Ashyby, A. M., and Fitt, B. D. L. 2002. Improved PCR-based assays for pre-symptomatic diagnosis of light leaf spot and determination of mating type of *Pyrenopeziza brassicae* on winter oilseed rape. *European Journal of Plant Pathology* 108:379-383.
- Fouly, H. M., and Wilkinson, H. T. 2000. Detection of *Gaeumannomyces graminis* varieties using polymerase chain reaction with variety-specific primers. *Plant Disease* 84:947-951.
- Fraaije, B. A., Lovell, D. J., Rohel, E. A., and Hollomon, D. W. 1999. Rapid detection and diagnosis of *Septoria tritici* epidemics in wheat using a polymerase chain reaction/PicoGreen assay. *Journal of Applied Microbiology* 86:701-708.
- Fraaije, B. A, Lovell, D. J., Coelho, J. M., Baldwin, S., and Hollomon, D. W. 2001. PCR-based assays to assess wheat varietal resistance to blotch (*Septoria tritici* and *Stagonospora*

*nodorum*) and rust (*Puccinia striiformis* and *Puccinia recondita*) diseases. *European Journal Plant Pathology* 107:905–917.

Frederick, R. D., Snyder, K. E., Tooley, P. W., Berthier-Schaad, Y., Peterson, G. L., Bonde, M. R., Schaad, N. W., and Knorr, D. A. 2000. Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. *Phytopathology* 90:951-960.

Gachon, C., and Saindrenan, P. 2004. Real-time PCR monitoring of fungal development in *Arabidopsis thaliana* infected by *Alternaria brassicicola* and *Botrytis cinerea*. *Plant Physiology and Biochemistry* 42:367-371.

Gair, R., Jenkins, J. E. E., and Lester, E. 1987. *Cereal pests and diseases*. Fourth Edition. Farming Press LTD, UK.

Ginzinger, D. G. 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Experimental Hematology* 30:503-512.

Godwin, J. R., Bartlett, D. W., and Heaney, S. P. 1999. Azoxystrobin: Implications of biological mode of action, pharmacokinetics and resistance management for spray programmes against *Septoria* diseases of wheat. In: *Septoria on cereals: a study of pathosystems*. Lucas, J. A., Bowyer, P., and Anderson, H. M. eds. CABI Publishing, Wallingford, Oxon; UK. pp. 299-315.

Goodwin, P. H., Hsiang, T., Xue, B. G., and Liu, H. W. 1995. Differentiation of *Gaeumannomyces graminis* from other turf-grass fungi by amplification with primers from ribosomal internal transcribed spacers. *Plant Pathology* 44:384-391.

Gorelenkov, V., Antipov, A., Lejnine, S., Darasella, N., and Yuryev, A. (2001). Set of novel tools for PCR primer design. *Biotechniques* 31:1326-1330.

Gough, F. J., and Lee, T. S. 1985. Moisture effects on the discharge and survival of conidia of *Septoria tritici*. *Phytopathology* 75:180-182.

Gunson, R., Gillespie, G., and Carman, W. F. 2003. Optimisation of PCR reactions using primer chessboarding. *Journal of Clinical Virology* 26:369-373.

Guo, J. R., Schnieder, F., and Verreet, J.-A. 2005. Rapid and efficient extraction of genomic DNA from different phytopathogenic fungi using DNAzol reagent. *Biotechnology Letters*. In press.

Guo, J. R., Abd-Elsalam, K. A., Schnieder, F., and Verreet, J.-A. 2003. A rapid and effective method for total RNA extraction from conidia and mycelium of *Septoria tritici*. *Journal of Rapid Methods and Automation in Microbiology* 11:53-59.

Hamer, L., Adachi, K., Montenegro-Chamorro, M. V., Tanzer, M. M., Mahanty, S. K., Lo, C., Tarpey, R. W., Skalchunes, A. R., Heiniger, R. W., Frank, S. A., Darveaux, B. A., Lampe, D. J., Slater, T. M., Ramamurthy, L., DeZwaan, T. M., Nelson, G. H., Shuster, J. R., Woessner, J., and Hamer, J. E. 2001. Gene discovery and gene function assignment in filamentous fungi. *Proceedings of the National Academy of Sciences of the United States of America* 98(9):5110-5115.

- Hartung, J. S., Daniel, J. F., and Pruvost, O. P. 1993. Detection of *Xanthomonas campestris* pv. *citri* using the polymerase chain reaction. *Applied and Environmental Microbiology* 59:1143-1148.
- He, Q., Marjamaki, M., Soini, H., Mertsola, J., and Viljanen, M. K. 1994. Primers are decisive for sensitivity of PCR. *BioTechniques*. 17:82-87.
- Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. 1996. Real time quantitative PCR. *Genome Research* 6:986-994.
- Henson, J. M., and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology* 31:81-109.
- Henson, J. M., Goins, T., Grey, W., Mathre, D. E., and Elliott, M. L. 1993. Use of polymerase chain reaction to detect *Gäumannomyces graminis* DNA in plants grown in artificially- and naturally-infested soil. *Phytopathology* 83:283-287.
- Hess, D. E., and Shaner, G., 1987. Effect of moisture and temperature on development of *Septoria tritici* blotch in wheat. *Phytopathology* 77:215-219.
- Hibbett, D. S. 1992. Ribosomal RNA and fungal systematics. –*Transactions of Japan Mycological Society* 33:533-556.
- Hietala, A. M., Eikenes, M., Kvaalen, H., Solheim, H., and Fossdal, C. G. 2003. Multiplex real-time PCR for monitoring *Heterobasidion annosum* colonization in Norway spruce clones that differ in disease resistance. *Applied and Environmental Microbiology* 69:4413-20.
- Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. 1992. Simultaneous amplification and detection of specific DNA sequences. *Biotechnology* 10:413-417.
- Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology* 11:1026-1030.
- Hilu, H. M., and Bever, W. M. 1957. Inoculation, overwintering and susceptible and pathogen relationship of *Septoria tritici* on *Triticum* diseases. *Phytopathology* 47:474-480.
- Hu, X., Nazar, R. N. and Robb, J. 1993. Quantification of *Verticillium* biomass in wilt disease development. *Physiological and Molecular Plant Pathology*, 42:23-36.
- Hunter, T., Coker, R. R., and Royle, D. J. 1999. The teleomorph stage, *Mycosphaerella graminicola*, in epidemics of *Septoria tritici* blotch on winter wheat in the UK. *Plant Pathology* 48:51-57.
- Ingle, C. A., and Kushner, S. R. 1996. Development of an *in vitro* mRNA decay system for *Escherichia coli*: poly(A) polymerase I is necessary to trigger degradation. *Proceedings of the National Academy of Sciences of the United States of America* 93:12926–12931.
- Innis, M. A. and Gelfand, D. H. 1990. Optimization of PCRs. In: *PCR Protocols*. Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds. Academic Press, New York. pp. 3-12.



- Ivanova, N. B., and Belyavsky, A. V. 1995. Identification of differentially expressed genes by restriction endonuclease-based gene expression fingerprint. *Nucleic Acids Research* 23:2954-2958.
- Jlibene, M., Gustafson, J. P., and Rajaram, S. 1992. A field disease evaluation method for selecting wheat resistant to *Mycosphaerella graminicola*. *Plant Breeding* 108:26-32.
- Jordan, V. W. L. , and Hutchoen, J. A. 1999. Disease management in less-intensive, integrated systems. In: *Septoria on cereals: A study of pathosystems*. Lucas, J. A., Bowyer, P., and Anderson, H. M. eds. CABI Publishing, Wallingford, Oxon, UK. pp. 263-272.
- Josephson, K. L., Gerba, C. P., and Pepper, I. L. 1993. Polymerase chain reaction of nonviable bacterial pathogens. *Applied and Environmental Microbiology* 59:3513-3515.
- Kajjalainen, S., Karhunen, P. J., Lalu, K., and Lindstrom, K. 1993. An alternative hot start technique for PCR in small volumes using beads of wax-embedded reaction components dried in trehalose. *Nucleic Acids Research* 21:2959–2960.
- Kema, G. H. J., Annone, J. G., Sayoud, R., and van Silfhout, C. H. 1996a. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. I. Interactions between pathogen isolates and host cultivars. *Phytopathology* 86:200-212.
- Kema, G. H. J., Dazhao, Y., Rijkenberg, F. H. J., Shaw, M. W., and Baayen, R. P. 1996b. Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. *Phytopathology* 86:777-786.
- Kema, G. H. J., Verstappen, E. C. P., and Waalwijk, C. 2000. Avirulence in the wheat *Septoria tritici* leaf blotch fungus *Mycosphaerella graminicola* is controlled by a single locus. *Molecular Plant-Microbe Interactions* 13:1375-1379.
- Kema, G. H. J., Goodwin, S. B., Hamza, S., Verstappena, E. C. P., Cavalettob, J. R., Van der Lee, T. A. J., de Weerdta, M., Bonantsa, P. J. M., and Waalwijk, C. 2002. A combined amplified fragment length polymorphism and randomly amplified polymorphism DNA genetic linkage map of *Mycosphaerella graminicola*, the *Septoria tritici* leaf blotch pathogen of wheat. *Genetics* 161:1497-1505.
- Kendall, S. J., Hollomon, D. W., and Selley, A. 1998. Immunodiagnosis as an aid to the timing of fungicide sprays for the control of *Mycosphaerella graminicola* on winter wheat in the UK. In: *Proceedings of the Brighton Crop Protection Conference – Pests and Diseases*. Vol. 2. pp. 701-706. Farnham, UK.
- Kermekchiev, M. B., Tzekov, A., and Barnes, W. M. 2003. Cold-sensitive mutants of *Taq* DNA polymerase provide a hot start for PCR. *Nucleic Acids Research* 31:6139-6147.
- Kim, S., and Mariani, K. J. 1995. DNA and RNA-DNA annealing activity associated with the tau subunit of the *Escherichia coli* DNA polymerase III holozyyme. *Nucleic Acids Research* 23:1374-1379.
- Knight, S. C., Athoney, V. M., Brady, A. M., Greenland, A. J., Heaney, S. P., Murray, D. C., Powell, K. A., Schulz, M. A., Spinks, C. A., Worthington, P. A., and Youle, D. 1997.

- Rationale and perspectives on the development of fungicides. *Annual Reviews of Phytopathology* 35:349-372.
- Kong, D., Shen, H., Huang, Y., and Mi, H. 2004. PCR hot-start using duplex primers. *Biotechnology Letter* 26:277-280.
- Kupfer, D.M., Reece, C. A., Clifton, S. W., Roe, B. A., and Prade, R. A. 1997. Multicellular ascomycetous fungal genomes contain more than 8000 genes. *Fungal Genetics and Biology* 21:364-372.
- Lee, M.S., LeMaistre, A., Kantarjian, H. M., Talpaz, M., Freireich, E. J., Trujillo, J. M., and Stass, S. A. 1989. Detection of two alternative bcr/abl mRNA junctions and minimal residual disease in *Philadelphia* chromosome positive chronic myelogenous leukemia by polymerase chain reaction. *Blood* 73:2165-2170.
- Lee, L. G., Connell, C. R., and Bloch, W. 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Research* 21:3761-3766.
- Lees, A. k., Cullen, D. W., Sullivan, L., and Nicolson, M. J. 2002. Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato in soil. *Plant Pathology* 51:293-302.
- Leonard, K. J, and Fry, W. E. 1986. *Plant disease epidemiology: population dynamics and management volume 1*. Macmillan Publishing Company, New York, USA, pp 153-179.
- Liew, E. C. Y., Maclean, D. J., and Irwin, J. A. G. 1998. PCR based detection of *Phytophthora medicaginis* using the intergenic spacer region of the ribosomal DNA. *Mycological Research* 102:73-80.
- Linde, C., Zhan, J., and McDonald, B. A.. 2002. Population structure of *Mycosphaerella graminicola*: from lesions to continents. *Phytopathology* 92:946-955.
- Livak, K. J., Flood, S. J. A., Marmaro, J. Giusti, W., and Deetz, K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic hybridisation. *PCR Methods and Applications* 4:357-362.
- Lovell, D. J., Hunter, T., Powers, S. J., Parker, S. R., and van den Bosch, F. 2004. Effect of temperature on latent period of *Septoria* leaf blotch on winter wheat under outdoor conditions. *Plant Pathology* 53:170-181.
- Luck, J. E., and Gillings, M. R. 1995. Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the polymerase chain reaction. *Mycological Research* 99:1482-1488.
- Maher, N., Vermund, S., Lasbury, M., and Lee, C. H. 2000. Development and evaluation of a molecular viability assay for *Pneumocystis carinii*. *Journal of Clinical Microbiology* 38:1947-1952.
- Mahuku, G. S., and Platt, H. W. 2002. Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. *American Journal of Potato Research* 79:107-117.

- Martin, R. R., James, D. J., and Lévesque C. A. 2000. Impacts of molecular diagnostics technologies on plant disease management. *Annual Review of Phytopathology* 38:207-239.
- McCartney, H. A., Foster, S. J., Fraaije, B. A., and Ward, E. 2003. Molecular diagnostics for fungal plant pathogens. *Pest Management Science* 59:129-142.
- McDonald, B. A., and Martinez, J. P. 1991. Chromosome length polymorphisms in a *Septoria tritici* population. *Current Genetics* 19:265-271.
- McDonald, B. A., Zhan, J., Yarden, O., Hogan, K., Garton, J., and Pettway, R. E. 1999. The population genetics of *Mycosphaerella graminicola* and *Phaeosphaeria nodorum*. In: *Septoria on Cereals: A Study of Pathosystems*. Lucas, J. A., Bowyer, P., Anderson, H. M. eds. CABI International, Wallingford, UK, pp. 44-69.
- McMaugh, S. J., and Lyon, B. R. 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. *Biotechniques* 34:982-986.
- McPherson, M. J., and Møller, S. G. 2000. PCR. BIOS Scientific Publisher Limited, Oxford, UK.
- Micheli, M. R., Bova, R., Pascale, E., and D'Ambrosio, E. 1994. Reproducible DNA fingerprint with the random amplified polymorphic DNA (RAPD) method. *Nucleic Acids Research* 22:1921-1922.
- Miller, S. A., and Martin, R. R. 1988. Molecular diagnosis of plant disease. *Annual Review of Phytopathology* 26:409-432.
- Mishra, P. K., Fox, R. T. V., and Culham, A. 2003. Development of a PCR-based assay for rapid and reliable identification of pathogenic Fusaria. *FEMS Microbiology Letters* 218:329-332.
- Möller, E. M., Bahnweg, G., Sandermann, H., and Geiger, H. H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamental fungi, fruit bodies and infected tissues. *Nucleic Acids Research* 20:6115-6116.
- Moravec, T., Cerovska, N., and Boonham, N. 2003. The detection of recombinant, tuber necrotizing isolates of potato virus Y (PVYNTN) using a three-primer PCR based in the coat protein gene. *Journal of Virological Methods* 109:63-68.
- Morrison, T. B., Weis, J. J., and Wittwer, C. T. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* 24:954-958, 960, 962.
- Nascimento, A. M., Goldman, G. H., Park, S., Marras, S. A., Delmas, G., Oza, U., Lolans, K., Dudley, M. N., Mann, P. A., and Perlin, D. S. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrobial Agents and Chemotherapy* 47(5):1719-1726.
- Nicholson, P., Lees, A. K., Maurin, N., Parry, D., and Rezanoor, H. N. 1996a. Development of a PCR assay to identify and quantify *Microdochum nivale* var. *nivale* and *Microdochum nivale* var. *majus* in wheat. *Physiological and Molecular Plant Pathology* 48:257-271.

- Nicholson, P., and Parry, D. W. 1996b. Development and use of a PCR assay to detect *Rhizoctonia cerealis*, the cause of sharp eyespot in wheat. *Plant Pathology* 45:872-883.
- Nicholson, P., Rezanoor, H. N., Simpson, D. R., and Joyce, D. 1997. Differentiation and quantification of the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuiformis* using a PCR assay. *Plant Pathology* 46:842-856.
- Nicholson, P., Simpson, D. R., Weston, G., Rezanoor, H. N., Lees, A. K., Parry, D. W., and Joyce, D. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assay. *Physiological and Molecular Plant Pathology* 53:17-37.
- Niessen, L., Schmidt, H., and Vogel, R. 2004. The use of *tri5* gene sequences for PCR detection and taxonomy of trichothecene-producing species in the *Fusarium* section *Sporotrichiella*. *International Journal of Food Microbiology* 95:305-319.
- Orsini, M., and Romanno-Spica, V. 2001. A microwave-based method for nucleic acid isolation from environmental samples. *Letters in Applied Microbiology* 33:17-20.
- Palmer, C. L., and Skinner, W. 2002. *Mycosphaerella graminicola*: latent infection, crop devastation and genomics. *Molecular Plant Pathology* 3:63-70.
- Parker, S. R., Lovell, D. J., Royle, D. J., and Paveley, N. D. 1999. Analyzing epidemics of *Septoria tritici* for improved estimates of disease risk. In: *Septoria on cereals: A study of pathosystems*. Lucas, J. A., Bowyer, P., Anderson, H. M. eds. CAB International, Wallingford, UK, pp. 44-69.
- Plasterer, T. N. 1997. PRIMERSELECT: Primer and probe design. *Methods in Molecular Biology* 70:291-302.
- Pnini-Cohen, S., Zilberstein, A., Schuster, S., Sharon, A., and Eyal, Z. 2000. Elucidation of *Septoria tritici* and wheat interactions using GUS-expressing isolates. *Phytopathology* 90:297-304.
- Poupard, P., Simonet, P., Cavelier, N., and Bardin R.. 1993. Molecular characterization of *Pseudocercospora herpotrichoides* isolates by amplification of ribosomal DNA internal transcribed spacers. *Plant Pathology* 42:873-881.
- Qi, M., and Yang, Y. 2002. Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and northern blot/phosphoimaging analyses. *Phytopathology* 92:870-876.
- Qin, L., Prins, P., Jones, J. T., Popeijus, H., Smant, G., Bakker, J., and Helder, J. 2001. GenEST, a powerful bidirectional link between cDNA sequence data and gene expression profiles generated by cDNA-AFLP. *Nucleic Acids Research* 29:1616-1622.
- Rachdawong, S., Cramer, C. L., Grabau, E. A., Stromberg, V. K., Lacy, G. H., and Stromberg, E. L. 2002. *Gaeumannomyces graminis* vars. *avenae*, *graminis*, and *tritici* identified using PCR amplification of avenacinase-like genes. *Plant Disease* 86:652-660.

- Raeymaekers, L. 2000. Basic principles of quantitative PCR. *Molecular Biotechnology* 15:115-122.
- Ray, S., Anderson, J. M., Urmeev, F. I., and Goodwin, S. B. 2003. Rapid induction of a protein disulfide isomerase and defense-related genes in wheat in response to the hemibiotrophic fungal pathogen *Mycosphaerella graminicola*. *Plant Molecular Biology* 53:701-714.
- Reisler, E. 1993. Actin molecular structure and function. *Current Opinion in Cell Biology* 5:41-47.
- Rigotti, S., Gindro, K., Richter, H., and Viret, O. 2002. Characterization of molecular markers for specific and sensitive detection of *Botrytis cinerea* Pers. Fr. in strawberry (*Fragaria x ananassa* Duch.) using PCR. *FEMS Microbiolgy Letters* 209:169-174.
- Rillo, A. O., and Caldwell, R. M. 1996. Inheritance of resistance to *Septoria tritici* in *Triticum aestivum* subsp. *vulgare*, Bulgaria 88. *Phytopathology* 56:597.
- Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* 245:154-160.
- Rochelle P. A., De Leon, R., and Stewart, M. H., and Wolfe, R. L. 1997. Comparison of primers and optimization of PCR conditions for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water. *Applied Environmental Microbiology* 63:106-114.
- Rohel, E. A., Cavelier, N., and Hollomon, D. W. 2001. Microscopic analysis of the effect of azoxystrobin treatments on *Mycosphaerella graminicola* infection using green fluorescent protein (GFP)-expressing transformants. *Pest Management Science* 57:1017-1022.
- Rohel, E. A., Laurent, P., and Fraaije, B. A., Cavelier, N., Hollomon, D. W. 2002. Quantitative PCR monitoring of the effect of azoxystrobin treatments on *Mycosphaerella graminicola* epidemics in the field. *Pest Management Science* 58:248-254.
- Rollo, F., Salvi, R., and Torchia, P. 1990. Highly sensitive and fast detection of *Phoma tracheiphila* by polymerase chain reaction. *Applied Microbiology and Biotechnology* 32:572-576.
- Royle, D. J. 1994. Understanding and predicting epidemics: a commentary based on selected pathosystems. *Plant Pathology* 43:777-789.
- Rychlik, W., Spencer, W. J., and Rhoads, R. E. 1990. Optimisation of annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Research* 18:6409-6412.
- Rye, H. S., Yue, S., Quesada, M. A., Haugland, R. P., Mathies, R. A., and Glazer, A. N. 1993. Picogram detection of stable dye-DNA intercalation complexes with two-color laser-excited confocal fluorescence gel scanner. *Methods in Enzymology* 217:414-431.
- Saade, M., Aparicio, F., Sánchez-Navarro, J. A., Herranz, M. C., Myrta, A., Di Terlizzi, B., and Pallás, V. 2000. Simultaneous detection of the three ilarviruses affecting stone fruit trees

- by nonisotopic molecular hybridization and multiplex reverse transcription-polymerase chain reaction. *Phytopathology* 90:1330-1336.
- Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arneheim, N. 1985. Enzymatic amplification of  $\beta$ -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350-1354.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.
- Salmon, M. A., Vendrame, M., Kummert, J., and Lepoivre, P. 2002. Rapid and homogeneous detection of apple stem pitting virus by RT-PCR and a fluorogenic 3' minor groove binder-DNA probe. *European Journal of Plant Pathology* 108:755-762.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.
- Sanderson, F. R. 1972. A *Mycosphaerella* species as the ascogenous state of *Septoria tritici* Rob.ex Desm. *New Zealand Journal of Botany* 10:707-709.
- Schaad, N. W., Opgenorth, D., and Gauth, P. 2002. Real-time polymerase chain reaction for one-hour on-site diagnosis of Pierce's disease of grape in early season asymptomatic vines. *Phytopathology* 92:721-728.
- Schena, L., Nigro, F., and Ippolito, A. 2002. Identification and detection of *Rosellina necatrix* by conventional and real-time Scorpion-PCR. *European Journal of Plant Pathology* 108:355-366.
- Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology* 110:893-908.
- Schenk, P. M., Kazan, K., Manners, J. M., Anderson, J. P., Simpson, R. S., Wilson, I. W., Somerville, S. C., and Maclean, D. J. 2003. Systemic gene expression in *Arabidopsis* during an incompatible interaction with *Alternaria brassicicola*. *Plant Physiology* 132:999-1010.
- Schesser, K., Luder, A., and Henson, J. M. 1991. Use of polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis*, in infected wheat plants. *Applied and Environmental Microbiology* 57:553-556.
- Schierwater, B., and Ender, A. 1993. Different thermostable DNA polymerases may amplify different RAPD products. *Nucleic Acids Research* 21:4647-4648.
- Schnerr, H., Niessen, L., and Vogel, R. F. 2001. Real time detection of tri5 gene in *Fusarium* species by LightCycler<sup>TM</sup>-PCR using SYBR<sup>®</sup> Green I for continuous fluorescence monitoring. *International Journal of Food Microbiology* 71:53-61.
- Schnieder, F., Koch, G., Jung, C., and Verreet, J.-A. 1998. The application of molecular markers for genetic characterization of *Septoria tritici* populations. *Journal of Plant Disease and Protection* 105:452-461.

- Schnieder, F. 1998. Molekulare Analyse der genetischen Struktur and Diversität deutscher and europäischer Populationen von *Septoria tritici*, dem Erreger einer Blattdürre an Weizen. Dissertation at the Institute of Phytopathology, University of Kiel.
- Schnieder, F., Koch, G., Jung, C., and Verreet, J.-A. 2001. Genotypic diversity of the wheat leaf blotch pathogen *Mycosphaerella graminicola* (anamorph) *Septoria tritici* in Germany. *European Journal Plant Pathology* 107:285-290.
- Shaner, G., and Finney, R. E. 1976. Weather and epidemics of *Septoria* leaf blotch of wheat. *Phytopathology* 66:781-785.
- Shaw, M. W., and Royle, D. J. 1989. Airborne inoculum as a major source of *Septoria tritici* (*Mycosphaerella graminicola*) infections in winter wheat crops in the UK. *Plant Pathology* 38:35-43.
- Shaw, M. W. 1990. Effects of temperature, leaf wetness and cultivar on the latent period of *Mycosphaerella graminicola* on winter wheat. *Plant Pathology* 39:255-268.
- Sheridan, G. E. C., Masters, C. I., Shallcross, J. A., and Mackey, B.M. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. *Applied and Environmental Microbiology* 64:1313-1318.
- Shipton, W. A., Boyd, W. R. J., Rosielle, A. A., and Shearer, B. J. 1971. The common *Septoria* diseases of wheat. *Botanical Review* 37:231-262.
- Simón, M. R., Ayala, F. M., Cordo, C. A. Röder, M. S., and Böner, A. 2004. Molecular mapping of quantitative trait loci determining resistance to *Septoria tritici* blotch caused by *Mycosphaerella graminicola* in wheat. *Euphytica* 138:41-48.
- Singh, V. K., and Kumar, A. 2001. PCR Primer Design. *Molecular Biology Today* 2: 27-32.
- Singer, V. L., Lawlor, T. E., and Yue, S. 1999. Comparison of SYBR Green I nucleic acid gel stain mutagenicity and ethidium bromide mutagenicity in the *Salmonella*/mammalian microsome reverse mutation assay (Ames test). *Mutation Research* 439:37-47.
- Skinner, W., Keon, J., and Hargreaves, J. 2001. Gene information for fungal plant pathogens from expressed sequences. *Current Opinion in Microbiology* 4:381-386.
- Smith, O. P., Peterson, G. L., Beck, R. J., Schaad, N. W., and Bonde, M. R. 1996. Development of a PCR-based method for identification of *Tilletia indica*, causal agent of Karnal bunt of wheat. *Phytopathology* 86:115-122.
- Soanes, D. M., Skinner, W., Keon, J., Hargreaves, J., and Talbot, N. J. 2002. Genomics of phytopathogenic fungi and the development of bioinformatic resources. *Molecular Plant-Microbe Interactions* 15:421-427
- Stewart, S. J., and Jehan-Byers, R. 1996. Development of a rapid on-site test for the presymptomatic detection of *Septoria tritici* and *Septoria nodorum*. Brighton Crop Protection Conference - Pests and Diseases 1:215-220.

- Suarez, M. C., Bernal, A., Gutierrez, J., Tohme, J., and Fregene, M. 2000. Developing expressed sequence tags (ESTs) from polymorphic transcript-derived fragments (TDFs) in cassava (*Manihot esculenta* Crantz). *Genome* 43:62-67.
- Teare, J. M., Islam, R., Flanagan, R., Gallagher, S., Davies, M. G., and Grabau, C. 1997. Measurement of nucleic acid concentrations using the DyNA Quant and the GeneQuant. *Biotechniques* 22:1170-1174.
- Tian, S., Weinert, J. and Wolf, G. A. 2002. Studies on fungicide effect to *Septoria tritici* and *Stagonospora nodorum* in wheat. In: Proceeding of the 53th German Plant Protection Congress, pp. 109-110. September 16-19, 2002, Bonn, Germany.
- Tian, S., Weinert, J., and Wolf, G. A. 2004. Application of BA-ELISAs to assess varietal resistance of wheat and triticale to *Septoria tritici* and *Stagonospora nodorum* under field conditions. In: Proceeding of the 54th German Plant Protection Congress, pp. 580-581. September 20-23, 2004, Hamburg, Germany.
- Tucker, S. L. and Talbot, N. J. 2001. Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annual Review of Phytopathology* 39:385-417.
- Tyagi, S., and Kramer, F. R. 1996. Molecular beacons: probes that fluorescence upon hybridisation. *Nature Biotechnology* 14:303-308.
- Van Ginkel, M., and Scharen, A. L. 1988. Host-pathogen relationships of wheat and *Septoria tritici*. *Phytopathology* 78:762-766.
- Vandemark G. J., and Barker B. M. 2003. Quantifying *Phytophthora medicaginis* in susceptible and resistant alfalfa with a real-time fluorescent PCR assay. *Journal of Phytopathology* 151:577-583.
- Verreet, J.-A., Hoffmann, G. M., and Portner, J. 1990. Nachweis des Teleomorph *Mycosphaerella graminicola* (Fuckel) Schroeter (Anamorph: *Septoria tritici* Rob. and Desm.) in der Bundesrepublik Deutschland. *Journal of Phytopathology* 130:105-113.
- Verreet, J. A., Klink, H., and Hoffmann, G. M. 2000. Regional monitoring for disease prediction and optimization of plant protection measures: The IPM wheat model. *Plant Disease* 84:816-826.
- Vet, J. A., and Marras, S. A. 2005. Design and optimization of molecular beacon real-time polymerase chain reaction assays. *Methods in Molecular Biology* 288:273-290.
- Vierstraete, A. 1999. Principle of the PCR. University of Ghent, Belgium. <http://allserv.rug.ac.be/~avierstr/principles/pcr.html>.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23:4407-4414.
- Waalwijk, C., van der Heide, R., de Vries, I., van der Lee, T., Schoen, C., Costrel-de Corainville, G., Häuser-Hahn, I., Kastelein, P., Köhl, J., Lonnet, P., Demarquet, T., and



- Kema, G. H. J. 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. *European Journal of Plant Pathology* 110:481-494.
- Wainshibaum, S. J., and Lipps, P. E. 1991. Effect of temperature and growth stage of wheat on development of leaf and glume blotch caused by *Septoria tritici* and *S. nodorum*. *Plant Disease* 75:993-998.
- Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., and Itakura, K. 1979. Hybridization of synthetic oligodeoxyribonucleotides to phi chi 174 DNA: the effect of single base pair mismatch. *Nucleic Acids Research* 6:3543-3557.
- Ward, E. 1995. Improved polymerase chain reaction (PCR) detection of *Gaeumannomyces graminis* including a safeguard against false negatives. *European Journal of Plant Pathology* 101:561-566.
- Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M., and Dunez, J. 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods* 39:27-37.
- Whitcombe, D., Theaker, J., Guy, S. P., Brown, T., and Little, S. 1999. Detection of PCR products using self-probing amplicons and fluorescence. *Nature Biotechnology* 17:804-807.
- Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, S. V., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18:6531-6353.
- Wittwer, C. T., Herrmann, M. G., Moss, A. A., and Rasmussen, R. P. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 22:130-131:134-138.
- Wu, D. Y. W., Ugozzoli, L., Pal, B. K., Qian, J., and Wallace, R. B. 1991. The effect of temperature and oligonucleotide primer length on specificity and efficiency of amplification by the polymerase chain reaction. *DNA and Cell Biology* 10:233-238.
- Yeates, C., and Gillings, M. R. 1998. Rapid purification of DNA from soil for molecular biodiversity analysis. *Letters in Applied Microbiology* 27:49-53.
- Yu, K., and Pauls, K. P. 1992. Optimization of the PCR program for RAPD analysis. *Nucleic Acids Research* 20:2606.
- Zadoks, J. C. 2003. Two wheat septorias, two emerging diseases from the past. Proceedings of the 6<sup>th</sup> International Symposium on *Septoria* and *Stagonospora* Diseases of Cereals: Global insight into the *Septoria* and *Stagonospora* diseases of cereals, pp. 1-12. December 8-12, 2003. Tunis, Tunisia.
- Zhan, J., Mundt, C. C., and McDonald, B. A. 2002. Local adaptation and effect of host genotype on the evolution of pathogens: an experimental test in a plant pathosystem. *Journal of Evolutionary Biology* 15:634-647.
- Zhan, J., Kema, G. H. J., and McDonald, B. A. 2004. Evidence for natural selection in the mitochondrial genome of *Mycosphaerella graminicola*. *Phytopathology* 94:261-267.

- Zhang, J., and Madden, T. L. 1997. PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation. *Genome Research* 7:649-656.
- Zwiers L-H, and De Waard, M. A. 2000. Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. *Fungal Genetics and Biology* 30:115–125.
- Zwiers, L. H., Stergiopoulos, I., Gielkens, M. M. C., Goodall, S. D., and De Waard, M. A. 2003. ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. *Molecular Genetics and Genomics* 269:499-507.

## 8. Appendix

### 8.1 Media, chemicals, solutions and buffers

#### 8.1.1 Media

##### LB medium

10 g tryptone, 5 g yeast extract, 5 g NaCl, 1,000 ml distilled H<sub>2</sub>O, pH 7.0, autoclaved.

##### LB<sub>ampicillin</sub> agar plate

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, 1,000 ml distilled H<sub>2</sub>O, pH 7.0, autoclaved. 100 µg/ml ampicillin were added before put into Petri dishes.

##### MYA and MYB medium

MYA: 5 g malt extract, 5 g yeast extract, 10 g glucose, 15 g agar, 1,000 ml distilled water, pH 6.0, autoclaved. 100 ppm ampicillin was added before use.

MYB: the same as MYA except agar.

##### PDA and PDB medium

PDA: 24 g potato dextrose broth, 15 g agar, 1,000 ml distilled H<sub>2</sub>O, autoclaved.

PDB: the same as PDA except agar.

##### SOC medium

2% (w/v) trypton, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose.

#### 8.1.2 Solutions and buffers

##### Autoclaved, DEPC-treated water

Add DEPC to a final concentration of 0.1% (v/v) to MiliQ water, autoclave for 1 h, open the autoclave door briefly to release steam, boil the water for at least 30 min in a heating bad.

##### DNA extraction buffer 1

200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS.

##### DNA extraction buffer 2

1% (w/v) CTAB, 100 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25mM EDTA, 0.5% (w/v) SDS.

##### 6x DNA loading dye for agarose gel

0.03% (w/v) orange G, 15% (w/v) Ficoll<sup>®</sup> 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA (pH 8.0).

##### DNA loading dye for polyacrylamide gel

98 ml formamide, deionized, 2 ml 0.5 M EDTA (pH 8.0), 25 mg Orange G (0.025% (w/v)), 25 mg bromophenolblue (0.025% (w/v)).

##### 0.1M IPTG stock solution

1.2 g IPTG, 50 ml MilliQ-H<sub>2</sub>O. Filter-sterilize and store at 4°C.

##### 10 M LiCl (200 ml)

67.82 g LiCl, add DEPC-treated H<sub>2</sub>O to 200 ml, DEPC-treat, autoclave and store at 4°C.

**5x RL-buffer**

10 mM Tris-acetate (pH 7.6), 10 mM Mg-acetate, 50 mM K-acetate, 5 mM DTT, 250 µg/ml BSA.

**RNA loading buffer 1 (5ml, for RNA electrophoresis on agarose gel)**

1 ml 10x TBE buffer, 13% (w/v) Ficoll (0.65 g), 10 µl 2.5% (w/v) BPB, 7 M Urea (2.1 g) and 2 µl ethidium bromide, add DEPC-treated water to 5 ml, sterile filtration.

**RNA loading buffer 2 (500 µl, for RNA electrophoresis in Northern hybridization)**

250 µl formamide, 83 µl 37% (w/v) formaldehyde, 50 µl 10 x MOPS Buffer, 10 µl 2.5% (w/v) BPB, 1.5 µl ethidium bromide (10 mg/ml), add to 500 µl with DEPC-treated H<sub>2</sub>O (prepare before use).

**50x TAE buffer**

2 M Tris-HCl, 50mM EDTA, 1M HAC, pH 8.0.

**5x TBE buffer**

54 g Tris-base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0), add H<sub>2</sub>O to 1,000 ml.

**1x TE buffer**

100 mM Tris-HCl, 1 mM EDTA, pH 7.5.

**X-Gal (2ml)**

100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 2 ml N,N'-dimethyl-formamide. Cover with aluminum foil and store at -20°C.

**8.1.3 Chemical solutions for Northern hybridization****10x MOPS buffer**

200 mM MOPS, 50 mM sodium acetate, 20 mM EDTA, pH 7.0. Sterile filtration. Store at 4°C.

**1x MOPS buffer**

100 ml 10x MOPS buffer to 1,000 ml DEPC-treated water, and autoclave at 121°C for 30 min before use.

**20x SSC**

3 M NaCl, 300 mM sodium citrate, pH 7.0, DEPC-treated and autoclaved.

**10% SDS**

5 g SDS in 50 ml DEPC-treated H<sub>2</sub>O, filtered.

**2x SSC, 0.1% (w/v) SDS (500ml)**

100 ml 20x SSC, 10 ml 10% (w/v) SDS, add DEPC-treated H<sub>2</sub>O to 1,000 ml.

**0.1x SSC, 0.1% (w/v) SDS (500 ml)**

2.5 ml 20 X SSC, 5 ml filtered 10% SDS, 492.5 ml DEPC-treated H<sub>2</sub>O.

**1x maleic acid buffer**

0.1 M maleic acid, 0.15 M NaCl, adjust with NaOH (solid) to pH 7.5.

1x Tween washing buffer

0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween-20.

10x Blocking solution

5 g blocking reagent (Roche) in 50 ml 1x maleic acid buffer, store at 4°C, stable for two weeks.

1x Blocking solution

Dilute 10x Blocking solution 1:10 with maleic acid buffer. Prepare it before use.

Antibody solution

Centrifuge anti-digoxigenin-AP for 5 min at 10,000 rpm in the original vial prior to each use, and pipette the necessary amount carefully from the surface. Dilute anti-digoxigenin-AP 1:10,000 (75 mU/ml) in 1x Blocking solution. Prepare it before use.

Detection buffer

0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C), stable at room temperature.

CSPD ready-to-use (Roche)

## 8.2 Tables

**Table A1** Random primers used in the PAPD-PCR

Primer	Sequence (5'--3')	Primer	Sequence (5'--3')
Roth-A 01	CAG GCC CTT C	OPAH-01	TCC GCA ACC A
Roth-A 02	TGC CGA GCT G	OPAH-02	CAC TTC CGC T
Roth-A 03	AGT CAG CCA C	OPAH-03	GGT TAC TGC C
Roth-A 04	AAT CGG GCT G	OPAH-04	CTC CCC AGA C
Roth-A 05	AGG GGT CTT G	OPAH-05	TTG CAG GCA G
Roth-A 06	GGT CCC TGA C	OPAH-06	GTA AGC CCC T
Roth-A 07	GAA ACG GGT G	OPAH-07	CCC TAC GGA G
Roth-A 08	GTG ACG TAG G	OPAH-08	TTC CCG TGC C
Roth-A 09	GGG TAA CGC C	OPAH-09	AGA ACC GAG G
Roth-A 10	GTG ATC GCA G	OPAH-10	GGG ATG ACC A
Roth-A 11	CAA TCG CCG T	OPAH-11	TCC GCT GAG A
Roth-A 12	TCG GCG ATA G	OPAH-12	TCC AAC GGC T
Roth-A 13	CAG CAC CCA C	OPAH-13	TGA GTC CGC A
Roth-A 14	TCT GTG CTG G	OPAH-14	TGT GGC CGA A
Roth-A 15	TTC CGA ACC C	OPAH-15	CTA CAG CGA G
Roth-A 16	AGC CAG CGA A	OPAH-16	CAA GGT GGG T
Roth-A 17	GAC CGC TTG T	OPAH-17	CAG TGG GGA G
Roth-A 18	AGG TGA CCG T	OPAH-18	GGG CTA GTC A
Roth-A 19	CAA ACG TCG G	OPAH-19	GGC AGT TCT C
Roth-A 20	GTT GCG ATC C	OPAH-20	GGA AGG TGA G
Roth-H 01	GGT CGG AGA A	OPAG-01	CTA CGG CTT C
Roth-H 02	TCG GAC GTG A	OPAG-02	CTG AGG TCC T
Roth-H 03	AGA CGT CCA C	OPAG-03	TGC GGG AGT G
Roth-H 04	GGA AGT CGC C	OPAG-04	GGA GCG TAC T
Roth-H 05	AGT CGT CCC C	OPAG-05	CCC ACT AGA C
Roth-H 06	ACG CAT CGC A	OPAG-06	GGT GGC CAA G
Roth-H 07	CTG CAT CGT G	OPAG-07	CAC AGA CCT G
Roth-H 08	GAA ACA CCC C	OPAG-08	AAG AGC CCT C
Roth-H 09	TGT AGC TGG G	OPAG-09	CCG AGG GGT TT
Roth-H 10	CCT ACG TCA G	OPAG-10	ACT GCC CGA C
Roth-H 11	CTT CCG CAG T	OPAG-11	TTA CGG TGG G
Roth-H 12	ACG CGC ATG T	OPAG-12	CTC CCA GGG T
Roth-H 13	GAC GCC ACA C	OPAG-13	GGC TTG GCG A
Roth-H 14	ACC AGG TTG G	OPAG-14	CTC TCG GCG A
Roth-H 15	AAT GGC GCA G	OPAG-15	CCC ACA CGC A
Roth-H 16	TCT CAG CTG G	OPAG-16	CCT GCG ACA G
Roth-H 17	CAC TCT CCT C	OPAG-17	AGC GGA AGT G
Roth-H 18	GAATCG GCC A	OPAG-18	GTG GGC ATA C
Roth-H 19	CTG ACC AGC C	OPAG-19	AGC CTC CGG TT
Roth-H 20	GGG AGA CAT C	OPAG-20	TGC GCT CCT C

**Table A2** Other primers used in this research

Primer	Sequence (5'--3')	Primer	Sequence (5'--3')
<i>Eco</i> RI-adapter	5-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'	<i>Mse</i> I-adapter	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
<i>Eco</i> RI-0	GACTGCGTACCAATTC	<i>Mse</i> I-0	GATGAGTCCTGAGTAA
<i>Eco</i> RI-A	GACTGCGTACCAATTCA	<i>Mse</i> I-A	GATGAGTCCTGAGTAAA
<i>Eco</i> RI-AA	GACTGCGTACCAATTCAA	<i>Mse</i> I-AA	GATGAGTCCTGAGTAAAA
<i>Eco</i> RI-AT	GACTGCGTACCAATTCAT	<i>Mse</i> I-AT	GATGAGTCCTGAGTAAAT
<i>Eco</i> RI-AC	GACTGCGTACCAATTCAC	<i>Mse</i> I-AC	GATGAGTCCTGAGTAAAC
<i>Eco</i> RI-AG	GACTGCGTACCAATTCAG	<i>Mse</i> I-AG	GATGAGTCCTGAGTAAAG
R3005-F	CGAACCCAGGAAGAAGAGTG	R3005-R	TTCCGAACCCCTAACCTAAGA
R3006-F	AGCCAGCGAAATAATTGGAC	R3006-1R	CGAAGGAGCGGGTTGAGT
R3006-1F	GGACGACGATAGTTGTGCTG	R3006-2R	TGTCGTCGTGGTTGTCATTT
R3009-F	AAACGTCGGTAATAGGGTGCT	R3009-R	CGGCATCGCATTAAAGACTA
R3009-1F	GGCCCGAGAGGGATACTAAC	R3009-1R	GCAAAAGCATGCGACCTTAT
R3009-2F	CGGTTCTACCCCGTCTTCTAC		
R3009-3F	TCCTTAGGCCCTTCCGTATT		
R3009-4F	CGGTTCTACCCCGTCTTCTA	R3009-4R	GGGGCCTAGCCTGTTAGACT
R3009-5F	TCGGGGGAAAACATAGAAGA	R3009-5R	GTCGGCATCGCATTAAAGAC
R5870-F	GTAGGGATCAGCGTTGCAG	R5870-R	ATCCCTGCAGAGACAACAGG
R5870-1F	CCACTCCTGCTCTCTTCACC	R5870-1R	TCTGTTCTGCGTGCCATTAG
R5870-2F	GTGTGGTAGCGCTCGAATTT	R5870-2R	CCATGAAGCATGAGGAAGGT
R5870-3F	AGTGGAGCAACGATCGAAGT		
ITS1	TCCGTAGGTGAACCTGCGGG	ITS4	TCCTCCGCTTATTGATATGC
M13-uni	AGGGTTTTCCAGTCACGACGTT	M13-rev	GAGCGGATAACAATTTACACAGG
ST-rRNA F	CTGCGTCGGAGTTACGAGT	ST-rRNA R	GTGAGGGCTCGTGA ACTCC
ST-act F	GGTCGTACCACCGGTATTGT	ST-act R	GATCGGATGGAGTCAGCAG
E1	CGGTATGGGAACACTTCTCATCAG	STSP2R	GTAACGACCGTTGCGGAAATCGCT
STIF2	ACTCACAATCCTCATTTCGACGCGA	BAF4ST	GACCAATTCGGCACCCCTCAGTGTA
ITS1	TCCGTAGGTGAACCTGCGGG	JB446	CGAGGCTGGAGTGGTGT

**Table A3** Cycle programmes of the four specific primer sets tested for real-time PCR

Programme	Cycles	Segment number	St-rRNA-F/R	St-act F/R 5870-1F and R5870-1R	R5870-1F R5870-2R	Slope (°C/s)	Acquisition
denaturing	1	1		15 min at 95°C		20	None
Amplification	40	1	15 s at 94°C	15 s at 94°C	15 s at 94°C	20	None
		2	20 s at 60°C	30 s at 60°C	20 s at 60°C	20	None
		3	20 s at 72°C	30 s at 72°C	25 s at 72°C	20	None
		4	5 s at 82°C	5 s at 80°C	5 s at 80°C	20	Single
Melting curve	1	1		0 s at 95°C		20	None
		2		15 s at 65°C		20	None
		3		0 s at 95°C		0.1	Continuous
Cooling	1			30 s at 40°C		20	None

**Table A4** Disease severity assessed visually by a disease classification in wheat plants inoculated with *Mycosphaerella graminicola*

Days after inoculation	Total leaves	Scale of disease severity						Disease incidence (%)	Disease index
		0	1	2	3	4	5		
8	34	17	12	5	0	0	0	50.00	12.94
10	33	4	15	4	6	2	2	81.82	35.76
12	25	0	4	5	4	2	10	100.00	67.20
14	23	0	3	2	4	2	12	100.00	75.65
16	25	0	1	3	4	3	14	100.00	80.80
18	23	0	2	1	1	1	18	100.00	87.83
20	23	0	1	1	1	2	18	100.00	90.43
22	20	0	0	0	2	2	16	100.00	94.00
24	21	0	0	0	1	0	21	100.00	98.18
26	23	0	0	0	1	1	21	100.00	97.39

**Table A5** Disease severity of *Mycosphaerella graminicola* leaf blotch assessed by disease classification in F, F1 and F2 leaves sampled weekly from a fungicide-untreated plot during the epidemic period<sup>a</sup>

Date	Leaf position	Scale of disease severity						Disease incidence (%)	Disease index
		0	1	2	3	4	5		
May 3	F1	30	0	0	0	0	0	0.00	0.00
	F2	28	2	0	0	0	0	6.67	1.33
May 10	F1	29	0	0	0	0	0	0.00	0.00
	F2	23	6	0	0	0	0	20.69	4.13
May 17	F	30	0	0	0	0	0	0.00	0
	F1	25	5	0	0	0	0	16.67	3.23
	F2	9	21	0	0	0	0	70.00	15.56
May 24	F	29	0	0	0	0	0	0.00	0
	F1	8	21	0	0	0	0	72.41	14.48
	F2	4	22	3	0	0	0	75.86	19.31
June 1	F	22	8	0	0	0	0	26.67	5.33
	F1	0	9	9	8	2	2	100.00	46.00
	F2	0	2	6	7	5	10	100.00	70.00
June 7	F	20	10	0	0	0	0	33.33	6.67
	F1		1	1	2	6	20	100.00	88.66
	F2	0	0	0	1	4	25	100.00	96.00
June 14	F	17	10	3				43.33	10.67
	F1	0	0	1	3	4	22	100.00	91.33
	F2	0	0	0	0	0	30	100.00	100.00
June 21	F	5	25	0	0	0	0	83.33	16.67
	F1	0	0	0	1	5	24	100.00	95.33
	F2	0	0	0	0	0	30	100.00	100.00

<sup>a</sup> Trial place: Grünhorst, Gettorf, Schleswig-Holstein. Sampling date: from May 3 to June 21, 2004.



**Table A6** Disease severity of *Mycosphaerella graminicola* leaf blotch assessed by disease classification and by counting pycnidial number in two fungicide-treated plots and the fungicide-untreated plot (CK)

Date	Treatment	Leaf position	Disease severity classification							Disease index	PNL <sup>a</sup>
			0	1	2	3	4	5			
May 17	Fandango	F	29	0	0	0	0	0	0.00	0.00	
	Input	F	29	0	0	0	0	0	0.00	0.00	
	CK	F	30	0	0	0	0	0	0.00	0.00	
	Fandango	F1	29	0	0	0	0	0	0.00	0.00	
	Input	F1	29	0	0	0	0	0	0.00	0.00	
	CK	F1	25	5	0	0	0	0	3.23	0.00	
	Fandango	F2	23	6	0	0	0	0	4.13	0.67	
	Input	F2	26	3	0	0	0	0	2.07	0.00	
	CK	F2	9	21	0	0	0	0	15.56	0.67	
	Fandango	F3	2	13	5	4	1	3	35.00	39.3	
	Input	F3	3	10	4	2	5	5	47.58	4.67	
	CK	F3	0	3	8	8	6	5	61.33	45.3	
June 14	Fandango	F4		5	6	4	4	11	66.67	338.00	
	Input	F4		1	3	3	1	21	83.33	89.00	
	CK	F4	0	0	0	3	2	25	74.00	284.70	
	Fandango	F	26	4	0	0	0	0	2.67	18.70	
	Input	F	29	1	0	0	0	0	0.67	0.30	
	CK	F	17	10	3	0	0	0	10.67	0.00	
	Fandango	F1	0	4	6	7	5	8	64.67	79.30	
	Input	F1	0	17	6	4	2	0	33.79	14.70	
	CK	F1	0	0	1	3	4	22	91.33	416.7	
	Fandango	F2	0	0	0	0	0	30	100.00	326.30	
	Input	F2	0	0	4	7	8	9	70.67	92.30	
	CK	F2	0	0	0	0	0	30	100.00	394.00	
Fandango	F3	0	0	0	0	0	30	100.00	0.00		
Input	F3	0	0	2	6	2	20	86.67	0.00		
CK	F3	0	0	0	0	0	30	100.00	0.00		

<sup>a</sup> PNL means pycnidial number per leaf. The value is the mean of 30 leaves. The PNL data were kindly provided by Dr. F. Schnieder.

**Table A7** Quantification of fungal DNA amount in inoculated wheat plants during the disease development<sup>a</sup>

Primer sets	Days	Cycle number				DNA amount (pg)			
		1	2	3	Mean ± SD	1	2	3	Mean ± SD
ST-rRNA	0	26.77	26.49		26.63 ± 0.20	9.26	11.39		10.32 ± 1.50
F/R	2	26.43	26.30	26.28	26.34 ± 0.08	11.90	13.10	13.30	12.77 ± 0.76
	4	25.68	25.47	25.73	25.63 ± 0.14	20.72	24.19	19.97	21.63 ± 2.26
	6	24.82	24.39	24.64	24.62 ± 0.22	39.11	53.73	44.67	45.83 ± 7.38
	8	22.49	22.44	22.29	22.41 ± 0.10	218.65	226.88	253.46	233.00 ± 18.20
	10	21.89	22.06	21.97	21.97 ± 0.09	340.60	300.40	321.05	320.68 ± 20.10
	12	21.00	21.13	21.06	21.06 ± 0.07	657.31	597.13	628.82	627.75 ± 30.11
	14	20.63	20.80	20.65	20.69 ± 0.09	863.93	761.97	851.26	825.72 ± 55.57
	16	19.9	19.99	20.05	19.98 ± 0.08	1481.42	1386.13	1326.03	1397.86 ± 78.35
	18	19.84	19.95	20.23	20.01 ± 0.20	1548.55	1427.70	1160.93	1379.06 ± 198.34
	20	19.93	19.72	20.09	19.91 ± 0.19	1448.95	1692.10	1287.42	1476.15 ± 203.70
	22	20.04	20.16	20.16	20.12 ± 0.07	1335.86	1222.54	1222.54	1260.31 ± 65.43
	24	20.17	20.02		20.10 ± 0.11	1213.54	1355.75		1248.64 ± 100.55
ST-act	0	30.50	31.16	31.16	30.94 ± 0.38	5.56	3.44	3.44	4.15 ± 1.22
F/R	2	30.67	30.48	30.50	30.55 ± 0.10	4.91	5.64	5.56	5.37 ± 0.40
	4	29.43	29.91	29.53	29.62 ± 0.25	12.10	8.54	11.25	10.63 ± 1.86
	6	28.37	28.56	28.54	28.49 ± 0.10	26.13	22.76	23.10	24.00 ± 1.86
	8	25.97	26.26	26.17	26.13 ± 0.15	149.45	121.06	129.24	133.25 ± 14.62
	10	25.46	25.82	25.56	25.61 ± 0.19	216.49	166.66	201.32	194.82 ± 25.54
	12	24.54	25.27	24.89	24.90 ± 0.37	422.42	248.54	327.57	332.84 ± 87.06
	14	24.57	24.44	24.35	24.45 ± 0.11	413.32	454.26	484.96	450.85 ± 35.94
	16	23.43	24.13	23.62	23.73 ± 0.36	946.28	569.02	824.26	779.85 ± 192.51
	18	23.32	23.40	23.55	23.42 ± 0.12	1025.01	967.13	867.26	953.13 ± 79.80
	20	23.33	23.33	23.73	23.46 ± 0.23	1017.59	1017.59	760.94	932.04 ± 148.18
	22	23.67	23.59	23.70	23.65 ± 0.06	794.85	842.42	777.71	804.99 ± 33.53
	24	23.47	23.86		23.67 ± 0.28	919.17	692.35		805.76 ± 160.38

<sup>a</sup>The experiment was performed with three replicates.

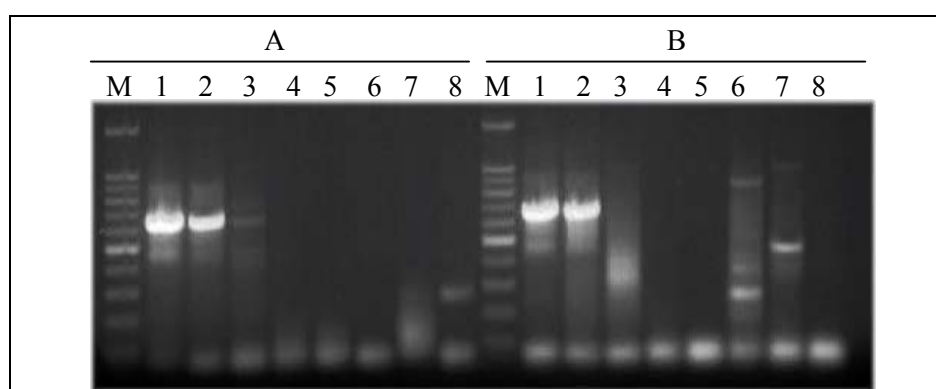
**Table A8** Quantification of *Mycosphaerella graminicola* DNA amount in F, F1 and F2 leaves sampled from a fungicide-untreated field during the epidemic period

Sample	Cycle number				DNA amount (pg)			
	1	2	3	Mean ± SD	1	2	3	Mean ± SD
ST-rRNA F/R								
5-17-F	30.4	30.61	30.98	30.66 ± 0.29	0.64	0.54	0.41	0.53 ± 0.12
5-24-F	30.28	30.49	30.77	30.51 ± 0.25	0.69	0.59	0.48	0.59 ± 0.11
6-1-F	31.05	31.25	30.05	30.78 ± 0.64	0.39	0.34		0.37 ± 0.04
6-7-F	27.79	27.75	27.54	27.69 ± 0.13	4.37	4.50	5.26	4.71 ± 0.48
6-14-F	25.10	24.91	25.00	25.00 ± 0.10	31.89	36.70	34.34	34.31 ± 2.41
6-21-F	24.32	24.96	24.51	24.60 ± 0.33	56.75	35.37	49.31	47.14 ± 10.85
5-3-F1	29.06	29.27	29.28	29.20 ± 0.12	1.71	1.47	1.45	1.54 ± 0.14
5-10-F1	29.78	29.78	29.38	29.65 ± 0.23	1.01	0.97	1.35	1.11 ± 0.21
5-17-F1	28.24	28.61	28.29	28.38 ± 0.20	3.14	2.39	1.84	2.46 ± 0.65
5-24-F1	27.04	27.26	27.04	27.11 ± 0.13	7.61	6.47	7.61	7.23 ± 0.66
6-1-F1	24.54	24.61	24.52	24.56 ± 0.05	48.24	45.80	49.95	48.00 ± 2.09
6-7-F1	22.01	21.89	22.48	22.13 ± 0.31	312.63	341.61	220.92	291.72 ± 63.00
6-14-F1	21.04	21.22	21.48	21.25 ± 0.22	640.06	560.37	462.45	554.29 ± 88.96
6-21-F1	20.81	20.88	20.82	20.84 ± 0.04	758.60	720.60	753.02	744.07 ± 20.52
5-3-F2	29.10	29.28	29.38	29.25 ± 0.14	1.66	1.45	1.35	1.49 ± 0.16
5-10-F2	27.34	27.31	27.32	27.32 ± 0.02	6.10	6.23	6.19	6.17 ± 0.07
5-17-F2	27.01	27.01	27.49	27.17 ± 0.28	7.78	7.78	5.44	7.00 ± 1.35
5-24-F2	27.01	27.20	27.50	27.24 ± 0.25	7.23	6.76	5.42	6.47 ± 0.94
6-1-F2	24.37	24.94	25.05	24.79 ± 0.37	54.53	35.79	32.99	41.10 ± 11.71
6-14-F2	20.88	21.10	21.19	21.06 ± 0.16	720.37	612.31	572.93	635.20 ± 76.34
6-7-F2	20.97	20.66	20.61	20.75 ± 0.20	674.03	847.49	879.38	800.30 ± 110.51
6-21-F2	20.76	20.88	21.07	20.90 ± 0.16	784.82	718.24	624.19	709.08 ± 80.71
ST-act F/R								
6-7-F		29.33	29.00	29.17 ± 0.23		13.01	16.53	14.77 ± 2.49
6-21-F	27.75	28.43	28.40	28.19 ± 0.38	41.00	25.02	25.57	30.53 ± 9.07
6-1-F1	29.31	28.84	29.01	29.05 ± 0.24	13.20	18.57	16.41	16.06 ± 2.70
6-7-F1	26.87	27.13	26.87	26.96 ± 0.15	77.71	64.34	77.71	73.26 ± 7.72
6-14-F1	24.95	25.87	25.51	25.44 ± 0.46	313.60	160.72	208.77	227.69 ± 78.18
6-21-F1	24.63	24.91	24.86	24.80 ± 0.15	395.68	322.84	334.79	351.11 ± 39.07
5-24-F2		29.99	29.82	29.91 ± 0.12		8.05	9.11	8.58 ± 0.75
6-1-F2		28.39	28.61	28.50 ± 0.16		25.75	21.95	23.85 ± 2.69
6-7-F2	25.81	25.42	25.35	25.53 ± 0.25	167.88	222.87	234.50	208.42 ± 35.59
6-14-F2	25.10	24.99	25.26	25.12 ± 0.14	281.21	304.61	250.35	278.73 ± 27.22
6-21-F2	25.27	25.31		25.29 ± 0.03	248.54	241.42		244.98 ± 5.03
R5870-1F/R5870-2R								
5-3-F1	36.13	35.93	35.80	35.95 ± 0.17	0.54	0.62	0.68	0.61 ± 0.07
5-10-F1	37.06	36.2	36.85	36.70 ± 0.45	0.27	0.51	0.32	0.37 ± 0.13
5-17-F1	34.93	34.68	34.80	34.80 ± 0.13	1.30	1.56	1.43	1.43 ± 0.13
5-24-F1	33.49	33.70	33.86	33.68 ± 0.19	3.74	3.20	2.85	3.26 ± 0.45
6-1-F1	32.54	32.55	32.49	32.53 ± 0.03	7.51	7.46	7.79	7.59 ± 0.18
6-7-F1	29.14	28.91	28.92	28.99 ± 0.13	91.49	108.34	107.55	102.46 ± 9.51
6-14-F1	27.34	27.57	27.28	27.40 ± 0.15	343.62	290.16	359.12	330.97 ± 36.18
6-21-F1	27.07		27.29	27.18 ± 0.16	419.07		356.49	387.78 ± 44.25

**Table A9** Quantification of *Mycosphaerella graminicola* DNA amount in wheat leaves sampled from two fungicide-treated plots on May 17 and June 14, 2004

Sample	Cycle number				DNA amount (pg)			
	1	2	3	Mean ± SD	1	2	3	Mean ± SD
<b>Fandango</b>								
5-17-F	30.16		30.42	30.29 ± 0.18	0.76		0.50	0.63 ± 0.18
5-17-F1	28.46	28.28	28.45	28.40 ± 0.10	2.66	3.04	2.68	2.79 ± 0.42
5-17-F2	28.76	28.79	28.95	28.83 ± 0.10	2.13	2.08	1.85	2.02 ± 0.15
5-17-F3	23.91	23.99	24.08	23.99 ± 0.09	76.59	73.27	67.55	72.47 ± 6.72
5-17-F4	23.76	23.97	23.75	23.83 ± 0.12	85.57	73.27	86.20	81.68 ± 7.29
6-14-F	31.32	31.51		31.42 ± 0.13	0.32	0.28		0.30 ± 0.03
6-14-F1	23.88	24.31	24.04	24.08 ± 0.22	78.31	57.00	69.58	68.30 ± 10.71
6-14-F2	21.44	21.41	21.41	21.42 ± 0.02	474.91	485.55	485.55	482.00 ± 6.14
6-14-F3	22.28	22.24	22.42	22.31 ± 0.09	255.34	263.00	230.25	249.53 ± 17.13
<b>Input</b>								
5-17-F								
5-17-F1	27.37	27.19	27.39	27.32 ± 0.11	5.94	6.79	5.86	6.20 ± 0.52
5-17-F2	27.45	27.41	27.58	27.48 ± 0.09	5.60	5.77	5.09	5.49 ± 0.35
5-17-F3	25.06	25.16		25.11 ± 0.07	32.75	30.42		31.59 ± 1.65
5-17-F4	23.79	23.72	23.58	23.70 ± 0.11	83.69	88.13	97.74	89.85 ± 7.18
6-14-F	30.22	30.77		30.50 ± 0.39	0.72	0.48		0.60 ± 0.17
6-14-F1	26.56	26.76	26.52	26.61 ± 0.13	10.81	9.33	11.14	10.43 ± 0.96
6-14-F2	22.68	22.68	22.78	22.71 ± 0.06	190.02	190.02	176.49	185.51 ± 7.81
6-14-F3	21.99	22.18	22.20	22.12 ± 0.12	316.34	274.92	270.89	287.38 ± 25.16
<b>Fungicide-untreated plot</b>								
5-17-F3	22.42	22.75	23.03	22.73 ± 0.31	230.25	180.44	146.73	185.81 ± 42.02
5-17-F4	23.54	23.44	23.33	23.44 ± 0.11	100.67	108.39	117.56	108.87 ± 8.46
6-14-F3	20.67	21.09	20.50	20.75 ± 0.30	838.77	615.03	951.01	801.61 ± 171.04

### 8.3 Figures



**Fig. A1** Specificity test of two primer sets R3009 F/R (A) and R5870 F/R (B) designed from RAPD-fragments. Lanes 1 to 8 are *Mycosphaerella graminicola*, *M. graminicola*-infected plants, healthy plants, *Erisiphe graminis* f.sp. *tritici* infected plants, *Septoria petroselini*, *Stagonospra nodorum*, *Microdochium nivale* and *Fusarium culmorum*, respectively. M is the 100 bp DNA ladder (Promega).

**R3005 sequence (520 bp)**

TTCCGAACCCAGGAAGAAGAGTGGTGAGAGAGCAATTCGCGAAGAGTTTGCAGCCGATGTGGA  
 TCTTGTATACGTGTTGCGCGGCGGTGGGCTTGGTGGTGATGTTCTTCGTGCAGAAGAAGGTGTT  
 GACGGCGAAGCATGAGGAGACGAAGACGGGGTTGGAGGCGGAGAGGGAGAATGCTGCGGCCA  
 GGAAGAGGGAGAAGGAGGAGAGGGGGGAAGGCCGAGGGGTGCGGTGGAGAAGGATGTTGA  
 GGCTGGGAGTTGAGGTGGAGGTGGATTTGGTTTTGGATATGTTAGCAAGGCGTTTTTGTATGT  
 ATAGTGTGCTGCCAGGTGGTAAAATATCGACAGGATAAGGAGGATTGGATGATAGTGGCATGCT  
 TGAGTGGCAGAGACAATGTAATAGTACCTCCTTCAGCTACTTCACGAGCAGGTTTCGAGGAAAC  
 GTGCATGAGGATTGCACTTCTTGGAAGACGTTATCTCGATGAAGCATCGTTCATGATGTCTTAG  
 GGTTAGGGTTTCGGAA

**R3006 sequence (636 bp)**

AGCCAGCGAAATAATTGGACGACGATAGTTGTGCTGTACCATCGCCGAAATGTGTGCAATGCA  
 CGATGGCGGGCGTCGTCGGGTGGTGGTGGGTTGAAACCGAGGACCCTCCCGTCATACCGCACG  
 AACCAAAGTTTGATAGCtCCGAGGAATGTGACATGCATTTCCCGGAGGTGAAATCCTCTACTCT  
 CACaAACCACCGATTGTCTGCCAGTGGAGTATGTCCATGTCCACGTCCACGTCCGAGGCTGCGG  
 TCAAATGACAACCACGACGACACATCATCTGCGACATCCACCATCTGCATGATGCGTCGCGCCC  
 CGCTGCTTCGCCGAGACATGGCCAGCGCATCTCCACCAACTCACTCCCAATGGACGCTCCCC  
 CCGTCAATGAGGAAATATCACCGCATCGGCCGACCCCTGAACCACAACCACATCTACATCGA  
 CACACCCGCTCCGTCTCACTGATCACCTCTCACCTCTACCACACAGCAGTGACACAAGCCTTTT  
 CCCGGCTGGCCCTGCGGTGGGACCACCACTCTTGAGTCTCTTCTCTCTGCTGCGCATCGCTC  
 TCTCACTGCCGACGGCGCGCAATCGCCGGGCTCCACGACTCAACCCGCTCTTCGCTGGCT

**R3009 sequence (649 bp)**

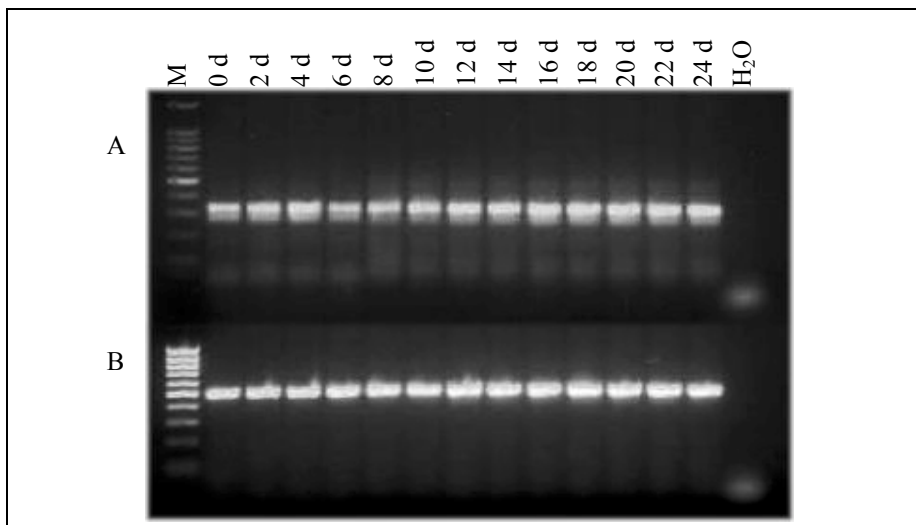
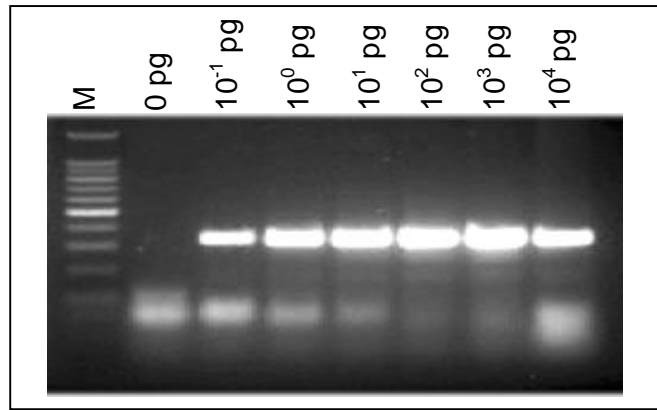
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 CCTCGACCTTCCGCTATCGATAAAGCGGTTCTACCCCGTCTTCTACGTATCCCTACTAGAGCTAT  
 ATCGGGCCCCGAGAGGGATACTAACTACCCCGGTGCATCCCTTAAGCCTAACGACAATAAG  
 AAGACGACTAGAGTAGAAGGGATCCTAGCTATAAGGGGCGACACGCGGAAACGAGGGAA  
 TACCTAGTACGATAGTTAGGGTACTCCCTAGAATAAATAAGTAGATATCGGGGGAAAACATA  
 GAAGAAGCGAGTGAACCTTGTTAAAGAGTTTGAATTAAGCAAGCCCGCTGCAGGCCGAAGGC  
 AGGCCTAAAACAGGCCCAATACTACTACAGGCAGGTATCCTACGCGACTAAAGAAGAAAAGA  
 ACTGCAGATAAAGGTCGCATGCTTTTGCCTTACCCTCTACGCTAGAAATTAAGGCTCTATAGC  
 CTAACAACAAGAACC GGACTAAGGAGTCTAACAGGCTAGGCCCGCTATAGTCTTTAATGCGA  
 TGCCGACGTTTG

**R5870 sequence (709 bp)**

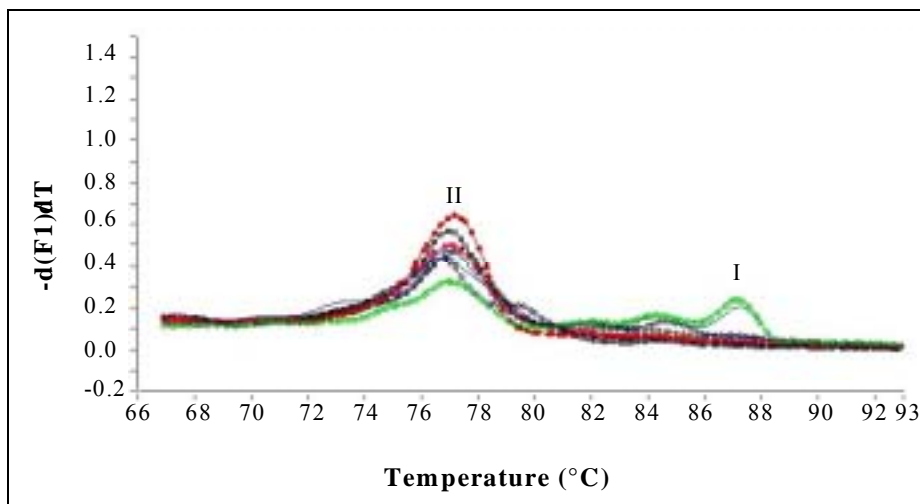
GTTGCGATCCAGGCGGGCGAGCACAAGCGCGTTCGCTGGTAGGGATCAGCGTTGCAGGGCTGC  
 ACATGTACAGTGGAGCAACGATCGAAGTAGGAGAAGACGATGGGCAGCGATGTCCTATAACTA  
 CATATACATGGCTTGCCTCGTTATGACCGTACCTTCAGAATCTTCAACTACGGATGCTTAGCTTT  
 CAAGATTGCCACTCCTGCTCTCTTACCAGCTGTTTCCACGATTGTTGCCCTCCGGAGCCTGT  
 TAGTACCGGTGTGATCGACAAGGTGCCACCTTGTCGGATCTCGATATAGGACAAATACAACCA  
 CGCTATTGTGTGGTAGCGCTCGAATTCGAACCTCCGATCCGACGAGGTCTTGCAATTCATACTG  
 GGAGCTCGTCGGGAATGCTTCCGCCCGCGTCTAGAAGTCGTGGCATCTGGTACCCTGATCCCTC  
 CCAAGTTCGACTGTCGATGTCAAGCCGGCGTTTGGAGTTTGCCTCCGCATCCAAACCTTCTCA  
**TGCTTCATGGA**ATATTCCGCTCGTTCTGCGCGGATGCAGCCcTGAGGCCAGAGGCCATGGgACG  
 nTCAAGGTCATATCAGGCAGTGAGCTTCACTAATGGCACGCAGAACAGAAAGATCCTGTCTCCTA  
 GAGTTCACGGGTTACGGGTATTGTGCAAGTTGATTAAATTCCTGTTGTCTCTGCAGGGATCGC  
 AAC

**Fig. A2** The sequences of four *Mycosphaerella graminicola*-specific fragments isolated using RAPD-PCR. The sequences of random primers are shaded. The underlined letters show the sequences of the primers R5870-1F and R5870-1R, and the fat letters show the sequence of the primer R5870-2R.

**Fig. A3** Visualization of real-time PCR products with the primer set ST-rRNA F/R and different amount of *Mycosphaerella graminicola* DNA input ranging from 0- $10^4$  pg. 20  $\mu$ l of each product are loaded on a 2% agarose gel. M is the 100 bp DNA ladder (Promega).



**Fig. A4** Visualization of real-time PCR products in the quantification of *Mycosphaerella graminicola* DNA in inoculated plants on 0-24 days after inoculation. Twenty  $\mu$ l of each product are loaded on a 2% agarose gel. M in A is the 100 bp DNA ladder (Promega) and M in B is the 100 pb DNA ladder (Roche).



**Fig A5** Melting curves of nine leaf samples collected from a fungicide-untreated control generated by real-time PCR using the primer set ST-act F/R, plotted  $[-d(F1)/dT]$  versus temperature. The curves reveal (I) target product, (II) non-specific products or primer dimers. The nine leaf samples are: two F leaves sampled on June 1 and June 14; four F1 leaves on May 3, 10, 17 and 24, three F2 leaves on May 3, 10 and 17. There were no cycle numbers obtained from real-time PCR.

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