FUNGICIDE RESISTANCE AND EFFICACY FOR CONTROL OF *PYRENOPHORA TERES* AND *MYCOSPHAERELLA GRAMINICOLA* ON BARLEY AND WHEAT

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Abstract

Barley net blotch (BNB) caused by *Pyrenophora teres*, and Septoria tritici blotch (STB) caused by *Mycosphaerella graminicola*, are destructive cereal diseases worldwide on barley and wheat respectively. Due to the lack of highly resistant cultivars, both diseases are widely controlled using fungicides. Systemic, site-specific modern fungicides have played an essential role in disease management in cereals. Triazole-based fungicides, which inhibit the C14 demethylation step in fungal ergosterol biosynthesis, known as demethylation inhibitors (DMIs) and strobilurins, known as quinine outside inhibitors (QoIs), which interfere with energy production in the fungal cell, by blocking electron transfer at site of quinone oxidation in the *cytochrome bc1* complex, are two major site-specific systemic groups of fungicides, currently used to control cereal diseases. Multiple, consecutive and extensive use of these fungicides has led to the emergence of fungicide resistance in these plant pathogens. The existence of G143A and F129L mutations has been found to be associated with resistance of many plant pathogens to QoIs. However, in *P. teres* only F129L was found to confer insensitivity. The presence of an intron in several fungi (including rusts and *P. teres*) determines that it is impossible for the G143A mutation to survive and thus be selected for. Alterations in *CYP51* gene in plant pathogens has also been found to be one of the major mechanisms resulting in reduced sensitivity towards DMIs. The aim of this research was to investigate the impact of the F129L mutation in isolates of *P. teres*, and mutations in the *CYP51* gene in *M. graminicola* isolates on the activity of QoI and DMI fungicides respectively.

Results revealed a high frequency of the F129L mutation within recent UK *P. teres* isolates. Furthermore, the common change (G143A) in *cytochrome b* was not found in *P. teres* strains. The results also showed a lack of any fitness penalty associated with the mutation. Bioassay tests indicated that inhibition of net blotch by QoIs was variable. Single QoI fungicides such as pyraclostrobin and picoxystrobin were found to be highly inhibitory whilst the efficacy of other QoIs was less pronounced. It has been found that efficacy of QoI fungicides varied amongst a population of isolates with the F129L mutation. This might suggest that some QoIs were compromised by the F129L mutation to some degree. However, the results obtained were in agreement

with previous reports that the F129L mutation in the *cytochrome b* gene generates lower levels of resistance and was not as serious as that posed by the G143A mutation in other plant pathogens. In addition, fungicide mixtures, comprising QoIs and DMIs or the novel SDHI formulations, were found to have great efficacy in net blotch disease management.

Sequence results of *CYP51* gene fragment indicated existence of 15 alterations in recent UK and German isolates of *M. graminicola*. Some of these mutations, such as Y137F, were found to be rare whilst the I381V mutation was found to be increasing with time. However, investigations indicated a lack of phenotypic fitness penalties associated with these alterations. Apical germ tube growth measurement was found an effective method to assess *in vitro* activity of DMI fungicides against *M. graminicola* isolates. Based on bioassay studies, six categories within *M. graminicola* isolates were detected, showing different sensitivities to azole fungicides. In general, genotypes characterised S, R3+ and R4 were sensitive to most azole fungicides. The R3+ variant, however, showed less sensitivity to tebuconazole and prochloraz. In *in vitro* studies, the R5 variants, exhibited sensitivity to many DMIs but were less sensitive to prochloraz. This supporting the results obtained from *in planta* assays, where this genotype was found to be sensitive to tebuconazole but less sensitive to prochloraz. On the other hand, genotypes characterised R6a, R7 and R8, containing I381V mutation, were resistant to tebuconazole but sensitive to prochloraz. The latter variant, however, were more sensitive to prochloraz. It can be suggested from results obtained in this study that *CYP51* alterations were differentially selected by different members of the azole class of fungicides.

Q-PCR was also used to evaluate *in planta* fungicide activity on both diseases. The method indicated similar pattern to that observed in visual assessments. Detection of medium to high correlation values between both assessments confirmed the validity of q-PCR assessment. This suggests that q-PCR assays may serve as an alternative method for accurate assessment of the fungicide effects on cereal diseases. The method can be a valuable tool to evaluate disease occurrence in pathogens with a long latent period, such as *M. graminicola*, as q-PCR could readily detect the pathogen during the asymptomatic latent period.

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Abbreviations

µg	.....	Microgram
µL	.....	Microliter
µm	.....	Micrometre
µM	.....	Micromole
a. i.	.....	Active ingredient
AB	.....	Alamar blue
ANOVA	.....	Analysis of variance
BLA	.....	Barley leaf agar
BMA	.....	Barley meal agar
BNB	.....	Barley net blotch
bp	.....	Base pair
BSE	.....	Barley straw extract
CDS	.....	Complete coding sequence
cm^2	.....	Square centimetre
CRD	.....	Completely randomised design
CT	.....	Cycle threshold
CTAB	.....	Cetyl trimethyl ammonium bromide
cyt b	.....	*Cytochrome b*
d	.....	Day
DAI	.....	Days after inoculations
DMIs	.....	Demethylation inhibitors
DW	.....	Distilled water
EC_{50}	.....	Concentration which inhibits growth by 50%
EDTA	.....	Ethylenediaminetetraacetic acid
EU	.....	European Union
fg	.....	Femtogram
FRAC	.....	Fungicide Resistance Action Committee
FRAG	.....	Fungicide Resistance Action Group
g	.....	Gram
h	.....	Hour
ha	.....	Hectare
HGCA	.....	Home-Grown Cereals Authority
HR	.....	High RF
L	.....	Litre
LR	.....	Low RF
LSD	.....	Least significant difference
m	.....	Metre

m^2	.....	Square metre
MBC	.....	Methyl benzimidazole carbamate
MCM	.....	Modified Czapek's medium
MEA	.....	Malt extract agar
mg	.....	Milligram
MIC	.....	Minimum inhibition concentration
min	.....	Minute
mL	.....	Millilitre
mm	.....	Millimetre
mM	.....	Millimole
mm^2	.....	Square millimetre
MR	.....	Medium RF
Mt	.....	Million tonnes
MT	.....	Mutant type
ND	.....	Not detected
ng	.....	Nanogram
nm	.....	Nanometre
NUV	.....	Near ultraviolet light
PCD	.....	Programmed cell death
PDA	.....	Potato dextrose agar
PDB	.....	Potato dextrose broth
POA	.....	Peanut oatmeal agar
Ptm	.....	*Pyrenophora teres* f. *maculata*
Ptt	.....	*Pyrenophora teres* f. *teres*
PVPP	.....	Polyvinylpyrrolidone
QoI	.....	Quinone outside inhibitor
q-PCR	.....	Quantitative PCR
r	.....	Correlation
R^2	.....	Coefficient of determination
RF	.....	Resistance factors
s	.....	Second
SASA	.....	Science and Advice for Scottish Agriculture
SBI	.....	Sterol biosynthesis inhibitor
SDHI	.....	Succinate dehydrogenase inhibitors
SDW	.....	Sterilised distilled water
SNP	.....	Single nucleotide polymorphism
SRS	.....	Substrate recognition site
STAR	.....	Strobilurin-type action and resistance

STB	.....	Septoria tritici blotch
TAG	.....	The Arable Group
TBE	.....	Tris-Borate-EDTA
Tm^{o}C	.....	Temperature in Celsius
TPA	.....	Tomato paste agar
U	.....	Unit
USDA	.....	United States Department of Agriculture
UV	.....	Ultra violet
V8JA	.....	V8 juice agar
WT	.....	Wild type

Chapter 1 General Introduction

1.1 Cereal crops

1.1.1 Barley

Barley (*Hordeum vulgare* L.) is an important cereal grain crop which ranks fifth globally among all crops in dry matter production. It comes behind maize (*Zea mays*), wheat (*Triticum aestivum*), rice (*Oryza sativa*) and soybean (*Glycine max*) and ahead of sugarcane (*Saccharum officinarum* L.), potato (*Solanum tuberosum* L.) and sorghum (*Sorghum vulgare* Pers.) (FAO, 2007). Barley together with wheat, pea (*Pisum sativum*) and lentil (*Lenis culinaris*) was one of the first crops domesticated from about 10,000 years ago, in the fertile Crescent of the Middle East (Harlan and Zohary, 1966; Smith, 1998). With the expansion of agriculture, cultivated barley had reached the Nile Valley in fifth millennium B. C. (Darby *et al.*, 1977) and then reached the highlands of Ethiopia (Lakev *et al.*, 1997). At the same time it was expanded to the eastern direction to the Caucasus and Transcaucasia regions (Lisitsina, 1984) and the highlands of Indian subcontinent (Costantini, 1984). The cultivation of barley further expanded to the western parts of the Mediterranean basin in fourth millennium B. C. (Hopf, 1991) and the Balkans and Northern Europe in the third millennium (Korber-Grohne, 1987).

The first utilisation of barley was thought to be as human nutrition (Fischbeck, 2002) but after the dominance of wheat and rice as alternatives, it changed later into a feed, malting and brewing grain. It is, however, a major food source for some cultures in areas of North Africa, the Near East in the highlands of central Asia and the Horn of Africa (Newman and Newman, 2006). Currently in the UK it is used mainly for brewing purposes (HGCA). Barley was well-known for its benefits as a source of energy and for maintaining health (Percival, 1921). The main advantage of incorporating barley in diets nowadays is due to its potential health benefits. Lowering of blood cholesterol, with b-glucans (Behall *et al.*, 2004), and the glycemic index (Cavallero *et al.*, 2002) by barley has been reported widely (Pins and Kaur, 2006).

Barley, the most genetically diverse cereal grain, is classified in to spring or winter types, two-row or six-row, hulled or hulless by presence or absence of a hull tightly adhering to the grain, and malting or feed end-use type (Baik and Ullrich, 2008). However, two-row ear types and hulled kernels characterize the early forms of cultivated barley (Zohary and Hopf, 1993).

The estimated world barley production in 2008/09 was 156 million tonnes (Mt), which is considered the highest on record. This peak was primarily due to the increase in area sown in Canada (HGCA). Barley production in 2008, compared to the previous years, increased in most of the main EU barley-producing member states. The majority of this increase was in the UK, France and Germany. However, the major UK competitor for the barley, Denmark experienced lower production due to the dry weather. According to the United States Department of Agriculture (USDA), estimated EU barley production in 2010 was 53,398 Mt (Figure 1.1) while in 1999 was 59,936 Mt, a decrease of 10.9% (Figure 1.2). In the UK, barley production was estimated in 2009 to be 6.2 Mt, but in 2010 barley production decreased by 22% to 5.2 Mt. This is primarily due to the reduction in the planted area of spring barley (Anonymous, 2010a).

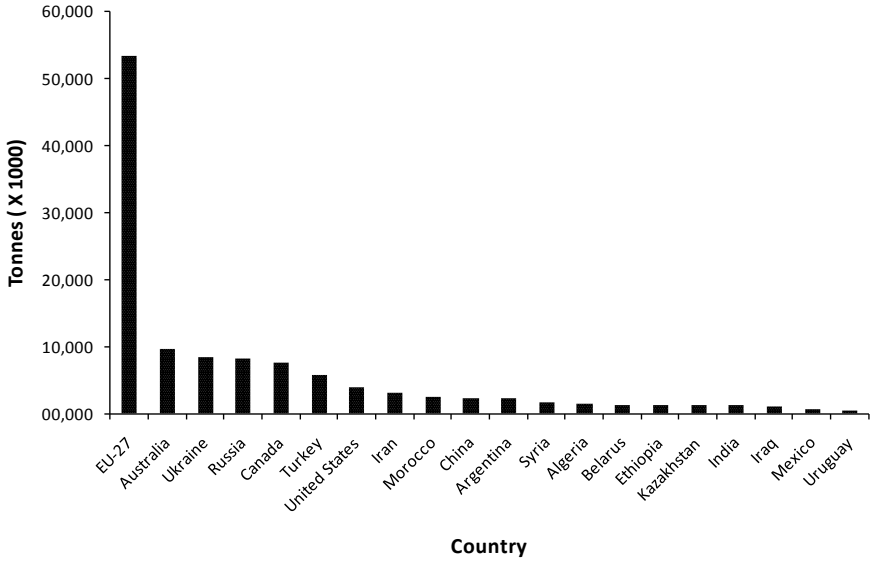


Figure 1.1 Barley production in 2010 by country - data from USDA.

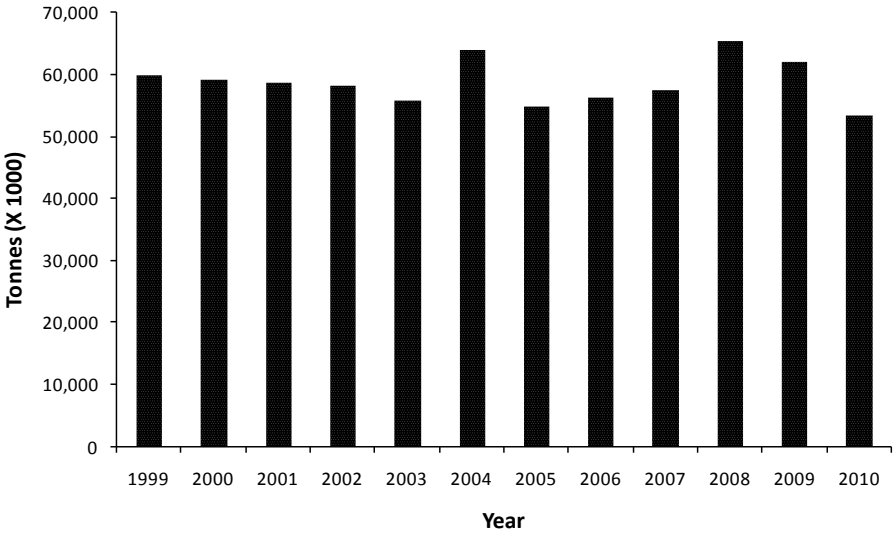


Figure 1.2 Barley production over the past 11 years in EU-27 - data from USDA.

1.1.2 Wheat

Wheat is a grass which belongs to plant family Graminae and is native to arid countries of western Asia (Cornell and Hoveling, 1998). The first primitive wheat (einkorn and emmer types) was harvested and cultivated in the so-called Fertile Crescent of south-western Iran, north-eastern Iraq, and south-eastern Turkey (Kurdistan) (Figure 1.3), where wild wheats can still be found growing. The domestication of wheat began with wild-types and then gradually shifted to the present day durums, club wheats and common wheats (Cook and Veseth, 1991). It is believed that the domestication of these varieties for use in agriculture in the UK dates to 6000 years ago.

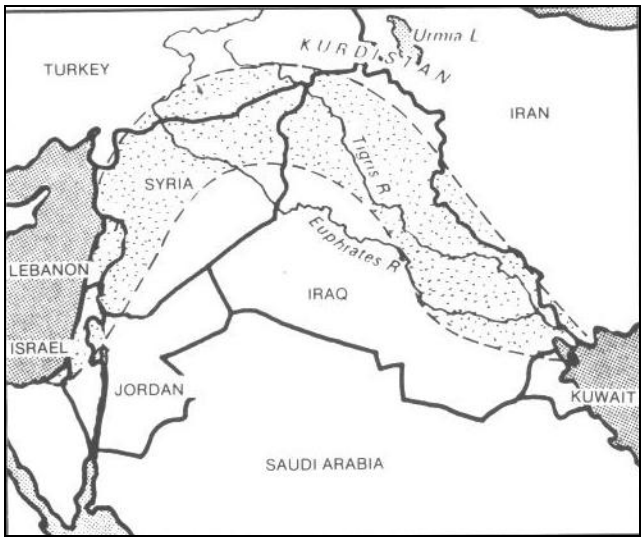


Figure 1.3 Fertile Crescent region, where wheat was first cultivated. Picture from Cook and Veseth (1991).

Within all cereal crops wheat has the widest adaptation globally. It is grown in some 100 countries around the world, starting from as far north as Finland to as far south as Argentina. The heaviest concentration is, however, located between the 30th and 60th latitudes in the temperate zone of the northern hemisphere, which includes the major wheat growing areas of North America, Europe, Asia and North Africa. There is also a lesser concentration area located between the 27th and 40th latitudes south, mainly in Australia, Argentina, Brazil and South Africa (Oleson, 1994).

The majority of varieties cultivated today are grouped together within a broad category called common or bread wheat, which accounts for approximately 95% of world production. Most of the remaining 5% of cultivated varieties are durum wheats (Oleson, 1994). There are variable classifications applied to wheat, depending on agronomic properties and the usage of the crop. Based on the suitability for baking bread, wheat is divided into two classes, hard and soft. Hard kernel is characterized for hard wheat that yields flour with high starchy gluten and protein and this is suitable for producing western style breads and some types of noodles. Soft wheat, on the other hand, has a lower protein and gluten level and is mostly dedicated for producing cakes, and biscuits, which do not require strong flour. Wheat-based foods, for many, are the major sources of energy, protein, vitamins and minerals. In some

societies, wheat-based foods supply two-thirds or more of the daily caloric intake (Ranhotra, 1994). Wheat alone will not provide all the essential amino acids in the amounts needed for proper growth and maintenance of good health and must be enriched with a small quantity of leguminous or animal protein. However, whole-wheat flour is a good source of fibre, especially water-soluble fibre, vitamins and minerals (Ranhotra *et al.*, 1990).

Wheat is the most popular crop which is widely grown, traded and consumed worldwide (Oleson, 1994). Wheat participates in nearly 35% of the staple food of the world population, contributing 28% of dry matter as the edible food and up to 60% of the daily calorie intake in several developing countries (FAOSTAT 2008; http://faostat.fao.org). Thus, wheat has a significant impact on human health in giving nutritional quality. Wheat is also used as a feed grain for poultry and for many classes of livestock, as alternate to maize. However, the usage of wheat for feed is variable depending on the price relationship between wheat and other feed grains and quality of the wheat in the given year. In account of this, it is estimated that nearly two-thirds of the wheat produced in the world is used for food; the remainder is used for feed, seed and non-food applications (Ranhotra, 1994). Although, since the green revolution, global cereal yields have increased dramatically, insufficient is provided to satisfy the global requirement (Welch and Graham, 2004). Furthermore, the demand for wheat is growing faster than any other crop, because of the ever-increasing global population. Fortunately, one of the remarkable achievements of the 20th century was the growth of world wheat production. Since 1960, the increase in world wheat production has been faster than population growth. In the time the world population nearly doubled, from 3 billion in 1960 to 5.3 billion in 1990, wheat production has almost tripled from the 1960 level. This steady growth of world wheat production from 1960 to 1990, ranging from a low of 225 Mt to 593 Mt in 1990, resulted in an increase in wheat production of over 100 Mt per decade (Oleson, 1994). World wheat production in 2009 was 681.9 Mt (Anonymous, 2010c). The anticipated global demand by the year 2020 will reach between 840 Mt (Rosegrant *et al.*, 1995) and 1050 Mt (Kronstad, 1998). The challenge of 21st century is to produce 70% more food to meet the demand of the increased population at a time of implementing more sustainable methods and

adaption to climate change (Tilman *et al.*, 2002). A major concern for feeding the world in 2050 relates to slower increases in yields of major cereal crops over the past three decades (Alston *et al.*, 2009). For instance, annual increase of wheat yield is declining and is now just below 1% (Fischer *et al.*, 2009).

Due to suitable soils and climate, wheat has become of primary importance within cereals grown in the UK (Figure 1.4). The domination of wheat became a phenomenon since farmers began large scale intensive production (Cook and Hardwick, 1990). This was accompanied by extensive mono-cropping of a few particularly high yielding cultivars, often associated with resistance to a single important disease (Yarham and Giltrap, 1989). The annual UK wheat production is around 15 Mt and nearly 25% of this is exported to countries around the world. The UK wheat production for the year 2010 was estimated 14.8 Mt, an increase of 5% over 2009 (Anonymous, 2010a).

Wheat in the UK is sown in either autumn or spring, albeit both sowing times being harvested in August (with the exception of Scotland which is harvested one month later). However, the autumn sowing is dominant and this is primarily because the temperate climate of the UK allows the plant to grow through the winter and produce a higher yield compared to spring sowing (Anonymous, 2011b).

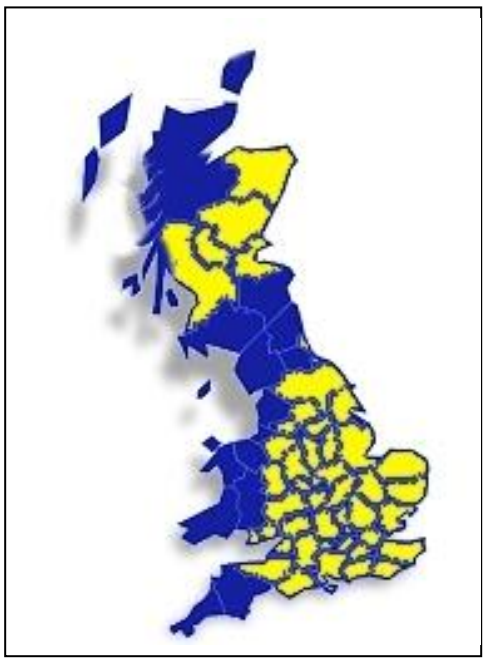


Figure 1.4 Wheat growing areas (yellow) in the UK
(From: ukagriculture.com)

1.2 Cereal diseases

Cereals are vulnerable to many biotic attacks including those by fungi, bacteria, viruses, nematodes and insects. Considerable reductions in grain yield and quality results from the damage they generate. The major threats to human food and crop production, since agriculture became the main source of human food supply, are from yield losses caused by plant pathogens. Depending on the nature of the pathogen and the severity of the attack, the extent of the damage is varied. An earlier study by Jones and Clifford (1983) estimated an annual reduction in yield of about 12% on a world basis. Many necessary and desirable changes in agricultural practices have participated in changes to the status of various diseases. For instance, monocultures or cropping systems with a small number of crop components are susceptible to abiotic (weather, soil conditions, etc.) and biotic (diseases, insects, etc.) stresses (Tanaka *et al.*, 2002). The highly simplified nature of these cropping systems often allows the best adapted pest species to multiply. This phenomenon, in particular, applies to leaf diseases of cereal crops, where noticeable annual losses from epidemics are developing (Barnes, 1964; Oerke *et al.*, 1994; Bockus *et al.*, 2001; Murray and Brennan, 2010).

Barley is vulnerable to many diseases on different parts of the plant, as summarised in Table 1.1. In addition, there are many abiotic stresses due to nutrient deficiencies and extreme environmental conditions causing stunning, uneven growth, abnormal patterns of colour on leaves and stems and poor yield (Neate and McMullen, 2005).

Table 1.1 Major barley diseases.

Disease name	Pathogen name
Net blotch	*Pyrenophora teres*
Spot blotch	*Bipolaris sorokiniana*
Stagnospora leaf blotch	*Stagnospora avenae* f. sp. *triticea*
Speckled leaf blotch	*Septoria passerinii*
Scald	*Rhynchosporium secalis*
Stem rust	*Puccinia graminis* f sp. *hordei*
Leaf rust	*Puccinia hordei*
Loose smut	*Ustilago nuda*
Covered smut	*Ustilago hordei*
Powdery mildew	*Blumeria graminis* f. sp. *hordei*
Head blight	*Fusarium* spp.
Ergot	*Claviceps purpurea*
Bacterial blight	*Xanthomonas transluscens* pv *transluscens*
Barley Yellow Dwarf Virus	BYDV
Common root rot	*Cochliobolus sativus*
Take-all disease	*Gaeumannomyces graminis* var *tritici*
Pythium root rot	*Pythium* spp.

Wheat is also susceptible to many biotic and abiotic diseases and disorders. Common diseases are summarised in Table 1.2. Disorders associated with nutrient deficiencies and extreme environmental conditions are as described for barley diseases (Prescott *et al.*, 1986; Duveiller *et al.*, 1997).

Table 1.2 Major wheat diseases.

Disease name	Pathogen name
Powdery mildew	*Blumeria graminis* f. sp. *tritici*
Septoria tritici blotch	*Mycosphaerella graminicola*
Septoria nodorum blotch	*Leptosphaeria nodorum* (*Stagonospora nodorum*)
Fusarium seedling blight	*Fusarium graminearum*
Seedling blight and foot rot	*Cochliobolus sativus*
Ergot	*Claviceps purpurea*
Tan spot	*Pyrenophora (Drechslera) tritici-repentis*
Eyespot	*Oculimacula acuformis*, *O. yallundae*
Take-all	*Gaeumannomyces graminis var. tritici*
Brown rust	*Puccinia triticina*
Stem rust (black rust)	*Puccinia graminis* f. sp. *tritici*
Stripe (yellow) rust	*Puccinia striiformis*
Common (bunt) smut	*Tilletia caries*, *T. foetida*, and *T. controversa*
Loose smut	*Ustilago tritici*
Flag smut	*Urocystis agropyri*
Fusarium head blight	*Fusarium* spp.
Barley yellow dwarf virus	BYDV
Leaf streak	*Xanthomonas translucens* pv. *undulosa*
Leaf blight	*Pseudomonas syringae pv. syringae*

Adapted from: The wheat disease management guide 2010 (HGCA).

1.2.1 Barley net blotch (BNB)

1.2.1.1 Importance

Net blotch of barley, caused by the ascomycete fungus *Pyrenophora teres* (Anamorph *Drechslera teres*), is one of the most important diseases, causing yield losses in all barley growing regions of the world (Wilcoxson *et al.*, 1992), occurring wherever the crop is grown in the temperate, humid regions (Dickson, 1956; Smedegard-Petersen, 1976). It was widely distributed in western Europe in the 1970s and early 1980s, where it caused severe yield losses (Skou and Haahr, 1987). It was an increasingly important pathogen in the UK during the eighties, particularly in the south-west of England (Jordan, 1981). Many countries have reported an increased incidence of the disease in the last decades, caused partly by the more common practice of growing

barley repeatedly in the field. In France, the disease reached epidemic proportions in 1992 resulting in yield losses of 15-25 % (Albertini *et al.*, 1995). An increased prevalence of the disease has also been reported in several North African and Middle Eastern countries (Douiyssi *et al.*, 1996). Mathre (1982) stated that losses due to this disease neared 100% in some highly susceptible barley cultivars, but losses ranging between 10-40% are more common. In Latin America, surveys from 1990-2000 have revealed that net blotch was the most important barley disease in Argentina, causing average losses of 20% (Carmona *et al.*, 1999). However, under suitable environmental conditions losses can reach up to 100%. Yield losses in susceptible cultivars can be up to 40-45 % (Steffenson *et al.*, 1991; Kashemirova, 1995). In Finland, net blotch is the most damaging disease in southern coastal areas of the Arctic Circle (Makela, 1975).

1.2.1.2 Taxonomy

Pyrenophora teres, the pathogen of net blotch of barley, is classified as follows (Liu *et al.*, 2011):

Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Dothidiomycetes
Order	Pleosporales
Family	Pleosporaceae
Genus	Pyrenophora
Species	teres
Form	teres

The perfect stage, *Pyrenophora teres*, was first described by Drechsler (1923). The imperfect stage is *Drechslera teres* (Sacc.) Shoem. (syn.: *Helminthosporium teres* Sacc.). The pathogen was known as *H. teres* until the late 1950s when the genus *Helminthosporium* was subdivided into *Dreschlera* and *Bipolaris* based on spore morphology (Shoemaker, 1959). *Pyrenophora teres* was subsequently subdivided into two forms by Smedegard-Petersen

(1971) based on the distinct disease symptoms produced on barley. *Ppyrenophora teres* f. *teres* (Ptt) produces the classic net-type symptoms while *P. teres* f. *maculata* (Ptm) causes spot-type lesions (Figure 1.5). The latter form was first recorded as a different species called *P. japonica* (Ito and Kuribayashi, 1931). However, after successful mating between *P. teres* and *P. japonica* by both Mcdonald (1967) and Smedegard-Petersen (1971) it was concluded that they represented the same species. Although there is evidence of recombination between net- and spot-type of *P. teres* isolates in the field (Campbell *et al.*, 2002), traditional methods to describe the differences have been overcome by using the molecular methods, which can distinguish both sub species easily. In an assay done by Leisova *et al*. (2005), AFLP-based PCR markers have been used successfully to distinguish between both sub-species of *P. teres*.

Figure 1.5 Net-like symptoms (top) caused by *P. teres* f. *teres* and spot type symptoms (bottom) caused by *P. teres* f. *maculata* (Beattie, 2006).

1.2.1.3 Life cycle

Pyrenophora teres is considered to be a seed-borne and a stubble-borne pathogen. It was believed that the seed-borne inoculum was the most frequent source of infection in Britain (Webster, 1951). However, Piening (1961) reported that the ascospores produced on the straw caused at least

half of net blotch infections which occurred in Alberta, western Canada. Many authors have since reported the importance of infected seeds as well as infected plant debris from the previous season in contributing to establishing the disease (Shipton *et al.*, 1973; Hampton, 1980; Carmona *et al.*, 2008; Nakova, 2009). In the UK, seed-borne inoculum is usually much less important than infected stubble, though infected seed can start early foliar epidemics which may damage yield (HGCA).

The net blotch pathogen *P. teres* has two life stages (Figure 1.6). The asexual state, which produces conidia (Figure 1.7), has a major role in initiation and spread of the disease and the sexual state associated with the formation of a pseudothecium, occurs in the late summer or the beginning of autumn before overwintering, leading to the subsequent release of ascospores (Piening, 1968; Shipton *et al.*, 1973).

During the growing season of barley, disease spread occurs by water splash, with droplets holding conidia causing new infections in humid conditions with temperatures ranging between 20-30^{o}C (Keon and Hargreaves, 1983). The latent period varies from 5 to 11 days (Peever and Milgroom, 1994). This short period leads to multiple-infections during the growing season. Although conidia have a limited viability (3 months) in plant debris, the pathogen can survive as a mycelium for up to 15 months (Shipton *et al.*, 1973). Thus, infected plant residue in the field is considered to be the primary source of inoculum in the following years, when the seed-borne infections are eliminated by seed-dressing fungicides.

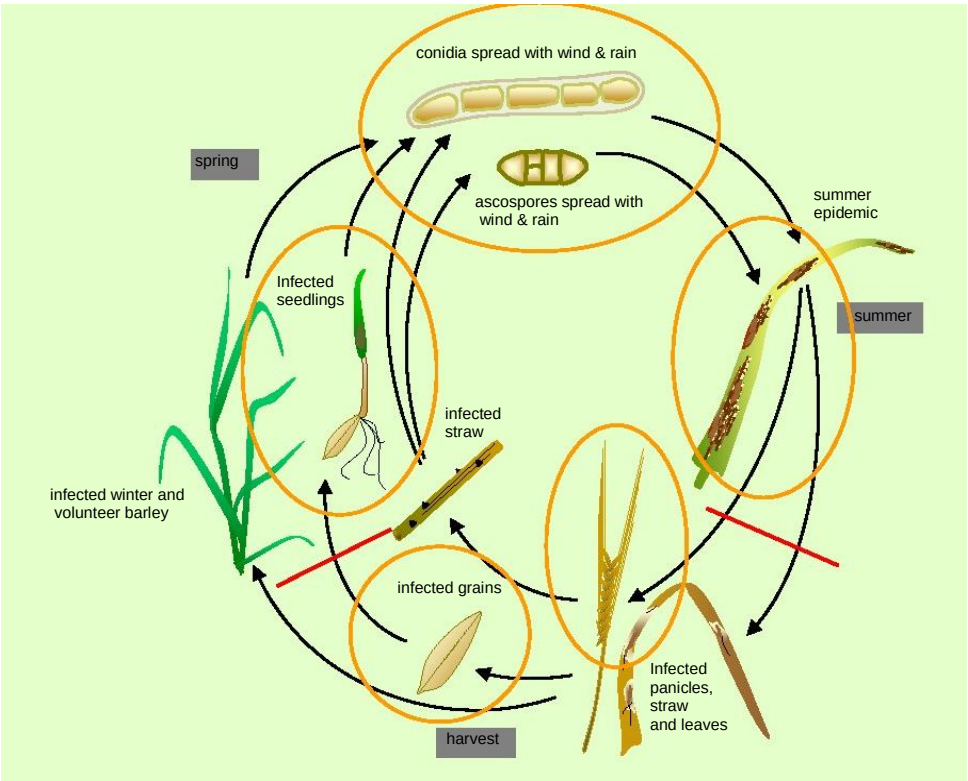


Figure 1.6 Life cycle of *P. teres* explaining initiation and spread of net blotch disease of barley. From: Jorgensen *et al*. (2004).

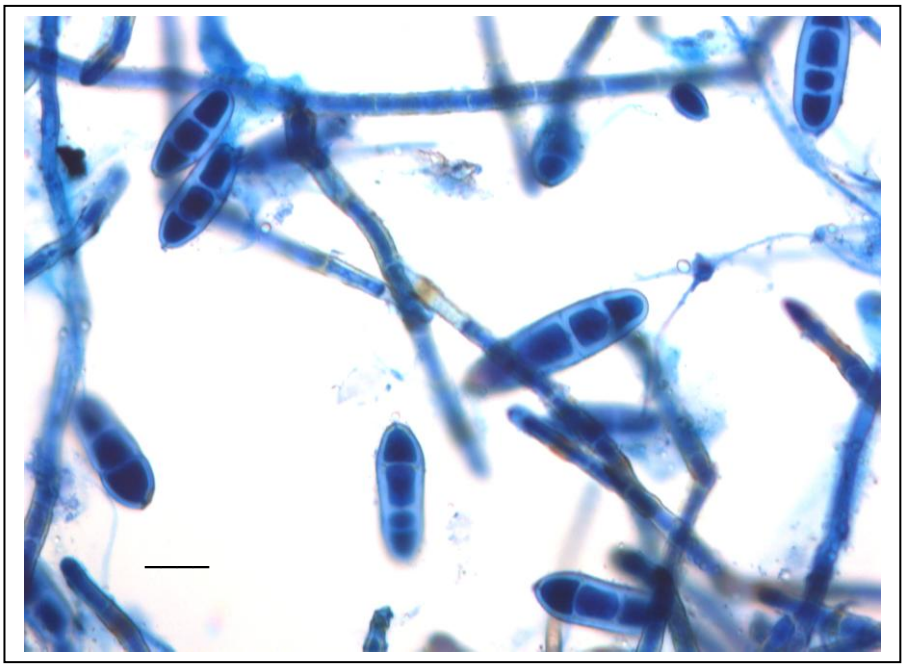


Figure 1.7 Conidia from *P. teres*, the asexual state of the fungus which spreads the disease during growing season (scale bar = 40 µm).

1.2.2 Septoria tritici blotch (STB)

1.2.2.1 Importance

Coalescence analysis of pathogen DNA sequence data indicates that *Mycosphaerella graminicola* (Fuckel) J. Schorot. in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz.), an important pathogen of wheat worldwide, emerged about 10500 years ago during the domestication of wheat in the Fertile Crescent of the Middle East, from an ancestral population which still exists and has a wide host range (Stukenbrock *et al.*, 2007). STB caused by *M. graminicola* is the most economically important foliar disease of wheat in the UK (Polley and Thomas, 1991; Hardwick *et al.*, 2001), France (Halama, 1996) and many other north western European countries with a temperate climate (Eyal, 1999). The disease is also reported worldwide in epidemic form in moist regions of South America, the Mediterranean basin, Africa, Asia and Australia (Serivastava and Tewari, 2002). Worldwide more than 50 million hectares of wheat, mainly grown in high rainfall areas, are affected (Gilchrist and Dubin, 2007). The economic losses, due to this disease, in the UK in 1998 were estimated at £35.5 million (Hardwick *et al.*, 2001). In epidemic occurrences the yield losses can reach 30-40% (Eyal, 1999; Palmer and Skinner, 2002). Similar losses also have been reported previously worldwide. In California, USA, yield losses ranging from 19 to 33% were reported (Brownell and Gilchrist, 1979). Losses from 21 to 37 % (Kraan and Nisi, 1993) and 20 to 50% have been detected in Argentina (Annone *et al.*, 1991). In other countries, yield reductions range from 31 to 54% (Eyal *et al.*, 1987), from 10 to 45% (Caldwell and Narvaes, 1960) and even more than 60% have been reported (Shipton *et al.*, 1971; Forrer and Zadoks, 1983; King *et al.*, 1983).

1.2.2.2 Taxonomy

Several amendments to the taxonomy and nomenclature of *Septoria* and *Stagonospora* have been made by many workers during the last four decades and not all researchers working on these fungi use the recent nomenclature. Therefore, the participants of the Fourth International Workshop on *Septoria* on cereals made suggestions to accept the most recent taxonomy of the fungus and to urge plant scientists to use the proper taxonomy and

nomenclature in research and other types of publications (Cunfer, 1997). *Mycosphaerella graminicola* is the teleomorph (sexual state) of *S. tritici* on wheat (Sanderson, 1976). It is the imperfect or conidial state (asexual state) which survives on wheat debris from previous season (Brokenshire, 1975). However, the current taxonomy status of the sexual state of *S. tritici* is as follows:

Kingdom: Fungi
Phylum: Ascomycota
Class: Loculoascomycetes
Order: Dothidiales
Family: Dothidiaceae
Genus: Mycosphaerella
Species: graminicola

1.2.2.3 Life cycle

Mycisphearella graminicola survives through the summer on residues of a previous wheat crop and initiates infections in the autumn (Holmes and Colhoun, 1975; Brown *et al.*, 1978; Serivastava and Tewari, 2002). There is some evidence that the fungus is able to survive in association with other grass hosts and wheat seed (Sprague, 1950; Prestes and Hendrix, 1977; Krupinsky, 1997)). These sources of the fungus are probably most important when wheat residues are absent. Regardless of rotation or residue management practices, there is usually enough inoculum to initiate autumn infections (Duczek *et al.*, 1999). Primary inoculum, as ascospores produced in pseudothecia, arises from infected crop debris (Sanderson and Hampton, 1978). It was shown to have an important role in establishment of epidemics during the months of August to October in the northern hemisphere and February to April in the Southern Hemisphere (Shaw and Royle, 1989; Arseniuk *et al.*, 1998). Local secondary infections primarily originate from the anamorphic conidia or pycnidiospores during the growing season, which are disseminated mainly by rain splash.

STB is favoured by cool, wet weather. The optimum temperature range is 16 to 21^{o}C (Eyal, 1971; Holmes and Colhoun, 1974). However, infections can

occur during the winter months at temperatures as low as 5°C. Infection requires at least 6 to up to 48 h of leaf wetness for maximum effect. Once infection has occurred, the fungus takes 21 to 28 d to develop the characteristic black fruiting bodies and produce a new generation of spores. The spores produced in these fruiting bodies are exuded in sticky masses and require rain to splash them onto the upper leaves and heads (De Wolf, 2008). Eyal *et al*. (1987) described the symptoms of leaf blotch on wheat leaves as irregular chlorotic lesions that usually appear 5-6 d after inoculation. However, the time of first expression is highly dependent on the cultivar and environmental conditions prevailing during the infection process. Three to six days later, at 18-24°C and high relative humidity, necrotic lesions develop at the chlorotic sites. Conidia formation occurs usually after 15 d on either upper or lower surfaces of the leaves. Pycnidiospores can be viable on infested debris for several months (Hilu and Bever, 1957). The overall *M. graminicola* life cycle is illustrated in Figure 1.8.

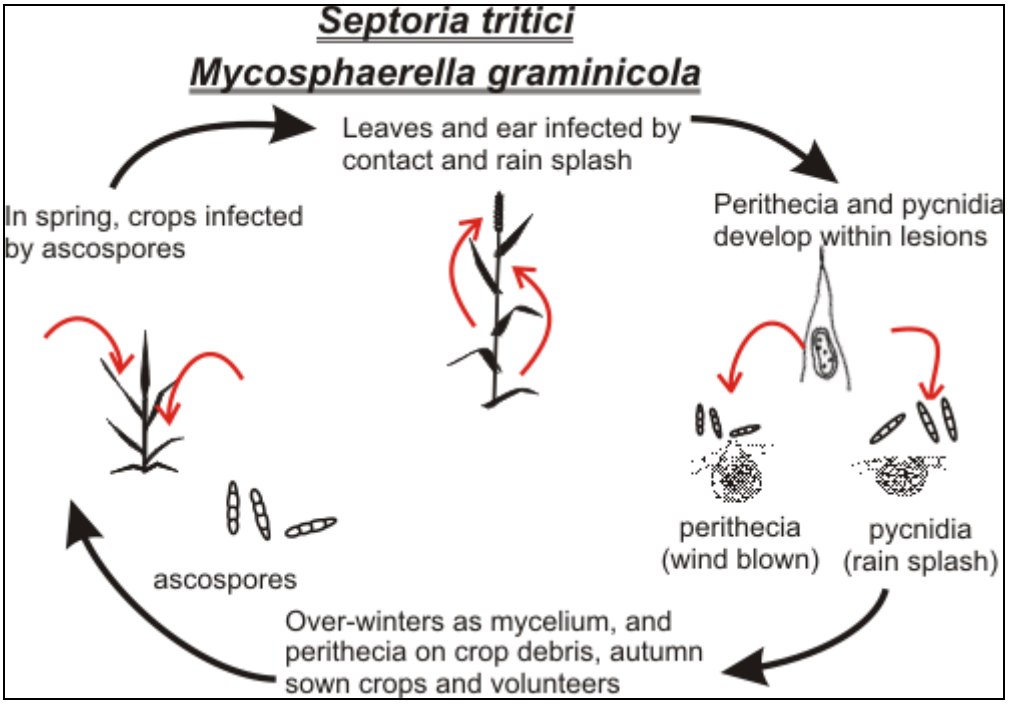


Figure 1.8 The life cycle of *M. graminicola* illustrating initiation and spread of the leaf blotch pathogen (www.hgca.com).

1.3 Disease management in cereals

1.3.1 Cultural practices

Cultural practices which include sanitation, tillage, crop rotation and change of sowing date, are considered key components in disease management. Sanitation is the process by which the initial inoculum from which epidemics start, is reduced, excluded or eliminated (VanderPlank, 1963). Sanitation by removal of infected crop material from the field, is one method to reduce inoculum and to prevent pathogen dissemination (Conway, 1996). Burying plant residue using tillage, although often contradictory to the benefit of moisture retention, is sometimes used as a method of sanitation to reduce disease (House and Brust, 1989). Additionally, incorporation of residues into soil often stimulates microbial activity that, in turn, biologically suppresses pathogen activity. Survival of many pathogens in the soil is a problematic issue in the management of many plant diseases. With *Colletotrichum acutatum*, leather leaf fern anthracnose, survival of conidia and sclerotia declined rapidly where infected leaf debris was buried in soil (Norman and Strandberg, 1997). In cereal pathogens such as *P. teres*, after about 9 months in the field, the inoculum produced on straw was still found capable of initiating net blotch of barley (Piening, 1968). Reduction of soil water, used for mobility of certain inocula can, for some pathogens, reduce the severity of the disease. For instance, in root disease caused by *Pythium* spp., which utilize water for zoospore movement, reduction of irrigation often lowers the severity of the disease (Kerr, 1964). Composting of plant residues is another method to eliminate the viability of plant pathogenic fungi and bacteria. This method was used by Suarez-Estrella *et al*. (2007) as a useful tool for recycling plant waste and eliminating phytopathogenic bacteria and fungi on vegetable residues.

Crop rotation is a natural mean of controlling plant pathogens (Cook, 1986). The occurrence of disease caused by fungi or bacteria can be reduced by growing unrelated crops and therefore avoiding an increase in pathogen inoculum in crop residues. For instance, in BNB, crop rotation with two seasons between barley crops would provide a degree of control of the disease (Shipton *et al.*, 1973). Turkington *et al*. (2005) have also reported that *P.*

teres disease severity was highest and yield lowest when barley was grown on its own residue, when compared to barley crops grown in rotation. They concluded that crop rotations, with alternative crops such as triticale, were a good strategy. Based on this principle, farmers in the UK sow a break crop (usually oilseed rape) every 4-5 years. Although this is a long way from traditional crop rotation programmes, it was found effective, causing considerable reduction of some cereal diseases, such as take-all of wheat caused by *Gaeumannomyces graminis.* However, a similar procedure may not apply to eyespot, caused by *Oculimacula yallundae* because it can survive for at least three years on straw on soil. However, by practicing crop rotation, inocula of pathogens surviving on crop residues can be reduced when the residues are buried in the soil. Degradation of litter by saprophytic microorganisms will deprive the pathogen of a food source (Carlile, 1998).

Sowing date also has a major impact on disease development. Early autumn sowing of cereals may allow infection of newly emerging crops from debris carrying diseases from a previous cereal crop. Cereal diseases such as septoria leaf spot of wheat, leaf blotch and scald of barley, barley yellow dwarf virus and eyespot of winter cereals may by readily transmitted to crops emerging in late August and early September (Carlile, 1998). This is reflected in the fact that net blotch of barley is a major pathogen of autumn-sown crops, but not an issue with spring-sown ones.

1.3.2 Host resistance

The development of disease-resistant plants is the most preferred method to combat plant pathogens. The method can minimize fungicide applications or even eliminate their use (Carlile, 1998). Plants organize multiple strategies to defend themselves against pathogen attack. The use of disease-resistant cultivars, instead of susceptible ones, will modify the disease triangle relationship and reduce the amount of disease developing in a crop (Conway, 1996). The method is considered one of the most effective and environmentally safe means in controlling cereal pathogens (Ali *et al.*, 2008). Plants have a range of defence mechanisms which are rapid and efficient against a wide variety of pathogens including bacteria, fungi, viruses and

nematodes. Plant defence mechanisms have been reviewed recently by Jones and Dangl (2006). One of the most common defence mechanisms against pathogen attack is the hypersensitive response, the rapid and localised programmed cell death (PCD) at the site of infection (Hammond-Kosack and Jones, 1996).

Inheritance of resistance in barley net blotch was found to occur in a Mendelian fashion in the very early studies of Geschele (1928). Genes providing incompletely dominant resistance, effective against *P. teres* isolates, were described by Schaller (1955) and Mode and Schaller (1958) in California, USA. However, based on later intensive studies and the accumulation of information on host resistance, durable resistance to this pathogen could be conferred by multiple resistance genes (Douiyssi *et al.*, 1998). Most single resistance sources were overcome by known pathotypes/biotypes (races) of the pathogen. Such resistance breakdown, due to virulence phenotype changes in the pathogen population, is more likely to happen when one or a few resistance genes are deployed over large areas (McDonald and Linde, 2002). Thus, the availability of germplasm with broad resistance to multiple diseases is important to the success of crop improvement programmes (Polak and Bartos, 2002). Incorporating multiple resistance genes would make breeding for resistance more complicated (Wolpert *et al.*, 2002). Therefore, before breeding for durable resistance can be successfully undertaken, more information is required on the virulence of the pathogen and susceptibility of the host (Liu *et al.*, 2011).

Although resistance genes effective against wheat pathogens causing leaf spot diseases such as STB and tan spot have been introduced (Adhikari *et al.*, 2004b; Singh and Hughes, 2005), the majority of wheat cultivars currently grown are susceptible to fungal leaf spot diseases (Singh *et al.*, 2006). Some resistance genes have been found to enhance wheat cultivar resistance to STB (Somasco *et al.*, 1996). Although some of these genes have remained effective for 15-25 years, reports have confirmed breakdown of some of them (Jackson *et al.*, 2000). The use of resistant varieties is, however, the least expensive, easiest, and safest and one of the most effective means of

controlling crop disease. Good management measures are required to prolong the resistance as long as possible.

1.3.3 Chemical control using fungicides

Since other disease control measures are inadequate to suppress pathogens sufficiently and cannot overcome yield losses alone, the use of chemicals is essential. Growers, therefore, often elect to use pesticides, although it increases the costs of cultivation and may raise environmental concerns. Chemical applications can be used in controlling fungal diseases, and some bacterial diseases, but little success has been obtained in controlling viruses (Baldwin and Rathmell, 1988); although control of the vector may sometimes provide indirect control. Antibiotics have been used, on rare occasions, to control some sensitive phytoplasmas (Davis and Whitcomb, 1971).

Fungicides have been used for many years to protect plants. The first uses were to protect major cereal crops and grapevines. Since the Second World War, a huge increase has taken place in the number of crops and crop diseases treated, the diversity of chemicals available, the purpose and the frequency of their use, and the potential of treatments. The emergence of fungicides has contributed to enhance improvements in quality and quantity of agricultural products (Oerke *et al.*, 1994). The lack of disease resistance against pathogens in many cereals, such as wheat, has led to use of fungicides as a major measure to manage the diseases (Verreet *et al.*, 2000).

Materials used as fungicides in the early years of application were naturally occurring compounds such as chalk, wood ash and sulphur. Those compounds were non-selective, persistent and toxic to many forms of life (Campbell, 1989). Copper and lime sulphur compounds, first produced in the 1800s, became commonly used on vegetables, fruits and ornamental plants and were preferred fungicides for control of mildew in England. Lime sulphur and copper compounds are still active and broadly used to protect crops. Later, compounds based on mercuric chlorides emerged to control soil-borne pathogens in the 1860s. In the 1900s, another generation of non-selective fungicides emerged from products of coal gas production or other industrial

processes, such as nitrophenols (Fent and Hunn, 1996), chlorophenols (Kahkonen *et al.*, 2007) and petroleum oil (Gupta, 2008) which were, unfortunately, also toxic to both users and non-target organisms (HaghighiPodeh and Bhattacharya, 1996). In 1930s, with the advent of methyl bromide used as a fumigant in France (Krikun *et al.*, 1974) and the introduction of pentacholorophenol as a wood preservative (Carey and Bravery, 1989), the modern era of synthetic organic fungicides began to take steps against fungi. However, their physicochemical properties and persistence in use determined they would eventually become an environmental hazard (Galassi *et al.*, 1996; Calvert *et al.*, 1998). After that, efforts to develop new chemicals with reduced persistence and environmentally friendly properties were initiated. Compounds such as benzimidazole, 2-amino-pyrimidines, carboxanilides, phosphorothio-lates, morpholines dicarboximates, and ergosterol demethylation inhibitors (DMIs) were introduced in 1960s and 1970s with more efficacy, followed by improvements of their properties later in the 1980s (Anonymous, 2002). The outcome was the development of a number of novel fungicides which were generally used in relatively small amounts due to their more potent action against plant pathogens. The new commercial fungicides launched were phenylpyroles, anilinopyrimidines, quinone outside inhibitors (QoIs) (also called strobilurins), benzamides and carboxylic acid amides (Gullino *et al.*, 2000). Consequently, systemic, single-site fungicides, since their introduction in the 1960s, have gradually replaced the older non-systemic, multi-site compounds, establishing higher levels of disease control.

Fungicides can be divided into several groups or classified in different ways. Important distinctions made are between single- or multi-site modes of action and between molecules with protectant and eradicant activities. Protectant (contact) fungicides (Table 1.3), which protect host plants against pathogens by acting against the inoculum landing on the surface, and which normally do not enter the plant and affect established infections, must be applied before penetration of the pathogen into the host plant.

Table 1.3 Main groups of protectant fungicides, with examples and mode of action.

Type	Example	Mode of action (where known)
Metal based fungicides		
Copper fungicides	Bordeaux mixture	Non-specific
Tin fungicides	Fentin acetate	Non-specific
Mercury fungicides	Phenyl mercury acetate	Non-specific
Sulphur fungicides		
Dithiocarbamates	Thiram	Thiol proteins
Others		
Pthalimides	Captan	Proteins
Dicarboximades	Iprodione	?

Table modified from (Lucas, 1998)

Protectant fungicides act by forming an exterior chemical blockade to prevent or protect against infection. Despite their effectiveness against a wide range of fungi, they have limitations in practical use. They must be applied in advance of pathogen penetration of the host and they remain active only with sufficient concentration on the plant surface. They are also subject to degradation and erosion by light, rain and other environmental factors. Hence, there is a need of reliable, early warning of an infection risk (Lucas, 1998). There is the risk of potential phytotoxicity and damage to the plant if absorbed, as reported with dicarboximide (iprodione) and phenylpyrrole (fludioxonil) (Brent and Hollomon, 2007). On the contrary, systemic fungicides (Table 1.4) enter the plant, distribute and render the plant tissues resistant to attack.

Therefore, systemic fungicides can act as eradicant compounds by entering the plant and, to some extent, killing established infections (Manners, 1993). Some systemic fungicides have preventive and curative activities affecting the pathogen before and after infection (Brent and Hollomon, 2007).

Table 1.4 Main groups of systemic fungicides, with examples and mode of action

Type	Example	Mode of action (where known)
Oxathiins	Carboxin	Succinate dehydrogenase
Hydroxypyrimidines	Ethirimol	Adenosine deaminase
Methyl benzimidazoles (MBC)	Carbendazim	β-tubulin
Azoles	Propiconazole	Sterol 14 α-demethylase
Imidazole	Prochloraz	Sterol 14 α-demethylase
Morpholines	Fenpropimorph	Sterol isomerase and reductase
Phenylamides	Metalaxyl	RNA polymerase
Phosphonates	Fosetyl-AI	?
Organophosphorous fungicides	Edifenphos	Membrane function
Melanin biosynthesis inhibitors	Tricyclazole	Inhibits polyketide pathway
Strobilurins	Azoxystrobin	Mitochondrial electron transport
Anilinopyrimidines	Pyrimethanil	Protein secretion? Methionine biosynthesis?
Defence activators	CGA 245704 (a benzothiadiazole)	Induces systemic acquired resistance (SAR)

Table modified from Lucas (1998).

Systemic, single-site fungicides are active against a defined metabolic target in a pathogen (Jane, 2001). They are specific in their toxicity, have little effect against most organisms and they can be safely absorbed and mobilized into plant tissues. These properties are required for systemic activity (Narayanasamy, 2002). Compared with non-systemics, systemic fungicides as a group are developing new fungicide markets and are approximately twice as valuable in terms of sales. Among these systemic fungicides, ergosterol biosynthesis inhibitors (SBIs) are a leading group and account for nearly 24% of the total fungicide sales (Hewitt, 1998). However, despite these considerable advances in systemic fungicides, the non-systemics such as mancozeb, chlorothalonil plus copper and sulphur-based products have a combined value equivalent to 18% of global fungicide sales. Nonetheless, the

popularity of systemic fungicides is increasing at the expense of non-systemics, particularly in cereals (Hewitt, 1998).

Two major site-specific systemic groups of fungicides are currently used to control cereal diseases. The triazole-based fungicides, which inhibit the C14 demethylation step in fungal ergosterol biosynthesis, belongs to demethylation inhibitors (DMIs) (Gisi *et al.*, 2000), and strobilurins (Quinone outside Inhibitors or QoIs), a recent group of fungicides which have been widely used for the control of cereal diseases (Chin *et al.*, 2001).

Sterol biosynthesis inhibitors (SBIs) are dominant compounds used as control agents in medical and agricultural fungal diseases (Leroux *et al.*, 2008a). They include 4 groups of inhibitors including 14α-demethylase inhibitors (DMIs). DMIs target P450-enzymes (*CYP51*) and are believed to inhibit cytochrome 450 by binding to the active site (cysteine pocket). Many of them are triazole derivates (e.g. epoxiconazole, propiconazole, prothioconazole, and tebuconazole), imidazole (e.g. prochloraz), pyrimidines (e.g. fenarimol) or pyridines (e.g. pyrifenox).

The first identification of strobilurins was within the framework of a programme begun in late 1976 aimed at discovering new compound agents from basidiomycetes (Sauter *et al.*, 1999), where the first compounds discovered were strobilurins A and B, obtained from fermentation of *Strobilurus tenacellus* (a wood-rotting fungus that grows on pinecones) (Anke *et al.*, 1977). They found powerful antibiotics (strobilurin A and B) active against a range of fungal species. Early studies revealed that these molecules inhibit the respiration of fungi (Anke *et al.*, 1979). Further studies confirmed the compounds interfere with energy production in the fungal cell by blocking electron transfer at the site of quinol oxidation in the *cytochrome bc1* complex, thereby preventing ATP formation (Sauter *et al.*, 1999). Furthermore, some strobilurins promote the growth of treated plants by delaying the senescence and having water-conserving effects (Clark, 2003). These natural products, due to their unique activities and simplicity of structures, attracted agrochemical companies to synthesize similar or more effective compounds. Many companies established intensive research and

trials to produce synthetic compounds, until the first product was launched by Zeneca onto the German market in February 1996 as azoxystrobin, under the trade name Amistar (Sauter *et al.*, 1999). QoIs were introduced in the UK in 1997 and due to their flexibility of use, efficacy against a range of diseases (including cereal diseases) and benefits in yield, quickly became leading compounds of choice in programmes for cereal disease control (Fraaije *et al.*, 2003).

Over 95% of winter cereal crops in the UK are treated with fungicides, with the mean number of applications of 2.53 in 2010 on winter wheat and 1.7 on winter barley (Anonymous, 2011a). The study undertaken by Oerke (1999), investigating the impact of actual disease control on crop productivity in different regions, has suggested that the prohibition of pesticides, especially fungicides, would cause considerably higher yield reductions in field crops in northern Europe, which currently have very intensive farming systems, than in southern Europe, where productivity per area is lower.

1.4 Evolution of Fungicide resistance

In modern agriculture, despite huge achievements, certain cultural practices have contributed to enhance the destructive potential of diseases. These include practicing monoculture, growth of cultivars susceptible to pathogens, and the use of nitrogenous fertilizers that increase disease susceptibility. Thus, plant disease control is now intensively dependant on fungicides (Schwinn, 1992). One of the most fundamental properties of living matter is the ability of an organism to adapt to changing environmental conditions and their ability to survive new adverse circumstances. Pesticide applications are one of these undesirable changes in the environment for an organism that make it adapt to such a new situation and become resistant. In microorganisms, changes from one form to another are possible and may be detrimental for the organism itself. This will be of little concern to the chemical control of pathogens, but the problem of resistance arises if those changes decrease sensitivity to the chemical group (Elliot, 1973).

Two main factors may confer resistance in microorganisms, physiological adaptation and gene mutation. The resistance due to physiological adaptation is unstable and disappears with no exposure to fungicides. Some organisms under stressed conditions enhance their ability to generate variants by, for example, stimulation of retrotransposon activity in pathogens such as *F. oxysporum*, *Ophiostoma ulmi*, and *O. nono-ulmi* (Anaya and Roncero, 1996; Bouvet *et al.*, 2008). Gene mutations remain the main mechanism for the stable and inheritable resistance, where the fungicide does not induce resistance but acts as selective agent (Chaube and Pundhir, 2005). Non-selective fungicides interfere with several metabolic processes in the fungal cell, hence, are called multisite inhibitors. On the contrary, site-specific fungicides are restricted to a single target for activity, commonly a biosynthetic enzyme essential for fungal growth. Thus, single gene mutations may result in the development of resistant strains against site-specific fungicides. As a consequence, the resistance problem is far more common in selective fungicides as compared to non-selective ones. On account of this, the build-up of resistance, based on experimental and practical experiences, is greatly favoured by sole use of site-specific fungicides (Brent, 1995). Under these circumstances, a potential of partial or total loss of efficacy is a major risk, due to intensive use of these fungicides over a large areas, resulting in the emergence of pathogen genotypes that have the ability to overcome the activity of the fungicides. The degree of this risk is mainly dependent on the mode of action of the fungicide, the way it is used and the evolutionary potential of the target fungi (Shaw, 2000). Thus, with the existence of genetic variation for resistance within the population of a pathogen, fungicide applications provide selective pressure on the population because resistant isolates have higher selective advantage in the presence of fungicide, compared to sensitive isolates. Eventually, resistant genotypes will increase in frequency in the whole pathogen population in subsequent generations and the effectiveness of fungicides may decline.

Fungicide resistance should be distinguished clearly from a temporary adaptation of a fungal pathogen to a fungicide. Adaptations are neither heritable nor stable and are not expected to cause severe problems. Furthermore, poor field performance of a fungicide is not necessarily related to

the presence of resistant strains in a field. Poor disease control might be caused by improper application, extremely high infection pressure, or other factors not related to resistance. Thus, the term "field resistance" should be used only when decreased fungicide efficacy is correlated with the increased frequency of resistant strains (Koller and Scheinpflug, 1987).

Development of resistance by organisms towards chemicals used to control harmful examples includes several examples, such as resistance of bacteria to antibiotics and insects to insecticides. Nevertheless, there were few problems of resistance to fungicides, even though some had been used on a large scale for control of fungal diseases for almost a century. However, after the advent of systemic fungicides, several problems with fungicide resistance occurred in practice (Dekker, 1982). Two main reasons made the problem of resistance common; the extensive use of fungicides in crop protection, such as on cereals, and the introduction of single-site inhibitors that have many benefits, but are more at risk of development of resistance than older, multi-site compounds (De Waard *et al.*, 1993; Lucas, 2006). Prior to the discovery and widespread use of systemic and selective fungicides, there were very few instances, when correctly applied protectant compounds failed to control a pathogen. In such a case, copper, sulphur and dithiocarbamate fungicides remained effective for decades. Despite this, examples of the development of resistance, such as *Pyrenophora* to mercury-based seed-dressings and *Venturia inaequalis* to dodine, are exceptions to this rule. The effect of reduced dose might be another issue that influences the evolution of fungicide resistance. It is, however, not yet confirmed whether reduced rate application of a single fungicide might increase or decrease the probability of evolution of fungicide resistance (Shaw and Pijls, 1994). FRAC investigations drew a conclusion that the effect of reduced application rates varies according to the fungicide in question. Lowering the dose of an at-risk fungicide (at normal spray frequency) can delay build-up of major gene resistance by decreasing the overall effectiveness. This will increase the numbers of sensitive survivors and thus slowing down the selection of resistant forms that can survive the full dose. However, with regard to multi-step resistance, lowering doses can enhance resistance development by allowing low level resistant forms to

survive, which would be inhibited by full rate application (Brent and Hollomon, 2007).

Practical fungicide resistance began to occur shortly after the introduction of single-site fungicides. Incidences of resistance to important diseases have been well-documented (Brent and Hollomon, 2007). Not only did the incidence of resistance increase greatly, but the time taken for resistance to emerge was also shortened, sometimes to within two years of the first commercial introduction, as was the case with benzimidazoles, phenylamides and QoIs (Table 1.5).

Table 1.5 History of fungicide resistance

Date fist observed (approx.)	Fungicide or fungicide class	Years before commercial use prior to resistance observed	Main crop disease and pathogens affected
1960	Aromatic hydrocarbons	20	Citrus storage rots, *Penicillium* spp.
1964	Organo-mercurials	40	Cereal leaf spot and stripe, *Pyrenophora* spp.
1969	Dodine	10	Apple scab, *V. inaequalis*
1970	Benzimidazoles	2	Many target diseases and pathogens
1971	2-Amino-pyrimidines	2	Cucurbit and barley powdery mildews, *Sphaerotheca fuliginea* & *Blumeria graminis*
1971	Kasugamycin	6	Rice blast, *Magnaporthe grisea*
1976	Phosphorothiolates	9	Rice blast, *Magnaporthe grisea*
1977	Triphenyltins	13	Sugar-beet leaf spot, *Cercospora betae*
1980	Phenylamides	2	Potato blight and grape downy mildew, *Phytophthora infestans* & *Plasmopara viticola*
1982	Dicarboximides	5	Grape grey mould, *Botrytis cinerea*
1982	Sterol demethylation inhibitors (DMIs)	7	Cucurbit and barley powdery mildews, *Sphaerotheca fuliginea* & *Blumeria graminis*
1985	Carboxanilides	15	Barley loose smut, *Ustilago nuda*
1998	Quinone outside inhibitors (QoIs; strobilurins)	2	Many target diseases and pathogens
2002	Melanin biosynthesis inhibitors (Dehydratase) (MBI-D)	2	Rice blast, *Magnaporthe grisea*

Table from: Brent and Hollomon (2007).

Fungicide resistance has been found in many pathogens and in different groups of fungicides. For instance, resistance was found in the eyespot pathogen, *O. yallundae* and in wheat leaf blotch, *M. graminicola*, in 1980s, after intensive use of carbendazim (MBC) in the UK (Jones and Clifford, 1983; Fraaije, 2007). It was also found in *Rhynchosporium secalis* against similar benzimidazole fungicides in Northern Ireland in 1993 (Taggart *et al.*, 1994) and in England and Wales also in 1993 (Phillips and Locke, 1994).

Many authors have confirmed the negative effect of multiple applications of fungicides on the development of fungicide resistance. Bateman (1994) reported the failure of control by carbendazim when used against the eyespot within 2 years of consecutive use. This was ascribed to the selection for resistance within the fungal population. However, in the same study, less sensitive isolates were found in plots after 8 years of treatment with prochloraz, either alone or in combination with carbendazim. Prochloraz, however, maintained good efficacy against eyespot for many years. This may reflect the lack of systemic activity of this compound. It is therefore not diluted at sites of stem base application by acropetal movement and would thus continue to provide a fungitoxic dose enhancing selection pressure in favour of in sensitive mutant isolates.

Resistance to QoIs occurred very rapidly after introduction of the first strobilurin fungicides, azoxystrobin and kresoxym-methyl, which were launched for agricultural disease management in 1996. In Europe, QoIs were introduced as cereal fungicides during the late 1990s. Soon after their introduction, resistance to QoIs developed in several cereal pathogens. Early detection of resistance was in diseases including wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*) and in barley powdery mildew (*B. graminis* f.sp. *hordei*) in Northern Germany (Heaney *et al.*, 2000), in *M. graminicola* in the UK and Ireland in 2002 (Fraaije *et al.*, 2003) and in populations of the banana pathogen *Mycosphaerella fijiensis* (Sierotzki *et al.*, 2000a).

Two major amino acid substitutions have been detected in the *cytochrome b* (*cyt b*) gene in plant pathogens that show resistance to QoI fungicides. One

such mutation leads to a substitution of glycine by alanine at amino acid position 143 (G143A). This is the main mechanism known to confer resistance to QoIs and is found in a broad range of pathogenic fungi and oomycetes, such as *Bl. graminis* and *M. graminicola* (Heaney *et al.*, 2000; Sierotzki *et al.*, 2000b; Fraaije *et al.*, 2003) and *Plasmopara viticola* (Wong and Wilcox, 2000). Another mutation at amino acid position 129, which leads to the substitution of phenylalanine by leucine (F129L), confers insensitivity in plant pathogens including *Alternaria solani* (Pasche *et al.*, 2005), *Pythium aphanidermatum* (Bartlett *et al.*, 2002), and *P. viticola* (Heaney *et al.*, 2000; Sierotzki *et al.*, 2005). There is also another substitution in the *cytochrome b* gene (glycine to arginine) at codon 137 (G137R) which was found in plant pathogens, such as *Pyrenophora tritici-repentis* (tan spot of wheat), at a very low frequency (2 out of 250 isolates from 2005 in Germany). This mutation conferred a similar level of resistance to F129L (Sierotzki *et al.*, 2007). The F129L mutation been found in *P. teres* in Europe since 2003 (Fraaije *et al.*, 2003; Yamaguchi and Fujimura, 2005). Since then, several investigations have focused on the presence of this alteration in populations of *P. teres* and its relationship with the efficacy of some QoI fungicides (Sierotzki *et al.*, 2007; Jorgensen, 2008).

In 2002, Septoria tritici blotch (*M. graminicola*) was severe in Western Europe, and there were reports of poor control by QoIs in some regions. Subsequently, there has been considerable research effort which has confirmed the rapid development of resistance to strobilurin (QoI) fungicides in *M. graminicola* populations (Fraaije *et al.*, 2003). Further studies have shown that isolates with the G143A mutation were recovered from untreated wheat plots at Rothamsted, suggesting that the mutation was already present in ascospores founding the 2002 epidemic. The incidence of G143A in UK *M. graminicola* populations increased from around 30% to 80% by the end of 2003. In 2004 this trend was repeated in other northern regions of Europe (Lucas, 2005). Eventually a total failure of control achieved by QoIs was reported against *M. graminicola* populations carrying the G143A mutation or resistant-conferring allele in approximately of 90% of the UK population of *M. graminicola* (Fraaije *et al.*, 2005; Lockley and Clark, 2005).

Reduced sensitivity to DMIs was reported by 1994 for at least 13 plant pathogens. In most cases the resistance was polygenic, although in some cases was monogenic (De Waard, 1994). It has also been found in *Uncinula necator*, grape powdery mildew (Delye *et al.*, 1997), and in *B. graminis* f. sp. *hordei* (Delye *et al.*, 1998) where, in both diseases, the resistance was found to be correlated with the Y136F substitution in the *CYP51* gene. Reduced sensitivity was also found in other cereal pathogens such as *O. yallundae* and *O. acuformis* as a result of intensive use of DMIs (Leroux and Gredt, 1997). A clear erosion in triazole efficacy against *M. graminicola* has shown that higher doses are now required to achieve effective disease control (Cools *et al.*, 2005).

1.5 Managing fungicide resistance

After the introduction of systemic organic fungicides the development of resistance became a wider practical problem in agriculture. Thereafter, discussions began about strategies that could be used to cope with this phenomenon (Schwinn, 1982). In 1970s, when the severe losses coincided with widespread resistance to fungicides, awareness in the industry evolved and it was realised that the problem had to be addressed. The foundation of the Fungicide Resistance Action Committee (FRAC) in 1981 was as a response to this imperative task and this body has, since then, played a leading role in shaping the fungicide resistance management strategies. This was primarily achieved by having the impact and authority to set strategies and by offering training and education (Highwood, 1989). Before establishment of any tactics, fungicide resistance must be detected and measured in various ways, depending on the fungus-fungicide combination. Firstly, the recognition of resistant strains of fungi must be made by comparison with data obtained with sensitive strains. Thus, it is essential to establish the base-line sensitivity, either by appropriate experiments with incontestable wild type strains or by the use of data from the literature (Georgopoulos, 1982). Secondly, two important parameters should be measured: the extent of resistance; the proportion of the population that no longer show the normal sensitivity and the degree of resistance; the magnitude of the differences in sensitivity. The success of any anti-resistance strategy depends on several factors, including the availability of rapid and reliable monitoring methods, by which efficacy of

control can be evaluated, and the availability of fungicide companion partners with different active mode of actions. Furthermore, anti-resistance strategies have to fit economical, ecological and legislative requirements (Kuck, 1994), meaning that effective resistance management could only be achieved with the cooperation of users in terms of preparation and implementation of the recommendations. Therefore, regulation, through both statutory action and by working with other interested parties, to help develop and encourage the adoption of effective strategies, has an important role in ensuring long term sustainability of product use and extension of timescales for product usefulness. Thus, resistance management strategies are considered within the perspective of wider demands for sustainable crop production (Macdonald, 2008).

Several commonsense anti-resistance strategies have been adopted against different pathogens. In *B. graminis* f. sp *tritici*, mixtures of fungicides with different resistance mechanisms have been tried by the SBI Working Group of FRAC and found to be the most appropriate strategy. They found a reduction of field application rates to be not recommended (Schulz, 1994). In contrast, in *U. necator*, fungicide mixtures with different modes of actions (triadimenol with sulphur) did not slow down the evolution of resistance in natural populations (Steva, 1994). However, the study claimed that reduction of the number of treatments, and use of sole use of sulphur were the only strategies helpful in slowing down the evolution of resistant phenotypes and keeping the disease under control. In managing resistance of *M. graminicola* isolates towards DMIs, Leroux *et al*. (2008c) have suggested a combination of DMI with multisite inhibitors such as chlorothalonil or boscalid to complement DMIs, as well as mixtures with other triazoles. Thus, use of some older, multisite fungicides now play key roles as partners in mixtures or as treatments in fungicide rotations (Lucas, 1998). The mixture of triazoles with prochloraz as an alternative anti-resistance strategy was also suggested, based on the fact that this imidazole derivative is especially active against field isolates exhibiting high resistance towards triazoles (Leroux *et al.*, 2008c). It is also widely accepted that within the triazole group levels of insensitivity to *CYP51* mutants varies considerably; some molecules (eg epoxiconazole) show more activity than others (eg tebuconazole).

Another strategy to reduce the evolution of resistance to fungicides is through the use of varieties with partial resistance to diseases. Growing varieties with good disease resistance properties was found to be a vital component in disease management in helping to minimize losses with less fungicide usage (Jorgensen *et al.*, 2008). In wheat, for instance, varieties with partial resistance to powdery mildew have been used by Iliev (1994) effectively to prolong the efficacy of the systemic fungicide, propiconazole, and to prevent the pathogen from developing resistance by increasing the generation time of the pathogen approximately six fold. Thus, growing varieties with partial resistance, in combination with a systemic fungicide, limits the number of reproductive generations of the pathogen and lengthens the period of protection against the pathogen. Based on the points described, general guidelines have been suggested by Fungicide Resistance Action Group-UK (FRAG-UK) to provide good resistance management aimed to minimize the level of exposure of the pathogen to the fungicide and therefore minimize the risk of resistance occurring (Anonymous, 2011c). This could be summarized as follows:

- Use of other control measures in parallel with fungicide input
- Use of varieties exhibiting a high degree of resistance to prevalent disease
- Avoidance of the growth of one variety in a large scale in a high disease risk areas where the variety is known to be susceptible
- Restrict use of fungicides only in situations where the risk of the disease warrants treatment
- Use of an appropriate fungicide dose that will give effective disease control and that are suitable for the variety and disease pressure
- Follow the full use of effective fungicides with different modes of action or as alternate sprays
- Use of fungicide partners at doses that give similar efficacy and persistence
- Follow a regular crop monitoring and treat before the establishment of any disease

- Avoid repeated applications of the same product or products with similar modes of action and never exceed the maximum recommended number of applications

1.6 Thesis objectives

The aims of this study was to determine the effect of fungicide resistance in net blotch of barley, associated with the F129L mutation, and in septoria leaf blotch of wheat, associated with *CYP51* changes. The study focused on the following main areas:

1. Development of reliable disease inoculation methods.
2. Detection of the F129L mutation in isolates of *P. teres* and *CYP51* alterations in *M. graminicola*.
3. Determination of fitness penalties associated with the mutations in the pathogens.
4. Application of *in vitro* methods for fungicide efficacy evaluation.
5. *In vitro* and *in planta* evaluation of single QoI fungicides against *P. teres* isolates associated with the F129L mutation. These were compared to epoxiconazole, mixture compounds comprising QoIs and DMIs and a novel SDHI fungicide product.
6. Detection of different genotypes in *M. graminicola* isolates based on sequence analysis and the response of the genotypes to DMIs.
7. Evaluation of activity of single and mixed active ingredient fungicide products, with different modes of action, and a novel SDHI product, against different *M. graminicola* isolates.
8. Measure of fungicide efficacy using PCR-based methods and compare with visual disease assessments.

Chapter 2 General Methods

2.1 General culture media

Where possible, all microbiological media were obtained from Sigma (Dorset, UK) or from Oxoid (Basingstoke, UK). For sterilisation, all media was autoclaved at 121^{o}C for 20 min.

2.1.1 Pre-prepared PDA

Full-strength pre-prepared PDA was routinely used for fungal growth, unless otherwise stated. The medium was prepared by suspending 39 g of PDA powder in 1 L of distilled water and dissolved by heating using a microwave prior to sterilisation by autoclaving.

2.1.2 V8 juice agar (V8JA)

V8 juice medium was prepared from 200 mL of V8 juice (Campbells Soups Ltd), 3.0 $CaCO_3$, 15 g agar, and distilled water (DW) to bring the total volume to 1000 mL. Before autoclaving, the pH was adjusted to 6.3.

2.1.3 Peanut oatmeal agar (POA)

Peanut leaves (60 g) were placed in 500 mL of water, heated to boiling point for 15 min and filtered through muslin. In a different beaker, 72 g oatmeal was placed in 500 mL water and boiled for 15 min and filtered. Both solutions were mixed together and after adding 18 g agar, DW was added to make the total volume 1 L.

2.1.4 Modified Czapek's medium (MCM)

MCM contained 0.5 KH_2PO_4, 0.5 $MgSO_4$, 0.5 KCL, 1.2 urea, 20 lactose, and 20 g L^{-1} agar and DW to the total volume of 1 L.

2.1.5 Malt extract agar (MEA)

MEA was made containing 20 g malt extract with 18 g agar, suspended in 500 mL of boiling water to allow dissolving. The mixture was cooled and the total volume brought to 1 L.

2.1.6 Barley leaf agar (BLA)

BLA was prepared from 100 g (FW) green barley leaves, ground using a blender and then filtered through muslin. Agar (20 g) was added and DW used to bring the total volume to 1 L.

2.1.7 Barley meal agar (BMA)

Barley seed meal (50 g) was boiled for 15 min, filtered with muslin and the resultant liquor collected. Agar (18 g) was added and DW used to achieve a total volume of 1 L.

2.1.8 Tomato paste agar (TPA)

TPA medium was prepared from 20 g tomato paste (30%), 13 g agar powder and DW to the total volume of 1 L.

2.1.9 Potato dextrose broth (PDB)

PDB powder (24 g) was suspended in 1 L of purified water, heated to boiling, with continuous agitation, until completely dissolved before autoclaving.

2.2 Chemicals

All chemicals and solvents used were of analytical grade where possible and were obtained from Sigma, unless otherwise stated.

2.3 Collection of isolates

2.3.1 *P. teres*

Initially, thirteen isolates of *P. teres* were obtained as cultures from different research centres in the UK and mainland Europe. Six isolates (3 purported F129L mutants and 3 purported wild types) were obtained from DuPont, France. Five isolates of unknown pedigree were from Science and Advice for Scottish Agriculture (SASA) and two unknown isolates from National Institute for Agricultural Botany (NIAB). The second group of isolates was obtained from barley leaf samples of growing season 2008-09, provided by members of The Arable Group (TAG). Leaf samples were received from 10 different areas of the Midlands and Eastern England.

2.3.2 *M. graminicola*

Six isolates were obtained from the culture collection of Dr. Stephen Rossall, Plant and Crop Sciences Division, University of Nottingham, Sutton Bonington Campus. They were maintained as stock cultures kept in 80% glycerine in 1.5 mL Eppendorf tubes at -80°C. All other isolates (12 isolates) obtained in this study were derived from infected wheat leaves from the 2008-09 season, received from England, Scotland and Germany.

2.4 Maintenance of isolates

2.4.1 *P. teres*

Pure cultures were transferred to slants of potato dextrose agar (PDA) in universal glass tubes, with leaving the lids slightly loose. Once a sufficient growth had occurred (within 7-10 d) at 20°C, the lids were tightened and then the slant stocks were stored at 4°C. To avoid bacterial contamination, PDA medium were amended with the antibiotics penicillin (30 mg L^{-1}) and streptomycin (133 mg L^{-1}). To keep the cultures viable, the slant stocks were sub-cultured from old cultures every 3-4 months. As a precaution against decline in pathogenicity of isolates, after many consecutive sub-culturing incidents, and to maintain aggressiveness, barley plants were regularly inoculated with a mixture of spore and mycelium fragments prepared from slant cultures. The fungus was re-isolated from visible, typical net-like lesions. The resultant cultures were maintained as described above.

2.4.2 *M. graminicola*

Spore suspensions were obtained by flooding 5-7 d-old PDA cultures with 15 mL sterilised 80% v/v glycerol and gently scraping with a sterile plastic inoculation loop under aseptic conditions. The spores were then filtered through four-layers of muslin gauze to avoid mycelium fragments into a sterile conical flask. From the crude suspension thus obtained for each isolate, 30-40 aliquots (1.5 mL) were pipetted into Eppendorf tubes. The tubes were then stored at -80°C to provide stock cultures for future experiments.

2.5 Spore preparation

2.5.1 *P. teres*

New cultures were prepared from slant stock cultures by inoculating either PDA or V8 juice agar (V8JA) media and incubating under 12 h near ultraviolet light (NUVL). After 10 d of incubation, plates were flooded with 10-15 mL of sterilised distilled water (SDW) and scraped to release spores. An additional step was required for poor-sporulating isolates, which was the use of an electrical hand-held blender (PHILIPS, Mexico) to macerate the mycelium into small fragments for spray inoculation.

Spore or mycelia suspensions were diluted with SDW according to the requirements of the experiment and quantified by haemocytometer counts and dilution (Improved Neubauer, Weber Scientific International, Sussex, UK).

2.5.2 *M. graminicola*

Frozen spore suspensions were removed from the freezer and defrosted at room temperature. Under aseptic conditions aliquots of each isolate were pipetted and spread onto the surface of PDA plates. After 5-7 d incubation, conidial suspensions of *M. graminicola* were prepared by flooding cultures with approximately 10 mL of SDW and gently scraping with a sterilised plastic inoculation loop. The spore suspensions were then filtered through four layers of sterile muslin to remove mycelial fragments. Resultant suspensions were diluted with SDW according to the requirement of the experiment after enumeration using haemocytometer counts.

2.6 Source of seed and plant growth

The winter barley cultivar Pearl was used for pathogen re-isolation, pathogenicity, and fungicide bioassays. The cultivar is susceptible to *P. teres* (with an HGCA resistance rating of 5.3 in 2010- 2011). Seed was kindly donated by Limagrain UK.

Wheat seed (cultivar Riband) was supplied by RAGT Seeds Ltd (RAGD Group, Cambridgeshire, UK). The cultivar, although no longer widely-grown, is highly susceptible to *M. graminicola* (with an HGCA resistance rating of 3 in 2008-2009).

Wheat and barley seeds were sown in 13 cm diameter pots containing John Innes No.3 compost at a rate of 15 seeds per pot. After germination, the seedlings were thinned down to 10 plants per pot. Plants were raised in a controlled environment room with a 20°C day temperature and 12°C night temperature and a 16 h photoperiod at a light intensity of 200 µmol m^{-2} s^{-1}. Experiments were routinely initiated when the plants reached growth stage 12 (Zadoks *et al.*, 1974). During the experiments, the plants were manually watered daily. To avoid unwanted, naturally-occurring powdery mildew infections, the controlled environment rooms were cleaned before start of each experiment with 2% Trigene solution (Medichem, Kent, UK). The mildew-specific fungicide, ethirimol 25% SC (10 mL L^{-1}), was also applied to the plants at a volume equivalent to 200 L water ha^{-1} at the first sign of mildew infection of plants.

2.7 Inoculation

Spray inoculations were undertaken using hand-held sprayers (Fisher Scientific, Loughborough, UK). Inocula were applied to barley or wheat plants at growth stage 12 with pathogen suspensions prepared and described in sections 2.3.1 and 2.3.2. Inocula were applied at 10^4 propagules mL^{-1} and 10^6 conidia mL^{-1} for *P. teres* and *M. graminicola* respectively. Barley and wheat plants were placed in transparent plastic bags immediately after inoculation and a layer of water was placed in to the trays containing the pots. After 48 h the bags were removed and the inoculated plants maintained under the conditions described in sections 2.4.1 and 2.4.2.

2.8 Disease assessment

2.8.1 Net blotch

Disease assessments of BNB were carried out 10 d after inoculation (DAI). The net-like necrosis was assessed visually using the rating scale of Tekauz (1985) as illustrated in Figure 2.1.

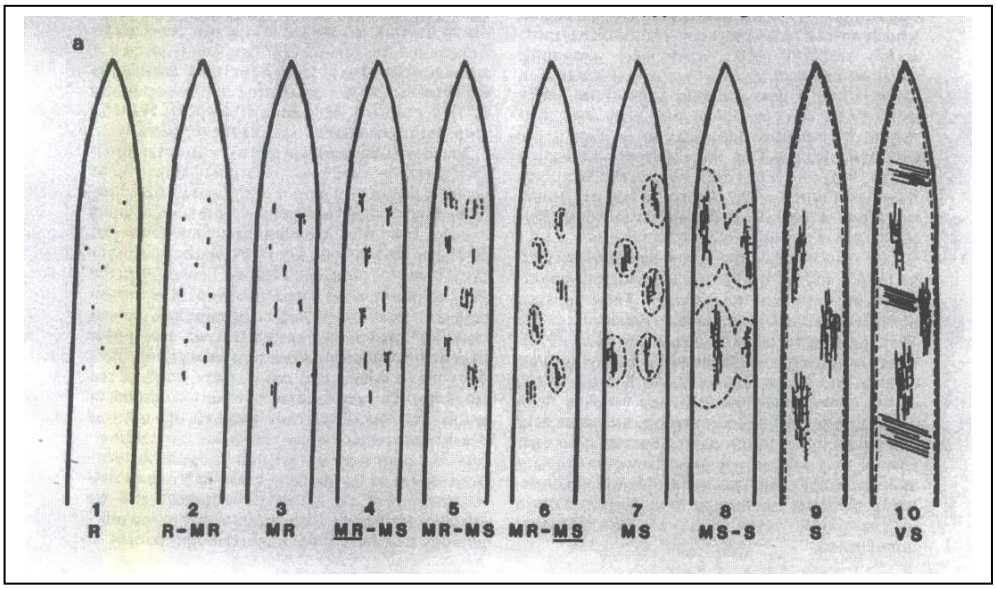


Figure 2.1 A numerical scale used for visual net blotch assessment on barley plants (Tekauz, 1985).

2.8.2 Septoria tritici blotch

Disease incidence of *S. tritici* was assessed visually 21 DAI. Disease assessments were carried out by evaluating the percentage area of necrotic lesions of inoculated leaves (2nd leaf from the bottom of the plant). The total area assessed (in %) was that covered with black pycnidia as well as the area showing chlorosis without sporulation (Figure 2.2).

Figure 2.2 Typical symptoms of STB caused by *M. graminicola*, including the area covered with pycnidia (centre) surrounded by chlorosis area.

2.9 Fungicides

Experimental fungicide samples were obtained from different agrochemical companies by Dr. Stephen Rossall and are described in Table 2.1.

Table 2.1 Fungicides used in studies with BNB and STB.

Product name	Active ingredient	Concentration (g L^{-1})	Class	Source
Twist	Trifloxystrobin	125	QoI	Bayer
Comet	Pyraclostrobin	250	QoI	BASF
Amistar	Azoxystrobin	250	QoI	Syngenta
Acanto	Picoxystrobin	250	QoI	Syngenta
Opus	Epoxiconazole	125	Triazole	BASF
Folicur	Tebuconazole	250	Triazole	Bayer
Proline	Prothioconazole	250	Triazole	Bayer
Warbler	Prochloraz	400	Imidazole	Nufarm
Unix	Cyprodinil	750	Anilinopyrimidine	Syngenta
New SDHI	Penthiopyrad	200	SDHI	DuPont
Fandango	Prothioconazole + fluaxostrobin	100+100	Triazole + QoI	Bayer
Prosaro	Prothioconazole + Tebuconazole	210 + 210	Triazole + triazole	Bayer
Tracker	Boscalid + epoxiconazole	233 + 67	SDHI + triazole	BASF
Joules	chlorothalonil	500	Chloronitriles	Nufarm

2.10 Calibrations of the hand pump spray for fungicide application

Fungicide applications were carried out using 200 mL hand-pumped aerosol spray bottles (Fisher Scientific, Loughborough, UK). To avoid cross contamination a separate sprayer was used for each treatment. All products were applied in a volume of water equivalent to 200 L ha^{-1}. This equates to 20 mL m^{-2}. Sprayers were calibrated and the time taken to apply this volume was determined. Plants were then placed in a 1 m^2 area before application of a 20 mL of spray, thus simulating field application rate.

2.11 DNA extractions

All extractions of genomic DNA from fungal pathogens and host plants were performed using an extraction kit (DNeasy® Plant Mini Kit (50), QIAGEN, GmbH) or the cetyl trimethyl ammonium bromide (CTAB) method (Allen *et al.*, 2006). To maintain a high quality, the extracted DNA was also purified using the Micro Bio-Spin Chromatography column purification method where poly vinylpolyrrolidione (PVPP) was used as a purification agent (Bio-Rad, UK).

2.12 Agarose gel preparation and electrophoresis

Preparation of agarose gels was achieved by suspending agarose at a rate 1-1.5% in the 1X Tris-Borate-EDTA (TBE) and dissolved using a microwave oven. Ethidium bromide (Fisher Scientific UK Limited, Loughborough, UK, 0.5 µg L^{-1}) was added to the solution and cooled to 60^{o}C. Subsequently, the solution was mixed well manually and gently poured into a plastic plate mounted with a comb. Instantly and before the gel solidification, the bubbles around the comb tips and on the surface of the gel were removed using pipette tips. After the solidification of the gel, the comb was gently removed to allow appropriate loading of dye, DNA or PCR products. Electrophoresis was performed at 90 V for 60 to 80min, after which it was visualised under ultra violet (UV) illumination and photographs taken.

2.13 Gene sequencing and alignment

Unless otherwise stated, all PCR fragments were sequenced using a CEQ 8000 Beckman Coulter sequencer (High Wycombe, UK) or by Eurofins MWG Operon, Germany. Sequences were aligned and analysed by using BioEdit software (Biological sequence alignment editor, version 7.0.9).

2.14 Data analysis

Initial data analysis was carried out using Microsoft Excel 2007. For general analysis of variance (ANOVA), GenStat version 11.0 was used. Fisher's least significant difference (LSD), with a significance level of 5%, was performed to determine significant differences between means. To avoid mis-comparisons, all data from *in vitro* and *in planta* fungicides performance evaluations were

manipulated to the percentage inhibition or disease control, relative to the untreated control of the same experiment.

For detecting EC_{50} values, probit analysis, with the aid of SPSS software version 19 (IBM Statistics, USA), was used. The statistical programme calculated the linear regression to fit the response versus the concentration.

To normalise the distribution of data angular, arcsine (ASIN) or square root (SQRT) transformations of values were undertaken as necessary.

Chapter 3 *Pyrenophora teres* isolation, growth, maintenance, inoculation, detection of F129L mutation, and fitness costs

3.1 Introduction

3.1.1 Isolation of *P. teres*

Pyrenophora teres, the causal agent of net blotch of barley, is a serious foliar disease, causing net-like symptoms. The fungus is a stubble- and also seed-borne pathogen but it is normally isolated from leaf lesions. Sierotzki *et al*. (2007) isolated the pathogen from leaf samples with necrotic symptoms. In their method, leaves with visible symptoms were cut into 2 cm long pieces and then surface sterilized with 2% sodium hypochlorite. After removing the disinfectant with sterilized water, the pieces were dried and then placed (adaxial side upwards) in Petri dishes on moist filter paper (3 mL water per dish with 8 cm diameter) and incubated at 20°C under black light (UV) for 2-4 d. Conidiophores emerged at the edges of the leaves. Single conidia were picked up under a binocular microscope with the aid of a fine needle and transferred to malt agar plates. The growing mycelia of isolates were transferred as mycelial discs to wheat or barley agar plates and incubated for 14 d under black light at 20°C. Infected seed, in parallel with dried infected leaves, were used by Jonsson *et al*. (1997) to obtain isolates of *P. teres*. In this isolation method leaves with disease symptoms were collected from barley plants grown in yield trials and from commercial fields. The leaves were placed in paper envelopes, dried and stored at 20-23°C. Dried leaves were surface sterilized with 50% ethanol for 30 s and sodium hypochlorite for 45 s. The leaf pieces (2-4 mm^2) were placed on water agar and incubated at 20°C with a 12 h photoperiod. After 2-7 d, single spores were collected and placed on 25% V8-juice agar. Spore suspensions were obtained ten days after incubation by flooding the surface of Petri dish with 6 mL of sterile water. The resulting suspension with spores and mycelia was mixed with 2 mL of glycerol and stored in 1 mL aliquots at -80°C. Surface sterilization with 50% ethanol for 15 s and 2% sodium hypochlorite for 30 s was also used by Robinson and Jalli (1997) to isolate *P. teres* from leaf tissue with net blotch lesions. Samples were collected from 9 sites in Finland during summer 1994. A similar isolation

method was utilised by Karakaya and Akyol (2006); they used 1% sodium hypochlorite to surface sterilize the barley leaves and then transferred them to Petri dishes containing moistened filter paper. After sporulation single conidia were harvested and placed onto PDA. Gupta and Loughman (2001) used 5-10 mm diameter leaf fragments with net blotch lesions taken from recently dried and old lyophilised samples, all originating from Western Australia. Leaves with net-like symptoms were cut into 5 to 10 mm diameter fragments, surface sterilized with 0.5% sodium hypochlorite solution for 2 min, and then double rinsed in sterile deionised water for 1 min. The sterilized fragments were dried and aseptically transferred to 2% water agar plates and incubated 15-18^{o}C with 12 h near-UV light alternating with 12 h dark. A different sodium hypochlorite concentration (5%) and time (5 min) was used by Arabi *et al*. (2003) and Tuohy *et al*. (2006) to surface sterilize barley leaves showing net blotch symptoms. These were then soaked three times in SDW for 5 min, cut into pieces (3-5 x 1-3 mm) and then dried between filter paper. Leaf fragments were then transferred on to V8-juice medium and incubated for 10 d at 22 ± 1^{o}C in continuous darkness to allow mycelium growth. A single spore technique was used by Leisova *et al*. (2006), where leaf segments with disease symptoms were excised and incubated at 20–23°C on potato lactose agar before single conidia were transferred to fresh plates and incubated for 10 d.

3.1.2 Sporulation

There are differences in sporulation between isolates of *P. teres* and each isolate responds individually to type of medium, light regimes and temperature. *Pyrenophora teres*, in comparison with other *Pyrenophora* species, often sporulates poorly in culture and much variation exists between isolates (Deadman and Cooke, 1985). In this regard, Clifford and Jones (1981) reported that 25% of isolates derived from leaf samples received by the UK Cereal Pathogen Virulence Survey in 1980 failed to sporulate in culture, and for the agar plates received the previous year nearly 50% did not produce spores on lima bean agar.

However, Sato and Takeda (1991) recommended that isolates of *P. teres* should be cultured on V8 agar medium at 25± 6^{o}C degrees under a diurnal,

near ultra violet (NUV) irradiation regime. Tomato paste agar (TPA) was proposed by Al-Tikrity (1987) for sporulation of isolates of *P. teres*. He claimed that a high level of sporulation was obtained when cultures were incubated at 21°C for 9 d in darkness. Abundant sporulation was obtained by Sanglard *et al*. (1998a) by using peanut oatmeal agar (POA) for isolates of *P. teres* incubated at 18°C with a 12 h photoperiod for 15 d. Using barley straw extract (BSE), Akins (2005) found significant differences in sporulation among isolates of *Drechslera graminea* from different areas of Canada, Montana, Germany and Syria and from isolates originating from the same field. They also found that incubating the culture plates at 16°C under fluorescent light (12 h light/12 h dark) for 5 days following incubation under NUV light for 7 days resulted in 40% higher conidial production. They further confirmed that seed extract, green leaves of barley and mature wheat straw did not induce sporulation.

3.1.3 Inoculation methods

An appropriate method is essential for the study of plant pathogens using artificially inoculated plants. Artificial inoculation of barley plants by *P. teres* is necessary in many bioassays, such as testing the pathogenicity of different isolates and evaluation of fungicide performance *in planta*. Optimum temperature and high humidity are major components for successful inoculation. Shipton *et al*. (1973) stated that under field conditions net blotch is prevalent when damp weather prevails. He also added that a wet period of 5-15 h is favoured for successful infection, mentioning that the optimum temperature for spore germination is approximately 25°C, while the best temperature for spore production in culture is 21°C. In this regard different methods have been followed in different circumstances. Sierotzki *et al*. (2007) used a hand sprayer to inoculate barley plants until a layer of fine droplets was formed on the surface of barley leaves and then, to maintain high humidity, inoculated plants were kept in fabric tents at 100% relative humidity maintained by a boom irrigation system for 48 h at 20°C, followed by transfer to normal glasshouse conditions for a further 3 d at 20°C. Tween 20 as a wetting agent has been used in many inoculation techniques and with many plant pathogens. A conidial suspension containing 0.1% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate) was used by Leisova *et al*. (2006) as a wetting agent to enhance inoculation efficacy.

Conidial concentration is another issue to consider for successful inoculation, where typical concentrations should be adjusted to produce typical disease symptoms. Densities of 5 x 10^3 to 1 x 10^4 mL^{-1} in sterile water were prepared by Leisova *et al*. (2006) to obtain efficient inoculation, while Karakaya and Akyol (2006) utilised a suspension of 15-20 x 10^4 mycelium parts mL^{-1}, with which they successfully produced infected barley plants. To enhance the inoculation method, a drop of Tween 20 was added to each 100 mL of the suspension and then the plants were kept in moisturised plastic bags for 72 h. Mycelial suspensions were also used as inoculum for artificial inoculation by Arabi *et al.* (2003). They prepared the inoculum by growing mycelium fragments in 50 mL of 10% V8 broth in 250 mL flasks for 10 d in darkness at 22±1°C. The mycelium was then filtered and 10 g (fresh weight) suspended in 100 mL of SDW and ground to create mycelium units. Tween 20 also added to the suspension (0.1 mL to each 100 mL) and then adjusted to 3 x 10^3 units mL^{-1}. They stated that this concentration was sufficient to provide uniform infection.

3.1.4 F129L mutation in *P. teres* isolates

Resistance to QoI fungicides was first detected in 1998, just two years after their introduction, in wheat powdery mildew and in 1999 in barley powdery mildew in northern Germany (Heaney *et al.*, 2000). In 2002 resistance in field isolates of *M. graminicola* in the UK and Ireland was reported (Fraaije *et al.*, 2003). Two common amino acid substitutions have been detected in the *cyt b* gene in plant pathogens that govern resistance to QoI fungicides. One mutation leads to a substitution of glycine by alanine at codon 143 (G143A) and is the main mechanism of resistance of QoIs. Another mutation at codon 129, which leads to the substitution of phenylalanine by leucine (F129L), results in generally less pronounced resistance levels and sensitivity studies have shown that the different QoIs are not equally affected by this mutation (Fisher *et al.*, 2004). The latter is present in less sensitive isolates with the nucleotide exchanges from TTC (coding for phenylalanine) to TTA, TTG or CTC (all coding for leucine) (Semar *et al.*, 2007). The Fungicide Resistance Action Committee (FRAC) has indicated that QoI fungicides form a cross-resistance

group, which is different to other commercially available fungicides (Anon, 1998). Thus, resistance in fungi to one compound within the STAR group will confer resistance to all STAR compounds, but not to compounds from different cross-resistance groups. First detection of *P. teres* resistance to QoI fungicides was in 2003 in France, Sweden and Denmark. Based on DNA sequence analysis, the F129L mutation was found in *P. teres* isolates resistant to QoI fungicides. The following year the frequency of F129L mutation increased in populations and in 2005 it further increased in incidence and distribution in France and the UK, but in Germany, Switzerland, Belgium and Ireland it remained below 2% (Sierotzki *et al.*, 2007).

3.1.5 Determining fitness costs of resistance mutations

Resistance towards pesticides is one of the most pressing problems facing the public, animal and plant health today. There are usually costs to pathogen adaptation that have an important impact on host-parasite evolution. Changes in fungicides sensitivity may be associated with loss of infectivity and other pathogenicity-associated traits (MitchellOlds and Bradley, 1996; Hall *et al.*, 2004; Bahri *et al.*, 2009), meaning that a single gene mutation can influence multiple phenotypic traits. Possible point mutations are likely to happen frequently, even during moderate epidemics, in pathogens with a large population size and rapid multiplication. The ability to overcome control measures, therefore, reflects the overall fitness of these mutants, and effort is being directed towards assessment of their fitness (Hollomon and Brent, 2009). Fungicide-resistant genes with SBI fungicides that have greater fitness in the presence of fungicide also have some associated fitness costs in the absence of fungicide. The fitness costs which correlate with fungicide resistance genes are important because of their evolutionary effects. This will allow selection against resistance in the absence of fungicide, leading to a decrease in the frequency of resistance genes in the pathogen population (Koller and Scheinpflug, 1987). Detecting fitness costs from laboratory-created isolates has been practiced by many researchers but this may not represent the fitness of resistant field isolates. Few studies have involved naturally occurring resistant isolates that were sampled from field populations (Kadish and Cohen, 1988). Chen *et al*. (2007) found that both field resistant and carbendazim-sensitive strains of *Fusarium graminearum* (wheat ear blight)

showed similar response in their temperature sensitivity, fitness and pathogenicity on ears. In contrast, in *V. inaequalis* (apple scab) populations, maximum lesion density in the resistant group was 20% lower and the latent period 7% longer, than in the sensitive group.

Fitness costs could be variable between pathogen populations because of different resistance genes causing different fitness penalties and there are also differences in genetic backgrounds between populations. Two fitness cost components were investigated by Peever and Milgroom (1994), which were latent period (the time from inoculation to the first appearance of a conidium) and sporulation (total sporulation per lesion). They quantitatively determined both parameters in a glasshouse experiment using detached barley leaf sections, inoculated with conidia of isolates of *P. teres*, resistant to triadimenol and propiconazole. A further study of fitness costs to *Botrytis cinerea*, associated with dicarboximide resistance, was conducted by Raposo *et al*. (2000). They measured the survival of isolates of the pathogen both inside and outside a greenhouse. The study measured the percentage survival of mycelia on artificially inoculated tomato stem species and as percentage of viable sclerotia produced on PDA.

3.1.6 Objectives

The aim of the work reported in this chapter was to isolate *P. teres* from infected plant material, enhance the sporulation of pathogen before *in planta* inoculations and to develop the inoculation methods. The developed inoculation methods were used as a standard technique in all subsequent work based on plant infection. This chapter also aims to detect the F129L mutation in isolates of *P. teres* and also to reveal possible fitness costs associated with the mutation.

3.2 Materials and methods

3.2.1 Isolation of *P. teres*

Thirteen isolates of *P. teres* were obtained from different research centres in the UK and mainland Europe. The first group of isolates obtained and their sources are shown in Table 3.1. Five isolates of unknown pedigree (458, 1782, 557, 83, and 18) were from SASA, six isolates (3 F129L mutants and 3 wild

types) were obtained from DuPont, France and two unknown isolates from National Institute for Agricultural Botany (NIAB). The second group of isolates were obtained from barley leaf samples collected in the 2008-09 growing season, and sent by The Arable Group (TAG). Leaf samples received from TAG were from different areas of the Midlands and Eastern England. One pathogen isolate was taken from each leaf and cultured on PDA (Table 3.2). Isolates obtained were from single leaves and therefore, only one isolate was taken from each sample. The following isolation method was used to obtain new isolates from the TAG leaf samples: leaves with visible net-like symptoms were cut into 1 x 1 cm sections, placed in 8% Domestos solution (0.5% sodium hypochlorite) for 5 min, washed 3 times with SDW and then dried on sterile filter papers. The sterilised plant pieces were put adaxial side down on the surface of either PDA or V8 medium and incubated for 5 days under continuous florescent light at 20°C. Pure cultures were obtained by sub-culturing on to fresh agar media. From these pure cultures agar slants were made in universal glass tubes, incubated for 5 days and then stored as stock cultures at 3-5°C. For isolates with good sporulation, spore suspensions were made in 50% glycerol and 1.5 mL aliquots placed in Eppendorf tubes and kept at -80°C as stock cultures.

Table 3.1 First group of isolates of *P. teres*, reported sensitivity and source.

Isolate ID	Barley cultivar	Purported sensitivity	Source[a]
H ½	Unknown	Wild type	DuPont (Hungary)
18	Unknown	Unknown	SASA (CABI – UK)
83	Pearl	Unknown	SASA (Hampshire – UK)
458	Unknown	Unknown	SASA (Suffolk – UK)
557	Unknown	Unknown	SASA (North Humberside – UK)
1782	Oxbridge	Unknown	SASA (East Lothian – UK)
Pt 01-02	Unknown	Unknown	NIAB (UK)
1522	Unknown	Wild type	DuPont (UK)
1539	Unknown	Mutant type (F129L)[b]	DuPont (France)
Pt 07-1	Unknown	Unknown	NIAB (UK)
1534	Unknown	Mutant type (F129L)	DuPont (Belgium)
F20/3	Unknown	Mutant type (F129L)	DuPont (France)
1530	Unknown	Wild type	DuPont (France)

[a] Isolates received as pure cultures in 2007.
[b] provided as F129L later shown not to have this mutation.

Table 3.2 Second group of isolates of *P. teres*, obtained in this study during growing season 2008-2009.

Isolate ID	Barley cultivar	Fungicide history	Source[a]
OTV-1	Cassata	Treated once with Fandango	Oxfordshire-Thames Valley
MR2-1	Pearl	Untreated	TAG- Morley
MR1-1	Cassata	Untreated	TAG- Morley
LN-2	Flagon	Untreated	Linby-Nottinghamshire
HSS-2	Pearl	Untreated	TAG Hampshire Sutton Scotney
GL-2	Flagon	Untreated	Glentham- Lincolnshire
CoL-2	Pearl	Untreated	Caythorpe-Lincolnshire
CayL-3	Pearl	Untreated	Caythorpe-Lincolnshire
BoT-1	Saffron	Untreated	Stapenhill, Burton on Trent
THM-2	Cassata	Untreated	TAG-Hampshire

[a] Isolated from samples provided as infected leaves from field-grown crops.

3.2.2 Induction of sporulation

According to the literature reviewed and based on culturing processes undertaken during this study, *P. teres* sporulates poorly on the common medium PDA and this was the main hindrance in artificial inoculation. In this regard, different media and different light regimes have been used by many researchers in order to enhance the sporulation of the net blotch pathogen. Media tested in the study reported here as shown in Table 3.3.

Agar media were evaluated for their ability to produce conidia for inoculation of barley plants. The experiment was arranged as a completely randomised design (CRD) with four replicates. Each replicate was a 9 cm Petri dish inoculated at 5 points with 1 cm^2 fungal culture blocks, taken from the edge of 7 d-old cultures. The culture blocks were placed with mycelium downwards and then incubated for 7-15 d, depending on the procedure used in the experiment. Separate procedures were followed including different light regimes described in Table 3.3.

Table 3.3 Media and light regimes used in the study to enhance sporulation of the *P. teres* isolates.

Media	Components L^{-1}	Light regimes (h)
Full strength PDA(1)	39 g	24 UV, 12 NUV
50% PDA	19.5 g	12 NUV
25% PDA	9.75 g	12 NUV
V8 Agar (20%)(2)	200 mL v8 + 3 g CaCo3 + 18 g agar	24 UV
V8 Agar (10%)	100 mL v8 + 3g CaCo3 + 18 g agar	12 NUV
POA(3)	50 g peanut leaflets + 15 g oatmeal + 20 g agar	12 NUV
Modified Czapek's medium(MCM)(4)	0.5 g KH2PO4 + 0.5 g MgSo4 + 0.5 g KCL + 1.2 g Urea + 20 g Lactose + 20 g Agar	24 UV, 12 NUV
Malt extract agar(MEA)(2)	1.5% malt extract + 2% agar	12 NUV
Barley leaf agar(BLA)(3)	100 g green barley leaflets + 20 g agar	12 NUV
Barley meal agar(BMA)	50 g barley seed meal + 18 g agar	12 NUV
TPA(5)	20 g tomato paste(30%) + 13 g agar	Dark

(1) Karakaya and Akyol (2006)
(2) Peever and Milgroom (1994)
(3) Speakman and Pommer (1998a)
(4) Ordon *et al.* (2007)
(5) Al-Tikrity (1987)

Disrupting of the surface of the culture and the effect of removal of aerial hyphae was also evaluated after 5 d of incubation, followed by re-incubation under uv-light for an additional 3 d. The spore production was measured by flooding the surface of the cultures with 10 mL of water and disruption with a sterile spatula. The suspension obtained was put into 50 mL Falcon tubes and then shaken vigorously to release spores. Spore concentration was measured using a haemocytometer. The results of sporulation are shown as averages of the spore production for all procedures tested.

3.2.3 Inoculation methods

3.2.3.1 Mycelium suspension

The net blotch-susceptible barley cultivar Pearl was grown in 9 cm pots at a density of 10 plants per pot. Plants were watered daily to maintain vigorous growth. At the growth stage 12 (Zadoks *et al.*, 1974), the plants were inoculated with spores, macerated mycelium fragments or with the combination of two. To prepare inoculum, fungal mycelium of isolates of *P. teres* was grown on either PDA or V8 agar amended with antibacterial antibiotics. Fresh plates were inoculated at 5 points with agar cubes taken from margins of 7 day-old *P. teres* cultures. The inoculated plates were incubated for 10 d at 20^{o}C with alternate 12 h near-UV light and dark, to enhance sporulation. After incubation, the surface of the 10 d old cultures was flooded with water and scraped with a spatula to release spores and mycelium. The spore and mycelium were macerated with a blender, filtered through 2 layers of muslin and then adjusted to 1 x 10^4 units mL^{-1} (comprising a mixture of mycelium fragments and conidia) with the aid of a haemocytometer. Tween 20 (10 µL) was added per 100 mL of inocula as a wetting agent. Barley plants were spray inoculated until run-off, using a hand sprayer and then the plants were bagged with transparent plastic bags for 24 h. A layer of water was also added to the bottom of the trays to keep a high humidity. Ten days after inoculation, net blotch disease was assessed on each isolate using the 1-10 rating scale described in section 2.6.1.

3.2.3.2 Mycelial plugs

To modify the inoculation procedure, due to the lack of sporulation of some isolates, fungal mycelium plugs were used to inoculate barley plants as an alternative method. Barley plants of two cultivars, Pearl and Cassata, at growth stage 12, were inoculated using mycelium plugs taken from 7 d old *P. teres* cultures of isolates F20/3 (Mutant F129L) and 1782 (Wild type) grown on PDA medium. For this purpose, the upper surface of the second leaf of plants was chosen and inoculated with 5 mm mycelium discs, which were placed mycelium downwards at 2-3 cm from each other (Figure 3.1). To maintain high relative humidity, the plants were covered with transparent plastic bags for 72 h and a layer of water put in the trays to maintain high humidity. The

inoculated plants were maintained in a growth room at a temperature of 20°C for 10 d and the disease was assessed visually as described before in section 3.2.3.1.

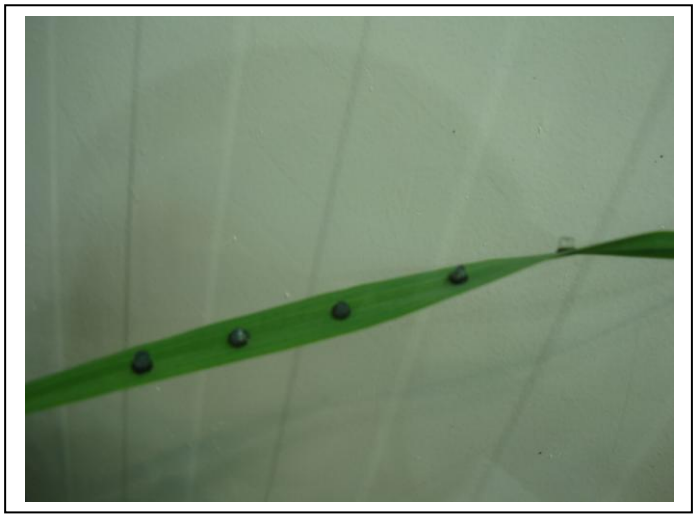


Figure 3.1 Mycelium plug as a method for artificial infection of barley plants with isolates of *P. teres*.

3.2.3.3 Growth of plants from artificially-inoculated seed

Artificially inoculated barley seed was prepared as a trial to find an alternative method to produce infected barley plants. To do this 5 d-old *P. teres* cultures were prepared in 9 cm Petri dishes. Seeds of barley cultivar Pearl were surface sterilised with 20% Domestos solution for 30 min, washed 3 times with sterilised water then dried with filter paper. The seeds were placed on the edges of growing colonies at a rate 5-10 seed per plate (Figure 3.2). The fungal cultures were incubated for further 3 d. Seeds with visible grown fungal mycelium were then grown in 9 cm pots in standard potting compost. The grown barley plants were monitored for the appearance of net blotch symptoms from the beginning of germination until growth stage 14.

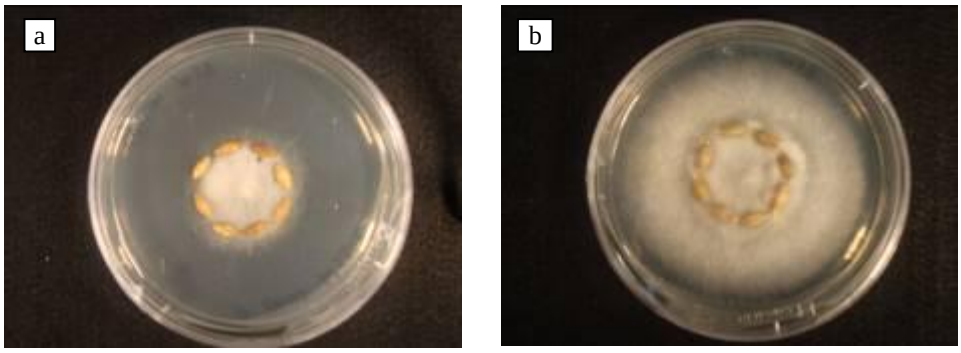


Figure 3.2 Barley seeds, cultivar Pearl, surface sterilised then put on the edges of *P. teres* mycelium culture; a) start of incubation, b) after 7 days of incubation.

3.2.4 Detection of the F129L mutation in *P. teres* isolates

To detect the F129L mutation in unknown-pedigree isolates and to confirm the presence of the mutation in other isolates received from different research centres, PCR methods were used. Fungal DNA extractions were done according to the following procedure: 100 mg of fresh fungal mycelium, grown on PDA medium, was taken from each isolate and put in microtubes (2 mL screw cap tubes) with 0.5 g of 2 mm glass beads and then placed in liquid nitrogen for 30 s. To disrupt the fungal tissue, the tubes placed in a tissue-lyser (FastPrep™ FP 120, Thermo Electron) and run at the highest speed (6.5 Hz) for 40 s. The fungal DNA was then extracted following the manufacturer's protocol for the mini extraction kit (DNeasy® Plant Mini Kit (50), QIAGEN, GmbH). A 351 bp PCR fragment was amplified following the procedure of Semar *et al*. (2007) with the primers shown in Table 3.4. Primers in the paper Semar *et al*. (2007), and used in this study, were site-specific (allele-unspecific). The 351 bp primer amplified part of *cyt b* gene sequence, which only included the target site for the F129L mutation and none of the other known sites for QoI resistance (eg G143A). This single exon target, which starts at 4315 and ends at 4665 (15 bp), is located between two introns (NCBI Genbank, accession No. DQ919067). The PCR products were sequenced to detect polymorphisms. Another group of allele-specific primers, derived from the paper of Sierotzki *et al*. (2007) were also tried to detect SNPs in DNA gene

sequences. After using the latter group of primers, DNA did not required sequencing; the PCR products were run on a gel to detect any differences.

Table 3.4 Primers used to amplify DNA of *P. teres* isolates.

Primer name	Priming direction	Sequence (5'-3')	Specificity	Tm°C
CytbC1[a]	Forward	TGGTGGGTGGCTGAATATGCTACT	F129L allele-unspecific	60
CytbC2[a]	Reverse	CAGACATTCCAAGACTATTTGAGGAAC	F129L allele-unspecific	60
PtCytF1[b]	Forward	AGGTTGTAGTTAGCCGGGAAC	F129L allele-unspecific	57.3
PtCytF2 [b]	Forward	AGATAAATTTAGGTTGTAGTTAGCC	F129L allele-unspecific	56.4
PtCytR1 [b]	Reverse	ACTTTTGTTAAACAGTCTTTTATTG	F129L allele-unspecific	53.1
PtF129Lun[c]	Forward	CCGCAAAATATCGGGBACTAA	F129L allele-unspecific	57.9
PtTTCsp[c]	Reverse	GCTATGTTGGTAACCCAGGCA	TTC allele-specific	59.8
PtTTAsp[c]	Reverse	TTTGTGCTATGTTGGTAACCCTGT	TTA allele-specific	59.3
PtTTGsp[c]	Reverse	TGTGCTATGTTGGTAACCCTGC	TTG allele-specific	60.3
PtCTCsp[c]	Reverse	GTGGCTATGTTGGGTAACCCAGGTG	CTC allele-specific	62.4

[a] primers used by Semar *et al*. (2007)
[b] primers designed in this study
[c] primers used by Sierotzki *et al*. (2007)

Amplifications were performed in a total volume of 25 µL working solution, comprising of 0.4 µM of each primer, 0.2 mM dNTPs, 1 x PCR reaction buffer (Promega, Madison, USA), 1.5 mM $MgCl_2$, 0.5 U polymerase (GoTaq® Flexi DNA Polymerase, Promega, Madison, USA). PCR was performed in Flexigene cycler (Flexigene, Cambridge, UK) under the following standard conditions: initial preheat for 3 min at 95°C, followed by 35 cycles at 95°C for 15 s, annealing temperature 60°C for 30 s and 72°C for 30 s followed by a final amplification step 72°C for 15 min. Amplified DNA fragments were resolved on 1.5% agarose gels (Bioline, UK) for 60 minutes at 90 volts. The gel was

prepared with 1 x TAE buffer and ethidium bromide was added to provide a final concentration of 0.5 µg mL^{-1}. Four microliters of each PCR product was loaded in to the gel well with 4 µL of DNA size marker (100 bp ladder) (Promega, Madison, USA). To eliminate multi-bands, PCR products were purified from the clear bands displayed on the gel (GenElute™, Gel Extraction Kit, Sigma) and then the products were purified with GenElute™ PCR CleanUp Kit (Sigma). The final purified products were quantified by using a NanoDrop® Nd-1000 spectrophotometer and then PCR fragments were sequenced (CEQ 8000 Beckman Coulter). Sequences were aligned and analysed by using BioEdit software (Biological sequence alignment editor, version 7.0.9) and the changes in the sequences were compared with sequence of *cyt b* gene (GenBank: DQ919067.1).

3.2.5 Detection of fitness costs

3.2.5.1 Measuring sporulation

Sporulation as one of the components of pathogen's fitness was measured using the procedure described in section 3.2.1. Isolates of *P. teres* were grown on either PDA or V8 medium under continuous fluorescent light with 12 h UV-light for 10 d. Petri dishes (9 cm) were inoculated at 5 points with 1 cm^2 fungal culture blocks taken from edges of 7 d old cultures. The culture blocks were placed upside down and then incubated for 10 d. Conidia production was measured by flooding the surface of the cultures with 10 mL of water and disruption with a sterile spatula. The resultant suspension was placed in 50 mL Falcon tubes and then shaken vigorously to release conidia. From the suspension thus prepared the number of spores recovered was measured using a haemocytometer. The experiment was a completely randomised design with 4 replicates.

3.2.5.2 Measuring growth rate

The growth rate of mycelium of *P. teres* isolates were tested on agar culture on 9 cm Petri dishes. Using a sterile cork borer, PDA medium was inoculated with 5 mm mycelium discs taken from edges of 7 day-old cultures of isolates of the pathogen grown on PDA. The discs were placed mycelium downwards on the centre of the Petri dishes and then incubated in the dark with a temperature of 20°C ± 2 for 10 d (Figure 3.3). The radial growth of the

pathogen was measured. The measurements were taken in two planes at 90^{o} to each other and averaged. After a deduction of 5 mm was made for the diameter of mycelial discs, the growth rate was measured and expressed in mm d^{-1}. Then the data was analysed by using GenStat version 11 software package.

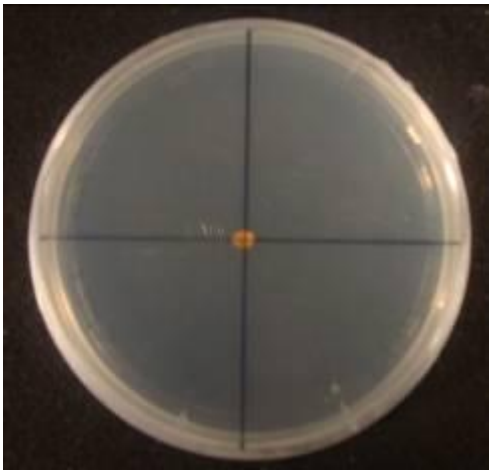


Figure 3.3 Potato dextrose agar medium inoculated in the centre with a 4 mm mycelial disc taken from edge of 7 d old cultures of *P. teres*

3.2.5.3 Pathogenicity

To investigate the disease aggressiveness of wild type and mutant isolates of *P. teres* and to establish possible fitness costs associated with the mutation, the susceptible barley cultivar Pearl was grown in 9 cm pots (10 plants per pot). The CRD experiment was arranged with 4 replicates. At the growth stage 12, the plants were inoculated with a mixture of mycelium and spores at 1 x 10^4 propagules mL^{-1}. Post-inoculation conditions and disease assessments were as described in section 3.2.3.

3.2.6 Data analysis

Data were analysed by using general analysis of variance (ANOVA) from Genstat (10th edition). Fisher's least significant difference (LSD) with a significant level of 5% was performed to determine significant differences between means.

3.3 Results

3.3.1 Induction of sporulation

The results of using different media and light regimes to induce sporulation showed that there was poor sporulation for many isolates of *P. teres*. Results given in Table 3.5 summarise the efficacy of 11 media tested.

Table 3.5 The effect of different media used to enhance sporulation of different *P. teres* isolates.

Isolate	PDA-full	50%PDA	25% PDA	V8-full	50% V8	POA	MCM	MEA	BLA	BMA	TPA
H ½	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1530	*	*	*	*	*	*	NS	NS	NS	NS	NS
1534	*	*	*	**	**	**	NS	NS	NS	NS	NS
1522	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1539	****	***	**	****	**	NS	NS	NS	NS	NS	NS
18	*	*	*	*	*	NS	NS	NS	NS	NS	NS
83	**	**	*	**	**	**	NS	NS	NS	NS	NS
458	*	*	*	*	*	*	NS	NS	NS	NS	NS
557	*	*	*	*	*	*	NS	NS	NS	NS	NS
1782	**	**	**	**	**	**	NS	NS	**	**	NS
Pt 01-02	**	**	**	**	**	**	NS	NS	**	**	NS
Pt 07-1	**	**	**	**	**	**	NS	NS	**	**	NS
F20/3	**	*	*	**	*	*	NS	NS	NS	NS	NS
Bot-1	*	*	*	*	*	*	NS	NS	NS	NS	NS
THM-2	**	*	*	**	*	*	NS	NS	NS	NS	NS
HSS-2	*	*	*	*	*	*	NS	NS	NS	NS	NS
Cayl-3	**	*	*	**	*	*	NS	NS	NS	NS	NS
Col-2	*	*	*	*	*	*	NS	NS	NS	NS	NS
MR-1-1	***	**	*	***	**	*	NS	NS	NS	NS	NS
OTV-1	**	**	*	*	*	*	NS	NS	NS	NS	NS
GL-2	**	*	*	**	*	*	NS	NS	NS	NS	NS
MR2-1	***	*	*	***	*	*	NS	NS	NS	NS	NS

**** Excellent
*** Good
** Moderate
* Poor
NS No sporulation

Good or partial sporulation occurred with using PDA, V8 JA and peanut oatmeal agar. Excellent sporulation occurred in isolate 1539 when full-strength PDA and V8 was used. PDA at 50% also supported good sporulation for isolate 1539. Both MR-1-1 and MR-2-1 produced good sporulation when grown on full-strength PDA and V8 JA. Moderate sporulation was obtained by using either PDA or full-strength or half-strength V8 JA with isolates, 1534, 83, 1782, pt01-02, pt01-07 and F20/3. No sporulation was obtained in both isolates H1/2 and 1522 with all media tested in the experiment. On the other hand, there was no sporulation of many isolates with using media MCM, MEA, BLA, BMA and TPA. Agar disruption and different light regimes did not give improved sporulation (data not shown).

3.3.2 Inoculation methods

3.3.2.1 Fungal suspension

The results of using a fungal suspension, comprising a mixture of mycelial fragments and conidia, showed the ability of all *P. teres* isolates tested to infect the susceptible barley cultivar, Pearl. A considerable difference ($F_{(20,42)}$=18.58, P<0.05) was found between the isolates used (Table 3.6). The results in Figure 3.4 show that the wild type isolate 1539, which has a greatest conidial production, produced the highest disease score on barley plants and showed a significant difference with the rest of the *P. teres* isolates evaluated. There were no significant differences between isolates MR-2, MR-1, 1534, THM-2, and F20/3. These isolates have degrees of conidia in the inoculum mixture ranging between moderate and good. On the other hand, no significant differences were found between 10 isolates four of them with moderate conidia production (1782, Cayl-3, Pt07-1, Otv-1) and six with a poor conidia production (557, 18, 1530, Col-1, 458, and Hss-2). However, infection with isolates 1522 and H1/2, which did not sporulate at all, were significantly lower.

Table 3.6 Statistical analysis of the difference in pathogenicity between *P. teres* isolates.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	P-Value
P. teres isolate	20	115.69	5.78	18.58	<0.001
Residual	42	13.07	0.31		
Total	62	128.8			

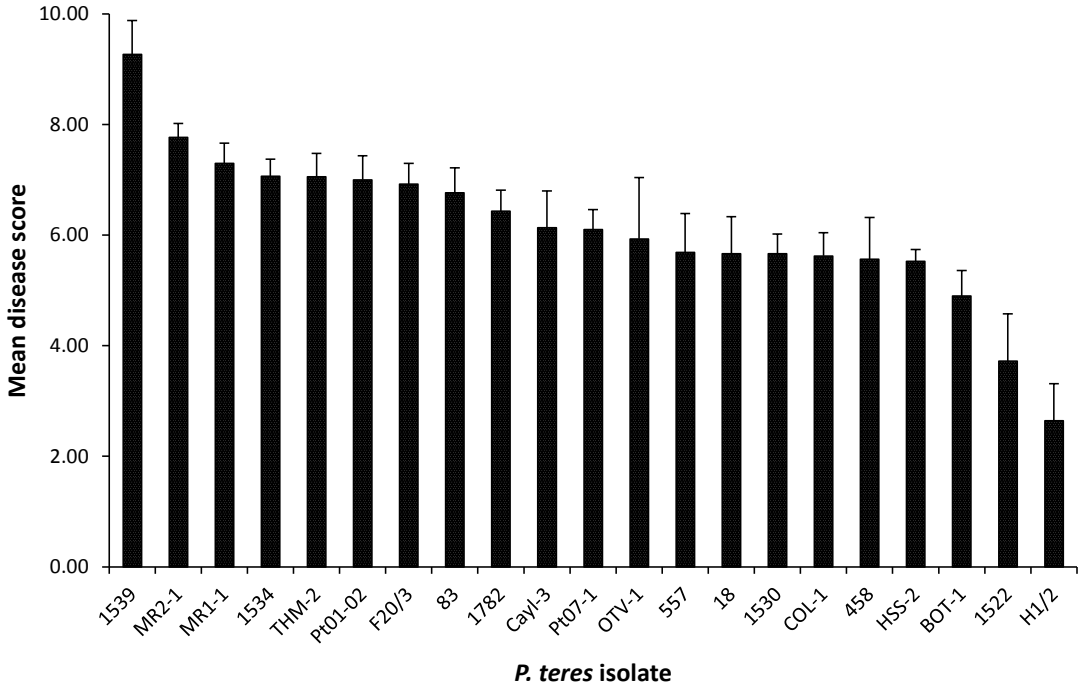


Figure 3.4 Infection of the barley cultivar Pearl with a mixed suspension of mycelium and conidia of isolates of *P. teres* assessed using the 1-10 scale of Tekuaz, (1985). Error bars are standard deviation.

3.3.2.2 Mycelial discs

Use of mycelial discs was an alternative method to attempt to infect barley, especially for isolates exhibiting poor sporulation. The results of using this technique revealed that the method could infect plants and provide visible symptoms. However, they were small and not typical of the symptoms

produced using suspensions sprayed on to plants (Figure 3.5 b and d). The lesions did not exhibit net-like symptoms of the type produced by *P. teres* either naturally or produced with spray inoculation.

However, using mycelial discs to inoculate 2 barley cultivars with 2 *P. teres* isolates showed that there was no significant differences in infection between Pearl and Cassata cultivars (Figure 3.6) and disease incidence induced by F20/3 (F129L mutant) isolate was significantly higher than that resulting from that with the wild type isolate 1782.

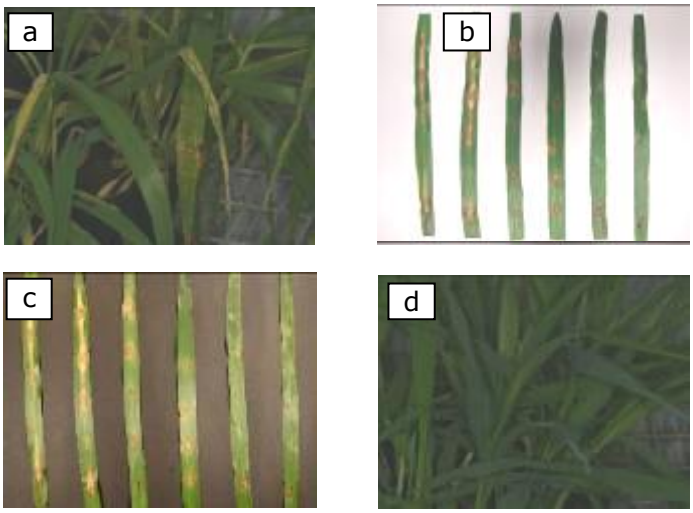


Figure 3.5 Barley net blotch symptoms; a) symptoms produced by inoculating with a mixture of conidia and mycelium fragments; b) symptoms produced by using mycelium plugs on barley cultivar Cassata; c) symptoms produced by using mycelial plugs on cultivar Pearl ;d) healthy barley plants.

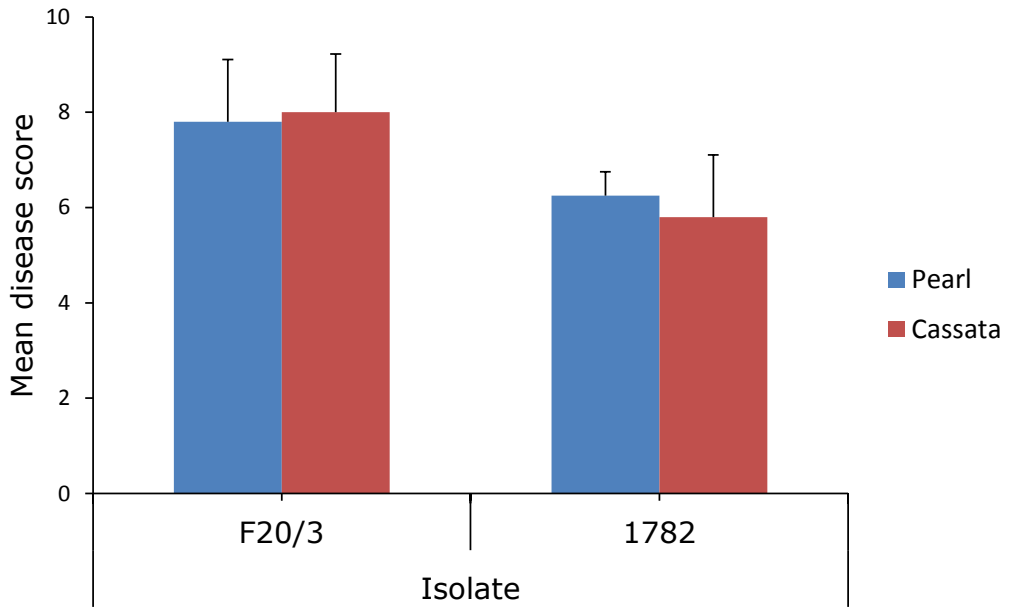


Figure 3.6 Disease development on two barley cultivars with two isolates of *P. teres* using mycelial plugs.

3.3.2.3 Artificially inoculated seeds

After the emergence of plants grown from artificially inoculated seed, they were monitored and inspected for any occurrence of disease incidence. The plants were allowed to grow in a conducive environment with daily observation, but by 3 weeks after emergence none of the plants were infected with net blotch.

3.3.3 Detection of F129L mutation in *P. teres* isolates

The *cyt b* gene from DNA isolated from *P. teres* was amplified using PCR. The resulting PCR products were run on agarose gel, visualized and the predicted DNA bands of 351 bp were clearly detected (Figure 3.7). DNA sequence analysis showed that 10 isolates out of 23 tested were QoI-insensitive, carrying the F129L mutation (Table 3.7). Figure 3.8 shows that the codon TTC (coding for phenylalanine in the wild type) was changed to TTG in isolate F20/3, to CTC in isolate 1534 and to TTA in the rest of mutant isolates tested (all coding for leucine in the mutant types). Sequence analysis also revealed that isolate 1539 obtained from DuPont, France, which was donated as mutant, showed the wild-type genotype. This was confirmed when the template DNA was sequenced in both forward and reverse directions.

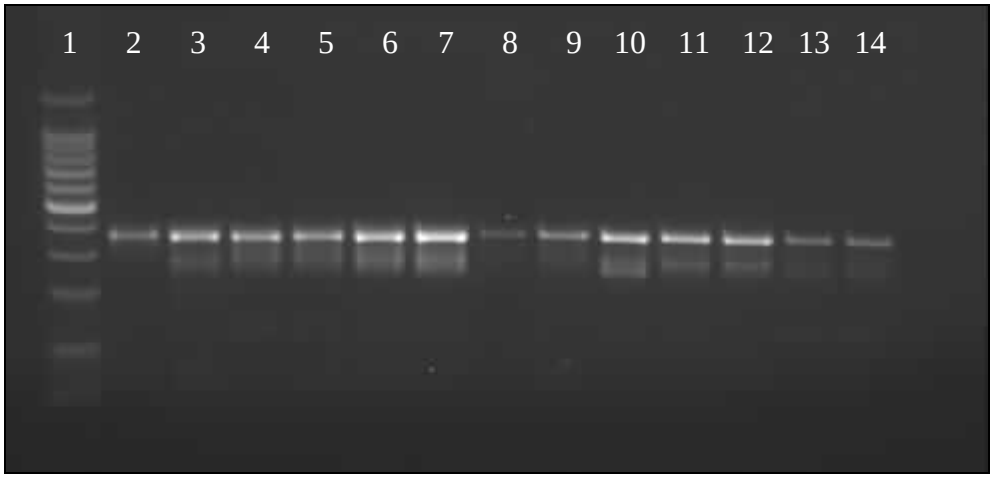


Figure 3.7 Visualisation of DNA fragments of 13 *P. teres* isolates on gel electrophoresis. Lane 1: 100 bp ladder, lane 2-14: *P. teres* isolates

a

H1/2	ACAGCCTTCCTGGGT
1522	.....
1530	.....
1534	.....C.....
1539	.....
F20/3	.....G.....
18	.....
83	.....
458	.....
557	.....
1782	.....
pt 01-02	.....
pt 07-1	.....
OTV-1	.....A.....
MR2-1	.....A.....
MR1-1	.....A.....
LN-2	.....A.....
HSS-2	.....
GL-2	.....A.....
COL-2	.....A.....
CAYL-3	.....A.....
BOT-1	.....
THM-2	.....A.....

b

H1/2	TAFLG
1522	.....
1530	.....
1534	..L..
1539	.....
F20/3	..L..
18	.....
83	.....
458	.....
557	.....
1782	.....
pt 01-02	.....
pt 07-1	.....
OTV-1	..L..
MR2-1	..L..
MR1-1	..L..
LN-2	..L..
HSS-2	.....
GL-2	..L..
COL-2	..L..
CAYL-3	..L..
BOT-1	.....
THM-2	..L..

Figure 3.8 Sequence alignment of a portion of the amplified fragments of the *cyt b* gene shows that the codon TTC (coding for phenylalanine of the wild type) is present as CTC, TTG and TTA all coding for leucine in the mutant types of *P. teres*; a) nucleotide alignment; b) translated amino acid alignment.

There were no ambiguities in the base calling from sequencing traces, with each chromatogram file showing clear, distinct peaks at the region of interest (Figure 3.9). In addition, further confirmation was made when the PCR products of isolates 1534, 1539 and F20/3 were sequenced by GATC Biotech Ltd., St John's Innovation centre, Cowley Road, Cambridge, UK.

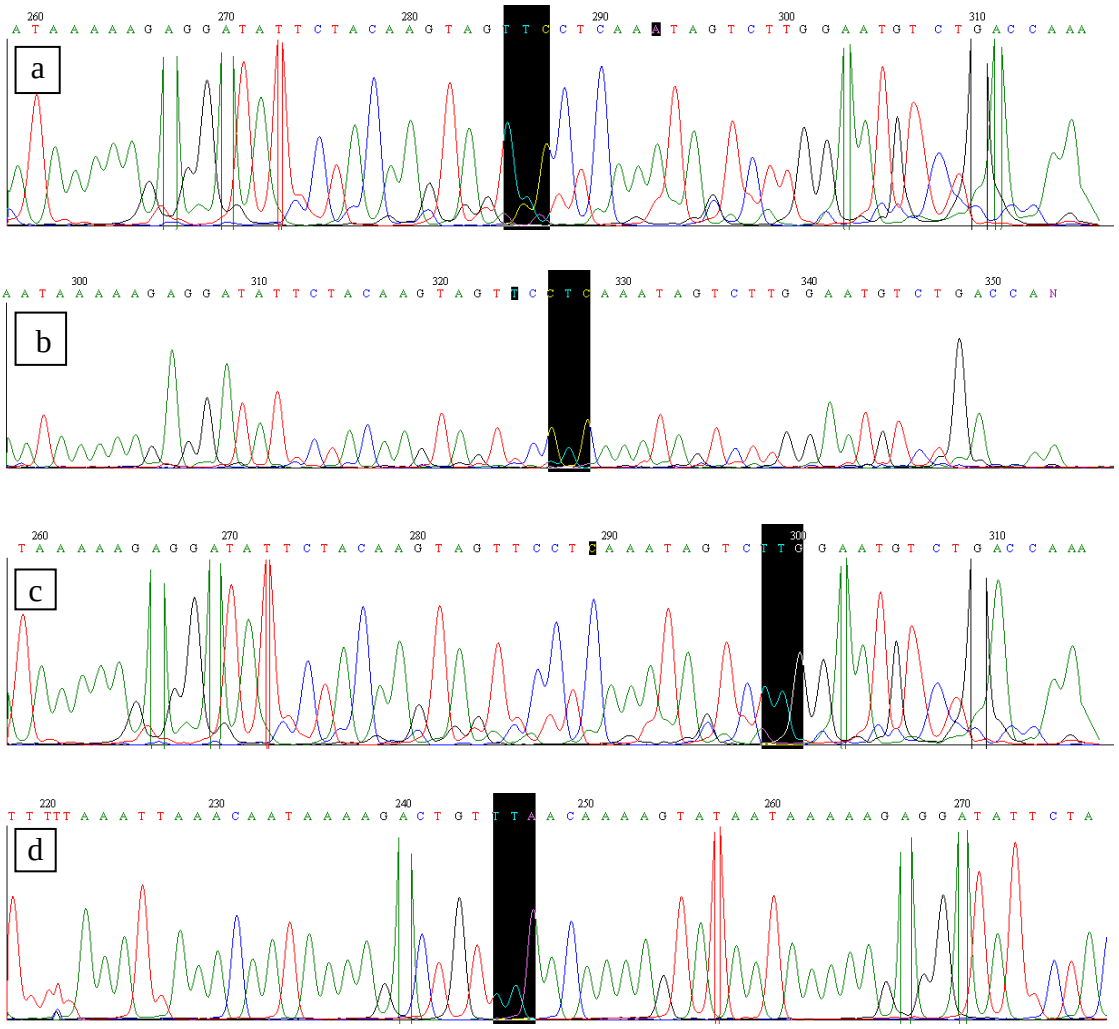


Figure 3.9 Chromatograms of DNA sequencing analyses showing clear distinct peaks at the region of interest; a) wild type isolate 1530 (TTC represents phenylalanine); b) mutant isolate 1534 (change to CTC); c) mutant isolate F20/3 (change to TTG) ; and d) mutant isolate GL-2 (change to TTA), all these changes represent leucine in mutant isolates of *P. teres*.

Table 3.7 Detection of change of phenylalanine to leucine at mutation site 129 in 23 *P. teres* isolates tested.

Isolate ID	Source	Sequence result
H1/2	DuPont (Hungary)	WT
18	SASA (CABI – UK)	WT
83	SASA(Hampshire – UK)	WT
458	SASA (Suffolk – UK)	WT
557	SASA (North Humberside – UK)	WT
1782	SASA (East Lothian – UK)	WT
Pt 01-02	NIAB (UK)	WT
1522	DuPont (UK)	WT
1539	DuPont (France)	WT
Pt 07-1	NIAB (UK)	WT
1534	DuPont (Belgium)	MT (F129L)
F20/3	DuPont-France	MT(F129L)
1530	DuPont (France)	WT
OTV-1	Oxfordshire-Thames Valley	MT(F129L)
MR2-1	TAG- Morley	MT(F129L)
MR1-1	TAG- Morley	MT(F129L)
LN-2	Linby-Nottinghamshire	MT(F129L)
HSS-2	TAG Hampshire Sutton Scotney	WT
GL-2	Glentham- Lincolnshire	MT(F129L)
COL-2	Caythorpe-Lincolnshire	MT(F129L)
CAYL-3	Caythorpe-Lincolnshire	MT(F129L)
BOT-1	Stapenhill, Burton on Trent	WT
THM-2	TAG-Hampshire	MT(F129L)

3.3.4 Fitness costs

3.3.4.1 Sporulation

The sporulation of isolates of *P. teres* was measured and a significant difference between isolates tested was detected ($F_{(21,66)}$=674.32, P<0.05) (Table 3.8). The results in Figure 3.10 show that wild type isolate 1539 gave the highest conidia production compared to other isolates. Two mutant F129L isolates, namely MR2-1 and MR1-1 came second ranking of sporulation and both differed significantly from the other isolates tested. Moderate sporulation was obtained with isolates THM-2(F129L), 1782, F20/3 (F129L), Cayl-

3(F129L), 83, GL-2 (F129L), 1534 (F129L), Pt07-1, Otv-1 (F129L) and Pt01-2. However, isolates Col-2, 18, 1530, HSS-2, 458, Bot-1, 557, H1/2 and 1522 exhibited poor or non-existent sporulation. However, no pattern was found between sporulation of isolates with respect to the presence of the F129L mutation.

Table 3.8 Statistical analysis of the difference in sporulation between *P. teres* isolates

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	P-Value
P. teres isolate	21	541.76	25.8	674.32	<0.05
Residual	66	2.53	0.038		
Total	87	544.28			

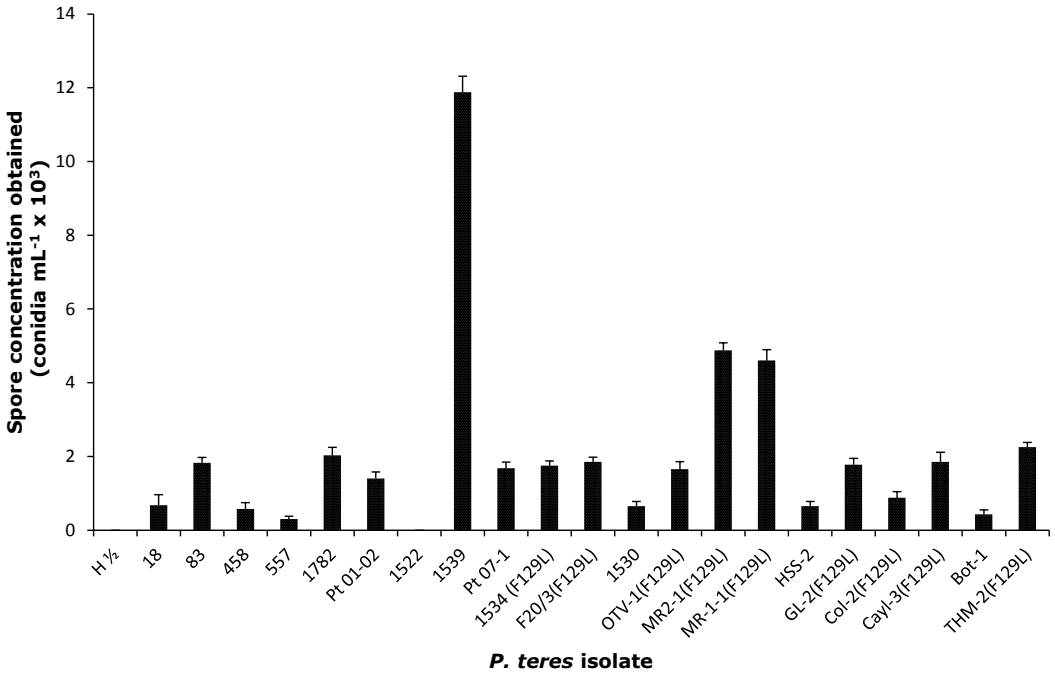


Figure 3.10 Comparison between 22 different *P. teres* isolates for their sporulation on PDA.

3.3.4.2 Growth rate

The total growth of 18 *P. teres* isolates was measured after incubation for 10 d and then the mean growth rate per day was calculated. The results in Figure 3.11 show that there was little difference between all isolates tested regardless of their sensitivity. However, the growth rate of isolate Col-2 was slow and H1/2 very slow.

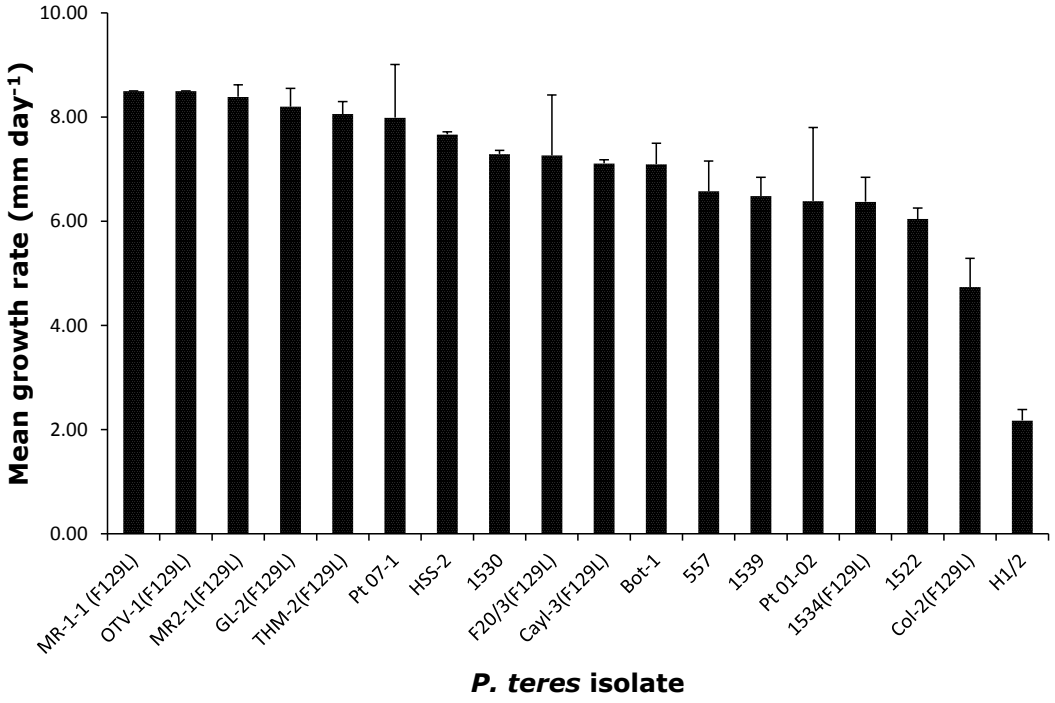


Figure 3.11 Growth rate of *P. teres* isolates grown on PDA. Each value is the average of four individual plates per isolate, error bars are standard deviations.

3.3.4.3 Pathogenicity

The optimum inoculation method developed was used to screen the pathogenicity of *P. teres* isolates. Statistical analysis showed highly significant differences ($F_{(20,42)}$=26.82, P<0.05) between isolates (Table 3.9). From the results shown in Figure 3.12 wild type isolates 83 and 1530 were the most pathogenic, compared to the rest of the isolates tested and have significant differences compared to other isolates. Other strains, which included wild-type

and F129L mutants, showed a range of pathogenicities, but again there was no pattern between genotypes with respect to the F129L mutation.

Table 3.9 Statistical analysis of the difference in pathogenicity between *P. teres* isolates.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	P-Value
P. teres isolate	20	342.43	17.12	26.82	<0.001
Residual	42	26.82	0.64		
Total	62	369.25			

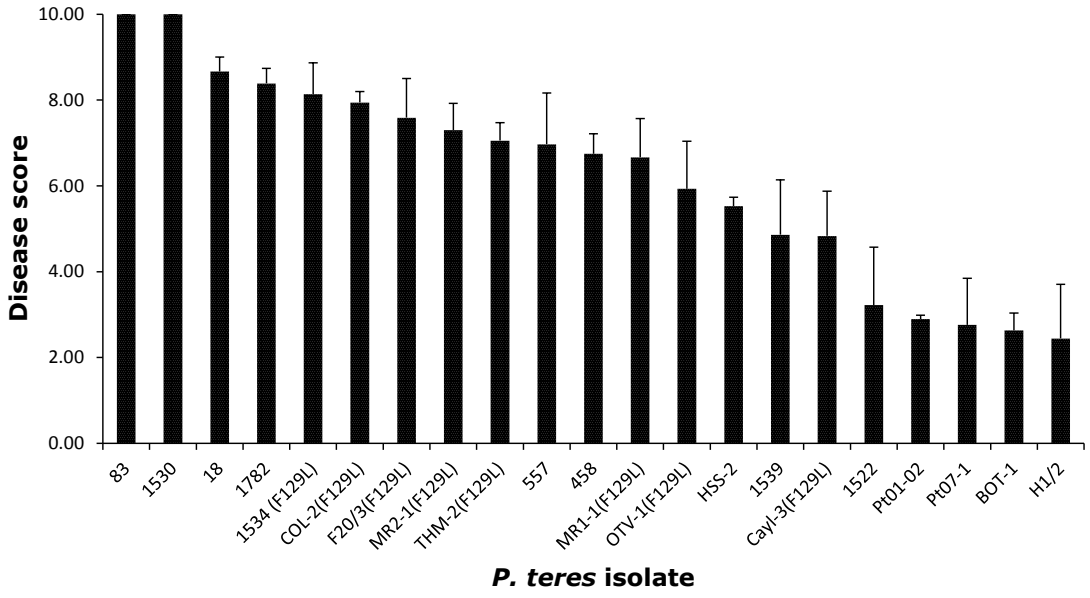


Figure 3.12 Pathogenicity of *P. teres* isolates towards barley cultivar, Pearl. Data taken 10 DAI.

3.3.5 Discussion

In this current research, in addition to obtaining cultures from research centres, new *P. teres* isolates were also obtained from leaf material provided by TAG. It is essential to a have a good set of isolates with different backgrounds to provide a sufficiently diverse population for subsequent analyses. A successful technique for isolation from infected barley plants was followed in this study. Development of a method for isolation from infected leaves was necessary because the process was continued up to the end of the study to maintain the virulence of the pathogen. Although the infected plant samples received were from different cultivars, the barley variety Pearl was used for maintaining isolates. The isolation technique, with slight modification, was broadly used by several previous researchers (Jonsson *et al.*, 1997; Robinson and Jalli, 1997; Gupta and Loughman, 2001; Arabi *et al.*, 2003; Karakaya and Akyol, 2006; Leisova *et al.*, 2006; Tuohy *et al.*, 2006; Sierotzki *et al.*, 2007) for isolating isolates of *P. teres*. However, in their techniques, there were differences in use of fresh or dried leaves and seed, sterilising agent (whether sodium hypochlorite or ethanol or both), media used, temperature, light, and incubation time.

Several agar media were utilised to attempt to produce conidia from *P. teres* cultures. However, few of them could enhance sporulation, which was very important for inoculation and provision of uniform, consistent disease symptoms. Despite that PDA and V8 medium gave the best sporulation for several isolates but they could not stimulate sporulation of several others. Alternating incubation temperature from 20 (daytime) to 12^{o}C (night time) did not affect conidia formation on agar media. However, the results revealed that alternating fluorescent light and UV light with dark periods increased conidial formation. Agar disruption to the growing mycelium after 5 days of incubation had no effect. However, many other researchers could produce conidia in different circumstances. Sato and Takeda (1991) recommended use of V8 agar medium at 25 + or – 6 degrees under a NUV irradiation regime. Al-Tikrity (1987) enhanced conidia production on tomato paste agar supplemented with calcium carbonate ($CaCO_3$) incubated at 21^{o}C for 9 days in dark. Similarly, Speakman and Pommer (1998a) found abundant sporulation using POA (peanut oatmeal agar) for isolates of *P. teres* incubated at 18^{o}C and in 12 h

dark and 12 h light cycle for 15 d. It could be concluded from results reported here that under identical conditions different isolates have variable sporulation. This conclusion is in strong agreement with Babadoost and Johnston (2005) who found significant differences in sporulation among isolates of *Pyrenophora graminea* from different areas of Canada, Montana, Germany and Syria and from isolates originating from the same field. They suggested 7 days of continuous NUV light resulted in 40% higher conidia production. However, Deadman and Cooke (1985) concluded that the fungus *P. teres*, in comparison with other *Pyrenophora* species, is traditionally a poor sporulator in culture and much variation exists between isolates.

The existence of variation in sporulation between isolates of *P. teres* led to the investigation of more than one inoculation method. In addition to attempting inoculation of barley plants with conidia and mycelium suspensions, mycelium discs and artificially inoculated barley seed were also tried. The results obtained indicated the traditional method of a conidial and mycelial suspension was superior to other methods tested. This therefore became the standard inoculation method used for artificial infection of barley with *P. teres*. The concentration of 1 x 10^4 units mL^{-1} was found sufficient to produce uniform symptoms. This concentration was consistent with that utilised by Leisova *et al*. (2006), where they used a conidia suspension concentration ranging from 5 x 10^3 to 1 x 10^4 per mL. A high humidity was critical to establish the disease on barley leaves. This was secured by putting a layer of water at the bottom of the trays used in the experiment. The necessity of providing high humidity is strongly supported by many researchers referred to in section 3.1.3. Those isolates which sporulated well and thus contributed a high conidial proportion to the inoculum tended to be more pathogenic. However, mycelial suspensions, as an alternative to conidia, were also reported to be successful in production of net blotch symptoms in earlier work by Arabi *et al*. (2003) and Karakaya and Akyol (2006).

The results of sequence analysis of the portion of the *cyt b* gene showed that the F129L mutation is widespread within the population of *P. teres* screened for F129L mutation (43% of 23 isolates). This is especially true for the second group of isolates collected in the 2008 season in the UK, where eight isolates

out of 10 were F129L mutants. The wild-type and mutant isolates in this sample set indicates the prevalence of the mutation in the UK population of *P. teres*. However, sequence results showed that isolate 1539, which was provided by DuPont as an F129L mutant isolate, did not carry this mutation. The original characterisation by DuPont was based on fungicide-sensitivity phenotype, rather than on genotype sequence analysis, and it was thus incorrectly identified, before being donated for this work. However, the primers used to amplify *cyt b* gene did not extend to cover the sites which contain other possible resistance mutations, such as G137R or G143A. Therefore, the insensitivity of this isolate (shown later in Chapter 4) may possibly be due to the presence of these mutations.

This widespread nature of the F129L mutation was confirmed by Jorgenson (2008), who reported that since 2008 the F129L mutation has been on the increase within UK and French populations of the net blotch pathogen. Sequence analyses also revealed that the change in the *cyt b* gene in the codon 129 is from TTC to TTG in isolate F20/3 and to CTC in isolate 1534 and for the rest of the mutant isolates the change was from TTC to TTA. The latter change seems more common than other changes, especially in recent collected strains from the UK. Finding the same codon for leucine in mutated UK isolates perhaps indicates that the F129L mutations did not occurr independently, suggesting that they may have arisen from one single mutation event, with subsequent further distribution. The existence of an intron directly after the position 143 is supported by worldwide extensive monitoring studies. Semar *et al*. (2007) and Sierotzki *et al*. (2007) reported that in *P.teres*, an intron in the *cyt b* gene, was present immediately after the codon for the amino acid in position 143. The G143A mutation would prevent splicing out of the intron, prior to transcription into mRNA, thereby disrupting functionality of the *cyt b* protein, leading to a lethal event. Thus the G143A mutation cannot occur in *P. teres*. The same phenomenon has also been found in other plant pathogens. Introns starting exactly after the codon 143 have been found and described in *Puccinia* spp. and *Phakopsora pachyrhizi* (Chen and Zhou, 2009) as well as in *Alternaria solani* (Yin *et al.*, 2009) and for these pathogens no G143A mutation has been detected to date, despite repetitive use of QoI fungicides. This intron was absent in pathogens such as *A.*

alternata, *Blumeria graminis*, *Pyricularia grisea*, *M. graminicola*, *M. fijiensis*, *V. inaequalis* and *P. viticola*, in which resistance to QoI fungicides has occurred and the glycine is replaced by alanine at position 143 in the resistant genotype. However, other conclusions did not agree with the above phenomenon. The research on field resistance of *Pososphaera fusca* (cucurbit powdery mildew) to QoI resisance done by (Perez-Garcia *et al.*, 2008) emphasised that the absence of G143A mutation is not due to the intron immediately after codon 143. This is also may be the case with other pathogens such as in *P. teres* ispite of the previous confirmations that this unlikely to happen.

Fitness costs due to the existence of the F129L mutation in terms of sporulation, growth rate and pathogenicity were investigated. Although the wild type isolate 1539 was the highest sporulator, compared to other isolates, some other wild-type isolates with poor or zero sporulation were also detected. Large diversity in spore production was, however, detected among isolates with the F129L mutation (Figure 3.10). The sporulation assay, used to detect a possible fitness penalty associated with the presence of the F129L mutation in different *P. teres* isolates, showed consistent results in both experiments reported (Table 3.5 and Figure 3.10). This may reflect consistency of environmental conditions used in both experiments. Results obtained in growth rate experiments, as an alternative parameter to measure fitness costs, demonstrated that there were no such penalties consistently associated with F129L mutant isolates. Five mutant isolates were found to have the highest growth rates.

However, the pathogenicity tests for the same group of *P. teres* isolates, reported in Figures 3.4 and 3.12, showed some inconsistency. Although similar results were obtained for the majority of isolates tested, some (eg 1539) showed considerable variability. This may reflect variation in the spore / mycelial fragment inocula, reduced environmental control in *in* planta experiments or loss of pathogecity with time in culture storage. Attempts to reduce the latter were, however, minimised by repeated re-isolation of the fungus from infected leaves throughout the course of the research programme. Although four wild-type isolates were found to be more

pathogenic, they did not differ significantly from some mutant isolates. Some other wild-type isolates were found to have low pathogenicity. Inferences from the pathogenicity tests suggest that there were no trade-offs for mutant isolates. The results of the three parameters used to measure fitness costs suggest that *P. teres* isolates behaved independently from the effect of their sensitivity towards QoI fungicides. A similar lack of correlation between fitness and resistance was supported by Peever and Milgroom (1994) who could not detect any fitness costs associated with resistance to triadimenol or propiconazole in isolates of *P. teres*.

Chapter 4 Net blotch of barley, *P. teres* and fungicide performance - bioassays

4.1 Introduction

4.1.1 Fungicide efficacy

Despite environmental concerns, fungicide applications remain essential, among other control methods, for maintaining healthy crops and reliable, high quality yields. The emergence of fungicides has contributed greatly to enhancement in quality and quantity of agricultural products (Oerke *et al.*, 1994). Fungicides also form a major contribution to integrated crop management and their effectiveness must be sustained as much as possible. It has been suggested that prohibition of pesticides, especially fungicides, would cause considerably higher yield reductions in field crops in northern Europe, where very intensive farming systems are used, than in southern Europe where productivity per area is lower (Oerke, 1999). Because of the lack of cereal cultivars highly resistant to all major fungal diseases, the application of fungicides remains a major factor in disease management (Verreet *et al.*, 2000).

Currently two major site-specific systemic groups of fungicides are widely used to control of cereal diseases. They are the triazoles and the strobilurins (QoIs). Triazoles dominate the cereal fungicide market, with application of single products accounting for as much as 40% of the total area to which foliar fungicides are applied in the UK since 1990 (Cools *et al.*, 2006). Strobilurins which have a broad spectrum activity against all major foliar cereal pathogens, are also important fungicides, and may have direct effects on plant physiology, resulting in higher yields of cereals (Beck *et al.*, 2002).

A major risk of intensive use of fungicides over large areas is the potential for partial or total loss of efficacy, due to the emergence of pathogen phenotypes that have the ability to overcome the activity of fungicides (Shaw, 2000). Resistance of cereal pathogens to fungicides is thus developing and has become a major constraint in agriculture, reducing the field performance of many products. Performance of most of the modern fungicides has been

affected to some degree and much evidence indicates that development of resistance is greatly favoured by the continued, exclusive use of fungicides with a specific mode of action (Brent, 1995). QoIs, since their launch in 1997, contributed to a substantial yield increase. However, just two years after their introduction, resistance was detected in many fungal plant pathogens (Heaney *et al.*, 2000; Fraaije *et al.*, 2003). Intensive studies of molecular mechanisms of QoI resistance have revealed that a single point mutation, which causes an amino acid change/substitution in *cyt b* is thought to govern the expression of resistance (Gisi *et al.*, 2002; Kuck, 2007). Insensitivity related to the F129L mutation has been found in the less sensitive isolates of some cereal pathogens, including *P. teres* (Semar *et al.*, 2007).

4.1.2 *In vitro* fungicide efficacy

Since the first development of pesticides different methods have been used to assess the activity of these compounds in solid culture (agar). *In vitro* fungal sensitivity, using amended agar with differing concentrations, is one of the most appropriate methods to evaluate fungicide activity (Georgopoulos, 1982). The method depends on measuring radial growth of mycelium of the target pathogen at selected concentrations. Determination of fungicide efficacy, or estimation of resistance level, can be measured by calculating an EC_{50} (concentration which inhibits growth by 50%) or by measuring the ratio of EC_{50}s for resistant and sensitive isolates. For this purpose, different media, depending on the pathogen, can be used. Duvert and Vives (1997) suggested that radial growth assays are quite convenient for small samples but less well adapted for monitoring of the sensitivity of fungal populations. In this regard, Serenius and Manninen (2006) used PDA amended with 0.1 and 1.0 µg mL^{-1} active ingredient prochloraz (Warbler) for testing 364 *P. teres* isolates originating from experimental work and farmers' fields. Measurement of radial mycelial growth was also used by Campbell and Crous (2002) in an assay evaluating the sensitivity of both net and spot type *Pyrenophora* to triadimenol, bromuconazole, flusilazole, propiconazole and tebuconazole. A different agar plate method to determine fungicide efficacy *in vitro* was followed by Sierotzki *et al.* (2007) who inoculated agar plates, amended with a series of fungicide concentrations, by spraying a suspension of conidia and mycelium fragments of *P. teres* and incubation for 5 d at 20°C. Growth of

mycelium was then assessed visually and compared with the unamended control. A single discriminatory dose of SBI fungicides was selected for bioassays by Peever and Milgroom (1994). Doses of fungicides approximating to the population EC_{50} values for each fungicide were shown to be appropriate for determining SBI-resistant phenotypes for *P. teres*. Serenius and Manninen (2006) used a radial growth assay to determine tolerance against prochloraz at concentrations of 0.1 and 1 μg mL^{-1} in PDA culture media. Prochloraz was added to cooled liquid media prior to pouring media into Petri dishes. Amended agar plates were inoculated with 7 mm mycelium plugs and incubated under NUV light at 18°C with a 12 h light period until the fungus reached the edges of the control dishes. Radial growth, relative to growth on control medium, was measured at this time. A microtitre method, as an alternative to agar plates, was proposed for *P. teres* by Duvert and Vives *(1997)* who prepared a range of concentrations in glucose-peptone liquid medium into microtitre plate wells. The wells were then inoculated with 100 μL of conidial suspension (2000 conidia mL^{-1}). After incubation of the plates in the dark for 3 days, the growth of the fungal colonies was determined by the measurement of the absorbance at 630 nm using a plate reader. Efficacy was calculated by comparison of the treatments with the untreated control.

4.1.3 *In planta* fungicide efficacy

In vitro assays may give an indication of the performance of a fungicide and the existence of resistance isolates, but may not reflect performance *in planta*. Therefore it is also necessary to ascertain fungicides performance either in field trials or in controlled environment greenhouses. Many such investigations have been undertaken. To investigate the practical impact of the F129L mutation on the field efficacy of the QoI fungicides, field trials were performed by Semar *et al.* (2007) at sites with different levels of F129L mutants in isolates of *P. teres*. Strobilurin fungicides used in these trials were Comet (250 g L^{-1} pyraclostrobin), Amistar (250 g L^{-1} azoxystrobin) and Opera (133 g L^{-1} pyraclostrobin + 50 g L^{-1} epoxiconazole). Field research was also undertaken by HGCA to provide an independent source of information about the activity of current and newly introduced fungicides against the major barley diseases. The diseases investigated were rhynchosporium, brown rust, powdery mildew, net blotch and ramularia. The evaluation included protectant and eradicant

properties of 13 fungicides in field trials carried out throughout the UK and Ireland under high disease pressure conditions (Oxley and Hunter, 2005). Field assessment were also done by Jayasena *et al.* (2002). Ten fungicides (pyraclostrobin, tebuconazole, flutriafol, epoxiconazole, propiconazole, triadimefon, azoxystrobin, trifloxystrobin, difenoconazole and a mixture of propiconazole with iprodione) were evaluated as single applications for control of spot-type net blotch of barley caused by *Pyrenophora teres* f. *maculata* at three locations during 1999 and 2000. Bayleton (triadimefon, 50% WP) and Tilt (propiconazole 42% EC) were assessed in a field trial by Johnston and Macleod (1987) where they investigated the foliar application of both fungicides on net blotch severity at two growth stages. They reported that net blotch was controlled by the fungicides adding that the overall protein content and grain yield did increase. *In planta* application of fungicides in combination with other parameters was investigated by Turkington *et al.* (2004), who tested six fungicide timings, in conjunction with three seedbed treatments, to evaluate the efficacy of propiconazole on the severity of net blotch and production of barley. Recently, the impact of the fungicide tebuconazole was tested by Soovali and Koppel (2010) in 2 treatment regimes in three spring barley varieties over three years on the control of major barley pathogens *P. teres* and *Cochliobolus sativus*. They concluded that the fungicide treatments had a strong impact on the control of infection *of P. teres* and increased kernel yield in variable disease infection conditions.

4.1.4 PCR-based assessment of fungicide activity

Disease assessment is essential in plant pathology. Conventional methods are time consuming and the results obtained might not always reflect the true extent of pathogen colonisation. PCR-based methods are an alternative strategy to ascertain the effects of compounds on fungal growth and may enable detection of pathogens in plant tissues before symptoms become visible (Henson and French, 1993). Advantages over traditional diagnostic methods include the points that the assays are more accurate, faster and can be used with little experience of plant pathology. Such methods are currently widely applied for early diagnosis and disease assessment of many plant diseases (Schena *et al.*, 2004). They have been used to recognize and

quantify pathogen DNA levels in many systems, including the barley fungal pathogen *Ramularia collo-cygni* (Heuser and Zimmer, 2002), *Puccinia striiformis*, the causal agent of yellow rust (Holtz *et al.*, 2010), and *Phytophthora infestans* (late blight of potato and tomato) (Alonso *et al.*, 2010). Real-time PCR (quantitative or qPCR) has also been also used to detect and quantify plant pathogens in soil, including *Ustilaginoidea virens*, the causal agent of false smut disease of rice (Ashizawa *et al.*, 2010) and to identify races of the tomato wilt pathogen *Fusarium oxysporum* f. sp. *Lycopersici* (Inami *et al.*, 2010). Quantification using qPCR can also overcome conventional methods for detection of seed-borne pathogens. Detection of the closely related pathogens *P. teres* and *P. graminea* was successfully achieved by Justesen *et al.* (2008). Furthermore, they confirmed that the new method could be an alternative to the tradition freezing blotter method. Quantitative PCR was also used by Bates and Taylor (2001), who emphasised the necessity of detecting closely related barley seed-borne pathogens before making disease control decisions. In their conclusion, they stated that different disease management strategies are made based on the presence and level of agriculturally important pathogens. Simultaneous detection, identification and quantification of multiple pathogens in plant tissues has been undertaken by many researchers. A real-time multiplex PCR approach based on TaqMan PCR was developed by Mathre (1997) to detect and quantify four *Phytophthora* species from samples originating from 11 hosts. The method proved its specificity in detecting target DNA and the detection limit was 100 femtogram (fg) for isolates tested, indicating the suitability of the method for qualitative and quantitative analyses.

Several quantitative PCR assays have been applied to assess the effects of fungicides. For example, Doohan *et al.* (1999) and Edwards *et al.* (2001) applied a competitive PCR assay to determine fungicide effects on fusarium head blight. Q-PCR may also serve as an alternative method for accurate assessment of fungicide effects on leaf blotch. Due to the advantages mentioned, q-PCR was used by Kianianmomeni *et al.* (2007) to monitor QoI resistant *cyt b* alleles in barley net blotch field samples.

4.1.5 Objectives

The objectives of this research were to evaluate the *in vitro* and *in planta* performances of fungicides against wild-type and F129L mutants of *P. teres* and to determine any correlation between both assays. Fungicides tested were single QoIs, penthiopyrad and with some other triazole fungicides included for comparative purposes. Assessment of fungicides using qPCR in comparison to conventional visual disease assessment was also performed.

4.2 Materials and methods

4.2.1 *In vitro* fungicides activity

4.2.1.1 Discriminative dose assay

To investigate the performance of QoIs and other fungicides, including triazoles, against wild-type and mutant isolates of *P. teres*, a group of fungicides (shown in Table 4.1) were tested *in vitro*. For this purpose, Petri dishes were used (25 well, 18 mm each well; Sterilin, Staffordshire, UK). PDA at 55^{o}C was amended with five concentrations of fungicides (10, 5, 1, 0.1, 0.0 mgL^{-1} active ingredient-a.i.) and continuously agitated while pouring to ensure even distribution in the wells of Petri dishes. The last well was left as an untreated control, filled with unamended PDA. Using a sterile cork borer, the amended media were inoculated with a circular mycelium plug of 4 mm diameter taken from the edges of 7 d-old cultures of isolates of the pathogen grown on PDA. The mycelial plugs were placed face-down on the centre of the wells and then incubated in the dark at a temperature of 20^{o}C ± 2. The growth of the fungus was monitored daily until the fungus in unamended control wells reached the edge of the well. Radial growth of the pathogen was measured using digital callipers at two different angles at 90^{o} to each other and the mean calculated. After a deduction of 4 mm was made to account for the mycelium plug, percentage inhibition for each treatment and at each concentration was calculated relative to the untreated control. The experiment was complete randomised design with 4 replicates. Data were analysed using general analysis of variance (ANOVA) and for comparisons multiple range tests (P=0.05) were made using SPSS software version 19.

Table 4.1 Fungicides used in both *in vitro* and *in planta* bioassays.

Product name	Active ingredient	Concentration (g L^{-1})	Chemical class	Field application rate (L ha^{-1})
Twist	Trifloxystrobin	125	Strobilurin	2
Comet	Pyraclostrobin	250	Strobilurin	1
Amistar	Azoxystrobin	250	Strobilurin	1
Acanto	Picoxystrobin	250	Strobilurin	1
Opus	Epoxiconazole	125	Triazole	1
Folicur	Tebuconazole	250	Triazole	1
Warbler	Prochloraz	400	Imidazole	1
Unix	Cyprodinil	750	Anilino-pyrimidine	0.67
Novel SDHI	Penthiopyrad	200	SDHI	1.5
Fandango	Prothioconazole + fluaxostrobin	100+100	Mixture	1.25

4.2.1.2 EC_{50} determination

Twenty five-well Petri dishes were used to determine EC_{50}s for isolates of *P. teres*. For this purpose, 10 fungicide concentrations were prepared ranging from 100 to 0 mg L^{-1} a.i. To achieve this, PDA medium was used and the fungicide was added the cooled liquid media at 50°C and before solidification prior to pouring media to Petri dishes. The highest concentration, 100 mg L^{-1}, was prepared and from this other concentrations were prepared by serial dilution. The concentrations used were: 100, 33.33, 11.11, 3.7, 1.24, 0.41, 0.14, 0.046, 0.015 and 0.00 mg L^{-1}.

The wells were inoculated with 4 mm mycelium plugs taken from the edges of 7 d-old cultures of *P. teres* grown on PDA. Two 25-well plates dedicated for a set of the 10 concentrations served as a replicates (Figure 4.1). The plates were incubated at 20°C ± 2 in the dark for 3-5 d, depending on the isolate, until the growth of the untreated control reached the edges of the wells. Radial

growth was measured by using digital callipers as described above in section 4.2.1.1. The percentage inhibition at each concentration was measured, relative to the untreated control, and from that EC_{50}s were determined using probit analysis with the aid of SPSS software. The statistical programme calculated the maximum likelihood to estimate the linear regression to fit the regression of the response versus the concentration.

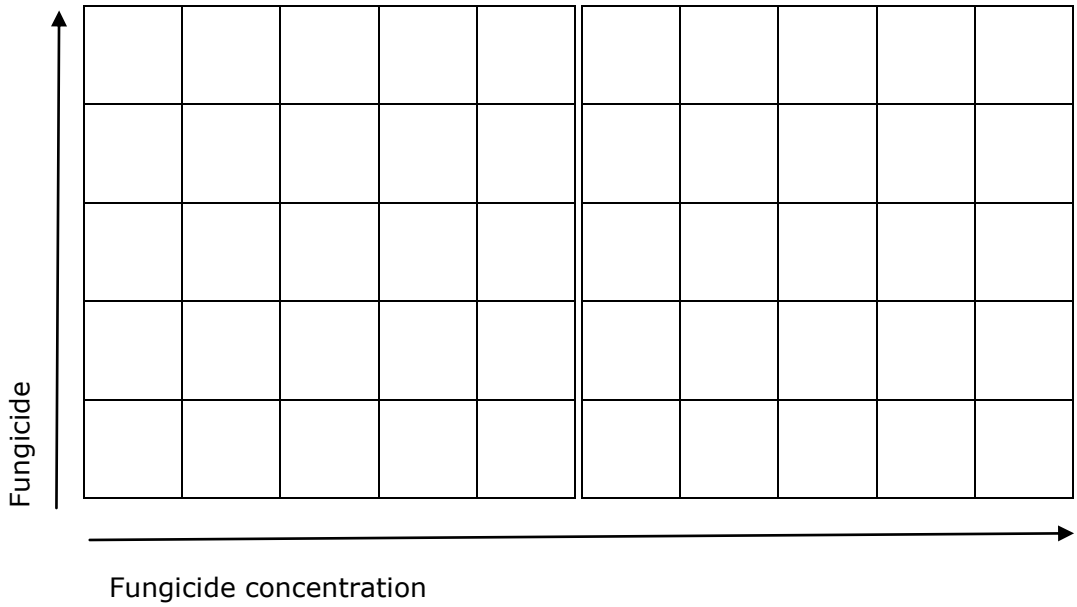


Figure 4.1 Layout of 25-well Petri dishes used for detection of EC_{50} for *P. teres* isolates towards fungicides used in the assay.

4.2.2 *In planta* fungicide activity

4.2.2.1 Visual disease assessment

To evaluate the efficacy of fungicides on barley plants, the *P. teres*-susceptible barley cultivar Pearl was grown in 9 cm pots at a density of 10 plants per pot. Ten days after emergence, at growth stage 12 (Zadoks *et al.*, 1974), the plants were sprayed with fungicides as a protective spray. The fungicide generic name, common name and chemical class are presented in Table 4.1, with the active ingredient concentration and the full field application rate. Two days later the plants were inoculated with 1 x 10^4 units mL^{-1} (comprising a mixture of mycelium fragments and conidia) until run-off, covered with plastic

bags for 48 h and then incubated in a controlled environment room at 20°C with the photoperiod of 16 h. To maintain high humidity, water was put in the bottom of trays. Control plants were treated the same as experimental plants but without fungicide application (water only). After 10 d, the disease incidence was assessed visually by evaluating necrosis using a 1-10 scale (Tekauz, 1985) and data manipulated to the percentage of fungicide efficacy (% of disease control) relative to the untreated control for each treatment using the following formula:

$$\% \text{ disease control} = \frac{\text{Disease degree of untreated control} - \text{disease degree of treated}}{\text{Disease degree of untreated control}} \times 100$$

4.2.2.2 Quantitative fungicide assessment using q-PCR

A q-PCR assay was used in *in planta* experiments to evaluate the effects of fungicide treatments on *P. teres* net blotch and compared to the traditional visual assessment. To do this, barley cultivar Pearl was grown, sprayed, inoculated as described in section 2.5 and the disease incidence assessed as described in section 2.6.1. After visual disease assessment, leaves (10 for each replicate) were stored at -80°C for later DNA extraction for assessment of fungal DNA using q-PCR.

Leaves which had been stored at -80°C were placed in liquid nitrogen and ground to powder with mortar and pestle. The ground plant material (around 5 g) was mixed well and then 200 mg of the ground plant material taken (as a representative) for DNA extraction. DNA was extracted following the CTAB DNA extraction method with some modifications of protocols used by Allen *et al*. (2006). The resultant DNA was purified using the Micro Bio-Spin Chromatography column purification method and then quantified using a Nanodrop spectrophotometer.

Pyrenophora teres specific primers were described by Bates *et al.* (2001). The plant-specific primers described in the above paper failed to amplify DNA from the variety Pearl, when used in the work reported here. This is possibly because the original primers were based on a cultivar-specific gene. Novel barley-specific plant primers were therefore designed, using Primer3 software, from the barley cultivar Pearl MADS-box protein BM5A (VRN-H1) gene, complete coding sequence (CDS) (NCBI, Accession No EF591645). Primer sequences and specifications are given in Table 4.2. For q-PCR, both fungal primers and barley primers were checked for their specificity (Figure 4.2). The extracted DNA of samples was also checked with standard PCR for confirmation of existence of plant and fungal DNA in extracts.

Plant DNA of experimental samples was adjusted to 10 ng μL^{-1} before being used for q-PCR. Plates (96 well, Starlab, UK) with transparent seals (Bio-Rad, UK) were used for running q-PCR. Test plates were loaded first to validate and optimise the standards, primer concentrations and conditions of q-PCR. For the construction of standards, twofold dilutions from pure DNA for both the pathogen and the plant were prepared from stocks of 10 ng μL^{-1} of pure genomic DNA.

The 25 µL mixture contained 12.5 µL 2x SYBR Green JumpStart Taq Ready Mix (Sigma), 0.4 µM of each primer, 2.5 µL of template and water to volume of 25 µL. Q-PCR was performed using a q-PCR system (BioRad-IQ5 multicolour Real-Time PCR Detection System) with operations of manufacturer's instructions. Thermal cycling conditions were: 2 min at 94°C, 40 cycles of 15 s at 94°C, 1 min at 60°C, and 30 s at 72°C. Reactions were performed in duplicate in the same run. Quantities of *P. teres* DNA were calculated relative to plant DNA using the regression equation of standard curves. The data were first analysed using the Bio-Rad-IQ5 instrument analysis software for detecting cycle threshold (CT) values. For the identification of target PCR product and non-specific products, such as primer dimers, a melting curve analysis was used. Other calculations and analysis were performed using Microsoft Excel 2007. For DNA quantification, a standard curve was generated by plotting the log of the DNA concentration of standards against the cycle number of each curve

within the log-linear stage of amplification (Figure 4.3). SPSS was used for the statistical analysis such as ANOVA and comparisons of means.

Table 4.2 Barley and *P. teres* primers used in quantification of fungal DNA in barley plants.

Primer name	Priming direction	Sequence (5'-3')	Specificity	Product length	Tm°C
ITSFF	Forward	GCAGATTGGGTAGTCCCCGCTTT	*P. teres*	94 bp	64.2
ITSR	Reverse	GAGCCCGCCAAGGAAACAAGTAGT	*P. teres*		64.4
VRN-F	Forward	GAAGCGGATCGAGAACAAG	barley	128 bp	58.5
VRN-R	Forward	TGGTGGAGAAGATGATGAGG	barley		58.5

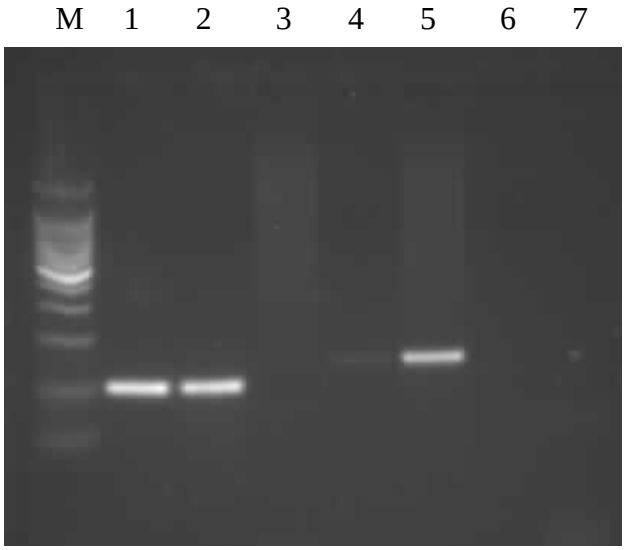


Figure 4.2 Detection of the specifity of primers used in q-PCR. PCR samples electrophoresed and visualised by staining with ethidium bromide on a 1.5% agarose gel. Samples amplified with the fungal primers ITSF and ITSR (lanes 1-3; where: lanes 1 and 2 are fungal DNA, lane 3 barley DNA); samples amplified with the plant primers VRN-F and VRN-R (lanes 4-6; where: lane 4 and 5 are barley DNA diluted and concentrated, respectively; lane 6 is fungal DNA); lane 7 is no template control sample; and M is the 100 bp PCR marker.

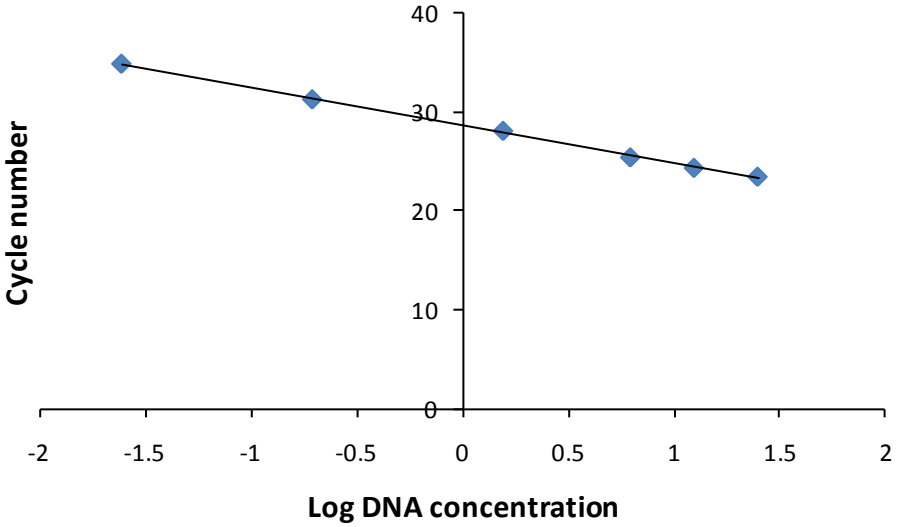


Figure 4.3 Standard curve for calculation of the fungal DNA concentration in the experimental samples.

4.3 Results

4.3.1 *In vitro* activity

4.3.1.1 Discriminative dose assay

4.3.1.1.1 QoIs compared to epoxiconazole

Five fungicide concentrations, as discriminative doses, were tested on different wild-type and F129L mutant *P. teres* isolates *in vitro*. The results showed significant differences in inhibition of mycelium growth between fungicides and concentrations used in the study. Percentage inhibition increased with increasing fungicide concentration in all fungicides used in the experiment.

As a general observation, wild type isolates, apart from isolate 1539, were sensitive to fungicides tested (Figure 4.4 and Figure 4.5). Pyraclostrobin was the most active QoI, causing high growth inhibition except for isolate 83

(Figure 4.5 c). Epoxiconazole generally was as effective as pyraclostrobin. Most QoIs and epoxiconazole showed low activity against isolate 1539 (Figure 4.5 e). The activity of most QoIs against mutant isolates was noticeably lower compared to wild-type isolates (Figure 4.6 a-c and Figure 4.7 d-e). Nevertheless, the performance of pyraclostrobin was as good as against wild-types. On the other hand, the efficacy of epoxiconazole, although lower than pyraclostrobin, showed higher activity than that exhibited by other QoIs against most mutant isolates, except for strain F20/3 (Figure 4.6 b). The efficacy of azoxystrobin, although lower than pyraclostrobin and epoxiconazole, was second best in the performance of the QoIs tested against most wild type isolates but showed lower activity against mutant isolates.

The minimum inhibition concentration (MIC), the concentration at which the growth is inhibited completely, varied between isolates. MIC for pyraclostrobin against isolates 1530, 1782 and mutant isolate 1534 was 10 mg L^{-1}, for mutant isolates F20/3 and MR1-1 was 5 mg L^{-1}, and for mutant isolates THM-2 and Cayl-3 was 1 mg L^{-1}. Epoxiconazole could reach the total inhibition point at 5 mg L^{-1} against two wild type isolates (1530 and 1782). However, none of the other QoIs, except azoxystrobin against isolate 1530 at 10 mg L^{-1}, reached MIC point for the concentrations tested against all isolates.

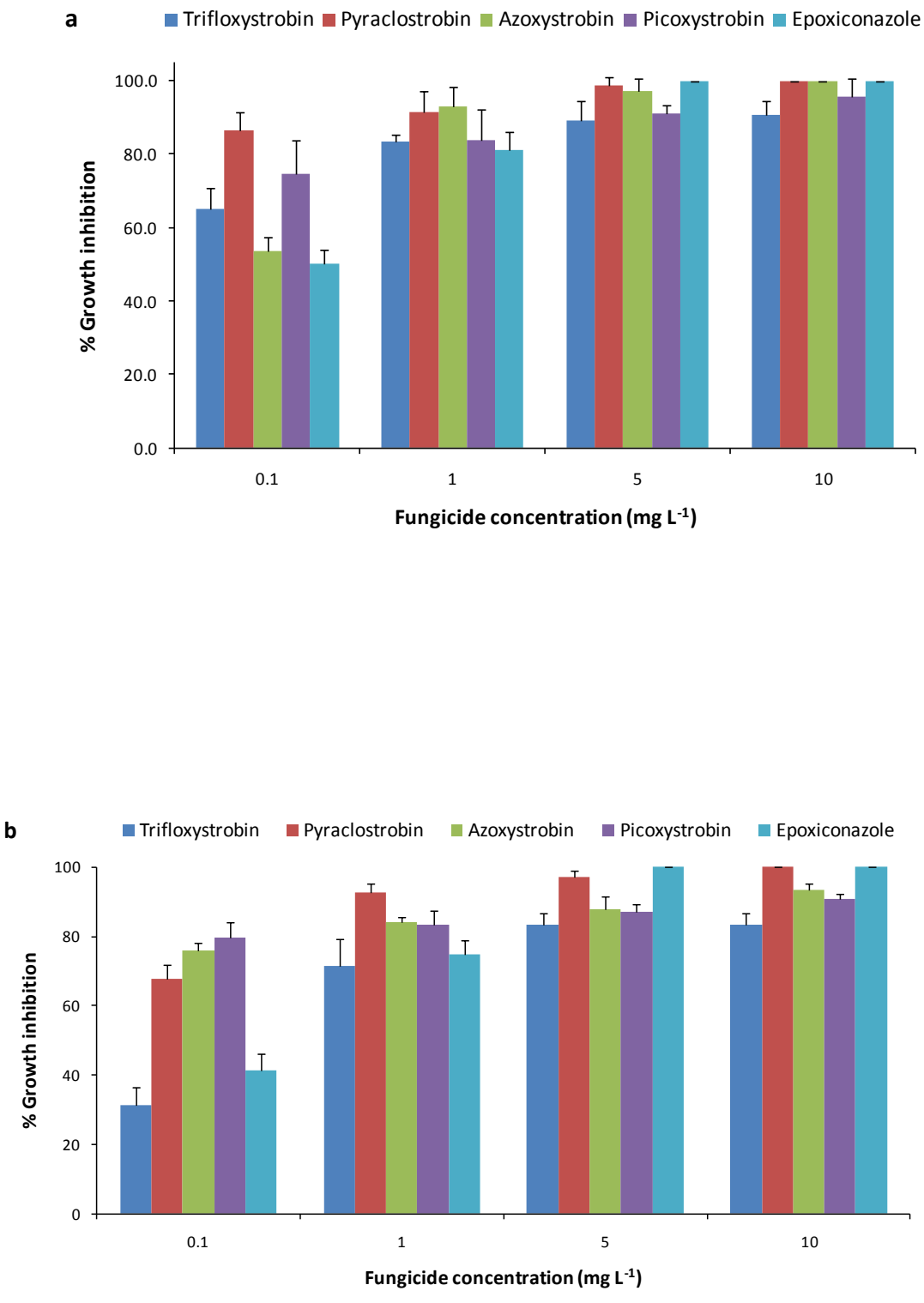


Figure 4.4 Percentage of growth inhibition of the *P. teres* wild type isolates on agar media amended with concentrations of 4 QoI fungicides and epoxiconazole. a) 1530, b) 1782.

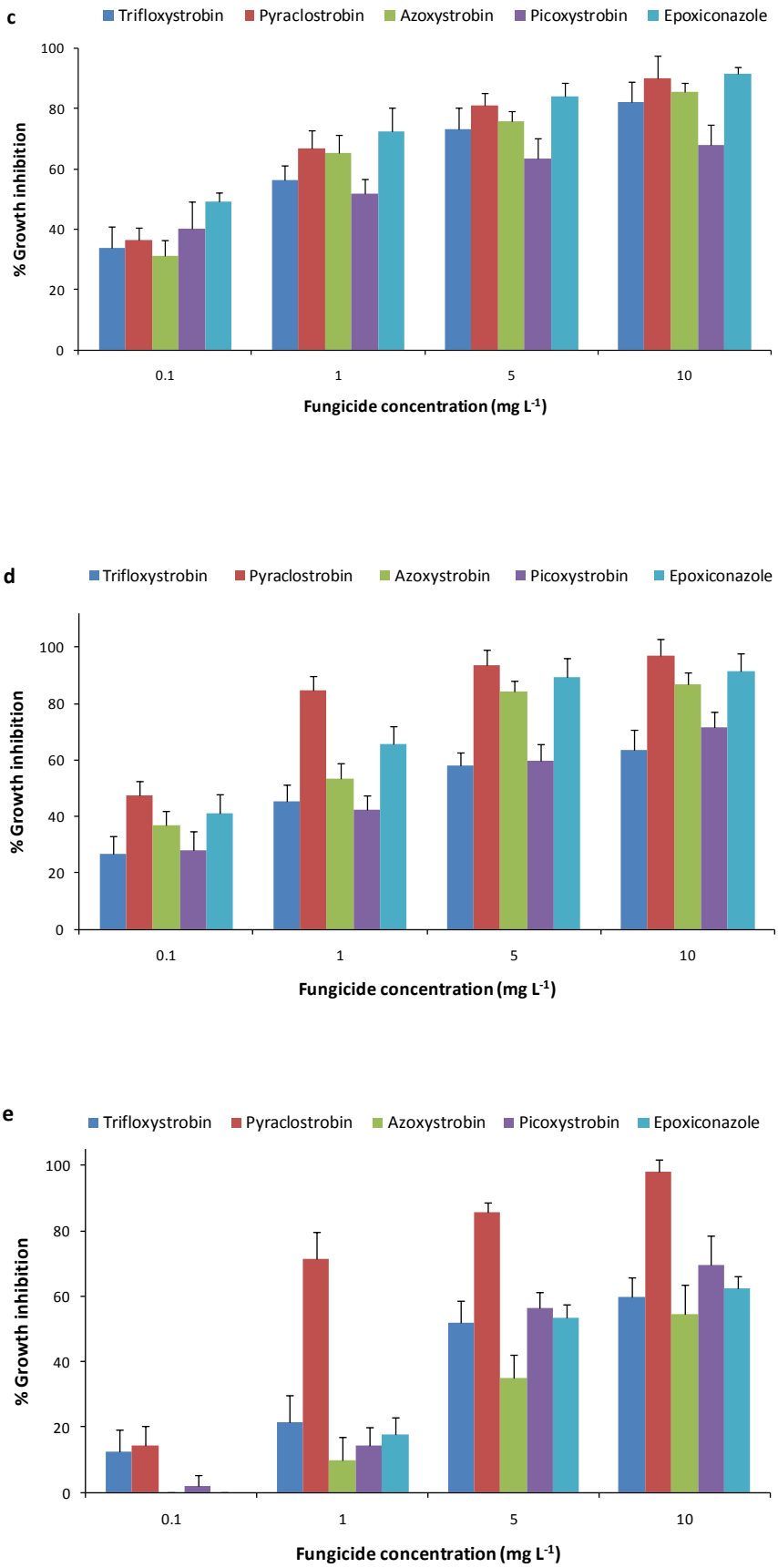


Figure 4.5 Percentage of growth inhibition of the *P. teres* wild type isolates on agar media amended with concentrations of 4 QoI fungicides and epoxiconazole. c) 83, d) 18 e) 1539.

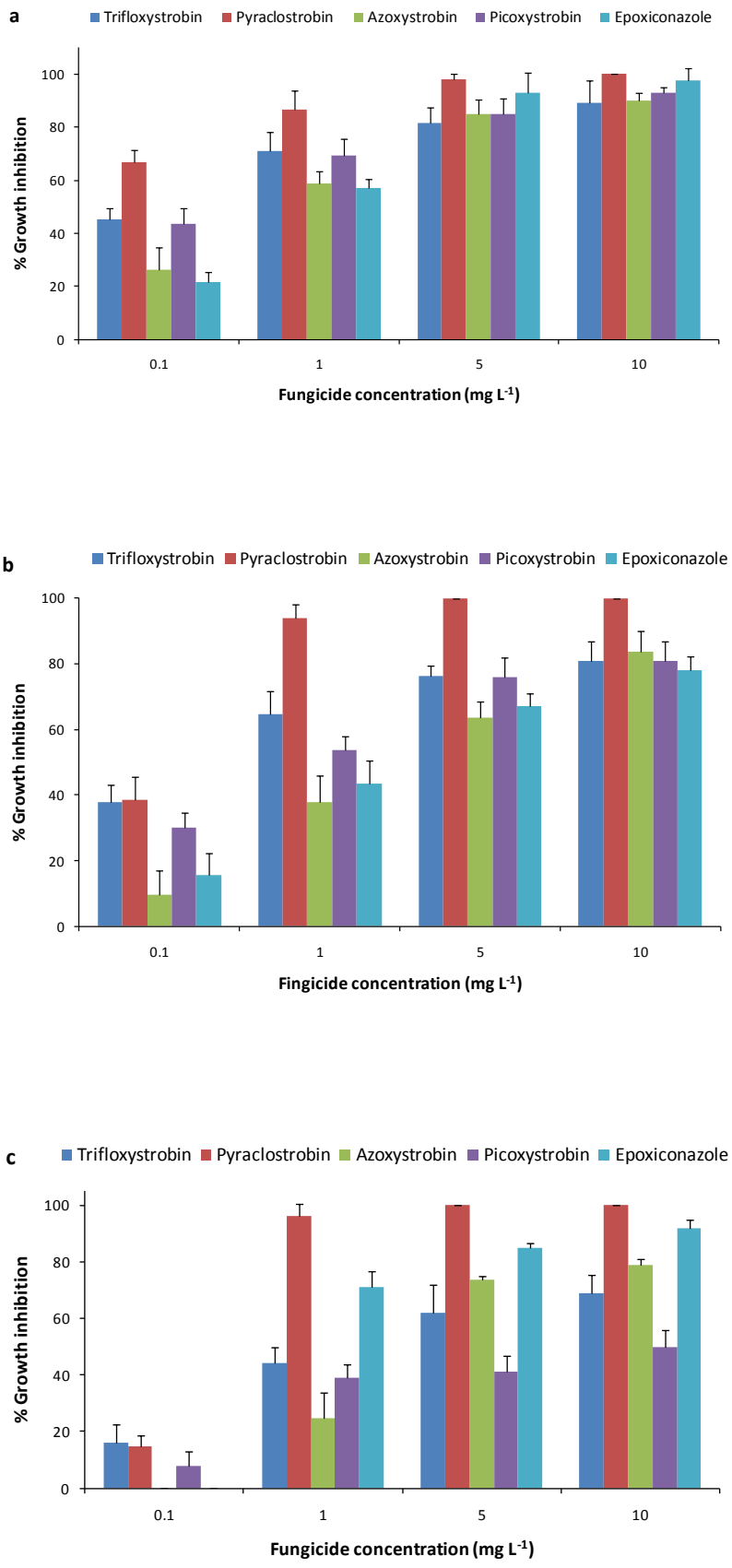
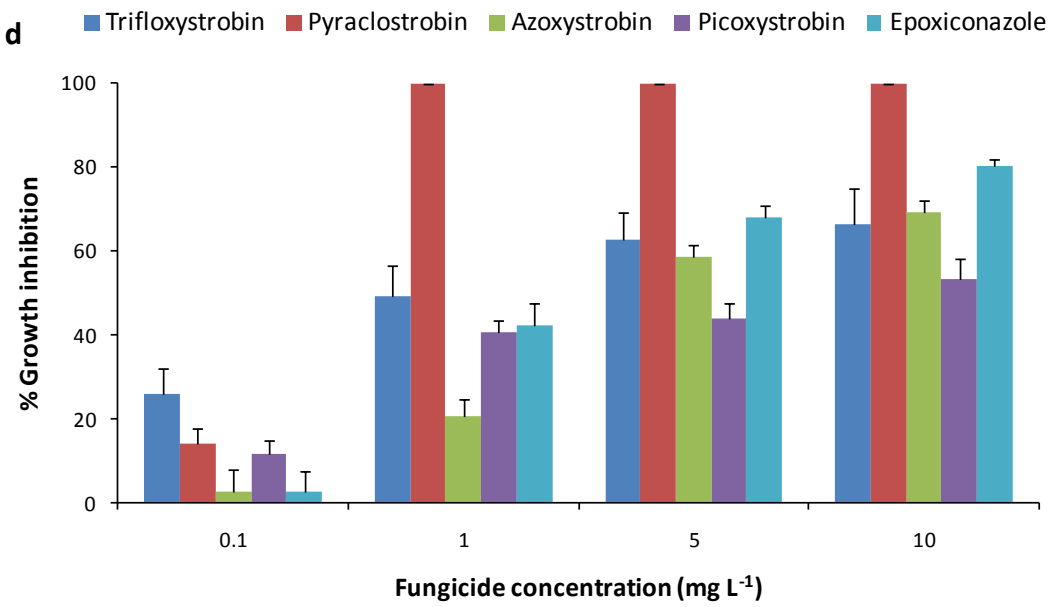


Figure 4.6 Percentage of growth inhibition of the *P. teres* mutant (F129L) isolates on agar media amended with concentrations of 4 QoI fungicides and epoxiconazole. a) 1534, b) F20/3, c) MR1-1.



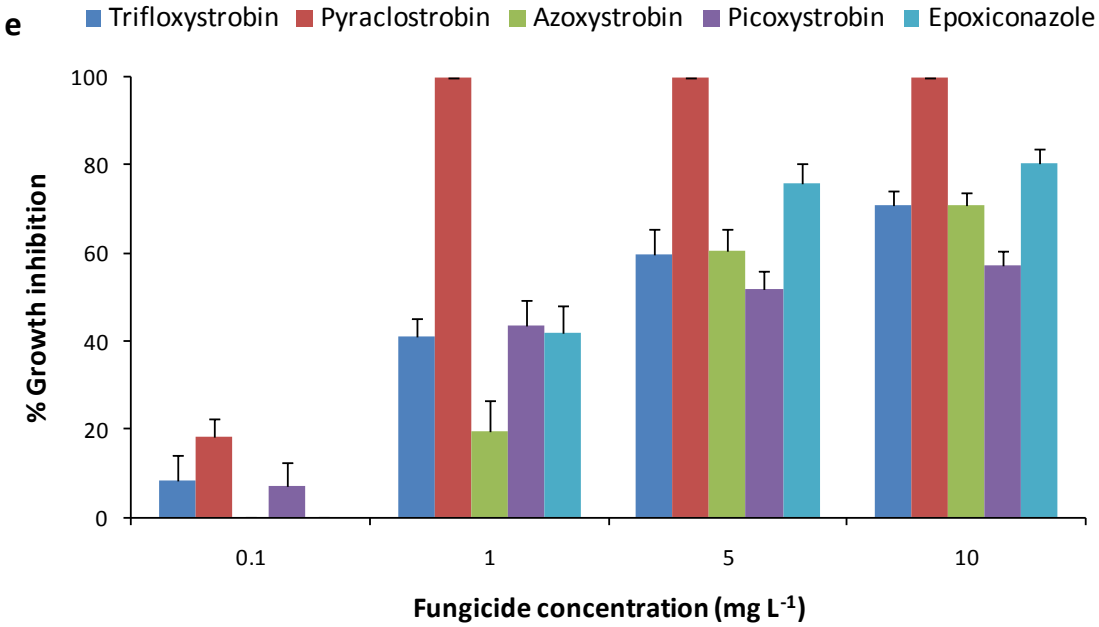
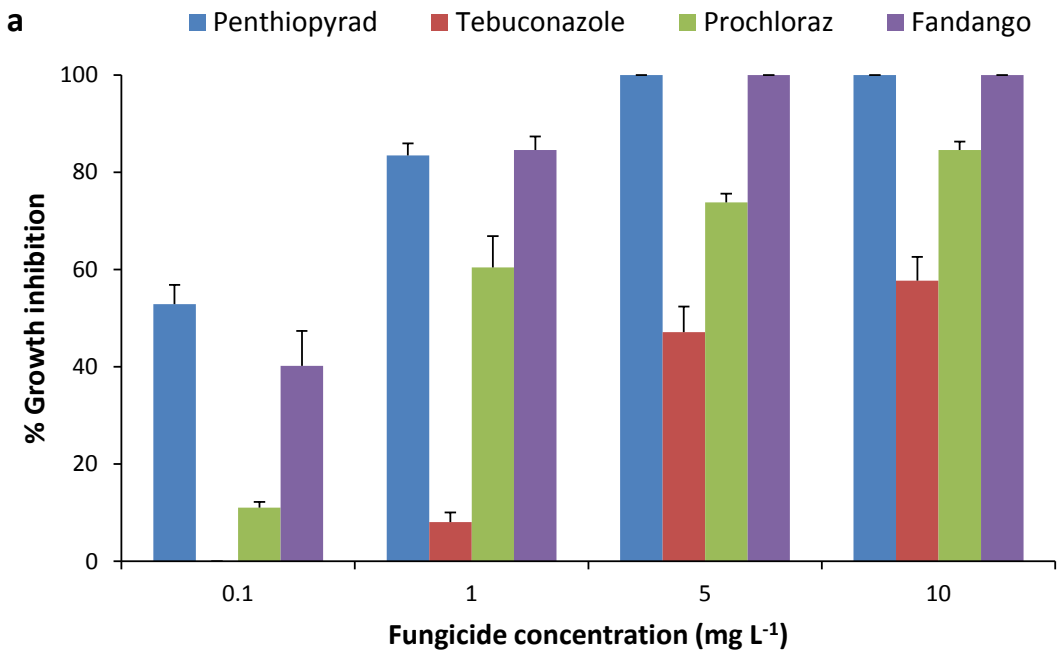


Figure 4.7 Percentage of growth inhibition of the *P. teres* mutant (F129L) isolates on agar media amended with concentrations of 4 QoI fungicides and epoxiconazole. d) THM-2, e) Cayl-3.

4.3.1.1.2 Penthiopyrad, Fandango and other triazoles

To extend information on fungicide activity, a range of other products were tested. These included an unlaunched SDHI (penthiopyrad), provided in confidence by DuPont, UK, Ltd, to allow evaluation of this new class of fungicides against *P. teres*. The efficacy of penthiopyrad, tebuconazole, prochloraz and Fandango (fluoxastrobin + prothioconazole) was evaluated *in vitro* against 4 wild-type and 5 mutant (F129L) *P. teres* isolates. As a general observation on the efficacy of this group of fungicides, penthiopyrad was most active in inhibiting the growth of isolates of *P. teres* regardless of the sensitivity of isolates (Figure 4.8 – 4.10). It did, however, show lower activity against wild type isolate Bot-1 (Figure 4.9 d). Tebuconazole, an older triazole, showed low activity against most wild type isolates, except isolate 83 (Figure 4.8 b), and all mutant isolates. The imidazole, prochloraz, was efficient against two wild type isolates (83 and Bot-1) and most mutant isolates. Fandango, a mixture of a QoI and a triazole, showed high activity against two wild types (1530 and HSS-2) and three mutant isolates (MR-1-1, THM-2 and Cayl-3) but it was less efficient against other wild type and mutant isolates.

The MIC of penthiopyrad was 5 mg L^{-1} against wild type isolates 1530 and 83 and mutant isolates 1534 and MR1-1 while it was 10 mg L^{-1} against wild type isolate HSS-2 and mutant isolates Cayl-3. Because the efficacy of tebuconazole was less pronounced, it reached the MIC point against isolate 83 only, while prochloraz showed better performance and inhibited the growth of 4 isolates completely, namely wild type isolate Bot-1 (at 1 mg L^{-1}) , wild type isolate 83 and mutant isolate 1534 (at 5 mg L^{-1}) and mutant isolate Cayl-3 (at 10 mg L^{-1}). Fandango, although showing an activity as good as prochloraz, achieved the MIC point at 5 mg L^{-1} only against isolate 1530.



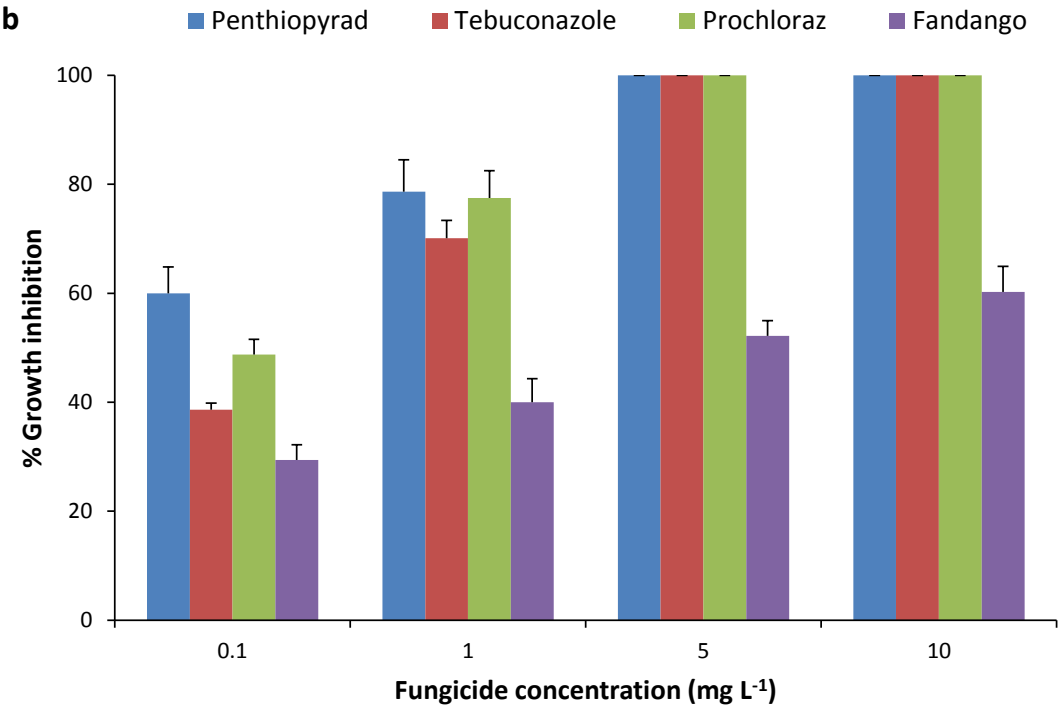


Figure 4.8 Percentage of growth inhibition of the *P. teres* wild type isolates on agar media amended with concentrations of penthiopyrad, Fandango, prochloraz and tebuconazole. a) 1530, b) 83.

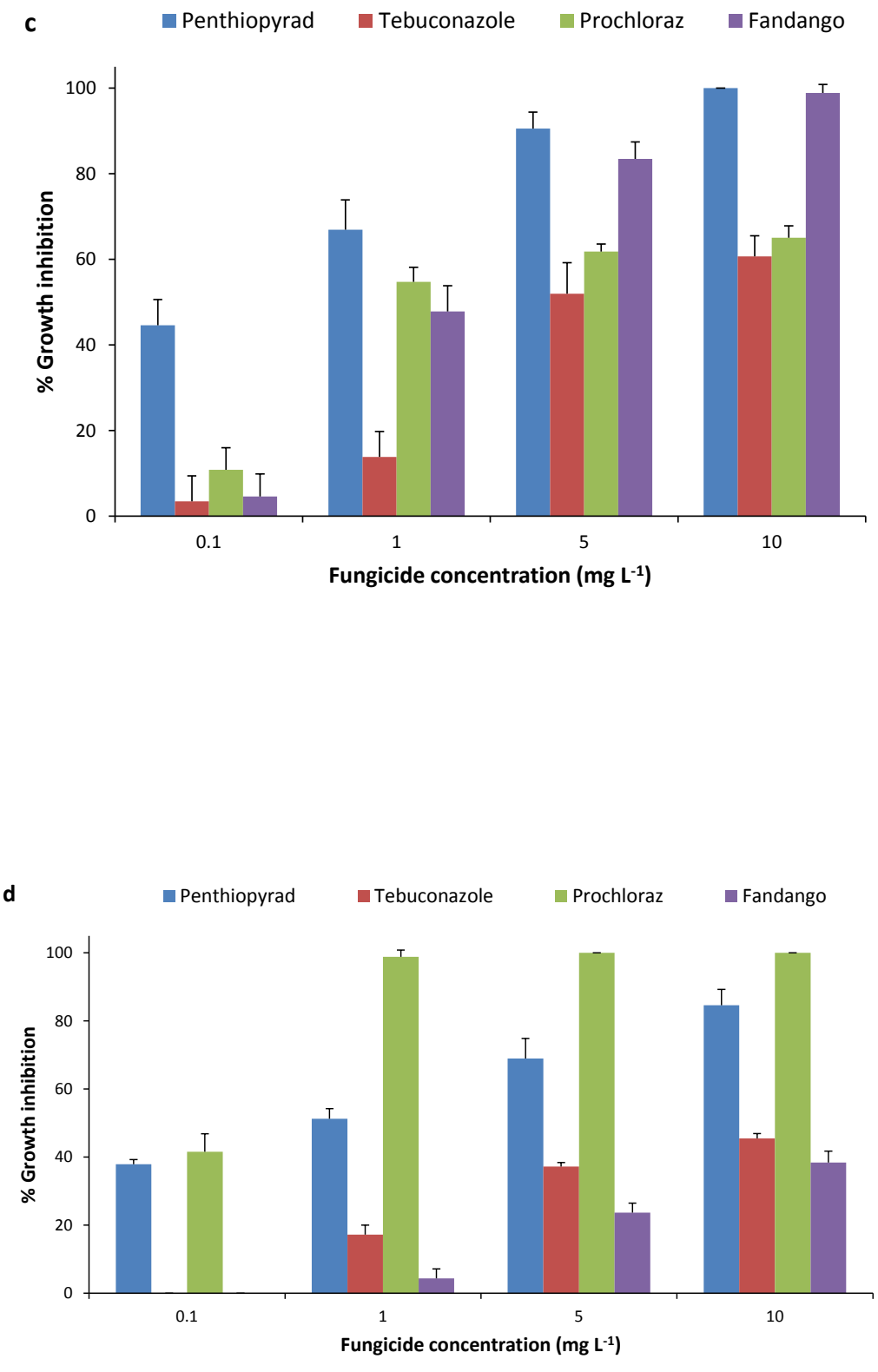


Figure 4.9 Percentage of growth inhibition of the *P. teres* wild type isolates on agar media amended with concentrations of penthiopyrad, Fandango, prochloraz and tebuconazole. c)) 83, d) Bot-1.

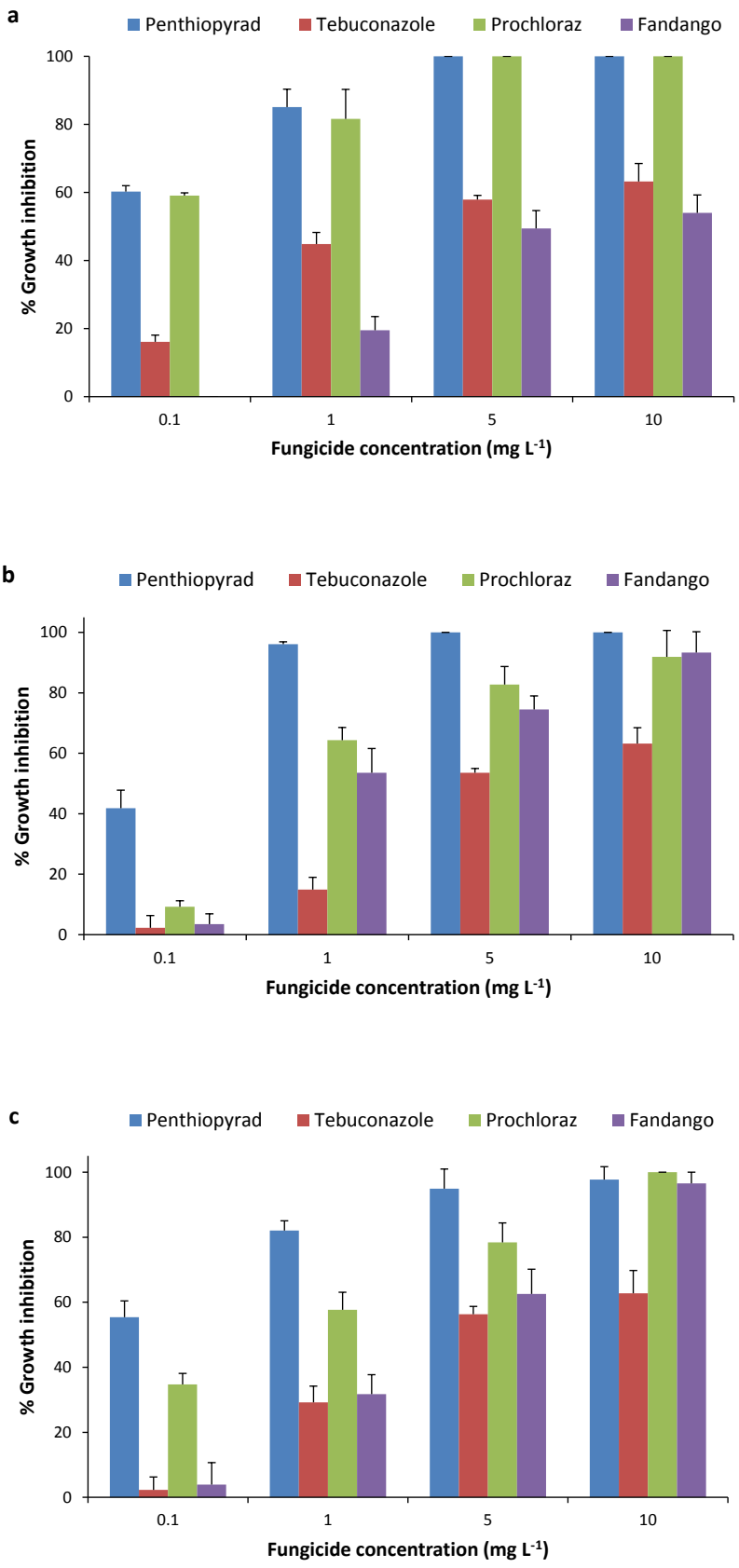
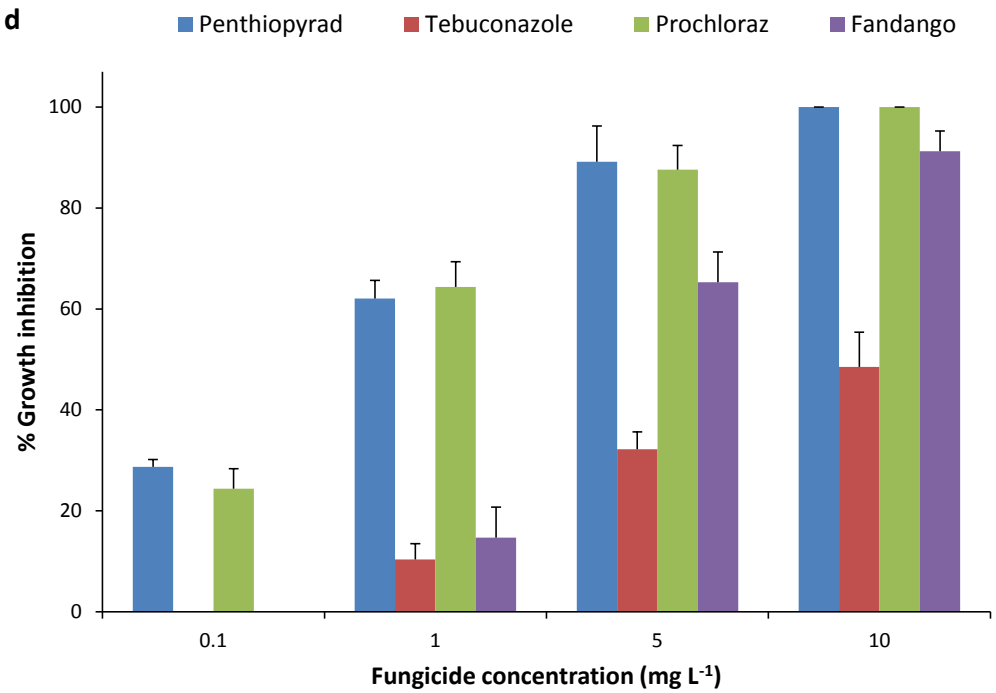


Figure 4.10 Percentage of growth inhibition of the *P. teres*, mutant isolates on agar media amended with concentrations of penthiopyrad, Fandango, prochloraz and tebuconazole. a) 1534, b) MR1-1, c) THM-2.



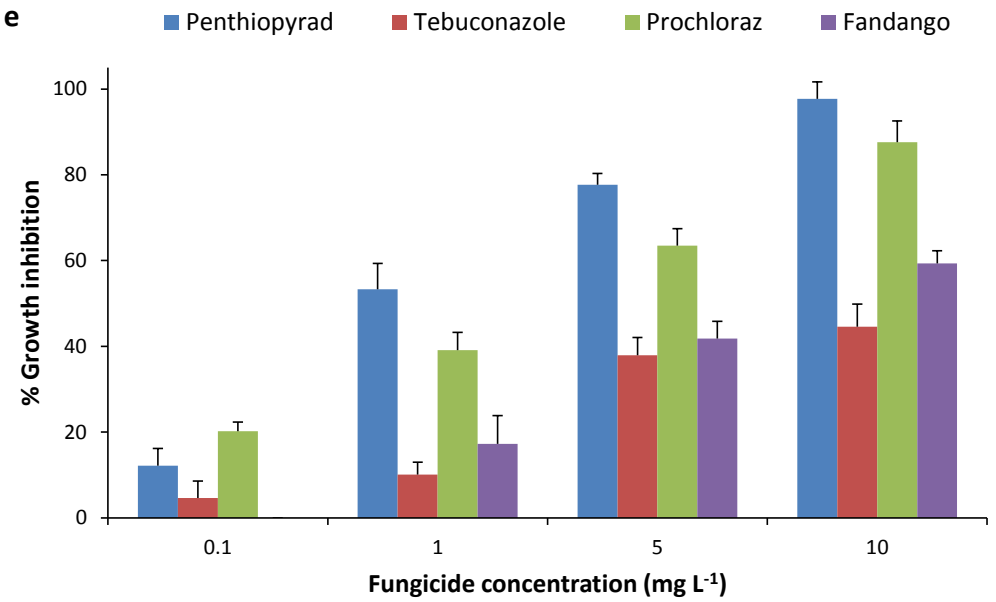


Figure 4.11 Percentage of growth inhibition of the *P. teres*, mutant isolates on agar media amended with concentrations of penthiopyrad, Fandango, prochloraz and tebuconazole. d) Cayl-3 e) F20/3.

4.3.1.2 EC_{50}

EC_{50}s were measured *in vitro* proportional to untreated control. This was to detect sensitivity of isolates of *P. teres* against QoIs and other groups of fungicides. The results showed that mutant isolates were found to have higher EC_{50} values towards QoIs than wild type isolates (Table 4.3). EC_{50} values of wild type isolates towards trifloxystrobin ranged from 0.02 mg L^{-1} (isolate 1530) to 1.11 mg L^{-1} (isolate 458) while for mutant isolates ranged from 1.25 (isolate F20/3) to 2.41 mg L^{-1} (isolate Cayl-3). A similar situation was observed with other QoIs. Mutant and wild type isolates showed lower EC_{50} values towards pyraclostrobin. It ranged from 0.1-0.22 for wild type isolates and 0.28-0.69 mg L^{-1} towards mutant isolates. Interestingly, some of mutant and wild type isolates showed little difference in their EC_{50} towards pyraclostrobin, which was very low and variable, reflecting the sensitivity of the fungus to this fungicide. It caused complete inhibition *in vitro* at 1 mg L^{-1} (Figure 4.7 d and e), at 5 mg L^{-1} (Figure 4.6 b and c), and at 10 mg L^{-1} (Figure 4.4 a and b, and Figure 4.6 a) in section 4.3.1.1.1. Mutant isolates also showed consistently higher EC_{50} values towards azoxystrobin and picoxystrobin compared to the wild-type.

Isolates showed a high degree of variability in EC_{50} values towards epoxiconazole, prochloraz, tebuconazole and prothioconazole regardless of their pedigree. However, prochloraz generally gave lower EC_{50} values towards all isolates compared to other azoles. An EC_{50} was also evaluated for penthiopyrad fungicide and found to be generally low, where the highest EC_{50} value (0.85 mg L^{-1}) was towards mutant isolate F20/3 and the lowest value (0.06 mg L^{-1}) was also with the mutant isolate OTV-1 (Table 4.4). The results in the Table 4.4 also shows EC_{50} values towards the mixture fungicide, Fandango, where if compared to QoIs, were considerably high against some mutant isolates, such as 1534 and F20/3, and even for wild-type isolates such as isolate 83.

Table 4.3 EC_{50} (mg L^{-1}) of isolates of *P. teres* with 4 QoI fungicides measured using an amended agar technique.

Isolate ID	Trifloxystrobin	Pyraclostrobin	Azoxystrobin	Picoxystrobin
H1/2	0.16	0.03	0.09	0.07
18	0.82	0.11	0.37	1.42
83	0.49	0.20	0.41	0.57
458	1.11	0.11	0.53	1.65
557	0.10	0.03	0.05	0.01
1782	0.032	0.04	0.001	0.001
1522	0.44	0.10	0.28	0.30
1539	5.36	0.52	0.62	1.34
1534 (F129L)	1.77	0.28	0.72	3.51
F20/3 (F129L)	1.25	0.69	2.41	2.40
1530	0.02	0.01	0.07	0.01
MR1-1 (F129L)	1.93	0.38	2.64	4.42
HSS-2	ND	0.3	ND	1.03
CAYL-3 (F129L)	2.41	0.49	3.85	3.95
BOT-1	ND	0.20	ND	1.22
THM-2(F129L)	1.37	0.56	3.88	6.39

Table 4.4 EC_{50}(mg L^{-1}) of isolates of *P. teres* with 4 triazole fungicides, penthiopyrad and Fandango measured by an amended agar technique.

Isolate ID	Epoxiconazole	Prochloraz	Tebuconazole	Prothioconazole	Penthiopyrad	Fandango
H1/2	0.57	ND[a]	ND	ND	ND	ND
18	0.22	ND	ND	ND	ND	ND
83	0.11	0.18	0.21	6.47	0.08	3.05
458	0.31	ND	ND	ND	ND	ND
557	0.11	ND	ND	ND	ND	ND
1782	0.18	ND	ND	ND	ND	ND
1522	1.90	ND	ND	ND	ND	ND
1539	5.01	ND	ND	ND	ND	ND
1534	0.49	0.08	2.44	10.57	0.07	6.35
F20/3	1.46	1.28	14.27	12.57	0.85	6.58
1530	0.12	0.97	6.63	ND	0.10	0.16
OTV-1	ND	1.48	8.12	ND	0.06	1.72
MR2-1	ND	1.76	5.77	ND	0.07	2.68
MR1-1	0.89	0.77	5.08	6.62	0.13	1.20
HSS-2	2.37	1.90	5.63	4.36	0.19	1.06
CAYL-3	1.89	0.42	10.70	2.88	0.37	3.00
BOT-1	7.19	0.12	10.73	8.97	0.48	1.83
THM-2	1.96	0.37	4.04	9.99	0.08	1.91

[a] = Not detected

4.3.2 *In planta* fungicide activity

4.3.2.1 Visual assessment

QoIs and other fungicides were assessed for their efficacy *in planta*, as a protective application (two days before inoculation), against *P. teres* isolates. The impact of QoI fungicides on different *P. teres* isolates *in planta* showed a different pattern from that of the *in vitro* assessments. Disease control obtained by most QoIs was greatly affected by the F129L mutation. Few of them gave high control of mutant isolates. Disease control achieved by QoIs tested here seemed to be more effective against wild type isolates than mutant isolates

From the results shown in Figure 4.12–15, as general observation, all QoIs tested showed low efficacy against two mutant isolates (1534 and THM-2) and high performance against three wild type isolates (HSS-2, 1782, and 83). However, their performances against other isolates were noticeably variable. Trifloxystrobin and azoxystrobin showed low activity against isolate 1539 and at the same time they were efficient against the mutant isolate MR1-1 (Figure 4.12 and 4.15). Furthermore, trifloxystrobin alone was less efficient against one mutant isolate (OTV-1). Pyraclostrobin, picoxystrobin and azoxystrobin, in addition to showing high efficacy against mutant isolates mentioned above, were also efficient against the mutant isolate OTV-1. In contrast to trifloxystrobin and azoxystrobin, both pyraclostrobin (Figure 4.13) and picoxystrobin (Figure 4.14) showed high performance against isolate 1539 and low performance against mutant isolate MR1-1. The efficacy of picoxystrobin, shown in Figure 4.14, suggests it is outperforming other QoIs *in planta*. The fungicide, in addition to providing high disease control against wild type isolates, also gave high activity against mutant isolates, higher than that shown by other QoIs. Despite the observation that the performance of this fungicide was relatively high against mutant isolates, it did, however, show lower activity against mutant isolates compared to wild-types. Pyraclostrobin was also efficient against all wild type isolates as well as the mutant isolate OTV-1. However, it showed low disease control against other mutant isolates (MR1-1, 1534, and THM-2).

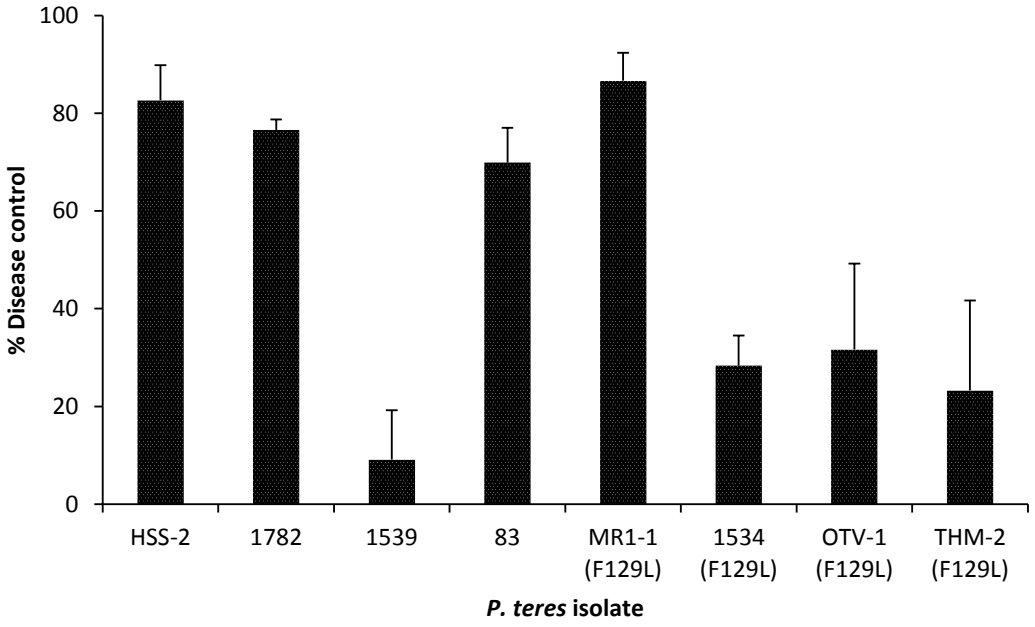


Figure 4.12 Percentage disease control achieved by trifloxystrobin *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

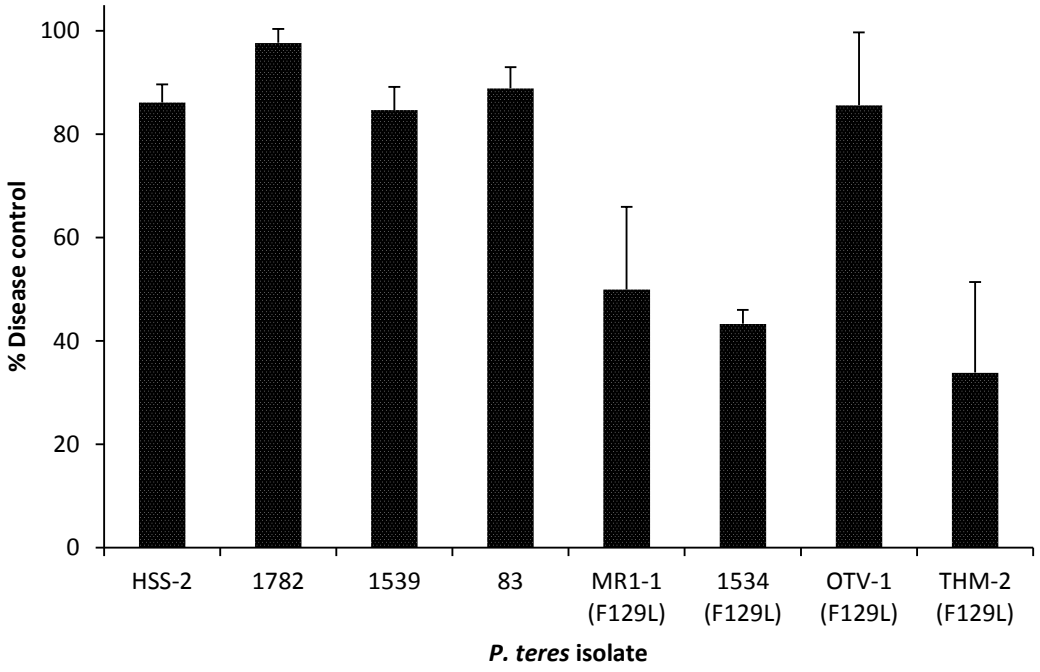


Figure 4.13 Percentage of disease control achieved by pyraclostrobin *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

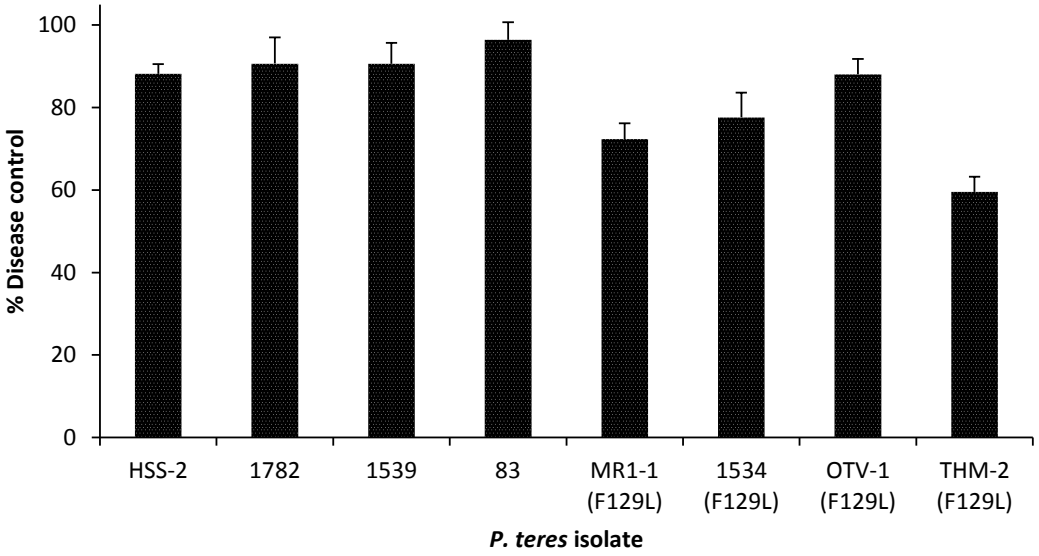


Figure 4.14 Percentage of disease control achieved by picoxystrobin *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

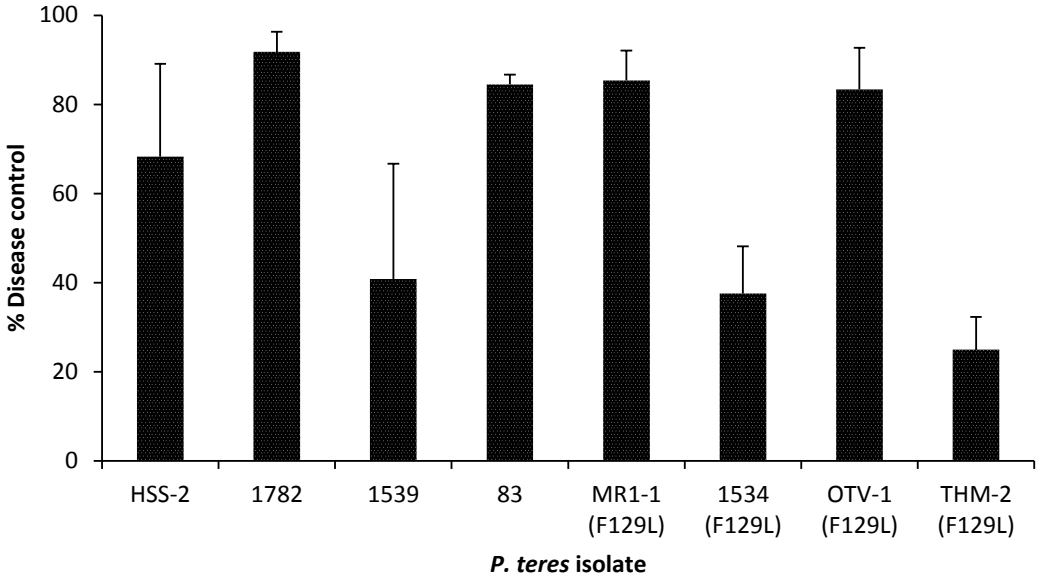


Figure 4.15 Percentage of disease control achieved by azoxystrobin *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

In planta assessments using other fungicides, penthiopyrad, other azoles, and Fandango, were also conducted on a range of *P. teres* isolates. It seemed that penthiopyrad, regardless of the sensitivity of isolates, outperformed other fungicides including QoIs (Figure 4.16). Although the highest performance of this fungicide was against the wild-type isolate HSS-2 (97.7%), its performance against mutant isolates was also high. The lowest performance achieved by penthiopyrad here was against wild-type isolate 1539 (81.1%) which was still a high level of activity.

The performance of triazoles (tebuconazole and epoxiconazole) and the imidazole (prochloraz) was generally low against most isolates. Tebuconazole (Figure 4.17), prochloraz (Figure 4.18) and epoxiconazole (Figure 4.20), although all providing high disease control against the wild-type isolate HSS-2, were all less efficient against 5 isolates (THM-2, Cayl-3, 1782, 1530, and MR1-1) regardless of the existence of the F129L mutation. Nevertheless, they exhibited high activity against some isolates, namely isolate 83 with tebuconazole and epoxiconazole and isolate OTV-1 with prochloraz and epoxiconazole. Prothioconazole outperformed prochloraz and tebuconazole in achieving disease control and showed better efficacy than that shown by some QoIs, such as trifloxystrobin and azoxystrobin against selected isolates (Figure 4.19). It showed high performance against most isolates regardless of their sensitivities. Nonetheless, lower performance was observed against isolates 1782 and 83. Fandango a mixture of fluoxastrobin and prothioconazole, although it showed low growth inhibition *in vitro* against some isolates (section 4.3.1.1.2), was as good as penthiopyrad and outperformed other fungicides such as triazoles and single QoIs *in planta* against all isolates regardless of their sensitivities (Figure 4.21).

In general there was no correlation between efficacy and the presence of the F129L mutation for the fungicides tested above. This is entirely consistent with this mutation affecting the activity of QoIs.

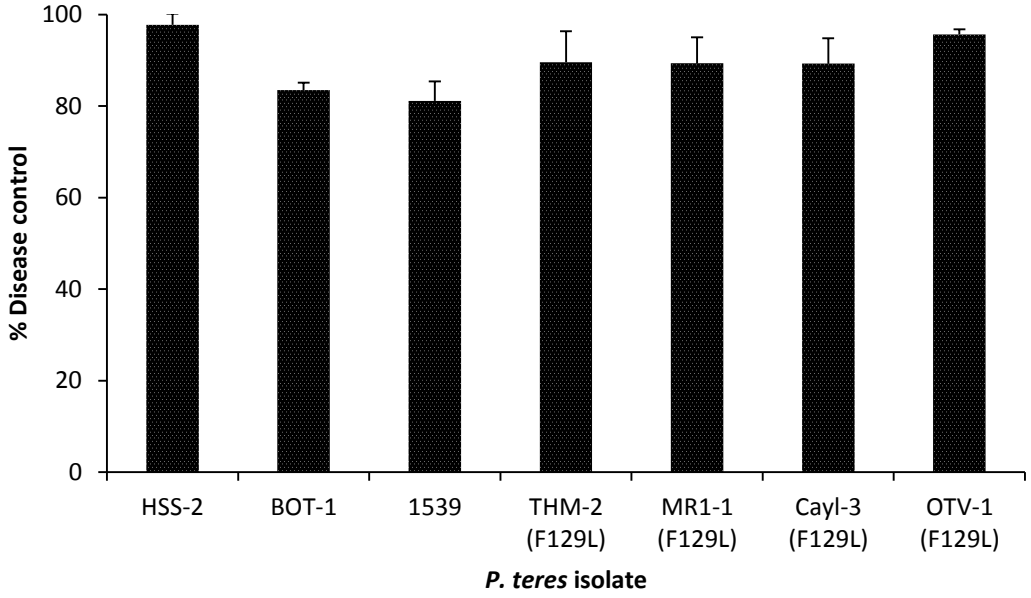


Figure 4.16 Percentage disease control achieved by penthiopyrad *in planta* against wild-type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

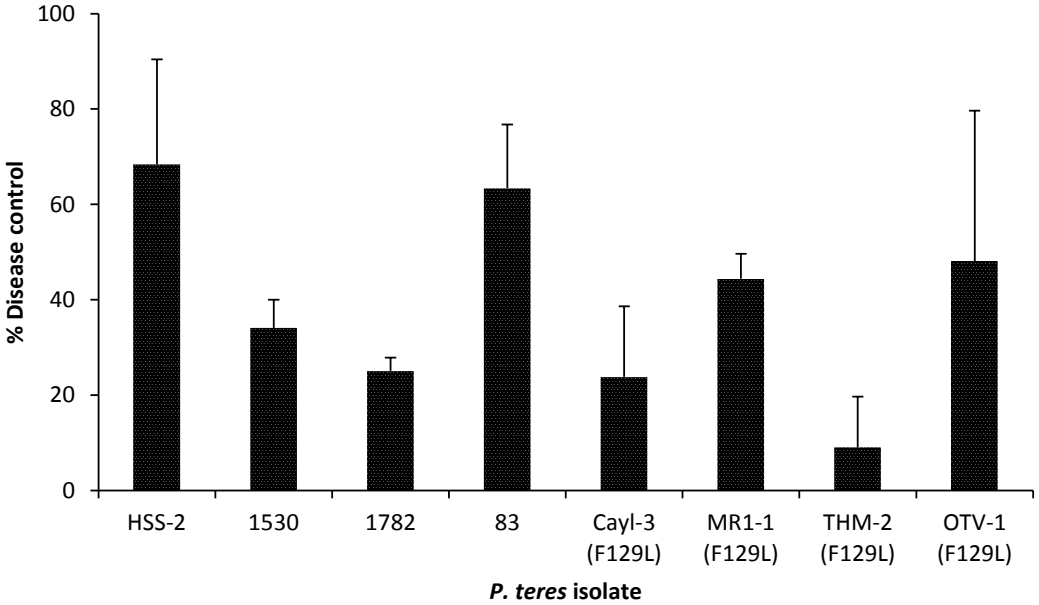


Figure 4.17 Percentage disease control achieved by tebuconazole *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

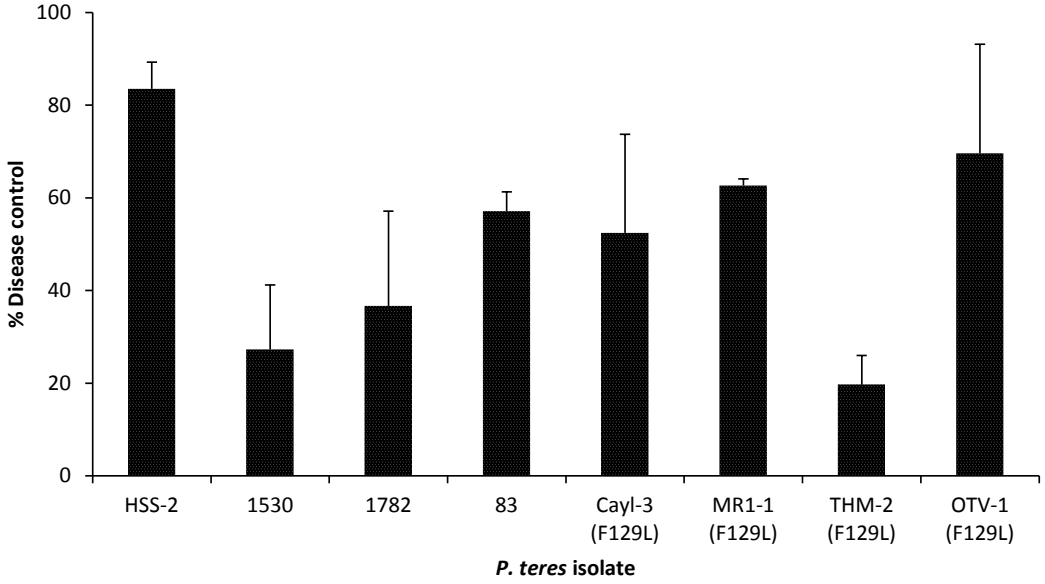


Figure 4.18 Percentage disease control achieved by prochloraz *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

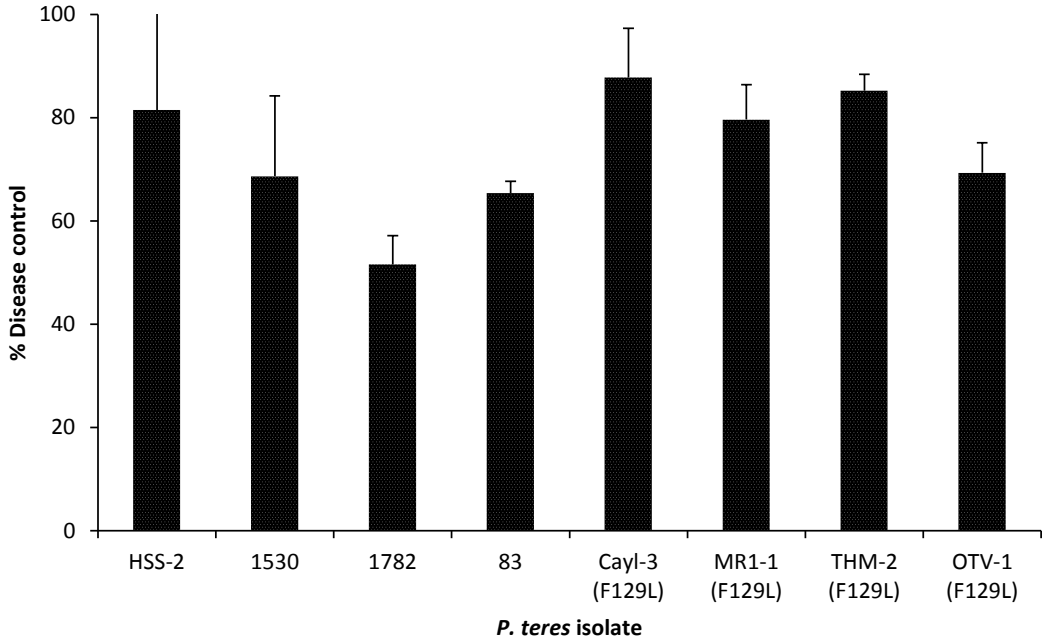


Figure 4.19 Percentage disease control achieved by prothioconazole *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

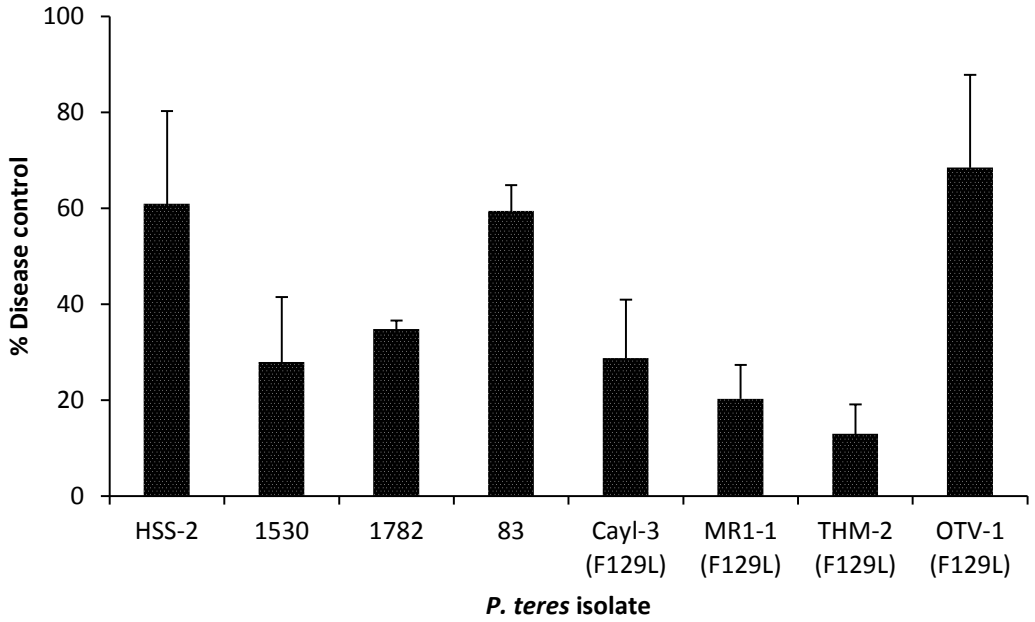


Figure 4.20 Percentage disease control achieved by epoxiconazole *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

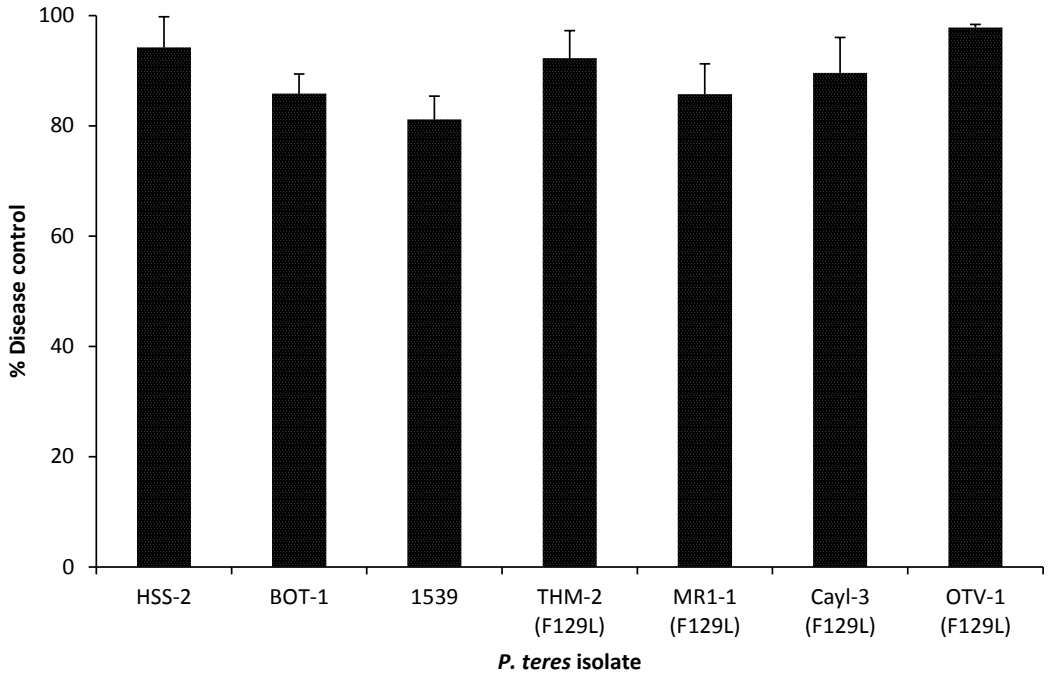


Figure 4.21 Percentage disease control achieved by Fandango *in planta* against wild type and mutant (F129L) *P. teres* isolates. Error bars are standard deviations.

4.3.2.1.1 Correlation between EC_{50} and *in planta*

To ascertain how EC_{50} values reflect fungicide performance *in planta*, a linear correlation (r) was determined for each fungicide and isolate by plotting EC_{50} values against percentage disease control. The equation and R^2 (coefficient of determination) was also calculated for each interaction. The correlations ranged from weak to strong according to the fungicide, all correlations being negative. In other words, fungicide: isolate interactions having a high EC_{50} did not exhibit high performance *in planta* and showed lower disease control, but in some instances the relationship was weak

By looking at Figure 4.22 it can be seen that a negative medium correlation (-0.65) with a weak R^2 (0.43) shows that the EC_{50} of the isolates did not reflect the *in planta* performance of trifloxystrobin consistently. However, a low EC_{50} for wild-type isolates 1782 and 83 correlated well with high *in planta* performance and for isolate 1539, although it is wild-type, the high EC_{50} value was well reflected the low *in planta* performance of trifloxystrobin towards this isolate. In the case of other isolates, the relationship was variable and their EC_{50}s did not reflect the *in planta* activity of the fungicide.

Negative medium correlation between EC_{50} values and disease control was also found in pyraclostrobin (Figure 4.23). It is noticeable again, as with trifloxystrobin, that low EC_{50} values related to high *in planta* performance in wild-type isolates 1782 and 83. By having high EC_{50} values in mutant isolates 1534, MR1-1 and THM-2, the *in planta* activity of pyraclostrobin decreased. However, wild-type isolates HSS-2 and 1539 which also had a high EC_{50} value did not exhibit corresponding low *in planta* performances.

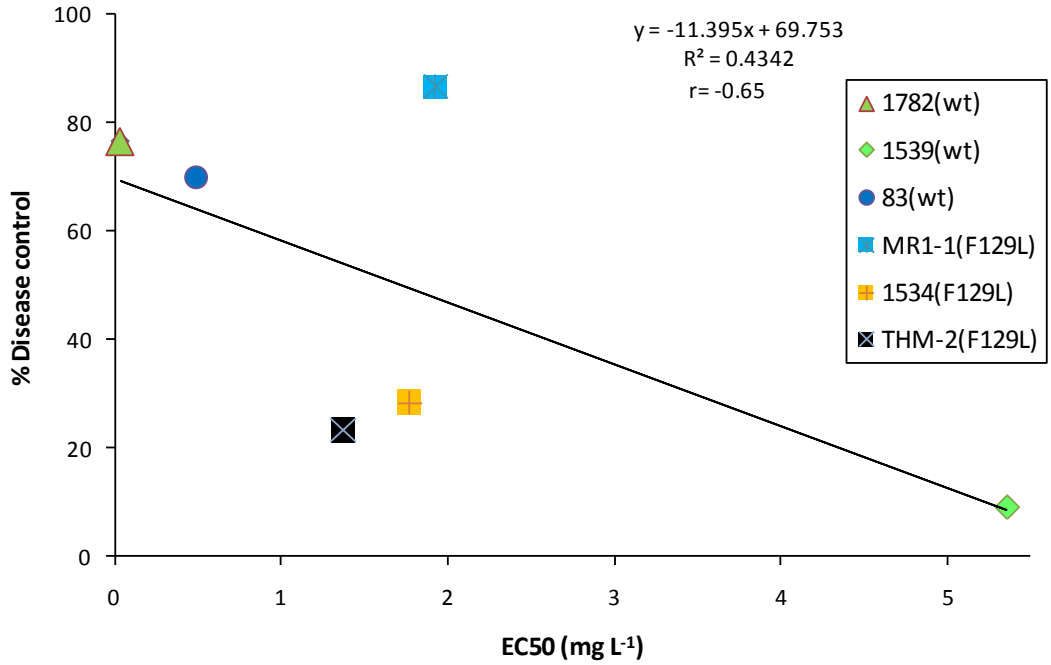


Figure 4.22 Correlation between EC_{50} values and *in planta* performance of trifloxystrobin in different *P. teres* isolates.

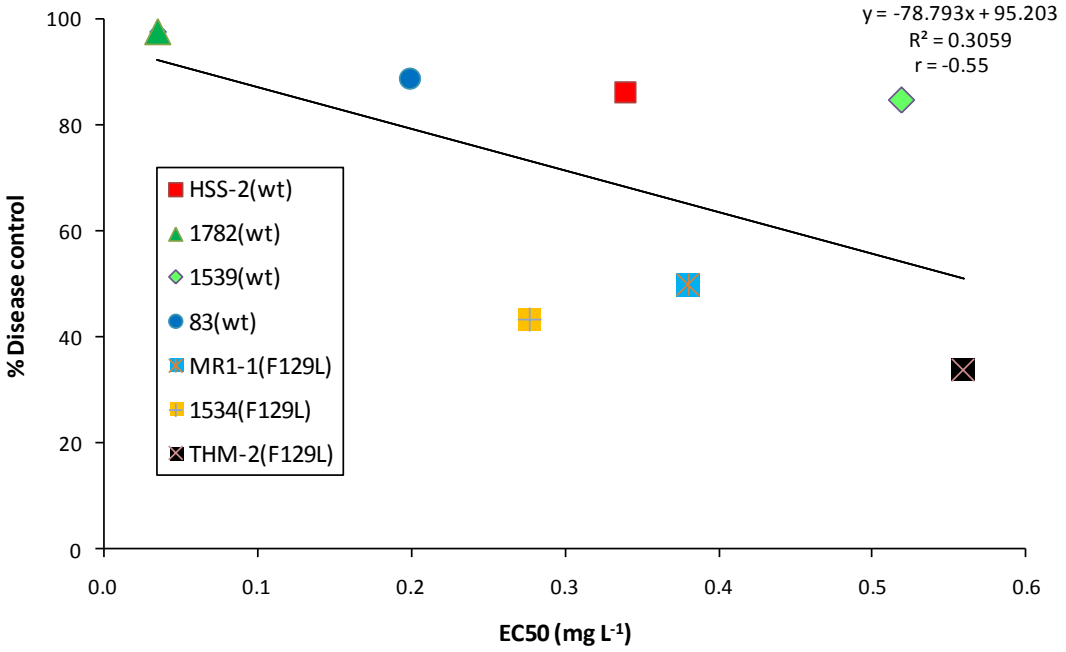


Figure 4.23 Correlation between EC_{50} values and *in planta* performance of pyraclostrobin in different *P. teres* isolates.

Different correlation results to those seen with trifloxystrobin and pyraclostrobin were seen with picoxystrobin in Figure 4.24 where a high strong negative correlation (-0.97) was found, which reflects that at low EC_{50} values high disease control was detected (with wild type isolates 1782, 83, HSS-2, and 1539). High EC_{50} values and low performance of the fungicide was observed with mutant isolates 1534, THM-2, and MR1-1. The Figure also shows a high R^2 value which reflects less data spread around the linear trendline.

In case of azoxystrobin, the *in planta* performance of two wild type isolates namely 1782 and 83 and mutant isolate THM-2 is well correlated with their EC_{50}s (Figure 4.25). However, this relation for isolates 1539, 1534, and MR1-1 was less pronounced and therefore a weak correlation and R^2 were found (r=-0.42, R^2=0.17).

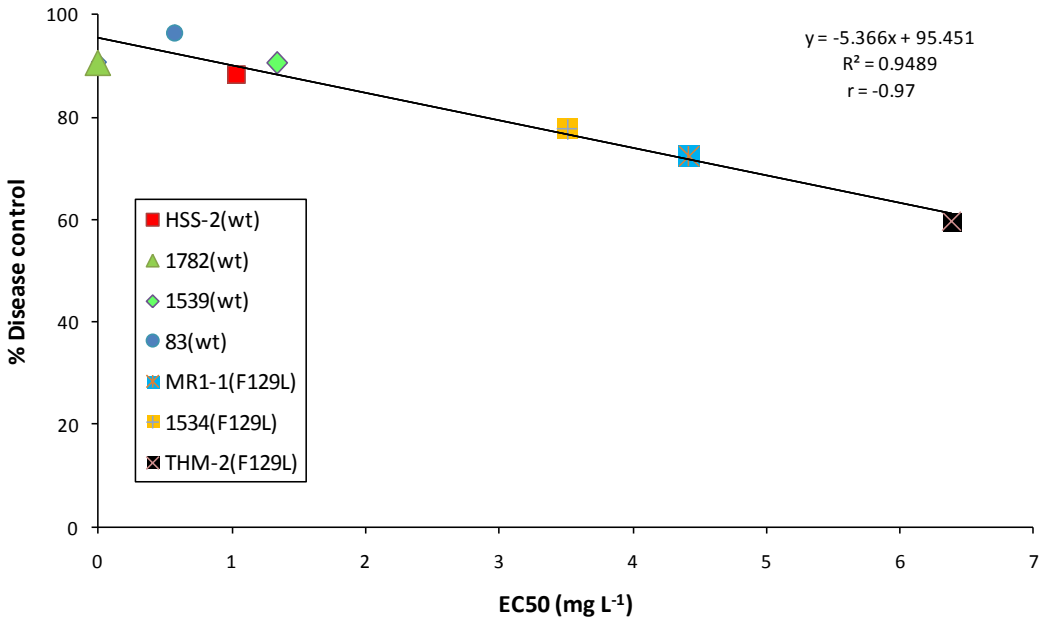


Figure 4.24 Correlation between EC_{50} values and *in planta* performance of picoxystrobin in different *P. teres* isolates.

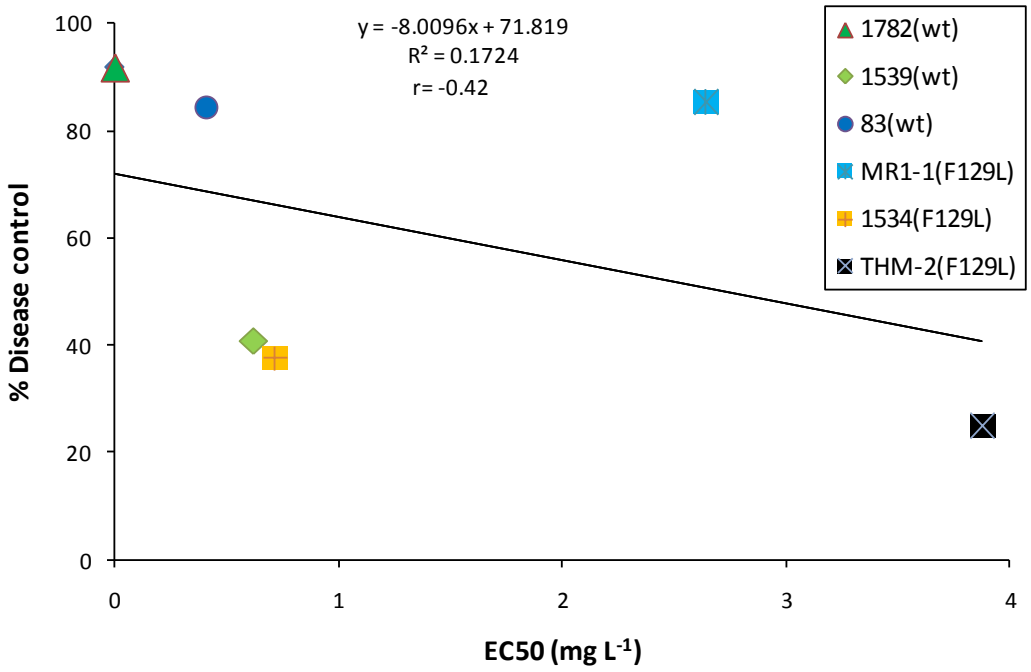


Figure 4.25 Correlation between EC_{50} values and *in planta* performance of azoxystrobin in different *P. teres* isolates.

4.3.2.2 Quantitative fungicide assessment using q-PCR

A comparison was made between visual disease assessment and quantitative disease assessment, using q-PCR to measure the amount of pathogen DNA present. The effect of two of the most active QoI fungicides (picoxystrobin and pyraclostrobin), two of most common triazoles (epoxiconazole and prothioconazole) and anilinopyrimidine (cyprodinil) were compared following artificial inoculation with a range of *P. teres* isolates.

As a general observation, the QoI fungicide, picoxystrobin, showed high performance with wild type isolates (Figure 4.26 and 4.27) as well as mutant isolates (Figure 4.28 - 4.30) either assessed visually or quantitatively using q-PCR. Picoxystrobin also showed high efficacy in previous *in planta* assessments presented in section 4.3.2.1. Pyraclostrobin was not as active as picoxystrobin, especially when assessed visually against most isolates, except for isolate 1539 where it showed high performance in both assessments.

Triazole fungicides, epoxiconazole and prothioconazole, were not efficient in most cases regardless of the sensitivity of the isolates. Moreover, in some cases epoxiconazole did not differ significantly with that of untreated control. This was also shown in another *in planta* experiment described in section 4.3.2.1, where triazoles showed low efficacy against a range of *P. teres* isolates. Cyprodinil on the other hand, showed high efficiency, whether assessed visually of quantitatively, against most isolates except for isolate 1539.

In most cases, quantitative assessments using q-PCR followed a similar pattern to that obtained using visual assessment. This was concluded after detecting the correlation between two assessment methods for each isolate. The results of correlations showed high positive correlations between visual and quantitative assessments ranging from 0.88 to 0.96. This indicates that q-PCR assessment was highly representative of the results obtained by visual disease assessment.

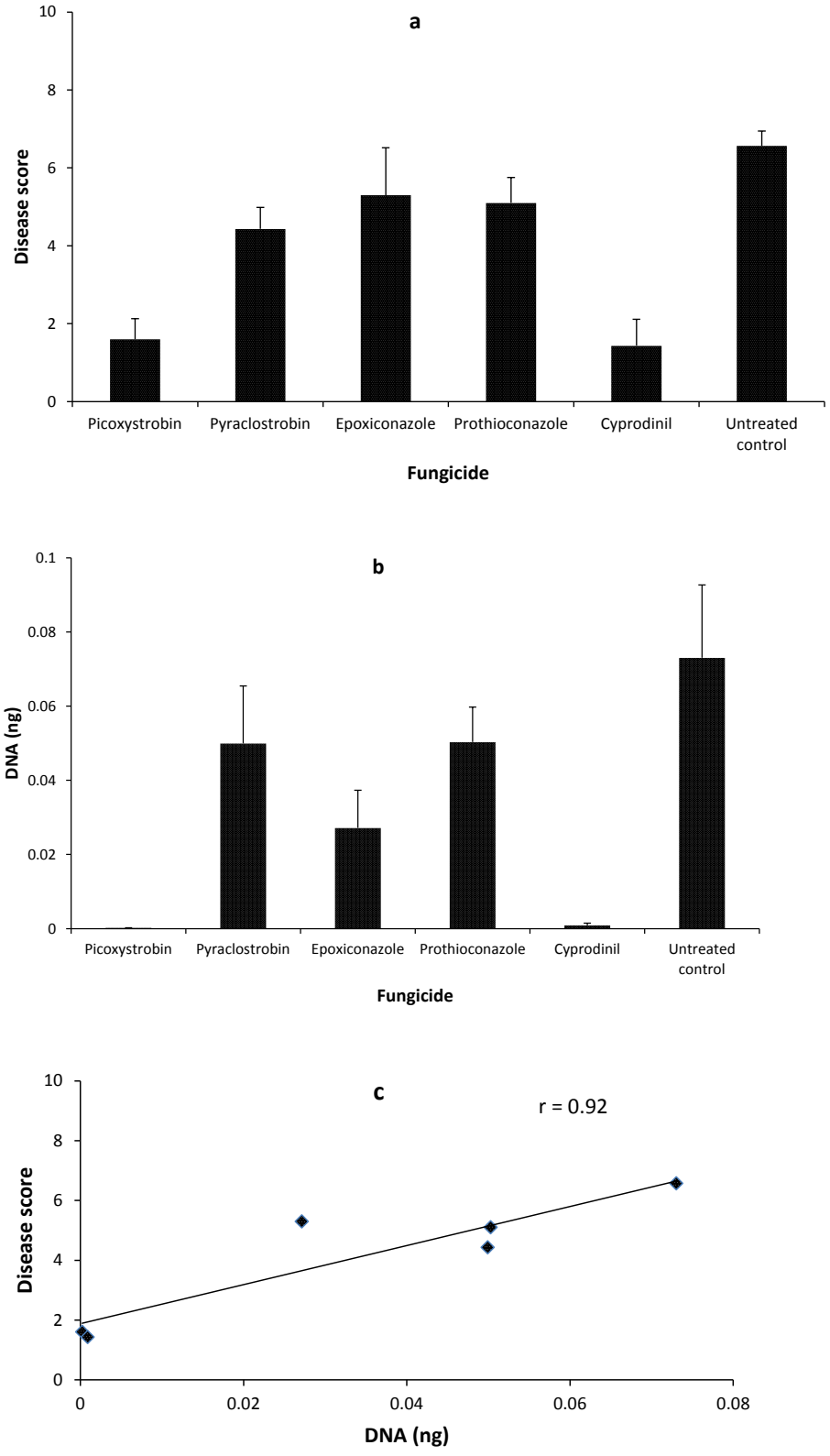


Figure 4.26 Assessment of fungicide efficacy on the disease incidence, caused by *P. teres*, wild type isolate HSS-2. a) visual assessment using the 0-10 rating scale, b) quantitative assessment using q-PCR, c) correlation between visual and quantitative assessments. Bars represent means of 3 replicates, error bars are standard deviation.

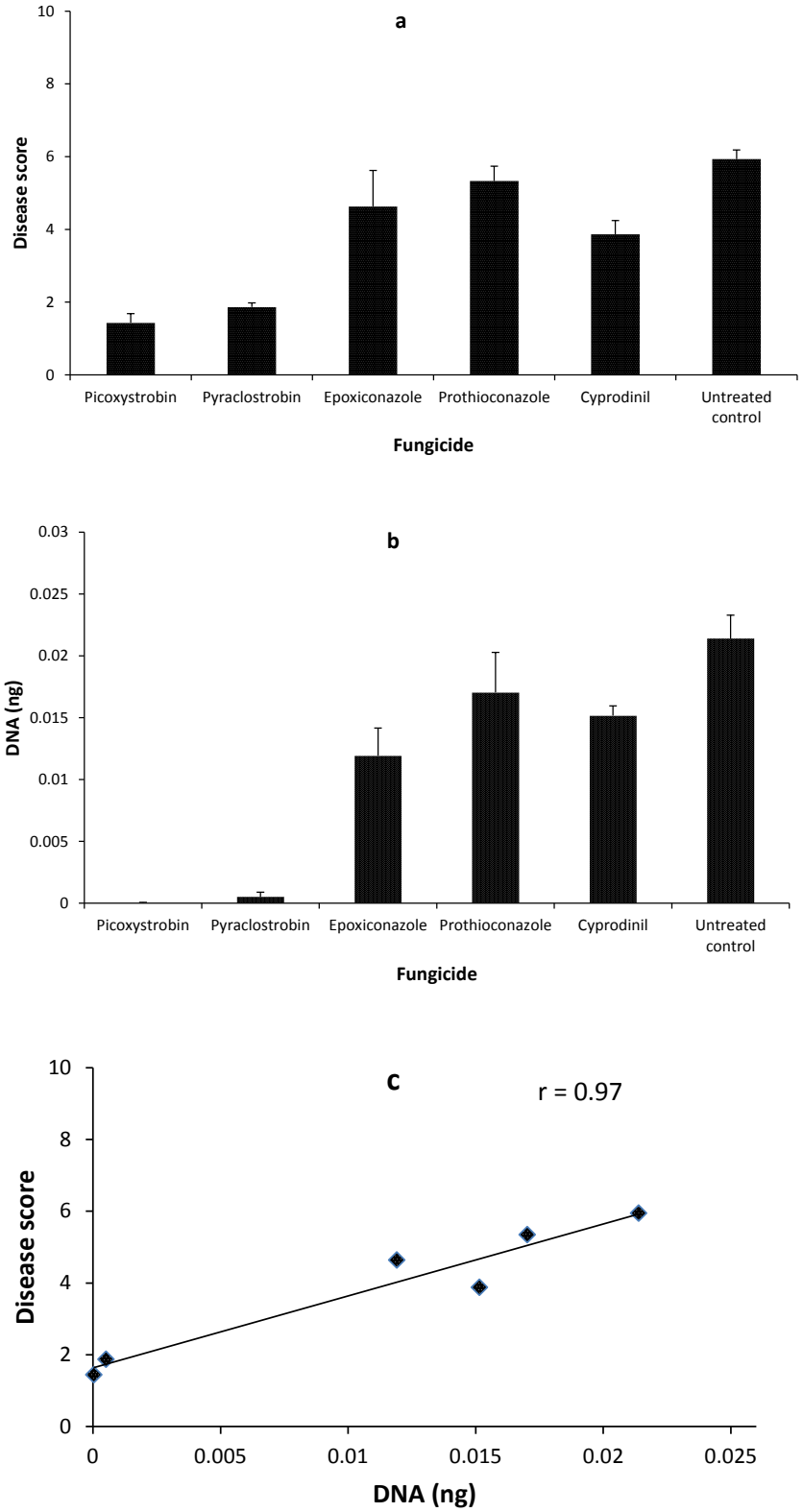


Figure 4.27 Assessment of fungicide efficacy on the disease incidence, caused by *P. teres* isolate 1539. a) visual assessment using the 0-10 rating scale, b) quantitative assessment using q-PCR, c) correlation between visual and quantitative assessments. Bars represent means of 3 replicates, error bars are standard deviation.

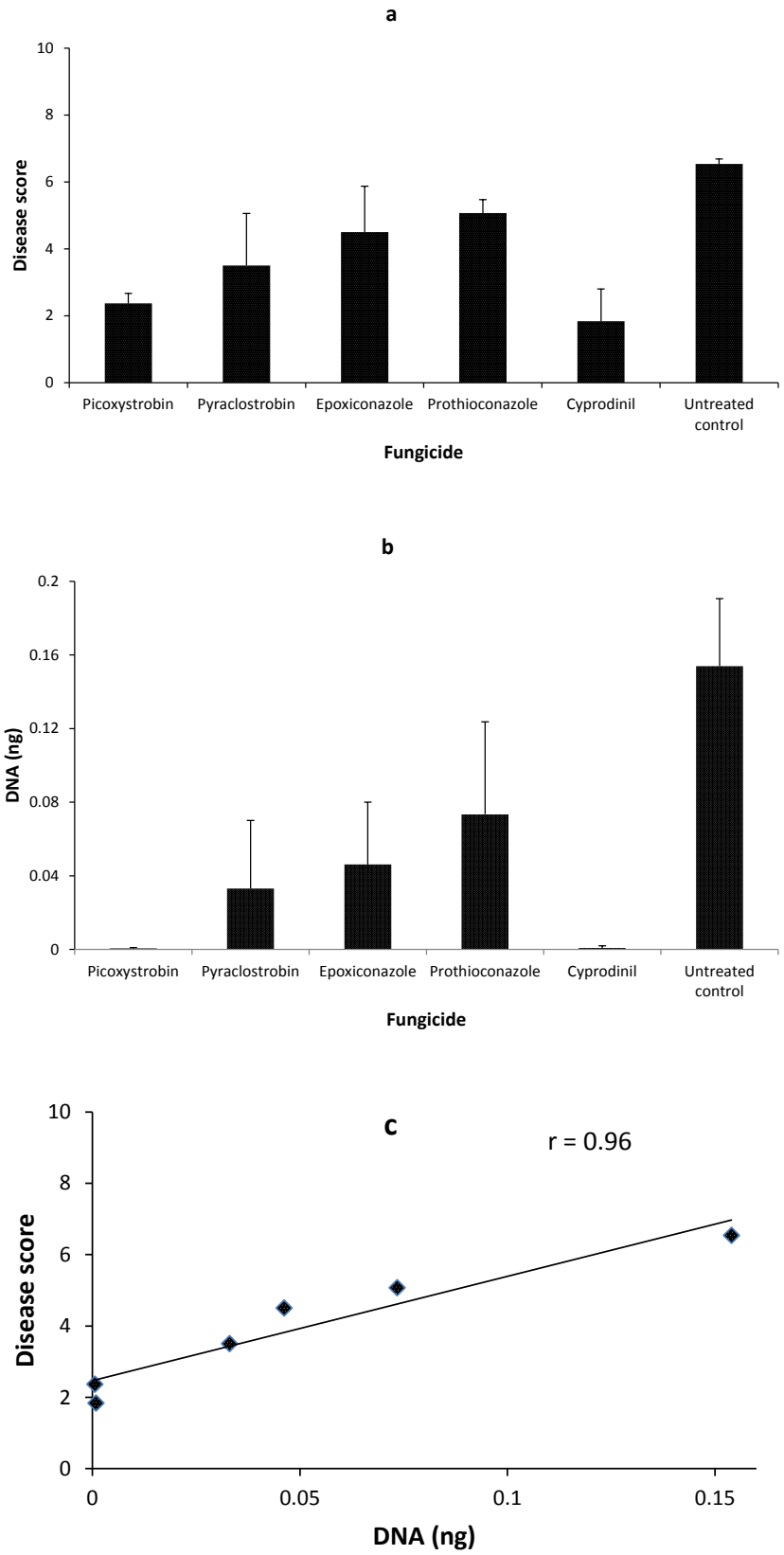


Figure 4.28 Assessment of fungicide efficacy on the disease incidence, caused by *P. teres* isolate 1534. a) visual assessment using the 0-10 rating scale, b) quantitative assessment using q-PCR, c) correlation between visual and quantitative assessments. Bars represent means of 3 replicates, error bars are standard deviation.

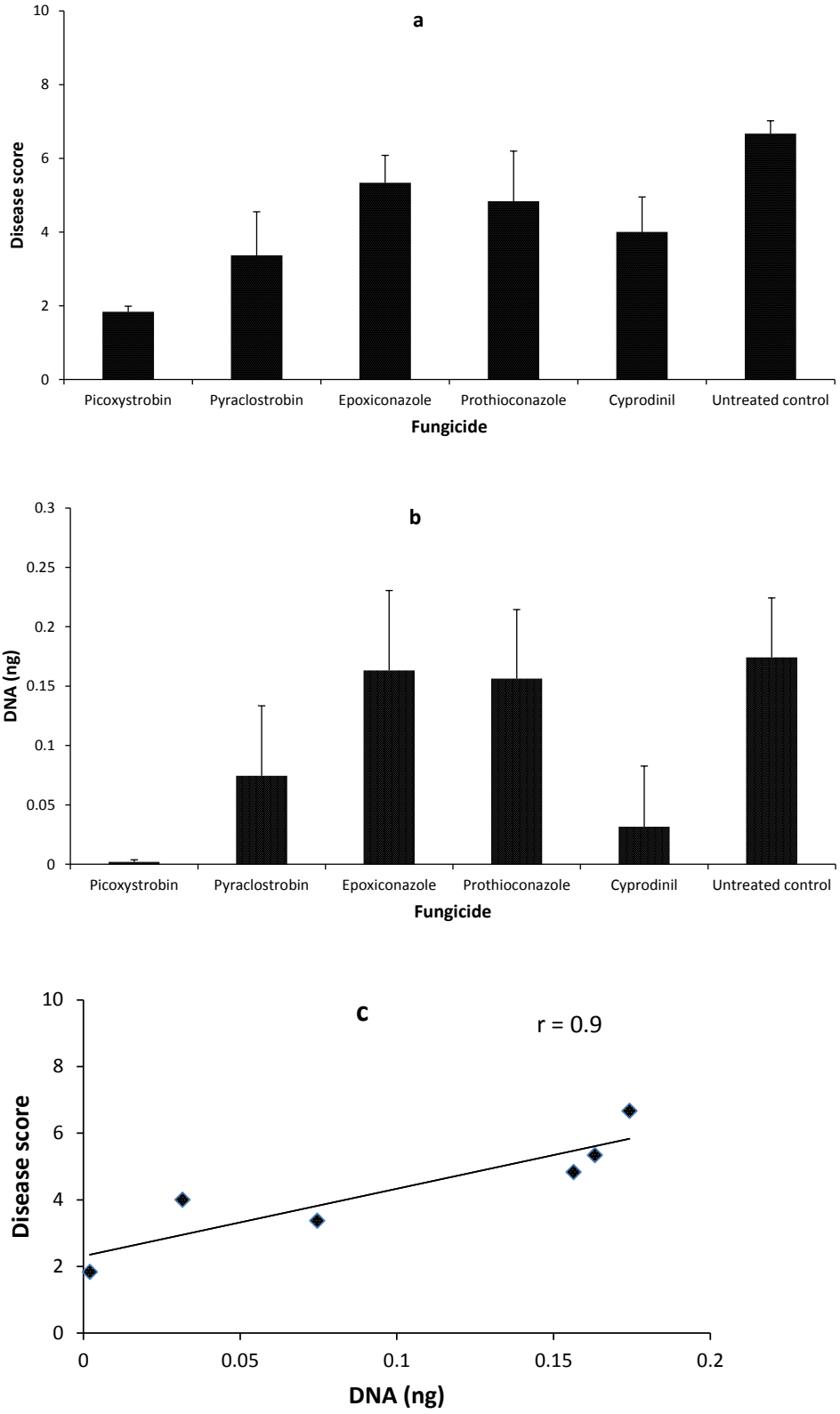


Figure 4.29 Assessment of fungicide efficacy on the disease incidence, caused by *P. teres* isolate MR1-1. a) visual assessment using the 0-10 rating scale, b) quantitative assessment using q-PCR, c) correlation between visual and quantitative assessments. Bars represent means of 3 replicates, error bars are standard deviation.

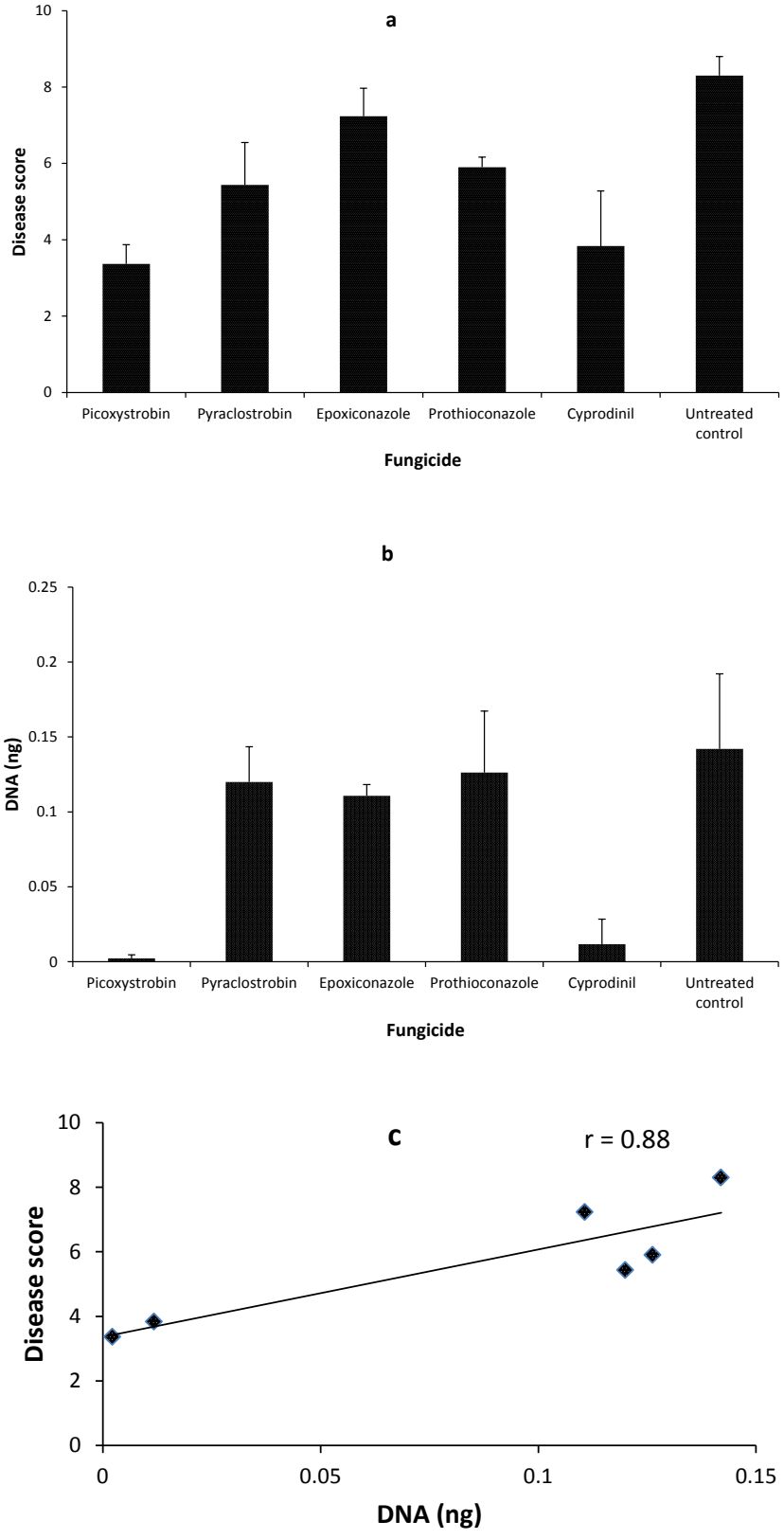


Figure 4.30 Assessment of fungicide efficacy on the disease incidence, caused by *P. teres* isolate THM-2. a) visual assessment using the 0-10 rating scale, b) quantitative assessment using q-PCR, c) correlation between visual and quantitative assessments. Bars represent means of 3 replicates, error bars are standard deviation.

4.4 Discussion

The aim of this chapter was to investigate the *in vitro* and *in planta* efficacy of single QoIs available, along with other fungicides, against wild-type isolates of *P. teres* and those carrying the F129L mutation in the *cyt b* gene. The performances of 4 QoIs, compared to epoxiconazole were assessed *in vitro*. The results in this research have shown that QoIs inhibited the growth of the fungal pathogen to some degree and to different extents, with, for instance, pyraclostrobin being more inhibitory than the other fungicides tested. However, the performance of other QoIs, trifloxystrobin, azoxystrobin and picoxystrobin was less pronounced. Lower performances of some of QoIs towards mutant isolates of *P. teres* suggest that they were compromised by the F129L mutation *in vitro*. Epoxiconazole, however, showed variable performances against isolates of *P. teres*. It was as high as pyraclostrobin, particularly at concentrations of 5 and 10 mg L^{-1}, against a range of wild type and mutant isolates, but in some cases, showed as low activity as other QoIs against other mutant isolates. This may reflect the presence of undetected (eg. in *CYP51* gene) mutations in isolates of *P. teres* used in this work, which may have conferred insensitivity to triazoles. However this pattern of performance of epoxiconazole, which is similar to some QoIs, is not expected to be correlated with the F129L mutation.

The activity of penthiopyrad *in vitro*, regardless of the genotype of *P. teres* isolates, outperformed QoIs and triazoles and was shown to be the strongest inhibitor of the fungal growth on agar medium. Interestingly, it showed a lower performance against the UK wild type isolate Bot-1.

Tebuconazole exhibited the lowest performance *in vitro* (44.5-63% at the highest concentration tested) against almost all *P. teres* isolates with the exception of isolate 83. In comparison prochloraz provided high growth inhibition of most isolates. This superiority of prochloraz over tebuconazole is also supported by Serenius and Manninen (2006), who found overall inhibition of radial growth, when testing 364 *P. teres* isolates, was 63 and 86% on media amended with 0.1 and 1.0 mg L^{-1} prochloraz, respectively. However, the performances of tebuconazole and prochloraz reported here were for

comparison purposes and their activity is not related to the F129L mutation. Low performance of these two fungicides may reflect other changes, such as *CYP51* mutations, known to confer resistance to triazoles in many plant pathogens. In agreement with these results, earlier research indicated the existence of resistance to DMI fungicides, such as triadimenol, among field populations of *P. teres* (Peever and Milgroom, 1992; Campbell and Crous, 2002). This evidence is also supported by Duvert *et al.* (1996) and Duvert and Vives (1997) when they found the variability in the efficacy of triazoles *in vitro* against *P. teres* isolates. In an assay evaluating the sensitivity of both net and spot type *Pyrenophora* to triadimenol, bromuconazole, flusilazole, propiconazole and tebuconazole, results of Campbell and Crous (2002) revealed that both net- and spot-type isolates had strong resistance to triadimenol with the mean of EC_{50} value of 25.7 mg mL^{-1}. The results of this study is further supported by Serenius and Manninen (2006) who stated that *P. teres* isolates originating from fields in which prochloraz was sprayed during the growing season displayed increased growth on prochloraz-amended medium. They added that such isolates may have been under strong selection pressure.

The *in vitro* performance of the mixed active ingredient fungicide, Fandango (fluoxastrobin + prothioconazole), was clearly variable against isolates of *P. teres*. Many previous reports have recommended the use of the mixture two active ingredients, instead of a single one. On account of this, Fandango was used as a comparison treatment to single QoIs and triazoles. The results obtained showed that Fandango gave a variable performance. It exhibited high activity against two wild types and three mutant isolates but it was less efficient against other wild type and mutant isolates. This might reflect the QoI component (fluoxastrobin) in the mixture being affected by the F129L mutation to some degree. The low performance of Fandango against the wild-type isolate Bot-1 (40%), and low performance of tebuconazole against the same isolate, may suggest that prothioconazole (the azole component of Fandango) might be affected by the existence of insensitivity towards triazoles in this isolate.

The results for EC_{50} values of QoIs showed that lower EC_{50} values of wild-type isolates were evident, indicating that higher fungicide concentrations were needed for isolates with the F129L mutation to achieve inhibition *in vitro*. Interestingly, some of the F129L mutant and wild-type isolates showed little difference in their EC_{50}s towards some QoI fungicides. Research by Sierotzki *et al*. (2007), which examined the sensitivity of a population of 2005 isolates to azoxystrobin, has shown that EC_{50} values of *P. teres* isolates from France, Switzerland, Belgium, the UK, Ireland and Germany were varied and ranged from 0.001 – 100 mg L^{-1}. They stated the majority of F129L isolates displayed greater EC_{50} values and found the threshold EC_{50} for presence or absence of the F129L mutation was 0.5mg azoxystrobin L^{-1}. Interestingly, they also found that a few isolates had the F129L mutation but were sensitive to azoxystrobin. In contrast to that observation, they reported some wild-type isolates with relatively high EC_{50} values, without possessing the F129L mutation. This phenomenon is also supported by Ypema (2005) who, in an American Phytopathological Society Conference abstract, suggested that the resistance (insensitivity) occurred, in some cases, with no detectable point mutation. This was also observed with isolate 1539, which was provided by DuPont as F129L isolate; however, sequence analysis showed this mutation was not present. The insensitivity of this isolate to single QoIs reported in this study is supported by the findings of Perez-Garcia *et al*. (2008) who found 13 QoI-resistant isolates in *Podosphaera fusca*, which did not possess amino acid mutations, such as G143A or F129L, conferring resistance in many fungal pathogens. The insensitivity of isolate 1539 towards single QoIs in the current study, however, may refer to the possibility of existence of other mutations such as G137R or G143A which are found recently in *P. teres* isolates. However, this current study did not detect these possible mutations in *P. teres* isolates tested due to the use of specific primers which targeted a small exon in the *cytochrome b* gene, covering position 129 only. There is also the possibility of a contribution from an alternative oxidase (AOX) in this isolate. Such enzymes have been found in other plant pathogens, conferring insensitivity to QoIs (Seyran *et al.*, 2010). In *M. gramnicola* isolates, in the presence of the QoI fungicides azoxystrobin, activation of AOX increased the flexibility in respiration, which allowed resistant strains to survive. In the case of triazoles they showed variable EC_{50}s towards each isolate. However, there is

no evidence that resistance to triazoles is associated with the F129L mutation in *P. teres*. Previous authors referred to resistance of *P. teres* isolates to triazoles expressed in EC_{50} values. For instance, Campbell and Crous (2002) reported a strong resistance shown by both net and spot type isolates to triadimenol and lower resistance to other triazoles. Nevertheless, spot-type isolates showed higher resistance than net-type isolates to five triazole fungicides screened in the study.

The results obtained in *in planta* trials demonstrated that some QoIs, such as trifloxystrobin, showed very low activity against almost all mutant isolates used in the study. Pyraclostrobin exhibited activity against a few F129L mutant strains. Picoxystrobin, however, showed low growth inhibition *in vitro* against most mutant isolates but displayed the best efficacy against mutant isolates *in planta*. Nevertheless, its activity against mutant isolates was lower than that shown against wild type isolates. The activity of azoxystrobin was reduced by the presence of the F129L mutation in isolates tested. The decline in the field efficacy of QoIs has been confirmed worldwide in several pathogens on a wide variety of crops. This resistance, depending on the pathogen, has either been associated with one of two distinct point mutations or, in some cases, no detectable mutation (Ypema, 2005; Perez-Garcia *et al.*, 2008). Consequently, it seems that the impact of the F129L mutation in the current study varied for each fungicide depending on the mutant isolate. This may indicate that different isolates with the F129L mutation behaved independently to each member of the QoI fungicide family. This concept is supported by sensitivity studies in transformed strains of *Saccharomyces cerevisiae*, reported by Fisher *et al.* (2004), where they demonstrated that the different QoIs are not equally affected by the F129L mutation.

Inferences from the *in planta* studies suggest that the F129L mutation is likely to have affected the disease control achieved by some members of QoIs tested here. Although the *in vitro* studies revealed that QoIs inhibited the growth of the fungal pathogen to some degree, their efficacy *in planta*, however, was less pronounced. The results obtained here are in agreement with those of Maumene *et al.* (2009) who reported that in spite of the relatively low frequency of the resistance mutation, reduction of the efficacy of

QoIs tested (azoxystrobin, pyraclostrobin, picoxystrobin, trifloxystrobin) was observed to various degrees. However, the findings of the current study are opposed to the results of previous studies reported by Semar *et al.* (2007), where they stated that the field performance of pyraclostrobin, conducted in 2005-2006 in France, is not affected by the F129L mutation and the fungicide provided a good control of net blotch of barley in fields with different frequencies of the F129L mutation. On the other hand, and in the same experiment, they supported the findings reported here by confirming the existence of the variation among QoI performance, when they found that pyraclostrobin outperformed azoxystrobin in controlling net blotch carrying the F129L mutation. It can be speculated that the F129L mutation generates lower levels of resistance which may be insufficient to cause a serious effect on the disease control (Lucas, 2005; Hollomon, 2007). There was not an observation of total failure of fungal control, as reported with the G143A mutation in *M. graminicola* (Lockley and Clark, 2005). Sierotzki *et al.* (2005) emphasised that different amino acid changes in the target protein can cause different levels of resistance. They further confirmed that the G143A mutation caused much higher levels of resistance to QoIs than the less common F129L mutation. Sierotzki *et al.* (2007) reported that in *P. teres*, an intron in the *cyt b* gene, immediately after the codon for the amino acid in position 143, was present. The G143A mutation would prevent splicing out of the intron, prior to transcription into mRNA, thereby disrupting functionality of the *cyt b* protein, leading to a lethal event. Thus the G143A mutation cannot occur in *P. teres*. According to FRAC reports, in 70 pathogens exhibiting a high level of resistance, this was shown to be the result of a single G143A mutation, while the F129L mutation generally caused a much lower degree of resistance (Brent and Hollomon, 2007). The results obtained in this study are similar of those obtained by Oxley and Hunter (2005) who reported that good field protection of barley plants against net blotch was achieved with QoI fungicides (picoxystrobin, pyraclostrobin, and azoxystrobin). They further added that, for eradication purposes, picoxystrobin and pyraclostrobin achieved the best control.

The comparison of the *in planta* activities shown by QoIs with that obtained *in vitro*, suggests that although a few isolates followed the same pattern and

perfectly matched the *in vitro* data, for many isolate: fungicide interactions the *in vitro* performance did not reflect the one seen *in planta*. In other words, many instances of high *in vitro* activity against *P. teres* isolates were associated with low *in planta* efficacy and *vice versa*. *In vitro* studies may provide results that do not reflect the complex interactions which occur with a living plant treated with a fungicide. *In planta* testing may be considered superior to that done *in vitro* because it provides a more-representative indication of true fungicide efficacy. *In vitro* studies have the potential to offer insights into the relative activity of different fungicidal molecules towards pathogen species and isolates of the same species. Results can often be obtained rapidly and reproducibly, but there in an inherent danger that such findings may not always reflect the true antifungal activity of compounds when used on plants to control disease. This may occur if the fungicide used to treat plants is inactive, only being converted to a fungicidal moiety after application. In this situation *in vitro* activity may be lower and not reflect the true *in planta* efficacy of the molecule. For instance, triadimefon, an early systemic triazole foliar fungicide, that acts by inhibiting steroid demethylation and was used against many plant pathogens such as powdery mildews and fungi on fruits, vegetables and other crops (Roberts and Huston, 1999), is enzymatically reduced in plants and fungi to triadimenol, a more fungi-active metabolite (Deas and Clifford, 1982; Deas *et al.*, 1986; Kenneke *et al.*, 2008). Similarly, benomyl and thiophanate-methyl are both transformed to the more active molecule carbendazim after application (Clemons and Sisler, 1969; Baude *et al.*, 1973). This phenomenon was also observed with other fungitoxic compounds. Working with *Sclerotinia sclerotiorum*, which causes sclerotinia stem and root rot of tomato and other economically important vegetable crops, Kurt *et al*. (2011) found that mycelial growth was completely inhibited *in vitro* by 3 naturally-occurring fungitoxic compounds (methyl, allyl and benzyl isothiocyanate). In an *in planta* assay, however, only allyl isothiocyanate showed a similarly high level of activity. The observation made in some of this work of reduced efficacy *in planta*, compared to *in vitro* activity, may reflect degradation of the active molecules in plant tissues. As a general observation conclusions drawn on the relative activity of fungicides would benefit from a combination of both *in vitro* and *in planta* evaluations.

Penthiopyrad, other azoles, and the mixed product Fandango were also assessed *in planta* and their efficacies were compared with QoIs. Penthiopyrad was noticeably effective *in planta* regardless of the sensitivity of isolates. It also perfectly matched the *in vitro* activity in inhibiting the mycelium growth and was also consistent with EC_{50} values. Penthiopyrad as a new active ingredient within SDHIs is launched recently into the market. The activity of this fungicide, although was high either *in vitro* or *in planta*, care must be taken in using this fungicide consistently. As site-specific fungicides, SDHIs are at medium to high risk of resistance (Anonymous, 2011d). The efficacy of the azoles tested was variable and some poor performances were detected. The activities of tebuconazole, prochloraz, and epoxiconazole, although is not related to the F129L mutation, were low and followed a similar pattern to some QoIs. Prothioconazole, however, showed good activity in controlling the disease *in planta*. The superiority of prothioconazole over other azoles tested here was similar to that found by Oxley and Hunter (2005) who stated that prothioconazole displayed better efficacy in protecting barley plants against net blotch compared to epoxiconazole. Low *in planta* activity of most azoles reported in this study may suggest the existence of resistance within *P. teres* isolates towards SBIs. This is in agreement with many previous researchers who reported resistance in *P. teres* isolates to members of triazoles. In this regard Duvert *et al.* (1996) demonstrated that under greenhouse and field conditions variable efficacy of triazole fungicides against net blotch and other diseases was observed. Fandango, the mixture of a QoI and a triazole, however, showed low performance *in vitro*, but high activity *in planta*, regardless of the sensitivity of *P. teres* isolates. This is in agreement with that of Oxley and Hunter (2005) where there reported that best protection of barley plants from net blotch was achieved by the Fandango and QoIs. The efficacy of the mixture of QoI and triazole fungicides was also supported by Semar *et al.* (2007) when they found that the combination product, Opera (133 g L^{-1} pyraclostrobin + 50 g L^{-1} epoxiconazole), outperformed some single QoIs and was as good as pyraclostrobin. The results of this study, however, showed that the *in planta* performance of Fandango was not consistent with the EC_{50} data.

A comparison was made between conventional and q-PCR assessment of fungicide activities *in planta*. Both assessments demonstrated that the best disease control, although was affected by the F129L mutation to some degree, was obtained with using picoxystrobin. However a second QoI, pyraclostrobin, was less effective in giving protection of barley plants, allowing for more disease occurrence and the F129L mutation has more adverse effect on this fungicide's activity. Cyprodinil, an anilinopyrimidine, was also an efficient fungicide used in these experiments and gave high protection against most of the isolates, regardless of their genotype. The results showed strong positive correlations between both assessment methods. This indicates the accuracy of the quantitative PCR method in assessing fungicide performance by measuring the amount of DNA of the pathogen in plant tissues. The method could thus be an alternative to symptom evaluation. This is mainly because the method is rapid to undertake and very sensitive, allowing pathogen detection before symptoms are visible. PCR-based methods, including q-PCR, allow fast accurate detection and quantification of plant pathogens and are now applied to practical problems (McCartney *et al.*, 2003). Thus, in addition to diagnosis of plant pathogens in host plants, PCR-based methods could also be used to evaluate fungicide performance by measuring the amount of pathogen DNA at a pre-symptomatic stage (Schena *et al.*, 2004; Guo *et al.*, 2006), and also to detect the resistance genotype status of the pathogen by detecting the resistant alleles within infected plants. The superiority of the q-PCR method over traditional assessments was strongly supported by Guo *et al.* (2007) where they could detect *M. graminicola* in wheat leaf layers when it was not detectable visually. They further added that q-PCR may provide an alternative method for an accurate assessment of the fungicide effects on plant pathogens. It was noticed, in some cases, that a high visual assessment corresponded to a very low detection of DNA using q-PCR. This difference might indicate that with visual assessment the size of lesions does not always reflect the fungal content in infected areas. Thus the symptomatic area may not the fully invaded by the pathogen but it might be caused by toxic events associated with tissue colonisation (Smedegard-Petersen, 1977; Bach *et al.*, 1979; Barrault *et al.*, 1982; Friis *et al.*, 1991). In contrast, low visible lesions were, in some cases, associated with high DNA concentrations. This was possibly because that assessment using q-PCR could detect the pathogen,

even during the symptomless latent period, which is not detectable by visual assessment.

Chapter 5 Septoria leaf blotch of wheat, isolation, detection of *CYP51* mutations and fitness costs

5.1 Introduction

Septoria leaf blotch caused by *M. graminicola* is the most economically important foliar disease of wheat in the UK, France and many other European countries (Polley and Thomas, 1991). Because of the lack of good source resistance in wheat cultivars, the main method to control the disease is by using fungicides. The widespread incidence of QoI-resistant isolates within *M. graminicola* populations in these countries has resulted in reduced field performance of strobilurins (Gisi *et al.*, 2002). Sterol 14α-demethylation inhibitors (DMIs), which belong to sterol biosynthesis inhibitor group (SBIs), also known as triazoles, have played an important role against *M. graminicola* for the last two decades (Leroux *et al.*, 2007). They are systemic fungicides with both protective and curative activity in disease control (Kuck and Scheinpflug, 1986). Despite their long term use, widespread resistance to azole fungicides in plant pathogenic fungi has not occurred. In contrast, in human fungal pathogens the resistance is widespread (Cools *et al.*, 2006). However, a decline in the efficacy of some azoles against *M. graminicola* has been reported recently (Cools and Fraaije, 2008).

In plant and human pathogens , three major mechanisms of resistance to DMIs have been reported. These include: 1) point mutations in the *CYP51* gene encoding the sterol 14α-demethylase that result in reduced affinity of DMIs for their target ; 2) over-expression of the *CYP51* gene , resulting in elevated levels of *CYP51*p; and 3) reduced accumulation of DMIs in fungal cells through up-regulation of active efflux proteins. The latter mechanism involves ABC (ATP-binding cassette) transporters or major facilitators and can mediate multidrug resistance to various classes of fungicides. A combination of these mechanisms leading to a polygenic regulation of DMI resistance, is commonly found in clinical isolates of *Candida albicans* (Morschhauser, 2002). In plant pathogens, such as *M. graminicola*, this similar phenomenon could also be responsible for resistant phenotypes (Stergiopoulos *et al.*, 2003). However, in

European countries, DMI resistance resulted mostly from changes in the *CYP51* gene, at least until 2007 (Leroux and Walker, 2011). To date, 22 different amino acid alterations (substitutions and deletions) have been detected in the *CYP51* gene in *M. graminicola* populations in Western Europe (Zhan *et al.*, 2006; Leroux *et al.*, 2007; Stammler *et al.*, 2008a; Cools *et al.*, 2010). Previous studies indicated the existence of 8 categories of *M. graminicola* strains (TriR1-TriR8) displaying reduced sensitivity to DMIs (Leroux *et al.*, 2006; Leroux *et al.*, 2007; Leroux *et al.*, 2008c). These different R-types are associated with either single or combinations of single nucleotide polymorphism (SNPs) or amino acid deletions in the *CYP51* gene. Changes from glycine to aspartate (G460D/S) at position 460, a tyrosine to phenylalanine (Y137F) at position 137, and valine to alanine (V136A) at position 136 have been described as R2, R3, and R5 phenotypes respectively. The R4 genotype is characterised by a mutation Y461S/H or ΔY459/G460, while genotypes R6, R7- and R7+ are characterised by a SNP that leads to substitution of valine for isoleucine at position 381 (I381V), in combination with either a point mutation Y459S/D/N or Y461S/H (R6), or the double amino acid deletion ΔY459/G460 with the mutation A379G (R7+) or without A379G (R7-) (Leroux *et al.*, 2007; Stammler *et al.*, 2008a). The mutations V136A and I381V occur only in combinations with mutations or a deletion of the amino acids tyrosine or glycine in the YGYG region (positions 459–461), while mutations or the YG-deletion at 459–462 could also occur as a single event (Stammler *et al.*, 2008a). There are also other single mutations such as D107V, D134G, S524T or combinations of them (V136A + I381V or I381V without a mutation at 459–462) described by Stammler *et al.*(2008a) for the first time in isolates of *M. graminicola* and have never been detected before in the *CYP51* gene. However, these classifications, with the new emerging mutations, have been modified recently by the Leroux group to include more R-groups ranging from R1-R12 (Leroux and Walker, 2011).

5.2 Objectives

The aim of this research was to isolate a collection of *M. graminicola* strains from infected leaves, derived mainly from the UK and Germany, for comparison with some older stock isolates, and to detect alterations in the *CYP51* gene, encoding the sterol 14α -demethylase target for triazole

fungicides. The effect of these SNP changes or deletions on the phenotypic fitness, expressed as pathogenicity and *in vitro* growth rate on agar media, was also undertaken.

5.3 Methods

5.3.1 Isolation

Wheat leaves, from the 2008-2009 season, were received from wheat fields in England, Scotland, and Germany. The leaves were surface sterilized with a 8% Domestos solution (Domestos®, Johnson Diversy Ltd., Northampton, UK) to give a sodium hypochlorite concentration of 0.5%, for 5 minutes, washed three times with sterile distilled water and then dried with sterile filter papers. Leaf segments were attached (pycnidia facing up) to glass slides with the aid of Vaseline, then placed in a sterile damp chamber for 24 h. Conidia oozing from pycnidia were picked up using a fine point glass needle and then transferred to fresh PDA, amended with antibiotics, by streaking the surface of medium with the inocula. The inoculated plates were incubated at 20°C for 3-5 d. From single colonies appearing on the PDA plates, three isolates, each from a separate leaf, were chosen from each region. Isolates were consecutively numbered and further sub-cultured for the purpose of making spore suspensions for glycerol stock cultures for long-term cold storage at -80°C. Older isolates, from previous years, were also included in the study as reference strains. All *M. graminicola* isolates used in this study are described in Table 5.1.

5.3.2 Detection of *CYP51* mutations

To detect the *CYP51* mutations in *M. graminicola* isolates, PCR-based methods were used. Fungal isolates were grown in 30 mL of potato dextrose broth (PDB), placed in 100 mL conical flasks. The inoculated liquid cultures were incubated in a controlled environment incubator shaker (New Brunswick Scientific, Edison, USA) at 20°C for 2-3 weeks depending on the isolate. The resultant mycelia were placed in Falcon tubes, centrifuged at 2065 g for 5 min, washed twice with water and then placed in a freezer at -80°C. Fungal samples were placed in liquid nitrogen and then freeze-dried for 48 h (Christ-Alpha 2-4 LD, Germany).

Table 5.1 *M. graminicola* isolates used in this study.

No	Isolate	Origin	Year	Fungicide history	Sensitivity to QoIs
1	Tibb-2	Tibbermore, Scotland	2008	Untreated	Unknown
2	Nuf-Un-2	Nufarm-England	2008	Untreated	Unknown
3	Nuf-Pz-2	Nufarm-England	2008	Prochloraz	Unknown
4	Roy-Un-2	Royston-England	2008	Untreated	Unknown
5	King-Un-2	Devon-England	2008	Untreated	Unknown
6	King-Pz-2	Devon-England	2008	Prochloraz	Unknown
7	Skedd-2	Fife-Scotland	2008	Untreated	Unknown
8	Head-2	Headly Hall, Yorkshire	2008	Untreated	Unknown
9	Ger-3-2	Germany	2008	Unknown	Unknown
10	Ger-4-2	Barlt-Germany	2008	Unknown	Unknown
11	Pittend	Kinross-Scotland	2008	Untreated	Unknown
12	Ire-3	Ireland	2003	Untreated	Wild type
13	HA-3	Harper Adams	2006	Unknown	G143A
14	G303	Rothamsted (Herts)	2003	Treated	G143A
15	Roy-Pz-1	Royston-England	2008	Prochloraz	Unknown
16	S331	Loughborough	1995	Unknown	Wild type
17	Ctrl-1	Rothamsted (Herts)	2001	Untreated	Wild type
18	Lars-37	Somerset	2003	Untreated	G143A

DNA extraction followed this procedure: 20 mg of freeze-dried mycelium was taken from each isolate, placed in microtubes (2 mL screw cap tubes) with 0.5 g of 2 mm glass beads and then placed in liquid nitrogen for 30 seconds. To disrupt the fungal tissue, the tubes were placed in a tissue-lyser (FastPrepTM FP 120, Thermo Electron) and run at the highest speed (6.5 Hz) for 40 seconds. The fungal DNA was then extracted following the manufacturer's protocol for the extraction kit (DNeasy® Plant Mini Kit (50), QIAGEN, GmbH) and quantified using a NanoDrop® Nd-1000 spectrophotometer (Thermo Scientific). Four distinct PCR reactions were performed to amplify the *CYP51* gene by using four primer sets (synthesized by Eurofins, UK) designed and used by Leroux *et al*. (2007), each primer was designed to amplify a part of the gene ranging from 555 to 622 bp, to make PCR products overlapping each other (Table 5.2). At the beginning of the *CYP51* gene, an additional upward 200 bp sequence was amplified with CYP1 and CYP2 primers. Amplifications

were performed in a total volume of 25 µL which consisted of 0.4 µM of each primer, 0.2 mM dNTPs, 1x GoTaq PCR reaction buffer (Promega, Madison, USA), 1.5 mM $MgCl_2$, 0.5 U DNA polymerase (GoTaq® Flexi DNA Polymerase, Promega). PCR was performed in Flexigene cycler using the following conditions: initial preheat for 2 min at 95°C, followed by 37 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min followed by a final step 72°C for 15 min. Amplified DNA fragments were resolved and visualized on a 1.5% agarose gel. The gel was prepared with 1x TAE buffer and ethidium bromide was added for a final concentration of 0.5 µg mL^{-1}. Four microliters of each PCR product was loaded into the gel well alongside 4 µL of a DNA size marker (100 bp ladder).

To detect the differences, the PCR products were sequenced (Eurofins, UK), the four sequence parts of the gene were then gathered and then the whole sequence of the *CYP51* gene for each isolate were aligned beside the sequence of the *CYP51* gene of wild type isolate IPO323 and analysed using BioEdit software.

Table 5.2 Primers used to amplify the four parts of *CYP51* gene in *M. graminicola*.

Primer name	Primer direction	Sequence(5'-3')	Product length	Tm(C)
CYP1(F)	Forward	GAAACAGCGTGTGTGAGAGC	564	59.4
CYP2(R)	Reverse	GCGTTGACGTCCTTCAGTTT		57.3
CYP3(F)	Forward	CTGCTGGGAAAGAAGACGAC	555	59.4
CYP4(R)	Reverse	TCTTCTTCTGCGCATAATCG		55.3
CYP5(F)	Forward	GGGATTCACACCGATCAACT	614	57.3
CYP6(R)	Reverse	AGTTTCGAGAGGTTGGCGTA		57.3
A(F)	Forward	CACTCTTCATCTGCGACCGAGTC	622	64.2
B(R)	Reverse	CTGCTGTAATCCGTACCCACCAC		64.2

5.3.3 Fitness costs

5.3.3.1 Pathogenicity

The susceptible wheat cultivar Riband was grown in 13 cm pots at a rate of 10 plants per pot. The experiment was arranged in CRD with three replicates. At the growth stage 12 (Zadoks *et al*., 1974), the plants were inoculated with a spore suspensions at 1 x 10^6 conidia mL^{-1} of each isolate of the pathogen. The inoculated plants were bagged with transparent plastic bags for 24 h. A layer of water was also added to the bottom of the trays to keep a high humidity. The plants were maintained in a controlled environment room at a day temperature of 20°C and at 12°C night temperature with 16 h photoperiod. After incubation for 21 d, disease occurrence as symptoms expressed for each isolate was assessed visually as the percentage necrotic leaf area.

5.3.3.2 Growth rate

The mycelial growth rates of *M. graminicola* isolates were tested on agar culture using 9 cm Petri dishes. Using a sterile cork borer, fresh PDA medium was inoculated with 5 mm circular mycelium discs. Mycelium discs were taken from 15 d cultures produced by inoculating fresh PDA plates with spore suspensions taken from glycerol stock cultures stored at -80°C. The discs were mycelium downwards placed on the centre of the Petri dishes and then incubated in darkness at 20°C ± 2 for 15 d (Figure 3.3, section 3.2.5.2). The radial growth of the pathogen was then measured. The measurements were taken in two planes at 90° to each other and averaged. After a deduction of 5 mm was made for the diameter of mycelial discs, the growth rate was measured and expressed in mm d^{-1}. Data were then analysed using the GenStat version 11 software package.

5.4 Results

5.4.1 Isolation

From the wheat leaves of the 2008 season, obtained from different areas of the UK (England and Scotland) and from Germany, three isolates, each from one leaf, were chosen from each geographic region. However, only one isolate was obtained from the Pittendreich area of Scotland (Table 5.1, section 5.3.1). The growth of *M. graminicola* isolates on the agar medium was yeast-like in

appearance and this state was maintained for 5-7 d at 20°C, depending on the isolate. It was observed that 5-7 d incubation was optimum to harvest spores from cultures for inoculation purposes. A decline in sporulation was observed when the cultures were incubated for a longer time.

5.4.2 Detection of *CYP51* mutations

Sequencing the *CYP51* gene, encoding the sterol 14α-demethylase target for triazole fungicides, identified several point mutations within 18 *M. graminicola* isolates. These mutations included SNPs and amino acid deletions. Amino acid changes were at positions 24 (valine to aspartate) in isolate S331, at position 50 (leucine to serine) in 15 isolates, change serine to tyrosine at position 51 also in isolate S331, and 9 isolates had changes from serine (S) to asparagine (N) at position 188. At the position 379, the change from alanine (A) to glycine (G) was observed in 5 isolates, the change from isoleucine (I) to valine (V) at position 381 dominated the changes, combined with other changes and deletions at positions 459, 460, 461, and 513 (Figure 5.1).

The overall SNP changes and deletions occurring in the *CYP51* gene for each isolate are shown in Table 5.3. Based on the changes and deletions of this study and by referring to the previous classifications of Leroux group (Leroux and Walker, 2011) which were based on genotyping and *in vitro* phenotyping, isolates can be categorised into 9 variants, which express phenotypic variation in sensitivity to triazoles. Sensitive isolates (S) included those with no important mutations mentioned previously by other researchers (included isolate S331 only). The Y137F mutation was found in one isolate only (isolate Ctrl-1) and therefore supposed to be classified as R3 genotype. However, the S524T mutation although not detected in this study because the primers used to amplify the *CYP51* gene did not extend to cover the 524 position of the gene, previous sequence results carried out by Cools *et al*. (2005) confirm the existence of this mutation in this isolate. Therefore with the existence of the S524T mutation, a new name (R3+) had to be given to this variant. The R4a variants included isolates with Y461H (isolate Ire-3) but when combined with V136C was given a different name as R4a+(isolate Roy-Pz-1). The combination of Y461H with V136A considered R5a variant included 2 isolates (Skedd-2 and Lars-37) while the latter mutation when combine with the Y459/G460 deletion is characterised as R5b variant (isolate Nuf-Pz-2).

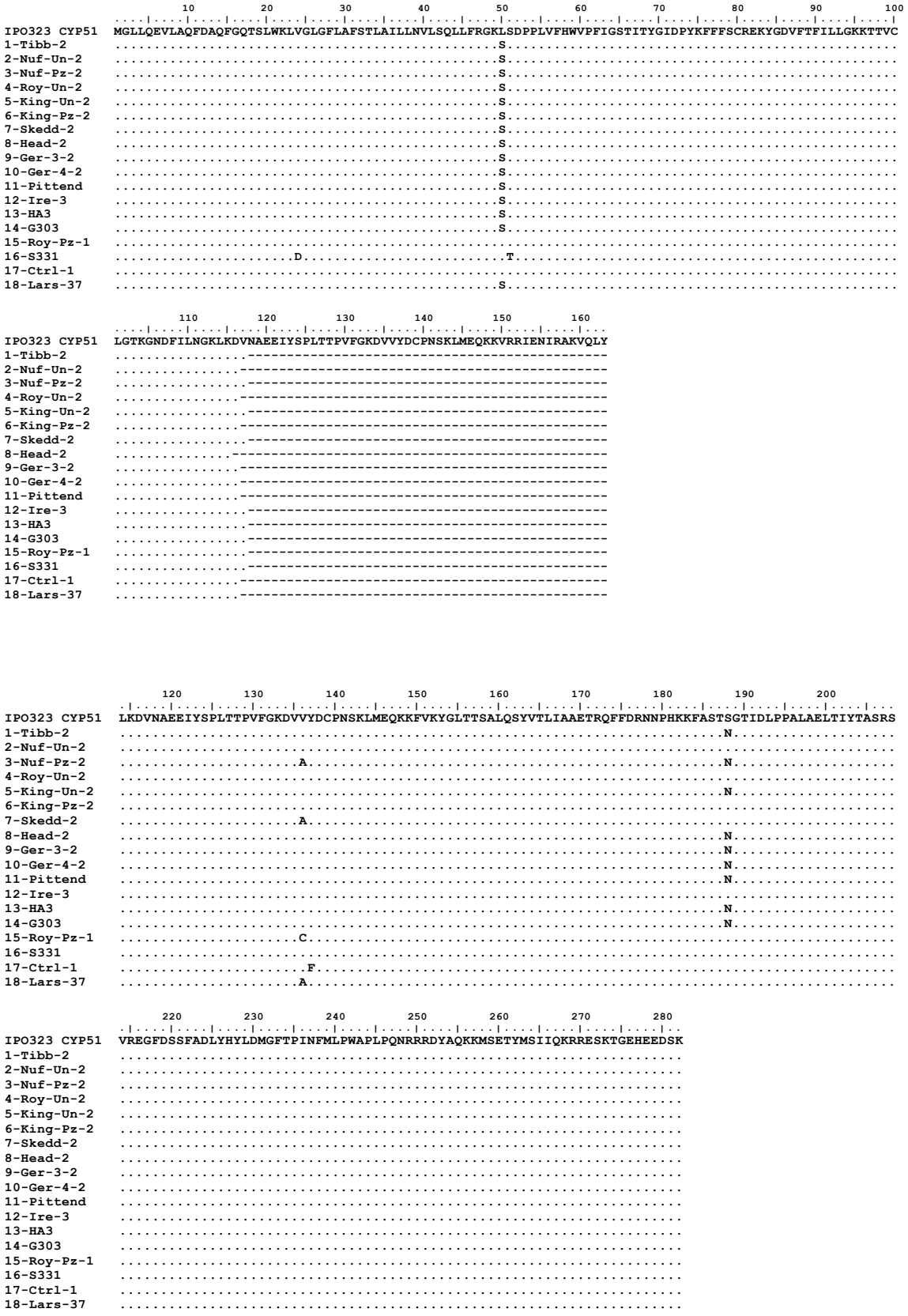


Figure 5.1 Amino acid sequences of the CYP51 gene of 18 *M. graminicola* isolates aligned with the wild type isolate IPO323 (continued in next page).

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                280        290        300        310        320        330        340        350        360
                 |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
IPO323 CYP51    DSKGANTRTAMPFPTRRLLILRCSWPASTLHLRPSPGSLSASHPAPTSKTNSSKNKRICSVTPTAVSRSSHTPTSRNSPSSIKSSKKPFV
1-Tibb-2        ..................................................................................................
2-Nuf-Un-2      ..................................................................................................
3-Nuf-Pz-2      ..................................................................................................
4-Roy-Un-2      ..................................................................................................
5-King-Un-2     ..................................................................................................
6-King-Pz-2     ..................................................................................................
7-Skedd-2       ..................................................................................................
8-Head-2        ..................................................................................................
9-Ger-3-2       ..................................................................................................
10-Ger-4-2      ..................................................................................................
11-Pittend      ..................................................................................................
12-Ire-3        ..................................................................................................
13-HA3          ..................................................................................................
14-G303         ..................................................................................................
15-Roy-Pz-1     ..................................................................................................
16-S331         ..................................................................................................
17-Ctrl-1       ..................................................................................................
18-Lars37       ..................................................................................................

                     370        380        390        400        410        420        430        440        450        460
                 .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
IPO323 CYP51    SKKPFVVVKETLRIHAPIHSILRKVKSPMPIEGTAYVIPTTHTLLAAPGTTSRMDEHFPDCLHWEPHRWDESPSEKYKHLSPTTALGSIAEEKEDYGYGL
1-Tibb-2        ...............G.V.....................................................................--...
2-Nuf-Un-2      ...............V.....................................................................H..
3-Nuf-Pz-2      ..................................................................................--...
4-Roy-Un-2      ...............V.....................................................................H..
5-King-Un-2     ...............G.V...................................................................--...
6-King-Pz-2     ...............V.....................................................................S....
7-Skedd-2       ..................................................................................S..
8-Head-2        ...............G.V...................................................................--...
9-Ger-3-2       ...............V.....................................................................--...
10-Ger-4-2      ...............G.V...................................................................--...
11-Pittend      ...............G.V...................................................................--...
12-Ire-3        ..................................................................................S..
13-HA3          ...............V.....................................................................--...
14-G303         ...............V.....................................................................--...
15-Roy-Pz-1     ..................................................................................H..
16-S331         ..................................................................................
17-Ctrl-1       ..................................................................................
18-Lars-37      ..................................................................................H..

                     470        480        490        500        510
                 .|....|....|....|....|....|....|....|....|....|....|
IPO323 CYP51    VSKGAASPYLPFGAGRHRCIGEQFAYVQLQTITATMVRDFKFYNVDGSDNVV
1-Tibb-2        ..................................................K..
2-Nuf-Un-2      ...................................................
3-Nuf-Pz-2      ..................................................K..
4-Roy-Un-2      ...................................................
5-King-Un-2     ..................................................K..
6-King-Pz-2     ...................................................
7-Skedd-2       ...................................................
8-Head-2        ..................................................K..
9-Ger-3-2       ..................................................K..
10-Ger-4-2      ..................................................K..
11-Pittend      ..................................................K..
12-Ire-3        ...................................................
13-HA3          ..................................................K..
14-G303         ..................................................K..
15-Roy-Pz-1     ...................................................
16-S331         ...................................................
17-Ctrl-1       ...................................................
18-Lars-37      ...................................................
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Figure 5.1 (continued) Amino acid sequences of the *CYP51* gene of 18 *M. graminicola* isolates aligned with the wild type isolate IPO323.

The R6a variants are characterised by the combination of the I381V with the mutations at positions 459 or 461. This includes isolate Nuf-Un-2, Roy-Un-2 and King-Pz-2. The R7 group represents isolates with the I381V mutation combined with the double deletions at 459 and 460 positions of the *CYP51* gene. The latter variant when combine with the A379G mutation is characterised as R8 variant (Table 5.3).

Table 5.3 SNPs and deletions in the *CYP51* gene of 18 *M. graminicola* isolates*.

Isolate	Amino acid position												Genotype
	24	50	51	136	137	188	379	381	459	460	461	513[a]	
Tibb-2	V	**S**[b]	S	V	Y	**N**	**G**	**V**	-[c]	-	Y	**K**	R8
Nuf-Un-2	V	**S**	S	V	Y	S	A	**V**	Y	G	**H**	N	R6a
Nuf-Pz-2	V	**S**	S	**A**	Y	**N**	A	I	-	-	Y	**K**	R5b
Roy-Un-2	V	**S**	S	V	Y	S	A	**V**	Y	G	**H**	N	R6a
King-Un-2	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
King-Pz-2	V	**S**	S	V	Y	S	A	**V**	**S**	G	Y	N	R6a
Skedd-2	V	**S**	S	**A**	Y	S	A	I	Y	G	**S**	N	R5a
Head-2	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
Ger-3-2	V	**S**	S	V	Y	**N**	A	**V**	-	-	Y	**K**	R7
Ger-4-2	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
Pittend	V	**S**	S	V	Y	**N**	**G**	**V**	-	-	Y	**K**	R8
Ire-3	V	**S**	S	V	Y	S	A	I	Y	G	**S**	N	R4a
HA-3	V	**S**	S	V	Y	**N**	A	**V**	-	-	Y	**K**	R7
G303	V	**S**	S	V	Y	**N**	A	**V**	-	-	Y	**K**	R7
Roy-Pz-1	V	L	S	**C**	Y	S	A	I	Y	G	**H**	N	R4a+
S331	**D**	L	**T**	V	Y	S	A	I	Y	G	Y	N	S
Ctrl-1	V	L	S	V	**F**	S	A	I	Y	G	Y	N	R3+
Lars-37	V	**S**	S	**A**	Y	S	A	I	Y	G	**H**	N	R5a

[a] primers used did not extend to cover further areas of the *CYP51* gene and therefore, the S524T not detected
[b] bold letters represent changes
[c] deletion of amino acid
* R group classification correct when research undertaken in 2009

5.4.3 Fitness costs

5.4.3.1 Pathogenicity

The results illustrating the pathogenicity of 18 *M. graminicola* isolates, shown in Figure 5.2, revealed that there were variable pathogenicities between isolates. The most pathogenic isolate was Tibb-2 (R8 with the diseased leaf area of 66.33%. However, isolates Ctrl-1, Ger-3-2, Nuf-Un-2, Pittend, and King-Pz-2, although slightly less pathogenic than Tibb-2, did not differ significantly in their pathogenicity when compared to the former. The rest of the isolates, however, showed lower pathogenicity, regardless of the existence of changes and deletions in their *CYP51* gene sequences.

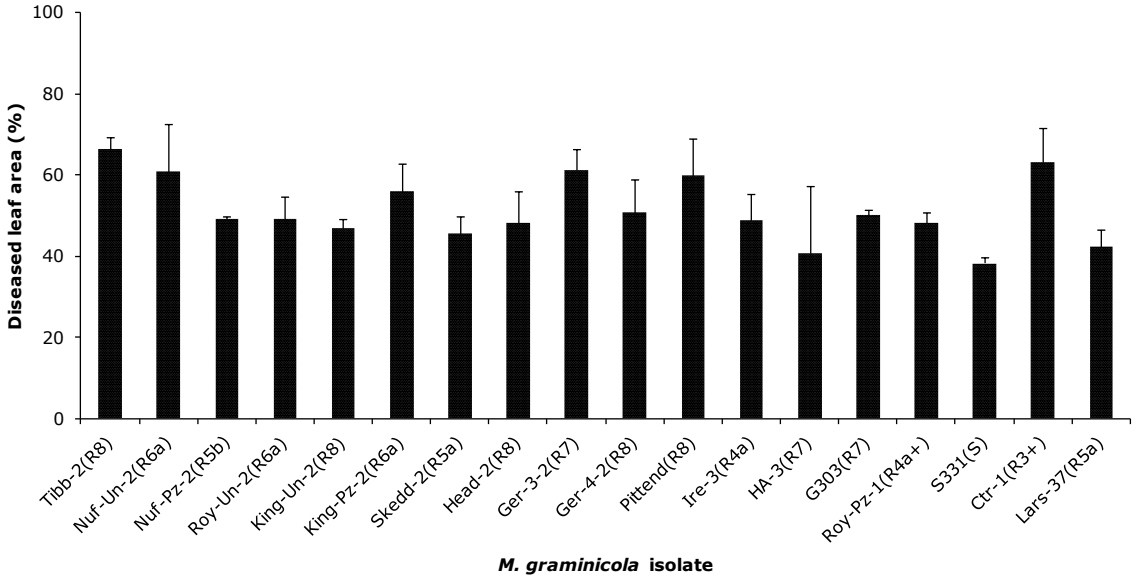

Figure 5.2 Pathogenicity of 18 *M. graminicola* isolates performed in a controlled environment condition. Error bars are standard deviations.

5.4.3.2 Growth rate

After 16 d of incubation, the mean growth rate per day was calculated for isolates of *M. graminicola* with different mutations or alterations. The results showed that mycelial growth rate varied considerably among isolates. Isolates

representing genotypes R8 (Tibb-2 and Ger-4-2) and R6a (Nuf-Un-2 and Roy-Un-2) grew at average rates of 1.30, 1.15, 1.14 and 1.05 mm d^{-1}, respectively, whilst isolates representing genotypes R7+ (Head-2), R5 (Lars-37) and R7 (Ger-3-2 and G303) had significantly slower growth ($P < 0.001$) with average growth rates 0.48, 0.55, 0.61 and 0.62 mm d^{-1}, respectively (Figure 5.3). Thus, growth rates were found to vary between isolates from the same category indicating no particular pattern related to phenotypic growth-rate differences in relation to *CYP51* alterations.

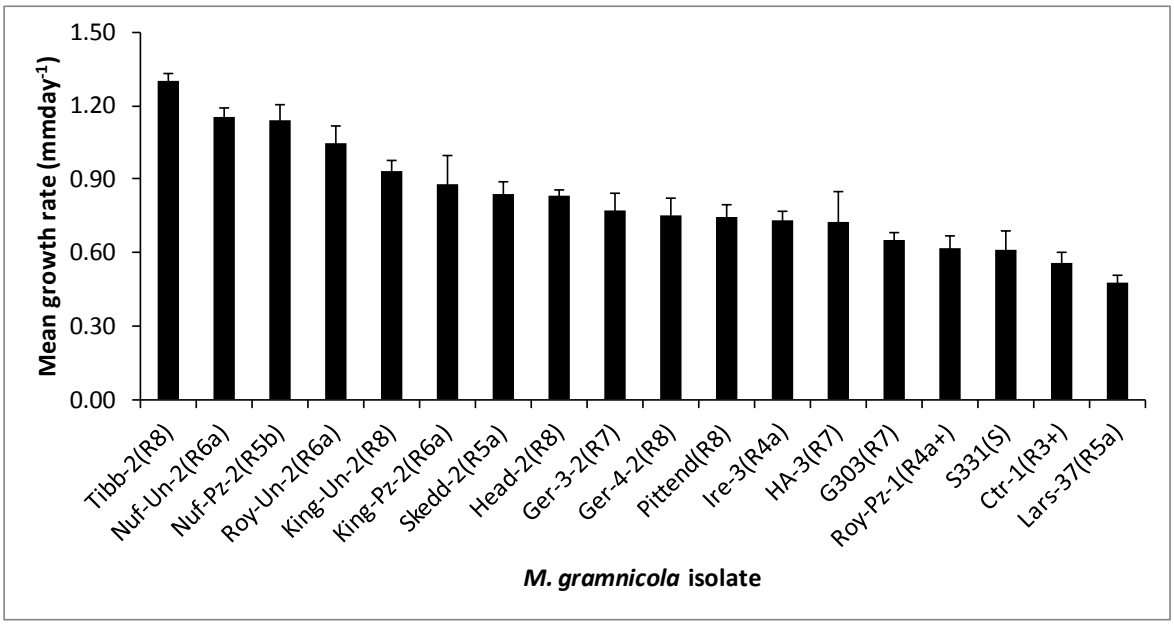

Figure 5.3 Average growth rates of *M. graminicola* grown on PDA. Each value is the average of four individual plates per isolate, error bars are standard deviations.

5.5 Discussion

The direct isolation method, using infected leaf segments, was successful for isolating *M. graminicola*. A continuous wet period at a temperature of approximately 20°C was found to the conducive for production of pycnidia. After 5-7 d, pinkish-orange, yeast-like colonies developed and from each single colony an isolate was produced. Earlier research by Eyal *et al*. (1987) also used a similar technique, where they stated the necessity of both

moisture and an optimum temperature of 18-20°C. However, they stated that the incubation time required for conidia production ranged from 7-10 d. Production of yeast-like spores on agar media was also reported by Stammler *et al*. (2008c) and found to be more practical for glasshouse studies.

The results of screening 18 *M. graminicola* isolates revealed that *CYP51* mutations are widespread across the UK as well as in German populations of *M. graminicola*. This was previously suggested by many authors in recent European populations of *M. graminicola* (Cools, 2007; Brunner *et al.*, 2008; Cools and Fraaije, 2008). In this study, screening of point mutations in the *CYP51* gene, revealed the existence of 9 genotypes (variants) of strains (S, R3+, R4a, R4a+, R5a, R5b, R6a, R7 and R8 displaying different sensitivities to DMIs. Previous studies have confirmed the presence of up to 1-12 different sub-populations that respond differently to different triazoles (Leroux *et al.*, 2006; Leroux *et al.*, 2007; Leroux *et al.*, 2008c; Stammler *et al.*, 2008a; Leroux and Walker, 2011). Other research groups, including the Rothamsted group led by Fraaije, however, do not agree with this R-group classification as it is based on multiple, unrelated parameters.

The results of current research have shown the possibility of 15 different alterations (substitutions or deletions) in the *CYP51* gene in positions from 24 to 513 (Table 5.3). Earlier work by Leroux *et al*. (2007) showed 16 different mutations and deletions in the same range of sequence and at the same positions. However, it would appear that mutations in the *CYP51* gene represent a continuous process which has continued over last 20 years. Since the process began new changes have emerged from year to year. To date, more than 20 different combinations of mutations have been detected and the trend continues to increase (Clark *et al.*, 2010). In the current study two new alterations, V24D and S51T (both in isolate S331) have been detected and their effects on the sensitivity of *M. graminicola* to DMIs is not known. Interestingly, within the population of isolates tested, it was also found that substitution Y137F was present in only one isolate (Ctrl-1), an older isolate, which was isolated in 2001 and donated much later to Dr Rossall, as a triazole-sensitive strain, by the Rothamsted research group. This finding was also supported by Leroux *et al*. (2007) where they stated that Y137F is rare or

even absent in modern *M. graminicola* populations. It has been suggested that isolates carrying Y137F are less sensitive to triadimenol, an azole fungicide introduced in the late 1970s and now no longer used for *M. graminicola* control. The substitution from isoleucine to valine at position 381 was also detected frequently. This was previously found to be unique to *M. graminicola* (Fraaije *et al.*, 2007) and is still the predominant substitution in Western Europe (Stammler *et al.*, 2008a). Furthermore, sequence results showed the high level of I381V genotypes (9 out of 12 of 2008 isolates, 75%) in samples screened. This is in agreement with that of Selim (2009) who observed a high frequency of I381V genotypes (70%) in samples screened *in planta* using allele-specific q-PCR. Similarly, Fraaije *et al*. (2007) found the prevalence of the I381V mutation in the *CYP51* gene in populations of *M. graminicola* and they added that this frequency increased from 40% in 2004 to 67% in 2006. Similar to these findings, Chassot *et al*. (2008) also confirmed the occurrence of a significant change in *M. graminicola* genotype composition over the last 2 decades; where wild type isolates disappeared while genotypes R3 to R6 predominated. However, the recently-emerged *CYP51* genotypes, carrying combinations of mutations D134G, V136A, Y461S, and S524T, revealed a substantial impact on sensitivity to the most widely-used triazoles, which include epoxiconazole and prothioconazole (Cools *et al.*, 2011). However, in this current study the primers used to amplify the *CYP51* gene did not extend to cover the 524 position of the gene and it is therefore not known whether this change exists in isolates that were screened for mutations in this work. With hindsight, use of more extensive primers to detect other mutations would have been beneficial to this work. The primers used were those which had been utilised previously by Leroux group (Leroux *et al.*, 2007).

Previous studies found four residues altered in *M. graminicola* isolates in regions predicted to impact on substrate/inhibitor recognition (Cools and Fraaije, 2008) with other alterations at non-conserved residues implicated in reduced azole sensitivity. In agreement with this, biological data obtained by Lepesheva and Waterman (2004) has demonstrated a clear relationship between substitutions in putative substrate recognition sites (SRSs), SRS-1 (V136A/C and Y137F) and SRS-5 (A379G and I381V) associated with isolate azole sensitivity. Therefore alterations at non-conserved residues are likely to

be compensatory, required to maintain enzyme activity when residues important for function are changed. In response to this, particular amino acid changes only occur consecutively, as A379G is only found in isolates carrying the I381V substitution. Some alterations are never found in combination such as V136A and I381V. This is in agreement with the results presented here and supports the same concept that was observed in the results obtained in screening all *M. graminicola* isolates. However, an exception to this rule was found by Stammler *et al*. (2008a), who found a UK isolate which had the V136A mutation, combined with I381V, Y461H and the new D134G mutation. Recently, Leroux and Walker (2011) have also found the V136A mutation combined with I381V in isolates of *M. graminicola* collected in 2009 in the UK and France.

The data on pathogenicity has revealed that there was no correlation between alterations in the *CYP51* gene and pathogenicity. High virulence was found in isolates within R8 genotypes (Tibb-2 and Pittend.), R7 (Ger-3-2) or R6a (Nuf-Un-2). Other isolates with different R-types, including R6a, R7 and R8, exhibited lower pathogenicity. It can be concluded that the pathogenicity of isolates of *M. graminicola* was not compromised by alterations or mutations in the *CYP51* gene. In agreement with these results, Stammler *et al*. (2008c) did not detect any changes in the pathogenicity, under glasshouse conditions, between isolates collected before and after 2000, irrespective of the presence of QoI resistance or not. Previous research on other diseases such as *P. teres*, net blotch of barley, undertaken by Peever and Milgroom (1994), did not detect any fitness costs associated with resistant to other triazoles, triadimenol and propiconazole, and they concluded that management of DMI resistance cannot depend on the existence of fitness costs. Nikou *et al*. (2009) also found no fitness penalties associated with resistance mutations in the highly triazole-resistant phenotypes of *Cercospora beticola*, the causal agent of Cercospora leaf spot disease of sugar beet, and most isolates retained their resistance levels even after four generations on fungicide free medium. Fitness costs associated with mycelial growth rates also confirmed no particular pattern related to *CYP51* mutations. Large differences in growth rates were detected within the same genotype category. For instance isolates belonging to genotype R8 were distributed among categories with the highest, intermediate and lowest growth rates.

Chapter 6 Fungicide performance associated with *CYP51* mutations

6.1 Introduction

Mycosphaerella graminicola, the causal agent of septoria leaf blotch in wheat, is considered the main constraint in wheat production in many European countries and also in many countries outside Europe (Eyal, 1999; Hardwick *et al.*, 2001; Palmer and Skinner, 2002). Owing to the lack of highly resistant cultivars, the application of fungicides is currently the major measure in disease management. Several fungicide families have been used to control *M. graminicola*, and within these, sterol 14α-demethylation inhibitors have been the key components for 3 decades (Bayles, 1999). After the confirmation of the existence of widespread QoI resistance within *M. graminicola* populations (Fraaije *et al.*, 2005), reduction in sensitivity towards DMI fungicides has emerged. Extensive European-wide monitoring studies have shown a shift towards lower sensitivity at the beginning of the 2000s. However, although this shift had been thought to have stabilised (Leadbeater and Gisi, 2009), further evolution of insensitive genotypes has been detected since 2008. Several European studies have also shown the significant shifts in the sensitivity of *M. graminicola* populations to this group of fungicides in the last 20 years (Leroux *et al.*, 2007). Mutations in the *CYP51* gene have been shown to confer resistance to azoles, although generally in combination with other mechanisms (Perea *et al.*, 2001). Studies conducted recently confirmed the importance of these alterations in development of azole resistance (Fraaije *et al.*, 2007; Leroux *et al.*, 2007).

6.2 Fungicides bioassays

6.2.1 *In vitro* assays

Many sensitivity test methods have been used to ascertain the shift of DMI sensitivity. Microtitre assays using plate readers is one of the methods used by many researchers to evaluate the *in vitro* sensitivity of *M. graminicola* isolates towards DMIs and other fungicide groups. Flat-bottomed microtitre plates were used by Fraaije *et al*. (2007) to evaluate epoxiconazole, tebuconazole, prochloraz and azoxystrobin. In the method they used 100 µL of Czapek Dox

liquid medium amended with 11 fungicide concentrations (3x geometry); aliquots of 100 µL of conidial suspensions (10^5 conidia mL^{-1}) of *M. graminicola* were then added to each well. Plates were then incubated for 4 days at 23°C, and growth measured by a plate reader at 630 nm. From the data obtained, EC_{50} values were calculated using a dose response relationship. A microtitre assay, using different epoxiconazole concentrations, was also used by Stammler *et al*. (2008a), where they used YBG-medium (1% yeast extract, 1% bacto peptone, 2% glycerol). The medium in each well was then inoculated with approximately 1000 conidia and incubated for 6 d at 18°C before evaluation of the growth using a photometer (405 nm). ED_{50} values were calculated by probit analysis. A similar microtitre method was used, but with the addition of the metabolic activity indicator Alamar Blue, to evaluate the sensitivity of many human and plant pathogenic agents. It was used with *Saccharomyces cerevisiae* (Fai and Grant, 2009) to evaluate a range of toxicants, with the human pathogenic bacterium *Staphylococcus epidermics* (Pettit *et al.*, 2005; Pettit *et al.*, 2009), with filamentous fungi (EspinelIngroff *et al.*, 1997), with plant pathogenic fungi, such as *Botrytis cinerea*, (Pelloux-Prayer *et al.*, 1998) and to evaluate the sensitivity of *M. graminicola* isolates towards QoIs (Siah *et al.*, 2010).

Measuring mycelium growth on agar media is another conventional *in vitro* method used by many authors. It was used to assess DMI activity against isolates of *Monilinia fructicola* (Schnabel *et al.*, 2004), to evaluate carbendazim performance against *Botrytis allii* (Viljanen-Rollinson *et al.*, 2007), and for *Septoria tritici* (Tvaruzek *et al.*, 2005). However, an alternative *in vitro* method based on germ tube elongation was used by Leroux *et al*. (2007), where they stated that the method was more accurate than other methods such as microtitre techniques.

6.2.2 *In planta* fungicide activity

6.2.2.1 Visual fungicide assessment

In vitro assays may give an indication of the performance of a fungicide and the existence of resistant isolates, but may not reflect performance *in planta*. Therefore it is also necessary to ascertain fungicide performance either in field trials or in controlled environment tests. Many such investigations have been undertaken. Different groups of fungicides have been applied against many

cereal diseases to assess their efficacy with or without the existence of resistant isolates within fungal populations. A field trial was performed by Guo *et al*. (2007) to assess the activity of 3 mixed fungicides, comprising QoIs and triazoles, in 2004 in Germany against *M. graminicola* isolates. In field experiments the effects of a range of QoI fungicides, in combination with the DMI epoxiconazole, or with chlorothalonil, were assessed by McCartney *et al.* (2007) in Northern Ireland in 2004 and 2005 using the winter wheat cultivars Robigus and Savannah, partially resistant and moderately susceptible respectively to STB.

Mixtures based on azoxystrobin were used by Maliniski (2004) for control of some winter wheat diseases. He found that the mixture of azoxystrobin and propiconazole was most effective for control of powdery mildew. Application of strobilurin fungicides independently, distinctly suppressed tan spot, while control of eyespot was maintained by carbendazim. QoI and DMI fungicides were also evaluated by Schurch *et al.* (2009) to determine the resistance levels in *M. graminicola* in 2008 for samples obtained from 17 fields in Switzerland. They found that the Swiss population is, on average, more sensitive to DMI fungicides compared to other European populations of this pathogen. The field performance of epoxiconazole in relation to the existence of *CYP51* mutations was evaluated by Stammler *et al.* (2008a); they found a limited influence of *CYP51* haplotypes on the sensitivity of 615 isolates from different European regions.

6.2.2.2 Quantitative fungicide assessment using q-PCR

Disease assessment is essential in plant pathology. Conventional methods tend to be time consuming and the results obtained might not always reflect the true extent of pathogen invasion. PCR-based methods are able to overcome the difficulties mentioned above. Additionally, PCR methods enable detection of pathogens in plant tissues before visible symptoms can be detected (Henson and French, 1993). Other advantages over traditional diagnostic methods include they are more precise, faster and can be used with a little experience of plant pathology. The methods currently are widely applied for early diagnosis and disease assessment of many plant diseases (Schena *et al.*, 2004). Real-time or quantitative PCR (q-PCR) was used

successfully by Adhikari *et al.* (2004a) to measure the amount of *M. graminicola* in inoculated resistant and susceptible wheat cultivars. They found that q-PCR was a valuable tool for discriminating between septoria-resistant and susceptible lines of wheat. Fraaije *et al.* (2002) used q-PCR in combination with visual assessment to identify factors involved in the onset and extent of disease development in a study investigating the effect of the crop height on the epidemics of *S. tritici* and *Stagnospora nodorum* (wheat glume blotch). Furthermore, q-PCR can be used for detection and quantification of fungal foliar pathogens, in resistance screening to measure the interaction between different pathogens and their hosts at different growth stages, and in specific tissues of wheat plants (Fraaije *et al.*, 2001). Quantitative PCR assays as an effective pre-symptomatic tool to diagnose *M. graminicola* at the very beginning stage of infection is desirable for monitoring the disease progression in infected wheat plants. In this regard, Guo *et al.* (2006) achieved immediate detection after inoculation and monitored the steady increase of *M. graminicola* in wheat before visible symptoms appeared. Much research has now focused on such alternative methods for assessment of fungicide activity in disease control. Quantitative PCR and visual monitoring of *M. graminicola* epidemics were performed to investigate the effect of curative and preventative applications of azoxystrobin in wheat field crops by Rohel *et al*. (2002). They found that azoxystrobin activity toward *M. graminicola* mainly resides in lengthening the time interval between the earliest PCR detection and the measurement of 10% necrotic leaf area. In another study by Guo *et al.* (2007) a q-PCR assay was applied to evaluate the effects of two fungicide treatments on *M. graminicola* leaf blotch in the field compared with two traditional assessments. The results showed the superiority of the quantitative assay over traditional visual assessment and also over those PCR assays estimating DNA input with end-point measurement.

6.3 Aim of the research

The aim of this research was to assess different triazole fungicides *in vitro* in relation to multiple changes in the *CYP51* gene within a group of *M. graminicola* isolates. The *in vitro* bioassays included using microtitre method and measurement of the apical growth of conidia on fungicide amended agar medium. The fungicides were also evaluated *in planta* and the disease

occurrence was measured visually and quantitatively using q-PCR. Finally, correlations were determined between visual and quantitative assessments of fungicide activities.

6.4 Methods

6.4.1 *In vitro* fungicide activity

6.4.1.1 Microtitre plate without growth indicator

Mycosphaerella graminicola isolates maintained as glycerol stocks at -80°C were grown on PDA amended with anti-bacterial antibiotics. After 5-7 d of incubation, spore suspensions were made and adjusted to 10^6 conidia mL^{-1}. To prevent spore germination during the work spore suspensions were kept on ice. Potato dextrose broth (PDB) was prepared and sterilised then amended with antibiotics to prevent bacterial contamination. Eleven different fungicide concentrations were made in PDB (2-fold fungicide dilutions) which were: 50, 25, 12.5. 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.098, 0.049 µg mL^{-1}. Each fungicide concentration represented one column of 96 well microtitre plates and the last column was left as a fungicide-free control. Aliquots (150 µL) of each fungicide concentration were added to each well of the microtitre plate. Fifty microlitres of spore suspension, which has a final concentration of 2.5 x 10^4 spores mL^{-1}, were then added to the wells that contain the fungicide concentrations. An 8-tipped multichannel pipette was used to deliver the amended medium and the spore suspensions. For each fungicide the plate was replicated three times. The fungicides tested are described in Table 6.1.

The lids of inoculated plates were closed and sealed to avoid evaporation and then incubated in the dark at 20°C for 72 h after which the optical densities were measured at 550 nm using a plate reader (Microplate Manager, Version 5.2.1, Bio-Rad Laboratories, UK). The absorbance data were saved as Excel data sheets and used to detect dose response regression curves, using Sigma plot Version 10, from which the EC_{50} value of each isolate was then calculated.

Data were also obtained using technical, non-formulated samples of pure fungicide active ingredients (tebuconazole and prochloraz) using the micro-

titre plate assay. This work was kindly undertaken by Dr Bart Fraaije's group at Rothamsted Research. The method used was as described by Mullins *et al.* (2011).

Table 6.1 Fungicides used in *in vitro* and *in planta* bioassays with *M. graminicola* isolates.

Product name	Active ingredient	Concentration (g L^{-1})	Chemical class	Field application rate (L ha^{-1})
Folicur	Tebuconazole	250	Triazole	1
Warbler	Prochloraz	400	Imidazole	1.25
Proline	Prothioconazole	250	Triazole	0.8
Opus	Epoxiconazole	125	Triazole	1
Joules	Chlorothalonil	500	Chloronitriles	2
Fandango	Prothioconazole + fluoxastrobin	100 + 100	Triazole + QoI	1.5
Tracker	Boscalid + epoxiconazole	233	SDHI + triazole	1.5
Prosaro	Prothioconazole + tebuconazole	250	Triazole + triazole	1.2
Novel SDHI	Penthiopyrad	200	SDHI	1.5

6.4.1.2 Microtitre plate with growth indicator

An alternative microtitre method using a fluorometric dye, Alamar Blue (AB), (Trek Diagnostic systems Ltd, UK) was used as a growth indicator. Use of AB was attempted to determine the growth of the fungus in fungicide amended liquid medium. Before undertaking experiments, the assay needed determination of standard conditions for optimum growth of the microorganism and for activity of the growth indicator. For these purposes, several buffers were tested with 2 liquid media by incubating different conidia concentrations in the presence of AB. The aim was to find out the lowest pH suitable for the growth of *S. tritici* and at the same time maintain the blue colour of AB. Preliminary results of this optimisation found that Czapek-Dox medium in a sodium phosphate buffer (pH value 6.91), with a conidial concentration of 1.6×10^6 conidia mL^{-1}, and 72 h incubation were the optimum conditions. The fluorometric method was carried out in 96 well plates. One hundred microlitres of double concentrated medium were placed in each well

then 80 µL of spore suspension was added, with three replicates. The final row, was with no spores and had 80 µL of water only added, thus providing a negative control which included medium and AB only; the positive control consisted of medium, AB and inoculum. AB (20 µL) added to all wells at a concentration of 10% based on previous studies and the manufacturer's recommendation. The final volume in the wells therefore became 200 µL. After a gentle shaking by hand to mix the dye, the plates were incubated in the dark at 20°C. After 72 h incubation, absorbance was measured at 570 and 600 nm using the plate reader, following the instructions of the manufacturer of AB. The calculations to determine percentage reduction were made using the following formula:

$$\text{Percentage reduction} = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where:
O1 = molar extinction coefficient (E) of oxidised AB (Blue) at 570 nm
O2 = E of oxidised AB at 600 nm
R1 = E of reduced AB (Red) at 570 nm
R2 = E of reduced AB at 600 nm
A1 = absorbance of test wells at 570 nm
A2 = absorbance of test wells at 600 nm
N1 = absorbance of negative control well (media plus AB but no spores) at 570 nm
N2 = absorbance of negative control well (media plus AB but no spores) at 600 nm

6.4.1.3 *In vitro*-measuring apical growth

The activity of fungicides against germ-tube elongation was performed as an alternative method to measure the EC_{50}. The method was modified from the method used by Leroux *et al*. (2007), where the solid medium was prepared from glucose 10, K_2HPO_4 2, KH_2PO_4 2 and agar 12.5 g L^{-1}, autoclaved, and then amended with fungicides at 50°C. For each fungicide, 10 concentrations were tested; starting from 100 mg L^{-1} as the highest concentration to 0.0051 mgL^{-1} as the lowest concentration (geometric progression X3), including plates with no fungicides as untreated controls. Media amended with fungicides were homogenized and then poured into 9 cm plastic Petri dishes. After solidification

of the media, 250 µL of conidia suspensions (2 x 10^5 conidia mL^{-1}) were pipetted on to the surface of the agar plates and spread with the sterilised plastic spreaders (Sterilin, Staffordshire, UK). The assay was a complete randomised design and repeated 3 times. After incubation for 48 h at 20°C in the dark, the lengths of apical germ-tubes (30 for each treatment) were measured (Figure 6.1) under a microscope using a micrometre (Graticules Ltd, Stonebridge, Kent, UK). The concentration causing 50% reduction in the germ-tube elongation (EC_{50}) was determined by linear regression of the germ-tube lengths (a percentage of control) against the log of fungicide concentration. To do this SPSS v16 was used to find probits at a 5% confidence limit. Finally the average resistance factors (RF) were estimated as ratios: EC_{50} of resistant phenotype / EC_{50} of sensitive phenotype. This assay was repeated using technical grade, non-formulated tebuconazole for comparison with the formulated commercial product.

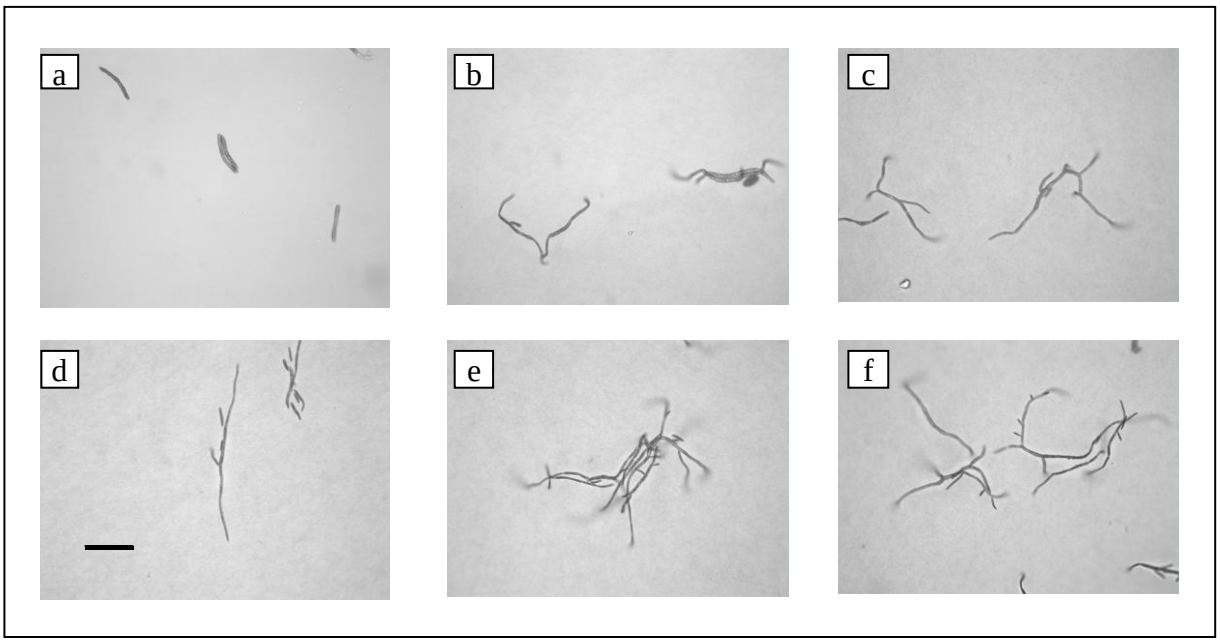

Figure 6.1 Conidial apical growth of *M. graminicola*, isolate G303, in epoxiconazole-amended agar medium, at concentrations a) 100, b) 11.11, c) 1.23, d) 0.14, e) 0.015 and f) 0.00 mg mL^{-1}. Scale bar = 40 µm (all images).

6.4.2 *In planta* fungicide activity

6.4.2.1 Visual disease assessment

The *Mycosphaerella*-susceptible wheat cultivar Riband was grown in 13 cm pots at a density of 10 plants per pot. Ten days after emergence, at growth stage 12 (Zadoks *et al.*, 1974), the plants were sprayed with fungicides as a protective spray. Three pots were used for each fungicide treatment. The fungicide generic name, common name and chemical class are presented in Table 6.1, with the active ingredient concentration and the full field application rate. Two days later, the plants were inoculated with 1 x 10^6 conidia mL^{-1} until run-off, covered with plastic bags for 48 h and then incubated in a controlled environment room at day temperature of 20°C and night temperature of 12°C with the photoperiod of 16 h. To maintain a high humidity, a layer of water was placed in the bottom of trays. Control plants were treated the same as experimental plants but without fungicide application (water only). After 21 days, the disease incidence was assessed visually for percentage diseased area with *M. graminicola* lesions and then the leaves were dried at room temperature and stored prior to DNA extraction. Data were manipulated to the percentage of fungicide efficacy (% of disease control) relative to the untreated control for each treatment using the following formula:

$$\% \text{ disease control} = \frac{\text{Disease degree of untreated control} - \text{disease degree of treated}}{\text{Disease degree of untreated control}} \times 100$$

6.4.2.2 Quantitative fungicide assessment using q-PCR

A q-PCR assay was used to assess the fungicide activity *in planta* and compared to the traditional visual assessment. Dried leaves were placed in liquid nitrogen and then ground to powder with mortar and pestle. The ground plant material (around 5 g) was mixed well and then 100 mg was taken for DNA extraction. DNA was extracted using a Plant Mini kit (QiaGen) following the manufacture's protocol. Specific primers (Table 6.2) for *M. graminicola*

were designed from *CYP51* gene sequence using Primer 3 software and were checked for their specificity. The samples were also checked with standard PCR for confirmation of existence of plant and fungal DNA in extracted DNA.

Plant DNA of unknown samples was adjusted to 10 ng uL^{-1} before being used for q-PCR. The 96 well plates with transparent seals were used for running q-PCR. For the construction of standards, twofold dilutions were prepared from a stock of 10 ng uL^{-1} of pure genomic DNA. Test plates were loaded first to validate and optimise the standards, primer concentrations and conditions of q-PCR.

The 25 µL mixture contained 12.5 µL 2x SYBER Green JumpStart Taq Ready Mix (Sigma), 0.4 µM of each primer, 2.5 µL of template and water to volume of 25 µL. Real-time PCR was performed using a light cycler system (BioRad-IQ5 multicolour Real-Time PCR Detection System) used according to the manufacturer's instructions. Thermal cycling conditions were: 2 min at 94°C, 40 cycles of 15 s at 94°C, 56 s at 56°C, 50 s at 60 and 1 min at 55°C. All tests were performed in duplicate in the same run. DNA amounts of *M. graminicola* were calculated using the regression equation of standard curves. Data were first analysed using the LightCycler analysis software. For the identification of target PCR product and non-specific products, such as primer dimers, a melting curve analysis was used. Other calculations and analysis were performed using Microsoft Excel 2007 and for the statistical analysis, such as ANOVA and comparisons, SPSS was used.

Table 6.2 *M. graminicola* primers used in q-PCR assessment of fungicide activity.

Primer name[a]	Priming direction	Sequence (5'-3')	Product length	Tm°C
Steu-2-F	Forward	GCCAACCTCTCGAAACTCAC	20	59.4
Steu-2-R	Reverse	GCATGGGAGACTTGAGGTTG	20	59.4

[a] Primers designed from *CYP51* gene sequence of *M. graminicola*

6.5 Results

6.5.1 *In vitro* fungicide activity

6.5.1.1 Microtitre plate without growth indicator

The results from microtitre-based methods, where liquid medium amended with fungicide concentrations without growth indicator, which were used to detect the *in vitro* activity of fungicides against *M. graminicola* strains, revealed the occurrence of contamination causing higher absorbance, which led to misleading and unreliable data that were not representative of reality. This may reflect contamination associated with the commercial fungicide products used in this work. Data for each treatment or single concentration were found to be variable, indicated by high standard deviations from the growth averages. Furthermore, in some cases, the values for untreated controls were higher than the values from treatments. The method was therefore abandoned. Subsequently, this work was repeated by Dr Bart Fraaije's group at Rothamsted Research, using technical, non-formulated samples of the fungicides tebuconazole and prochloraz. Results from this evaluation, in comparison to obtained data using a germ-tube extension assay (for formulated and non-formulated tebuconazole) is given in Table 6.4.

6.5.1.2 Microtitre plate with growth indicator

The second microtitre method, using a colorimetric technique, did not function with azole fungicides, although it had been used before with QoIs for detecting insensitive *M. graminicola* isolates possessing the G143A mutation. This may reflect the association between Alamar Blue and metabolic processes which are energy-dependent; triazoles affect membrane integrity and may not have an effect on respiration, which QoIs clearly inhibit. Similar to the microtitre method without growth indicator, the same problem of contamination and data with high standard deviations also occurred. This led to the abandonment of the method and a search for an alternative.

6.5.1.3 *In vitro*-measuring apical growth

The sensitivity of 18 *M. graminicola* isolates was tested against triazoles, mixtures of triazoles and a new SDHI, penthiopyrad. As a general observation,

the results in Table 6.3 shows that most isolates (9 out of 11) having I381V mutations (R6a, R7 and R8) exhibited higher EC_{50}s to tebuconazole but lower EC_{50}s to prochloraz. Interestingly, isolates with the combination of alterations I381V and A379G (R8) showed high EC_{50} values towards tebuconazole and much lower EC_{50} towards prochloraz. However, isolates Nuf-Un-2 and Roy-Un-2 (both R6a), although having the I381V mutation, showed low and similar EC_{50}s to tebuconazole and prochloraz. The old isolate Ire-3 (R4a), showed high EC_{50} to tebuconazole and low values to prochloraz. The most sensitive isolate was S331 (S) showing very low EC_{50}s to all fungicides tested. Other old isolates, Ctrl-1 (R3+) and Lars-37 (R5a), exhibited a slightly higher EC_{50}s to tebuconazole compared to prochloraz. Notably, old isolates HA3 and G303 (both R7 genotype that have the I381V mutation) showed higher EC_{50} values against tebuconazole and low EC_{50}s to prochloraz.

In vitro toxicities of prothioconazole and epoxiconazole against isolates tested were variable regardless of the type of mutation. However, it is noticeable that isolate Nuf-Pz-2 which has combinations of alterations V136A, S188N, double deletion ΔY459/ΔG460 and N513K (R5b) exhibited high EC_{50} values 1.24 and 1.18 mg L^{-1} against prothioconazole and epoxiconazole respectively.

The results showed that the multisite fungicide chlorothalonil is highly effective, exhibiting low EC_{50} values against most isolates (EC_{50} from 0.03 to 0.34 mg L^{-1}). Likewise, Fandango, a mixture of triazole and QoI, also showed similar toxicity towards most isolates tested in the assay (EC_{50} values between 0.001 and 0.22 mg L^{-1}). The activity of Tracker (a mixture of boscalid and epoxiconazole) was variable. However, higher EC_{50} values were observed towards one isolate with resistant type R7 and most R8. In the case of Prosaro (mixture of prothioconazole and tebuconazole), relatively high EC_{50} values were found towards most of the isolates that included R5, R6, and R8. Penthiopyrad showed the highest toxicity for conidial germ tube growth by providing very low EC_{50} values, not exceeding 0.21 mg L^{-1} towards all isolates tested. Isolates characterised as S, R3+, and R4 gave low EC_{50} values towards triazoles, mixtures and other fungicides.

Table 6.3 EC_{50} values of *M. graminicola* isolates measured as germ tube elongation using an amended agar technique.

		Fungicide								
Isolate ID	R-Type	Tebuconazole	Prochloraz	Prothioconazole	Epoxiconazole	Chlorothalonil	Fandango	Tracker	Prosaro	Penthiopyrad
Tibb-2	R8	1.14	0.09	0.39	0.23	0.17	0.12	1.29	0.53	0.19
Nuf-Un-2	R6a	0.29	0.30	0.09	0.22	0.03	0.001	0.45	0.49	0.09
Nuf-Pz-2	R5b	0.38	0.05	1.24	1.18	0.04	0.001	0.57	1.63	0.05
Roy-Un-2	R6a	0.21	0.25	0.33	1.02	0.07	0.02	0.31	1.38	0.07
King-Un-2	R8	0.56	0.11	0.38	0.16	0.03	0.02	0.82	1.17	0.10
King-Pz-2	R6a	1.64	0.12	0.63	4.26	0.34	0.11	0.24	1.21	0.06
Skedd-2	R5a	0.25	0.20	0.27	0.47	0.11	0.04	0.23	0.39	0.07
Head-2	R8	0.89	0.48	0.37	0.77	0.15	0.22	1.12	1.08	0.21
Ger-3-2	R7	2.12	0.52	0.50	0.10	0.13	0.04	1.27	0.54	0.10
Ger4-2	R8	0.52	0.22	0.22	1.49	0.19	0.22	1.58	0.48	0.12
Pittend	R8	0.98	0.001	0.12	0.10	0.18	0.01	0.23	0.24	0.07
Ire-3	R4a	0.31	0.03	0.11	0.03	0.09	0.03	0.09	0.15	0.07
HA-3	R7	0.51	0.13	0.08	0.66	0.06	0.05	0.22	0.13	0.09
G303	R7	0.87	0.25	0.06	0.10	0.07	0.02	0.13	0.10	0.08
Roy-Pz-1	R4a+	0.22	0.04	0.02	0.39	0.05	0.04	0.13	0.22	0.07
S331	S	0.09	0.01	0.02	0.02	0.04	0.03	0.05	0.05	0.04
Ctrl-1	R3+	0.24	0.15	0.05	0.02	0.06	0.03	0.15	0.19	0.07
Lars-37	R5a	0.16	0.19	0.28	0.20	0.03	0.01	0.22	0.10	0.08

A comparison of the results obtained using the apical growth assay, done with commercial and technical grade tebuconazole, and those derived using a micro-titre plate assay (from Fraaije's group), using technical tebuconazole and prochloraz, is given in Table 6.4.

In general, the results obtained for tebuconazole using the micro-titre plate and apical germ tube growth assays followed a similar trend.

Table 6.4 A comparison between the apical growth assay and micro-titre plate assay in detecting EC_{50}.

Isolate	*CYP51* variant	Mutation	Germ-tube length assay		Microtitre plate assay (RES)	
			Tebuconazole (formulated)	Tebuconazole (tech.grade)	Tebuconazole (tech.grade)	Prochloraz (tech.grade)
Tibb-2	R8	L50S, S188N, A379G, I381V, Δ[a], N513K	1.14	1.06	3.50	0.0021
Nuf-Un-2	R6a	L50S, I381V, Y461H	0.29	0.31	2.80	0.0431
Nuf-Pz-2	R5b	L50S, V136A, S188N, Δ, N513K	0.38	0.41	0.040	0.150
Roy-Un-2	R6a	L50S, I381V, Y461H	0.21	0.47	Contaminated[b]	Contaminated
King-Un-2	R8	L50S, S188N, A379G, I381V, Δ, N513K	0.56	0.67	Contaminated	Contaminated
King-Pz-2	R6a	L50S, I381V, Y459S	1.64	1.04	1.77	0.0124
Skedd-2	R5a	L50S, V136A, Y461S	0.25	0.32	0.0339	0.0969
Head-2	R8	L50S, S188N, A379G, I381V, Δ, N513K	0.89	0.95	3.55	0.0015
Ger-3-2	R7	L50S, S188N, I381V, Δ, N513K	2.12	1.06	2.76	0.0324
Ger4-2	R8	L50S, S188N, A379G, I381V, Δ, N513K	0.52	0.66	3.91	0.0041
Pittend	R8	L50S, S188N, A379G, I381V, Δ, N513K	0.98	0.76	4.36	0.0003
Ire-3	R4a	L50S, Y461S	0.31	0.13	0.626	0.0274
HA-3	R7	L50S, S188N, I381V, Δ, N513K	0.51	0.43	1.90	0.0323
G303	R7	L50S, S188N, I381V, Δ, N513K	0.87	0.73	2.50	0.0638
Roy-Pz-1	R4a+	V136C, Y461H	0.22	0.27	3.86	0.0549
S331	S	V24D, S51T	0.09	0.05	0.0132	0.0010
Ctrl-1	R3+	Y137F, S524T	0.24	0.16	0.454	0.0874
Lars-37	R5a	L50S, V136A, Y461H	0.16	0.22	0.0241	0.0658
IPO323		wt			0.0695	0.0001

[a] Δ Deletions at positions 459 and 460

[b] Isolate culture contaminated with bacteria upon arrival at Rothamsted Research.

Insensitive variants could be detected with either assay. Three principal exceptions were, however, detected; Nuf-Pz-2, Skedd-2 and Lars-37. In these cases lower EC_{50} values were obtained using the micro-titre assay. Prochloraz gave low EC_{50} values for most isolates tested, suggesting this molecule could provide useful field efficacy. Relatively high EC_{50} values were obtained for a small number of isolates, principally those designated within the R5 grouping.

Resistance factors for each R-type were also calculated for fungicides and the results in Table 6.5 show that RFs for R3 isolate were generally low except for prochloraz, which exhibited a high value (RF=15). Similarly, the RFs for isolates of the R4 group were also low towards all fungicides, with the exception of epoxiconazole (RF=10.5). Both R5 and R6 isolates showed higher RF values towards prochloraz, prothioconazole, epoxiconazole and Prosaro. The R8 isolates exhibited high RF values towards prochloraz, prothioconazole, Tracker and Prosaro, while R7 isolates, in addition to having high RF values towards prochloraz, prothioconazole and Tracker, also showed high RF towards tebuconazole. RF values of all R-types towards Fandango and penthiopyrad were generally low; however the highest RF values were with R8 isolates.

Table 6.5 Detection of resistance factors of 6 R-types of *M. graminicola* towards fungicides including DMIs.

	Resistance factors[a]					
Fungicide	R3+	R4	R5	R6	R7	R8
Tebuconazole	2.7±0	2.9±0.7	2.9±1.2	7.9±8.9	13±9.4	9.1±3
Tebuconazole[b]	34.4±0	169.9±173.2	2.5±0.5	173.1±55.2	180.8±33.4	290.2±30
Prochloraz	15±0	3.5±0.7	14.7±8.4	22.3±9.3	30±20	18±18.5
Prochloraz[b]	87.4±0	41.2±19.5	104.2±42.6	27.8±21.7	42.8±18.2	2±1.6
Prothioconazole	2.5±0	3.3±3.2	29.8±27.9	17.5±13.5	14.3±16.2	14.8±6
Epoxiconazole	1.0±0	10.5±12.7	30.8±25.3	26.3±21.6	4.3±1.9	7.2±3.3
Fandango	1.0±0	1.2±0.2	0.6±0.7	1.5±1.9	1.2±0.5	3.9±3.4
Tracker	3.0±0	2.2±0.6	6.8±4	6.7±3.4	10.8±12.7	20.2±12.1
Prosaro	3.8±0	3.7±1	14.1±16.3	20.5±9.4	5.1±4.9	14±8.1
Penthiopyrad	1.8±0	1.8±0	1.7±0.4	1.8±0.4	2.3±0.3	3.5±1.5

[a]Resistant factors (calculated as ratios: EC_{50} of resistant genotype / EC_{50} of sensitive genotype) were from the average EC_{50} values from Table 6.3.
[b]RF values detected from EC50 values detected using microtitre plate assay from Table 6.4..

6.5.2 *In planta* fungicide activity

6.5.2.1 Visual disease assessment

Triazoles and other fungicides were assessed for their efficacy *in planta* as a protective application against *M. graminicola* (two days before inoculation). From the triazoles tested, the impact of tebuconazole on *M. graminicola* isolates was variable regardless of the type of mutation of the isolate (Figure 6.2). The disease control achieved by tebuconazole was significantly higher ($F_{(17, 36)} = 5.49$, $P<0.05$) on sensitive and low resistant isolates S331 (S), Nuf-Pz-2 (R5b), Ire-3 (R4a), Skedd-2 and Lars-37 (both R5a) and the R7 isolate Ger-3-2. However, other isolates belonging to R6a, R7 and R8 groups were less sensitive towards tebuconazole.

The activity of prochloraz *in planta* was significantly higher ($F_{(17, 36)} = 4.8$, $P<0.05$) against a wider range of isolates compared with that achieved by tebuconazole (Figure 6.3). Isolates Ire-3 (R4a), Roy-un-2 (R6a), Head-2 (R8) Skedd-2 (R5a), Ctrl-1 (R3+), Roy-Pz-1 (R4a+), and Ger-4-2 (R8) were sensitive to prochloraz. However, its performance was variably lower on other isolates with different resistant genotypes.

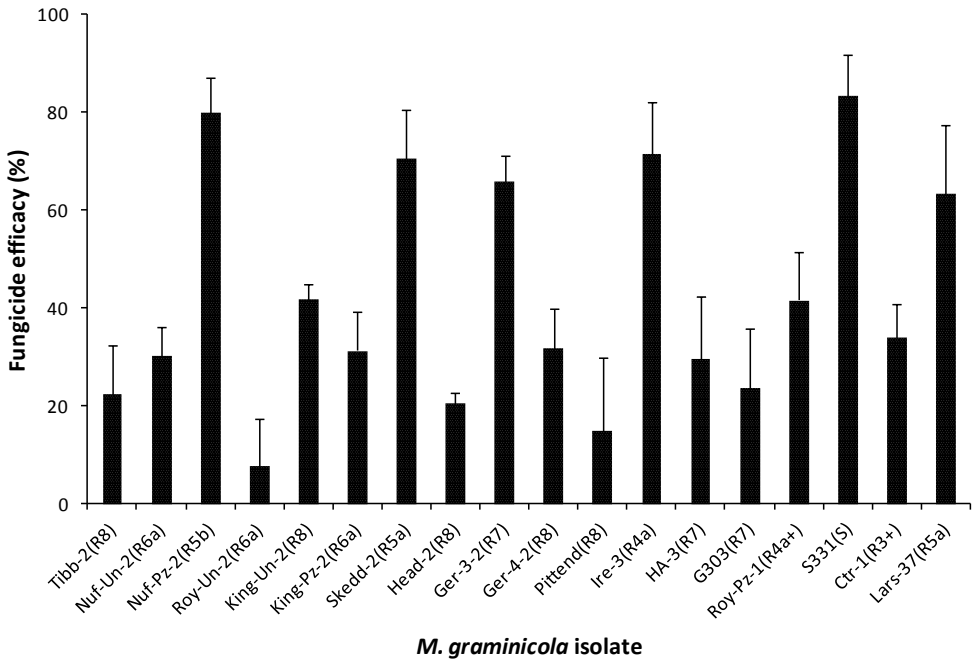

Figure 6.2 The *in planta* efficacy of tebuconazole towards *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

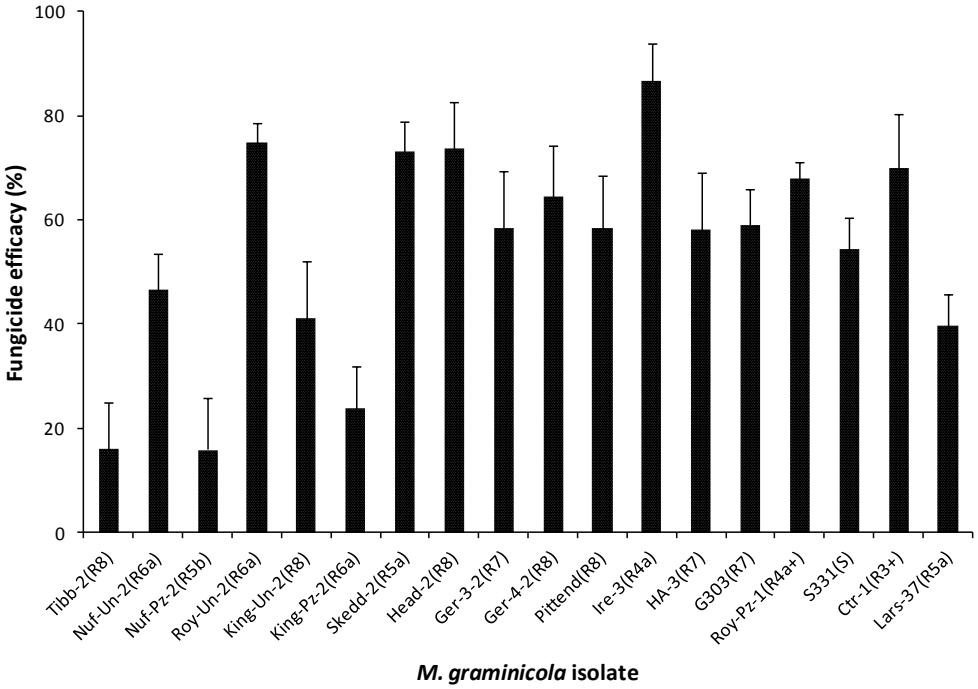

Figure 6.3 The *in planta* efficacy of prochloraz towards *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

The efficacy of prothioconazole was significantly higher ($F_{(17, 36)}$ = 8.05, P<0.05) towards isolates Ire-3 (R4a) and Ger-3-2 (R7). Nevertheless, its *in planta* activity on other isolates was variable and did not follow a specific pattern (Figure 6.4). It was noticed that the lowest efficacy was against isolates Roy-Un-2 (R6a) (3.42%) and Nuf-Pz-2 (R5b) (6.93%). The activity of epoxiconazole was significantly variable ($F_{(17, 36)}$ = 3.96, P<0.05). It was more effective against isolates S331 (S-type), Ctrl-1 (R3+), Ire-3 (R4a) and Roy-Pz-1 (R4a+) than isolates within the R7 group (HA-3, G303, and Ger-3-2) (Figure 6.5). However, it showed lower activity against isolates with R6a (Roy-Un-2) and R8 (Ger-4-2, Head-2 and Tibb-2) or with R5a (Lars-37).

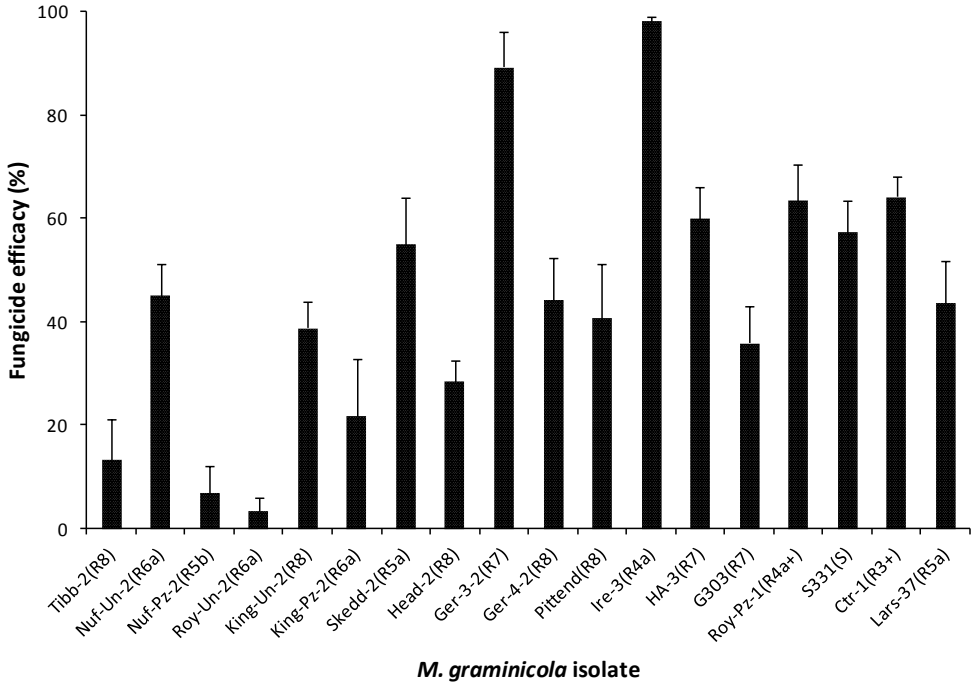

Figure 6.4 The *in planta* efficacy of prothioconazole towards *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

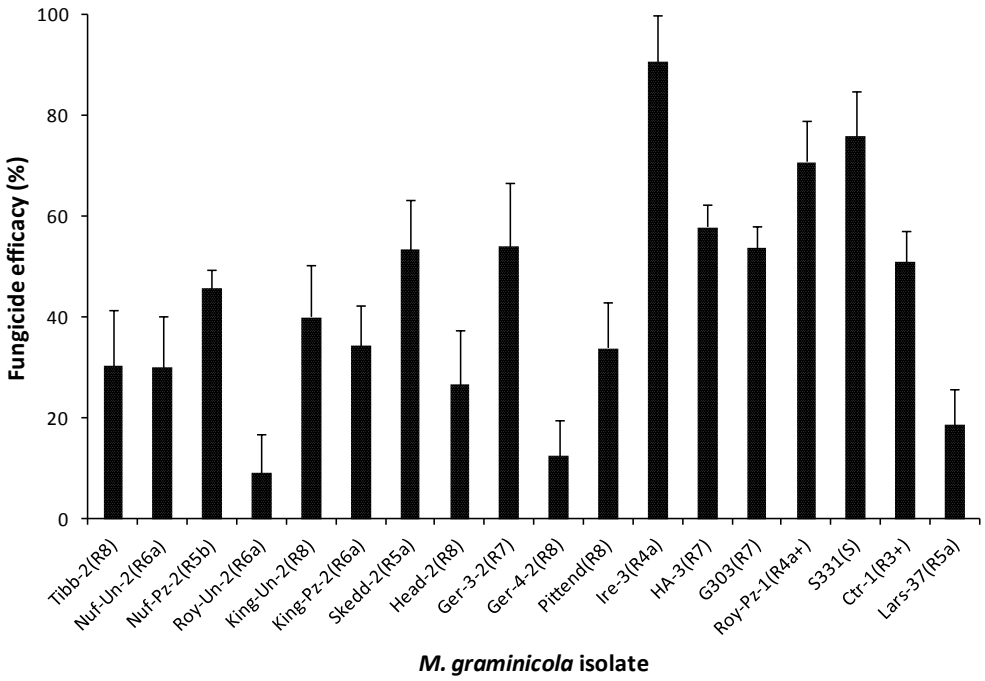

Figure 6.5 The *in planta* efficacy of epoxiconazole towards *M. graminicola* isolates with of *CYP51* mutations. Error bars represent standard deviation.

The efficacy of the multisite fungicide, chlorothalonil, was variable on *M. graminicola* isolates (Figure 6.6). It showed significantly higher activity ($F_{(17, 36)}$ = 4.77, P<0.05) against isolates Ger-3-2, HA-3, Lars-37, and Nuf-Un-2. Isolates G303, S331, Ire-3, Roy-Un-2, King-Un-2 and Ctrl-1 showed less sensitivity, while the remainder were the least sensitive towards this fungicide. Fandango, a mixture of a QoI and a triazole, exhibited high performance against 4 isolates namely Ire-3 (R4a), S331(S), Pittend (R8), and Ger-3-2 (R7). However, its activity on other isolates ranged from moderate to very low (Figure 6.7).

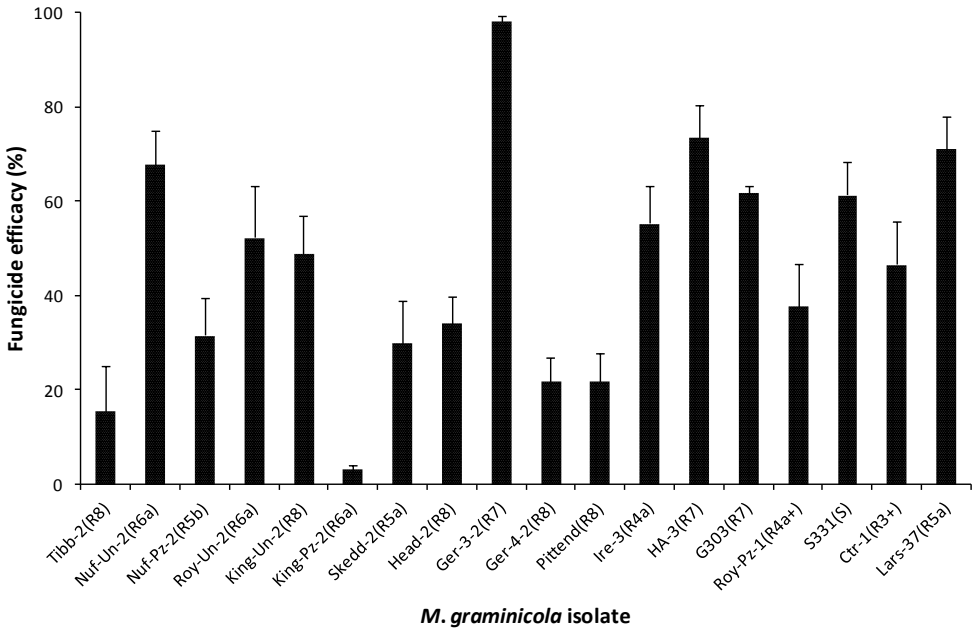

Figure 6.6 The *in planta* efficacy of chlorothalonil against *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

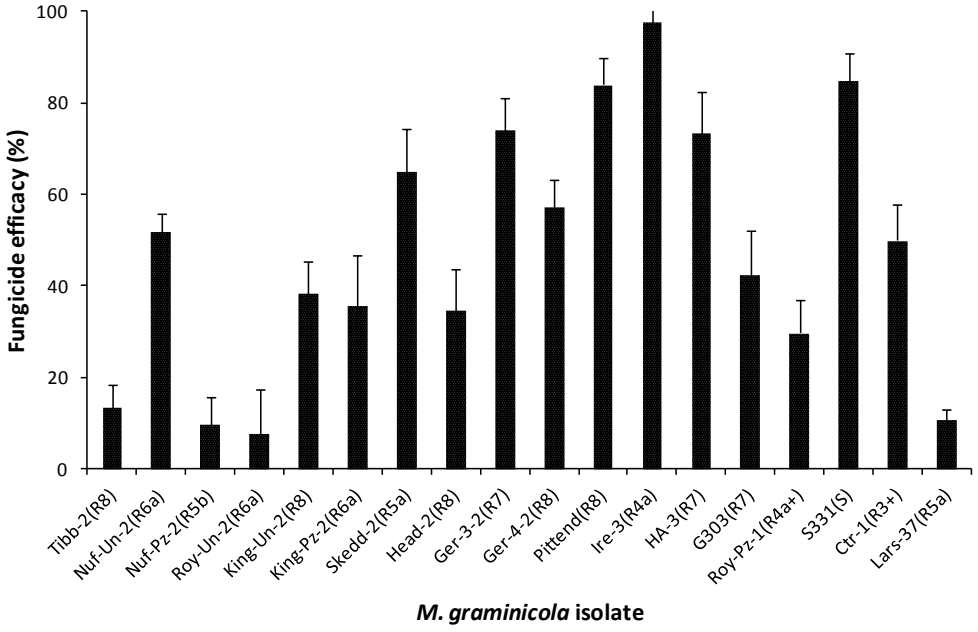

Figure 6.7 The *in planta* efficacy of Fandango against *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

A similar performance was observed with Tracker and Prosaro; both showed high activity against isolates Ire-3, S331, Ger-3-2 and HA-3 and low activity against isolates Roy-Un-2, Tibb-2, King-Pz-2 and Pittend (Figure 6.8 and Figure 6.9).

The activity of the novel SDHI (penthiopyrad), regardless of the existence of different mutations, was high against all *M. graminicola* isolates. The results shown in Figure 6.10 demonstrate that the lowest disease-control efficacy by this product was 89% against isolate Tibb-2, which was considered a high performance compared to the other fungicides tested.

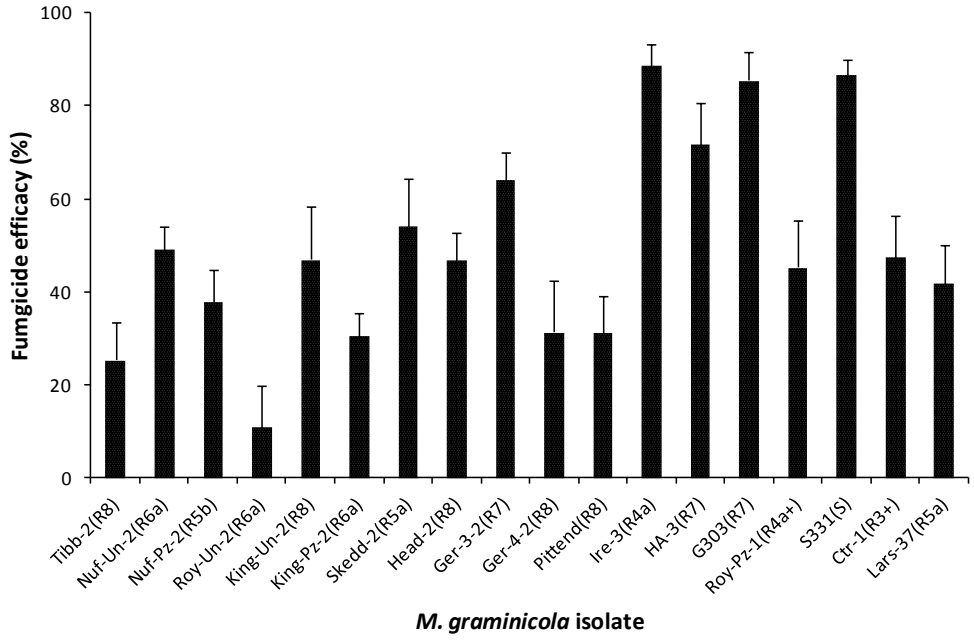

Figure 6.8 The *in planta* efficacy of Tracker against *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

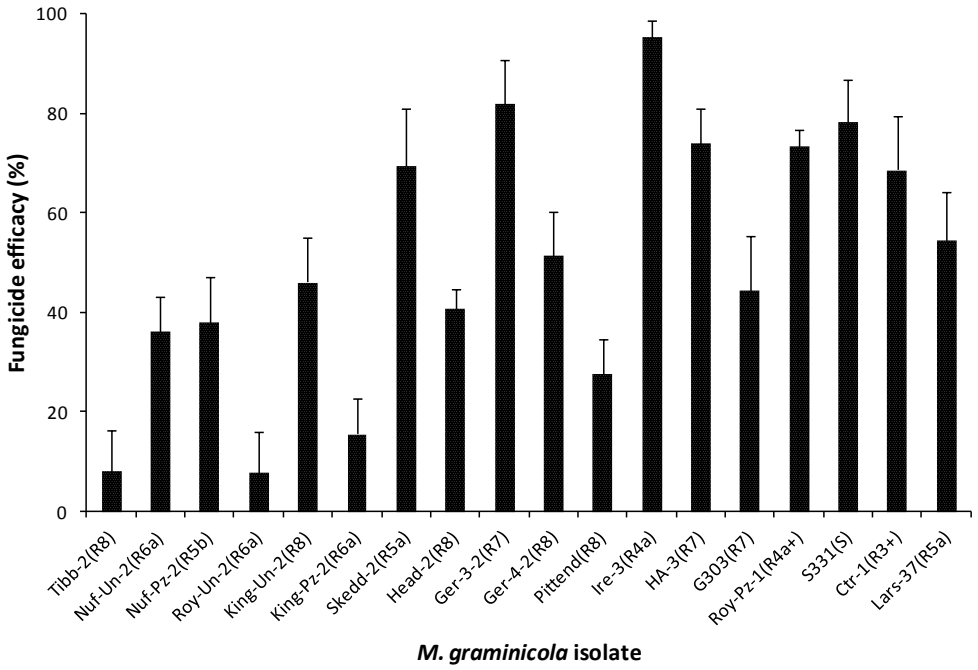

Figure 6.9 The in planta efficacy of Prosaro against *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

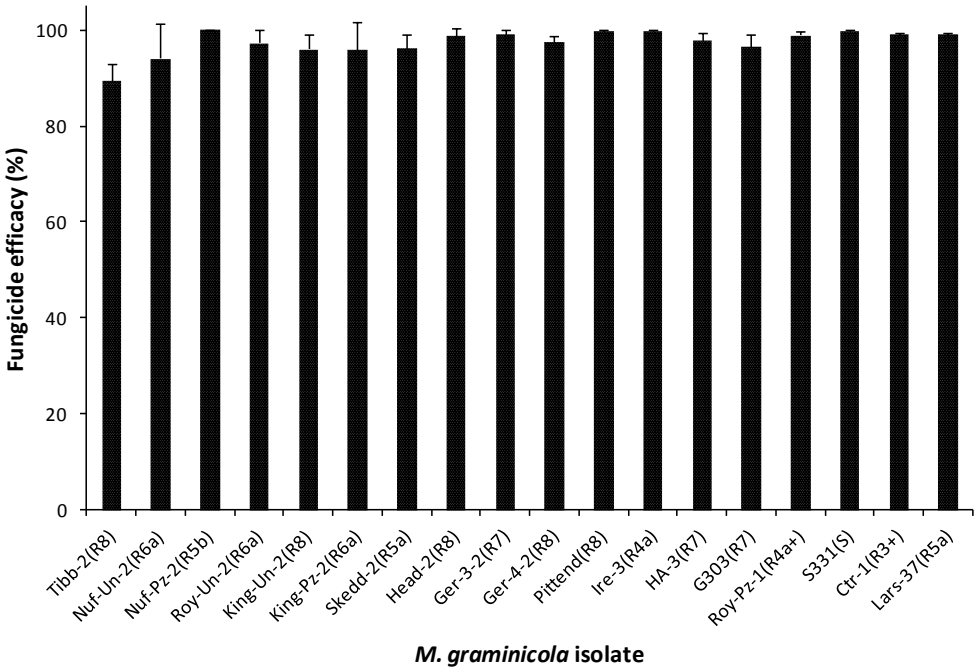

Figure 6.10 The in planta efficacy of penthiopyrad against *M. graminicola* isolates with *CYP51* mutations. Error bars represent standard deviation.

6.5.2.2 Quantitative fungicide assessment using q-PCR

Four fungicides, tebuconazole, epoxiconazole, prochloraz and penthiopyrad that were visually assessed *in planta*, were also assessed quantitatively using q-PCR. The assessment used 7 *M. graminicola* isolates with different R-types. Visual assessment of the activities exhibited by these fungicides on isolate Ire-3 (R4a) is shown in Figure 6.11; where it can be seen that penthiopyrad significantly outperformed other fungicides in decreasing the disease incidence. Prochloraz and epoxiconazole also showed good activity whilst tebuconazole exhibited the lowest performance. A similar pattern was shown when the fungicides were assessed quantitatively using q-PCR with no significant differences between penthiopyrad, prochloraz and epoxiconazole. However, the activity of tebuconazole when assessed quantitatively did not differ significantly with that of the untreated control (Figure 6.12). The results also showed a positive medium correlation (r=0.73) between the two assessment methods (Figure 6.13).

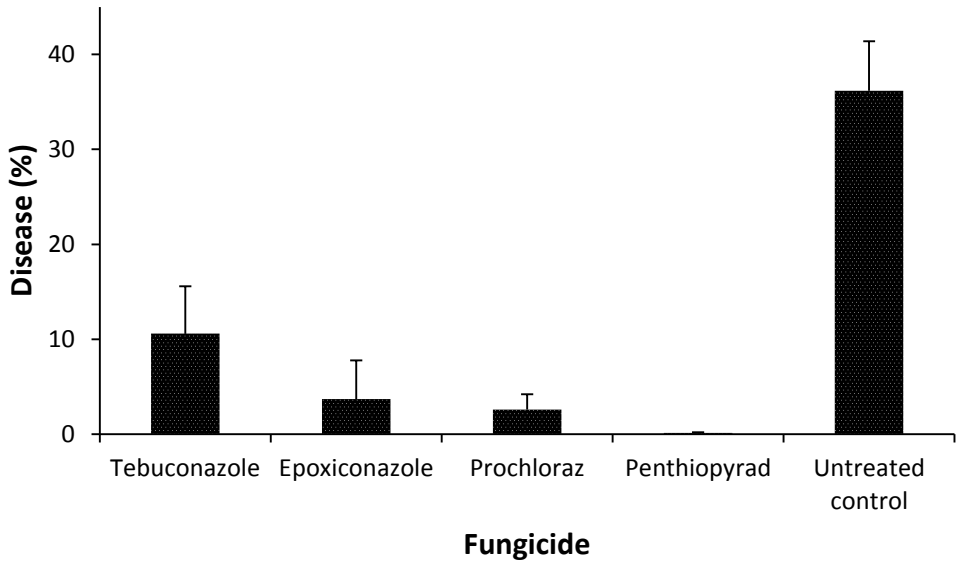

Figure 6.11 Visual assessment of fungicides on *M. graminicola* isolate Ire-3 (R4a). Error bars represent standard deviation.

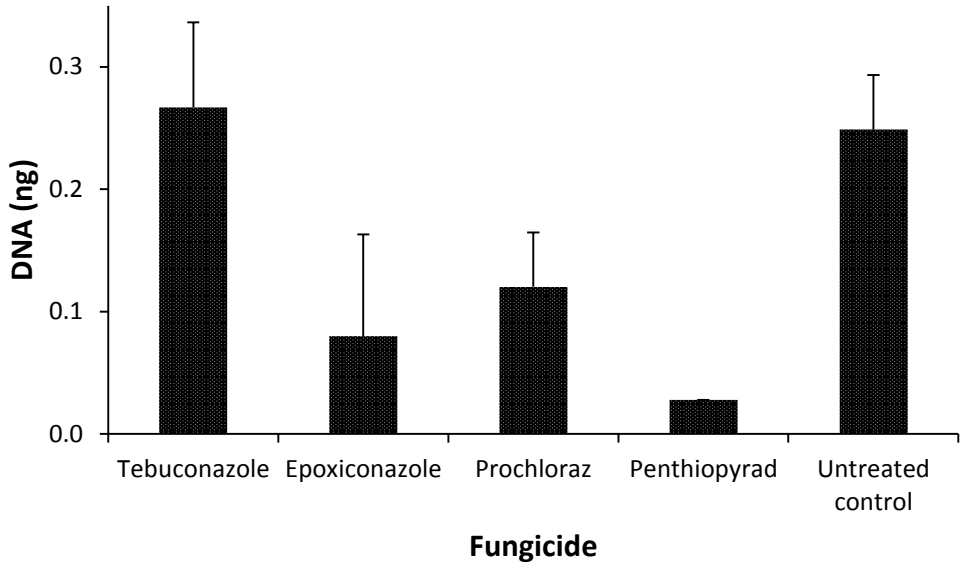

Figure 6.12 Quantitative assessment of fungicides on *M. graminicola* isolate Ire-3 (R4a). Error bars represent standard deviation.

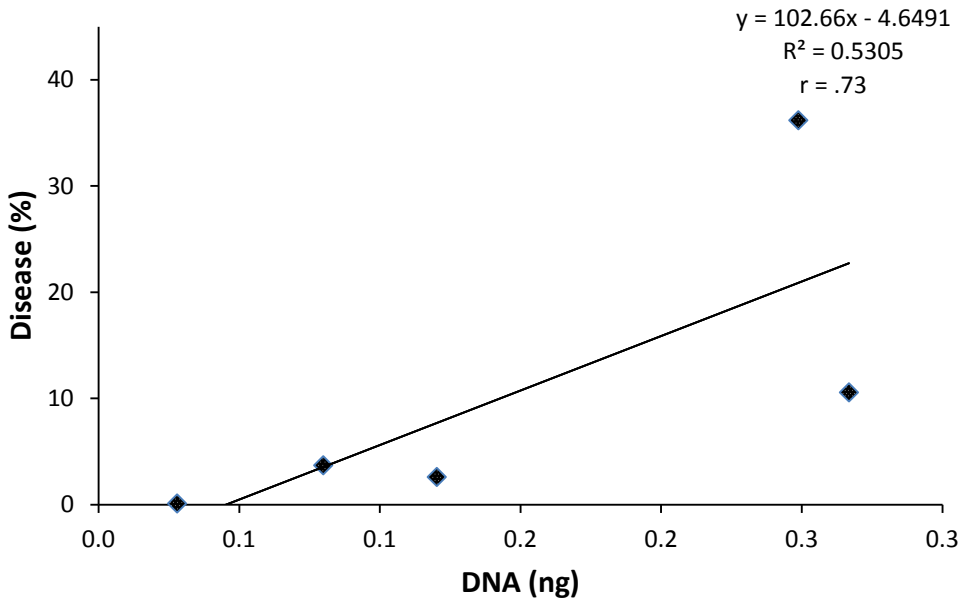

Figure 6.13 Correlation between visual and quantitative assessment of fungicides on isolate Ire-3 (R4a).

Fungicide efficacy *in planta* was assessed on isolate Ctrl-1 (R3+) and the results given in Figure 6.14 show that, again, penthiopyrad outperformed other fungicides. Prochloraz and epoxiconazole exhibited lower activities with no significant differences between them. However, tebuconazole showed the lowest performance as shown previously with isolate Ire-3. Quantitative assessment of fungicides on the same isolate followed a similar pattern (Figure 6.15), and therefore, a strong positive correlation (r = 0.93) was found between both assessments (Figure 6.16).

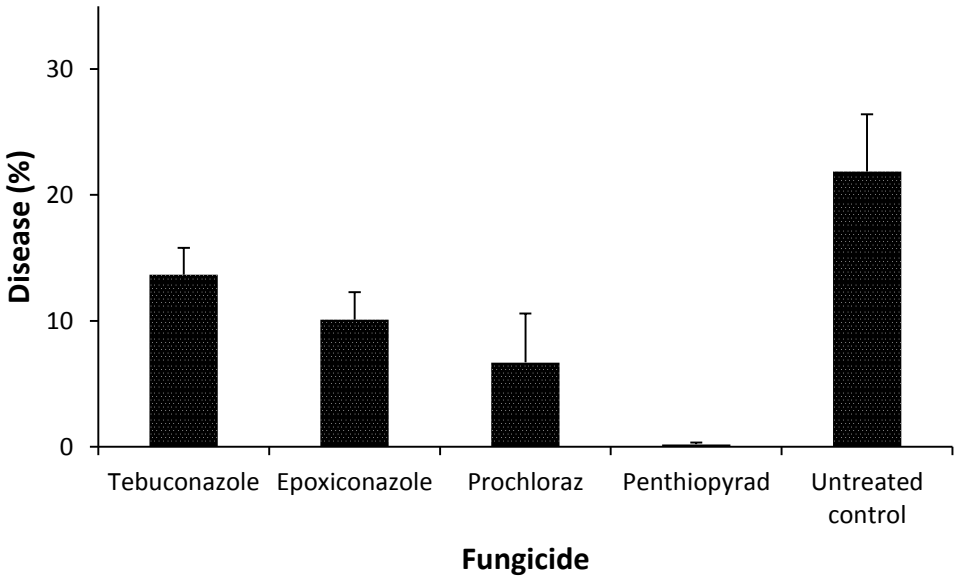

Figure 6.14 Visual assessment of fungicides on *M. graminicola* isolate Ctrl-1 (R3+). Error bars represent standard deviation.

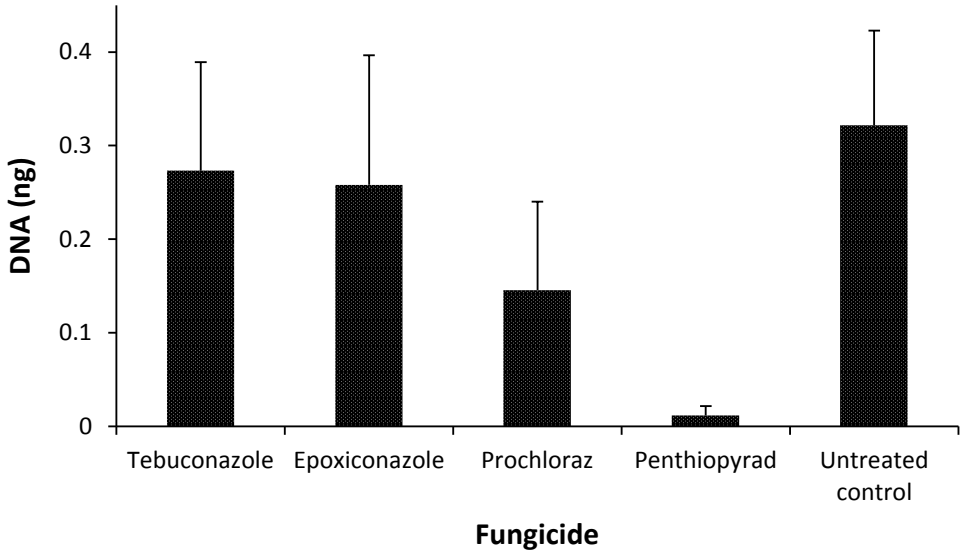

Figure 6.15 Quantitative assessment of fungicides on *M. graminicola* isolate Ctrl-1 (R3+). Error bars represent standard deviation.

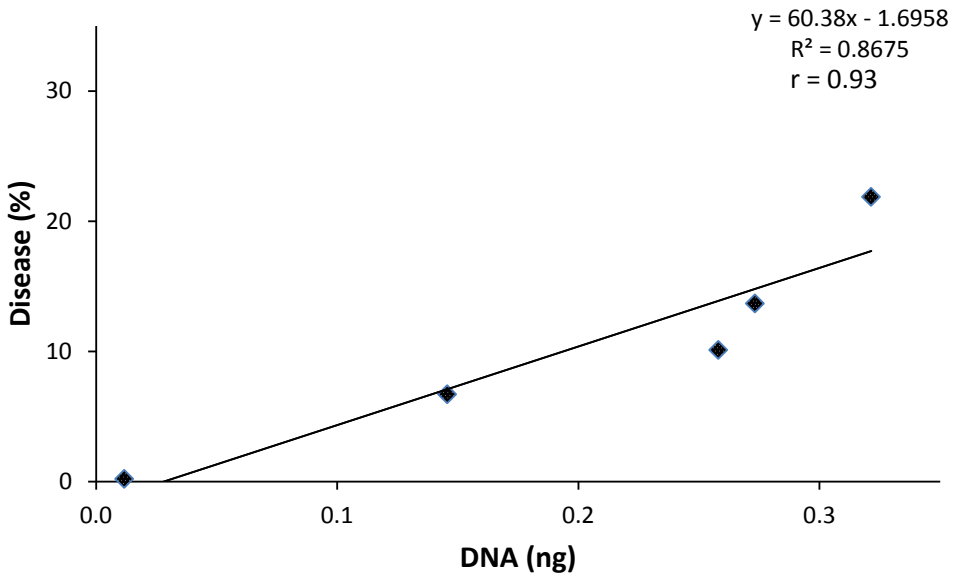

Figure 6.16 Correlation between visual and quantitative assessment of fungicides on isolate Ctrl-1 (R3+).

The efficacy of penthiopyrad was also pronounced with isolate Skedd-2 (R5a) when assessed visually (Figure 6.17). Significantly lower efficacies, however, were exhibited by prochloraz, tebuconazole and epoxiconazole. Quantitative assessment, although following a similar pattern to that of visual assessment, showed that tebuconazole also exhibited an activity similar to the penthiopyrad (Figure 6.18). Q-PCR assessment also showed that the activities of prochloraz and epoxiconazole were low and did not differ significantly from the untreated control. Nevertheless, a strong positive correlation (r = 0.84) was found between visual and quantitative assessment (Figure 6.19).

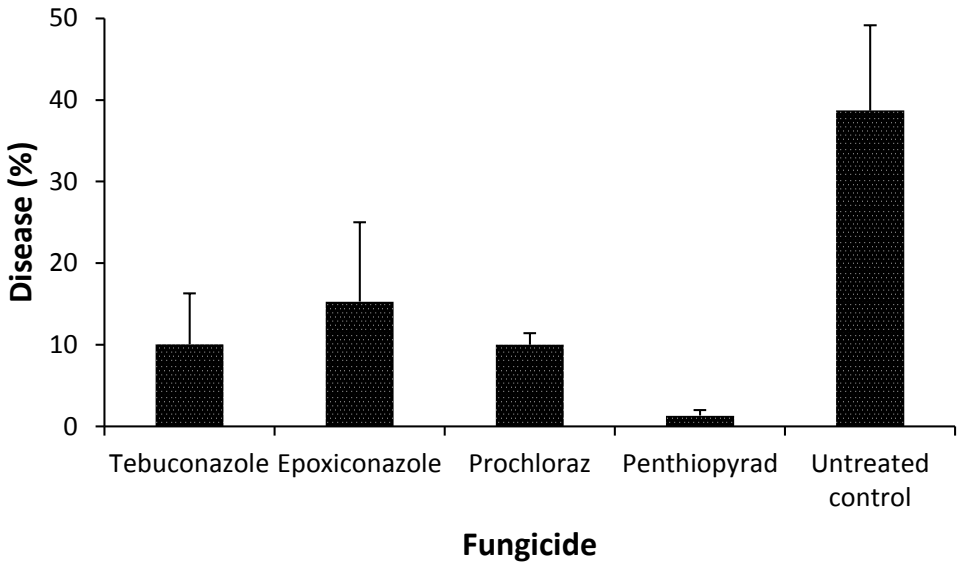

Figure 6.17 Visual assessment of fungicides on *M. graminicola* isolate Skedd-2 (R5a). Error bars represent standard deviation.

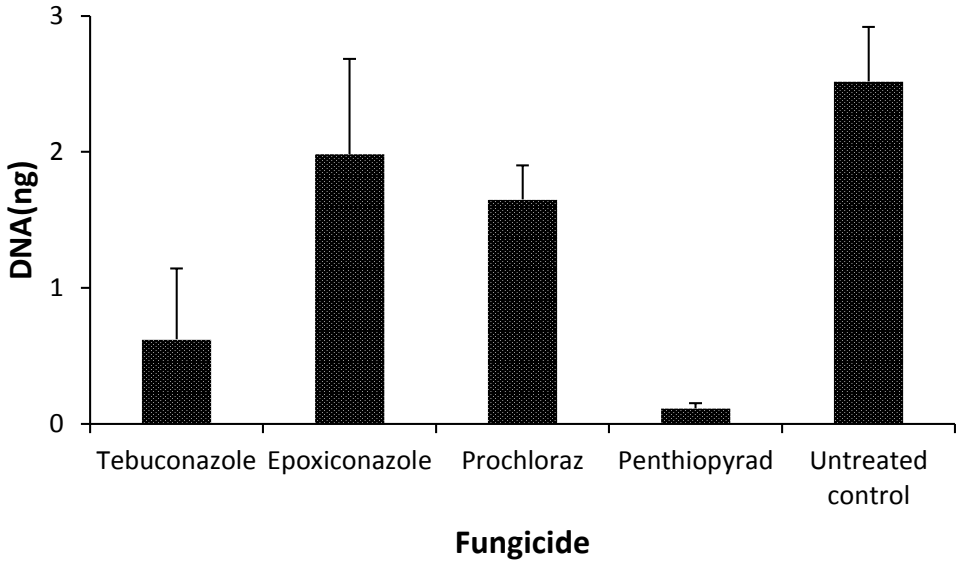

Figure 6.18 Quantitative assessment of fungicides on *M. graminicola* isolate Skedd-2 (R5a). Error bars represent standard deviation.

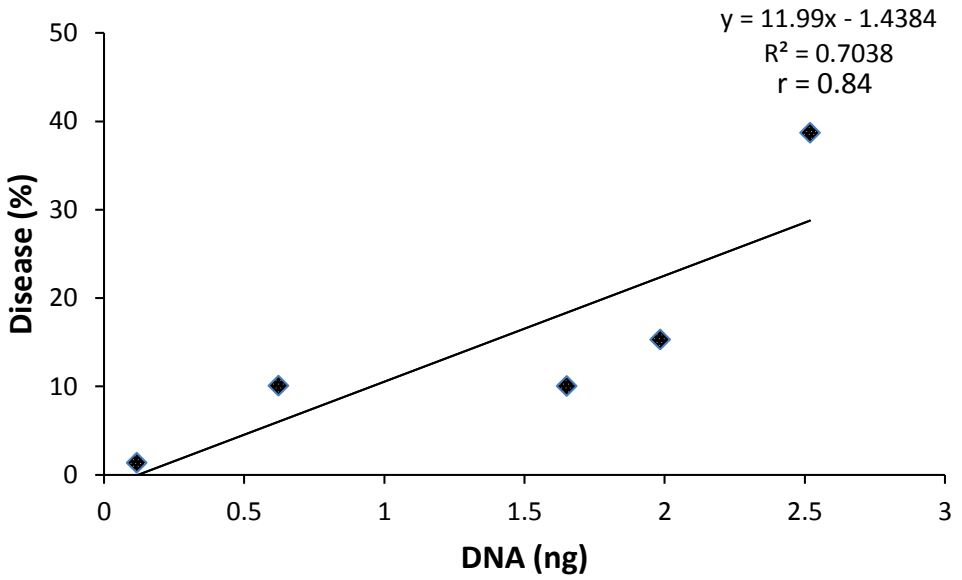

Figure 6.19 Correlation between visual and quantitative assessment of fungicides on isolate skedd-2 (R5a).

Visual assessment of fungicides was performed on R6+ isolate (Roy-Un-2) and the results showed that the lowest disease occurrence was by using penthiopyrad as a protective fungicide (Figure 6.20). Prochloraz also showed good activity against this isolate. Tebuconazole and epoxiconazole, by showing no significant differences with the untreated control, did not give good protection against this isolate. The quantitative assessment, however, revealed that penthiopyrad was the only fungicide to give high protection against this pathogen strain. All other fungicides did not differ significantly from the untreated control (Figure 6.21). However, the strong positive correlation (r = 0.82) between the two assessments indicates that the quantitative assessment well-represented the one assessed visually (Figure 6.22).

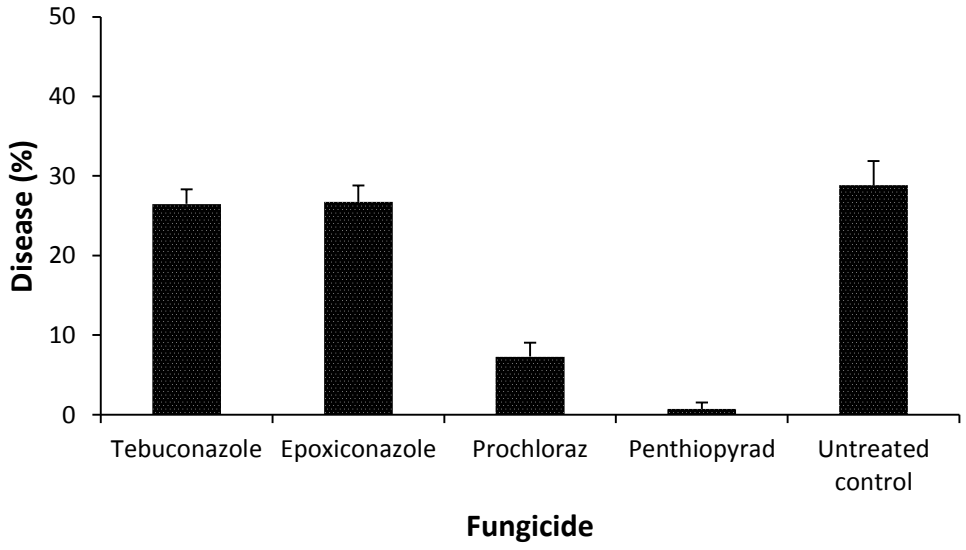

Figure 6.20 Visual assessment of fungicides on *M. graminicola* isolate Roy-Un-2 (R6a). Error bars represent standard deviation.

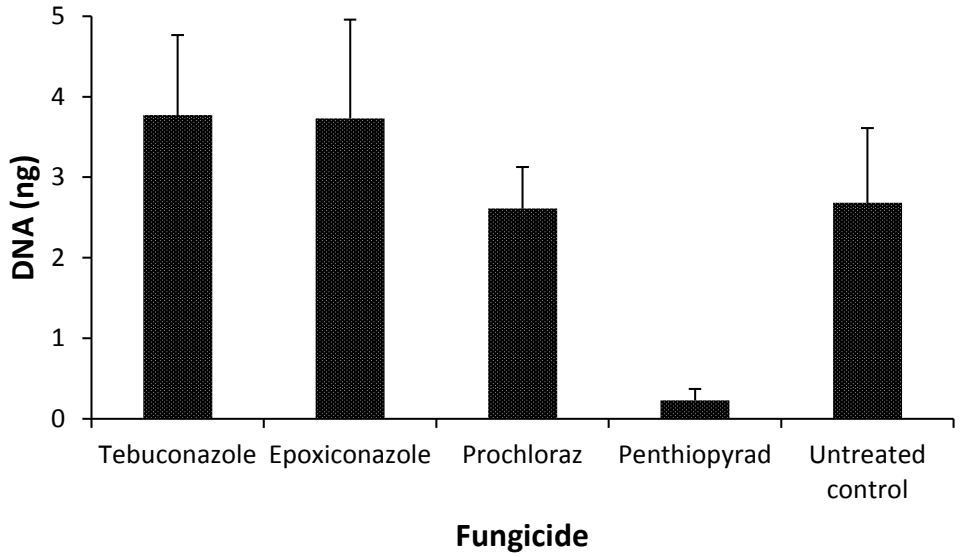

Figure 6.21 Quantitative assessment of fungicides on *M. graminicola* isolate Roy-un-2 (R6a). Error bars represent standard deviation.

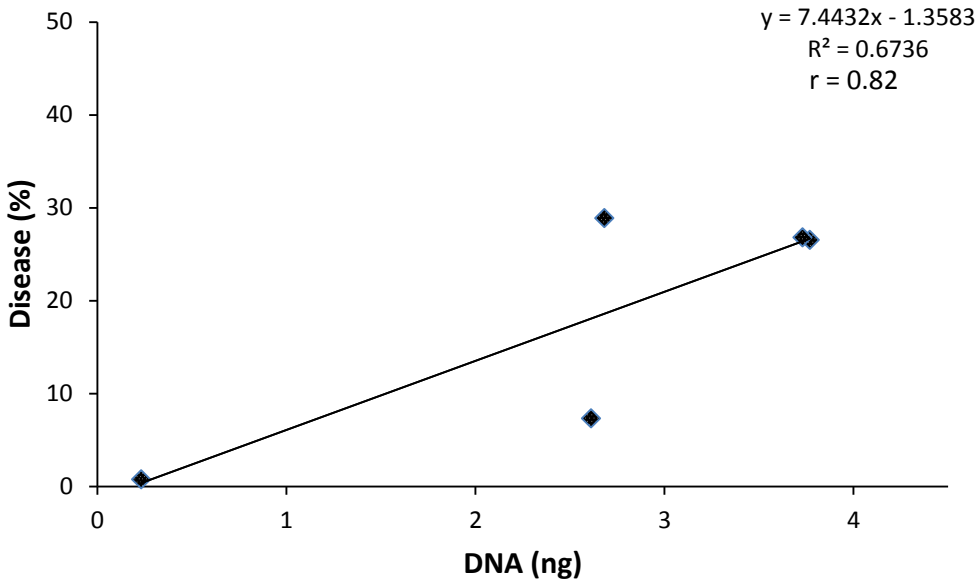

Figure 6.22 Correlation between visual and quantitative assessment of fungicides on isolate Roy-Un-2 (R6a).

Fungicides were also assessed visually and quantitatively on R8 isolate King-Un-2 and the results demonstrated a similarity between the two assessments (Figure 6.23 and Figure 6.24). In both assessments, penthiopyrad was significantly the best in protecting wheat plants from *M. graminicola* isolate King-Un-2, while other fungicides showed lower activities. Arising out of this a strong correlation between two assessment methods was observed (Figure 6.25).

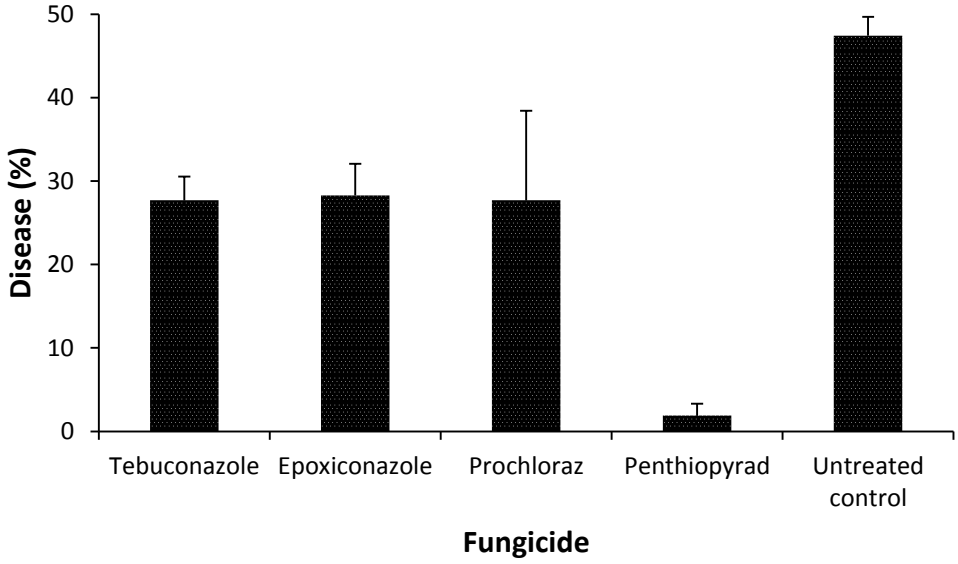

Figure 6.23 Visual assessment of fungicides on *M. graminicola* isolate King-Un-2 (R8). Error bars represent standard deviation.

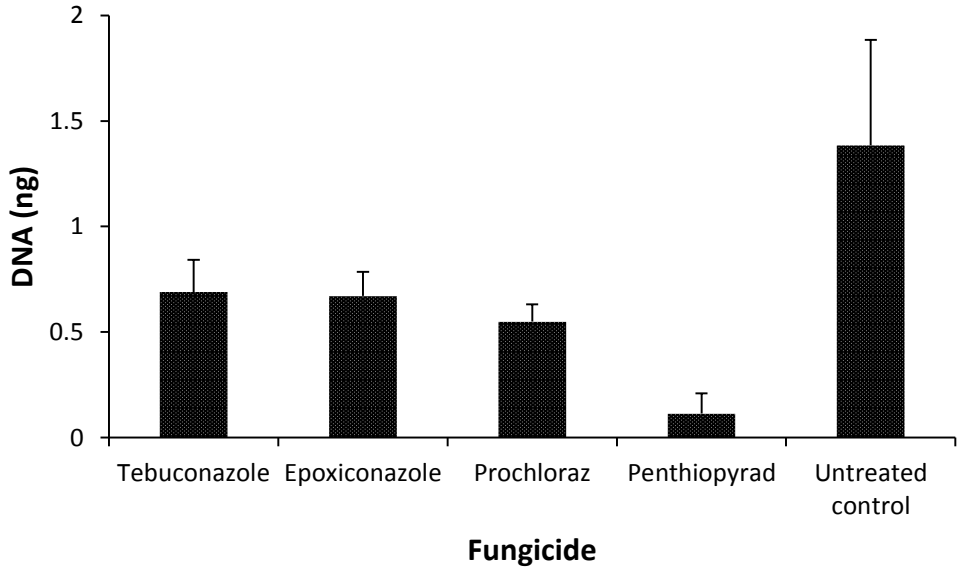

Figure 6.24 Quantitative assessment of fungicides on *M. graminicola* isolate King-un-2 (R8). Error bars represent standard deviation.

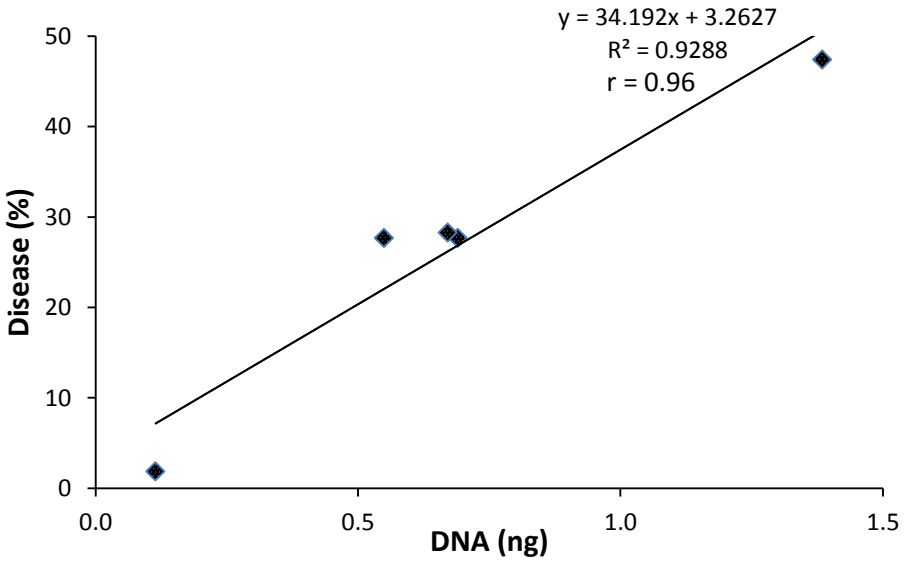

Figure 6.25 Correlation between visual and quantitative assessment of fungicides on isolate King-Un-2 (R8).

A similar situation was found with isolate Ger-3-2 (R7) for both assessments (Figure 6.26 and Figure 6.27), having a high positive correlation (Figure 6.28). Fungicide efficiencies were also assessed using both measurements on isolate HA-3 (R7). The visual assessment revealed high efficacy of penthiopyrad and low activity of prochloraz and epoxiconazole and very low efficacy of tebuconazole (Figure 6.29). However, when the same fungicides were assessed quantitatively, penthiopyrad was highly effective while all triazoles had low efficacy (Figure 6.30). The medium positive correlation between the two assessments indicated good representation of q-PCR measurement with that of visual assessment (Figure 6.31).

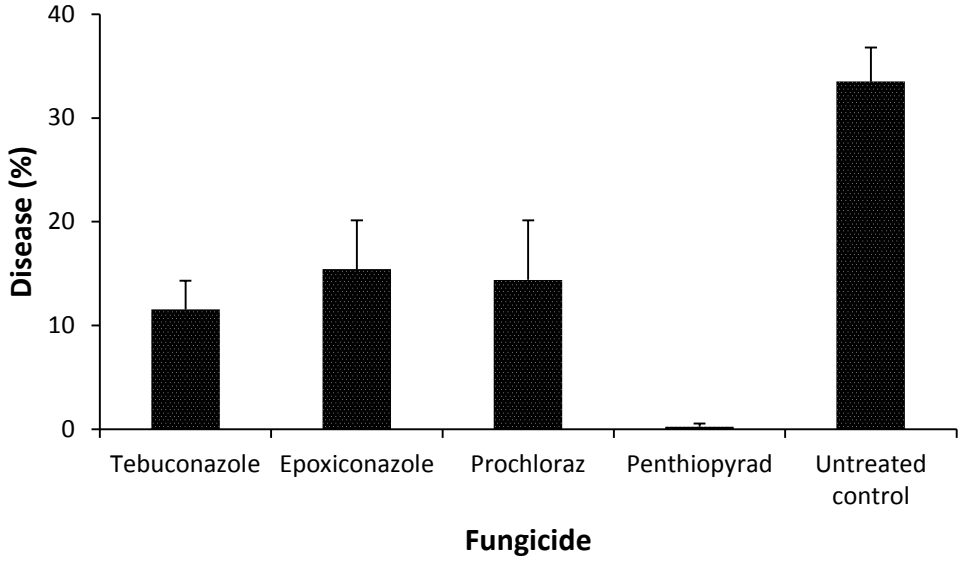

Figure 6.26 Visual assessment of fungicides on *M. graminicola* isolate Ger-3-2 (R7). Error bars represent standard deviation.

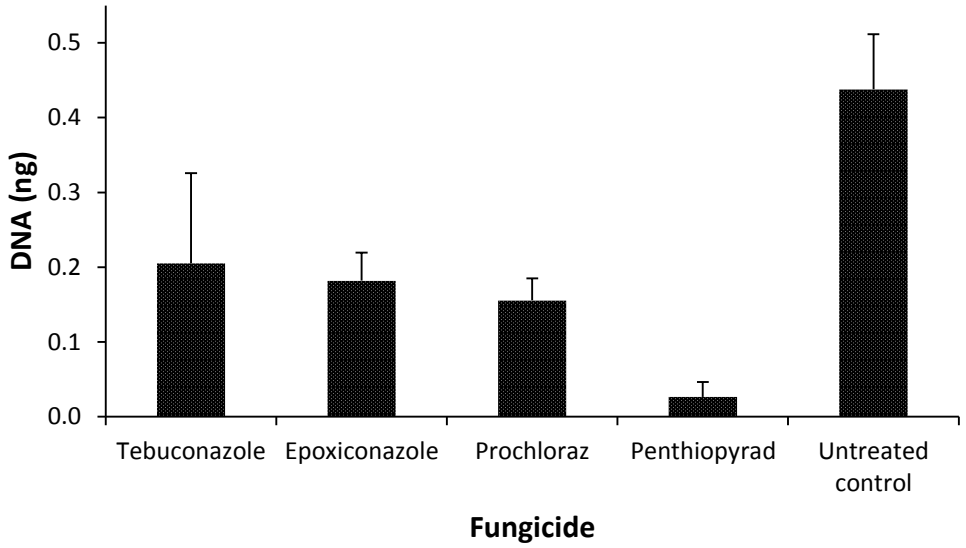

Figure 6.27 Quantitative assessment of fungicides on *M. graminicola* isolate Ger-3-2 (R7). Error bars represent standard deviation.

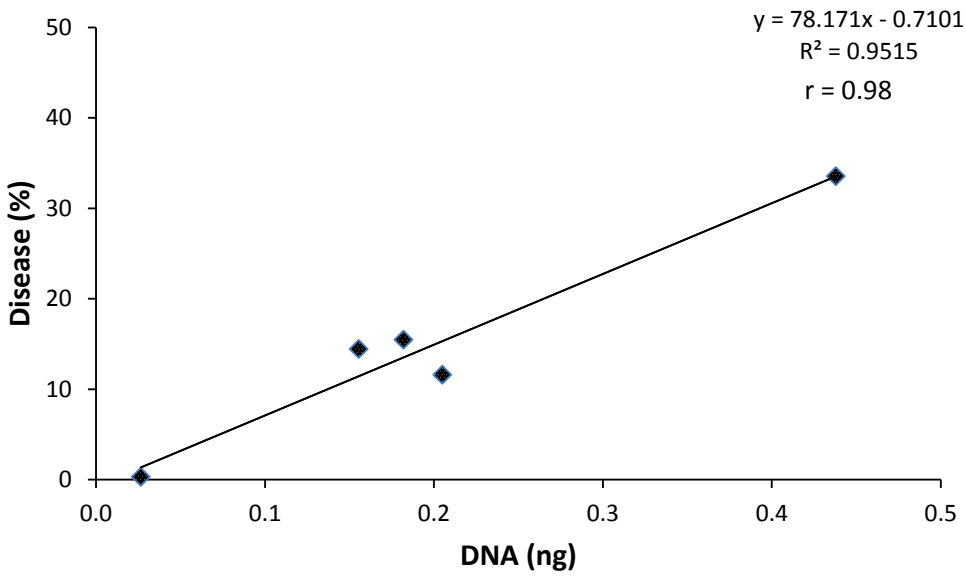

Figure 6.28 Correlation between visual and quantitative assessment of fungicides on isolate Ger-3-2 (R7).

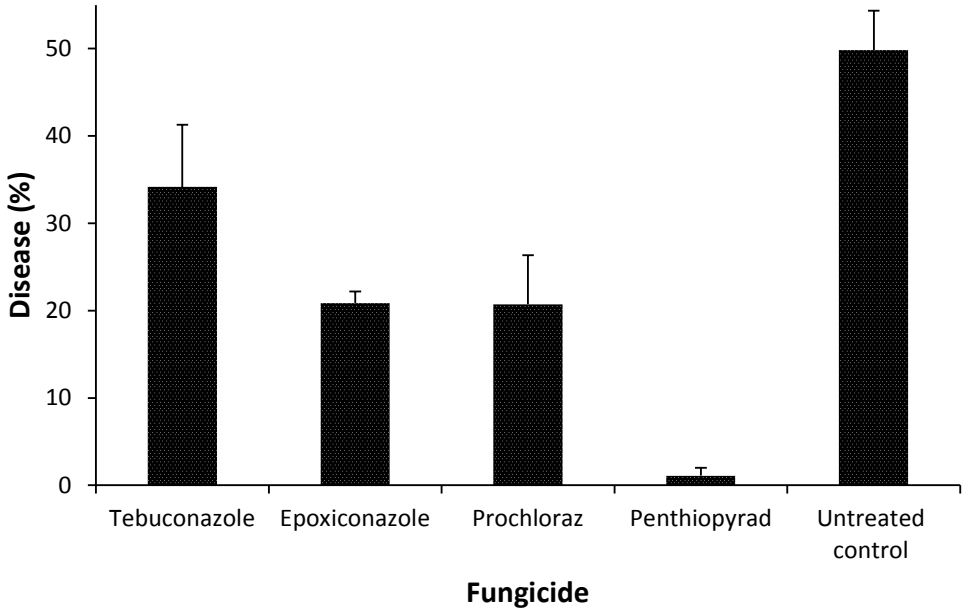

Figure 6.29 Visual assessment of fungicides on *M. graminicola* isolate HA-3 (R7). Error bars represent standard deviation.

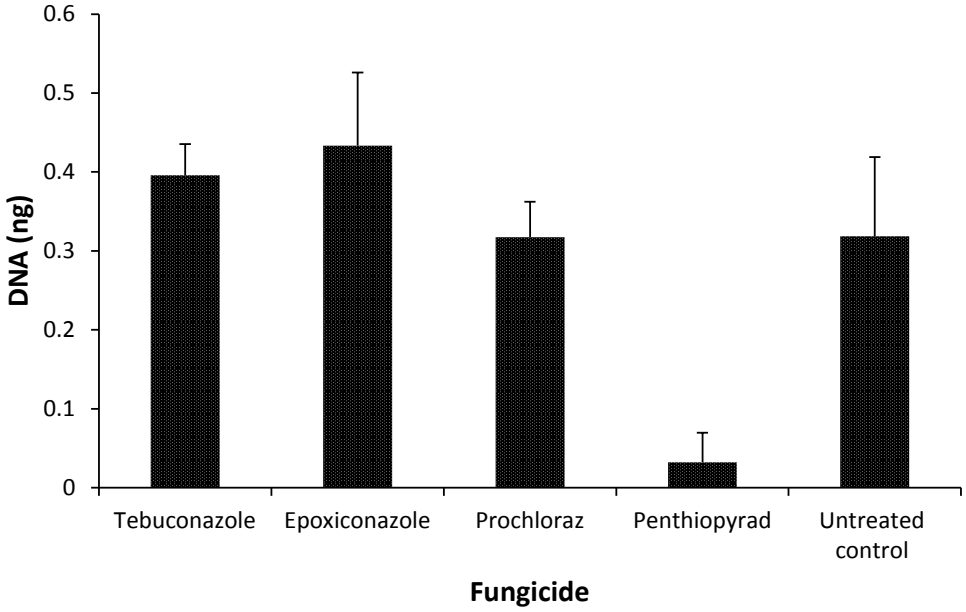

Figure 6.30 Quantitative assessment of fungicides on *M. graminicola* isolate HA-3 (R7). Error bars represent standard deviation.

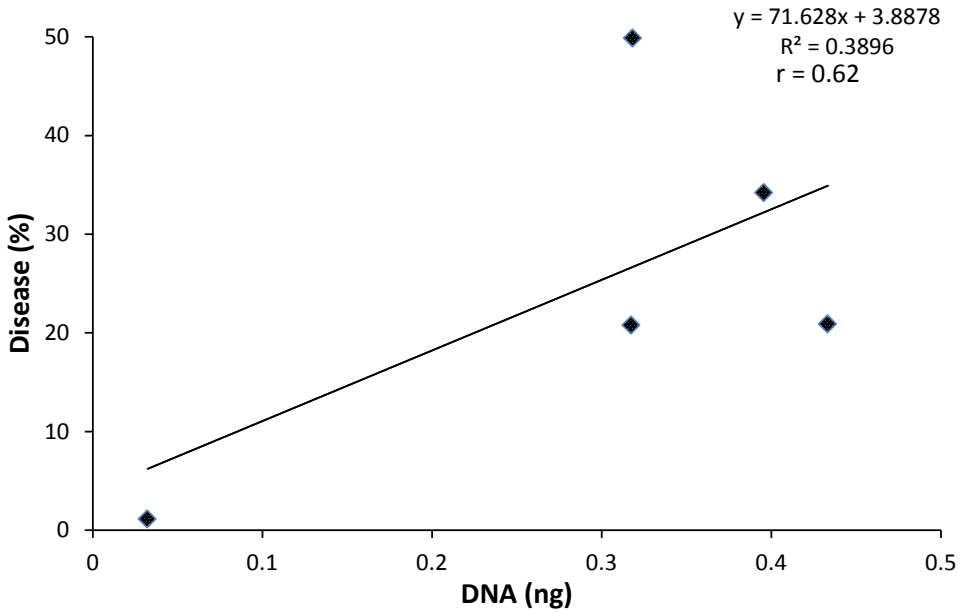

Figure 6.31 Correlation between visual and quantitative assessment of fungicides on isolate HA-3 (R7).

6.6 Discussion

In vitro fungicide activity against *M. graminicola* was assessed using three different methods. The microtitre plates methods, with or without a growth indicator, have been widely and successfully used by many researchers. However, in this work, unfortunately the method gave high data variability between replicates of the same treatment. This was probably the result of bacterial contamination that led to detection of higher absorbance values. Bacterial contamination was also detected by previous researchers who used the same method. For instance, Pijls *et al*. (1994) found contamination in an entire row of a microtitre plate used to assess activity of fungicides against *M. graminicola*, causing higher absorbance measured by the plate reader. However, other reasons, such as use of commercially-formulated fungicide products instead of pure technical grade materials, might have been involved. Using active ingredients contained in commercial products, that included components such as emulsifiers, may give turbidity to the liquid media compared to the technical materials, which tend to give clear solutions at the concentrations used. This added further errors to the absorbance values obtained by plate readers, giving lack of reliability to data obtained.

To address the unreliability of data obtained from microtitre methods in this work, a different technique, based on measuring conidial germ-tube growth, was used to assess the *in vitro* sensitivity of *M. graminicola* isolates to fungicides. The method, although was laborious to implement, was used as an alternative to microtitre plate assays, in which fungal growth is measured in liquid medium after incubation (4-10 days), using a spectrophotometer (Pijls *et al.*, 1994; Mavroeidi and Shaw, 2005). EC_{50} values obtained from previous studies claimed that the germ tube growth test was up to 10 times more sensitive than the microtitre techniques (Leroux *et al.*, 2007).

The relative speed of the two methods used to assess EC_{50} values must also be considered. Experienced workers can evaluate up to 5 fungicides with 48 pathogen strains in 1.5 working days spread over 5 d. Apical germ tube growth assessments would take approximately 15 working days to achieve the same results and is thus much slower. The problems associated with the microtitre plate assays used in this work were many due to microbial contamination associated with non-sterile commercial fungicide formulations. This can be overcome using technical-grade materials, which are effectively sterilised by dissolution of stocks in acetone or ethanol, before incorporation into aqueous media at low solvent concentrations.

Mycosphaerella graminicola isolates, based on fungicide sensitivity tests and according to previous characterisations (Leroux *et al.*, 2007; Stammler *et al.*, 2008a), were classified in this study to 9 variants (S, R3+, R4a,R4a+, R5a, R5b, R6a, R7, and R8). The results showed great differences in fungicide resistance levels among these isolates. The S-genotype includes isolate S331 with not common mutations. However, two new mutations (V24D and S51T) which are not mentioned before were identified in this isolate. This isolate exhibited the lowest EC_{50} value towards all triazoles tested. Isolates with Y137F mutation is characterised as R3 variant by Leroux group. Sequence results of this study also detected this mutation in Ctrl-1, the isolate originated from Rothamsted Research. However, the S524T mutation, although was not detected in this study because the primers used to amplify the *CYP51* gene did not extend to cover the 524 position of the gene, previous sequence results

carried out by Cools *et al*. (2005) confirm the existence of this mutation in this isolate. Therefore a new name (R3+) had to be given to this variant. *In vitro* data from microtitre assay, using technical grade, undertaken kindly by Dr Bart Fraaije group, also support the effect of this variant in decreasing the sensitivity to tebuconazole and prochloraz (Table 6.4). Previous results have also shown that this combination can have a substantial impact on azole fungicide sensitivity (Cools *et al.*, 2011). The R4a variants were considered isolates with Y461H; when combined with V136C was given a different name as R4a+. These variants, R4a+ in particular, displayed a relatively high EC_{50}s towards tebuconazole. A combination of the Y461H mutation with the V136A mutation considered R5a variant included 2 isolates (Skedd-2 and Lars-37) while the latter mutation when combine with the Y459/G460 deletion is characterised as R5b variant (isolate Nuf-Pz-2). This variant showed a slightly higher EC_{50} towards prochloraz and had low EC_{50} values towards tebuconazole following the observation made by many other researchers (Fraaije *et al.*, 2007; Leroux *et al.*, 2007). Data from a microtitre plate assay kindly provided by Dr. Bart Fraaije of Rothamsted Research have also confirmed this pattern (Table 6.4). However, EC_{50}s from apical germ-tube, using either formulated or technical grade, did not support this pattern. Two of the R5 isolates (Nuf-Pz-2 and Skedd-2) exhibited higher EC_{50} values towards prothioconazole and epoxiconazole. However the other R5 isolate (Lars-37) had showed similar EC_{50} to both fungicides. According to Leroux classification, the variants R3, R4 and R5 are gathered within a larger group showing low resistance to triazoles called TriLR.

The R6a variants are characterised by the combination of the I381V with the mutations at positions 459 or 461. This includes isolate Nuf-Un-2, Roy-Un-2 and King-Pz-2. The R7 group represents isolates with the I381V mutation combined with the double deletions at 459 and 460 positions of the *CYP51* gene. The latter variant when combine with the A379G mutation is characterised as R8 variant. These three variants (R6, R7 and R8) are showing a moderate resistance to triazoles (TriMR). On the other hand, EC_{50}s from both the germ-tube length assay and the microtitre plate assay for the R6, R7 and R8 genotypes were higher to tebuconazole compared with that shown to prochloraz. This is in agreement with previous findings that isolates carrying

I381V (R6, R7 and R8) are less sensitive to tebuconazole but sensitive to prochloraz (Fraaije *et al.*, 2007; Leroux *et al.*, 2007; Stammler *et al.*, 2008b). Interestingly, data obtained from microtitre assay showed that the R8 variants (L50S, S188N, A379G, I381V, DY459/G460, N513K) were more sensitive to prochloraz compared with R7 variants (L50S, S188N, I381V, DY459/G460, N513K). This is in agreement with the findings of Mullins *et al*. (2011) where they stated that the inclusion of the A379G mutation in the combination of L50S, S188N, I381V, DY459/G460, N513K were doubled the sensitivity to prochloraz compared with that of lacking this mutation.

Most of the R6, R7 and R8 genotypes were also generally less sensitive (showed higher EC_{50} values) towards other DMIs and mixtures of DMIs (Table 6.3). The results obtained by *in vitro* (EC_{50}) trials demonstrate that there was not always a cross-resistance between all tested triazoles (R3+, R4, R5, R6, and R8), as some were sensitive to a triazole but were resistant to another one. The same phenomenon was observed with the imidazole fungicide, prochloraz, as previously observed in *Oculimacula* sp., the causal agents of wheat eye spot (Leroux and Gredt, 1997). However, in a study determining the *in vitro* sensitivity of over 120 *M. graminicola* isolates, from throughout England and Wales, to 8 DMI fungicides and to examine cross-sensitivity relationships, Elcock *et al*. (2000) found a positive cross-sensitivity between some of the DMI fungicides tested. Earlier results by Gisi and Herman (1994) also detected a positive cross-resistance between cyproconazole and flutriafol for the entire population of samples of *M. graminicola* collected in a sensitivity monitoring programme in the wheat fields in the UK.

In the case of resistance factor (RF) values, the genotypes could be categorised into 3 triazole-resistant phenotypes: low RF (LR), RF less than 10, medium RF (MR), RF from 10 to 20, and high RF (HR), RF values more than 20 (Table 6.5). For tebuconazole, RF values were either below 10 (genotypes R3+, R4, R5, R6 and R8 or greater than 10 (R7 only). For prothioconazole, R3+ and R4 were located within LR group, R6, R7, and R8 located within MR group, and R5 located under HR group. High differences were observed with epoxiconazole, with RFs below 10 for genotypes R3+, R7 and R78, and RF values between 10-20 (R4) or above 20 (R5 and R6). For prochloraz, R4 was

located within LR phenotypes while all other genotypes were located under MR or HR group. R3+ variant (Y137F + S524T) had the highest RF to prochloraz compared to other triazoles (Table 6.5). In agreement of this result Cools *et al*. (2011) found this variant not only further reduces sensitivity to triadimenol but also decreases sensitivity to other azoles such as prochloraz. For most DMIs, the highest RFs were recorded in genotypes exhibiting Δ459/G460 deletions, with substitutions I381V and/or A379G (R6, R7, and R8 and genotypes with the V136A mutation (R5). This is in agreement with findings of Leroux *et al*. (2008b) who found that strains with Δ459/G460 deletions or alterations, with substitutions I381V and A379G, exhibited the highest resistance factors to most DMIs. In the same way, great differences were also found with Tracker and Prosaro ranging from LR to HR groups. Finally, the smallest differences were observed with Fandango and penthiopyrad where all RF values locate under the LR group. Calculation of RF values provides a rapid and easily-understood method of describing fungicide sensitivity.

From the results of visual assessment of *in planta* efficacy of fungicides it was evident that triazoles and mixtures were efficient, and gave high disease control against sensitive isolate S331 and isolate Ire-3 (R4a) which is also supported by *in vitro* assay. However, the performance of tebuconazole was different from that of prochloraz; it exhibited high activity against R5 genotypes and at the same time its efficacy was very low against isolates carrying the I381V mutation (R6, R7 and R8). Additionally, this pattern was also supported by RF values in (Table 6.5) where it can be seen that the R5 genotypes have low RF values whereas R6, R7 and R8 have higher values towards tebuconazole. This finding is strongly supported by previous reports confirming that the R5 genotypes are sensitive to tebuconazole but resistant to prochloraz, whereas genotypes carrying I381V are less sensitive to tebuconazole but sensitive to prochloraz (Fraaije *et al.*, 2007; Leroux *et al.*, 2007). *In planta* resistance of isolates with I381V mutation (R6, R7, and R8 to tebuconazole and sensitivity to prochloraz is also supported by *in vitro* data (Table 6.3 and Table 6.4). Fungicide sensitivity work carried out at Rothamsted Research by Fraaije *et al*. (2008) also confirmed that *CYP51* variants with I381V were much less sensitive to tebuconazole and accumulate in fields which have been treated with this fungicide. They further showed that

CYP51 alterations, particularly A136V and I381V, were differentially selected by different members of the azole class of fungicides. On the other hand, prochloraz also showed high activity *in planta* against a range of isolates with I381V mutations such as Roy-Un-2 (R6a), Head-2 and Ger-4-2 (R8), Ger3-2 (R7), Pittend (R8) and a slightly lower efficacy against G303 and HA3 (R7). This also supported by previous findings of many researchers, but it is, however, not supported with high EC_{50} values of some of isolates. However, the *in planta* resistant of R5 isolates (Nuf-Pz-2, Skedd-2 and Lars-37) to prochloraz was evident and in agreement with the report described above. It is interesting that isolate Nuf-Pz-2 (R5b) originated from prochloraz-treated plants (Table 5.1). This suggests that the R5 genotype was differentially selected by application of this fungicide before leaf sampling to isolate the pathogen. Contradicting this phenomenon, prochloraz also exhibited low activity against two R8 isolates (Tibb-2 and King-Un-2) and two R6a isolates (King-Pz-2 and Nuf-Un-2). The low performance of prochloraz against R5 isolates *in planta*, although in agreement with previous findings and with the EC_{50} values obtained from microtitre assay done by Dr Bart Fraaije at Rothamsted Research, is not strongly supported by the *in vitro* data of this study using apical growth assay.

Prothioconazole and epoxiconazole also showed low *in planta* activity against most R5, R6, R7 and R8 genotypes; however, both displayed high activity against R4 genotypes. Contrary to the low activity of epoxiconazole against most *CYP51* genotypes reported in this study, Stammler *et al.* (2008a) stated that the influence of *CYP51* genotypes on the sensitivity was limited and they further suggested that there were no correlations between the *in vitro* sensitivity pattern and field performance of epoxiconazole. Interestingly, prothioconazole alone gave high disease control against the R7 genotype (Ger-3-2). The most recent study confirms that prothioconazole behaved differently from other triazoles in its mechanism of inhibition. It was found to be a competitive inhibitor of substrate binding to MgCYP51 with 840-fold less affinity than epoxiconazole and tebuconazole (Parker *et al.*, 2011).

Comparison between *in planta* efficacy data and EC_{50} values for tebuconazole, derived from microtitre assays and germ tube growth assays using commercial

and technical grade materials, are also interesting. In general, there is a good correlation between results derived from all assays; most isolates (R6, 7 and 8) with high EC_{50} values, derived from both methods, were poorly-controlled by a tebuconazole-based product *in planta*. At the same time, tebuconazole showed high *in planta* efficacy against *Mycosphaerella* variants Nuf-Pz-2, Skedd-2 and Lars 37 (all R5 group) and the EC_{50} values obtained by both methods, the apical growth assay and the microtitre assay, were well-correlated with *in planta* activity results. In combination with earlier comments on the relative speed of the assays, data obtained by the microtitre method showed better support and therefore must be considered superior.

It would appear that mutations in the *CYP51* gene represent a continuous process which has continued over last 20 years. Since the process began new changes have emerged from year to year. To date, more than 20 different combinations of mutations have been detected and the trend continues to increase (Clark *et al.*, 2010). The S524T mutation has recently been reported as a new change linked to a further reduction in sensitivity to azoles. However, when Rothamsted Broadbalk archive samples were analysed it was discovered that this mutation was already present in 1999. This finding was confirmed with the detection of S524T in the *CYP51* protein of a Rothamsted strain isolated in 2001. In the UK *M. graminicola* population, the S524T mutation is not considered important in affecting field performance of any azole fungicides (Clark *et al.*, 2010). Although the gene sequencing of this current study did not cover the 524 site of the *CYP51* gene, to reveal the existence of S524T change, the mutation was previously found by (Stammler *et al.*, 2008a) in some isolates of *M. graminicola*. Recent research by (Cools *et al.*, 2011), however, observed that the *CYP51* genotypes carrying combinations of alterations D134G, V136A, Y461S, and S524T have a substantial impact on sensitivity to the most widely used triazoles, which includes epoxiconazole and prothioconazole.

Chlorothalonil a broad-spectrum, multisite fungicide, showed good activity against a range of genotypes including sensitive (S), R5, R6 and R7; however, it showed low performance against a wider range of isolates belonging to different R-types. Nevertheless, there is no evidence that the activity of this

fungicide has any relation with *CYP51* mutations but previous studies support the fact that chlorothalonil alone exhibited low performance against *M. graminicola* isolates (McCartney *et al.*, 2007). Fandango, although exhibiting high activity against a wide range of isolates belonging to S, R5, R7 and R8 genotypes, showed low performance against some isolates belonging to R5, R6 and R8 genotypes. Low protections against other isolates belonging to different genotypes all were located within LR group (RF less than 3.9). Interestingly, Tracker (a mixture of boscalid and epoxiconazole), when compared to epoxiconazole, showed a slightly higher performance against three R7 genotypes (G303, HA-3 and Ger-3-2) located under the MR group but was less efficient towards isolates belonging to R6, R8 or R5 genotypes. Prosaro a mixture of two triazoles (prothioconazole and tebuconazole), gave low protection against isolates belong to R6 and R8 genotypes but exhibited a slightly higher activity against a wider range of isolates, ranging from sensitive to highly resistant (S, R3, R4, R5, and R7) with different RF values. This wider activity of Prosaro might reflect the existence of sub-populations in *M. graminicola* with different sensitivities to triazoles (Jorgensen, 2008). Penthiopyrad, however, showed the highest *in planta* efficacy towards all isolates tested, regardless of the genotype group of the isolate. This correlates with the low EC_{50} and RF values for each group of isolates.

Results obtained from germ tube growth *in vitro* assays did not always correlate well with those obtained using *in planta* tests. This phenomenon was also observed in fungicide efficacy experiments with net blotch of barley; *in vitro* activities were not always consistent with *in planta* activities of same fungicides (see section 4.4). In conclusion, a combination of *in vitro* and *in planta* assays to evaluate fungicide performance may be a sensible recommendation to make.

It can be concluded in this current research that triazoles provided variable activities against *CYP51* variants. This is probably because there is more than one mechanism conferring resistance to DMIs. This multiple resistance mechanism that accounts for variation in sensitivity to azole fungicides was reported by many authors. Cools *et al.* (2008) have noticed that isolates of *M. gramnicola* with the same *CYP51* sequence often have a wide range of

sensitivities to the most effective azoles, suggesting a contribution of mechanisms other than target site change to the final phenotype. With reference to this, the results of Leroux and Walker (2011) suggest that 11 possible changes in the *CYP51* gene encoding 14α-demethylase is the basic mechanism in weakly, moderately and highly resistant strains but these changes, when combined with over-expression of drug efflux transporters, probably result in multidrug resistance in some of the most resistant phenotypes. Mutations recorded at positions 50, 188, 379, and 513 did not seem to be correlated to DMI resistance (Leroux *et al.*, 2008b).

The quantitative PCR assessment of *in planta* activity of fungicides showed a similar pattern to that observed in visual assessments. Detecting medium to high correlation values between both assessments confirm the accuracy of q-PCR assessment. However, in some cases, such as in isolates HA-3 and Ire-3, where the correlations between both assessments were 0.62 and 0.73 respectively, a slightly different pattern was noticed between the methods. This might be because the molecular methods can detect infections with no visible symptoms. This is strongly supported by Guo *et al*. (2006) who could detect *M. graminicola* DNA directly after inoculation. A steady increase was also detected before visible symptoms appeared at 8 d. The results of q-PCR were significantly correlated with the disease incidences measured visually (r=0.90). This indicates that q-PCR assays may serve as an alternative method for accurate assessment of the fungicide effects on *M. graminicola* leaf blotch (Guo *et al.*, 2007). Other researchers have also stated the superiority of this technology over traditional methods to detect the fungal content (Fraaije *et al.*, 2002). Such alternatives include the onset of disease development and measuring fungal biomass, estimating expression of host genes that are associated with disease resistance (Goodwin, 2007).

Chapter 7 General discussion and conclusions

The aims of this study were to ascertain the fungicide resistance levels in two cereal pathogens, net blotch of barley and septoria leaf blotch of wheat, both economically important in the UK and worldwide. Concerns have been recently raised about the poor activity of QoIs against many plant pathogens, including *P. teres* and *M. graminicola*. Resistance of *M. graminicola* populations to QoIs, associated with the G143A mutation in the *cytochrome b* gene, is now widespread, resulting in total failure of these fungicides in many European countries including the UK. Reports in France and the UK suggested that there is also partial resistance to QoIs in *P. teres* isolates associated with the F129L mutation. The initial focus of this work was therefore on the effect of the F129L mutation in *cytochrome b* in isolates of *P. teres*. In addition, in *M. graminicola*, there has been a significant decline in the efficacy of triazoles in several countries and this drop in activity has been related by many authors to multiple mechanisms, including alterations in the *CYP51* gene (Stergiopoulos *et al.*, 2003; Cools *et al.*, 2005; Chassot *et al.*, 2008; Cools and Fraaije, 2008; Leroux and Walker, 2011).

Based on the resistance situations described above, the development of fungicide resistance in both *P. teres* and *M. graminicola* has been investigated. Such data could, in the future, be valuable for resistance-management strategies.

7.1 *Pyrenophora teres*; detection of F129L mutation and fitness costs

This research investigated the presence of the F129L mutation in a total of 23 isolates obtained. The results revealed that the mutation was found more frequently in recent isolates, compared with old isolates of *P. teres*, derived from culture collections. In the UK isolates of *P. teres* collected in the 2008 season, it was found that eight isolates out of 10 carried the F129L mutation. In comparison, only 3 out of 13 isolates that were collected in previous years (most of them from UK) had this mutation. This widespread nature of the F129L mutation was confirmed by Jorgenson (2008), who reported that since 2008 it has been on the increase within UK and French populations of the net blotch pathogen. This increase in the proportions of *P. teres* isolates carrying

the F129L mutation reflect selective pressure by exposure to QoI fungicides, since their introduction to control net blotch of barley provided an advantage to insensitive mutants within the pathogen population. Sequence analysis of the *cyt b* gene also revealed that the change in SNPs were from TTC to TTA in all recent UK isolates. This perhaps indicates that the F129L mutations had not occurred independently, suggesting that they may have arisen from one single mutation event, with subsequent further distribution. Sequence of the *cyt b* gene in the current research, however, did extend to cover to the whole *cyt b* gene of *P. teres* and did not amplify the remainder of the gene fragments which might contained the G137R or G143A mutations. However, the FRAC QoI working group (Leadbeater *et al.*, 2010) reported that the G137R mutation, although observed in other pathogens, has only recently found in *P. teres* in Germany and Ireland. Previous studies, however, indicated that in *P.teres*, an intron in the *cyt b* gene, immediately after the codon for the amino acid in position 143, is present. The G143A mutation would prevent splicing out of the intron, prior to transcription into mRNA, thereby disrupting functionality of the *cyt b* protein, leading to a lethal event. Thus the G143A mutation is unlikely to occur in *P. teres* (Semar *et al.*, 2007; Sierotzki *et al.*, 2007). QoI resistance was found in isolates without an intron between codons 143 and 144. This observation is supported by structural analysis of the *cytb* gene in field isolates of *B. cinerea*, which was classified into two groups: genes with an intron at 143 and those without an intron (Banno *et al.*, 2009).

Fitness costs due to the existence of the F129L mutation in terms of sporulation, growth rate and pathogenicity were investigated. Detection of a large diversity in these parameters demonstrated that there were no such penalties consistently associated with F129L mutant isolates. The results suggested that the overall phenotypic fitness of *P. teres* isolates was independent from the existence of this mutation. This lack of correlation between fitness and resistance was also found in previous studies in isolates of *P. teres* towards triazoles such as in triadimenol or propiconazole (Peever and Milgroom, 1994).

7.2 Fungicide activity associated with F129L in *P. teres*

The *in vitro* activity of QoIs suggests that there were some fungicides, such as pyraclostrobin, still active against isolates with the F129L mutation. This was also clear from detection of low EC_{50} values of this fungicide for mutant isolates. However, these results were not consistent with other QoIs because they showed lower activities, having greater EC_{50}s. This might suggest that other QoIs are compromised by the F129L mutation *in vitro*. However, different results *in vitro* and *in planta* have been seen, where picoxystrobin, albiet affected to some degree, showed the best activity against mutant isolates compared to other QoIs. Pyraclostrobin which was the most active *in vitro*, exhibited less *in planta* efficacy against most mutant isolates. Based on the results obtained in both *in vitro* and *in planta* assays, it can be concluded that the performance of some QoIs was affected by the F129L mutation in isolates of *P. teres*. Nevertheless, it seemed that the impact of this mutation varied for each fungicide, depending on the isolate. This indicates that different isolates with the F129L mutation behaved independently with each member of the QoI fungicide group. This is supported by sensitivity studies in transformed strains of *S. cerevisiae* reported by Fisher *et al.* (2004), where they have shown that different QoIs are not equally affected by the F129L mutation. However, the results of previous studies obtained by Semar *et al.* (2007) revealed that the field performance of pyraclostrobin, in experiments conducted in 2005-2006 in France, was not affected by the F129L mutation and the fungicide provided good control of net blotch of barley in fields with different frequencies of the mutation. On the other hand and in the same experiment they supported findings reported here by confirming the existence of variation among QoI efficacies when they found that pyraclostrobin outperformed azoxystrobin in controlling net blotch carrying the F129L mutation.

The performance of triazoles was also assessed both *in vitro* and *in planta*. Most triazoles, except epoxiconazole which was as good as pyraclostrobin *in vitro*, showed low activity, both *in vitro* and *in planta*, against most *P. teres* isolates regardless of the presence of the F129L mutation. However, other factors may have caused low activity of triazoles towards *P. teres* isolates.

Previous studies have confirmed this observation in many plant pathogens. For instance, resistance to azoles has been found in field isolates of *P. teres* towards triadimenol (Peever and Milgroom, 1992; Campbell and Crous, 2002) and to prochloraz (Serenius and Manninen, 2006).

Using fungicide mixtures comprising QoIs and DMIs may be an alternative to the use of a single fungicide. The application of Fandango (fluoxastrobin plus prothioconazole), as an example of such mixtures, was tested and it was found that in spite of low activity *in vitro*, this fungicide exhibited high efficacy *in planta*. Previous reports also stated that the best protection of barley plants from net blotch was achieved by the mixtures such as Fandango (Oxley and Hunter, 2005) and Opera (epoxiconazole plus pyraclostrobin) (Semar *et al.*, 2007). On the other hand the experimental SDHI fungicide formulation (based on penthiopyrad) was used in this study, and outperformed all other fungicides tested, both *in vitro* and *in planta*, achieving a very high performance against isolates of *P. teres* with the F129L mutation.

A comparative study of fungicide efficacy *in planta*, in association with the existence of the F129L mutation, was performed between conventional (visual) and quantitative (using q-PCR) assessment. A strong positive correlation between both assessments indicated the accuracy of the PCR-based method in assessing fungicide efficacy by quantitative assessment of pathogen DNA in the plant tissues. The method could be used as an alternative to conventional assessment. This is mainly because it is fast and measures very low amounts of fungal DNA, which might not result in visible lesions (McCartney *et al.*, 2003; Guo *et al.*, 2007). This could therefore measure fungicide efficacy and disease progression before visible symptoms are apparent (Schena *et al.*, 2004; Guo *et al.*, 2006).

From the results of this study it is apparent that *in vitro* studies do not always reflect fungicide performance *in planta*. Therefore, care is needed in evaluating fungicide performance from genotyping, *in vitro* and/or *in planta* experiments. A combination of these approaches is important. It can be speculated that the effect of F129L mutations in *P. teres* is moderate and not as serious as G143A in other plant pathogens such as *M. graminicola*. Furthermore, the impact of

the F129L mutation varied between QoI members. Some QoI fungicides still give good protection of barley plants against net blotch, despite the presence of the F129L mutation. Mixtures and new formulations may be alternatives to single QoIs. Triazoles, for unknown reasons, did not consistently show high activity.

7.3 *Mycospharella graminicola*, *CYP51* alterations and fitness costs

The results of genotyping using PCR methods indicated that mutations in the *CYP51* gene are frequent in the newly-obtained *M. graminicola* strains, compared to older isolates. Alterations and deletions occurred at 12 positions. Genotypes were in most cases, characterised by combinations of several mutations (Table 5.3). These combinations were used to classify isolates in genotypes as previously suggested by Leroux *et al*., (2006) and Leroux *et al*., (2007). A high frequency of genotypes with the I381V mutations (R6, R7, and R8 was observed. This may suggest that this change occurred because of the selection pressures from continuous use of azole fungicides on isolates of *M. graminicola*. This high level of I381V genotypes (75%) was also found in recent studies on European *M. graminicola* populations, where the frequency was increased from 40% in 2004 to 67% in 2006 (Fraaije *et al.*, 2007) and to 70% in samples screened later *in planta* using allele-specific q-PCR (Selim, 2009). This trend may therefore have started several years ago. Chassot *et al*. (2008) confirmed the occurrence of a significant change in *M. graminicola* genotype composition over the last 2 decades. Wild-type isolates disappeared while the genotypes R3 to R6 predominated. The rarity of isolates with Y137F is another outcome of current research, reflecting the effect of fungicide pressure on the emergence or disappearance of genotypes, where a decline of genotypes with Y137F was found in recent populations of *M. graminicola*. It has been suggested that isolates carrying the Y137F SNP are less sensitive to triadimenol, an azole fungicide introduced in the late 1970s and now no longer used for *M. graminicola* control (Leroux *et al.*, 2007). Due to these frequent alterations and changes in recent European populations of *M. graminicola*, 12 R-groups that respond differently to different triazoles have been found (Cools *et al.*, 2011; Leroux and Walker, 2011). However, the diversity of these

mutations seemed not to have any effect on the pathogenicity and on mycelial growth rates.

7.4 Fungicide activity associated with *CYP51* mutations in *M. graminicola*

Three *in vitro* methods were employed to measure EC_{50}s of *M. graminicola* isolates towards azole fungicides. The microtitre plates methods, with or without the growth indicator Alamar Blue, gave high data variability between replicates of the same treatment. This was probably the result of bacterial contamination that led to detection of higher absorbance values. In this regard, Pijls *et al*. (1994) also found the occurrence of bacterial contaminations in an entire microtitre plate row when inoculated with pycnidiospores of *M. graminicola* resulting in higher absorbance using a plate reader. However, other reasons such as use of commercially-formulated fungicide products instead of pure technical grade materials might have been involved. Using active ingredients contained in commercial products, that included components such as emulsifiers, may give turbidity to the liquid media compared to the pure technical materials, which tend to give clear solutions at the concentrations used. This added further errors to the absorbance values obtained by plate readers, giving lack of reliability to data obtained. Technical samples of all pure active ingredients were not readily available for this research programme. The second microtitre method using a colourimetric method, did not work with azole fungicides, although it was used previously with QoIs, in detecting insensitive *M. graminicola* isolates possessing the G143A mutation (Professor John Lucas, personal communication). Other researchers also did not recommend the use of microtitre methods incorporating Alamar Blue, due to the resultant data having high standard deviations from the growth averages, reflecting a lack of reliability of this method (Siah *et al.*, 2008; Siah *et al.*, 2010). As the indicator detects metabolic activity of organisms, it may be more suitable for fungicides which inhibit energy production (eg QoIs and SDHIs) rather than those which interfere with membrane integrity, such as SBIs. In contrast, the third method attempted, which depended on measuring apical germ tube growth on solid media amended with fungicides, was found to detect successfully sensitivity of isolates to fungicides. The method, which was found

in this current study to be laborious and time consuming, used as an alternative to microtitre method which was used widely and successfully for *in vitro* assays for a large number of isolates with many plant pathogens. However, previous work by Leroux *et al*. (2007) claimed that the method, measuring apical germ tube growth grown on solid medium, was found to be more sensitive than the microtitre tests.

From the results obtained in this study it was apparent that genotypes characterised as S were sensitive *in vitro* showing low EC_{50}s and also in disease control *in planta*. The R3+ and R4 genotypes, although exhibiting a slightly higher EC_{50}s than the S genotype, were also sensitive towards all azoles tested in *in planta* assays. On the other hand, the R5 genotypes were sensitive towards azoles, such as tebuconazole, but less sensitive to prochloraz *in vitro*, supporting the results obtained from *in planta* assays, where this genotype was found to be sensitive to tebuconazole but less sensitive to prochloraz. The results also confirm that most genotypes with I381V (R6a, R7 and R8) were less sensitive to tebuconazole but sensitive to prochloraz either *in planta*. This was entirely in agreement with the results of many researchers confirming the same fact (Fraaije *et al.*, 2007; Leroux *et al.*, 2007). In support of this, Fraaije *et al*. (2008) found high I381V frequency (>95%) in tebuconazole-treated plots but much lower frequency (16-22%) in plots treated with prochloraz. This could confirm the concept that genotypes with the I381V mutation were selected by tebuconazole and has a tight relationship with use of this fungicide. As an exception of the above concept, prothioconazole was found to give higher disease control against the R7 isolate (Ger-3-2) compared to tebuconazole and epoxiconazole. A recent study confirmed that prothioconazole behaved differently from other triazoles in its mechanism of inhibition. It was found to be a competitive inhibitor of substrate binding to MgCyp51 with 840-fold less affinity than epoxiconazole and tebuconazole (Parker *et al.*, 2011).

In relation to reductions in DMI efficacy, Clark (2006) stated that not all azoles are equally affected by mutations in *CYP51* and resistance has developed slowly, although this group of fungicide targets the single protein, sterol-14α-demethylase. This slow development of resistance in DMIs might be because

combinations of alterations in the *CYP51* gene are responsible for resistance, instead of single-target site, where a single amino acid substitution confers a high level of resistance (Sanglard *et al.*, 1998b). However, the recently-emerged *CYP51* genotypes carrying combinations of alterations D134G, V136A, Y461S, and S524T revealed a substantial impact on sensitivity to most widely used triazoles which include epoxiconazole and prothioconazole (Cools *et al.*, 2011). However, the site includes position 524 of the *CYP51* gene, which was not sequenced in this study. Other fungicides that were used for comparison to azoles showed variable activities. Chlorothalonil was found to be less effective. This observation was supported by previous authors who found that chlorothalonil exhibited low activity when applied as sole fungicide against *M. graminicola* isolates compared with the application of a mixture of azoxystrobin and epoxiconazole (McCartney *et al.*, 2007).

The efficacy of mixed formulations, such as Fandango, Tracker and Prosaro, were variable. Interestingly, Prosaro as a mixture of two triazoles (prothioconazole and tebuconazole), exhibited high activity against a wider range of isolates ranging from sensitive to highly resistant (S, R3+, R4, R5, and R7) with different RF values. This might be because of the potential of broadened activity exhibited by the mixture of two triazoles combined in Prosaro against *M. graminicola* strains belonging to sub-populations, with different sensitivity to triazoles (Jorgensen, 2008). The use of single DMIs may select for specific genotypes, whilst mixtures of DMIs with small variation in sensitivity range between genotypes may minimize the preferential selection of resistant strains and ensure consistent disease control (Chassot *et al.*, 2008). On the other hand, mixtures of triazoles with prochloraz may be adopted as an anti-resistance strategy based on the fact that this imidazole derivative is active towards isolates exhibiting resistance towards triazoles (Leroux *et al.*, 2008c). FRAC also suggested the use of mixtures instead of a sole product. According to the recommendations, the mixtures can broaden the scale of disease control of a product. The combination of specific characteristics of the components of a mixture will increase the activity of the product. The components of the mixture must have activity against the field populations of the target pathogen when used alone. The activity profiles of the components should also be combined in such a way that effective disease

management is achieved (Anonymous, 2010b). The interaction between field populations of *M. graminicola* and triazole fungicides is typical in this respect. Because of the presence of sub-populations of *M. graminicola* with differing substitutions to different SBIs, combination of fungicides from this one class may still provide beneficial effects in management of fungicide resistance.

The novel pyrazole carboximide SDHI (penthiopyrad), as described earlier in the net blotch section of this thesis, where it gave a very high performance against isolates with the F129L mutation, outperformed other fungicides. This suggests the use of new products of this type may provide an alternative measure to control *M. graminicola* isolates with prevalent *CYP51* mutations. Care must be taken, however, in adoption of effective strategies to manage resistance to this new chemistry, to ensure they do not suffer the same fate as QoIs. Such issues are high on the FRAC agenda.

The first use of SDHI fungicides (eg carboxin, an oxathiin carboximide), which were launched in 1960s, was against a limited group of plant pathogens belonging to basidiomycetes, such as Rhizoctonia diseases (Zhang *et al.*, 2009). In contrast to original SDHIs, newer active ingredients, such as boscalid (a pyridine carboximide, launched in 2003), have broad spectrum activity against a wide range of pathogens. This molecule was, however, not market as a single active ingredient product for cereal disease control, but was later combined with epoxiconazole in products such as Tracker for use in this market. The latest generation of pyrazole carboximide SDHIs has just been or is about to be launched. These include bixafen (Bayer), isopyrazam (Syngenta), fluxapyroxad (BASF) and penthiopyrad (DuPont; used in this work). Such molecules have significantly greater activity against a broad spectrum of cereal pathogens than the earlier generations of SDHIs and are likely to have a very important role in cereal disease crop protection.

Resistance to older SDHIs, such a carboxin, and also to the new generation of SDHIs have been observed in several pathogens (Keon *et al.*, 1991; Avenot *et al.*, 2009; Miyamoto *et al.*, 2010). Their highly-specific mode of action dictates that resistance is possible from a single point mutation affecting the binding site. The risk of resistance evolving to this class of fungicides should thus be

considered to be high. However, due to a unique mode and site of action of SDHIs, no cross resistance with other chemical classes has been observed (Avenot *et al.*, 2008). The development of products or tank mixes which combine SDHIs with triazoles or with multi-site products such as chlorothalonil will be part of a recommended strategy to prevent (or delay) the development of resistance to this important new chemistry. This concept is supported by FRAC and HGCA in their recommendations to farmers (Anonymous 2011).

It was found that resistance of *M. graminicola* isolates tested in this study towards triazoles was variable and this was possibly because of the presence of more than one mechanism for insensitivity (Stergiopoulos *et al.*, 2003; Cools *et al.*, 2004; Cools, 2007). These mechanisms include: alterations in *CYP51* gene, resulting in decrease of the affinity of DMIs for their target site, *CYP51* overexpression, causing high levels of sterol 14α demethylase, and an increase in the efflux of DMIs due to the up-regulation of ABC (ATP-binding cassette) or MFS (major facilitator superfamily) transporters in the membrane (Sanglard *et al.*, 1998a; Akins, 2005). In several previous studies isolates of *M. graminicola* with reduced sensitivity to triazoles, such as epoxiconazole, have been identified but all resistance mechanisms operating in these isolates were not fully defined (Cools *et al.*, 2005). However, the results of this study indicated that *CYP51* gene alterations can be considered one of the mechanisms conferring resistance in *M. graminicola* isolates; this was also reported by Leroux and Walker (2011) where they stated that *CYP51* mutations were the main mechanism to alter sensitivity in isolates of *M. graminicola,* at least until 2007. Due to the effect of multiple mechanisms to account for resistance of *M. graminicola* isolates to DMIs, which is of polygenic nature, the resistance risk is thus considered moderate (Chassot *et al.*, 2008).

Assessing the fungicide performance with q-PCR in the presence of fungicide resistance was found to be a very useful tool, especially in diseases with long latent periods, such as STB, where visible symptoms can be slow to develop.

7.5 Conclusions and future work

This study found a widespread occurrence of the F129L mutation in recent *P. teres* isolates in the UK. This rapid increase is due to the continuous fungicide

selection pressure by use of QoIs, which has selected mutant isolates with increasing time of use. However, bioassay results found that the mutation was not as serious as the G143A mutation, present in other plant pathogens, and QoIs should continue to give effective control of *P. teres*. There were also no phenotypic fitness costs in relation to the mutation.

In *M. graminicola* isolates, multi-allele alterations (substitutions and deletions) were detected. A total of 15 alterations were detected in 12 positions in *CYP51* gene. The substitution characterised V136A was found to be selected by prochloraz while genotypes characterised as I381V were differentially selected by tebuconazole. The study confirmed previous findings that these alterations contribute as major factors to cause resistance in the azole group of fungicides. Nevertheless, fungicide bioassays revealed variability in the activity within DMIs. A mixture of compounds comprising different modes of actions will play an essential role in disease management programmes. The introduction of new classes of chemistry also offers opportunities for more effective resistance management.

Future research to extend the programme reported here might include:

- Examination of isolates of *P. teres* which differ in triazole sensitivity, for modifications associated with the *CYP51* gene.
- Determination of the effect of other mutations in *M. graminicola* associated with over-expression of the *CYP51* gene and the activity of ABC (ATP-binding cassette) transporters efflux systems, to further understand variability in SBI efficacy.
- Extension of the research to include a larger population of isolates of both *P. teres* and *M. graminicola*, collected from different locations over a larger time scale.
- Further evaluation of other SDHI fungicides, alone and in combination with other active groups, to provide more information on future protection of cereal crops from these important pathogens.

Bibliography

Adhikari, T B, Balaji, B, Breeden, J, Crane, C F, Anderson, J M, Goodwin, S B (2004a). Real-time PCR analysis of genes expressed during wheat-*Mycosphaerella graminicola* interactions. *Phytopathology* **94**: S2-S3.

Adhikari, T B, Cavaletto, J R, Dubcovsky, J, Gieco, J O, Schlatter, A R, Goodwin, S B (2004b). Molecular mapping of the Stb4 gene for resistance to *Septoria tritici* blotch in wheat. *Phytopathology* **94**: 1198-1206.

Akins, R A (2005). An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Medical Mycology* **43**: 285-318.

Al-Tikrity, M N (1987). A simple technique for production of *Drechslera teres* spores. *Transactions of the British Mycological Society* **89**: 402-402.

Albertini, L, Barrault, G, Sarrafi, A, Caron, D (1995). Investigations in the ethiology , biology , epidemiology and control of the causal agents of barley leaf blights in France. *Rachis* **14**: 13-25.

Ali, S, Singh, P K, McMullen, M P, Mergoum, M, Adhikari, T B (2008). Resistance to multiple leaf spot diseases in wheat. *Euphytica* **159**: 167-179.

Allen, G C, Flores-Vergara, M A, Krasnyanski, S, Kumar, S, Thompson, W F (2006). A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. *Nature Protocols* **1**: 2320-2325.

Alonso, G D, Llorente, B, Bravo-Almonacid, F, Cvitanich, C, Orlowska, E, Torres, H N, Flawia, M M (2010). A quantitative real-time PCR method for *in planta* monitoring of *Phytophthora infestans* growth. *Letters in Applied Microbiology* **51**: 603-610.

Alston, J M, Beddow, J M, Pardey, P G (2009). Agricultural Research, Productivity, and Food Prices in the Long Run. *Science* **325**: 1209-1210.

Anaya, N, Roncero, M I G (1996). Stress-induced rearrangement of Fusarium retrotransposon sequences. *Molecular & General Genetics* **253**: 89-94.

Anke, T, Hecht, H J, Schramm, G, Steglich, W (1979). Antibiotics from Basidiomycetes .9. Oudemansin, an Antifungal Antibiotic from *Oudemansiella mucida* (Schrader Ex Fr) Hoehnel (Agaricales). *Journal of Antibiotics* **32**: 1112-1117.

Anke, T, Oberwinkler, F, Steglich, W, Schramm, G (1977). Strobilurins - new antifungal antibiotics from basidiomycete *Strobilurus tenacellus* (Pers Ex Fr) Sing. *Journal of Antibiotics* **30**: 806-810.

Annone, J A, Calzolari, O, Polidoro, O, Conta, H (1991). Efecto de la mancha de la hoja causada por *Septoria tritici* sobre el rendimiento. INTA EEA Pergamino Informe. 122, 4.

Anon (1998). Strobilurins. Fungicide Resistance Action Committee (FRAC) Fungicide Use Guidelines (Principles for Effective Resistance Management), July 1998., Global Crop Protection Federation, Brussels, Belgium, 7pp.

Anonymous. (2002). A History of Crop Protection and Pest Control in our Society: Analyzing the risks, balancing the benefits: the facts on pesticides and human safety., from www.croplife.ca/.

Anonymous. (2010a). Farming and Food Brief. Retrieved 6/6/2011, from <http://www.defra.gov.uk>

Anonymous (2010b). FRAC recommendations for fungicide mixtures designed to delay resistance evolution, FRAC**:** 7pp.

Anonymous. (2010c). International wheat production statistics. from http://en.wikipedia.org/wiki/International_wheat_production_statistics.

Anonymous. (2011a). Disease results-Winter wheat commercial crops survey 2009/2010. Retrieved 28/8/2011, 2011, from http://www.cropmonitor.co.uk/wwheat/surveys/highlight2010.cfm.

Anonymous. (2011b). Farming and the Countryside - What's going on and Why. Retrieved 20 May, 2011, from http://www.ukagriculture.com/crops/wheat.cfm.

Anonymous. (2011c). Fungicide Resistance Management in Cereals. Retrieved 17 May, 2011, from www.pesticides.gov.uk.

Anonymous. (2011d). Wheat disease management guide, HGCA. Retrieved 19/8/2011, 2011, from http://www.hgca.com/document.aspx?fn=load&media_id=6943&publicationId=4406.

Arabi, M I E, Al-Safadi, B, Charbaji, T (2003). Pathogenic variation among isolates of *Pyrenophora teres*, the causal agent of barley net blotch. *Journal of Phytopathology-Phytopathologische Zeitschrift* **151**: 376-382.

Arseniuk, E, Goral, T, Scharen, A L (1998). Seasonal patterns of spore dispersal of *Phaeosphaeria* spp. and *Stagonospora* spp. *Plant Disease* **82**: 187-194.

Ashizawa, T, Takahashi, M, Moriwaki, J, Hirayae, K (2010). Quantification of the rice false smut pathogen *Ustilaginoidea virens* from soil in Japan using real-time PCR. *European Journal of Plant Pathology* **128**: 221-232.

Avenot, H, Morgan, D P, Michailides, T J (2008). Resistance to pyraclostrobin, boscalid and multiple resistance to Pristine (R) (pyraclostrobin plus boscalid) fungicide in *Alternaria alternata* causing alternaria late blight of pistachios in California. *Plant Pathology* **57**: 135-140.

Avenot, H, Sellam, A, Michailides, T (2009). Characterization of mutations in the membrane-anchored subunits AaSDHC and AaSDHD of succinate dehydrogenase from *Alternaria alternata* isolates conferring field resistance to the fungicide boscalid. *Plant Pathology* **58**: 1134-1143.

Bach, E, Christensen, S, Dalgaard, L, Larsen, P O, Olsen, C E (1979). Structures, properties and relationship to the aspergillomarasmines of toxins produced by *Pyrenophora teres*. *Physiological Plant Pathology* **14**: 41-46.

Bahri, B, Kaltz, O, Leconte, M, de Vallavieille-Pope, C, Enjalbert, J (2009). Tracking costs of virulence in natural populations of the wheat pathogen, *Puccinia striiformis* f.sp.*tritici*. *Bmc Evolutionary Biology* **9**.

Baik, B K, Ullrich, S E (2008). Barley for food: Characteristics, improvement, and renewed interest. *Journal of Cereal Science* **48**: 233-242.

Baldwin, B C, Rathmell, W G (1988). Evolution of Concepts for Chemical Control of Plant-Disease. *Annual Review of Phytopathology* **26**: 265-283.

Banno, S, Yamashita, K, Fukumori, F, Okada, K, Uekusa, H, Takagaki, M, Kimura, M, Fujimura, M (2009). Characterization of QoI resistance in Botrytis cinerea and identification of two types of mitochondrial cytochrome b gene. *Plant Pathology* **58**: 120-129.

Barnes, E H (1964). Changing Plant Disease Losses in Changing Agriculture. *Phytopathology* **54**: 1314-1319.

Barrault, G, Alali, B, Petitprez, M, Albertini, L (1982). Contribution to the study of the toxic activity of *Helminthosporium teres*, a parasite on barley (*Hordeum vulgare*). *Canadian Journal of Botany-Revue Canadienne De Botanique* **60**: 330-339.

Bartlett, D W, Clough, J M, Godwin, J R, Hall, A A, Hamer, M, Parr-Dobrzanski, B (2002). The strobilurin fungicides. *Pest Management Science* **58**: 649-662.

Bateman, G L (1994). Selection in Populations of the Eyespot Fungus in Continuous Wheat by Repeated Applications on Carbendazim and Prochloraz. *Fungicide Resistance*: 219-224.

Bates, J A, Taylor, E J A (2001). Scorpion ARMS primers for SNP real-time PCR detection and quantification of *Pyrenophora teres*. *Molecular Plant Pathology* **2**: 275-280.

Bates, J A, Taylor, E J A, Kenyon, D M, Thomas, J E (2001). The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. *Molecular Plant Pathology*. 2**:** 49-57.

Baude, F J, Gardiner, J A, Han, J C Y (1973). Characterization of residues on plants following foliar spray applications of Benomyl. *Journal of Agricultural and Food Chemistry* **21**: 1084-1090.

Bayles, R (1999). The interaction of strobilurin fungicides with cereal varieties. *Plant Varieties and Seeds* **12**: 129-140.

Beattie, A D (2006). Genomic analysis of *Pyrenophora teres*: A virulence gene mapping, karyotyping and genetic map construction. Department of Plant Sciences, University of Saskatchewan. PhD thesis**:** 150pp.

Beck, C, Oerke, E C, Dehne, H W (2002). Impact of strobilurins on physiology and yield formation of wheat. *Mededelingen - Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent.* **67**: 181-187.

Behall, K M, Scholfield, D J, Hallfrisch, J (2004). Diets containing barley significantly reduce lipids in mildly hypercholesterolemic men and women. *American Journal of Clinical Nutrition* **80**: 1185-1193.

Bockus, W W, Appel, J A, Bowden, R L, Fritz, A K, Gill, B S, Martin, T J, Sears, R G, Seifers, D L, Brown-Guedira, G L, Eversmeyer, M G (2001). Success stories: Breeding for wheat disease resistance in Kansas. *Plant Disease* **85**: 453-461.

Bouvet, G F, Jacobi, V, Plourde, K V, Bernier, L (2008). Stress-induced mobility of OPHIO1 and OPHIO2, DNA transposons of the Dutch elm disease fungi. *Fungal Genetics and Biology* **45**: 565-578.

Brent, K J (1995). Fungicide resistance in crop pathogens: how can it be managed? Brussels, Global Crop Protection Federation (GCPF), Brussels, reprinted in the United Kingdom 1999. p3

Brent, K J, Hollomon, D W (2007). Fungicide resistance in crop pathogens: How can it be managed? Fungicide Resistance Action Committee, FRAC, 2nd revised edition, Brussels, Belgium.

Brokenshire, T (1975). Wheat debris as an inoculum source for seedling infection by *Septoria tritici*. *Plant Pathology* **24**: 202-207.

Brown, J S, Kellock, A W, Paddick, R G (1978). Distribution and Dissemination of *Mycosphaerella graminicola* (Fuckel) Schroeter in Relation to Epidemiology of Speckled Leaf Blotch of Wheat. *Australian Journal of Agricultural Research* **29**: 1139-1145.

Brownell, K H, Gilchrist, D G (1979). Assessment of yield loss in wheat cultivars infected by *Septoria tritici* in California. *Phytopathology* **69**: 1022-1023.

Brunner, P C, Stefanato, F L, McDonald, B A (2008). Evolution of the *CYP51* gene in *Mycosphaerella graminicola*: evidence for intragenic recombination and selective replacement. *Molecular Plant Pathology* **9**: 305-316.

Caldwell, R M, Narvaes, I (1960). Losses to winter wheat from infection by *Septoria tritici*. *Phytopathology* **50**: 630-630.

Calvert, G M, Talaska, G, Mueller, C A, Ammenheuser, M M, Au, W W, Fajen, J M, Fleming, L E, Briggle, T, Ward, E (1998). Genotoxicity in workers exposed to methyl bromide. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis* **417**: 115-128.

Campbell, G F, Crous, P W (2002). Fungicide sensitivity of South African net- and spot-type isolates of *Pyrenophora teres* to ergosterol biosynthesis inhibitors. *Australasian Plant Pathology* **31**: 151-155.

Campbell, G F, Lucas, J A, Crous, P W (2002). Evidence of recombination between net- and spot-type populations of *Pyrenophora teres* as determined by RAPD analysis. *Mycological Research* **106**: 602-608.

Campbell, R (1989). Biological control of microbial plant pathogens., Cambridge University Press, Cambridge, UK. pp. 70-76.

Carey, J K, Bravery, A F (1989). A Technique for Assessing the Preventative Efficacy against Decay Fungi of Preservative Treatments Applied to Wood. *International Biodeterioration* **25**: 439-444.

Carlile, W R (1998). New Studies in Biology: Control of Crop Diseases, Edward Arnold, UK. 100p.

Carmona, M, Barreto, D, Moschini, R, Reis, E (2008). Epidemiology and Control of Seed-borne *Drechslera teres* on Barley. *Cereal Research Communications* **36**: 637-645.

Carmona, M A, Barreto, D E, Reis, E M (1999). Detection, transmission and control of *Drechslera teres* in barley seed. *Seed Science and Technology* **27**: 761-769.

Cavallero, A, Empilli, S, Brighenti, F, Stanca, A M (2002). High (1 -> 3,1 -> 4)-beta-glucan barley fractions in bread making and their effects on human glycemic response. *Journal of Cereal Science* **36**: 59-66.

Chassot, C, Hugelshofer, U, Sierotzki, H (2008). Sensitivity of *CYP51* Genotypes to DMI Fungicides in *Mycosphaerella graminicola*. Modern Fungicides and Antifungul Compounds. H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russell and H. Lyr (eds.), DPG, Selbstverlag, Germany. 5**:** 129-136.

Chaube, H S, Pundhir, V S (2005). Crop Diseases and Their Management, Prentice-Hall of India Private Limited, New Delhi. p308

Chen, C J, Wang, J X, Luo, Q Q, Yuan, S K, Zhou, M G (2007). Characterization and fitness of carbendazim-resistant strains of *Fusarium graminearum* (wheat scab). *Pest Management Science* **63**: 1201-1207.

Chen, Y, Zhou, M G (2009). Characterization of *Fusarium graminearum* Isolates Resistant to Both Carbendazim and a New Fungicide JS399-19. *Phytopathology* **99**: 441-446.

Chin, K M, Chavaillaz, D, Kaesbohrer, M, Staub, T, Felsenstein, F G (2001). Characterizing resistance risk of *Erysiphe graminis* f.sp *tritici* to strobilurins. *Crop Protection* **20**: 87-96.

Clark, B, Fraaije, B, Lucas, J, Cools, H J (2010). Septoria resistance and azole use 2010. RRA Newsletter**:** p4.

Clark, D C (2003). Agronomic implications of some morphological and biochemical effects of trifloxystrobin in wheat growing. *Pflanzenschutz-Nachrichten* **56**: 281-296.

Clark, W S (2006). *Septoria tritici* and azole performance. In: Fungicide Resistance: Are we winning the battle but lossing the ware? R. J. Bryson, F. J. Burnett, V. Foster, B. A. Fraaije and R. Kennedy, Aspects of Applied Biology. 78**:** 127-132.

Clemons, G P, Sisler, H D (1969). Formation of a fungiotoxic derivative from Benlate. *Phytopathology* **59**: 705-&.

Clifford, B C, Jones, D (1981). Net Blotch of Barley. UK Cereal Pathogen Virulence Survey 1980 Annual Report**:** 71-77.

Conway, K E (1996). An overview of the influence of sustainable agricultural systems on plant diseases. *Crop Protection* **15**: 223-228.

Cook, R J (1986). Interrelationships of the plant health and the sustainability of agriculture, with special reference to plant diseases. *American Journal of Alternative Agriculture* **1**: 19-24.

Cook, R J, Hardwick, N V (1990). Disease-control in combinable crops - meeting the challenge of the 1990s. Brighton Crop Protection Conference - Pests and Diseases, 1990 : Proceedings, Vols 1-3**:** 477-486.

Cook, R J, Veseth, R J (1991). Wheat Health Management. Minnesota, USA, The American Phytopathological Society, 152p.

Cools, H J (2007). Molecular mechanisms correlated with reduced azole sensitivity in *Mycosphaerella graminicola*. *Phytopathology* **97**: S24-S24.

Cools, H J, Fraaije, B A (2008). Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Management Science* **64**: 681-684.

Cools, H J, Fraaije, B A, Bean, T P, Lucas, J A (2008). Characterization of mechanisms corralated with reduced azole sensitivity in Mycosphaerella graminicola. *Journal of Plant Pathology* **90**: S2.136.

Cools, H J, Fraaije, B A, Kim, S H, Lucas, J A (2006). Impact of changes in the target P450CYP51 enzyme associated with altered triazole-sensitivity in fungal pathogens of cereal crops. *Biochemical Society Transactions* **34**: 1219-1222.

Cools, H J, Fraaije, B A, Lucas, J A (2004). Molecular mechanisms conferring reduced sensitivities to triazoles in UK isolates of *Septoria tritici*. *Phytopathology* **94**: S20-S21.

Cools, H J, Fraaije, B A, Lucas, J A (2005). Molecular examination of *Septoria tritici* isolates with reduced sensitivities to triazoles. Modern Fungicides and Antifungal Compounds. H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russell and H. Lyr. Alton, UK. IV**:** p. 103–114.

Cools, H J, Mullins, J G L, Fraaije, B A, Parker, J E, Kelly, D E, Lucas, J A, Kelly, S L (2011). Impact of Recently Emerged Sterol 14 alpha-Demethylase (CYP51) Variants of Mycosphaerella graminicola on Azole Fungicide Sensitivity. *Applied and Environmental Microbiology* **77**: 3830-3837.

Cools, H J, Parker, J E, Kelly, D E, Lucas, J A, Fraaije, B A, Kelly, S L (2010). Heterologous Expression of Mutated Eburicol 14 alpha-Demethylase (*CYP51*) Proteins of *Mycosphaerella graminicola* To Assess Effects on Azole Fungicide Sensitivity and Intrinsic Protein Function. *Applied and Environmental Microbiology* **76**: 2866-2872.

Cornell, H J, Hoveling, A W (1998). Wheat: Chemistry and Utilization. Lancaster, Pennsylvania, USA, Technomic Publishing Company, 426p.

Costantini, L (1984). The begening of agriculture in the Kachi Plain: The evidence of Mehrgarts. South Asian Archaeology 1981. Proceedings 6th International Conference Association of South Asian Archaelogists in Western Europe, , Cambridge, Cambridge University Press, pp.29-33.

Cunfer, B M (1997). Taxonomy and nomenclature of *Septoria* and *Stagonospora* species on small grain cereals. *Plant Disease* **81**: 427-428.

Darby, W J, Ghaliounugi, P, Grivetti, L (1977). Food-The gift of Osiris, Volumes 1 and 2, Academic Press, London.

Davis, R E, Whitcomb, R F (1971). Mycoplasmas, Rickettsiae, and Chlamydiae - Possible Relation to Yellows Diseases and Other Disorders of Plants and Insects. *Annual Review of Phytopathology* **9**: 119-&.

De Waard, M A (1994). Resistance to fungicides which inhibit sterol 14a-demethylation, a historical perspective. Fungicide Resistance. S. P. Heaney, D. Slawson, W. D. Hollomonet al, (eds.), British Crop Protection Council (BCPC) monograh, . 60**:** 3-10.

De Waard, M A, Georgopoulos, S G, Hollomon, D W, Ishii, H, Leroux, P, Ragsdale, N N, Schwinn, F J (1993). Chemical control of plant-diseases - problems and prospects. *Annual Review of Phytopathology* **31**: 403-421.

De Wolf, E. (2008). *Septoria tritici* blotch. Kansas State University Agricultural Experiment Station and Cooperative Extension Service. Kensas State plant pathology EP-133. Retrieved 15th May, 2011, from http://www.ksre.ksu.edu/library/plant2/ep133.pdf.

Deadman, M L, Cooke, B M (1985). A method of spore production for *Drechslera teres* using detached barley leaves. *Transactions of the British Mycological Society* **85**: 489-493.

Deas, A H B, Carter, G A, Clark, T, Clifford, D R, James, C S (1986). The Enantiomeric Composition of Triadimenol Produced during Metabolism of Triadimefon by Fungi .3. Relationship with Sensitivity to Triadimefon. *Pesticide Biochemistry and Physiology* **26**: 10-21.

Deas, A H B, Clifford, D R (1982). Metabolism of the 1,2,4-Triazolylmethane Fungicides, Triadimefon, Triadimenol, and Diclobutrazol, by *Aspergillus niger* (Vantiegh). *Pesticide Biochemistry and Physiology* **17**: 120-133.

Dekker, J (1982). Introduction in Fungicide Resistance in Crop Protection. In:. Fungicide Resistance in Crop Protection. J. Dekker and S. G. Georgopoulos, Centre for Agricultural Publishing and Documentation, Wageningen**:** p1-6.

Delye, C, Bousset, L, Corio-Costet, M F (1998). PCR cloning and detection of point mutations in the eburicol 14 alpha-demethylase (*CYP51*) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus. *Current Genetics* **34**: 399-403.

Delye, C, Laigret, F, CorioCostet, M F (1997). A mutation in the 14 alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. *Applied and Environmental Microbiology* **63**: 2966-2970.

Dickson, J G (1956). Diseases of field crops, 2nd edition. New york, McGraw-Hill Book Company, Inc. New York, NY. 517 pp.

Doohan, F M, Parry, D W, 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.

Douiyssi, A, Rasmusson, D C, Roelfs, A P (1998). Responses of barley cultivars and lines to isolates of *Pyrenophora teres*. *Plant Disease* **82**: 316-321.

Douiyssi, A, Rasmusson, D C, Wilcoxson, R D (1996). Inheritance of resistance to net blotch in barley in Morocco. *Plant Disease* **80**: 1269-1272.

Drechsler, C (1923). Some graminicolous species of *Helminthosporium*: I. *Journal of Agricultural Research* **24**: 0641-0740.

Duczek, L J, Sutherland, K A, Reed, S L, Bailey, K L, Lanford, G P (1999). Survival of leaf spot pathogens on crop residues of wheat and barley in Saskatchewan. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **21**: 165-173.

Duveiller, E, Fucikovsky, L, Rudolph, K (1997). The Bacterial Diseases of Wheat : Concepts and Methods of Disease Management. Mexico, D.F., CIMMYT, 78pp.

Duvert, P, Lacombe, J P, Machefer, G, Baudoin, P (1996). Efficacy of bromuconazole plus iprodione against the brown spot disease complex of barley. *Agro Food Industry Hi-Tech* **7**: 34-36.

Duvert, P, Vives, F (1997). A proposed microtiter method for the assessment on *in vitro* sensitivity of *Pyrenophora teres* to triazoles. *Med. Fac. Landbouww. Univ. Gent* **62**: 1097-1102.

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.

Elcock, S J, Turner, J A, Kendall, S J, Hollomon, D W, Jones, D, Black, L, Cooke, L R (2000). Potential for the development of reduced sensitivity to DMI fungicides in current control practices for *Mycosphaerella graminicola* in winter wheat in the UK. *Bcpc Conference: Pests & Diseases 2000, Vols 1-3, Proceedings* **1-3**: 407-414.

Elliot, R W (1973). Genetics of drug resistance. In:. Drug resistance and selectivity. E. Mihnih, (ed.), Academic Press, New York**:** p41-71.

EspinelIngroff, A, Bartlett, M, Bowden, R, Chin, N X, Cooper, C, Fothergill, A, McGinnis, M R, Menezes, P, Messer, S A, Nelson, P W, Odds, F C, Pasarell, L, Peter, J, Pfaller, M A, Rex, J H, Rinaldi, M G, Shankland, G S, Walsh, T J, Weitzman, I (1997). Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. *Journal of Clinical Microbiology* **35**: 139-143.

Eyal, Z (1971). Kinetics of Pycnospore Liberation in *Septoria tritici*. *Canadian Journal of Botany* **49**: 1095-&.

Eyal, Z (1999). The *Septoria tritici* and *Stagonospora nodorum* blotch diseases of wheat. *European Journal of Plant Pathology* **105**: 629-641.

Eyal, Z, Scharen, A L, Prescott, J M, Van Ginkel, M (1987). The septoria disease of wheat: concepts and methods of disease management. CIMMYT, Mexico City**:** 46pp.

Fai, P B, Grant, A (2009). A comparative study of *Saccharomyces cerevisiae* sensitivity against eight yeast species sensitivities to a range of toxicants. *Chemosphere* **75**: 289-296.

Fent, K, Hunn, J (1996). Cytotoxicity of organic environmental chemicals to fish liver cells (PLHC-1). *Marine Environmental Research* **42**: 377-382.

Fischbeck, G (2002). Contribution of Barley to agriculture: A Brief Overview. In: . Barley Science: Recent Advances from Molecular Biology to Agronomy of Yield and Quality. G. A. Salfer, J. L. Molina-Cano, R. Savin, J. L. Araus and I. Romagosa. (eds.), New York, Food Products Press, an Imprint of The Haworth Press**:** p1-5.

Fischer, R A, Byerlee, D, Edmesdes, G O (2009). Can Technology Deliver on the Yield Challenge to 2050? Paper presented as part of the: Expert Meeting on How to Feed the World in 2050, FAO, Rome.

Fisher, N, Brown, A C, Sexton, G, Cook, A, Windass, J, Meunier, B (2004). Modeling the Q(o) site of crop pathogens in *Saccharomyces cerevisiae* cytochrome b. *European Journal of Biochemistry* **271**: 2264-2271.

Forrer, H R, Zadoks, J C (1983). Yield reduction in wheat in relation to leaf necrosis caused by *Septoria tritici*. *Netherlands Journal of Plant Pathology* **89**: 87-98.

Fraaije, B (2007). Dynamics of fungicide resistant alleles in field populations of *Mycosphaerella graminicola*. *Phytopathology* **97**: S36-S36.

Fraaije, B A, Cools, H J, Fountaine, J, Lovell, D J, Motteram, J, West, J S, Lucas, J A (2005). Role of ascospores in further spread of QoI-resistant cytochrome b alleles (G143A) in field populations of *Mycosphaerella graminicola*. *Phytopathology* **95**: 933-941.

Fraaije, B A, Cools, H J, Kim, S H, Motteram, J, Clark, W S, Lucas, J A (2007). A novel substitution I381V in the sterol 14 alpha-demethylase (*CYP51*) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. *Molecular Plant Pathology* **8**: 245-254.

Fraaije, B A, Cools, H J, Motteram, J, Gilbert, S R, Kim, S H, Lucas, J A (2008). Adaptation of *Mycosphaerella garaminicola* Populations to Azole Fungicides in th UK. Modern Fungicides and Antifungal Compounds. H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russell and H. Lyr, DPG, Selbstverlag, Germany. V**:** p. 121-127.

Fraaije, B A, Lovell, D J, Baldwin, S (2002). Septoria epidemics on wheat: Combined use of visual assessment and PCR-based diagnostics to identify mechanisms of disease escape. *Plant Protection Science* **38**: 421-424.

Fraaije, B A, Lovell, D J, Coelho, J M, Baldwin, S, 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 of Plant Pathology* **107**: 905-917.

Fraaije, B A, Lucas, J A, Clark, W S, Burnett, F (2003). QoI resistance development in populations of cereal pathogens in the UK. The BCPC Conference Pests and Diseases. The British Crop Protection Council, Alton, Hampshire, UK**:** pp.689-694.

Friis, P, Olsen, C E, Moller, B L (1991). Toxin Production in *Pyrenophora teres*, the Ascomycete Causing the Net-Spot Blotch Disease of Barley (*Hordeum vulgare* L). *Journal of Biological Chemistry* **266**: 13329-13335.

Galassi, S, Vigano, L, Sanna, M (1996). Bioconcentration of organochlorine pesticides in rainbow trout caged in the river Po. *Chemosphere* **32**: 1729-1739.

Georgopoulos, S G (1982). Detection and measurement of fungicide resistance. In:. Fungicide Resistance in Crop Protection. J. Dekker and S. G. Georgopoulos, Centre for Agricultural Publishing and Documentation, Wageningen**:** p24.

Geschele, E E (1928). The response of barley to parasitic fungi *Helminthosporium teres* Sacc. *Bulletin of Applied Botany of Genetics and Plant-Breeding* **19**: 371-384 (in Rev. Appl. Mycol. 8, 165).

Gilchrist, L, Dubin, H J. (2007). Septoria diseases of wheat. from http://www.fao.org/DOCREP/006/Y4011E/y4011e0i.htm.

Gisi, U, Chin, K M, Knapova, G, Farber, R K, Mohr, U, Parisi, S, Sierotzki, H, Steinfeld, U (2000). Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. *Crop Protection* **19**: 863-872.

Gisi, U, Hermann, D (1994). Sensitivity behaviour of *Septoria tritici* population on wheat to cyproconazole. *British Crop Protection Council Monograph; Fungicide resistance* **6**: 11-18.

Gisi, U, Sierotzki, H, Cook, A, McCaffery, A (2002). Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science* **58**: 859-867.

Goodwin, S B (2007). Back to basics and beyond: increasing the level of resistance to Septoria tritici blotch in wheat. *Australasian Plant Pathology* **36**: 532-538.

Gullino, M L, Leroux, P, Smith, C M (2000). Uses and challenges of novel compounds for plant disease control. *Crop Protection* **19**: 1-11.

Guo, J R, Schnieder, F, Verreet, J A (2006). Presymptomatic and quantitative detection of *Mycosphaerella graminicola* development in wheat using a real-time PCR assay. *Fems Microbiology Letters* **262**: 223-229.

Guo, J R, Schnieder, F, Verreet, J A (2007). A real-time PCR assay for quantitative and accurate assessment of fungicide effects on *Mycosphaewella graminicola* leaf blotch. *Journal of Phytopathology* **155**: 482-487.

Gupta, E (2008). Oil vulnerability index of oil-importing countries. *Energy Policy* **36**: 1195-1211.

Gupta, S, Loughman, R (2001). Current virulence of *Pyrenophora teres* on barley in Western Australia. *Plant Disease* **85**: 960-966.

HaghighiPodeh, M R, Bhattacharya, S K (1996). Fate and toxic effects of nitrophenols on anaerobic treatment systems. *Water Science and Technology* **34**: 345-350.

Halama, P (1996). The occurrence of *Mycosphaerella graminicola*, teleomorph of *Septoria tritici* in France. *Plant Pathology* **45**: 135-138.

Hall, R J, Gubbins, S, Gilligan, C A (2004). Invasion of drug and pesticide resistance is determined by a trade-off between treatment efficacy and relative fitness. *Bulletin of Mathematical Biology* **66**: 825-840.

Hammond-Kosack, K E, Jones, J D G (1996). Resistance gene-dependent plant defense responses. *Plant Cell* **8**: 1773-1791.

Hampton, J G (1980). The role of seed-borne inoculum in the epidemiology of net blotch of barley in New Zealand. *New Zealand Journal of Experimental Agriculture* **8**: 297-299.

Hardwick, N V, Jones, D R, Slough, J E (2001). Factors affecting diseases of winter wheat in England and Wales, 1989-98. *Plant Pathology* **50**: 453-462.

Harlan, J R, Zohary, D (1966). Distribution of Wild Wheats and Barley. *Science* **153**: 1074-&.

Heaney, S P, Hall, A A, Davies, S A, Playa, G (2000). Resistance to fungicides in the QoI-STAR cross resistance group: Current perspectives. The BCPC Conference Pests and Diseases. The British Crop Protection Council, Alton, Hampshire, UK**:** 755-762.

Henson, J M, French, R (1993). The polymerase chain-reaction and plant-disease diagnosis. *Annual Review of Phytopathology* **31**: 81-109.

Heuser, T, Zimmer, W (2002). Quantitative analysis of phytopathogenic ascomycota on leaves of pedunculate oaks (Quercus robur L.) by real-time PCR. *Fems Microbiology Letters* **209**: 295-299.

Hewitt, H G (1998). Fungicides in Crop Protection, CAB International, Wallingford, UK, 221pp.

Highwood, D P (1989). Fungicide Resistance Action Committee. *Pesticide Outlook* **1**.

Hilu, H M, Bever, W M (1957). Inoculation, oversummering, and suspect-pathogen relationship of *Septoria tritici* on *Triticum* species. *Phytopathology* **47**: 474-480.

Hollomon, D (2007). Editorial - Are some diseases unlikely to develop QoI resistance? *Pest Management Science* **63**: 217-218.

Hollomon, D W, Brent, K J (2009). Combating plant diseases - the Darwin connection. *Pest Management Science* **65**: 1156-1163.

Holmes, S J I, Colhoun, J (1974). Infection of wheat by *Septoria nodorum* and *Septoria tritici* in relation to plant age, air temperature and relative humidity. *Transactions of the British Mycological Society* **63**: 329-338.

Holmes, S J I, Colhoun, J (1975). Straw-borne Inoculum of *Septoria nodorum* and *S. tritici* in relation to incidence of disease on wheat plants. *Plant Pathology* **24**: 63-66.

Holtz, M D, Xi, K, Kumar, K, Zantinge, J (2010). Molecular detection of *Puccinia striiformis* using conventional and real-time PCR. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **32**: 407-407.

Hopf, M (1991). South and Southwest Europe. In:. Progress in Old World Paleoethnobotany. W. Van Zeist, K. Wasilikowa and K. E. Behre. (eds.), Rotterdam, Balkema**:** pp.241-277.

House, G J, Brust, G E (1989). Ecology of Low-Input, No-Tillage Agroecosystems. *Agriculture Ecosystems & Environment* **27**: 331-345.

Iliev, I (1994). Partial resistance of wheat varieties to powdery mildew - A factor preventing resistance to fungicides. *British Crop Protection Council Monograph; Fungicide resistance* 111-115.

Inami, K, Yoshioka, C, Hirano, Y, Kawabe, M, Tsushima, S, Teraoka, T, Arie, T (2010). Real-time PCR for differential determination of the tomato wilt fungus, *Fusarium oxysporum* f. sp. *lycopersici*, and its races. *Journal of General Plant Pathology* **76**: 116-121.

Ito, S, Kuribayashi, K (1931). The ascigerous forms of some graminicolous species of *Helminthosporium* in Japan. *Journal Faculty of Agriculture* **29**: 85-125.

Jackson, L F, Dubcovsky, J, Gallagher, L W, Wennig, R L, Heaton, J, Vogt, H, Gibbs, L K, Kirby, D, Canevari, M, Carlson, H, Kearney, T, Marsh, B, Munier, D, Mutters, C, Orloff, S, Schmierer, J, Vargas, R, Williams, J, Wright, S (2000). Regional barley and common and durum wheat performance tests in California. *Agron Prog Rep* **272**: 1-56.

Jane, T (2001). Microorganisms and Biotechnology, Nelson-Thornes Publisher, UK, pp. 168-169. .

Jayasena, K W, Loughman, R, Majewski, J (2002). Evaluation of fungicides in control of spot-type net blotch on barley. *Crop Protection* **21**: 63-69.

Johnston, H W, Macleod, J A (1987). Response of spring barley to fungicides, plant-growth regulators, and supplemental nitrogen
Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie **9**: 255-259.

Jones, D G, Clifford, B C (1983). Cereal Diseases: their pathology and control. 2nd edition,, John Wiley and Sons Ltd. UK.

Jones, J D G, Dangl, J L (2006). The plant immune system. *Nature* **444**: 323-329.

Jonsson, R, Bryngelsson, T, Gustafsson, M (1997). Virulence studies of Swedish net blotch isolates (*Drechslera teres*) and identification of resistant barley lines. *Euphytica* **94**: 209-218.

Jordan, V W L (1981). Etiology of barley net blotch caused by *Pyrenophora teres* and some effects on yield. *Plant Pathology* **30**: 77-87.

Jorgensen, H J L, Orum, J E, Pinnschmidt, H O, Nielsen, G C (2008). Integrating Disease Control in Winer Wheat-Optimizing Fungicide Input. In:. Modern Fungicides and Antifungal compounds. D. W. Dehne, H. B. Deising, U. Gisiet al, (eds.), DPG, Selbstverlag, Germanay. V**:** 197-209.

Jorgensen, L N (2008). Resistance situation with fungicides in cereals. *Zemdirbyste-Agriculture* **95**: 373-378.

Jorgensen, L N, Pinnschmidt, H, Nielsen, B J, Nielsen, G C (2004). Bygbladplet, biologi og bekæmpelse. [Biology and control of Barley Net Blotch (*Pyrenophora teres*).]. *Grøn Viden Markbrug*: 289.

Justesen, A F, Hansen, H J, Pinnschmidt, H O (2008). Quantification of *Pyrenophora graminea* in barley seed using real-time PCR. *European Journal of Plant Pathology* **122**: 253-263.

Kadish, D, Cohen, Y (1988). Fitness of *Phytophthora infestans* Isolates from Metalaxyl-Sensitive and Metalaxyl-Resistant Populations. *Phytopathology* **78**: 912-915.

Kahkonen, M A, Tuomela, M, Hatakka, A (2007). Microbial activities in soils of a former sawmill area. *Chemosphere* **67**: 521-526.

Karakaya, A, Akyol, A (2006). Determination of the seedling reactions of some Turkish barley cultivars to the net blotch. *Plant Pathology Journal* **5**: 113-114.

Kashemirova, L A (1995). Phytosanitary forecasting systems of spring barley protection against spot and net blotches, Bolshie Vazemi. Abstract of PhD thesis (in Russian)**:** 33p.

Kenneke, J F, Mazur, C S, Ritger, S E, Sack, T J (2008). Mechanistic Investigation of the Noncytochrome P450-Mediated Metabolism of Triadimefon to Triadimenol in Hepatic Microsomes. *Chemical Research in Toxicology* **21**: 1997-2004.

Keon, J P R, Hargreaves, J A (1983). A cytological study of the net blotch disease of barley caused by *Pyrenophora teres*. *Physiological Plant Pathology* **22**: 321-329.

Keon, J P R, White, G A, Hargreaves, J A (1991). Isolation, Characterization and Sequence of a Gene Conferring Resistance to the Systemic Fungicide Carboxin from the Maize Smut Pathogen, *Ustilago maydis*. *Current Genetics* **19**: 475-481.

Kerr, A (1964). Influence of soil moisture on infection of peas by *Pythium ultimum*. *Australian Journal of Biological Sciences* **17**: 676-685.

Kianianmomeni, A, Schwarz, G, Felsenstein, F G, Wenzel, G (2007). Validation of a real-time PCR for the quantitative estimation of a G143A mutation in the cytochrome bc(1), gene of *Pyrenophora teres*. *Pest Management Science* **63**: 219-224.

King, J E, Jenkins, J E L, Morgan, W A (1983). The estimation of yield losses in wheat from severity of infection by *Septoria* species. *Plant Pathology* **32**: 239-249.

Koller, W, Scheinpflug, H (1987). Fungal Resistance to Sterol Biosynthesis Inhibitors - a New Challenge. *Plant Disease* **71**: 1066-1074.

Korber-Grohne, U (1987). Nutzpflanzen in Deutschland. Stuttgart**:** Thesis: pp.48-52.

Kraan, G, Nisi, J E (1993). Septoriosis del trigo en la Republica Argentina. Situacion del cultivo frent a la enfermended. Page 1-8 in: Proceedings of the *Septoria tritici* Workshop, CIMMYT, Mexico city.

Krikun, J, Netzer, D, Sofer, M (1974). The role of soil fumigation under conditions of intensive agriculture. *Agro-Ecosystems*: 117-122.

Kronstad, W E (1998). Agricultural development and wheat breeding in the 20th century. Wheat: Prospects for Global Improvement. Proceedings of the 5th International Wheat Conference. H. J. Braun, F. Altay, W. E. Kronstad, S. P. S. Beniwal and A. McNab. Ankara, Turkey, Developments in Plant Breeding, Kluwer Academic Publishers, Dordrecht. 6**:** 1-10.

Krupinsky, J M (1997). Aggressiveness of *Stagonospora nodorum* isolates obtained from wheat in the northern Great Plains. *Plant Disease* **81**: 1027-1031.

Kuck, K H (1994). Evaluation of Anti-Resistance Strategies. *British Crop Protection Council Monograph; Fungicide resistance*: 43-46.

Kuck, K H (2007). QoI Fungicides: resistance mechanisms and its practical importance. In:. Pesticide Chemistry. Crop Protection, Public Health, Environmental Safety. H. Ohkawa, H. Miyagawa and P. W. Lee, (eds.), WILEY-VCH, Weinheim, Germany**:** 275-283.

Kuck, K H, Scheinpflug, H (1986). Biology of sterol-biosynthesis inhibiting fungicides. *Bowers, W. S. Et Al (Ed.). Chemistry of Plant Protection, Vol. 1. Sterol Biosynthesis, Inhibitors and Anti-Feeding Compounds. Ix+151p. Springer-Verlag: Berlin, West Germany; New York, N.Y., USA. Illus*: 65-96.

Kurt, S, Gunes, U, Soylu, E (2011). *In vitro* and *in vivo* antifungal activity of synthetic pure isothiocyanates against *Sclerotinia sclerotiorum*. *Pest Managment Science* **67**: 869-875.

Lakev, B, Semane, Y, Alemayehu, F, Gehre, H, Grando, S, Van Leur, A J, Ceccarelli, S (1997). Exploiting and diversity in barley landraces in Ethiopia. *Genetic Resources and Crop Evolution* **44**: 2.

Leadbeater, A, Gisi, U (2009). The Challenges of Chemical Control of Plant Diseases. *Recent Developments in Management of Plant Diseases* **1**: 3-17.

Leadbeater, A, Sierotzki, H, Jain, S, Mehl, A, Viollet, D, Raupach, G, Bird, R, Genet, J, Stammler, G, Semar, M, Gold, R (2010). QoI working group of FRAC, minutes of the meeting all crops, DuPont, Frankfurt, Germany**:** 12p.

Leisova, L, Kucera, L, Minarikova, V, Ovesna, J (2005). AFLP-based PCR markers that differentiate spot and net forms of *Pyrenophora teres*. *Plant Pathology* **54**: 66-73.

Leisova, L, Minarikova, V, Kucera, L, Ovesna, J (2006). Quantification of *Pyrenophora teres* in infected barley leaves using real-time PCR. *Journal of Microbiological Methods* **67**: 446-455.

Lepesheva, G I, Waterman, M R (2004). *CYP51* - the omnipotent P450. *Molecular and Cellular Endocrinology* **215**: 165-170.

Leroux, P, Albertini, C, Gautier, A, Gredt, M, Walker, A S (2007). Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14 alpha-demethylation inhibitors in field isolates of *Mycosphaerelia graminicola*. *Pest Management Science* **63**: 688-698.

Leroux, P, Bach, J, Debieu, D, Fillinger, S, Fritz, R, Walker, A S (2008a). Mode of action of sterol biosynthesis inhibitors and resistance phenomena in fungi. In:. Modern Fungicides and Antifungal Comounds V. H. W. Dehne, H. B. Deising, U. Gisiet al, (eds.), DPG, Selbstverlag, Germany**:** 85-92.

Leroux, P, Gredt, M (1997). Evolution of fungicide resistance in the cereal eyespot fungi Tapesia yallundae and Tapesia acuformis in France. *Pesticide Science* **51**: 321-327.

Leroux, P, Gredt, M, Walker, A S (2008b). Resisance to DMI Fungicides in *Mycosphaerella graminiwcolla* correlates with Mutations in the *CYP51* Gene. In:. Modern Fungicides and Antifungal Compounds V. H. W. Dehne, H. B. Deising, U. Gisiet al, (eds.), DPG, Selbstverlag, Germany**:** p105.

Leroux, P, Walker, A-S (2011). Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* **67**: 44-59.

Leroux, P, Walker, A S, Albertini, C, Gredt, M (2006). Resistance to fungicides in French populations of *Septoria tritici*, the causal agent of wheat leaf blotch. *Aspects of Applied Biology* **78**: 153-162.

Leroux, P, Walker, A S, Couleaud, G, Maumen'e, C, Le H´enaff, G (2008c). Field strategies to manage fungicide resistance in *Mycosphaerella graminicola*, the causal agent of wheat leaf blotch. Modern Fungicides and Antifungal Compounds. D. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russell and H. Lyr, DPG Seebstverlag, Brauschweig, Germany. V**:** pp. 143–149.

Lisitsina, G N (1984). The caucasus-A centre of ancient farming in Eurasia. In: Plants and Ancient Man. W. van Zeist and W. A. Casparie, (eds.), Balkema, Roterdam, pp 285-292.

Liu, Z, Ellwood, S R, Oliver, R P, Friesen, T L (2011). *Pyrenophora teres*: profile of an increasingly damaging barley pathogen. *Molecular Plant Pathology* **12**: 1-19.

Lockley, D, Clark, W S (2005). Fungicide dose-response trials in wheat: the basis for choosing 'appropriate dose'. London, UK: Home-Grown Cereals Authority: HGCA Project Report. HGCA Project Report no.373.

Lucas, J (1998). Plant Pathology and Plant Pathogens., Blackwell Science. 274p.

Lucas, J (2006). Adaptation of fungi to fungicides: an historical perspectvie. In: Fungicide Resistance: are we winning the battle but losing the war? R. G. Bryson, F. J. Burnett, V. Foster, B. A. Fraaije and R. Kennedy, (eds.), *Aspects of Appied Biology*. 78**:** 1-2.

Lucas, J A (2005). QoI resistance in cereal pathogens: The European experience. *Phytopathology* **95**: S143-S143.

Macdonald, O C (2008). Regulatory Aspects of Resistance Management. In: Modern Fungicides and Antifungal Compounds 5. H. W. Dehne, H. B. Deising, U. Gisiet al, (eds.), DPG, Selbstverlag, Germany**:** p113-120.

Makela, K (1975). Occurrence of *Helminthosporium* species on cereals in finland in 1971-73. *Journal of Scientific Agricultural Society of Finland* **47**: 171-217.

Maliniski, Z T (2004). Preliminary studies on efficacy of fungicide mixtures with azoxystrobin in control of some winter wheat diseases. *Pestycydy* **1**: 83-90.

Manners, J G (1993). Principles of Plant Pathology, Cambridge University Press, UK. 343p.

Mathre, D E (1982). Compendium of Barley disease. St Paul, Minnesota, American Phytopathological Society, 78p.

Mathre, D E (1997). Compendium of barley diseases, Second edition, 90p.

Maumene, C, Couleaud, G, Maufras, J Y (2009). Barley performances state of the resistance of Barley's Helminthosporiose to the strobilurines and impact on the efficiency. 9th International conference on plant diseases. Tours, France**:** p761-770.

Mavroeidi, V I, Shaw, M W (2005). Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquinconazole, prochloraz and azoxystrobin over a period of 9 years. *Crop Protection* **24**: 259-266.

McCartney, C, Mercer, P C, Cooke, L R, Fraaije, B A (2007). Effects of a strobilurin-based spray programme on disease control, green leaf area, yield and development of fungicide-resistance in *Mycosphaerella graminicola* in Northern Ireland. *Crop Protection* **26**: 1272-1280.

McCartney, H A, Foster, S J, Fraaije, B A, Ward, E (2003). Molecular diagnostics for fungal plant pathogens. *Pest Management Science* **59**: 129-142.

McDonald, B A, Linde, C (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* **40**: 349-379.

Mcdonald, W C (1967). Variability and inheritance of morphological mutants in *Pyrenophora teres*. *Phytopathology* **57**: 747-755.

MitchellOlds, T, Bradley, D (1996). Genetics of *Brassica rapa* .3. Costs of disease resistance to three fungal pathogens. *Evolution* **50**: 1859-1865.

Miyamoto, T, Ishii, H, Stammler, G, Koch, A, Ogawara, T, Tomita, Y, Fountaine, J M, Ushio, S, Seko, T, Kobori, S (2010). Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. *Plant Pathology* **59**: 873-881.

Mode, C J, Schaller, C W (1958). Two additional factors for host resistance to net blotch in barley. *Agronomy Journal* **50**: 15-18.

Morschhauser, J (2002). The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **1587**: 240-248.

Mullins, J G L, Parker, J E, Cools, H J, Togawa, R C, Lucas, J A, Fraaije, B A, Kelly, D E, Kelly, S L (2011). Molecular Modelling of the Emergence of Azole Resistance in Mycosphaerella graminicola. *Plos One* **6**.

Murray, G M, Brennan, J P (2010). Estimating disease losses to the Australian barley industry. *Australasian Plant Pathology* **39**: 85-96.

Nakova, M (2009). Barley net blotch. *Agricultural Sciences* **1**: 45-49.

Narayanasamy, P (2002). Microbial plant pathogens and crop disease management, Science Publishers Inc., Enfield, USA. 553pp.

Neate, S, McMullen, M (2005). Barley Diseases Handbook, North Dakota State University, 52pp.

Newman, C W, Newman, R K (2006). A brief history of barley foods. *Cereal Foods World* **51**: 4-7.

Nikou, D, Malandrakis, A, Konstantakaki, M, Vontas, J, Markoglou, A, Ziogas, B (2009). Molecular characterization and detection of overexpressed C-14 alpha-demethylase-based DMI resistance in *Cercospora beticola* field isolates. *Pesticide Biochemistry and Physiology* **95**: 18-27.

Norman, D J, Strandberg, J O (1997). Survival of *Colletotrichum acutatum* in soil and plant debris of leatherleaf fern. *Plant Disease* **81**: 1177-1180.

Oerke, E C (1999). The importance of disease control in modern plant production. Modern fungicides and antifungal compounds II. 12th International Reinhardsbrunn Symposium. Friedrichroda, Thuringia, Germany**:** 11-17.

Oerke, E C, Dehne, H W, Schunbeck, F, Wber, A. (1994). Crop Production and Crop Protection: Estimated Losses in Major Food and Cash Crops. Elsevier Science, Amesterdam, 808pp.

Oleson, B T (1994). World wheat production, utilization and trade. Wheat: Production, Properties and Quality. W. Bushuk and V. F. Rasper. (eds.), London, Chapman & Hall**:** pp.1-11.

Ordon, F, Afanasenko, O, Mironenko, N, Filatova, O, Kopahnke, D, Kramer, I (2007). Genetics of host-pathogen interactions in the *Pyrenophora teres* f. *teres* (net form) - barley (*Hordeum vulgare*) pathosystem. *European Journal of Plant Pathology* **117**: 267-280.

Oxley, S J P, Hunter, E A (2005). Appropriate fungicide doses on winter barley: producing dose-response data for a decision guide. HGCA project report No.366. www.hgca.co.uk.

Palmer, C L, Skinner, W (2002). *Mycosphaerella graminicola*: latent infection, crop devastation and genomics. *Molecular Plant Pathology* **3**: 63-70.

Parker, J E, Warrilow, A G S, Cools, H J, Martel, C M, Nes, W D, Fraaije, B A, Lucas, J A, Kelly, D E, Kelly, S L (2011). Mechanism of Binding of Prothioconazole to *Mycosphaerella graminicola CYP51* Differs from That of Other Azole Antifungals. *Applied and Environmental Microbiology* **77**: 1460-1465.

Pasche, J S, Piche, L M, Gudmestad, N C (2005). Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Disease* **89**: 269-278.

Peever, T L, Milgroom, M G (1992). Inheritance of triadimenol resistance in *Pyrenophora teres*. *Phytopathology* **82**: 821-828.

Peever, T L, Milgroom, M G (1994). Lack of correlation between fitness and resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. *Phytopathology* **84**: 515-519.

Pelloux-Prayer, A L, Priem, B, Joseleau, J P (1998). Kinetic evaluation of conidial germination of *Botrytis cinerea* by a spectrofluorometric method. *Mycological Research* **102**: 320-322.

Percival, J (1921). The Wheat Plant., Duckworth Publishers, London.

Perea, S, Lopez-Ribot, J L, Kirkpatrick, W R, McAtee, R K, Santillan, R A, Martinez, M, Calabrese, D, Sanglard, D, Patterson, T F (2001). Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrobial Agents and Chemotherapy* **45**: 2676-2684.

Perez-Garcia, A, Fernandez-Ortuno, D, Tores, J A, de Vicente, A (2008). Field resistance to QoI fungicides in *Podosphaera fusca* is not supported by typical mutations in the mitochondrial cytochrome b gene. *Pest Management Science* **64**: 694-702.

Pettit, R K, Weber, C A, Kean, M J, Hoffmann, H, Pettit, G R, Tan, R, Franks, K S, Horton, M L (2005). Microplate Alamar Blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrobial Agents and Chemotherapy* **49**: 2612-2617.

Pettit, R K, Weber, C A, Pettit, G R (2009). Application of a high throughput Alamar Blue biofilm susceptibility assay to *Staphylococcus aureus* biofilms. *Annals of Clinical Microbiology and Antimicrobials* **8**: 28.

Phillips, A N, Locke, T (1994). Carbendazim resistance in *Rhyncosorium secalis* in England and Wales. *Fungicide Resistance*: 251-254.

Piening, L (1968). Development of barley net blotch from infested straw and seed. *Canadian Journal of Plant Science* **48**: 623-&.

Piening, L J (1961). The occurence of *Pyrenophora teres* on barley straw in Alberta. *Canadian Plant Disease Survey* **41**: 299-300.

Pijls, C F N, Shaw, M W, Parker, A (1994). A rapid test to evaluate *in vitro* sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. *Plant Pathology* **43**: 726-732.

Pins, J J, Kaur, H (2006). A review of the effects of barley beta-glucan on cardiovascular and diabetic risk. *Cereal Foods World* **51**: 8-11.

Polak, J, Bartos, P (2002). Natural sources of plant disease resistance and their importance in the breeding. *Czech Journal of Genetics and Plant Breeding* **38**: 146-149.

Polley, R W, Thomas, M R (1991). Surveys of diseases of winter-wheat in England and Wales, 1976 -1988. *Annals of Applied Biology* **119**: 1-20.

Prescott, J M, Burnett, P A, Saari, E E, Ranson, J, Bowman, J, de Milliano, W, Singh, R P, Bekele, G (1986). Wheat Diseases and Pests: A Guide for Field Identification, CIMMYT. Mexico, D. F., Mexico. 143p.

Prestes, A M, Hendrix, J W (1977). *Septoria tritici* Rob. Ex Desm.: Relaqao patogeno-hospedeiro, reposta varietal e influancia no sistema radicular do trigo. *Suppl. Ciencia Cult* **29**: 23.

Ranhotra, G S (1994). Wheat: contribution to world food supply and human nutrition. Wheat: Production, Properties and Quality. W. Bushuk and V. F. Rasper. London, (eds.), Chapman & Hall**:** pp12-24.

Ranhotra, G S, Gelroth, J A, Astroth, K (1990). Total and Soluble Fiber in Selected Bakery and Other Cereal Products. *Cereal Chemistry* **67**: 499-501.

Raposo, R, Gomez, V, Urrutia, T, Melgarejo, P (2000). Fitness of *Botrytis cinerea* associated with dicarboximide resistance. *Phytopathology* **90**: 1246-1249.

Roberts, T R, Huston, D H (1999). Metabolic pathway of agrochemicals, part 2. Insecticides and fungicides. Cambridge, UK, The Royal Society of Chemistry**:** p1090.

Robinson, J, Jalli, M (1997). Quantitative resistance to *Pyrenophora teres* in six Nordic spring barley accessions. *Euphytica* **94**: 201-208.

Rohel, E A, Laurent, P, 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.

Rosegrant, M W, Agcaoili- Sombilla, A, Perez, N (1995). Global Food Projections to 2020. Discussion paper. Washington, D.C., IFPRI. 5.

Sanderson, F R (1976). *Mycosphaerella graminicola* the ascogenous state of *Septoria tritici*. *New Zealand Journal of Botany* **14**: 359-360.

Sanderson, F R, Hampton, J G (1978). Role of Perfect States in Epidemiology of Common Septoria Diseases of Wheat. *New Zealand Journal of Agricultural Research* **21**: 277-281.

Sanglard, D, Ischer, F, Calabrese, D, de Micheli, M, Bille, J (1998a). Multiple resistance mechanisms to azole antifungals in yeast clinical isolates. *Drug Resistance Updates* **1**: 255-265.

Sanglard, D, Ischer, F, Koymans, L, Bille, J (1998b). Amino acid substitutions in the cytochrome P-450 lanosterol 14 alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrobial Agents and Chemotherapy* **42**: 241-253.

Sato, K, Takeda, K (1991). Studies on the conidia formation of *Pyrenophora teres* Drechs. II. Effects of day length, medium and temperature under near ultraviolet radiation. *Nagaku Kenkyu* **62**: 165-176.

Sauter, H, Steglich, W, Anke, T (1999). Strobilurins: Evolution of a new class of active substances. *Angewandte Chemie-International Edition* **38**: 1329-1349.

Schaller, C W (1955). Inheritance of Resistance to Net Blotch of Barley. *Phytopathology* **45**: 174-176.

Schena, L, Nigro, F, Ippolito, A, 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.

Schnabel, G, Bryson, P K, Bridges, W C, Brannen, P M (2004). Reduced sensitivity in *Monilinia fructicola* to propiconazole in Georgia and implications for disease management. *Plant Disease* **88**: 1000-1004.

Schulz, U (1994). Evaluating Anti-Resistance Strategies for Control of *Erysiphe graminis* f. sp. *tritici*. *British Crop Protection Council Monograph; Fungicide resistance*: 55-58.

Schurch, S, Frei, P, Frey, R, Wullschleger, J, Sierotzki, H (2009). Septoria leaf blotch of wheat: sensitivity to fungicides of the Swiss *Mycosphaerella graminicola* population. *Agrarforschung* **16**: 420-424.

Schwinn, F J (1982). Socio-economic impact of fungicide resistance. In: Fungicide Resistance in Crop Protection. J. Dekker and S. G. Georgopoulos, Centre for Agricultural Publishing and Documentation, Wageningen**:** p16.

Schwinn, F J (1992). Significance of fungal pathogens in crop production. *Pesticide Outlook*: 18-25.

Selim, S (2009). Allele-specific real-time PCR for quantification and discrimination of sterol 14 alpha-demethylation-inhibitor-resistant genotypes of *Mycosphaerella graminicola*. *Journal of Plant Pathology* **91**: 391-400.

Semar, M, Strobel, D, Koch, A, Klappach, K, Stammler, G (2007). Field efficacy of pyraclostrobin against populations of *Pyrenophora teres* containing the F129L mutation in the *cytochrome b* gene. *Journal of Plant Diseases and Protection* **114**: 117-119.

Serenius, M, Manninen, O (2006). Prochloraz tolerance of *Pyrenophora teres* population in Finland. *Agricultural and Food Science* **15**: 35-42.

Serivastava, K D, Tewari, A K (2002). Fungal Diseases of Wheat and Barley: Foliar Diseases. In: Diseases of Field Crops. V. K. Gupta and Y. S. Paul, (eds.), INDUS, New Delhi, India**:** p 58-78.

Seyran, M, Brenneman, T B, Stevenson, K L (2010). In vitro toxicity of alternative oxidase inhibitors salicylhydroxamic acid and propyl gallate on Fusicladium effusum. *Journal of Pest Science* **83**: 421-427.

Shaw, M W (2000). Models of the effects of dose heterogeneity and escape on selection pressure for pesticide resistance. *Phytopathology* **90**: 333-339.

Shaw, M W, Pijls, C F N (1994). The effect of reduced dose on the evolution of fungicide resistance in *Septoria tritici*. *British Crop Protection Council Monograph; Fungicide resistance*: 47-54.

Shaw, M W, 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.

Shipton, W A, Boyd, S R J, Rosielle, A A, Shearer, B I (1971). Common septoria diseases of wheat. *Botanical Review* **37**: 231-262.

Shipton, W A, Khan, T N, Boyd, W J R (1973). Net blotch of barley. *Review of Plant Pathology* **52**: 269-290.

Shoemaker, R A (1959). Nomenclature of *Drechslera* and *Bipolaris*, grass parasites segregated from *Helminthosporium*. *Canadian Journal of Botany* **37**: 879-887.

Siah, A, Deweer, C, Morand, E, Reignault, P, Halama, P (2008). Assessment of *Mycosphaerella graminicola* resistance to azoxystrobin. *Communications in Agricultural and Applied Biological Sciences* **73**: 41-9.

Siah, A, Deweer, C, Morand, E, Reignault, P, Halama, P (2010). Azoxystrobin resistance of French *Mycosphaerella graminicola* strains assessed by four in vitro bioassays and by screening of G143A substitution. *Crop Protection* **29**: 737-743.

Sierotzki, H, Frey, R, Wullschleger, J, Palermo, S, Karlin, S, Godwin, J, Gisi, U (2007). *Cytochrome b* gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Management Science* **63**: 225-233.

Sierotzki, H, Kraus, N, Assemat, P, Stanger, C, Cleere, S, Windass, J (2005). Evaluation of resistance to QoI fungicides in *Plasmopara viticola* populations in Europe. Modern Fungicides and Antifungal Compounds IV. Gisi U ed. by Dehne HW, Kuck KH, Russell PE and Lyr H, BCPC, Alton, UK**:** 73-80.

Sierotzki, H, Parisi, S, Steinfeld, U, Tenzer, I, Poirey, S, Gisi, U (2000a). Mode of resistance to respiration inhibitors at the *cytochrome bc*(1) enzyme complex of *Mycosphaerella fijiensis* field isolates. *Pest Management Science* **56**: 833-841.

Sierotzki, H, Wullschleger, J, Gisi, U (2000b). Point mutation in cytochrome b gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f. sp *tritici* field isolates. *Pesticide Biochemistry and Physiology* **68**: 107-112.

Singh, P K, Hughes, G R (2005). Genetic control of resistance to tan necrosis induced by *Pyrenophora tritici-repentis*, races 1 and 2, in spring and winter wheat genotypes. *Phytopathology* **95**: 172-177.

Singh, P K, Mergoum, M, Ali, S, Adhikari, T B, Elias, E M, Anderson, J A, Glover, K D, Berzonsky, W A (2006). Evaluation of elite wheat germ plasm for resistance to tan spot. *Plant Disease* **90**: 1320-1325.

Skou, J P, Haahr, V (1987). Field screening for resistance to barley net blotch. *Annals of Applied Biology* **111**: 617-627.

Smedegard-Petersen, V (1971). *Pyrenophora teres* f. *maculata* f. *nov*. and *Pyrenophora teres* f. *teres* on barley in Denmark. Royal Veterinary and Agricultural University Yearbook. Copenhagen**:** pp124-144.

Smedegard-Petersen, V (1976). Pathogenesis and genetics of net spot blotch and leaf stripe of barley caused by *Pyrenophora teres* and *Pyrenophora graminea*, DSR Copenhagen. DSc**:** 176p.

Smedegard-Petersen, V (1977). Isolation of 2 toxins produced by *Pyrenophora teres* and their significance in disease development of net-spot blotch of barley. *Physiological Plant Pathology* **10**: 203-&.

Smith, B D (1998). The Emergence of Agriculture. *Scientific American Library, HPHLP,*: 231pp.

Somasco, O A, Qualset, C O, Gilchrist, D G (1996). Single-gene resistance to *Septoria tritici* blotch in the spring wheat cultivar 'Tadinia'. *Plant Breeding* **115**: 261-267.

Soovali, P, Koppel, M (2010). Efficacy of fungicide tebuconazole in barley varieties with different resistance level. *Agricultural and Food Science* **19**: 34-42.

Sprague, R (1950). Diseases of cereals and grasses in North America. New York, Ronald Press, 538pp.

Stammler, G, Carstensen, M, Koch, A, Semar, M, Strobel, D, Schlehuber, S (2008a). Frequency of different *CYP51*-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole sensitivity and field efficacy. *Crop Protection* **27**: 1448-1456.

Stammler, G, Kern, L, Semar, M, Glaettli, A, Schoefl, U (2008b). Sensitivity of *Mycosphaerella graminicola* to DMI fungicides related to mutations in the target gene cyp51 (14 alfa demethylase). In: Modern Fungicides and Antifungal Compounds V. 15th International Reinhardsbrunn Symposium. H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russell and H. Lyr. BCBC, UK. 5**:** 137-142.

Stammler, G, Semar, M, Strobel, D, Schoefel, U (2008c). Studies on potential factors affecting the control of *Mycosphaerella graminicolla* in the field. Modern Fungicides and Antifungal Comounds. H. W. Dehne, H. B. Deising, U. Gisiet al, (eds.), DPG, Selbstverlag, Germany. V**:** 187-191.

Steffenson, B J, Webster, R K, Jackson, L F (1991). Reduction in yield loss using incomplete resistance to *Pyrenophora teres* f. *teres* in barley. *Plant Disease* **75**: 96-100.

Stergiopoulos, I, Van Nistelrooy, J G M, Kema, G H J, De Waard, M A (2003). Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* **59**: 1333-1343.

Steva, H (1994). Evaluating Anti-Resistance Strategies for Control of *Uncenula necator*. *British Crop Protection Council Monograph; Fungicide resistance* 59-66.

Stukenbrock, E H, Banke, S, Javan-Nikkhah, M, McDonald, B A (2007). Origin and domestication of the fungal wheat pathogen *Mycosphaerella graminicola* via sympatric speciation. *Molecular Biology and Evolution* **24**: 398-411.

Suarez-Estrella, F, Vargas-Garcia, M C, Lopez, M J, Moreno, J (2007). Effect of horticultural waste composting on infected plant residues with pathogenic bacteria and fungi: Integrated and localized sanitation. *Waste Management* **27**: 886-892.

Taggart, P J, Cooke, L R, Mercer, P C (1994). Benzimidazole resistance in *Rhynchosporium secalis* in Northern Ireland and Its implication for disease control. *Fungicide Resistance*: 243-246.

Tanaka, D L, Krupinsky, J M, Liebig, M A, Merrill, S D, Ries, R E, Hendrickson, J R, Johnson, H A, Hanson, J D (2002). Dynamic cropping systems: An adaptable approach to crop production in the great plains. *Agronomy Journal* **94**: 957-961.

Tekauz, A (1985). A numerical scale to classify reactions of barley to *Pyrenophora teres*. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **7**: 181-183.

Tilman, D, Cassman, K G, Matson, P A, Naylor, R, Polasky, S (2002). Agricultural sustainability and intensive production practices. *Nature* **418**: 671-677.

Tuohy, J M, Jalli, M, Cooke, B M, Sullivan, E O (2006). Pathogenic variation in populations of *Drechslera teres* f. *teres* and *D. teres* f. *maculata* and differences in host cultivar responses. *European Journal of Plant Pathology* **116**: 177-185.

Turkington, T K, Kutcher, H R, Clayton, G W, O'Donovan, J T, Johnston, A M, Harker, K N, Xi, K, Stevenson, F C (2004). Impact of seedbed utilization and fungicide application on severity of net blotch *Pyrenophora teres* and production of barley. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **26**: 533-547.

Turkington, T K, Xi, K, Tewari, J P, Lee, H K, Clayton, G W, Harker, K N (2005). Cultivar rotation as a strategy to reduce leaf diseases under barley monoculture. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **27**: 283-290.

Tvaruzek, L, Horakova, P, Ji, L (2005). Resistance behaviour of *Septoria tritici* to some fungicides in the territory of the Czech Republic. *Acta Agrobotanica* **58**: 79-84.

VanderPlank, J E (1963). Disease: Epidemics and control. Academic Press, New York, 349pp.

Verreet, J A, Klink, H, Hoffmann, G M (2000). Regional monitoring for disease prediction and optimization of plant protection measures: The IPM wheat model. *Plant Disease* **84**: 816-826.

Viljanen-Rollinson, S L H, Marroni, M V, Butler, R C (2007). Reduced sensitivity to carbendazim in isolates of *Botrytis allii*. *New Zealand Plant Protection* **60**: 108-113.

Webster, J (1951). Graminicolous pyrenomycetes. IV. The occurence of *Microthyrium culmigenum* on grasses in Britain. *Transactions of the British mycological society* **34**: 309-317.

Welch, R M, Graham, R D (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany* **55**: 353-364.

Wilcoxson, R D, Rasmusson, D C, Treeful, L M (1992). Inheritance of resistance to *Pyrenophora teres* in Minnesota barley. *Plant Disease* **76**: 367-369.

Wolpert, T J, Dunkle, L D, Ciuffetti, L M (2002). Host-selective toxins and avirulence determinants: What's in a name? *Annual Review of Phytopathology* **40**: 251-+.

Wong, F P, Wilcox, W F (2000). Distribution of baseline sensitivities to azoxystrobin among isolates of *Plasmopara viticola*. *Plant Disease* **84**: 275-281.

Yamaguchi, I, Fujimura, M (2005). Recent topics on action mechanisms of fungicides. *Journal of Pesticide Science* **30**: 67-74.

Yarham, D J, Giltrap, N J (1989). Crop diseases in a changing agriculture - Arable crops in the UK - A review. *Plant Pathology* **38**: 459-477.

Yin, Y, Liu, X, Li, B, Ma, Z (2009). Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. *Phytopathology* **99**: 487-497.

Ypema, H L (2005). QoI resistance mechanisms and occurrences. *Phytopathology* **95**: S142-S143.

Zadoks, J C, Chang, T T, Konzak, C F (1974). Decimal code for growth stages of cereals. *Weed Research* **14**: 415-421.

Zhan, J, Stefanato, F L, McDonald, B A (2006). Selection for increased cyproconazole tolerance in *Mycosphaerella graminicola* through local adaptation and in response to host resistance. *Molecular Plant Pathology* **7**: 259-268.

Zhang, C Q, Liu, Y H, Ma, X Y, Feng, Z, Ma, Z H (2009). Characterization of sensitivity of Rhizoctonia solani, causing rice sheath blight, to mepronil and boscalid. *Crop Protection* **28**: 381-386.

Zohary, D, Hopf, M (1993). Domestication of plants in the Old World, Second edition, Oxford Science Publications, Clarendon Press, Oxford, pp33-64.