Triazole fungicide sensitivity among South African *Puccinia graminis* f. sp. *tritici* isolates

by

Isabella du Toit

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Magister Scientiae in Botany
Department Plant Sciences
Faculty of Natural and Agricultural Sciences
University of the Free State, Bloemfontein, South Africa

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Supervisor: Prof. B. Visser
Department of Plant Sciences, University of the Free State, Bloemfontein, South Africa

Co-supervisor: Prof. W.H.P. Boshoff
Department of Plant Sciences, University of the Free State, Bloemfontein, South Africa

Co-supervisor: Dr L.A. Rothmann
Department of Plant Sciences, University of the Free State, Bloemfontein, South Africa

Ethical clearance letter

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10-Mar-2021

Dear **Ms Isabella Du Toit**

Ethics Clearance: **Investigating triazole sensitivity among Puccinia graminis f. sp. tritici isolates from South Africa.**

Principal Investigator: **Ms Isabella Du Toit**

CONDITIONALLY APPROVED

This letter confirms that this research proposal with tracking number: **UFS-ESD2021/0030** and title: **Investigating triazole sensitivity among Puccinia graminis f. sp. tritici isolates from South Africa.** was given ethical clearance by the Ethics Committee pending clarification of the following:

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Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Biosafety & Environmental Research Ethics Committee
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Directorate: Research Development
T: +27 (0)51 401 9388 | +27 (0)51 401 2075 | E: smithann@ufs.ac.za
Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa
P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za

Declaration

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Isabella du Toit 24 November 2022

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Abbreviations

a. i.	Active ingredient
ANOVA	Analysis of variance
ARC-SG	Agricultural Research Council - Small Grain
Bgh	*Blumeria graminis* f. sp. *hordei*
Bgt	*Blumeria graminis* f. sp. *tritici*
bp	Base pairs
$CHCl_3$	Chloroform
CTAB	Cetyl trimethylammonium bromide
d.f.	Degrees of freedom
DMIs	Demethylation inhibitors
EC_{50}	Effective inhibition of half-maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
FRAC	Fungicide Resistance Action Committee
IAA	Isoamylalcohol
IPM	Integrated pest management
IPTG	Isopropyl β-d-1-thiogalactopyranoside
LB	Lysogeny broth
LSD	Least significant difference
m.s.	Mean squares
NA	North American
PCR	Polymerase chain reaction
Pg	*Puccinia graminis*
Pgt	*Puccinia graminis* f. sp. *tritici*
Pr	Probability value (p value)
Pst	*Puccinia striiformis*
Pt	*Puccinia triticina*
QoIs	Quinone-outside inhibitors
s.s.	Sum of squares
SA	South Africa
SDHIs	Succinate-dehydrogenase inhibitors
SNP	Single-nucleotide polymorphism

Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UFS	University of the Free State
USA	United States of America
UV	Ultra-violet
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
i.e.	‘That is’
n.d.	No date

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Chapter 1: General introduction

Wheat is an important crop and a key component in the diet of people, particularly in developing countries such as South Africa (SA; Pathak and Shrivastav, 2015). The crop is produced worldwide and is considered the most important source of food (Igrejas and Branlard, 2020). Two closely related species *Triticum aestivum* L. (bread wheat) and *T. durum* (durum wheat) are widely cultivated with global production dominated by countries including China, India, Russia, Ukraine and the United States of America (USA; OECD-FAO, 2020). During the 2019 to 2021 production seasons, SA produced an average of 1.8 million metric tons of bread wheat (CEC-Sagis, 2021), with nearly 42.2% of the total wheat yield contributed by spring wheat cultivars planted under dry land in the Western Cape province (Galal, 2021).

Rust diseases are present in most areas where wheat is cultivated (Kolmer, 2005; Pretorius *et al*., 2020). Stem rust of wheat and barley (*Hordeum vulgare* L.), caused by *Puccinia graminis* f. sp. *tritici* Eriks. and E. Henn. (*Pgt*), is considered the most damaging wheat disease in history (Pretorius *et al*., 2017). Recent reports indicate this pathogen's re-emergence in areas after decades of absence (Lewis *et al*., 2018; Tsushima *et al*., 2022). Kernels harvested from rust infected wheat are nutrient poor and low in test weight, while healthy kernels are plump and nutrient rich (Agrios, 2005). Disease control is important to maintain the production of high yielding wheat and is achieved through different control strategies, including fungicide application, a strategy widely practiced in SA (Roelfs *et al*., 1992; Soko *et al*., 2018; Anonymous, 2020b).

Rust pathogens are known for their ability to overcome monogenic sources of resistance, rapid dispersal over thousands of kilometres (Visser *et al*., 2019) and exponential rate of development into epidemics under favourable conditions. Stem rust mainly affects the stems of wheat during grain fill, as such fungicide applications are considered less effective if not applied preventatively (Wanyera *et al*., 2009; Tadesse *et al*., 2010). The effective chemical control of stem rust depends on application timing, adequate downward translocation of the active ingredient (a. i.) or coverage of the stem area (Bayer Crop Science, n.d.).

The occurrence of fungicide insensitivity in populations of fungal pathogens limits the efficacy and period during which fungicides remain effective. Loss in fungicide efficiency contributes to increased costs associated with new fungicide development (Ma and Michailides, 2005). Fungicide insensitivity is the acquired and heritable decrease in the sensitivity of isolates to the

a. i. present within a fungicide formulation (Brent and Hollomon, 2007). The widespread, repeated, and incorrect fungicide application as well as overreliance on a particular a. i. increases the risk of fungicide insensitivity. However, it may also occur without prior exposure in a phenomenon known as cross-resistance where insensitivity develops for different fungicides having a. i. with similar modes of action (Kang *et al*., 2019; McGrath, 2001). This insensitivity is usually to the mode of action of the a. i. of the specific fungicide and is a result of selection pressure exerted on the fungal population (Brent and Hollomon, 2007).

Fungicide sensitivity tests are conducted to determine pathogen sensitivity levels to specific fungicides, where a range of doses are used to determine the effective inhibition of half-maximal effective concentration (absolute EC_{50}; Russel, 2020). EC_{50} values determined for urediniospores of a single isolate of South African *Pgt* race 2SA88 (UVPgt55) to propiconazole and tebuconazole were recorded as 18.973 mg/L and 0.312 mg/L, respectively (Komen, 2007). Tebuconazole has a high residual effect and can kill quiescent urediniospores after 120 min of exposure, completely inhibiting germination. Compounds like this reduce spore viability and therefore slows disease progression and the development of fungicide resistance (Mueller *et al*., 2005).

Genetic mechanisms underlie the development of fungicide insensitivity in fungal populations. For example, variants of the *CYP51* gene have been associated with varying levels of triazole sensitivity (Tian *et al*., 2019). The *CYP51* gene, which forms part of the cytochrome P450 family, codes for the sterol 14α-demethylase enzyme. This enzyme plays an essential role in sterol biosynthesis (Lepesheva and Waterman, 2004; Lepesheva *et al*., 2010), including ergosterol, a sterol present in the plasma-membranes of fungi responsible for maintaining membrane fluidity and stability (Rodriguez *et al*., 1985; Yoshida, 1993; Parks and Casey, 1995).

The aim of the study was to determine whether a correlation exists between possible sequence variants of the *CYP51* gene and sensitivity to triazole-based fungicides among 45 *Pgt* isolates collected between 1981 and 2020 in SA. The first objective was to determine whether isolate sensitivity levels deviate between historically collected *Pgt* isolates when compared to recently collected field isolates to the triazole fungicides, propiconazole and tebuconazole. During the second objective, the *CYP51* gene was sequenced to identify sequence variants that might explain any differences in fungicide sensitivity.

Chapter 2: Literature study

2.1 The significance of stem rust of wheat caused by *Puccinia graminis* f. sp. *tritici*

Global food production demand is expected to increase 60% by 2050 (Dahm, 2020). This includes a staple crop such as wheat, which is the second most produced crop in the world and contributes 19% of human caloric intake (Atchison *et al*., 2010). The increased production demand is only one of the challenges that wheat producers face, along with climate change and increased disease pressure (Aggarwal *et al*., 2019). Wheat plants can be attacked by many different pathogens that cause disease (Singroha *et al*., 2017), which include biotrophic and necrotrophic fungi, nematodes, bacteria and viruses. Among these, the most prominent fungal diseases, such as Karnal bunt, loose smut, powdery mildew and rust, threaten wheat production.

Three highly specialised obligate fungi target wheat, i.e. stem rust caused by *Pgt*, leaf rust by *P. triticina* Eriks. (*Pt*) and stripe rust by *P. striiformis* Westend. f. sp. *tritici* Eriks. (*Pst*; Lorrain *et al*., 2019), although stem rust is considered the most damaging (Pretorius *et al*., 2017). Similar visible signs and symptoms of the host plant are presented by the above-mentioned *Puccinia* species., of which the target regions and adaptation to climatic conditions of the causal organisms vary (Marsalis and Goldberg, 2016). Infection occurs on the above-ground plant parts and pustules (uredinia) ranging from yellow-orange to reddish-brown or even black are produced upon successful colonisation. These pustules give the appearance of "rust" on the host plant, hence the associated common disease name.

The life cycle of wheat rusts includes both a sexual and an asexual stage (Schumann and Leonard, 2000). *Puccinia graminis* (*Pg*) is a heteroecious pathogen, requiring the infection of two unrelated host plants to complete both stages of its life cycle. While the asexual stage occurs on wheat, the sexual stage takes place on the alternate host, *Berberis* (Jin *et al*., 2010). The sexual stage is an important mechanism to introduce genetic recombination that leads to the generation of new virulence gene combinations with devastating consequences for cereal crop production (Jin, 2011). *Pg* is macrocyclic, producing five different spore stages during the completion of its life cycle (Schumann and Leonard, 2000), of which one is associated with the asexual stage and four with the sexual stage. These five spore stages are urediniospores, teliospores, basidiospores, pycniospores (spermatia) and aeciospores. The long-distance dispersal of wheat rusts is largely through the aerial movement of urediniospores produced during the asexual reproduction of the fungus (Brown and Hovmøller, 2002). Wind dispersal

facilitates the spread of urediniospores over hundreds to thousands of kilometres from the initial source of infection, thus enabling rust diseases to occur on a continental scale (Visser *et al*., 2019).

Wheat rust has been reported in all wheat producing regions of the world (Kolmer, 2005). Wheat producers have experienced significant yield losses due to wheat stem rust outbreaks in the past. Infection of the stems restricts nutrient flow to the wheat heads, which results in the development of small and nutrient poor wheat kernels (Leonard and Szabo, 2005). Stems weakened by infection tend to result in further yield losses through lodging which makes the mechanical harvesting of crops difficult (Schumann and Leonard, 2000). Severe stem rust outbreaks in the past resulted in yield losses of 9-33% in Scandinavia in 1951, 5-20% in eastern and central Europe in 1932 (Zadoks, 1963), as well as severe outbreaks in Queensland and New South Wales in Australia (Rees, 1972). In China during 1948, 1951, 1952 and 1956, severe outbreaks were recorded (Roelfs, 1977). During 1980 and 1981 in SA, studies indicated that stem rust of wheat caused an average grain loss of 35% across a range of genotypes (Pretorius, 1983).

A new virulent *Pgt* race, Ug99 (North American (NA) race name TTKSK; Jin *et al*., 2008), was first detected in Uganda in 1998 and described in 1999 (Pretorius *et al*., 2000). Since this first description, the race has given rise to an asexual lineage that has spread through Africa and the Middle East where severe stem rust outbreaks consequently occurred (Singh *et al*., 2015). To date, another fifteen race variants considered to be part of the Ug99 lineage, have been identified from different countries with broad virulence to commercially deployed resistance genes (Singh *et al*., 2015; Nirmala *et al*., 2017; Anonymous, 2021c).

In the wheat production region of southern Ethiopia, severe stem rust outbreaks occurred in 2013 on Digalu, the most widely grown wheat cultivar (Olivera *et al*., 2015). *Pgt* race TKTTF, first detected in Ethiopia in August of 2012 and confirmed in October 2013 caused this outbreak (Newcomb *et al*., 2013). Digalu wheat, which carries the *SrTmp* resistance gene (Olivera *et al*., 2015) and conferred resistance to the Ug99 race group, was ineffective against this new highly virulent race. Despite early warnings and the subsequent extensive use of fungicides, the absence of cultivars with genetic resistance against this race made control efforts challenging during 2014-2015. Similarly, the wheat cultivar Robin released in 2011 also carried the *SrTmp* resistance gene (Singh *et al*., 2015). Stem rust occurred in some fields planted with this cultivar during 2014 in Kenya and small-scale farmers experienced significant

losses as they were unable to rely on the use of fungicides for protection when the resistance gene became ineffective against the new race.

New virulent wheat rust races continue to emerge and spread (Kolmer, 2005; Singh *et al*., 2015) resulting in outbreaks in distant geographical areas. Urgent control measures are then needed to mitigate losses (Hovmøller *et al*., 2010). The introduction of newly emerged races into new areas can have severe consequences for wheat production (Kolmer, 2005). When the new race is virulent to a resistance gene deployed in currently planted wheat cultivars, the gene is rendered ineffective and this shortens the effective life-span of the gene. To prevent yield losses due to the development of new virulent wheat rust races, a clear understanding of the epidemiology of the disease as well as disease control, is required. Disease control tactics include genetic resistance, cultural practices, the eradication of the alternate host species, *Berberis*, agricultural products that induce systemic acquired resistance within treated plants and chemical control achieved through fungicide application (Roelfs *et al*., 1992; McGrath, 2004).

Cereal cultivation was introduced to SA in the 17th Century by Dutch settlers. By 1726, the first documented wheat stem rust epidemic was recorded in the south-western parts of the Western Cape province (McCall, 1908). As wheat production expanded, isolated rust outbreaks have occurred repeatedly in SA (Pretorius *et al*., 2020). These outbreaks are most severe under the cool and humid conditions of the rainfall seasons of the Western Cape and Free State provinces (Pretorius *et al*., 2007).

This review provides an overview on fungicide sensitivity, its significance, and mechanisms of development. The effectiveness and implications of fungicide application are considered as well as the classification of the different fungicide groups. The genetic mechanisms that underlie the development of insensitivity are explored with a focus on genetic variation within the *CYP51* gene and its association with the development of triazole insensitivity. The review aims to report on the development of fungicide insensitivity in wheat rust pathogens and whether crop production can continue effectively without the use of timely fungicide applications.

2.2 Fungicide application to reduce wheat rust occurrence

Fungicides are substances that inhibit fungal growth with the subsequent prevention of disease (Anonymous, 2020a). Active ingredients within fungicides function through various mechanisms, i.e., damaging cellular membranes, interrupting pivotal processes like energy production and respiration, negatively impacting metabolic processes and inactivating critical enzymes or proteins (McGrath, 2004). Although fungicide application provides effective crop protection against fungal diseases, chemical products are expensive, potentially polluting and, if not applied in a timely manner according to the recommendation, have an increased risk of fungicide insensitivity developing amongst the target organisms (Gomes *et al*., 2018).

There are 46 registered classes of fungicides according to the Fungicide Resistance Action Committee (FRAC; Oliver, 2014). Fungicides are classified according to either their chemical group, physical characteristics, or their general or specific mode of action (Latin, 2017). The mode of action describes the effects that the specific a. i. within a fungicide has on the pathogenic fungus to ultimately prevent disease (Latin, 2017). Many fungicides classified within one group share a common mode of action.

The most frequently used fungicide groups to control wheat rust fungi (Table 2.1) include demethylation inhibitors (DMIs), succinate-dehydrogenase inhibitors (SDHIs) and quinone-outside inhibitors (QoIs; FRAC, 2022). DMIs inhibit the biosynthesis of ergosterol, a key component of the plasma membrane of fungi, which inhibits subsequent fungal growth (Wyenandt, 2020). SDHIs inhibit fungal respiration by blocking ubiquinone-binding sites in the mitochondrial complex II during cellular respiration (Avenot and Michailides, 2010). QoI-fungicides inhibit fungal energy production that ultimately leads to the inhibition of fungal growth (Anonymous, 2020b).

Results from field trials conducted in the highlands of Ethiopia, confirmed that near complete disease control of wheat stem rust can be achieved when propiconazole, a DMI triazole-based fungicide, is applied weekly (Tadesse *et al*., 2010). Treated plots were sprayed immediately after stem rust symptoms were visible for four consecutive weeks with propiconazole at 500 mL/ha. The yield from treated plots was significantly higher than the yield from untreated plots. Consequently, the time of disease onset and the early detection of the disease are the most important factors to keep in mind when applying fungicides to control stem rust (Roelfs, 1985; Beard *et al*., 2004).

Table 2.1 Classification of fungicide groups used for disease control in wheat (FRAC, 2022). Fungicides are classified into groups according to their modes of action. A code is assigned by the Fungicide Resistance Action Committee (FRAC) to differentiate between fungicide groups. Different chemical families contain different active ingredients that are present within fungicides.

Fungicide groups	**FRAC code**	**Chemical families**	**Common name of active ingredient**
Demethylation inhibitors (DMI-fungicides) (SBI: Class 1)	3	Imidazole	Imazalil
			Prochloraz
		Triazole	Cyproconazole
			Difenoconazole
			Epoxiconazole
			Fenbuconazole
			Fluquinconazole
			Flutriafol
			Ipconazole
			Metconazole
			Propiconazole
			Prothioconazole
			Tebuconazole
			Triadimenol
			Triticonazole
Amines (Morpholines) (SBI: Class 2)	5	Morpholine	Fenpropimorph
		Piperidine	Fenpropidin
		Spiroketalamine	Spiroxamine
Succinate-dehydrogenase inhibitors (SDHI)	7	Oxathiin carboxamide	Carboxin
		Pyridine carboxamide	Boscalid

Table 2.1 (cont.) Classification of fungicide groups used for disease control in wheat (FRAC, 2022).

Fungicide groups	**FRAC code**	**Chemical families**	**Common name of active ingredient**
Succinate-dehydrogenase inhibitors (SDHI)	7	Pyrazole carboxamide	Bixafen
			Fluxapyroxad
			Isopyrazam
			Penthiopyrad
Quinone-outside inhibitors (QoI-fungicides)	11	Oxazolidinedione	Famoxadone
		Strobilurin	Azoxystrobin
			Dimoxystrobin
			Fluoxastrobin
			Kresoxim-methyl
			Picoxystrobin
			Pyraclostrobin
			Trifloxystrobin
Thriophene-carboxamides	38	Thiophene-carboxamide	Silthiofam
Anilino-Pyrimidines (AP-fungicides)	9	Anilino-pyrimidine	Cyprodinil
Azanaphthalene	13	Aryloxyquinolines	Quinoxyfen
		Quinazolinones	Proquinazid
Phenylpyrroles (PP-fungicides)	12	Phenylpyrrole	Fludioxonil
Dicarboximides	2	Dicarboximide	Iprodione
Benzo-thiadiazole BTH	P1	Benzothiadiazole	Acibenzolar-S-methyl
Methyl Benzimidazole Carbamates (MBC-fungicides)	1	Benzimidazole	Carbendazim

Table 2.1 (cont.) Classification of fungicide groups used for disease control in wheat (FRAC, 2022).

Fungicide groups	**FRAC code**	**Chemical families**	**Common name of active ingredient**
Methyl Benzimidazole Carbamates (MBC-fungicides)	1	Thiophanate	Fuberidazole
			Thiophanate-methyl
Aryl-phenyl-ketones	U8	Aryl-phenyl-ketone	Metrafenone
			Pyriofenone
Inorganics - carbonates	NC	Inorganic	Potassium hydrogen carbonate
Inorganic - copper	M1	Copper	Cupric ammonium carbonate
Inorganics - sulphur	M2	Sulphur	Sulphur
Dithiocarbamates and relatives	M3	Dithiocarbamate	Mancozeb
			Maneb
			Thiram
			Ziram
Phthalimide	M4	Folpet	Folpet
Chloronitriles (phthalonitriles)	M5	Phthalonitrile	Chlorothalonil
Guanidines	M7	Guanidine	Guazatine

Although the proactive treatment was effective, the number of fungicide applications used in this trial is unlikely to be economically viable for commercial wheat planted in lower yield potential areas. Additionally, field trials conducted in Australia confirmed that triazole-based DMI fungicide treatments with either 145 mL/ha or 290 mL/ha tebuconazole or 250 mL/ha or 500 mL/ha flutriafol reduced disease severity of wheat stem rust (Loughman *et al*., 2005). However, fungicides applied at first detection were more effective than those applied three weeks after disease appearance, especially when accompanied with moderate levels of infection.

Disease pressure has significant implications for the effectiveness of fungicides applications. Under conditions of low disease pressure, the combined application of strobilurin and triazole increased yield (Da Costa *et al*., 2012; Tedford *et al*., 2017). The low disease pressure of gray leafspot in maize, caused by *Cercospora zeae-maydis*, was achieved by growing hybrids with high levels of genetic resistance against the disease and conducting field trials in locations with a history of low disease pressure where maize was not grown in the previous season (Tedford *et al*., 2017). This highlighted the importance of integrating disease management strategies such as site and cultivar selection as well as fungicide application. However, Woore and Holland (2020) reported that the effects of this strategy were negligible on yield in the absence of considerable disease pressure, i.e., disease scored as a 0 for complete plant death. A mean score of 7.7 was achieved in the field, indicating low levels of disease and can be ascribed to the integrated approach of disease control that was followed.

Although effective for disease management, certain disadvantages of fungicide application can be observed. The application of fungicides could adversely affect non-target fungi in the phyllosphere (Karlsson *et al*., 2014), which perform important ecological functions while interacting with the host plant. The most frequently used fungicides significantly affect the fungal community composition that might subsequently affect plant vigour (Karlsson *et al*., 2014).

Furthermore, the use of fungicides in agriculture results in the persistence of toxic residues in the soil with potential environmental pollution (Stefani *et al*., 2012). The use of certain substances results in disturbances of soil microbial activity that might influence important biogeochemical processes that occur in the plant rhizosphere. Toxic residues might also migrate off-site and contaminate waterways (Wightwick *et al*., 2010), potentially resulting in adverse impacts on the health of both aquatic and terrestrial ecosystems. The detrimental effect

of fungicide application on the environment has promoted research into alternative or natural fungicides that are less harmful than the widely used synthetic fungicides (Stefani *et al*., 2012).

2.3 Mechanisms and significance of the development of fungicide insensitivity

The development of fungicide insensitivity is a serious concern as fungicides are important to control crop diseases and subsequently secure food production under current intensive agricultural practices (Hollomon, 2012). Loss of fungicide efficiency contributes to increased costs associated with the development of new fungicides, which becomes necessary when the period of fungicide effectiveness is shortened by insensitivity of the target pathogen (Ma and Michailides, 2005).

Fungicide insensitivity is the acquired and heritable decrease in sensitivity of pathogenic fungi to fungicides. In contrast, fungicide resistance refers to the ability of the pathogen to survive fungicide treatment due to its acquired level of insensitivity (Brent and Hollomon, 2007). This tends to occur within fungal populations following the widespread, continued and often incorrect application of fungicides (Kang *et al*., 2019). The development of fungicide insensitivity is a significant problem in modern agriculture occurring in many major crop pathogens (Oliver, 2014). Fungicide insensitivity has developed for nine different fungicide classes, of which six are now obsolete to control rust pathogens (Table 2.2; Oliver, 2014). The three remaining effective classes are QoI, DMI and SDHI fungicides.

The development of fungicide insensitivity is usually targeted against the mode of action of the specific fungicide (Brent and Hollomon, 2007), and is the result of selection pressure exerted by the fungicide on the fungal population through the continuous exposure of the pathogen to the active ingredient. The sensitive wild type population is eradicated during treatment, while the insensitive mutant population is not affected, ultimately causing a change in the frequency of the mutant population. Insensitivity can develop towards a single a. i., but also towards several a. i., simultaneously (Yang *et al*., 2019). This is described as cross-resistance and can occur in fungicides with a. i. that share a common mode of action, or in fungicides containing a. i. with distinct modes of action (Karaoglanidis and Thanassoulopoulos, 2003; Chowdhary *et al*., 2013; Avenot *et al*., 2016).

Table 2.2 The nine high- and medium-risk fungicide classes for the development of fungicide insensitivity in rust fungi (Oliver, 2014)[1]. Examples of fungicides within these different fungicide classes are given as classified according to the Fungicide Resistance Action Committee (FRAC) as well as whether insensitivity was detected in rust fungi.

Risk level	**Class**	**Example**	**Insensitivity in rust fungi**
High	A1	Metalaxyl	Yes
	B1	Benomyl	Yes
	C3	Azoxystrobin	No
	E3	Iprodione	Yes
Medium	C2	Carboxin	Yes
		Bixafen	No
	G1	Tebuconazole	No[1]
	E2	Fludioxonil	Yes
	D1	Cyprodinil	Yes
	I2	Carpropamid	Yes

[1] Although not listed by Oliver, 2014, insensitivity has been reported (Ardium *et al*., 2012).

A decrease in the efficiency of wheat leaf rust control was detected in production areas where DMIs were applied after 16-, 14- and 7-years of tebuconazole, cyproconazole and epoxiconazole use, respectively (Reis, 1991; Reis *et al*., 1997; Ardium *et al*., 2012). Research confirmed that the decrease in efficiency was caused by the development of insensitivity to DMIs (Ardium *et al*., 2012). The increased insensitivity led to producers being unable to achieve complete control of leaf rust through the application of DMIs alone. Disease control was only achieved in commercial fields where QoI-fungicides were used in combination with DMIs.

Mechanisms that explain how fungicide insensitivity develops are described as either qualitative or quantitative in nature (Deising *et al*., 2008). Qualitative resistance is based on the acquisition of mutations in the target gene of the fungicide. These mutations could be acquired through ultra-violet (UV) irradiation in the case of wind-borne urediniospores. Genetic variation in wheat rust pathogens also occur as a result of natural mutations (such as by UV irradiation) and somatic recombination (Burdon, 1993). The efficacy of the fungicide is reduced when the gene(s) that encodes the a. i. target is mutated, resulting in the substitution of amino acids required for binding. The a. i. is unable to bind to the mutated target protein and, therefore, unable to inhibit fungal growth as usual.

In contrast, quantitative resistance can be caused by many different mechanisms but is mostly due to mechanisms that keep the intracellular concentrations of the a. i. of the fungicide low (Deising *et al*., 2008). This type of resistance is based on individuals within a population expressing genes that render the pathogen insensitive to fungicide application in some way. The stress exerted by the a. i. of the fungicide results in induced expression of these genes leading to insensitivity. Over time an adjusted fungal population with increased insensitivity to the specific a. i. of the fungicide develops.

The establishment of fungicide insensitivity can occur at varying rates, either very rapidly or quite slowly, depending on certain factors (Staub and Sozzi, 1984). Fungicides are classified as being either single or multi-site (Mueller, 2006), with the former targeting a single aspect of the metabolic pathway of the fungal pathogen, while the latter targets multiple metabolic sites. Fungal isolates insensitive to single-site fungicides, such as the benzimidazoles, have developed within two years after the release of such fungicide, onto the market (Deising *et al*., 2008). In contrast, isolates insensitive to treatment with multi-site fungicides were only discovered after more than 30 years of application. As a result, multi-site fungicides pose a

lesser risk for the development of fungicide insensitivity (Staub and Sozzi, 1984). There are four main mechanisms involved in the development of insensitivity towards the a. i. contained by single-site fungicides (Lucas *et al*., 2015). These are the alteration of the pathogenic target protein, which inhibits fungicide binding, the overexpression of target proteins causing an increase in the fungicide concentrations needed to inhibit fungal growth, the export of the fungicide from pathogenic cells via efflux pumps and the degradation of the fungicide by metabolic enzymes. The rapid development of fungicide insensitivity towards single-site fungicides, such as DMIs, is indicated in Table 2.3 and can be compared to the slow development of insensitivity in *Pyrenophora avenae* reported in 1964 towards a multi-site fungicide.

Pathogen response levels to specific fungicides are determined by conducting fungicide sensitivity tests, by testing a range of doses to determine the EC_{50} estimate (Russel, 2020). This value indicates the concentration of the a. i. on a dose response curve where 50% maximal growth occurs (Noel *et al*., 2018). The quantification of pathogen population responses to fungicides, requires establishing a baseline sensitivity response, defined as the profile of the sensitivity response of the fungal isolates to the specific fungicide (Russel, 2020). The baseline response is determined to measure the response of a fungal isolate previously unexposed to the fungicide. When the sensitivity responses of fungal isolates exposed to the fungicide deviate strongly from the established baseline response, it may imply that the commercial rate of fungicide application no longer effectively controls the isolate.

2.4 Fungicide insensitivity in South Africa

Fungicide application is a widely practiced strategy in SA to control fungal diseases and maintain the production of high yielding cereal crops, which are chiefly produced in the Southern Cape and Swartland regions of the Western Cape province (Anonymous, 2020b; Anonymous, 2021a). Fungicides are applied as foliar sprays during the seven-leaf and flag-leaf stages of wheat development (Anonymous, 2020b). As barley and wheat production involves fungicides with similar a. i. (Agri-Intel, 2021) the need to monitor the use of fungicides and the development of potential insensitivity, is critical for pathogens like *Pgt* with the ability to infect both host plants.

Table 2.3 Occurrence of fungicide insensitivity in different fungal pathogens towards single-site and multi-site fungicides[1]. Single-site fungicides are indicated as having one target protein while a multi-site fungicide is indicated as having multiple target proteins. The first report of the occurrence of fungicide insensitivity is given, as well as the number of years of fungicide exposure before insensitivity occurred.

Fungicide class	**Number of target sites**	**First fungicide insensitivity report**	**Years until insensitivity detected**	**Pathogen**
Organomercurials	Many	1964	40	*Pyrenophora avenae*
Benzimidazoles	1	1970	2	*Venturia inaequalis*, *Botrytis cinerea*
Phenylamides	1	1980	2	*Phytophthora infestans*, *Plasmopara viticola*
Dicarboximides	1	1982	5	*Botrytis cinerea*
DMIs	1	1982	4	*Blumeria graminis*
Carboxanilides	1	1986	14	*Ustilago nuda*
Morpholines	2	1994	34	*Blumeria graminis*
Strobilurins	1	1998	2	*Blumeria graminis*

[1] Retrieved from Deising *et al.*, (2008) as compiled from Hewitt, H. G. Fungicides in Crop Protection. Wallingford, United Kingdom: Centre for Agriculture and Bioscience International; 1998. p. 221.

The estimated total cereal production in 2021 was 19.7 million tonnes, with wheat contributing ~10.7% (Anonymous, 2021b). Barley production in the Western Cape province has been subjected to continual fungicide application programs for many years. An investigation into the sensitivity of South African net- and spot-type *P. teres* isolates affecting barley, revealed significant differences in fungicide sensitivity levels to five commonly used DMI fungicides, namely bromuconazole, flusilazole, propiconazole and tebuconazole and triadimenol (Campbell and Crous, 2002). The baseline sensitivity of *P. teres* populations to DMIs could not be effectively determined as a result of prolonged fungicide application programs. An overall higher degree of insensitivity was found in spot-type isolates compared to net-type isolates due to a higher proportion of the spot-type populations in the Western Cape being subjected to fungicide control. Additionally, cross-resistance in *P. teres* was reported for some DMIs, which masks the actual sensitivity of more recently introduced DMIs, such as bromuconazole introduced in 1996 (Peever and Milgroom, 1993; Campbell and Crous, 2002).

Isolates of *Tapesia yallundae*, causing eyespot disease in wheat, collected from 15 wheat fields in the Western Cape province (Ntushelo and Crous, 2004), showed shifts in triazole sensitivity from the baseline measured in a previously unexposed field. This shift in sensitivity was detected for all triazoles, including propiconazole and tebuconazole. Subsequently, resistance management strategies were recommended to prevent further increases in fungicide insensitivity.

The efficacy of different triazole-based fungicides was investigated as foliar and seed treatments to control stripe rust in SA (Boshoff *et al*., 2003). At that time, the only registered seed treatment was triticonazole, but results indicated the protection provided by triadimenol was more effective. Triazole seed treatments were effective in the prevention of inoculum build-up in the early developmental stages of susceptible wheat cultivars. This reduced the costs associated with the application of foliar sprays as well as the risk of severe early outbreaks. The potential risks associated with fungicide treatment, whether foliar or seed treatment, need to be considered and how to mitigate these risks.

Records of fungicide insensitivity in SA are difficult to obtain since baseline studies to determine sensitivity levels are lacking. Because fungicide application is widely used for disease control in SA, it is important to be cautious and consider the threat that the development of fungicide insensitivity might hold in the future.

2.5 Genes involved in the development of triazole insensitivity

The occurrence of fungicide insensitivity limits the efficacy and period during which fungicides remain useful for disease control (Ma and Michailides, 2005). The identification of gene variants associated with increased insensitivity will enable the extension of the period during which fungicides are effective through applying early resistance management strategies.

Ergosterol, a sterol in the plasma membranes of eukaryotic organisms, is responsible for the maintenance of membrane fluidity and stability enabling fungal growth (Rodriguez *et al*., 1985; Parks and Casey, 1995). The *CYP51* gene codes for the sterol 14α-demethylase, which is the target protein for DMI fungicides, including azole-based fungicides (Hamamoto *et al*., 2000; Lepesheva and Waterman, 2004). The sterol 14α-demethylase enzyme, part of the cytochrome P450 family, is essential in the biosynthetic pathway of ergosterol (Yoshida, 1993; Lepesheva and Waterman, 2004; Lepesheva *et al*., 2010). The protein is anchored within the cellular membrane by a single N-terminal transmembrane helix domain with certain parts of its globular domain embedded in the bilayer (Yu *et al*., 2015). Two views of the protein can be seen in Figure 2.1 where the position of the protein is indicated within the bilayer of the plasma membrane.

Molecular mechanisms associated with fungal insensitivity to azole-based fungicides include point mutations within the *CYP51* gene sequence, the overexpression of the gene and the overexpression of other genes that encode efflux pump proteins (Lucas *et al*., 2015). Variants of the *CYP51* gene have been associated with varying levels of triazole sensitivity in plant pathogenic fungi (Tian *et al*., 2019).

Reduced fungicide efficiency was previously observed in *Zymoseptoria tritici*, causing septoria leaf blotch of wheat, in Australia (McDonald *et al*., 2019). Most isolates collected in European countries were insensitive to both azole and strobilurin chemical families, forming part of the DMI and QoI fungicide groups, respectively. When the *CYP51* alleles from Australian *Z. tritici* isolates were sequenced, haplotypes insensitive to azole treatment were detected. These haplotypes emerged within ten years following the widespread use of azole fungicides. Although insensitivity to azoles in *Z. tritici* was observed across Europe, disease control was achieved through mixed applications of compounds of the azole group and combining azoles with fungicides with different modes of action (Jørgensen *et al*., 2018).

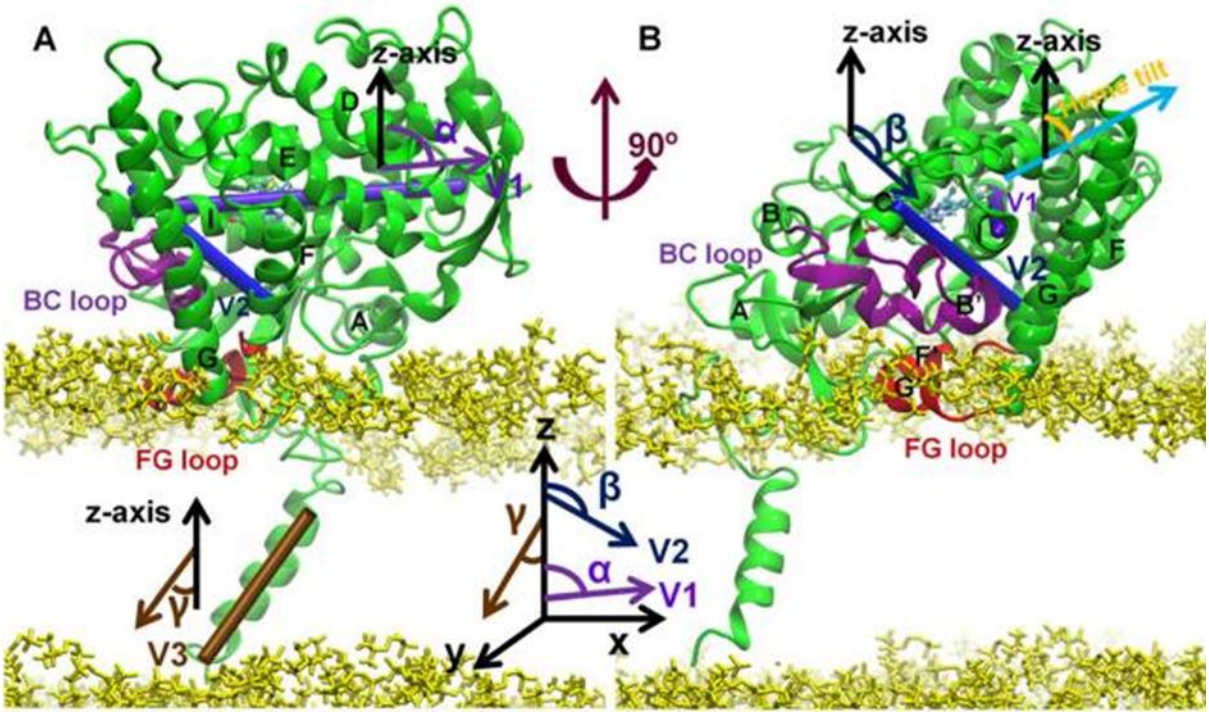

Figure 2.1 Two views of the CYP51 protein within the bilayer of ligand-free *Trypanosoma brucei* (Yu *et al*., 2015).

Within *Blumeria graminis*, which causes powdery mildew in cereals, the *CYP51* gene occurs as a single copy with three exons interrupted by two short introns (Wynand and Brown, 2005). Since *B. graminis* f. sp. *hordei* (*Bgh*) and *B. graminis* f. sp. *tritici* (*Bgt*) differ in their response to DMIs, the *CYP51* gene was sequenced to confirm whether detected nucleotide variations within the gene were associated with variations in fungicide sensitivity. Two single-nucleotide polymorphism (SNP) mutations were detected in the gene when compared to the published *Bgh CYP51* gene sequence. These two SNPs resulted in the amino acid substitution Y136F in both *Bgh* and *Bgt* isolates and K147Q in *Bgh* isolates. As a result, the Y136F substitution caused a reduction in the affinity of the enzyme for its fungicide inhibitor substrate due to the increased hydrophobicity (Délye *et al*., 1997). This substitution was present in the CC66 pathotype that showed low levels of insensitivity to DMIs (Blatter *et al*., 1998), suggesting that this mutation only confers a low level of insensitivity (Wynand and Brown, 2005). The K147Q substitution expressed high levels of insensitivity to the DMIs (Wynand and Brown, 2005). This mutation occurred within the B'-helix of the protein, which might prevent inhibitor binding by reducing the net positive charge of the helix while still allowing endogenous substrate binding. Additionally, the Y136F mutation within the *CYP51* gene and increased *CYP51* gene expression were responsible for azole insensitivity observed in *Erysiphe necator*, the causal agent for powdery mildew of grapes, from populations in the eastern USA (Frenkel *et al*., 2015). This increased insensitivity to myclobutanil was associated with both Y136F and A1119C mutations within the *CYP51* gene.

Additional *CYP51* gene mutations were observed in *Pt* isolates with increased insensitivity to azole fungicides (Stammler *et al*., 2009). The Y134F mutation resulted in an amino acid substitution of phenylalanine with tyrosine, which occurred at a frequency of 4.5% in European field isolates. This substitution did not seem to affect the sensitivity levels of *Pt* towards epoxiconazole. Additional mechanisms might therefore be involved in the mediation of increased insensitivity.

Many European countries rely on DMI fungicides to control *Mycosphaerella graminicola* – causing wheat leaf blotch (Leroux *et al*., 2007). In *M. graminicola*, DMI insensitivity is associated with mutations in the *CYP51* gene (Leroux *et al*., 2007), where amino acid changes at codon positions 459, 460 and 461 were associated with low insensitivity to the fungicide. A change at codon position 381 in combination with the previous changes were associated with the highest insensitivity to all DMIs excluding prochloraz. Mutations at codon positions 316

and 317 were also associated with low insensitivity in some of the isolates towards most DMIs. Seventeen amino acid changes have been detected in insensitive *M. graminicola* isolates. The impact of these alterations has not been described biochemically, but studies have revealed that *CYP51* gene variants are differentially selected by azole fungicides (Cools and Fraaije, 2008).

Azole fungicides within the DMI group used to control *M. graminicola* and *Pt* have shown a rapid reduction in efficiency (Cools and Fraaije, 2008). Mutations within the *CYP51* gene resulted in amino acid changes that affected the binding affinity of the enzymes with the azoles in the pathogenic yeast *Candida albicans* (Lamb *et al*., 2000). As a result, enzyme activity was maintained, and the pathogen survived the fungicide treatment.

The mechanism of increased insensitivity to DMIs was also investigated in six isolates of *Penicillium digitatum*, responsible for post-harvest decay in citrus, by sequencing the *CYP51* gene (Hamamoto *et al*., 2000). Three isolates were insensitive to DMIs, while three were sensitive, although the protein-coding regions of the *CYP51* gene were identical in all six. Within the promoter region of the *CYP51* gene, DMI-insensitive isolates contained a 126-base pair (bp) sequence that was tandemly repeated five times. In contrast, the sequence was not repeated in the DMI-sensitive isolates. The constitutive expression of the *CYP51* gene was 100-fold higher in the three insensitive isolates compared to the sensitive isolates (Hamamoto *et al*., 2000). When a *CYP51* gene from an insensitive isolate including the promoter region, was introduced into a sensitive isolate, the resulting transformants showed increased *CYP51* expression while also being insensitive to DMI treatment. Reducing the number of tandem repeats to two, led to decreased *CYP51* expression and increased sensitivity. The results confirmed that the 126 bp tandem repeat acted as an enhancer for *CYP51* expression, which in turn resulted in DMI insensitivity.

The Y134F substitution, detected when the *CYP51* gene was sequenced from *Pst* isolates, was orthologous to the Y137F substitution detected in *Z. tritici* isolates (Woodman *et al*., 2021). This substitution was observed in at least fourteen other fungal species where it has been associated with insensitivity to DMIs, including *Phakopsora pachyrhizi*, causing soybean rust, and *Pt* (Stammler *et al*., 2009; Klosowski *et al*., 2016; Mair *et al*., 2016; Tian *et al*., 2019).

The Y137F substitution confers only moderate DMI insensitivity but paves the way for the development of other mutations that can severely affect control provided by fungicide application when carried in combination with the Y137F substitution (Woodman *et al*., 2021).

An example of this is the *CYP51* Y137F substitution in *Bgh* that was replaced after 2 years by a double mutant carrying S524T additionally (Tucker *et al*., 2019). The resulting mutant showed high resistance and insensitivity to tebuconazole and propiconazole.

It has been proposed that mutations in the *M. graminicola CYP51* gene, leading to fungicide insensitivity, have arisen in either Denmark or the United Kingdom (UK), with a subsequent eastward spread across Europe through wind (Brunner *et al*., 2008). Selection pressure exerted by azole fungicides, will result in an increased frequency of mutants with high levels of insensitivity in the future. The widespread gene flow obtained from wind dispersal will spread these spores carrying mutant alleles to new regions where outbreaks will occur if fungicide applications persist as in the past.

Recently, a Y134F substitution in the *CYP51* gene was reported in a high proportion (50% and 60%, respectively) of *Pst* isolates from China and New Zealand (Cook *et al*., 2021). Fungicide sensitivity testing revealed that the identified substitution was associated with low resistance factors. The substitution in two geographically distinct locations suggests that the mutation arose multiple times under the selection pressure exerted by DMI fungicide applications. There is a possibility that the mutation will become more widespread in *Pst* in the future through either independent evolution or movement resulting from human activity because of the acquisition of the mutation through multiple events.

2.6 Management of fungicide insensitivity

Efforts aimed at preventing fungicide insensitivity from occurring within pathogen populations are beneficial to extend the period of fungicide efficiency. Alleviating the pressure on the continuous need to develop new fungicides, which is expensive and time-consuming, is critical.

To effectively manage fungicide insensitivity, it is important to limit repeated exposure of the target pathogen to the same active ingredient (Brent and Hollomon, 2007). If the factors that determine the selection of resistant mutants in a pathogen population can be identified, the management of fungicide insensitivity can utilise the same principles to become more effective (Van den Bosch *et al*., 2014). The reduction of the product selection coefficient and limited exposure of the product to the pathogen, will decrease the selection pressure exerted by the fungicide on the fungal population (Van den Bosch *et al*., 2014; Brent and Hollomon, 2007).

Tactics to reduce fungicide insensitivity must thus aim to limit the exposure time of the pathogen to the fungicide. This can be accomplished by implementing several integrated control measures to combat pathogen establishment, dispersal and survival. Integrated pest management (IPM) tactics include properly disposing of crop debris, controlling volunteer plants that might carry disease, and selecting cultivars with a high degree of resistance to diseases. Disease resistance in cultivars is obtained through breeding and selection, which eliminates the need for a repetitive fungicide application to prevent disease. The planting of monocultures in areas where the disease is known to occur, should be avoided, as well as the unnecessary use of fungicide application and improper dosages of the fungicide. The recommended commercial dose of the fungicide indicated in the manufacturer's instructions should be strictly adhered to, and producers should be made aware of the consequences of inappropriate fungicide use (Brent and Hollomon, 2007).

Fungicides containing a. i. with different modes of action should be used in combination with each other instead of a single fungicide with a single a. i. being applied alone (Brent and Hollomon, 2007). Evidence suggests that fungicides with different modes of action should be applied as mixtures rather than in alternation with one another, as this is the best resistance management tactic (Elderfield *et al*., 2018). Crops must be monitored regularly to ensure that disease is detected early and treatment can be initiated as soon as possible, thereby reducing yield loss and preventing the build-up and subsequent spread of the disease. The use of repeated applications of the same fungicide should also be avoided, as well as exceeding the recommended number of fungicide applications within a particular season.

The available evidence of fungicide insensitivity focusses on cases where a single-mutation resulted in high levels of fungicide insensitivity (Van den Bosch *et al*., 2014). Evidence of fungicide insensitivity caused by a range of mutations that result in a shift in sensitivity over long periods of time is therefore lacking. The available data is also primarily from foliar fungicide spray experiments with little evidence of the selection for insensitivity that results from seed treatments. These limitations in the available information around fungicide insensitivity need to be considered to improve the management of fungicide insensitivity. In the past, the focus was on the development and application of single-site fungicides, but higher research priority should be given to the development of multi-site fungicides (Deising *et al*., 2008). Single-site fungicides exert a strong selection pressure for insensitive mutants whereas

multi-site fungicides do not and thus allow a longer period of effectiveness. Resistance management is thus key for the effective prevention of fungicide insensitivity.

2.7 Conclusions

Despite the occurrence of fungicide insensitivity and the potential negative effects on the environment, producers revert to fungicide use when resistance in cultivars fails, often resulting in no workable alternatives once the crop has been established (Russel, 2006). It is, therefore, important to either prevent the development of fungicide insensitivity or to increase the lifespan of effective fungicides by implementing resistance management strategies. When new fungicides are released, the development of insensitivity is important to consider (Russel, 2006). Many fungicides that carry only one a. i. are at higher risk for fungicide insensitivity developing. These fungicides are currently recommended for use with clearly defined resistance management strategies.

The development of fungicide insensitivity is a significant problem in modern agriculture, occurring in many pathogens of major crops (Oliver, 2014). In the past, it was believed that wheat rust fungi were immune to the development of fungicide insensitivity due to limited previous exposure to fungicides for which resistance was a risk, which resulted in a heavy reliance on fungicides for rust control (Scherm *et al*., 2009; Murray and Brennan, 2010; Oliver, 2014). Pathogens that have developed insensitivity rapidly, are classified as high-risk species. While wheat rust fungi are classified as low-risk species, certain properties, such as being polycyclic, abundant and wind-borne, are shared with high-risk fungi like *Botrytis* and *Blumeria* species. Therefore, being observant of the emergence of fungicide insensitivity developing in wheat rust pathogen populations should be considered a cornerstone of pathogen and fungicide monitoring.

Molecular research into fungicide insensitivity led to the advancement of our knowledge of the mechanisms involved in the acquisition of mutations associated with fungicide insensitivity (Ma and Michailides, 2005). The development of effective and fast-acting methods to detect genes that contribute to fungicide insensitivity in pathogen populations has been facilitated through molecular tools. The continual analysis of genes, and associated biochemical responses, in pathogenic fungi, are required to further enhance our understanding and effective management of fungicide insensitivity.

In conclusion, there is a risk of fungicide insensitivity developing in wheat rust pathogen populations and steps need to be taken to detect and avoid its development. The successful production of many crops is not possible without disease monitoring and the timeous application of fungicides. When genetic resistance fails, or resistant cultivars are not available, or under conditions of high disease pressure producers need access to alternative control strategies such as fungicide application. The long-term efficacy of fungicide applications can be ensured when proper fungicide resistance management tactics are implemented.

Baseline studies to determine sensitivity levels are currently lacking in SA, and the extensive reliance on fungicide applications for disease control of cereals, marks the importance of routine monitoring of fungicide sensitivity. In this study, the deviation in sensitivity levels to propiconazole and tebuconazole were determined among historically collected and recently *Pgt* collected field isolates, and sequence variants of the *CYP51* gene were identified. The results will serve as a point of reference for future studies and confirm the genetic potential of *Pgt* to develop fungicide insensitivity in SA.

Chapter 3: Materials and methods

3.1 Fungicide sensitivity trials

3.1.1 *Puccinia graminis* f. sp. *tritici* isolates

Forty-five *Pgt* isolates representative of 12 South African races, were selected for fungicide sensitivity response tests (Table 3.1; Figure 3.1). These isolates were collected between 1981 and 2020, and represented both historical, as well as recently collected race isolates, which originated from diverse localities with good representation of the different wheat production areas in SA. Ten isolates collected between 1981 and 2016 from wheat and triticale cultivars represented the historically collected *Pgt* races 2SA4, 2SA55A, 2SA55B, 2SA88, 2SA104, 2SA105, 2SA106, 2SA107, 2SA88+ and 2SA108. More recent isolate collections were made in the Free State and Western Cape provinces, which included isolates of the two new races 2SA5 and 2SA42. In the Free State, fourteen isolates were collected in the Bethlehem wheat production area during 2016, 2017 and 2020 from the wheat cultivars SST 0177, SST 374, PAN 3161 and McNair (trap nursery). Two isolates were collected from a farm southwest of Bloemfontein in 2020 from the cultivar SST 356. In the Western Cape province, collections were made near Suurbraak in 2017 from the hosts McNair and SST 88, in the Swellendam area in 2017 from SST 88, at the Tygerhoek research farm outside Riviersonderend in 2017 from the triticale cultivar Coorong, and in the Riversdal and Albertina districts in 2020 from SST 88 and SST 0177. Most isolates originated from the 2017 and 2020 seasons, with 16 isolates collected each year. Collections were made from nine different hosts of which the most were made from SST 88 (eight collections) and the least from Coorong and SST 356 (two collections each). Isolates were representative of 12 *Pgt* races with 11 representing 2SA5.

The different races are indicated by their Agricultural Research Council - Small Grain (ARC-SG) "2SA", University of the Free State (UFS) and NA race names (Terefe *et al*., 2016; Table 3.1). The NA race classification system applies the letter codes "B" to "T" based on the seedling response (low to high) of *Pgt* isolates on twenty differential wheat lines. The differential lines are subdivided into five subsets of four lines, each carrying a known and unique stem rust resistance gene (Fetch *et al*., 2009).

Table 3.1 Forty-five isolates representative of twelve *Puccinia graminis* f. sp. *tritici* (*Pgt*) races selected for fungicide sensitivity response tests. Included are the isolate number, different race names, origin and cultivar collected from, and the year of first detection or collection.

Isolate no.	ARC-SG[1] race name	UFS[2] race name	NA[3] race name	Origin	Cultivar	Year detected
1	2SA104	Pgt56	BPGSC+*Sr27, Kw*	Bethlehem	SST 0177	2020
2	2SA42	Pgt63	PTKSK	Bethlehem	SST 374	2020
3	2SA42	Pgt63	PTKSK	Bethlehem	PAN 3161	2020
4	2SA42	Pgt63	PTKSK	Bethlehem	PAN 3161	2020
5	2SA42	Pgt63	PTKSK	Bethlehem	PAN 3161	2020
6	2SA107	Pgt60	PTKST	Bethlehem	PAN 3161	2020
7	2SA107	Pgt60	PTKST	Bethlehem	PAN 3161	2020
8	2SA107	Pgt60	PTKST	Bethlehem	PAN 3161	2020
9	2SA107	Pgt60	PTKST	Bethlehem	PAN 3161	2020
10	2SA88+	Pgt61	TTKSF+*Sr9h*	Bethlehem	McNair	2016
11	2SA104	Pgt56	BPGSC+*Sr27, Kw*	Suurbraak	McNair	2017
12	2SA104	Pgt56	BPGSC+*Sr27, Kw*	Suurbraak	McNair	2017
13	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Tygerhoek	Coorong	2017
14	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Tygerhoek	Coorong	2017
15	2SA88	Pgt55	TTKSF	Bethlehem	SST 374	2017
16	2SA88	Pgt55	TTKSF	Bethlehem	SST 374	2017
17	2SA88	Pgt55	TTKSF	Bethlehem	SST 374	2017
18	2SA88	Pgt55	TTKSF	Bethlehem	SST 374	2017
19	2SA88	Pgt55	TTKSF	Suurbraak	McNair	2017
20	2SA88	Pgt55	TTKSF	Suurbraak	McNair	2017
21	2SA104	Pgt56	BPGSC+*Sr27, Kw*	Suurbraak	McNair	2018
22	2SA104	Pgt56	BPGSC+*Sr27, Kw*	Suurbraak	McNair	2018
23	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Swellendam	SST 88	2017
24	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Swellendam	SST 88	2017

Table 3.1 (cont.) Forty-five isolates representative of twelve *Puccinia graminis* f. sp. *tritici* (*Pgt*) races selected for fungicide sensitivity response tests. Included are the isolate number, different race names, origin and cultivar collected from, and the year of first detection or collection.

Isolate no.	ARC-SG[1] race name	UFS[2] race name	NA[3] race name	Origin	Cultivar	Year detected
25	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Suurbraak	SST 88	2017
26	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Suurbraak	SST 88	2017
27	2SA42	Pgt63	PTKSK	Bloemfontein	SST 356	2020
28	2SA42	Pgt63	PTKSK	Bloemfontein	SST 356	2020
29	2SA104	Pgt56	BPGSC+*Sr27, Kw*	Suurbraak	SST 88	2017
30	2SA104	Pgt56	BPGSC+*Sr27, Kw*	Suurbraak	SST 88	2017
31	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Albertina	SST 88	2020
32	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Riversdal	SST 88	2020
33	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Albertina	SST 0177	2020
34	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Riversdal	SST 0177	2020
35	2SA5	Pgt64	BFGSC+*Sr27, Kw*	Riversdal	SST 0177	2020
36	2SA4	UVPgt50	PSKSC	Western Cape	Wheat	1981
37	2SA55A	UVPgt54	BNGSC	Riviersonderend	Triticale	2000
38	2SA55B	UVPgt54+*Sr9g*	BPGSC	Riviersonderend	Triticale	2000
39	2SA88	UVPgt55	TTKSF	Riviersonderend	Wheat	2000
40	2SA104	UVPgt56	BPGSC+*Sr27, Kw*	KwaZulu-Natal	Triticale	2003
41	2SA105	UVPgt57	BPGSC+*Sr27, Kw, Satu*	Western Cape	Triticale	2005
42	2SA106	UVPgt59	TTKSP	Western Cape	Wheat	2007
43	2SA107	UVPgt60	PTKST	KwaZulu-Natal	Wheat	2009
44	2SA88+	UVPgt61	TTKSF+*Sr9h*	Afrikaskop	Wheat	2010
45	2SA108	UVPgt62	BFBSC+*Sr27*	Transvaal	Triticale	2016

[1] Agricultural Research Council – Small Grain; [2] University of the Free State; [3] North American

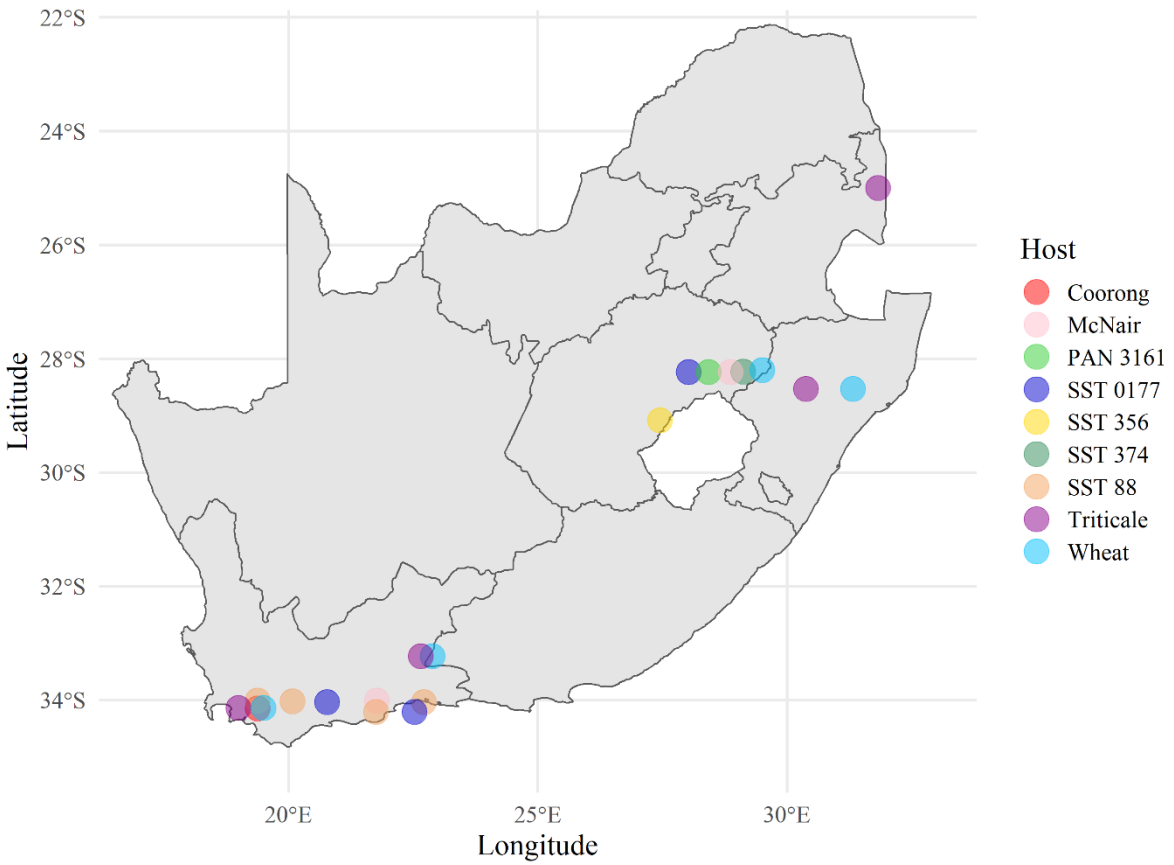

Figure 3.1 Map indicating the origin of *Puccinia graminis* f. sp. *tritici* race isolates that were collected from different wheat production areas in South Africa.

3.1.2 Increase of *Puccinia graminis* f. sp. *tritici* (*Pgt*) urediniospores

Approximately 50 seeds of the universally susceptible differential McNair were planted in ninety 10 cm pots, each filled with Mikskaar Professional Potting Soil 70 (Hygrotech, Pretoria, SA). The seeds were germinated in a growth cabinet at 25°C for four days, where after the seedlings were transferred to a rust-free greenhouse cubicle with a day/night temperature regime of 22 and 18°C, respectively. The seedlings were drenched with 0.03% (w/v) maleic hydrazide Reagent Plus® (Sigma-Aldrich, St. Louis, Missouri, USA) upon coleoptile emergence and watered daily with reverse-osmosis purified water. Seedlings were fertilized by adding 100 ml 0.2% (w/v) Multifeed-Classic water-soluble fertilizer [Effekto®, NPK 19:8:16 (43)] per tray of 7 pots upon transfer to the greenhouse and again following inoculation.

Two pots of McNair seedlings were inoculated seven days after planting with urediniospores of each of the 45 selected *Pgt* isolates. Prior to inoculation, the urediniospores were removed from the -80°C freezer, and heat shocked at 46°C for 6 min. Primary leaves were inoculated with 1 mg/ml urediniospores suspended in 0.3 ml Soltrol® 130 isoparaffinic oil using a pressure pump at a 25 kPa pressure setting (Vacuubrand® pump - model MZ2) connected to an inoculation device (Pretorius *et al.*, 2019). Inoculated seedlings were first placed in an illuminated growth cabinet (200 μE/m^2/s light; 25°C) for 45 min to dry off, before transferring them to a dew simulation chamber at ±18°C for 18 h. The plants were then again dried off in the growth cabinet for 3 h before being transferred to the greenhouse and placed in separate compartments to prevent contamination between isolates. Twelve to thirteen days after inoculation, fresh urediniospores were harvested from the infected plants for use in germination tests.

3.1.3 Fungicides

The sensitivity of the *Pgt* isolates was tested against the triazole-based fungicides propiconazole (Tilt 250 EC provided by Syngenta, Centurion, SA) and tebuconazole (Folicur 250 EW provided by Bayer Crop Science, Kempton Park, SA; Table 3.2).

Table 3.2 Propiconazole and tebuconazole fungicide dilutions used in *Puccinia graminis* f. sp. *tritici* (*Pgt*) urediniospore germination tests. Fungicide dilutions, µl fungicide/ml 2xdistilled water required to achieve the specified concentrations and the gram (g) active ingredient (a. i.)/ litre (L) at selected concentrations, are given. The full-recommended commercial dose for propiconazole is 1.66 ml/L water (0.41667 g/L a. i.) and 2.75 ml/L water (0.6875 g/L a. i.) for tebuconazole both at an application rate of 300 L per hectare.

Propiconazole			**Tebuconazole**		
Final dilution	**µl/ml**	**a. i. (g/L)**	**Final dilution**	**µl/ml**	**a. i. (g/L)**
x0.000	0.0000	0.0000	x0.000	0.0000	0.0000
x0.075	0.1245	0.0313	x0.010	0.0275	0.0069
x0.100	0.1660	0.0417	x0.015	0.0413	0.0103
x0.200	0.3320	0.0833	x0.020	0.0550	0.0138
x0.400	0.6640	0.1667	x0.030	0.0825	0.0206

3.1.4 Germination tests

Propiconazole was diluted with 2x distilled water to final concentrations of x0.075, x0.100, x0.200 and x0.400. The lowest dilution was well below the full-recommended commercial dose of 1.66 ml/L water at an application rate of 300 L per hectare (Table 3.2). Similarly, tebuconazole was diluted to x0.010, x0.015, x0.020 and x0.030, with the lowest dilution being well below the full-recommended commercial dose of 2.75 ml/L water at an application rate of 300 L per hectare (Table 3.2).

Final concentrations were determined based on the results from preliminary experiments using one *Pgt* isolate (results not given). The diluted fungicide solutions were mixed into 10 ml molten 1.5% (w/v) 2x distilled water agar. The control treatment consisted of 2x distilled water.

Approximately 1.75 ml of the amended water agar were transferred into the wells of a 48-well Multidish (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The different fungicide dilutions were organized in a completely randomized design within the Multidish with three replications for each isolate at each fungicide concentration (n = 1350).

A settling tower, with a fixed turntable (14 rpm) at the base, was used to evenly distribute approximately 0.013 g fresh urediniospores of each *Pgt* isolate onto the prepared Multidishes with a 3 min spore settling time (Negussie *et al*., 2005). The Multidishes were incubated for 3 h at 18°C to facilitate spore germination.

Germinating urediniospores for all treatments and replications were photographed with an Olympus SZX10 stereomicroscope (x3.02 magnification). The germination percentage for each treatment was determined by counting 50 spores per agar well. Germination was considered positive when the length of the germ tube was greater than double the length of the short diameter of the spore (Zadoks, 1961). Germination percentages were calculated by dividing the number of germinated spores by the total number of counted spores, multiplied by 100.

3.1.5 Data analysis

The relative inhibition rate of urediniospore germination was calculated as follows:

$$Inhibition\ rate = (1 - \left(\frac{mean\ germination\ rate\ of\ treatment}{mean\ germination\ rate\ of\ control}\right)\ x\ 100$$

Summarized dose response curves were constructed where the germination rates of the urediniospores were plotted against the fungicide concentrations using the R package "ggplot2" v4.05 (Wickham, 2016). Two-way analysis of variance (ANOVA) was performed for the fungicides to determine their individual effects and whether significant differences could be observed between replicates, isolates, concentrations and the interactions between isolates and concentrations at the 5% significance level. Least significant difference (LSD) tests were performed for the two fungicides using the R package "agricolae" v4.0.5 (Mendiburu, 2020) to identify significant differences between groups of isolates.

The absolute EC_{50} for the isolates were calculated using the R package "ec50estimator" v4.0.5 (Alves, 2020) and by fitting the germination rate against the log transformed fungicide concentrations using the best fit model (LL.3) determined using the R package "drc" v4.0.5 (Ritz *et al*., 2015). Analyses were conducted using R version 4.0.5 (R Core Team, 2021) in the R Studio version 1.4.1103 (RStudio Team, 2020) platform.

3.2 Sequence analysis of the *CYP51* gene

3.2.1 DNA extraction from selected *Pgt* isolates

Twenty-three isolates were selected for *CYP51* sequence analysis based on their collection history and the calculated absolute EC_{50} values (3.1.5). This included ten isolates representing the historically collected race isolates and thirteen isolates with either high or low EC_{50} estimates for one or both of the individual fungicides.

DNA was extracted from the selected isolates using a modified cetyl trimethylammonium bromide (CTAB) method (Saghai-Maroof *et al*., 1984). Approximately 50-100 µl urediniospores were freeze dried and ground to a fine powder with a Qiagen Tissue-Lyser (Haan, Germany) at 30 r/s for 1 min. The DNA was extracted using 750 µl CTAB extraction buffer (0.1 M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8.0, 20 mM

ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 2% (w/v) CTAB). The samples were incubated for 1 h at 65°C and then extracted once with 500 µl chloroform ($CHCl_3$)/ isoamylalcohol (IAA, 24:1 v/v) before being centrifuged at 12000 *g* for 10 min at 4°C. The DNA was precipitated from the aqueous phase with 500 µl isopropanol and then incubated at room temperature for 20 min before being centrifuged again at 12000 *g* for 10 min at 4°C. The precipitate was washed with ice-cold 70% (v/v) ethanol, incubated for another 20 min at room temperature and then centrifuged for a further 5 min at 12000 *g* for 10 min at 4°C. The resulting pellet was air-dried for 1 h at room temperature before the addition of 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 2 µl RNase (10 mg/ml). The pellet was dissolved overnight at 4°C, before incubation at 37°C for 1 h. The DNA was extracted with 20 µl 7.5 M ammonium acetate and 200 µl $CHCl_3$ / IAA (24:1) before 10 min of centrifugation at 12000 *g* at 4°C. The DNA was precipitated from the aqueous phase for 1 h at -20ºC with 500 µl ice-cold 100% (v/v) ethanol. After centrifugation at 12000 *g* at 4°C for 10 min, the DNA was washed with 70% (v/v) ethanol and centrifuged at 12000 *g* for 10 min at 4°C. The pellet was air-dried and dissolved overnight at 4°C in 50 µl TE buffer.

The DNA concentration and purity were determined using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, Massachusetts, USA). DNA stocks were diluted with DNAase and RNAase free Sabax water to a final concentration of 10 ng/µl.

3.2.2 Polymerase chain reaction (PCR) amplification of the *CYP51* gene fragment

Primers specific to the *CYP51* gene (Ensembl accession number VDEP01000442) were designed using the Primer3 online tool (https://primer3.org/) (Table 3.3; Figure 3.2). Gradient PCR was performed to determine the optimal annealing temperature for all *Pgt*_CYP51 primer sets using *Pgt* isolate 34 (2SA5) DNA.

The PCR comprised of 20 ng DNA, 1 µM of each primer set and 1x KAPA-Taq Ready-Mix concentration (KapaBiosystems, Sigma-Aldrich, USA). Cycling conditions were 3 min at 95°C, followed by 40 cycles of a 30 sec denaturation step at 95°C, a 30 sec primer annealing step at temperatures ranging from 52°C to 62°C and a 2 min extension step at 72°C, followed by a single 5 min step at 72°C.

A second PCR optimization was performed to determine the optimal concentration of all primer sets in terms of the least amount of visible primer dimers. These reactions comprised of 20 ng DNA, 1x KAPA-Taq Ready-Mix and primers at final concentrations of 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 µM, respectively. Cycling reactions were 3 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 62°C and 2 min at 72°C, followed by 5 min at 72°C.

All subsequent PCRs were performed using the optimal annealing temperature of 62°C and a primer concentration of 0.25 µM. A no-template control was included in all reactions (Aaij and Borst, 1972).

The entire *CYP51* gene fragment was PCR amplified for cloning from the isolates using the F1R1 primer combination (Table 3.3). Two subsequent PCRs, using the F1R2 and F2R1 primer combinations, were used to PCR amplify two overlapping *CYP51* gene fragments that made up the entire *CYP51* gene (Figure 3.2).

Finally, the F3R1 primer combination was used to amplify the second *CYP51* gene region in several isolates where the F2R1 primer combination was unsuccessful.

3.2.3 Agarose gel electrophoresis

PCR amplicons were separated on a 1.2% (w/v) agarose gel prepared in 0.5x TAE (20 mM Tris-acetate, 0.5 mM EDTA pH 8.0) containing 10 µg/ml ethidium bromide (Sambrook *et al*., 1989). Eight µl loading buffer (15% (w/v) Ficoll, 2.5 mg/ml Orange G), was added to each PCR reaction before the DNA was separated at 10 V/cm for 30 min using 0.5x TAE as running buffer. The DNA was visualized under UV light and photographed with a Gel Doc™ EZ Imager (Bio-Rad, Hercules, California, USA).

The PCR products were purified using the Favorgen Gel/PCR purification kit from Favorgen Biotech (Pingtung, Taiwan) according to the manufacturer's instructions. The DNA was eluted in 40 µl elution buffer of which 10 µl was separated on a 1.2% (w/v) agarose gel as described, to confirm successful recovery.

Table 3.3 Primers used to amplify and sequence the *CYP51* gene from selected *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates. The sequences of the different primers are indicated in the 5'-3' direction.

Primers	**Bovis name**	**Sequence (5'-3')**
*Pgt*_CYP51 forward (F1)	Bovis 1294	CTCATCGACCCACTGATCG
*Pgt*_CYP51 reverse (R1)	Bovis 1295	TACGAGTGGATGTTCCTCCTAGTAA
Internal *Pgt*_CYP51 forward (F2)	Bovis 1349	TTTGCAGAACCGAAAACGCA
Internal *Pgt*_CYP51 reverse (R2)	Bovis 1348	CTGTTCCTGCCTCAATTCGG
Internal *Pgt*_CYP51 forward (F3)	Bovis 1959	CAAACCAAGATTCAAAATCCTCACA

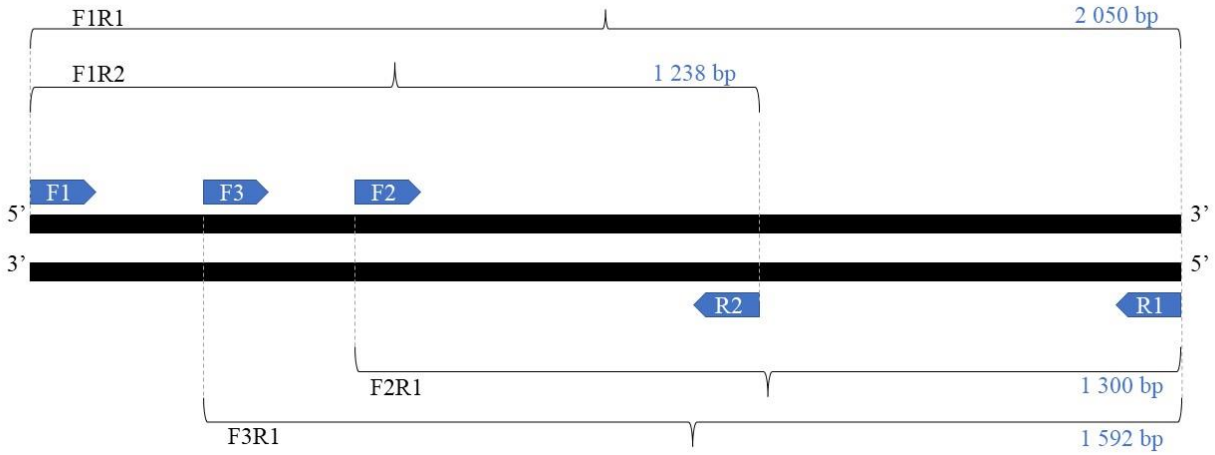

Figure 3.2 Gene specific primer attachment regions on the *CYP51* gene fragment and the resulting amplified DNA fragments produced with PCR.

3.2.4 Cloning of the amplified *CYP51* gene fragments

The recovered *CYP51* gene fragments were cloned into the pGemT-Easy plasmid vector (Promega, Madison, Wisconsin, USA). The ligation reaction contained 5 ng DNA, 1x Rapid ligase buffer, 10 ng pGemT-Easy plasmid DNA and 1.5 U T4 DNA ligase, which was incubated at 4°C overnight. The plasmid vectors were transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, Wisconsin, USA) by mixing 5 µl ligation mixture with 65 µl JM109 competent cells and incubating it for 30 min on ice. After a 45 sec heat shock at 42°C, 1 ml Lysogeny broth (LB; 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) was added to the *E. coli* cells before incubation for 60 min on a shaking platform at 37°C.

After centrifugation at 12000 *g* for 5 min, the pellet was resuspended in 100 µl growth medium and the cells plated on LB plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 2% (w/v) agar) containing 50 µg/ml ampicillin. The plates were previously treated with 40 µl 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (20 mg/ml) and 40 µl isopropyl β-d-1-thiogalactopyranoside (IPTG; 2 mg/ml). The plates were incubated overnight at 37°C. White colonies were selected and transferred to a reference LB plate containing 50 µg/ml ampicillin that was previously treated with X-gal and IPTG. Eight white colonies for each isolate were inoculated from this reference plate in 1 ml LB medium containing 50 µl/ml ampicillin and grown overnight at 37°C on a shaking platform.

The *E. coli* cells were pelleted through a 5 min centrifugation at 10000 *g*, at 4°C, and the pellet resuspended in 100 µl 2x distilled water and incubated at 94°C for 5 min. After a final centrifugation for 5 min at 10000 *g*, the extracted plasmid DNA in the resulting supernatant was directly used for PCR to amplify the cloned insert (3.2.2). After purification, the inserts were bi-directionally sequenced using the respective forward and reverse primers.

3.2.5 Sequencing of the *CYP51* gene from the selected *Pgt* isolates

Eight cloned *CYP51* gene fragments for each *Pgt* isolate were bidirectionally sequenced using the BigDye^{TM} Terminator sequencing kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each 10 µl sequence reaction comprised of 0.5 µl reaction mix (1/16th), 3.2 pmol of the respective primer, and 2 µl (1x) sequencing buffer. The cycling conditions for the reactions were 1 min at 96°C, followed by 25 cycles of 10 sec at 96°C, 5 sec at 56°C and 4 min at 60°C.

After sequencing, 10 µl Sabax water, 15 µl of a 125 mM EDTA solution and 60 µl 100% (v/v) ethanol were added to each sequence reaction. The reactions were incubated at -20°C for 5 min, whereafter they were centrifuged for 80 min at 2 300 *g* at 4°C. The supernatant was removed, and 200 µl 70% (v/v) ethanol was added. After centrifugation at 2 300 *g* for 20 min at 4°C, the supernatant was again removed and the pellet dried for 30 sec in an open PCR machine at 96°C. The sequenced DNA was finally resolved in a 3130x1 Genetic Analyzer (Applied Biosystems, 46 Foster City, California, USA) using the StdSeq50_POP7 sequencing run module.

3.2.6 Bioinformatic analysis of the sequenced *CYP51* gene

The resulting sequences were subjected to bioinformatic analysis. The forward and reverse complement sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and any discrepancies that were detected were resolved. Contigs representing the full-length *CYP51* gene using both the F1R2 and F2R1 (F3R1) fragments were constructed using the Cap3 online tool (http://doua.prabi.fr/software/cap3). The contigs were aligned to identify possible allelic variants of the genes in the different isolates. The allelic variant genes were translated into amino acid sequences (https://web.expasy.org/translate/).

The resulting amino acid sequences were aligned using MUSCLE (Edgar, 2004) and a maximum likelihood phylogenetic tree at 1000 bootstraps constructed using MEGA X (Kumar *et al*., 2018). Reference CYP51 protein sequences of *Pgt* (KAA1068883.1, KAA1081585.1, XP 003325369.2, GMQ09617T0 and KAA1080186.1), *Pt* (ACS37521.1 and PTTG05595), *P. horiana* P. Hennings (AHK06541.1), *P. coronata* f. sp. *avenae* Urban & Marková (PLW06402.1) and *Pst* (XP 047804605.1, AEM55575.1, KNE90962 and PSTCY32 09949) were included from NCBI (https://www.ncbi.nlm.nih.gov/), while the reference protein sequence PSTCY32 09949 was received from Tian *et al*., (2019). The CYP51 protein sequences from *Melampsora americana* Arthur (KAH9815292.1) and *P. pachyrhizi* H. Syd & P. Syd (KAI8452430.1) were used as outgroups.

Chapter: 4 Results

4.1 Fungicide sensitivity trials

The use of a settling tower was efficient to evenly distribute *Pgt* urediniospores on the Multidishes which allowed microscopic observation and counting of individual spores. The 3 h incubation period of isolates at 18°C resulted in excellent urediniospore germination percentages for the untreated control treatments, with mean germination percentages exceeding 95% in both fungicide trials (Appendices 21 and 22). Germ tubes were easily identifiable at the required length for germination with no distinguishability problems experienced due to overgrowth.

4.1.1 Urediniospore germination percentages

4.1.1.1 Propiconazole

The *Pgt* urediniospores from the 45 isolates were highly viable as evident by their strong germination responses and clear microscopic differences observed in germ tube length between fungicide treated and untreated controls (Figure 4.1). The LSDs in germination percentages for isolates were 1% for dilutions, 4% for *Pgt* isolates and 9% for the interactions between isolates and concentrations. The mean germination percentages of the 45 *Pgt* isolates recorded for the control (96.98%) and propiconazole dilutions differed significantly ($p < 0.05$ Figure 4.2; Table 4.1) with no significant differences ($p < 0.05$) detected between replicates. Means for the x0.075 (25.03%), x0.10 (6.04%) and x0.20 (0.34%) propiconazole dilutions differed significantly ($p < 0.05$) from each other. Although lower than the mean germination percentage recorded for the x0.20 dilution, the x0.40 (0.03%) propiconazole dilution did not vary significantly from the x0.20 dilution ($p > 0.05$; Figure 4.2).

A significant ($p < 0.05$) interaction was detected for the *Pgt* isolates among fungicide dilutions. Therefore, one-way ANOVAs were performed for the control and each propiconazole dilution to separate means per treatment (Appendices 1 to 5). Excellent germination percentages were observed for the x0.00 propiconazole (untreated control) treatment (mean germination = 96.98%; Appendices 1 and 21). Among the *Pgt* isolates, the mean germination percentages ranged from 92% (isolate 30) to 100% (isolates 3, 4 and 14) forming three significantly

different groups. The LSD value recorded was 6.92% ($p < 0.05$) between isolates (Appendices 1, 6 and 21).

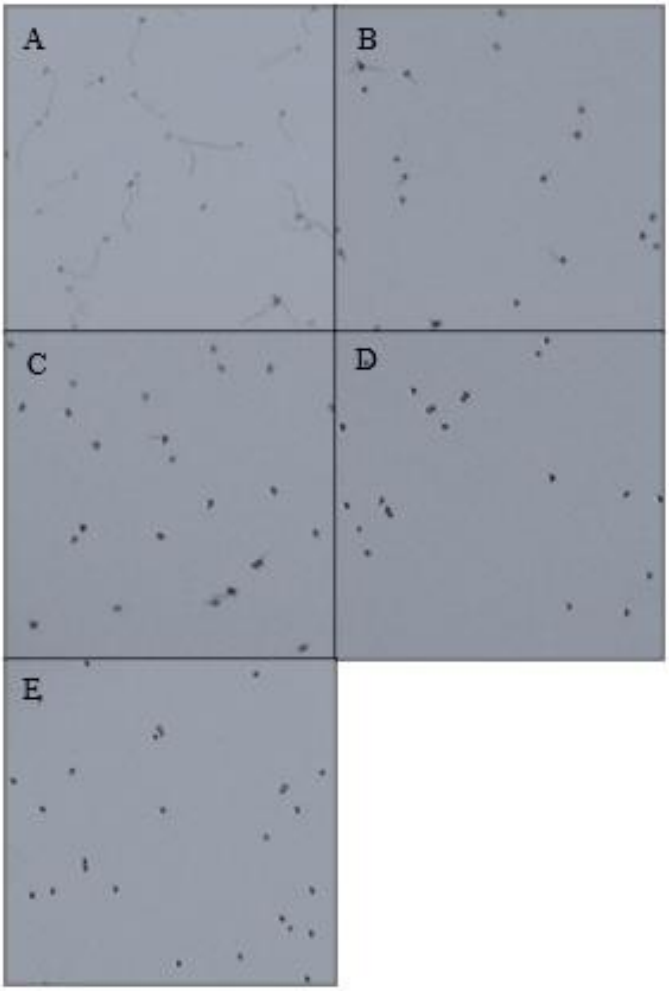

Figure 4.1 Photo plates (A to E) illustrating germination of *Puccinia graminis* f. sp. *tritici* (*Pgt*) urediniospores of isolate 20 between the control (A) and four (B to E) dilutions (x0.075, x0.10, x0.20 and x0.40) for the active ingredient propiconazole.

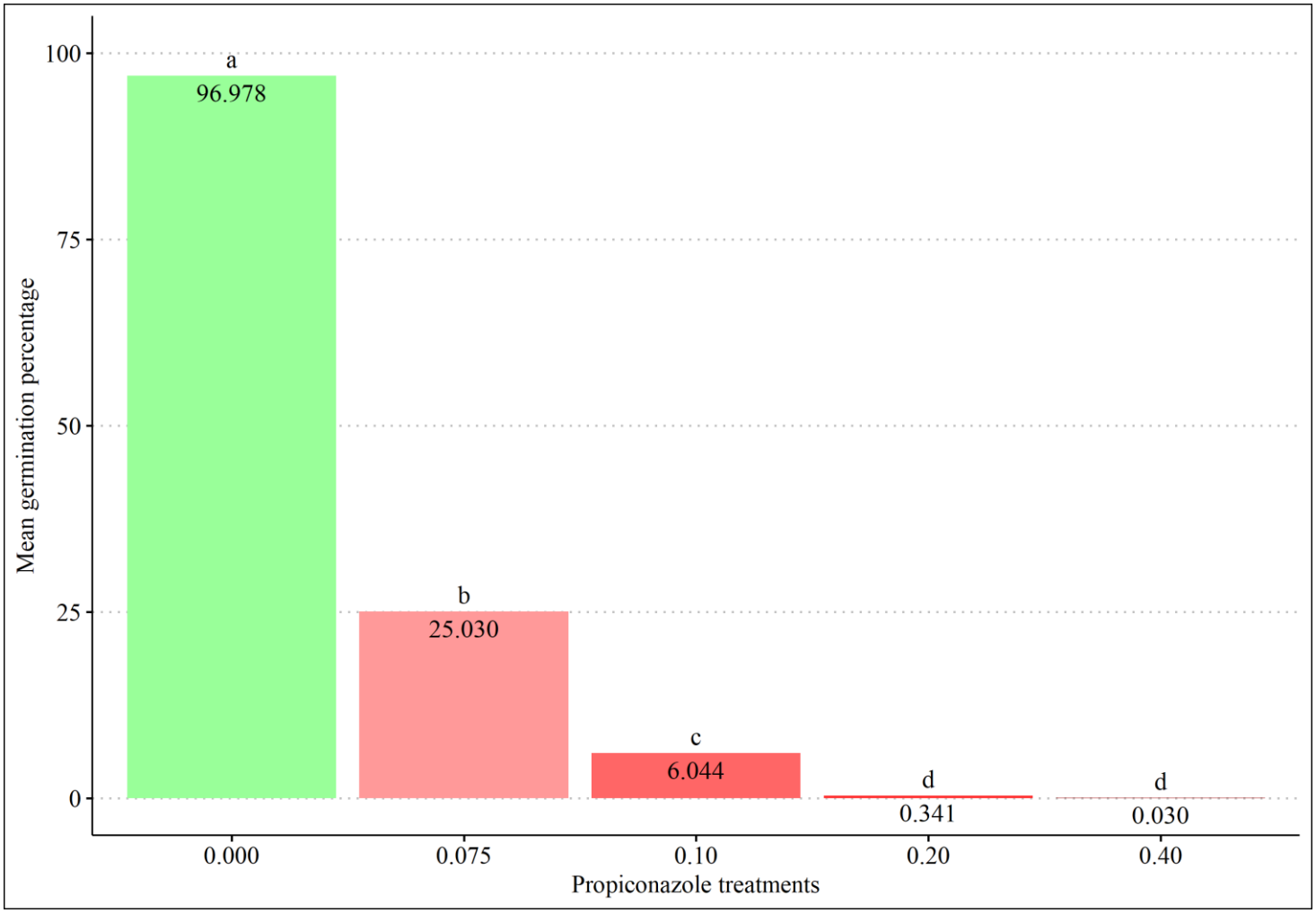

Figure 4.2 Mean urediniospore germination percentages recorded for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the untreated control and four propiconazole dilutions. The least significant difference (LSD) among treatments was calculated as 1%, i.e., means with the same LSD letter do not differ significantly from each other ($p < 0.05$, ANOVA, Table 4.1).

Table 4.1 Analysis of variance (ANOVA) results for urediniospore germination percentages recorded for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates. Treatments included an untreated control and four propiconazole dilutions (x0.075, x0.10, x0.20 and x0.40).

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	19662	447	13.214	<2e-16*
Dilution	4	913865	228466	6756.099	<2e-16*
Isolate x dilution interaction	176	59508	338	9.999	<2e-16*
Residuals	450	15217	34		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 4%, dilutions = 1% and isolate x dilution interaction = 9%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

The urediniospore germination percentage for the x0.075 propiconazole dilution revealed significant variation among the *Pgt* isolates forming ten significantly different groups with a minimum significant difference of 35.85% among the groups (Appendices 2, 7 and 21). The mean germination percentage ranged from 0.67% (isolates 11 and 44) and 1.33% (isolate 21) to 97.33% (isolate 41), where the overall isolate mean germination was 25.03%.

The germination at the x0.10 propiconazole dilution resulted in less significant variation in germination percentages among the isolates, with only four groups differing significantly (LSD = 24.58%, Appendices 3, 8 and 21). The total mean germination percentage at the dilution was 6.04%. Nine isolates (isolates 3, 6, 7, 8, 13, 28, 29, 30 and 33) showed no germination at the x0.1 dilution. The highest mean germination percentages recorded were 48% and 62.67%, for isolates 31 and 32, respectively. For isolates that germinated, the mean germination percentages for the remaining isolates ranged between 24.67% (isolate 24) and 0.67% (isolates 2, 5, 11, 18 and 19), with no significant difference observed in the latter group of isolates. Nine isolates were highly sensitive at the x0.10 dilution with 0% germination (isolates 3, 6, 7, 8, 13, 28, 29, 30 and 33) and remained at 0% for lower dilutions, except for isolate 33 with 0.67% at the x0.20 dilution.

The mean germination at the x0.20 propiconazole dilution was 0.34% forming two statistically distinct groups with an LSD of 2.64% (Appendices 4, 9 and 21). Variation within germination responses ranged from a minimum of 0% recorded for 33 *Pgt* isolates, to 4% recorded for isolate 31. The remaining isolates ranged from 2% (isolate 41) to 0.67% (isolates 25, 33, 37, 42 and 44).

Isolate 41 was the only isolate to germinate at the x0.40 propiconazole dilution, with the remaining 44 *Pgt* isolates showing 0% germination (Appendices 5, 10 and 21). A mean germination of 1.33% was observed for urediniospores of isolate 41, and the LSD value of 1.20% clearly delineated the germinated isolate from those that did not germinate.

Comparing *Pgt* isolate germination responses across two propiconazole dilutions (x0.075 and x0.10), indicated a strong decline in germination percentages as the concentration of the a. i. increased (Figure 4.3). Most isolates showed a strong decline (including isolates 15, 28, 29, 40, 41, 42, 43 and 45) in their germination percentage at the x0.10 dilution compared to the x0.075

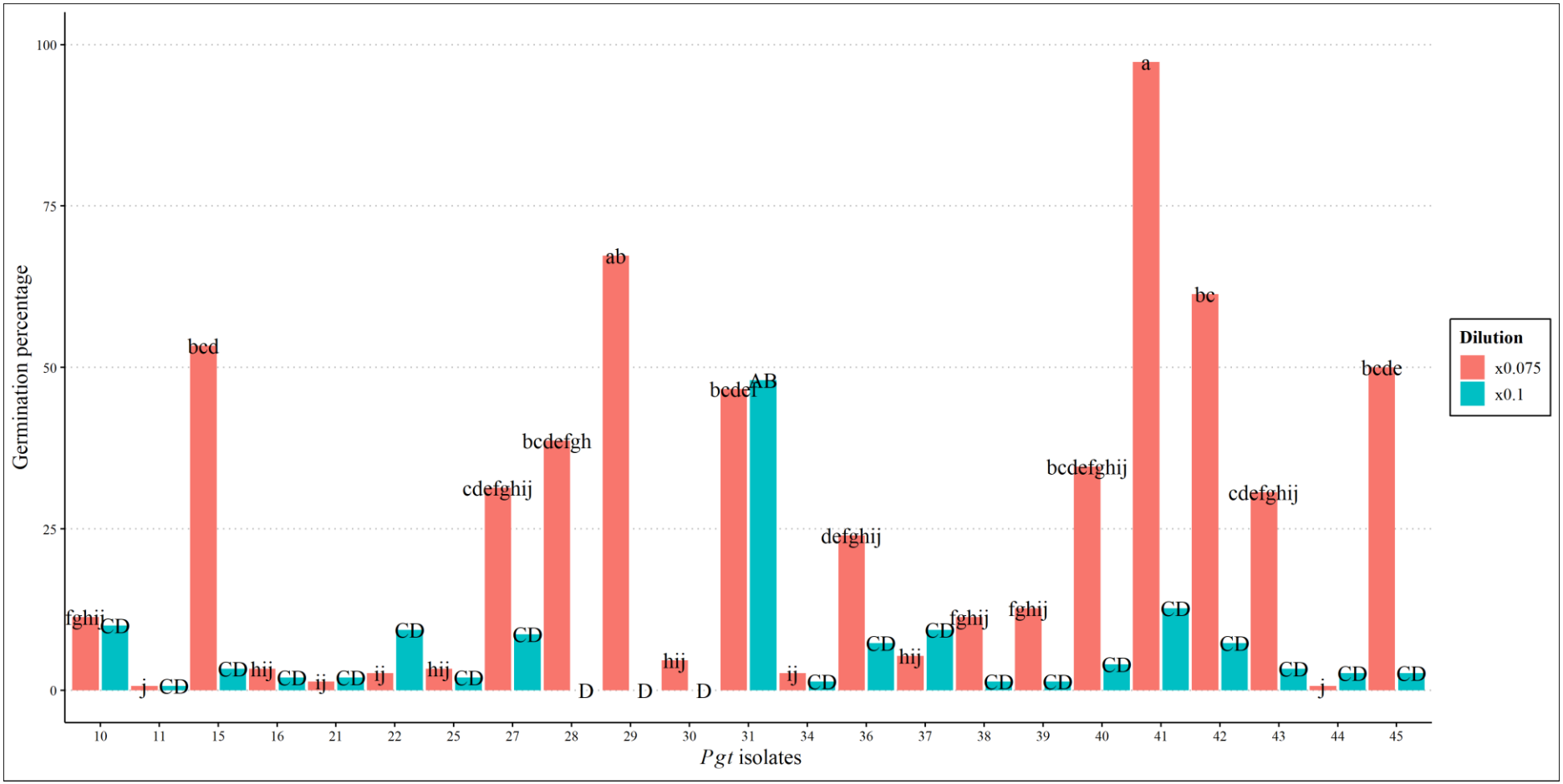

Figure 4.3 Comparison of the mean *Puccinia graminis* f. sp. *tritici* (*Pgt*) urediniospore germination percentages for 23 isolates selected for *CYP51* gene sequence analysis at the x0.075 and x0.10 propiconazole dilutions ($p < 0.05$, ANOVA, Appendices 2 and 3).

dilution or remained very low at both dilutions (including isolates 11, 16, 21, 25, 30 and 34). Exceptions were isolates 22, 31, 37 and 44, which showed slightly higher germination percentages at the x0.10 dilution compared to the x0.075 dilution. Isolate 22 had a germination of 2.67% at the x0.075 propiconazole dilution and 9.33% at the x0.10 dilution. In the same order, germination recorded for isolate 31 was 46.67% and 48%, isolate 37 5.33% and 9.33% and isolate 44 0.67% and 2.7%. *Pgt* isolates 28, 29 and 30 achieved 0% germination at the x0.10 dilution.

4.1.1.2 Tebuconazole

When tested with different dilutions of tebuconazole, the urediniospores from the 45 isolates showed clear differences in germ tube length between the fungicide treated and untreated controls (Figure 4.4). The LSDs in germination percentages between isolates were 2% for dilutions, 5% for *Pgt* isolates and 12% for the interactions. The mean germination percentages of the 45 *Pgt* isolates recorded for the control and tebuconazole dilutions differed significantly ($p < 0.05$) from each other (Figure 4.5; Table 4.2). Mean urediniospore mean germination percentages for the x0.01 (73.60%), x0.015 (34.59%), x0.02 (7.20%) and x0.03 (0.56%) tebuconazole dilutions differed significantly ($p < 0.05$) from each other. No significant differences ($p > 0.05$) were detected between the replicates of the dilutions. A significant ($p < 0.05$) interaction was detected for the *Pgt* isolates among tebuconazole dilutions. Therefore, one-way ANOVAs were performed for the control and each tebuconazole dilution to separate means per treatment (Appendices 11 to 15).

Germination for the x0.00 tebuconazole treatment (untreated control) treatment was excellent (mean germination = 95.48%; Appendices 11, 16 and 22). Among the *Pgt* isolates, the mean germination percentage ranged from 64.00% (isolate 33) to 100% (isolates 4, 5, 8 and 15). Urediniospores of two isolates appeared less viable, with lowest mean germination percentages recorded at 64% (isolate 33) and 78% (isolate 35). There were little significant differences among the mean germination percentages of the remaining isolates (LSD = 7.83%, $p < 0.05$).

The urediniospore germination percentage for the x0.01 tebuconazole dilution revealed significant variation among the *Pgt* isolates forming nine different groups (Appendices 12, 17 and 22; LSD = 24.79%). The lowest mean germination percentage at x0.01 was 15.33% (isolate

22), while the greatest was 92% (isolate 18). Most isolates ranged between 83.33% (isolate 15) and 89.33% (isolate 29) and did not differ significantly from each other. A total mean germination of 73.60% over the *Pgt* isolates at the x0.01 tebuconazole dilution was observed.

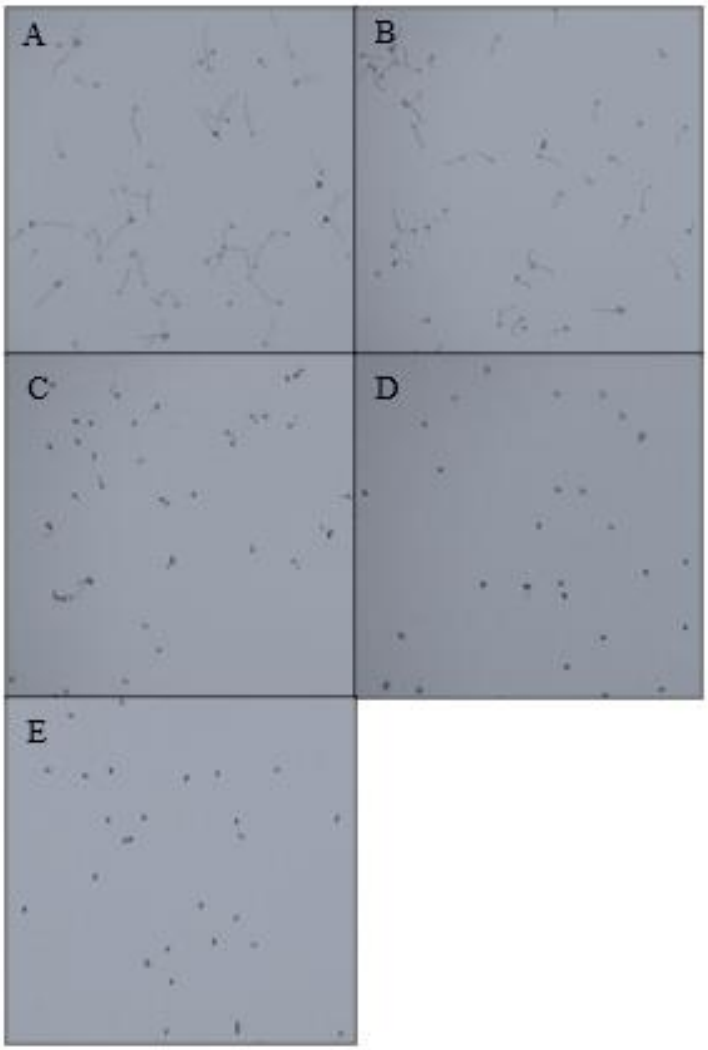

Figure 4.4 Photo plates (A to E) illustrating germination of *Puccinia graminis* f. sp. *tritici* (*Pgt*) urediniospores of isolate 20 between the control (A) and four (B to E) dilutions (x0.01, x0.015, x0.02 and x0.03) for the active ingredient tebuconazole.

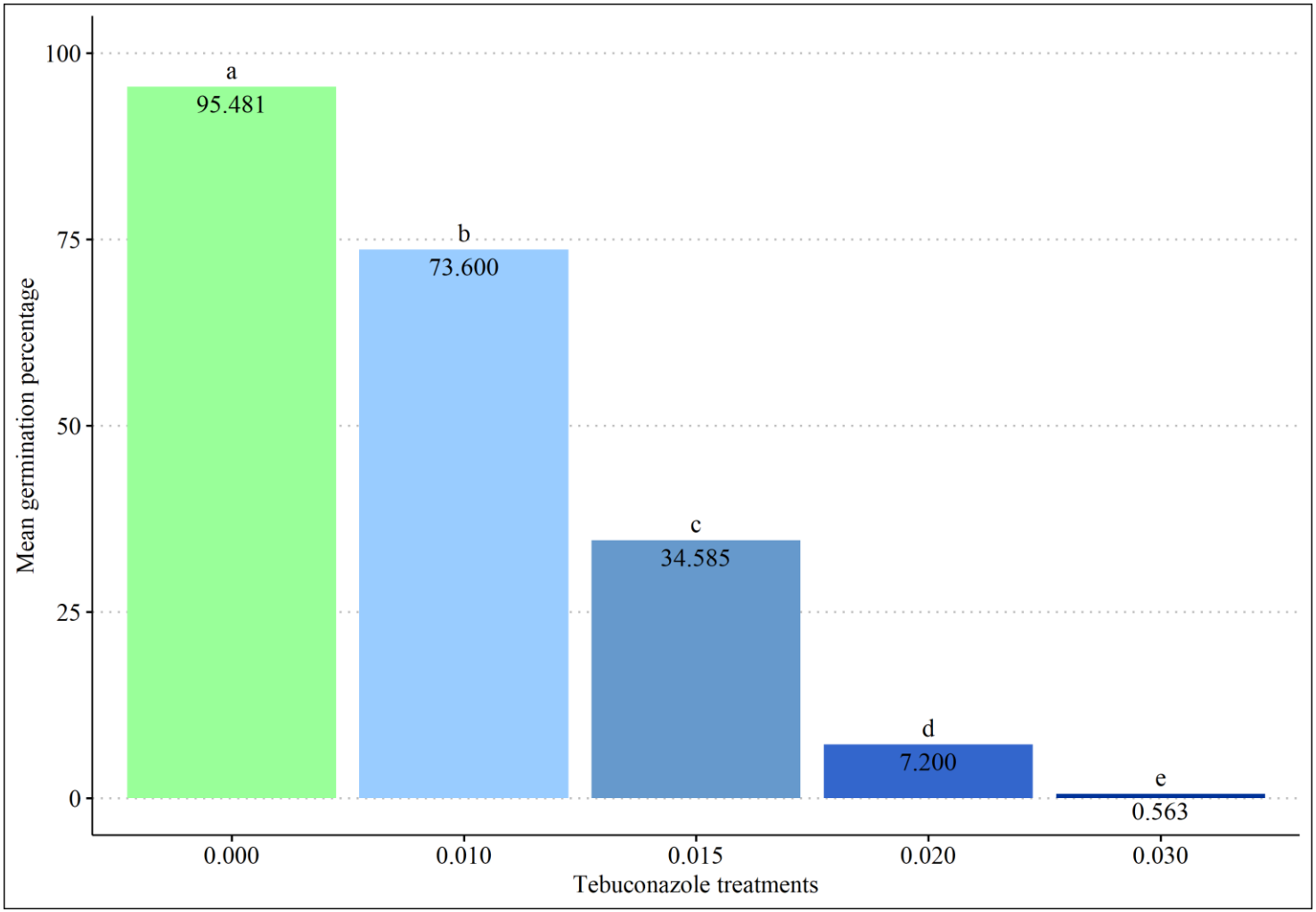

Figure 4.5 Mean urediniospore germination percentages recorded for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the untreated control and four tebuconazole dilutions. The least significant difference (LSD) among treatments was calculated as 2%, i.e., means with the same LSD letter do not differ significantly from each other ($p < 0.05$, ANOVA, Table 4.2).

Table 4.2 Analysis of variance (ANOVA) results for urediniospore germination percentages recorded for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates. Treatments included an untreated control and four tebuconazole dilutions (x0.01, x0.015, x0.02 and x0.03).

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	48043	1092	6.095	<2e-16*
Dilution	4	923598	230900	1288.952	<2e-16*
Isolate x dilution interaction	176	86315	490	8.597	<2e-16*
Residuals	450	25671	57		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 5%, dilutions = 2% and isolate x dilution interaction = 12%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

At the x0.015 tebuconazole dilution, the mean germination percentages recorded for the *Pgt* isolates resulted in more significant variation with nine distinct groups differing significantly (LSD = 49.71%, Appendices 13, 18 and 22). A total mean germination of 34.15% at x0.015 dilution was observed. The lowest mean germination percentage was 0.67% (isolate 33), while the highest mean germination percentage was 82.67% (isolate 42). Most isolates had mean germination percentages that ranged between 22.7% (isolate 3) and 30.7% (isolate 12), with no significance difference observed among these isolates.

The mean germination percentages at the x0.02 tebuconazole dilution formed five significantly distinct groups of isolates (LSD = 17.90%; Appendices 14, 19 and 22). The total mean germination percentage at the x0.02 dilution was 7.20%. Variation in germination ranged from a minimum of 0% recorded for two *Pgt* isolates (isolates 33 and 35) to 63.33% recorded for isolate 29. Most isolates did not respond significantly differently from each other and ranged between 1.33% (isolates 11, 13, 16, 21 and 37) and 14% (isolate 36).

Urediniospores of 36 *Pgt* isolates showed 0% germination at the x0.03 tebuconazole dilution, (Appendices 15, 20 and 22). The mean germination percentage over isolates was calculated at 0.56% (LSD = 3.08%). Isolates that germinated at this dilution were isolates 28, 30, 39, 45 (1.33%), 22, 27, 29 (2%), 31 (2.67%) and isolate 24 with the highest germination (11.33%).

Overall, *Pgt* isolates had the highest urediniospore germination percentages at the lowest tebuconazole dilution (x0.01) with a gradual decline in germination as the concentration of the a. i. in the dilution increased to x0.03.

Figure 4.6 presents a comparison of the mean urediniospore germination percentages for 23 *Pgt* isolates selected for molecular analysis at the x0.01 and x0.015 tebuconazole dilutions. All isolates showed a decline in their germination percentage at the x0.015 dilution compared to the x0.01 dilution with the exception of isolate 22, which showed a slight increase to 24.70% (x0.015) from 15.30% (x0.10). Isolates 21, 29, 34, 37 and 43 were very sensitive to the increase in tebuconazole, with higher mean germination percentages observed at the x0.01 than at the x0.015 dilution. *Pgt* isolate 42 was the least sensitive to the increase in tebuconazole responding with a mean germination percentage of 84.67% at the x0.01, which only slightly decreased to 82.67% at x0.015.

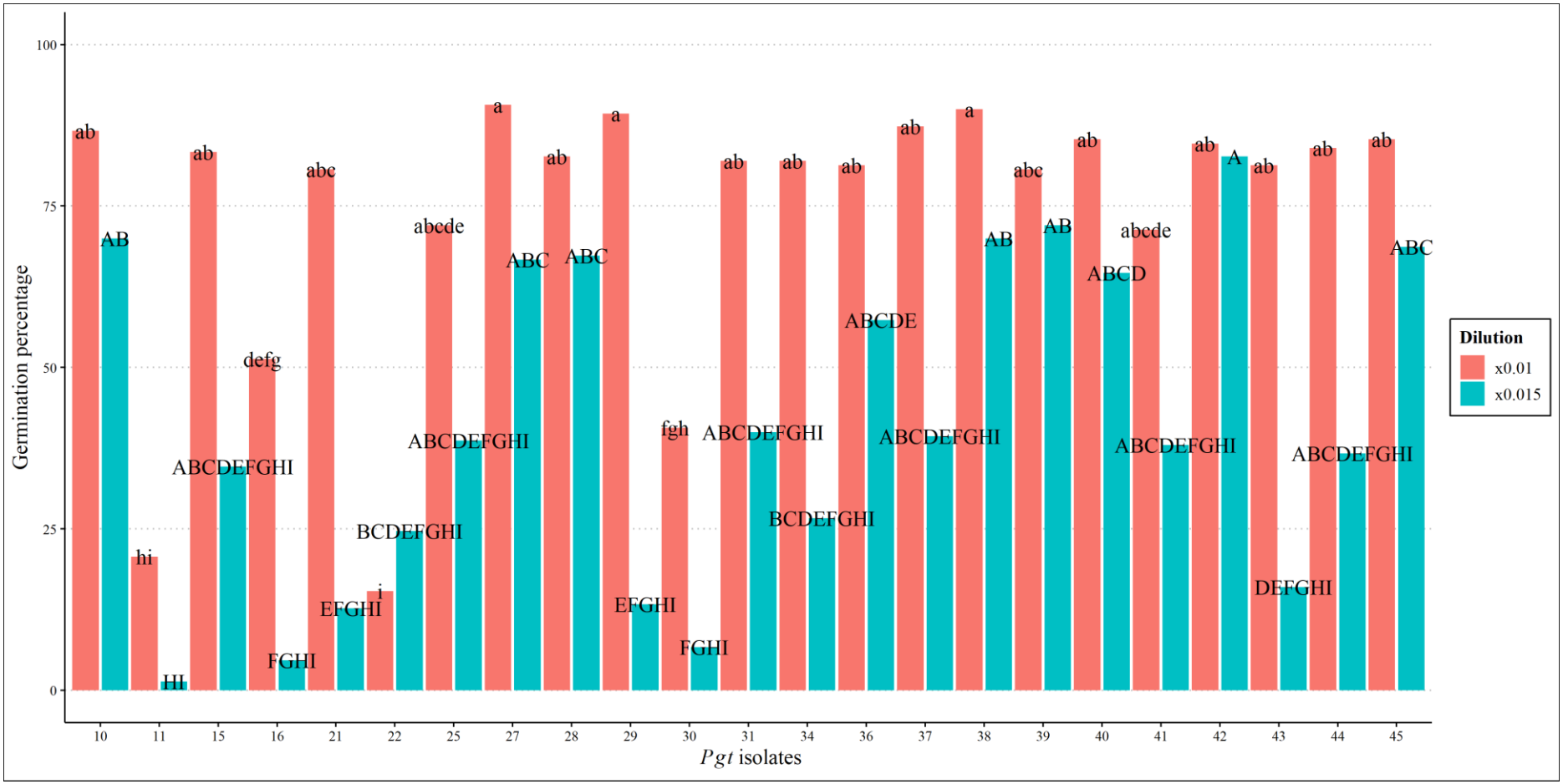

Figure 4.6 Comparison of the mean *Puccinia graminis* f. sp. *tritici* (*Pgt*) germination percentages for urediniospores from 23 selected isolates at the x0.01 and x0.015 tebuconazole dilutions ($p < 0.05$, ANOVA, Appendices 12 and 13).

4.1.2 EC_{50} estimates

4.1.2.1 Propiconazole

The absolute EC_{50} estimates of propiconazole were determined for the 45 *Pgt* isolates with propiconazole as an active ingredient (Table 4.3). The EC_{50} values covered a large range with a low of 0.0002 (isolate 44) and a high of 0.0937 (isolate 41). The mean EC_{50} determined for propiconazole was 0.057. Similar values to the mean were observed for isolates 2 (EC_{50} estimate 0.0560), 38 (0.0579), 39 (0.0594) and 36 (0.0599). Isolate 44 responded similarly to isolates 37 (EC_{50} estimate 0.0098), 21 (0.0038) and 11 (0.0006), indicating low EC_{50} values and, thus, high sensitivity to propiconazole. Additional sensitive isolates included isolates 25 (EC_{50} estimate 0.0120), 16 (0.0189) and 34 (0.0212). The highest EC_{50} was recorded for isolates 41, 15 (EC_{50} estimate 0.0759), 29 (0.0770), 42 (0.0791) and 31 (0.0838).

Contrasting observations were made between recently collected isolates 11 (0.0006; year collected in 2017), 21 (0.0038; 2018), 25 (0.0120; 2017), 16 (0.0189; 2017) and 34 (0.0212; 2020) that were sensitive to propiconazole, while isolates 15 (0.0759; 2017), 29 (0.0770; 2017) and 31 (0.0838; 2020) were less sensitive. Similar deviations in responses were observed for the historically collected *Pgt* isolates (representing the UFS reference isolates for each *Pgt* race) with EC_{50} estimates that ranged from 0.002 (isolate 44; year collected 2010) and 0.0098 (isolate 37; 2000) to 0.0937 (isolate 41; 2005), 0.0791 (isolate 42; 2007) and 0.0755 (isolate 45; 2016). Two historic isolates (44 and 41) represented both the most and the least sensitive isolates to the propiconazole treatment, respectively. The other historically collected isolates had EC_{50} estimates that were similar to the mean (0.057) calculated over isolates. These were isolates 38 (EC_{50} estimate 0.0579; year collected 2000), 39 (0.0594; 2000), 36 (0.0599; 1981), 43 (0.0687; 2009) and 40 (0.0703; 2003).

4.1.2.2 Tebuconazole

The absolute EC_{50} values were calculated for the *Pgt* isolates for tebuconazole (Table 4.4). The EC_{50} values ranged from the lowest value of 0.004 (isolate 22) to the highest value of 0.018 (isolate 28), with a mean EC_{50} of 0.013 calculated over isolates. Isolates 5, 3, 29, 17, 8, 19, 29, 7, 41, 1, 6, 12 and 15 (EC_{50} estimates 0.013) all had the same response as the mean response. In contrast, isolates 33 (EC_{50} estimate 0.009), 30 (0.009), 22 (0.004), 16 (0.010), 13 (0.009)

and 11 (0.008) showed low EC_{50} values and, thus, high sensitivity to tebuconazole. Other sensitive isolates included isolates 16 (EC_{50} estimate 0.010) and isolates 2 and 35 (0.011). In addition to isolate 28, other isolates for which higher EC_{50} estimates were recorded, included isolates 42 and 39 (EC_{50} estimate 0.017) and isolates 32, 34, 40, 36, 38, 27, 10 and 45 (0.016).

From the more recently collected *Pgt* isolates, isolates 22 (EC_{50} estimate 0.004; 2018), 11 (0.008; 2017), 13 (0.009; 2017), 30 (0.009; 2017) and 33 (0.009; 2020), were more sensitive to tebuconazole while isolates 27 (0.016; 2022), 10 (0.016; 2016) and 28 (0.018; 2020) were less sensitive. Isolate 28 represented the overall lowest sensitivity to tebuconazole.

Most of the historically collected *Pgt* isolates appeared less sensitive to tebuconazole compared to the recently collected isolates with higher EC_{50} estimates that ranged from 0.012 (isolate 43; 2009) and 0.013 (isolate 41; 2005) to 0.016 (isolates 40; 2003, 36; 1981, 38; 2000, and 45; 2016) and 0.017 (isolates 39; 2000, and 42; 2007). Isolate 43 was the only historically collected isolate more sensitive than the mean response.

4.1.3 Isolate responses to both fungicides

Considering the results obtained for both a. i., a higher propiconazole concentration was required to achieve the same amount of urediniospore germination inhibition (50%) compared to tebuconazole (Figure 4.7). *Pgt* isolates 11, 21 and 16 were more sensitive to both propiconazole and tebuconazole compared to other isolates. Similarly, the historically collected isolate 41 was less sensitive to propiconazole with a more moderate response to tebuconazole. Two of the less sensitive isolates included isolate 28 and 31, with EC_{50} estimates of 0.0736 and 0.0838 for propiconazole and 0.018 and 0.014 for tebuconazole, respectively.

Historically collected *Pgt* isolates, 44 and 41, represented both the most (isolate 44) and least (isolate 41) sensitive isolates to propiconazole. All the historically collected *Pgt* isolates, except isolates 43 and 41, produced EC_{50} estimates higher than the 0.013 mean EC_{50} for tebuconazole. The *Pgt* isolates produced EC_{50} estimates that ranged from 0.012 (isolate 43) and 0.013 (isolate 41) to 0.016 (isolates 36, 38, 40 and 45) and 0.017 (isolates 39 and 42) for tebuconazole.

Table 4.3 The effective inhibition of half-maximal effective concentration (absolute EC_{50}) estimates (mg/L) measured for urediniospore germination percentages for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates against the fungicide active ingredient propiconazole. Isolates in rows highlighted in grey represent those selected for *CYP51* sequence analysis.

Isolate no.	UFS race name	EC50 estimate	Std. Error	Lower limit	Upper limit
1	Pgt56	0.0677	0.0027	0.0619	0.0740
2	Pgt63	0.0560	0.0083	0.0380	0.0740
3	Pgt63	0.0646	0.0124	0.0376	0.0920
4	Pgt63	0.0698	0.0026	0.0642	0.0750
5	Pgt63	0.0632	0.0116	0.0380	0.0880
6	Pgt60	0.0691	0.0073	0.0532	0.0850
7	Pgt60	0.0678	0.0273	0.0083	0.1270
8	Pgt60	0.0740	0.0040	0.0652	0.0830
9	Pgt60	0.0648	0.0061	0.0515	0.0780
10	Pgt61	0.0239	0.0088	0.0048	0.0430
11	Pgt56	0.0006	0.0035	-0.0069	0.0080
12	Pgt56	0.0295	0.0071	0.0139	0.0450
13	Pgt64	0.0675	0.0175	0.0293	0.1060
14	Pgt64	0.0712	0.0009	0.0692	0.0730
15	Pgt55	0.0759	0.0008	0.0742	0.0780
16	Pgt55	0.0189	0.0226	-0.0305	0.0680
17	Pgt55	0.0546	0.0052	0.0432	0.0660
18	Pgt55	0.0692	0.0102	0.0471	0.0910

Table 4.3 (cont.) The effective inhibition of half-maximal effective concentration (absolute EC_{50}) estimates (mg/L) measured for urediniospore germination percentages for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates against the fungicide active ingredient propiconazole. Isolates in rows highlighted in grey represent those selected for *CYP51* sequence analysis.

Isolate no.	UFS race name	EC50 estimate	Std. Error	Lower limit	Upper limit
19	Pgt55	0.0725	0.0012	0.0698	0.0750
20	Pgt55	0.0653	0.0015	0.0621	0.0690
21	Pgt56	0.0038	0.0081	-0.0139	0.0210
22	Pgt56	0.0607	0.0394	-0.0250	0.1460
23	Pgt64	0.0755	0.0027	0.0697	0.0810
24	Pgt64	0.0741	0.0017	0.0704	0.0780
25	Pgt64	0.0120	0.0281	-0.0493	0.0730
26	Pgt64	0.0610	0.0026	0.0553	0.0670
27	Pgt63	0.0651	0.0023	0.0601	0.0700
28	Pgt63	0.0736	0.0032	0.0666	0.0810
29	Pgt56	0.0770	0.0015	0.0737	0.0800
30	Pgt56	0.0634	0.0443	-0.0330	0.1600
31	Pgt64	0.0838	0.0119	0.0578	0.1100
32	Pgt64	0.0494	0.0091	0.0295	0.0690
33	Pgt64	0.0751	0.0006	0.0738	0.0760
34	Pgt64	0.0212	0.0271	-0.0271	0.0800
35	Pgt64	0.0399	0.0103	0.0174	0.0620
36	UVPgt50	0.0599	0.0075	0.0436	0.0760
37	UVPgt54	0.0098	0.0091	-0.0100	0.0300

Table 4.3 (cont.) The effective inhibition of half-maximal effective concentration (absolute EC_{50}) estimates (mg/L) measured for urediniospore germination percentages for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates against the fungicide active ingredient propiconazole. Isolates in rows highlighted in grey represent those selected for *CYP51* sequence analysis.

Isolate no.	UFS race name	EC50 estimate	Std. Error	Lower limit	Upper limit
38	UVPgt54+*Sr9g*	0.0579	0.0035	0.0503	0.0660
39	UVPgt55	0.0594	0.0182	0.0198	0.0990
40	UVPgt56	0.0703	0.0058	0.0576	0.0830
41	UVPgt57	0.0937	0.0145	0.0622	0.1250
42	UVPgt59	0.0791	0.0006	0.0778	0.0800
43	UVPgt60	0.0687	0.0029	0.0624	0.0750
44	UVPgt61	0.0002	0.0004	-0.0006	0.0010
45	UVPgt62	0.0755	0.0007	0.0740	0.0770

Table 4.4 The effective inhibition of half-maximal effective concentration (absolute EC_{50}) estimates (mg/L) measured for urediniospore germination percentages for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates against the fungicide active ingredient tebuconazole. Isolates in rows highlighted in grey represent those selected for *CYP51* sequence analysis.

Isolate no.	UFS race name	EC50 estimate	Std. Error	Lower limit	Upper limit
1	Pgt56	0.0130	0.0019	0.0089	0.0170
2	Pgt63	0.0110	0.0004	0.0096	0.0110
3	Pgt63	0.013	0.00024	0.012	0.013
4	Pgt63	0.0120	0.0006	0.0110	0.0140
5	Pgt63	0.0130	0.0003	0.0118	0.0130
6	Pgt60	0.0130	0.0007	0.0117	0.0150
7	Pgt60	0.0130	0.0003	0.0123	0.0140
8	Pgt60	0.0130	0.0003	0.0121	0.0130
9	Pgt60	0.0140	0.0009	0.0124	0.0160
10	Pgt61	0.0160	0.0005	0.0154	0.0170
11	Pgt56	0.0080	0.0007	0.0068	0.0100
12	Pgt56	0.0130	0.0007	0.0117	0.0150
13	Pgt64	0.0090	0.0002	0.0088	0.0100
14	Pgt64	0.0120	0.0001	0.0122	0.0130
15	Pgt55	0.0130	0.0009	0.0115	0.0150
16	Pgt55	0.0100	0.0002	0.0096	0.0110
17	Pgt55	0.0130	0.0001	0.0125	0.0130
18	Pgt55	0.0140	0.0005	0.0125	0.0150
19	Pgt55	0.0130	0.0004	0.0120	0.0140
20	Pgt55	0.0150	0.0003	0.0145	0.0160

Table 4.4 (cont.) The effective inhibition of half-maximal effective concentration (absolute EC_{50}) estimates (mg/L) measured for urediniospore germination percentages for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates against the fungicide active ingredient tebuconazole. Isolates in rows highlighted in grey represent those selected for *CYP51* sequence analysis.

Isolate no.	UFS race name	EC50 estimate	Std. Error	Lower limit	Upper limit
21	Pgt56	0.0120	0.0002	0.0116	0.0120
22	Pgt56	0.0040	0.0021	-0.0008	0.0080
23	Pgt64	0.0120	0.0004	0.0111	0.0130
24	Pgt64	0.0140	0.0004	0.0130	0.0150
25	Pgt64	0.0140	0.0007	0.0127	0.0160
26	Pgt64	0.0140	0.0007	0.0128	0.0160
27	Pgt63	0.0160	0.0002	0.0159	0.0170
28	Pgt63	0.0180	0.0006	0.0164	0.0190
29	Pgt56	0.0130	0.0002	0.0122	0.0130
30	Pgt56	0.0090	0.0008	0.0073	0.0110
31	Pgt64	0.0140	0.0011	0.0119	0.0170
32	Pgt64	0.0160	0.0004	0.0147	0.0170
33	Pgt64	0.0090	0.0005	0.0081	0.0100
34	Pgt64	0.0160	0.0003	0.0123	0.0140
35	Pgt64	0.0110	0.0002	0.0103	0.0110
36	UVPgt50	0.0160	0.0006	0.0146	0.0170
37	UVPgt54	0.0140	0.0006	0.0130	0.0150
38	UVPgt54+*Sr9g*	0.0160	0.0002	0.0159	0.0170
39	UVPgt55	0.0170	0.0004	0.0157	0.0170
40	UVPgt56	0.0160	0.0006	0.0145	0.0170

Table 4.4 (cont.) The effective inhibition of half-maximal effective concentration (absolute EC_{50}) estimates (mg/L) measured for urediniospore germination percentages for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates against the fungicide active ingredient tebuconazole. Isolates in rows highlighted in grey represent those selected for *CYP51* sequence analysis.

Isolate no.	UFS race name	EC50 estimate	Std. Error	Lower limit	Upper limit
41	UVPgt57	0.0130	0.0004	0.0122	0.0140
42	UVPgt59	0.0170	0.0004	0.0163	0.0180
43	UVPgt60	0.0120	0.0002	0.0118	0.0120
44	UVPgt61	0.0140	0.0008	0.0122	0.0160
45	UVPgt62	0.0160	0.0003	0.0159	0.0170

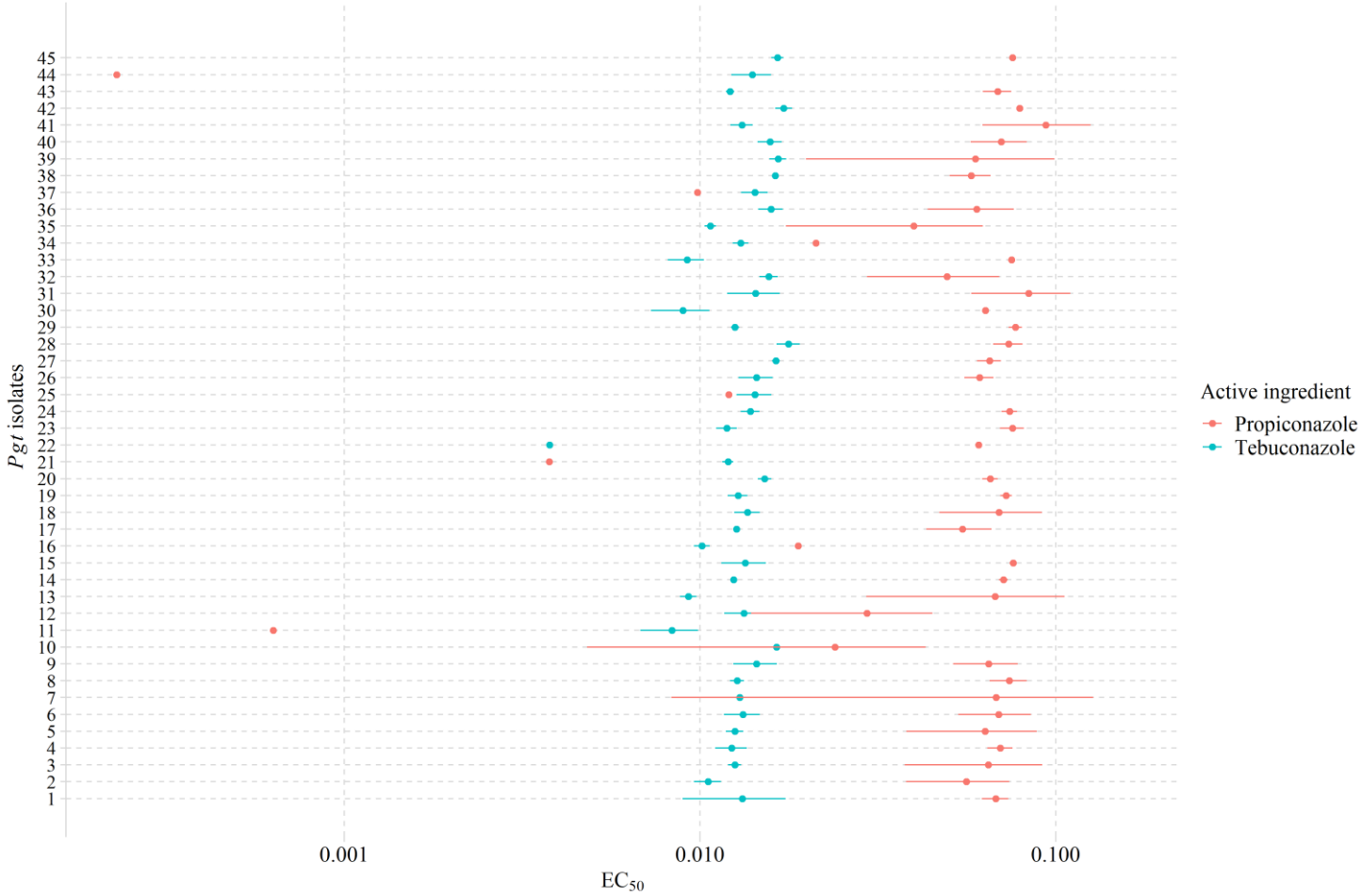

Figure 4.7 The effective inhibition of half-maximal effective concentration (absolute EC_{50}) estimates measured for urediniospore germination percentages for 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates to the fungicide active ingredients propiconazole (mean EC_{50} 0.057) and tebuconazole (mean EC_{50} 0.013; Isolate 1 to 35 collected from 2016 onwards, isolate 36 to 45 were first race collections made between 1981 and 2016). The error bars represent a confidence interval of 95%.

The EC_{50} estimates determined for *Pgt* race 2SA104 (isolates of UVPgt56), revealed that isolate 11 (EC_{50} estimate 0.0006; collected during 2017) was the most sensitive to propiconazole followed by isolate 21 (0.0038; 2018). The least sensitive isolate to propiconazole was isolate 29 (0.077; 2017), followed by historically collected isolate 40 (0.0703; 2003). Isolate 22 (0.004; 2018) was the most sensitive to tebuconazole, followed by isolate 11 (0.008; 2017). The historically collected isolate 40 with EC_{50} estimate of 0.016 was the least sensitive isolate of this race to tebuconazole.

4.2 Sequence analysis of the *CYP51* gene

4.2.1 Isolate selection for sequence analysis

Based on the EC_{50} estimate results, 23 *Pgt* isolates were selected for *CYP51* gene sequence analysis. All ten historically collected isolates, which were considered previously unexposed to the two fungicides, were included. The remaining thirteen isolates were selected due to having high and low EC_{50} estimates for either both or to the individual active ingredient (Table 4.3; 4.4).

4.2.2 PCR amplification of the *CYP51* gene

Total DNA extraction was successful, as DNA from five randomly selected *Pgt* isolates selected for molecular analysis revealed fragments larger than 10 kb in length (Figure 4.8). While the entire *CYP51* gene region was successfully amplified using the *Pgt*_CYP51 forward (F1) and *Pgt*_CYP51 reverse (R1) primer set, the successful cloning of the fragment into pGemT-Easy plasmid proved difficult.

Consequently, two overlapping *CYP51* gene fragments that make up the entire *CYP51* gene region, were separately amplified and cloned. The amplification of the F1R2 *CYP51* gene region resulted in a 1 238 bp fragment, while the amplification of the F2R1 *CYP51* gene region resulted in a 1 300 bp fragment (Figure 4.9). The majority of fragments were successfully amplified, although some reactions failed. The PCR reaction was thus repeated for fragments that were not initially amplified. Both the F1R2 and F2R1 fragments were successfully amplified the second time for these *Pgt* isolates, with the exception of the F2R1 fragment from *Pgt* isolates 15, 16, 21, 22 and 27 (Figure 4.10).

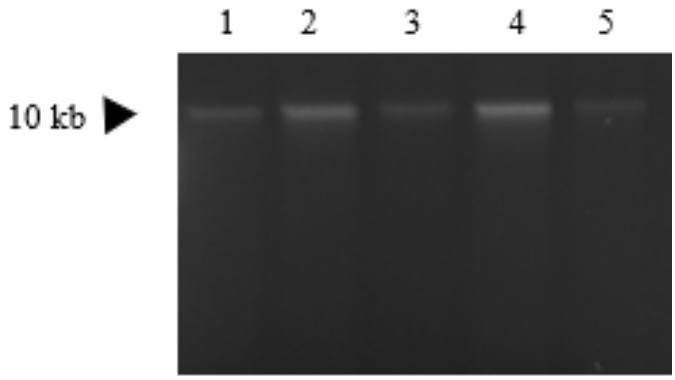

Figure 4.8 Total genomic DNA extracted from *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates (from left to right) 10, 28, 31, 29 and 36.

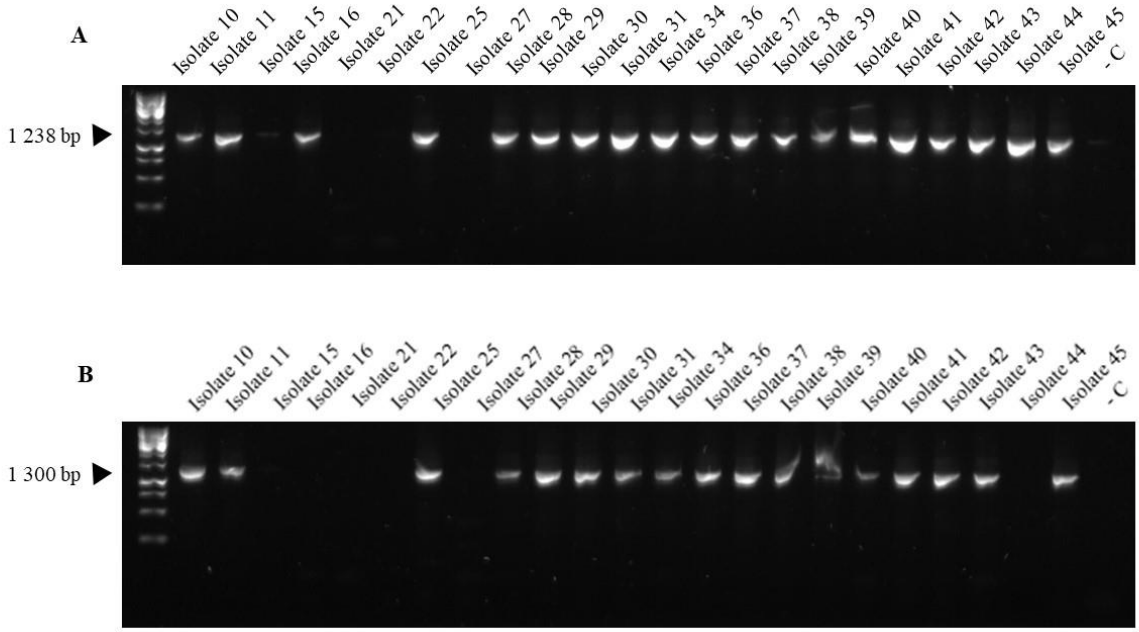

Figure 4.9 PCR amplification of two gene fragments that make up the complete *CYP51* gene from 23 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates using (A) the *Pgt*_CYP51 forward (F1) and internal *Pgt*_CYP51 reverse (R2) primer set, and (B) the internal *Pgt*_CYP51 forward (F2) and *Pgt*_CYP51 reverse (R1) primer set. A non-template control (- C) was included.

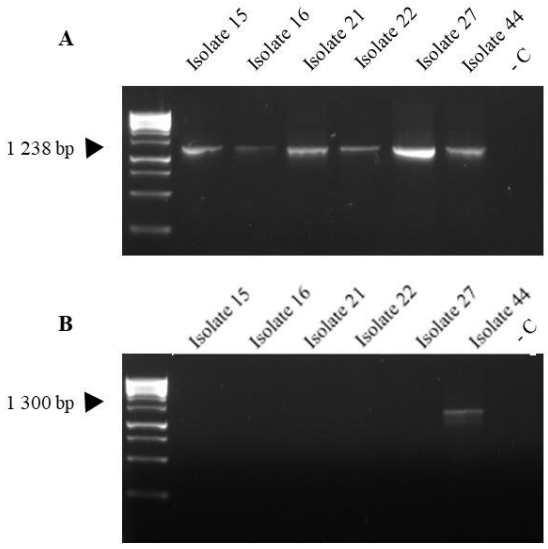

Figure 4.10 PCR amplification of the two fragments that make up the complete *CYP51* gene from six *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates, using (A) the *Pgt*_CYP51 forward (F1) and internal *Pgt*_CYP51 reverse (R2) primer set, and (B) the internal *Pgt*_CYP51 forward (F2) and *Pgt*_CYP51 reverse (R1) primer set. A non-template control was included (-C).

New internal *Pgt*_CYP51 forward (F3) primers were designed to anneal upstream from the internal *Pgt*_CYP51 forward (F2) primers (Figure 3.2). An additional PCR reaction was performed using the internal *Pgt*_CYP51 forward (F3) and *Pgt*_CYP51 reverse (R1) primer set to successfully amplify the F3R1 gene region from the remaining five *Pgt* isolates. The resulting fragment was 1 592 bp in length (Figure 4.11).

4.2.3 Cloning of the *CYP51* gene fragment

The F1R2, F2R1 and F3R1 amplification products were successfully cloned into the pGemT-Easy plasmid vector and eight recombinant plasmids were selected for each *Pgt* isolate for DNA sequencing.

In Figure 4.12 (A), cloning results for *Pgt* isolate 25 showed the presence of the 1 238 bp F1R2 insert at lanes 3 and 8, while Figure 4.12 (B) showed the presence of the 1 300 bp F2R1 insert in all eight recombinant plasmids for *Pgt* isolate 30. Subsequently, more recombinant plasmids were PCR screened for isolate 25 until eight recombinant plasmids were obtained for both gene fragments of all isolates. The amplified gene fragments were purified and used as templates in subsequent sequencing reactions.

4.2.4 Sequencing of the *CYP51* gene from the selected *Pgt* isolates

The *CYP51* contigs constructed from the two sequenced gene fragments were used to identify the two alleles present in the 23 dikaryotic individuals. When compared to the wild type *CYP51* exome sequence (Genbank accession KAA1080186), only two alleles were found in all 23 individuals. Allele 1 was identical to that of accession KAA1080186, while allele 2 contained many nucleotide changes (Table 4.5). All these cumulative changes separated the *CYP51* gene into the two identified alleles (Figure 4.13).

The five exons of the *CYP51* gene were highly conserved across the 23 isolates as all but one change in nucleotide sequence from the wild type resulted in silent mutations with no effect on the resulting protein. Only one change (G1528A) resulted in a missense mutation (D510N) in allele 2 on exon 5 (Figure 4.13; Table 4.5). Within the five introns of the two alleles, 25 single nucleotide changes, as well as deletions and insertions were detected, when the nucleotide sequences were compared to that of the wild type *CYP51* gene sequence.

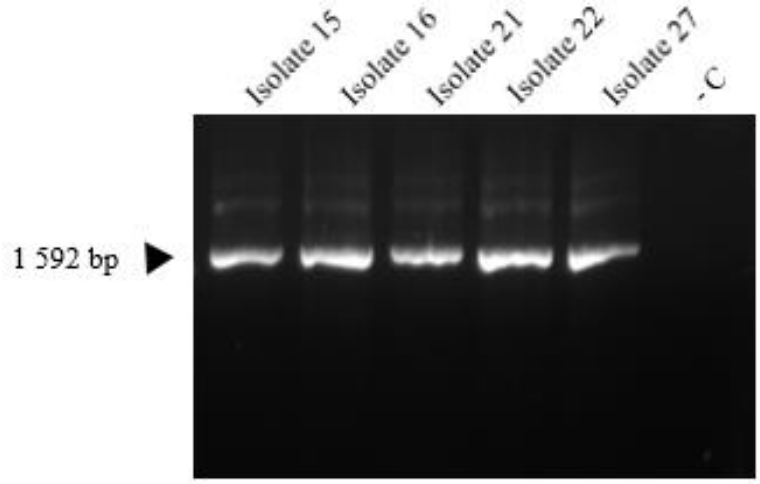

Figure 4.11 PCR amplification of the second *CYP51* gene fragment from five *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates, using the internal *Pgt*_CYP51 forward (F3) and *Pgt*_CYP51 reverse primer set. A non-template control was included (-C).

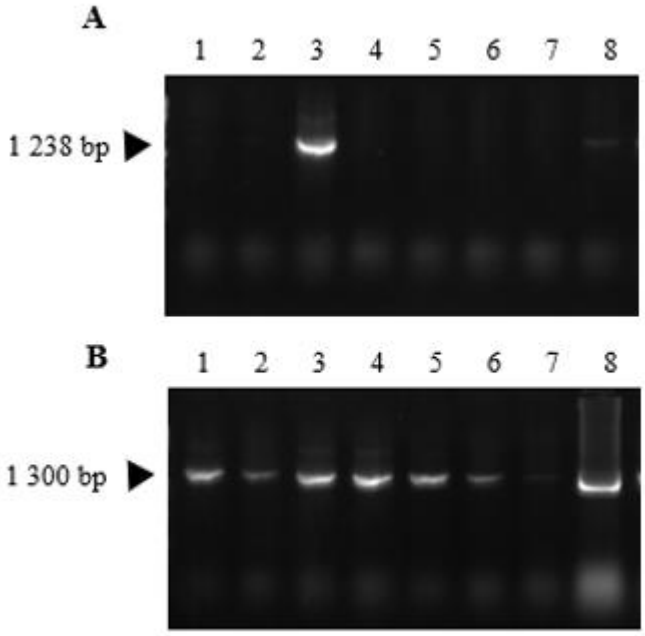

Figure 4.12 PCR amplification of the two *CYP51* gene fragments from recombinant plasmid DNA using (A) the *Pgt*_CYP51 forward (F1) and internal *Pgt*_CYP51 reverse (R2) primer set, and (B) the internal *Pgt*_CYP51 forward (F2) and *Pgt*_CYP51 reverse (R1) primer set.

Figure 4.13 Amino acid sequence alignment of the partial *Puccinia graminis* f. sp. *tritici* (*Pgt*) CYP51 wildtype protein (KAA1080186) with two alleles identified from the 23 selected isolates. The D510N missense mutation in allele 2 is indicated with an arrow. *Pgt* isolate 11 was heterozygous for both alleles (A1 and A2), while isolate 16 was homozygous for allele 1 (A1) and isolate 29 was homozygous for allele 2 (A2).

Table 4.5 Nucleotide and resulting amino acid sequence changes within the *CYP51* gene from 23 South African *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates when the wild type *CYP51* (KAA1080186) allele was compared to the generated allele 2.

Mutation type	**Position**	**Nucleotide change**	**Amino acid change**
Silent	Exon 1	C84G	-
Silent	Exon 2	T282C	-
Silent	Exon 2	G324A	-
Silent	Exon 2	G360A	-
Silent	Exon 3	G538A	-
Silent	Exon 3	A538G	-
Silent	Exon 3	T607C	-
Silent	Exon 4	C981G	-
Silent	Exon 4	T1047C	-
Silent	Exon 4	A1101G	-
Silent	Exon 4	C1125T	-
Silent	Exon 4	T1131C	-
Silent	Exon 4	A1140C	-
Silent	Exon 4	C1143T	-
Silent	Exon 4	A1194C	-
Silent	Exon 4	T1197C	
Silent	Exon 4	C1308T	-
Silent	Exon 4	A1365C	-
Silent	Exon 5	C1613A	-
Silent	Exon 5	G1724A	-
Missense	Exon 5	G1866A	D510N
-	Intron 2	2bp deletion	-
-	Intron 4	6bp deletion	-
-	Intron 4	6bp insertion	-
-	Intron 5	2bp insertion	-

Pgt isolates 10, 29, 30, 31 and 34 were homozygous for allele 2 (Figure 4.13), while *Pgt* isolates 15, 16, 22, 27 and 28 and historically collected isolates 36, 37, 38, 39, 40, 41, 42, 43 and 44 were homozygous for allele 1. Heterozygous individuals for the two alleles were isolates 11, 25 and historically collected isolate 45. Sequencing results from *Pgt* isolate 21 were overall of poor quality and the isolates was thus excluded from the study. However, since recently collected *Pgt* isolates 11, 22, 29 and 30, were of the same race, and their results were of good quality, sequencing analyses were successfully completed.

A maximum likelihood phylogenetic tree was constructed using *CYP51* allelic variants (Figure 4.14). Identified allele 1 shared 100% homology with two other *Pgt* CYP51 protein sequences (GMQ09617T0 and KAA1080186.1), while allele 2 shared lower homology with four other *Pgt* sequences. All the CYP51 protein sequences from *Pgt* grouped together in a single clade with significant bootstrap support, as did the protein sequences from *Pt* and *Pst*, respectively. The *Pst* protein sequences shared 100% sequence homology with each other.

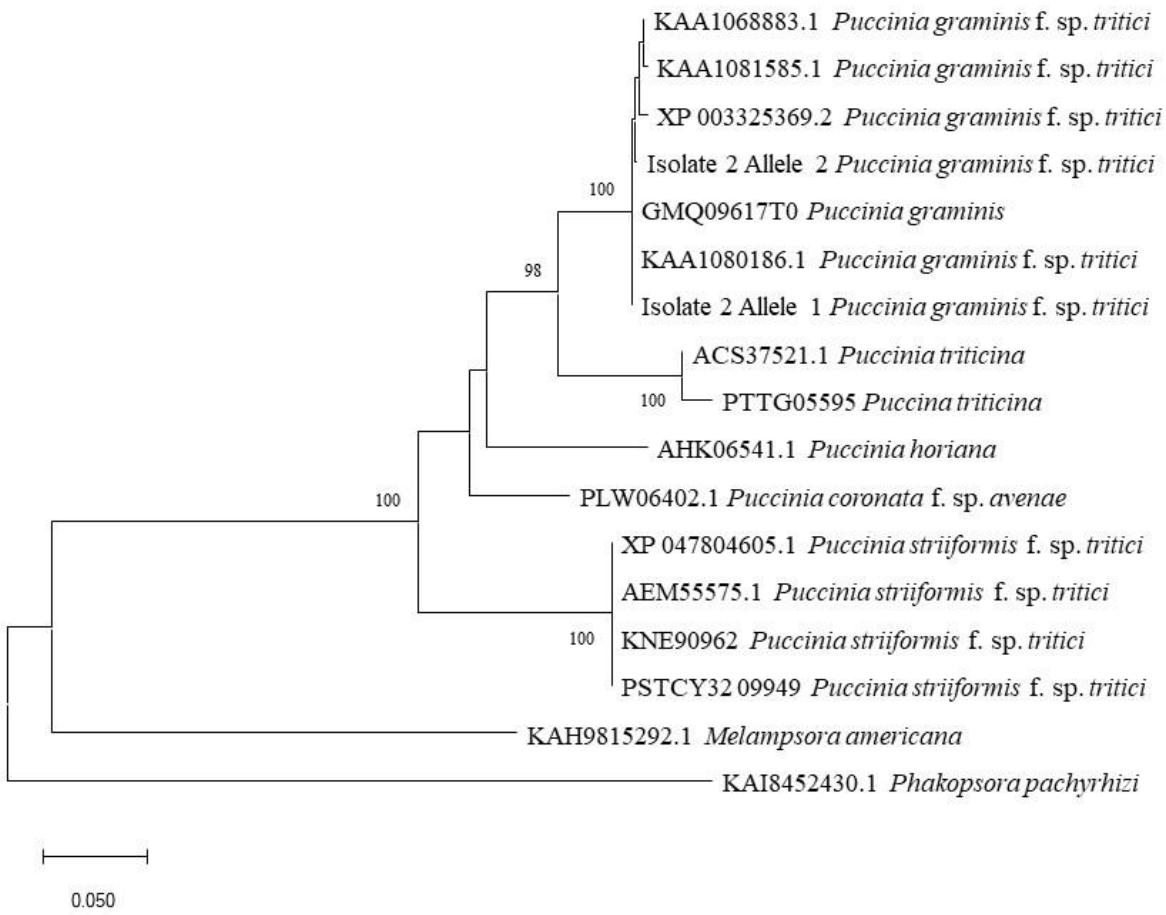

Figure 4.14 Maximum likelihood phylogenetic tree of *CYP51* allelic variants. Accessions from *Phakopsora pachyrhizi* and *Melampsora americana* were used as outgroups. Bootstrap values above 90% are indicated.

Chapter 5: Discussion

Wheat rust, such as *Pgt*, remains a threat to wheat production despite efforts to develop cultivars with durable sources of genetic resistance (Ellis *et al*., 2014) and the application of fungicides (Chen, 2020). Fungicides are commonly applied on wheat and barley in SA to control a complex of foliar diseases, including outbreaks caused by rust pathogens (Anonymous, 2020b). Furthermore, *Pgt* can infect wheat and barley (Prins *et al*., 2020), which are commonly planted in adjacent fields in the Rûens production area of the Western Cape province, SA. Propiconazole or tebuconazole are the a. i. in more than 15 fungicide formulations registered to the control *Pgt* on wheat in SA (Agri-Intel, 2021). Of these fungicides, propiconazole has been banned in European Union countries since 2019 (Minut *et al*., 2020) and tebuconazole will likely also be banned under the European Commission pesticides review (Hillocks, 2012). Since the introduction of site-specific fungicide inhibitors in the 1970s, which include DMI fungicides like Tilt (a. i. propiconazole) and Folicur (a. i. tebuconazole), the emergence of fungicide insensitivity has become a concern (Cook *et al*., 2021).

Rust pathogens are generally considered low-risk of developing fungicide tolerance (Brent and Hollomon, 2007). However, they share features such as short life cycles, airborne dispersal and different modes of reproduction associated with high-risk pathogens, including fungi causing powdery mildew (Grimmer *et al*., 2015). In support of a higher risk factor, fungicide resistance associated mutations have been reported for several rust fungi. These include a G143A mutation in the *CytB* gene (Oliver, 2014), and the detection a DMI resistance-associated substitution (Y134F) in the CYP51 protein among *Pst* isolates from China and New Zealand (Cook *et al*., 2021). While a shift to increased insensitivity to tebuconazole was detected in *Pt* and *Pst*, there was no evidence of fungicide insensitivity in *Pgt* (Park and Clark, 2020). More recently, DMI insensitivity among isolates of *P. hordei* Otth. (*Ph*) has been detected in Australia, which was due to increased *CYP51* gene copy numbers (Prof. Robert Park, personal communication, March 2022). These reports, together with the often-routine application of fungicides in especially the Western Cape wheat and barley production areas, confirm the risk of fungicide resistance developing among isolates of cereal rust pathogens, which prompted the current study.

The sensitivity of *Pgt* isolates to propiconazole and tebuconazole was measured for the first time in SA to determine whether a correlation exists between possible *CYP51* sequence variants

and fungicide insensitivity. From *in vitro* urediniospore germination tests on fungicide amended water agar, significant differences in germination percentages were detected between the untreated control and propiconazole dilutions. Furthermore, the propiconazole dilutions differed significantly from each other, except for the x0.20 and x0.40 dilutions. These two dilutions were highly efficient in suppressing *Pgt* urediniospore germination. Similarly, the mean germination percentages for the untreated control and the tebuconazole dilutions differed significantly among all the tested tebuconazole dilutions. The germination inhibition was directly proportional to the increase in concentration. Overall, the highest fungicide dilutions (x0.075 propiconazole and x0.010 tebuconazole) yielded the highest mean germination percentage with a subsequent decrease over dilutions with the lowest mean germination percentage at the lowest fungicide dilutions (x0.40 propiconazole and x0.030 tebuconazole). Although a few isolates produced significantly lower germination percentages with the untreated control treatment, it did not impact the results as the relative urediniospore inhibition rate was calculated. Furthermore, no significant differences in germination percentage were detected among the replicates of the untreated controls and the dilutions for both propiconazole and tebuconazole. Therefore, the results produced following our protocol were sound and yielded consistent and repeatable results.

The *Pgt* isolates showed significant differences in their urediniospore sensitivity to the different propiconazole and tebuconazole dilutions in contrast to the consistent high viability observed for the untreated controls. Minimum significant difference percentages at each fungicide dilution revealed groups of *Pgt* isolates differing significantly from each other in terms of their sensitivity responses. The x0.075 propiconazole dilution revealed ten statistically distinct groups among the 45 *Pgt* isolates based on mean urediniospore germination percentages. The x0.10 propiconazole dilution revealed four statistically distinct groups of isolates, while each of the x0.20 and x0.40 dilutions revealed two statistically distinct groups. The x0.01 and x0.015 tebuconazole dilutions revealed nine statistically distinct groups of *Pgt* isolates each, while the x0.02 revealed five groups and the x0.03 dilutions two groups.

The differences detected in germination percentages showed that variation in sensitivity exists among *Pgt* isolates. This variation was, however, at dilutions well below the recommended commercial doses for both active ingredients. In this study, the lowest dilutions where sensitivity was measured was 0.1667 g/L a. i. for propiconazole and 0.0206 g/L a. i. for tebuconazole compared to the registered commercial doses of 0.41667 g/L (recommended rate

per hectare for ground application of 500 ml per 300 L of water) and 0.6875 g/L (825 ml per 300 L of water), respectively (Syngenta, 2022; Bayer Crop Science, n.d.). Although the lowest measured fungicide dilutions were effective to inhibit *Pgt* urediniospore germination, the significant variation in sensitivity among the isolates indicated the existence of an underlying genetic potential to develop fungicide insensitivity within the *Pgt* population. Reports of field failure of fungicides to cereal rust pathogens are only occasional in literature (Bayles *et al*., 2000; Stammler *et al*., 2009; Kang *et al*., 2019; Tian *et al*., 2019). However, the risk of fungicide insensitivity developing in rust species has been confirmed in literature (Park and Clark, 2020; Cook *et al*., 2021). The current findings further emphasized the need for producers to be cautious when relying on fungicide application, as well as the importance to follow fungicide management strategies to extend the period of effectiveness of fungicides.

The DMI fungicide Tilt (propiconazole) was first registered in SA in 1986, while Folicur (tebuconazole) was first registered in 1990 (Personnel communication Mr Francois van Deventer, CPD Technical Lead – Fungicides, Syngenta, SA and Mr David Mamogobo, CP Regulatory Affairs Manager Africa, Bayer (Proprietary) Ltd, Isando, SA). Considering the timeline of fungicide registration and isolate collection (Figure 5.1), only 2SA4 (UVPgt50 isolate 36) can be considered as previously unexposed to the a. i. of these two fungicides. Overall, the fungicide sensitivity results did not reveal strong evidence of a decrease in fungicide sensitivity over time when the recently collected isolates were compared to historic isolates, for both fungicides.

To determine whether fungicide insensitivity has developed, sensitivity tests are performed using a range of doses to determine the EC_{50} value (Russel, 2020). The EC_{50} value indicates the dose of the a. i. of a particular fungicide that achieves a 50% inhibition of urediniospore germination compared to the control treatment. Hereby, a baseline sensitivity response is established that acts as a reference point for what can be considered the accepted response of isolates of a particular fungus to a specific fungicide treatment (Russel, 2020). The EC_{50} results from this study will therefore be valuable in future studies to identify *Pgt* isolates with increased tolerance to the two active ingredients.

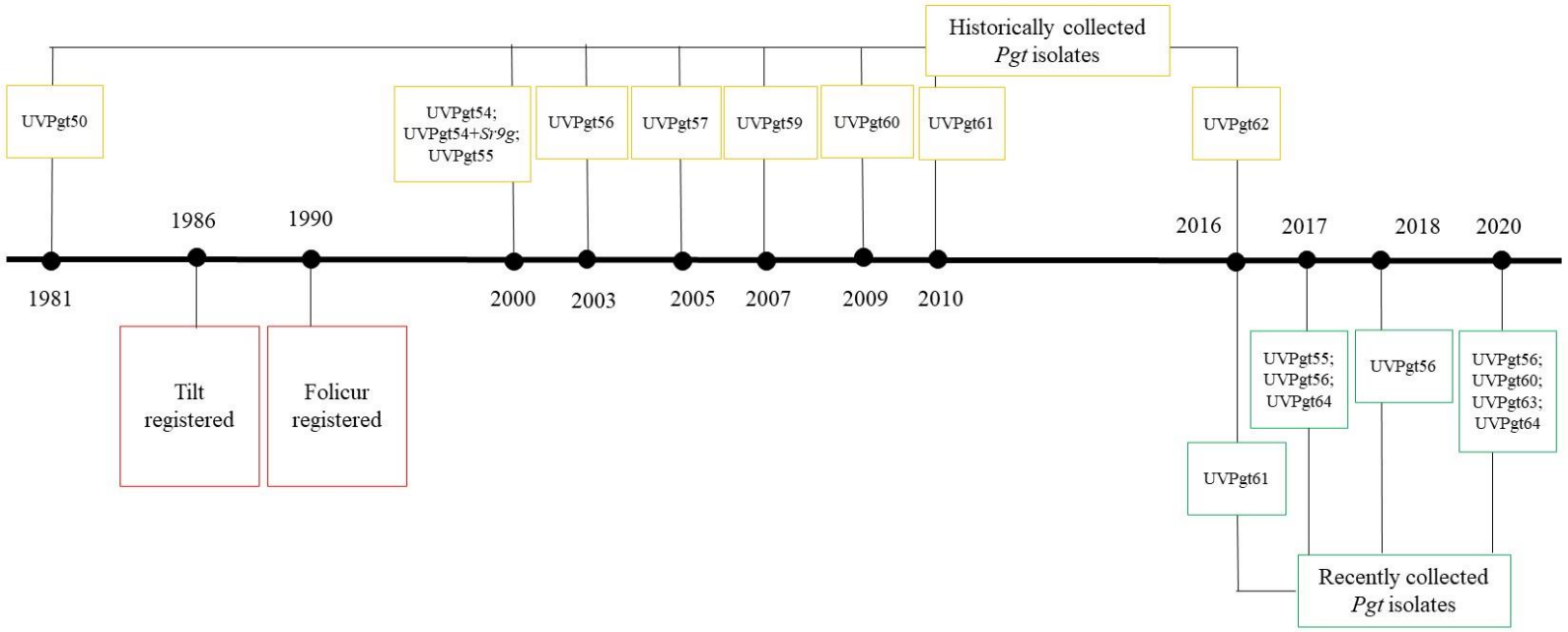

Figure 5.1 Timeline for *Puccinia graminis* f. sp. *tritici* (*Pgt*) race isolates collected and years of first registration of DMI fungicides Tilt (a. i. propiconazole) and Folicur (a. i. tebuconazole).

Race 2SA104 (isolate 40 of UVPgt56) was less sensitive to both a. i. propiconazole and tebuconazole compared to some of the more recently collected isolates. Importantly, 2SA104 is closely related, phenotypically and genotypically, to older *Pgt* races like 2SA102 (not included in this study), first detected in 1988, and if evolved from an isolate of this older race, isolate 40 can strictly not be considered as previously unexposed to the two a. i. used in this study (Smith and Leroux, 1992; Boshoff *et al*., 2018b). The same argument is valid for recently collected isolates of *Pgt* race 2SA55A (UVPgt54), 2SA55B (UVPgt54+*Sr9g*), 2SA105 (UVPgt57), 2SA88+ (UVPgt61), 2SA108 (UVPgt62) and 2SA42 (UVPgt63) as they are predicted to have evolved locally through mutations from existing *Pgt* race isolates (Terefe *et al*., 2016; Boshoff *et al*., 2018b; Terefe *et al*., 2019).

The EC_{50} estimates determined for 2SA42 (isolates of Pgt63) revealed that all six isolates (2020) responded similarly to propiconazole with a narrow range of estimates (0.056 for isolate 2 to 0.0736 for isolate 28) recorded. This could act as evidence that variation in sensitivity can somehow be accounted for by the collection date, as all these isolates were collected during the 2020 season. The range in EC_{50} responses determined for tebuconazole for these isolates varied between 0.011 for isolate 2 to 0.018 for isolate 28.

For *Pgt* race 2SA107 (isolates of UVPgt60), the EC_{50} estimates also revealed similar responses considering the range in EC_{50} estimates (propiconazole 0.0648 for isolate 9 to 0.0740 for isolate 8; tebuconazole 0.012 for isolate 43 to 0.014 for isolate 9) despite the time differences of collections between 2016 and 2020, compared to the historic isolate 43 collected during 2009. This indicated that the collection date was not a factor in the development of varying levels of sensitivity among these isolates.

For *Pgt* race (isolates of UVPgt61), the two isolates (isolate 10 from 2016 and isolate 44 from 2010) revealed a strong change in sensitivity over time for propiconazole (0.0002 for isolate 44 to 0.0239 for isolate 10). However, isolates of this race appeared more similar in sensitivity to tebuconazole considering the range in values produced (0.012 for isolate 4 to 0.016 for isolate 10).

The EC_{50} estimates determined for *Pgt* race 2SA5 (isolates of Pgt64) revealed that isolates 25 (2017), 32 (2020), 34 (2020) and 35 (2020) were more sensitive to propiconazole whereas isolates 13 (2017), 33 (2020) and 35 (2020) produced the lowest values for tebuconazole.

Isolate 31 (2020) was the least sensitive to propiconazole (EC_{50} estimate of 0.0838) and tebuconazole (0.016).

Considering *Pgt* race 2SA88 (isolates of UVPgt55), the EC_{50} estimates revealed that historically collected isolate 39 (0.0594; collected 2000) was more sensitive to propiconazole along with isolate 17 (0.0594; 2017), but the most sensitive isolate was isolate 16 (0.0189; 2017). The least sensitive isolates to propiconazole were isolates 15 and 19, with EC_{50} estimates of 0.0759 and 0.0725 (both from 2017), respectively. The range in EC_{50} estimates for tebuconazole varied from 0.01 for isolate 16 (2017) to 0.017 for isolate 39 (2000).

Other factors, such as the host the *Pgt* isolate was collected from, and the physical origin of the collections did not provide clear explanations for the differences in observed sensitivity. This suggests that factors outside what was included in the study may contribute to the incidence of isolates with varying levels of sensitivity. These could include differences in virulence and fitness factors among *Pgt* isolates as well as differences in sensitivity to other a. i. like strobulurin containing fungicides commonly used by cereal producers. These factors may impact the survival and incidence of *Pgt* isolates over time irrespective of their sensitivity to the two a. i. used in this study.

Random mutations of the target gene might also have influenced the detected variation in sensitivity, by causing variability in the population (Loewe and Hill, 2010). Variation among *Pgt* populations has been linked to the effects of genetic drift, migration and demographic events (Ali *et al*., 2014; Stefansson *et al*., 2014). What can, however, be assumed from the variation in sensitivity detected, is that the genetic potential to develop increased insensitivity exists among *Pgt* isolates and that the continued selection pressure exerted on the *Pgt* population by the overreliance on specific a. i. may result in the development of stronger insensitivity or even resistance in future.

Sequencing results identified two allelic variants of the *CYP51* gene among the selected *Pgt* isolates. While alleles 1 and 2 differed from each other, both were identical in their sequences across the isolates, even regarding the single mutations and deletions present within the introns. This indicates a single probable origin for both alleles occurring in all the different isolates.

Most nucleotide changes were contained within the introns of the gene, which will therefore have no effect on the resulting CYP51 protein. No SNP's within the 4 bp boundary of the intron/exon region was detected. Additionally, most nucleotide changes detected within the

exons resulted in silent mutations, which would also not affect the resulting CYP51 protein. The single nucleotide change, from G to A at nucleotide position 1 866, resulted in a missense mutation where aspartic acid (D) in the resulting protein was exchanged with an asparagine (N) at position 510. Both amino acids are polar and play similar roles in some proteins (Betts and Russel, 2003). They only differ in that aspartic acid contains an oxygen molecule in place of the amide group present in asparagine. Other amino acids were also present at position 510 on the other reference *CYP51* genes (proline for ACS3752.1 and PTTG05695; lysine for KAI8452430.1; glutamic acid for KAH9815292.1). This suggests that the D to N change occurred in a non-essential region of the protein that might not affect the functionality of the protein.

The identification of different alleles of the *CYP51* gene indicated that genetic differences do exist within the *Pgt* population, but the resulting changes in the amino acid sequence indicated that the encoded protein remained highly conserved, as slight changes in the CYP51 protein sequence might affect the fitness and consequent survival of affected *Pgt* isolates (Kiran *et al*., 2021). The ratio of synonymous to nonsynonymous changes observed in nature favours synonymous mutations in a type of selection known as purifying selection (Carroll, 2006). While some structural changes are allowed to occur within the CYP51 protein, other changes could negatively affect the enzymatic activity of the protein. These 'injured' variations will most likely be selected against (Becher and Wirsel, 2012; Carroll, 2006). This may explain the high number of synonymous compared to nonsynonymous changes seen within the two alleles of the *CYP51* gene.

Furthermore, the high conservation of the CYP51 amino acid sequences in all the tested *Pgt* isolates, supports the lack of evidence of increased fungicide insensitivity over time measured among the studied *Pgt* isolates. Although more isolates need to be assessed in future, the current results are in support of findings by Park and Clark (2020), where no evidence of fungicide insensitivity was detected for *Pgt* in Australia. *Pgt* isolates homozygous for allele 1 ranged in EC_{50} estimates from 0.0002 (isolate 44) to 0.0937 (isolate 41) for propiconazole, while they ranged from 0.004 (isolate 22) to 0.0180 (isolate 28) for tebuconazole. The average EC_{50} estimate across these homozygotes was 0.057 for propiconazole, which was identical to the overall propiconazole average EC_{50} estimate, while the average was 0.014 for tebuconazole, in close resemblance of the overall tebuconazole average of 0.013. *Pgt* isolates homozygous for allele 2 ranged in EC_{50} estimates from 0.0212 (isolate 34) to 0.0838 (isolate 31) for

propiconazole, and from 0.009 (isolate 30) to 0.016 (isolates 10 and 34) for tebuconazole. The average EC_{50} estimates for these homozygotes were 0.054 for propiconazole and 0.013 for tebuconazole. The heterozygous *Pgt* isolates ranged in EC_{50} estimates from 0.0006 (isolate 11) to 0.0755 (isolate 45) for propiconazole, and from 0.008 (isolate 11) to 0.016 (isolate 45) for tebuconazole. The average EC_{50} estimate for these heterozygotes was 0.029 for propiconazole, due to the outlying low EC_{50} estimate of 0.0002 (isolate 44), and 0.013 for tebuconazole.

No current evidence of fungicide insensitivity in *Pgt* was detected in SA and although some allelic variation existed among the isolates, it can be assumed that none resulted in any profound alterations of the CYP51 protein. The identification of genetic variation in fungicide target genes, such as the *CYP51* gene from the current study, confirms that the potential exists for cereal rusts to develop fungicide insensitivity (Cook *et al*., 2021).

The development of fungicide insensitive isolates cannot only be expected to occur locally, since records show that *Pgt* race dispersal can occur over vast distances (Visser *et al*., 2019) with several examples of confirmed incursions of rust races into SA (Terefe *et al*., 2016; Terefe *et al*., 2022). This emphasizes the equally important large-scale routine monitoring of fungicide sensitivity required along with the annual rust surveys currently only focusing on virulence and the impact that new races with wider virulence have on cultivar responses (Boshoff *et al*., 2018a; Terefe *et al*., 2019; Terefe *et al*., 2022). The data from this study serves as a baseline for future research on the development of fungicide insensitivity among *Pgt* isolates in SA and globally to fungicides containing the a. i. propiconazole and tebuconazole.

Chapter 6: Conclusions and recommendations

In this study, the sensitivity of *Pgt* isolates to the commonly used triazole based fungicides propiconazole and tebuconazole, was measured. The included isolates were collected over a long time, and were representative of two of the main wheat production areas, as well as of the most commonly found *Pgt* races, in SA. The results will serve as a point of reference for future studies on the fungicide sensitivity of *Pgt* to these a. i. in SA, and will be relevant to similar international studies.

The methodology developed to determine *Pgt* urediniospore germination percentages yielded consistent and repeatable results with low variation detected over replicates. The *Pgt* isolates showed significant differences in their sensitivity at the different fungicide dilutions for both propiconazole and tebuconazole in contrast to the untreated controls, where mostly excellent germination percentages were observed for all isolates. The results did not reveal strong evidence of a decrease in fungicide sensitivity over time, when responses from recently collected isolates (after 2016) were compared to the historic isolates. Although the *Pgt* isolates varied significantly in their germination percentages to both a. i., it was at dilutions well below the recommended commercial doses. Thus, from the assessment of the current selection of *Pgt* isolates, there is no reason to expect field failure due to fungicide resistance when applying formulations containing either of these two a. i. to control stem rust. However, in future studies the number of *Pgt* isolates should be increased with a more concerted effort to collect isolates from fields following fungicide applications and the use of other a. i. like strobulurins.

Two allelic variants of the *CYP51* gene were identified among the 23 *Pgt* isolates selected for molecular analysis. A single nucleotide change (G1866A) resulted in a missense mutation where aspartic acid (D) was exchanged for asparagine (N) at amino acid position 510 in the resulting protein. Evidence suggests that the mutation will probably not affect the functionality of the protein. While the two alleles differed from each other, both were identical in their sequences across the different isolates, even regarding the mutations and deletions present within the introns. A single origin for both alleles is thus a probability. However, further research is required to confirm this using a larger collection of *Pgt* isolates. Although genetic variation existed amongst the *Pgt* isolates, the encoded protein remained highly conserved, which supported the lack of evidence of increased fungicide insensitivity observed for these isolates.

Despite these findings, the existence of genetic variation in the *CYP51* gene, and the observed significant differences in sensitivity levels to propiconazole and tebuconazole, confirms the underlying genetic potential for *Pgt* to develop fungicide insensitivity. Therefore, going forward the routine monitoring of fungicide sensitivity on a larger scale, application of newer technologies and the inclusion of more relevant target genes, are considered as important as the annual *Pgt* surveys in SA currently only focus on race composition, virulence and cultivar responses.

The results from this study underlined certain aspects to be considered for further research. While the outcomes served as a baseline for future research on *Pgt* sensitivity to fungicides containing propiconazole and tebuconazole, there is still a lack of baseline studies to determine fungicide sensitivity to other a. i. used to control *Pgt* in SA. Because of the extensive reliance on fungicide applications for disease control on cereals, routine monitoring of fungicide sensitivity in fungal populations will remain an important consideration. The germination test protocol established to measure especially propiconazole sensitivity, can be improved in future studies by adjusting fungicide dilutions to measure a more gradual change in urediniospore germination. This will contribute to a more accurate assessment of the absolute EC_{50} measurement to this active ingredient.

The inclusion of more *Pgt* isolates for *CYP51* sequence analysis would enable future studies to investigate the probable single origin for both alleles, which was not possible to resolve during the current study. The use of next generation sequencing to detect genetic variation within fungicide target genes in further studies would enable the sequencing of multiple genes simultaneously, thereby addressing the limitations of Sanger sequencing that was used in the current study to sequence a single gene fragment at a time. This will enable research to expand insensitivity tests to fungicides containing a. i. with different modes of action.

In conclusion, results from the current study, as well as the much-needed research capacity that was generated, will enable future research to be more inclusive considering the rust species involved and the a. i. relevant to disease control of rusts on cereals in SA.

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Appendices

Appendix 1 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.00 propiconazole (untreated control) treatment.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	484	11.01	2.78	2.3e-05*
Replicate	2	30	15.20	3.84	0.025
Residuals	88	348	3.96		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 6.92%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 2 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.075 propiconazole dilution.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	60224	1369	12.90	<2e-16*
Replicate	2	948	474	4.47	0.014
Residuals	88	9336	106		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 35.85%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 3 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.10 propiconazole dilution.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	18382	418	8.38	<2e-16
Replicate	2	106	53	1.06	0.35
Residuals	88	4387	50		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 24.58%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 4 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.20 propiconazole dilution.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	73.7	1.674	2.91	1e-05*
Replicate	2	0.1	0.030	0.05	0.95
Residuals	88	50.6	0.575		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 2.64%.

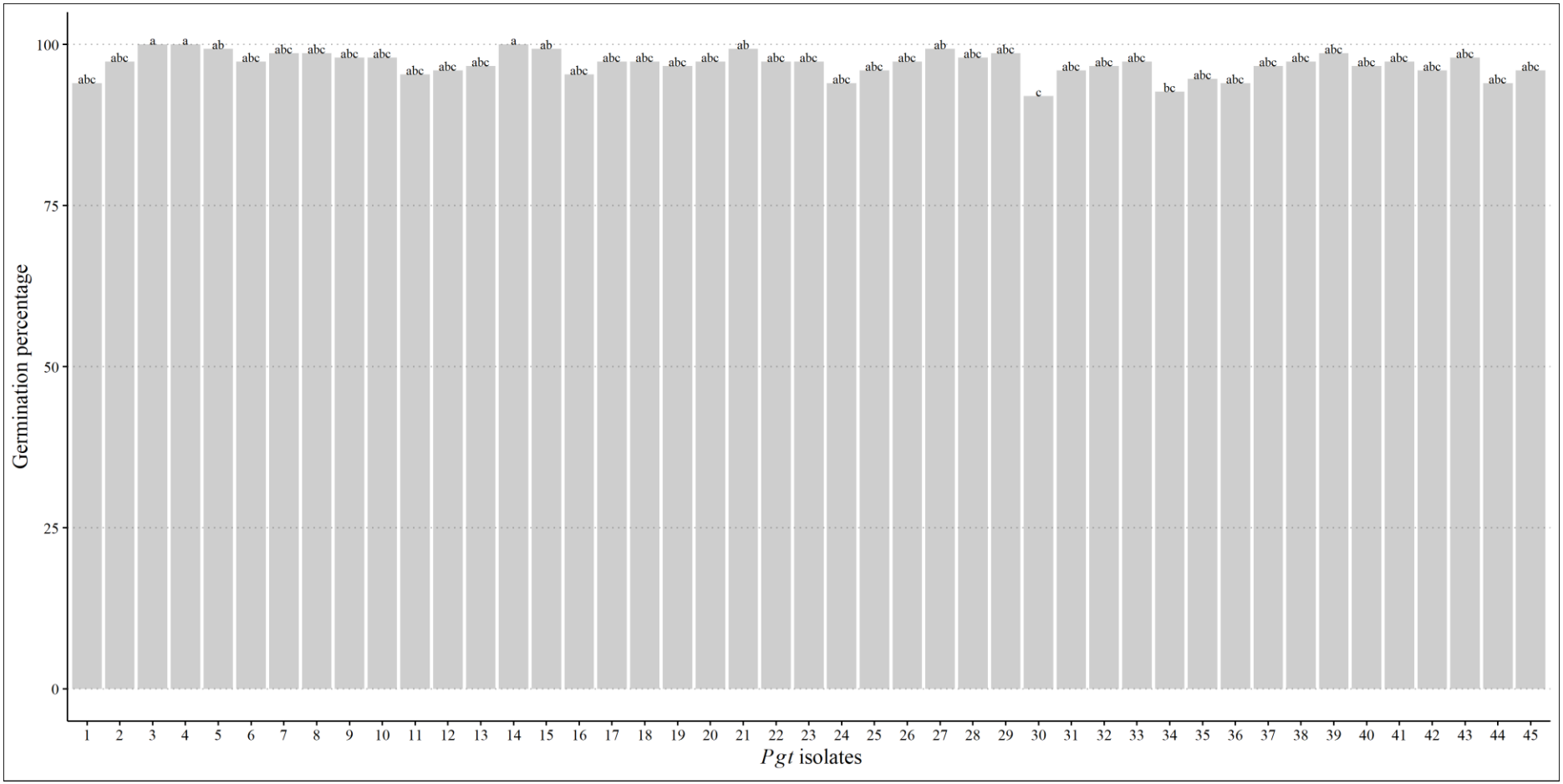
Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 5 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.40 propiconazole dilution.

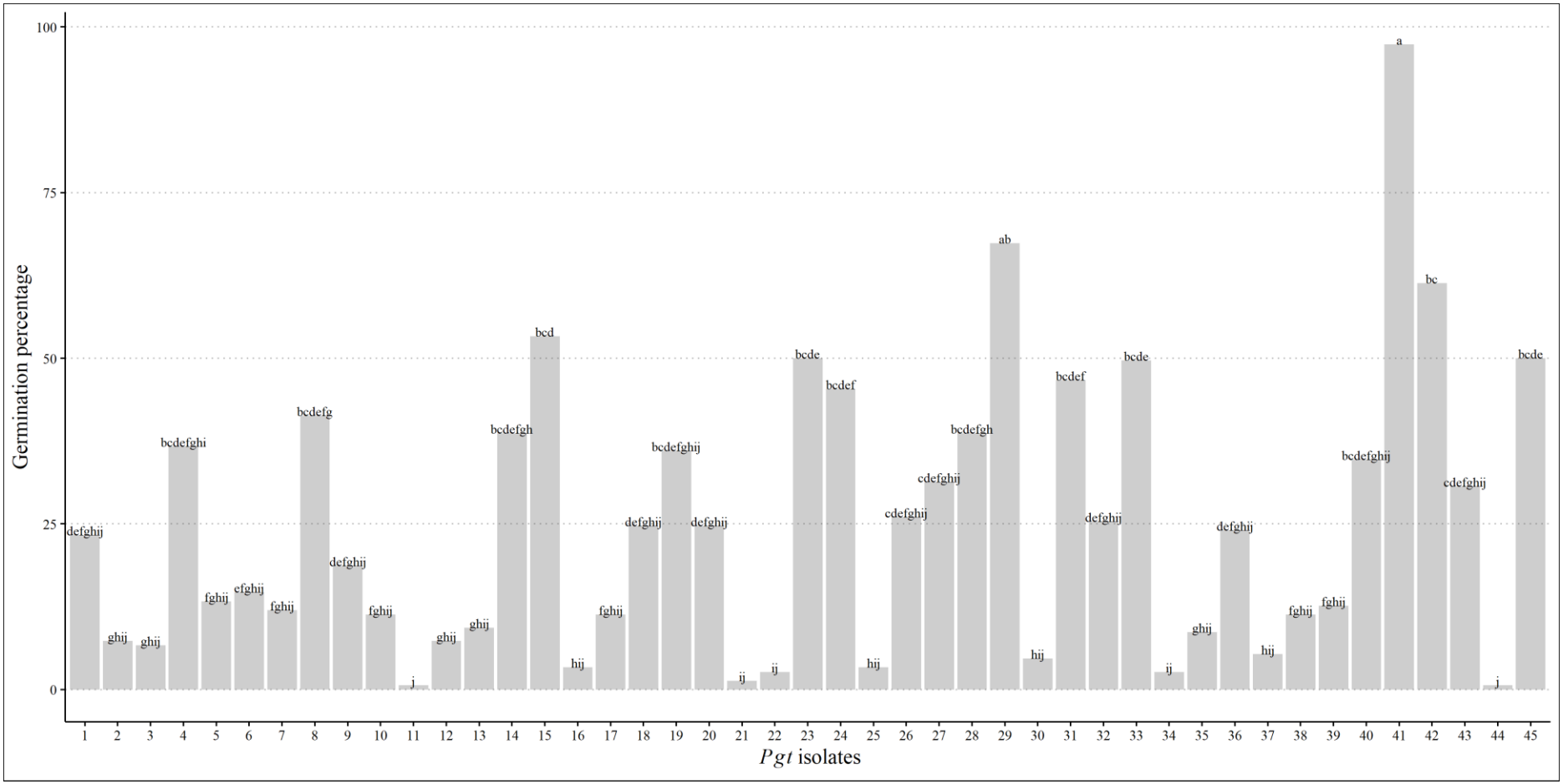
Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	5.21	0.118	1	0.49
Replicate	2	0.24	0.118	1	0.37
Residuals	88	10.43	0.118		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 1.20%.

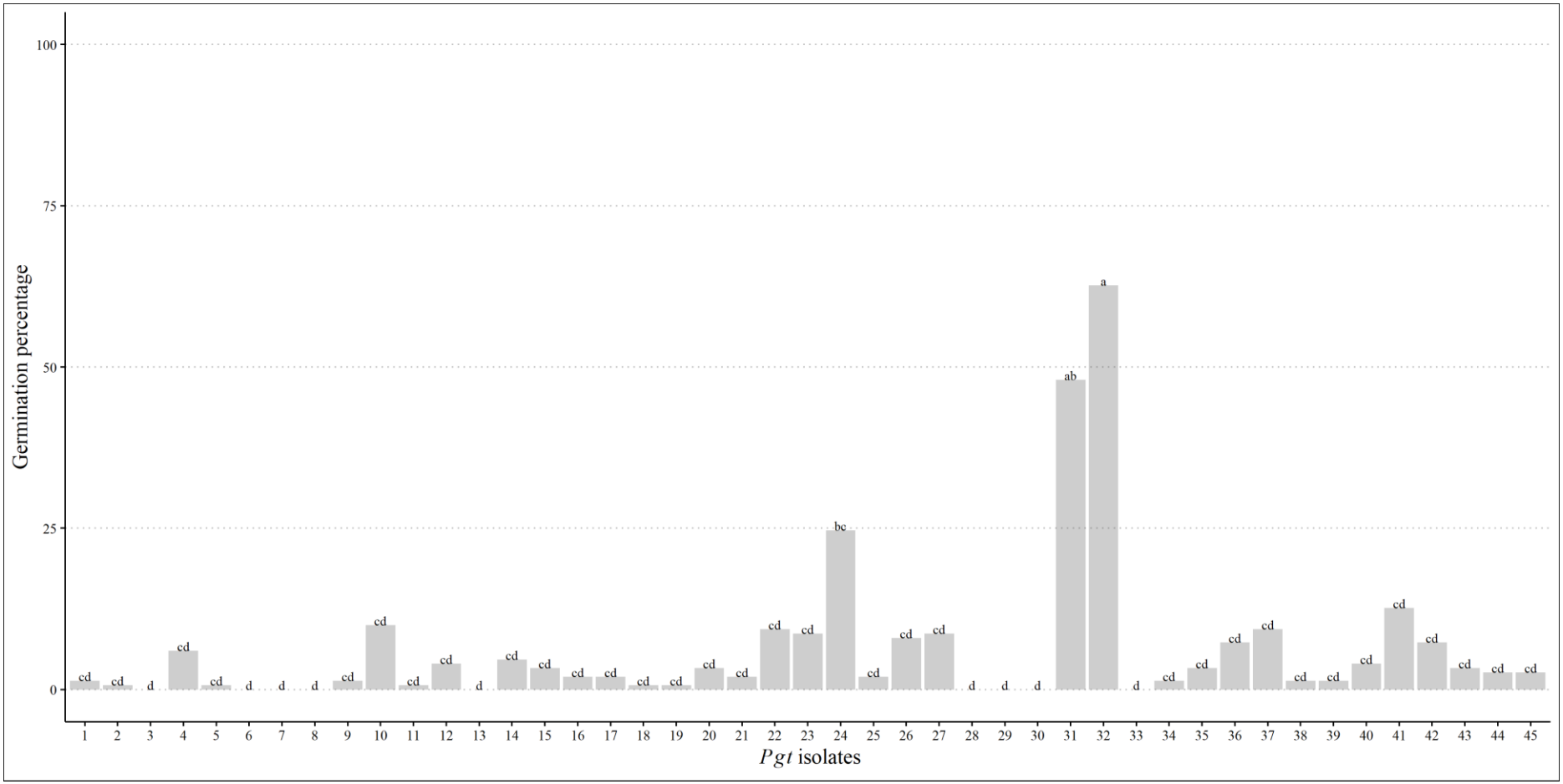
Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).



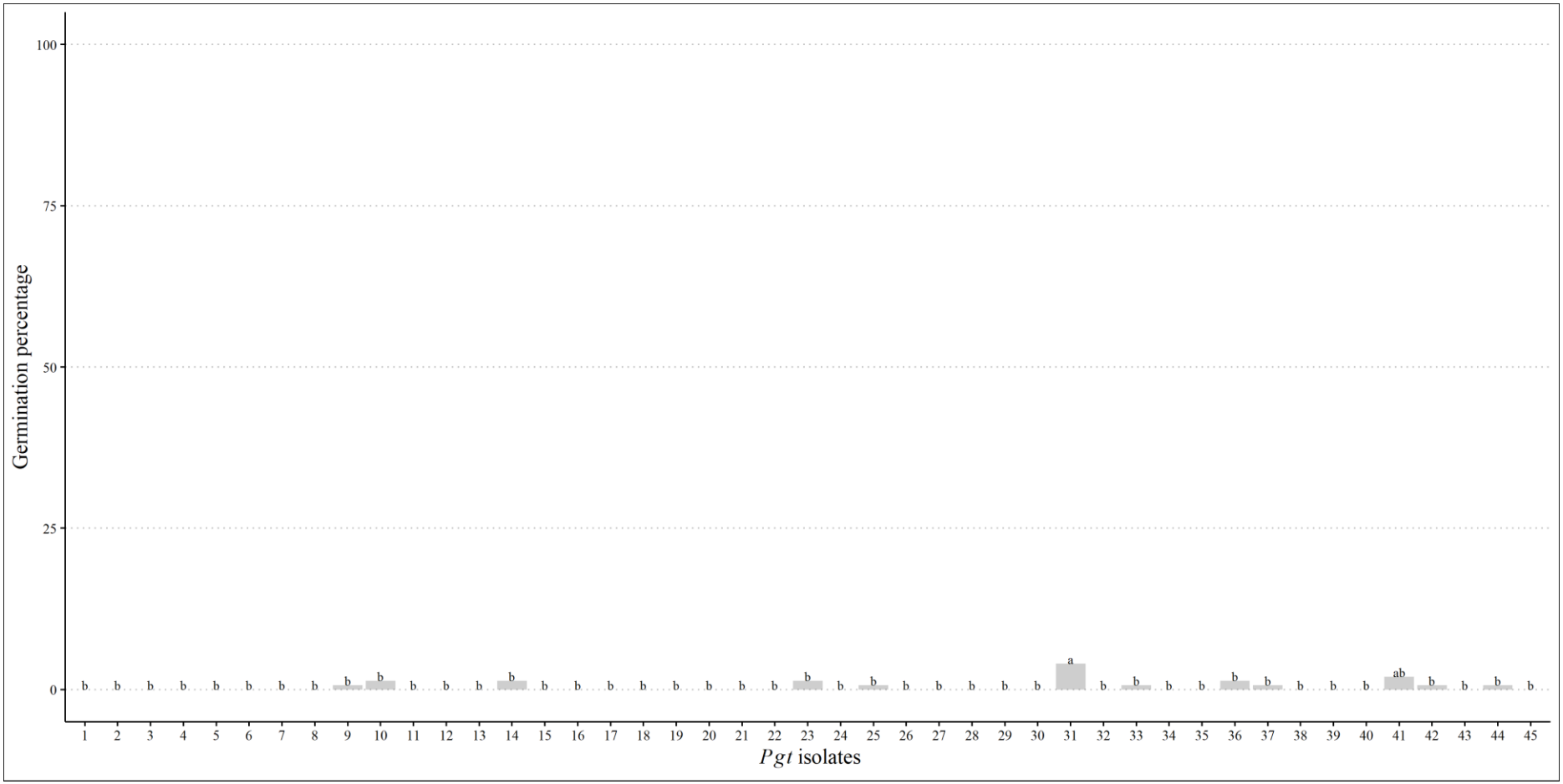
Appendix 6 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.00 propiconazole (untreated control) treatment. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 6.92%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 1).



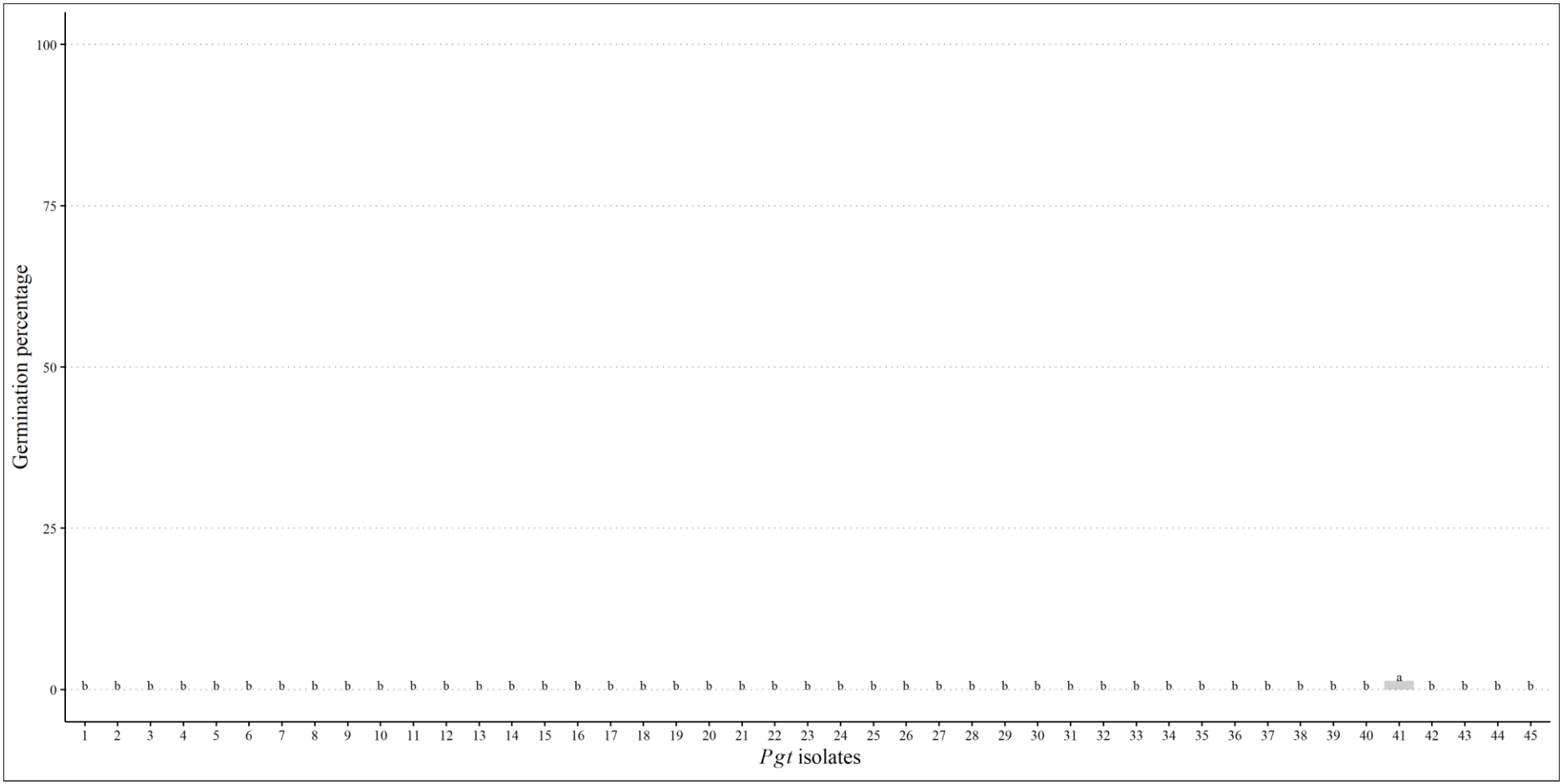
Appendix 7 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.075 propiconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 35.85% i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 2).



Appendix 8 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.10 propiconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 24.58%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 3).



Appendix 9 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.20 propiconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 2.64%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 4).



Appendix 10 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.40 propiconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 1.20%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 5).

Appendix 11 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.00 tebuconazole (untreated control) treatment.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	4890	111.1	21.97	2e-16*
Replicate	2	8	4.1	0.81	0.45
Residuals	88	445	5.1		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 7.83%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 12 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.01 tebuconazole dilution.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	50012	1137	22.40	<2e-16*
Replicate	2	362	181	3.57	0.032
Residuals	88	4465	51		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 24.79%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 13 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.015 tebuconazole dilution.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	64900	1475	7.23	2.5e-15*
Replicate	2	105	53	0.26	0.77
Residuals	88	17950	204		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 49.71%.

Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 14 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.02 tebuconazole dilution.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	5381	122.3	4.93	1.1e-10*
Replicate	2	5	2.3	0.09	0.91
Residuals	88	2185	24.8		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 17.90%.

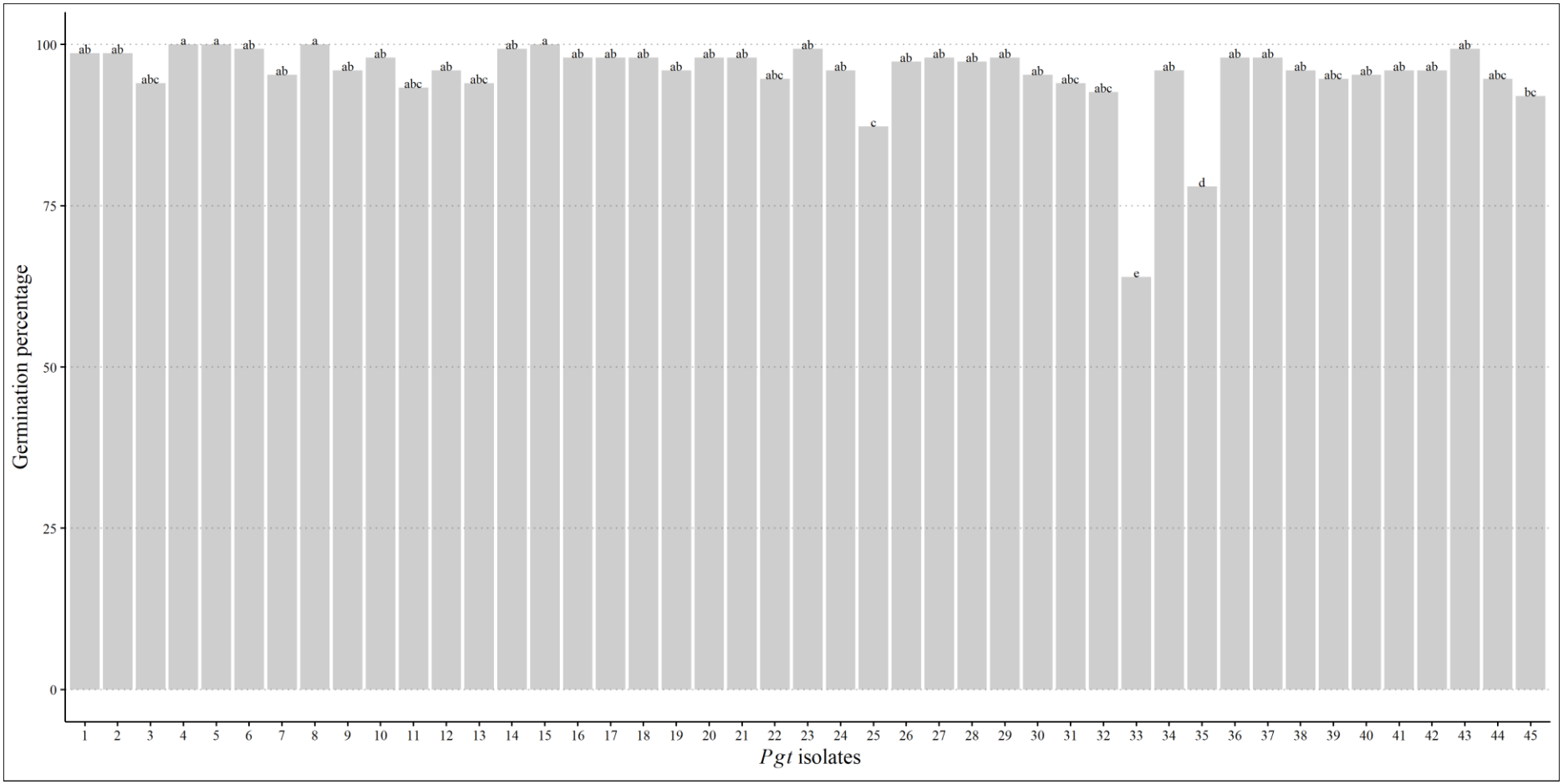
Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).

Appendix 15 Analysis of variance (ANOVA) results for urediniospore germination percentages of 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.03 tebuconazole dilution.

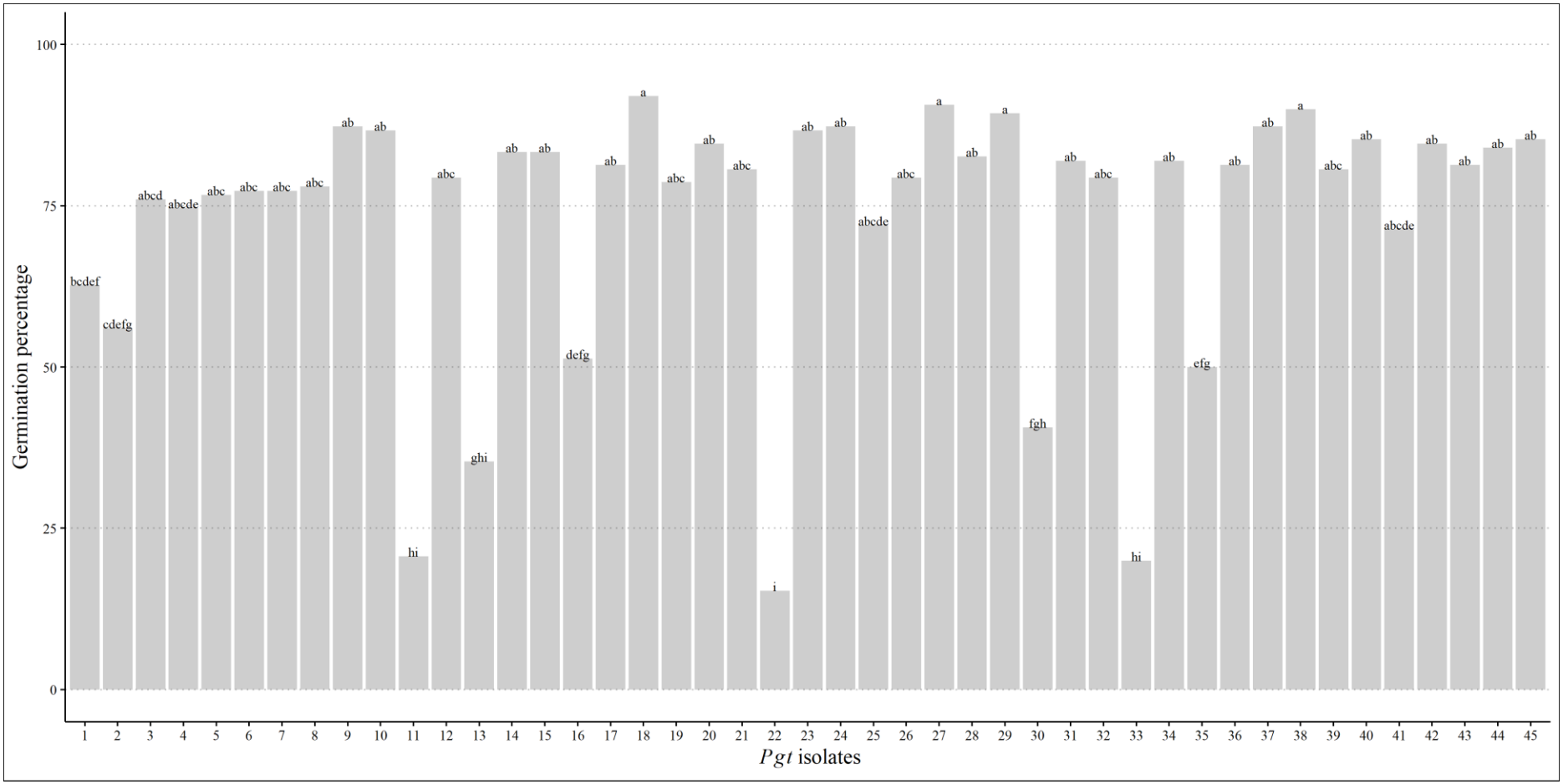
Source of variation	**d.f.**	**s.s.**	**m.s.**	**F value**	**Pr (>F)**
Isolate	44	421	9.57	12.19	2e-16*
Replicate	2	3	1.45	1.85	0.16
Residuals	88	69	0.79		

*Term significant at alpha = 0.05; Least significant difference (LSD) between isolates = 3.08%.

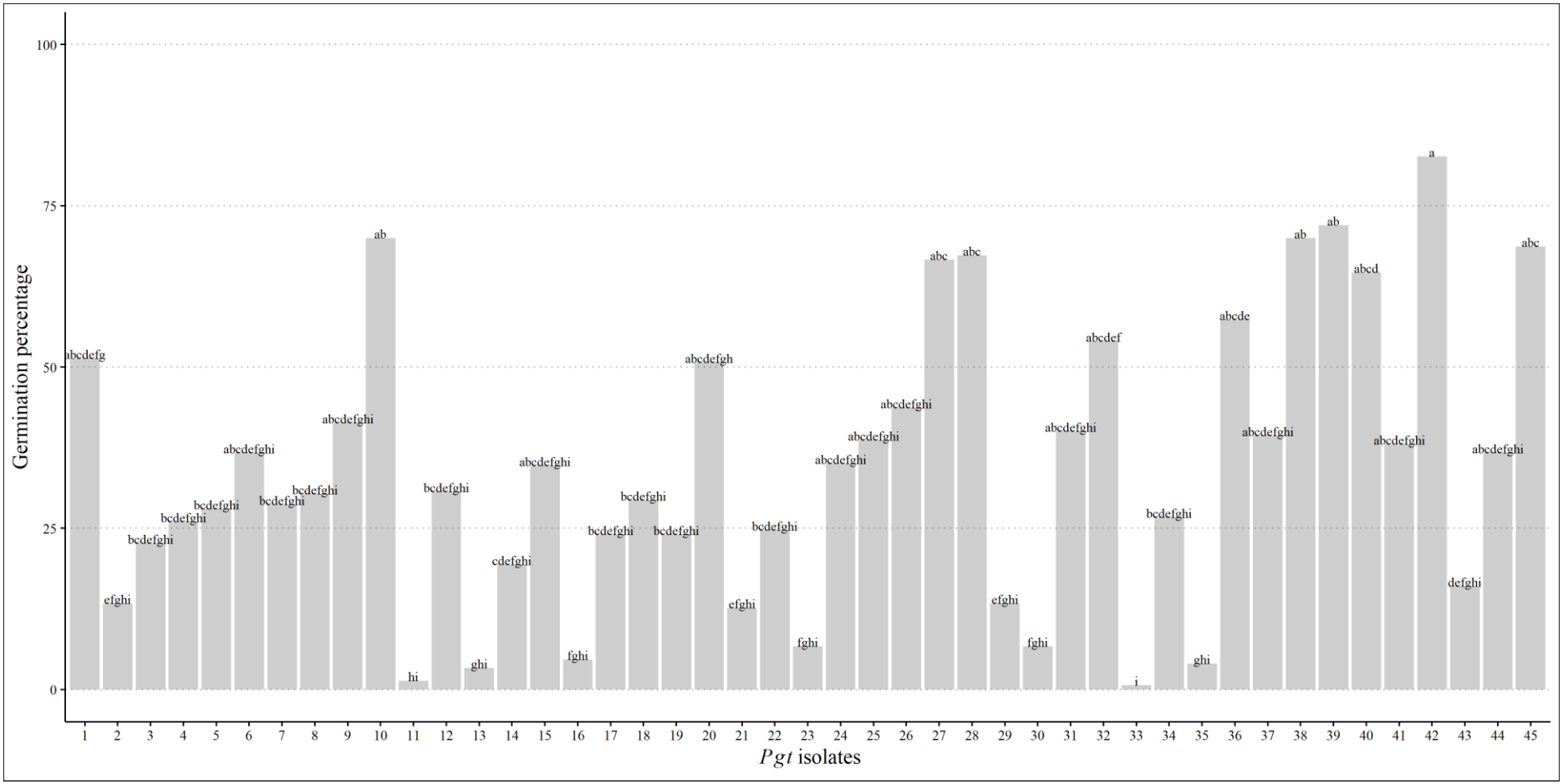
Variate: Urediniospore germination percentage. d.f. = Degrees of freedom; s.s. = sum of squares; m.s. = mean squares; Pr = probability value (p value).



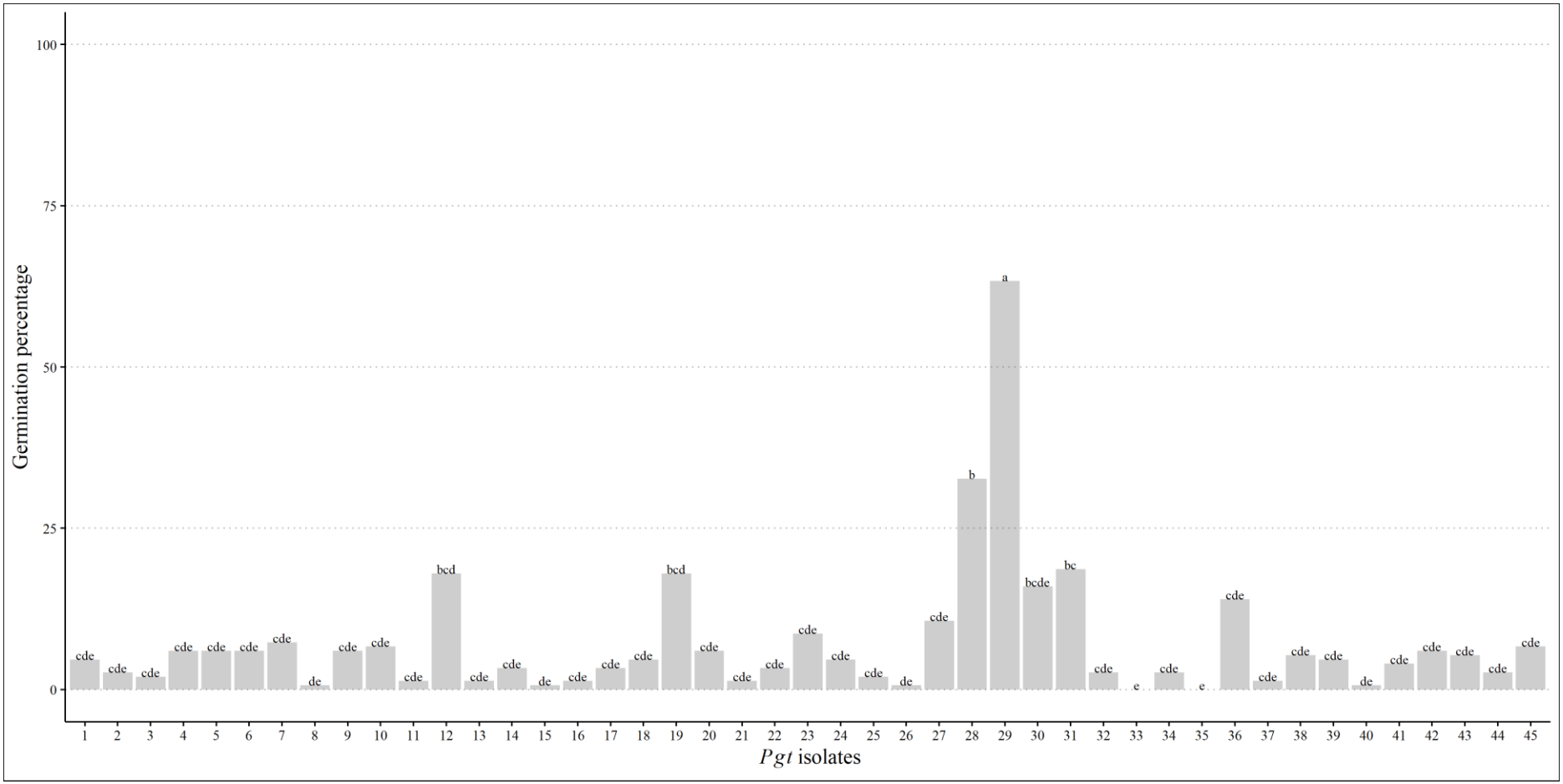
Appendix 16 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.00 tebuconazole (untreated control) treatment. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 7.83%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 11).



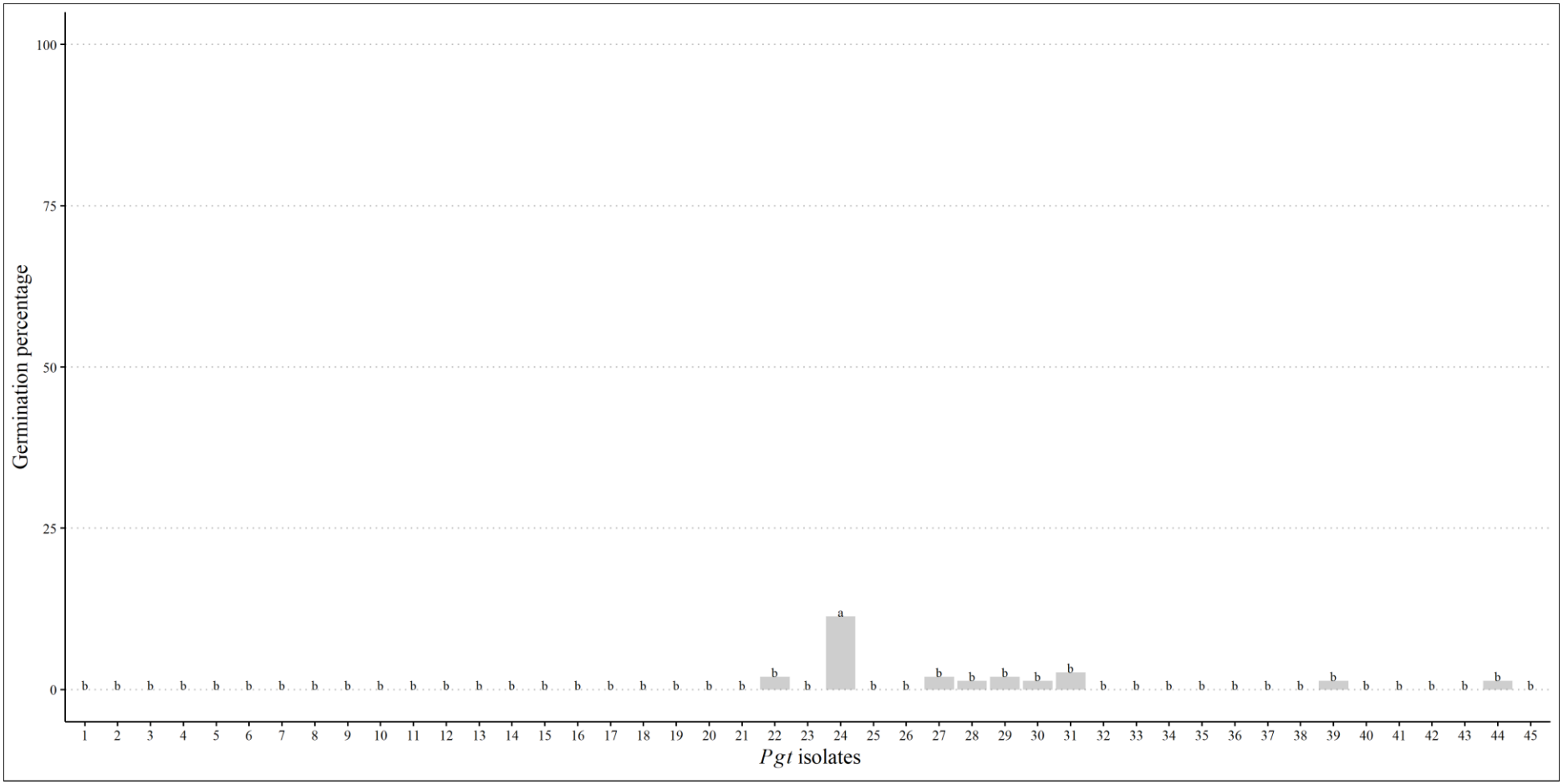
Appendix 17 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates for the x0.01 tebuconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 24.79%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 12).



Appendix 18 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates at x0.015 tebuconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 49.71%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 13).



Appendix 19 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates at x0.020 tebuconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 17.90%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 14).



Appendix 20 Mean germination percentage recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates at x0.030 tebuconazole dilution. Least significant difference (LSD) between urediniospore germination percentage means of different isolates = 3.08%, i.e., entries with the same LSD symbol do not differ significantly from each other ($p < 0.05$ ANOVA, Appendix 15).

Appendix 21 Mean germination percentages recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates over the four different fungicide dilutions (x0.075, x0.10, x0.20 and x0.40) with propiconazole as active ingredient and an untreated control.

	Germination percentage over five different fungicide dilutions using propiconazole as active ingredient				
Isolate nr.	**x0.00**	**x0.075**	**x0.10**	**x0.20**	**x0.40**
1	94.00^{abc}	23.33^{defghij}	1.33^{cd}	0.00^{b}	0.00^{b}
2	97.33^{abc}	7.33^{ghij}	0.67^{cd}	0.00^{b}	0.00^{b}
3	100.00^{a}	6.67^{ghij}	0.00^{d}	0.00^{b}	0.00^{b}
4	100.00^{a}	36.67^{bcdefghi}	6.00^{cd}	0.00^{b}	0.00^{b}
5	99.33^{ab}	13.33^{fghij}	0.67^{cd}	0.00^{b}	0.00^{b}
6	97.33^{abc}	14.67^{efghij}	0.00^{d}	0.00^{b}	0.00^{b}
7	98.67^{abc}	12.00^{fghij}	0.00^{d}	0.00^{b}	0.00^{b}
8	98.67^{abc}	41.33^{bcdefg}	0.00^{d}	0.00^{b}	0.00^{b}
9	98.00^{abc}	18.67^{defghij}	1.33^{cd}	1.33^{b}	0.00^{b}
10	98.00^{abc}	11.33^{fghij}	10.00^{cd}	1.33^{b}	0.00^{b}
11	95.33^{abc}	0.67^{j}	0.67^{cd}	0.00^{b}	0.00^{b}
12	96.00^{abc}	7.33^{ghij}	4.00^{cd}	0.00^{b}	0.00^{b}
13	96.67^{abc}	9.33^{ghij}	0.00^{d}	0.00^{b}	0.00^{b}
14	100.00^{a}	38.67^{bcdefgh}	4.67^{cd}	1.33^{b}	0.00^{b}
15	99.33^{ab}	53.33^{bcd}	3.33^{cd}	0.00^{b}	0.00^{b}
16	95.33^{abc}	3.33^{hij}	2.00^{cd}	0.00^{b}	0.00^{b}
17	97.33^{abc}	11.33^{fghij}	2.00^{cd}	0.00^{b}	0.00^{b}
18	97.33^{abc}	24.67^{defghij}	0.67^{cd}	0.00^{b}	0.00^{b}
19	96.67^{abc}	36.00^{bcdefghij}	0.67^{cd}	0.00^{b}	0.00^{b}
20	97.33^{abc}	24.67^{defghij}	3.33^{cd}	0.00^{b}	0.00^{b}
21	99.33^{ab}	1.33^{ij}	2.00^{cd}	0.00^{b}	0.00^{b}

Appendix 21 (cont.) Mean germination percentages recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates over the four different fungicide dilutions (x0.075, x0.10, x0.20 and x0.40) with propiconazole as active ingredient and an untreated control.

	Germination percentage over five different fungicide dilutions using propiconazole as active ingredient				
Isolate nr.	**x0.00**	**x0.075**	**x0.10**	**x0.20**	**x0.40**
22	97.33^{abc}	2.67^{ij}	9.33^{cd}	0.00^{b}	0.00^{b}
23	97.33^{abc}	50.00^{bcde}	8.67^{cd}	1.33^{b}	0.00^{b}
24	94.00^{abc}	45.33^{bcdef}	24.67^{bc}	0.00^{b}	0.00^{b}
25	96.00^{abc}	3.33^{hij}	2.00^{cd}	0.67^{b}	0.00^{b}
26	97.33^{abc}	26.00^{cdefghij}	8.00^{cd}	0.00^{b}	0.00^{b}
27	99.33^{ab}	31.33^{cdefghij}	8.67^{cd}	0.00^{b}	0.00^{b}
28	98.00^{abc}	38.67^{bcdefgh}	0.00^{d}	0.00^{b}	0.00^{b}
29	98.67^{abc}	67.33^{ab}	0.00^{d}	0.00^{b}	0.00^{b}
30	92.00^{c}	4.67^{hij}	0.00^{d}	0.00^{b}	0.00^{b}
31	96.00^{abc}	46.67^{bcdef}	48.00^{ab}	4.00^{a}	0.00^{b}
32	96.67^{abc}	25.33^{defghij}	62.67^{a}	0.00^{b}	0.00^{b}
33	97.33^{abc}	49.67^{bcde}	0.00^{d}	0.67^{b}	0.00^{b}
34	92.67^{bc}	2.67^{ij}	1.33^{cd}	0.00^{b}	0.00^{b}
35	94.67^{abc}	8.67^{ghij}	3.33^{cd}	0.00^{b}	0.00^{b}
36	94.00^{abc}	24.00^{defghij}	7.33^{cd}	1.33^{b}	0.00^{b}
37	96.67^{abc}	5.33^{hij}	9.33^{cd}	0.67^{b}	0.00^{b}
38	97.33^{abc}	11.33^{fghij}	1.33^{cd}	0.00^{b}	0.00^{b}
39	98.67^{abc}	12.67^{fghij}	1.33^{cd}	0.00^{b}	0.00^{b}
40	96.67^{abc}	34.67^{bcdefghij}	4.00^{cd}	0.00^{b}	0.00^{b}
41	97.33^{abc}	97.33^{a}	12.67^{cd}	2.00^{ab}	1.33^{a}
42	96.00^{abc}	61.33^{bc}	7.33^{cd}	0.67^{b}	0.00^{b}

Appendix 21 (cont.) Mean germination percentages recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates over the four different fungicide dilutions (x0.075, x0.10, x0.20 and x0.40) with propiconazole as active ingredient and an untreated control.

	Germination percentage over five different fungicide dilutions using propiconazole as active ingredient				
Isolate nr.	**x0.00**	**x0.075**	**x0.10**	**x0.20**	**x0.40**
43	98.00^{abc}	30.67^{cdefghij}	3.33^{cd}	0.00^{b}	0.00^{b}
44	94.00^{abc}	0.67^{j}	2.67^{cd}	0.67^{b}	0.00^{b}
45	96.00^{abc}	50.00^{bcde}	2.67^{cd}	0.00^{b}	0.00^{b}
Mean %	96.98^{a}	25.03^{b}	6.04^{c}	0.34^{d}	0.03^{d}

*Main effects: Least significant difference (LSD) between isolates = 4%, dilutions = 1% and isolate x dilution interaction = 9%, $LSD_{x0.00}$ = 6.92% $LSD_{0.075}$ = 35.85%, $LSD_{0.10}$ = 24.58%, $LSD_{0.20}$ = 2.64%, $LSD_{0.40}$ = 1.20%.

Appendix 22 Mean germination percentages recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates over the four different fungicide dilutions (x0.01, x0.015, x0.02 and x0.03) with tebuconazole as active ingredient and an untreated control.

	Germination percentage over five different fungicide dilutions using tebuconazole as active ingredient				
Isolate nr.	**x0.00**	**x0.01**	**x0.015**	**x0.02**	**x0.03**
1	99.33^{ab}	62.67^{bcdef}	51.33^{abcdefg}	4.67^{cde}	0.00^{b}
2	99.33^{ab}	56.00^{cdefg}	13.33^{efghi}	2.67^{cde}	0.00^{b}
3	94.00^{abc}	76.00^{abcd}	22.67^{bcdefghi}	2.00^{cde}	0.00^{b}
4	100.00^{a}	74.67^{abcde}	26.00^{bcdefghi}	6.00^{cde}	0.00^{b}
5	100.00^{a}	76.67^{abc}	28.00^{bcdefghi}	6.00^{cde}	0.00^{b}
6	99.33^{ab}	77.30^{abc}	36.67^{abcdefghi}	6.00^{cde}	0.00^{b}
7	95.33^{ab}	77.30^{abc}	28.67^{bcdefghi}	7.33^{cde}	0.00^{b}
8	100.00^{a}	78.00^{abc}	10.33^{bcdefghi}	0.67^{de}	0.00^{b}
9	96.00^{ab}	87.33^{ab}	41.33^{abcdefghi}	6.00^{cde}	0.00^{b}
10	98.00^{ab}	86.67^{ab}	70.00^{ab}	6.67^{cde}	0.00^{b}
11	93.33^{abc}	20.67^{hi}	1.33^{hi}	1.33^{cde}	0.00^{b}
12	96.00^{ab}	79.33^{abc}	30.67^{bcdefghi}	18.00^{bcd}	0.00^{b}
13	94.00^{abc}	35.33^{ghi}	3.33^{ghi}	1.33^{cde}	0.00^{b}
14	99.33^{ab}	83.33^{ab}	19.33^{cdefghi}	3.33^{cde}	0.00^{b}
15	100.00^{a}	83.33^{ab}	34.67^{abcdefghi}	0.67^{de}	0.00^{b}
16	98.00^{ab}	51.33^{defg}	4.67^{fghi}	1.33^{cde}	0.00^{b}
17	98.00^{ab}	81.33^{ab}	24.00^{bcdefghi}	3.33^{cde}	0.00^{b}
18	98.00^{ab}	92.00^{a}	29.33^{bcdefghi}	4.67^{cde}	0.00^{b}
19	96.00^{ab}	78.67^{abc}	24.00^{bcdefghi}	18.00^{bcd}	0.00^{b}
20	98.00^{ab}	84.67^{ab}	50.67^{abcdefgh}	6.00^{cde}	0.00^{b}
21	98.00^{ab}	80.67^{abc}	12.67^{efghi}	1.33^{cde}	0.00^{b}

Appendix 22 (cont.) Mean germination percentages recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates over the four different fungicide dilutions (x0.01, x0.015, x0.02 and x0.03) with tebuconazole as active ingredient and an untreated control.

	Germination percentage over five different fungicide dilutions using tebuconazole as active ingredient				
Isolate nr.	**x0.00**	**x0.01**	**x0.015**	**x0.02**	**x0.03**
22	94.67^{abc}	15.33^{i}	24.67^{bcdefghi}	3.33^{cde}	2.00^{b}
23	99.33^{ab}	86.67^{ab}	6.767^{fghi}	8.67^{cde}	0.00^{b}
24	96.00^{ab}	87.33^{ab}	35.00^{abcdefghi}	4.67^{cde}	11.33^{a}
25	87.33^{c}	72.00^{abcde}	38.67^{abcdefghi}	2.00^{cde}	0.00^{b}
26	97.33^{ab}	79.33^{abc}	43.67^{abcdefghi}	0.67^{de}	0.00^{b}
27	98.00^{ab}	90.67^{a}	66.67^{abc}	10.67^{cde}	2.00^{b}
28	97.33^{ab}	82.67^{ab}	67.33^{abc}	32.67^{b}	1.33^{b}
29	98.00^{ab}	89.33^{a}	13.33^{efghi}	63.33^{a}	2.00^{b}
30	95.33^{ab}	40.67^{fgh}	6.67^{fghi}	16.00^{bcde}	1.33^{b}
31	94.00^{abc}	82.00^{ab}	40.00^{abcdefghi}	18.67^{bc}	2.67^{b}
32	92.67^{abc}	79.33^{abc}	54.00^{abcdef}	2.67^{cde}	0.00^{b}
33	64.00^{e}	20.00^{hi}	0.67^{i}	0.00^{e}	0.00^{b}
34	96.00^{ab}	82.00^{ab}	26.67^{bcdefghi}	2.67^{cde}	0.00^{b}
35	78.00^{d}	50.00^{efg}	4.00^{ghi}	0.00^{e}	0.00^{b}
36	98.00^{ab}	81.33^{ab}	57.33^{abcde}	14.00^{cde}	0.00^{b}
37	98.00^{ab}	87.33^{ab}	39.33^{abcdefghi}	1.33^{cde}	0.00^{b}
38	96.00^{ab}	90.00^{a}	70.00^{ab}	5.33^{cde}	0.00^{b}
39	94.67^{abc}	80.67^{abc}	72.00^{ab}	4.67^{cde}	1.33^{b}
40	95.33^{ab}	85.33^{ab}	64.67^{abcd}	0.67^{de}	0.00^{b}
41	96.00^{ab}	71.33^{abcde}	38.00^{abcdefghi}	4.00^{cde}	0.00^{b}
42	96.00^{ab}	84.67^{ab}	82.67^{a}	6.00^{cde}	0.00^{b}

Appendix 22 (cont.) Mean germination percentages recorded for urediniospores from 45 *Puccinia graminis* f. sp. *tritici* (*Pgt*) isolates over the four different fungicide dilutions (x0.01, x0.015, x0.02 and x0.03) with tebuconazole as active ingredient and an untreated control.

	Germination percentage over five different fungicide dilutions using tebuconazole as active ingredient				
Isolate nr.	**x0.00**	**x0.01**	**x0.015**	**x0.02**	**x0.03**
43	99.33^{ab}	81.33^{ab}	16.00^{defghi}	5.33^{cde}	0.00^{b}
44	94.67^{abc}	84.00^{ab}	$36.67^{abcdefghi}$	2.67^{cde}	0.00^{b}
45	92.00^{bc}	85.33^{ab}	68.67^{abc}	6.67^{cde}	1.33^{b}
Mean %	95.48^{a}	73.60^{b}	34.59^{c}	7.20^{d}	0.56^{e}

*Main effects: Least significant difference (LSD) between isolates = 5%, dilutions = 2% and isolate x dilution interaction = 12%, $LSD_{0.00}$ = 7.83%, $LSD_{0.01}$ = 24.79%, $LSD_{0.015}$ = 49.71%, $LSD_{0.02}$ = 17.90%, $LSD_{0.03}$ = 3.08%.