University of Southern Queensland

BREEDING FOR DURABLE RESISTANCE TO *PUCCINIA SORGHI*: STATUS AND STRATEGIES

A Thesis submitted by

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ABSTRACT

Australia has experienced an increase in incidence and severity of maize common rust (CR), caused by *Puccinia sorghi*, which could threaten the maize industry. Over a 120 isolates collected from seven maize growing regions were stored and maintained their viability and infectivity over 24 months at -80°C. The isolates were inoculated on the main commercial maize lines and it was found that they do not carry any resistance to CR and that the main resistance gene to *P. sorghi* (*Rp* gene), *Rp1_D*, is no longer effective. Maize lines carrying single or compound resistance genes were identified as carrying effective resistance to CR, *Rp1_G*, *Rp5*, *Rp1_E*, *Rp1_L*, *RpGDJ*, *RpGJF*, and *Rp5GCJ*. And other lines were selected as part of a proposed differential set *Rp1_B*, *Rp1_C*, *Rp1_D*, *Rp1_F*, *Rp1_K*, *Rp3_D* and *Rp4_A*. An hybrid assembly of the first collected isolate was created with assembling short and long reads. The hybrid assembly of Isolate 1 was also mapped against the short read of 17 isolates. The five most diverse isolates, in terms of collection location, year and host, were phenotyped against a set of maize lines carrying no resistance gene, from one and up to four resistances genes. A QUAST analysis revealed a draft genome of 145,963,792 bp and total length of 145,815,044 bp (>= 1000 bp), compared to 99,534,058 bp in Rochi et al. (2018). The prediction from the Braker pipeline identified 20,438 genes model. No clones were detected among the 17 isolates sequenced and they represented unique genotypes grouped into three clusters. No pattern of association of virulence was detected in the phenotyping study based on location of collection. This suggested that the variation in infection level on the differential set was not related to agro-ecological region of origin. This study successfully identified key strategies for maize

breeding programs to develop durable resistance and meet national production targets.

CERTIFICATION OF THESIS

I, Aurelie Quade, declare that the PhD Thesis entitled "Breeding for Durable Resistance to *Puccinia sorghi*: Status and Strategies" is not more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references, and footnotes.

This Thesis is the work of Aurelie Quade, except where otherwise acknowledged, with the majority of the contribution to the papers presented as a Thesis by Publication undertaken by the student. The work is original and has not previously been submitted for any other award, except where acknowledged.

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STATEMENT OF CONTRIBUTION

Manuscript 1:
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Student contributed 90% to this paper. Collectively Park, R. F., Stodart, B. J. and Ash, G. J. contributed to the remainder.

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DEDICATION

This thesis is dedicated to my grandparents, Micheline and Raymond Clerc, to my father, Philippe Clerc, and to all the people that do not lack the intelligence, but the opportunity.

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ABBREVIATIONS

AVPMA	Australian Pesticides and Veterinary Medicines Authority
CR	common rust
CRM	corn relative maturity
DAP	days after planting
DF	degrees of freedom
DPI	days post inoculation
GM	genetically modified
GRDC	Grains Research and Development Corporation
HR	hypersensitive reaction
IT	infection type
LN	liquid nitrogen
LRRs	leucine-rich repeats
MAS	marker assisted selection
NLRs	nucleotide binding LRRs proteins
PCR	polymerase chain reaction
Pst Sr	*Puccinia striiformis* resistance gene
QR	quantitative resistance
QTLs	quantitative trait loci
R-gene	resistance gene
Rlm gene	resistance *Leptosphaeria maculans* gene
Rp gene	resistance *Puccinia* gene
SCR	southern corn rust
SR	wheat stem rust
Sr	stem rust resistance gene
TEs	transposable elements
USQ	University of Southern Queensland
WUE	water use efficiency

CHAPTER 1: INTRODUCTION

Global human population is predicted to reach 9.8 billion by 2050 (Searchinger et al., 2018). Continued population growth predictions are combined with the changes in food consumption patterns. There is an increasing demand for calories in poorer nations and food waste in richer nations, and the pressure on agricultural systems and natural resources globally is unprecedented. The supply of food is one of the greatest challenges that humankind must face in the 21st Century. Current global food production relies on three crop types, (cereals, legumes and root/tubers), to supply 90% of the world food energy intake (Su et al., 2017). Future increases in production will unquestionably require crop protection strategies with minimal environmental impacts.

Maize is the third most important crop behind wheat and rice globally, in terms of tonnage produced, Anon (2021). It is a staple food in many regions of the world and is also used as an animal fodder crop. In Australia, maize is planted from Northern Queensland to Tasmania but, unlike Australia's main grain crops used for human consumption, wheat and barley, it is grown during the summer months (O'Keeffe, 2009; Cogswell, 2012). Only 6 to 7% of Australia's maize production is exported (approximately 80,000 tons annually) (Cogswell, 2012). The potential exists to increase maize production to 3.47 million tonnes, pushing Australia toward becoming a competitive exporter in the Asia-Pacific region, a market which was worth AUD $4.1 billion in 2012 (Cogswell, 2012) (recent data not available). A staggering 20% to 40% of crop production is lost each year to pests, and plant pathogens are costing the global economy an estimated $220 billion, Anon (2021). These figures do not include the impact of conventional chemical controls on the environment. One of the main approaches to plant disease management, breeding for resistance, is also one of the most ecologically sustainable.

In their natural environments, plant populations have evolved and maintain a diversity of resistance genes. Plant breeding programs seek to identify plant genotypes carrying adequate levels of resistance to plant pathogens for use in plant improvement and commercialisation. However, the use of specific resistances on a large scale inadvertently increases selection pressure on pathogen populations. This is also true in the maize/rust pathosystem. To reach its production potential in Australia, sustainable management of maize diseases is paramount.

In Australia in recent years, maize common rust (CR), caused by *Puccinia sorghi* Schwein., has increased in incidence and severity. Common rust has the potential to cause yield losses of up to 40% in susceptible varieties and yield losses of 60% recorded under extreme disease pressure (Pataky, et al., 2001). Maize possesses at least 24 single "resistance to *Puccinia*" (*Rp*) genes, each conferring resistance to specific races of *P. sorghi* (Hooker, 1985). Lines with different *Rp* genes have been used to assess different virulence in isolates present in specific maize-growing regions across in the United States (Hulbert et al., 1991; Groth et al., 1992) the Argentinian Corn Belt Region (Darino et al., 2016) and South Africa (Fato, 2000). Differences in virulence were observed in each country, as might be expected, given that the coevolution of pathogens and their hosts generates a dynamic system (Chavan et al., 2015). In Australia, the level of resistance of the major commercial lines and the effectiveness of *Rp* genes against local isolates of *P. sorghi* has not been evaluated for many years, and the cause of the recent rust outbreaks is unknown.

The biotrophic nature of rusts leads to the development of intricate mechanisms to cope with the pressure exerted by their dependence on a live host to extract nutrients. Often host specific, they have developed the ability to disperse over large distances (Milus et al., 2009; Hovmoller et al., 2011). However, their most remarkable feature is their ability to generate new virulence, and/or new combination of existing virulence, which enables them to by-pass host defence (Schwessinger et al., 2018). Rusts possess

a complex life cycle with up to 5 spore stages: urediniospores, teliospores, basidiospores, spermatia and aeciospores (Bettgenhaeuser et al., 2014). Most genetic diversity originates from recombination of genes, which are reshuffled at the sexual stage. At the urediniospore or clonal stage, exponential spore production increases the population size, therefore the likelihood of mutant genotypes arising. The clonal stage is often the costliest to the agricultural industry, overcoming crop resistance genes, often as rapidly as they are commercialised (Park, 2015b).

The characterisation of the maize/rust pathosystem relies on an understanding of both the diversity of virulence in the pathogen, as well as the identity and effectiveness of the resistance genes in the host. The ongoing monitoring of CR virulence requires the maintenance of a collection of rust isolates (as urediniospores) carrying known virulence. The conditions required for long-term storage of these spores is species specific. Some rust species have been stored for decades at ultra-cold temperatures, whilst these conditions are lethal for other species. Isolates with known virulence are utilised to identify and characterise CR resistance genes in maize. Due to the constantly evolving/mutating nature of the rust pathogen populations, these collections reflect the pathotypes likely to be present in defined agro-ecological regions through phenotyping. To identify resistance genes suitable for deployment in Australia, we must first characterise rust isolates present in the main maize growing regions.

Resistance conferred by single major genes is often short-lived, particularly when faced with a diverse pathogen population. Resistance breeding for the long-term must be conducted in conjunction with determining the virulence alleles in the pathogen population that the resistance genes in the crop genes will encounter. . The level of protection provided by resistance genes lies in the choice of recombination of resistance genes. The capacity to deploy new resistances is often much slower than the rate at which pathogens evolve virulence. Although several studies have demonstrated that *Puccinia* resistance loci in maize have the capacity to generate new

resistance specificities against pathogen infection (Chavan et al., 2015), no study has been conducted to determine the diversity present in the population of *P. sorghi* in Australia and its potential to overcome current resistance genes in commercial maize lines. Once candidate resistance genes are identified, further assessments are required to understand the level of diversity of the CR population in Australia and to estimate the durability of the candidate resistance genes in agriculture. The breeding of maize lines for durable resistance is achievable if we can tailor strategies to anticipate the pathogen potential for diversification.

The aim of this research is to produce foundational information for maize breeding programs to develop durable resistance to CR in Australia using a curated collection of rust isolates, identifying suitable candidate resistance genes and determining the potential for *P. sorghi* to compromise the durability of resistance gene in Australia.

CHAPTER 2: LITERATURE REVIEW

2.1 Maize crop

Maize or corn, *Zea mays* L. subspecies *(*ssp*.) mays,* and their close relatives the teosintes, *Zea mays*, belong to the genus *Zea*, in the tribe *Andropogoneae* in the subfamily *Panicoideae* in the family *Poaceae* (Doebley & Iltis, 1980; Sánchez G et al., 2011). The *Andropogoneae* tribe has 86 genera and five species belong to the genus *Zea*, though only *Zea mays* is cultivated. Exact genealogy remains uncertain, but domestication began 6000 years ago in the Mesoamerican region, according to archaeological records and phylogenetic analysis (Piperno & Flannery, 2001).

Maize is the third most important crop behind wheat and rice (O'Keeffe, 2009). Corn kernels are used for human consumption, industrial food products and in the pharmaceutical industry (Huang et al., 2006). The high nutritional value of kernels is prized as animal feed, though the whole plant is often used in silage. The use of corn has been extended to the production of starch, ethanol, plastic substitutes and in antibiotics (O'Keeffe, 2009).

The composition of kernel determines maize use (Purseglove, 1972) (

Table 0-1).

Table 0-1: Maize types and their usage, Anon (2008)

Maize type	Properties	Usage
Flint	High percentage of hard endosperm around a soft small centre	Food
Dent	Hard endosperm, filled with soft starch	Grain and silage, number one maize type grown in the USA
Floury	Endosperm mainly composed of soft starch	Easier to grind and mostly grown in the Andean region
Waxy	Starch composed entirely of amylopectin (rather than the usual ratio of 70% amylopectin, 30% amylose)	Consumed in East Asia and grown for some industrial use (e.g.: corn syrup in soft drinks)
Pop	High proportion of hard endosperm	Though grown on a small scale, it is a worldwide snack.
Sweet	Harvest early when kernel moisture is at 70%, higher sugar content to starch ratio	Fresh vegetable

Soil type is not a limiting factor to grow maize, though the crop does best on deep, free draining, heavier soils that are slightly acidic (O'Keeffe, 2009). Maize can be grown in environments receiving between 400-800 mm of rainfall during the growing season or where irrigation is available (Ekins et al., 2007). The water use efficiency (WUE) of maize plants is higher than wheat and canola (Huang et al., 2006), making it an ideal crop during the warmer months. Maize has a transpiration rate of 388 molecules of water lost per molecule of carbon dioxide fixed. By comparison, the transpiration ratios of soybean and wheat under the same conditions are 704 and 613, respectively. Maize is, however, more susceptible to drought stress than wheat and lack of water at key developmental stages results in higher yield penalties.

The life cycle of maize is characterised by vegetative growth or V stages, from emergence to V16, and reproductive developmental or R-stage, from R1 to R5 (O'Keeffe, 2009). The germination of corn is dependent on adequate soil moisture and suitable temperatures. At soil temperatures of 25°C seedlings emerge in 4 to 5 days, whereas 14 days, or more, may be required at temperatures below 12°C. Growth rate increases at temperatures between 10 to 30°C. Below 10°C the growth ceases and it plateaus above 30°C. Hence, the prediction of growth rate based on accumulation of thermal units or "growing degree days" is commonly used by growers to estimate time to maturity. These requirements will dictate planting time, which in Australia usually occurs between September and December, Anon (2018). However, tropical regions of Australia may be planting from early August up to late March.

Varieties are selected based on their end use quality, time to maturity requirements and pest and disease resistance (Du Plessis, 2003; Moore et al., 2014; 2017). Corn relative maturity (CRM) determines the time from

planting to harvest maturity. Most Australian hybrids range from 90 to 126 days.

2.2 Domestic market

Unlike other major Australian crops, such as wheat, canola and cotton, corn is grown from Northern Queensland to Tasmania (O'Keeffe, 2009; 2017). On average, 440,000 tonnes are produced annually, on 64,000 thousand hectares. In 2018, the production was 380,000 tonnes and 393,003 in 2021, Anon (2021; 2018).

In Australia, corn is grown for stock feed (grain and silage), human consumption and industrial processing (O'Keeffe, 2009). To supply industry needs and usage different maize types are grown: soft, starchy grain, semi-dent, semi-flint and hard flint, Anon (2008). The principal difference between grain types is their protein to starch ratio: harder types contain more protein and softer types more starch. In 2015, just under 85% of the corn produced was grit types (36%) and soft dent (46%), Anon (2015). Grit corn is used for breakfast cereals, confectionary products or corn chips, Anon (2008). Food processing industry require the softer grain type and popcorn is made from the hard type.

2.3 Production and export market

In 2021, the three largest corn producing countries worldwide were the USA, China and Brazil, Anon (2021). Their estimated production amounted to 352,709, 242,852 and 82,744 thousand metric tonnes, respectively, Anon (2021).

In the same period, the three major corn exporters were the USA, Argentina and Brazil, with estimates of 70,041, 36,912 and 20,429 thousand metric tonnes, respectively, Anon (2021).

Amongst the three major corn importers were Japan, Mexico and South Korea with a net estimated import of 15,239, 17,396 and 11,653 and thousand metric tonnes, respectively, Anon (2021). Interestingly, in the year 2017-2016 the USA and China imported 1.45 and 2.46 thousand metric tonnes, respectively, Anon (2018). And in the year 2017-2018, an estimated 1.02 and 4 thousand metric tonnes of corn were imported by the two countries.

A GRDC Report in 2012 found that the combined tonnage for Japanese and South Korean maize import was 25 million tonnes, of which Australia supplied 3.47 million tonne (Cogswell, 2012).

Australia is therefore in an advantageous position to become a competitive export player in the Asia-Pacific region. Its growing season allows the supply of grain when the USA, Europe and Russia do not, and its proximity to an export market worth AUD $4.1 billion, with reduced transportation cost. The demand for Australian products in Asia is rising, as experienced by the cosmetic, meat and dairy industries (Enright, 2017).

Few countries worldwide grow corn under strict regulations in term of genetically modified (GM) free status, chemical applications and consumer safety. The Australian maize industry combines all three and growers are seeking to minimize and, when possible, eliminate, environmental impact. Australian corn returned $20 to $30 a tonne premium over GM corn with pick premium reaching $60 a tonne in 2013 (Clarry, 2013).

Currently, only 6 to 7% of Australian maize production is exported, about 80 000 tonnes annually, Anon (2017). In 2021, Australia exported 86,902 tonnes, Anon (2021).

Australia has the potential to occupy 14% of the export market for Japan and South Korea combined. A report produced by the Maize Association of Australia is urging the Australian government to negotiate export contracts with both countries (Cogswell, 2012). If we consider predicted population

growth worldwide, it is a low-risk investment by the Australian Government.

2.4 Industry threats

Cultivation of maize requires wide row spacing favouring the development of summer weeds, Anon (2008). Water loss through weed competition, particularly summer grass, is a problem during seedling establishment, especially within 3 weeks of crop emergence (O'Keeffe, 2009).

Several insect pests cause damage to aerial and root parts of the plants. Insects present in the soil may cause up to 30% yield losses and re-sowing of the crop may be necessary, Anon (2008).

The impact of the main diseases affecting maize vary based on the climate, seasonal conditions, deployment of genetic resistance and access to chemical control. Worldwide the main diseases of maize are northern leaf blight, tropical rust and Southern Corn Rust (SCR), Anon (2016).

Corn is the number one agricultural industry in the USA (Green et al., 2018). In a survey conducted between 2012 and 2015, and representing 96.7% of the total corn produced in the USA, an average of 10.6% was lost to disease (Mueller et al., 2016). The estimated loss to corn diseases in the US for that period was US$189.1 per hectares. More specifically, the estimated corn yield loss to above ground and foliar diseases combined was 77.5 million tonnes: of which CR accounted for 5.8 million tonne and 6.9 million tonnes for SCR. By comparison, the disease that caused the most significant yield loss in the US was grey leaf spot (caused by *Cercospora zeae-maydis*) with a loss of 14.5 million tonnes. However, it is suspected that chemical control also plays an important part at controlling maize foliar diseases. However, the cost of diseases focuses on yield loss, which are easily measurable, rather than a comprehensive costing of hybrid seeds carrying resistance genes, fungicides and fungicide application, and cultural management. For instance, a report by Murray and Brennan (2009) found

that in the Northern Region disease control ranged from $156/ha, for stripe rust, to $10.67/ha, for loose smut. The cost of spray alone averages $10 per hectare.

In Australia, the costliest diseases of maize are northern leaf blight, caused by caused by *Exserohilum turcicum* (Luttr.) K. J. Leonard & Suggs (1974), Anon (2017). Maize is also affected by stalk and cob rot, caused by *Fusarium verticillioides* (Sacc.) Nirenberg (1976) and fusarium ear rot, caused by *F. graminearum* (Schwein.) Petch (1936), and other *Fusarium* species*,* and diplodia cob rot or ear rot, caused by several *Diplodia* species.

Other minor diseases of maize in Australia include boil smut or common smut, caused by *Ustilago maydis* (D.C.) Corda (1842), and head smut, caused by *Sporisorium reilianum* (J. G. Kühn) Langdon & Full. (1978).

Southern corn rust is found in tropical regions of Australia, as hot and humid conditions are required to induce disease development, Anon (2016). However, CR is also an important foliar disease of maize worldwide (Ramirez-Cabral et al., 2017), and is likely to hinder the production targets of the Australian maize industry. It is suspected that epidemics could cause up 40% yield loss on susceptible varieties, though no research has been conducted on the subject in Australia.

Current strategies to control CR rely on fungicide application, the use of resistant lines and a combination of both (Darino et al., 2015). The resistance is classified as being either quantitative or qualitative (Olukolu et al., 2016). Maize plants possessing quantitative resistance, also called partial resistance, still express the symptoms of infection. However, the rate of disease spread is moderately reduced to completely halted. This resistance is considered non-race specific and durable. The second resistance type is based on major gene resistance. Qualitative resistance is race-specific and non-durable (Stuthman et al., 2007). Several genes and QTL's have been identified as contributing to rust resistance and tolerance on hybrids from across the world (Olukolu et al., 2016). In Australia in

recent years, cultivars of maize, previously resistant, have been displaying alarming levels of infection of CR, raising concern over the effectiveness of the current control measures.

2.5 The rusts

2.5.1 Introduction

Rusts are obligate biotrophic parasites, a group of pathogenic fungi belonging to the order *Pucciniales* that comprises more than 300 genera (Sharma, 2006; Aime et al., 2017). Biotrophs require continuous infection of live plants to extract nutrients from living tissues (Aime et al., 2017). There are over 8000 species of *Pucciniales* spread across 125 genera and 11-15 families (Cummins & Hiratsuka, 2004) and new taxa are continuously added (Aime et al., 2017). Of the 300 genera, *Puccinia* is the largest, *Uromyces* second and *Ravenelia* third largest. The rust fungi of the order *Pucciniales* are pathogenic to all vascular plant groups (McTaggart et al., 2016) and affect several plants of agronomic importance, such as coffee, wheat, barley, maize and sugar cane (Schumann & D'Arcy, 2012).

The members of the Pucciniales order share one common characteristic: a narrow host range (Bushnell & Roelfs, 1984; McTaggart et al., 2016). One exception to this is soybean rust, caused by *Phakopsora pachyrhizi* Syd. & P. Syd. (1914), which can infect over 31 species in 17 genera of leguminous plants (Ono et al., 1992). Wheat stem rust, caused by *Puccinia graminis* Pers. (1794), can infect over 365 species of plants in 54 genera (Bettgenhaeuser et al., 2014). Most Pucciniales rust fungi are restricted to one or two host species, such as coffee rust, maize common rust and wheat leaf rust *Puccinia triticina* Erikss. (1899) (Agrios, 2005).

2.5.2 Rust life cycle

Rusts possess the most complex life cycle of all fungi (Aime et al., 2017). Up to five distinct stages are described in species termed macrocyclic

(Bushnell & Roelfs, 1984). At each stage, a different type of spore is produced in structures called sori: spermatia, aeciospores, urediniospores, teliospores and basidiospores (Aime et al., 2017). In demicyclic species, the stage producing the urediniospores is missing and occasionally the spermatia. In microcyclic species, only teliospores and spermatia are produced (Petersen, 1974). Though the spore stages of most species of rust are easily distinguishable, others possess nomenclatural issues (Aime et al., 2017). Most well-known species of rust such as *Puccinia polysora* Underw. (1897), *Puccinia sorghi* Schwein (1832), *P. graminis* and *Hemileia vastatrix* Berk and Broome (1869) are classic examples of macrocyclic, heteroecious members of the Pucciniales.

Heteroecious fungi require two unrelated host plants to complete their life cycle, whilst autoecious fungi require one host (Bettgenhaeuser et al., 2014). In heteroecious rust species, clonal reproduction occurs on the primary host and sexual reproduction is completed on the alternate host (Rochi et al., 2018). Spermatia and aeciospores are produced on the alternate host, whilst urediniospores and teliospores are produced on the primary host (Aime et al., 2017). Heteroecious and autoecious rust species may be macrocyclic or demicyclic (Petersen, 1974). However, monocyclic forms are all autoecious.

Two phases comprise the life cycle of heteroecious rust: a repetition and a sexually phase of spore production (Bettgenhaeuser et al., 2014). The first phase is marked by lower genetic diversity, but high dispersibility, and the second by greater genetic diversity, but lower dispersibility. The production of urediniospores in the repetition stage is clonal and has a rapid turnover of generations. The second stage occurs in the spring, when the cold induced dormancy of the teliospores is overcome. Upon germination teliospores are not expelled in air current, but produce haploid basidiospores through meiosis (Aime et al., 2018). However, it is on the alternate host species that the greatest genetic variation is achieved through sexual reproduction.

Life cycle plasticity in rust fungi is determined by their ability to complete their life cycles in the absence of some spore stages (Aime et al., 2017). Stages can be omitted depending on the availability of a suitable host and/or environmental conditions (Bushnell & Roelfs, 1984). For instance, *P. graminis*, can either exhibit a monoecious or heteroecious behaviour. In the tropics, little fluctuation in temperatures is observed. Such that most rust species solely rely on the clonal stage of their life cycles without deploying sexual reproduction (Petersen, 1974; Kim et al., 1988).

Though, sexual reproduction is necessary to achieve greatest genetic diversity (Drenth et al., 2019). Mutation, genetic copy error and rare case of sexual hybridization at the clonal stage still result in high genetic diversity within a rust population (Park et al., 1999). However, under colder climates, the infection of a secondary host and the production of overwintering spores are required (Petersen, 1974). In North America, wheat stem rust teliospores germinate to produce basidiospores that infect *Berberis* species on which the pycnidial and aecial stages are completed (Leppik, 1961).

2.5.3 Case study: Wheat stem rust

Wheat stem rust (SR) is the most devastating and costly of all wheat rusts (Stokstad, 2007). Pardey et al. (2013) conducted agro-ecological modelling of the pathogen based on climatic requirements for infection. The research revealed that 1.4 million km² or 66% of the world's wheat growing regions are suitable for disease development. Fortunately, in developed countries major epidemics have become a problem of the past (McIntosh et al., 1995). For instance, in the United States of America the last major outbreak occurred in 1950, where 40% of the spring crop was affected (Stokstad, 2007). Agronomic advances during the green revolution in 1960 led scientists to believe that wheat stem rust was eradicated. In 1980's, the *Sr31* resistance gene from rye was translocated to wheat lines and conferred good levels of resistance to stem rust (Park, 2015b). However,

wheat lines carrying *Sr31* also possess poorer dough quality and for that reason, such lines were not commonly grown in Australia. Australian growers preferred to occupy a market segment on the international stage that supplies high quality grain. Hence, they relied on wheat stem rust resistance genes other than *Sr31* (Park, 2015a). The *Sr31* gene remained effective for nearly 20 years, until a virulent pathogen isolate emerged in Uganda in 1999 (Stokstad, 2007). Soon the isolate, coined Ug99, rapidly spread to other African countries such as Kenya, Ethiopia, Sudan and was found in Yemen by 2006. Breeding programs began to develop wheat lines resistant to Ug99. However, new races of Ug99 defeated the newly deployed resistance genes. For instance, the TTKS race caused 71% yield losses in experimental field trials and its race variants are virulent to 90% of world's wheat cultivars (Singh et al., 2008). A variant of Ug99 caused severe epidemics in Kenya in 2007 affecting half of all previously resistant wheat varieties. In Ethiopia, race TKTTF was responsible for 100% yield losses in the season 2013-2014 (Olivera et al., 2015). Isolates of this race have also been observed in Lebanon and Iran (Singh & Dhaliwal, 2014).

In Southern Somalia, the wheat variety Digalu was released in 2005 for its resistance to stripe rust and stem rust strains of Ug99 (Olivera et al., 2015). By 2013-2014, an estimated 500,000 ha of Digalu was planted. In 2013, high incidence of stem rust with low severity was reported. By early 2014, Digalu had become highly susceptible with 80 to 100% disease severity. Ug99 is a reminder to us all to keep identifying durable resistance genes on a global scale, as new virulent strains are constantly emerging.

In 2013, Germany experienced a stem rust outbreak and suspected that Ug99 had reached Europe (Olivera Firpo et al., 2017). Prior to 2013, Germany had not seen stem rust outbreaks for decades. The outbreak was linked to favourable environmental conditions for disease development. The study by Olivera Firpo et al. (2017) confirmed that of all the isolates collected that year, though phenotypically similar to the TKTTF race of Ug 99, were genetically different to those found in Africa. Further studies

revealed that the German strains were virulent against *Sr* genes effective against Ug99.

In the rust pathosystem, changes in virulence are more frequent in the presence of the alternate host for sexual recombination (Olivera Firpo et al., 2017). Though, the break-down of major resistance genes is said to be slower where the pathogen relies on the clonal stage of its life cycle, it occurs nonetheless (Johnson & Newton, 1946).

In Australia, annual surveys of wheat rust populations have been carried out since 1922 with the objectives of identifying rust races (Park, 2015b). In recent years, the genotyping of rust isolates confirmed that sexual reproduction is rare, understandably due to the lack of the alternate host barberry for SR in Australia. The genetic diversity is generated by mutation, introduction of exotic isolates and more rarely by somatic hybridization (Park & Wellings, 2012). In the last 30 years, few new pathotypes have been recorded (Park, 2015b). The lack of sexual recombination is not sole responsible. Driving the deployment of resistant inbred varieties, the durable resistance gene against SR and pathotyping programs have kept pathogen population levels very low, reducing mutation rate to a near null level. Prior to the deployment of resistance genes and pathotyping programs Australia experienced SR epidemics in 1889, 1916, 1947 and 1973 (Park, 2007). The use of monogenic resistance alone resulted in the following pattern: Variety Eureka resistant to SR was released in 1938 and its resistance was broken down by 1942, cultivar (cv) Gabo was released in 1942 and broken down by 1948, and cv Warigo was released in 1943 and broken down by 1959 (Watson & Luig, 1963).

New races are constantly emerging in various parts of the world (Park, 2008). A SR outbreak occurred in 2001 in Western Australia with the breakdown of *Sr38*. Following the West-East Australian wind current, the strain emerged in South Australia in 2003, in Victoria in 2004 and had reached Queensland and Tasmania by 2006 (Park, 2015b).

Without adequate genetic resistance, SR in Western Australia may cause 10% to 50% yield loss on susceptible cultivars and up to 90% on highly susceptible cultivars (Keed & White, 1971). Stem rust also infects triticale, and barley can act as a summer host providing a green bridge between cropping seasons (Loughman et al., 2005). Though, the wheat rust survey revealed that SR diversity and level of inoculum have declined since the 1970 from 40 pathotypes identified in the year 1973, to 14 pathotypes identified between 2000-2013 (Park, 2015b). Park (2008) stressed the importance of mapping the rust pathogens population and targeted breeding efforts to offer adequate levels of protection, especially in agro-ecological zones where application of fungicides is uneconomical due to low yield farming systems. Brennan and Murray (1988) estimated that the concerted efforts of resistance breeding saved growers $128 million annually.

Close monitoring of the SR population in Australia and the deployment of durable resistance genes have controlled the pathogen population. Professor Robert Park and his team at the Institute of Plant Breeding in Western Sydney have been testing and monitoring the efficacy of wheat stem rust resistance genes for decades. By preventing major outbreaks, they reduced the likelihood of a mutation occurring to extremely low levels (Park, 2018). The resistance deployed is therefore not subjected to high levels of positive selection on the pathogen population and likely to remain effective in the long term.

Australian and worldwide wheat materials are currently being screened for SR resistance to develop resistant material to strains of Ug99, before their further spread across the globe (Park, 2007). Wheat stem rust is one example amongst many of the devastating consequences of rust outbreaks in cropping system worldwide.

2.6 Overcoming biotrophic constraints

As obligate biotrophic pathogens, rusts totally depend on their host plant to complete their life cycles (Bettgenhaeuser et al., 2014). They have therefore developed intricate mechanisms to secure contact with the host, overcome their host defence mechanisms and infect new hosts.

A spore landing on a plant must germinate, identify the plant as a host/non-host and circumvent immune responses to infect (Deising et al., 1992). An array of genetic exchanges is utilised by rusts to enhance their ability to infect (Petersen, 1974). Following the hypothesis of the gene for gene interaction (Kaur et al., 2021), population with advanced infectious potential also possess the greatest genetic diversification (McDonald & Linde, 2002).

Genetic variations occur at each spore stages and allow for a uniquely high levels of plasticity in the rust life cycles (Aime et al., 2018). Not only do they switch from a micro- to a macro-cyclic life cycle, but spore production occurs on completely unrelated host plants. Many have studied the evolutionary forces behind heteroecious behaviour (Leppik, 1965, 1967; Hart, 1988; McTaggart et al., 2016; Aime et al., 2018).

Rusts rely on airborne spores for dispersal over hundreds often thousands of kilometres (Roelfs & Bushnell, 1985). Interestingly, the ability of spores to disperse appears to be concurrent with their functions in the pathogen life cycle. Not all spore types rely on air currents for dispersal and some only spread over relatively short distances.

2.6.1 Spore dispersal

Further than requiring living tissues for their survival, most rusts only infect a specific host or a narrow range of hosts (Aime et al., 2018). Therefore, it becomes critical for a spore to come into contact with a suitable host.

The large number of spores produced at the clonal stage explains the rapid spread of rust infection at field level (Roelfs & Bushnell, 1985). One microgram (µg) of urediniospores contains about 450 spores (Rowell & Olien, 1957). Daily, a mature pustule of *P. graminis* can produce 10 350 spores or 23 µg of urediniospores per day (Katsuya & Green, 1967). Taken to the field level, Rowell and Roelfs (1971) determined that 5 kg of spores can be produced per hectare per day with infection rate of 50 pustules per tiller, representing 5% disease severity. The size and shape of the urediniospores of CR are similar to those of wheat rust (Roelfs & Bushnell, 1985) and it is expected to produce the same number and quantity of spores per hectare.

What a large number of spores at the field level fails to explain is the movement of spores, hundreds often thousands of kilometres away from their centre of origin (Hermansen, 1973; Milus et al., 2009; Hovmoller et al., 2011). Though most spores are trapped within the plant canopy or deposited within 100 m of the source (Wingen et al., 2012), a notable amount has been found at altitudes of 3000 m (Roelfs & Bushnell, 1985). Voegele et al. (2009) reported spores present at altitudes of 12 000 m. Weinhold (1955) calculated the terminal velocity of spores, or rate at which they fall, to range between 0.94 to 1.25 cm/sec in still air. It would take a urediniospore 67 hours to fall from an altitude of 3000 m at a rate of 1.25 cm/sec. Further aided by the dominant winds, spores are displaced thousands of kilometres from the infection site of origin (Hovmoller et al., 2011). At an altitude of 1500 m with a wind speed of 13.3 m per second a spore would travel 1760 km (Uk-kelberg, 1933). Coffee rust, *H. vastatrix*, infected a plantation in Angola in 1966 and was found in Brazil in 1970 (Brown & Hovmøller, 2002). In 2000, a new aggressive strain of *Puccinia. striiformis* f. sp*. tritici* Westend (1854), *PstS1*, characterized by a shorter latent phase and increased spore germination percentage emerged in the USA (Hovmoller et al., 2011). A closely related strain of *PstS1* and tolerant of warmer temperatures was found in Europe in 2000. By 2002, *PstS1* was

detected in Western Australia, over 17,000km away. The spread of this pathotype was most likely the result of human movement.

Long distance dispersal of spores, and subsequent epidemic on the primary host, most commonly occurs with urediniospores (Helfer, 2013). The size of these spores ranges from 15-30 µm in diameter (Roelfs & Bushnell, 1985). They also possess a thick cell wall and fine spines that facilitate air movement, as well as human assisted movement.

Other rust spore types utilise air currents for dispersal, but the distances covered are not as great as those of urediniospores (Helfer, 2013). For instance, the length of the chestnut brown two celled teliospores ranges from 50-80 µm (Roelfs & Bushnell, 1985). Unlike other spores, teliospores are long-lived and possess a thick wall that prevents their desiccation and damage by ultraviolet radiation (Helfer, 2013). From the teliospores in the spring germinate basidiospores (Aime et al., 2018). According to Kinlock (2003) basidiospores generally disperse within 300 m of the site of release. The size of basidiospores ranges from 10-20 µm (Cummins & Hiratsuka, 2004) and require high humidity and a narrow temperature range to survive (Bushnell & Roelfs, 1984). However, Van Arsdel (1967) reported that moist turbulent air stream may carry the spores to distances of up to 8 km.

Though similar in size to urediniospores, about 25 µm, Barnes et al. (2005) described aeciospores dispersibility to be limited to a few dozen metres due to their relatively large diameter. They also reported that a dispersal of 400 m from the site of origin and an altitude of 300 m as extreme for this spore type.

Spermatia cannot infect plant tissue and cause disease and their role is limited to sexual reproduction and to fertilise receptive hyphae on nearby spermagonia (Aime et al., 2018). Thus, their longevity is affected by UV light and a dry atmosphere (Helfer, 2013).

The unique voyage undertaken by a multitude of urediniospores, over thousands of kilometres, is nothing but accidental. The high dispersibility of this spore type appears to be part of a strategy utilised by the pathogen to secure contact with the host plant.

Urediniospores can cope with extremes of cold temperatures found at high altitude and the thermal shock occasioned upon descent into warmer atmosphere layer. The longevity of urediniospores has been found to be superior when kept at temperatures of -15°C compared to spores kept at 5°C (Mahindapala, 1978). Mahindapala (1978) found that spores stored at 5°C lost all viability after 11 weeks, whereas spores stored at - 15°C retain their infectious potential after one year. He also demonstrated that spore viability was reduced under certain temperature/relative humidity regime of 0%, 38%, 76% and 100%. Viability was highest at 38% relative humidity for all temperatures tested: 10°C, 15°C, 20°C and 25°C. The combination, under which the spores remained viable the longest, 24 weeks, was at 10°C and 38% relative humidity. Compared to viability of 1 week to 5 weeks under other all other temperature/relative humidity combination.

Furthermore, urediniospore germinability remains higher when stored at ultra-cold temperature compared to spores stored at room temperatures (Mederick & Sackston, 1972). The cold temperature storage induces a cold dormancy, similar to what would happen at higher altitudes. The hypothesis of adaptation to high elevation transport is further supported by the heat shock requirement needed by spores to revert the cold induced dormancy (Bushnell & Roelfs, 1984). We suspect that the heat shock requirement described in laboratory experiments occurs in nature as the spores descend to lower altitudes at a time when the primary host is being cultivated and temperatures are warmer.

There is a difference of 9.8°C from sea level for every 1000 m increase in height (Stull, 1950). An ideal day for maize growth is 30°C ±5°C (Du Plessis, 2003). Therefore, with a decrease of 9.8°C, the temperatures at an altitude of 3000 m would be down to 0.6°C, a drop of 29.4°C. At an altitude

of 12 000 m, temperatures of –87.6°C would be recorded, a drop of 117.6°C. Two minutes at temperatures of 4°C or lower induce a cold dormancy (Mahindapala, 1978). This sheds light on how spores not only survive cold storage temperature from room temperatures to 2-4 minutes in liquid nitrogen (LN) at -196°C and require ultra-cold treatment to retain higher germinability (Staples & Wynn, 1965).

2.6.2 Securing host infection

Once a spore lands on a suitable host, it must by-pass the host immune system to cause infection (Mendgen & Deising, 1993). Therefore, unlike necrotrophs such as *Sclerotinia sclerotiorum* de Bary (1884), which exploit to their advantage the responses of the immune systems (Guimarães & Stotz, 2004), rusts rely on high mutation rate to overcome their host resistance (Hart, 1988; Aime et al., 2018). Qualitative resistance is based on disrupting pathogen recognition patterns, until a virulent population emerges able to defeat the major gene resistance. In quantitative resistance, the host plant immune systems deploy an array response, each with limited effects, but, when combined, provide the host plants with sufficient protection to ensure survival and reproduction (French et al., 2016). For instance, in the maize-rust pathosystem, the centre of co-evolution is the highland of South America (Piperno & Flannery, 2001). This co-evolution ensured the survival of the host and the pathogen (Flangas & Dickson, 1961). However, in modern agriculture disease outbreaks rather than being curbed are often of epidemic proportion (Schumann & D'Arcy, 2012). Though inadvertently, monoculture selects for virulence within a pathogen population. Environments allowing year-round cropping results in the pathogen ongoing asexual life cycle among seasons (Kim et al., 1988). With each new generation of rust spores, mutation rate is accelerated and disease pressure results in severe yield losses (McDonald et al., 2013).

To infect their host plant, rust fungi maintain high genetic variation with the use of stratagems, such as sexual hybridization and somatic

recombination (Drenth et al., 2019). Early work by Eriksson (1898) failed to describe observable differences between fungi infecting different plant species. He therefore described botanical species of fungi possessing similar morphological characteristics but capable of infecting different host plants as *formae speciales* (f*.* spp*.*), singular *forma specialis* (f. sp.) (Anikster & Wahl, 1979).

These subunits are further subdivided into races based on their ability to infect varieties of a single species. External factors play an important part in the creation of *formae speciales* (Aime et al., 2017). A change of virulence between plants at various growth stages and race virulence behaviour is known to be influenced by environmental factors, such as temperatures, humidity levels and light intensity (Hurkman, 2002). However, these factors act upon a virulence that is already present.

Experimental research has been reported to successfully cross teliospores of different *formae speciales* under controlled environments (Johnson & Newton, 1946; Bushnell & Roelfs, 1984). Stakman et al. (1930) transferred pycniospores from *Puccinia graminis* f. sp. *agrostidis* to *Puccinia graminis* f. sp. *tritici* pycnia and succeeded in obtaining aecia. The resulting F1 hybrids showed a decrease in virulence against wheat and could not infect *Agrostis alba*. Interestingly, three of the races were new to science. In some successful sexual hybridization cases, the F1 and F2 hybrids have a wider host range than their parents (Roelfs & Bushnell, 1985). However, in all cases, hybridity caused deleterious effects in F1 and F2 generations, such as a loss of virulence and/or unusual life cycle disruptions (Johnson, 1949). Urediniospores and teliospores were observed in old pycnial lesions, suggesting an abnormal spore production.

Lastly, genetic variability is achieved by somatic recombination, the process by which dikaryotic vegetative hyphae fuse either by exchanging nucleic information and/or by parasexual recombination (Park et al., 1999). Somatic recombination has occurred under controlled and natural environmental conditions for several rust species. Though, Park and

Wellings (2012) admitted that it was difficult to observe and prove, they did not reject somatic recombination as a source of genetic variation.

The ability to overcome plants defence mechanisms and infect a wide range of primary host through genetic diversification is a common feature of rust pathogens. Mutation, sexual hybridization and somatic hybridization all generate new virulence potential on a current selection of plant species, but also on host previously unaffected.

2.6.3 Life cycle plasticity

Leppik (1965) developed the hypothesis of the hologenetic ladder, whereby rust fungi create new species by sourcing a new host to avoid extinction of a lineage adapted to ancient host. In his theory, Leppik (1965) insisted that co-evolution was the driving force behind a change of host. Rust sources living tissues from an ancestral and a more recent host to overcome the constraint imposed by biotrophy. Leppik (1953) theorised new host acts as a green bridge, whilst the older host guaranties lineages persistence through sexual reproduction (Hiratsuka & Sato, 1982).

However, neither study by McTaggart et al. (2016) and Aime et al. (2018) found clear genetic evidence to support Leppik's theory of the hologenetic ladder. Though, McTaggart et al. (2016) found few specific examples of co-evolution, his work on *Pucciniales* was in agreement with Hart (1988) hypothesis of host jumps. In his 2016 research, he found genetic evidence that the diversification of *Pucciniales* rust fungi occurred later than previously thought, and after host diversification. Aime et al. (2018) further explained that the selection pressure imposed on the sexual stage (aecial stage) to conserve host association is greater than on survival stage (telial stage), leading to a heteroecious behaviour and host jump.

Biological specialisation defines fungi exploiting a narrow host range (Leppik, 1965). This specialisation modelled the interaction patterns between rusts species and their aecial hosts. High selection pressure

between the host and pathogen lead to specialisation (Aime et al., 2018). However, in biogenic radiation a rust radiates from an alternate host to a primary host (Leppik, 1967). The lower selection pressure at the telial stage leads to diversification by host jumps.

The diversification at the telial stage allows for plasticity of the rust life cycle, particularly in the Pucciniales. If most rust pathogens undergo five distinct phases in their life cycle, not all phases must be completed for survival (Bushnell & Roelfs, 1984). The spermatia and aeciospores have been reported in the rust agro-ecological region of origin, but may not always be produced in other part of the world (Dillard & Seem, 1990). It is often the case when the secondary host is not available or environmental conditions are dictating the production of certain spore types. For instance, *P. sorghi* aecial stage occurs on 30 species of *Oxalis*, most of which are present worldwide (Dunhin et al., 2004). However, the fungi often rely on the uredinial stage for survival, such as in Hawaii where maize is grown year-round (Kim et al., 1988). This is also true of ancestral rust species found in humid tropics and characterised by an autoecious life cycle and short generation succession (Hart, 1988). Under colder climates, rusts produce a second spore type that is long lived and capable of overwintering: teliospores (Mahindapala, 1978). Though, *P. sorghi* completes all five stages of its life cycle in the highland of Mexico and in South Africa, it too displays plasticity (Roelfs & Bushnell, 1985; Dunhin et al., 2004).

Our understanding is that biological specialisation prevents the loss of the aecial sexual reproduction stage to the more efficient uredinial and mass producer spore stage (Leppik, 1965, 1967). And that biogenic radiation maintains the ability of rust to infect new hosts. The loss of the sexual stage would lead to a great loss of genetic diversity (Drenth et al., 2019). Though genetic diversity through parasexual activities, mutation, hybridisation and somatic hybridization maintained a diverse population with adequate aggressiveness. Such as is reported in Hawaii where elite maize and sweet corn lines become susceptible to CR within two to three years of release

(Kim et al., 1988). These mechanisms for creating genetic diversity are not as efficient as the sexual reproduction stage under conditions where primary host is not present year-round.

2.7 Maize common rust

2.7.1 Hosts and symptoms

Puccinia sorghi is the causal agent of CR of maize (*Zea mays*), sweet corn (*Zea mays* subsp. *mays*), highland teosinte (*Zea mays* subsp*. mexicana*) and teosinte (*Zea perennis*) at the uredial and telial stages (Mederick, 1968). As for most rust pathogens, CR is highly specialised and no other hosts have been reported. Schweinitz was the first to describe *P. sorghi*

Fig 1: Pustules caused by *Puccinia sorghi* on a maize experimental line grown in a field in Allora, Queensland, Australia (photo taken by Aurelie Quade on the 27th of March 2018, using a canon EOS 600D, lens EFS 18-55mm)

Common rust pustules may appear on any aerial part of the host. Each pustule on maize plants contains thousands of spores (Jackson-Ziems, 2014). Common rust is distinguished from SCR by the colour and location of its pustules on the leaves (2016) (Fig. 2.7.1). In the early stages of infection, light cinnamon to brown colour pustules containing urediniospores appear on both side of the leaves (Mederick, 1968). The shape and size of the pustule are expected to be round to oval becoming elongated when infection occurred at early developmental stages of maize (Mederick, 1968; 2016). Symptom expression may vary depending on environmental conditions, the host resistance and the race of the pathogen (Mederick, 1968).

As the growing season is nearing the end, black to dark brown pustules are the sign that urediniospore production is being replaced by the production of longer-lived teliospores.

2.7.2 Life cycle of *Puccinia sorghi*

The life cycle of CR in Australia has been described and studied (Waterhouse, 1955) and is characterised by five spore stages: spermatia, aeciospores, urediniospores, teliospores and basidiospores (Aime et al., 2017). Spore stages exhibit a high level of plasticity as encountered in most macrocyclic rust pathogens. Though heteroecious in its native South American environment and in parts of the world where the secondary host is found (Bushnell & Roelfs, 1984), the behaviour of CR becomes autoecious like, under climates where maize is grown all year round, producing urediniospores only (Aime et al., 2017). Sexually produced spermatia and aeciospores have been described in South Africa, India, Mexico and Switzerland (Bushnell & Roelfs, 1984).

Though, gene recombination during prophase 1 of sexual reproduction achieves the greatest genetic diversity, as homologous chromosomes pair and exchange segments of DNA. Meiosis is not the sole source of genetic variation. Mutation, genetic copy error and rare case of sexual hybridization

at the clonal stage still result in variation in virulence under tropical conditions (Kim et al., 1988).

In North America, CR does not overwinter on *Oxalis* species (Pataky Gonzalez, et al., 2001). Maize infections result from secondary infection by urediniospores and aeciospores blown northward from tropical region (2016). Spermagonial and aecial stages occur on oxalis in neighbouring Mexico.

The uredinial stage is also called the clonal stage. Mass copies of a strain produce large spore load, guaranteeing infection and multiplication (Utpal, Harlapur, Dhutraj, Dibakar and Pawar, 2015). As the growing season finishes, maize plants senesce triggering the production of black overwintering teliospores (Anikster, 1986).

Teliospores not only survive cold temperatures on dead maize debris, but a period of cold temperatures and weathering is required to break their dormancy and initiate their germination. Unlike urediniospores, teliospores cannot infect maize plants and are the product of karyogamy, the fusion of two haploid nuclei (Aime et al., 2017). The dormancy of teliospores is overcome and germination initiated by temperature warming. Basidiospores arising from germinating teliospores are the product of meiosis (Sharma, 2006; Aime et al., 2017). Though, basidiospores can infect several species of *Oxalis*, they are most commonly found on *Oxalis corniculata* L., *O. stricta* L. and *O. europaea* Jord (Roelfs & Bushnell, 1985). Released in air currents, basidiospores infect species of *Oxalis*, not maize. The production of basidiospores is the first step toward completing the sexual cycle. The second step occurs upon infection of the secondary host in a lesion called a spermagonium.

A spermagonium contain receptive hyphae that are fertilised by a male spermatia (also known as pycniospores) (Sharma, 2006). It is at this stage of the rust life cycle that sexual reproduction occurs. Once fertilised, spermatia produce dikaryotic hyphae that grow through the leaf tissue of

the secondary host plant producing aecia in which aeciospores are born. Aeciospores cannot infect the secondary host, *Oxalis*. Released in the air current, aeciospores are deposited on maize plants to produce pustules bearing urediniospores carrying novel combinations of alleles present in the pathogen population, at which point the cycle resumes.

2.7.3 Epidemiology

The small size of urediniospores, 23-29 x 26-32 µm and globoid shape favours aerial transportation (Hooker, 1985). They are easily deposited on a maize plant and optimal germination occurs at temperatures between 15°C to 25°C, with 100% relative humidity. Germination is inhibited at 98% relative humidity or lower. Though slower, the germination of urediniospores has been reported at temperatures ranging from 4° to 31°C. Under suitable weather conditions, new pustules appear on average every seven days at temperatures between 15°C to 22°C (Bushnell & Roelfs, 1984). New pustules are produced in susceptible host plant tissue every 16, 10, 7 and 5 days at 10, 15, 20 and 25°C, respectively. Though, temperatures of 20°C are best suited for spore production (Mueller et al., 2016).

Teliospores are brownish-black, two celled with a size of 16-23 x 29-54 µm. The cold induced dormancy of the teliospores is overcome by temperatures of 19°C to 21°C and germination occurs (Anikster, 1986).

2.7.4 Infection process

Voegele et al. (2009) attributed five infectious characteristics to obligate biotrophs: highly differentiated infection structures, lower enzyme activity compared to necrotrophs, close contact between pathogen and host tissues, suppression of host defence throughout the infection process and the use of haustoria structures.

Following contact with their host plants, urediniospores re-hydrate, grow an adhesion pad and germinate (Deising et al., 1992). Attachment of the spore to the host surface is a determining event to the success of infection (Kunoh et al., 1991). The germinating spores develop a germ-tube, or hyphae, and produce a limited amount of cell wall degrading enzymes (Mendgen & Deising, 1993) (Fig 2). In contrast to necrotrophic fungi, obligate fungi rely on complex structures for host infection (Deising et al., 1994). Infection by obligate fungi tends to maintain host tissue and cell integrity, as nutrients can only be retrieved from living cells.

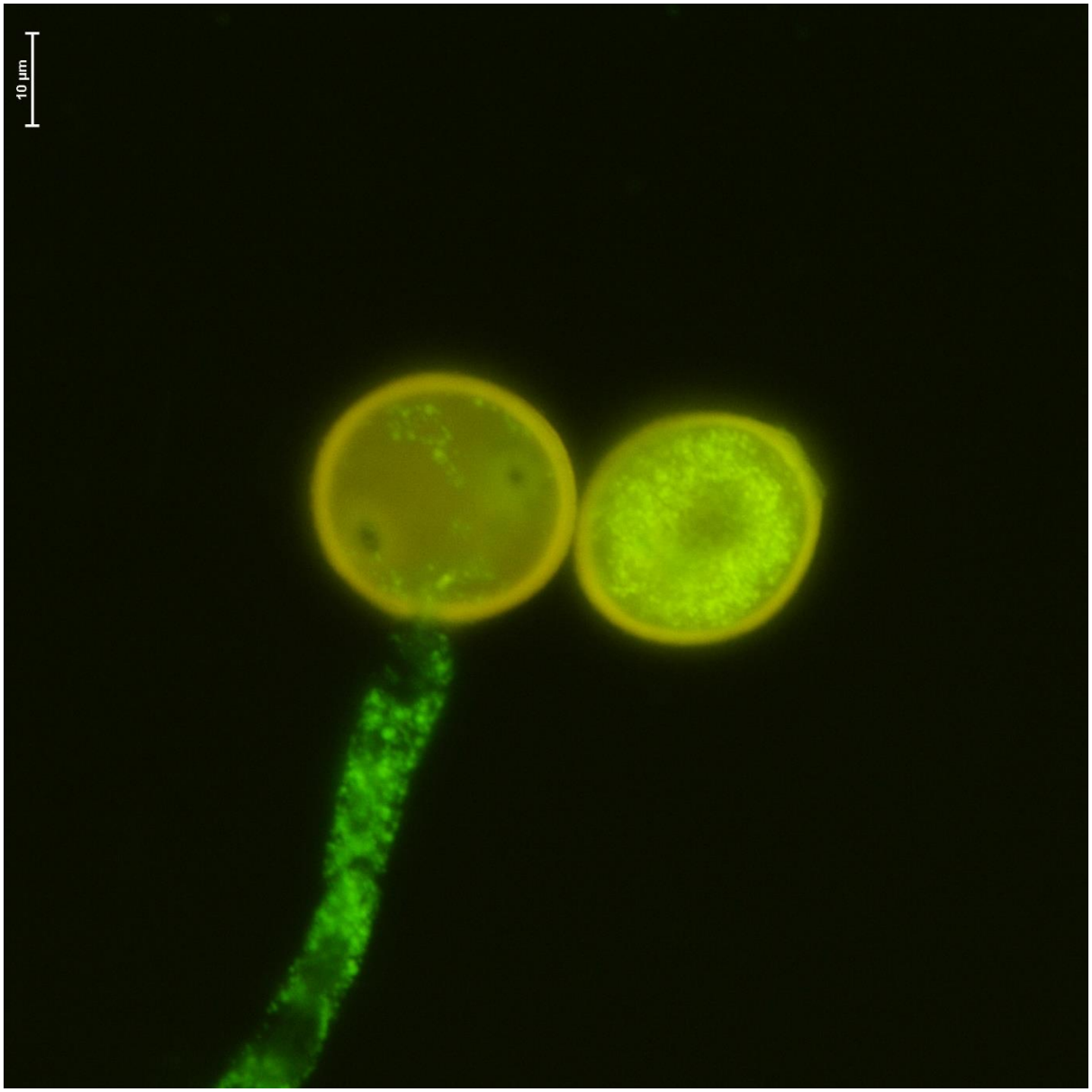


Fig 2: A germinating urediniospore of *Puccinia sorghi* compared to an ungerminated spore, notice the nutrient relocation from the spore to the elongating germ-tube (photo taken at Charles Sturt University, NSW, Australia, by Aurelie Quade, magnification x40, under florescent light).

The formation of specialised structures is an essential part of the infection process of many fungi, including rusts (Allen et al., 1991). On the leaf surface, germ tubes, or hyphae, receive chemical and physical clues from their environment. The hyphal tip is suggested to sense and recognise the host plant (Hoch & Staples, 1991). Similar to other rust fungi, the germ-tube of *P. sorghi* spores grows until it comes into contact with a stomata, prompting the development of an appressorium (Allen et al., 1991; Deising et al., 1992).

Research conducted by Ekpa et al. (2018) recorded the formation of appressoria of 27 different rust fungi, on surfaces with ridges of different height. Allen et al. (1991) hypothesised that height variation sent topographical clues to initiate appressorium formation. His study found that with all three rusts of maize, appressoria continued to develop on ridges with increased heights, compared with fungi that developed an appressorium at lower ridge heights, perhaps mimicking topographical clues similar to the stomatal mesophyll cells found on maize leaves.

The pressure exerted by the appressorium facilitates the invasion of a penetrating hyphae composed of a penetration peg and an infection peg (Mendgen & Deising, 1993). The secretion of chitosan appears to elicit plant defence (Deising et al., 1994). Failed infections are characterised by a hypersensitive response from the host plant triggering a rapid cell death (Wulff et al., 2011). The pathogen must therefore avoid detection by recognition R proteins of the plant, colonise the plant tissue and maintain the clonal stage of spore production for as long as the host lives to access nutrients.

Penetrating hyphae change to infection hyphae to access nutrients within the plant cells (Deising et al., 1994). Specialised cell-wall degrading

enzymes are also involved in the infection process. The production of proteases, cellulases and pectic acid has been observed. However, the reliance and amount of enzyme produced by biotrophs is limited to prevent cell degradation that would lead to cell death. On the contrary, the infection process in necrotroph fungi relies primarily on chemical degradation of the cell wall (Garg et al., 2010). At this point onward, the infection process of biotrophs has only been studied *in planta* and cannot be induced *in vitro* (Voegele & Mendgen, 2003). Haustoria are specialised structures by which fungi extract nutrients whilst maintaining plant cell full function. They grow inside the plant cell and each rust species appears to have its unique structural specificities.

The role of haustoria in the infection process also includes biosynthetic pathways, suppression of immune reaction and modifying the flow of host metabolites (Voegele & Mendgen, 2003).

2.8 Other maize rusts

2.8.1 Tropical rust

Tropical rust, caused by the fungus *Physopella zeae* (Mains.) Cummins and Ramachar (syn. *Angiospora zeae* Mains), is autoecious and microcyclic (Bushnell & Roelfs, 1984). Outbreaks of this pathogen are usually confined to the warm and humid parts of Central, South America and the Caribbean countries (Mederick, 1968). Though, one outbreak was recorded in the USA in 1969 (Dolezal, 2011).

Crops are more susceptible to outbreaks when planted late in the season and at low altitudes (Sanches et al., 2011). Losses of up to 60% have been reported in susceptible hybrids (Von Pinho et al., 2000). Maize lines possessing a narrower genetic base often have poorer agronomic traits. For instance, popcorn lines appear to be more susceptible to most diseases (Ziegler & Ashman, 1994).

The germination of spores of tropical rust occurs at relatively elevated temperatures, 22°C to 30°C, high humidity and high levels of solar radiation, Anon (2016). The germination of spores of *P. zeae* require 1 to 2 h of free moisture on the leaf surface and is induced at temperatures of 22°C (Bonde et al., 1982). Following inoculation, Bonde et al. (1982) observed leaf penetration at 12h and pustule formation after 6 to 7 days.

Once erupted through the epidermis, pustules vary in shape from round to oval and may be black rimmed (Bonde et al., 1982; De Leon, 1984). The lesion in the centre of the pustules are white to pale yellow.

It is thought that the pathogen is taxonomically related to the genus *Phakopsora*, which includes the causal agent of soybean rust, *P. pachyrhizi* (Bonde et al., 1982). According to Bonde et al. (1982), the two pathogens differ in their mode of infection. Once the epidermal cell is penetrated, tropical rust forms primary hyphae that develop as secondary intracellular hyphae in the epidermis and mesophyll tissues. *Phakopsora pachyrhizi*, in contrast, colonises the leaf mesophyll tissue as intercellular hyphae.

Although, races of *P. zeae* have been identified and it is an important disease of maize in South and Central America, studies of genetic resistance to tropical rust are limited (Sanches et al., 2011).

2.8.2 Southern corn rust

Of the three corn rusts, SCR, caused by *P. polysora*, causes the most substantial losses (Ji, 2006). The disease can cause yield losses of more than 45% (Bhavani et al., 2018). Losses of 60-80% were reported in Pennsylvania and Maryland in the USA, 50-70% in West Africa and 42-53% in northern China. This is concurrent with projection work by Ramirez-Cabral et al. (2017) on the global risk level for corn rusts predicting an increase in SCR outbreaks.

The pathogen is found in the tropical and subtropical parts of the Americas, Asia, Australia and Africa (Ji, 2006; 2016). Altitudes below 1200 m,

temperatures of 27°C and high relative humidity are optimum conditions for infection and disease development. At these latitudes the host plant is present all year round and *P. polysora* is microcyclic and autoecious, producing urediniospores and teliospores only (Roelfs & Bushnell, 1985). Other spore stages are unknown. The spores can infect maize (*Zea Mays* L.), the maize teosinte relative and *Tripsacum* species (Ji, 2006).

Under microscopic inspection, the spores of SCR are oblong in shape, compared to tropical rust and CR whose spores are round (Hooker, 1985). In the field, SCR is easily identifiable by the shape, colour and location of the pustules, Anon (2016). Southern corn rust lesions are small, light orange to light brown in colour and found on the upper side of the leaves.

Up until 1949, *P. polysora* was considered a minor disease in the corn belt region of the USA (Futrell, 1975). Minor occurrences were recorded in corn growing regions in 1949, 1958, 1972 and 1973. However, due to an increase in corn demand in 1970, a second crop was planted later in the season. The change in cultural practices resulted in severe outbreaks and substantial yield losses. In the top 10 of the most serious corn disease, SCR was positioned 7th in 2013 and 5th in 2015 in the Northern States (Mueller et al., 2016). In the Southern state, SCR came 7th in 2013, 4th in 2014 and 2015.

The disease outbreaks were also blamed on a lack of genetic diversity to SCR resistance (Bhavani et al., 2018). In 1975, Futrell (1975) reported that three inbred maize lines comprised 50% of hybrid production in the USA and all three were susceptible to SCR. Though, initially three single dominant and race specific genes were identified, such resistance types have not proved durable (Bhavani et al., 2018). Since then, other candidate genes for SCR resistance have been identified and novel races of SCR were identified across the globe with the potential to overcome resistance genes. Control in the form of durable resistance is best to prevent severe epiphytotic episodes (Mei et al, 2023; Stuthman et al., 2007).

2.9 Management of common rust in maize

2.9.1 Genetic resistance

To sustain the current rate of population growth, crops are increasingly grown outside their usual agro-ecological range (Boyd et al., 2013). Growing crops under a broader range of environmental conditions comes with its own challenges. Broad acre farming and monoculture practices offer pathogens a continual source of host plants (McDonald & Linde, 2002). Furthermore, commercially available varieties with improved resistance exert positive selection on pathogen populations.

The complexity of the maize rust resistance loci has been studied at length (Bennetzen et al., 1988; Sudupak et al., 1993; Chavan et al., 2015; Yang et al., 2017). Durable genetic resistance of the maize host plant is also achievable, so long as qualitative and quantitative resistance are carefully balanced (Yang 2017). The rate at which a more virulent isolate emerges depends on the rate at which the pathogen adapts/mutate within a population, the size of the population (Brun et al., 2010) and on the nature of the host plant resistance (Sudupak et al., 1993). Aggressive strains of CR can cause yield losses of up to 49% on susceptible hybrids (Groth et al., 1983). Adequate and long-term controls target the early stages of a pathogen life cycle to prevent sporulation and the emergence of a virulent population (Park, 2008). Efforts to control SR in Australia are based on the development and deployment of genetically resistant cultivars. This strategy successfully reduced the pathogen population to the point where very few strains occur and it is presently hardly found in commercial wheat crops (Park, 2007). .

2.9.2 Qualitative or major gene resistance

The selection of single major resistant gene in a crop has proven a short-term management option for diseases (Brun et al., 2010). The R-gene (resistant gene) in a plant selects for a virulent strain within a pathogen

population (Bennetzen et al., 1988). The 'boom and bust' cycle has been described as a typical pattern when major gene resistance has been deployed (McDonald & Linde, 2002; Mundt, 2009; Schumann & D'Arcy, 2012). It begins with the adoption of a resistant cultivar by the agricultural community. As the acreage planted with the resistant cultivar increases, rare virulent alleles are selected for and their occurrence increases in the pathogen population (Brun et al., 2010) so that the resistance of the cultivar becomes non-effective: the bust.

Resistance to biotrophic fungi is complex and resistance genes often occur in clusters (Sudupak et al., 1993). In the rust-maize pathosystem, the *Rp* genes, resistance to *Puccinia*, are a typical example of such clustering (Hooker, 1985).

The *Rp1* genes have a high recombination potential (Sudupak et al., 1993). The mispairing of genes during reproduction has helped maize increase the rate at which new resistance genes are created (Chavan et al., 2015). The complexity of the loci carrying the R genes and the meiotically unstable alleles have maintained the resistance of maize as diverse as the rust pathogen.

Hooker (1985) characterised 24 *Rp* genes, 16 of which were located in the *Rp1* area on the short arm of chromosome 10. Of the 16 genes described, 14 were a series of alleles at a single locus and were given the name of *Rp1* (Sudupak et al., 1993; Yang et al., 2017). The genes identified on chromosome 10 are clustered at the same locus and were designated *Rp1_A* to *Rp1_N* (Pataky, et al., 2001). Recent genomic research by Olukolu et al. (2016) estimate that most resistance genes are located on chromosomes 3, 4 and 10. Hooker (1985) and his team described six genes located near the centromere on chromosome 3, namely *Rp3_A* to *Rp3_F*, and isolated 61 *Rp1* genes from *Rp1_B*, *Rp1_M* and two maize inbred lines using polymerase chain reaction (PCR) techniques. The presence of such genes is confirmed phenotypically by the level of resistance conferred to a

particular *P. sorghi* isolates. Why has maize such a pronounced recombining ability?

Maize was cultivated as an open-pollinated crop, ever since its domestication and cultivation about 6000 years ago (Bushnell & Roelfs, 1984). According to Yang et al. (2017), maize is unlike most other cereal crops, such as rice and wheat. It requires constant outbreeding enhancement to maintain hybrid vigour. Self-pollinating cereals have a stable genetic architecture that provides plant breeders with more control over the make-up of a new cereal line. However, the heterosis requirement of maize dictates to plant breeders the strategy to be adopted. As breeders cross parent plants as genetically distant as possible, quantitative resistance is deployed over qualitative resistance. For the last 50 years, breeding programs have attempted to stack several major R genes into elite lines (Wulff et al., 2011). The process known as gene pyramiding often results in a loss of other desirable traits, such as high yield and resistance to other pathogens.

The high gene recombination potential of maize and the protection it confers to most major diseases rationalise the lack of interest for *P. sorghi* pathogenicity worldwide. Indeed, the most common and effective gene used against common rust is the *Rp1_D* gene (Pataky, et al, 2001), which remained effective in continental USA against all races of common rust for over 35 years. Groth et al. (1992) assessed the resistance of 24 alleles in sweet corn and determined that only *Rp1_D* and *Rp3_D* had remained totally effective. However, in 1999 yield losses of up to 40% were recorded on sweet corn hybrids carrying the *Rp1_D* resistance gene (Pataky, et al, 2001). Though, the *Rp1_D* resistance remained effective in maize on continental USA up to 1999, it had been defeated in Hawaii much earlier (Pataky, Pate, et al., 2001). Optimal conditions to grown corn year-round increased rust populations and in turn increased the number of mutants, and the ability of the pathogen to overcome resistance (Sserumaga et al., 2020; Kim et al., 1988). The research carried out by Kim et al. (1988) and

Milus et al. (2009) came to the same conclusion. They both highlight the risks of relying on major gene resistance when faced with ever changing pathogen populations and in environments conducive to disease outbreaks.

The unstable behaviour and adaptiveness of *P. sorghi* remain independent of its population size. Under less-than-ideal conditions the population size may be reduced and population proliferation might slow down, however, the potential to adapt to new environments remains. Major gene resistance is not for long term use, the differences between an avirulent and a virulent pathogen are trivial, but lethal (Wulff et al., 2011). R proteins, involved in resistance mechanisms, bind or detect a specific race of a pathogen (Yang et al., 2017). The role of pathogen effectors is to by-pass, suppress host defence and/or influence host cell metabolism in their favour. And R proteins linked to major gene resistance cannot detect new effectors.

In broad terms, there are two most common types of major resistance genes. Leucine-rich repeats (LRRs) detect pathogens on the outside of the cells and nucleotide binding LRR proteins (NLRs) in the intracellular environment (Chavan et al., 2015). Most R genes triggering NLRs reactions are responsible for cell death and hypersensitive response (HR) (Wulff et al., 2011). Cell death is an effective response of the plant immune system to control biotrophic pathogens, as these pathogens require living tissues for development (Yang et al., 2017). However, one may hypothesize that the hypersensitive response creates a source of food for necrotrophic pathogens. This has been demonstrated in the canola-*Sclerotinia sclerotiorum* pathosystem (Joshi et al., 2016). Their developing hyphae releases compounds similar to those produced by the host plants and whose function is to trigger cell death. Yang et al. (2017) promoted the role of quantitative resistance to control most of the important diseases of maize. The R genes have not provided an effective mean of control of complex pathogen such as rusts (French et al., 2016), blackleg of canola (Brun et al., 2010) and potato blight (Schumann & D'Arcy, 2012). The broad-spectrum effect of quantitative resistance differs from the NRLs mode of

action in its mechanical nature. Quantitative resistance directly affects the development of pathogens, rather than elicits detection (McDonald & Linde, 2002). Plant breeders are now incorporating quantitative resistance traits in their programs in the pursuit of long-term disease management. Although, minor genes only provide partial resistance, they are effective across all races of a pathogen population (Wulff et al., 2011).

2.9.3 Quantitative or minor gene resistance

Quantitative resistance (QR) pathways do not follow the gene-for-gene hypothesis found in qualitative resistance (McDonald & Linde, 2002). In maize, and in other crops, past research focused on major gene resistance for ease of segregation between resistant and susceptible material (Hooker, 1985; Sudupak et al., 1993; Pataky, Pate, et al., 2001). Quantitative resistance fails to observe segregation ratios. The desirable traits are difficult to detect and/or have low heritability, conferring QR an array of immune responses from broad to specific (French et al., 2016). Our understanding is that QR can be divided into macro and micro immunity. Macro immunity engenders a broader range of responses that combat several pathological organisms whilst micro immunity triggers specific biochemical pathways that are ultra-targeted. In the maize-rust pathosystem, QR delays disease onset by diminishing the frequency of infection and the spore production (increased incubation period, the latent period and slower growth within the plant tissue) (Gingera et al., 1995).

Though, the quantitative trait loci (QTL's) involved in QR have minor effects, the macro immunity provides resistance not only to all races of a pathogen, but in some cases to pathogens of different phylum (McDonald & Linde, 2002). For instance, maize line carrying qNLB1.02B73, a QTL on the short arm of chromosome 1, demonstrated superior resistance to several pathogens such as northern leaf blight, Stewart's wilt and common rust (Jamann et al., 2016). The locus also controls flowering time.

Durable resistance is often promoted based on tolerance rather than resistance. French et al. (2016) suggest that tolerance is deployed to reduce selection pressure within the pathogen population. Macro immunity is characterised by a series of cascading events triggering for instance the production of lignin, flavonoids and salicylic acid (Wulff et al., 2011). These compounds alter the ability of the pathogen to cause severe disease outbreak (Milus et al., 2009).

The emergence of a virulent strain is intensified in environments that provide pathogens with ideal developmental conditions (Kim et al., 1988). Mutation within pathogens often results in the breakdown of QR and eventually macro resistance is ineffective (Fu et al., 2009).

Specific spatio-temporal and environmental cues affect and/or effect micro-immunity (French et al., 2016). A change in resistance in different agro-ecological zones and ontological stages (developmental stages) has been documented in several crops. In wheat, the pleiotropic locus *Lr*34 is effective at seedling stages at low temperatures and in adult plants at around 20°C (Wulff et al., 2011). The *Lr*34 locus has provided resistance for over 50 years to multiple diseases. Quantitative resistance is being at the right place at the right time and the *Yr*36 locus in wheat is a typical example of environment-specific immunity (French et al., 2016). The locus provides partial resistance to *Puccinia striiformis* in adult plants at temperatures between 25°C to 35°C, but exhibits susceptibility around temperatures of 15°C (Fu et al., 2009).

In maize, the vegetative phase from juvenile to adult is associated with various levels of disease resistance (Hurkman, 2002). Susceptibility to CR is higher during the vegetative phase and lower at the reproductive stage (Hurkman, 2002; Chandler & Tracy, 2007; Riedeman & Tracy, 2010).

The maize genome couples meiotic instability and a complex set of resistant genes (Sudupak et al., 1993). The arsenal of responses deployed by maize originates from, but is not limited to, specific gene(s), epistasis (Phillips,

2008), the deletion of allele and possibly the residual effect of defeated R genes (French et al., 2016). The durability of the resistance of *Rp* loci to rust is an illustration of the effectiveness of QR (Sudupak et al., 1993). In its natural habitats, pressure from CR resulted in an instability in the maize genome at the *Rp* locus, accelerating the creation of new genes and/or new gene assortments (Chavan et al., 2015).

In the maize rust pathosystem, both the effectiveness of major and the durability of minor genes improved resistance when used in combination (Badu-Apraku & Fakorede, 2017). Similarly, lines of *Brassica napus* combining *Rlm6* (resistance *Leptosphaeria maculans*) with minor genes remained resistant after five years of field trials, while the resistance of lines carrying only the *Rlm6* gene was overcome within three years of release (Brun et al., 2010). In the last decade, advances in genetic engineering have enabled plant breeders to precisely target major and minor genes of interest. The increasing use of Market Assisted Selection (MAS) enables pyramiding of genes by transfer of targeted loci (Kou & Wang, 2010). However, the division between qualitative and quantitative resistance remains unclear. R genes often co-localise with resistance QTL's (French et al., 2016). Kou and Wang (2010) suspected that defeated R-genes still provided moderate levels of resistance. The multitude of R genes present in the maize genome imparted long-term resistance to CR. However, in environments that provide ideal conditions for the multiplication of the pathogen and a continuous host supply the mutation rate of rust is a pace ahead of plant defence. In Australia, the lack of sexual recombination of wheat rust and the effective deployment of resistant varieties in the field has slowed the development of new virulence and the *Lr13* gene remained effective for over 20 years (Park, 2008). However, *Lr13* was rendered ineffective within a few years of release in the USA, Canada, South Africa, Europe, Mexico and South America. And the rapid break down of all maize/corn resistance in Hawaii demonstrates the fast-evolving nature of the pathogen (Kim et al., 1988).

Genetic control of CR is the most cost effective and reliable way to control the disease, providing that the resistance has a broad and diverse genetic base (Santos et al., 2017). In the case of sweet corn and popcorn lines, a lower number of alleles per locus increases susceptibility to CR. This highlights the necessity to implement breeding strategies that focus on strengthening quantitative resistance in concert with an array of qualitative genes.

Although, genetic resistance is the main form of effective control to all rust pathogens worldwide, in some cases fungicides may be used. In situations of high disease pressure, break-down of resistance can be prevented with timely application of a fungicide of the appropriate chemistry (Pataky & Campana, 2007) and only when the risks of epidemic and associated yield losses are high (Pataky & Eastburn, 1993).

2.9.4 Chemical control

Polycyclic diseases, such as rust, produce several generations of spores during one growing season (Dillard & Seem, 1990). Under adequate environmental conditions new pustules appear every 5 to 10 days, depending on temperature and humidity levels (Roelfs & Bushnell, 1985). Rusts reach epidemic levels rapidly and application of fungicides must be initiated whilst the crop is most susceptible, at the onset of foliar infection and using the correct chemistry (Wright et al., 2014).

Maize is most susceptible to CR infection during the vegetative phase (Hurkman, 2002; Chandler & Tracy, 2007). The soft, juvenile leaf tissues are easily penetrated by the germinating spores and weather conditions earlier in the growing season are often conducive to disease development. However, the greatest yield penalty occurs when infection takes place during pollination (Utpal, et al., 2015). Depletion of photosynthetic compounds during silking curbs the grain nutrient uptake resulting in yield losses of up to 30% on susceptible varieties (Utpal, 2011).

Timely application of fungicide is critical to control spore load and delay the onset of an epidemic. Dillard and Seem (1990) established a spraying action threshold of 2% disease severity or 6-uredinia-per-leaf with 80% disease incidence. Pataky and Eastburn (1993) also recommended the Dillard and Seem (1990) action threshold. In New-Zealand, Wright et al. (2014) established a similar action threshold at 3% and 6% disease severity for sweet corn.

If left untreated, moderate disease severity combined with high incidence requires several foliar applications to manage secondary infections throughout the growing season (UtpalHarlapurDhutrajSuryawanshi, et al., 2015). Such practice is uneconomical to growers, as the cost of application of fungicides outgrows return potential. Furthermore, later infections often result in negligible yield losses. Indeed, maize plants possess varying levels of adult plant resistance, nutrient uptake is completed and environmental conditions are not typically as conducive to disease development (Dey & Bhinder, 1993).

In the USA, several systemic and non-systemic fungicide chemistries are available that effectively control CR of maize in the field (Utpal, 2011). However, systemic fungicides absorbed within the plant tissue resulted in lowest germination of spores of CR *in-vitro* and in the field.

Utpal (2011) tested the efficacy of eight types of systemic fungicides: Hexaconazole, Propiconazole, Tebuconazole, Penconazole, Triadimefon, Difenconazole, Myclobutanol and Azoxystrobilurin. For all chemistries the lowest spore germination was achieved at 0.1% concentration. Tebuconazole, Difenconazole and Propiconazole gave the lowest spore germination with percentages of 12%, 18% and 20%, respectively. By comparison, the spore germination of the untreated control was 80.47%. The non-systemic fungicides tested by Utpal (2011) were: Mancozeb, Zineb, Kresoxim-methyl and Mancozeb plus Phyton. At a rate similar to that of systemic fungicides, the lowest spore germination was obtained with Mancozeb plus Phyton, Mancozeb and Zineb with percentages of 16%, 38%

and 42%, respectively. Though, non-systemic fungicides performed best at 0.25% concentration, their efficacies in controlling the germination of spores of CR remained lower than systemic compounds.

In the field, foliar application of Tebuconazole at 0.1% concentration at 35 and 50 days after planting (DAP) translated into a yield increase of 29% over the untreated plots (Utpal, 2011). At a similar rate and days after application (DAP), disease control using Hexaconazole generated a yield increase of 25% over the untreated plots. Utpal, et al. (2015) also found that Tebuconazole at 0.1%concentration still provided adequate protection. However, yield increase was achieved with six spray applications, starting 3 days after inoculation, and applied every 10 days. Wegulo et al. (1998) also recorded yield increase, over the unsprayed control, with five applications of chlorothalonil or two to four applications of Chlorothalonil, Mancozeb, or Propiconazole. Repeated spraying is only recommended for seed production, the protection of susceptible popcorn and sweet corn, but not more than two applications (Subedi, 2015).

Several fungicides with different mode of action are available to control CR, such as strobilurin, triazole, chlorothalonil and mancozeb (Utpal, et al., 2015). In Argentina, Sartori et al. (2017) listed triazole and strobilurin as effective control means. Other countries apply drastic restrictions. In New-Zealand, one registered product was available for the years 2007-2009 to control CR in maize and sweet corn, Azoxystrobin (Wright et al., 2014). There is currently no data available on the fungicide chemistries authorized by the Australian Government to control common rust. Several attempts were made to contact the Australian Pesticides and Veterinary Medicines Authorities (APVMA) without success.

Chemical control of CR in the field may be beneficial when conducted in the early stages of infection and/or as an added layer of protection to partially resistant lines (Wegulo et al., 1998). However, the success of application also depends on crop sowing date, plant growth stage, weather conditions and the level of infection of nearby crops (Wright et al., 2014). In

environments conducive to disease development up to three fungicide applications are required to achieve maximum yield potential (Wegulo et al., 1998). The cost of repeated application is uneconomical in lower yielding environments, such as Australia (Park, 2008). Groth et al. (1983) also reported on the negative environmental effects. Other control methods are available with varying degrees of effectiveness compared to genetic resistance and chemical application and/or with low economic return.

2.9.5 Cultural practices

Disease incidence is highest under monoculture farming systems (Wulff et al., 2011). In mixed intercropping, significant reductions in bean rust incidence of 51% and 25% was observed when compared to sole cropping and row intercropping, respectively. A reduction in bean diseases was achieved when using maize as an intercrop (Fininsa, 1996). In its native environment, maize cultivated alongside climbing beans and squash provides adequate levels of disease protection (Thurston, 1990).

Though effective, mixed and row intercropping practices are lower yielding. The production levels under such practices remain well below predicted population growth (Schumann & D'Arcy, 2012). Thus, the use traditional farming practices is restricted to small scale farming (Thurston, 1990).

An understanding of the whole farm system can help growers manage CR by incorporating outbreak likelihood with hybrid selection, date of planting and irrigation management (Wright et al., 2014). However, under weather conditions conducive to disease development, all of the above act as preventative measures and are not curative.

Similarly, soil care and an optimum fertilisation regime may reduce disease onset, but will have no effect on epidemics unless combined with plant resistance (Wright et al., 2014).

2.9.6 Biological control

Antagonistic bacteria and fungi have been tested for their efficacy at controlling CR *in-vitro* and in the field (Utpal, 2011; Sartori et al., 2017). Utpal (2011) recorded lower urediniospores germination ranging from 27% to 61% with biological control, compared to 97% for the control. *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* achieved significant reductions in spore germination of 27%, 34% and 46%, respectively. *Pseudomonas fluorescens* was found to be least effective (61%) at 5% concentration as compared to control (97%). Sartori et al. (2017) also obtained promising control levels of northern leaf blight and CR using *Pantoea* ssp. and *Bacillus* ssp. in the field. The greatest yield increased was achieved with *Bacillus* ssp. when compared to the untreated control. However, more than one application was necessary and the amount required and the lack of effective control make those options uneconomical for farmers. Furthermore, under laboratory conditions, antagonistic organisms are tested under optimal growing conditions that are not repeatable in the field (Utpal et al., 2015). The results obtained in a laboratory setting are seldom replicated under natural field conditions.

2.10 Maize rust: a worldwide impact

Southern and tropical rust can kill their maize host plants and result in 100% infection levels (Ji, 2006), though epidemics are restricted to tropical and sub-tropical conditions, Anon (2016). Common rust occurs on a much broader geographic range and yield losses may reach up to 40%, though infection levels are often lower (Ramirez-Cabral et al., 2017). We must develop an understanding of the cause of disease outbreaks to reach our agricultural production targets.

The human population has more than doubled over the last 50 years and is predicted to reach 9.8 billion by 2050 (Searchinger et al., 2018). The maize industry must assume all scenarios of disease outbreaks to maintain

its productivity target and remain a viable cropping option for farmers. Not only should CR management strategies incorporate methods from other rust programs, such as the wheat-rust program, but they should also compare outbreaks from other diseases possessing similar traits, such as environmental adaptability, mutational ability and long-distance dispersal of spores.

Common rust affects all aerial parts of corn, causing yield reduction of approximately six percent for each 10 percent in rust incidence (Pataky, Pate, et al., 2001). Reduction in seed size accounts for most of the yield losses (Roelfs & Bushnell, 1985). Common rust appeared in the top 10 of maize disease of Northern United States (Mueller et al., 2016). The disease ranked 8th as the most destructive disease in 2013 and 4th in 2014. In countries lacking management strategies epidemics of CR have caused substantial yield losses. For instance, in Nepal losses of 6% to 32% have been recorded since 1970 (Subedi, 2015). In Nigeria, Akinbode et al. (2014) visited 40 farms in the year 2013 scattered across five states and reported that 65% had various levels of CR infection in their maize crop.

Ramirez-Cabral et al. (2017) anticipated that under the current climate predictions epidemics of SCR are likely to increase, whilst those of CR are likely to decrease. The high adaptability of the rust pathogens and an increase in area of planted maize, a scenario envisioning a decrease in common rust is yet to be proven.

In Australia, no research papers were found on the disease and one can conclude that the occurrence of common rust is rare and its incidence low. In 2017, reports from industry professionals and agronomists prompted from the Grain Research and Development Corporation (GRDC) and the University of Southern Queensland (USQ) to release funding to conduct research on the common rust-maize pathosystem (Somes, 2018).

2.11 Summary and project aims

Major diseases inherently have a large population, high rates of genetic recombination, high fitness and high dissemination potential (McDonald & Linde, 2002). As a large pathogen population emerges, so does their ability to mutate and undergo recombination. The variety of genotypes augment the fitness pool to select from. Leveraging on their ability to disseminate genotype/genes, individuals with higher success rate of plant infection contribute extensively to population expansion. This is similar to the re-establishment, endemic, epidemic, and crash phases theory brought forward by Burdon (1993). Rusts are such pathogens.

The long-distance dispersal of spores ensures contact with the host plant. In Australia, Watson and de Sousa (1983) suspected that spores were coming from Africa. Park (2007) later confirmed that hybridization occurred between African and Australian race of leaf rust. In addition to human assisted spread, spores are blown from Africa to Western Australia. Wind current carry the spores of new races in a West to East fashion across 1500km of desert. Spores from African maize rust could be blown into Australia and or come from tropical regions in neighbouring Pacific islands where maize is grown year-round.

Rusts have evolved to overcome the constraint imposed by their biotrophic requirements to become highly successful plant pathogens worldwide (Bushnell & Roelfs, 1984; Roelfs & Bushnell, 1985). Therefore, rust necessitate close monitoring of the virulence of their population and recurrent identification and deployment of their host resistance. No control programs, whether genetic and/or using fungicides, will be cost-effective without such knowledge (Park, 2018).

In Australia, CR in maize was not considered an issue, resulting in no investment made to research the maize-rust pathosystem. Unsurprisingly, the constantly evolving nature of the pathogen resulted in a breakdown of the deployed resistance. Furthermore, our lack of knowledge of the

diversity of the CR population is affecting breeding strategies. As, we do not know where to direct breeding efforts to develop new resistant genes, we also need to assess the effectiveness of the current resistant genes and the yield losses incurred based on disease severity. Unfortunately, there is currently no differential maize set available in Australia against which we can determine the virulence of CR isolates, but also rate resistant genes against several other diseases.

For the Australian maize industry to develop disease resistant material for its growers and to preserve its image as a quality grain exporter on the international scene.

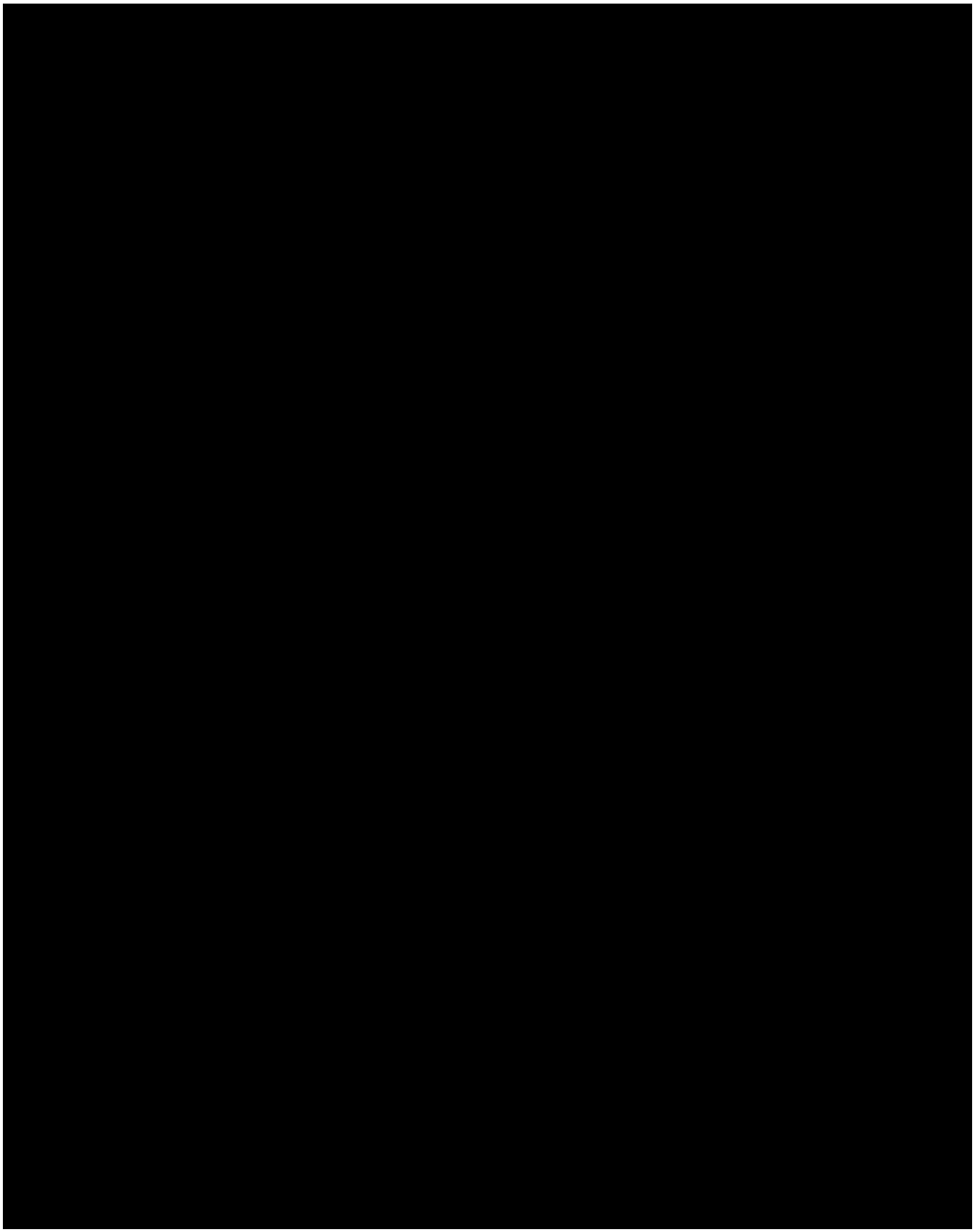
Therefore, this project proposes to:

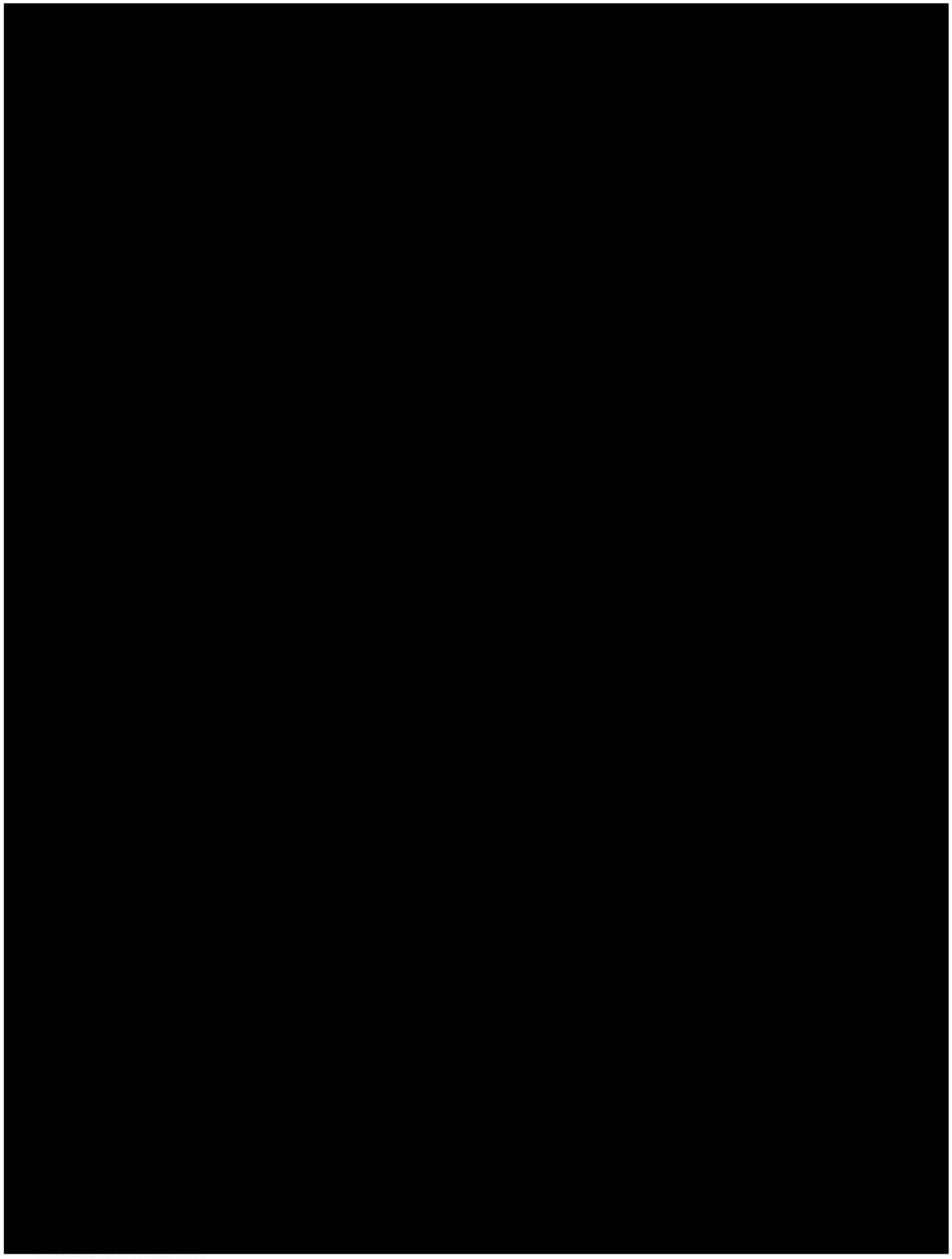
1- Collect and curate CR samples across Eastern Australia
2- Identify *Rp* genes resistant to isolate of CR collected in Eastern Australia, and create a differential set of maize and sweet corn lines carrying known resistant genes
3- Determine the diversity within the population of CR in Eastern Australia and implications for maize breeding programs.

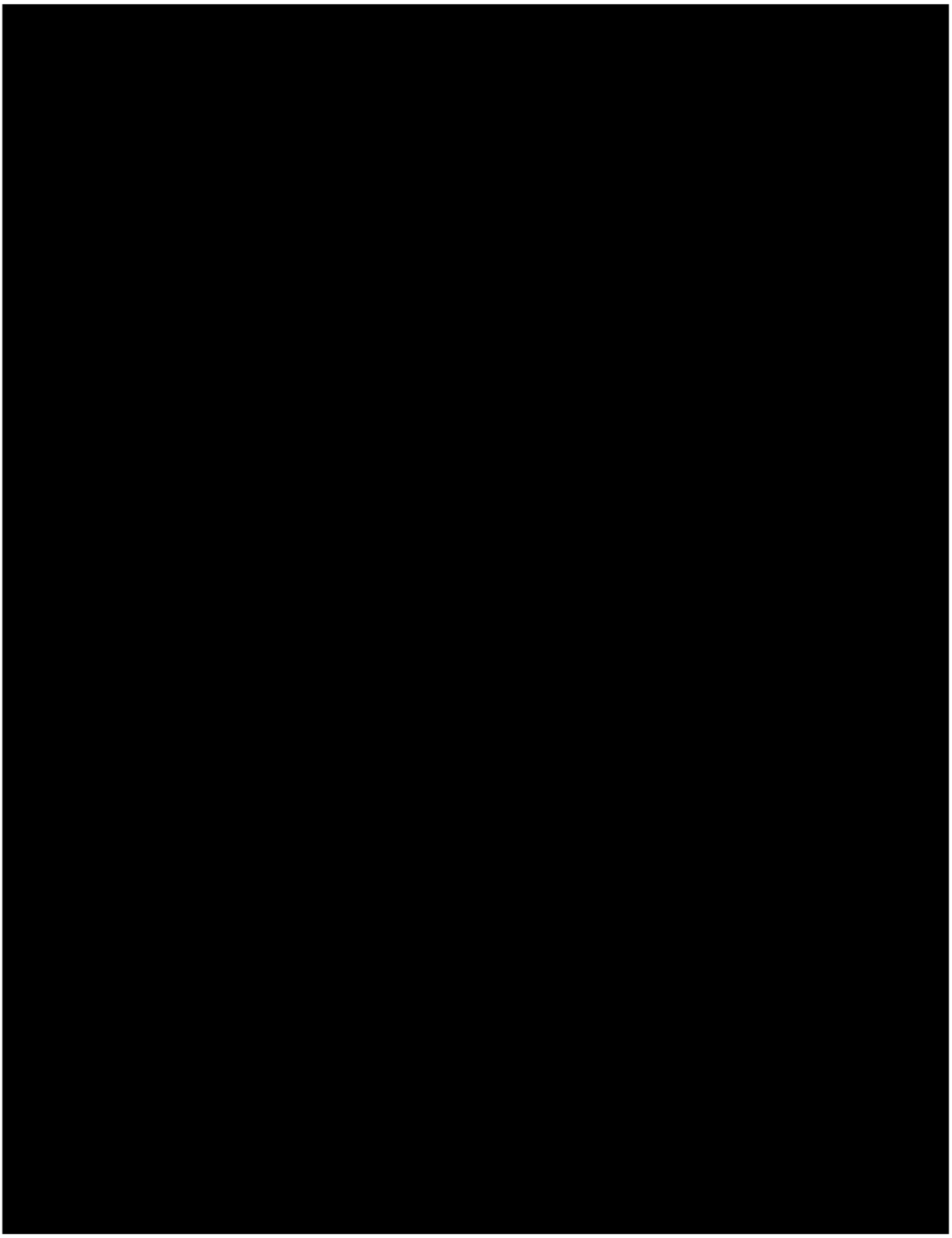
CHAPTER 3: PAPER 1 - UREDINIOSPORES OF *PUCCINIA SORGHI*: PRE- AND POST-COLD STORAGE REQUIREMENTS

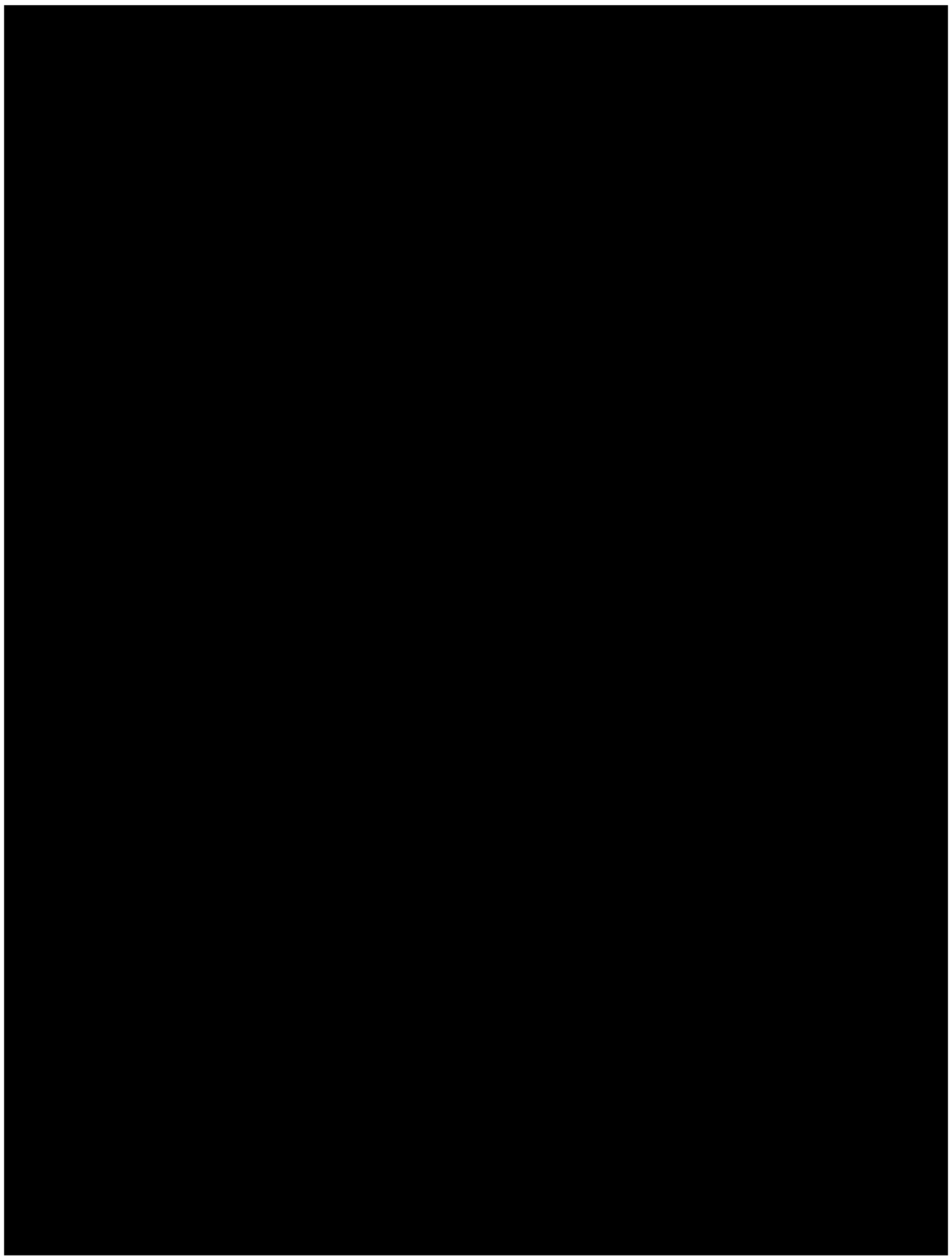
3.1 Introduction

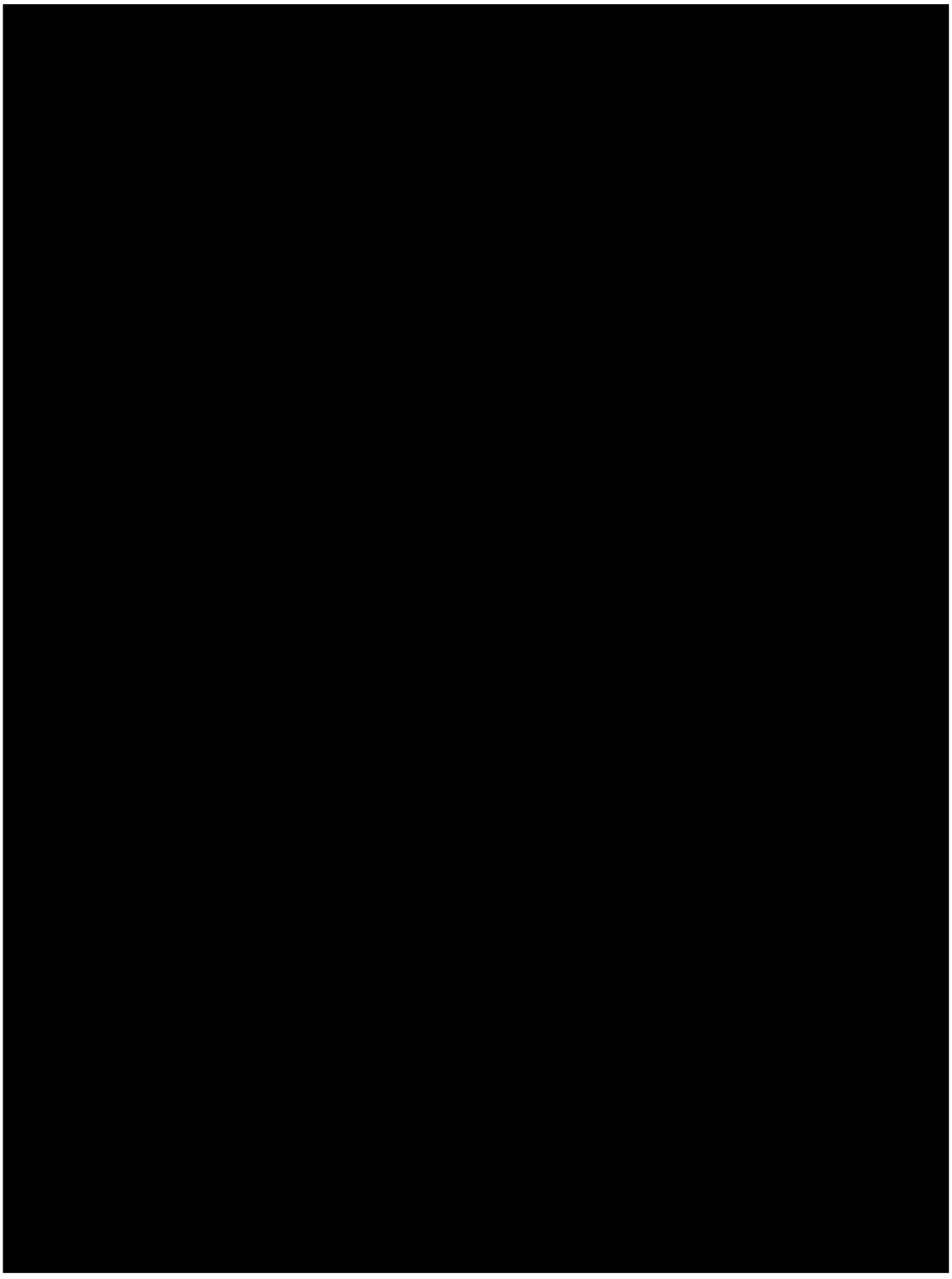
Maize common rust is a biotroph and studies can only be conducted on a live host using urediniospores. Storage requirements for the long-term preservation of urediniospores of *P. sorghi* was seldom studied. Most of the literature cited focused on the germination of spores, but not on the germination of spores following cold storage. The following study proposed to examine pre- and post-cold storage requirements of *P. sorghi* urediniospores to maintain a viable set of spores carrying known virulence.

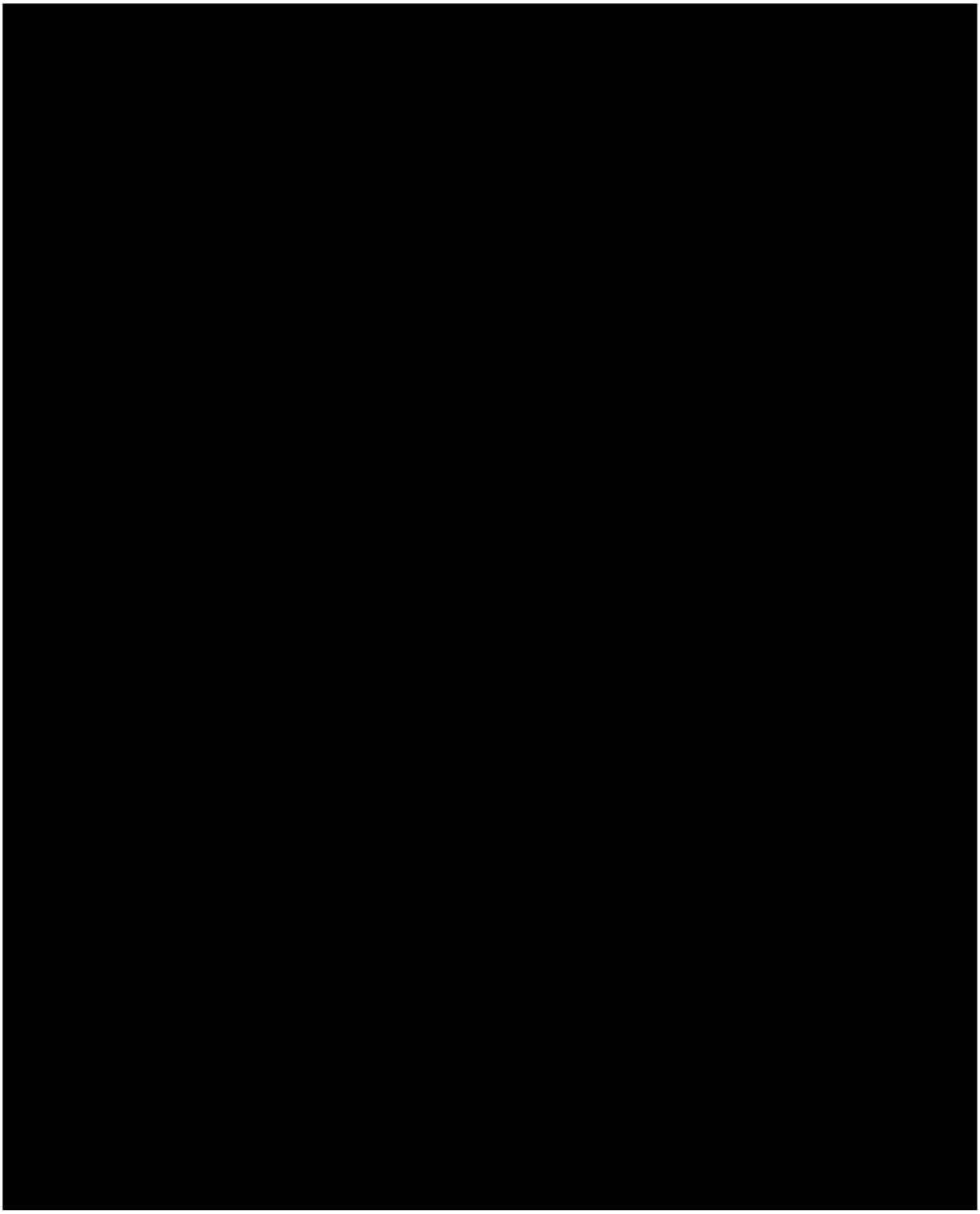


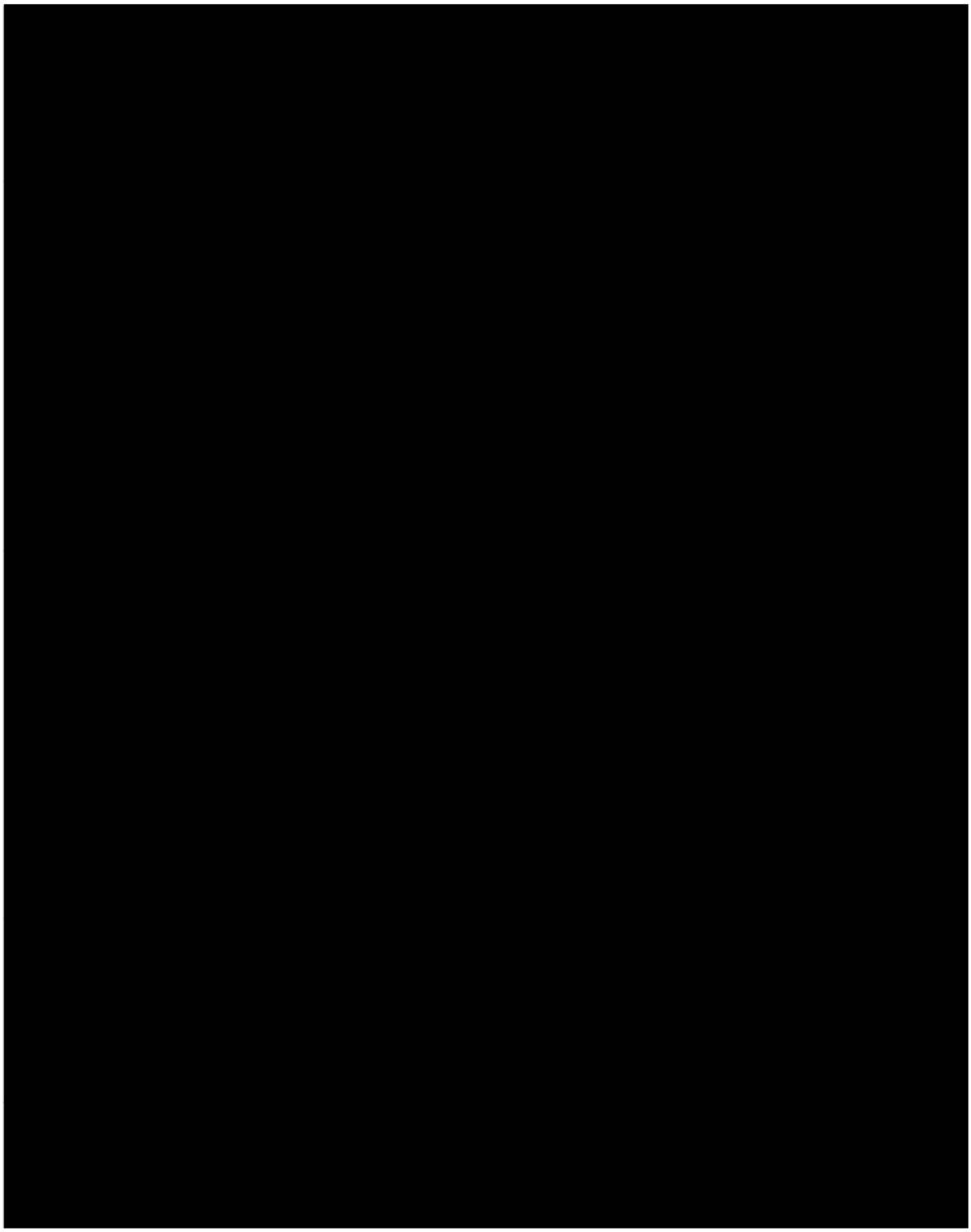












3.2 Links and implications

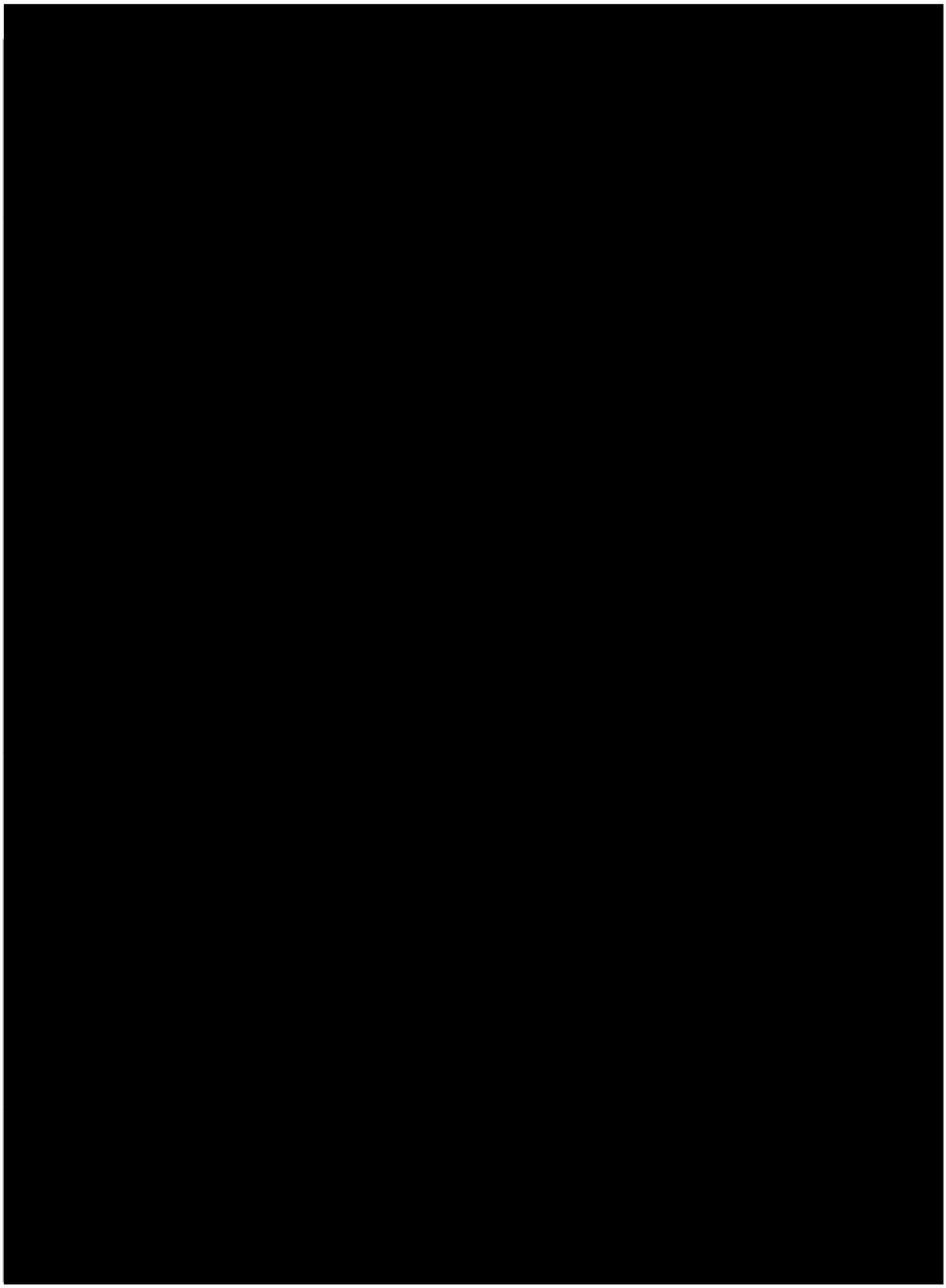
Urediniospores of *P. sorghi* were successfully stored at -80°C for over 2 years and retained infectivity after 3 years (data not shown). This allows for pathotyping and genotyping studies to be conducted by maintaining a long-term collection of isolates carrying known virulence. The pilot study on the effect of thawing temperature on spore germination from different agro-ecological regions each temperature regime was replicated 6 times (with 3 different isolates), the whole experiment was repeated 3 times. The drying experiment had 4 replications of drying time and the whole experiment was repeated 3 times (over time). The heat shock experiment/liquid nitrogen had 6 replications for each treatment and the experiment was repeated twice (over time). The long-term storage experiment had 6 replications at -80°C (in 2 different fridges) and 3 replications at -20°C (in one fridge). All experiments were split-plot design, except for the long-term storage experiment, which did not have a design allocated. All statistical models and data analysis were applied based on experimental designs.

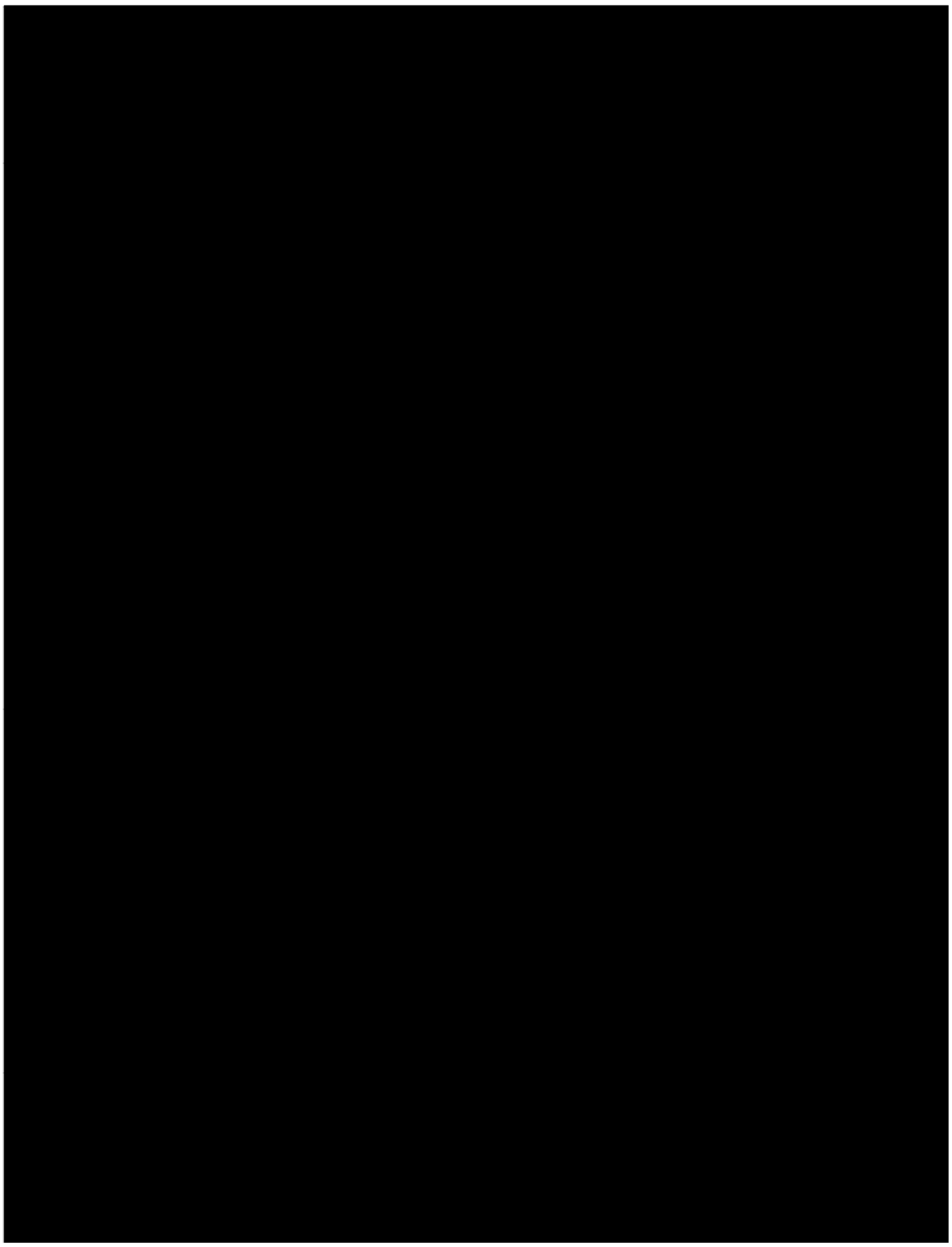
This paper led to the publication of article 2 in chapter 4.

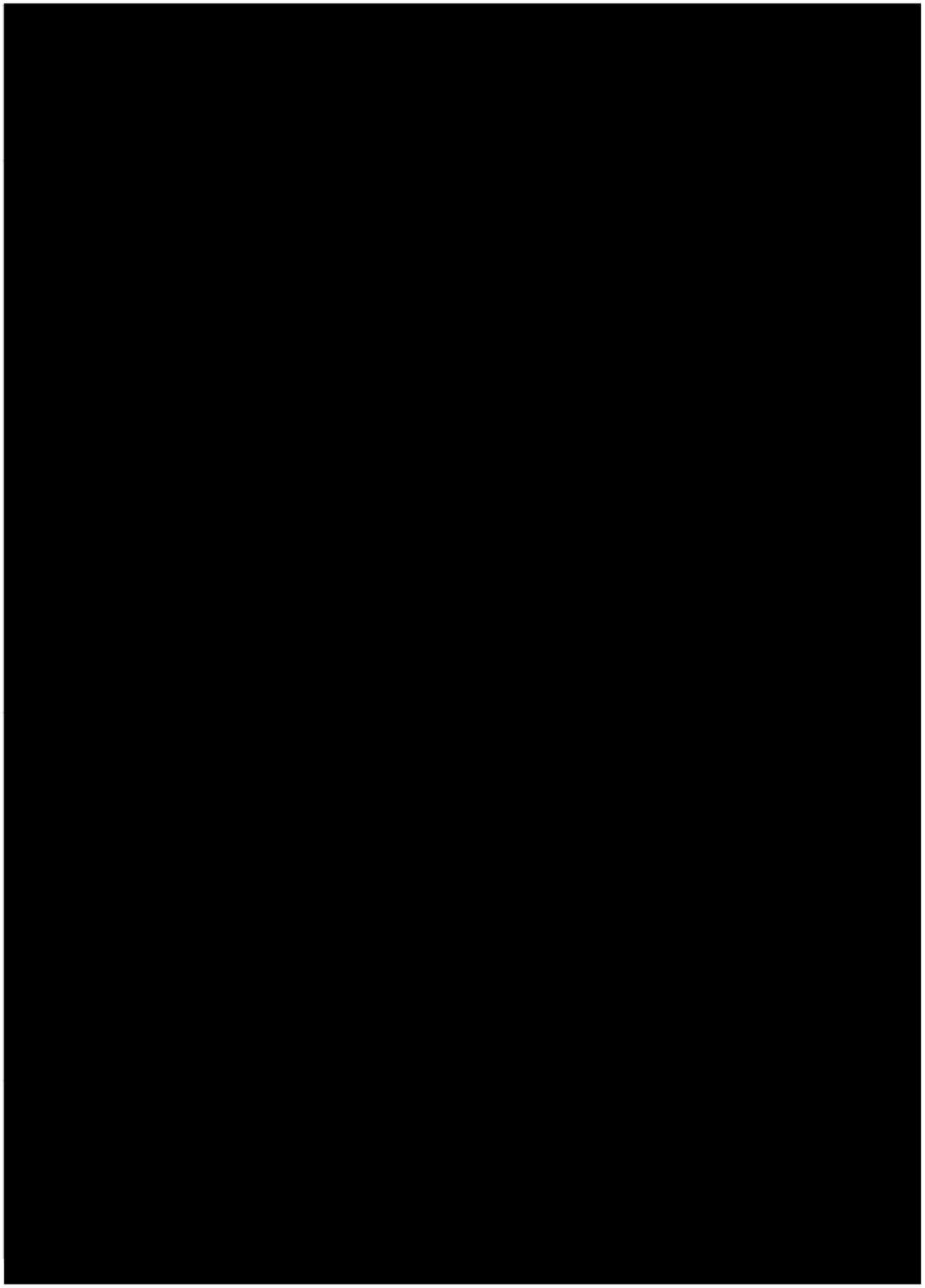
CHAPTER 4: PAPER 2 - RESISTANCE IN MAIZE (*ZEA MAYS*) TO ISOLATES OF *PUCCINIA SORGHI* FROM EASTERN AUSTRALIA

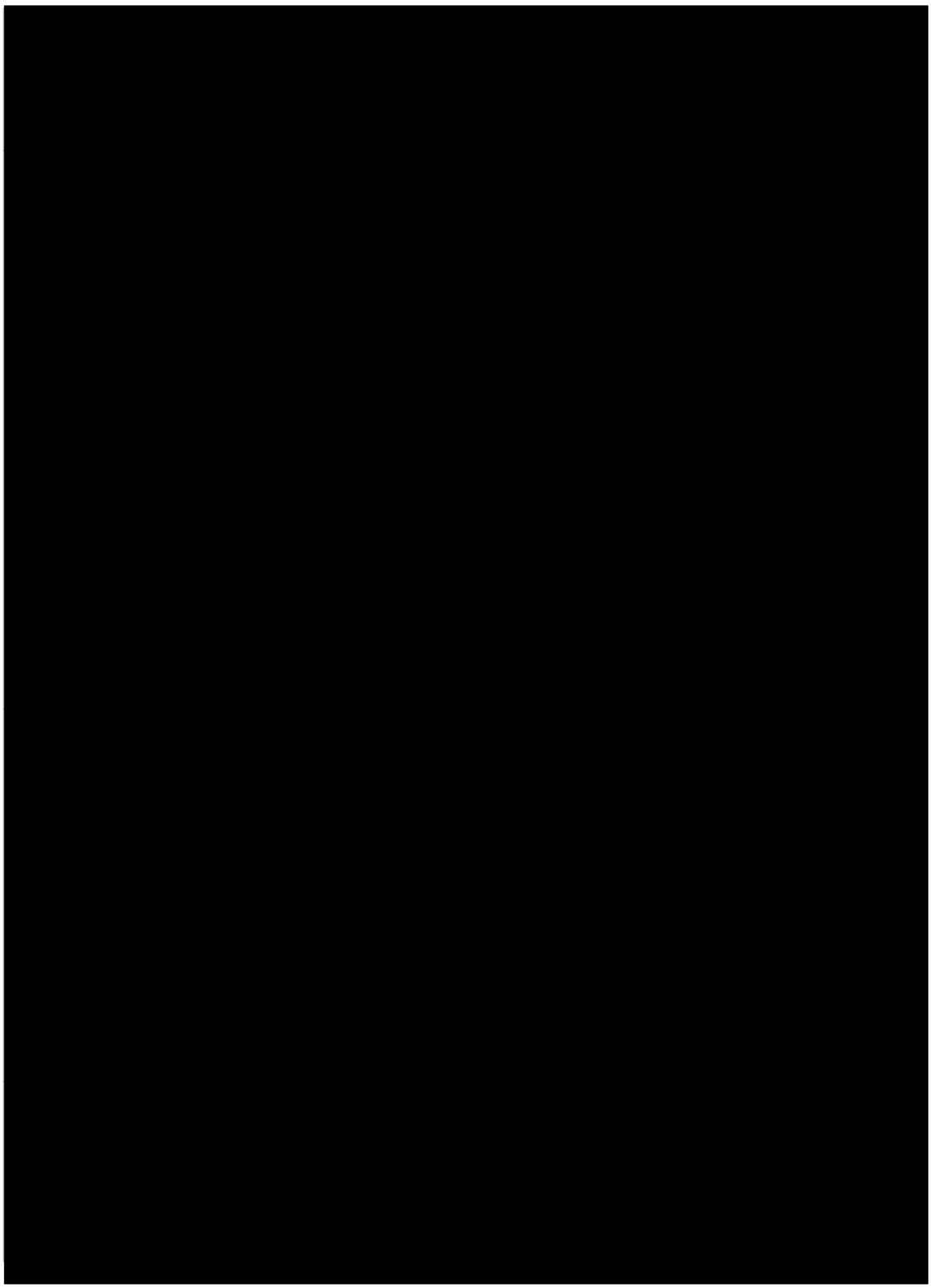
4.1 Introduction

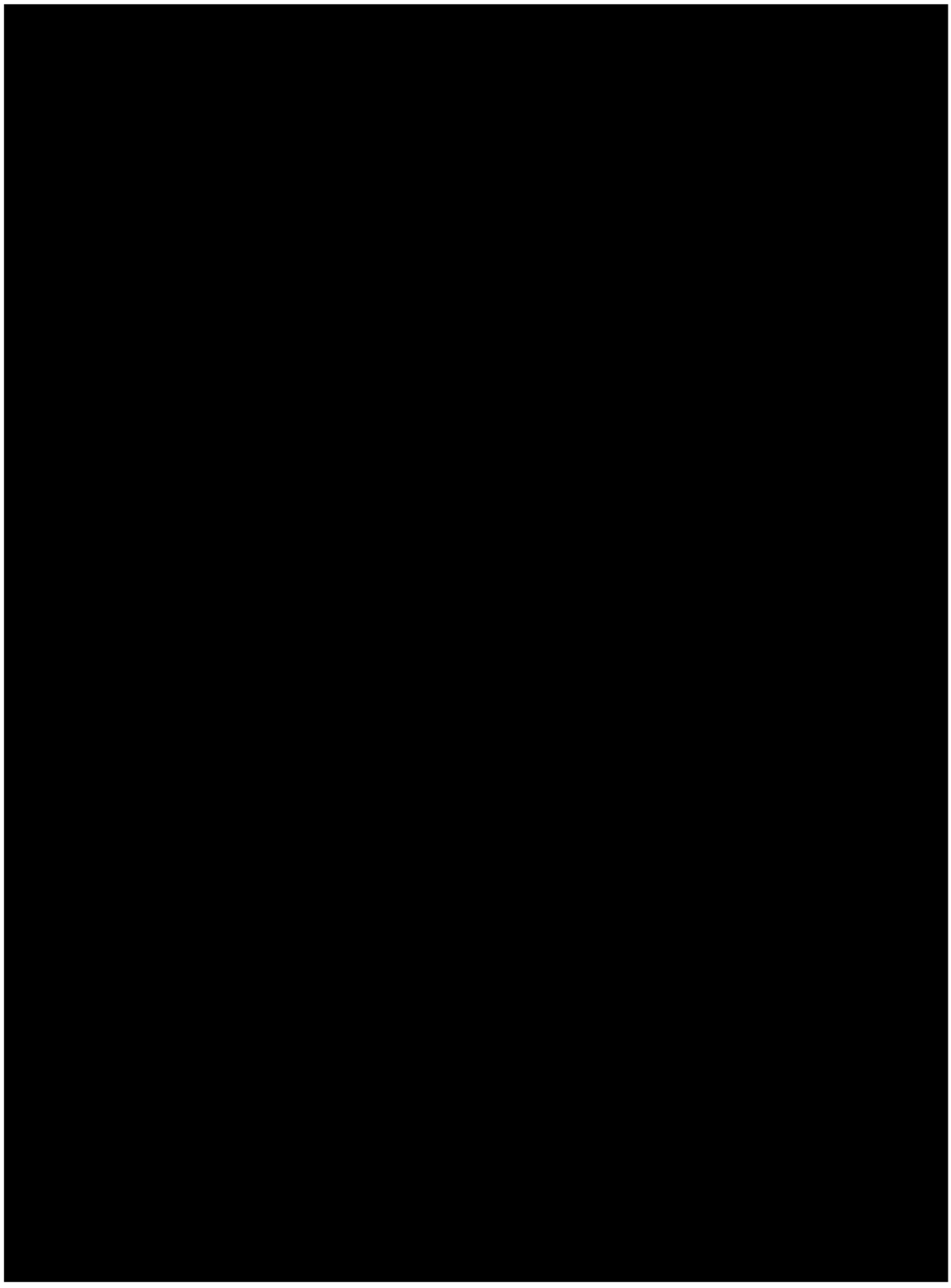
By iInoculating young seedlings of maize with viable urediniospores, it was possible to assess the level of resistance of the commonly grown commercial lines of maize. If the resistance of the main commercial lines proved unsatisfactory, the main purpose of the research was also to determine the initial level of resistance of the main resistance gene, *Rp1_D,* assess candidate effective resistance genes and genes suited to the development of a differential set.

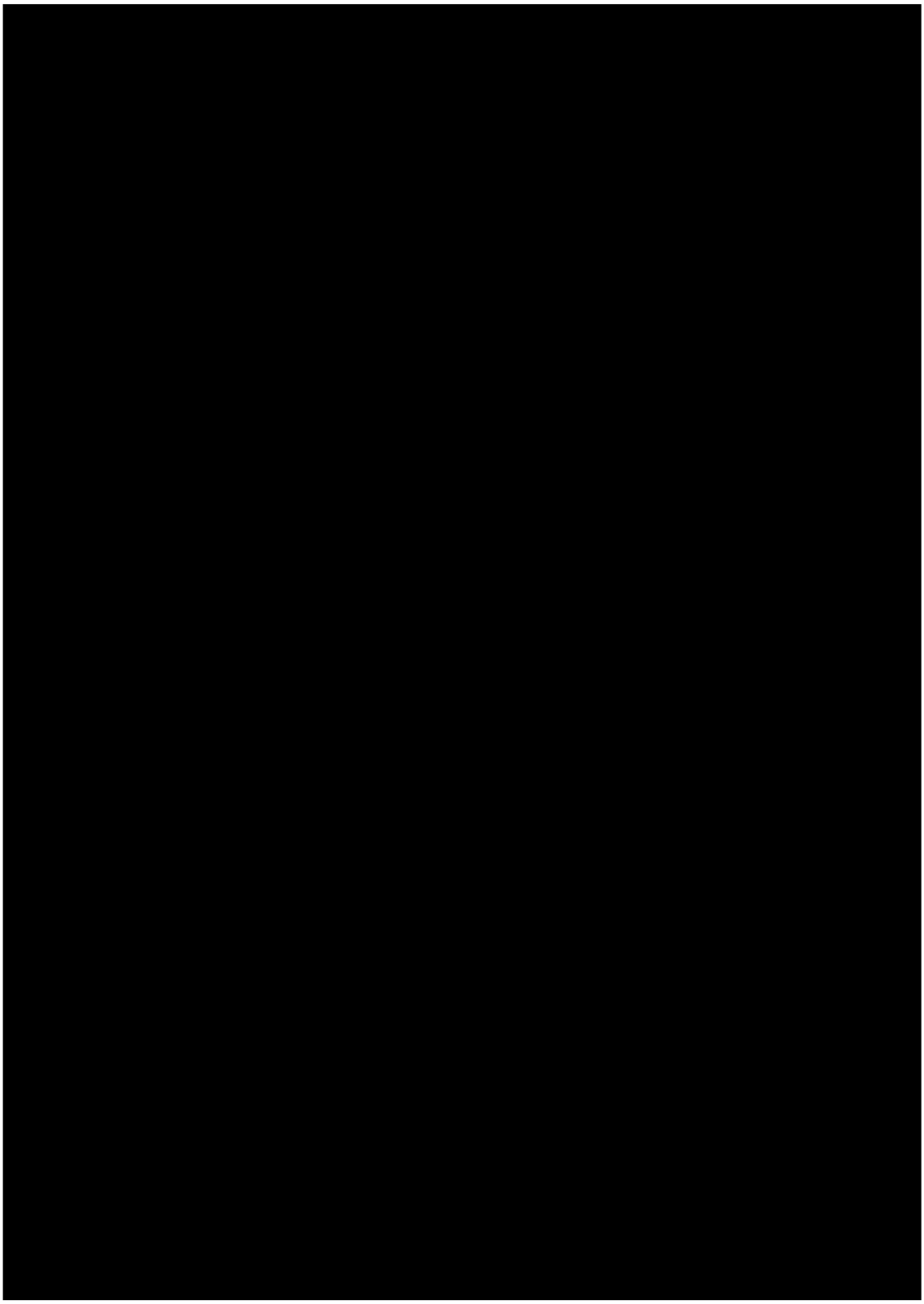


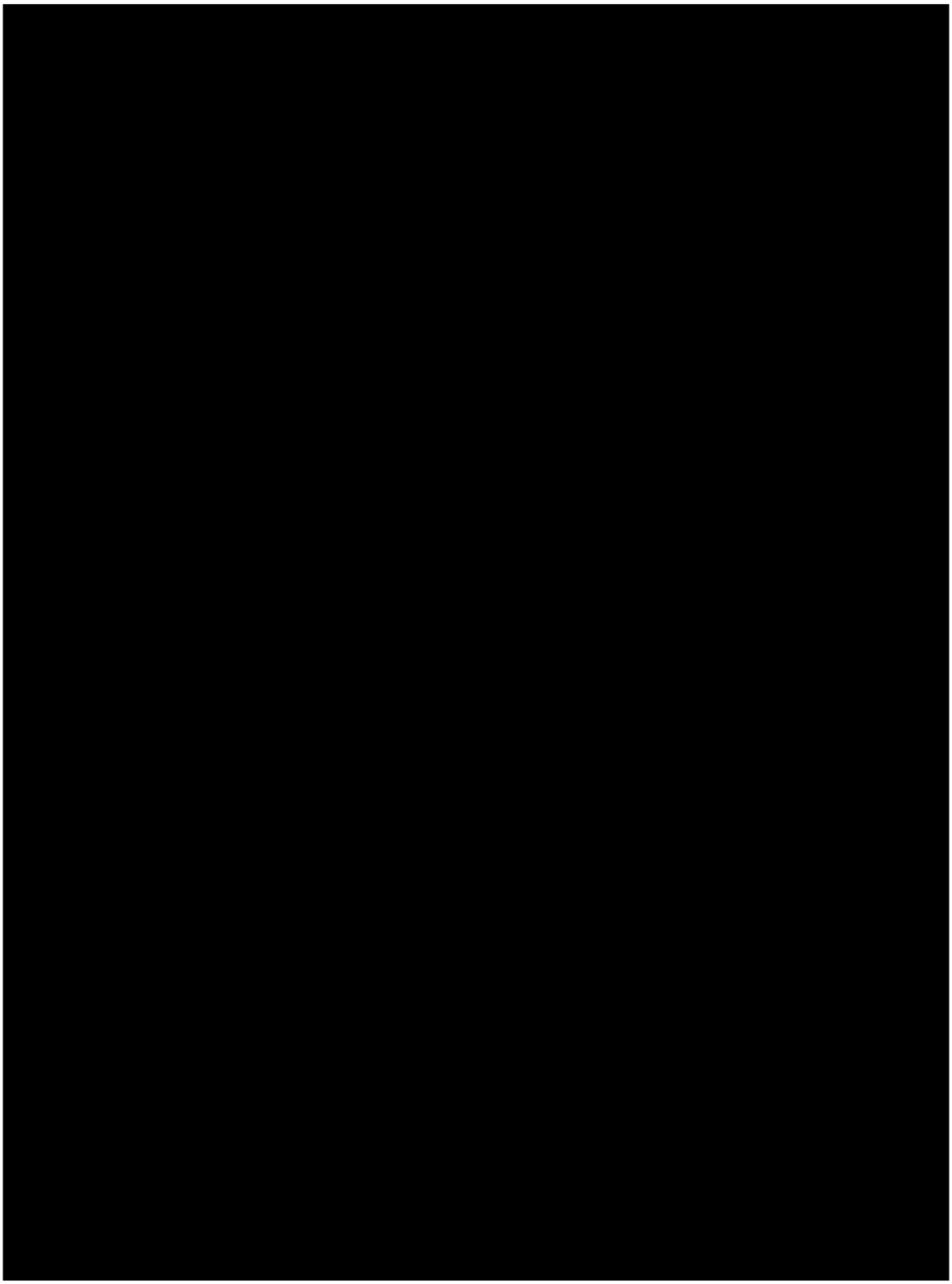












4.2 Links and implications

The main commercial lines grown in Eastern Australia carried no resistance to *P. sorghi* and partly explained the increase in disease incidence and severity observed by growers. Eight candidate maize lines were identified as carrying effective resistance to *P. sorghi* and the resistance was effective against isolates collected across Eastern Australia. Maize lines carrying resistance genes with varied response to isolates of the Australian CR population were proposed as candidate lines for a differential set. Such lines are critical to virulence studies. Further study is required to determine the extent of the pathogen diversity and implications for the durability of newly deployed resistance. Though eight single and compound resistance genes were identified as carrying effective resistance, it is important to determine whether these lines will be faced with a genetically stable pathogen population or a pathogen population capable of mutating rapidly.

CHAPTER 5: PAPER 3 - PHENOTYPIC AND GENOTYPIC DIVERSITY OF *PUCCINIA SORGHI* IN EASTERN AUSTRALIA: IMPLICATIONS FOR MAIZE BREEDING PROGRAMS

5.1 Introduction

Biotrophic pathogen populations often exhibit high genetic plasticity, combined with exponential spore production and the ability to disseminate spores over long distances. This ensures contact with the host plant and increases the chance of overcoming the resistance gene of the host plant. Breeding programs can benefit from understanding the diversity of a pathogen population. This allows for the identification, introgression and deployment of durable resistance genes.

Phenotypic and genotypic diversity of *Puccinia sorghi* in Eastern Australia: implications for maize breeding programs, 2023, accepted for publication in Phytofrontier.

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ABSTRACT

Common rust of maize (CR), caused by *Puccinia sorghi* Schwein., is found in all maize growing regions across the globe. In recent years, an increase in incidence and severity of CR has been observed in Australia. The challenges posed by a diverse population of *P. sorghi* to maize breeding programs and the strategies required to deploy durable resistance were explored. Preliminary studies of diversity were conducted by phenotyping the five most diverse isolates against maize lines carrying no resistance genes, and from one and up to four resistance genes. Further genotyping studies were required. Short reads of 17 CR isolates were collected in an array of agro-ecological regions in Eastern Australia and these were mapped back against the hybrid assembly of Isolate 1. A hybrid assembly between short (Illumina paired-end) and long (PacBio) reads was conducted in MaSuRCA of the first isolate collected. A draft genome of 145,963,792 bp was obtained after QUAST analysis and a total length of 145,815,044 bp (when removing base pair below ≥ 1000), compared to 99,534,058 bp in Rochi et al. (2018). The prediction from the BRAKER pipeline identified 20,438 genes model. All 17 isolates sequenced represented unique genotypes, and no clones were detected. The 17 isolates grouped into three clusters. The diversity observed within the population of *P. sorghi* in Australia suggests that monogenic resistance may not be durable.

Keywords: *Zea mays*, rust, Pucciniales*,* hybrid assembly, short reads, long reads, genome assembly, fungal pathogen diversity.

Puccinia sorghi Schwein., causal agent of maize common rust (CR), belongs to the Pucciniales. Most species within this large group of biotrophic fungi have evolved to have narrow host ranges. In order to survive and reproduce, rust fungi must infect living plant tissues and colonise plant cells via the production of haustoria. To adapt to the constraints imposed by a biotrophic lifestyle, rusts possess a genome with great plasticity. A key contributor to genetic diversity of rust fungi is their complex life cycle, which may comprise up to five different spore stages: urediniospores, teliospores, basidiospores, spermacia, and aeciospores (Bettgenhaeuser et al., 2014). Basidiospores and aeciospores are produced through meiosis, more specifically though, aeciospores are dikaryotic and the product of plasmogamy. The recombination of alleles in the pathogen population is achieved at the aecial stage on the alternate host for heteroecious rust species, such as *P. sorghi*. Exceptions aside, clonally produced urediniospores, at the uredinial stage, cause economic losses to agricultural crops and are the focus of this study.

The success of rust fungi as pathogens are their high rate of sporulation, which maintains genome plasticity, and the rapid spread of new virulence over long distances (Roelfs & Bushnell, 1985). A study of the wheat stem rust pathogen *Puccinia graminis* f. sp. *tritici* estimated that at an infection rate of 50 pustules per wheat tiller in the field, approximately 10^{12} spores per hectare per day were produced (Rowell & Roelfs, 1971). Potentially, one in every 100,000 to 10 million cells/spores will carry mutations beneficial to the pathogen, as determined in other pathosystems involving fungi (Stam & McDonald, 2018). On any given day, at similar infection levels, every infected hectare may contain about 225,000 spores carrying mutations beneficial to the pathogen. However, rust species genomic diversity is more likely to result in mutation occurring every 100,000 spores, resulting in approximately 10^{7} spores per hectare per day with beneficial mutations (Stam & McDonald, 2018).

The virulence of rust pathogens has been studied by phenotyping the responses of host plants, carrying specific resistance genes, when challenged with specific rust isolates. This information has guided resistance breeding programs by allowing selection of effective resistance genes (Park, 2015b). In the maize-common rust pathosystem, changes in virulence have led to known resistance genes being overcome in the US (Groth et al., 1992), South America (Darino et al., 2016), South Africa (Fato, 2000) and Australia (Quade et al., 2021). Pre-emptive breeding studies have contributed to the identification of future virulence and/or future virulence combinations based on the data provided by existing virulence profiles. However, without such data these studies are limited to examining virulence already present in the pathogen population. The potential for pathogens to develop new virulence is often linked to the extent of their genetic diversity. Studies of rust species have determined that intricate genetic features are involved to ensure successful infection and access to host plant resources (Barsoum et al., 2019). The genomes of rust fungi sequenced to date range from 90 Mbp to 1 Gbp in size, over 50% of their large genome is repetitive in nature, as rust urediniospores carry two nuclei (Miller et al., 2018; Tobias et al., 2020). Rust genomes contain a large number of transposable elements (TEs), for example, 33% of the genome of *P. sorghi* consists of TEs (Rochi et al., 2018). Although the role of TEs in creating new virulence specificities in *P. sorghi* is not well understood, the ability to shift sections of DNA certainly plays a role in the emergence of new pathotypes (Rochi et al., 2018). The relationship between pathogenicity and specific TEs has been documented in other fungal plant pathogens such as *Botrytis cinerea* (Porquier et al., 2021).

The high number of repetitive regions in rust genomes presents difficulties in *de novo* assemblies using short-read sequencing only, as errors such as high fragmentation, misassembly, and merging of two distinct haplotype sequences are likely to occur. Rochi et al. (2018) generated an *ab initio* draft genome sequence for *P. sorghi*, which was followed by a short-read

comparative genome analysis to improve the predictive secretomes based on three wheat rust pathogens and the maize boil smut pathogen *Ustilago maydis.* The limitations of short-read based assemblies can be mitigated through long-read sequencing, which significantly improves the assembly of repeat-rich genomes. However, Single Molecule Real Time (SMRT) long-read sequencing possesses a higher error rate compared to short-read sequencing. Potential errors in the genome structure created with the long reads can be corrected using high quality short reads, improving the quality of the genome assembly (De Maio et al., 2019). The cost of long-read sequencing remains high; therefore, a limited number of isolates can be processed while short-read sequencing allows for the sequencing of a relatively large number of samples. A comprehensive genome assembly with long reads, with errors corrected with short reads to obtain a hybrid assembly of *P. sorghi* has not been conducted. The genome of *P. sorghi* available on the National Center for Biotechnology Information (NCBI) uses short reads only and is highly fragmented (Rochi et al., 2018).

In recent years, Australia has experienced an increase in the incidence and severity of CR. This is due to a lack of resistance in Australian commercial maize hybrids (Quade et al., 2021) and favourable environmental conditions. The aim of this research was to gain an understanding of the evolutionary potential for *P. sorghi* populations in Australia. It was hypothesised that: *i)* phenotyping of the most varied isolates in terms of hosts and agro-ecological region of collection was a suitable preliminary study to confirm or refute virulence diversity, *ii)* genotyping a set of isolates would reveal the presence of population subdivision based on agro-ecological region of collection, and *iii)* produce an improved hybrid genome assembly for *P. sorghi*. Specifically, we explored the challenges a diverse population of *P. sorghi* would pose to maize breeding programs and the strategies that would be required to deploy durable resistance.

MATERIALS AND METHODS

Phenotyping of a diverse set of isolates on a maize differential set. The focus of this experiment was to determine the overall effect of each isolate over time on a set of maize lines carrying a range of resistance genes. The maize lines in the differential set included genotypes carrying no known resistance gene and up to four different resistance genes. Isolates causing infection levels between 0 to 30% were considered as possessing no to low virulence, between 30% and 60% moderate virulence, and greater than 60% high virulence.

Virulence patterns were assessed by inoculating 3-week-old seedlings carrying known resistance genes with the following five isolates. Single pustule Isolates 1, 9, 24, 31 and 73 were arbitrarily selected from a collection of 89 isolates based on their diversity in terms of geographic origin (Victoria versus Queensland), year of collection (2018 versus 2019), and host (either maize or sweet corn) (Table 1). The single pustule increase, maize growth and inoculation were conducted as described in Quade et al. (2021). The maize lines tested either carried single resistance gene *Rp1_B, Rp1_C, Rp1_E, Rp1_F, Rp1_G, Rp1_I, Rp1_J, Rp1_K, Rp1_L, Rp1_M, Rp3_D, Rp4_A, Rp5, Rp7, Rp8_A, Rp8_B, Rpp9* (conferring resistance to *P. polysora* Underw.), or multiple *Rp* genes *Rp1_FJ* (*Rp1_F* and *Rp1_J*), *Rp5D* (*Rp5* and *Rp1_D*), *RpGDJ* (*Rp1_D, Rp1_G* and *Rp_J*) and *Rp5GCJ* (*Rp5, Rp1_C, Rp1_G* and *Rp1_J*), all in unknown backgrounds. Three sweet corn lines were also used and carried no resistance genes, *Rp1_D* or *RpGJF* (*Rp1_G, Rp1_J* and *Rp1_F*) (Table 2). Infection scores were recorded every 48h from 6 days post inoculation (DPI) until 16 DPI.

The assessments of the resistance of the commercial hybrids and the *Rp* lines were conducted in a split-plot design, blocked by isolates and maize lines as the plot. When experimental constraints prevented replication, the whole experiment was replicated two or three times over time. The number of replications was determined based on the degrees of freedom (df) at the residual level, where 15 df and over were considered satisfactory.

The data was treated as continuous and disease severity scores were recorded as percentage of diseased area in the range of 0, 1%, 5%, 10%, followed by 10% increments to 100%. NA were given to a completely dead leaf or a missing plant.

Quantitative data were analysed using ASreml v.4 in RStudio v.1.2.5033 by fitting a linear mixed model (Butler et al., 2018; RStudio, 2018). Continuous data received a logic-transformation (Welham et al., 2004) and data reported in this paper were back-transformed. Differences in predicted values were reported to be significant when a *P* value <0.05 was recorded, after which *post hoc* analysis using Tukey's test was applied. The plot of the residuals and the test for normal distribution of the residuals were satisfactory in all the analyses of all experiments.

Fungal samples, DNA extraction and sequencing. From a collection of 89 *P. sorghi* samples collected between 2018 and 2020 from naturally occurring populations (Quade et al., 2021), Isolate 1 was selected for PacBio Sequencing as it was the first isolate collected (in 2018) and it was hypothesized that changes within the pathogen population between 2018 and 2019, and location would be recorded. DNA was extracted from 75 mg of un-germinated urediniospores using a modified CTAB procedure (Schwessinger et al., 2018). The method described by Vaser et al. (2017) was used to remove impurities and low molecular weight DNA. A 20K library was prepared and sequenced on PacBio RSII platform, using one SMRT cell at Novogen (HK).

The first isolate collected, Isolate 1, and 16 other isolates were sequenced using the Illumina Novaseq (S4) PE150 platform to represent a wide range of hosts and locations (Table 1). All isolates were established from samples collected in 2018, with the exception of Isolate 73, which originated from a sample collected in 2019. Isolates 43_1 and 43_2 were established from the same leaf sample from maize in Queensland, but from different pustules (Table 1). Prior to Illumina short-read sequencing, all 17 samples were purified by increasing urediniospores from a single pustule. DNA from

0.10 mg of urediniospores was extracted following a CTAB extraction method as described by Bailey et al. (2013). All DNA samples were concentrated and purified. DNA concentration was quantified using Qubit 3 Fluorometer (Thermo Fisher Scientific) and a NanoDrop ND-1000 (Thermo Fisher Scientific). Genomic DNA was sent to Novogen for library preparation and Illumina sequencing at 8 Gb of raw data per sample.

Table 1: Details of *P. sorghi* isolates used in the experiments.

Isolate [a]	Collection date	Region	State [b]	Agro-ecological region climate	Latitude	Longitude	BBCH [c]	Host[d]	Rust score (infection type)[e]
1*	**15/02/2018**	**Darling Downs**	**QLD**	**Subhumid, subtropical slopes and plains**	**-27.509568**	**151.389859**	**18**	**Maize**	**NA**
9*	**1/03/2018**	**East Gippsland**	**VIC**	**Wet temperate coast**	**-37.722071**	**148.414755**	**83**	**Sweet corn**	**9**
12	1/03/2018	East Gippsland	VIC	Wet temperate coast	-37.722071	148.414755	79	Sweet corn	5
13	1/03/2018	East Gippsland	VIC	Wet temperate coast	-37.722071	148.414755	80	Sweet corn	4
15	1/03/2018	East Gippsland	VIC	Wet temperate coast	-37.722071	148.414755	79	Sweet corn	9
24*	**27/03/2018**	**Southern Downs**	**QLD**	**Wet subtropical coast**	**-28.046435**	**151.962135**	**73**	**Maize**	**7**
27	27/03/2018	Southern Downs	QLD	Wet subtropical coast	-28.046435	151.962135	73	Maize	7
31*	**28/03/2018**	**Lockyer Valley**	**QLD**	**Wet tropical Coast and Tableland**	**-27.431075**	**152.584398**	**79**	**Sweet corn**	**2**
39	27/03/2018	Darling Downs	QLD	Subhumid, subtropical slopes and plains	-27.641913	151.869093	83	Maize	2
43_1	29/03/2018	Darling Downs	QLD	Subhumid, subtropical slopes and plains	-27.751234	151.447163	79	Maize	3
43_2	29/03/2018	Darling Downs	QLD	Subhumid, subtropical slopes and plains	-27.751234	151.447163	79	Maize	3
51	30/04/2018	East Gippsland	VIC	Wet temperate coast	-37.724023	148.433945	83	Missing value	7
55	6/05/2018	North Burnett	QLD	Wet subtropical coast	-24.792465	151.123412	87	Maize	4
56	25/05/2018	Southeast QLD	QLD	Wet subtropical coast	-27.375303	152.443875	87	Maize	7

57	22/05/2018	Southeast QLD	QLD	Wet subtropical coast	-27.419467	152.470468	87	Maize	7
58	22/05/2018	Southeast	QLD	Wet subtropical coast	-27.419467	152.470468	87	Maize	5
73*	**9/05/2019**	**East Gippsland**	**VIC**	**Wet temperate coast**	**-37.722071**	**148.414755**	**89**	**Maize**	**3**

[a] 43_1 and 43_2 were retrieved from the same leaf sample, but from different pustules.

[b] QLD: Queensland, VIC: Victoria.

[c] BBCH: **B**iologische **B**undesanstalt, Bundessortenamt und **CH**emische Industrie (Codifies key phenological developmental stages of agricultural plants).

[d] Maize is classified as an agricultural crop, whereas sweet corn is classified as a horticultural crop.

[e] Disease scores taken in the field ranged from 1 to 9, where 1 was no disease and 9 was dead.

* and bold indicates phenotyped isolates.

Short read preparation and assembly. The raw Illumina paired-end reads were uploaded in the publicly available Galaxy Platform (Afgan et al., 2018). The quality of the raw reads was checked using FastQC v.0.72 (Andrews, 2010) and trimmed in Trimmomatic v.0.36.6 (Bolger et al., 2014) with parameters for paired-end, using a sliding window of 4 bp and average quality of 30.

Long-read DNA sequencing and *de novo* assembly. A hybrid genome of Isolate 1 was assembled *de novo* using two pipelines MaSuRCA v.4.0.3 (Zimin et al., 2013) and Flye v.2.9 (Kolmogorov et al., 2019). The raw reads of PacBio and Illumina were assembled in MaSuRCA v. 4.0.3 with two rounds of polishing with POLCA. The pre-assembly polishing for Flye was conducted using FMLRC v.2 (Wang et al., 2018), followed by Flye assembly with default parameters in Galaxy v.2.8.3 (Lin et al., 2016). SAM files were created with BWA-mem v.0.7.17.2, and post-assembly polishing using RACON Galaxy v.1.4.20 (Vaser et al., 2017) with default parameters. Genome completeness was assessed by identifying Benchmarking Universal Single-Copy Orthologs (BUSCOs) based on the Basidiomycota_odb9-29222 database using BUSCO v.3 (Seppey et al., 2019).

Annotation of hybrid assembly. For the hybrid assembly of Isolate 1, all steps leading to annotation were performed *de novo* in Gensas (Humann et al., 2019). Families of TEs were identified using RepeatModeler v.1.0.11 (Smit & Hubley, 2008). Regions of all assembled contigs possessing similarities between sequences were identified in Basic Local Alignment Search Tool (BLAST) (NCBI Resource Coordinators, 2016). Sequences from rRNA and tRNA were identified using RNAmmer v.1.2 (Lagesen et al., 2007) and tRNAScan v.2.0 (Chan & Lowe, 2019). Eukaryotic genes were predicted in GeneMarkES v.4.33 (max contig = 5,000,000 bp, min contig = 5,000 bp and max gap = 5,000 bp) (Borodovsky & Lomsadze, 2011) and a consensus of gene structures was obtained based on *ab initio* gene prediction and protein from GeneMarkES and BLAST.

Orthology to Basidiomycota_odb9-29222 database was measured with BUSCO v.3 for completeness and assessment of gene set. Protein-coding genes found with GeneMarkES were aligned with DIAMOND v.0.9.22 (Buchfink et al., 2015) against the NCBI refseq fungi (Protein) (O'Leary et al., 2016). Another pipeline was followed for gene prediction. BRAKER v2.1.6 (Hoff et al., 2019) using ProtHint and GeneMark-EP (Brůna et al., 2020) with an in-house modified OrthoDB fungal protein database (Kriventseva et al., 2018), followed by fungal training with AUGUSTUS (Stanke & Morgenstern, 2005). Lastly, a gene prediction with contig evidence files from *P. striiformis,* BioSample SAMN00025370 (Cantu et al., 2011) was performed in gensas.

Variant calling and analysis of genetic distance. Adapter- and quality-filtered Illumina paired-end reads of the 17 sequenced isolates were independently mapped to the hybrid assembly of Isolate 1 using BWA-mem v.0.7.17.2. After coordinate based sorting, duplicates were removed in sortSAMtools view v.2.0.3 galaxy (Li & Durbin, 2009). Alignments from all isolates were merged and variant calling was conducted in FreeBayes parallel v.1.3.5 (Tange, 2011; Garrison & Marth, 2012) using default settings (*--min-mapping-quality* 30 *--min-base-quality* 20 *--pvar* 0.0001). The vcf file was filtered using VCFfilter v.1.0.0 (Garrison, 2015) using parameters DP > 10 and QUAL > 20. The identified single nucleotide polymorphisms (SNPs) were further filtered in TASSEL v.5.2.75 (Bradbury et al., 2007) for minimum allele frequency of 5% and no missing data in order to maintain informative loci. This resulted in the identification of 928,211 SNPs for the 17 isolates. Due to the small number of isolates (n = 17), many routine population analyses of genetic diversity and population differentiation could not be carried out, however, genetic distance among isolates and population structuring were investigated as follows. Nei's genetic distance was used to calculate pairwise comparisons of genetic distance between isolates to obtain a distance matrix. The matrix was then used in the UPGMA method with 1,000 bootstrap replicates using *aboot*

function from *poppr* v.2.9.1 package in R to generate a dendrogram (Kamvar et al., 2014). SplitsTree v4.18.3 (Moulton, 2003), which is more suitable to depict genetic relationships in the presence of recombination and was used to construct a neighbour-net network to visualise the genetic relationship among isolates with 1000 bootstraps. The existence of an underlying population structure without *a priori* population assignment was investigated using Discriminant Analysis of Principal Components (DAPC) in R package *adegenet* v.2.1.5 (Jombart, 2008). The optimal number of clusters was determined using the function *find.clusters* and DAPC was used to assign individuals to clusters, retaining the number of principal components encompassing 86% of the cumulative variance.

TABLE 2. Maize differential set entry name and corresponding Resistance *Puccinia* genes (*Rp*)

Maize line entry	Resistance gene
E8*	NA
E9*	*Rp1_D*
E10*	*Rp1_G, Rp1_J, Rp1_F*
E11	*Rp1_B*
E12	*Rp1_C*
E13	*Rp1_E*
E14	*Rp1_F*
E15	*Rp1_G*
E16	*Rp1_I*
E17	*Rp1_J*
E18	*Rp1_K*
E19	*Rp1_L*
E20	*Rp1_M*
E21	*Rp3_D*
E22	*Rp4_A*
E23	*Rp5*
E24	*Rp7*
E25	*Rp8_A*
E26	*Rp8_B*
E27	*Rpp9*
E28	*Rp1_F, Rp1_J*
E29	*Rp5, Rp1_D*
E30	*Rp1_D, Rp1_G, Rp1_J*
E31	*Rp5, Rp1_C, Rp1_G, Rp1_J*

* Resistance genes carried in sweet corn, all other genes carried in maize.

RESULTS

Phenotypic diversity. Pustule initiations were visible with all five isolates at 6 DPI (Fig. 1). By 8 DPI, it was possible to classify the isolates as having low or high virulence on the differential maize set for each resistance gene (Fig. 1). From an agronomy perspective, the overall infection level/pattern for Isolate 73 and 1 was over 60%, by comparison infection levels remained below 40% from 10 DPI for most maize lines. On the contrary, Isolates 9, 24 and 31 displayed diverse infection levels on the differential set.

At 10 DPI, Isolate 31 infection levels were above 25% for *Rp1_K,* whereas infection levels remained below 8% for Isolates 73, 24, 9 and 1. Isolate 31 had infection levels of 10% on sweet corn line E9 carrying *Rp1_D,* whereas infections over 20% for Isolate 24, 73 and 1. Isolate 24 was able to cause infection on sweet corn lines E10 that carry resistance genes *Rp1_G, Rp1_J, Rp1_F,* whereas Isolates 73, 31, 9 and 1 did not. Isolate 1 and 73 infection levels for the maize line E29 carrying *Rp5 and Rp1_D* were above 38%, however remained below 25% for Isolate 24 and 9.

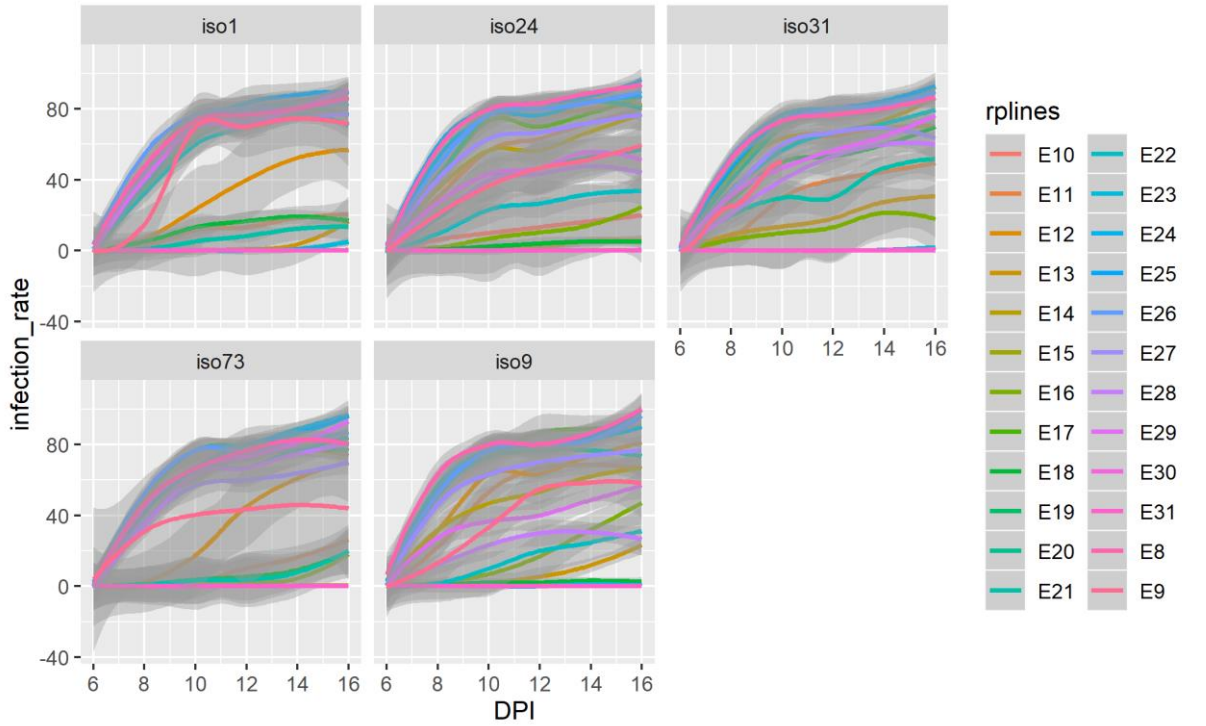


Fig. 1. The percentage of infection from 6 to 16 days post inoculation (DPI) for the five most diverse *Puccinia sorghi* isolates, in terms of collection location, year and host (maize versus sweet corn) on a maize differential set carrying known resistance genes in an experiment conducted in Wagga Wagga, NSW, Australia.

There was no pattern of association of virulence with location of collection, suggesting that the variation in virulence on the differential set and infection levels over time were not related to agro-ecological region of origin. Similarly, there was no association between patterns of virulence and year of collection. Rust samples collected in 2018 had contrasting virulence pattern on the *Rp* genes investigated. Interestingly, Isolate 1 and 73 displayed the most similar patterns of infection, despite being collected from locations separated by over 1,300 km and during different years and belonged to the same clade. The only differences were reported on Isolate 24 causing infection on *Rp1_E* and Isolate 73 on *Rp1_I.* Although early phenotyping data determined that some diversity was present between the isolates, it did not indicate the extent of the genomic divergence between the isolates.

Population subdivision. All 17 isolates sequenced represented unique genotypes, and no clones were detected (genotypic diversity = 1). Following clustering of genotypes based on genetic distance, the 17 isolates grouped into three different clusters (Fig. 2 and Fig. 3). Isolates 9, 12, 13, and 58 belonged to clade one; Isolates 9, 12, and 13 all collected on the same date and in the same field in Victoria (VIC), were most closely related. Isolate 58 was collected in 2018 from Queensland (QLD). Interestingly, Isolate 15 was also collected at the same location as Isolates 9, 12, and 13 but belonged to the third clade. Isolate 15 was most closely related to Isolate 73, despite the two originating from different hosts, sweet corn and maize, respectively. These two isolates originated from the same field, though 14 months apart. The second clade comprised the most diverse group of isolates. Most originated from diverse agro-ecological regions in QLD, except for Isolate 51 (VIC): Sub-humid, subtropical slopes and wet subtropical coast. The third clade comprised two isolates from VIC (Isolates 15 and 73) and two from QLD (Isolates 1 and 56). Results from the DAPC analysis was in line with the genetic distance tree, identifying three main clusters (Supplementary Fig. 3). Cluster 1, the main cluster, consisted of Isolates 24, 27, 31, 39, 43-1, 43-2, 51, 55, 57; Cluster 2 included Isolates 1, 15, 56 and 73; and Cluster 3 consisted of Isolates 9, 12, 13, 58. The same clusters were identified using the neighbour-net algorithm implemented in SplitTree (Fig. 3). Reticulation of the network in the main cluster (Cluster 1) indicated presence of recombination among isolates, while no reticulation was detected in Clusters 2 and 3.

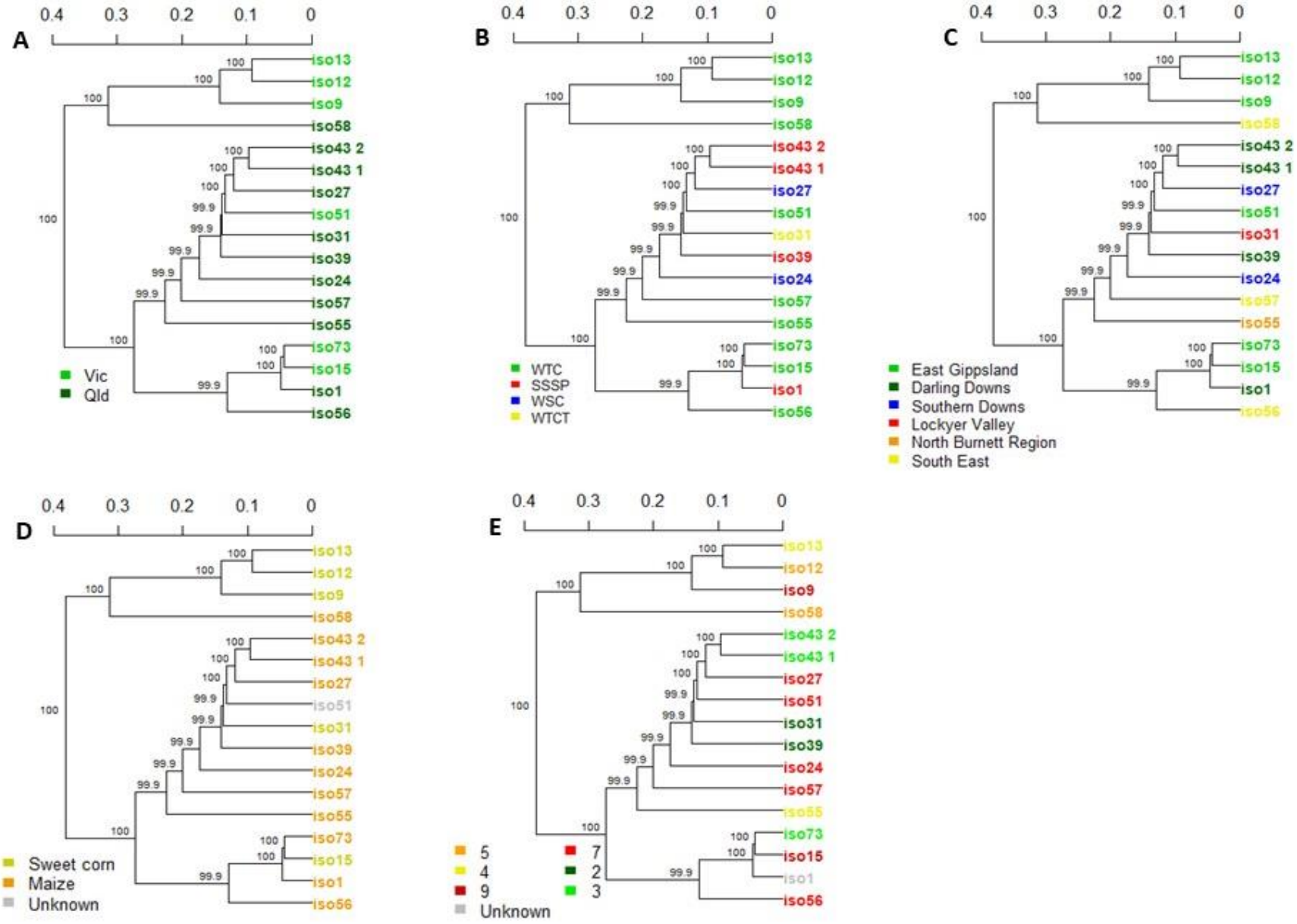

A
0.4 0.3 0.2 0.1 0
iso13
iso12
iso9
iso58
iso43 2
iso43 1
iso27
iso51
iso31
iso39
iso24
iso57
iso55
iso73
iso15
iso1
iso56
Vic
Qld
B
WTC
SSSP
WSC
WTCT
C
East Gippsland
Darling Downs
Southern Downs
Lockyer Valley
North Burnett Region
South East
D
Sweet corn
Maize
Unknown
E
5
4
9
Unknown
7
2
3

Fig. 2. UPGMA dendrogram constructed based on Nei's distance among *Puccinia sorghi* isolates collected from *Zea maydis* in Australia. Bootstrap support values greater than 50 are shown above the branches. The colours of the isolates correspond to the **A**, State of collection (Qld: Queensland, Vic: Victoria); **B**, climate (WTC: wet temperate coast; SSSP: subhumid, subtropical slopes and plains; WSC: wet subtropical coast; WTCT: wet tropical coast and tableland), **C**, region, **D**, host, and **E**, virulence**.**

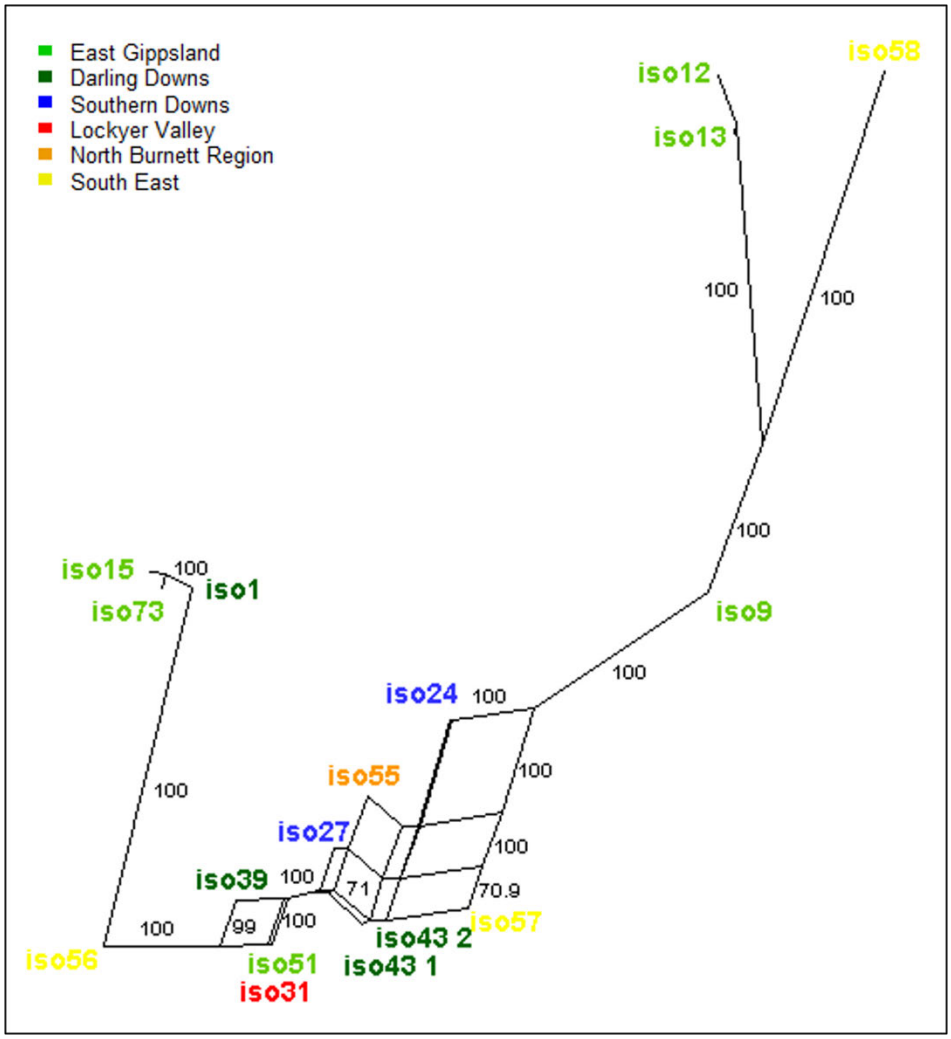
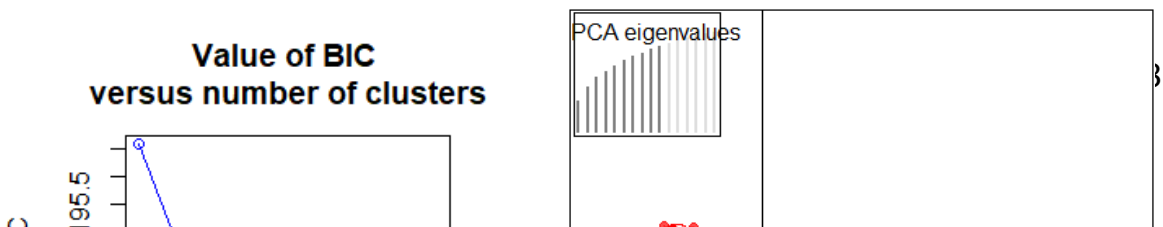


Fig. 3. Neighbour-net network constructed for *Puccinia sorghi* isolates collected from *Zea maydis* in Australia, with 1000 bootstraps replicates, constructed using genome-wide Single Nucleotide Polymorphism data. Colour of isolates correspond to the region where the isolates were collected.



Supplementary Fig. 3. Discriminant Analysis of Principal Components (DAPC) for *Puccinia sorghi* isolates collected from *Zea maydis* in Australia, without *a priori* assumption of population subdivision. The graph on the left shows Bayesian Information Criterion (BIC) plotted against the number of inferred clusters. The graph on the right depicts ordination plot of three assigned genetically distinct groups of individuals.

***De novo* hybrid assembly of Isolate 1.** In MaSuRCA, the total number of contigs was 2476, compared to 2627 for Flye (Table 3). In BUSCO, the completeness of the *P. sorghi* draft genome constructed using MaSuRCA was estimated to be 90.2%, compared to 89% using Flye, and was therefore retained. Following the 20x *de novo* hybrid assembly of Isolate 1 in MaSuRCA a draft genome of 145,963,792 bp was obtained after QUAST analysis (Table 3) and total length of 145,815,044 bp (≥ 1000 bp), compared to 99,534,058 bp in Rochi et al. (2018). The draft genome comprised 2,702 contigs for the long read assembly, compared to 28,117 contigs in the short read assembly generated by Rochi et al. (2018). Of the total number of contigs, 2,476 were over 1,000 bp. The N50 was 123,460 and 4,987 for Rochi et al. (2018) and the L50 was 354, compared to 3829 for Rochi et al. (2018).

TABLE 3. Genome statistics for the draft hybrid assembly of *P. sorghi* Isolate 1.

Isolate 1 statistics	MaSuRCA assembly	Flye assembly
# contigs (>= 0 bp)	2,702	2,728
# contigs (>= 1000 bp)	2,476	2,627
Total length (>= 0 bp)	145,963,792	155,488,878
Total length (>= 1000 bp)	145,815,044	155,417,575
# contigs	2,702	2,723
Largest contig	685,541	981,868
Total length	145,963,792	155,486,859
Estimated reference length	77,000,000	155,486,859
GC (%)	44.38	44.58
N50	123,460	165,816
NG50	203,904	na
N75	67,607	75,180
NG75	152,782	na
L50	354	269
LG50	134	na
L75	750	612
LG75	243	na
# N's per 100 kbp	0.2	0.23

N50 = 50% of the total assembled sequences is on contigs larger than that size.

NG50 = The size of the smallest contig in the set of largest contigs that make up 50% of the estimated genome size.

N75 = 75% of the total assembled sequences is on contigs larger than that size.

NG75 = The size of the smallest contig in the set of largest contigs that make up 75% of the estimated genome size.

L50 = Count of the smallest number of contigs whose length makes up 50% of the genome assembly size.

LG50 = Count of the smallest number of contigs whose length makes up 50% of the estimated genome size.

L75 = Count of the smallest number of contigs whose length makes up 75% of the genome assembly size.

LG75 = Count of the smallest number of contigs whose length makes up 75% of the estimated genome size.

na = not available

Following Repeat Masker, a majority of the TEs were classified as LTR 28.32% (Table 4). When LTR repeats (40,828,212 bp) were removed, the resulting total genome length was 104,986,831 bp. The collapse of abundant repeats of rust genome in short read assembly often obscures their "true" genome size. The *de novo* assembly resulted in a total interspersed repeats content of 72.77%. RNAmmer (Lagesen et al., 2007) and tRNAScan (Chan & Lowe, 2019) identified 13 rRNA and 1007 tRNA, respectively. DNA elements accounted for 9.70%, and 34.20% remained unclassified. Gene prediction was achieved *de novo* in Gensas with GeneMarkes and resulted in 10,357 predicted genes.

The gene prediction from the BRAKER pipeline identified 20,438 genes model and gave the most prediction. However, the gene prediction with evidence file from *P. striiformis* resulted in 5,536 predicted features.

The hybrid assembly and gene predictions were conducted *de novo*, resulting in lower gene identification than in the assembly by Rochi et al. (2018).

TABLE 4. Proportions of repeated sequence content in *P. sorghi* hybrid assembly.

Repeat class	**Percentage (%)**
SINEs	0
LINES	0.55
LTR elements	28.32
DNA elements	9.70
Unclassified	34.20
Total Interspersed repeats	72.77
Small RNA	0
Satellites	0
Simple repeats	0.42
Low complexity	0

DISCUSSION

Diversity in pathogen populations is the primary cause of resistance breakdown in hundreds of commercial crops (Schumann & D'Arcy, 2012). The *P. sorghi* inoculum load found in Australia is likely to drive the emergence of new virulent strains. Phenotyping of the five most diverse isolates demonstrated that the rust population is diverse in Eastern Australia. The effect of the isolates not only varied on a genetic basis for their maize hosts, but also between 2018 and 2019, i.e., not all isolates produced urediniospores at 8 DPI on all the maize lines. Most isolates produced some pustules on all maize lines. The risk of the occurrence of a beneficial mutation in the pathogen remains low, at infection levels of 1 to 30%. The phenotyping data also suggest that virulence patterns were not correlated to a particular agro-ecological region. The level of diversity within a field was not examined.

Maintaining an up-to-date and well characterised collection of *P. sorghi* isolates with different virulence profiles is critical to assess the resistance in elite breeding lines and novel commercial lines. The data from the long-read sequence and mapped short-reads of the 17 isolates can assist with the identification of *P. sorghi* secretomes and candidate effectors. For instance, it was suspected that the gene prediction of 20,438 genes could potentially be higher, if the gene identification had not been conducted *de novo* and more evidence files from other fungi had been used.

The diversity observed within the population of *P. sorghi* in Australia suggests that maize breeding programs are at higher risks of experiencing the break-down of monogenic resistance in the short to medium term. Although gene pyramiding offers great hope to increase the longevity of resistance in commercial crops, stacking an increasing number of resistance genes into one line comes at the expense of other desirable traits and is time consuming and costly. In maize, resistance genes located in the *Rp1* complex may have the ability to create new resistance specificities as

rapidly as *P. sorghi* engenders new virulence. The level of diversity in the *P. sorghi* population in Australia has the potential to overcome any newly deployed resistance genes soon after they are deployed in the field. The likelihood that one spore may carry a beneficial mutation for the pathogen is 10^7 per day, per hectare and under optimal conditions for disease development (Stam & McDonald, 2018). According to Stam and McDonald (2018), the likelihood that a spore carries mutations that would overcome two *R* genes is $10^{-6} \times 10^{-6} = 10^{-12}$, representing 10 double mutants per day, per hectare. And if three genes are to be overcome, one mutant would be found in every 10,000 hectares of infected maize fields. However, for the above to be true, the pathogen must be avirulent to all three resistance genes. In the lines carrying three resistance genes *RpGDJ* (*Rp1_D, Rp1_G* and *Rp1_J*), *RpGJF* (*Rp1_G, Rp1_J* and *Rp1_F*) and four resistance genes *Rp5GCJ* (*Rp5, Rp1_C, Rp1_G* and *Rp1_J*), only two resistance genes were effective, i.e., *Rp1_G* and *Rp5* (Quade et al., 2021). All other genes were ineffective. It is unknown whether the combination of those genes into one plant would have a synergistic effect and provides durable resistance. This leaves the maize industry in Australia with few candidates for long-term protection.

The high level of genetic diversity among genotypes in 2018 suggests that the population of *P. sorghi* in Eastern Australia is genetically diverse and may also supports the theory of long-distance dispersal of genotypes between states. In 2018, weather conditions were not conducive to disease development, and yet sporulation occurred and three distinct clades were identified following mapping (Fig. 2 and Fig. 3). We previously thought that the three clades would group rust isolates collected from the same field and/or in the same year. However, this was not the case. For instance, Isolates 9, 12 and 13 from VIC belonged to Clade 1 together with Isolate 58, which was collected from QLD two months later. Clade 2 comprised the most diverse group of isolates. Although most isolates in Clade 2 originated from QLD, except for Isolate 51, they all came from different locations of

diverse agroecology. Clade 3 comprised of two isolates from VIC (Isolates 15 and 73) and isolates from QLD (Isolates 1 and 56). Isolate 15 was expected to be genetically more closely related to Isolates 9, 12, and 13, but belonged to Clade 3 together with Isolates 1, 56, and 73. Isolates 15 and 73 originated from different hosts. As demonstrated by other studies (Shah & Dillard, 2004), *P. sorghi* infects maize and sweet corn indiscriminately. Although collected 14 months apart, isolates 15 and 73 also originated from the same field. It is suspected that individual strains may persist from one year to the next, but this remains to be confirmed.

Such high levels of genotypic diversity and lack of clones in the isolates suggest that the *P. sorghi* population in Australia may undergo sexual reproduction and/or experience incursions from overseas. The alternative host, *Oxalis* spp., is present in Australia. If the sexual cycle is not active in Australia, it would have been expected that clonally produced isolates would group within a clade based on year and/or location. It was originally hypothesised that rust isolates collected from the same field or the same region would belong to the same cluster, but this was obviously not the case. Surprisingly, these genetic differences were observed even in a year of reduced disease pressure and therefore low sporulation. In years of environmental condition being conducive to urediniospore production, it is expected that the likelihood of virulence favourable to the pathogen will increase, as the number of spores produced increases. The results also warrant a closer look at the occurrence of the alternate host, *oxalis* spp, growing in maize producing area in Australia.

Isolates with emerging virulence will have the potential to produce more spores and spread from new infection sites. The question remains as to why Isolate 58 from QLD belonged to the same clade along three isolates from VIC, but Isolate 51 from VIC was in the Clade 2 with isolates from QLD. It is unclear whether these isolates migrated in wind currents or human intervention transported them from one state to the next.

The hybrid assembly resulted in an increase in read length. Rochi et al. (2018) obtained a genome size of 78 Mb for *P. sorghi* after assembly with SPAdes, once most of the reads had been assembled. A total length of 145,815,044 bp (≥ 1000 bp) was obtained. This is similar to long-read based on *de novo* assemblies of several other rust fungi, for example, *P. triticina* 140 Mb (Wu et al., 2020), *P. coronata* f. sp. *avenae* 99.2 Mb (Miller et al., 2018), and Rochi et al. (2018) estimated that the genome size of *P. sorghi* 102 Mb. The higher percentage of LTR found by Rochi et al. (2018) (15.76%) compared to 28.32% in the findings of this research suggests that the short-read assembly is less effective in handling repeat regions. The repetitive nature of the genome of rust species limits the ability to estimate their true genome size based on assembled sequence data. LTR are responsible, amongst other mechanisms, for the integration of the newly created cDNA into another location and contribute to genome plasticity. DNA elements accounted for 9.70% of the repeats, compared to 7.41% for Rochi et al. (2018). However, it was unclear why 34.20% remained unclassified, compared to 22.13% in the study by Rochi et al. (2018) and 16.02% in the study by Miller et al (2018). It is suspected that the higher quality genome assembly resulted in the identification of a greater number of repeat regions that are yet to be categorised. The total interspersed repeat (IR) content of 72.77% found inthe *de novo* assembly is close to that found by Tobias et al. (2020) in their study of *Austropuccinia psidii* with over 80% IR.

Maize breeding programs are urged to orientate their research towards multigenic resistance and/or quantitative resistance. The likelihood of single gene to continue to provide effective resistance is extremely low considering the diversity present in the Australian population of *P. sorghi.* Ongoing monitoring of the rust population across maize growing regions to make informed decisions on which maize lines to be removed from the market as soon as they become susceptible to prevent exponential spore production. A targeted effort to understand the role of the alternate host,

Oxalis spp., in the emergence of new pathotypes is required. Virulence studies will also help us understand the origin of new virulence and alert us to potential overseas incursions, i.e.: transported by human and/or wind currents.

ACKNOWLEDGEMENTS

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5.2 Links and implications

Preliminary phenotyping studies revealed that all five isolates infected the maize line carrying known resistance genes differently. Genotyping of 17 isolates of CR collected across Eastern Australia confirmed that population diversity was great. Therefore, maize breeding programs cannot rely on monogenic resistance and should carefully select compound resistance genes for durable resistance.

CHAPTER 6: DISCUSSION AND CONCLUSION

The pressure imposed by biotrophy on rust fungi has shaped them to become some of the most successful pathogens. Their ability to adapt to a constantly evolving environment and the deployment of host resistance in agriculture has also made them some of the most economically damaging pathogens worldwide.

This research determined that undried urediniospores had an 87% increase in germination compared to dried spores and as such were suitable for long-term cold storage of isolates. Glasshouse conditions did not provide extra moisture and urediniospores did not require further drying. Further testing is necessary before placing spores in long-term storage, as glasshouse environments can be variable. Urediniospores of *P. sorghi* were successfully stored at -80°C for 24 months and remained infectious. Further testing should be conducted to study the viability and infectivity of *P. sorghi* urediniospore after 24 months. Unlike other wheat rust species, *P. sorghi* did not require cold shock in liquid nitrogen pre-cold storage, nor a heat shock to induce/revive them from dormancy. Urediniospore removed from a -80°C freezer germinated readily between 22°C and 55°C.

The resistance of the current commercial lines grown in Easter Australia were found to be overcome by *Puccinia sorghi*. These lines do not carry effective resistance genes and are a likely source of urediniospores production in all maize agro-ecological regions. The high spore load maintained across agro-ecological regions is likely to make the deployment of genes for resistance more challenging. In the wheat-rust pathosystem, susceptible lines are removed from commercialisation and replaced with lines possessing effective resistance (Park, 2015a). This prevents inoculum build-up and reduces the likelihood of mutations beneficial to the pathogen

to arise. However, no such monitoring is conducted for the common rust population.

No information was found regarding the ability of *P. sorghi* to infect the current commercial maize lines, nor regarding the resistance genes they carry. Though, no recurrent epidemics were observed in the field during 2018-2020, this was most likely due to environmental conditions not being conducive to disease development. Glasshouse trials showed that compatibility between the main commercial lines and 14 isolates obtained from different agro-ecological regions in Eastern Australia. All commercial lines tested were susceptible to all isolates. The level of infection observed will not only affect the maize industry, but also the sweet corn industry. Sweet corn is a horticultural commodity, whose cobs are consumed fresh and the industry standards for human consumption require that no pustules be found on the husk and the kernels.

The major source of resistance to common rust in maize and sweet corn, *Rp1_D*, was ineffective to 7 out of the 14 isolates tested and all isolates produced pustules. The seven isolates, virulent on *Rp1_D,* originated in Queensland and southern Victoria, showing that virulence is already present in Northern and Southern maize growing regions. It is certain that if lines carrying *Rp1_D* continue to be planted, the population of common rust carrying virulence to *Rp1_D* will reach 100 % of the population in a relatively short period of time. The resistance of newly commercialised maize line must be monitored by assessing the effectiveness of the gene(s) they carry. In the short term, maize line carrying ineffective genes can be removed from commercialisation. In the long-term, the ongoing collection of data in Eastern Australia would support modelling of virulence occurrence and likely spread. The virulence to *Rp1_D* also appears to be ongoing as virulence was found in 2018 and again in 2019. This suggests that once a virulent pathotype emerges it will persist in subsequent years.

Eight lines carrying single or compound resistance genes were identified as suitable candidates for resistance breeding: *Rp1_E, Rp1_I, Rp1_L, Rp1_G,*

Rp5, RpGDJ, RpGFJ and *Rp5GCJ*. These genes were effective against all isolates retrieved between 2018 and 2020. In the short to medium term, introgressing these resistance genes into maize lines carrying other desirable traits is critical:

1)to prevent yield losses and to maintain productivity,

2)to prevent spore production and disrupt the disease life cycle and,

3)to reduce the likelihood of a new infective pathogen strains emerging.

The eight lines identified as carrying effective resistance may not be candidates for long-term resistance. The effective resistance identified in some single genes has been defeated in other countries. For instance, *Rp5* and *Rp1_G* rapidly became ineffective in North America (Hulbert et al, 1995), suggesting that they may not be durable in Australia. Compound resistance is more likely to be durable than monogenic resistance, if the resistance genes have a synergistic effect and when faced with a stable pathogen population. The likelihood of a spore carrying a beneficial mutation is in the order of 10^{7} per day, per hectare under optimal conditions for disease development (Stam & McDonald, 2018). According to Stam and McDonald (2018), the likelihood that a spore carries mutations that would overcome 2 *R* genes is $10^{-6} \times 10^{-6} = 10^{-12}$, representing 10 double mutants per day, per hectare. And if three genes, are to be overcome one mutant would be found in every 10 000 hectares of infected maize fields. However, all three resistance genes must be effective. The maize lines with the compound resistance genes *RpGDJ, RpGFJ* and *Rp5GCJ,* carried at most two fully resistant genes. Indeed, *Rp1_J* was ineffective to all five isolates, and *Rp1_D, Rp1_F and Rp1_C* were identified as suitable differential lines to assess the virulence of *P. sorghi.* The former three lines had compatible reaction types with 1 and 2 isolates out of the 5 tested, respectively. Furthermore, an IT of 1 was recorded on the eight candidate lines for resistance at 20 DPI. Undoubtedly, the extreme disease pressure in a glasshouse environment is the likely cause of uredinia development. If the

spores produced had been collected, increased and re-inoculated onto these 8 lines several times:

1) What would have been the IT after the second or third round?

2) What would have been the percent infection?

If a deployed resistance gene is likely to break down, we should aim at finding and introgressing new resistance genes suited to the Australian maize industry that are effective against an ever-evolving *P. sorghi* population.

Maize is an outcrossing species, and unlike wheat and barley, possesses complex resistance loci. Several resistance genes, varying copies of genes and allelic diversity all contribute to frequent gene and allele recombination. In this highly unstable resistance complex, "ineffective genes" can still contribute to the creation of novel specificities by mispairing during gene reassortment. The lines carrying *Rp1_J, Rp1_M, Rp7, Rp8_A, Rp8_B* and *Rpp9* were ineffective against the five isolates and since 1991 in other countries. They may contribute to the creation of novel resistance specificities and/or differential lines, whose resistance varies based on isolate.

From a plant pathology perspective, it is important to maintain a diverse genetic base of maize with reactions ranging from resistant, to intermediate and susceptible. However, it must be pointed out that IT, which determines whether a pathogen and its host are compatible, is not correlated to percent infection, i.e., potential yield losses. In some instances, incompatibility was detected, however a high percentage of infections were recorded. For instance, *Rp1_D* recorded IT of 2 with Isolate 1, 9, 24 and 31, however, percent infections were 50%, 48%, 24% and 58% respectively. These percentages would account for yield losses of 30%, 28.8%, 14.4% and 34.8%, respectively. It is therefore recommended that the resistance genes deployed for agricultural purposes be scored on percent infection and infection type.

The high percentage of infection on the susceptible maize lines was observed on 3-week-old seedlings. The adult plant experiment indicated that as susceptible maize plants mature, lower disease levels were observed. This is in contrast with field observations where high disease incidence and severity are observed as the crop canopy closes, creating a suitable environment for disease development. Interestingly, an IT score of 3 remained on all growth stages. Adult plant resistance is an added crop protection strategy and planting outside the disease window of opportunity may only diminish the risk of infection, it will not eliminate that risk.

Compound (multiple) resistance genes gave the highest levels of protection across a large suit of isolates. However, the durability of these genes is guaranteed only if the CR population is and remains stable. The durability of resistance genes lies in the biotic interaction between the host and the pathogen. Often plants resistance loci do not have the capacity to engender new resistance specificities to match the new virulence of the pathogen. However, the pathosystem between *P. sorghi* and maize is dynamic and both have the potential to evolve as rapidly as the other.

The high level of urediniospores load is likely to result in new virulent isolates. Diversity in pathogen populations is the primary cause of resistance break down of hundreds of commercial crops (Agrios, 2005). Preliminary phenotyping studies revealed that some variation was present within *P. sorghi* populations. Differences in virulence over time for five isolates were observed on all the maize lines carrying known *Rp* genes. Worryingly, the phenotyping data suggests that virulence patterns were not correlated to a particular agro-ecological region and appears to be the results of regional diversity. The diversity within a field was not examined; however, this could shed light on diversity at the micro-level.

The molecular work conducted on the first isolate collected in 2018 and subsequent isolates confirmed that *P. sorghi* has the potential to mutate and overcome resistance genes as rapidly as they are deployed. The data from the long read and mapped short read of the 17 isolates can assist with

the identification of *P. sorghi* secretomes and candidate effectors. It is suspected that the gene prediction of 10,357 could potentially be higher. It was unclear whether some genes may belong to *P. sorghi* only and therefore have not yet been characterised for that species. Similarly, the total TE of 72% is near the 80% found for *Austropuccinia psidii* by Tobias et al. (2020), above the 58.39% found for *P. triticina* (Wu et al., 2020) and above the 33% found for *P. sorghi* by Rochi et al. (2018). Again, it was not possible to distinguish between limited genomic data for *P. sorghi* or assembly errors.

The Australian *P. sorghi* population has the potential to overcome any newly deployed resistance gene soon after they are deployed. This leaves the maize industry in Australia with few options for durable protection. The likelihood of single genes remaining effective is extremely low considering the diversity present in the Australian population of *P. sorghi.* There needs to be an ongoing monitoring of the population across several maize growing regions to inform breeders and deployment of resistance genes, and to remove maize lines from production as soon as they become susceptible to prevent exponential spore production. Virulence studies would also help us understand the origin of new virulence, whether from Australia or coming from overseas incursion.

In conclusion, it was demonstrated that:

1- *Puccinia sorghi* urediniospores were present during the three-year study. Two years of which were in drought condition. Though spore levels were not causing significant yield losses, it is a worry that spores were produced, nonetheless. As soon as weather conditions become suitable for disease outbreaks, yield losses of up to 40% will no doubt occur if inoculum persists in the maize growing regions.

2- *Puccinia sorghi* urediniospores were successfully stored for 24 months while maintaining germinability and infectivity.

3- The commercial maize lines grown across Eastern Australia do not carry resistance to *P. sorghi* and are a source of urediniospore production.

4- Maize lines were identified that carried effective resistance genes to Australian isolates collected from six agro-ecological regions in Eastern Australia.

5- The level of variation within the common rust population has the potential to impact the longevity of the deployed resistance genes.

The following recommendations are aimed at implementing strategies to control maize common rust in the short, medium and long term.

The sweet corn and the maize industries must combine their efforts to control maize common rust. The high levels of infection found in maize are affecting the efficacy and durability of resistance genes deployed in sweet corn lines. The high infection levels found in maize are acting as a source of new isolates carrying new virulence. Furthermore, concerted efforts and control programs are required to maintain qualitative and quantitative export targets.

Currently, maize growers have neither fungicides nor resistant maize lines available for the control of maize common rust. There is a need to register a chemical to prevent the development of uredinia early in the infection process. Perhaps the labels of existing fungicides, applied on wheat and barley rusts, could be extended to maize common rust.

Suitable candidate genes for resistance that are readily available to introgress into new commercial lines were identified. However, the effectiveness and durability of candidate genes must also be assessed. It was not determined whether the three to four resistance genes carried by some maize lines would have a synergistic effect or if the resistance is only provided by one, or at most two genes.

More research is required to understand the source of variation of *P. sorghi* in Australia. It is unclear whether sexual reproduction and/or high mutation

rate at the clonal stage are responsible. Furthermore, we must identify the spore source of inoculum from one maize growing season to the next. Are the early season epidemics caused by aecial spores and/or urediniospores and where is the source of either spore stages? It is possible that spores originating from neighbouring countries initiate epidemics in Australia. If so, we must work in close collaboration with growers and breeders to control inoculum load. Under tropical conditions, where maize is grown all year round, resistance genes are overcome within two to three years of commercial release under high disease pressure.

The ongoing monitoring of wheat stem rust pathotypes has allowed the Australian wheat industry to flourish with no major epidemics since 1973 (Park, 2007). The careful rotation between resistance genes, before they are completely overcome, prevented the production of urediniospores and the emergence of new infective pathotypes. In the long-term, increasing the pool of available resistance genes would achieve comparable results for the maize and the sweet corn industries. The Maize Genetic Cooperation Stock Center carries a valuable collection of resistance genes against *P. sorghi*, most of the resistance genes developed where tested in the USA with endemic isolates and under their prevailing weather conditions. Australia should aim at creating and maintaining its own collection of maize lines carrying resistance genes against the Australian *P. sorghi* population and with efficacy tested under Australian conditions.

Resistance genes could be used in conjunction with biocontrol agents. Several strategies already exist such as parasitism, predatory and plant health enhancers. However, at present no effective biocontrol agents were available to control rust pathogen on plants. Though, no data could be found for the control of maize common rust, other research have been conducted on bacterial endophytes with potential as biocontrol agent in wheat rust (Huang & Pang, 2017). Biocontrol may not achieve a level of protection comparable to that of fungicidal chemicals, but they may help to

slow down epidemics in general and they have no harmful effects on the environment (Fravel, 2005).

Another avenue for research with no environmental impact would be to investigate virulence effectors in the rust population. The philosophy of plant protection, since the early days of agriculture, has been to select and breed for crops with increased resistance. Molecular advances of the last 20 years have enabled us to understand the genes and gene groups involved in the infection process. To attain durable resistance, the current philosophy must change to reduce the infectivity of plant pathogens, rather than increasing the resistance of the host plant. Such advances are already used to human advantage in animals, perhaps the same technology could be used against plant pathogens (Johnsson et al., 2019). The transmission of heritable elements such as male sterility in mosquitoes, has prevented the transmission of many human pathogens, saving the lives of millions of people. Recombinant DNA methods are the next generation technologies (Alphey et al., 2013), allowing the creation of powerful genetic systems, where rust pathogen of major crops could be genetically engineered to lose their infectivity or increase infectivity on weeds only.

This research successfully provided the maize industry with the necessary information to support its ongoing productivity and profitability. Implementing the strategies for the control of CR in the short, medium and long term, as proposed in this report will no doubt help to manage the disease.

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