# Histopathology of rust infection in wheat and barley

by

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## Declaration

I, Gerrie Johanna Maree, declare that the Master's Degree research dissertation that I herewith submit for the qualification, MSc Agric, at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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Gerrie Johanna Maree Date

## Contents

## List of Tables



## List of Figures

## List of Abbreviations

| | |
|---|---|
| ABC | adenosine triphosphate (ATP)-binding cassette |
| AFLP | amplified fragment length polymorphism |
| ANOVA | analysis of variance |
| APR | adult plant resistance |
| ASR | all-stage resistance |
| Avr | avirulence |
| BLAST | basic local alignment search tool |
| bp | base pairs |
| CAP | cleaved amplified polymorphism |
| cDNA | complementary DNA |
| CI | coefficient of infection |
| CNRQ | calibrated normalized relative quantity |

| | |
|---|---|
| $CO_2$ | carbon dioxide |
| Cq | quantification cycle |
| DArT | diversity array technology |
| DH | doubled haploid |
| cm | centimetre(s) |
| DMDC | dimethyldicarbonate |
| DNA | deoxyribonucleic acid |
| dpi | days post-inoculation |
| e.g. | *exempli gratia* (for example) |
| EDTA | ethylene-diamine-tetraacetic acid |
| *et al.* | *et alii* (and others) |
| ETI | effector-triggered immunity |
| f. sp. | *forma specialis* |
| *g* | relative centrifugal force |
| g | gram(s) |
| GLM | general linear model |
| *H.* | *Hordeum* |
| h | hour(s) |
| ha | hectare(s) |
| HCL | hydrochloric acid |
| HCN | host cell necrosis |
| H-index | hypersensitivity index |
| HMC | haustorial mother cell(s) |
| hpi | hours post-inoculation |
| HR | hypersensitive response |
| HTAP | high-temperature adult-plant |
| *HvAdf* | *Hordeum vulgare* actin depolymerizing factor |

| | |
|---|---|
| *HvRga* | *Hordeum vulgare* resistance gene analog |
| i.e. | *id est* (that is) |
| IT | infection type |
| KOH | potassium hydroxide |
| kPa | kilopascal |
| kV | kilovolt(s) |
| L | litre(s) |
| LAI | leaf area infected |
| *Lr* | leaf rust resistance gene |
| LSD | least significant difference |
| M | Molar |
| m | metre(s) |
| MAS | marker-assisted selection |
| mg | milligram(s) |
| min | minute(s) |
| mL | millilitre(s) |
| mM | millimolar |
| mol | mole |
| MP | mapping population |
| MR | moderately resistance |
| MS | moderately susceptibility |
| NDVI | normalized difference vegetation index |
| nm | nanometre(s) |
| ng | nanogram(s) |
| NPK | nitrogen, phosphorus and potassium |
| PAMP/MAMP | pathogen/microbe-associated molecular pattern |
| *P.* | *Puccinia* |

| | |
|---|---|
| *Pg* | *Puccinia graminis* |
| *Pgs* | *Puccinia graminis* f. sp. *secalis* |
| *Pgt* | *Puccinia graminis* f. sp. *tritici* |
| pH | potential hydrogen |
| *Ps* | *Puccinia striiformis* |
| *Pst* | *Puccinia striiformis* f. sp. *tritici* |
| PTI | PAMP-triggered immunity |
| QTL | quantitative trait loci |
| $R^2$ | coefficient of determination |
| R | resistance |
| $r_s$ | Spearman rank-order correlation coefficient |
| RAPD | random amplified polymorphic DNA |
| RFLP | restriction fragment length polymorphism |
| RIL | recombinant inbred line |
| RNA | ribonucleic acid |
| *Rpg*/*rpg* | reaction to *Puccinia graminis* (dominant/recessive) |
| RT | reaction type |
| RT-qPCR | quantitative reverse transcription polymerase chain reaction |
| S | susceptibility |
| SCAR | sequence characterized amplified region |
| SNP | single nucleotide polymorphism |
| spp. | species |
| *Sr* | stem rust resistance gene |
| SSR | simple sequence repeat |
| SSV | substomatal vesicle |
| STS | sequence tagged sites |
| subsp. | subspecies |

| | |
|---|---|
| *T.* | *Triticum* |
| T | trace |
| TAE | Tris-acetate-EDTA |
| Tris | trisaminomethane |
| USA | United States of America |
| UVPgs | Universiteit Vrystaat *Puccinia graminis* f. sp. *secalis* |
| UVPgt | Universiteit Vrystaat *Puccinia graminis* f. sp. *tritici* |
| v | volume |
| w | weight |
| WGA | wheat germ agglutinin |
| *Yr* | stripe rust resistance gene |
| % | percentage |
| °C | degrees Celsius |
| µg | microgram(s) |
| µL | microliter(s) |
| µm | micrometre(s) |
| *18S* | *18S ribosomal RNA* |
| *β-TUB* | *β-tubulin* |

ACRONYMS IN FIGURES

| | |
|---|---|
| A | appendix |
| AP | appressorium |
| GT | germ tube |
| HMC | haustorial mother cell |
| PIH | primary infection hypha |
| S | septum |
| SIH | secondary infection hypha |

| | |
|---|---|
| SSV | substomatal vesicle |
| US | urediniospore |

## List of Addendums

## General Introduction

Wheat and barley are high-priority cereal crops in terms of food security. Besides the increasing demand, fluctuating weather conditions and reduced area available for production, the cereal industry is under persistent pressure from destructive biotic factors. Pathogens in the *Puccinia* genus causing rust diseases on cereals are counted among the most serious threats in terms of crop losses. An increasing need for durable, race-nonspecific defences against these organisms has been realized due to their adaptable nature.

Part of this study was the continuance of resistance assessment in two wheat varieties previously identified to carry complete adult plant resistance (APR) to stripe rust. Here, the underlying premise was that histological assessments should provide a better understanding of how different sources of resistance restrict stripe rust development. A doubled haploid population derived from Kariega, a South African wheat cultivar carrying the APR gene *Lr34/Yr18/Sr57* and contributing quantitative trait loci (QTL) *QYr.sgi-2B* and *QYr.sgi-4A* (Ramburan *et al.*, 2004), was constructed (Ramburan *et al.*, 2004; Prins *et al.*, 2005) and initial analysis of resistance mechanisms of the single QTL/gene performed (Moldenhauer *et al.*, 2006, 2008). Subsequent investigation in this study (Chapter 2) involved the histological analysis of various QTL/gene combinations under field conditions in 2011 and 2012. This study completed and expanded preliminary assessments made by Dr Negussie Tadesse, a post-doctoral fellow at the University of the Free State, during 2011.

Through QTL mapping, Agenbag *et al.* (2012) studied the resistance in a segregating recombinant inbred line population retaining different combinations of stripe rust APR loci from the French variety Cappelle-Desprez. To improve the accuracy of predicting which locus/loci is expressed in specific lines, more stringent criteria for the presence of QTL were applied (R. Prins, personal communication) and selected lines were analysed here (Chapter 3). Carriers of different QTL combinations, exposed to field stripe rust epidemic conditions, were assessed through phenotypic, histological and molecular methods.

Chapter 4 aimed to elucidate whether the inherent stem rust resistance of barley might be related to the early infection and/or colonization processes. Stem rust infected

barley lines/cultivars were examined and compared to control wheat lines at histological level and transcriptional stage at different time points post inoculation.

# Chapter 1: Literature review

## CEREALS

Cereal grains are an essential food source, representing 60% of the proteins and calories consumed by humans worldwide. Additionally, cereals are staple to people in poverty stricken countries, providing 75% and 67% of their total caloric and protein intake, respectively (FAOSTAT, 2009).

Global cereal utilization for 2017/18 is estimated to rise 0.5% from the previous year, to a record level of 2 584 million tonnes. The proportions for human consumption, animal feed and other uses are set at 43%, 35% and 22% of total usage, respectively. Cereal production worldwide is only projected slightly more than total utilization at 2 593.7 million tonnes (FAO, 2017)

Wheat is the cereal grain cultivated on the largest area worldwide and the second most produced grain after maize for the 2016 crop year, calculated at 760.1 million tonnes globally. Leading wheat producers include the European Union, China and India. Barley is ranked fourth after rice in amount of grain produced worldwide with 148.6 million tonnes, 41% of which by the European Union, followed by Russia and Australia (Statista 2016; FAO, 2017). The global forecast for both wheat and barley production is set to decrease during 2017 with 2.2% and 4.2%, respectively (FAO, 2017).

The wheat cultivated today, hexaploid bread wheat (*Triticum aestivum* L.) and tetraploid hard or durum-type wheat (*T. turgidum* L.) is derived from einkorn (*T. monococcum* L.) and emmer wheat (*T. dicoccoides*) (Zohary, 1969) that grew wild in the cradle of cereal agriculture (Dvořák *et al.,* 1998) geographically situated at modern time south-eastern Turkey and northern Syria (Lev-Yadun *et al.,* 2000). It is proposed that common barley (*Hordeum vulgare* L.) was brought into culture in the Israel-Jordan region from a wild relative, *H. spontaneum* (Zohary, 1969). Evidence suggests that some diversification of barley might have occurred in the Himalayas (Badr *et al.,* 2000).

Over the past 20 years, the area cultivated to wheat in South Africa has decreased from 1 946 000 to 508 000 ha. Although highly fluctuating from year to year, production saw a decline from 2 333 000 to 1 918 000 tonnes over the same period, while total consumption has risen from 2 236 000 to 3 255 000 tonnes. Wheat delivered in the

2016/2017 cropping season was mostly produced in the Western Cape (57%), Free State (16%) and Northern Cape (14%). During the same season, 354 000 tonnes of barley was produced on 89 000 ha, 89% of which in the Western Cape (DAFF, 2017).

## RUST DISEASES

Largely comprised in the genus *Puccinia* (Cummins and Hiratsuka, 2003), rust fungi cause among the most destructive diseases in global cereal production including wheat, barley, oat, rye and maize (Kleinhofs *et al.,* 2009). Wheat is host to three rust diseases, capable of causing yield losses up to 60% for leaf and stripe rust, and 100% for stem rust (Park, 2007; Dubin and Brennan, 2009).

Rust epidemics can be dated back to biblical times (Kislev, 1982), and have been documented in all major cereal producing countries ever since (Dubin and Brennan, 2009). Recent outbreaks have occurred in Ethiopia (Olivera *et al.,* 2015), Sicily, Afghanistan, Uzbekistan (http://www.rusttracker.org, 30/09/2017) and Siberia (Shamanin *et al.*, 2016). Besides the resulting food shortages, epidemics also have a substantial impact on seed availability for the next planting season (Shean, 2010), market value and hence food prices, as well as the welfare of farmers and employees.

While leaf rust appears to be more significant endemically, both stem and stripe rust cause more devastation during epidemics (Dubin and Brennan, 2009). Stripe rust outbreaks were traditionally associated with cool, moist weather, while warm humid seasons customarily favour stem and leaf rust. However, in recent times, aggressive stripe rust strains, tolerant to higher temperatures, have emerged and advanced into warmer, non-traditional regions (Hovmøller *et al.,* 2008; Milus *et al.,* 2009).

In addition to the mutable nature of rusts regarding virulence (Leonard, 2001) and alarming rates of reproduction, they are able to disperse over long distances on wind and by anthropogenic activities (Wellings, 2007), putting global cereal production under constant threat.

STRIPE RUST

The first description of stripe (yellow) rust was given by Gadd in 1777, but only designated in 1896 by Eriksson and Henning (1896) as *P. glumarum*. Hylander *et al.* (1953) renamed the stripe rust fungus as it is known today, i.e. *P. striiformis* Westend. (*Ps*).

Several grasses within the Poaceae family have been known to host the asexual, urediniospore stage of *Ps*, after which the association was made between host specialization and *Ps* isolates from certain grass species. Subsequently, five special forms (Latin: '*formae speciales*') were named based on the genus of the host source. *Ps* f. sp. *tritici* (*Pst*) specializes on wheat, *Ps* f. sp. *hordei* on barley (Eriksson, 1894; Farr *et al.,* 1995), *Ps* f. sp. *secalis* on rye, *Ps* f. sp. *elymi* on *Elymus* spp. and *Ps* f. sp. *agropyron* on *Agropyron* spp. (Eriksson, 1894; Hovmøller *et al.*, 2011). Host specialization has been reported to occur on orchard grass (*Ps* f. sp. *dactylidis*) (Manners, 1960; Tollenaar, 1967), Kentucky blue grass (*P. pseudostriiformis* formerly *Ps* f. sp. *poae*) (Britton and Cummins, 1956; Tollenaar, 1967; Abbasi *et al.*, 2004), *Leymus secalinus* (*Ps* f. sp. *leymi*) (Niu *et al.,* 1991) and *Hordeum* spp. in Australia (*Ps* f. sp. *pseudo-hordei*) (Wellings, 2007). Even more, specialization occurs within a single host genus at the cultivar level (Anikster, 1985) often based on a gene-for-gene relationship (Flor, 1971). These races/pathotypes can be identified through a set of host differentials carrying individual resistance genes (Line *et al.*, 1970; Wan and Chen, 2014; Wan *et al.*, 2016).

Until recently, the geographical region across Armenia, Azerbaijan and Georgia, was believed to be the centre of origin of *Pst*, occurring on wild grass species as the primary host (Hassebrauk, 1965; Leppik, 1970). Analysis of the global population genetic structure however, revealed that *Pst* most likely originated in the Himalayan and near-Himalayan region (Ali *et al.*, 2014; Thach *et al.*, 2016). This locality revealed higher genotypic diversity, sexual recombination potential as well as the independent maintenance of a differentiated *Pst* population structure compared to the mainly clonal populations in other regions (Ali *et al.*, 2014).

*Ps* was accepted as an autoecious microcyclic rust pathogen of cereals for more than a century, with unsuccessful attempts to identify possible sexual hosts dating back to 1894 (Stubbs, 1985). Though recently, Jin *et al.* (2010) identified *Berberis* spp.

(*B. chinensis*, *B. holstii*, *B. koreana* and *B. vulgaris*) as alternate hosts. In addition, Wang and Chen (2013) reported that Oregon grape (*Mahonia aquifolium*) also hosts the *Pst* sexual phase. *Pst* is now officially classified as a heteroecious rust pathogen with a macrocyclic life cycle comprised of five spore stages, similar to that of *P. graminis* (Jin *et al.,* 2010).

Evidence suggests that virulence recombination of *Pst* (Mboup *et al.*, 2009; Duan *et al.,* 2010) is likely to take place through the sexual phase in the alternate host (Jin *et al.,* 2010). However, the lack of dormancy in teliospores and swift production of basidiospores implicates the regular event of disease escape in the face of depleted basidiospore inoculum. This led Rapilly (1979) to propose that the alternate host would provide the pathogen with insignificant means to survive between cropping seasons. To date, natural infection of barberry has only been reported at very low frequencies in China (Wang *et al.*, 2015; Zhao *et al.*, 2016). The lack of involvement of *Berberis* spp. in natural stripe rust epidemics occurring in the United States of America (USA) (Chen *et al.*, 2012) was ascribed to the rapid degradation of teliospores and small window of vulnerability of barberry (Wang and Chen, 2015).

*Pst* has the renowned ability to migrate across long distances, seeing as low genetic diversity persists in the mostly clonal population worldwide (Chen *et al.,* 1993; Hovmøller *et al.,* 2002; Enjalbert *et al.,* 2005; Wellings, 2007), apart from high diversity detected in populations in the Middle East (Bahri *et al.,* 2009), Pakistan (Bahri *et al.,* 2011) and a recombinant population structure in China (Mboup *et al.,* 2009; Ali *et al.*, 2014).

The aggressive nature of local *Pst* populations as well as discerning advantages of migrants, such as virulence towards widely deployed resistance genes or an increased tolerance to commonly occurring stresses like high temperatures, will be key determining factors in the success of a newly introduced pathotype (Ali *et al.*, 2014).

Brown and Hovmøller (2002) found that *Pst* populations are repeatedly re-establishing in the main wheat-growing regions in north-eastern China. Recurrent introduction of migrants appears to coexist and are in fact dominated by older Asian populations specific to regions (Ali *et al.*, 2014). These recombinant *Pst* populations in Himalayan (Nepal and Pakistan) and near-Himalayan (China) regions may possibly provide new

sources of virulent strains with the ability to replace distant clonal populations (Hovmøller *et al.*, 2016).

The emergence of two high temperature-adapted aggressive strains, possibly from East Africa (Walter *et al.*, 2016), recently extended the geographic range of *Pst* to areas not previously classified as at risk and very distinct from local populations (Hovmøller *et al.*, 2011). The *PstS1* aggressive strain, originally from the East African-Middle Eastern region, completely replaced pre-existing populations in the USA in the twenty-first century (Milus *et al.*, 2009). This was the first documentation of an emerging *Pst* population adapted to warmer temperatures, although the concept was already proposed in the 1970's (Macer, 1972; Zadoks, 1979). *PstS1* was detected in the south-eastern USA in 2000 and in Western Australia in 2002 (Chen, 2005; Milus *et al.*, 2009). *PstS2*, derived from *PstS1*, became prevalent in the Middle East and Central Asia (Hovmøller *et al.*, 2008). It was detected in Europe between 2000 and 2004, but usually at low frequencies (Hovmøller *et al.*, 2008; De Vallavieille-Pope *et al.*, 2012) and confirmed to be avirulent on several European wheat cultivars (Hovmøller, 2007).

Recently three exotic *Pst* race invasions into Europe have been reported which very rapidly became widespread. The triticale attacking race was first detected in 2006 on the Bornholm island and in the following years in Germany, Scandinavia (Hovmøller *et al.*, 2011) and France. Originated through sexual recombination in the near-Himalayan region (Hovmøller *et al.*, 2016), isolates of the "Warrior" and "Kranich" strains emerged in 2011, causing population sweeps that replaced the original NW European populations (Hubbard *et al.*, 2015; Hovmøller *et al.*, 2016).

The universal dispersal of *Pst* has occurred fairly recently, with most cases of emergence accounted for within the last decades. Dispersal can occur through successive jumps between neighbouring fields throughout the season, as reported in the USA (Kolmer, 2005), while winds allow direct spread of the pathogen across long distances, which transpired between England and Denmark (Justesen *et al.,* 2002). Pathogen dispersal can also occur through humans travelling between continents and unintentionally transferring spores as reported by Wellings *et al.* (1987) and Wellings (2007) for the introduction of *Pst* into Australia in 1979 through contaminated clothing and/or goods from Europe.

Within two years of the first detection of *Pst* in South Africa in 1996 on cultivar Palmiet (Pretorius *et al.*, 1997), it became endemic to all leading wheat producing regions in the country (Boshoff *et al.*, 2002a). Thus far, four races have been reported in South Africa: 6E16A- (1996) (Pretorius *et al.*, 1997; Boshoff *et al.*, 2002b), 6E22A- (1998), 7E22A- (2002) (Pretorius *et al.*, 2007) and 6E22A+ (2005) (Visser *et al*., 2016). Each pathotype revealed an increase in virulence to stripe rust resistance genes compared to the preceding race. South African *Pst* isolates were confirmed to cluster with isolates from Europe, Central and Western Asia (Hovmøller *et al.*, 2008).

STEM RUST

The initial in-depth reports of wheat stem rust by Fontana and Tozetti in 1767 were published by the American Phytopathological Society in 1932 and 1952, respectively. The causal pathogen was named *Puccinia graminis* (*Pg*) in 1797 by Persoon, according to Roelfs *et al.* (1992). In 1815, de Candolle recognized that leaf rust was caused by an altogether different fungus, initially believed to be another form of the stem rust pathogen (Chester, 1946), and defined it as *Uredo rubigo-vera* (De Candolle, 1815). Subject to multiple name changes, the leaf rust pathogen was designated in 1956 as *P. recondita* (Cummins and Caldwell), until recently when it changed back to *P. triticina* (Savile, 1984); originally described by Eriksson (1899).

More than 400 graminaceous species have been reported to act as hosts for *Pg* (Cummins, 1971; Gäumann, 1959; Gerechter-Amitai, 1973), ensuing in the division of the stem rust fungus into several groups. A wide range of grass species and cultivated cereals can be attacked by *Pg* subsp. *graminis* (Gerechter-Amitai, 1973), further allocated into different forms on account of host specialization. *Pg* f. sp. *tritici* Erikss. and Henning (*Pgt*), and *Pg* f. sp. *secalis* Erikss. and Henning (*Pgs*) attack primarily wheat and rye, respectively, in addition to barley and several grass species. A somatic hybrid between these two specialized forms (*Pgt* and *Pgs*) has been reported in Australia, known as ‘Scabrum’ rust (Park, 2007) and able to attack barley (Park *et al.*, 2015). Another form, *Pg* f. sp. *avenae* Erikss. and Henning, primarily attacks oat but is found on other grass species as well (Farr *et al.,* 1995).

According to Leppik (1970), both the stem rust fungus and its aecial *Berberis* host originated in central Africa. To date, a vast range of *Pgt* pathotypes have been

identified from across the world, varying in virulence and subsequent damage caused. The detection of the 'Ug99' African race exhibiting increased virulence to widely used resistance genes (Pretorius *et al.*, 2000), now designated as TTKSK (Jin *et al.*, 2008), poses a serious global threat to cereal production. To date, the 'Ug99' race group comprises of 13 variants which have been detected in several African countries as well as in Yemen and Iran (http://www.rusttracker.org, 30/09/2017).

Genetic variability in rust populations leading to the detection of new pathotypes can be the result of mutations or sexual recombination (Knott, 1989). Migration, another pathway of rust introduction, is believed to be the result of a *Pgt* race discovered in South Africa in 2000. This isolate proved to be a close relative of the original TTKSK isolate (Visser *et al.*, 2009, 2011).

## *Puccinia* LIFE CYCLE

Nearing the end of a rust epidemic phase and/or cropping season, telia structures break through the cereal host epidermis to produce thick-walled, two-celled teliospores. In the case of *Pg*, teliospores present the pathogen with a resting phase during unfavourable environmental conditions when primary and ancillary hosts are no longer available. Conversely, teliospores of *Ps* do not act as resting spores, germinating rapidly under free water conditions. Upon germination, rust teliospores undergo karyogamy to form a diploid nucleus (2n), followed by meiosis resulting in a promycelium of four cells, differentiating into single haploid basidiospores. Ejected from the sterigmata, basidiospores require a minimum dew period of 40 h to infect the alternate host (Chen *et al.,* 2014), resulting in the development of pycnia on the upper side of the leaf. Pycniospores and receptive pycnial hyphae of different mating types fuse to form aecia on the lower side of the leaf (Craigie, 1927, 1931). Dikaryotic aeciospores are produced followed by their dispersal, and completing the sexual life cycle upon successfully infecting the cereal host.

A dense mat of hyphae becomes established beneath the host epidermis called uredinia, bearing masses of dikaryotic urediniospores on sporophores that ruptures the host epidermis. This results in the respective pustules associated with each disease. Stem rust is recognized by brick-red, diamond-shaped uredinial pustules mainly on stems and leaf sheaths. The symptoms of stripe rust involve characteristic

yellow to orange rust pustules growing systemically in long stripes, mainly between veins on leaf blades (Jin *et al.,* 2010). This asexual phase of repeated production and infection cycles of urediniospores on the primary host give rise to wide-scale epidemics occurring on cereal crops.

In addition to the sexual and asexual phases, and depending on the region, rust populations might also rely on volunteer, ancillary grasses to host the urediniospores for survival during stressed times in the summer (Arthaud *et al.*, 1966; Azbukina, 1980; Nazari *et al.*,1996).

## *Puccinia* INFECTION PROCESS

Classified as obligate biotrophs, rust pathogens require a living host to grow and reproduce. The majority of research focuses on the economically important asexual urediniospores, which include a number of resulting infection structures (Leonard and Szabo, 2005; Chen *et al.,* 2014), all essential for successful establishment of the rust pathogen in the cereal host (Staples and Macko, 1984; Wiethölter *et al.*, 2003).

Germination of the urediniospore is usually initiated within 3 h of contact with free water, including an optimal temperature range and several hours of darkness. Optimal conditions may vary considerable between rust taxa, e.g. germination of *Ps* urediniospores specifically is greatly influenced by the presence of air pollutants (Sharp, 1967). The germ tube grows mainly at right angles to the long axis of the epidermal cells on the host leaf/stem surface (Kang *et al.,* 1997, 2002), until a stoma is reached (Moldenhauer *et al.,* 2006, 2008).

*Pst* infection of the cereal host occurs through the germ tube's direct penetration of a stoma (De Vallavieille-Pope *et al.*, 1995). A globular substomatal vesicle (SSV) within the stomatal cavity then develops, followed by two or three thick primary infection hyphae (PIH) (Swertz, 1994; Moldenhauer *et al.,* 2006) from one pole. The PIH of *Pst* mostly grow horizontal and tends to thicken at the tip where a haustorial mother cell (HMC) develops (Niks, 1986).

When a stoma is reached in the case of many other rusts, including *Pgt*, the germ tube elongation halts and generally forms an appressorium over the opening (Emmett and Parberry, 1975), later delimited by a septum. The lower surface of the appressorium

produces a narrow penetration peg, growing through the stoma and give rise to a fusiform SSV in the substomatal cavity. According to literature, the subsequent infection structures include a longitudinal, horizontally orientated PIH produced at one end of the SSV while a short appendix forms at the other end; both able to differentiate into the next infection structure (Swertz, 1994). The SSV of the *Pg* fungus, however, has been revealed as capable of producing a HMC directly without the differentiation of a PIH or threadlike appendix (Niks, 1986).

As a general rule, rust pathogens form a HMC upon contact with a host mesophyll cell, which is delimited by a septum from the preceding hypha. Most of the cytoplasm moves into the HMC (Kang *et al.,* 2002), leaving the earlier structures vacuolated. A thick, multi-layered wall enables the firm attachment of the HMC to the host cell wall before a slender neck penetrates, invaginating the plasma membrane (Mares, 1979; Heath and Skalamera, 1997; Ma and Shang, 2009). The primary parasitic interface between pathogen and host is provided by the resulting haustorium (Kang *et al.,* 1997, 2003; Hovmøller *et al.,* 2011). Haustoria are highly specialized feeding structures drawing nutrients and water from host cells (Mendgen, 1981; Voegele *et al.,* 2009), in addition to playing roles in vitamin synthesis (Sohn *et al.,* 2000) and signalling between host and pathogen through effector molecules (Kamoun, 2007; Voegele *et al.,* 2009).

Although haustoria mostly occur in the host mesophyll cells, it has been found that 15% are located in epidermal cells (Sørensen C, unpublished information). Hovmøller *et al.* (2011) reported that spherical young haustoria become more branched when older, resulting in an increased interface region between fungus and host and possibly more efficient nutrient uptake.

During haustorium formation, the primary infection hyphae may branch out near the first HMC to form a hyphal network producing more HMC and haustoria. Subsequently, the fungal mycelium develops inter- and intracellularly, forming a pustule bed within the host tissue, which later differentiates into an uredinium (Chen *et al.,* 2014).

*Pgt*, unlike *Pst*, does not grow systemically in the cereal host. Thus, by the time a third *Pgt* haustorium is produced, the reserves from the urediniospore have been depleted. Further colonization will largely depend on the success of initial haustoria to extract nutrients from host cells without triggering a resistant response (Leonard and Szabo, 2005).

## HOST-PATHOGEN INTERACTIONS

According to the 'zigzag' model of Jones and Dangl (2006), active plant defence involves two phases: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). With PTI, the first line of defence, pattern recognition receptors recognize alien pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) at the plant cell surface, activating basal/general defence genes. One of the most well-studied PAMPs is a basic element in fungal cell walls, i.e. chitin. PAMPs are promptly detected in non-hosts, where a variety of constitutive and induced defence mechanisms, including lignification and hypersensitive cell death, are employed. These processes are not necessarily similar to that associated with ETI-induced host resistance (Christopher-Kozjan and Heath, 2003). Loosely defined non-host resistance against cereal rust applies to most of the plant forms which, in fact, do not accommodate the causal pathogens.

Some adapted pathogens are able to suppress PTI and counteract the basal host defences by releasing signal molecules called effectors. Effector proteins can either promote virulence (virulence factors) or induce a defence response (avirulence factors, Avr). Virulent effectors in fungi are released from haustoria, altering the function, metabolism and structure of plant cells in order to promote pathogenesis (Hogenhout *et al.,* 2009). During the second defence phase, ETI is triggered upon direct or indirect recognition of the effector Avr proteins by plant resistance (*R*) genes. Localized programmed cell death, labelled as the hypersensitive response (HR), is commonly associated with amplified PTI responses triggered by ETI (Tao *et al.,* 2003).

Further invasion of host tissue by biotrophs is inhibited by the HR, as the required living host cells no longer supply nutrients and water (Glazebrook *et al.,* 1997). Furthermore, the HR elicits systemic acquired resistance entailing long-term enhanced resistance against a variety of pathogens in distant tissue (Durrant and Dong, 2004), activation of pathogenesis-related gene expression as well as reprogramming defence-related genes (Jones and Dangl, 2006).

Flor (1942, 1971) proposed a gene-for-gene model, founded on the recognition between reciprocal pairs of dominant genes from both the host (*R* genes) and pathogen (*Avr* gene) in order for resistance to occur, i.e. an incompatible interaction. Compatibility will take effect in the case of mutation or loss of host *R* gene and/or

pathogenic *Avr* gene, resulting in disease (Hammond-Kosack and Jones, 1997). The success in active plant defence lies further in the timely recognition of pathogenic effectors and induction of applicable defence mechanisms (Demirci *et al.,* 2016).

This gene-for-gene model applies to many of the pathogen race-specific resistance which can quickly lead to the removal of compromising effectors or the release of additional effectors to suppress ETI. In contrast, the basal defense of PTI is not governed by the recognition of a specific *Avr* gene, characterized as pathogen race-nonspecific resistance (Wolter *et al.*, 1993; Piffanelli *et al.*, 2002). By avoiding and suppressing PTI and ETI, pathogens acquire virulence (Jones and Dangl, 2006).

## RESISTANCE

Although fungicide applications can lower the damaging effects of stem rust, the extra input costs and negative environmental effects of chemical treatments necessitate the use of host resistance to effectively control rust diseases of agricultural crops. Incorporating *R* genes into cultivars has proven to be the most economical means for combatting rusts, and thus has been the primary management strategy (Steffenson, 1992; Kolmer, 2001; Kleinhofs *et al.,* 2009).

The first program, with regards to breeding for rust resistance, was launched in the USA in 1905. The goal was to develop stem rust resistant spring wheat, following a severe epidemic in 1904 (Stakman, 1955). During the same time at Cambridge in the United Kingdom, the Mendelian inheritance of stripe rust resistance was discovered by Biffen (1905).

The typical practice for rust resistance involved the release of cultivars containing a single gene. Whenever a new gene was deployed, high levels of resistance are imparted onto the cultivar, increasing its popularity which in turn puts strong selection pressure on the pathogen population. The frequency of virulence to the single resistance gene increases, which soon becomes ineffective. This led to susceptibility in commercial cultivars making them less desirable for farmers, i.e. a ‘boom and bust cycle’ (Singh *et al.,* 2004).

In most cases, resistance genes overcome by the adapting rust pathogen cannot be employed for future use. This undesirable occurrence can be avoided by responsibly

managing resistance genes to conserve their effective nature, a practice termed gene stewardship. Combining resistance sources in a single host genotype, known as stacking or pyramiding, is the favoured strategy to effectively steward genes. Resistance genes in combinations are known to provide protection for each other. First mentioned by Watson and Singh (1952), the approach of combining several genes has been included in many breeding programmes (Pretorius *et al.,* 2017a).

Durability was later defined as resistance deployed in varieties covering large areas, remaining effective for a prolonged period under high pathogen pressure (Johnson and Law, 1975; Johnson, 1984).

Rust resistance can be broadly categorized into all-stage resistance (ASR) or adult plant resistance (APR) (Ellis *et al.,* 2014). ASR, previously known as seedling resistance, is conferred by major genes expressed throughout plant growth, present individually or in simple combinations. The majority of catalogued rust resistance genes are grouped under ASR, and vulnerable to 'boom-and-bust' events in the face of increased virulence in a mutated pathogen, seeing as these genes provide resistance against specific races. Ribeiro Do Vale *et al.* (2001) stated that race-specificity is the consequence and not cause of monogenic resistance.

APR genes typically confer partial resistance (Caldwell, 1968) during later stages of plant development, which can be either specific or non-specific to a pathotype. Race-nonspecific APR provide broad-spectrum resistance to one or more species of a pathogen. This is characteristic of a slow rusting response, displaying a susceptible infection type (IT) at first, but effectively reducing further infections and severity throughout disease occurrence (Nelson, 1978; Parlevliet, 1979). In the field, disease development is slowed down rather than prevented, which can greatly benefit the control of cereal rust diseases (Shaner *et al.,* 1978).

Some race-nonspecific APR genes provide inadequate disease protection on their own, much the same as a defeated race-specific APR gene. Theoretically, combinations comprised of APR genes, or APR in conjunction with ASR genes will exhibit increased levels of resistance, though this is not always the case in practice. It is important not to assume all APR genes are durable and additive in their effect. Complementary genes providing additive resistance effects, and ideally combinations

including both APR and ASR, have been instrumental in effective crop protection and conservation of durable resistance (Ellis *et al.,* 2014).

Quantitative trait loci (QTL) conferring resistance is an important, exploitable source of genetic plant defence. Although smaller in effect individually, combinations of QTL can additively contribute in imparting increased levels of resistance. Numerous QTL have been identified to provide APR to cereal rusts (Rosewarne *et al.,* 2013; Yu *et al.,* 2014).

Genetic diversity is of equal importance as sustainable resistance, and achieved by the continual exploitation of additional sources supporting durability. All designated resistance genes to date have been obtained from wheat and related genera or species (Pretorius *et al.*, 2017a).

## STRIPE RUST RESISTANCE GENES

To date, a total of 78 stripe rust resistance genes have been catalogued, a large portion of which conferring ASR (McIntosh *et al.*, 2013, 2017). At present several temporarily designated resistance genes are being reviewed and further studied.

Some of the durable genes are expressed when plants mature and daily temperatures average above 21°C (Chen, 2007). This high-temperature adult-plant (HTAP) resistance contributes to ‘inoculum decline’ by reducing initial severity and inhibiting subsequent infections (Chen *et al.,* 2014). Unlike slow-rusting resistance, generally exhibiting a compatible host-pathogen interaction associated with lower rust severity, HTAP resistance usually displays lower ITs symptomatic of an incompatible interaction. Due to the sensitive nature of HTAP resistance to environmental conditions, adequate resistance levels may be imparted in one region while proving insufficient in another region. Several HTAP sources conferring race-nonspecific resistance and deployed in specific areas, have remained effective for more than 60 years (Chen, 2013). Highly durable resistance can be attained by combining HTAP resistance with valuable ASR genes (Lin and Chen, 2009), providing near-complete protection from disease damage in genotypes carrying both types of resistance. Since it is masked by ASR, it is advisable to firstly select for HTAP resistance and thereafter incorporating it into elite cultivars (Chen, 2013)

Additionally, *Pst* resistance QTL have been mapped to all wheat chromosomes, apart from 1D (Rosewarne *et al.*, 2013; Chen *et al.,* 2014; McIntosh *et al.,* 2017) with the prospect of multiple others to be detected and described in the future (Rosewarne *et al.*, 2013). These QTL contribute diverse levels of resistance to the overall phenotype.

## STEM RUST RESISTANCE GENES

In the past, stem rust resistance in wheat has only remained effective for five to six years (Kilpatrick, 1975; Dubin and Brennan, 2009) due to implementing single major resistance genes, easily overcome by the evolving pathogen (Kleinhofs *et al.,* 2009). Since the mid-1950's, wheat losses due to stem rust have been kept to a minimum by incorporating several resistance genes into cultivars (Kleinhofs *et al.,* 2009) in major wheat producing areas globally (Singh *et al.,* 2004; Leonard and Szabo, 2005; Park, 2007). In contrast, the release of barley cultivars containing one major resistance gene, *Rpg1*, has been instrumental in the regulation of barley stem rust since the 1940's (Steffenson, 1992; Kleinhofs *et al.,* 2009; Dubin and Brennan, 2009). A mostly resistant wheat crop and the shorter maturation period of barley possibly contributed to the abiding disease control. The eradication program of *Berberis spp.*, initiated in 1917 in several cereal-producing USA states to break the rust cycle, was instrumental in reducing the frequency of epidemics by way of decreasing initial inoculum levels as well as the number of pathogenic rust races (Roelfs, 1982).

Roelfs (1978) reported that apart from times when wheat was severely infected, epidemics of barley stem rust rarely occurred in the past. The durable *Rpg1* has shielded barley against stem rust losses since 1942 when cultivar Kindred was introduced (Steffenson, 1992). A new *Pgt* race (QCCJB) appeared in 1988 (Martens *et al.,* 1989; Steffenson *et al.,* 2017) and caused minor stem rust epidemics in the USA and Canada until 1991 (Steffenson, 1992; Roelfs *et al.,* 1993). At this time, the majority of commercial field samples belonged to the QCCJB pathotype, not only attacking *Rpg1*-containing barley cultivars but several wheat cultivars as well (Roelfs *et al.,* 1993). However, by 1997 there was an absence of pathotype QCCJB in rust surveys due to the removal of these susceptible wheat cultivars from the market (McVey *et al.,* 2002).

In 1998, the widely virulent *Pgt* race, TTKSK, was detected in Uganda (Pretorius *et al.,* 2000) and has since spread to several countries in Africa (Kenya, Ethiopia, Sudan, Tanzania, South Africa, Zimbabwe, Mozambique, and Eritrea) and recently the Middle East as well (Yemen and Iran) (Singh *et al.,* 2008, 2010, 2015; Mukoyi *et al.,* 2011; Nazari *et al.,* 2009; Pretorius *et al.,* 2010, 2012; Visser *et al.,* 2011; Wanyera *et al.,* 2006; Wolday *et al.,* 2011). The magnitude of TTKSK was realized as virulence against numerous commonly used *R* genes became known, including broadly deployed *Sr31* in wheat (Jin and Singh, 2006; Pretorius *et al.,* 2000) and *Rpg1* in barley (Steffenson *et al.,* 2009).

The TTKSK race was considered the most serious threat to global cereal production in more than 50 years. At the time of detection, it was capable of attacking more than 90% of wheat cultivars grown worldwide (Singh *et al.,* 2008) while over 96% of global barley varieties, cultivated and wild, are at risk (Steffenson *et al.,* 2017). According to Hodson *et al.* (2012), the universal spread of TTKSK and its variants to other cereal-producing regions is imminent in the near future.

Identification of additional resistance genes is a necessity to counter new rust races and preventing subsequent devastation to cereal crops. To date, 63 designated stem rust *R* genes as well as numerous QTL have been catalogued in wheat (McIntosh *et al.,* 2013, 2017). Eight genes conferring stem rust resistance have been identified in the barley germplasm following its comprehensive screening (Mamo *et al.,* 2015).

The *Rpg1* gene was detected in barley accessions derived from a Swiss landrace, ‘Chevron’ (CIho 1111) and ‘Peatland’ (CIho 5267), as well as in an individual plant of the cultivar Wisconsin 37 released as ‘Kindred’ (CIho 6969) (Powers and Hines, 1933; Shands, 1939; Steffenson, 1992). The gene is located on chromosome 7H and encodes a functional protein enzyme with twofold kinase domains, namely an active and a pseudo-kinase. Cloning of *Rpg1* revealed an identical allele present in barley cultivars Chevron, Peatland and Kindred (Brueggeman *et al.,* 2002).

Due to the durable resistance imparted against a wide scope of known *Pgt* races, *Rpg1* is the only major *R* gene deployed on a large scale in barley varieties for more than 70 years (Steffenson, 1992; Mamo *et al.,* 2015). However, *Rpg1* is completely ineffective to *Pgt* races QCCJB (Sun and Steffenson, 2005) and TTKSK (Mamo *et al.,* 2015), and similarly offers no protection against *Pgs* pathotypes (Steffenson *et al.,* 1982).

Genes *Rpg2* and *Rpg3* were recognized in barley accessions 'Hietpas-5' (CIho 7124) (Patterson *et al.,* 1957) and 'PI 382313' (Jedel, 1990; Jedel *et al.,* 1989), respectively. Low and insufficient levels of stem rust resistance, specifically against the TTKSK *Pgt* race, are conditioned by *Rpg2* and *Rpg3* (Steffenson *et al.,* 2013).

Identified in breeding line 'Q21861' ('PI 584766'), the recessive *rpg4* stem rust *R* gene imparts resistance against *Pgt* race QCCJB (Jin *et al.,* 1994; Mamo *et al.,* 2015). Considered as highly temperature sensitive, *rpg4* is only expressed at relatively low temperatures (17–22°C), while entirely ineffective once the temperature rises above 27°C (Jin *et al.,* 1994). By all accounts, *rpg4* is thought to control a gene-for-gene interaction, encoding an actin depolymerizing factor which is involved in reorganizing fungal cytoskeleton (Brueggeman *et al.,* 2008). Another gene, temporarily described as *RpgU*, was identified in 'Peatland' and accounted for moderate field resistance against *Pgt* pathotype QCCJB (Fox and Harder, 1995).

Fetch *et al.* (2009) recently identified a recessive stem rust *R* gene (*rpg6*) in '212Y1'; a barley line with a translocation from *Hordeum bulbosum* L. It is located on chromosome 6H and imparts resistance against *Pgt* race QCCJB (Kleinhofs *et al.,* 2009).

Three of the eight known stem rust resistance genes identified in barley, confers resistance to *Pgs*. Luig (1957) reported the presence of a dominant resistance gene in barley line 'Skinless', while *rpgBH*, described from 'Black Hulless' (CIho 666) and previously known as the *S* gene, is inherited in a recessive manner (Steffenson *et al.,* 1984; Sun and Steffenson, 2005). The dominant *Rpg5* gene (previously categorized as *RpgQ*) was also discovered in 'Q21861' and described based on its reaction to the rye stem rust pathogen (Sun *et al.,* 1996). It encodes a unique resistance protein comprising of three domains: the nucleotide-binding site, leucine rich repeat, and serine threonine protein kinase domain (Brueggeman *et al.,* 2008).

Until recently, *Rpg5* was considered entirely separate from *rpg4*. However, through comprehensive mapping and positional cloning, Brueggeman *et al.* (2008) deduced tight linkage between the distinct *rpg4* and *Rpg5* genes on barley chromosome 5HL. In the same study, detailed genetic analysis and post-transcriptionally silencing the *Rpg5* gene, indicated that *rpg4*-mediated stem rust resistance is only possible with co-occurrence of three tightly linked genes (*Rpg5*, *HvRga1*, and *HvAdf3*). The *rpg4/Rpg5*

complex locus is the only highly effective source of resistance described in barley to date, conferring ASR and APR against the broadly virulent TTKSK lineage (Arora *et al.*, 2013; Mamo *et al.*, 2015; Gill *et al.,* 2016; Steffenson *et al.*, 2017). However, non-functional *Rpg5* proteins provided evidence of susceptibility in lines that do in fact carry the complex (Arora *et al.,* 2013). The authors theorized that *Pgt* and *Pgs* interact differently with the *Rpg5* gene based on the fact that wheat and rye stem rust resistance is in essence recessive and dominant, respectively, and make use of different resistance mechanisms. Dracatos *et al.* (2015) suggested that the gene conferring resistance to *Pg* f. sp. *avenae* and 'Scabrum' rust may possibly be the same *Rpg5* gene or either located near the *Rpg5* locus.

## BREEDING FOR RESISTANCE

Although gene pyramiding is possible using conventional breeding methods, it is a complicated process to combine genes exhibiting similar phenotypic reactions. Nowadays, tagging individual resistance genes with molecular/DNA markers, significantly benefits the process of stacking genes into desired combinations which has become the preferred approach for long-lasting control of cereal rusts (Ellis *et al.*, 2014). In addition, a vital breeding objective is to lay genetic foundations imparting high, steady levels of rust resistance which can be achieved through efficient marker-assisted selection of several durable genes.

The indirect marker-assisted selection (MAS) process forms part of the technologically advanced molecular breeding discipline, accelerating the development of elite cultivars with supreme resistance, yield, quality, and agronomic traits. The desired loci, in this case associated with rust resistance, can be identified through linkage/biparental mapping or association mapping, the latter being the preferred method, reaching higher resolution of resistance polymorphism with reduced effects of linkage drag (Prins *et al.*, 2005; Bertrand *et al.*, 2008; Agenbag *et al.*, 2012).

Resistance QTL, segregating according to Mendel's laws (Singh *et al.,* 2000), form various recombinants during meiosis. The detection of genes and QTL is preferred through doubled haploid (DH) or recombinant inbred line (RIL) populations allowing for repeatable trials that can be thoroughly assessed for the complexities of durable APR (Rosewarne *et al.*, 2013; Yu *et al.*, 2014).

Additional selling points of DNA markers linked to desired genes include their convenient utilization, increased level of predictability over phenotypic screening of the disease, the fact that races do not have to be present to select for genes imparting resistance against them, as well as the reduction in amount of lines that need testing early in the breeding process (Bertrand *et al.*, 2008).

The diminished use of earlier markers, such as RFLPs, RAPDs, SCARs, CAPs, AFLPs and STSs, can be attributed to laborious techniques with poor reproducibility and/or population specificity. More advanced marker systems include microsatellites/SSRs as well as the latest technologies with increased throughput capacities, i.e. SNPs and DArTs, surpassing the preceding marker technologies.

Stacking several resistance sources in a single host genotype to achieve durability encompasses several difficulties. The expansion of population sizes to accommodate more gene combinations complicates the detection of rare recombinants. In order for the potential durable cultivar to compare with others commercially available, the pyramid of rust resistance genes should be further combined with other desirable traits regarding yield, quality, abiotic and other biotic resistance.

Another concern of gene stacking is presented as fitness costs and trade-offs, i.e. genes with significant pleiotropic effects may influence other desirable traits to some extent. Conversely, partial resistance which is commonly polygenic, may have potentially low fitness cost. Increasing evidence suggests that resistance to one disease often results in the cost of susceptibility to other diseases. Therefore, the improvement of multiple traits simultaneously remains a challenging task for plant breeders. Another proposal outlines the possibility that genes that survived selection, either natural or artificially applied by breeders, might be those with benefits exceeding total costs (Brown and Rant, 2013).

## ASSESSING RESISTANCE

Selection and evaluation of rust resistance necessitate its measurement, usually achieved by measuring the amount of pathogen present at a given time relative to that in a susceptible control. This has particular relevance to the assessment of quantitative or partial resistance which cannot be done in absolute terms (Ribeiro Do Vale *et al.*, 2001). If undetectable, Parlevliet (1993) proposed the evaluation of direct or indirect

pathogenic effects on the host. The amount of plant tissue affected is generally a good reflection of the amount of pathogen present, which in turn depends on a number of factors besides the level of host resistance of the cultivar; such as interplot interference (Parlevliet and Van Ommeren, 1975), inoculum density (Parlevliet, 1989) and plant growth stage (Ribeiro Do Vale *et al.*, 2001).

Traditional phenotypic scoring, entailing the disease severity and/or host IT, is the most commonly used method and remain an integral part of assessment, especially under field conditions. Since quantitatively expressed genes have relatively small effects on disease response, phenotypic assessment under epidemic field conditions is required for subsequent selection. Additionally, more reliable APR expression has been reported in the field as opposed to controlled conditions in the greenhouse or growth chambers (Boshoff, 2000; Ramburan *et al.*, 2004). Fluctuations may occur between seasonal IT scores owing to the environmental sensitivity of rust pathogens and their additional interaction with the host (Pretorius *et al.*, 2007) and have been suggested to be an unstable trait, especially for genotypes with intermediate ITs (Danial, 1995).

The time-consuming aspect of field screening with the added complexity to discern between APR (Boshoff, 2000), has fronted scientists to explore more rapid means to expand the capacity of phenotyping. Spectral crop sensors have been proven useful in accurately distinguishing stripe rust severity and host IT as well as mapping APR QTL in wheat populations (Pretorius *et al.*, 2017b). The infection levels are determined through the measurement of reflected wavelengths with regards to normalized difference vegetation index (NDVI). This method is however dependent on uniform and severe stripe rust infections which will, in theory, also be effective in the assessment for leaf rust. It was however suggested that low stem rust levels might not be detected.

Histology is one of the most pertinent approaches for fungal detection and analyses of processes associated with its growth, differentiation, infection and other cellular functions. These have proven valuable in the understanding of the interaction between biotrophic rust pathogens and host (and non-host) plants (Niks and Dekens, 1991; Swertz, 1994). Fluorescence microscopy is considered a valuable technique to appraise early infection stages of pathogenic rust fungi (Zurn *et al.*, 2015) and can be instrumental in elucidating mechanisms linked to specific resistance genes or QTL.

The progress of rust infection during early stages of development has been difficult to analyse since disease symptoms only appear 8 to 9 days post inoculation (Zurn *et al.*, 2015). Quantitative real-time PCR is a relatively novel way to investigate the timing of resistance expression by quantifying the fungal biomass accumulation in host plants over time (Ayliffe *et al.*, 2013) and additionally, has been valuable in the mapping of APR QTL (Acevedo *et al.*, 2010a). This molecular-based approach has been applied to several pathosystems, contributing to the insight of cultivar resistance before symptoms become visible (Atallah and Stevenson, 2007; Acevedo *et al.*, 2010b; Hu *et al.*, 2014).

The magnitude of accurate and reliable disease screening and assessment, which is sometimes overlooked, is a necessity (Bock *et al.*, 2015) for small differences in resistance to be detected.

REFERENCES

Abbasi M, Hedjaroude GA, Scholler M and Goodwin SB. 2004. Taxonomy of *Puccinia striiformis* s. l. in Iran. *Rostaniha* 5: 71-82.

Acevedo M, Jackson EW, Chong J, Rines HW, Harrison S and Bonman JM. 2010a. Identification and validation of quantitative trait loci for partial resistance to crown rust in oat. *Phytopathology* 100: 511-521.

Acevedo M, Jackson E, Sturbaum A, Ohm H and Bonman J. 2010b. An improved method to quantify *Puccinia coronate* f. sp. *avenae* DNA in the host *Avena sativa*. *Canadian Journal of Plant Pathology* 32: 215-224.

Agenbag GM, Pretorius ZA, Boyd LA, Bender CM and Prins R. 2012. Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez. *Theoretical and Applied Genetics* 125: 109-120.

Ali S, Gladieux P, Leconte M, Gautier A, Justesen AF, Hovmøller MS, Enjalbert J and De Vallavieille-Pope C. 2014. Origin, migration routes and worldwide population genetic structure of the wheat yellow rust pathogen *Puccinia striiformis* f. sp. *tritici*. *PLoS Pathogens* 10(1): e1003903. doi: 10.1371/journal.ppat.1003903

American Phytopathological Society. 1932. Observations on the rust of grain by Felice Fontana, 1767. In: P.P. Pirone, transl., *Phytopathological Classics*, No. 2, *American Phytopathological Society*, Hayworth Printing Company, Washington, DC, 40 pp.

American Phytopathological Society. 1952. V. Alimurgia: True nature, causes and sad effects of the rusts, the bunts, the smuts, and other maladies of wheat and oats in the field by GT Tozetti, 1767. In: L.R. Tehon, transl., *Phytopathological Classics*, No. 9, *American Phytopathological Society*, St. Paul, Minnesota, 139 pp.

Anikster Y. 1985. The formae speciales. In: The *Cereal Rusts*, Vol. II: *Diseases, Distribution, Epidemiology, and Control* (Roelfs AP and Bushnell WR, eds). Orlando, FL: Academic Press, pp. 115-130.

Arora D, Gross T and Brueggeman R. 2013. Allele characterization of genes required for *rpg4*-mediated wheat stem rust resistance identifies *Rpg5* as the *R* gene. *Phytopathology* 103: 1153-1161.

Arthaud J, Guyot L and Malencon G. 1966. Comparative biometric studies of the formae speciales of black rust (*Puccinia graminis* Pers.) living on the wild grasses of the Moroccan Atlas. In: *Proceedings of the Cereal Rusts Conference*, Cambridge, 1964, pp. 204-206.

Atallah Z and Stevenson W. 2007. A methodology to detect and quantify five pathogens causing potato tuber decay using real-time quantitative PCR. *Phytopathology* 97: 865-872.

Ayliffe M, Singh D, Park R, Moscou M and Pryor T. 2013. Infection of *Brachypodium distachyon* with selected grass rust pathogens. *Molecular Plant-Microbe Interactions* 26: 946-957.

Azbukina Z. 1980. Economical importance of aecial hosts of rust fungi of cereals in the Soviet Far East. In: *Proceedings of the 5th European and Mediterranean Cereal Rusts Conference*, Bari and Rome, pp. 199-201.

Badr A, Müller K, Schäfer-Pregl R, El Rabey H, Effgen S, Ibrahim HH, Pozzi C, Rohde W and Salamini F. 2000. On the origin and domestication history of Barley (H*ordeum vulgare*). *Molecular Biology and Evolution* 17(4): 499-510.

Bahri B, Leconte M, Ouffroukh A, De Vallavieille-Pope C and Enjalbert J. 2009. Geographic limits of a clonal population of wheat yellow rust in the Mediterranean region. *Molecular Ecology* 18: 4165-4179.

Bahri B, Shah SJA, Hussain S, Leconte M, Enjalbert J and De Vallavieille-Pope C. 2011. Genetic diversity of wheat yellow rust population in Pakistan and its relationship with host resistance. *Plant Pathology* 60: 649-660.

Bertrand C, Collar Y and Mackill DJ. 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences* 363: 557-572.

Biffen RH. 1905. Mendel's law of inheritance and wheat breeding. *Journal of Agricultural Science* 1: 4-48.

Bock CH, El Jarroudi M, Kouadio LA, Mackels C, Chiang KS and Delfosse P. 2015. Disease severity estimates: Effects of rater accuracy and assessment methods for comparing treatments. *Plant Disease* 99: 1104-1112.

Boshoff WHP. 2000. Control of foliar rusts of wheat in South Africa with special emphasis on *Puccinia striiformis* f. sp. *tritici*. PhD Thesis, University of the Free State, South Africa.

Boshoff WHP, Pretorius ZA and Van Niekerk BD. 2002a. Establishment, distribution, and pathogenicity of *Puccinia striiformis* f. sp. *tritici* in South Africa. *Plant Disease* 86: 485-492.

Boshoff WHP, Pretorius ZA and Van Niekerk BD. 2002b. Resistance in South African and foreign wheat cultivars to pathotypes 6E16A- and 6E22A- of *Puccinia striiformis* f. sp. *tritici*. *South African Journal of Plant and Soil* 19: 27-36.

Britton M and Cummins GB. 1956. The reaction of species of *Poa* and grasses to *Puccinia striiformis*. *Plant Disease Report* 40: 643-645.

Brown JKM and Hovmøller MS. 2002. Aerial dispersal of fungi on the global and continental scales and its consequences for plant disease. *Science* 297: 537-541.

Brown JKM and Rant JC. 2013. Fitness costs and trade-offs of disease resistance and their consequences for breeding arable crops. *Plant Pathology* 62: 83-95. doi: 10.1111/ppa.12163

Brueggeman R, Druka A, Nirmala J, Cavileer T, Drader T, Rostoks N, Mirlohi A, Bennypaul H, Gill U and Kudrna D. 2008. The stem rust resistance gene *Rpg5* encodes a protein with nucleotide-binding-site, leucine-rich, and protein kinase domains. *Proceedings of the National Academy of Sciences USA* 105: 14970-14975.

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B and Kleinhofs A. 2002. The barley stem rust resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. *Proceedings of the National Academy of Sciences USA* 99: 9328-9333.

Caldwell RM. 1968. Breeding for general and/or specific plant disease resistance. In: *Proceedings of the 3rd International Wheat Genetics Symposium* (Findlay KW and Shepherd KW, eds). Australian Academy of Science, Canberra, Australia, pp. 263-272.

Chen W, Wellings C, Chen X, Kang Z and Liu T. 2014. Wheat stripe (yellow) rust caused by *Puccinia striiformis* f. sp. *tritici*. *Molecular Plant Pathology* 15: 433-446.

Chen X. 2013. High-temperature adult-plant resistance, key for sustainable control of stripe rust, Review article. *American Journal of Plant Sciences* 4: 608-627. doi: 10.4236/ajps.2013.43080

Chen X, Line RF and Leung H. 1993. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. *Phytopathology* 83: 1489-1497.

Chen XM. 2005. Epidemiology and control of stripe rust *Puccinia striiformis* f. sp. *tritici* on wheat. *Canadian Journal of Plant Pathology* 27: 314-337.

Chen XM. 2007. Challenges and solutions for stripe rust control in the United States. *Australian Journal of Agricultural Research* 58: 648-655.

Chen XM, Wang MN, Wan AM, Cheng P and Cheng JJ. 2012. Sexual or asexual reproduction, which one is more important for stripe rust. In: *Disease Risk and Food Security. Proceedings of the 13th International Cereal Rust and Powdery Mildew Conference* (Chen WQ, ed). Beijing: China Agricultural Science and Technology Press, pp. 36-37.

Chester KS. 1946. The nature and prevention of the cereal rusts as exemplified in the leaf rust of wheat. Chronica Botanica, Waltham, Massachusetts, 269 pp.

Christopher-Kozjan R and Heath MC. 2003. Cytological and pharmacological evidence that biotrophic fungi trigger different cell death execution processes in host and nonhost cells during the hypersensitive response. *Physiological and Molecular Plant Pathology* 62: 265-275.

Craigie JH. 1927. Discovery of the function of the pycnia of the rust fungi. *Nature* (London) 120: 765-767.

Craigie JH. 1931. An experimental investigation of sex in the rust fungi. *Phytopathology* 21: 1001-1040.

Cummins GB. 1971. The rust fungi of cereals, grasses, and bamboos. Berlin and New York: Springer-Verlag.

Cummins GB and Caldwell RM. 1956. The validity of binomials in the leaf rust complex of cereals and grasses. *Phytopathology* 46: 81-82.

Cummins GB and Hiratsuka Y. 2003. Illustrated genera of rust fungi. *American Phytopathological Society*, St. Paul, Minnesota, USA, 3rd edition, 223 pp.

DAFF 2017. Department of Agriculture, Forestry and Fisheries, South Africa. Abstract of Agricultural Statistics. http://www.daff.gov.za accessed 23/08/2017.

Danial DL. 1995. Durable resistance in wheat to yellow rust in Kenya. Breeding for disease resistance with emphasis on durability. *Proceedings of a Regional Workshop for Eastern, Central and Southern Africa*, Njoro, Kenya, October 2-6, 1994, 274 pp.

De Candolle A. 1815. Uredo rouille des cereales. In: *Flora Francaise, Famille des Champignons*, 83 pp.

Demirci YE, Inan C, Günel A, Maytalman D, Mert Z, Baykal AT, Korkut SV, Arda N and Hasançebi S. 2016. Proteome profiling of the compatible interaction between wheat and stripe rust. *European Journal of Plant Pathology* 145: 941-962. doi: 10.1007/s10658-016-0882-1

De Vallavieille-Pope C, Ali S, Leconte M, Enjalbert J, Delos M and Rouzet J. 2012. Virulence dynamics and regional structuring of *Puccinia striiformis* f. sp. *tritici* in France between 1984 and 2009. *Plant Disease* 96: 131-140.

De Vallavieille-Pope C, Huber L, Leconte M and Goyeau H. 1995. Comparative effects of temperature and interrupted wet periods on germination, penetration and infection of *Puccinia recondita* f. sp. *tritici* and *P. striiformis* on wheat seedlings. *Phytopathology* 85: 409-415.

Dracatos P, Singh D, Fetch T Jr and Park RF. 2015. Resistance to *Puccinia graminis* f. sp. *avenae* in barley is associated with the *Rpg5* locus. *Phytopathology* 105(4): 490-494. doi: 10.1094/PHYTO-08-14-0224-R

Duan X, Tellier A, Wan A, Leconte M, De Vallavieille-Pope C and Enjalbert J. 2010. *Puccinia striiformis* f. sp. *tritici* presents high diversity and recombination in the over-summering zone of Gansu, China. *Mycologia* 102: 44-53.

Dubin HJ and Brennan JP. 2009. Combating stem and leaf rust of wheat: Historical perspective, impacts, and lessons learned. IFPRI Discussion Paper 910. Washington, DC: International Food Policy Research Institute.

Durrant WE and Dong X. 2004. Systemic acquired resistance. *Annual Review of Phytopathology* 42: 185-209.

Dvořák J, Luo MC, Yang ZL and Zhang HB. 1998. The structure of *Aegilops tauschii* genepool and the evolution of hexaploid wheat. *Theoretical and Applied Genetics* 97: 657-670.

Ellis JG, Lagudah ES, Spielmeyer W and Dodds PN. 2014. The past, present and future of breeding rust resistant wheat. *Frontiers in Plant Science* 5: 641.

Emmett RW and Parberry DG. 1975. Appressoria. *Annual Review of Phytopathology* 13(1): 147-167.

Enjalbert J, Duan X, Leconte M, Hovmøller MS and De Vallavieille-Pope C. 2005. Genetic evidence of local adaptation of wheat yellow rust (*Puccinia striiformis* f. sp. *tritici*) within France. *Molecular Ecology* 14: 2065-2073.

Eriksson J. 1894. Über die spezialisierung des parasitsimus bei den getreiderostpilzen. *Berichte Der Deutschen Botanischen Gesellschaft* 12: 292-331.

Eriksson J. 1899. Nouvelles études sur la rouille brune des céréales. *Annales des Sciences Naturelles Botanique*. Eighth Série 9: 241-288.

Eriksson J and Henning E. 1896. Die Getreideroste. Ihre Geschichte und Natur sowie Massregein gegen dieselben, Stockholm, PA Norstedt and Soner, 463 pp.

FAO 2017. Food and Agriculture Organization of the United Nations, Food Outlook: Biannual report on global food markets, June 2017. http://www.fao.org/giews/ accessed 30/08/2017.

FAOSTAT 2009. http://faostat.fao.org

Farr DF, Bills GF, Chamuris GP and Rossman AY. 1995. Fungi on plants and plant products in the United States. APS Press, St. Paul, MN.

Fetch T Jr, Johnston P and Pickering R. 2009. Chromosomal location and inheritance of stem rust resistance transferred from *Hordeum bulbosum* into cultivated barley *(H. vulgare*). *Phytopathology* 99: 339-343.

Flor HH. 1942. Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* 28: 365-391.

Flor HH. 1971. Current status of the gene-for-gene concept. *Annual Review of Phytopathology* 9: 275-296.

Fox S and Harder D. 1995. Resistance to stem rust in barley and inheritance of resistance to race QCC. *Canadian Journal of Plant Science* 75: 781-788.

Gäumann E. 1959. Die Rostpilze Mitteleuropas. *Biträge zur Kryptogamenflora der Schweiz*, Vol. Band XII. Buchdruckeri Büchler and Co., Bern, Switzerland.

Gerechter-Amitai ZK. 1973. Stem rust on cultivated and wild grasses in Israel. PhD Thesis, Hebrew University, Jerusalem.

Gill U, Brueggeman R, Nirmala J, Chai Y, Steffenson B and Kleinhofs A. 2016. Molecular and genetic characterization of barley mutants and genetic mapping of mutant *rpr2* required for *Rpg1*-mediated resistance against stem rust. *Theoretical and Applied Genetics* 129: 1519-1529.

Glazebrook J, Rogers EE and Ausubel FM. 1997. Use of *Arabidopsis* for genetic dissection of plant defense responses. *Annual Review of Genetics* 31(1): 547-569.

Hammond-Kosack KE and Jones JDG. 1997. Plant disease resistance genes. *Annual Review in Plant Physiology and Plant Molecular Biology* 48: 575-607.

Hassebrauk K. 1965. Nomenklatur, geographische Verbreitung und Wirtsbereich des Gelbrostes, *Puccinia striiformis* West. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem 116: 1-75.

Heath MC and Skalamera D. 1997. Cellular interactions between plants and biotrophic fungal parasites. *Advances in Botanical Research* 24: 195-225.

Hodson DP, Grønbech-Hansen J, Lassen P, Alemayehu Y, Arista J, Sonder K, Kosina P, Moncada P, Nazari K, Park RF, Pretorius ZA, Szabo LJ, Fetch T and Jin Y. 2012. Tracking the wheat rust pathogens. In: *Proceedings BGRI 2012 Technical Workshop*, Beijing (McIntosh R, ed), pp. 11-22.

Hogenhout SA, Van der Hoorn RAL, Terauchi R and Kamoun S. 2009. Emerging concepts in effector biology of plant-associated organisms. *Molecular Plant-Microbe Interactions* 22: 115-122.

Hovmøller MS. 2007. Sources of seedling and adult plant resistance to *Puccinia striiformis* f. sp. *tritici* in European wheats. *Plant Breeding* 126: 225-233.

Hovmøller MS, Justesen AF and Brown JKM. 2002. Clonality and long-distance migration of *Puccinia striiformis* f. sp. *tritici* in north-west Europe. *Plant Pathology* 51: 24-32.

Hovmøller MS, Sørenson SK, Walter S and Justesen AF. 2011. Diversity of *Puccinia striiformis* on cereals and grasses. *Annual Review of Phytopathology* 49: 197-217.

Hovmøller MS, Walter S, Bayles RA, Hubbard A, Flath K, Sommerfeldt N, Leconte M, Czembor P, Rodriguez-Algaba J, Thach T, Hansen JG, Lassen P, Justesen AF, Ali S and De Vallavieille-Pope C. 2016. Replacement of the European wheat yellow rust population by new races from the centre of diversity in the near-Himalayan region. *Plant Pathology* 65: 402-411.

Hovmøller MS, Yahyaoui AH, Milus EA and Justesen AF. 2008. Rapid global spread of two aggressive strains of a wheat rust fungus. *Molecular Ecology* 17: 3818-3826.

Hu J, Johnson EG, Wang N, Davoglio T and Dewdney MM. 2014. qPCR quantification of pathogenic *Guignardia citricarpa* and non-pathogenic *G. mangiferae* in citrus. *Plant Disea*se 98: 112-120.

Hubbard A, Lewis CM, Yoshida K, Ramirez-Gonzales RH, De Vallavieille-Pope C, Thomas J, Kamoun S, Bayles R, Uauy C and Saunders DGO. 2015. Field pathogenomics reveals the emergence of a diverse wheat yellow rust population. *Genome Biology* 16: 23.

Hylander N, Jørstad I and Nannfeldt JA. 1953. Enumeratio uredionearum Scandinavicarum. *Opera Botanica* 1:1-102.

Jedel P. 1990. A gene for resistance to *Puccinia graminis* f. sp. *tritici* in PI382313. *Barley Genetics Newsletter* 20: 43-44.

Jedel P, Metcalfe D and Martens J. 1989. Assessment of barley accessions PI 382313, PI 382474, PI 382915, and PI 382976 for stem rust resistance. *Crop Science* 29: 1473-1477.

Jin Y and Singh R. 2006. Resistance in US wheat to recent eastern African isolates of *Puccinia graminis* f. sp. *tritici* with virulence to resistance gene *Sr31*. *Plant Disease* 90: 476-480.

Jin Y, Steffenson B and Miller J. 1994. Inheritance of resistance to pathotypes QCC and MCC of *Puccinia graminis* f. sp. *tritici* in barley line Q21861 and temperature effects on the expression of resistance. *Phytopathology* 84: 452-455.

Jin Y, Szabo LJ and Carson M. 2010. Century-old mystery of *Puccinia striiformis* life history solved with the identification of *Berberis* as an alternate host. *Phytopathology* 100: 432-435.

Jin Y, Szabo LJ, Pretorius ZA, Singh RP, Ward R and Fetch T. 2008. Detection of virulence to resistance gene *Sr24* within race TTKS of *Puccinia graminis* f. sp. *tritici*. *Plant Disease* 92: 923-926.

Johnson R. 1984. A critical analysis of durable resistance. *Annual Review of Phytopathology* 22: 309-330.

Johnson R and Law CN. 1975. Genetic control of durable resistance to stripe rust (*Puccinia striiformis*) in the wheat cultivar Hybride de Berse. *Annals of Applied Biology* 81: 385-391.

Jones JDG and Dangl JL. 2006. The plant immune system. *Nature* 444: 323-329.

Justesen AF, Ridoutb CJ and Hovmøller MS. 2002. The recent history of P*uccinia striiformis* f. sp. *tritici* in Denmark as revealed by disease incidence and AFLP markers. *Plant Pathology* 51: 13-23.

Kamoun S. 2007. Groovy times: filamentous pathogen effectors revealed. *Current Opinion in Plant Biology* 10: 358-365.

Kang ZS, Huang LL and Buchenauer H. 2002. Ultrastructural changes and localization of lignin and callose in compatible and incompatible interactions between wheat and *Puccinia striiformis*. *Journal of Plant Diseases and Protection* 109: 25-37.

Kang ZS, Huang LL and Li JY. 1997. *Morphology of plant pathogenic fungi under scanning electron microscope.* Beijing, China Agriculture Press.

Kang ZS, Wang Y, Huang LL, Wei GR and Zhao J. 2003. Histology and ultrastructure of incompatible combination between *Puccinia striiformis* and wheat with low reaction type resistance. *Agricultural Sciences in China* 2: 1102-1113.

Kilpatrick RA. 1975. New wheat cultivars and longevity of rust resistance, 1971–75. *United States Agricultural Research Service*, Northeastern Region [Report] ARS-NE 64: 3-20.

Kislev ME. 1982. Stem rust of wheat 3300 years old found in Israel. *Science* 216: 993-994.

Kleinhofs A, Brueggeman R, Nirmala J, Zhang L, Mirlohi A, Druka A, Rostoks N and Steffenson BJ. 2009. Barley stem rust resistance genes: structure and function. *The Plant Genome* 2: 109-120.

Knott DR. 1989. The wheat rust pathogens. In: *The wheat rusts; breeding for resistance* (Knott DR, ed). Berlin, Heidelberg: Springer-Verlag, pp. 1-37.

Kolmer JA. 2001. Early research on the genetics of *Puccinia graminis* and stem rust resistance in wheat in Canada and the United States. In: *Stem Rust of Wheat from Ancient Enemy to Modern Foe* (Peterson PD, ed). *The American Phytopathological Society*, St. Paul, MN, pp. 51-82.

Kolmer JA. 2005. Tracking wheat rust on a continental scale. *Current Opinion in Plant Biology* 8: 441-449.

Leonard KJ. 2001. Stem rust—future enemy? In: *Stem Rust of Wheat: from Ancient Enemy to Modern Foe* (Peterson PD, ed). *The American Phytopathological Society*, St. Paul, MN, pp. 119-146.

Leonard KJ and Szabo L. 2005. Pathogen profile: stem rust of small grains and grasses caused by *Puccinia graminis*. *Molecular Plant Pathology* 6: 99-111. doi: 10.1111/J.1364-3703.2004.00273.X

Leppik EE. 1970. Gene centers of plants as sources of disease resistance. *Annual Review of Phytopathology* 8: 323-344.

Lev-Yadun S, Gopher A and Abbo S. 2000. The cradle of agriculture. *Science* 288: 1602-1603.

Lin F and Chen XM. 2009. Quantitative trait loci for non- race-specific, high-temperature adult-plant resistance to stripe rust in wheat cultivar express. *Theoretical and Applied Genetics* 118(4): 631-642. doi: 10.1007/s00122-008-0894-0

Line RF, Sharp E and Powelson R. 1970. A system for differentiating races of *Puccinia striiformis* in the United States. *Plant Disease Report* 54: 992–994.

Luig NH. 1957. Inheritance studies of barley in relation to disease resistance. M.S. Thesis, University of Sydney, Australia.

Ma Q and Shang HS. 2009. Ultrastructure of stripe rust (*Puccinia striiformis* f. sp. *tritici*) interacting with slow-rusting, highly resistant, and susceptible wheat cultivars. *Journal of Plant Pathology* 91: 597-606.

Macer RCF. 1972. The resistance of cereals to yellow rust and its exploitation in plant breeding. *Proceedings of the Royal Society of London* 181: 281-301.

Mamo BE, Smith KP, Brueggeman RS and Steffenson BJ. 2015. Genetic characterization of resistance to wheat stem rust race TTKSK in landrace and wild barley accessions identifies the *rpg4/Rpg5* locus. *Phytopathology* 105: 99-109.

Manners JG. 1960. *Puccinia striiformis* Westend. var. *dactylidis* var. nov. *Transactions of the British Mycological Society* 43: 65-68.

Mares DJ. 1979. Microscopic study of the development of yellow rust (*Puccinia striiformis*) in a wheat cultivar showing adult-plant resistance. *Physiological Plant Pathology* 15: 289-292.

Martens JW, Dunsmore KM and Harder DE. 1989. Incidence and virulence of *Puccinia graminis* in Canada on wheat and barley in 1988. *Canadian Journal of Plant Pathology* 11: 424-430.

Mboup M, Leconte M, Gautier A, Wan AM, Chen W, De Vallavieille-Pope C and Enjalbert J. 2009. Evidence of genetic recombination in wheat yellow rust populations of a Chinese oversummering area. *Fungal Genetics and Biology* 46: 299-307.

McIntosh RA, Dubcovsky J, Rogers WJ, Morris C, Appels R and Xia XC. 2013. Catalogue of gene symbols for wheat. http://wheat.pw.usda.gov/GG2/Triticum/wgc/2013/

McIntosh RA, Dubcovsky J, Rogers WJ, Morris C and Xia XC. 2017. Catalogue of gene symbols for wheat. https://shigen.nig.ac.jp/wheat/komugi/genes/macgene/supplement2017.pdf

McVey DV, Long DL and Roberts JJ. 2002. Races of *Puccinia graminis* in the United States during 1997 and 1998. *Plant Disease* 86: 568-572.

Mendgen K. 1981. Nutrient uptake in rust fungi. *Phytopathology* 71: 983-989.

Milus E, Kristensen K and Hovmøller M. 2009. Evidence for increased aggressiveness in a recent widespread strain of *Puccinia striiformis* f. sp. *tritici* causing stripe rust of wheat. *Phytopathology* 99: 89-94.

Moldenhauer J, Moerschbacher BM and Van der Westhuizen AJ. 2006. Histological investigation of stripe rust (*Puccinia striiformis* f. sp. *tritici*) development in resistant and susceptible wheat cultivars. *Plant Pathology* 55: 469-474.

Moldenhauer J, Pretorius ZA, Moerschbacher BM, Prins R and Van der Westhuizen AJ. 2008. Histopathology and PR-protein markers provide insight into adult plant resistance to stripe rust of wheat. *Molecular Plant Pathology* 9(2): 137-145.

Mukoyi F, Soko T, Mulima E, Mutari B, Hodson D, Herselman L, Visser B and Pretorius Z. 2011. Detection of variants of wheat stem rust race Ug99 (*Puccinia graminis* f. sp. *tritici*) in Zimbabwe and Mozambique. *Plant Disease* 95: 1188.

Nazari K, Mafi M, Yahyaoui A, Singh RP and Park RF. 2009. Detection of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) race TTKSK (Ug99) in Iran. *Plant Disease* 93: 317.

Nazari K, Torabi M and Mardoukhi V. 1996. Wild grass species as oversummering hosts of *Puccinia striiformis* f. sp. *tritici* in Iran. *Cereal Rusts Powdery Mildews Bulletin* 24: 105-107.

Nelson RR. 1978. Genetics of horizontal resistance to plant disease. *Annual Review of Phytopathology* 16: 359-378.

Niks RE. 1986. Variation of mycelial morphology between species and formae speciales of rust fungi of cereals and grasses. *Canadian Journal of Botany* 64: 2976-2983.

Niks RE and Dekens RG. 1991. Prehaustorial and posthaustorial resistance to wheat leaf rust in diploid wheat seedlings. *Phytopathology* 81: 847-851.

Niu YC, Li ZQ and Shang HS. 1991. *Puccinia striiformis* West. f. sp. *leymi* and f. sp. *elymi*, two new formae speciales. *Acta Universitatis Agriculturae Boreali-Occidentalis* 19: 58-62.

Olivera P, Newcomb M, Szabo LJ, Rouse M, Johnson J, Gale S, Luster DG, Hodson D, Cox JA, Burgin L, Hort M, Gilligan CA, Patpour M, Justesen AF, Hovmøller MS, Woldeab G, Hailu E, Hundie B, Tadesse K, Pumphrey M, Singh RP and Jin Y. 2015. Phenotypic and genotypic characterization of race TKTTF of *Puccinia graminis* f. sp. *tritici* that caused a wheat stem rust epidemic in southern Ethiopia in 2013-14. *Phytopathology* 105: 917-928.

Park RF. 2007. Stem rust of wheat in Australia. *Australian Journal of Agricultural Research* 58: 558-566.

Park RF, Golegaonkar PG, Derevnina L, Sandhu KS, Karaoglu H, Elmansour HM, Dracatos PM and Singh D. 2015. Leaf rust of cultivated barley: Pathology and Control. *Annual Review of Phytopathology* 53: 26.1-26.25. doi: 10.1146/annurev-phyto-080614-120324

Parlevliet JE. 1979. Components of resistance that reduce the rate of epidemic development. *Annual Review of Phytopathology* 17: 203-222.

Parlevliet JE. 1989. Identification and evaluation of quantitative resistance. In: *Plant disease epidemiology - Genetics, Resistance and Management*, Vol. 2 (Leonard KJ and Fry WE, eds). McGraw-Hill Publishing Company, New York, pp. 215-248.

Parlevliet JE. 1993. What is durable resistance, a general outline. In: *Durability of disease resistance* (Jacobs TH and Parlevliet JE, eds). Kluwe Academic Publisher, pp. 23-39.

Parlevliet JE and Van Ommeren A. 1975. Partial resistance of barley leaf rust, *Puccinia hordei*. II. Relationship between field trials, micro - plot tests and latent period. *Euphytica* 24: 293-303.

Patterson F, Shands R and Dickson J. 1957. Temperature and seasonal effects on seedling reactions of barley varieties to three races of *Puccinia graminis* f. sp. *tritici*. *Phytopathology* 47: 395-402.

Piffanelli P, Zhou F, Casais C, Orme J, Jarosch B, Schaffrath U, Collins NC, Panstruga R and Schultze-Lefert P. 2002. The barley MLO modulator of defence and cell death is responsive to biotic and abiotic stress stimuli. *Plant Physiology* 129: 1076-1085.

Powers L and Hines L. 1933. Inheritance of reaction to stem rust and barbing of awns in barley crosses. *Journal of Agricultural Research* 46: 12.

Pretorius ZA, Ayliffe M, Bowden RL, Boyd LA, DePauw RM, Jin Y, Knox RE, McIntosh RA, Park RF, Prins R and Lagudah ES. 2017a. Advances in control of wheat rusts. In: *Achieving sustainable cultivation of wheat*, Vol. 1: *Breeding, quality traits, pests and diseases* (Langridge P, ed). Burleigh Dodds Science Publishing, Cambridge, UK, pp. 295-343. ISBN: 978 1 78676 016 6; www.bdspublishing.com

Pretorius ZA, Bender CM, Visser B and Terefe T. 2010. First report of a *Puccinia graminis* f. sp. *tritici* race virulent to the *Sr24* and *Sr31* wheat stem rust resistance genes in South Africa. *Plant Disease* 94: 784.

Pretorius ZA, Boshoff WH and Kema GH. 1997. First report of *Puccinia striiformis* f. sp. *tritici* on wheat in South Africa. *Plant Disease* 81: 424.

Pretorius ZA, Lan CX, Prins R, Knight V, McLaren NW, Singh RP, Bender CM and Kloppers FJ. 2017b. Application of remote sensing to identify adult plant resistance loci

to stripe rust in two bread wheat mapping populations. *Precision Agriculture* 18: 411-428. doi: 10.1007/s11119-016-9461-x

Pretorius ZA, Pienaar L and Prins R. 2007. Greenhouse and field assessment of adult plant resistance in wheat to *Puccinia striiformis* f. sp. *tritici*. *Australasian Plant Pathology* 36: 552-559.

Pretorius ZA, Singh RP, Wagoire W and Payne T. 2000. Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Plant Disease* 84: 203.

Pretorius ZA, Szabo LJ, Boshoff WHP, Herselman L and Visser B. 2012. First report of a new TTKSF race of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) in South Africa and Zimbabwe. *Plant Disease* 96: 590.

Prins R, Ramburan VP, Pretorius ZA, Boyd LA, Boshoff WHP, Smith PH and Louw JH. 2005. Development of a doubled haploid mapping population and linkage map for the bread wheat cross Kariega × Avocet S. *South African Journal of Plant and Soil* 22: 1-8. doi: 10.1080/02571862.2005.10634672

Ramburan VP, Pretorius ZA, Louw JH, Boyd LA, Smith PH, Boshoff WHP and Prins R. 2004. A genetic analysis of adult plant resistance to stripe rust in the wheat cultivar Kariega. *Theoretical and Applied Genetics* 108: 1426-1433.

Rapilly F. 1979. Yellow rust epidemiology. *Annual Review of Phytopathology* 17: 59-73.

Ribeiro Do Vale FX, Parlevliet JE and Zambolim L. 2001. Concepts in plant disease resistance. *Fitopatologia Basileira* 26: 577-589.

Roelfs AP. 1978. Estimated losses caused by rust in small grain cereals in the United States, 1918-1976. *USDA Miscellaneous Publications* 1363: 85.

Roelfs AP. 1982. Effects of Barberry Eradication on Stem Rust in the United States. Cereal Rust Laboratory, ARS, USDA, University of Minnesota, St. Paul. *Plant Disease* 66: 177-181. *The American Phytopathological Society*. doi: 10.1094/PD-66-177

Roelfs AP, Long DL and Roberts JJ. 1993. Races of *Puccinia graminis* in the United States during 1990. *Plant Disease* 77: 125-128.

Roelfs AP, Singh RP and Saari EE. 1992. Rust diseases of wheat: concepts and methods of disease management. Centro International de Mejoramiento de Maiz y Trigo (CIMMYT), DF Mexico.

Rosewarne GM, Herrera-Foessel SA, Singh RP, Huerta-Espino J, Lan CX and He ZH. 2013. Quantitative trait loci of stripe rust resistance in wheat. *Theoretical and Applied Genetics* 126: 2427-2449.

Savile DBO. 1984. Taxonomy of the cereal rust fungi. In: *The Cereal Rusts*, Vol. I: *Origins, Specificity, Structure, and Physiology* (Bushnell WR and Roelfs AP, eds). Academic Press, Orlando, FL, pp. 79-114.

Shamanin V, Salina E, Wanyera R, Zelenskiy Y, Olivera P and Morgounov A. 2016. Genetic diversity of spring wheat from Kazakhstan and Russia for resistance to stem rust Ug99. *Euphytica* 212: 287. doi: 10.1007/s10681-016-1769-0

Shands R. 1939. Chevron, a barley variety resistant to stem rust and other diseases. *Phytopathology* 29: 209-211.

Shaner DL, Isom WH and Lyon JL. 1978. Effect of seeding depth on yield and competition with weeds of two wheat varieties. *Proceedings of the Western Society of Weed Science* 31: 88-95.

Sharp EL. 1967. Atmospheric ions and germination of urediospores of *Puccinia striiformis*. *Science* 156: 1359-1360.

Shean M. 2010. Middle East: Yellow rust epidemic affects regional wheat crops. United States Department of Agriculture.

Singh RP, Hodson DP, Huerta-Espino J, Jin Y, Bhavani S, Njau P, Herrera-Foessel S, Singh PK, Singh S and Govindan V. 2010. The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. *Annual Review of Phytopathology* 49: 465-481.

Singh RP, Hodson DP, Huerta-Espino J, Jin Y, Njau P, Wanyera R, Herrera-Foessel SA and Ward RW. 2008. Will stem rust destroy the world's wheat crop? *Advances in Agronomy* 98: 271-309.

Singh RP, Hodson DP, Jin Y, Lagudah ES, Ayliffe MA, Bhavani S, Rouse MN, Pretorius ZA, Szabo LJ, Huerta-Espino J and Basnet BR. 2015. Emergence and spread of new races of wheat stem rust fungus: continued threat to food security and prospects of genetic control. *Phytopathology* 105: 872-884.

Singh RP, Nelson JC and Sorrells ME. 2000. Mapping *Yr28* and other genes for resistance to stripe rust in wheat. *Crop Science* 40: 1148-1155.

Singh RP, William HM, Huerta-Espino J and Rosewarne G. 2004. Wheat rust in Asia: Meeting the challenges with old and new technologies. In: *New directions for a diverse planet: Proceedings of the 4th International Crop Science Congress*, Brisbane, Australia, September 26-October 1, 2004. Madison, Wisconsin, USA: Crop Science Society of America.

Sohn J, Voegele RT, Mendgen K and Hahn M. 2000. High level activation of vitamin B1 biosynthesis genes in haustoria of the rust fungus *Uromyces fabae*. *Molecular Plant-Microbe Interactions* 13: 629-636.

Stakman EC. 1955. Progress and problems in plant pathology. *Annals of Applied Biology* 42: 22-33.

Staples RC and Macko V. 1984. Germination of urediospores and differentiation of infection structures. In: *The Cereal Rusts,* Vol. I: *Origins, Specificity, Structure, and Physiology* (Roelfs AP and Bushnell WR, eds). Orlando, FL: Academic Press, pp. 255-289.

Statista 2016. www.statista.com accessed 23/08/2017.

Steffenson BJ. 1992. Analysis of durable resistance to stem rust in barley. *Euphytica* 63: 153-167.

Steffenson BJ, Case AJ, Pretorius ZA, Coetzee V, Kloppers FJ, Zhou H, Chai Y, Wanyera R, Macharia G, Bhavani S and Grando S. 2017. Vulnerability of barley to African pathotypes of *Puccinia graminis* f. sp. *tritici* and sources of resistance. *Phytopathology* 107(8): 950-962.

Steffenson BJ, Jin Y, Brueggeman RS, Kleinhofs A and Sun Y. 2009. Resistance to stem rust race TTKSK maps to the *rpg4/Rpg5* complex of chromosome 5H of barley. *Phytopathology* 99: 1135-1141.

Steffenson BJ, Wilcoxson RD and Roelfs AP. 1982. Field reaction of selected barleys to *Puccinia graminis*. *Phytopathology* 72: 1002.

Steffenson BJ, Wilcoxson RD and Roelfs AP. 1984. Inheritance of resistance to *Puccinia graminis* f. sp. *secalis* in barley. *Plant Disease* 68: 762-763.

Steffenson BJ, Zhou H, Chai Y and Grando S. 2013. Vulnerability of cultivated and wild barley to African stem rust race TTKSK. In: *Advance in Barley Sciences. Proceedings of 11th International Barley Genetics Symposium* (Zhang G, Li C and Liu X, eds). Zhejiang University Press, Hangzhou, China, and Springer, Berlin, pp. 243-255.

Stubbs RW. 1985. Stripe rust. In: The *Cereal Rusts*, Vol. II: *Diseases, Distribution, Epidemiology, and Control* (Roelfs AP and Bushnell WR, eds). New York: Academic Press, pp. 61-101.

Sun Y, Steffenson BJ and Jin Y. 1996. Genetics of resistance to *Puccinia graminis* f. sp. *secalis* in barley line Q21861. *Phytopathology* 86: 1299-1302.

Sun Y and Steffenson BJ. 2005. Reaction of barley seedlings with different stem rust resistance genes to *Puccinia graminis* f. sp. *tritici* and *Puccinia graminis* f. sp. *secalis*. *Canadian Journal of Plant Pathology* 27: 80-89.

Swertz CA. 1994. Morphology of germlings of urediospores and its value for the identification and classification of grass rust fungi. *Studies in Mycology* 36: 1-80.

Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, Han B, Zhu T, Zou G and Katagiri F. 2003. Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15(2): 317-330.

Thach T, Ali S, De Vallavieille-Pope C, Justesen AF and Hovmøller MS. 2016. Worldwide population structure of the wheat rust fungus *Puccinia striiformis* in the past. *Fungal Genetics and Biology* 87: 1-8.

Tollenaar H. 1967. A comparison of *Puccinia striiformis* f. sp. *poae* on bluegrass with P. *striiformis* f. sp. *tritici* and f. sp. *dactylidis*. *Phytopathology* 57: 418-420.

Visser B, Herselman L and Pretorius ZA. 2009. Genetic comparison of Ug99 with selected South African races of P*. graminis* f. sp. *tritici*. *Molecular Plant Pathology* 10: 213-222.

Visser B, Herselman L and Pretorius ZA. 2016. Microsatellite characterisation of South African *P. striiformis* races. *South African Journal of Plant and Soil* 33: 161-166.

Visser B, Herselman L, Park RF, Karaoglu H, Bender CM and Pretorius ZA. 2011. Characterization of two new *Puccinia graminis* f. sp. *tritici* races within the Ug99 lineage in South Africa. *Euphytica* 179: 119-127.

Voegele RT, Hahn M and Mendgen K. 2009. The Uredinales: cytology, biochemistry, and molecular biology. In: *The Mycota*, 5 (Deising HB, ed). Berlin, Springer/Heidelberg, pp. 69-98.

Walter S, Ali S, Kemen E, Nazari K, Bahri BA, Enjalbert J, Hansen JG, Brown JKM, Sicheritz-Pontén, Jones J, De Vallavieille-Pope C, Hovmøller MS and Justesen AF. 2016. Molecular markers for tracking the origin and worldwide distribution of invasive strains of *Puccinia striiformis*. *Ecology and Evolution* 6(9): 2790-2804. doi: 10.1002/ece3.2069

Wan A and Chen X. 2014. Virulence characterization of *Puccinia striiformis* f. sp. *tritici* using a new set of *Yr* single-gene line differentials in the United States in 2010. *Plant Disease* 98: 1534-1542.

Wan AM, Chen XM and Yuen J. 2016. Races of *Puccinia striiformis* f. sp. *tritici* in the United States in 2011 and 2012 and comparison with races in 2010. *Plant Disease* 100: 966-975.

Wang MN and Chen XM. 2013. First report of Oregon grape (*Mahonia aquifolium*) as an alternate host for the wheat stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*) under artificial inoculation. *Plant Disease* 97: 839.

Wang MN and Chen XM. 2015. Barberry does not function as an alternate host for *Puccinia striiformis* f. sp. *tritici* in the U.S. Pacific Northwest due to teliospore degradation and barberry phenology. *Plant Disease* 99: 1500-1506.

Wang MN, Wan AM and Chen XM. 2015. Barberry as alternate host is important for *Puccinia graminis* f. sp. *tritici* but not for *Puccinia striiformis* f. sp. *tritici* in the U.S. Pacific Northwest. *Plant Disease* 99: 1507-1516.

Wanyera R, Kinyua M, Jin Y and Singh R. 2006. The spread of stem rust caused by *Puccinia graminis* f. sp. *tritici*, with virulence on *Sr31* in wheat in Eastern Africa. *Plant Disease* 90: 113.

Watson IA and Singh D. 1952. The future for rust resistant wheat in Australia. *Journal of the Australian Institute of Agricultural Science* 18: 190-197.

Wellings CR. 2007. *Puccinia striiformis* in Australia: A review of the incursion, evolution and adaptation of stripe rust in the period 1979–2006. *Australian Journal of Agricultural Research* 58: 567-575.

Wellings CR, McIntosh RA and Walker J. 1987. *Puccinia striiformis* f. sp. *tritici* in Eastern Australia possible means of entry and implications for plant quarantine. *Plant Pathology* 36(3): 239-241.

Wiethölter N, Horn S, Reisige K, Beike U and Moerschbacher BM. 2003. In vitro differentiation of haustorial mother cells of the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*, triggered by synergistic action of chemical and physical signals. *Fungal Genetics and Biology* 38: 320-326.

Wolday A, Fetch T, Hodson D, Cao W and Briere S. 2011. First report of *Puccinia graminis* f. sp. *tritici* races with virulence to wheat stem rust resistance genes *Sr31* and *Sr24* in Eritrea. *Plant Disease* 95: 1591-1591.

Wolter M, Hollricher K, Salamini F and Schulze-Lefert P. 1993. The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defense mimic phenotype. *Molecular Genetics and Genomics* 239: 122-128.

Yu LX, Barbier H, Rouse MN, Singh S, Singh RP, Bhavani S, Huerta Espino J and Sorrells ME. 2014. A consensus map for Ug99 stem rust resistance loci in wheat. *Theoretical and Applied Genetics* 127: 1561-1581.

Zadoks JC. 1979. Strategies in combating cereal diseases in Europe, with special reference to yellow rust of wheat. In: *Proceedings of the Rice Blast Workshop*, Laguna, Philippines, pp. 183-198.

Zhao J, Wang M, Chen X and Kang Z. 2016. Role of alternate hosts in epidemiology and pathogen variation of cereal rusts. *Annual Review of Phytopathology* 54(1): 207-228.

Zohary D. 1969. The progenitors of wheat and barley in relation to domestication and agricultural dispersal in the Old World. In: *The domestication and exploitation of plants and animals* (Ucko PJ and Dimbley GW, eds). Duckworth, London, UK, pp. 47-66.

Zurn JD, Dugyala S, Borowicz P, Brueggeman R and Acevedo M. 2015. Unraveling the wheat stem rust infection process on barley genotypes through relative qPCR and fluorescence microscopy. *Phytopathology* 105: 707-712.

# Chapter 2: Histological assessment of adult plant resistance to stripe rust in Kariega x Avocet S doubled haploid lines

## INTRODUCTION

Stripe rust, caused by *Puccinia striiformis* Westend. f. sp. *tritici* (*Pst*), is a serious threat to the production of wheat (*Triticum aestivum* L.) worldwide. Since the first detection of stripe rust in South Africa in 1996 (Pretorius *et al.*, 1997), it has spread from the Western Cape to all wheat producing areas and has become endemic to the country (Pretorius *et al.*, 2007).

Genetic resistance continues to be the most efficient and preferred stripe rust control measure (Knott, 1989; Chen, 2005), with increasing emphasis on durable resistance to support sustainability of productive cultivars, effective resistance sources and breeding.

Seedling or all stage resistance (ASR), conferred by a single major resistance gene, is usually race-specific (Boyd, 2005) and easily defeated by new pathogenic races which can arise through mutation or recombination (Knott, 1989). Races are also regularly introduced to new regions by means of migration (Ali *et al.*, 2014). Adult plant resistance (APR) on the other hand, provides potentially durable sources of resistance (Johnson, 1988) and expression develops gradually at later growth stages (Johnson, 1992; McIntosh, 1992) by slowing down the development of the pathogen, rather than completely preventing infection (Singh *et al.*, 2005). It was proposed that the effects of durability might be further prolonged by combining multiple genes with additive and/or complementary resistance features, resulting in new wheat varieties with more intricate genetic protection (McIntosh, 1992; Boyd, 2005).

A targeted research effort was initiated in 1998 with the aim of analysing stripe rust resistance in South Africa. The project involved a cross between Kariega, a high yielding South African bread wheat cultivar exhibiting complete APR to stripe rust, and the fully susceptible Australian cultivar, Avocet S (Prins *et al.*, 2005). With the aim of better comprehending the genetics of Kariega, a doubled haploid (DH) wheat population of 150 lines was developed from the $F_1$ using the wheat-maize pollination procedure (Ramburan *et al.*, 2004; Prins *et al.*, 2005). Thorough genome coverage was obtained following the mapping approach of simple sequence repeats as anchor

loci in conjunction with amplified fragment length polymorphisms (Prins *et al.*, 2005) and a set of Diversity Array Technology (DArT) markers for the extended population size of 254 DH lines (Prins *et al.*, 2011).

After genetic mapping of field and marker data, Ramburan *et al.* (2004) identified quantitative trait loci (QTL) on three chromosomes mediating APR to stripe rust in Kariega. Main effect QTL were traced to chromosomes 2B (*QYr.sgi-2B.1* and *-2B.2*) and 7D (*QYr.sgi-7D*) along with a minor QTL, *QYr.sgi-4A.1*, detected on chromosome 4A.

Growth of the stripe rust pathogen was similar in Kariega and Avocet S up to 5 days post inoculation (dpi), but increased exponentially only in the susceptible line at 7 dpi (Moldenhauer *et al.,* 2006). Furthermore, the collective effect of the major and minor QTL was observed when lignified tissue, induced at 4 dpi in Kariega, completely surrounded the fungal colonies by 6 dpi in the resistant line. At the same time, long, unbranched fungal hyhae outgrew isolated lignified host cells in the susceptible line.

The effect of individual QTL in six DH lines varying in their resistance genotypes was studied by Moldenhauer *et al.* (2008). Histological analysis indicated that the two major QTL differed qualitatively in their interaction with *Pst*. Upon infection, *QYr.sgi-2B.1* appeared to conveyed a chlorotic and/or necrotic response in the host plant, although too slow to effectively prevent fungal growth. Klement (1982) and He (1996) described this host response as normosensitive plant cell death which is not associated with local resistance. Additionally, lignification was induced in a Kariega x Avocet S DH line (coded to mapping population [MP]) 51 (containing only *QYr.sgi-2B.1*), but also failed to restrict fungal growth. The contribution of *QYr.sgi-2B.1* to stripe rust resistance conditioned by Kariega is higher in host infection type (33–46%) than in disease severity (17–30%) (Ramburan *et al.*, 2004). Conversely, MP 35 (*QYr.sgi-7D*) restricted *Pst* growth without lignified host tissue. The QTL on chromosome 7D was identified as the APR *Lr34/Yr18/Sr57* gene complex (Krattinger *et al.*, 2009; Prins *et al.*, 2011), and appeared to convey resistance that does not involve necrosis (Moldenhauer *et al.*, 2008). It was suggested that resistance responses, other than lignification and necrosis, are simultaneously and perhaps synergistically induced which contributes to the stripe rust APR in Kariega (Kang *et al.*, 2015).

*QYr.sgi-4A.1* accounted for a slow rusting response during early stages of disease, but lost its importance as the disease progressed (Moldenhauer *et al.*, 2008; Prins *et al.*, 2011). It explained 28% of the disease severity and 14% of the host reaction type. Moreover, evidence suggests that *QYr.sgi-4A.1* is only effectively expressed under field conditions (Prins *et al.*, 2011) since it was previously undetected in growth chamber (in dwarf adult plants) (Ramburan *et al.*, 2004) and controlled greenhouse conditions (Moldenhauer *et al.*, 2008) and subsequently associated with little phenotypic variation in greenhouse tests (Prins *et al.*, 2011).

The aim of this project was to investigate the wheat host-*Pst* pathogen interaction at a cellular level to obtain a better understanding of resistance gene expression. This continuance of studies by Moldenhauer *et al.* (2006, 2008) will account for a wider range of MP entries representing all possible combinations of Kariega APR. In addition, the interacting effects of different QTL and/or gene will be evaluated under field grown conditions. It was proposed that combinations of resistance loci in a single wheat variety will provide higher levels of stripe rust resistance.

## MATERIALS AND METHODS

### Plant and pathogen materials

Sixteen wheat lines (Table 2.1) were selected from a DH population derived from a cross between parental lines Kariega (pedigree: SST44// K4500.2/SapsuckerS) and Avocet S (pedigree: Thatcher-*Ag. elongatum*/3*Pinnacle//WW15/3/Egret) (Prins *et al.*, 2005). The lines were selected based on the different stripe rust resistance QTL/gene combinations (regarding *QYr.sgi-2B.1*, *QYr.sgi-4A.1* and *Yr18*) derived from Kariega (Ramburan *et al.*, 2004; Prins *et al.*, 2011).

For the purpose of the present study, leaf material sampled from field trials in 2011 and 2012 was analysed. However, the entire population was also grown in other seasons and data from 2006 and 2014 (obtained from Z.A. Pretorius, Plant Sciences, UFS) will be reviewed for relationships over years. Trials were planted in rust screening nurseries at field sites of Pannar Seed (Pty) Ltd. near Greytown, KwaZulu-Natal, South Africa.

Entries were planted in single 1-m rows, spaced 76 cm apart. To facilitate sufficient inoculum development within the nursery, rows of stripe rust spreader (a mixture of Morocco and line JIC871) were planted in pathways at right angles to plots and as every tenth entry in each trial.

Emerging seedlings (cultivar Morocco) planted for inoculum multiplication under greenhouse conditions, were drenched with 99% maleic hydrazide Reagent Plus® [Sigma-Aldrich (0.3 g/L water, 50 mL per 10-cm pot)]. Seven days after planting, urediniospores of *Pst* pathotype 6E22A+ were retrieved from storage at -80°C and suspended in Soltrol® 130 (Phillips Petroleum, Bartlesville, OK, USA) isoparaffinic solvent in a gelatin capsule. The spore suspension was sprayed onto seedlings using a pressure pump connected to a custom-made inoculation device (Browder, 1971), followed by a 1 h incubation period at 25°C. Dried off seedlings were incubated at 10°C in a high humidity (> 96%) chamber for a 24 h dew period. Thereafter, a temperature of 17°C was maintained when seedlings were placed under fluorescent lights in a growth cabinet for 2 h before being returned to the greenhouse. When sporulation on seedlings was sufficient, fresh urediniospores were collected for inoculation of spreader rows (tillering stage) in the field (Zadoks *et al.*, 1974).

Using an ultra-low volume sprayer, spreaders were inoculated with *Pst* spores suspended in Soltrol® 130. Following inoculation, several 1-m sections of spreader plants were covered with plastic sheeting overnight to provide high humidity for maximum infection. Phenotypic data were documented on 9 October 2006, 3 October 2011, 8 October 2012 and 2 October 2014. Leaf tissue was sampled on 3 October 2011 and 28 September 2012.

**Phenotypic analysis**

Mean flag leaf severity and adult plant reaction type per test line were recorded during 2011 and 2012, and compared with stripe rust scores of 2006 and 2014. Disease severity was rated in accordance with the modified Cobb scale (Peterson *et al.*, 1948) where the numerical value corresponds to the percentage flag leaf area infected (LAI) visible as either sporulating uredinia or chlorotic and/or necrotic leaf tissue. For convenience, a LAI of 1% was assigned in the case of trace infection while 100% being the entire flag leaf rusted. Accompanying host reaction types (RT) specify resistance (R), moderate resistance (MR), moderate susceptibility (MS), and susceptibility (S).

A coefficient of infection (CI) was calculated by multiplying disease LAI and constant values assigned to host RT, namely: R=0.2, MR=0.4, MRMS=0.6, MS=0.8 and S=1.0 (Stubbs *et al.*, 1986), in addition to intermediate categories RMR=0.3 and MSS=0.9.

**Fluorescence microscopy**

After phenotypic evaluation of the test lines during the 2011 and 2012 cropping seasons, 3-5 cm long segments were cut from three flag leaves representative of each line. Leaf segments were fixed and cleared in ethanol:dichloromethane (3:1, v/v) containing 0.15% (w/v) trichloroacetic acid for 24 h.

The Uvitex stain for fungal structures and host cell reaction was based on a modified Rohringer *et al.* (1977) procedure. Specimens were drenched twice in 50% ethanol for 15 min, twice for 15 min in 0.05 M sodium hydroxide, and washed down three times with distilled water. Leaf segments were then immersed in 0.1 M Tris (hydroxymethyl) aminomethane/hydrochloric acid buffer (pH 8.5) and stained for 5 min in 0.1% (w/v) Uvitex 2B (Ciba-Geigy, Syngenta) in the preceding buffer (Niks and Dekens, 1987; Moldenhauer *et al.*, 2006). Samples were rinsed four times with water before saturated with 25% (v/v) aqueous glycerol for 30 min. Stained leaves were stored in 50% (v/v) glycerol with a trace lactophenol to prevent deterioration of fungal material and drying of tissue.

Variables studied by means of fluorescence microscopy to measure resistance, entailed the size of individual *Pst* colonies, whether infection sites were associated with host cell necrosis (HCN, an indication of the hypersensitivity reaction), and the presence of other mechanisms of resistance e.g. lignification associated with resistance loci. To compare the degree of *Pst* colonisation among the DH lines, the number of haustorial mother cells (HMC) per fungal colony in addition to the length (µm) of individual colonies growing parallel to the veins within the flag leaf of each line were quantified. Observations on 60 infection sites per DH line (20 sites on each of three leaf samples) were carried out with an Olympus AX70 microscope (Tokyo, Japan) magnifying from X4 up to X100. The filter combination UV-1A (330–380 nm excitation filter and 420 nm barrier filter) was used to observe fungal structures, while HCN associated with fungal colonies were observed and measured using the B-2A filter combination (excitation filter 450–490 nm and barrier filter 520 nm). Images of

fungal structures and HCN were captured using a CC12 camera and AnalySIS LS Research version 2.2 software (Olympus Soft Imaging System, Japan).

The hypersensitivity index (H-index) was calculated by dividing the length of necrosis with the colony length. An H-index greater than one will indicate that necrosis is larger than the fungal colony, which possibly inhibits fungal growth to some extent (Kloppers and Pretorius, 1997).

Lignification was tested in leaves pre-stained with Uvitex 2B according to the Wiesner test (Sherwood and Vance, 1976; Moldenhauer *et al.*, 2008). An S-7 England Finder slide (SPI Supplies, Structure Probe, Inc.) was used to identify the exact location of infection sites where fungal structures and HCN were observed to capture images of subsequent lignin stain. Samples were immersed in 2% phloroglucinol (diluted in 96% ethanol) for 1-2 h. A drop of HCl (37% in 2011, 30% for 2012 material) was added to the leaf specimen, after which the slide was gradually heated to 25°C on a hot plate until the veins turned red in colour. Leaf segments were then gently washed down with distilled water before microscopic inspection of red lignin colouration. Specimens were examined for any positive phloroglucinol/HCl-test (i.e. formation of red colour) including intensities through transmitted light microscopy using the X20 to X60 magnification on the Olympus AX70 microscope.

Only the 2012 leaf material was used for quantification of HCN (and subsequently H-index) and the qualitative analysis of lignification. HMC up to 30 were counted per colony for the 2012 cropping season.

**Statistical analysis**

Stripe rust data for the DH lines and parents were evaluated with analysis of variance (ANOVA) using General Linear Models (GLM) in NCSS 2007. The effects of genotypes and cropping seasons were accounted for in the model for analysis of phenotypic CI. In addition, the impact of biological replicates was included for the assessment of histological parameters (number of HMC and colony length). The effects of genotypes and biologic replicates were considered in H-index analysis. Calculated values, according to Fisher's protected least significant difference (LSD), were added to histograms where applicable.

Correspondence of data gathered across years were assessed using the Linear Regression and Correlation tool in NCSS 2007. Spearman's rank correlation ($r_s$) was used for evaluation of parameters quantified across different techniques.

## RESULTS

### Phenotypic analysis

CI values enabled the quantitative analysis of the combined LAI of the pathogen and host RT, documented over four years (Table 2.1). ANOVA results (Addendum 2.1) indicated highly significant differences in CI values among DH lines ($P<0.05$). Despite variation detected across cropping seasons in ANOVA, CI values were highly parallel with correlation factors calculated above 0.95 amongst the four cropping seasons.

**Table 2.1:** Leaf area infected and host reaction type of the parental lines and selected Kariega x Avocet S doubled haploid (MP) lines inoculated with *Puccinia striiformis* f. sp. *tritici* during four seasons.

| Doubled haploid line | QTL/gene | LAI and host RT | | | |
|---|---|---|---|---|---|
| | | 2006 | 2011 | 2012 | 2014 |
| Kariega * | | TR | TR | TR | 0R |
| MP 45 | 2B+4A+*Yr18* | TR | TR | TR | 0R |
| MP 65 | 2B+4A+*Yr18* | 0R | TR | TR | 0R |
| MP 10 | 2B+4A | 10RMR | 10R | 5R | N/A |
| MP 70 | 2B+4A | 5R | TRMR | TR | TR |
| MP 22 | 2B+*Yr18* | 5R | TR | TR | 0R |
| MP 49 | 2B+*Yr18* | 0R | 5R | TR | 0R |
| MP 68 | 4A+*Yr18* | 0R | 5R | TR | 0R |
| MP 108 | 4A+*Yr18* | TR | 20MS | 40MS | 0R |
| MP 51 | 2B | 40MR | 10R | 5R | 10MR |
| MP 142 | 2B | 60MR | 20R | 15R | TR |
| MP 148 | 4A | 80MSS | 60MS | 80MSS | 30S |
| MP 152 | 4A | 60MR | 40MR | 30MS | 20MS |
| MP 35 | *Yr18* | 15RMR | 40RMR | 20MR | 15R |

| | | | | | |
|---|---|---|---|---|---|
| MP 223 | *Yr18* | 15R | 10R | 5R | 0R |
| MP 16 | None | 100S | 90S | 100S | 100S |
| MP 145 | None | 100S | 100S | 100S | 90S |
| Avocet S * | | 100S | 90S | 100S | 90S |

* Parents of double haploid population.
QTL = Quantitative trait loci where 2B = *QYr.sgi-2B.1* and 4A = *QYr.sgi-4A.1*.
LAI = Leaf area infected (%) indicated by numerical value where T = Trace infection.
RT = Reaction type of host plant where R = Resistant, MR = Moderately Resistant, MS = Moderately Susceptible and S = Susceptible.

Similar to Kariega, all DH lines containing two or more resistance loci, except MP 108 carrying *QYr.sgi-4A.1+Yr18*, displayed very low CI values (< 5) across all four cropping seasons (Figure 2.1). Comparable resistance was observed for one of the entries containing *Yr18* (MP 223). Moreover, test lines containing at least one resistance QTL/gene, except carriers of a single *QYr.sgi-4A.1* (MP 148 and MP 152), had CI values below 10 for at least two of the appraised years. This was due to much higher LAI scores and less resistant host RT during 2011 and 2012 for a *QYr.sgi-4A.1+Yr18* entry and during 2006 for both carriers of *QYr.sgi-2B.1*, contrary to other cropping seasons. The susceptible parent Avocet S as well as MP 16 and MP 145 (lines with no resistance alleles) showed fully compatible symptoms and signs with slightly less LAI (90%) documented in 2011 and/or 2014.

Comparing infection over cropping seasons, entries with more substantial CI ratings (above 10), generally had slightly higher CI scores in 2006 and/or 2012 than in 2011 and 2014. Results were similar for 2011 and 2012 (field trials analysed in depth for this study) confirmed by a $R^2$-value of 0.94 for both LAI and host RT (Addendum 2.2).

Individual stripe rust resistance QTL/gene on chromosomes 2B, 4A and 7D had diverse plant responses in the 2012 field trial, as observed for MP 51, MP 152 and MP 35 DH lines, respectively (Figure 2.2). Matching TR scores were documented for lines containing three loci and most of the two-loci combinations, such as *QYr.sgi-2B.1+QYr.sgi-4A.1* in MP 70 and *QYr.sgi-4A.1+Yr18* in MP 68 (Figure 2.2F and G, respectively), while line MP 145 containing no resistance allele, responded similarly to the susceptible parent Avocet S (Figure 2.2H and B, respectively).

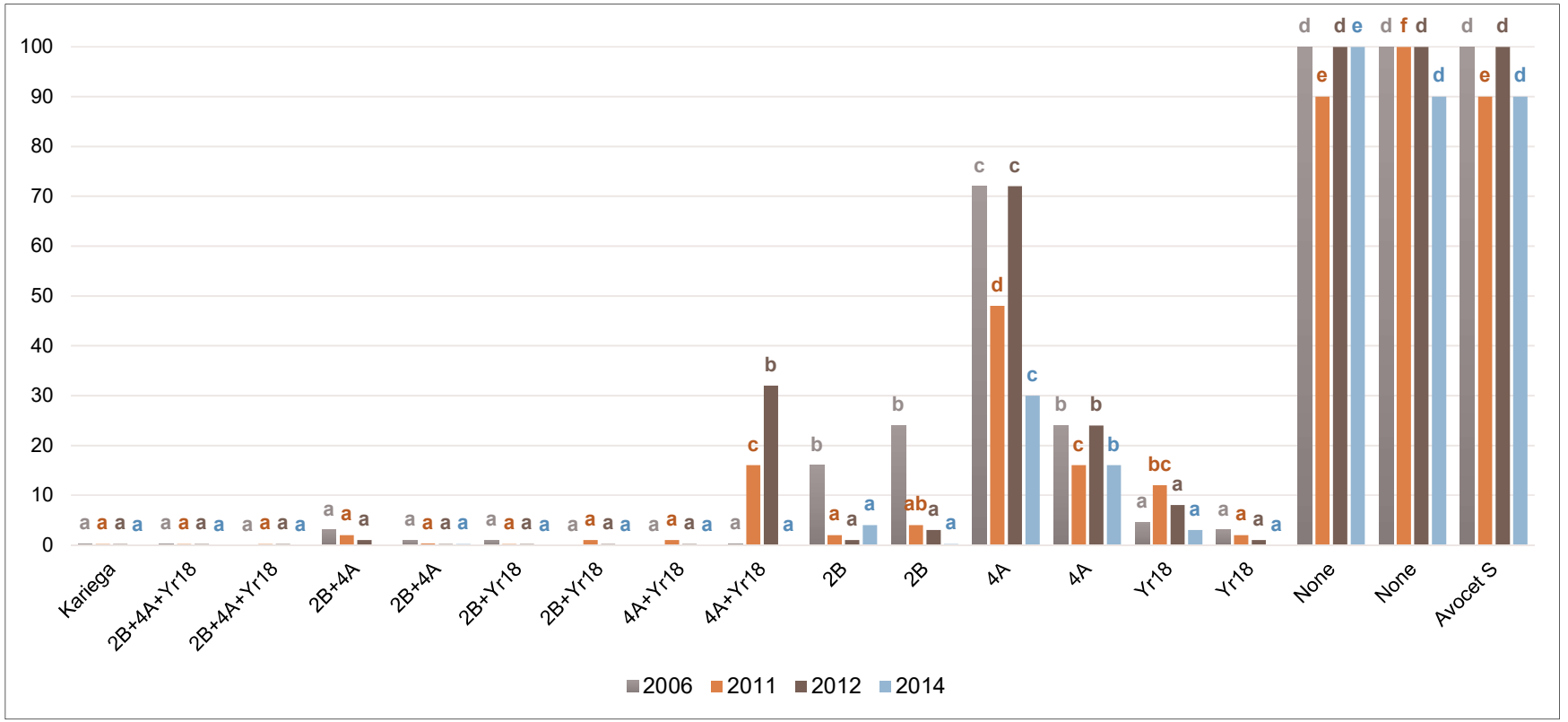



**Figure 2.1:** Coefficient of infection of parents (Kariega and Avocet S) and doubled haploid lines containing different combinations of stripe rust resistance quantitative trait loci 2B = *QYr.sgi-2B.1*, 4A = *QYr.sgi-4A.1* and gene *Yr18*. Data was recorded during four cropping seasons post inoculation with *Puccinia striiformis* f. sp. *tritici*. Least significant difference (LSD) between lines = 9.74, where means with the same LSD symbol do not differ significantly (P>0.05 ANOVA, Addendum 2.1).

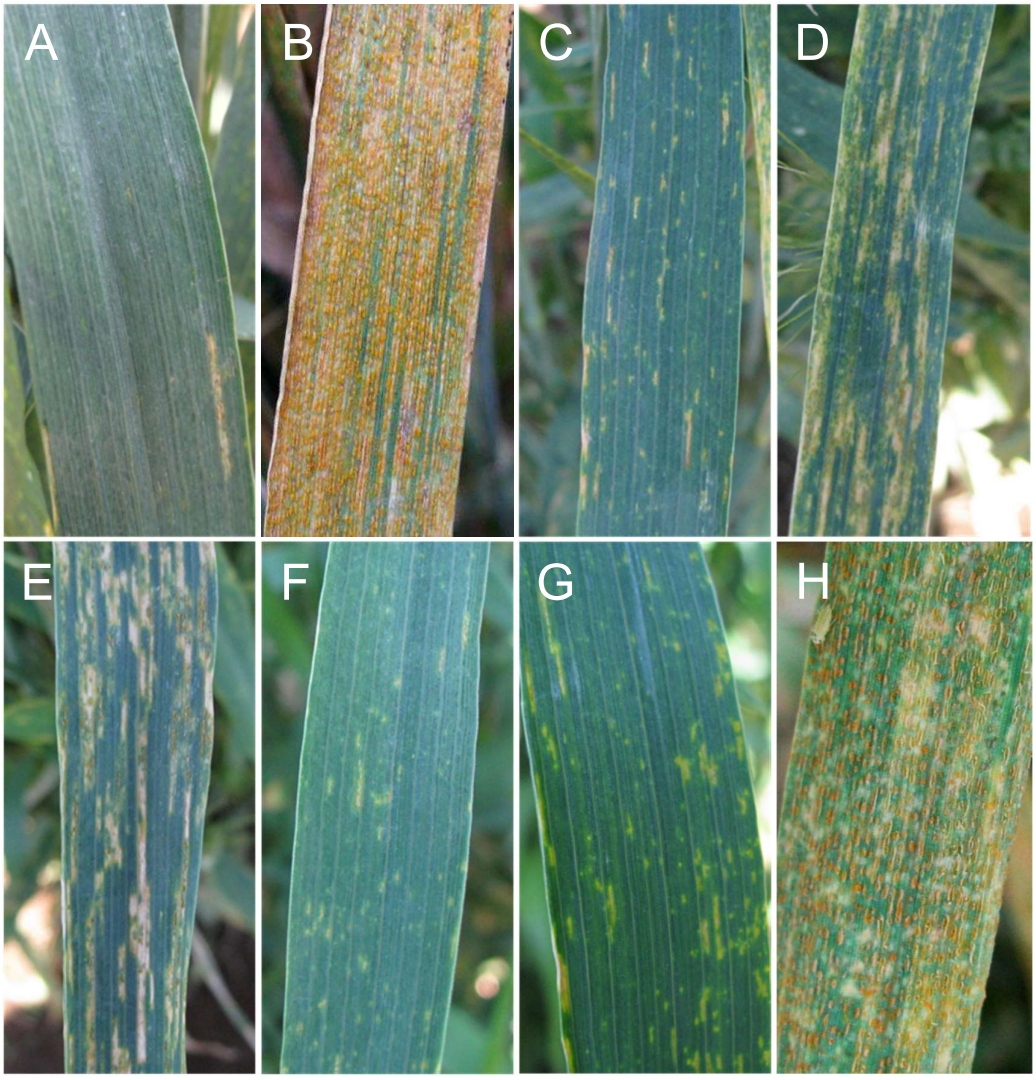



**Figure 2.2:** Adult field host response to stripe rust infection of parental wheat lines **A)** Kariega (TR), **B)** Avocet S (100S) and of doubled haploid lines carrying adult plant resistance QTL/gene **C)** 2B (5R), **D)** 4A (30MS), **E)** *Yr18* (20MR), **F)** 2B+4A (TR), **G)** 4A+*Yr18* (TR), and **H)** none (100S). QTL = Quantitative trait loci where 2B = *QYr.sgi-2B.1* and 4A = *QYr.sgi-4A.1*.

**Fluorescence microscopy**

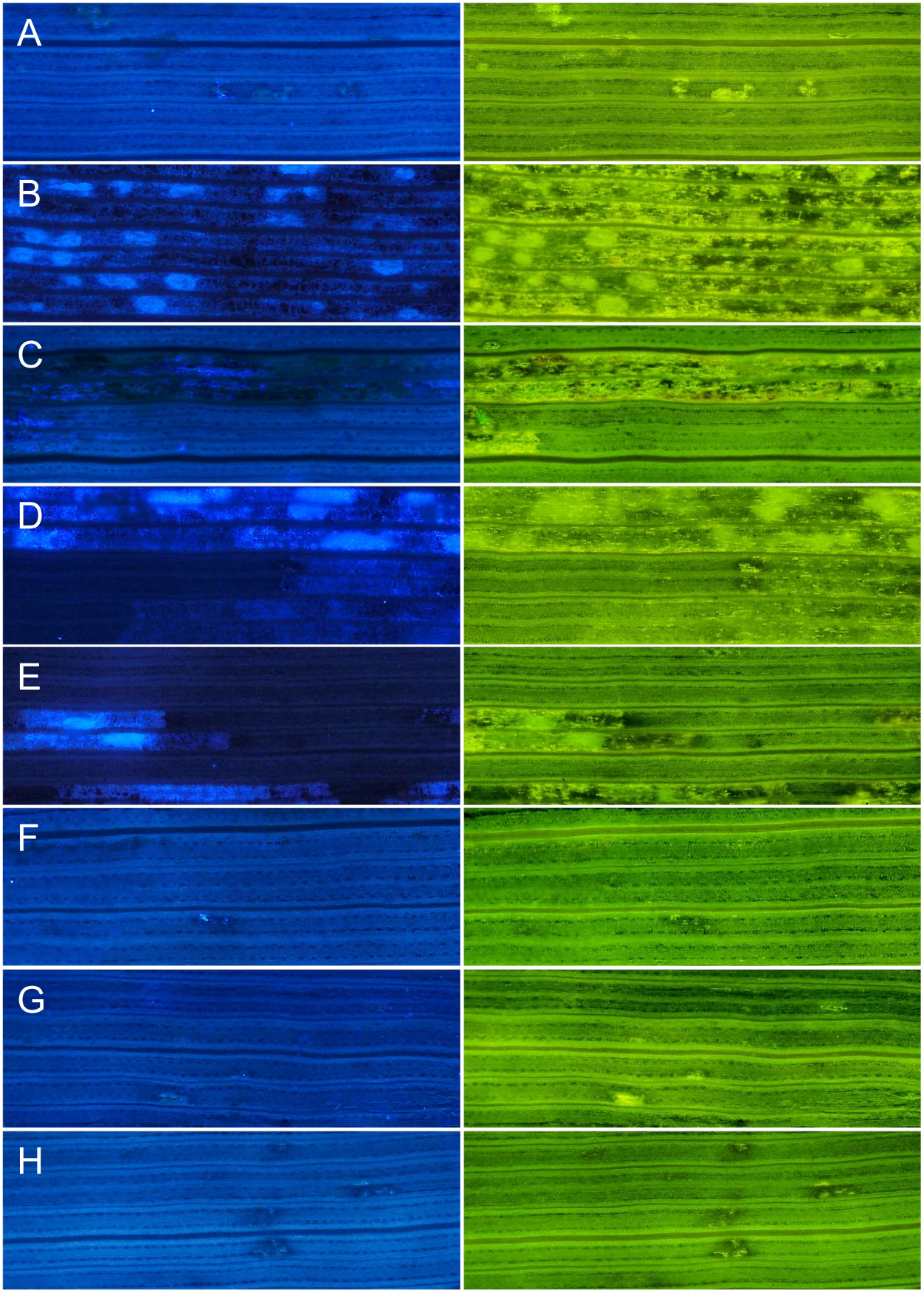
*Pst* detection was enabled by Uvitex 2B fluorochrome binding to chitin in the fungal cell wall (Coleman *et al.*, 1989) and resulting in the light blue fluorescence of infection structures. Although haustoria in the plant mesophyll cells were not visible, HMC and infection hyphae networks fluoresced extremely bright (left in Figure 2.3). HCN associated with fungal colonies appeared light green to yellow in colour (right in Figure 2.3).

At the time of sampling, sporulation was observed exclusively for the more susceptible lines, i.e. carriers of single *QYr.sgi-4A.1* (MP 148 and MP 152), one of the entries containing solely *Yr18* (MP 35), a *QYr.sgi-4A.1+Yr18* combination (MP 108), lines with no QTL/gene (MP 16 and MP 145) and the susceptible parent, all of which exhibited significant ranges in colony lengths. The large colonies, likely the result of initial infection, were however extensively coalesced which complicated reliable quantification of individual colony sizes and number of HMC per colony. Thus, only smaller assessable infection points were considered for these entries.

Slight overlapping of colonies also occurred in MP 51 and MP 142 (carrying solely *QYr.sgi-2B.1*) ensuing the same approach followed with the much smaller but noticed range in size of colonies. Conversely, a very narrow scope of colony lengths was observed for the remaining lines, i.e. the resistant parental line, entries carrying two QTL with *Yr18* (MP 45 and MP 65), most combinations of two loci (MP 10, MP 70, MP 22, MP 49 and MP 68) and MP 223 carrying a single *Yr18*.

Regarding number of HMC and colony length, ANOVA (Addendums 2.3 and 2.4, respectively) indicated very little variation between the 2011 and 2012 cropping seasons as well as among leaf replicates ($P>0.05$). However, highly significant differences were detected among DH lines in addition to a statistically significant interaction between test lines and cropping seasons ($P<0.05$).

Trends in number of HMC per colony and colony lengths were similar for 2011 and 2012 (Figure 2.4A and B, respectively) with a 0.83 repeatability calculated for number of HMC and 0.97 for *Pst* colony lengths.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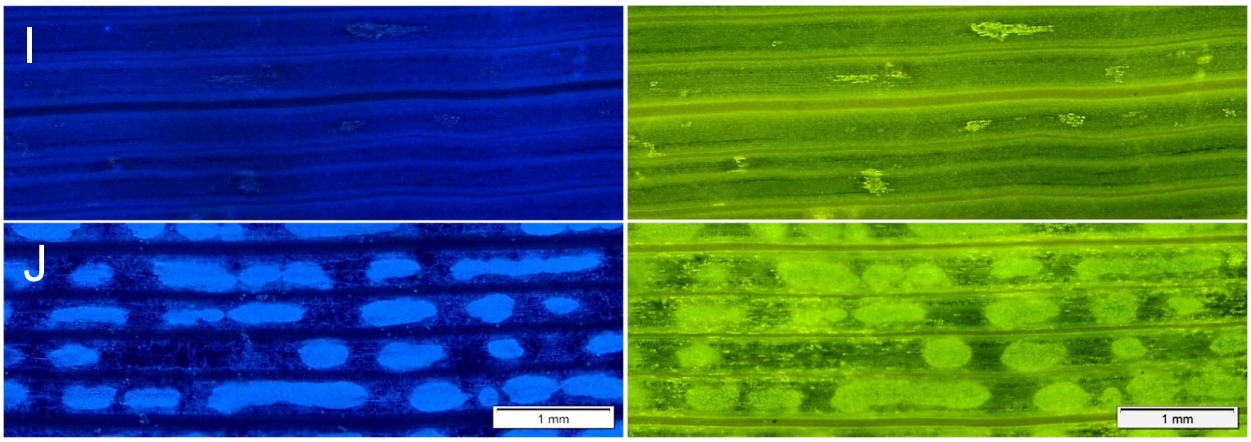
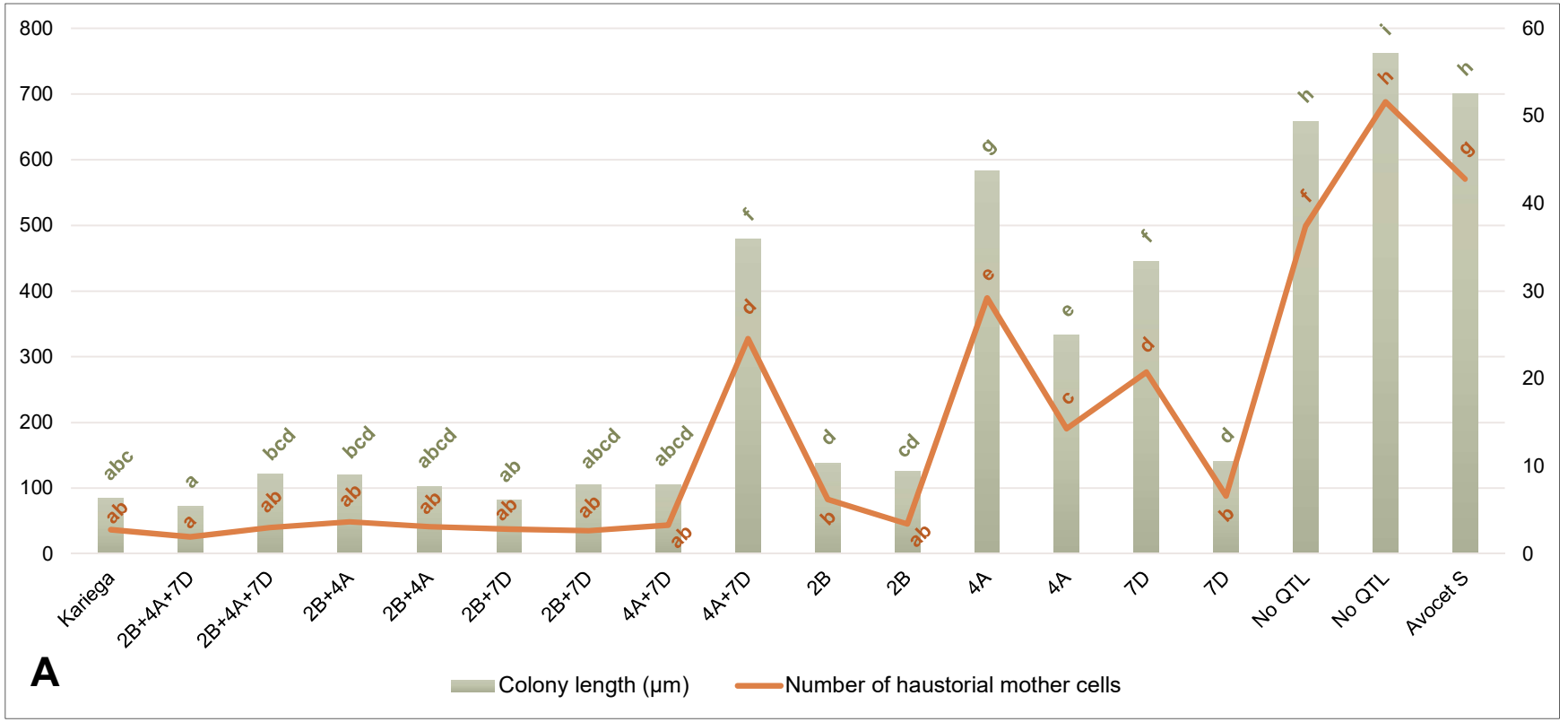



**Figure 2.3:** *Puccinia striiformis* f. sp. *tritici* fungal colonies (left) and associated host cell necrosis (right) following inoculation of a doubled haploid wheat mapping population (MP) varying in the resistance QTL/gene they contain. Parental lines **A)** Kariega (resistant) and **B)** Avocet S (susceptible) of MP lines carrying **C)** 2B in MP 51, **D)** 4A in MP 148, **E)** *Yr18* in MP 35, **F)** 2B+4A in MP 10, **G)** 2B+*Yr18* in MP 22, **H)** 4A+*Yr18* in MP 68, **I)** 2B+4A+*Yr18* in MP 45, and **J)** none in MP 16. Scale bar represents 1 mm on the flag leaf. QTL = Quantitative trait loci where 2B = *QYr.sgi-2B.1* and 4A = *QYr.sgi-4A.1*.

Excluding MP 108 containing the *QYr.sgi-4A.1*+*Yr18* combination, small individual colonies were measured for the resistant parent as well as all entries with more than one loci (smaller than 125 and 140 µm for 2011 and 2012 material, respectively) comprising of less than seven HMC per colony. Slightly larger colonies with less than 10 HMC per colony were observed for both lines carrying a single *QYr.sgi-2B.1* (MP 51 and MP 142) as well as MP 223 carrying solely *Yr18* (colonies smaller than 140 and 220 µm for 2011 and 2012 material, respectively).

MP 108 (*QYr.sgi-4A.1*+*Yr18*), excluded from above mentioned group, exhibited the same degree of resistance as both lines carrying solely *QYr.sgi-4A.1* (MP 148 and MP 152) and one of the entries carrying the single *Yr18* (MP 35) (colony sizes measured between 330 and 580 µm in 2011, and from 400 to 520 µm in 2012).

The susceptible parental line as well as MP 16 and MP 145 (lines with no resistance loci) produced the largest colonies during both cropping seasons (> 660 µm in 2011, > 600 µm in 2012) and more than 30 HMC were counted per colony.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Colony length (µm)
Number of haustorial mother cells

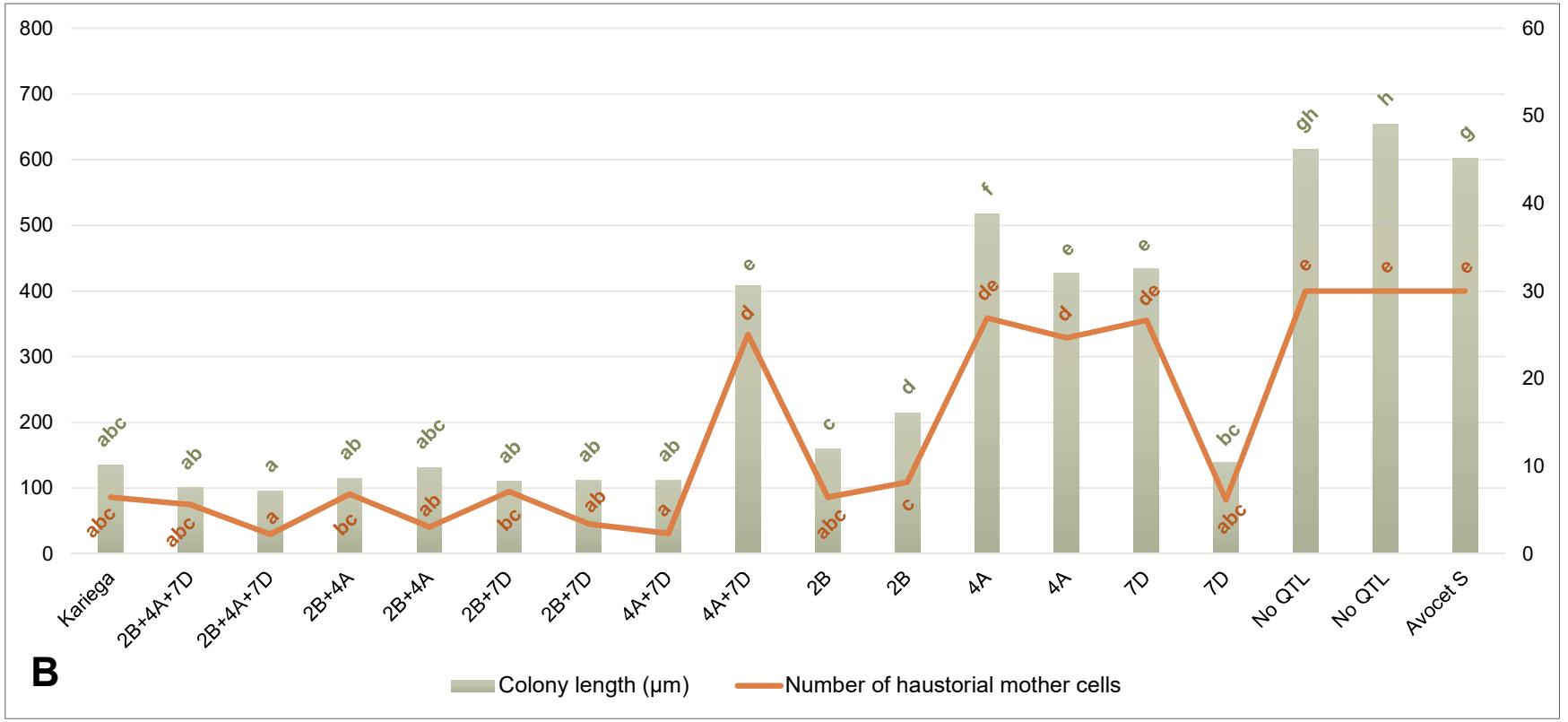



**Figure 2.4:** Average colony length and number of haustorial mother cells of individual *Puccinia striiformis* f. sp. *tritici* colonies quantified on parents (Kariega and Avocet S) and doubled haploid lines carrying different combinations of stripe rust resistance quantitative trait loci 2B = *QYr.sgi-2B.1*, 4A = *QYr.sgi-4A.1* and gene *Yr18*. Material was collected during the **A)** 2011 and **B)** 2012 cropping seasons. Least significant difference (LSD) between colony lengths and number of haustorial mother cells of different lines = 42.53 and 4.28, respectively, i.e. means with the same LSD symbol do not differ significantly from each other (P>0.05 ANOVA, Addendums 2.4 and 2.3, respectively).

In addition, substantial differences were observed between some of the lines containing the same APR locus/loci, which consistently grouped in outer margins of the range set by the LSD value. Considering entries with the *QYr.sgi-4A.1+Yr18* complex, MP 68 only allowed, on average, development of less than four HMC per colony and restricted individual colonies to 110 µm, while MP 108 produced colonies larger than 400 µm and at least 25 HMC per colony during both cropping seasons (Figures 2.3H and 2.5, respectively). Similarly, differences of 300 µm in colony lengths and 15 HMC per colony were observed in both cropping seasons between two lines containing a single *Yr18* (MP 35 and MP 223). In the 2011 cropping season, there was a 250 µm difference in colony lengths and a difference of 15 HMC between carriers of a single *QYr.sgi-4A.1* (MP 148 and MP 152). However, the variation between these two lines was much less in 2012 (90 µm between colony lengths and two HMC per colony).

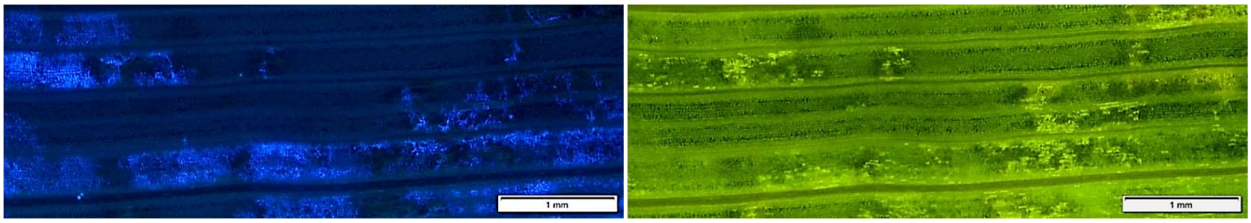



**Figure 2.5:** *Puccinia striiformis* f. sp. *tritici* fungal colonies (left) and associated host cell necrosis (right) of another line carrying the *QYr.sgi-4A.1+Yr18* combination (MP 108). Scale bar represents 1 mm on the flag leaf.

Prominent differences in HCN were observed among lines carrying different resistance combinations (Figure 2.3). Large, fully necrotic areas associated with *Pst* colonies were noted for Kariega and *QYr.sgi-2B.1+Yr18* combination, while the *QYr.sgi-2B.1+QYr.sgi-4A.1+Yr18* combination exhibited distinctly isolated necrotic host cells. Although host response was not completely necrotic for single *QYr.sgi-2B.1* and to lesser extent *Yr18*, necrotic host cells stretched across large areas associated with fungal colonies. Yet it has been reported that the restriction of fungal growth in *Yr18* is not a result of hypersensitive necrosis (Moldenhauer *et al.*, 2008). In contrast, isolated necrotic host cells were rare and sporadic along the length of the fungal colonies on lines carrying the single *QYr.sgi-4A.1*.

ANOVA (Addendum 2.5) indicated that H-index values calculated for 2012 data were significantly different among DH lines ($P<0.05$) with very little variation among the three leaf replicates sampled for each line ($P>0.05$).

The mean H-index values ranged from 0.7 for a line containing a single *QYr.sgi-4A.1*, to 2.5 for the resistant parental line as well as one of the entries containing the single *Yr18* (Figure 2.6). Six lines, including the susceptible parent, displayed an H-index less than or equal to one: MP 108, MP 148, MP 152, MP 35, MP 16 and MP 145. This indicates a smaller necrotic area than *Pst* colony for the most part, which made it unable for the host to inhibit fungal growth, resulting in larger colonies measured for these lines.

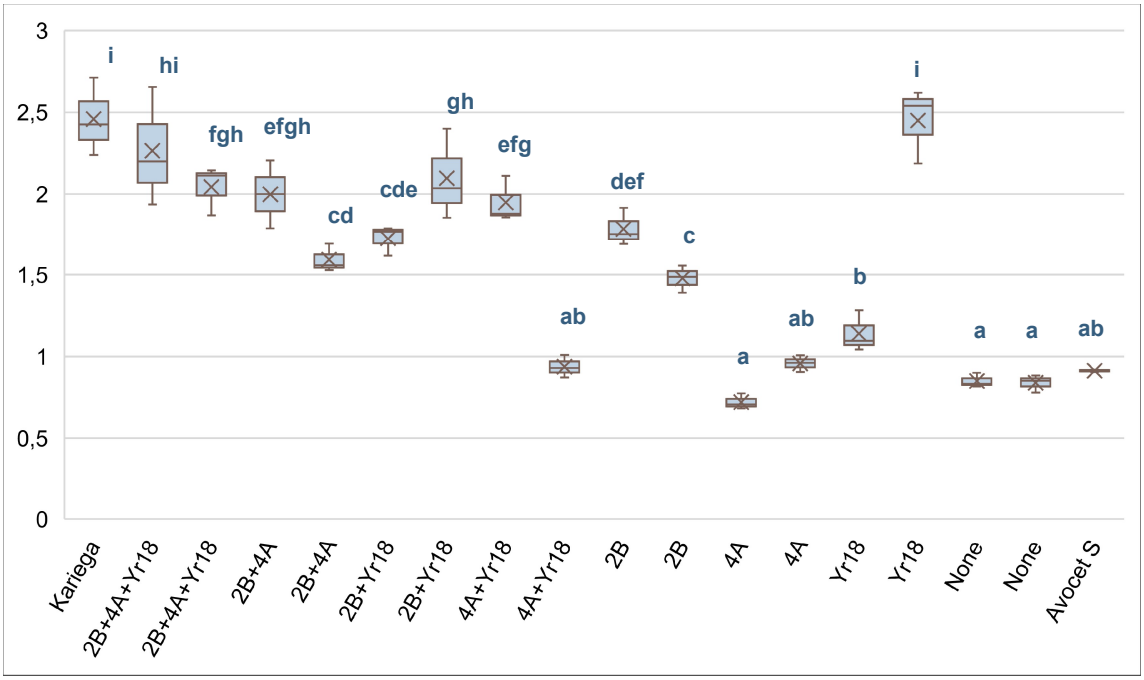



**Figure 2.6:** A box plot representing the hypersensitivity index of a Kariega x Avocet S doubled haploid population during the 2012 cropping season, with lines carrying different combinations of stripe rust resistance quantitative trait loci 2B = *QYr.sgi-2B.1*, 4A = *QYr.sgi-4A.1* and gene *Yr18*. Means are marked with X and error bars indicate data falling outside the upper and lower quartiles. Least significant difference (LSD) between hypersensitivity index means of different lines = 0.29, i.e. entries with the same LSD symbol do not differ significantly from each other ($P>0.05$ ANOVA, Addendum 2.5).

The H-index values correlated with other 2012 histology variables, given that all test lines where *Pst* colonies smaller than 200 µm and each containing less than 10 HMC, resulted in HCN of at least 1.5 times the size of individual colonies. This applied to the resistant parental line, both test lines carrying the single *QYr.sgi-2B.1* QTL, one entry containing the single *Yr18*, as well as the entries carrying more than one loci, excluding MP 108 with the *QYr.sgi-4A.1+Yr18* combination.

Conversely, most of the test lines where more than 25 HMC were counted per individual colony, which was larger than 400 µm in length, had H-index values less than 1. This was true for the susceptible parental line, lines with no resistance loci, carriers of the single *QYr.sgi-4A.1* as well as MP 108 (*QYr.sgi-4A.1+Yr18*). Except for a slightly higher H-index value of 1.14, MP 35 carrying the single *Yr18* can also be included in this group. Additionally, high correlation values (> 0.85) were computed when field data (CI values) and data obtained from histological analysis (number of HMC per colony and colony lengths) were compared (Addendum 2.7).

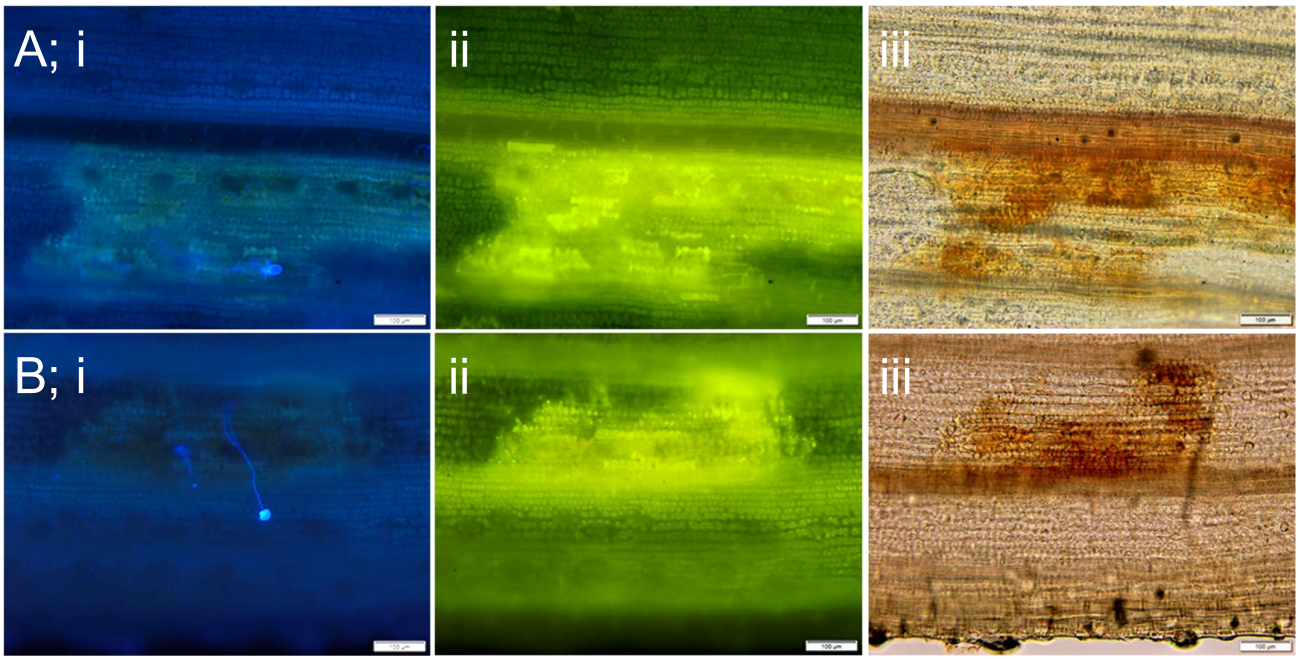



**Figure 2.7:** *Puccinia striiformis* f. sp. *tritici* infection points, host cell necrosis associated with fungal colonies and lignification defence response (i, ii and iii, respectively) of **A)** doubled haploid wheat line MP 51 retaining the single *QYr.sgi-2B.1* QTL responsible for the necrotic response in the host plant, and **B)** resistant parental line Kariega exhibiting complete adult plant resistance. Scale bars represent 100 µm on the flag leaf.

Although not quantified, deposition of lignin or lignin-like material (Figures 2.7 and 2.8) were observed in all lines containing at least one of the Kariega APR. Lignification was pronounced for *QYr.sgi-2B.1* and even more in the resistant parent, Kariega (Figure 2.7A and B, respectively), although single carriers of *QYr.sgi-4A.1* and *Yr18* also showed some degree of lignification (Figure 2.8A and B, respectively). Avocet S and lines with none of the APR loci (MP 16 and MP 145) displayed a negative phloroglucinol/HCl-stain (Figure 2.8C).

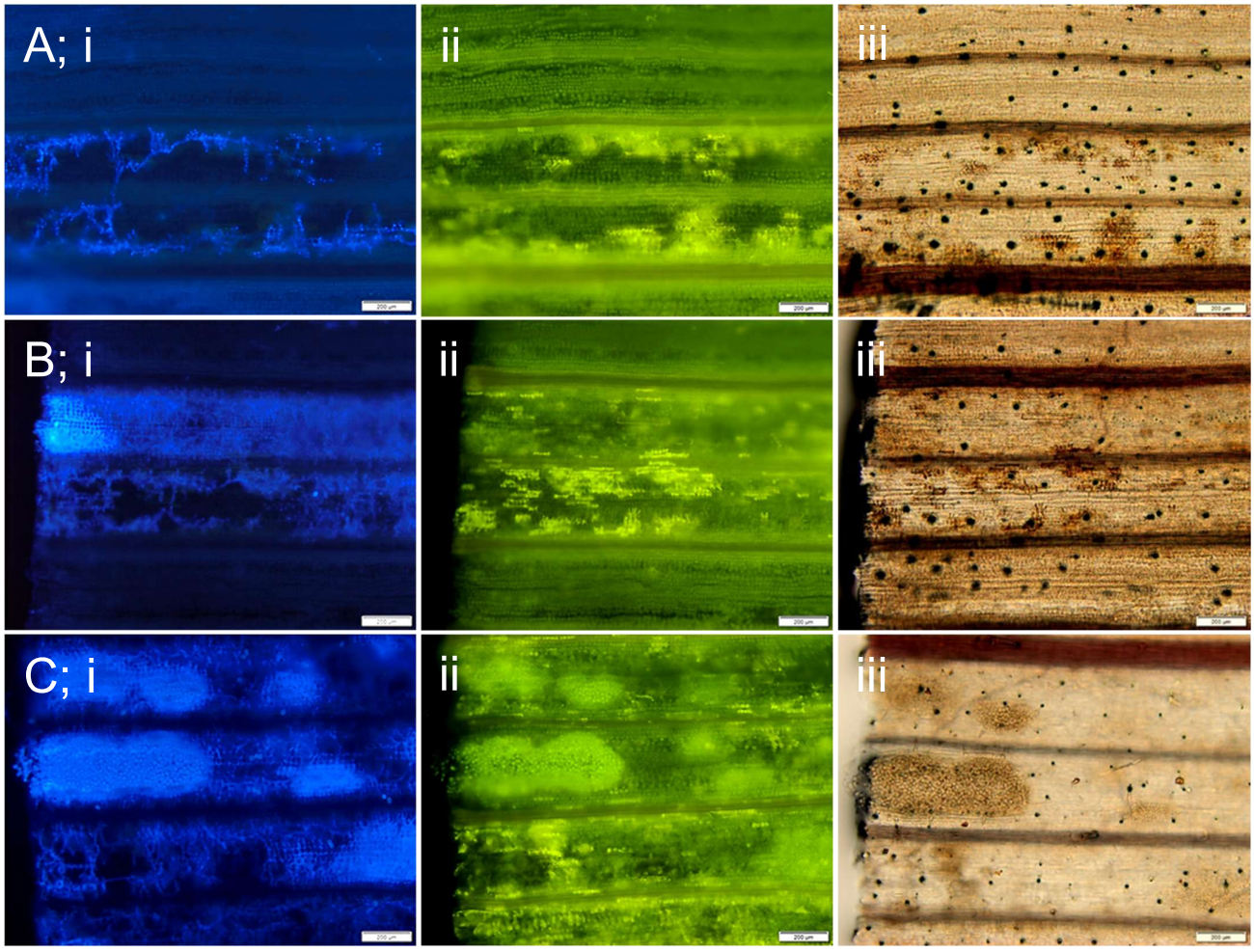



**Figure 2.8:** *Puccinia striiformis* f. sp. *tritici* colonies and associated host cell necrosis (i and ii, respectively) in doubled haploid wheat lines carrying different resistance quantitative trait loci. The test for presence of lignification (iii) was positive in **A)** MP 35 carrying *Yr18* and **B)** MP 148 containing *QYr.sgi-4A.1*, while **C)** MP 16 with no allele tested negative for lignin. Scale bars represent 200 µm on the flag leaf.

## DISCUSSION

Most if not all histological analyses of cereal rust infection are done on host plants inoculated in a controlled environment (Moldenhauer *et al.*, 2006; 2008; Chen *et al.*, 2015; Dugyala *et al.*, 2015). This implies one infection cycle and the absence of variation in histological parameters due to secondary infections and environmental changes. Since commercial cultivars are exposed to stripe rust infection under field conditions, it is appropriate to study the effectiveness of resistance APR, and their additive effects if any, in such an environment. The fact that multiple infections of the same leaf will have occurred at the time of sampling, poses a challenge to accurate measurements of representative colonies and host response types. This is even more pronounced for studying the development of the stripe rust pathogen which systemically colonises leaf tissue (Emge *et al.*, 1975).

Infection in the more resistant test lines was inhibited shortly after penetration, resulting in distinct, similar-sized colonies from multiple infections at the time of assessment. Conversely, the wide colony ranges observed in the more susceptible lines can be attributed to unsuccessful suppression of initial *Pst* infections which continued to grow even after secondary infections occurred later in the season.

Despite the technical challenges and depending on the APR loci, significant differences were observed in DH lines containing a single QTL/gene compared to those with more than one loci. At the time of sampling/phenotyping, little difference was observed between lines carrying two and those containing three loci. Thus, based on the resistance level observed, two APR loci may be sufficient from a host plant protection point of view. From a durability perspective, however, more genes may be needed.

Entries containing the single *QYr.sgi-2B.1* responsible for the necrotic response (MP 51 and MP 142) only had slightly higher LAI scores and, as expected, larger *Pst* colonies than entries where *QYr.sgi-2B.1* was in combination with other APR loci. However, all lines containing *QYr.sgi-2B.1* (single or in combination) had a resistant field RT, substantiating the higher proportion of the variance in RT (33–46%) than LAI (17–30%) associated with this QTL (Ramburan *et al.*, 2004). Full resistance was observed for *QYr.sgi-2B.1+QYr.sgi-4A.1+Yr18* and *QYr.sgi-2B.1+Yr18* combinations

across the four years, while the occasional lower host response was seen for *QYr.sgi-2B.1+QYr.sgi-4A.1* (RMR) and *QYr.sgi-2B.1* (MR).

While low CI values were calculated during the 2011 and 2012 seasons for both lines carrying only the 7D chromosome loci (containing the *Lr34/Yr18/Sr57* gene complex), moderate to large colonies were measured for the more susceptible MP 35. Similar sized *Pst* colonies and number of HMC per colony were quantified for MP 108 carrying *QYr.sgi-4A.1+Yr18.* Histological results obtained from the other line carrying *Yr18* (MP 223) were parallel to that of the remaining *Yr18* combinations, including MP 68 also containing the *QYr.sgi-4A.1+Yr18* combination. Completely resistant field responses were documented across the four appraised years for lines containing solely *Yr18*, except for the above-mentioned MP 35 (RMR to MR) and MP 108 (MS).

Much larger colonies and more HMC were quantified for both MP 148 and MP 152, containing the single slow rusting *QYr.sgi-4A.1* QTL, than for lines where it was combined with another resistance loci (excluding MP 108 containing *QYr.sgi-4A.1+Yr18*). Noteworthy is the little HCN associated with the unaccompanied *QYr.sgi-4A.1* QTL, while H-index values as well as host response types were significantly higher for entries where the 4A chromosome QTL appeared in combinations with other resistance loci. Parallel data was obtained from number of HMC per colony and colony length parameters, correlating 0.99 and 0.98 in the 2011 (Addendum 2.6) and 2012 (Addendum 2.7) trials, respectively.

Although a negative correlation ($r_s$ = -0.77) occurred between the H-index and general host response type (Addendum 2.7), consistently high H-index values were obtained for *QYr.sgi-2B.1* carriers. This was in agreement with previous reports of hypersensitivity associated with the *QYr.sgi-2B.1* QTL transferred from Kariega (Moldenhauer *et al*., 2008; Prins *et al*., 2011). Conversely, lines carrying single *QYr.sgi-4A.1* and *Yr18* revealed low and inconsistent H-index values, respectively. The unpredictable nature of hypersensitivity in the latter lines could point to environmental sensitivity of the *QYr.sgi-4A.1* and *Yr18* loci.

While stress-induced cellular lignification is recognized as an important resistance mechanism against rust fungi (Moerschbacher *et al.*, 1990; Tiburzy and Reisener, 1990), lignin is also fundamentally deposited in the secondary thickened plant cell wall. Pronounced synthesis and accumulation of lignin did not appear to be linked to a

specific Kariega APR QTL, as all entries containing at least one APR loci exhibited a lignification defence response in addition to negative tests for lines without resistance alleles.

The significant differences observed between some of the lines containing the same resistance APR (*QYr.sgi-4A.1+Yr18* combination, single *QYr.sgi-4A.1* and single *Yr18*) could be due to unknown resistance factor(s) inherited in the more resistant lines, or even interactions of a particular genotype with environment. Moreover, differences in growth period could have resulted in the earlier exposure of flag leaves of earlier lines to infection, with consequent higher severity levels compared to late entries. Future investigation into these similar Kariega APR-containing genotypes may further broaden the understanding of the inheritance of rust resistance.

REFERENCES

Ali S, Gladieux P, Leconte M, Gautier A, Justesen AF, Hovmøller MS, Enjalbert J and De Vallavieille-Pope C. 2014. Origin, migration routes and worldwide population genetic structure of the wheat yellow rust pathogen *Puccinia striiformis* f. sp. *tritici*. *PLoS Pathogens* 10(1): e1003903. doi: 10.1371/journal.ppat.1003903

Boyd LA. 2005. Centenary review: can Robigus defeat an old enemy? – Yellow rust of wheat. *Journal of Agricultural Science* 143: 233-243. doi: 10.1017/S0021859605005095

Browder LE. 1971. Pathogenic specialization in cereal rust fungi, especially *Puccinia recondita* f. sp. *tritici*, concepts, methods of study, and application. *Agricultural Research Service Technical Bulletin*, No.1432: 1-51. United States Department of Agriculture: Washington, DC.

Chen XM. 2005. Epidemiology and control of stripe rust (*Puccinia striiformis* f. sp. *tritici*) on wheat. *Canadian Journal of Plant Pathology* 27: 314-337.

Chen YE, Cui JM, Su YQ, Yuan S, Yuan M and Zhang H. 2015. Influence of stripe rust infection on the photosynthetic characteristics and antioxidant system of susceptible and resistant wheat cultivars at the adult plant stage. *Frontiers in Plant Science* 6: 779. doi: 10.3389/fpls.2015.00779

Coleman T, Madassery JV, Kobayashi GS, Nahm MH and Little JR. 1989. New fluorescence assay for the quantitation of fungi. *Journal of Clinical Microbiology* 27(9): 2003-2007.

Dugyala S, Borowicz P and Acevedo M. 2015. Rapid protocol for visualization of rust fungi structures using fluorochrome Uvitex 2B. *Plant Methods* 11: 54. doi: 10.1186/s13007-015-0096-0

Emge RG, Kingsolver CH and Johnson DR. 1975. Growth of the sporulating zone of *Puccinia striiformis* and its relationship to stripe rust epiphytology. *Phytopathology* 65: 679-681.

He SY. 1996. Elicitation of plant hypersensitive response to bacteria. *Plant Physiology* 112: 865-869.

Johnson R. 1988. Durable resistance to yellow (stripe) rust in wheat and its implications in plant breeding. In: *Breeding Strategies for Resistance to the Rusts of Wheat* (Simmonds NW and Rajaram S, eds). CIMMYT, Mexico, pp. 63-75.

Johnson R. 1992. Past, present and future opportunities in breeding for disease resistance, with examples from wheat. *Euphytica* 63: 3-22. doi: 10.1007/BF00023908

Kang Z, Tang C, Zhao J, Cheng Y, Liu J, Guo J, Wang X and Chen X. 2015. Wheat-*Puccinia striiformis* Interactions. In: *Stripe rust* (Chen X and Kang Z, eds). Springer pp. 155-282.

Klement Z. 1982. Hypersensitivity. In: *Phytopathogenic Prokaryotes* (Mount MS and Lacy GH, eds). New York: Academic Press, pp. 149-177.

Kloppers FJ and Pretorius ZA. 1997. Effects of combinations amongst genes *Lr13*, *Lr34* and *Lr37* on components of resistance in wheat to leaf rust. *Plant Pathology* 46: 737-750. doi: 10.1046/j.1365-3059.1997.d01-58.x

Knott DR. 1989. The wheat rusts: breeding for resistance. In: *Monographs on Theoretical and Applied Genetics*, No. 12 (Frankel R, Grossman M, Linskens HF, Maliga P and Riley R, eds). Berlin: Springer-Verlag, pp. 1-201.

Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta- Espino J, McFadden H, Bossolini E, Selter LL and Keller B. 2009. A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science* 323: 1360-1363.

McIntosh RA. 1992. Pre-emptive breeding to control wheat rusts. *Euphytica* 63: 103-113. doi: 10.1007/BF00023916

Moerschbacher BM, Noll U, Gorrichon L and Reisener HJ. 1990. Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. *Plant Physiology* 93: 465-470.

Moldenhauer J, Moerschbacher BM and Van der Westhuizen AJ. 2006. Histological investigation of stripe rust (*Puccinia striiformis* f. sp. *tritici*) development in resistant and susceptible wheat cultivars. *Plant Pathology* 55: 469-474.

Moldenhauer J, Pretorius ZA, Moerschbacher BM, Prins R and Van der Westhuizen AJ. 2008. Histopathology and PR-protein markers provide insight into adult plant resistance to stripe rust of wheat. *Molecular Plant Pathology* 9(2): 137-145.

Niks RE and Dekens RG. 1987. Histological studies on the infection of triticale, wheat and rye by *Puccinia recondita* f. sp. *tritici* and *P. recondita* f. sp. *recondita*. *Euphytica* 36: 275-285.

Peterson RF, Champbell AB and Hannah AE. 1948. A diagramatic scale for estimating rust intensity of leaves and stem of cereals. *Canadian Journal of Research* 26(5): 496-500.

Pretorius ZA, Boshoff WHP and Kema GHJ. 1997. First report of *Puccinia striiformis* f. sp. *tritici* on wheat in South Africa. *Plant Disease* 81: 424. doi: 10.1094/PDIS.1997.81.4.424D

Pretorius ZA, Pakendorf KW, Marais GF, Prins R and Komen JS. 2007. Challenges for sustainable cereal rust control in South Africa. *Australian Journal of Agricultural Research* 58: 593-601.

Prins R, Pretorius ZA, Bender CM and Lehmensiek A. 2011. QTL mapping of stripe, leaf and stem rust resistance genes in a Kariega x Avocet S doubled haploid wheat population. *Molecular Breeding* 27: 259-270. doi: 10.1007/s11032-010-9428-y

Prins R, Ramburan VP, Pretorius ZA, Boyd LA, Boshoff WHP, Smith PH and Louw JH. 2005. Development of a doubled haploid mapping population and linkage map for the bread wheat cross Kariega × Avocet S. *South African Journal of Plant and Soil* 22: 1-8. doi: 10.1080/02571862.2005.10634672

Ramburan VP, Pretorius ZA, Louw JH, Boyd LA, Smith PH, Boshoff WHP and Prins R. 2004. A genetic analysis of adult plant resistance to stripe rust in the wheat cultivar Kariega. *Theoretical and Applied Genetics* 108: 1426-1433.

Rohringer R, Kim WK, Samborsky DJ and Howes NK. 1977. Calcofluor: an optical brightener for fluorescence microscopy of fungal plant parasites in leaves. *Phytopathology* 67: 808–810.

Sherwood RT and Vance CP. 1976. Histochemistry of papillae formed in reed canary grass leaves in response to noninfecting pathogenic fungi. *Phytopathology* 66: 503-551.

Singh RP, Huerta-Espino J and William HM. 2005. Genetics and breeding for durable resistance to leaf and stripe rusts in wheat. *Turkish Journal of Agriculture and Forestry* 29: 121-127.

Stubbs RW, Prescott JM, Saari EE and Dubin HJ. 1986. Cereal Disease Methodology Manual. Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT), Mexico.

Tiburzy R and Reisener HJ. 1990. Resistance of wheat to *Puccinia graminis* f. sp. *tritici*: association of the hypersensitive reaction with the cellular accumulation of lignin-like material and callose. *Physiological and Molecular Plant Pathology* 36: 109-120.

Zadoks JC, Chang TT and Konzak CF. 1974. A decimal code for the growth stages of cereals. *Weed Research* 14: 415-421.

# Chapter 3: Quantification of stripe rust development in wheat lines with adult plant resistance derived from Cappelle-Desprez

## INTRODUCTION

Stripe rust, a disease caused by the fungus *Puccinia striiformis* Westend. f. sp. *tritici* (*Pst*), is currently a serious threat to global wheat (*Triticum aestivum* L.) production, an industry under pressure due to increasing demand. Costly fungicide applications are dependent on knowledge of spray technology and availability of associated infrastructure and products. Moreover, chemical management often is impractical under severe stripe rust epidemics that occur late in the growing season. This makes the use of resistant cultivars the most effective approach to reduce damage caused by *Pst* (Chen, 2005; Dodds and Rathjen, 2010).

Resistance to *Pst* is largely considered as either seedling resistance, i.e. all-stage resistance (ASR), or adult plant resistance (APR) expressed at advanced growth stages (Chen, 2005; Lagudah, 2011). Controlled by major-effect genes, ASR is typically race-specific (Boyd, 2005) and frequently succumbs to new pathogen races. *Pst* races evolve due to mutation, recombination and adaptation for increased aggressiveness and tolerance to high temperatures (Knott, 1989; Line and Chen, 1995; Thrall and Burdon, 2003; Ali *et al.*, 2014). The wind-borne nature of *Pst* urediniospores further assists in migration of new, virulent races.

More emphasis is placed on race non-specific, durable APR when developing cultivars (Line and Chen, 1995; Line, 2002; Chen, 2005, 2013). Combinations of minor, slow rusting APR genes have enabled cultivars to remain resistant for many years after being commercialized over large areas (Singh *et al.*, 2005, 2011; Chen, 2013).

However, the majority of more than 70 wheat stripe rust resistance genes catalogued to date (McIntosh *et al.*, 2017) are categorized as ASR, most of which are ineffective against current races of *Pst*. The continuous process of identifying and introgressing multiple sources of resistance with complementary and/or additive effects in single varieties is paramount in the attainment of durability.

Due to the importance of stripe rust in South Africa, wheat breeders in collaboration with plant pathologists and geneticists are constantly tasked with introducing durable

resistance into new lines and varieties. The evaluation of diverse sources of resistance is an important aspect in the effort towards durability.

A source of durable stripe rust APR has been recognized in the French wheat variety Cappelle-Desprez (Lupton and Macer, 1962; Powell, 2010; Feng *et al.*, 2011; Jagger *et al.*, 2011). In South Africa, high levels of resistance have been associated with Cappelle-Desprez since its first evaluation in 1998 (Boshoff *et al.*, 2002; Agenbag *et al.*, 2012).

Cappelle-Desprez carries the ASR genes *Yr3a* and *Yr4a* (Lupton and Macer, 1962; Lupton *et al.*, 1971; De Vallavieille-Pope *et al.*, 1990) on chromosomes 1B and 6B, respectively (Chen *et al.*, 1996). Early studies have indicated that APR in Cappelle-Desprez is imparted by *Yr16* located on chromosome 2D (Worland and Law, 1986), as well as the 5BS-7BS translocation (Law and Worland, 1997). Lagudah *et al.* (2009) proposed a yet undescribed locus imparting leaf and stripe rust APR to Cappelle-Desprez, after specific markers confirmed the absence of *Lr34/Yr18/Pm38* previously suspected to contribute to the leaf rust APR in this variety (McIntosch, 1992).

Derived from Cappelle-Desprez a breeding line Yr16DH70, which lacks the seedling resistance genes, was selected based on its APR to *Pst* and adaption to South African wheat growing conditions (Agenbag *et al.*, 2012). Following several years of testing, a major effect quantitative trait loci (QTL) on chromosome 2A (*QYr.ufs-2A*), in addition to three minor effect QTL on chromosomes 2D (*QYr.ufs-2D*), 5B (*QYr.ufs-5B*) and 6D (*QYr.ufs-6D*), were mapped in Yr16DH70 (Agenbag *et al.*, 2012). The contribution of *QYr.ufs-2A* to the phenotypic variation in Yr16DH70 amounted to 53.2% for reaction type and 47.8% for leaf area infected (Agenbag *et al.*, 2012); the necrotic response typical of race-specific resistance to stripe rust. Although a considerable proportion of the resistance can be ascribed to *QYr.ufs-2A*, its effect can be further enhanced through the combination with smaller QTL, demonstrated by its significant interactions with the three minor QTL from Yr16DH70. This locus is of particular interest, as multiple cases have been reported of stripe rust resistance QTL located within a common interval on the short arm of chromosome 2A (Mallard *et al.*, 2005; Boukhatem *et al.*, 2002; Bariana *et al.*, 2010; Lowe *et al.*, 2011; Hao *et al.*, 2011).

*QYr.ufs-2D* in Cappelle-Desprez covers the centromeric region of chromosome 2D, including the locus of *Yr16* (Worland and Law, 1986), with seemingly less effective

resistance under severe disease pressure. Genetic background of the variety as well as environmental conditions may add to the sensitive expressive nature of this minor QTL (Boukhatem *et al.*, 2002; Agenbag *et al*., 2012). Up to 10.3% of the phenotypic variance in Yr16DH70 was ascribed to *QYr.ufs-2D* (Agenbag *et al.*, 2012).

Cappelle-Desprez contains the 5BL-7BL and 5BS-7BS translocations, with the 5BS-7BS chromosome (Riley *et al.*, 1967; Badaeva *et al.*, 2007), later pinpointed to 5BS (Law *et al.*, 1978), implicated as the main contributor of the translocation resistance effect. Although the 5BS-7BS translocation in Yr16DH70 (Pretorius ZA, unpublished data) had little input in its stripe rust resistance (a maximum of 5.7%), the combination of *QYr.ufs-5B* with minor QTL *QYr.ufs-2D* resulted in lower disease scores (Agenbag *et al.*, 2012).

Apart from the ASR genes *Yr20* and *Yr23* (Chen *et al.*, 1995), no APR loci have been delineated to chromosome 6D. *QYr.ufs-6D* on the long arm of 6D expresses a small effect QTL in Yr16DH70, contributing to a maximum of 7.6% of the phenotypic variation (Agenbag *et al.*, 2012).

Yr16DH70 was crossed with the moderately susceptible South African cultivar Palmiet, carrying a minor QTL on chromosome 4B. However, *QYr.ufs-4B* was only detected with the host reaction type (RT) phenotypic data, explaining a maximum of 11.7% of the phenotypic variance and depended on disease pressure and environmental factors. A resultant segregating recombinant inbred line (RIL) mapping population (MP), consisting of 201 entries, was established (Agenbag *et al.*, 2012).

Previous groupings of QTL carriers were done based on closest QTL marker (Agenbag *et al.*, 2012). The data was reassessed prior to host-pathogen interaction studies, employing all the markers across the QTL interval for initial selection on still segregating $F_6$ RILs and validated on resultant $F_8$ (QTL areas mostly fixed). After eliminating *QYr.ufs-4B* carriers, eighteen lines were selected for further evaluation, carrying different putative combinations of Yr16DH70 resistance QTL (R. Prins, personal communication).

The present study aimed to shed light on interacting effects between different APR QTL derived from Cappelle-Desprez, by quantifying components of the expressed resistance in selected RILs. The traditional phenotypic assessment along with a novel

molecular technique was combined with histological analysis for further insight into host-pathogen interactions.

## MATERIALS AND METHODS

### Plant and pathogen materials

$F_8$ Palmiet (pedigree: SST3*//Scout*5/Agent) x Yr16DH70 (pedigree: Cappelle-Desprez/*2Palmiet) RILs along with the parental lines were planted in a rust screening nursery at the Pannar Redgates research facility near Greytown, KwaZulu-Natal, South Africa in 2015.

Entries were planted in single 1-m rows, spaced 76 cm apart, in two replications. To facilitate sufficient inoculum development within the nursery, rows of stripe rust spreader (a mixture of Morocco and line JIC871) were planted in right-angled rows to plots and as every tenth entry in the trial.

For inoculum multiplication, wheat cultivar Morocco was planted in Mikskaar® potting substrate MPS2. After seedling emergence, 99% maleic hydrazide ReagentPlus® (Sigma-Aldrich (0.3 g/L water, 50 mL per pot)) was applied to suppress plant growth. Seven days after planting, urediniospores of *Pst* pathotype 6E22A+ were retrieved from storage at -80°C and suspended in Soltrol® 130 (Phillips Petroleum, Bartlesville, OK, USA) isoparaffinic solvent in a gelatin capsule. Using a pressure pump connected to a custom-made inoculation device (Browder, 1971) the spore suspension was sprayed onto seedlings followed by a 1 h incubation period at 25°C. Dried off seedlings were incubated at 10°C in a high humidity (> 96%) chamber for a 24 h dew period. Thereafter, a temperature of 17°C was maintained when seedlings were placed under fluorescent lights in a growth cabinet for 2 h before being returned to the greenhouse. When sporulation on seedlings was sufficient, fresh urediniospores were collected for inoculation of spreader rows (tillering stage) in the field.

Using an ultra-low volume sprayer, spreaders were inoculated with *Pst* spores suspended in Soltrol® 130. Following inoculation, several 1-m sections of spreader plants were covered with plastic sheeting overnight to provide high humidity for maximum infection. Data and/or leaf tissue were collected on 17 September 2015 (first sampling) and 1 October 2015 (second sampling).

**Phenotypical analysis**

Mean flag leaf severity and adult plant reaction type per line were recorded at both time points. Disease severity was estimated in accordance with the modified Cobb scale (Peterson *et al.*, 1948) where the numerical value corresponds to the extent of the flag leaf area infected (LAI) in percentage (100% signifies the flag leaf blade being completely rusted). In addition, host reaction types (RT) are specified as resistance (R), moderate resistance (MR), moderate susceptibility (MS), and susceptibility (S). Coefficient of infection (CI) was calculated by multiplying LAI and constant values assigned to host RT, namely: R=0.2, MR=0.4, MRMS=0.6, MS=0.8 and S=1.0 (Stubbs *et al.*, 1986), as well as to intermediate categories RMR=0.3 and MSS=0.9.

Once the test lines were phenotypically evaluated, entries were sampled for histological (first sampling) and molecular analysis (first and second sampling). At the first sampling time, the same flag leaves were used to cut segments for the respective techniques.

**Fluorescence microscopy**

Growth of *Pst* colonies and associated host cell necrosis (HCN) were viewed and quantified on the flag leaves of RILs by means of fluorescence microscopy.

During the first sampling, two flag leaves representative of each entry were cut in 3-5 cm segments and transferred to 3:1 (v/v) ethanol:dichloromethane containing 0.15% (w/v) trichloroacetic acid. Leaves were fixed and cleared in this solution for 24 h.

The Uvitex stain for fungal structures and host cell reaction was based on a modified Rohringer *et al.* (1977) procedure. Specimens were drenched twice in 50% ethanol for 15 min, twice for 15 min in 0.05 M sodium hydroxide, and washed down three times with distilled water. Leaf segments were then immersed in 0.1 M Tris (hydroxymethyl) aminomethane/hydrochloric acid buffer (pH 8.5) and stained with preheated 0.1% (w/v) Uvitex 2B (Ciba-Geigy, Syngenta) (Niks and Dekens, 1987; Moldenhauer *et al.*, 2006) in the preceding buffer (65°C for 5 min; Dugyala *et al.*, 2015). Samples were then rinsed four times with water and saturated with 25% (v/v) aqueous glycerol for 30 min. Stained leaves were kept in 50% (v/v) glycerol with a trace lactophenol, preventing deterioration of fungal material and drying of tissue.

To compare the degree of *Pst* colonisation among the RILs, the length (µm) of individual fungal colonies growing parallel to the veins within the flag leaf of each line was measured. Observations on 40 infection sites per line (20 on each of two leaf samples) were carried out for each field replicate with an Olympus AX70 microscope (Tokyo, Japan) magnifying from X4 up to X100. The WB epifluorescence cube (330-385 nm excitation filter and 420 nm barrier filter) was used to observe fluorescing fungal tissue. The autofluorescence in wheat tissue, indicative of cell death, was perceived via the WU epifluorescence cube (450-480 nm excitation filter and 515 nm barrier filter). Images of fungal structures and HCN were captured using a CC12 digital camera and AnalySIS LS Research version 2.2 software (Olympus Soft Imaging System, Japan).

The hypersensitivity index (H-index) was calculated by dividing the length of necrosis with the colony length. An H-index greater than one will indicate that necrosis is larger than the fungal colony, and fungal growth possibly inhibited by a strong hypersensitive response (Kloppers and Pretorius, 1997).

Leaves were not collected for histology during the second sampling as infection levels were too advanced to allow microscopic discernment of individual colonies.

**Molecular analysis**

The development in fungal biomass was quantified at two time points, 14 days apart, according to a method based on relative gene expression (Coram *et al.*, 2008). This approach entails the expression of an experimental gene relative towards a reference gene. The remaining segments of the same flag leaves not used for histology during the first sampling, were submerged in RNA*later*™ (Ambion Inc, Sigma Aldrich) and stored at 4°C. The same method was followed at the second sampling time to measure the development of rust infection over time. Leaf segments were powdered in liquid nitrogen and stored at -80°C for subsequent analysis.

Ribonuclease free water was used for the RNA extraction and ensuing testing. The preparation entails 0.1% (v/v) dimethyldicarbonate (DMDC) added to deionised water, incubated overnight at room temperature and autoclaved twice to inactivate the DMDC.

Trizol reagent (Invitrogen™, Carlsbad, California, USA) was used in line with the manufacturer's instructions to extract total RNA from 100 µL flag leaf tissue

(Chomczynski and Sacchi, 1987). The frozen tissue was partially thawed and submerged in 500 µL Trizol reagent at room temperature for 10 min before 100 µL chloroform was added. Following 5 min of incubation, samples were centrifuged at 12 000 *g* for 15 min at 4°C. The cleared supernatant was transferred to 250 µL isopropanol for the precipitation of RNA. Following a 10-min incubation period, tubes were centrifuged at 12 000 *g* for 10 min at 4°C. Once the RNA pellet was retrieved using a water jet pump, it was rinsed with 70% (v/v) ethanol. The air-dried RNA was then dissolved in 100 µL DMDC treated water.

Due to the number of samples and limited resources, RNA of the two leaves of each line harvested at the time was pooled, while the two sampling times and field replicates remained separate.

The RNA concentration of the samples was calculated using a Nanodrop2000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA), with DMDC treated water as blank (Sambrook and Russell, 2000).

The integrity of the RNA was confirmed through electrophoresis by separating 100 ng on a 1.2% (w/v) agarose gel (Sambrook *et al.*, 1989) prepared in 0.5 x TAE (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.28% (v/v) acetic acid) containing 0.5 µg/mL ethidium bromide. Exposed to ultraviolet light illumination (302 nm), the gel was photographed utilizing the GelDoc XR+ System (BioRad, Hercules, California, USA) and Quantity One 4.6 program. Although the RNA concentrations were very low, intact RNA bands were observed.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was carried out with a Bio-Rad C1000 thermal cycler connected to a CFX96 real-time attachment, using KAPA™ SYBR® FAST Universal One-Step qRT-PCR Kit (Lasec).

For assessment of *Pst* biomass and development, the constitutively expressed *Pst β-tubulin* (Ling *et al.*, 2007) was quantified in reference to the constitutive expression of *18S ribosomal RNA* (*18S*) in wheat over the time course for each RIL.

The forward and reverse primer sequence information of *18S* in wheat, validated as acceptable for *Pst* gene expression (Scholtz and Visser, 2013), was used for the amplification of the reference gene. The RT-qPCR primer pair for *Pst β-tubulin* was developed using the web-based program Primer3, laid out in Table 3.1.

To standardize the primers for RT-qPCR, a temperature gradient and subsequent electrophoresis was carried out to decide on annealing temperatures optimal for each primer set. A standard curve reaction was performed making use of a 1:2 dilution series. Amplification efficiency of each primer pair was determined and the resulting amplicon confirmed by separating the standard curve reactions on 1.2% (w/v) agarose gel (as described before). The primers for both *18S* and *Pst β-tubulin* showed the most accurate amplification at 60°C with $R^2$ values of 0.952 and 0.986, respectively (Table 3.1).

Once the primer sets were optimized, expression of the experimental *Pst β-tubulin* gene was tested. Each 10 µL RT-qPCR reaction contained 10 ng total RNA, 10 µM of both forward and reverse primer (Table 3.1), 5 µL KAPA Master Mix buffer, 0.2 µL KAPA Reverse Transcriptase Mix and 1.8 µL DMDC treated water. The RT-qPCR protocol entails an initial step of 42°C for 10 min and a denaturing step of 95 °C for 3 min. The following steps were repeated 40 times for the synthesis of cDNA: an additional 10-sec denaturing step at 95°C and 30 sec of specific annealing temperature (Table 3.1) for primer annealing and elongation. Upon completion of each cycle, the plate was read for quantity SYBR green bound to double stranded nucleic acid. The incorporation of a melt curve, with 0.5°C increments every 5 sec from 65-95°C, confirmed the absence of non-specific amplicons. The plate was read again once the reaction was completed.

Using qBase+ software (version 3.1 Biogazelle, Zwijnaarde, Belgium), RT-qPCR data analysis of 20 wheat lines inoculated with *Pst* isolate 6E22A+ was done with every experiment consisting of two biological replicates at each of the two time points. The relative gene expression, expressed in the form of CNRQ (Calibrated Normalized Relative Quantity) values, and standard errors were calculated after quantification cycle (Cq) values were imported. Significant outliers were excluded for each set of four technical replicates.

**Statistical analysis**

RILs and parents were evaluated with analysis of variance (ANOVA) using General Linear Models (GLM) in NCSS 2007. The effects of genotypes and field replications were accounted for in the model for analysis of the histological parameters (colony

length, HCN and H-index). Additionally, the impact of sampling times was included for the assessment of phenotypic CI scores and relative *Pst β-tubulin* expression.

The correspondence of data across different techniques was assessed using Spearman's rank correlation ($r_s$). Fisher's protected least significant difference (LSD) values were calculated and used in histograms where applicable.

## RESULTS

The initial selection of specific RILs were done on the marker data generated on the $F_6$ RILs (Agenbag *et al.*, 2012). The $F_8$ RILs derived from these selections were then again genotyped with the markers spanning the respective QTL intervals, revealing predicted QTL (Addendum 3.1, obtained from R. Prins, CenGen (Pty) Ltd). In view of the wide coverage of resistance QTL combinations appraised in this study and for the sake of simplicity, the QTL will be abbreviated to its chromosome location, e.g. 2A refers to a RIL carrying resistance QTL *QYr.ufs-2A*, and 2A+2D mentions a RIL carrying the QTL combination *QYr.ufs-2A* and *QYr.ufs-2D*, etc.

**Table 3.1:** Nucleotide sequence, annealing temperature and amplification efficiency for primer pairs used during RT-qPCR.

| Gene symbol | Accession nr (NCBI) * | Primer sequence (5'-3') | Amplicon size (bp) | Annealing temp (°C) | RT-qPCR efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| *18S* | AH001810.2 | F: GTGACGGGTGACGGAGAATT<br>R: GACACTAATGCGCCCGGTAT | 151 | 60 | 109 | Jarošová and Kundu (2010) |
| *Pst β-TUB* | EF570842.1 | F: CTCGGACGAAACCTTCTGTATC<br>R: CTGAGGTAGGTGTAGCCAATTT | 106 | 60 | 98.5 | |

RT-qPCR = quantitative reverse transcription polymerase chain reaction.
* http://www.ncbi.nlm.nih.gov/, NCBI = National Centre for Biotechnology Information.
bp = base pairs.
*18S = 18S ribosomal RNA*.
*Pst β-TUB = Puccinia striiformis* f. sp. *tritici β-tubulin*.

**Table 3.2:** LAI, host RT and CI of a $F_8$ Palmiet x Yr16DH70 RIL wheat population inoculated with *Puccinia striiformis* f. sp. *tritici*. Two field replicates were phenotypically evaluated during a first and second sampling time.

| Recombinant inbred line (RIL) | Predicted quantitative trait loci (QTL) | First field replicate | | | | Second field replicate | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1st sampling | | 2nd sampling | | 1st sampling | | 2nd sampling | |
| | | LAI and RT | CI | LAI and RT | CI | LAI and RT | CI | LAI and RT | CI |
| Palmiet * | 4B | 30MRMS | 18 | 70MRMS | 42 | 30MRMS | 18 | 70MRMS | 42 |
| Yr16DH70 * | 2A+2D+5B+6D | 10MR | 4 | 20R | 4 | 10MR | 4 | 20R | 4 |
| 20 | 2A+2D+5B+6D | 20MRMS | 12 | 20RMR | 6 | 25MRMS | 15 | 30R | 6 |
| 148 | 2A+2D+5B+6D | 10MR | 4 | 20RMR | 6 | 20MRMS | 12 | 30RMR | 9 |
| 39 | 2A+2D+5B | 10MRMS | 6 | 30MR | 12 | 15MRMS | 9 | 40R | 8 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 34 | 2A+2D+6D | 15MRMS | 9 | 50MRMS | 30 | 20MRMS | 12 | 60R | 12 |
| 142 | 2A+2D+6D | 15MRMS | 9 | 25RMR | 7.5 | 20MRMS | 12 | 30RMR | 9 |
| 33 | 2A+5B+6D | 20MRMS | 12 | 40MRMS | 24 | 30MRMS | 18 | 40RMR | 12 |
| 10 | 2A+2D | 5RMR | 1.5 | 30R | 6 | 15MR | 6 | 25RMR | 7.5 |
| 144 | 2A+6D | 25MRMS | 15 | 40MRMS | 24 | 30MRMS | 18 | 40MR | 16 |
| 153 | 2D+5B | 20MRMS | 12 | 70R | 14 | 25MRMS | 15 | 50R | 10 |
| 17 | 2D+6D | 15MRMS | 9 | 30RMR | 9 | 20MRMS | 12 | 35RMR | 10.5 |
| 45 | 5B+6D | 40MRMS | 24 | 70MRMS | 42 | 30MRMS | 18 | 60MRMS | 36 |
| 112 | 2A | 40MS | 32 | 70MS | 56 | 40MRMS | 24 | 40MRMS | 24 |
| 178 | 2A | 40MRMS | 24 | 60MRMS | 36 | 35MRMS | 21 | 60MR | 24 |
| 15 | 2D | 30MS | 24 | 80MRMS | 48 | 50MSS | 45 | 90MRMS | 54 |
| 190 | 2D | 30MRMS | 18 | 60MRMS | 36 | 20MRMS | 12 | 50MR | 20 |
| 31 | 5B | 30MRMS | 18 | 50MRMS | 30 | 40MRMS | 24 | 60MRMS | 36 |
| 149 | 6D | 40MS | 32 | 50MRMS | 30 | 40MRMS | 24 | 60MRMS | 36 |
| 52 | None | 50MSS | 45 | 90MS | 72 | 40MRMS | 24 | 70MRMS | 42 |

LAI = Leaf area infected (%) indicated by numerical value.
RT = Host reaction type indicated by R = Resistant, MR = Moderately Resistant, MS = Moderately Susceptible and S = Susceptible.
CI = Coefficient of infection.
* Parents of recombinant inbred population.
QTL: 2A = *QYr.ufs-2A*, 2D = *QYr.ufs-2D*, 5B = *QYr.ufs-5B* and 6D = *QYr.ufs-6D*.
Brackets: lines with matching predicted QTL profiles.

**Phenotypic analysis**

The extent of disease severity, quantitatively measured through LAI (McIntosh *et al.*, 1995), was documented between 5 and 50% in the first sampling and 20 to 90% during the second sampling (Table 3.2). Host RT ranged from RMR to MSS during the first sampling and, overall, more resistant field reactions observed during the second sampling (R to MS).

In spite of a field effect noted between the replications, distinct differences in adult plant responses were seen among entries carrying different QTL combinations. Figures 3.1 and 3.2 reflect the range of phenotypes observed for different QTL profiles across replications. During the first sampling (Figure 3.1), 30MRMS was assigned to Palmiet (4B), while Yr16DH70 and RIL 148 carrying all four QTL (2A+2D+5B+6D) showed a 10MR reaction. Interestingly, the 2A+2D QTL combination had an even stronger plant response of 5RMR, while 30MRMS was documented for the combination of the other two QTL (5B+6D), and 50MSS for RIL 15 carrying the single 2D QTL. Variance among lines carrying resistance loci on different chromosomes was even more evident during the second sampling, regardless of the decline in sporulation (Figure 3.2). Some of the documented field responses include 20R (Yr16DH70), 25RMR (2A+2D), 30RMR (2A+2D+5B+6D), 40MR (2A+6D), 60MRMS (5B+6D combination and single 6D), 70MRMS [Palmiet (4B) and RIL 52 containing no QTL], and 90MRMS (RIL 15 carrying a single 2D QTL).

Quantitative analysis of the combined LAI and host plant RT, documented at two sampling points, was performed through CI values. ANOVA results (Addendum 3.2) pointed to highly significant differences in CI values among test lines and across sampling times ($P<0.05$), whereas the line-sampling time interaction proved insignificant ($P>0.05$). The statistical insignificant variation between the two field replications was also noticed in mostly similar trends of CI values obtained from the two trial repetitions, with variation most likely ascribed to field effects (Figure 3.3).

Most entries showed an increase in their CI values from the first to the second sampling, while exceptions in some of the more resistant genotypes entailed CI values that remained the same or marginally decreased. Since the host RT for all lines were either the same or more resistant during the second scoring, the increased CI values can only be attributed to escalation of LAI. The biggest change in field reaction over

the two-week interval was observed for Palmiet (4B), the 5B+6D combination, lines carrying only one (2A, 2D, 5B or 6D) or none of the Yr16DH70 stripe rust resistance QTL.

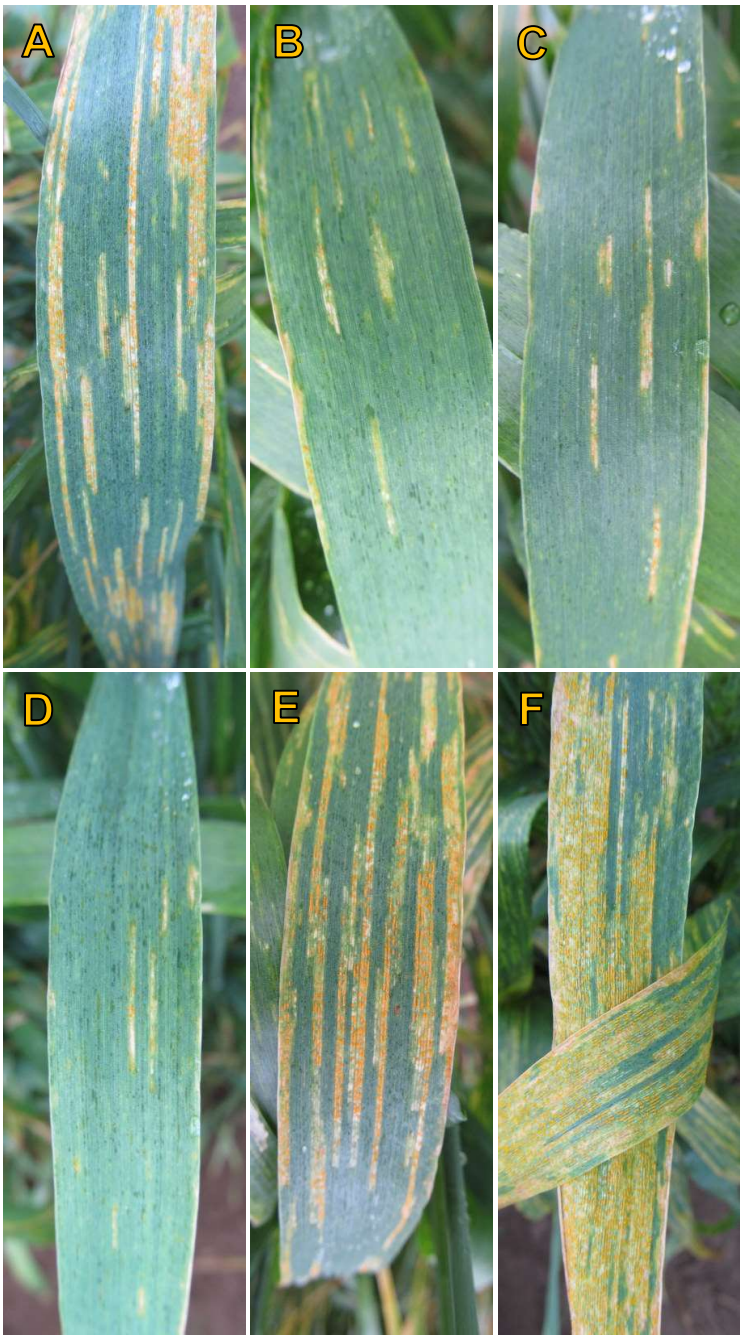
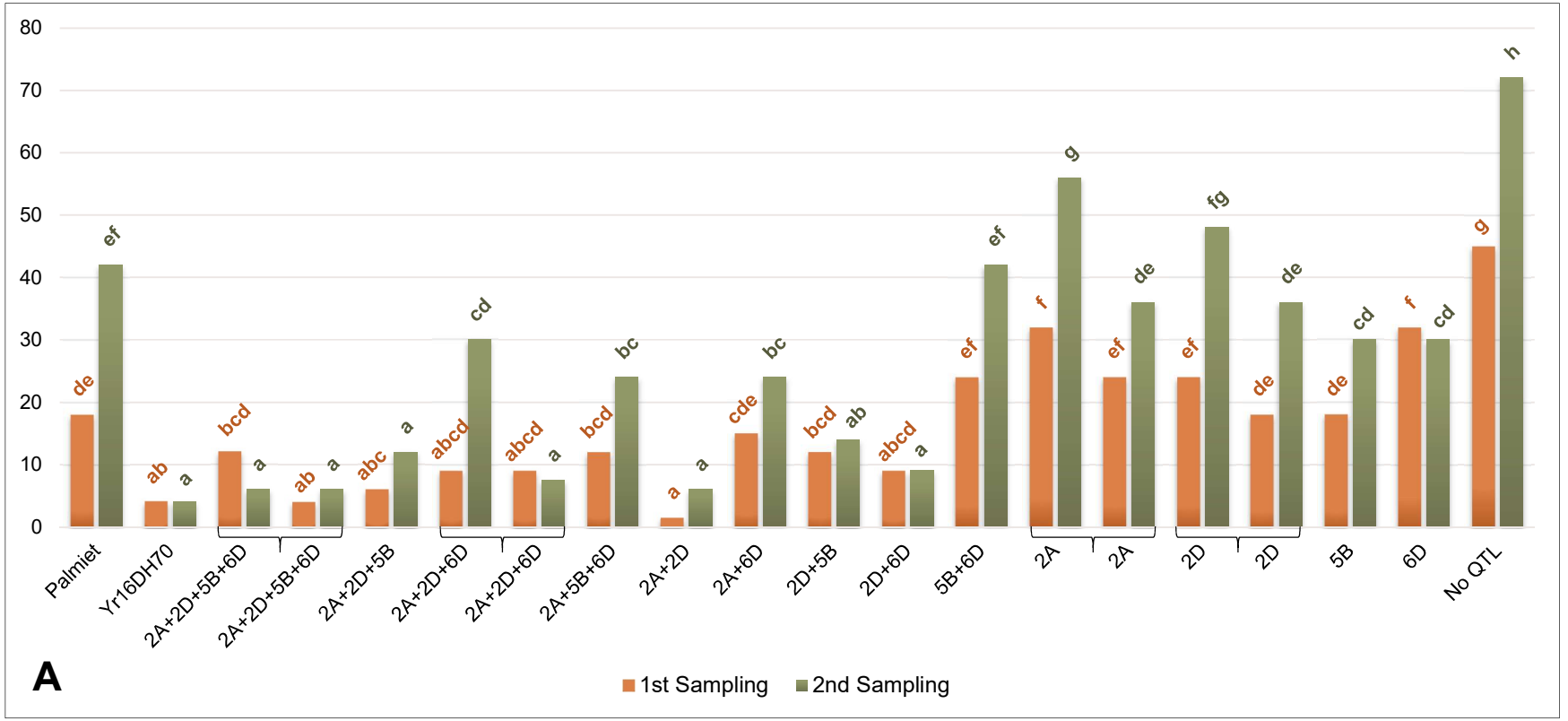



**Figure 3.1:** Stripe rust infection types of parents **A)** Palmiet (4B; 30MRMS) and **B)** Yr16DH70 (2A+2D+5B+6D; 10MR) and recombinant inbred lines containing resistance quantitative trait loci on chromosome(s) **C)** 2A+2D+5B+6D (10MR), **D)** 2A+2D (5RMR), **E)** 5B+6D (30MRMS) and **F)** 2D (50MSS), during the first sampling time across different replications.


**Figure 3.2:** Stripe rust infection types of parents **A)** Palmiet and **B)** Yr16DH70 and recombinant inbred lines containing resistance quantitative trait loci on chromosome(s) **C)** 2A+2D+5B+6D, **D)** 2A+2D, **E)** 2A+6D, **F)** 5B+6D, **G)** 2D, **H)** 6D and **I)** none, during the second sampling time across different replicates.


80
70
60
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Palmiet
Yr16DH70
2A+2D+5B+6D
2A+2D+5B+6D
2A+2D+5B
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2A+2D
2A+6D
2D+5B
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6D
No QTL
A
1st Sampling
2nd Sampling

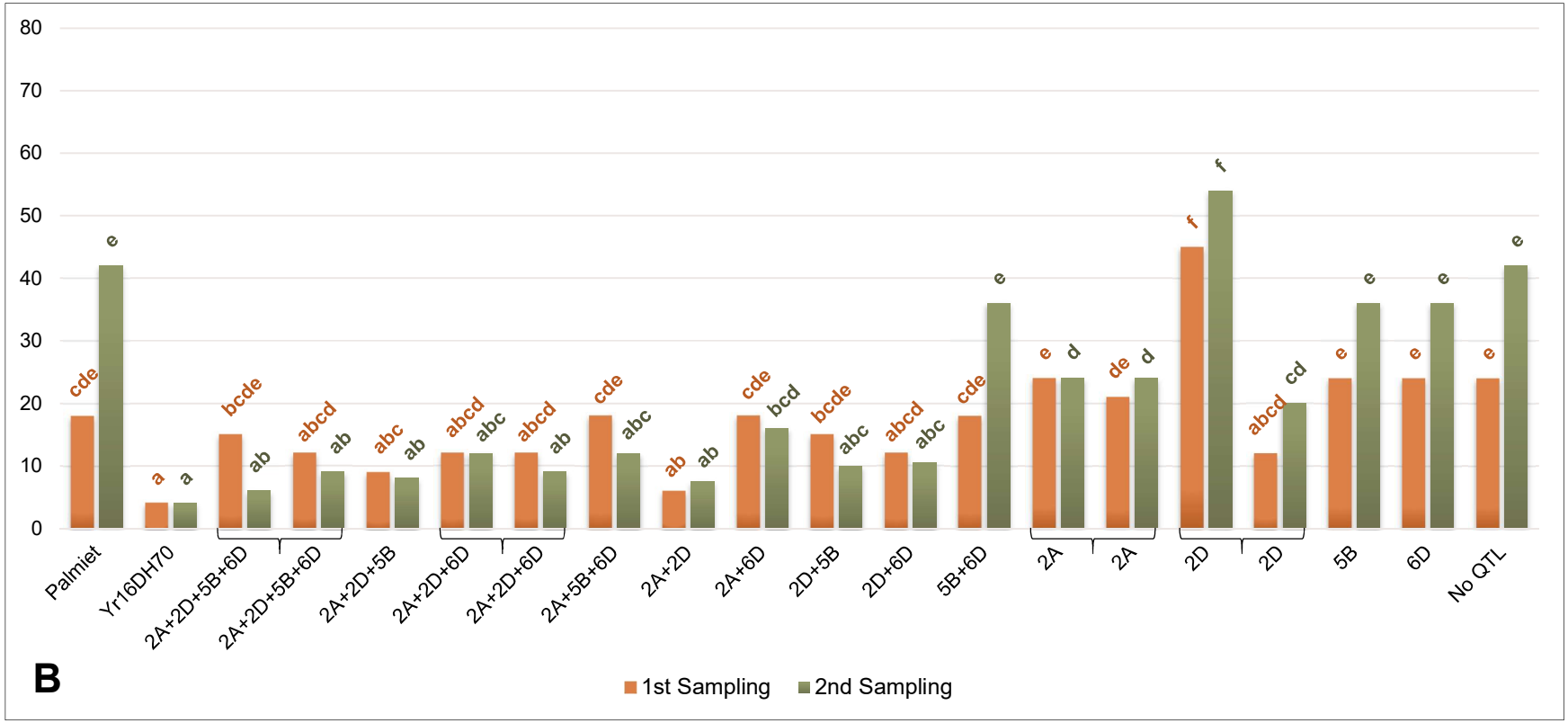



**Figure 3.3:** Coefficient of infection of parents (Palmiet and Yr16DH70) and $F_8$ wheat lines of a resulting recombinant inbred population containing different combinations of stripe rust resistance quantitative trait loci (QTL) occurring on chromosomes 2A, 2D, 5B and 6D. Data was recorded at two sampling times after inoculation with *Puccinia striiformis* f. sp. *tritici* in the **A)** first and **B)** second field replicate. Brackets indicate lines with matching predicted QTL profiles. Least significant difference = 10.47.

There was a clear distinction between the combined phenotypic parameters (i.e. CI) of above mentioned group and lines containing either QTL 2A or 2D in combination with at least one other resistance QTL. Lines in the former group revealed visibly larger CI values (relatively high during the first sampling and/or substantial increases reaching at least 30) than the latter group comprised of QTL occurring in combinations (aside from 5B+6D) where little change occurred in CI values; remaining below 16 at the second sampling. Inconsistent exceptions not reflecting the previous statement include 2A+2D+6D, 2A+5B+6D and 2A+6D in the more resistant group where CI values showed significant increases in the first trial, while unusual low CI scores were obtained for one of the single 2D QTL-carrying lines (RIL 190) in the second replication.

A consistent, unchanged CI value of 4 was observed for the resistant parent in both replications. Similarly, low scores (< 8) were repeatable for the 2A+2D QTL combination, with insignificant variation between RIL 10 and the resistant parent (LSD = 10.47).

Both replicates of Palmiet (4B), the 5B+6D combination, RIL 15 carrying a single 2D and RIL 52 without resistance QTL had high CI values with steep increases (exceeding 40 at the time of the second sampling). Initial high CI scores (between 20 and 32) were obtained for lines carrying a single QTL on chromosome 2A (RIL 112 and 178) and 6D (RIL 149) in both replications but only escalated in one of the replications. While the first replicate of RIL 52 carrying no QTL had visibly higher CI values than lines containing single QTL, similar scores were mostly calculated for these lines in the second replication.

Lines containing single 2A (RIL 112 and 178) and 2D (RIL 15 and 190) QTL had significantly higher CI values than the 2A+2D combination (RIL 10). Although to a lower extent, this has also manifested for the QTL combinations 2A+6D, 2D+5B and 2D+6D compared with respective single QTL carriers. However, the CI values calculated for the 5B+6D combination (RIL 45) were either higher or differed insignificantly from that of lines carrying a single QTL (RIL 31 with the single 5B and RIL 149 carrying 6D), across replications. The variation in CI values between the 5B+6D combination and that of the single QTL can be ascribed to LAI, as mostly similar host RT were documented (MRMS).

**Fluorescence microscopy**

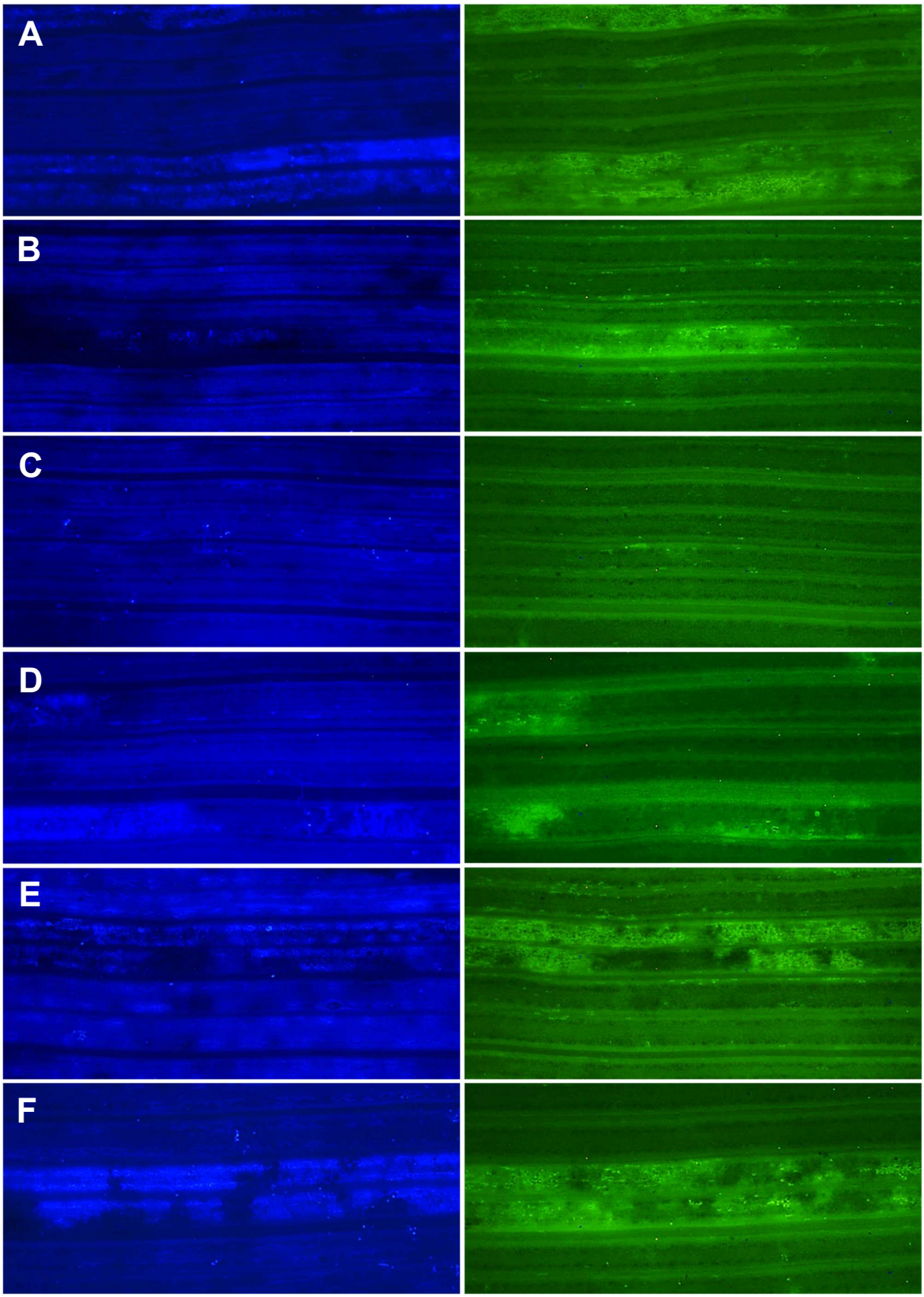
The Uvitex 2B fluorochrome that binds to chitin in the fungal cell wall allowed for detection of infection structures (Coleman *et al.*, 1989). Haustorial mother cells (HMC) and networks of infection hyphae fluoresced bright blue (left in Figure 3.4), and HCN associated with fungal colonies light green (right in Figure 3.4). Individual necrotic cells were often observed across the *Pst* colony area that did not result in larger necrotic areas. In these cases, HCN was measured between the most distant necrotic cells associated with the length of a colony.

Distinct variation in the degree of fungal growth and HCN were noted among RILs in different QTL groups. At the point of sampling, fully integrated colonies from the initial inoculation complicated the reliable quantification of individual colony sizes and even more that of associated HCN. Therefore, mostly smaller secondary infection points were measured.

ANOVA results indicated highly significant differences in colony lengths (Addendum 3.3) and H-indexes (Addendum 3.5) among lines ($P<0.05$), with little variation in HCN (Addendum 3.4) ($P>0.05$). Similar trends regarding colony lengths and H-index values were observed for the two replications (Figure 3.5), and confirmed by statistical insignificant variation in colony lengths and HCN across replications ($P>0.05$). Less variation occurred in H-index values among the second replicate of RILs than observed in the first replication, also evident in statistical significant differences between field replicates ($P<0.05$).

Fungal colony lengths in Palmiet (4B) were almost double that of the resistant parent (2A+2D+5B+6D) (averages of 250 and 140 µm, respectively), while the reverse was seen regarding H-index values of the first replicates and to lesser extent second replicates.

Most lines containing chromosome 2A or 2D QTL in combination with at least one other resistance QTL, showed small colony lengths (< 180 µm) plus H-index values > 1. Exceptions were seen for one replicate of QTL combinations 2A+5B+6D, 2A+6D and 2D+6D where H-indexes just below one or slightly larger colonies were produced. Small colonies (< 150 µm) were repeatedly measured for the resistant parent as well as lines in the 2A+2D+5B+6D and 2A+2D QTL groups.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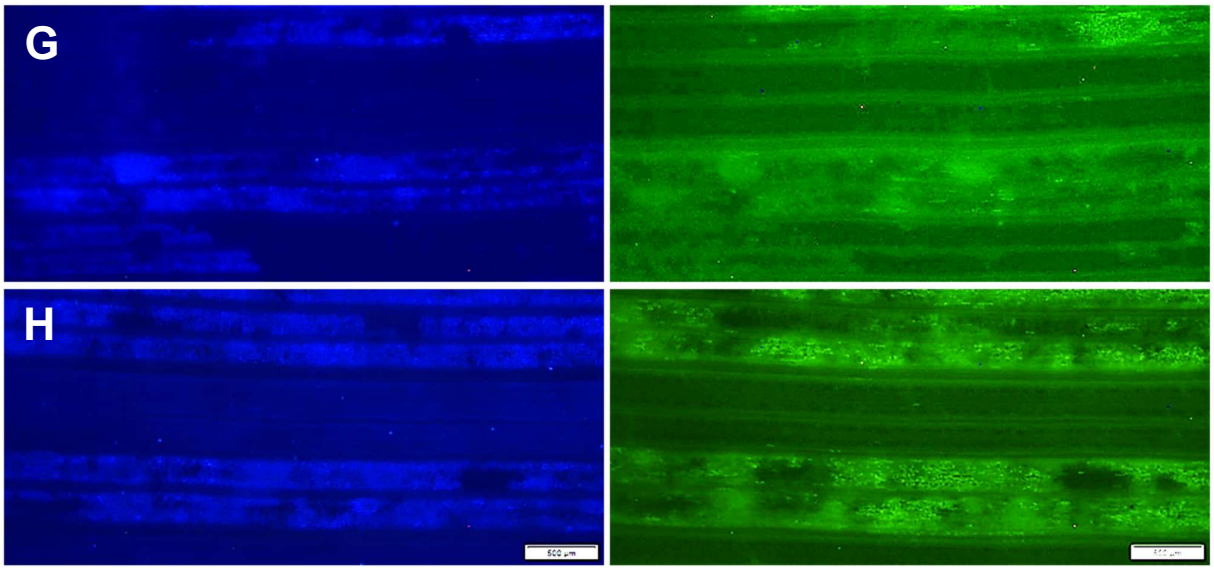
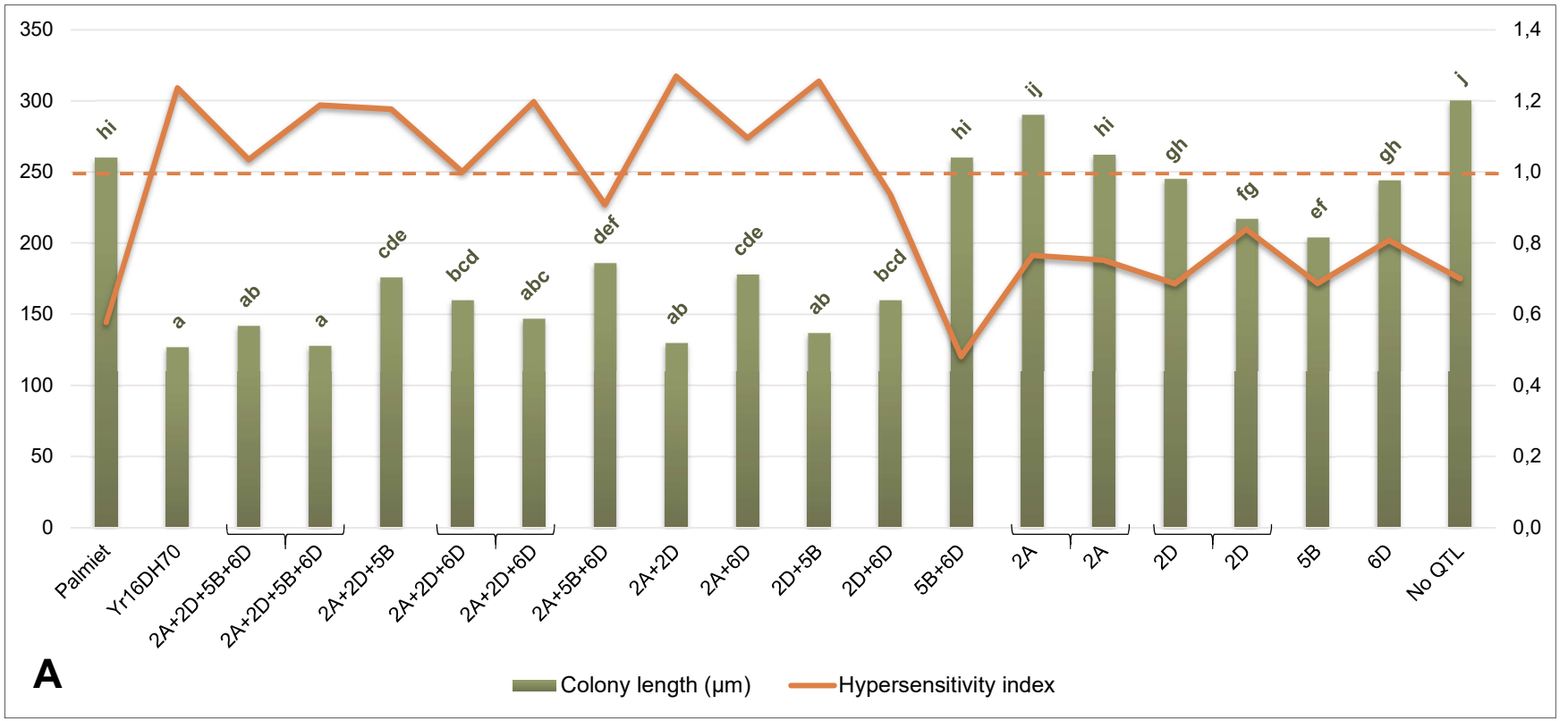



**Figure 3.4:** Microscopic images of leaves from the first sampling, showing fluoresced *Puccinia striiformis* f. sp. *tritici* colonies (left) and associated host cell necrosis (right) in parents **A)** Palmiet and **B)** Yr16DH70 and recombinant inbred lines containing resistance quantitative trait loci on chromosome(s) **C)** 2A+2D, **D)** 2A+6D, **E)** 5B+6D, **F)** 2D, **G)** 6D, and **H)** none. Scale bars represent 500 µm.

In contrast, entries transcribing a combination resistance QTL not including either 2A or 2D (i.e. 5B+6D), only one or no QTL, presented H-index values < 1. There was little variation in colony lengths measured for the majority of these lines, ranging from 250 to 300 µm in the first and 200 to 250 µm in the second replication. Exceptions include lines carrying solely 2D and 5B QTL which produced smaller colonies, yet H-index values well below or equal to one.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hi
gh
fg
ef
gh
j
Palmiet
Yr16DH70
2A+2D+5B+6D
2A+2D+5B+6D
2A+2D+5B
2A+2D+6D
2A+2D+6D
2A+5B+6D
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2A+6D
2D+5B
2D+6D
5B+6D
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2A
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2D
5B
6D
No QTL
A
Colony length (µm)
Hypersensitivity index

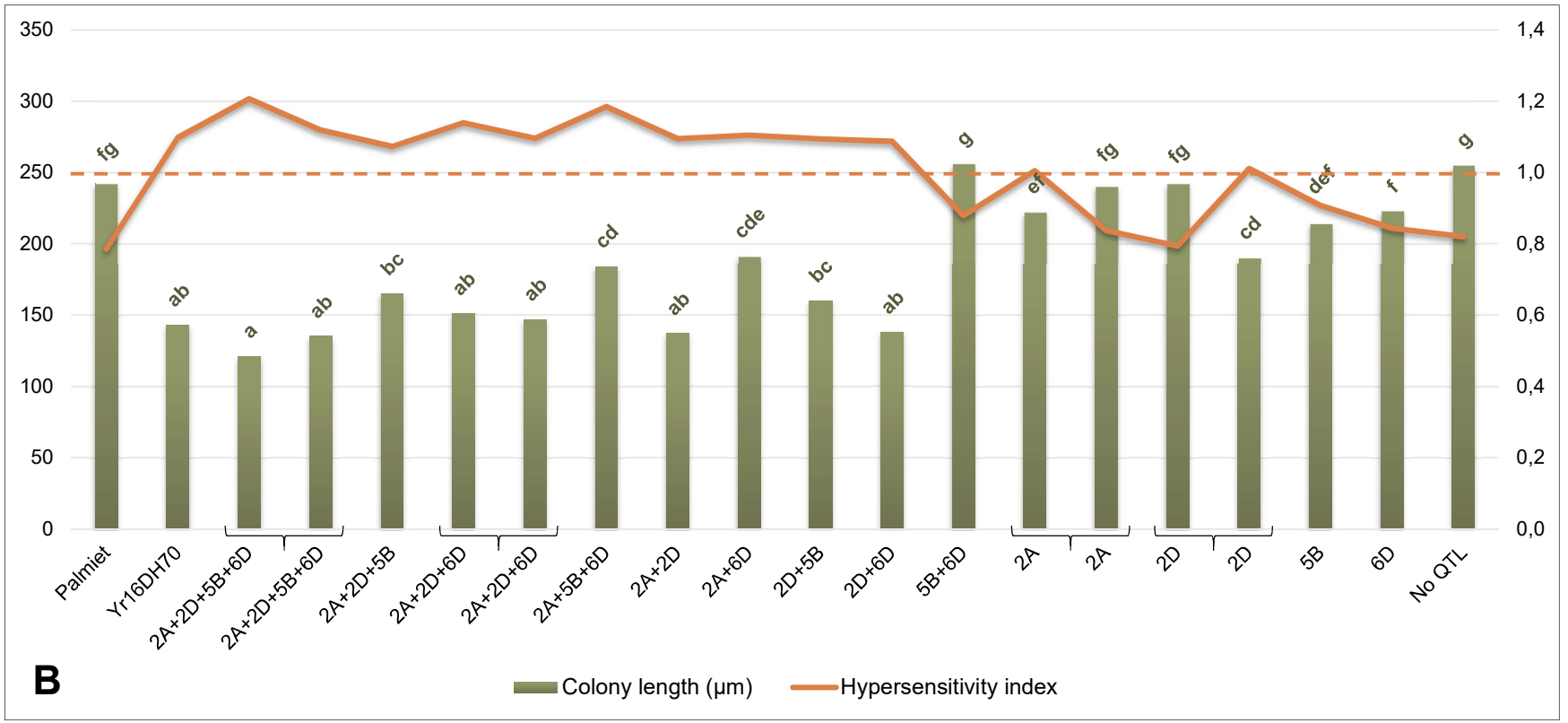
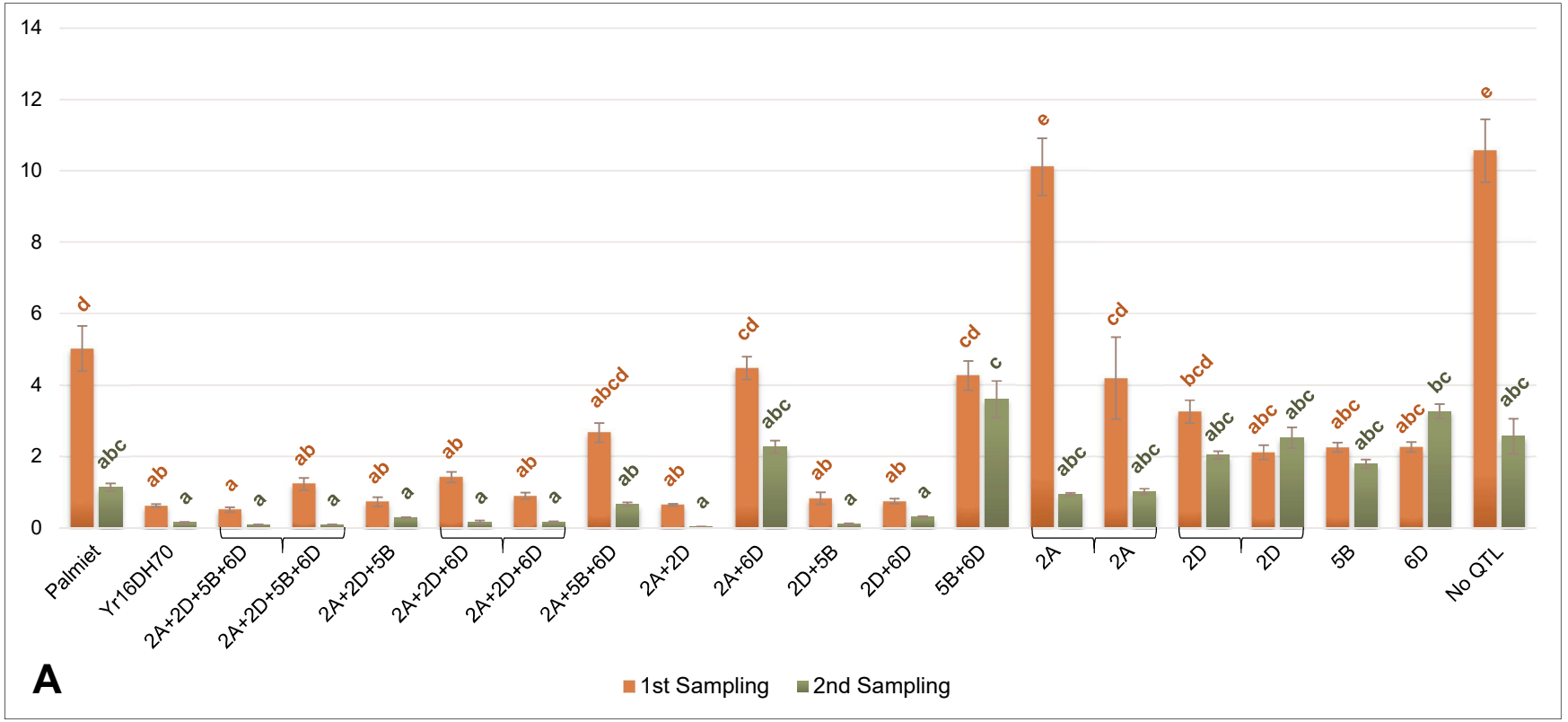



**Figure 3.5:** Average *Puccinia striiformis* f. sp. *tritici* colony length and hypersensitivity index of recombinant inbred wheat lines with different combinations of stripe rust resistance quantitative trait loci (QTL) located on different chromosomes in the **A)** first and **B)** second replication. Brackets indicate lines with matching predicted QTL profiles. Least significant differences (LSD) among lines for colony length and hypersensitivity index were calculated as 31.37 and 0.2, respectively, i.e. means with the same LSD symbol do not differ significantly from each other (P>0.05 ANOVA, Addendums 3.3 and 3.5, respectively).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Palmiet
Yr16DH70
2A+2D+5B+6D
2A+2D+5B+6D
2A+2D+5B
2A+2D+6D
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No QTL
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1st Sampling
2nd Sampling

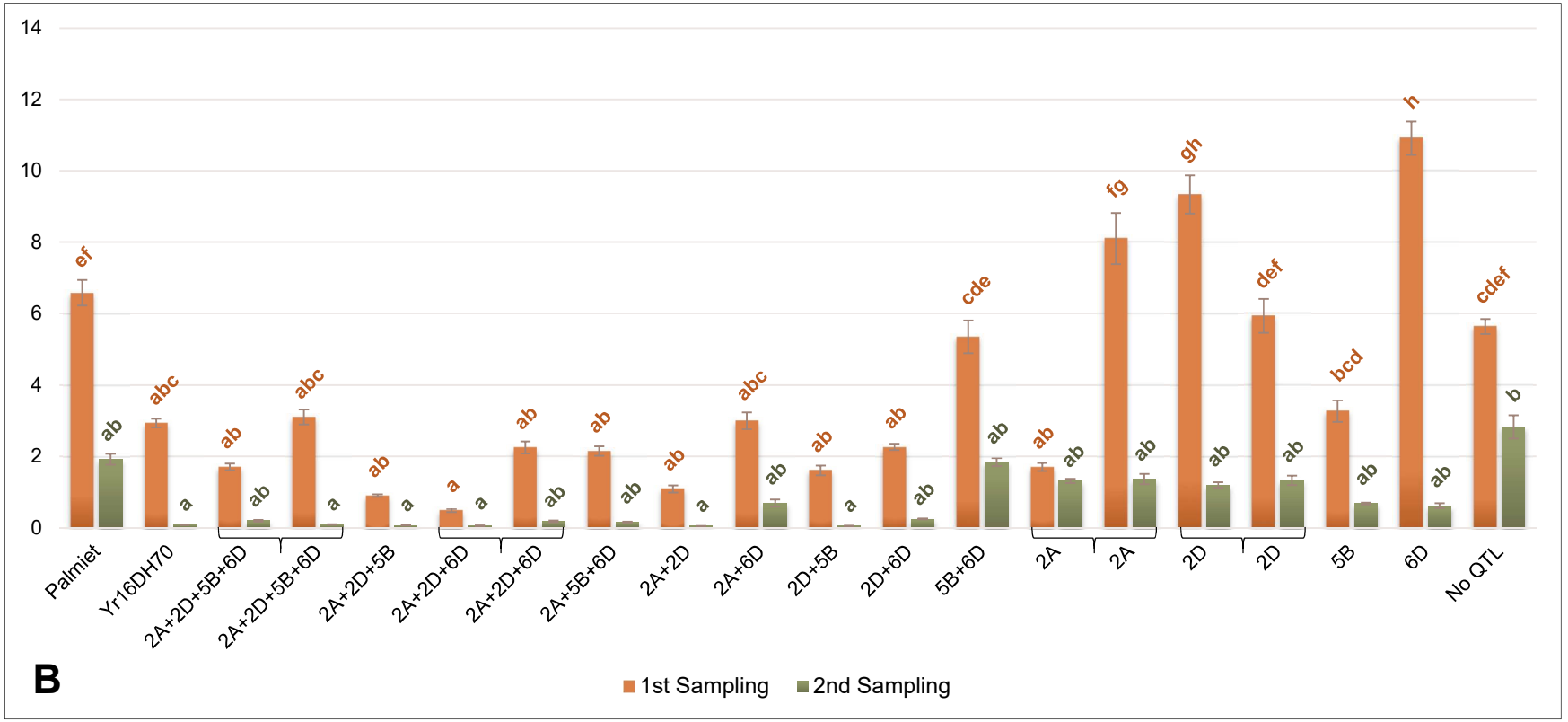



**Figure 3.6:** Relative *β-tubulin* expression of *Puccinia striiformis* f. sp. *tritici* in the wheat parents and recombinant inbred lines containing different combinations of stripe rust resistance quantitative trait loci (QTL) located on chromosomes 2A, 2D, 5B and 6D. Expression was measured at two sampling times for the **A)** first and **B)** second field replication. Brackets indicate lines with matching predicted QTL profiles. Error bars represent positive and negative standard errors. Least significant differences (LSD) among lines for relative *β-tubulin* expression were calculated as 2.71, i.e. means with the same LSD symbol do not differ significantly from each other (P>0.05 ANOVA, Addendum 3.6).

**Molecular analysis**

RT-qPCR enabled the rapid quantification of accumulated *Pst* fungal biomass in host plants. A single peak during melt curve analysis along with one ±100 bp amplicon on the agarose gel, confirmed the specific amplification of a single product. ANOVA (Addendum 3.6) showed significant differences in relative *Pst β-tubulin* expression across lines as well as the two-week time period ($P<0.05$). However, there was no significant interaction between test lines and the time points ($P>0.05$).

Some of the RILs revealed extreme levels of relative *Pst β-tubulin* expression with significant standard errors during the first sampling, although in only one of the replications. These include carriers of single QTL 2A (RIL 112 and 178), 2D (RIL 15 and 190), 6D (RIL 149) and a line containing no resistance QTL (RIL 52). These cases made it difficult to determine trends, even though insignificant statistical variation was observed between field replications ($P>0.05$).

The majority of lines experienced a sharp decrease in relative *Pst β-tubulin* expression from the first to the second sampling, except slight increases observed for two entries containing single resistance QTL (2D and 6D) (Figure 3.6).

Apart from the first replicate of the 2A+6D combination, very little *Pst β-tubulin* expression was detected in lines carrying either 2A or 2D in combination with at least one other QTL. The first sampling levels were typically lower in these lines compared to the more susceptible RILs mentioned below, in addition to barely detectable levels in the second sampling while susceptible lines accounted for high relative *Pst β-tubulin* expression. The more susceptible entries included the susceptible parent, lines containing 2A+6D, 5B+6D, a single (2A, 2D, 5B or 6D) or no resistance QTL.

## DISCUSSION

Mechanisms of durable resistance typically involve slowing down the development of the pathogen, instead of completely preventing infection (Singh *et al.*, 2005). However, depending on the environment, large-effect, slow rusting genes often provide insufficient APR in the field. The success of resistance can be greatly enhanced, to the point of near-immunity, by combining several small effect genes with slow rusting resistance (Singh *et al.*, 2000).

Through segregating mapping populations, minor QTL can be detected and studied in the absence of major genes, which typically increase the overall resistance of a genotype (Boyd, 2005). Periyannan *et al.* (2017) mentioned that it is important to recognise the most effective combinations of rust resistance genes that may interact in an additive way. Despite the many literature reports proposing gene combinations as a resistance breeding strategy, few contemporary studies have actually investigated complementary effects of gene combinations at histological level. For instance, enhanced leaf rust resistance was expressed when *Lr34* was combined with other resistance genes (German and Kolmer, 1992; Kolmer, 1992), with evident interactive effects reported when combined with *Lr13* and *Lr37* individually (Kloppers and Pretorius, 1997). Similarly, *Sr2* is known to raise the level of stem rust resistance mediated by minor resistance genes with additive effects (Knott, 1968), which have led to the stacking of many different '*Sr2*-complexes' (McIntosh *et al.*, 1995; Ellis *et al.*, 2014). Even though *Lr34* has contributed to long-lasting leaf rust resistance, as the '*Sr2*-complex' continues to do against stem rust, resistance conferred by interacting gene pairs is not always long-lasting. A case of non-durable resistance was reported for the complementary genes *Lr27* and *Lr31* in the Australian cultivar 'Gatcher' (Singh and McIntosh, 1984).

All parameters taken into account, the overall trend in this study confirmed the findings of Agenbag *et al.* (2012) of an increase in stripe rust resistance conferred by an increasing number of QTL retained from Cappelle-Desprez, and conversely, reduced resistance effects with only one or no QTL. This supports the assumption that resistance based on several genes and/or QTL, such as in Cappelle-Desprez, will significantly reduce disease levels and remain effective for longer periods. However, a noteworthy exception was observed with the 5B+6D combination, which represents the two QTL intervals that need to be improved to clarify their value (Agenbag *et al.*, 2012). The need to delimit QTL intervals to smaller regions is further attested by the diverse responses documented across parameters for RIL 15 and RIL 190, both predicted to carry the 2D-chromosome QTL. RIL 190 had one of the 5B Yr16DH70-alleles (186 bp) for one of the three markers spanning this QTL interval (gwm371), although not reflecting in the predicted presence of the QTL which requires the detection of all three marker loci (Addendum 3.1; data obtained from R. Prins, CenGen (Pty) Ltd). It is thus possible that RIL 190 could have retained 5B.

The diverse reactions observed across parameters for RIL 112 and RIL 178 with similar predicted genotypes in terms of Yr16DH70-derived QTL (a single 2A), might be the result of field effects since dissimilarities only reflected in the first replication and no other QTL marker was detected for either RIL. Similarly, slight variations were seen for the two representatives of 2A+2D+5B+6D- (RIL 20 and RIL 148) as well as 2A+2D+6D- (RIL 34 and RIL 142) QTL profiles. The rating of entries using the Zadoks growth scale will benefit future field trials to identify differences, if any, among lines in terms of growth period, earliness and consequently disease onset.

A clear distinction was made between more resistant RILs carrying either 2A or 2D combined with at least one other resistance QTL, and more susceptible RILs bearing no, only one, or a QTL combination not including 2A or 2D (i.e. 5B+6D). Along with the resistant parent, RILs containing 2A+2D+5B+6D and 2A+2D QTL profiles resulted in the highest resistance responses observed. This accentuates the fact that different QTL combinations vary significantly in the level of resistance imparted to the respective RILs as was found for severity and reaction type scores across 32 different QTL groups previously analysed (Agenbag *et al.*, 2012).

The most ineffective combination proved to be 5B+6D which had either similar or less success in withstanding disease development over the individual 5B or 6D QTL. Applying stricter criteria for QTL presence in a single RIL for each QTL profile, this finding corroborates results obtained through multiple RILs per QTL profile tested using the closest QTL marker (Agenbag *et al.*, 2012). The enhanced resistance effect with an additional chromosome 2A locus (2A+5B+6D) was slightly less than that conditioned by the other combinations comprised of three QTL (all of which included both 2A and 2D).

Part of the more resistant group, the 2A+6D combination showed slightly more disease development than the rest of the combinations comprised of two QTL (excluding 5B+6D mentioned above). Moreover, insignificant variation occurred with an additional 5B QTL (2A+6D+5B), while slightly lower disease scores were consistent for the 2A+2D+6D combination (i.e. an additional 2D QTL). Combinations 2D+5B and 2D+6D had visibly more resistant responses than that of the respective individual QTL, and further enhanced resistance effects with the addition of major QTL 2A (i.e. 2A+2D+5B and 2A+2D+6D). The 2A+2D QTL combination seemed highly beneficial in providing

stripe rust resistance, with little improvement and often even diminished effects with the addition of another QTL(s). These observations were consistent across all parameters measured, although in most cases based on a single RIL carrying a specific QTL profile.

Significant coefficients of correlation occurred among data sets generated from the different variables used to measure resistance in the first field replicate ($r_s$>0.8) (Addendum 3.7). Less correspondence was observed in the second replication (Addendum 3.8); a factor of 0.7 was calculated for the relationship between colony length with both CI and relative *Pst β-tubulin* expression, while CI and relative *Pst β-tubulin* expression showed an even smaller factor of 0.63. Conclusive evidence indicated an increase in HCN with more resistant lines, confirmed by a high negative correlation between H-index and colony length (-0.87 and -0.89 for the first and second replicates, respectively). While most of the entries included in the resistant group are carriers of *QYr.ufs-2A* conditioning a necrotic response (Agenbag *et al.*, 2012), high H-index values were also calculated for combinations 2D+5B and 2D+6D.

Although not used in previous analyses of Yr16DH70-derived QTL (Agenbag *et al.*, 2012), CI values enabled the quantitative assessment of the combined effect of two disease scores. Data generated from CI values appeared similar to that obtained from analysing LAI and RT separately.

Although field trials reflect authentic conditions experienced by commercial cultivars during which parallel data were generated across different parameters, challenges were faced when studying them. Even though replications did not differ significantly (P>0.05, Addendums 3.2 to 3.4 and 3.6), variation was noted between replicates of the same entry. This could be explained by the position in the trial, as the first replication was planted on the outskirts of the field while the second replication had a more central position where the inoculum as well as the humidity might be higher and more beneficial for disease. Difficulties were experienced with fluorescence microscopy, starting with the staining process that was complicated by hardy leaf material grown under field conditions. This possibly also resulted in the little success achieved attempting a rapid staining method (Dugyala *et al.*, 2015). Secondary *Pst* infections as well as the presence of other microorganisms with chitin structural components had to be managed.

The extreme cases of relative *Pst β-tubulin* expression detected during the first sampling, which also resulted in substantial standard errors (Figure 3.6), could be explained by excessively infected leaves that were sampled for these entries. This points to uneven rust distribution within a large field nursery despite experimental design and inoculation efforts to create a uniform rust epidemic.

Notwithstanding the challenges faced, valuable data in terms of QTL combinations were obtained. The assessment of disease at a single time point might have been inconclusive as the two sampling times contributed significantly to trend analysis, even though diminished symptoms occurred by the time of the second sampling. This most likely resulted from a 4.2°C increase in maximum temperatures recorded between September and October 2015 (F.J. Kloppers, personal communication). The deteriorated fungal material resulted in the considerable decrease in *Pst* biomass from the first to the second sampling. This decline coincides with results obtained from a trial performed under controlled conditions, aimed to identify when wheat rust pathogens reach their highest level of expression. The outcome indicated that gene expression in rust pathogens peaked between 10 and 15 days after infecting the adult host plant, followed by a rapid decline (Maré, 2017).

More illustrative data can possibly be obtained if assessment occurs at advanced times in disease development, i.e. before the decline in fungal material and coalescing of individual colonies, increasing the possibility of a second, usable histological sampling. However, this would be impractical for field trials as disease development is governed by weather conditions and thus does not transpire at stipulated times after inoculation.

REFERENCES

Agenbag GM, Pretorius ZA, Boyd LA, Bender CM and Prins R. 2012. Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez. *Theoretical and Applied Genetics* 125: 109–120.

Ali S, Gladieux P, Leconte M, Gautier A, Justesen AF, Hovmøller MS, Enjalbert J and De Vallavieille-Pope C. 2014. Origin, migration routes and worldwide population genetic structure of the wheat yellow rust pathogen *Puccinia striiformis* f. sp. *tritici*. *PLoS Pathogens* 10(1): e1003903. doi: 10.1371/journal.ppat.1003903

Badaeva ED, Dedkova OS, Gay G, Pukhalskyi VA, Zelenin AV, Bernard S and Bernard M. 2007. Chromosomal rearrangements in wheat: their types and distribution. *Genome* 50: 907-926.

Bariana HS, Bansal UK, Schmidt A, Lehmensiek A, Kaur J, Miah H, Howes N and McIntyre CL. 2010. Molecular mapping of adult plant stripe rust resistance in wheat and identification of pyramided QTL genotypes. *Euphytica* 176: 251–260.

Boshoff WHP, Pretorius ZA and Van Niekerk BD. 2002. Resistance in South African and foreign wheat cultivars to pathotypes 6E16A- and 6E22A- of *Puccinia striiformis* f. sp. *tritici*. *South African Journal of Plant and Soil* 19: 27-36.

Boukhatem N, Baret PV, Mingeot D and Jacquemin JM. 2002. Quantitative trait loci for resistance against yellow rust in two wheat derived recombinant inbred line populations. *Theoretical and Applied Genetics* 104: 111–118.

Boyd LA. 2005. Can Robigus defeat an old enemy? - Yellow rust of wheat. *Journal of Agricultural Science* 143: 1-11.

Browder LE. 1971. Pathogenic specialization in cereal rust fungi, especially *Puccinia recondita* f. sp. *tritici*, concepts, methods of study, and application. *Agricultural Research Service Technical Bulletin* No.1432, pp. 1-51. United States Department of Agriculture: Washington, DC.

Chen X. 2013. Review article: High-temperature adult-plant resistance, key for sustainable control of stripe rust. *American Journal of Plant Sciences* 4: 608.

Chen X, Jones SS and Line RF. 1996. Chromosomal location of genes for resistance to *Puccinia striiformis* in seven wheat cultivars with resistance genes at the *Yr3* and *Yr4* loci. *Phytopathology* 86: 1228–1233.

Chen XM. 2005. Epidemiology and control of stripe rust (*Puccinia striiformis* f. sp. *tritici*) on wheat. *Canadian Journal of Plant Pathology* 27: 314-337.

Chen XM, Jones SS and Line RF. 1995. Chromosomal location of genes for stripe rust resistance in spring wheat cultivars Compair, Fielder, Lee, and Lemhi and interactions of aneuploid wheats with races of *Puccinia striiformis*. *Phytopathology* 85: 375–381.

Chomczynski P and Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162(1): 156-159. ISSN 0003-2697. doi: 10.1016/0003-2697(87)90021-2

Coleman T, Madassery JV, Kobayashi GS, Nahm MH and Little JR. 1989. New fluorescence assay for the quantitation of fungi. *Journal of Clinical Microbiology* 27(9): 2003-2007.

Coram TE, Settles ML and Chen X. 2008. Transcriptome analysis of high-temperature adult-plant resistance conditioned by *Yr39* during the wheat– *Puccinia striiformis* f. sp. *tritici* interaction. *Molecular Plant Pathology* 9(4): 479–493.

De Vallavieille-Pope C, Picard-Formery H, Radulovic S and Johnson R. 1990. Specific resistance factors to yellow rust in seedlings of some French wheat varieties and races of *Puccinia striiformis* Westend in France. *Agronomie* 2: 103–113.

Dodds PN and Rathjen JP. 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics* 11: 539–548. doi: 10.1038/nrg2812

Dugyala S, Borowicz P and Acevedo M. 2015. Rapid protocol for visualization of rust fungi structures using fluorochrome Uvitex 2B. *Plant Methods* 11: 54. doi: 10.1186/s13007-015-0096-0

Ellis JG, Lagudah ES, Spielmeyer W and Dodds PN. 2014. The past, present and future of breeding rust resistant wheat. *Frontiers in Plant Science* 5: 641. doi: 10.3389/fpls.2014.00641

Feng J, Zuo LL, Zhang ZY, Lin RM, Cao YY and Xu SC. 2011. Quantitative trait loci for temperature-sensitive resistance to *Puccinia striiformis* f. sp. *tritici* in wheat cultivar Flinor. *Euphytica* 178: 321–329.

German S and Kolmer JA. 1992. Effect of gene *Lr34* in the enhancement of resistance to leaf rust of wheat. *Theoretical and Applied Genetics* 84: 97-105.

Hao Y, Chen Z, Wang Y, Bland D, Buck J, Brown-Guedira G and Johnson J. 2011. Characterization of a major QTL for adult plant resistance to stripe rust in US soft red winter wheat. *Theoretical and Applied Genetics* 123: 1401-1411.

Jagger IJ, Newell C, Berry ST, MacCormack R and Boyd LA. 2011. The genetic characterisation of stripe rust resistance in the German wheat cultivar Alcedo. *Theoretical and Applied Genetics* 122: 723–733.

Jarošová J and Kundu JK. 2010. Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR. *Plant Biology* 10: 146. doi: 10.1186/1471-2229-10-146

Kloppers FJ and Pretorius ZA. 1997. Effects of combinations amongst genes *Lr13*, *Lr34* and *Lr37* on components of resistance in wheat to leaf rust. *Plant Pathology* 46: 737–750. doi: 10.1046/j.1365-3059.1997.d01-58.x

Knott D. 1968.The inheritance of resistance to stem rust races 56 and 15B-1L (Can.) in the wheat varieties Hope and H-44. *Canadian Journal of Genetics and Cytology* 10: 311–320.

Knott DR. 1989. The Wheat Rusts - Breeding for Resistance. Monographs on *Theoretical and Applied Genetics* 12. Springer Verlag: Berlin.

Kolmer JA. 1992. Enhanced leaf rust resistance in wheat conditioned by resistance gene pairs with *Lr13*. *Euphytica* 61: 123-130.

Lagudah ES. 2011. Molecular genetics of race non-specific rust resistance in wheat. *Euphytica* 179: 81-91.

Lagudah ES, Krattinger SG, Herrera-Foessel S, Singh RP, Huerta-Espino J, Spielmeyer W, Brown-Guedira G, Selter LL and Keller B. 2009. Gene-specific markers

for the wheat gene *Lr34/Yr18/Pm38* which confers resistance to multiple fungal pathogens. *Theoretical and Applied Genetics* 119: 889-898.

Law CN, Sutka J and Worland AJ. 1978. A genetic study of day-length response in wheat. *Heredity* 41: 185-191.

Law CN and Worland AJ. 1997. The control of adult-plant resistance to yellow rust by the translocated chromosome 5BS-7BS of bread wheat. *Plant Breeding* 116: 59–63.

Line RF. 2002. Stripe rust of wheat and barley in North America: a retrospective historical review. *Annual Review of Phytopathology* 40: 75–118.

Line RF and Chen X. 1995. Successes in breeding for and managing durable resistance to wheat rusts. *Plant Disease* 79(12): 1254–1255.

Ling P, Wang MN, Chen XM and Campbell KG. 2007. Construction and characterization of a full-length cDNA library for the wheat stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*). *BMC Genomics* 8: 145.

Lowe I, Jankuloski L, Chao S, Chen X, See D and Dubcovsky J. 2011. Mapping and validation of QTL which confer partial resistance to broadly virulent post-2000 North American races of stripe rust in hexaploid wheat. *Theoretical and Applied Genetics* 123: 143–157.

Lupton FGH and Macer RCF. 1962. Inheritance of resistance to yellow rust (*Puccinia glumarum* Erikss. and Henn.) in seven varieties of wheat. *Transactions of the British Mycological Society* 45: 21-45.

Lupton FGH, Wilson FE and Bingham J. 1971. Breeding for nonrace-specific resistance to yellow rust and to mildew. In: *Annual Report - Plant Breeding Institute*, Cambridge, pp 70.

Mallard S, Gaudet D, Aldeia A, Abelard C, Besnard AL, Sourdille P and Dedryver F. 2005. Genetic analysis of durable resistance to yellow rust in bread wheat. *Theoretical and Applied Genetics* 110: 1401–1409.

Maré A. 2017. Development of wheat lines with complex resistance to rusts and Fusarium head blight. PhD Thesis, University of the Free State, South Africa.

McIntosh RA. 1992. Close genetic linkage of genes conferring adult plant resistance to leaf rust and stripe rust in wheat. *Plant Pathology* 41: 523-527.

McIntosh RA, Dubcovsky J, Rogers WJ, Morris C and Xia XC. 2017. Catalogue of Gene symbols for wheat. https://shigen.nig.ac.jp/wheat/komugi/genes/macgene/supplement2017.pdf

McIntosh RA, Wellings CR and Park RF. 1995. Wheat rusts: An atlas of resistance genes. CSIRO, Australia.

Moldenhauer J, Moerschbacher BM and van der Westhuizen AJ. 2006. Histological investigation of stripe rust (*Puccinia striiformis* f. sp. *tritici*) development in resistant and susceptible wheat cultivars. *Plant Pathology* 55: 469–474.

Niks RE and Dekens RG. 1987. Histological studies on the infection of triticale, wheat and rye by *Puccinia recondita* f. sp. *tritici* and *P. recondita* f. sp. *recondita*. *Euphytica* 36: 275–285.

Periyannan S, Milne RJ, Figueroa M, Lagudah ES and Dodds PN. 2017. An overview of genetic rust resistance: From broad to specific mechanisms. *PLoS Pathogens* 13(7): e1006380. doi: 10.1371/journal.ppat.1006380

Peterson RF, Champbell AB and Hannah AE. 1948. A diagrammatic scale for estimating rust intensity of leaves and stem of cereals. *Canadian Journal of Research* 26c: 496-500. doi: 10.1139/cjr48c-033

Powell NM. 2010. Phenotyping and genetic analysis of yellow rust resistance in the UK winter wheat cultivar Claire. PhD Thesis, University of East Anglia, UK.

Riley R, Coucoli H and Chapman V. 1967. Chromosomal interchanges and the phylogeny of wheat. *Heredity* 22: 233-247.

Rohringer R, Kim WK, Samborsky DJ and Howes NK. 1977. Calcofluor: an optical brightener for fluorescence microscopy of fungal plant parasites in leaves. *Phytopathology* 67: 808–810.

Sambrook J and Russell DW. 2000. Molecular Cloning: A Laboratory Manual, 3rd edition. Cold Spring Harbor Laboratory Press, New York, pp. A8.19-8.21; 7.31-7.34.

Sambrook J, Fritsch EF and Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press, New York.

Scholtz JJ and Visser B. 2013. Reference gene selection for qPCR gene expression analysis of rust-infected wheat. *Physiological and Molecular Plant Pathology* 81: 22-25.

Singh RP and McIntosh RA. 1984. Complementary genes for reaction to *Puccinia recondita tritici* in *Triticum aestivum*. Vol. I. Genetic and linkage studies. *Canadian Journal of Genetics and Cytology* 26: 723-735.

Singh RP, Huerta-Espino J and William HM. 2005. Genetics and breeding for durable resistance to leaf and stripe rusts in wheat. *Turkish Journal of Agriculture and Forestry* 29: 121-127.

Singh RP, Huerta-Espino J, Bhavani S, Herrera-Foessel SA, Singh D, Singh PK, Velu G, Mason RE, Jin Y, Njau P and Crossa J. 2011. Race non-specific resistance to rust diseases in CIMMYT spring wheats. *Euphytica* 179: 175-186.

Singh RP, Nelson JC and Sorrells ME. 2000. Mapping *Yr28* and other genes for resistance to stripe rust in wheat. *Crop Science* 40: 1148-1155.

Stubbs RW, Prescott JM, Saari EE and Dubin HJ. 1986. Cereal Disease Methodology Manual. Centro Internacional de Mejoramiento de Maíz yTrigo (CIMMYT), Mexico.

Thrall PH and Burdon JJ. 2003. Evolution of virulence in a plant host-pathogen metapopulation. *Science* 299(5613): 1735–1737.

Worland AJ and Law CN. 1986. Genetic analysis of chromosome 2D of wheat. *Zeischrift fur Pflanzenzuchtung* 96: 331–345.

# Chapter 4: Infection and colonisation of *Puccinia graminis* in barley

## INTRODUCTION

Stem rust is one of the most destructive diseases in small grain crops grown around the world, where substantial to complete yield losses can occur during epidemic years. The causal pathogenic fungus, *Puccinia graminis* Pers. (*Pg*), is able to spread over long distances under natural airborne conditions (Brown and Hovmøller, 2002), while mutation and recombination of existing races can also give rise to new virulence in the population (Knott, 1989).

The fungus is adapted to infect and complete its macrocyclic life cycle in a host range comprised of primary, secondary and ancillary hosts (Arthaud *et al.*, 1966). Most research efforts have been focused on the primary cereal hosts of the asexual stage, commercially produced on a global scale. The economically important wheat (*Triticum aestivum* L.) host has been well-studied regarding genetic resistance and development of durably resistant cultivars, whereas barley (*Hordeum vulgare* L.) has received comparatively less attention.

Barley is a host for the wheat (*P. graminis* f. sp. *tritici* Erikss. and Henning) (*Pgt*) and rye (*P. graminis* f. sp. *secalis* Erikss. and Henning) (*Pgs*) attacking forms of the pathogen (McIntosh *et al.*, 1995), with less extensive damage reported compared to that on wheat. The shorter maturation period of barley probably contributes to this observation, since the onset of rust infection frequently coincided with the ripening of the crop (Steffenson, 1992). Additionally, barley has acquired an underlying basal level of stem rust resistance conditioned by the *Rpg1* gene (Steffenson *et al.*, 2017) that has protected the crop in North America from substantial stem rust losses for more than 70 years (Steffenson, 1992).

This extensive implementation of a single resistance gene made the crop extremely susceptible to the emergence of new races of *Pgt* (Keiper *et al.*, 2003). Indeed, virulence was reported for *Rpg1* with the appearance of pathotypes QCCJB in 1988, causing crop losses in Northern America (Martens *et al.*, 1989; McVey *et al.*, 2002; Steffenson *et al.*, 2017) as well as TTKSK a decade later in East Africa (Pretorius *et al.*, 2000). With broad-spectrum virulence to many resistance genes (Pretorius *et al.*, 2000; Wanyera *et al.*, 2006; Jin *et al.*, 2007) and its rapid adaptive capacity to

overcome new resistance loci (Jin *et al.*, 2008, 2009), TTKSK and variants, grouped in the 'Ug99' lineage, have become one of the most endangering biotic factors to food security in over 5 decades (Singh *et al.*, 2006; Jin *et al.*, 2007). It was estimated that the 'Ug99' group is capable of attacking wheat crops cultivated over 80 to 95% of the total global wheat area (Singh *et al.,* 2008, 2015) while more than 96% of barley varieties grown worldwide are at risk (Steffenson *et al.,* 2017).

In addition to *Rpg1*, the 'Ug99' race group possesses virulence against the majority of barley stem rust resistance genes. The *rpg4*/*Rpg5* gene complex identified in breeding line Q21861 (Steffenson *et al.*, 2009; Wang *et al.*, 2013) is the only locus imparting highly effective resistance against 'Ug99' races at both seedling (Steffenson *et al.*, 2009; Case, 2017) and adult plant stages (Mamo *et al.*, 2015). Yet, Steffenson *et al.* (2017) reported the unexpected vulnerability of the gene complex under high disease pressure and elevated temperatures.

The recessive *rpg4* gene is markedly sensitive to higher temperatures and only expressed between 17 and 22°C (Jin *et al.,* 1994). Believed to regulate a gene-for-gene interaction, *rpg4* encodes an actin depolymerizing component in barley associated with the reorganization of fungal cytoskeleton. Though not to all, *rpg4* confers resistance to several *Pgt* races (Brueggeman *et al.,* 2009), which is determined by slight polymorphisms as was demonstrated in the resistant (*rpg4*+) and susceptible (*Rpg4*-) alleles for pathotype QCCJB, varying at only two amino acid sites. Derived from a cross between Q21861 (*rpg4*/*Rpg5* complex) and susceptible Steptoe (lacking *Rpg5* and expressing three amino acids different from Q21861 in the *rpg4* allele), progeny line SQ41 carries the *Rpg5* allele and a recombinant *Rpg4*- transcribing amino acid glutamine at the 39th position as in Q21861, while positions 101 and 135 express threonine and glycine, respectively, similar to Steptoe (Brueggeman *et al.*, 2009).

The *Rpg5* gene is tightly linked to *rpg4* and established as the key polymorphic gene in *rpg4*-mediated *Pgt* resistance (Brueggeman *et al.*, 2008; Wang *et al.*, 2013). The *Rpg5*-mediated resistance conditioned against isolates of *Pgs* is however not dependent on *rpg4*, as was proven for *Pgs* isolate 92-MN-90 (Brueggeman *et al.*, 2008, 2009; Mamo *et al.*, 2015).

*Rpg5* is thought to express a unique resistance protein comprised of a nucleotide-binding site and leucine rich repeat in conjunction with a serine threonine protein kinase

domain (Brueggeman *et al.*, 2008). It was suggested that *Rpg5* detects *Pgs* and *Pgt* in this pathosystem but interacts with the two stem rust pathogens using different resistance mechanisms (Arora *et al.*, 2013).

The majority of research regarding *Pgt* resistance in barley has been done in the United States of America where restrictions have been implemented against the utilisation of TTKSK in phenotyping. The use of pathotype QCCJB as a substitute is validated as evidence suggested that resistance against different *Pgt* races (including QCCJB and TTKSK) is conditioned by genetically similar mechanisms at the *rpg4*/*Rpg5* locus (Wang *et al.*, 2013).

Known as a stress tolerant crop, barley seems inherently more resistant to stem rust infection than wheat (Sun and Steffenson, 2005; Steffenson *et al.*, 2017). This elevated basal resistance may, under most circumstances, provide sufficient protection against *Pg* (Steffenson *et al.*, 2017).

To investigate whether the reduced receptivity of barley to stem rust is in any way related to the infection and early colonisation process, barley lines carrying different resistance genes along with wheat control entries were inoculated at the adult plant stage. Two *Pgt* pathotypes and one *Pgs* pathotype were used to perform histological and molecular analysis during onset and early establishment of disease.

## MATERIALS AND METHODS

### Plant and pathogen materials

Seed of barley lines Q21861, Q/SM20, SQ41, Hietpas-5, and Chevron, containing different stem rust resistance genes (*Rpg_;* **R**eaction to ***P****uccinia* ***g****raminis*), and Hiproly and PI 532013 (stem rust susceptible) were received from the University of Minnesota (Table 4.1). Two wheat checks were included namely line 37-07 (susceptible to wheat stem rust, Prins *et al.*, 2016) and local variety SST047 which is resistant to South African stem rust races.

**Table 4.1:** Selected barley lines and wheat entries.

| Crop | Line | Resistance gene(s) | Reference |
|---|---|---|---|
| Barley | Chevron | *Rpg1* | Shands, 1939 |
| | Q21861 | *Rpg1*, *rpg4*/*Rpg5* complex | Jin *et al.*, 1994; Steffenson *et al.*, 2009 |
| | Hietpas-5 | *Rpg2* | Patterson *et al.*, 1957 |
| | Q/SM20 | *rpg4*/*Rpg5* complex | Steffenson *et al.*, 2009 |
| | SQ41 | *Rpg5* | Brueggeman *et al.*, 2009 |
| | Hiproly | Susceptible | Steffenson BJ, unpublished |
| | PI 532013 | Susceptible | Steffenson BJ, unpublished |
| Wheat | SST047 | *Sr36* | Pretorius *et al.*, 2012 |
| | Line 37-07 | Susceptible | Prins *et al.*, 2016 |

Plants were grown in a disease-free greenhouse cubicle under controlled temperature conditions of 18 to 25°C. Two plants were grown per 2 L pot in steam-sterilized soil. A sufficient number were planted for three *Pg* isolates and two biological replications. Considering the different crops and cultivars, provision was also made for varying growth habits. Plants were watered twice a day with reverse osmosis water. Multifeed-Classic fertilizer [Effekto®, NPK Analysis 19:8:16 (43)] was applied twice a week at a concentration of 2.5 g/L water.

Wheat lines Federation4*/Kavkaz and McNair 701, and rye cultivar Pan233, were planted in Mikskaar® potting substrate MPS2, for multiplication of the spores of *Pg* pathotypes PTKST (‘Ug99’ race group, local isolate UVPgt60), BNGSC (non-‘Ug99’ local isolate UVPgt54, originally collected from barley [Boshoff *et al.*, 2002]) and UVPgs01, respectively. After seedling emergence, 99% maleic hydrazide ReagentPlus® [Sigma-Aldrich (0.3 g/L water, 50 mL per pot)] was applied to suppress plant growth. Seven days after planting, urediniospores of the *Pg* isolates were retrieved from storage at -80°C and heat shocked at 46°C for 6 min. The *Pg* pathotypes were suspended in Soltrol® 130 (Phillips Petroleum, Bartlesville, OK, USA) isoparaffinic solvent in gelatin capsules and sprayed onto the respective seedlings using a pressure pump connected to a custom-made inoculation device (Browder, 1971). The inoculation booth was flushed with water for at least 1 min between isolates.

Inoculated seedlings were dried-off in a growth cabinet for 1 h at 25°C prior to incubation at 20-23°C and > 96% relative humidity in a dew chamber. After a 16 h dew period seedlings were placed under fluorescent lights in the growth cabinet for 2 h and returned to the greenhouse. When sporulation on seedlings was sufficient, fresh urediniospores were collected for inoculation of adult plants at heading stage.

For each isolate a concentration of 1 mg spores per 1 mL water containing Tween20® surfactant was used. The spore suspension was applied as uniformly as possible with a low-pressure spray gun connected to a compressor operating at 250 kPa. The spray gun was thoroughly rinsed with water between the application of different isolates. To observe stomatal behaviour on stem leaf sheaths that underwent a dew period but without rust infection, the last internode of one stem per line was covered with household cling wrap before inoculation. When the inoculation process was complete the cling wrap was removed from the dew-controls and plants were placed in a plastic dew chamber at >96% humidity for 24 h. The incubation procedure for adult plants was described by Bender *et al.* (2016). A minimum of 14°C was measured for the incubation period in both replicates, while the first and second replications reached a maximum of 25°C and 28°C, respectively. The plants were then returned to greenhouse conditions at 18-25°C.

**Histological analysis**

Scanning electron microscopy

Four segments (5 mm in length) of the flag leaf sheath on the last stem internode of each treatment were cut before inoculation, at 24 hours post-inoculation (hpi) and 48 hpi. The protocol, according to Glauert (1974), entailed submerging stem segments in 3% (v/v) glutardialdehyde (in a 0.1 M sodium phosphate buffer at pH 7.0), as the primary fixative, for at least 24 h. The material was washed with fresh buffer solution for 5-10 min, immersed in the secondary fixative 1% osmium tetroxide for 1-2 h, followed by two steps of rinsing with the fixing buffer for 5-10 min each. Subsequently, dehydration was done in an ethanol series of 30%, 50%, 70% and 95% for 10-30 min each. The last step of the series was repeated with 100% ethanol for 15-30 min each. The material was dried using a Tousimis critical point dryer, replacing the ethanol with pressurised liquid $CO_2$ at 31.5°C. During this step the liquid $CO_2$ is converted to the gaseous phase that ensures maximum structural preservation.

For observations on the outer surface of the stems, the dried specimens were mounted on metal stubs. To observe the substomatal vesicle (SSV) from the inner epidermal surface, the epidermal layer was stripped with a needle after the dried stems were rolled open and mounted on metal stubs. With the use of a sputter coater, the dried stems were coated with gold, making them electrically conductive. A Shimadzu SSX-550 scanning electron microscope was used (accelerating voltage of 0.5 to 30kV 10V step) for inspection of fungal development on the outside of the stems (magnifying up to X1200) and inside the substomatal cavity (using magnification up to X3600).

Fluorescence microscopy

Growth of *Pg* colonies were viewed and quantified on the stems of selected barley and wheat entries through fluorescence microscopy.

Stems were sampled at 120 hpi. Four segments (3 cm in length) of the last stem internode of each treatment were submerged in 1 M KOH (56.11 g/mol + 1 mL Silwet L77 wetting agent) and incubated overnight at 37°C to extract the chlorophyll. Samples were washed twice with 50 mM Tris-HCl buffer (121.14 g/mol at pH 7.0) for 10 min each. Samples were kept in Tris-HCl buffer at 4°C until stems turned yellow and semi-transparent. Leaf sheaths of each sample were carefully removed from stems and stained overnight at room temperature with a Wheat Germ Agglutinin (WGA) Lectin FITC probe [Sigma-Aldrich (200 µL/10 mL new Tris-HCl buffer)]. Using the WU epifluorescence cube (450-480 nm excitation filter and 515 nm barrier filter) on an Olympus AX70 microscope (Tokyo, Japan), the area exhibiting fungal growth of 15 to 20 infection sites per stem segment was measured in square micrometres ($\mu m^2$), and a mean was calculated for each treatment. Images were captured using the fitted CC12 digital camera and AnalySIS LS Research version 2.2 software (Olympus Soft Imaging System, Japan).

**Molecular analysis**

The development in fungal biomass was quantified at two time points, five days apart, based on relative gene expression (Coram *et al.*, 2008). This approach entails the expression of an experimental gene relative towards a reference gene. Flag leaf sheaths on the last stem internode of the plants were cut at 120 and 240 hpi, immediately placed in liquid nitrogen and stored at -80°C for following analysis.

Ribonuclease free water was used for RNA extraction and subsequent testing. The preparation entails 0.1% (v/v) dimethyldicarbonate (DMDC) added to deionised water, incubated overnight at room temperature and autoclaved twice to inactivate the DMDC.

Frozen samples were transferred to liquid nitrogen and ground to a fine powder. Trizol reagent (Invitrogen™, Carlsbad, California, USA) was used in line with the manufacturer's instructions to extract total RNA from 100 µL leaf sheath tissue (Chomczynski and Sacchi, 1987). The frozen tissue was partially thawed and suspended in 500 µL Trizol reagent at room temperature for 10 min before 100 µL chloroform was added. Following 5 min of incubation, samples were centrifuged at 12 000 *g* for 15 min at 4°C. The cleared supernatant was recovered and added to 250 µL isopropanol for the precipitation of RNA. After a 10-min incubation period, tubes were centrifuged at 12 000 *g* for 10 min at 4°C. Once the RNA pellet was retrieved using a water jet pump, it was rinsed with 70% (v/v) ethanol. Finally, the air-dried RNA was dissolved in 200 µL DMDC treated water.

Using a Nanodrop2000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA), the RNA concentration of the samples was determined with DMDC treated water as blank (Sambrook and Russell, 2000).

Electrophoresis was performed to analyze extracted RNA quality by separating 400 ng on a 1.2% (w/v) agarose gel (Sambrook *et al.*, 1989) prepared in 0.5x TAE (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.28% (v/v) acetic acid) containing 0.5 µg/mL ethidium bromide. The gel was exposed to ultraviolet light illumination (302 nm) and photographed using the GelDoc XR+ System (BioRad, Hercules, California, USA) and Quantity One 4.6 program.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was effected with the Bio-Rad C1000 thermal cycler connected to a CFX96 real-time attachment, using KAPA™ SYBR® FAST Universal One-Step qRT-PCR Kit (Lasec).

Fungal biomass and development was assessed by quantification of the constitutively expressed fungal *β-tubulin* in reference to the constitutively expressed *18S ribosomal RNA* (*18S*) in host plants over the time course. The same reference gene was considered in both barley and wheat to facilitate normalization of RT-qPCR data.

The forward and reverse primer sequences of *18S* in wheat, validated as the most efficient reference gene for *Pgt* expression analysis (Scholtz and Visser, 2013), had a 100% alignment against barley *18S* using Basic Local Alignment Search Tool (BLAST), enabling its use for reference gene amplification in both crops. The RT-qPCR primer pair for *Pgt β-tubulin* was developed using the web-based program Primer3. The primer sequences for *β-tubulin* in *Pgt* was used to track the expression of this gene in *Pgs*. Blasting confirmed *β-tubulin* as highly conserved in *Pg* and both primer pair sequences showed complete homology. Any difference in nucleotide sequence for *β-tubulin* between the *formae speciales* was assumed negligible (Table 4.2).

To standardize the primers for RT-qPCR, a temperature gradient and subsequent electrophoresis was performed for determination of annealing temperatures optimal for each primer set. A standard curve reaction was performed making use of a 1:10 dilution series. Amplification efficiency of each primer pair was determined and amplicon confirmed by separating standard curve reactions on 1.2% (w/v) agarose gel (as described before). The most accurate amplification with *18S* was seen at 60°C with an $R^2$ of 0.998 and 0.996 for barley and wheat, respectively (Table 4.2), whereas the best gradient for *Pgt β- tubulin* was 66°C ($R^2$=0.917).

Once the primer sets were optimized, expression of the experimental *β-tubulin* gene was evaluated. Each 10 µL RT-qPCR reaction contained 10 ng total RNA, 10 µM of both forward and reverse primer (Table 4.2), 5 µL KAPA Master Mix buffer, 0.2 µL KAPA Reverse Transcriptase Mix and 1.8 µL DMDC treated water. The RT-qPCR protocol entails an initial step of 42°C for 10 min and a denaturing step of 95 °C for 3 min.

**Table 4.2:** Nucleotide sequence, annealing temperature and amplification efficiency for primer pairs used during RT-qPCR.

| Gene symbol | Accession nr. (NCBI) * | Primer sequence (5'-3') | Amplicon size (bp) | Annealing temp (°C) | RT-qPCR efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| *18S* | Wheat: AH001810.2 | F: GTGACGGGTGACGGAGAATT<br>R: GACACTAATGCGCCCGGTAT | 151 | 60 | 103 | Jarošová and Kundu, 2010 |
| | Barley: AH001585.2 | | | | 98 | |
| *Pgt β-TUB* | XM_003330619.2 | F: CTCGATCGTGATGAGTGGGA<br>R: AGTGCAATCGAGGGAAAGGA | 106 | 66 | 153 | |

RT-qPCR = quantitative reverse transcription polymerase chain reaction.
* http://www.ncbi.nlm.nih.gov/, NCBI = National Centre for Biotechnology Information.
bp = base pairs.
*18S = 18S ribosomal RNA*.
*Pgt β-TUB = Puccinia graminis* f. sp. *tritici β-tubulin*.

The following steps were repeated 40 times for the synthesis of cDNA: an additional 10 sec denaturing step at 95°C and 30 sec of specific annealing temperature (Table 4.2) for primer annealing and elongation. Upon completion of each cycle, the plate was read for quantity SYBR green bound to double stranded nucleic acid. A melt curve was included from 65-95°C with 0.5°C increments every 5 sec to confirm the absence of non-specific amplification products. The plate was read again once the reaction was completed.

Using qBase+ software (version 3.1 Biogazelle, Zwijnaarde, Belgium), RT-qPCR data analysis of nine host lines inoculated respectively with three *Pg* isolates was done with each experiment consisting of two biological replicates at two time points. The relative expression, expressed in the form of CNRQ (Calibrated Normalized Relative Quantity) values, and standard error were calculated after quantification cycle (Cq) values were imported. Significant outliers were excluded for each set of four technical replicates.

**Phenotypical analysis**

Considering at least four stems per accession, plants were scored for infection types (IT), 17 days post-inoculation according to the scale of R = minute to small uredinia surrounded by chlorosis or necrosis, MR = medium-sized uredinia often surrounded by chlorosis, MS = medium to large erumpent uredinia with little or no chlorosis, and S = very large erumpent uredinia with little or no chlorosis (Roelfs *et al.*, 1992). ITs were documented in order of their frequency when more than one was observed per accession.

**Statistical analysis**

Host lines and *Pg* isolates were evaluated with analysis of variance (ANOVA) using General Linear Models (GLM) in NCSS 2007. The effects of host entries and replicates were accounted for in analysis of colony length. The impact of sampling times was included for the assessment of relative *Pg β-tubulin* expression. Spearman's rank correlation ($r_s$) was used to estimate the correspondence of data measured via different techniques. Fisher's protected least significant difference (LSD) values were calculated and used in histograms where applicable.

RESULTS

Although the probability of varying growth habits was taken into account, the growth stage of barley cultivar SQ41 did not synchronize with other entries at the time of the first replication and could only be included in the second trial.

**Histological analysis**

Scanning electron microscopy

Only UVPgt60 treatments were sampled for scanning electron microscopy as representative of the stem rust infection process at the early stages of 24 and 48 hpi. The outer surface of barley and wheat stems was considered at 24 hpi, where detected structures were similar across crops and lines. Germination occurred on both longitudinal sides of the urediniospore with a commonly delayed response in one side. Directional germ tube growth, mainly perpendicular to the long axis of the epidermal cells on the stem surface, occurred whilst germ tubes extensively branched out. Usually, an individual appressorium formed above a stomatal opening (Figure 4.1), while two appressoria on one stoma was occasionally observed.

Likewise, no obvious differences in early infection structure development were observed from the inner epidermal surface of barley and wheat. Cylindrical in shape, SSV appeared at 24 hpi parallel to the stomatal slit (Figure 4.2). At 48 hpi, primary infection hyphae (PIH) extended from the pole of one end of the SSV, although more often the SSV produced a haustorial mother cell (HMC) directly without first differentiating into PIH (Niks, 1986) (Figure 4.3). This seemed like a programmed occurrence before contact was made with a host mesophyll/epidermal cell (R.E. Niks, personal communication). The HMC is recognized as the long, slender structure delimited by a ridge-like septum from the preceding PIH or SSV, frequently with an extended tip. Usually delayed, a short or threadlike appendix occasionally occurred at the pole of the opposite SSV end, also able to produce HMC. There was no clear tendency in appendix formation and the lack of a PIH among the barley and wheat lines. In addition, the production of secondary infection hyphae was visible at 48 hpi, branching from PIH or developing directly from the SSV (Figure 4.4). This frequently occurred before the first HMC and subsequent haustorium was establish.

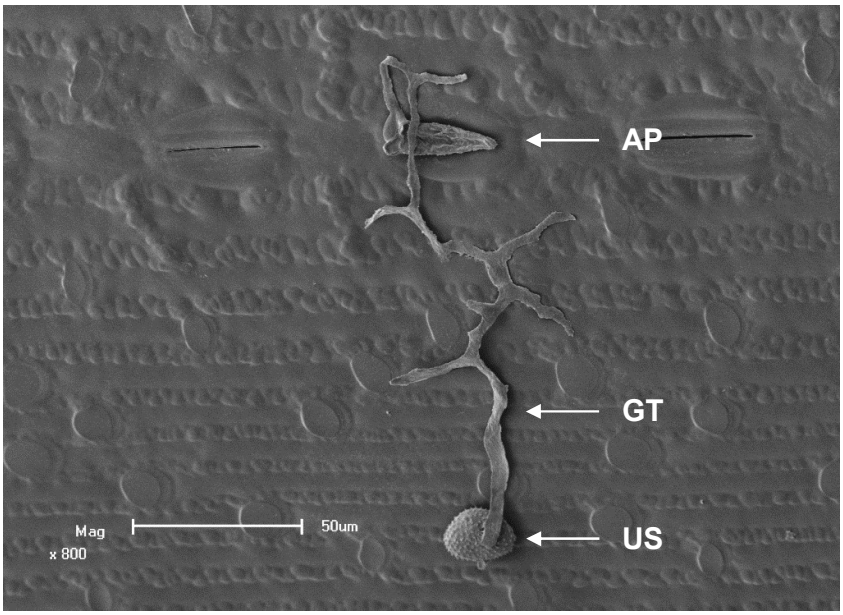



**Figure 4.1:** A scanning electron micrograph of a germinating *Puccinia graminis* f. sp. *tritici* urediniospore (**US**); the resulting germ tube (**GT**) forming an appressorium (**AP**) over the stoma on a barley plant 24 hours post-inoculation (X800 magnification).

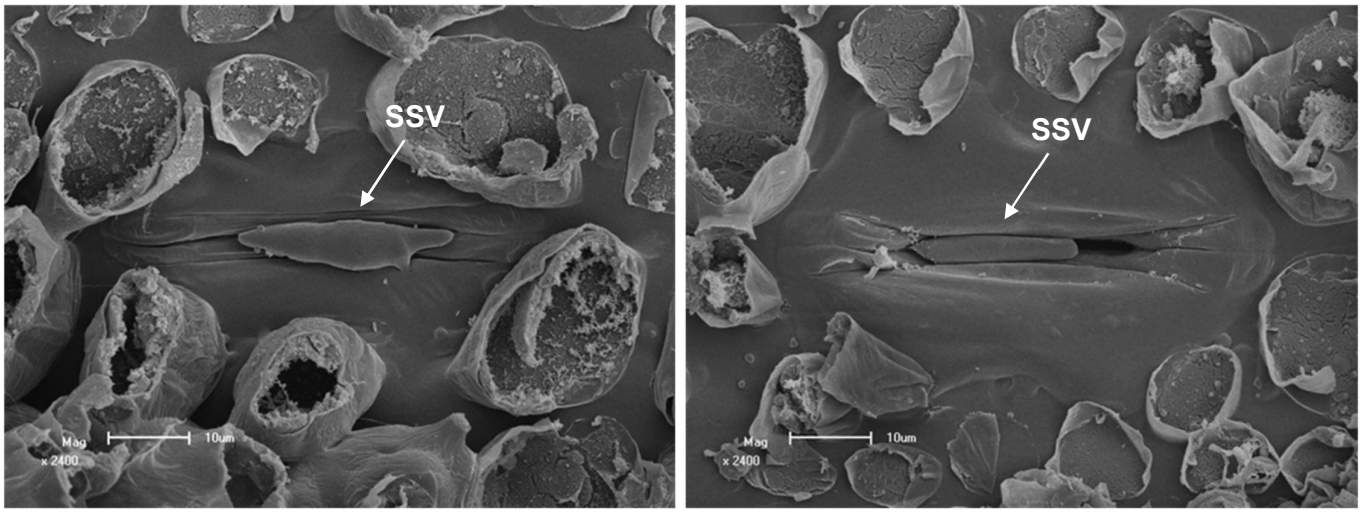



**Figure 4.2:** Scanning electron micrographs of substomatal vesicle (**SSV**) appearance at 24 hours post-inoculation following invasion through the stoma of barley (left) and wheat (right) (X2400 magnification).

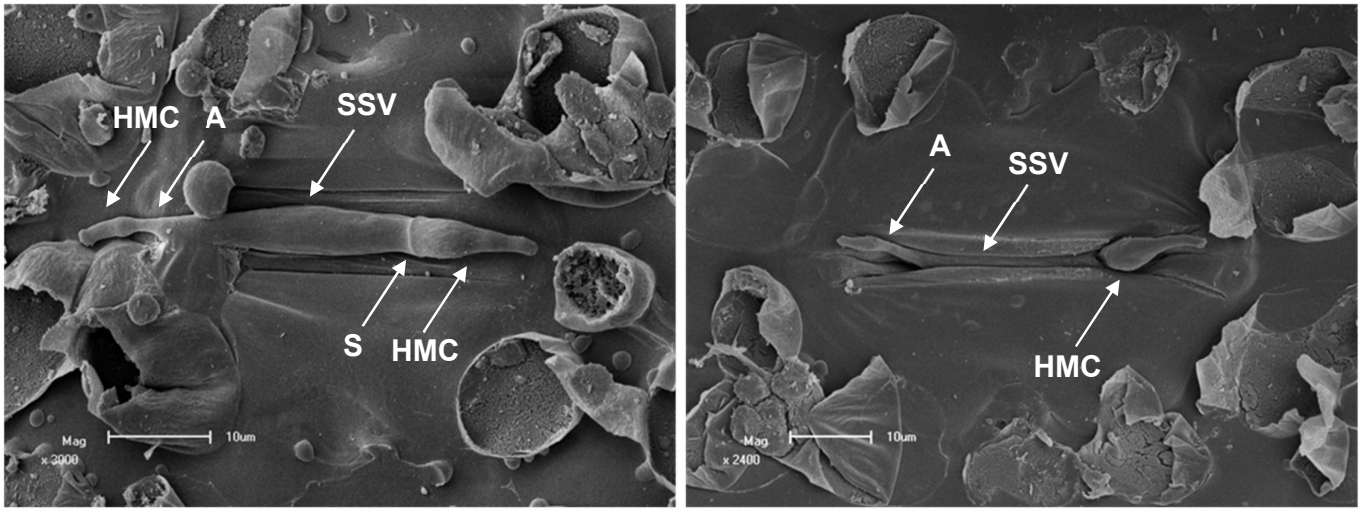



**Figure 4.3:** Scanning electron micrographs showing substomatal vesicles (**SSV**) directly forming haustorial mother cells (**HMC**) at 48 hours post-inoculation in barley (left, delimited by a septum (**S**)) and wheat (right) (X3000 and X2400 magnification, respectively). A short appendix (**A**) is produced at the other SSV end, differentiating into a HMC in barley (left).

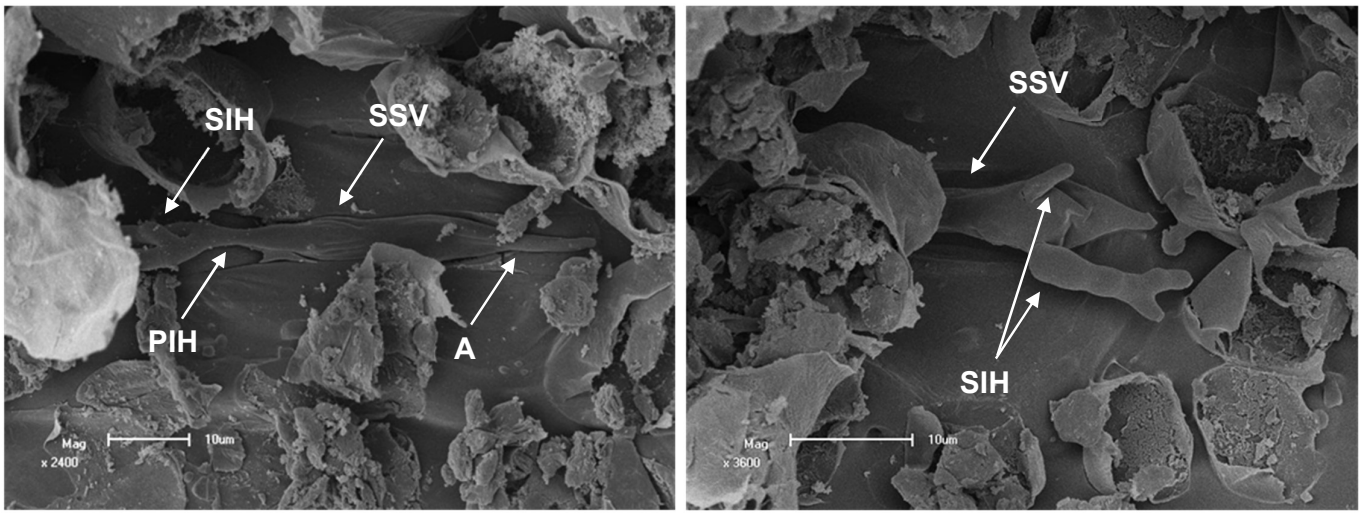



**Figure 4.4:** Scanning electron micrographs indicating the differentiation of secondary infection hyphae (**SIH**) from the primary infection hypha (**PIH**) or from the substomatal vesicle (**SSV**) at 48 hours post-inoculation, in barley (left) and wheat (right) (X2400 and X3600 magnification, respectively). A short appendix (**A**) extended from the opposite SSV pole (left).

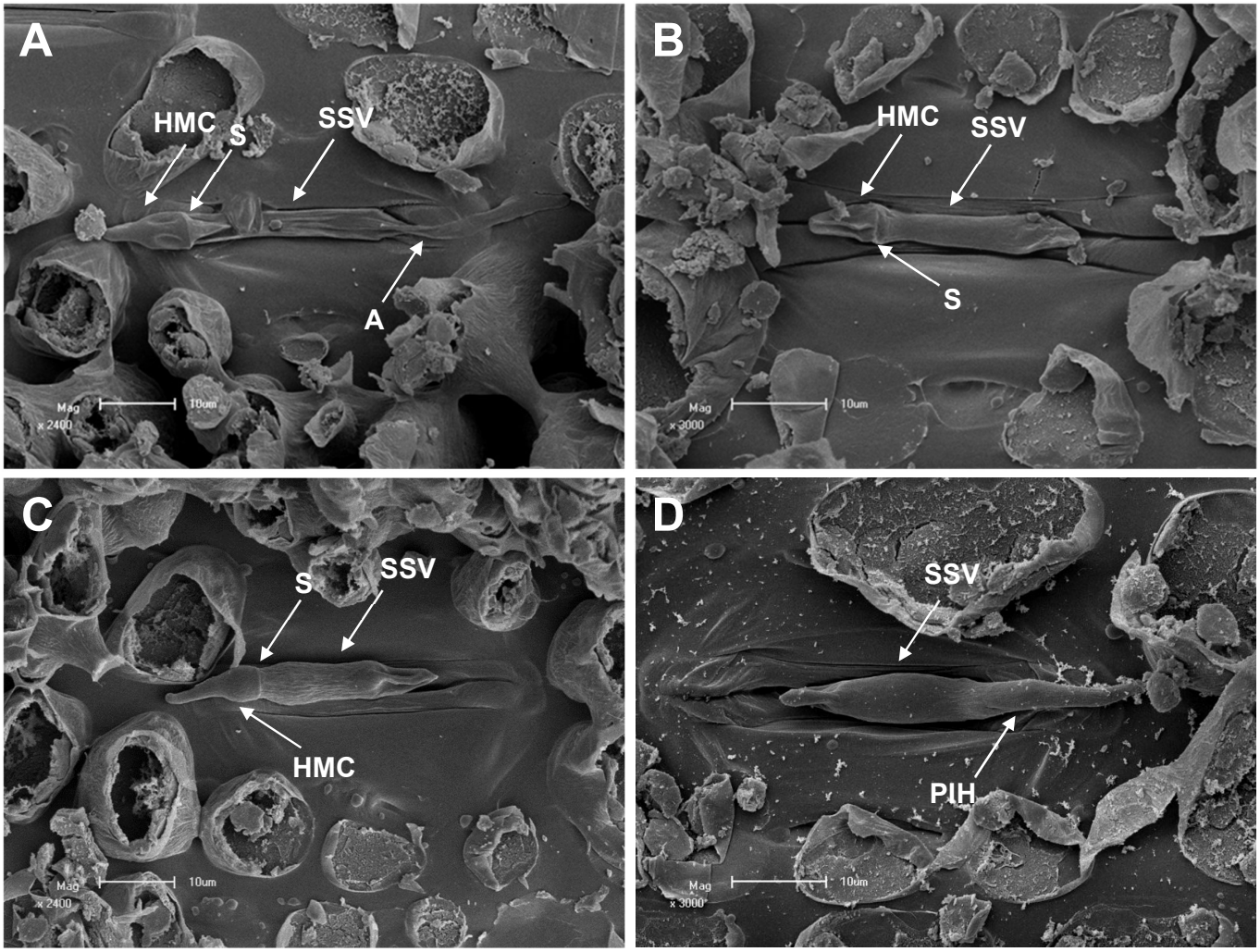



**Figure 4.5:** Scanning electron micrographs of early infection structures of *Puccinia graminis* f. sp. *tritici*, i.e. substomatal vesicle (**SSV**), a septum (**S**)-delimited haustorial mother cell (**HMC**), a threadlike appendix (**A**) and/or a primary infection hypha (**PIH**), collapsed in A) Q21861 (*Rpg1*, *rpg4/Rpg5* complex) and B) Q/SM20 (*rpg4/Rpg5* complex), and partly deflated in C) susceptible Hiproly and D) Chevron (*Rpg1*) (X2400 to X3000 magnification).

The lines containing the *rpg4/Rpg5* complex (Q21861 and Q/SM20) differed from the rest in that a substantial proportion of infection structures visible in the substomatal cavity were partially or completely collapsed by 48 hpi (Figure 4.5). Deflation only occurred in a small percentage and to a lesser extent in the infection structures of other barley and wheat entries, typically at the opposite end from the emerging PIH/HMC.

No abnormalities were observed in stomatal behaviour in the rust infected stems compared to control stems that received no treatment, as well as dew-control stems not inoculated with the spore suspension but subjected to the same dew conditions.

Fluorescence microscopy

ANOVA results (Addendum 4.1) indicated that colony sizes differed significantly among host lines ($P<0.05$), while *Pg* isolates and the line-isolate interaction were statistically insignificant sources of variation ($P>0.05$). In spite of the significant variation between replications ($P<0.05$) due to smaller colonies measured during the second replication, similar trends were observed for the two trials (Figure 4.6). Fluorescence microscopy images, displaying the differences in colony sizes of UVPgt54, UVPgt60 and UVPgs01 among lines, are presented in Figures 4.7, 4.8 and 4.9 respectively.

No HMC formation was observed in the highly resistant SST047 wheat and since SSVs were the only structures seen at infection points, colony size could not be determined for this line.

Susceptible barley accession PI 532013 consistently exhibited significantly larger *Pgt* colonies than the rest of the barley and wheat entries, including additional controls, at the time of sampling, i.e. 120 hpi (> 200 000 $\mu m^2$ and > 65 000 $\mu m^2$ in the first and second replication, respectively). Of the two *Pgt* isolates, UVPgt54 produced meaningfully larger colonies in PI 532013 than UVPgt60 with differences of more than 50 000 $\mu m^2$ and 10 000 $\mu m^2$ detected in the first and second replication, respectively. In addition to PI 532013, similarly large UVPgs01 colonies were measured on SQ41 (> 65 000 $\mu m^2$ in the second replication).

PI 532013 had significantly larger colonies than the susceptible lines Hiproly and 37-07. Somewhat smaller colonies were measured for wheat line 37-07 compared to barley control Hiproly and even Chevron (*Rpg1*) and Hietpas-5 (*Rpg2*) in the first trial. In the second replication, similar sized colonies were measured for control lines 37-07 and Hiproly, in this case slightly larger than that of Chevron and Hietpas-5.

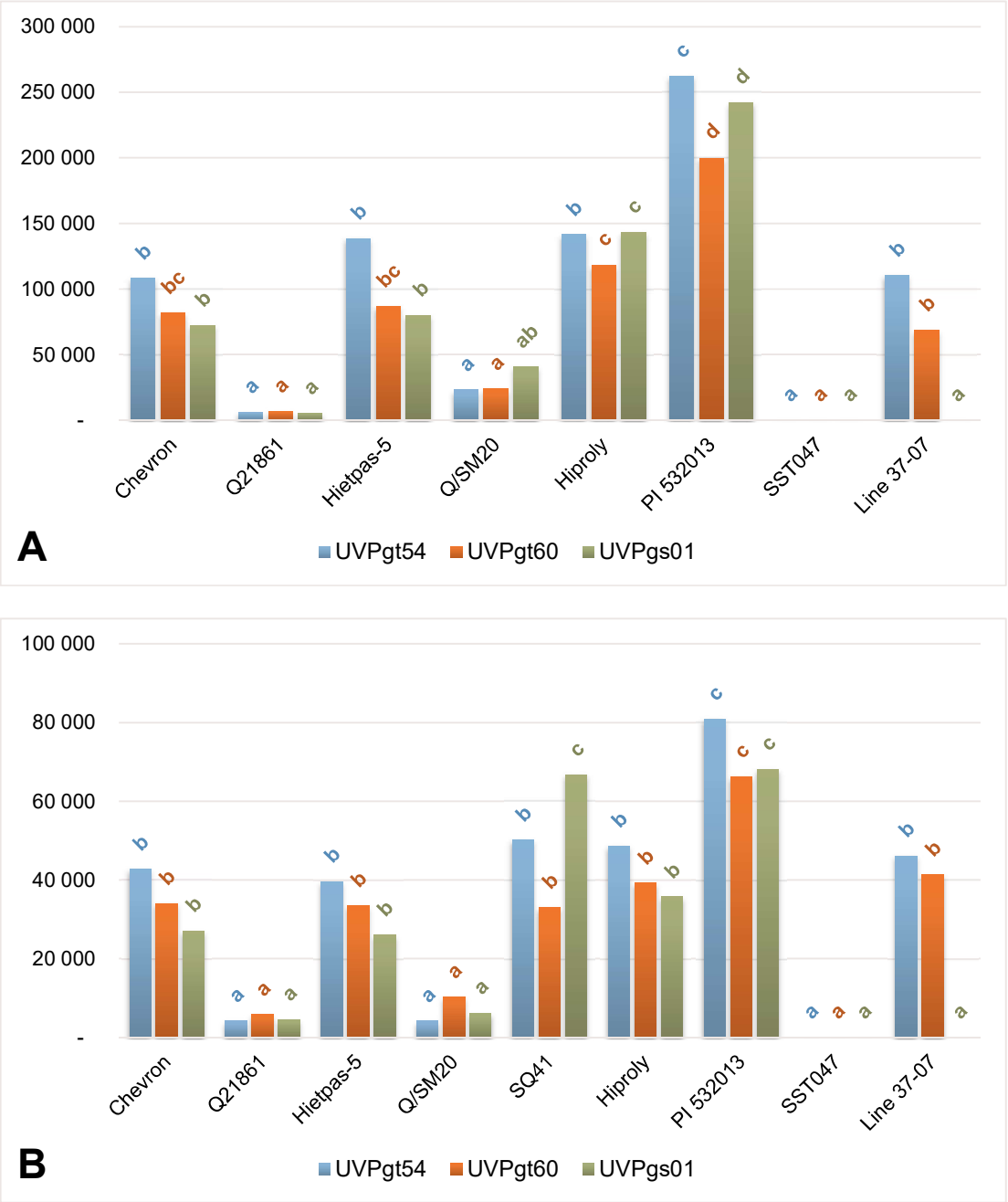



**Figure 4.6:** Average colony sizes (µm$^2$) of different *Puccinia graminis* isolates measured 120 hours post-inoculation on selected barley and wheat lines in the **A)** first and **B)** second replication. UVPgt54 and UVPgt60 are *P. graminis* f. sp. *tritici* and UVPgs01 *P. graminis* f. sp. *secalis*. Least significant differences (LSD) among lines for colony sizes were calculated as 45993.91, i.e. means with the same LSD symbol do not differ significantly from each other (P>0.05 ANOVA, Addendum 4.1).

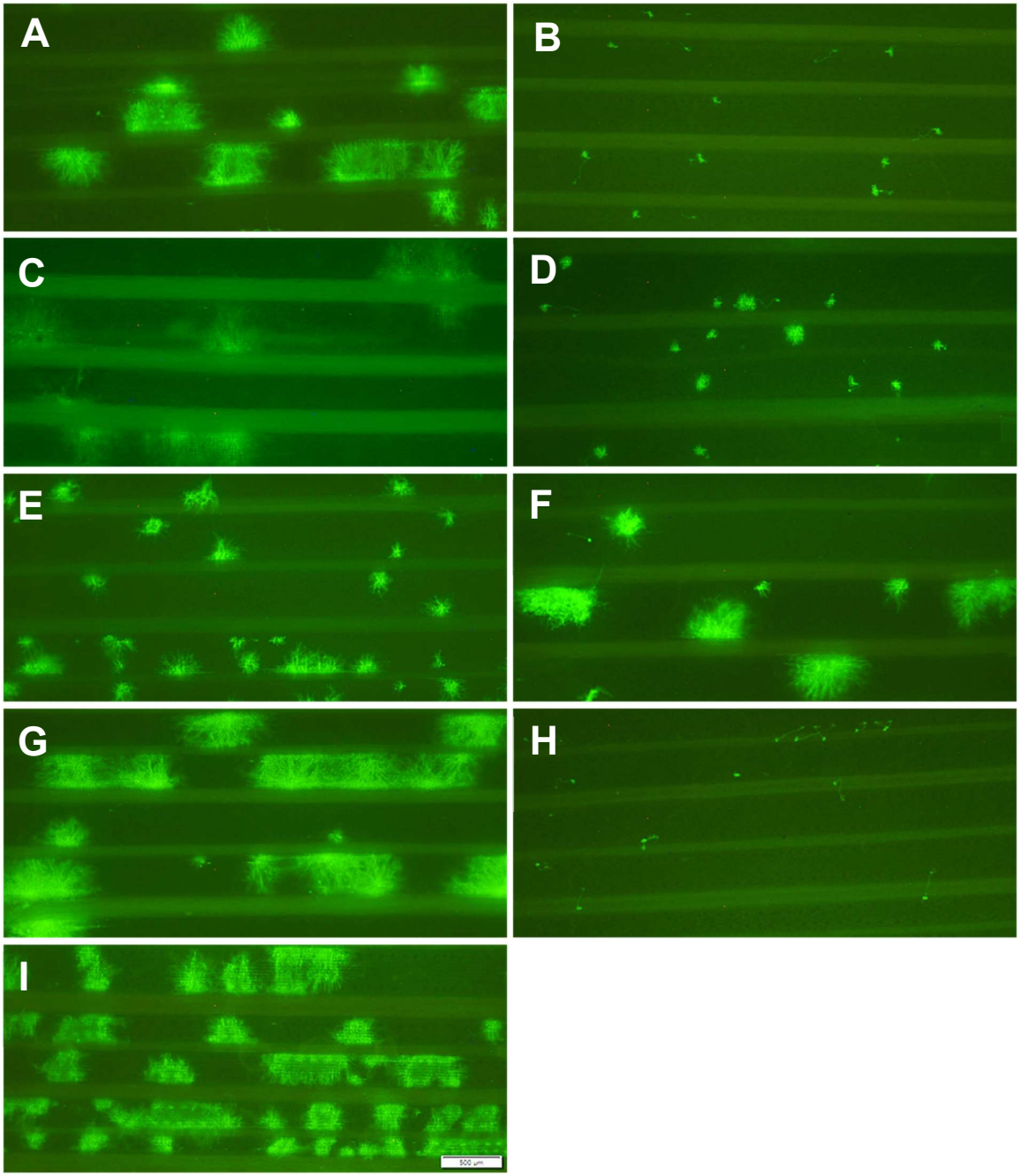



**Figure 4.7:** Microscope images, taken at 120 hours post-inoculation, showing differences in the size of fluoresced colonies of *Puccinia graminis* f. sp. *tritici* isolate UVPgt54 in **A)** Chevron, **B)** Q21861, **C)** Hietpas-5, **D)** Q/SM20, **E)** SQ41, **F)** Hiproly, **G)** PI 532013, **H)** SST047 and **I)** line 37-07. Scale bar represents 500 µm.

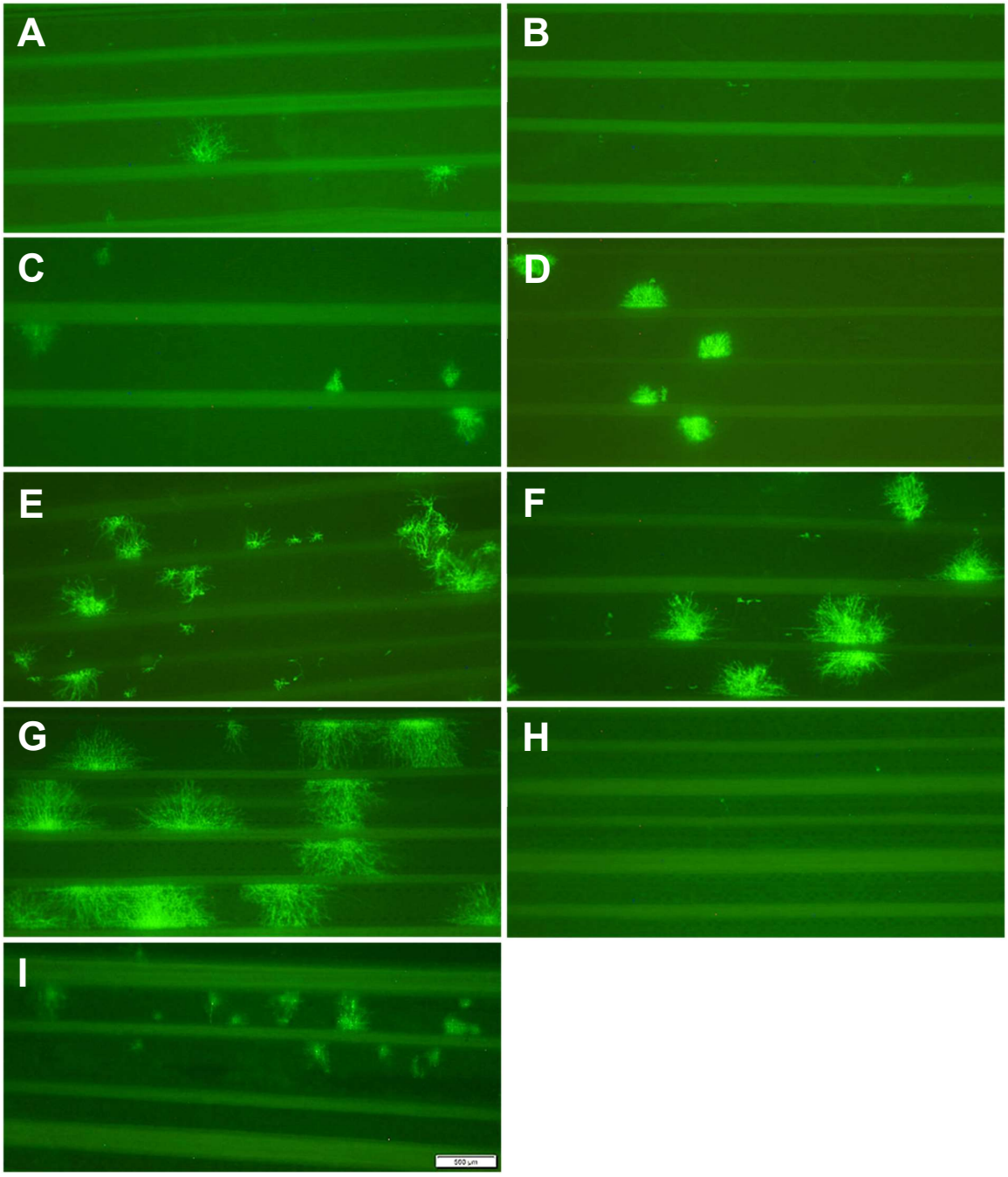



**Figure 4.8:** Microscope images, taken at 120 hours post-inoculation, showing differences in the size of fluoresced colonies of *Puccinia graminis* f. sp. *tritici* isolate UVPgt60 in **A)** Chevron, **B)** Q21861, **C)** Hietpas-5, **D)** Q/SM20, **E)** SQ41, **F)** Hiproly, **G)** PI 532013, **H)** SST047 and **I)** line 37-07. Scale bar represents 500 µm.

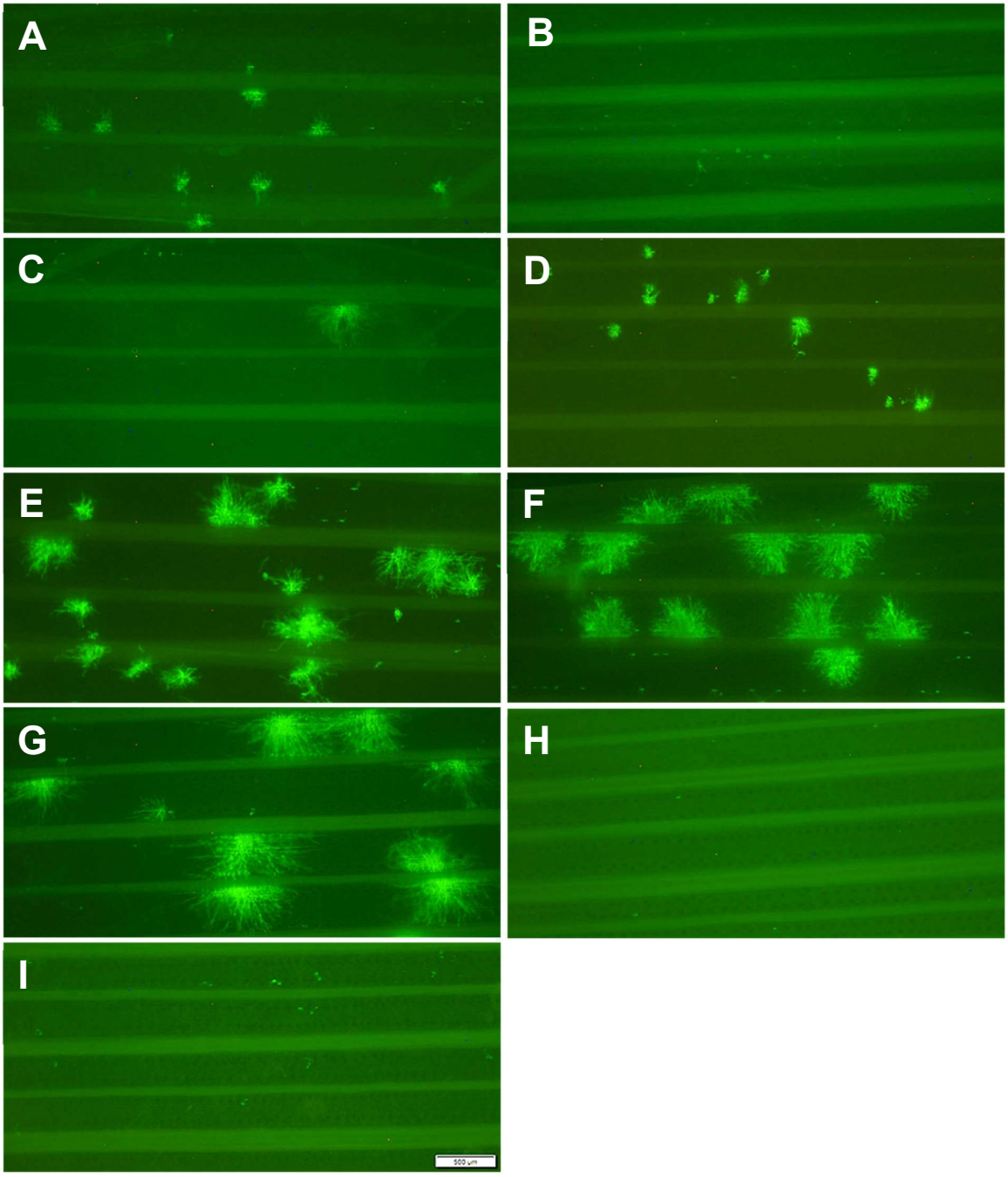



**Figure 4.9:** Microscope images, taken at 120 hours post-inoculation, showing differences in the size of fluoresced colonies of *Puccinia graminis* f. sp. *secalis* isolate UVPgs01 in **A)** Chevron, **B)** Q21861, **C)** Hietpas-5, **D)** Q/SM20, **E)** SQ41, **F)** Hiproly, **G)** PI 532013, **H)** SST047 and **I)** line 37-07. Scale bar represents 500 µm.

The UVPgt54 treatment grouped the additional barley (Hiproly) and wheat (line 37-07) controls with Chevron (*Rpg1*), Hietpas-5 (*Rpg2*) and SQ41 (*Rpg5*) where colonies ranged from 100 000 to 150 000 $\mu m^2$ in the first replication and 40 000 to 50 000 $\mu m^2$ in the second replication. Similarly, these lines were roughly grouped together regarding both UVPgt60 and UVPgs01 colony sizes (60 000 to 90 000 $\mu m^2$ in the first and 25 000 to 40 000 $\mu m^2$ in the second replication). Exceptions included Hiproly which produced somewhat larger *Pg* colonies in the first replicate (> 120 000 $\mu m^2$) and SQ41 that exhibited significantly larger UVPgs01 colonies in the second replication (65 000 $\mu m^2$). In addition, a colony size of 0 $\mu m^2$ was assigned to the UVPgs01 treatment of wheat line 37-07, as infection of the avirulent rye isolate stopped shortly after the formation of SSVs in line 37-07. However, striking similarities in colony sizes were consistent between the *Rpg1* source (Chevron) and the line containing *Rpg2* (Hietpas-5) for all three *Pg* isolates.

Very small *Pg* colonies were detected in both lines carrying the *rpg4*/*Rpg5* complex. In the first replication, noticeably smaller colonies were measured in Q21861, which additionally carries the *Rpg1* gene (< 7 000 $\mu m^2$ for all *Pg* isolates), compared to Q/SM20 (> 20 000 $\mu m^2$ for both *Pg* pathotypes, and differences even more evident for the rye isolate, measured larger than 40 000 $\mu m^2$). More similar sized colonies were measured for the second replicates of these lines; colonies of all three isolates were smaller than 10 000 $\mu m^2$.

There was a substantial difference between colonies measured in SQ41, carrying *Rpg5*, and lines carrying the *rpg4/Rpg5* complex (Q21861 and Q/SM20). Colonies smaller than 10 000 $\mu m^2$ were measured for all *Pg* isolates in Q21861 and Q/SM20. In contrast, UVPgs01 colonies in SQ41 (66 625 $\mu m^2$) were comparable to the 68 112 $\mu m^2$ measured for PI 532013. In addition, SQ41 inoculated with UVPgt54 and UVPgt60 had colony averages of 50 088 and 32 986 $\mu m^2$ respectively, which grouped with the other control lines showing less susceptibility at 120 hpi (Hiproly and line 37-07).

The overall trend for most entries was a decrease in average colony size produced, in descending order, by the *Pg* isolates UVPgt54, UVPgt60 and UVPgs01. Little variation was observed between UVPgt60 and UVPgs01 for most entries, excluding wheat line

37-07 and barley cultivar SQ41. These were in fact the only cases where significant variation occurred among *Pg* isolates.

**Molecular analysis**

RT-qPCR enabled the rapid quantification of accumulated *Pg* fungal biomass in host plants. A single peak during melt curve analysis along with one ±100 bp amplicon through gel electrophoresis, verified the specific amplification of a single product. The putative *β-tubulin* amplicon from *Pgs* was also confirmed identical in size to the *β-tubulin* amplicon from *Pgt* (106 bp), and will therefore be collectively referred to as *Pg β-tubulin*. Similar to the ANOVA results for colony size, the relative *Pg β-tubulin* expression differed significantly among lines ($P<0.05$) (Addendum 4.2), with little difference detected among *Pg* isolates as well as an insignificant interaction between lines and *Pg* isolates ($P>0.05$).

As expected, significant variation was observed in relative *Pg β-tubulin* expression between sampling times, i.e. 120 and 240 hpi. Interestingly, the sampling time had contrasting relations with the other variables; highly significant interacting effects occurred with different lines ($P<0.05$), while the sampling time-*Pg* isolate interaction presented insignificant sources of variation in terms of the relative expression of *Pg β-tubulin* ($P>0.05$). Extreme expression levels of relative *Pg β-tubulin*, inconsistently detected for the three control lines across all three isolates and two sampling times, complicated possible trend analysis with meaningful statistical variation occurring between the replications ($P>0.05$).

Extremely high relative *Pg β-tubulin* expression levels in the first replication sampled at 120 hpi were seen in susceptible wheat line 37-07 and barley accession PI 532013 inoculated with UVPgt54 and UVPgt60, respectively (Figure 4.10). Significant standard errors were calculated for these treatments, and to a lesser extent for PI 532013 inoculated with UVPgs01 and UVPgt54 where relatively high expression levels were detected. Conversely, minor standard errors were calculated for all the other treatments, showing low gene expression relative to above mentioned treatments. *Pg β-tubulin* expression of all isolates was barely detectable in the *rpg4/Rpg5* complex containing barley accessions (Q21861 and Q/SM20) as well as resistant wheat SST047. This was also the case for the incompatible interaction of wheat line 37-07 and UVPgs01.

Insignificant increase in the fungal biomass of all three isolates, from 120 to 240 hpi, occurred in SST047 (*Sr36*) and Q21861 (*Rpg1*, *rpg4/Rpg5* complex) (Figure 4.10). At 240 hpi accession Q/SM20, containing the *rpg4/Rpg5* complex, showed slight increases in expression levels for *Pgt* isolates, with little change for isolate UVPgs01. Chevron on the other hand, containing solely *Rpg1*, experienced considerable increases in fungal biomass for all three isolates.

The relative expression detected for UVPgt60 in Hiproly was higher than that of the other susceptible controls PI 532013 and line 37-07. Hiproly inoculated with UVPgt54 showed little fungal development from 120 hpi to 240 hpi, compared to the extensive gene expression in UVPgs01 at 240 hpi ensuing a substantial standard error calculated for this treatment. Parallel levels of fungal biomass were quantified for *Pgt* isolates UVPgt54 and UVPgt60 in barley accession PI 532013 and wheat line 37-07. At 240 hpi, relative expression of isolate UVPgs01 remained undetected in line 37-07, while 40-times the *Pg β-tubulin* expression was seen for this treatment in PI 532013. There was little *Pg* multiplication in Hietpas-5 (*Rpg2*) from 120 hpi to 240 hpi, with relative expression comparable to Q/SM20 (*rpg4/Rpg5*).

The expression levels of susceptible lines read in the second trial, were two-to-three-fold less at 120 hpi (Figure 4.11) than the first replication, while the opposite was true at 240 hpi. Consequently, much higher expression increases were quantified for the more susceptible lines over the time interval in the second replication.

Diverging expression was observed at 120 hpi for barley control PI 532013, with UVPgt54 *β-tubulin* expression exceeding the other treatments; almost double that of wheat control line 37-07. All other treatments resulted in marginal relative gene expression at 120 hpi. A steep increase from 120 to 240 hpi (Figure 4.11) in relative *β-tubulin* expression in PI 532013 and line 37-07 also produced major standard errors for these treatments. Little change was seen from 120 to 240 hpi in the already insignificant fungal biomass of all isolates in Q21861 and SST047. All the Q/SM20 treatments as well as line 37-07 inoculated with UVPgs01 had very low expression levels, while slightly higher expressions of all three treatments were detected in Chevron, Hietpas-5, SQ41 and Hiproly. Moreover, little variation was detected between UVPgt54 and UVPgs01; which was either more (SQ41 and Hiproly) or less (Chevron, Hietpas-5 and Q/SM20) than UVPgt60 expression levels.

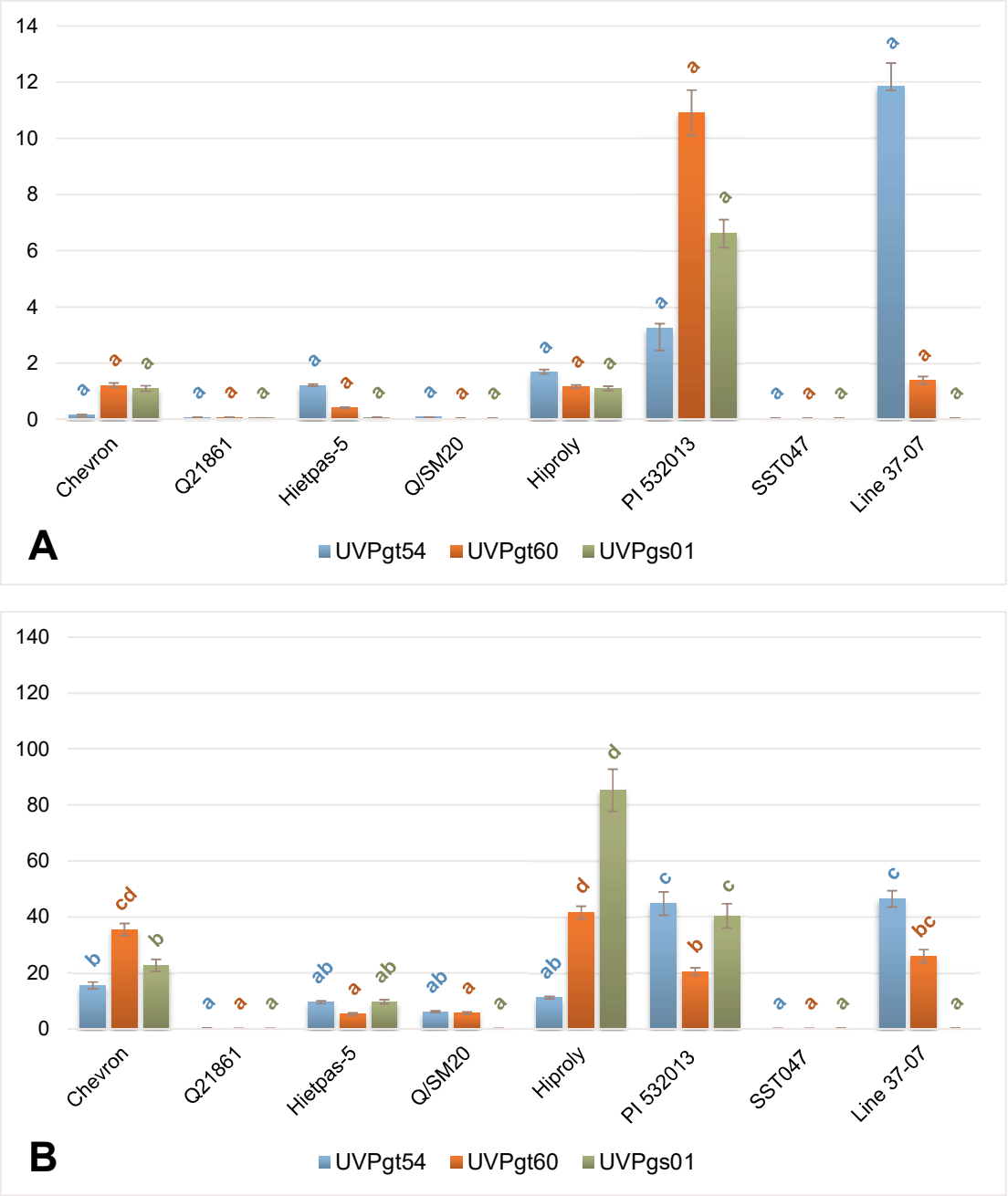



**Figure 4.10:** Relative *β-tubulin* expression of different *Puccinia graminis* isolates in selected barley and wheat lines sampled **A)** 120 and **B)** 240 hours post-inoculation in the first replication. Error bars represent positive and negative standard errors. UVPgt54 and UVPgt60 are *P. graminis* f. sp. *tritici* and UVPgs01 *P. graminis* f. sp. *secalis*. Least significant differences (LSD) among lines for relative *β-tubulin* expression were calculated as 13.29, i.e. means with the same LSD symbol do not differ significantly from each other (P>0.05 ANOVA, Addendum 4.2).

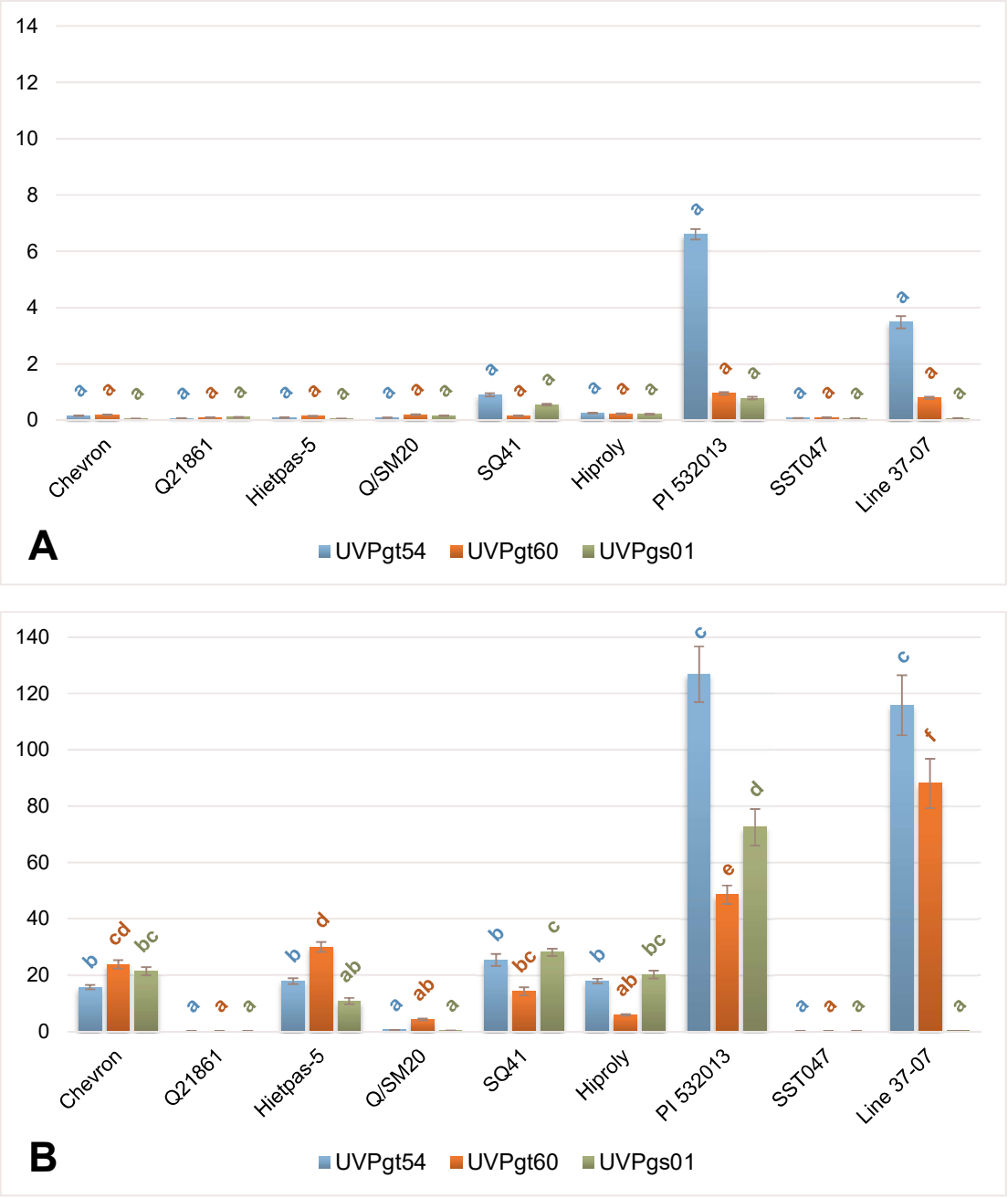



**Figure 4.11:** Relative *β-tubulin* expression of different *Puccinia graminis* isolates in selected barley and wheat lines sampled **A)** 120 and **B)** 240 hours post-inoculation in the second replication. Error bars represent positive and negative standard errors. UVPgt54 and UVPgt60 are *P. graminis* f. sp. *tritici* and UVPgs01 *P. graminis* f. sp. *secalis*. Least significant differences (LSD) among lines for relative *β-tubulin* expression were calculated as 13.29, i.e. means with the same LSD symbol do not differ significantly from each other (P>0.05 ANOVA, Addendum 4.2).

**Phenotypical analysis**

A final evaluation of ITs was taken 17 days post-inoculation for the first and second replication (Table 4.3). Corresponding images displaying the representative phenotypic reaction of lines inoculated with the three isolates are compiled in Figure 4.12.

PI 532013 was fully susceptible to all isolates except in the first replication where a moderate susceptible reaction was recorded for UVPgt60. Hiproly had a range of susceptible reactions, showing more disease symptoms towards pathotype UVPgt54. Wheat control line 37-07 exhibited fully susceptible symptoms for *Pgt* isolates and expected zero reactions towards the incompatible *Pgs* isolate. Wheat variety SST047 (*Sr36*) as well as barley line Q21861 carrying *Rpg1* and the *rpg4/Rpg5* complex, both had highly resistant reactions to all isolates.

**Table 4.3:** Infection types of adult barley and wheat lines inoculated with different *Puccinia graminis* isolates.

| Entry | First replication | | | Second replication | | |
|---|---|---|---|---|---|---|
| | UVPgt54 | UVPgt60 | UVPgs01 | UVPgt54 | UVPgt60 | UVPgs01 |
| Chevron | MRMS | MR | MRMS | RMR | RMR, MR | MRMS |
| Q21861 | R | 0R | R | 0R, R | 0R, R | 0R, TR |
| Hietpas-5 | S | MR | MRMS | S | MR | MS |
| Q/SM20 | MR, R | RMR | TR, R | R, RMR | R | 0R, TR |
| SQ41 | N/A | N/A | N/A | MRMS | MS, MR | MSS |
| Hiproly | S | MSS | MS | MSS | MRMS | MS |
| PI 532013 | S | MS | S | S | S | S |
| SST047 | 0R | 0R | 0R | 0R | 0R | 0R |
| Line 37-07 | S | S | 0R | S | S | 0 |

Adult plant reaction was assessed according to documented infection responses, where R = Resistant, MR = Moderately Resistant, MS = Moderately Susceptible and S = Susceptible (Roelfs *et al.*, 1992). UVPgt54 and UVPgt60 are *P. graminis* f. sp. *tritici* and UVPgs01 *P. graminis* f. sp. *secalis*.

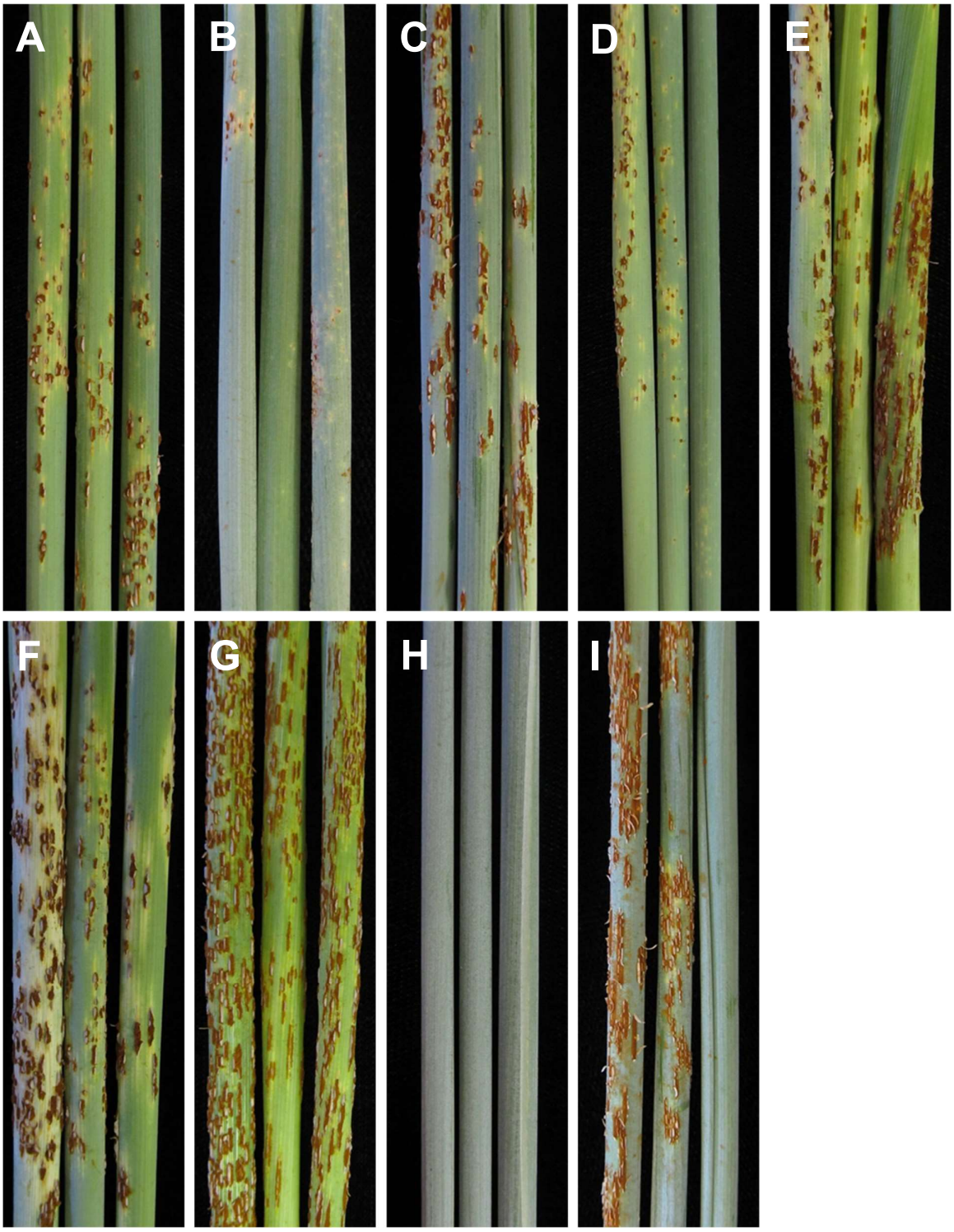



**Figure 4.12:** Infection types of **A)** Chevron (*Rpg1*), **B)** Q21861 (*Rpg1*, *rpg4/Rpg5* complex), **C)** Hietpas-5 (*Rpg2*), **D)** Q/SM20 (*rpg4/Rpg5* complex), **E)** SQ41 (*Rpg5*), **F)** Hiproly, **G)** PI 532013, **H)** SST047 (*Sr36*) and **I)** line 37-07; 17 days post-inoculation with UVPgt54, UVPgt60 and UVPgs01 (left, middle and right of each individual picture).

Carrying only the *rpg4/Rpg5* complex, Q/SM20 was highly resistant towards UVPgs01, resistant to UVPgt60 while resistant to moderately resistant reactions were documented towards UVPgt54. Chevron (*Rpg1*) had moderate reactions in the first and second replication, with ITs ranging from MRMS to RMR for UVPgt54, MR to RMR for UVPgt60, and MRMS for UVPgs01. Line SQ41, carrying the *Rpg5* gene, exhibited significantly more wheat as well as rye stem rust symptoms than lines containing the *rpg4/Rpg5* complex: MRMS for UVPgt54, MS to MR for UVPgt60 and MSS for UVPgs01. Hietpas-5 (*Rpg2*) were fully susceptible to UVPgt54, moderately resistant to UVPgt60 while moderate resistant and susceptible symptoms were observed against UVPgs01. Overall, more resistant phenotypic scores were documented for the entries during the second replication.

## DISCUSSION

Cultivated barley can naturally be affected by *P. hordei* (leaf rust), *P. coronata* var. *hordei* (crown rust), *P. striiformis* f. sp. *hordei* (stripe rust) and *P. graminis* (stem rust) (Park *et al.*, 2015), but is considered a near-nonhost or marginal host to other rust species and *formae speciales* (Atienza *et al.*, 2004; Jafary *et al.*, 2006). In addition, a general understanding drawn from stem rust levels in the field, affirms the more resistant nature of barley towards *Pg* as opposed to wheat (Steffenson *et al.*, 2017). This enhanced level of basal resistance was attributed to the comprehensive incorporation of *Rpg1* into breeding material since the mid-1940s (Steffenson, 1992). Despite the observations of a lower receptivity in barley to stem rust, the *Pgt* ‘Ug99’ race group threatens the global production of both wheat and barley, with virulence reported for widely deployed resistance genes including the durable *Rpg1* gene in barley (Steffenson *et al*., 2007).

In addition to the *tritici* and *secalis* forms of the stem rust pathogen, a somatic hybrid between these two *formae speciales* has been reported in Australia (Park, 2007). This hybrid, known as Scabrum rust, also attacks barley (Park *et al.*, 2015).

Deising *et al.* (1996) mentioned that the narrow host range of rust pathogens may be attributed to their sequential order of differentiation. Even though early infection stages are driven by parallel sequences of events, the morphology and behaviour of infection

structures vary significantly; enough to distinguish different rust species from one another (Swertz, 1994).

Following the germination of *Pgt* spores, protruded germ tubes advanced in a similar fashion on both barley and wheat stem surfaces, i.e. mainly at right angles to the long axis of the epidermal cells. Directional germ tube growth has been reported for many cereal rust fungi (Johnson, 1934; Dickinson, 1969; Wynn, 1976), while the germ tubes of *P. sorghi* (Hughes and Rijkenberg, 1985) and *Hemileia vastatrix* (Coutinho *et al.*, 1993) randomly elongates towards respective host stomata.

Apart from *P. striiformis* (Pole Evans, 1907; Swertz, 1994), all rust fungi typically form appressoria (Emmett and Parberry, 1975) with little morphological variation among species. In this study, no obvious differences in appressorial formation were observed among different barley and wheat entries.

Substomatal vesicles of *Pgt* isolate UVPgt60 appeared morphologically similar among the selected barley and wheat entries at 24 hpi, i.e. cylindrical/fusiform. By 48 hpi, a PIH was occasionally observed at one end of the SSV, following the delimitation of a HMC by a septum. More frequently, a PIH was absent and a HMC was directly formed from the SSV, similar to observations made by Niks (1986). At the same time, some SSVs produced a short/threadlike appendix at the opposite end. Secondary infection hyphae emerged from SSVs and/or along the PIH at 48 hpi. A noteworthy observation made during this study was the high proportion of entirely collapsed infection structures observed at 48 hpi in barley lines carrying the *rpg4/Rpg5* complex (Q21861 and Q/SM20). On the contrary, very little deflation in SSVs was seen in other entries, including the highly resistant wheat cultivar SST047, which usually occurred at the opposite end from the emerging PIH/HMC. The abortion of SSVs in barley may indicate the early onset of resistance mechanisms in certain genotypes.

Considerable variation in SSV shape has been recorded between *P. graminis* and other rust fungi. For instance, SSVs can range from rectangular (*P. coronata*), spheroid (*P. striiformis*) (Swertz, 1994), ellipsoid (*P. triticina*: Niks, 1982 and *Uromyces phaseoli* var. *vignae*: Heath and Heath, 1979), triangular (*P. sorghi*: Hurd-Karrer and Rodenhiser, 1947) with each end producing a PIH (Hughes and Rijkenberg, 1983), or H-shaped with two or more infection hyphae (*P. helianthi*: Sood and Sackston, 1970 and *P. melanocephala*: Sotomayor *et al.*, 1983). The majority of cereal rust fungi have

longitudinal, horizontally-orientated SSV. Transversely-orientated SSV have been reported for *P. hordei* (Swertz, 1994) and *U. transversalis* (Ferreira and Rijkenberg, 1989) where infection hyphae grew more deeply into the mesophyll. It has been suggested that the fungal SSV orientation is likely adapted to the orientation of the host substomatal chamber (Hu, 1996).

Swertz (1994) reported variation in rust fungal HMC structures from relatively short and unlobed (*P. brachypodii*) to long, slender and unlobed (*P. graminis*, *P. hordei* and *P. coronata*, the latter being frequently hooked). The majority of *Pgt* HMC observed in this study were formed directly from the SSV regardless of the presence of host mesophyll/epidermal cells (R.E. Niks, personal communication).

No structures beyond the HMC with an elongated tip (Swertz, 1994) were witnessed during the early infection process. The stripping technique used to remove the epidermal surface likely detached some of the infection structures in the cavity and surrounding host cells, as damaged tissue (plant and fungal) was frequently observed.

As stated by Swertz (1994), small quantitative differences occur among germlings from different *formae speciales* of *P. graminis*. These observations referred to the length of PIH and proportion secondary hyphae; *Pg* f. sp. *avenae* had the highest values for these parameters, *Pg* f. sp. *secalis* the lowest, and *Pg* f. sp. *tritici* ranked intermediate. The morphology of *Pgt* infection structures does not seem to vary among different host plant varieties, i.e. resistant versus susceptible lines, similar to descriptions of other pathosystems (Sood and Stackston, 1970; Swertz, 1994).

In a previous barley trial, artefacts were observed from the substomatal cavity on stomatal slits. It was thought to be a possible resistance mechanism in barley, as it has not been reported in previous wheat studies. Wheat controls were included in this trial in attempt to differentiate the two crops regarding the presence of these articles. Similar structures were in fact observed in both wheat and barley, presumable cuticular discharge or a product left during inoculation or sample preparation. Nevertheless, these proved insignificant in the study to elucidate the innate stem rust resistance in barley.

The widely deployed *Rpg1* gene encodes a functional protein kinase protein with dual kinase domains (Brueggeman *et al.*, 2002). Nirmala *et al.* (2007) suggested both pre-

and post- haustorial mechanisms to be involved in *Rpg1*-mediated resistance, though evidence did not point to haustorial formation being delayed (Zurn *et al.*, 2015).

Up to now, the *rpg4/Rpg5* gene complex has been the only locus described in barley to impart highly effective stem rust resistance against pathotype TTKSK at both seedling (Steffenson *et al.*, 2009) and adult plant stages (Steffenson *et al.*, 2017). The recessive *rpg4* resistance gene (Jin *et al.*, 1994) is believed to play a role in race-specific disease resistance through cytoskeleton rearrangement, while *Rpg5* may be involved in pathogen perception and signal transduction (Brueggeman *et al.*, 2008, 2009). Yet *rpg4*-mediated stem rust resistance requires the collective action of at least four tightly linked genes, the partially dominant *Rpg5* gene being the major determining factor (Brueggeman *et al.*, 2008; Wang *et al.*, 2013; Mamo *et al.*, 2015). Arora *et al.* (2013) confirmed that non-functional RPG5 proteins led to *Pgt* susceptibility in lines carrying the *rpg4/Rpg5* complex. Additionally, the expression of the complex is considerably influenced by the genetic background of the genotype, noted in the customary higher rust severity during both seedling and adult plant stages documented for the unaccompanied *rpg4/Rpg5* complex (Steffenson *et al.*, 2017). The authors suggested either a possible epistatic effect of *Rpg1* occurring with *rpg4/Rpg5* in Q21861, or hidden resistance still present in this line.

Conclusive results via fluorescence microscopy and molecular gene expression indicated that up to 120 hpi, barley did not exhibit stem rust resistance different to wheat. Larger *Pgt* colonies and higher *Pgt β-tubulin* expression of both isolates were quantified for the two barley susceptible lines (PI 532013 and Hiproly) compared to the susceptible wheat line 37-07. By 240 hpi, *β-tubulin* expression of both *Pgt* isolates in the wheat control increased to the same or higher levels than that of the susceptible barley controls. Hiproly proved to be the less susceptible barley control across parameters and time points studied.

Barley lines could be clearly discerned in regards to resistance gene(s) they carry. While the *rpg4/Rpg5* gene complex undoubtedly conferred the highest level of stem rust resistance (Q/SM20), the effect was amplified with the addition of *Rpg1* gene (Q21861). Conversely, Chevron (*Rpg1*) indicated significantly more disease symptoms and signs, which, up to 240 hpi, were similar and/or more severe than in Hietpas-5 (*Rpg2*). Phenotypic analysis at 17 days post-inoculation however, showed a more

resistant effect in Chevron than Hietpas-5 towards all *Pg* isolates. SQ41, carrying the sole *Rpg5* gene, revealed much higher susceptibility against both *Pgt* isolates compared to Q/SM20 (*rpg4/Rpg5* complex) at all time points. This difference in response between *Rpg5* and *rpg4*/*Rpg5* complex was augmented for the *Pgs* isolate, which is peculiar since *Rpg5* is thought to function independently from *rpg4* to confer resistance against *Pgs* isolates (Brueggeman *et al.*, 2008). This might point to some race specific *Pgs* resistance conferred by *Rpg5*. Another explanation, given by Arora *et al.* (2013), suggested that resistance to both *Pgt* and *Pgs* might be conditioned by a functional *rpg4*/*Rpg5* resistance locus based on similar general reactions documented for *Pgt* race QCCJB and *Pgs* isolate 92-MN-90.

Overall, mostly smaller colonies were noted for UVPgt60 and UVPgs01 compared to UVPgt54, and, furthermore, little variation existed between UVPgt60 and UVPgs01. Deviations were observed in the much larger UVPgs01 than UVPgt60 colonies on SQ41 (not confirmed with gene expression results), and the opposite for wheat line 37-07 which is incompatible with UVPgs01.

Less consistent gene expression data was obtained over the two replications, the result of extreme levels irregularly quantified for some of the control treatments associated with relatively high standard errors. This accentuates the importance of unselective sampling, and not being inclined to tissue where symptoms are visible. The exclusion of these extremities with high standard errors, revealed significant correlation between parameters studied at 120 hpi ($r_s$ = 0.82, 0.80 and 0.88 obtained for UVPgt54, UVPgt60 and UVPgs01, respectively).

Apart from extreme relative *Pg β-tubulin* expression in susceptible lines, the second replication resulted in significantly less colonization compared to the first, noted across all parameters. This might be the result of different maximum temperatures recorded during the dew period post inoculation, i.e. 25°C and 28°C during the first and second replications, respectively.

The mesothetic reactions of barley seedlings to *Pg* infections (mixed infection types) (Sun and Steffenson, 2005), makes adult plant assessment an important factor in obtaining reliable resistance data. Another consideration is the possibility of variation that may occur between greenhouse and field trials. All barley lines except SQ41 were tested in a rust screening nursery of Pannar Seed (Pty) Ltd. near Greytown, South

Africa in 2016 (Steffenson *et al.*, 2017). Overall, lines exhibited slightly more susceptibility towards UVPgs60 under field conditions compared to scores documented here under controlled conditions.

According to ANOVA results, the relative *Pg β-tubulin* expression differed significantly over the time-course from 120 to 240 hpi. There was a prominent interaction between host lines and the time-course, while the interaction between isolates and the time course was insignificant. Thus, from 120 to 240 hpi, the lines changed in their rankings regarding susceptibility when at the same time little variation seemed to occur in the ranking of pathogenicity of the *Pg* isolates.

REFERENCES

Arora D, Gross T and Brueggeman R. 2013. Allele characterization of genes required for *rpg4*-mediated wheat stem rust resistance identifies *Rpg5* as the *R* gene. *Phytopathology* 103: 1153–1161.

Arthaud J, Guyot L and Malencon G. 1966. Comparative biometric studies of the formae speciales of black rust (*Puccinia graminis* Pers.) living on the wild grasses of the Moroccan Atlas. In: *Proceedings of the Cereal Rusts Conference*, Cambridge, 1964, pp. 204-206.

Atienza SG, Jafary H and Niks RE. 2004. Accumulation of genes for susceptibility to rust fungi for which barley is nearly a non-host results in two barley lines with extreme multiple susceptibility. *Planta* 220: 71-79.

Bender CM, Prins R and Pretorius ZA. 2016. Development of a greenhouse screening method for adult plant response in wheat to stem rust. *Plant Disease* 100(1): 627-633.

Boshoff WHP, Pretorius ZA, Van Niekerk BD and Komen JS. 2002. First report of virulence in *Puccinia graminis* f. sp. *tritici* to wheat stem rust resistance genes *Sr8b* and *Sr38* in South Africa. *Plant Disease* 86(8): 922.

Browder LE. 1971. Pathogenic specialization in cereal rust fungi, especially *Puccinia recondita* f. sp. *tritici*, concepts, methods of study, and application. *Agricultural Research Service Technical Bulletin* No.1432, pp. 1-51. United States Department of Agriculture: Washington, DC.

Brown JKM and Hovmøller MS. 2002. Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science* 297: 537–541.

Brueggeman R, Druka A, Nirmala J, Cavileer T, Drader T, Rostoks N, Mirlohi A, Bennypaul H, Gill U, Kudrna D, Whitelaw C, Kilian A, Han F, Sun Y, Gill K, Steffenson B and Kleinhofs A. 2008. The stem rust resistance gene *Rpg5* encodes a protein with nucleotide binding site, leucine-rich, and protein kinase domains. *Proceedings of the National Academy of Sciences USA* 105: 14970-14975.

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B and Kleinhofs A. 2002. The barley stem rust-resistance gene *Rpg1* is a novel disease-

resistance gene with homology to receptor kinases. *Proceedings of the National Academy of Sciences USA* 99: 9328–9333.

Brueggeman R, Steffenson BJ and Kleinhofs A. 2009. The *rpg4/Rpg5* stem rust resistance locus in barley; resistance genes and cytoskeleton dynamics. *Cell Cycle* 8: 977–981.

Case AJ. 2017. Genetics, sources, and mapping of stem rust resistance in barley. PhD Thesis, University of Minnesota, St. Paul.

Chomczynski P and Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162(1): 156-159. ISSN 0003-2697. doi: 10.1016/0003-2697(87)90021-2

Coram TE, Settles ML and Chen X. 2008. Transcriptome analysis of high-temperature adult-plant resistance conditioned by *Yr39* during the wheat–*Puccinia striiformis* f. sp. *tritici* interaction. *Molecular Plant Pathology* 9(4): 479–493.

Coutinho TA, Rijkenberg FHJ and Van Asch MAJ. 1993. Development of infection structures by *Hemileia vastatrix* in resistant and susceptible selections of *Coffea* and in *Phaseolus vulgaris*. *Canadian Journal of Botany* 71: 1001-1008.

Deising H, Heiler S, Rauscher M, Xu H and Mendgen K. 1996. Cellular aspects of rust infection structure differentiation. In: *Histology, ultrastructure and molecular cytology of plant-microorganism interactions* (Nicole M and Gianinazzi-Pearson V, eds). *Developments in Plant Pathology* 7: 135-156. Springer, Dordrecht. doi: 10.1007/978-94-009-0189-6_8

Dickinson S. 1969. Studies in the physiology of obligate parasitism. VI. Directed growth. *Journal of Phytopathology* 66: 38-49. doi: 10.1111/j.1439-0434.1969.tb03084.x

Emmett RW and Parberry DG. 1975. Appressoria. *Annual Review of Phytopathology* 13(1): 147-167.

Ferreira JF and Rijkenberg FHJ. 1989. Development of infection structures of *Uromyces transversalis* in leaves of the host and a non-host. *Canadian Journal of Botany* 67: 429-433.

Glauert AM. 1974. Fixation, dehydration and embedding of biological specimens. In: *Practical methods in electron microscopy*, Vol. 3 (Glauert AM, ed). North Holland Publishing Company, Amsterdam.

Heath IB and Heath MC. 1979. Structural studies of the development of infection structures of cowpea rust, *Uromyces phaseoli var. vignae*. II. Vacuoles. *Canadian Journal of Botany* 57: 1830-1837. doi: 10.1139/b79-228

Hu G. 1996. Ultrastructure, cytochemistry and immunocytochemistry of the interaction between wheat (*Triticum aestivum*) and leaf rust (*Puccinia recondita* f. sp. *tritici*). PhD Thesis, University of Natal, South Africa.

Hughes FL and Rijkenberg FHJ. 1983. Epidermis removal technique for studying infection processes of *Puccinia sorghi* in maize leaves. *EMSSA Proc* 13: 17-18.

Hughes FL and Rijkenberg FHJ. 1985. Scanning electron microscopy of early infection in the uredial stage of *Puccinia sorghi* in *Zea mays*. *Plant Pathology* 34: 61-68.

Hurd-Karrer AM and Rodenhiser HA. 1947. Structures corresponding to appressoria and substomatal vesicles produced on nutrient-solution agar by cereal rusts. *American Journal of Botany* 34: 377-384.

Jafary H, Szabo LJ and Niks RE. 2006. Innate non-host immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities. *Molecular Plant-Microbe Interactions* 19: 1270-1279.

Jarošová J and Kundu JK. 2010. Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR. *Plant Biology* 10: 146. doi: 10.1186/1471-2229-10-146

Jin Y, Singh RP, Ward RW, Wanyera R, Kinyua M, Njau P, Fetch T, Pretorius ZA and Yahyaoui A. 2007. Characterization of seedling infection types and adult plant infection responses of monogenic *Sr* gene lines to race TTKS of *Puccinia graminis* f. sp. *tritici*. *Plant Disease* 91: 1096–1099.

Jin Y, Steffenson BJ and Miller JD. 1994. Inheritance of resistance to pathotypes QCC and MCC of *Puccinia graminis* f. sp. *tritici* in barley line Q21861 and temperature effects on the expression of resistance. *Phytopathology* 84: 452-455.

Jin Y, Szabo LJ, Pretorius ZA, Singh RP, Ward R and Fetch T. 2008. Detection of virulence to resistance gene *Sr24* within race TTKS of *Puccinia graminis* f. sp. *tritici*. *Plant Disease* 92: 923–926.

Jin Y, Szabo LJ, Rouse MN, Fetch T, Pretorius ZA, Wanyera R and Njau P. 2009. Detection of virulence to resistance gene *Sr36* within the TTKS race lineage of *Puccinia graminis* f. sp. *tritici*. *Plant Disease* 93: 367–370.

Johnson T. 1934. A tropic response in germ tubes of urediospores of *Puccinia graminis tritici*. *Phytopathology* 24: 80-82.

Keiper FJ, Hayden MJ, Park RF and Wellings CR. 2003. Molecular genetic variability of Australian isolates of five cereal rust pathogens. *Mycological Research* 107: 545-556.

Knott DR. 1989. The wheat rust pathogens. In: *The wheat rusts; breeding for resistance* (Knott DR, ed). Berlin, Heidelberg: Springer-Verlag, pp. 1-37.

Mamo BE, Brueggeman RS, Smith KP and Steffenson BJ. 2015. Genetic characterization of resistance to wheat stem rust race TTKSK in landrace and wild barley accessions identifies the *rpg4/Rpg5* locus. *Phytopathology* 105: 99–109. doi: 10.1094/PHYTO-12 13- 0340-R

Martens JW, Dunsmore KM and Harder DE. 1989. Incidence and virulence of *Puccinia graminis* in Canada on wheat and barley in 1988. *Canadian Journal of Plant Pathology* 11: 424-430.

McIntosh RA, Wellings CR and Park RF. 1995. Wheat Rusts: An Atlas of Resistance Genes. CSIRO, Australia.

McVey DV, Long DL and Roberts JJ. 2002. Races of *Puccinia graminis* in the United States during 1997 and 1998. *Plant Disease* 86: 568-572.

Niks RE. 1982. Early abortion of colonies of leaf rust, *Puccinia hordei*, in partially resistant barley seedlings. *Canadian Journal of Botany* 60: 714-723.

Niks RE. 1986. Variation of mycelial morphology between species and formae speciales of rust fungi of cereals and grasses. *Canadian Journal of Botany* 64: 2976-2983.

Nirmala J, Dahl S, Steffenson BJ, Kannangara CG, von Wettstein D, Chen X and Kleinhofs A. 2007. Proteolysis of the barley receptor-like protein kinase RPG1 by a proteasome pathway is correlated with *Rpg1*-mediated stem rust resistance. *Proceedings of the National Academy of Sciences USA* 104(24): 10276–10281.

Park RF. 2007. Stem rust of wheat in Australia. *Australian Journal of Agricultural Research* 58: 558–566.

Park RF, Golegaonkar PG, Derevnina L, Sandhu KS, Karaoglu H, Elmansour HM, Dracatos PM and Singh D. 2015. Leaf rust of cultivated barley: pathology and control. *Annual Review of Phytopathology* 53: 565-589. doi: 10.1146/annurev-phyto-080614-120324

Patterson F, Shands R and Dickson J. 1957. Temperature and seasonal effects on seedling reactions of barley varieties to three races of *Puccinia graminis* f. sp. *tritici*. *Phytopathology* 47: 395-402.

Pole Evans IB. 1907. The cereal rusts. I. The development of their uredo mycelia. *Annals of Botany* 21: 441-466.

Pretorius ZA, Jin Y, Bender CM, Herselman L and Prins R. 2012. Seedling resistance to stem rust race Ug99 and marker analysis for *Sr2*, *Sr24* and *Sr31* in South African wheat cultivars and lines. *Euphytica* 186: 15–23. doi: 10.1007/s10681-011-0476-0

Pretorius ZA, Singh RP, Wagoire WW and Payne TS. 2000. Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Plant Disease* 84: 203.

Prins R, Dreisigacker S, Pretorius Z, van Schalkwyk H, Wessels E, Smit C, Bender C, Singh D and Boyd LA. 2016. Stem rust resistance in a geographically diverse collection of spring wheat lines collected from across Africa. *Frontiers in Plant Science* 7: 973. doi: 10.3389/fpls.2016.00973

Roelfs AP, Singh RP and Saari EE. 1992. Rust diseases of wheat: concepts and methods of disease management. Centro International de Mejoramiento de Maiz y Trigo (CIMMYT), DF Mexico.

Sambrook J and Russell DW. 2000. Molecular cloning: A laboratory manual. 3rd edition. Cold Spring Harbor Laboratory, New York, pp. A8.19 – 8.21; 7.31 – 7.34.

Sambrook J, Fritsch EF and Maniatis T. 1989. Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Scholtz JJ and Visser B. 2013. Reference gene selection for qPCR gene expression analysis of rust-infected wheat. *Physiological and Molecular Plant Pathology* 81: 22-25.

Shands R. 1939. Chevron, a barley variety resistant to stem rust and other diseases. *Phytopathology* 29: 209-211.

Singh RP, Hodson DP, Huerta-Espino J, Jin Y, Njau P, Wanyera R, Herrera-Foessel SA and Ward RW. 2008. Will stem rust destroy the world's wheat crop? *Advances in Agronomy* 98: 271-309.

Singh RP, Hodson DP, Jin Y, Huerta-Espino J, Kinyua MG, Wanyera R, Njau P and Ward RW. 2006. Current status, likely migration and strategies to mitigate the threat to wheat production from race Ug99 (TTKS) of stem rust pathogen. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 1: 1-13.

Singh RP, Hodson DP, Jin Y, Lagudah ES, Ayliffe MA, Bhavani S, Rouse MN, Pretorius ZA, Szabo LJ, Huerta-Espino J, Basnet BR, Lan C and Hovmøller MS. 2015. Emergence and spread of new races of wheat stem rust fungus: continued threat to food security and prospects of genetic control. *Phytopathology* 105: 872–884.

Sood PN and Sackston WE. 1970. Studies on sunflower rust. VI. Penetration and infection of sunflowers susceptible and resistant to *Puccinia helianthi* race 1. *Canadian Journal of Botany* 48: 2179–2181.

Sotomayor IA, Purdy LH and Trese AT. 1983. Infection of sugarcane leaves by *Puccinia melanocephala*. *Phytopathology* 73: 695–699.

Steffenson BJ. 1992. Analysis of durable resistance to stem rust in barley. *Euphytica* 63: 153-167.

Steffenson BJ, Case AJ, Pretorius ZA, Coetzee V, Kloppers FJ, Zhou H, Chai Y, Wanyera R, Macharia G, Bhavani S and Grando S. 2017. Vulnerability of barley to

African pathotypes of *Puccinia graminis* f. sp. *tritici* and sources of resistance. *Phytopathology* PHYTO11160400R. doi: 10.1094/PHYTO-11-16-0400-R

Steffenson BJ, Jin Y, Brueggeman RS, Kleinhofs A and Sun Y. 2009. Resistance to stem rust race TTKSK maps to the *rpg4/Rpg5* complex of chromosome 5H of barley. *Phytopathology* 99: 1135–1141.

Steffenson BJ, Olivera P, Roy JK, Jin Y, Smith KP and Muehlbauer GJ. 2007. A walk on the wild side: Mining wild wheat and barley collections for rust resistance genes. *Australian Journal of Agricultural Research* 58: 532-544.

Sun Y and Steffenson BJ. 2005. Reaction of barley seedlings with different stem rust resistance genes to *Puccinia graminis* f. sp. *tritici* and *Puccinia graminis* f. sp. *secalis*. *Canadian journal of Plant Pathology* 27: 80-89.

Swertz CA. 1994. Morphology of germlings of urediniospores and its value for the identification and classification of grass rust fungi. *Studies in Mycology* 36: 1-152.

Wang X, Richards J, Gross T, Druka A, Kleinhofs A, Steffenson B, Acevedo M and Brueggeman R. 2013. The *rpg4*-mediated resistance to wheat stem rust (*Puccinia graminis*) in barley (*Hordeum vulgare*) requires *Rpg5*, a second NBS-LRR gene, and an actin depolymerization factor. *Molecular Plant-Microbe Interactions Journal* 26: 407–418. doi: 10.1094/MPMI-06-12-0146-R

Wanyera R, Kinyua MG, Jin Y and Singh RP. 2006. The spread of stem rust caused by *Puccinia graminis* f. sp. *tritici* with virulence on *Sr31* in Eastern Africa. *Plant Disease* 90: 113.

Wynn WK. 1976. Appressorium formation over stomata by the bean rust fungus: response to a surface contact stimulus. *Phytopathology* 66: 136-146.

Zurn JD, Dugyala S, Borowicz P, Brueggeman R and Acevedo M. 2015. Unravelling the wheat stem rust infection process on barley genotypes through relative qPCR and fluorescence microscopy. *Phytopathology* 105: 707-712.

## Conclusions

This study investigated rust resistance in wheat and barley at cellular level. A major objective was to avoid the common approach of studying resistance expression in seedlings and focus on more representative responses observed in adult plants. In the wheat-stripe rust pathosystem the emphasis was on characterising resistance QTL, either individually or in combination, derived from two sources. Data from field trials generally have an authenticity advantage over trials grown under controlled conditions, yet some challenges exist performing histological analysis. Aside from secondary *Puccinia striiformis* Westend. f. sp. *tritici* (*Pst*) infections, granting favourable weather conditions occur, the distinction between infection points is further complicated by contamination of other cereal rusts which are also tested in the same field nursery. Although stripe rust was the dominant disease at the time of sampling in the appraised DH and RIL populations, it was necessary to recognise and discard colonies of *P. triticina* in particular. Additionally, leaf material sampled in the field is much hardier than leaves grown under controlled conditions, lengthening the time needed for adequate clearing and staining of leaf samples.

Even though fluorescence microscopy had the same end results as visual scoring, histological analysis provides noteworthy information about plant response and underlying mechanisms not visually perceived at field level.

According to QTL data from DH lines containing Kariega-derived APR loci, *QYr.sgi-2B.1* was associated with up to 45% of the field RT variance documented over three scoring dates (Prins *et al.*, 2011). At the same time, little variation was documented for single and complexes of *QYr.sgi-2B.1* during visual scoring in 2011 and 2012. Microscopic observations indicated that fungal colonies from initial infection, eventually restricted by large necrotic areas, were visibly larger on entries carrying the *QYr.sgi-2B.1* region than those containing *QYr.sgi-2B.1* in combinations. These somewhat integrated colonies could not be quantified and thus did not reflect in the mean colony length of *QYr.sgi-2B.1*. Nonetheless, this attests to the fact that a single resistance QTL / gene is not as effective as combinations.

The small colonies measured on the *QYr.sgi-2B.1*-containing MP 51-line (only slightly larger than in the resistant parent) and intermediate to large colonies on MP 35

containing *Yr18* for 2011 and 2012 material, were contradictory to a previous study (Moldenhauer *et al.*, 2008). However, since sampling took place at two to eight days post inoculation in the aforementioned study and much later in the 2011 and 2012 trials, histological evidence confirms that major QTL *QYr.sgi-2B.1* plays a more important role than *Yr18* as the plants aged (Prins *et al.*, 2011).

Conclusive results stipulate that combinations of APR in a single variety had greater success in slowing down/restricting pathogen growth than each individual QTL region, with diverse resistance responses noticed among DH lines at all levels of assessment. Up to this point in disease development, little difference was seen between combinations of two and three APR loci.

The variation that exists between lines with the same genotype regarding Kariega stripe rust APR, suggests additional resistance yet to be described. Although Kariega is the obvious source, the susceptible parent might also contain potentially unsolved resistance. According to Ramburan *et al.* (2004), one of the significant QTL (*QYr.sgi-4A.2*) detected under growth chamber conditions, was retained from susceptible parent Avocet S. Singh *et al.* (2001) reported that Avocet S carries a minor effect stripe rust resistance QTL on chromosome 6A, though concealed in the Kariega x Avocet S DH population. The difference observed between Avocet S and the even more susceptible line MP 145 lacking Kariega resistance loci, could be the result of a minor Avocet S-QTL for APR not retained by MP 145. Alternatively, the variation in resistance between seemingly identical genotypes could be the result of gene interactions that were altered in the more susceptible background (Moldenhauer *et al.*, 2008).

With regards to the Palmiet x Yr16DH70 RIL population, Agenbag *et al.* (2012) reported that resistance QTL derived from Cappelle-Desprez significantly reduced disease only when *QYr.ufs-2D* and *QYr.ufs-5B* were combined as well as when the major effect *QYr.ufs-2A* QTL was paired with a minor QTL (*QYr.ufs-2D*, *QYr.ufs-5B* or *QYr.ufs-6D*). In the present study, repeatable results clearly indicated all lines carrying *QYr.ufs-2A* or *QYr.ufs-2D* in combination with at least one other QTL showed higher resistance levels, generally with enhanced effects for lines carrying an increasing number of resistance loci.

Different QTL combinations conditioned varying levels of resistance. The *QYr.ufs-5B+QYr.ufs-6D* QTL combination entailed a degree of complexity since it did not

perform well, exhibiting parallel and in some cases less resistant effects than the individual 5B- or 6D-chromosome QTL. The *QYr.ufs-2A+QYr.ufs-2D* combination contributed consistently high levels of resistance, and evidently performed sufficiently well without additional QTL, more so in some cases. This is an important finding as it shows that the identity of the gene or QTL may be more important than the number of genes in reducing stripe rust. The identity of resistance loci may relate to underlying defense mechanisms of a specific gene/QTL, as was reported for leaf rust resistance gene *Lr34* forming part of a ABC transporter gene with broad-spectrum resistance against multiple wheat fungal pathogens (Krattinger *et al.*, 2009). A better understanding of defense mechanisms can be achieved by cloning these resistance genes/QTL to ultimately identify the best loci to combine. The combination of Kariega and Cappelle-Desprez stripe rust resistance QTL in single lines should provide further insight in how complex genotypes restrict infection and fungal development.

Stacking four to five rust resistance loci in a single genotype will be advisable in terms of stability across environments (Singh *et al.*, 2011) and responsible gene stewardship. From a durability perspective, a more complex resistance genotype may be preferred in a commercial variety. However, the fitness cost of resistance genes in agricultural crops should also be considered. Brown and Rant (2013) mentioned that although the trade-off effects of disease resistance in crops are complex and difficult to measure, there is evidence that an increase in resistance will result in a small but measurable cost in productivity. The authors concluded that genes with large defence effects may subsequently have large pleiotropic effects while the fitness costs of partial, polygenic resistance may be substantially lower. When stacking several stripe rust resistance genes / QTL in a single variety, their effects on other desirable traits should be taken in consideration.

It is likely that only a fraction of resistance, within the wide range of diversity that exists, has been studied by plant breeders. Even so, genes and alleles which have been found useful in breeding programs, surviving natural as well as the more recent artificial selection processes, may be those that confer a benefit in controlling disease which exceeds any costs they might have retained (Brown and Rant, 2013).

In addition to the connection between defence systems and other components in metabolic and developmental networks within the plant (Robert-Seilaniantz *et al.*,

2011), interactions may also occur among stacked resistance loci. The resulting phenotype will rely on the mechanism and effectiveness imparted by each gene (German and Kolmer, 1992). Gene interactions can lead to enhanced resistance conditioned by the combination compared to that of the individual genes (Dyck and Samborski, 1982), either due to additive or epistatic effects.

Even though the extensive deployment of resistance gene *Rpg1* over a long time is believed to have played a role in the inherent stem rust resistance in barley (Steffenson *et al.*, 2017), virulence against this gene has been described in the recent emergence of the aggressive TTKSK *Puccinia graminis* f. sp. *tritici* Erikss. and Henning (*Pgt*) pathotype (Pretorius *et al.*, 2000). Steffenson *et al.* (2017) recounted the extreme vulnerability of the barley crop to the 'Ug99' race group of the wheat stem rust pathogen. Only 1.7% of cultivars and 1.4% wild barley accessions evaluated exhibited consistent moderately to highly resistant reactions, while a striking 37.5% and 84.6% of these resistant accessions (cultivated and wild, respectively), carried the only effective *rpg4/Rpg5* resistance locus described to date. Furthermore, although *Rpg5* is considered the determining factor of *rpg4*-mediated *Pgt* resistance (Mamo *et al.*, 2015), results from this study indicated that the individual *Rpg5* gene (i.e. susceptible *Rpg4*- allele) provide insufficient resistance to the wheat and, surprisingly, the isolate of the rye stem rust pathogen. This might point to the *Rpg5* gene not necessarily conferring broad-spectrum *Pgs* resistance. Furthermore, the vulnerability of the *rpg4/Rpg5* gene complex as a whole was exposed during high disease pressure conditions and elevated temperatures (Steffenson *et al.*, 2017).

Considering the current status of *Hordeum* spp. regarding their narrow genetic resistance basis, it is imperative to identify new sources of resistance that can be transferred into commercial cultivars. Quantitative trait loci conferring resistance against TTKSK have been identified by Moscou *et al.* (2011) and Zhou *et al.* (2014) contributing to the overall phenotypic resistance of a genotype when co-occurring with a major-effect resistance locus such as *Rpg1* or *rpg4/Rpg5*.

According to Jafary *et al.* (2006), rust pathogens likely have less success in supressing the basal defense in partially resistant barley genotypes, conditioned by non-hypersensitive, polygenic resistance loci. The possible contribution of a barley host genotype to basal stem rust resistance in seedlings was proposed based on

differences in resistance found between 24 and 48 hpi suggesting pre- and post-haustorial defense mechanisms (Zurn *et al.*, 2015). This is comparable with results from this study where aborted SSVs commonly occurred at 48 hpi in barley lines carrying the *rpg4/Rpg5* gene complex, pointing to the possible early elicitation of defense mechanisms in certain genotypes.

Although defence responses in barley are seemingly activated upon penetration of rust fungi into the substomatal cavity (Sellam and Wilcoxson, 1976; Lin *et al.*, 1998) and basal defence against rust in general expressed as a failed haustorium (Mellersh and Heath, 2001), the inherent basal resistance of barley against stem rust could not be ascribed to the early infection process, which was similar to that in wheat in this study. Up to 120 hpi, barley did not seem to exhibit less stem rust susceptibility than wheat, while some fungal suppression possibly occurred in barley between 120 and 240 hpi, as opposed to wheat.

Notwithstanding, both host plant and pathogen hold a variety of specific elements affecting the extent to which basal resistance can be supressed (Jafary *et al.*, 2006). Although *Pgt* is the most common stem rust form found on barley (Steffenson, 1992), its susceptibility towards *Pgs* was obvious in these trials. Furthermore, the existence of variation in virulence between strains from the same *forma specialis* was clearly detected in UVPgt54, originally isolated from barley, which was consistently more aggressive compared to UVPgt60.

It was suggested that basal and single gene dependent resistance only differs in the level of expression of the same pathway (Ellis *et al.*, 2007). Nevertheless, elucidating the mechanisms associated with the enhanced basal defence in barley against stem rust infections may provide valuable insight and contributions to the continuity in development of durable protection in cereals against rust pathogens.

REFERENCES

Agenbag GM, Pretorius ZA, Boyd LA, Bender CM and Prins R. 2012. Identification of adult plant resistance to stripe rust in the wheat cultivar Cappelle-Desprez. *Theoretical and Applied Genetics* 125: 109–120.

Brown JKM and Rant JC. 2013. Fitness costs and trade-offs of disease resistance and their consequences for breeding arable crops. *Plant Pathology* 62: 83–95. doi: 10.1111/ppa.12163

Dyck PL and Samborski DJ. 1982. The inheritance of resistance to *Puccinia* in a group of common wheat cultivars. *Canadian Journal of Genetics and Cytology* 24: 273-283.

Ellis JG, Dodds PN and Lawrence GJ. 2007. The role of secreted proteins in diseases of plants caused by rusts, powdery mildew and smut fungi. *Current Opinion in Microbiology* 10: 326-331.

German S and Kolmer JA. 1992. Effect of gene *Lr34* in the enhancement of resistance to leaf rust of wheat. *Theoretical and Applied Genetics* 84: 97-105.

Jafary H, Szabo LJ and Niks RE. 2006. Innate non-host immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities. *Molecular Plant-Microbe Interactions* 19: 1270-1279.

Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL and Keller B. 2009. A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science* 323: 1360-1363. doi: 10.1126/science.1166453

Lin K, Bushnell W, Smith A and Szabo L. 1998. Temporal accumulation patterns of defence response gene transcripts in relation to resistant reactions in oat inoculated with *Puccinia graminis*. *Physiological and Molecular Plant Pathology* 52: 95–114.

Mamo BE, Brueggeman RS, Smith KP and Steffenson BJ. 2015. Genetic characterization of resistance to wheat stem rust race TTKSK in landrace and wild barley accessions identifies the *rpg4/Rpg5* locus. *Phytopathology* 105: 99–109. doi: 10.1094/PHYTO-12 13- 0340-R

Mellersh DG and Heath MC. 2001. Plasma membrane-cell wall adhesion is required for expression of plant defence responses during fungal penetration. *Plant Cell* 13: 413-424.

Moldenhauer J, Pretorius ZA, Moerschbacher BM, Prins R and Van der Westhuizen AJ. 2008. Histopathology and PR-protein markers provide insight into adult plant resistance to stripe rust of wheat. *Molecular Plant Pathology* 9(2): 137–145.

Moscou MJ, Lauter N, Steffenson BJ and Wise RP. 2011. Quantitative and qualitative stem rust resistance factors in barley are associated with transcriptional suppression of defense regulons. *PLoS Genetics* 7(7): e1002208.

Pretorius ZA, Singh RP, Wagoire WW and Payne TS. 2000. Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Plant Disease* 84: 203.

Prins R, Pretorius ZA, Bender CM and Lehmensiek A. 2011. QTL mapping of stripe, leaf and stem rust resistance genes in a Kariega x Avocet S doubled haploid wheat population. *Molecular Breeding* 27: 259–270. doi: 10.1007/s11032-010-9428-y

Ramburan VP, Pretorius ZA, Louw JH, Boyd LA, Smith PH, Boshoff WHP and Prins R. 2004. A genetic analysis of adult plant resistance to stripe rust in the wheat cultivar Kariega. *Theoretical and Applied Genetics* 108: 1426–1433.

Robert-Seilaniantz A, Grant M and Jones JDG. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate–salicylate antagonism. *Annual Review of Phytopathology* 49: 317–343.

Sellam M and Wilcoxson R. 1976. Development of *Puccinia graminis* f. sp. *tritici* on resistant and susceptible barley cultivars. *Phytopathology* 66: 667–668.

Singh RP, Huerta-Espino J and William M. 2001. Slow rusting gene based resistance to leaf and yellow rusts in wheat: genetics and breeding at CIMMYT. In: *Proceedings of the Wheat Breeding Society of Australia* (Eastwood R, Hollamby G, Rathjen T and Gororo N, eds). Mildura, Australia, September 16–21, 2001. Australia: Wheat Breeding Society of Australia Inc, pp. 103–108.

Singh RP, Huerta-Espino J, Bhavani S, Herrera-Foessel SA, Singh D, Singh PK, Velu G, Mason RE, Jin Y, Njau P and Crossa J. 2011. Race non-specific resistance to rust diseases in CIMMYT spring wheats. *Euphytica* 179: 175-186.

Steffenson BJ. 1992. Analysis of durable resistance to stem rust in barley. *Euphytica* 63: 153-167.

Steffenson BJ, Case AJ, Pretorius ZA, Coetzee V, Kloppers FJ, Zhou H, Chai Y, Wanyera R, Macharia G, Bhavani S and Grando S. 2017. Vulnerability of barley to African pathotypes of *Puccinia graminis* f. sp. *tritici* and sources of resistance. *Phytopathology* PHYTO11160400R. doi: 10.1094/PHYTO-11-16-0400-R

Zhou H, Steffenson BJ, Muehlbauer G, Wanyera R, Njau P and Ndeda S. 2014. Association mapping of stem rust race TTKSK resistance in US barley breeding germplasm. *Theoretical and Applied Genetics* 127(6): 1293-1304. doi: 10.1007/s00122-014-2297-8

Zurn JD, Dugyala S, Borowicz P, Brueggeman R and Acevedo M. 2015. Unravelling the wheat stem rust infection process on barley genotypes through relative qPCR and fluorescence microscopy. *Phytopathology* 105: 707-712.

## Summary

The rate at which new virulent rust races emerge continues to leave global cereal production vulnerable. Since genetic defence is the most effective method to combat rust pathogens and minimize crop losses, the continuous identification and incorporation of diverse, durable resistance sources in new varieties are imperative for sustainable cereal production. In addition to visual scoring, histological analysis is a valuable method to investigate resistance mechanisms whether conferred by a single gene or several quantitative trait loci (QTL).

Earlier, assessments of wheat (*Triticum aestivum* L.) stripe rust [caused by *Puccinia striiformis* Westend. f. sp. *tritici* (*Pst*)] resistance in South Africa resulted in identification of the durable adult plant resistance (APR) gene *Lr34*/*Yr18*/*Sr57* and QTL *QYr.sgi-2B* and *QYr.sgi-4A* in the cultivar Kariega. In the present study, stripe rust colonization in Kariega x Avocet S doubled haploid (DH) lines, carrying different gene and/or QTL combinations, was compared through fluorescence microscopy. The flag leaves of field-infected adult plants stained with fluorophore Uvitex 2B, attested to the increased resistance effects of multiple loci appearing in a single host genotype compared to individual resistance loci. Additionally, the importance of gene/QTL identity in specific combinations was highlighted, with significant variation displayed among DH lines in terms of colony length (µm), number of haustorial mother cells per colony and hypersensitivity indexes ($P<0.05$).

To diversify the sources of stripe rust resistance in South African wheat germplasm, Palmiet (*QYr.ufs-4B*) was previously crossed with breeding line Yr16DH70, retaining contributing APR QTL *QYr.ufs-2A*, *QYr.ufs-2D*, *QYr.ufs-5B* and *QYr.ufs-6D* from Cappelle-Desprez. Evaluation of recombinant inbred lines under field conditions included *Pst* colony length visualised with fluorescence microscopy and accumulation of fungal biomass through RT-qPCR. Besides the confirmation of enhanced resistance of co-occurring resistance loci, the choice of QTL was accentuated in the varying levels of defence conditioned by different QTL combinations. Carriers of *QYr.ufs-2A* or *QYr.ufs-2D* accompanied by at least one other QTL exhibited higher resistance levels than single QTL and combinations not including either the 2A- or 2D-chromosome QTL, with significant variation found among lines across parameters ($P<0.05$).

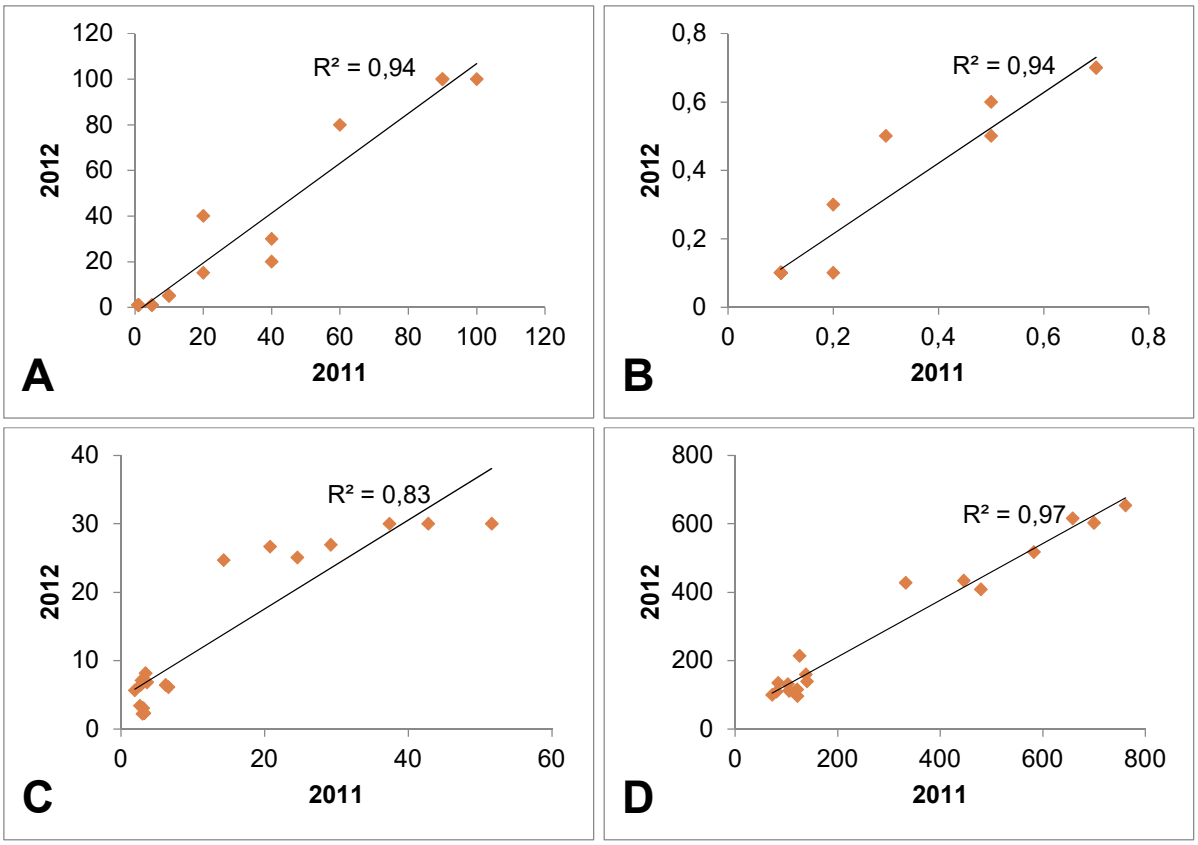
Barley (*Hordeum vulgare*), host to the wheat and rye attacking forms of the stem rust pathogen, *Puccinia graminis* (*Pg*), is considered inherently more resistant to *Pg* than wheat. To investigate whether this enhanced basal defence is associated with the early infection or colonization processes, adult plants of selected barley lines and wheat control entries were inoculated with *Pg* f. sp. *tritici* Erikss. and Henning pathotypes UVPgt54 and UVPgt60, and *Pg* f. sp. *secalis* Erikss. and Henning pathotype UVPgs01. Flag leaf sheaths on the last stem internode were sampled for analysis. Using scanning electron microscopy and epidermal stripping, no obvious differences in early infection structure development were observed between barley and wheat. Sub-stomatal vesicle appearance and production of haustorial mother cells were similar at 24 and 48 hours post-inoculation (hpi), respectively. Aborted *Pg* infection structures were regularly detected in, specifically, the *rpg4*/*Rpg5* gene complex-containing barley entries, and may be an indication of early onset of defences in certain host genotypes. Parallel data was obtained at 120 hpi using the WGA-FITC probe to measure colony sizes ($\mu m^2$) and RT-qPCR for assessment of accumulated fungal biomass. Significant variation occurred among entries ($P<0.05$) while the difference between rust pathotypes as well as the interaction between the two factors proved insignificant ($P>0.05$). The latent period between 120 and 240 hpi may hold some explanation, as a possibly steeper increase in accumulated fungal biomass in the susceptible wheat line did not reflect in opposing susceptible barley entries at 240 hpi.

## Addendums

**Addendum 2.1:** Analysis of variance for coefficient of infection of the Kariega x Avocet S doubled haploid wheat population inoculated with *Puccinia striiformis* f. sp. *tritici*, repeated over four cropping seasons.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 17 | 90835.07 | 5343.24 | 114.96 | 0.000000* | 1 |
| B: Year | 3 | 500.46 | 166.82 | 3.59 | 0.019912* | 0.759066 |
| Error | 50 | 2323.88 | 46.48 | | | |
| Total (Adjusted) | 70 | 93510.16 | | | | |
| Total | 71 | | | | | |

df = degrees of freedom.
Prob Level = Probability level.
* Term significant at alpha = 0.05
Least significant difference between lines = 9.74.
Least significant difference between years' = 4.59.




**Addendum 2.2:** Linear regressions between 2011 and 2012 cropping seasons in terms of A) stripe rust severity quantified through leaf area infected, B) host reaction type, C) number of *Puccinia striiformis* f. sp. *tritici* (*Pst*) haustorial mother cells per colony, and D) *Pst* colony lengths among wheat lines from the Kariega x Avocet S doubled haploid population. Repeatability indicated by $R^2$ value.

**Addendum 2.3:** Analysis of variance for number of haustorial mother cells among wheat lines from the Kariega x Avocet S doubled haploid population inoculated with *Puccinia striiformis* f. sp. *tritici* during the 2011 and 2012 cropping seasons.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 17 | 16200.94 | 953.00 | 69.48 | 0.000000* | 1 |
| B: Year | 1 | 4.97 | 4.97 | 0.36 | 0.549115 | 0.09127 |
| AB | 17 | 701.80 | 41.28 | 3.01 | 0.000617* | 0.995792 |
| C: Replicate | 2 | 30.72 | 15.36 | 1.12 | 0.332146 | 0.239485 |
| Error | 70 | 960.12 | 13.72 | | | |
| Total (Adjusted) | 107 | 17898.55 | | | | |
| Total | 108 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 4.28.

**Addendum 2.4:** Analysis of variance of *Puccinia striiformis* f. sp. *tritici* colony lengths in the Kariega x Avocet S doubled haploid wheat lines post inoculation during the 2011 and 2012 cropping seasons.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 17 | 4680746 | 275338 | 202.96 | 0.000000* | 1 |
| B: Year | 1 | 2797.87 | 2797.87 | 2.06 | 0.15543 | 0.293743 |
| AB | 17 | 69681.86 | 4098.93 | 3.02 | 0.000592* | 0.995928 |
| C: Replicate | 2 | 3721.48 | 1860.74 | 1.37 | 0.260438 | 0.285855 |
| Error | 70 | 94964.45 | 1356.64 | | | |
| Total (Adjusted) | 107 | 4851912 | | | | |
| Total | 108 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 42.53.

**Addendum 2.5:** Analysis of variance regarding the hypersensitivity index among the Kariega x Avocet S doubled haploid wheat lines, post inoculation with *Puccinia striiformis* f. sp. *tritici* in the 2012 cropping season.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 17 | 18.37 | 1.08 | 40.41 | 0.000000* | 1 |
| B: Replicate | 2 | 0.03 | 0.02 | 0.63 | 0.541206 | 0.145792 |
| Error | 34 | 0.91 | 0.03 | | | |
| Total (Adjusted) | 53 | 19.32 | | | | |
| Total | 54 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 0.29.

**Addendum 2.6:** Spearman's rank correlation factors between parameters used for quantification of resistance of Kariega x Avocet S doubled haploid wheat lines in the 2011 cropping season, post inoculation with *Puccinia striiformis* f. sp. *tritici* (*Pst*).

| | Coefficient of infection | Number of *Pst* HMC | *Pst* colony length |
|---|---|---|---|
| Coefficient of infection | 1 | | |
| Number of *Pst* HMC | 0.95 | 1 | |
| *Pst* colony length | 0.92 | 0.99 | 1 |

HMC = Haustorial mother cell(s).

**Addendum 2.7:** Spearman's rank correlation factors between parameters used for quantification of resistance of Kariega x Avocet S doubled haploid wheat lines in the 2012 cropping season, post inoculation with *Puccinia striiformis* f. sp. *tritici* (*Pst*).

| | Coefficient of infection | Number of *Pst* HMC | *Pst* colony length | H-index |
|---|---|---|---|---|
| Coefficient of infection | 1 | | | |
| Number of *Pst* HMC | 0.85 | 1 | | |
| *Pst* colony length | 0.92 | 0.98 | 1 | |
| H-index | -0.77 | -0.89 | -0.90 | 1 |

HMC = Haustorial mother cell(s).
H-index = Hypersensitivity index.

**Addendum 3.1:** Predicted stripe rust adult plant resistance QTL retained by selected Palmiet x Yr16DH70 $F_8$ RILs, scanned for all DNA markers across each QTL interval (obtained from R. Prins, CenGen (Pty) Ltd).

| | ***QYr.ufs-2A*** | | | ***QYr.ufs-2D*** | | | ***QYr.ufs-5B*** | | | ***QYr.ufs-6D*** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID Name** | **wmc 407** | **wmc 177** | **gwm 636*** | **wmc 245*** | **gwm 539** | **wmc 18** | **gwm 371** | **gwm 335*** | **barc 74** | **gwm 325*** | **barc 175** | **cfd 47** | **barc 202** | **Predicted QTL** |
| Palmiet#1 | 131 | 190 | 80 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | |
| Palmiet#2 | 131 | 190 | 80 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | 4B |
| Yr16DH70#1 | 135 | 192 | 108 | 149 | 134 | 221 | 186 | 210 | 180 | 141 | 226 | 191 | 304 | |
| Yr16DH70#2 | 135 | 192 | 108 | 149 | 134 | 221 | 186 | 210 | 180 | 141 | 226 | 191 | 304 | 2A+2D+5B+6D |
| RIL20#1_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 186 | 210 | 180 | 141 | 226 | 191 | 304 | |
| RIL20#2_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 186 | 210 | 180 | 141 | 226 | 191 | 304 | 2A+2D+5B+6D |
| RIL148#1_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 186 | 210 | 180 | 141 | 226 | 191 | 304 | |
| RIL148#2_$F_8$ | N/A | N/A | 108 | 149 | N/A | N/A | 186 | 210 | 180 | 141 | 226 | 191 | 304 | 2A+2D+5B+6D |
| RIL39#1_$F_8$ | 135 | 190 | 108 | 149 | 134 | 221 | 186 | 210 | 180 | 143 | 223 | 176 | 308 | |
| RIL39#2_$F_8$ | 135 | 190 | 108 | 149 | 134 | 221 | 186 | 210 | 180 | 143 | 223 | 176 | 308 | 2A+2D+5B |
| RIL34#1_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | |
| RIL34#2_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 182 | 218 | 177 | 141 | N/A | 191 | 304 | 2A+2D+6D |
| RIL142#1_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | |
| RIL142#2_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | 2A+2D+6D |
| RIL33#1_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 186 | 210 | 180 | 141 | 226 | 191 | 304 | |
| RIL33#2_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 186 | 210 | 180 | 141 | 226 | 191 | 304 | 2A+5B+6D |
| RIL10#1_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | |
| RIL10#2_$F_8$ | 135 | 192 | 108 | 149 | 134 | 221 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | 2A+2D |
| RIL144#1_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | |
| RIL144#2_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | 2A+6D |
| RIL153#1_$F_8$ | 131 | 192 | 80 | 149 | 134 | 221 | 186 | 210 | 180 | 143 | 223 | 176 | 308 | |
| RIL153#2_$F_8$ | 131 | 192 | 80 | 149 | 134 | 221 | 186 | 210 | 180 | 143 | 223 | 176 | 308 | 2D+5B |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RIL17#1_$F_8$ | 131 | 192 | 80 | 149 | 134 | 221 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | |
| RIL17#2_$F_8$ | 131 | 192 | 80 | 149 | 134 | 221 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | 2D+6D |
| RIL45#1_$F_8$ | 131 | 190 | 80 | 147 | 136 | 245 | N/A | 210 | 180 | 141 | 226 | 191 | 304 | |
| RIL45#2_$F_8$ | 131 | 190 | 80 | 147 | 136 | 245 | N/A | 210 | 180 | 141 | 226 | 191 | 304 | 5B+6D |
| RIL112#1_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | |
| RIL112#2_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | 2A |
| RIL178#1_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | |
| RIL178#2_$F_8$ | 135 | 192 | 108 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | 2A |
| RIL15#1_$F_8$ | 131 | 190 | 80 | 149 | 134 | 221 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | |
| RIL15#2_$F_8$ | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | 2D |
| RIL190#1_$F_8$ | 131 | 190 | 80 | 149 | 134 | 221 | 186 | 218 | 177 | 143 | 223 | 176 | 308 | |
| RIL190#2_$F_8$ | 131 | 190 | 80 | 149 | 134 | 221 | N/A | N/A | 177 | 143 | 223 | 176 | N/A | 2D |
| RIL31#1_$F_8$ | 131 | 190 | 80 | 147 | 136 | 245 | N/A | 210 | 180 | 143 | 223 | 176 | 308 | |
| RIL31#2_$F_8$ | 131 | 190 | 80 | 147 | 136 | 245 | N/A | 210 | 180 | 143 | 223 | 176 | 308 | 5B |
| RIL149#1_$F_8$ | 131 | 190 | 80 | 147 | 134 | 245 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | |
| RIL149#2_$F_8$ | 131 | 190 | 80 | 147 | 134 | 245 | 182 | 218 | 177 | 141 | 226 | 191 | 304 | 6D |
| RIL52#1_$F_8$ | 131 | 192 | 80 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | |
| RIL52#2_$F_8$ | 131 | 192 | 80 | 147 | 136 | 245 | 182 | 218 | 177 | 143 | 223 | 176 | 308 | None |

RIL = recombinant inbred line: two plants tested per RIL/cultivar, i.e. #1 and #2.
QTL = quantitative trait loci: 2A = *QYr.ufs-2A*, 2D = *QYr.ufs-2D*, 5B = *QYr.ufs-5B* and 6D = *QYr.ufs-6D*.
* Marker closest to QTL.
Highlighted = Yr16DH70 marker allele detected.
N/A = no amplification.

**Addendum 3.2:** Analysis of variance for coefficient of infection in Palmiet x Yr16DH70 $F_8$ recombinant inbred lines inoculated with *Puccinia striiformis* f. sp. *tritici*.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 19 | 11707.92 | 616.21 | 11.73 | 0.000000* | 1 |
| B: Time course | 1 | 987.01 | 987.01 | 18.79 | 0.000100* | 0.988257 |
| AB | 19 | 1440.86 | 75.83 | 1.44 | 0.162984 | 0.776086 |
| C: Replication | 1 | 130.05 | 130.05 | 2.48 | 0.123719 | 0.335541 |
| Error | 39 | 2048.95 | 52.54 | | | |
| Total (Adjusted) | 79 | 16314.8 | | | | |
| Total | 80 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 10.47.
Least significant difference between sampling times = 3.31.

**Addendum 3.3:** Analysis of variance of *Puccinia striiformis* f. sp. *tritici* colony lengths in Palmiet x Yr16DH70 $F_8$ recombinant inbred lines.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 19 | 98334.48 | 5175.5 | 21.87 | 0.000000* | 1 |
| B: Replication | 1 | 970.23 | 970.23 | 4.1 | 0.057196 | 0.484888 |
| Error | 19 | 4497.28 | 236.7 | | | |
| Total (Adjusted) | 39 | 103802 | | | | |
| Total | 40 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 31.37.

**Addendum 3.4:** Analysis of variance for host cell necrosis associated with *Puccinia striiformis* f. sp. *tritici* after inoculation of Palmiet x Yr16DH70 $F_8$ recombinant inbred lines.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 19 | 17116.28 | 900.86 | 2.07 | 0.060937 | 0.816797 |
| B: Replication | 1 | 1452.03 | 1452.03 | 3.33 | 0.083613 | 0.410603 |
| Error | 19 | 8274.48 | 435.5 | | | |
| Total (Adjusted) | 39 | 26842.78 | | | | |
| Total | 40 | | | | | |

* Term significant at alpha = 0.05

**Addendum 3.5:** Analysis of variance for hypersensitivity index among Palmiet x Yr16DH70 $F_8$ recombinant inbred lines, post inoculation with *Puccinia striiformis* f. sp. *tritici*.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 19 | 1.25 | 0.07 | 5 | 0.000486* | 0.998513 |
| B: Replication | 1 | 0.06 | 0.06 | 4.43 | 0.048944* | 0.514809 |
| Error | 19 | 0.25 | 0.01 | | | |
| Total (Adjusted) | 39 | 1.56 | | | | |
| Total | 40 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 0.24.
Least significant difference between replications = 0.08.

**Addendum 3.6:** Analysis of variance for relative *β-tubulin* expression of *Puccinia striiformis* f. sp. *tritici* after inoculation of Palmiet x Yr16DH70 $F_8$ recombinant inbred lines.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 19 | 189.47 | 9.97 | 2.84 | 0.002872* | 0.987485 |
| B: Time course | 1 | 122.54 | 122.54 | 34.87 | 0.000001* | 0.999926 |
| AB | 19 | 50.8 | 2.67 | 0.76 | 0.734825 | 0.43092 |
| C: Replication | 1 | 1.6 | 1.6 | 0.46 | 0.503219 | 0.101076 |
| Error | 39 | 137.07 | 3.51 | | | |
| Total (Adjusted) | 79 | 501.48 | | | | |
| Total | 80 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 2.71.
Least significant difference between sampling times = 0.86.

**Addendum 3.7:** Spearman's rank correlation factors between parameters used for quantification of resistance of the first field replication of Palmiet x Yr16DH70 $F_8$ recombinant inbred lines, post inoculation with *Puccinia striiformis* f. sp. *tritici* (*Pst*).

| | Coefficient of infection | Relative *β-tubulin* expression | Pst colony length | H-index |
|---|---|---|---|---|
| Coefficient of infection | 1 | | | |
| Relative *β-tubulin* expression | 0.83 | 1 | | |
| *Pst* colony length | 0.91 | 0.83 | 1 | |
| H-index | -0.73 | -0.60 | -0.87 | 1 |

H-index = Hypersensitivity index.

**Addendum 3.8:** Spearman's rank correlation factors between parameters used for quantification of resistance of the second field replication of Palmiet x Yr16DH70 $F_8$ recombinant inbred lines, post inoculation with *Puccinia striiformis* f. sp. *tritici* (*Pst*).

| | Coefficient of infection | Relative *β-tubulin* expression | Pst colony length | H-index |
|---|---|---|---|---|
| Coefficient of infection | 1 | | | |
| Relative *β-tubulin* expression | 0.63 | 1 | | |
| *Pst* colony length | 0.70 | 0.71 | 1 | |
| H-index | -0.65 | -0.82 | -0.89 | 1 |

H-index = Hypersensitivity index.

**Addendum 4.1:** Analysis of variance for *Puccinia graminis* colony sizes in selected barley and wheat entries.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 8 | 1.08E+11 | 1.36E+10 | 9.14 | 0.000014* | 0.999958 |
| B: Isolate | 2 | 2.48E+09 | 1.24E+09 | 0.84 | 0.445441 | 0.175809 |
| AB | 16 | 8.10E+09 | 5.06E+08 | 0.34 | 0.984669 | 0.154473 |
| C: Replication | 1 | 3.50E+10 | 3.50E+10 | 23.63 | 0.000066* | 0.996428 |
| Error | 23 | 3.41E+10 | 1.48E+09 | | | |
| Total (Adjusted) | 50 | 1.87E+11 | | | | |
| Total | 51 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 45993.91.
Least significant difference between replications = 21681.74.

**Addendum 4.2:** Analysis of variance for relative *β-tubulin* expression of *Puccinia graminis* post inoculation of selected barley and wheat entries.

| Source Term | df | Sum of Squares | Mean Square | F-Ratio | Prob Level | Power (Alpha=0.05) |
|---|---|---|---|---|---|---|
| A: Line | 8 | 11798.94 | 1474.87 | 5.57 | 0.000024* | 0.998835 |
| B: Isolate | 2 | 353.03 | 176.51 | 0.67 | 0.517049 | 0.157236 |
| AB | 16 | 5914.48 | 369.66 | 1.4 | 0.173112 | 0.776519 |
| C: Time course | 1 | 10486.4 | 10486.4 | 39.6 | 0.000000* | 0.999989 |
| AC | 8 | 8775.06 | 1096.88 | 4.14 | 0.000507* | 0.987488 |
| BC | 2 | 240.18 | 120.089 | 0.45 | 0.637463 | 0.120785 |
| D: Replication | 1 | 292.5 | 292.5 | 1.1 | 0.297283 | 0.178868 |
| Error | 63 | 16682.95 | 264.81 | | | |
| Total (Adjusted) | 101 | 55020 | | | | |
| Total | 102 | | | | | |

* Term significant at alpha = 0.05
Least significant difference between lines = 13.29.
Least significant difference between sampling times = 6.26.