Unravelling the diversity and distribution of the soybean rust fungus *Phakopsora pachyrhizi* in East Africa

Harun Muthuri Murithi

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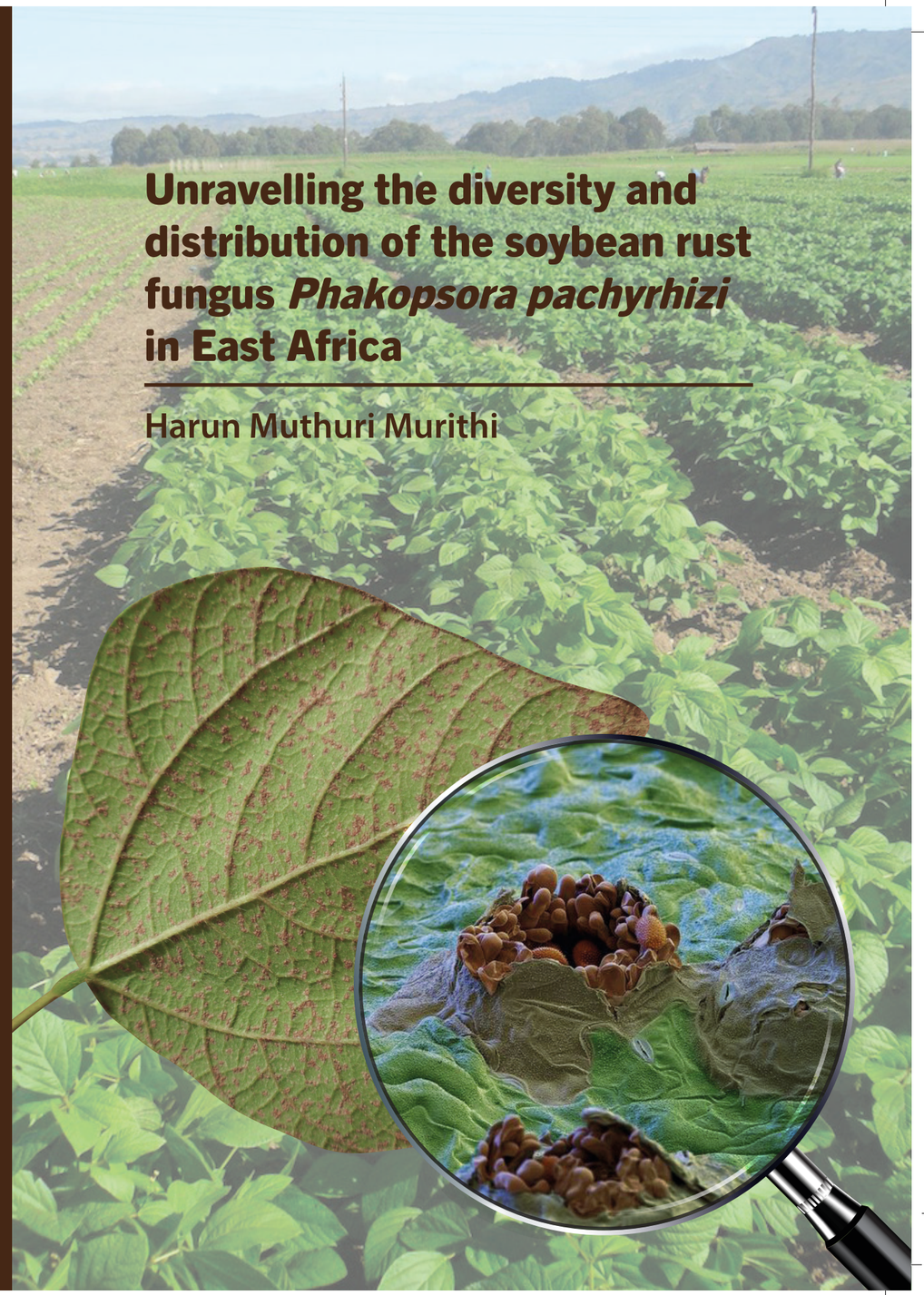

INVITATION

To attend the defence of
my PhD thesis entitled:

Unravelling the diversity and distribution of the soybean rust fungus *Phakopsora pachyrhizi* in East Africa

The defence will take place on
Monday
25th February 2019
at 1:30 pm
in the Aula of
Wageningen University
Generaal Foulkesweg 1a,
Wageningen

You are kindly invited to attend the reception that will be held in the Aula after the defence

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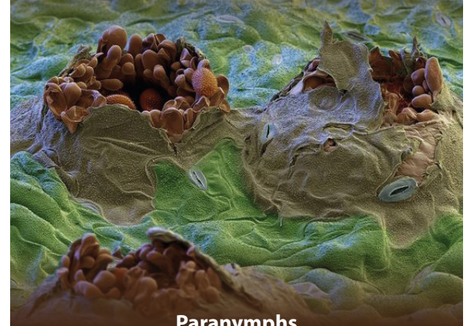

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Propositions

1. Deployment of soybean rust-resistance genes should depend on the structure of the local pathogen population rather than on a "one fits all" approach.
   (this thesis)

2. Stacking of three or more resistance genes may result in a long-term resistance to Phakopsora pachyrhizi, but for this approach a careful selection of the resistance gene combination is important.
   (this thesis)

3. To achieve food security and promote sustainable agriculture, small holder farmers in sub Saharan Africa must be empowered to adopt novel technologies and access resources that improve agricultural production.

4. Underestimation of prevalent asymptomatic infections hinders malaria elimination from Africa.

5. Open access publishing will enhance research in developing countries through access to high quality research information from across the globe.

6. The best time to plant a tree was 20 years ago, the second best time is now.

Propositions belonging to the thesis, entitled:
'Unravelling the diversity and distribution of the soybean rust fungus Phakopsora pachyrhizi in East Africa'

Harun Muthuri Murithi
Wageningen, 25th February 2019

Unravelling the diversity and distribution of the soybean rust fungus *Phakopsora pachyrhizi* in East Africa

Harun Muthuri Murithi

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This research was conducted under the auspices of the Graduate School Experimental Plant Sciences (EPS).

Unravelling the diversity and distribution of the soybean rust fungus *Phakopsora pachyrhizi* in East Africa

Harun Muthuri Murithi

Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr A. P. J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Monday February 25, 2019

at 1.30 p.m. in the Aula.

Harun Muthuri Murithi

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CHAPTER 1

General introduction

INTRODUCTION

The bulk of nutritional requirement for humans is provided by only a few major crops. Unfortunately, all these crops are prone to abiotic and biotic constraints that can compromise food security, resulting in global food crises that have regularly occurred in the past (Goss *et al*., 2014; Flood 2010; Strange *et al*., 2005). Biotic stress agents, including bacteria, fungi, oomycetes and viruses, cause significant yield losses in crops worldwide. Disease epidemics threaten food security and crop failures affect incomes, especially for the resource-poor smallholder farmers (Flood 2010). Globalization, increased international trade and climate change are some of the factors contributing to the introduction and spread of plant pathogens. For instance, the arrival of the oomycete pathogen *Phytophthora infestans* into Ireland, coupled with the practice of monoculture, led to a severe late blight epidemic on potato in the 19th century resulting into a massive food crisis that affected more than three million people. Nowadays, the emergence and rapid spread of a virulent isolate (Ug99) of the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* from Africa to the Middle East and further afield, poses a significant threat to food security (Singh *et al*., 2015). The production of major food crops in Africa, such as cassava and banana, is also significantly threatened by pathogens. For instance, the outbreak of the tropical race 4 of the fungus *Fusarium oxysporum* f. sp. *cubense* that causes Fusarium wilt, threatens banana production in Mozambique (Butler, 2013) and in other banana-growing countries in Africa (Garcia-Bastidas *et al*., 2014). Likewise, the rapid spread of cassava brown streak disease in East and Central Africa (Mulimbi *et al*., 2012), combined with its potential to move into west Africa, threatens cassava production (Casinga *et al*., 2018; Tomlinson *et al*., 2018; Patil *et al*., 2015). Knowledge on the pathogen distribution and methods for a rapid diagnosis of the disease are a prerequisite for efficient disease management. Furthermore, a better understanding of the mechanisms governing the interactions of pathogens with their hosts, is required in order to guide on how to best manage the threats posed by those pathogens.

Soybean rust; a threat to soybean production

The production of soybean (*Glycine max* (L). Merr), a leguminous plant that contains 40% of protein and 20% of oil in its seeds (Ali *et al*., 2010), has increased world-wide over the years. Currently, the global annual soybean production is approximately 335 million tonnes (FAOSTAT, 2018) (Fig. 1). In sub-Saharan Africa, soybean production is rapidly increasing as it

provides valuable protein and oil, and forms a significant potential as a source of income to more than six million households (Rusike *et al*., 2013; Abate *et al*., 2012).

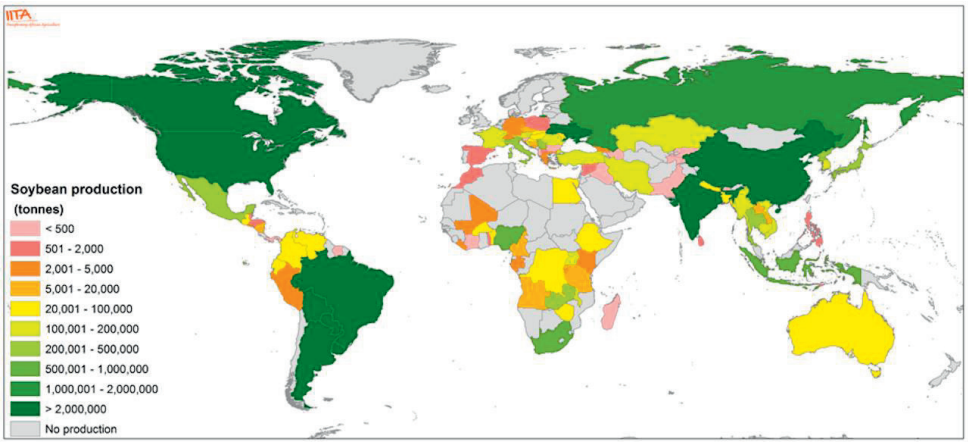

Figure 1. World soybean production by country (FAOSTAT, 2018). See text for details.

At least 25 countries produce soybean in Africa, amounting to about 2.1 million tonnes annually (Fig. 2) (FAOSTAT, 2018). However, the potential for the expansion of soybean production is threatened by numerous biotic and abiotic constrains (Wrather *et al*., 1997). One of such threats is formed by the plant-pathogenic basidiomycete fungi *Phakopsora pachyrhizi* (Sydow) and *Phakopsora meibomiae* (Arthur) that cause rust on soybean plants. *P. pachyrhizi* is the more aggressive of the two and is found in the eastern and western hemispheres. *P. meibomiae* is less aggressive and is restricted to the western hemisphere (Ono *et al*., 1992). The two fungal species can be distinguished based on their morphology and the characteristics of their internal transcribed spacer (ITS) regions within their ribosomal DNA (Fredrick *et al*., 2002).

P. pachyrhizi causes yield losses of 10-80% (Akinsami *et al*., 2001; Pretorius *et al*., 2001; Levy, 2005; Yorinori, 2005; Oloka, *et al.*, 2008). These severe yield losses are due to the numerous lesions formed on the infected soybean leaves that reduce photosynthetic efficiency (Fig. 3A). Mild infections result in a decreased size of the plant, lower numbers of seeds per pod and empty pods (Fig. 3B), while severe infection results in a complete defoliation of the plant, often with no seed production at all (Kumundini *et al.*, 2008). In 2006,

soybean rust accounted for the highest percentage of yield loss among other soybean diseases in the top eight of soybean-producing countries (Wrather *et al*., 2010).

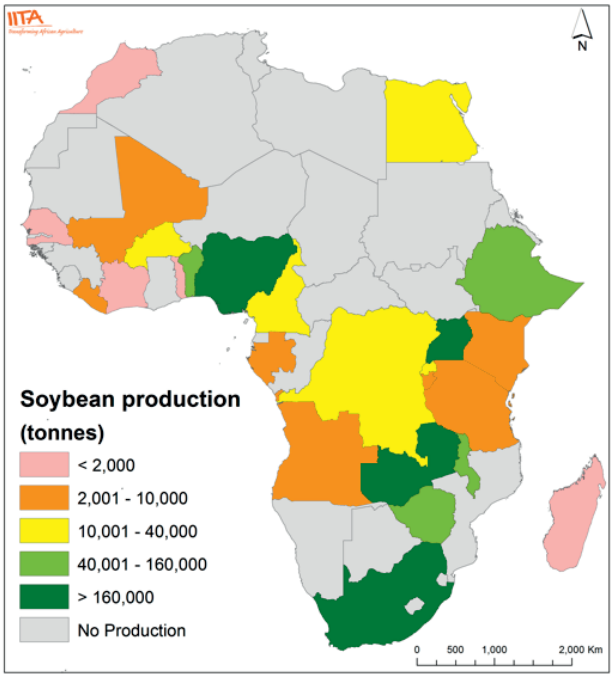

Figure 2. Map providing an overview of soybean production in Africa, (FAOSTAT, 2017).

The *P. pachyrhizi* infection process

Rust fungi may produce up to five different spore types during their life cycle if they are macrocyclic, namely pycniospores (spermatia), aeciospores, urediniospores, teliospores and basidiospores. The complete life cycle of macrocyclic rust fungi typically involves alternation between two unrelated hosts (Kolmer *et al*., 2009). Generally, haploid pycniospores are produced on the alternate host and after fertilization, the fungus produces dikaryotic aeciospores. These aeciospores infect the primary host and produce dikaryotic urediniospores that are involved in asexual reproduction of the rust fungus and contribute to further infections of primary host plants during the growing season. Towards the end of the season, when the weather conditions become unfavourable, dikaryotic teliospores are produced that are often melanised and form the resting stage of the rust fungus. At the start of the following season, the two nuclei in the teliospores fuse and subsequently undergo

meiosis, resulting in the production of haploid basidiospores that can again infect the alternate host to complete the life cycle (Zhao *et al*., 2016; Kolmer *et al*., 2009).

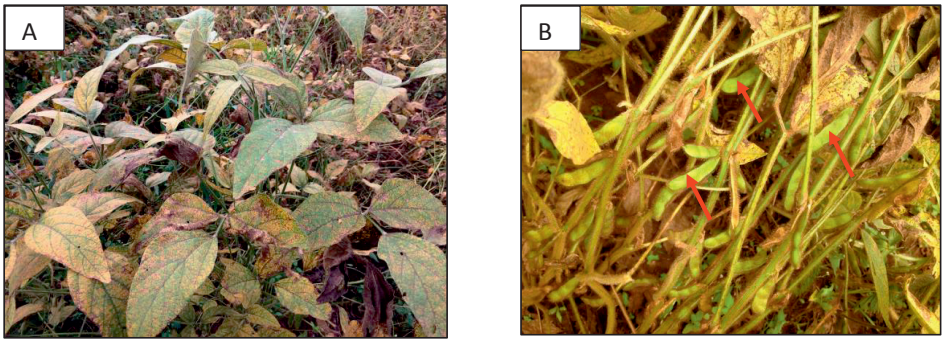

Figure 3. (A) Severely infected soybean leaves. (B) Empty pods (red arrows) associated with soybean rust infection.

Thus far, only three spore types have been observed for *P. pachyrhizi*, namely urediniospores, teliospores and basidiospores. Pycniospores and aeciospores have not yet been found, and thus this fungus is considered to be microcyclic (CABI, 2018). *P. pachyrhizi* urediniospores play an important role in disease development because, as explained above, they are the primary means of propagation and dispersal of the pathogen that are produced in large amounts. Uredinia develop on the lower side of the leaves and are pale brown, round-shaped with a central upper opening through which urediniospores are released. Upon landing on a suitable susceptible soybean plant and under suitable environmental conditions, urediniospores germinate to produce a germ tube that grows across the leaf surface and eventually forms an appressorium. With this appressorium, the fungus directly penetrates into an epidermal cell, using an appressorial peg similar to rice blast and powdery mildew pathogens. *P. pachyrhizi* utilizes a combination of mechanical force and cuticle-digesting enzymes to penetrate the soybean leaf surface. This infection strategy is a-typical for rust fungi, as they normally utilize stomatal penetration (Bonde *et al*., 1976; Goellner *et al*., 2010).

Teliospores have occasionally been described in Asia on soybean and on kudzu (*Pueraria* spp) (Harmon *et al*., 2006; Bromfield, 1984). They are typically heavily melanised and are usually produced to bridge unfavourable conditions in the absence of the host. *P. pachyrhizi* telia appear on the bottom side of the leaf as dark-brown to black specks (Harmon *et al*.,

2006). The teliospores have pale yellowish brown to colourless walls, about 1 µm thick or slightly thicker apically in the uppermost spore (Hartman *et al*., 2011). Although they are rarely found in nature, they have been successfully produced under controlled conditions (Saksirirat and Hoppe, 1991). Teliospores germinate to produce basidiospores that are thin-walled, hyaline and oval to cylindrical in shape. The role of basidiospores in the lifecycle of soybean rust remains unclear, since no alternate host has been identified (Goellner *et al*., 2010).

P. pachyrhizi distribution

P. pachyrhizi was first observed in Japan in 1902 (Hennings, 1903) and had spread to most of the countries of Asia and to Australia by 1934 (Bromfield, 1984). Early, unconfirmed reports of soybean rust in sub-Saharan Africa were posted in the 1960s and 1970s (Jarvaid and Ashraf, 1978; Levy, 2005). A number of confirmed disease cases were reported between 1996 and 2001 (Levy, 2005; Kawuki *et al*., 2003), followed by new reports of soybean rust in the Americas between 2001 and 2005 (Schneider *et al*., 2005; Yorinori *et al*., 2005). Urediniospores are small and light, and thus are perfectly adapted to long distance dispersal through wind currents, which can result in epidemics in new and disperse geographical locations (Isard *et al*., 2006). Spores of *P. pachyrhizi* may for example have arrived into the eastern coastal areas of Africa from Western India by north-eastern monsoon winds (Levy, 2005). Wind currents are also believed to have carried rust spores over long distances from South America into the United States through Hurricane Ivan (Isard *et al*., 2006).

The soybean – *P. pachyrhizi* interaction

Defence responses to pathogen invasion can be separated in two major phenomena. Plants are able to recognize conserved pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), with the help of transmembrane pattern recognition receptors (PRRs), which in most cases are receptor-like proteins (RLPs) or receptor-like kinases (RLKs) (Couto and Zipfel, 2016). Recognition of these patterns, such as fungal chitin or bacterial flagellin, leads to PAMP- or MAMP-triggered immunity (PTI or MTI), aimed at limiting further colonization of the host (Jones and Dangl, 2006). Besides PAMPs or MAMPs, plants are able to recognize particular virulence-related molecules that are secreted by the pathogen. These are so-called effectors, many of which are transferred to the cytoplasm to counter PTI/MTI (Jones and

Dangl, 2006; Dodds and Rathjen, 2010). Whereas successful suppression of PTI/MTI by effectors results in effector-triggered susceptibility (ETS), perception of these effectors by intracellular receptors, many of which contain a nucleotide binding site and leucine-rich repeats (NB-LRRs), leads to the activation of effector-triggered immunity (ETI) in resistant plants. ETI involves rapid and strong defence responses that may culminate into a hypersensitive response (HR), which is a form of programmed local cell death that is thought to prevent further host colonization. In response to the selection pressure that is posed on the pathogen upon effector recognition, pathogen lineages in which the gene that encodes the recognized effector is mutated or lost, or in which a novel effector evolved that is able to suppress the ETI that is triggered, may arise (Jones and Dangl, 2006). Such effectors again mediate ETS, while in the evolutionary arms race that takes place between the plant and the pathogen, the plant population will evolve novel resistance genes to recognize these novel effectors, again resulting in ETI (Jones and Dangl, 2006).

Effectors play a functional role in both pathogen attack and host immunity, and therefore understanding the processes that control (a)virulence and pathogenicity may eventually enhance our ability to protect crops from diseases. The advent of next generation sequencing (NGS) platforms and genome analysis tools has facilitated research into the molecular basis of plant-pathogen interactions. With RNA-Seq based on NGS, cost-efficient assembly, mapping and quantification of transcriptomes has become feasible (Wang *et al*., 2009). Using RNA-Seq, effector candidates from various fungal rust pathogens have now been identified (Saunders *et al*., 2012; Cantu *et al*., 2013; Nemri *et al*., 2014; Petre *et al*., 2016; de Carvalho, *et al*. 2017).

Soybean resistance genes against *P. pachyrhizi*

Resistance of soybean to *P. pachyrhizi* is governed by so-called *Rpp* genes, for resistance to *P. pachyrhizi*, which genetically interact with pathogen avirulence genes to activate ETI (Dodds & Rathjen, 2010). The interaction between the various soybean genotypes and *P. pachyrhizi* isolates is both plant genotype- and pathogen pathotype-specific, suggesting that the fungus is rapidly evolving in a host-specific manner (Bromfield, 1984; Hartman *et al*., 2011). This strict co-evolution between plant and pathogen has resulted in *P. pachyrhizi* races that differ in virulence towards specific soybean genotypes carrying different resistance genes. The differential interaction between various *P. pachyrhizi* isolates and different soybean

genotypes was first demonstrated in 1966 in Taiwan, where nine different soybean rust isolates produced different lesion types on soybean and other legume hosts (Lin, 1966). A series of soybean lines containing *Rpp* resistance genes *Rpp1* to *Rpp7* (McLean & Byth, 1980; Bromfield & Hartwig, 1980; Bromfield & Melching, 1982; Hartwig, 1986; Garcia *et al*., 2008; Li *et al*., 2012; Childs *et al*., 2018), is currently being used for classification of *P. pachyrhizi* pathotypes. Evaluation of *P. pachyrhizi* virulence is based on the infection types that are produced by a particular isolate of the fungus on the differential soybean set carrying the various *Rpp* genes. Bromfield (1984) described the three common infection types that are generally observed as (i) an immune (IM) infection type without the formation of any visible lesions, (ii) a reddish brown (RB) infection type with no to little spore formation, and (iii) a TAN infection type with tan-coloured lesions with many uredinia and abundant sporulation. IM and RB infection types represent incompatibility of the interaction and a resistant host, while a TAN infection type is regarded as a compatible interaction and a susceptible host. Many *P. pachyrhizi* races/pathotypes have currently been reported worldwide based on their differential compatibility on the soybean differential set, demonstrating that the virulence spectrum of the fungus is highly variable and dynamic (Murithi *et al*., 2016; Hartman *et al*., 2011).

To date, only a few studies have examined the variability of *P. pachyrhizi* virulence on soybean in Africa. Seven different pathotypes were identified among isolates collected from three different agro-ecological zones in Nigeria (Twizeyimana *et al*. 2009), while three pathotypes were described among isolates collected in Uganda (Tukamuhabwa and Maphosa, 2012). Further studies with isolates obtained from other African countries are required to investigate the virulence variability that may exist among the rust populations in this region. This knowledge is important for soybean breeders, as they can use the information to develop region-specific soybean varieties with durable resistance.

P. pachyrhizi isolates from different regions have been shown to differ genetically using different markers, including microsatellites and the analysis of conserved DNA sequences (Anderson *et al*. 2008; Freire *et al*., 2008; Abate *et al*., 2012; Rocha *et al*., 2015; Twizeyimana *et al*., 2011). Sequencing of total RNA extracted from haustoria of *P. pachyrhizi*-infected leaves enabled the identification of putative *P. pachyrhizi* effector proteins (Link *et al*., 2014; Kunjeti *et al*., 2016), some of which have been further characterized (Qi *et al*., 2016). Candidate effector proteins were recently also discovered among the 851 putatively secreted

proteins identified in *P. pachyrhizi* urediniospores collected in Brazil (de Carvalho, *et al*. 2017). The knowledge gained from these studies enhances our understanding of the population structure of the fungus, which is important for developing durable resistance. For instance, effectors could be used for assessing the specificity of resistance genes to identify effective combinations for gene pyramiding to develop more durable resistance.

Soybean rust management

A number of different control strategies are currently being used against soybean rust, including cultural control, the use of resistant soybean cultivars and chemical control (Hartman *et al*., 2005). Fungicide application is the primary means of managing the fungus and mostly takes place prior to, or at, flowering of the soybean plants (Levy, 2005; Miles *et al*., 2007; Mueller *et al*., 2009). Fungicides provide protection and delay soybean epidemics, as long as they are applied timely and in sufficient concentrations. About two to three spray applications are required in one season (Levy, 2005; Mueller *et al*., 2009; Scherm *et al*., 2009). Although fungicides are widely used, they are expensive and thus contribute to an increase in the total costs of soybean production. For instance, in Brazil, the cost of fungicide treatment against soybean rust was approximately US$ 2.2 billion in the 2013/2014 season, up from US$ 177 million in 2001/2002 when rust was first detected (Godoy *et al*., 2016). The rapid development of fungicide resistance, arising from selection pressure on the rust fungus, leads to loss of fungicide efficacy and is of serious concern nowadays (Dorrance *et al*., 2008; Godoy *et al*., 2016). Furthermore, the use of fungicides poses environmental and health risks.

Cultural control measures comprise the adjustment of planting date (Twizeyimana *et al*., 2011) and plant density. A soybean-free period of between two to three months is currently being implemented in Brazil to try and break the fungal infection cycle and delay the onset of novel infections (Godoy *et al*., 2016). During this period, volunteer soybean is also destroyed to minimize the sources of inoculum.

Nutrition plays a major role in plant physiological functions and is also important for plant disease management. For example, the application of micronutrients such as silicon reduces soybean rust onset by three days (Rodrigues *et al*., 2009; Lemos *et al*. 2011). Silicon treatment slows down disease development by affecting direct penetration by appressoria at the initial stages of the disease epidemic. Silicon also enhances disease resistance in plants through increased accumulation of phytoalexins and phenolic compounds that form a chemical

penetration barrier (Lemos *et al*., 2011). Many other micronutrients, including zinc, molybdenum and boron are also used for soybean rust disease control (Dordas, 2008).

Beneficial microbes are widely used for controlling various plant diseases. Micro-organisms that are antagonistic to rust fungi have been identified and include *Bacillus* spp. (Dorighello *et al*., 2015), *Lecanicillium psalliotae* (Saksirirat & Hoppe, 1990)*, Simpilicillium lanosoniveum* (Ward, 2012) and *Tricothesium roseum* (Kumar and Jha, 2012). These microorganisms colonize urediniospores, interfere with uredinium development and inhibit spore germination. Eventually, they significantly reduce inoculum build up, limit reinfection and also delay disease development.

Among all other disease control methods, resistance breeding has been identified as the most effective and economically feasible solution, and this approach has been used for the management of soybean rust already for a long time (Hartman *et al.*, 2005; de Souza *et al*., 2011). However, the seven single *Rpp* genes confer resistance to only a limited set of *P. pachyrhizi* isolates, as resistance provided by some of these genes became ineffective within a short period after their introduction (Hartman *et al.*, 2005). Pathotype-specific resistance is often short-lived, as pathogens have the capacity to rapidly evolve into new populations that overcome the deployed resistance genes (Boyd, 2006).

Screening of additional soybean accessions to identify novel sources of resistance to *P. pachyrhizi* has been conducted over the last years. For example, testing of over 16,000 soybean accessions in the United States identified about 800 accessions as potential novel sources of resistance (Miles *et al*. 2006). Although similar studies have been conducted worldwide to identify new sources of soybean rust resistance (Miles *et al*., 2008; Twizeyimana *et al*., 2008; Oloka *et al*., 2008; Pham *et al*., 2010; Walker *et al*., 2011), no commercial varieties with adequate resistance levels are available yet. *P. pachyrhizi* has numerous alternative hosts among the legume family that might be potential sources of novel resistance genes, which can be introgressed into soybean. For instance, the recently discovered pigeon pea (*Cajanus cajan*) resistance gene *CcRpp1* was found to confer full resistance to more than 80 *P. pachyrhizi* isolates from Brazil, Japan and the United States (Kawashima *et al*., 2016). This implies that resistance genes from other legumes, outside the soybean gene pool, can be useful sources of resistance against *P. pachyrhizi*. Efforts to stack multiple resistance genes to obtain more durable resistance against *P. pachyrhizi* are also ongoing and promising results have been reported (Lemos *et al*., 2011; Maphosa *et al*., 2012; Yamanaka *et al*., 2015).

Furthermore, partial resistance has also been explored against soybean rust (Tschanz & Wang, 1985; Hartman *et al*., 2005). Whereas partial resistance does not completely halt colonization of the host by the pathogen and still allows the fungus to sporulate, it often reduces the damage that is caused and reduces yield losses. This approach is limited in its applications for resistance breeding, as it is tedious and difficult to incorporate into advanced breeding programs (Hartman *et al.*, 2011). An alternative to partial resistance is tolerance, which comprises selection of high-yielding soybean genotypes under severe disease conditions. Studies on tolerance to *P. pachyrhizi* have obtained considerable success and the method appears to be feasible for disease management (Kawuki *et al*., 2004; Jarvie and Shanahan, 2009; Oloka *et al*., 2009).

Non-host resistance has been explored as an alternative source for resistance to *P. pachyrhizi.* A plant is considered a non-host for a given pathogen when all known accessions of that plant species are resistant to all isolates of the pathogen (Bettgenhaeuser *et al*., 2014). Non-host resistance is non-race-specific and therefore it has been proposed to be more durable (Gill *et al*., 2015). *Arabidopsis thaliana*, a non-host weed and model plant that is widely used in academia, has been investigated as an alternative source of resistance to plant pathogens (Stein *et al*., 2006; Lipka *et al*., 2008). The transfer of particular immunity genes from *A. thaliana* to soybean plants was observed to enhance resistance to *P. pachyrhizi* under greenhouse conditions (Langenbach *et al.,* 2016).

The rapid spread of *P. pachyrhizi* across the globe necessitates the generation of knowledge on its dispersal and monitoring of its introduction into new areas. Despite the impact of rust on soybean production, there is only limited information on rust distribution, diversity of the pathogen in particular regions and management practices, especially in Africa. This knowledge is vital, as it can guide breeding for durable rust-resistant soybean varieties. Identifying the most dominant rust pathotypes, and their geographical distribution, in addition to evaluation of soybean germplasm and resistant cultivar development, will contribute to a more sustainable soybean production.

OVERALL GOAL AND OBJECTIVES OF THIS THESIS

The overall goal of this PhD study was to investigate the presence, distribution and virulence diversity of soybean rust in four countries in eastern Africa: Kenya, Malawi, Tanzania and Uganda. In addition, it was investigated whether virulence changes occur among soybean rust

populations collected in this region over different years, whether such diversity is country-specific, and whether there are dominant pathotypes present across countries.

To accomplish this goal, surveys were conducted in soybean fields across different agro-ecological areas, samples were collected from *P. pachyrhizi*-infected soybean plots and data were generated on the existing soybean rust diversity in the four eastern African countries. The virulence spectrum of the collected soybean rust isolates on a differential set of soybean plants carrying different (combinations of) *Rpp* genes was evaluated. Eventually, to gain more insight into the interaction between *P. pachyrhizi* and soybean, RNA-Seq was employed to compare the transcriptome of selected *P. pachyrhizi* isolates and candidate effectors were identified, possibly matching particular *Rpp* genes.

The knowledge gathered from this research should facilitate monitoring of the distribution of *P. pachyrhizi* pathotypes. Soybean breeders may use this information to develop and deploy resistant cultivars with specific resistance to dominant *P. pachyrhizi* pathotypes. Agronomists and pathologists can use this information for administering appropriate disease control measures. The results described in this thesis are expected to improve soybean rust management and should lead to increased soybean productivity in eastern Africa, resulting in improved livelihoods for smallholder farmers.

THESIS OUTLINE

After the introduction to the thesis given in **Chapter 1, Chapter 2** is an overview of the economic importance of the soybean crop, its production and utilization in eastern and southern Africa. The chapter highlights the constraints facing soybean production and focusses on the single, most important pathogen threatening soybean production: *P. pachyrhizi*. The epidemiology, host range and the status of virulence variation of the pathogen are presented. The chapter concludes by highlighting possible soybean rust management strategies and describes the current research that is conducted on the disease in eastern and southern Africa.

In **Chapter 3,** a differential set of ten soybean varieties that contain (combinations of) pathotype-specific *P. pachyrhizi* resistance genes was used to evaluate the virulence of 17 *P. pachyrhizi* isolates and compare this to the virulence of isolates from a global collection.

Chapter 4 examines the virulence diversity of *P. pachyrhizi* isolates obtained from single pustules and reports on the pathotype distribution in East Africa. A set of 11 soybean host

differential varieties was used to identify the different pathotypes that occur, in addition to their dominance in the region. The effectiveness of the *Rpp* genes against isolates collected in the region was also examined. This is the first study of its kind involving a large number of *P. pachyrhizi* isolates from multiple countries in East Africa. The study identified various pathotypes that break resistance in most of the soybean cultivars carrying known resistance genes.

In **Chapter 5**, soybean accessions from both local and international sources were screened at multiple locations in Tanzania and Uganda to identify rust resistance. Through this study, a number of resistant soybean cultivars was identified that can be deployed in regional soybean breeding programs.

A comparative analysis of the transcriptomes of ten differential *P. pachyrhizi* isolates is presented in **Chapter 6**. The ten isolates were selected based on their virulence spectrum on the soybean varieties carrying different (combinations of) *Rpp* genes. Total RNA was extracted from susceptible soybean that had been inoculated with the ten different isolates and was analysed by Illumina sequencing. A total of 7,061 sequences of putatively secreted proteins were generated for all the ten isolates and among those, proteins that can be regarded as products of potential avirulence gene candidates, possibly matching the *Rpp* genes, were identified.

Finally, **Chapter 7** discusses the major findings that are presented in this thesis and also puts forward a perspective on the distribution of the soybean rust pathogen, its dynamics and the diversity in its virulence, together with possible soybean rust management strategies for Africa and the rest of the world.

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CHAPTER 2

Soybean production in eastern and southern Africa and threat of yield loss due to soybean rust caused by *Phakopsora pachyrhizi*

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ABSTRACT

Soybean is a major source of oil and proteins worldwide. The demand for soybean has increased in Africa, driven by the growing feed industry for poultry, aquaculture and home consumption in the form of processed milk, baked beans and for blending with maize and wheat flour. Soybean, in addition to being a major source of cooking oil, is also used in other industrial processes such as in the production of paints and candle wax. The demand for soybean in Africa so far outweighs the supply, hence the deficit is mainly covered through imports of soybean products such as soybean meal. The area under soybean production has increased in response to the growing demand, a trend that is expected to continue in the coming years. As the production area increases, diseases and insect pests, declining soil fertility and other abiotic factors pose a major challenge. Soybean rust disease, caused by the fungus *Phakopsora pachyrhizi*, presents one of the major threats to soybean production in Africa due to its rapid spread as a result of the ease by which its spores are dispersed by the wind. Disease control by introducing resistant soybean varieties has been difficult due to the presence of different populations of the fungus that vary in pathogenicity, virulence and genetic composition. Improved understanding of the dynamics of rust ecology, epidemiology and population genetics will enhance the effectiveness of targeted interventions that, in turn, will safeguard soybean productivity.

Soybean: its general use and economic importance

Soybean (*Glycine max*) is an important legume plant that is cultivated all over the world, not only as a major source of oil and protein in livestock feeds but also for human consumption, soil fertility improvement and, amongst others, for producing industrial products such as soy inks, non-toxic adhesives, candles and paints (Hartman *et al*., 1999; 2011b). Soybean is produced on about 6% of the world's arable land, representing an estimated total area of more than 92.5 million ha, giving 217.6 million tonnes of production each year. Soybean has a high protein content (about 40%) of good nutritional quality, and a high oil content (about 20%) which, together with numerous beneficial nutrients and bioactive factors, make soybean the crop of choice for improving the diets of millions of people in developing countries (Ali, 2010). Soybean can be used blended with maize and wheat flour as a source of protein, or as soymilk, but soybean is also eaten as baked beans or in the form of soy paste or fermented soybean curd (tofu). Full-fat soy flour is used in bakery and dietetic foods and

in novel products, such as tofu-based ice cream and soybean yogurt. Due to its protein content it can help to reduce malnutrition among children and nursing mothers when incorporated into other meals, hence enhancing nutrition in the developing world. Additionally, soybean plays a major role in improving soil fertility due to its ability to fix in the range of 44–103 kg of atmospheric nitrogen per hectare per year, thereby alleviating the need to apply large amounts of nitrogen fertilizer (Sanginga *et al*., 2003). This advantage is especially important for crop production in Africa due to the economic limitations in the use of fertilizers.

Soybean and its derivatives are among the most important agricultural products traded in the world market. Global soybean production rose nearly ten-fold, from 27 million tonnes in 1961 to 276 million tonnes in 2013 (FAOSTAT, 2013). The USA is the leading producer of soybean, accounting for about 32% of the global production, followed closely by Brazil (29%) and Argentina (17%) (FAOSTAT, 2013). The USA is also the main exporter of soybean, accounting for 44% of global exports, followed by Brazil with 34%. China accounts for nearly 59% of the total world import of soybean, followed by the EU (16%). The world trade for the six major legumes was estimated to be more than $21.8 billion in export, with soybean accounting for 84% of the total, followed by common bean (8.8%), groundnut (4.9%) and chickpea (2.4%) (Abate *et al*., 2012). As a major source of oil and protein, soybean accounts for about 56 and 67% of the total global oilseed production and world supply of protein to be consumed, respectively (USDA, 2014).

Soybean diseases, such as bacterial pustule, frogeye leaf spot, red leaf blotch, soybean rust and bacterial blight have been reported to cause massive yield losses in sub-Saharan Africa (SSA) (Kawuki *et al*., 2003). Soybean rust is rapidly spreading and establishing in the eastern and southern African region, thereby threatening soybean production (Murithi *et al*., 2014). Soybean resistance is difficult to obtain, due to the high degree of genetic variability of the pathogen (Levy, 2005; Yorinori, 2008; Yamaoka *et al*., 2014). Currently, different pathotypes of the fungus have been described across the major soybean growing regions worldwide (Lin, 1966; Yeh, 1983; Bromfield, 1984; Twizeyimana *et al*., 2009; Akamatsu *et al*., 2013).

With the current massive increase of the area under soybean production, soybean rust is an important disease that cannot be ignored. This review highlights the current trends concerning soybean production and developments concerning soybean demand in the

eastern and southern African region. Here, the status of soybean rust and its diversity at a global and regional level are also reviewed. Furthermore, the current research being performed on soybean rust is described, together with the control measures that can be implemented to secure soybean yields in the eastern and southern African region.

Soybean production and use in Africa

Soybean production in Africa occupies 1.3% of the total world area under soybean production representing 0.6% of the total production. In 2011, soybean was planted on 1.1 million ha of land in SSA, which is approximately 1% of the total arable land. Major production is concentrated in South Africa, which is the leading producer in Africa, contributing about 35% of the total production, followed by Nigeria (27%) and Uganda (8.5%) (FAOSTAT, 2013). Zambia, Zimbabwe and Malawi also produce substantial amounts of soybean. About 6.8 million households in SSA, representing about 28.6 million people, grow soybean. Soybean production in this area is projected to grow from about 1.5 million tonnes in 2010 to about 2 million tonnes in 2020, representing a growth rate of 2.3% per annum, to meet the predicted demand (Abate *et al*., 2012). The major factors that are expected to drive soybean production include land availability, the investment by private equities, international developmental organizations and banks into corporate farms, growth of the poultry market and the development of household consumption (Technoserve, 2011b).

Soybean production in SSA has doubled over a period of 15 years, driven by significant increases in the land planted with soybeans over the years. The soybean market has also grown rapidly over the last decade, driven by the rapid growth of the poultry market and demand for household consumption. The demand outweighs the production, leading to increases in imports of soybean and soybean products from India, Argentina and Brazil. Imports of soybean into SSA in 2011 were estimated at nearly 1.6 million tonnes, valued at $1.22 billion. South Africa, Nigeria and Kenya account for nearly 43, 21 and 18% of the total import volume in this region, respectively. Other countries, including Ethiopia, Zambia, Zimbabwe, Seychelles, Botswana, Tanzania and Gabon also import significant amounts of soybean each year. Exports from Uganda and Zambia to the neighbouring countries are about 29,000 tonnes per year (Abate *et al*., 2012).

Soybean production and use in eastern and southern Africa

Soybean has now been identified as the most preferred legume across eastern and southern Africa, as compared to common bean and cowpea, based on its preference by growers (Rusike *et al*., 2013). Uganda is the leading producer of soybean in eastern Africa, with an increase in production from 158,000 tonnes in 2005 to 213,300 tonnes in 2011. During the same period, the area under production increased from 144,000 to 150,000 ha (FAOSTAT, 2011). The upward trend in production is attributed to improved soybean research by the government, learning institutions and developmental organizations, which has resulted in the release of high-yielding varieties with increased tolerance to diseases such as frogeye leaf spot, bacterial pustule and soybean rust. Uganda is now among the key exporters of soybean products at the regional markets. Furthermore, dissemination of soybean processing and cooking methods by non-governmental organizations among women's groups has facilitated the adoption of soybean among smallholder households. This has led to an increase in the use of soymilk and soy flour among households in Uganda.

There is a substantial demand for soybean and soybean products, amounting to about 150,000 tonnes per year, in Kenya where production is dominated by smallholder farmers (Chianu *et al*., 2009). This is mainly attributed to an increasing demand for human consumption and from the rapidly growing feed manufacturing industry (Rusike *et al*., 2013). Production increased from 2,000 tonnes in 2009 to about 4,500 tonnes in 2012 (FAOSTAT, 2012). The climatic conditions in Kenya are suitable for soybean production; however, the potential for soybean production is not maximized because cultivation takes place only in a few areas in the west and east, and in the Rift Valley only on a small scale (Chianu *et al*., 2009). Efforts by the government, developmental organizations and the private sector have led to an increase in interest in the crop among small-scale farmers and processors, especially in the western region.

In Rwanda, soybean is planted in an area of over 47,000 ha, producing about 37,000 tonnes (FAOSTAT, 2011). Here, about 62% of all households producing soybean consume their total harvest. Consumption is mainly through blending with maize, sorghum and cassava flour, as roasted beans, soymilk and as a paste mixed with local vegetables. A large-scale investment in a soybean oil extraction plant (now under construction in Rwanda), with a capacity of 36,000 tonnes of oil per year, is expected to further increase the demand for soybean in the region (Rusike *et al*., 2013).

In the past, a lack of links between producers and buyers in Tanzania resulted in production of soybean being abandoned. Recent efforts by developmental organizations to increase soybean productivity and to link farmers to the market have seen an increase in the number of farmers producing soybean (Wilson, 2015). A total of 4,000–8,000 tonnes of soybean is now produced annually, with production concentrating mainly in the Southern Highlands. Rising incomes and urbanization have contributed to the growing demand, with the feed industry accounting for about 150,000 tonnes annually. The demand far outweighs the supply, which is met by imports from neighbouring countries such as Zambia and Uganda. Soybean meal is also imported from India and Argentina (Rusike *et al*., 2013).

The soybean industry is well established in the southern African region, with a total production of 861,000 tonnes in 2010 and a demand of 2 million tonnes (Technoserve, 2011a). In this region the demand for soybean for human consumption is usually in the form of flavoured textured soy protein (TSP), made from edible grade defatted soybean flour containing 50% protein (Ali, 2010). UNICEF and the World Food Programme (WFP) purchase corn soya blend (CSB), which normally forms part of feeding programmes and is supplied to vulnerable groups such as children and nursing women. More than 600 tonnes of CSB produced monthly in South Africa comprises 75% maize and 24% extruded soybean, supplemented with vitamins. The growing demand for soybeans offers a significant opportunity for smallholder farmers to increase incomes (Lubungu *et al*., 2013).

South Africa dominates both production and demand in the southern African region, with production expanding rapidly over the past 5 years and with the area under production more than doubling compared to other major cereal and oilseed crops (NAMC report, 2011). Zambia is the second largest producer in the southern African region with a total production of about 260,000 tonnes and an estimated growth of 14% per annum (FAOSTAT, 2013). Soybean production is largely concentrated in the eastern, central and northern zones. Zambia is a net exporter of soybean, with about 45% exported to Zimbabwe and 10% to Botswana. Malawi and Mozambique have had rapid increases in soybean production as well, due to the involvement of the government, international research organizations and NGOs in these countries. Since 1997, soybean production has diffused into smallholder farming communities in Zimbabwe, helping to diversify cropping systems and to overcome soil fertility constraints. The rapidly expanding local and regional markets for soybean provide an opportunity for value addition and product diversification that can lead to better livelihoods

and nutrition (Giller *et al*., 2011). Overall, increase in soybean production and demand in eastern and southern Africa will continue with the rising of incomes, increased urbanization and expansion of the livestock sector to cater for the increasing demand of poultry and other livestock products.

Constraints to soybean production

Numerous biotic and abiotic constraints affect soybean production all over the world. Abiotic factors related to poor soil fertility, poor nodulation and seed longevity are the major problems in the tropics. Biotic factors, particularly diseases, insect pests and weeds, have consistently contributed to severe yield losses and affected the quality of soybeans. Among the important soybean diseases known worldwide are bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*), bacterial pustule (*Xanthomonas axanapodis* pv. *glycines*), wild fire (*Pseudomonas syringae*), anthracnose (*Colletotrichum truncatum*), brown spot (*Septoria glycines*), charcoal rot (*Macrophomina phaseolina*), downy mildew (*Peronospora manshurica*), frogeye leaf spot (*Cercospora sojina*), red leaf blotch (*Phoma glycinicola*), soybean rust (*Phakopsora pachyrhizi*) and rhizoctonia foliar blight (*Rhizoctonia solani*) (Wrather *et al*., 1997). Among them, soybean rust, bacterial pustule, frogeye leaf spot, red leaf blotch and bacterial blight have been identified as the major soybean diseases in SSA (Kawuki *et al*., 2003).

Soybean rust particularly has been singled out as a major threat to soybean production globally, and its entry and establishment in Africa has caused major yield losses (Levy, 2005; Oloka *et al*., 2008; Dean *et al*., 2012). As experienced in Brazil, Argentina and Paraguay, spread and further establishment of soybean rust is expected to increase as soybean production intensifies (Yorinori *et al*., 2005). Yield losses ranging from 10 to 90% have been reported across the globe (Akinsanmi *et al*., 2001; Levy, 2005; Yorinori *et al*., 2005; Oloka *et al*., 2008). This impact is linked to the high specialization and significant variation that exists in the population of this obligate pathogen, concerning its virulence on soybean cultivars carrying specific resistance genes. Soybean cultivars available so far lack durable resistance and growers are left with using fungicides to control the pathogen as the only option for disease control.

The impact of soybean rust is similar to that exhibited by wheat stem rust (*Puccinia graminis* f. sp. *tritici*) races in which virulence evolves so rapidly that host race specific

resistance genes generally exploited in plant breeding are usually overcome within 5 years after introduction of a resistant cultivar (Singh *et al*., 2011). The spread of soybean rust spores through wind currents (Isard *et al*., 2007) facilitates its movement and the pathogen can easily enter new soybean production areas, while the high variability of pathogenicity of this fungus makes it difficult to control by specific culture methods.

Spread, establishment and host range of soybean rust

Soybean rust can be caused by two obligate biotrophic *basidiomycete fungi: Phakopsora meibomiae* and *P. pachyrhizi*. *P. pachyrhizi* is more aggressive than *P. meibomiae* and has established in the eastern and western hemisphere due to its ability to sporulate profusely, thereby enhancing its dispersal (Bromfield, 1984; Miles *et al*., 2003). The less invasive fungus *P. meibomiae* has not been reported outside the Americas. Under favourable conditions, with a temperature in the range of 15–28°C and the presence of moisture on the leaf surface for a period of 6–12 h, uredinia develop 5–7 days after infection, while urediniospores can be produced 2 days later (Marchetti *et al*., 1979; Melching *et al*., 1989). A relative humidity of 75–80% is necessary for spore germination and leaf infection. A single pustule can produce hundreds of urediniospores continuously for about 3 weeks after the onset of sporulation. The urediniospores are then dispersed by wind, resulting in new infections near the initial disease focus. The urediniospores can also be transported over long distances by the wind and may remain viable in the air for many days, as long as they are protected from ultraviolet radiation by a cloud cover, resulting in new infections outside the local area (Goellner *et al*., 2010). The disease cycle continues until the plant is defoliated or environmental conditions no longer favour disease development. Teliospores have been reported on kudzu (*Pueraria* spp.), as well as on soybean (Yeh *et al*., 1982; Harmon *et al*., 2006). Teliospores are generally over-seasoning structures and have been germinated under laboratory conditions to produce basidiospores (Saksirirat & Hoppe, 1991). The importance of the telial stage in the development of soybean rust in the field is unknown. They are not generally considered the primary source of inoculum and are not often observed in the field (Ono *et al*., 1992; Tan *et al*., 2001).

Symptoms due to soybean rust infection may be observed at any developmental stage of the plant, but losses are mostly associated with infection at the flowering (R1) stage through to pod filling (R6) stage (Hartman *et al*., 1991). Symptoms that manifest on the lower side of

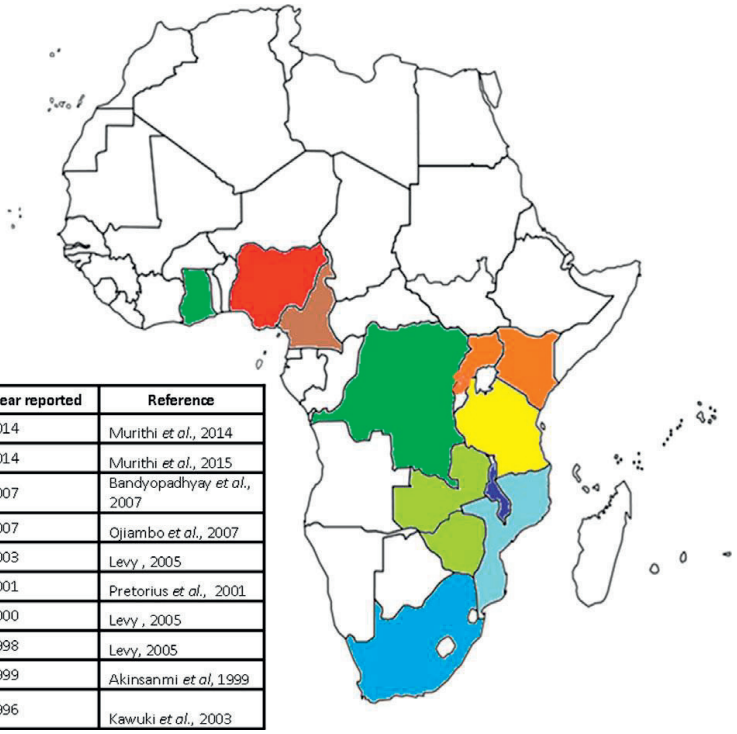
the leaf are usually grey-green, tan to dark brown, or reddish brown lesions with one or many yellowish brown to cream uredinia (Fig. 1; Ono *et al*., 1992; Hartman *et al*., 1999). Soybean rust infection lowers yields mainly through reducing the photosynthetic activity of the infected leaves. This is caused by a reduction in green leaf area due to lesion formation and premature defoliation, resulting in reduced dry matter accumulation, a decreased number of filled pods and a reduced size and weight of the seeds (Kumudini *et al*., 2008).

Figure 1. *Phakopsora pachyrhizi* symptoms as observed on soybean leaves. (a) Sporulation of *P. pachyrhizi* from uredinia on the lower side of the leaves. (b) Severely infected soybean leaves.

P. pachyrhizi has an exceptionally broad host range, comprising more than 150 species in about 53 genera of the legume family, the largest family of flowering plants (Ono *et al*., 1992; Hartman *et al*., 2011a). Wild hosts include kudzu (*Pueraria lobata*) and beggar weed (*Desmodium tortuosum*) (Isakeit *et al*., 2006; Sconyers *et al*., 2006). Common cultivated legumes that serve as hosts include *Phaseolus vulgaris* (common bean), *Phaseolus coccineus* (scarlet runner bean), *Vigna unguiculata* (cowpea), *Cajanus cajan* (pigeon pea), *Pisum sativum* (field pea), *Lens culinaris* (lentil) and the fodder legume *Neonotonia wightii* (Lynch *et al*., 2006; Nunkumar *et al*., 2008). These legumes are widely cultivated throughout the year as a major source of food in developing countries. Due to their cultivation at different periods throughout the year, *P. pachyrhizi* may overwinter on these hosts, which may later act as sources of primary inoculum that may be available to infect soybean fields at the start of the growing season (Tukamuhabwa & Maphosa, 2012). Recent reports of new hosts of *P. pachyrhizi* include 12 new genera of legumes in the USA (Slaminko *et al*., 2008) and black rosewood (*Afzelia xylocarpa*) in Thailand (Seemadua *et al*., 2012).

Since the first report of its occurrence on yam bean (*Pachyrhizus erosus*) in Japan in 1902,

subsequent reports of the occurrence of *P. pachyrhizi* in China, Taiwan, Australia (1934), India (1951) and Hawaii (1994) have followed. The earliest, unconfirmed report (Fig. 2) of soybean rust in Africa was in 1978 in Zambia (Javaid & Ashraf, 1978; Miles *et al*., 2003) on soybean plants, and on Bambara nut (*Vigna subterranea*) in Tanzania in 1981 (Teri & Keswani, 1981).



	Country	Year reported	Reference
	Tanzania	2014	Murithi *et al*., 2014
	Malawi	2014	Murithi *et al*., 2015
	Ghana	2007	Bandyopadhyay *et al*., 2007
	DRC	2007	Ojiambo *et al*., 2007
	Cameroon	2003	Levy , 2005
	South Africa	2001	Pretorius *et al*., 2001
	Mozambique	2000	Levy , 2005
	Zimbabwe, Zambia	1998	Levy, 2005
	Nigeria	1999	Akinsanmi *et al*, 1999
	Kenya, Uganda, Rwanda	1996	Kawuki *et al*., 2003

Figure 2. Year of the first reports on *Phakopsora pachyrhizi* on soybean in various countries in Africa.

Its introduction into Africa was proposed to have occurred through urediniospores blowing from western India to the east African coastal areas by moist northeast monsoon winds (Levy, 2005). The disease became prevalent in Africa in 1996 when it was first confirmed in Uganda on experimental plots and thereafter on farmer's fields throughout the country. All commercial cultivars were found to be susceptible (Kawuki *et al*., 2003). Within the same period (1998), the disease was reported in the major soybean growing regions in Kenya, Rwanda, Zimbabwe (Jarvie, 2009) and Zambia (Levy, 2005), in Nigeria in 1999 (Akinsanmi *et al*., 2001), in Mozambique in 2000, in South Africa in 2001 (Pretorius *et al*., 2001) and in Cameroon in 2003 (Levy, 2005). Other reports of the disease on soybean followed in 2007 in Ghana and the Democratic Republic of Congo in central Africa (Bandyopadhyay *et al*., 2007;

Ojiambo *et al*., 2007), and recently in Tanzania and Malawi (Murithi *et al*., 2014, 2015; Fig. 2).

The American continent was free of rust until the 2000/1 season, when it was reported in Paraguay in 2001 (Yorinori *et al*., 2005), and was established in Brazil and Argentina in 2002 and in Uruguay in 2004 (Rossi, 2003; Stewart *et al*., 2005). The disease was reported in the USA in Louisiana in 2004 (Schneider *et al*., 2005) and in Cuba in 2009 (Perez-Vicente *et al*., 2010); however, it was put on check in the USA through regular monitoring using sentinel plots, spore traps and variety screening. Strobilurin and triazole fungicides are widely used in the Americas for controlling soybean rust, but their use leads to high production costs and environmental concerns in addition to increasing tolerance of the fungus to some fungicides (Mueller *et al*., 2009). In 2003 Brazil used more than $590 million to control soybean rust on more than 18 million ha, with an average of two different fungicides per application (Yorinori *et al*., 2005).

Pathogenic variation of *P. pachyrhizi*

Being restricted to a parasexual cycle only may limit the variability and plasticity of a pathogen. However, significant variability in the pathogenicity on various hosts and virulence on susceptible plants has been observed in *P. pachyrhizi* populations, known for having asexual reproduction only (Akamatsu *et al*., 2013). *P. pachyrhizi* infection produces different infection types, depending on resistance or susceptibility of the soybean genotypes (Bromfield & Hartwig, 1980; Pham *et al*., 2009). Generally the reddish brown (RB)-infection type, consisting of reddish brown lesions showing meagre or no sporulation and the immune (IM)-infection type, characterized by the absence of visible symptoms, imply the presence of an incompatible interaction in which the pathogen is avirulent and the plant is resistant. Compatible interactions are characterized by a TAN-type of infection, consisting of tan coloured lesions with multiple actively sporulating uredinia. In this case the host genotype is considered to be susceptible and the pathogen virulent.

Pathotypes and races of *P. pachyrhizi* have traditionally been assessed based on the infection types caused by different isolates on various host differentials. Genes conferring resistance to *P. pachyrhizi* have been identified as *Rpp1* (for resistance to *P. pachyrhizi*) (McLean & Byth, 1980), *Rpp2* (Bromfield & Hartwig, 1980), *Rpp3* (Bromfield & Melching, 1982), *Rpp4* (Hartwig, 1986), *Rpp5* (Garcia *et al*., 2008) and *Rpp6* (Li *et al*., 2012). The genes have been mapped on the various soybean chromosomes; *Rpp1* is located on chromosome

18, *Rpp2* on 16, *Rpp3* on 6, *Rpp4* on 18, *Rpp5* on 3 and *Rpp6* on 18 (Hyten *et al*., 2007, 2009; Garcia *et al*., 2008; Silva *et al*., 2008). Significant progress has been made to characterize these genes using virus-induced gene silencing (VIGS), in which soybean is challenged with recombinant Bean pod mottle virus (BPMV) targeting endogenous genes (Pandey *et al*., 2011; Morales *et al*., 2012).

The earliest report of pathogenic variation in *P. pachyrhizi* was from Taiwan in 1966, when different infection phenotypes were observed on six different soybean genotypes and an additional five different legumes, in response to inoculation with nine different *P. pachyrhizi* isolates (Lin, 1966). In Australia two pathotypes were identified, with one being virulent on a particular soybean accession but avirulent on another one, while the other isolate was virulent on both soybean accessions (McLean & Byth, 1976). Since then, different pathotypes encompassing different isolates of *P. pachyrhizi* have been identified globally (Table 1).

The durability of the *Rpp* genes has already been challenged, because they confer resistance to only a limited set of specific *P. pachyrhizi* isolates, and these single gene sources have not been durable when used in commercial cultivars (Yeh, 1983; Bromfield, 1984; Hartman *et al*., 2005; Miles *et al*., 2011). For instance, cultivar PI 230970 (*Rpp2*), identified as resistant in field evaluations from 1971 to 1973, exhibited some TAN lesions in the field in 1976, indicating a loss of full resistance. By 1978, most of the lesions found on the plants were TAN-type lesions. Soybean cultivar PI 200492 (*Rpp1*), identified as resistant from 1961 to 1963, had become susceptible by the mid-1970s (Bromfield, 1984). Similarly, the *Rpp3* gene, present in cultivar PI 462312 and identified early in the 1970s, had become ineffective in the late 1970s (Bromfield, 1984). Cultivar PI 459025B (*Rpp4*) is known to still show resistance, but field trials have revealed susceptibility to some *P. pachyrhizi* isolates (Hartman *et al*., 2005). In Brazil, *Rpp1* to *Rpp4* were effective against rust in 2001; however, both *Rpp1* and *Rpp3* succumbed to the pathogen within 2 years of their introduction (Yorinori, 2008).

Table 1. Characterization of the virulence spectrum of *Phakopsora pachyrhizi* isolates from different geographical regions on differential sets of soybean

Country	Year	Number of isolates tested	Lines used	Pathotypes/Races identified	Reference
Taiwan	1966	9	11 legume accessions, 6 accessions of soybean and 5 *Phaseolus* species.	6	Lin, 1966
Australia	1977	2	cultivar Willis and PI 200492 (*Rpp1*)	2	McLean and Byth, 1976
Australia, India, Puerto Rico & Taiwan	1983	4	PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312(*Rpp3*)	4	Bromfield 1980
Taiwan	1983	50	PI 200492 (*Rpp1*), PI 462312 (*Rpp* 3), PI 230971, TK 5 and TN 4	3	Yeh *et al*., 1983
Taiwan	1983	42	AVRDC differential lines: PI 200492 (*Rpp* 1), PI 230970 (*Rpp* 2), PI 462312 (*Rpp* 3), PI 230971, PI 239871A, PI 239871B, PI 459024 and PI 459025B, TK-5, TN-4 and Wayne	9	AVRDC, 1983
Australia	1984	8	257 accessions of *Glycine* spp namely: *G. canescens, G. clandestine , G. tabacina* and *G. tomentella*	6	Burdon and speer 1984
China	1989	7	PI 200492 (*Rpp1*), PI 462312 (*Rpp3*), PI 459025B (*Rpp4*) and 5 other accessions	4	Tan and Sun, 1989
Japan	2000	45	AVRDC differential lines	18	Yamaoka *et al*, 2002
South Africa	2001	one composite population	AVRDC differential lines	0	Caldwell and McLaren, 2004
USA	2001	12 international	PI 200492 (*Rpp1*), PI 230970 *(Rpp2*), PI 462312 (*Rpp3*) and PI 459025B (*Rpp4*)	6	Bonde *et al*., 2006
USA		4 bulked international	PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*) and PI 459025B (*Rpp4*)	2	Miles *et al*., 2006
Paraguay	2003	1 composite field population	PI 462312 *(Rpp3*), PI 459025B (*Rpp4*) and 528 other accessions	2	Miles *et al*., 2008
USA		6	PI 200492 (*Rpp1*), PI 594538A (*Rpp1b*), PI 462312 (*Rpp3*), 459025B (*Rpp* 4) and 23 other accessions	2	Paul and Hartman, 2009
USA		10 international	PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), PI 459025B (*Rpp4*) and 16 others	8	Pham *et al*, 2009
Uganda	2004	19 lines	AVRDC differential lines	3	Tukamuhabwa *et al*., 2012

Nigeria	2009	116	PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), PI 459025B (*Rpp4*), PI 594538A, UG-5,TGx 1485-1D and TGx 1844-4F	7	Twizeyimana *et al*., 2009
USA	2010	8 international	PI 200492 (*Rpp1*), PI 594538A (*Rpp1b*), PI 587866 and PI 587880A	3	Ray *et al.*, 2009
Vietnam	2010	1 composite field population	PI 200492 (*Rpp1*), PI 594538A (*Rpp1b*), PI 462312 (*Rpp3*), 459025B (*Rpp4*) and 85 other accessions	7	Pham *et al*, 2010
Brazil	2011	3	13 accessions including sources of *Rpp1–5*	3	Yamanaka *et al*., 2010
USA	2011	8	PI 462312 (*Rpp3*), Hyuuga (*Rpp?*) and 12 other accessions	6	Kendrick *et al*., 2011
USA	2011	4	34 accessions including resistance sources- *Rpp1*-4		Miles *et al.*, 2011
USA	2011	field populations	PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), PI 459025B (*Rpp4*) and over 500 other accessions	2	Walker *et al*., 2011
USA	2011	72	PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), PI 506764 (*Rpp3*-Hyuuga), PI 459025B (*Rpp4*), and PI 200526 (*Rpp5*); UG-5	3	Twizeyimana *et al*., 2011
Japan	2014	26	9 soybean accessions, including resistance sources *Rpp1-5* and Kudzu (*Pueraria lobata*)	6	Yamaoka *et al*, 2014

Adapted and modified from Hartman *et al*., 2011a. PI, plant introduction; AVRDC, Asian Vegetable Research and Development Centre; *Rpp*, resistance to *Phakopsora pachyrhizi*.

The virulence of *P. pachyrhizi* populations differs based on the geographical regions from where they are collected (Twizeyimana *et al*., 2009; Yamanaka *et al*., 2010; Akamatsu *et al*., 2013; Table 1). Furthermore, the responses of the host genotypes differ (Bromfield, 1984) and there are differences between new and old isolates (Bonde *et al*., 2006). Concerning the latter, a comparison between isolates collected from different geographical locations in different periods in Asia, Australia, Africa and South America in 2001 and older isolates collected in the 1970s, revealed that the isolates collected in 2001 were more virulent. Newer isolates caused a lower frequency of RB reactions and in most cases, there was a complete absence of immune reactions on the various host differentials (Bonde *et al*., 2006).

Comparison of the pathogenicity profiles of 59 different rust populations obtained from Brazil, Argentina and Paraguay, which were tested on 16 soybean differentials, revealed a significant variation in pathogenicity among the populations. Only two pairs among the 59 *P. pachyrhizi* populations displayed identical pathogenicity profiles, indicating substantial variation in the rust populations studied (Akamatsu *et al*., 2013). Brazilian isolates exhibited a higher virulence, reflected by higher levels of sporulation when tested on four varieties carrying *Rpp1*, as compared to Japanese isolates. Fungal virulence can also vary over time, as was demonstrated by two Brazilian rust populations that showed a similar virulence on a set of differentials in 2005, but exhibited a different virulence spectrum on another set of differentials in 2008 (Yamanaka *et al*., 2010). In the USA, isolates collected from Florida in 2006, 2009 and 2011/12 were compared for their virulence on two soybean accessions PI 200492 (*Rpp1*) and PI 567102B (*Rpp6*). More sporulation was observed on the genotypes inoculated with the isolates that were collected in 2011/12, as compared to the ones collected in earlier years, suggesting the appearance of a pathotype that had become more virulent towards the normally resistant genotypes, as compared to the *P. pachyrhizi* pathotypes present among earlier populations (Paul *et al*., 2013).

A high level of virulence among several isolates of *P. pachyrhizi* has further been demonstrated by production of mixed reactions of RB and TAN lesions on particular soybean genotypes. One isolate, 72-1 from Australia, induced both RB and TAN lesions on the same leaflets of eight different soybean accessions (Bromfield *et al*., 1980). Other studies have reported mixed reactions in different rust populations, especially when using a bulk pathogen population or a mixture of isolates (Miles *et al*., 2006; Yamanaka *et al*., 2010; Maphosa *et al*.,

2013). These reactions could result from a mixture of races in the inoculum (Bonde *et al*., 2006) that may imply more diverse virulence of the different isolates in a given population.

Virulence variation of *P. pachyrhizi* isolates in Africa

In Africa, the pathogenicity and virulence of soybean rust has only been tested for a limited number of soybean rust isolates. *P. pachyrhizi* isolates collected from Zimbabwe in 2001 produced a TAN-type of infection on all soybean differentials carrying resistance genes (*Rpp1–Rpp4*), as compared to isolates originating from Taiwan, India and South America. In contrast, an isolate from South Africa in the same study produced RB infection types on *Rpp2*, *Rpp4* and *Rpp1+*, suggesting the presence of different pathotypes in Africa (Bonde *et al*., 2006). In Uganda, none of the 196 soybean varieties that were screened for resistance against soybean rust between 1996 and 1998 were found to be immune. Furthermore, eight of the varieties initially found to be resistant succumbed to rust in the subsequent seasons (Kawuki *et al*., 2003). Three virulent races were identified out of 45 different isolates that were tested on 19 soybean lines from the Asian Vegetable Research and Development Centre (AVRDC) in Uganda in 2004 (Tukamuhabwa & Maphosa, 2012). In the 2005 and 2006 growing seasons, 25 different soybean accessions, four among them bearing *Rpp1* to *Rpp4*, were found to be susceptible to rust populations originating from Uganda, except for accession PI 230970 (*Rpp2*) (Oloka *et al*., 2008). However, TAN-type lesions were recently observed on PI 230970 (*Rpp2*) when inoculated with five isolates of *P. pachyrhizi* in field trials in Uganda (Maphosa *et al*., 2013), suggesting a change in virulence of Ugandan rust populations within a period of less than ten years. In other studies, Twizeyimana *et al*. (2009) identified seven different pathotypes out of 116 representative isolates collected in three different agro-ecological zones in Nigeria and inoculated on eight different accessions, some of which had resistance genes *Rpp1* to *Rpp4*.

Recently, variable reactions were observed on 12 soybean lines inoculated with five different isolates from five different locations in Uganda. Three of the lines produced TAN-type lesions in the five different locations, while four of the resistant lines produced RB-type lesions (Maphosa *et al*., 2013). This short-term durability of resistance genes reflects virulence variability among *P. pachyrhizi* populations and the development of new physiological races in field populations. Presumably, the variable populations of rust in a given area allow for new populations to become dominant that are not targeted by the resistance mechanisms

effective against previously dominant forms. More research is needed to understand the virulence profile of soybean rust populations in other countries in Africa.

Soybean rust control strategies and current research.

The knowledge on pathogen variability of *P. pachyrhizi* in a given region is essential because it helps to guide deployment, screening and/or introduction of novel resistance genes against the currently prevailing pathotype groups. Furthermore, it will help in monitoring the dynamics and changes that occur in the population of existing pathotypes through the entry of new pathotypes from other regions. Thus, pathogen variability studies provide a means of monitoring the present state of the interaction with respect to pathogen virulence and plant resistance for a given pathogen and host population (Ramstedt *et al*., 2002).

Virulence characterization of the soybean rust populations in eastern and southern Africa is geared towards understanding the variability and dynamic plasticity of the rust population and aims to guide targeted breeding for resistance. Moderately resistant soybean cultivars, namely Maksoy 1N, Maksoy 2N, Maksoy 3N and Namsoy 4M, all developed in 2005 in Uganda, have been used in the management of soybean rust (Oloka *et al*., 2005). These cultivars contain partial resistance to soybean rust; however, some of the cultivars (Maksoy 1N, Maksoy 2N and Namsoy 4M) have recently been reported to succumb to this disease (Maphosa *et al*., 2013). This suggests the existence of variable virulence patterns among rust populations in Uganda and therefore a regular screening of germplasm is necessary to monitor virulence changes of the pathogen.

Tolerant soybean varieties have been developed in Zimbabwe based on the stability of their yields, or a tolerance approach that was followed by selecting genotypes with high yield potential even when infected by soybean rust (Tichagwa, 2004). Screening for yield stability to soybean rust involves determining yields from paired plots, with and without fungicides. High-yielding genotypes with relatively low yield loss under conditions of severe rust infestation are considered to be tolerant. Varieties identified through this method have been released and are currently being used in eastern and southern Africa to reduce yield losses due to soybean rust epidemics. Evolution of new virulence spectra through migration, mutation, recombination of existing pathogenicity and virulence genes and their subsequent selection on susceptible plants has been more frequent in rust as compared to necrotrophic pathogens (Singh *et al*., 2011). Therefore, successful breeding and deployment of resistance

genes against soybean rust, in combination with knowledge on the virulence spectrum of rust populations and the reaction of soybean lines to field isolates from different regions, is paramount. Because the major resistance genes are considered to be race-specific, soybean breeding lines and cultivars cannot be used without prior knowledge of the differences in virulence and race composition of a given rust population. Germplasm from the United States Department of Agriculture (USDA) is currently being tested in Malawi to identify novel resistant germplasm that can be used in national breeding programmes. Through the extensive breeding programme at the International Institute of Tropical Agriculture (IITA), soybean rust-resistant lines have been tested and released across Africa (Hartman *et al*., 2011a). Two resistant varieties, TGx 9 1988-5F and TGx 9 1989-19F (NCRISOY-1 and -2, respectively), were recently released in Nigeria (IITA, 2015). A rapid spread of soybean rust in farmers' fields was observed in field surveys conducted during the 2011– 2014 growing seasons in soybean producing areas in Kenya, Uganda, Tanzania and Malawi. Rust is spread across different agro-ecological zones (Fig. 3) and causes symptoms on up to 80% of the leaf area. Leaf samples with and without symptoms collected from these countries were subjected to quantitative PCR (qPCR) using *P. pachyrhizi*-specific primers to confirm the disease (Frederick *et al*., 2002). In vivo cultures were established in detached leaf assays (Twizeyimana *et al*., 2007) of soybean rust differential sets to determine the virulence and distribution of the pathotypes present in this region.

To understand the population biology of pathogenic fungi, the amount and distribution of genetic variation among and within the population is important; however, little is known of *P. pachyrhizi* populations in eastern and southern Africa. Genes encoding effector proteins secreted by pathogens during infection can be used as molecular markers to understand the biology of rust pathogen interactions and further identification of new resistance genes (Saunders *et al*., 2012). Molecular markers are used for the assessment of genetic diversity, phylogenetic relationships and characterization of pathotypes (Keiper *et al*., 2003).

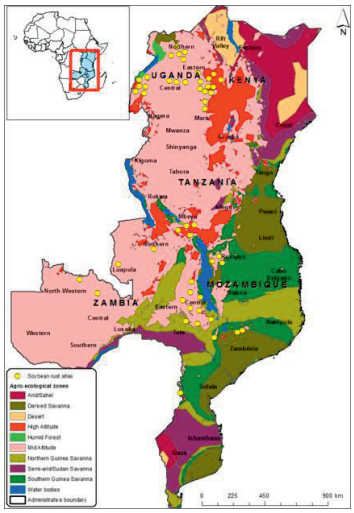


Figure 3. Soybean rust distribution over different agro-ecological zones in eastern and southern Africa.

Anderson *et al*. (2008) developed highly polymorphic microsatellite markers for the characterization of different strains of *P. pachyrhizi*. Eighty-four distinct genotypes were revealed among 116 isolates collected from three different agro-ecological zones in Nigeria, suggesting a high genetic variability of the pathogen (Twizeyimana *et al*., 2011). These microsatellite markers are currently being used to establish the genetic diversity of *P. pachyrhizi* populations in eastern and southern Africa in order to guide the deployment of resistant cultivars in breeding programmes.

Monitoring and surveillance of *P. pachyrhizi* development and dispersal is heavily influenced by environmental conditions, plant age and host species. Tracking of soybean rust races and monitoring the disease status globally is absolutely a priority. Monitoring factors, such as the prevailing wind patterns and climatic factors that favour its survival, sporulation and distribution in soybean producing areas, can help in controlling the disease. Monitoring of soybean rust is necessary to alert growers of significant dispersal before symptom development. For chemical control of soybean rust, it is critical for the grower to decide on whether and when to apply a fungicide. Early application of a fungicide when the pathogen has not yet established leads to waste, while when a fungicide is applied too late, yield losses are likely to occur due to disease development. Sentinel plots have been successfully used in the USA in a national network system aimed at monitoring the appearance of soybean rust (Geisler *et al*., 2007; Young *et al*., 2011). The sentinel plots are used to detect soybean rust

already present at low densities and are established in multiple locations in the soybean growing regions. Typically, they are planted earlier than commercial soybeans to provide an early warning system for commercial soybean fields. The plots use a variety of soybean maturity groups to extend monitoring throughout the growing season. Soybean rust monitoring begins with collecting and observing leaves from sentinel plots at regular intervals throughout the season. Spore traps and rain collectors are used in the sentinel plots to capture spores that are present in the air and that may lead to the development of disease (Dufault *et al*., 2010; Vittal *et al*., 2013). Fluorescent antisera specific for detection of *P. pachyrhizi* urediniospores are regularly used to detect airborne spores collected from the traps (Baysal-Gurel *et al*., 2008). Samples collected from the spores are also detected using qPCR to confirm the disease and to quantify the relative amounts throughout the season. These methods have already been used successfully in the USA to generate accounts on the real-time status of soybean rust spores in the atmosphere and alert growers about the risk of soybean rust establishment on their soybean fields in a particular season.

These monitoring efforts in the USA have saved the soybean industry millions of dollars in fungicide costs, as a result of the availability of accurate disease forecasting based on pathogen surveillance and environmental data. Other monitoring information tools, such as RUSTMAPPER application, have also been successfully used in monitoring wheat stem rust globally. This is a Google Earth-based application that provides up to date information on the current status and the potential spread of wheat stem rust (Hodson *et al*., 2012). Such applications can be developed for monitoring soybean rust across the globe, showing the current survey sites and the wind patterns that can influence spore dispersal. Sentinel plots and spore trap monitoring methods will be tested on a small scale in eastern and southern Africa to evaluate their workability. These methods will be optimized for adaptation to the conditions in the region to contribute to controlling soybean rust.

Concluding remarks and further perspectives

The substantial contribution of soybean to human nutrition, its use in animal feeds and its potential source of cash income for small farmers from selling the crop, are some of the factors contributing to the adoption of the crop among smallholder producers in eastern and southern Africa. Soybean production will continue to increase in eastern and southern Africa, driven by an increased production per acre and an expansion of the production area,

especially through increased intercropping and crop rotation. The threat of soybean rust to the soybean industry in eastern and southern Africa is serious and the variability of the virulence spectrum of the pathogen around the globe confirms the challenges it poses to crop protectors as they search for effective management tools. Although a variety of fungicides effective against soybean rust are available, the use of such fungicides is limited due to the high costs of the product and its application, as well as environmental concerns. Due to this restricted fungicide use, an early monitoring system for detection of rust threats for steering fungicide might only be relevant for large-scale producers in eastern and southern Africa.

Host plant resistance provides a cheaper, environmentally friendly, and much more sustainable approach for managing soybean rust among smallholder agriculture that characterizes the agricultural landscape of eastern and southern Africa. Identification of dominant pathotypes will therefore guide breeding for resistance to specific *P. pachyrhizi* populations. Furthermore, determining genetic diversity of *P. pachyrhizi* populations will provide vital information for breeders to develop resistant germplasm. Identifying, screening and deploying high yielding disease-resistant varieties in the soybean growing regions of eastern and southern Africa will help in reducing the yield losses due to soybean rust. A continuous monitoring of the *P. pachyrhizi* population using sentinel plots and spore traps, in combination with a consistent screening of *P. pachyrhizi* isolates, will be essential to understand the pathogenic differentiation of the rust population in eastern and southern Africa.

The use of single gene resistance may not be sustainable, whereas pyramiding of soybean rust resistance genes in a single soybean cultivar may provide more durable resistance against the highly variable rust populations in the field (Lemos *et al*., 2011; Maphosa *et al*., 2012). The loci of the six resistance genes (*Rpp1–Rpp6*) have been mapped with molecular markers and can thus be tagged and pyramided by making use of the linked molecular markers. For instance, a genotype with three resistance genes (*Rpp2, Rpp4* and *Rpp5*) potentially has more durable resistance than genotypes with single resistance genes (Yamanaka *et al*., 2010). These lines can be used in breeding programmes to deploy stable resistance. In addition to gene pyramiding, selection for novel sources of resistance to *P. pachyrhizi* is desirable. Mid-term interventions should include breeding for tolerance and/ or partial resistance. These methods can be incorporated into regional breeding programmes to develop slow rusting cultivars. Use of molecular techniques, such as marker- assisted selection of resistance genes and genetic

transformation, will ease pre-breeding efforts in the long run. Due to the enormous potential of the soybean crop to improve the diet of people and its significant contribution to better incomes and livelihoods in eastern and southern Africa, efforts to protect the crop from abiotic and biotic constraints, among which soybean rust poses a serious threat, are required to ensure sustainable soybean production.

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CHAPTER 3

Virulence diversity of Phakopsora pachyrhizi isolates from East Africa compared to a geographically diverse collection

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ABSTRACT

Soybean rust, caused by the biotrophic pathogen *Phakopsora pachyrhizi*, is a highly destructive disease causing substantial yield losses in many soybean producing regions throughout the world. Knowledge about *P. pachyrhizi* virulence is needed to guide development and deployment of soybean germplasm with durable resistance against all pathogen populations. To assess the virulence diversity of *P. pachyrhizi*, 25 isolates from eight countries, including 17 isolates from Africa, were characterized on 11 soybean genotypes serving as differentials. All the isolates induced tan lesions with abundant sporulation on genotypes without any known resistance genes and on soybean genotypes with resistance genes Rpp4 and Rpp5b. The most durable gene was *Rpp2*, where 96% of the isolates induced reddish brown lesions with little or no sporulation. Of the African isolates tested, the South African isolate was the most virulent, whereas those from Kenya, Malawi, and some of the isolates from Tanzania had the lowest virulence. An Argentinian isolate was virulent on most host differentials, including two cultivars carrying multiple resistance genes. Ten distinct pathotypes were identified, four of which comprised the African isolates representing considerable P. pachyrhizi virulence. Soybean genotypes carrying *Rpp1b*, *Rpp2*, *Rpp3*, and *Rpp5* resistance genes and cultivars Hyuuga and UG5 were observed to be resistant against most of the African isolates and therefore may be useful for soybean breeding programs in Africa or elsewhere.

INTRODUCTION

Soybean (Glycine max (L.) Merr.) production in eastern Africa has increased, driven by the high demand from feed millers and for human consumption (Murithi *et al.,* 2016; Rusike *et al.,* 2013). This trend is expected to continue as human population increases, and as income and soybean cultivars improve. However, this potential increase in production is under threat from soybean rust, a foliar disease caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd.. Soybean rust is one of the most economically important foliar diseases of soybean worldwide. Yield losses due to *P. pachyrhizi* of up to 80% were reported in experimental plots in Taiwan (Hartman *et al.,* 1991), 30 to 75% in Brazil (Yorinori *et al.,* 2005), up to 60% in experimental plots in the United States (Mueller *et al.,* 2009), and a review of the literature predicted potential yield losses in commercial fields in the United States that could exceed

80% (Sikora *et al.,* 2014). In Africa, losses of up to 80% have been reported in Zimbabwe (Levy 2005), and up to 45% in Uganda (Kawuki *et al.,* 2003; Oloka *et al.,* 2008). Leaf lesions caused by *P. pachyrhizi* reduce photosynthetic activity, leading to reduced flowering, fewer pods, smaller seeds, and premature defoliation (Kumudini *et al.,* 2008). Soybean rust was first reported in Japan in 1902 (Hennings 1903), and spreads by fungal spores transferred by wind currents (Isard *et al.,* 2007). The first confirmed report in Africa was in 1996 in Uganda, shortly after in Kenya and Rwanda in 1998 (Levy 2005), followed by Nigeria (Akinsanmi *et al.,* 2001), South Africa (Pretorius *et al.,* 2001), and Cameroon (Levy 2005). Later, the disease was reported in Ghana (Bandyopadhyay *et al.,* 2007) and the Democratic Republic of Congo (Ojiambo *et al.,* 2007), and most recently in Tanzania (Murithi *et al.,* 2014) and Malawi (Murithi *et al.,* 2015). Favourable temperatures of 15 to 28°C and high humidity during the soybean growing season in most African countries contribute to the establishment and spread of *P. pachyrhizi* (Hartman *et al.,* 2011).

The recent spread of soybean rust in eastern Africa poses a major challenge to the regional soybean sector as most of the soybean varieties grown are susceptible to this disease (Kawuki *et al.,* 2003; Murithi *et al.,* 2016; Oloka *et al.,* 2008). Effective control measures are required to safeguard soybean production posed by the potential threat of *P. pachyrhizi* in Africa. Disease management measures include early planting (Twizeyimana *et al.,* 2011), use of resistant cultivars (Hartman *et al.,* 2005), and fungicides (Levy 2005; Miles *et al.,* 2007; Mueller *et al.,* 2009). Scouting for soybean rust may also be critical for best management practices and several reviews have outlined their importance (Kelly *et al.,* 2015; Sikora *et al.,* 2014). Although fungicides are widely used in other locations, they are seldom used on soybean in East Africa.

Breeding soybean for resistance to soybean rust is considered one of the best management options to limit yield losses from the disease (Hartman *et al.,* 2005). Soybean rust resistance has been identified in a number of soybean genotypes bearing single resistance genes (Goellner *et al.,* 2010; Hartman *et al.,* 2011). For example, the genes *Rpp1* to *Rpp6* have been mapped to six different loci and are known to confer resistance to specific isolates of the pathogen (Garcia *et al.,* 2008; Li *et al.,* 2012). However, resistance breakdown has been reported across the globe (Hartman *et al.,* 2005; Miles *et al.,* 2008) and new *P. pachyrhizi* pathotypes emerge as a result of selection pressure, making it difficult to develop soybean cultivars with durable resistance to soybean rust.

Isolates of *P. pachyrhizi* collected from various countries have been reported to differ in virulence based on their reactions on differential hosts (Bonde *et al.,* 2006; Miles *et al.,* 2006; Pham *et al.,* 2009). Differential virulence of *P. pachyrhizi* was first demonstrated in Taiwan where six pathotypes were described (Lin 1966). Subsequently, 18 pathotypes were described in Japan (Yamaoka *et al.,* 2002, 2014), three in Brazil (Yamanaka *et al.,* 2010), and three in the United States (Twizeyimana and Hartman 2012). In Africa, seven pathotypes were described in Nigeria (Twizeyimana *et al.,* 2009) and three in Uganda (Tukamuhabwa and Maphosa 2012). A pathotype refers to a class of pathogen isolates whose members express a similar pattern of host-specific virulence toward individual plant clones or species within a specified set, and pathogen virulence in combination with plant resistance may be monitored through pathotyping (Ramstedt *et al.,* 2002).

The virulence of different isolates on host differentials carrying specific resistance genes are commonly used to determine the virulence spectrum of the pathogen. Evaluation of *P. pachyrhizi* virulence is based on three reaction types produced on soybean host differentials: Immune (IM), representing no observable reaction; reddish brown (RB), which represents reddish brown lesions with little or no sporulation and implying an incompatible interaction in which the plant is resistant and the pathogen is avirulent; and tan reaction (TAN), which represents tan lesions with abundant sporulation in which the plant is susceptible and the pathogen is virulent (Bromfield and Hartwig 1980; McLean and Byth 1980). Virulence of the *P. pachyrhizi* population for any geographic location may be dependent on virulence of prominent pathotypes. Knowledge of virulence dynamics should aid the development of soybean cultivars with effective resistance genes. Currently, only a limited number of *P. pachyrhizi* isolates from Africa have been evaluated for virulence (Bonde *et al.,* 2006; Tukamuhabwa and Maphosa 2012; Twizeyimana *et al.,* 2009). For example, an isolate collected from Zimbabwe produced a TAN reaction on all soybean differentials carrying resistance genes *Rpp1* to *Rpp4*; in contrast, an isolate from South Africa produced RB reactions on soybean carrying *Rpp1*, *Rpp2*, and *Rpp4* (Bonde *et al.,* 2006). In addition, Ugandan populations were virulent on *Rpp1* and *Rpp4* (Oloka *et al.,* 2008), and virulent on PI 230970 (*Rpp2*) (Maphosa *et al.,* 2013).

With the recent rapid spread of *P. pachyrhizi* in East Africa, it is necessary to characterize the virulence spectrum and evaluate the effectiveness of the various *Rpp* genes against the present *P. pachyrhizi* population in order to guide their deployment in breeding

programs. The goal of the study was to characterize the virulence spectrum of rust isolates from Africa in comparison with isolates from other continents and identify soybean resistance genes that would be effective against *P. pachyrhizi* pathotypes in Africa. The specific objectives were to (i) characterize the virulence diversity of *P. pachyrhizi* populations from eastern and southern Africa on a defined set of host differentials, (ii) compare the virulence spectrum of these populations with populations from other African regions and other continents, and (iii) evaluate the effectiveness of multiple resistance genes against the various isolates. The information gained may be useful for guiding screening methods and deployment of cultivars with durable resistance to all predominant pathotypes of *P. pachyrhizi* in eastern Africa.

MATERIALS AND METHODS

Field surveys and sample collections.

A total of 12 soybean fields in central (Dowa, Kasungu, and Mchinji) and southern Malawi (Thyolo) and 31 fields in the southern highlands (Iringa, Mbeya, Morogoro, and Ruvuma regions) of Tanzania were surveyed for the presence of soybean rust in the 2013–14 growing season. Out of the total fields surveyed, soybean rust was observed in 23 fields from which about 10 symptomatic leaf samples were collected from different soybean plants. In addition, 23 samples from different fields in the northwest and northeast regions of Pampa, Argentina, and one sample each from Australia (New South Wales), Kenya (western region), and South Africa (KwaZulu Natal) were sent to the University of Illinois under an APHIS permit. Two samples from the United States (Florida and Texas) and one from Taiwan were included in the study to give a total of 52 samples.

Soybean rust recovery and multiplication of inoculum.

To establish cultures of *P. pachyrhizi*, spores of infected leaf samples from each location were used to inoculate the abaxial surface of healthy detached leaves of Williams 82 (Twizeyimana and Hartman 2010), a fully susceptible soybean cultivar lacking resistance genes against all pathotypes of *P. pachyrhizi*. The inoculated leaves were placed on a paper towel saturated with 5 ml sterile distilled water in a 13.7 × 22.0 × 3.2 cm transparent plastic container; an additional 4 ml/container of distilled water was added as needed at 6-day intervals to keep the paper towel saturated. The containers first were placed in a tissue chamber in the dark

for 24 h at 23°C, followed by alternating dark and light periods of 12 h each at temperatures between 22°C and 25°C and humidity between 65 to 75% (Percival Scientific, Inc., Boone, IA) for 14 days. To obtain sufficient spores, recovered isolates were further multiplied on the cultivar Williams 82 under the same conditions.

Isolate infection types on soybean differentials.

Eleven soybean host differentials obtained from the USDA Soybean Germplasm Collection were selected based on prior knowledge of their resistance to soybean rust and the differential reaction to various *P. pachyrhizi* isolates (Table 1). Seven seeds of each of the 11 differential lines were sown in a plastic tray filled with Sunshine LCI mix (Sun Grow Horticulture Inc., Belleview, WA) mixed with 10 mg 14:14:14 N/P/K Osmocote fertilizer (Scotts-Sierra Horticultural Products Co., Marysville, OH). Trays were placed in the growth chamber in alternating dark and light periods of 12 h each at temperatures between 22°C and 25°C and humidity between 65 and 75% and under rust-free conditions.

Table 1. Soybean host differentials used to classify *Phakopsora pachyrhizi* isolates

Genotype	Resistance Gene(s)	Reference for gene designation and/or source of plant material
LD09-16057	*Rpp1*	Diers *et al*. 2014; McLean and Byth 1980
LD10-30052	*Rpp1b*	Chakraborty *et al*. 2009; Diers *et al*. 2014
PI 417125	*Rpp2*	Hartwig and Bromfield 1983; Laperuta *et al*. 2008
PI 462312	*Rpp3*	Bromfield and Melching 1982
PI 459025B	*Rpp4*	Hartwig 1986
LD10-14274	*Rpp5a*	Diers *et al*. 2014
PI 200526	*Rpp5b*	Garcia *et al*. 2008
PI 567102B	*Rpp6*	Li *et al*. 2012
LD10-14205 (Hyuuga)	*Rpp3,5*	Kendrick *et al*. 2011; Diers *et al*. 2014
UG5	*Rpp1,3*	Not published
Williams 82	Susceptible	Miles *et al*. 2006

After 3 to 4 weeks, leaflets from the 2nd and/or 3rd trifoliate leaves were used for inoculation. Each container, containing one leaflet from each of the 11 soybean differentials, was inoculated with a single *P. pachyrhizi* isolate. The containers were placed in a randomized complete block design with three replications for each isolate. Inoculation was done by

tapping two to three leaves bearing sporulating uredinia from each isolate to dislodge the spores onto the abaxial side of the leaflets of the differential. The leaflets were initially incubated in the dark for a period of 24 h followed by 12 h photoperiods at 22 to 25°C, and humidity of 65 to 75% in a tissue chamber for 14 days.

Pathotype evaluation and analysis.

Soybean leaves were examined by means of a stereomicroscope at 80× magnification to determine the infection types induced by each isolate 14 to 17 days after inoculation. The infection types were recorded as IM, RB, or TAN. IM and RB were grouped as a resistant response and TAN was regarded as a susceptible response. These categories were used to assign the pathotypes for each isolate. Inoculation tests were repeated and if a reaction was not clearly resistant or susceptible, it was repeated again.

For pathotype classification, the octal nomenclature system was used (Goodwin *et al.,* 1990). TAN reactions were coded as 1, and IM and RB as 0 (Twizeyimana *et al.,* 2009). Isolate virulence data were summarized using the HaGiS (Habgood-Gilmour spreadsheet) V.3.1 program (Herrmann *et al.,* 1999) that converts virulence data into octal format. Briefly, the 11 host differentials were arranged in triplets as follows, first octal digit comprising LD09-16057 (*Rpp1*), LD10-3005 (Rpplb), and PI 417125 (*Rpp2*). The second octal digit included PI 462312 (*Rpp3*), PI 459025B (*Rpp4*), and PI 200526 (*Rpp5a*). The third octal digit consisted of LD10-14274 (*Rpp5b*), PI 567102B (*Rpp6*), and cultivar Hyuuga carrying *Rpp3* and Rpp5 (Kendrick *et al.,* 2011). The fourth octal digit was represented by cultivar UG5. For each collection, the virulence frequency and mean virulence complexity or average number of soybean differentials with which an isolate has a susceptible interaction were calculated.

The Euclidean distance matrix was calculated using R version 3.2.0 in default settings (R Development Core Team 2008). The resulting matrices were entered into a hierarchical clustering function of the software. A dendrogram was generated using the unweighted pair group method with arithmetic mean (UPGMA; 31). The R package “pvclust” was executed to assess the uncertainty in hierarchical cluster analysis, which calculates P-values for each cluster using bootstrap resampling techniques.

RESULTS

Isolate infection types on soybean differentials.

All isolates induced a TAN infection type on cultivars LD09-16057 (*Rpp1*), PI 459025B (*Rpp4*), and PI 200526 (*Rpp5b*) and on the susceptible soybean cultivar Williams 82 (Table 2), except for isolate FL-07-01, which produced an IM infection type on *Rpp1*. RB and IM infection types were induced on PI 417125 (*Rpp2*) by all isolates, except for the isolate from South Africa. RB and IM infection types were induced on *Rpp1b* by all isolates except KE-12-01 and FL-07-01, which induced a TAN infection type. ARG-14-01 and FL-07-01 induced a TAN infection type on LD10-14274 (*Rpp5a*) while all the other isolates induced an RB infection type. All isolates induced an RB infection type on Hyuuga (except AUS-14-01, ARG-14-01, ARG-14-02, ARG-14-04, and FL-07-01) and on UG5 (except for the four isolates from Argentina).

The Argentinian isolate (ARG-14-01) was the most virulent on eight of the 11 differentials, including cultivars Hyuuga and UG5 carrying multiple resistance genes. Likewise, the other Argentinian isolates were also virulent on the two cultivars except isolate ARG-14-03, which was avirulent on Hyuuga. The Australian isolate (AUS-14-01) was the least virulent of the isolates from outside Africa, but was virulent on cultivar Hyuuga. FL-07-01 from the United States was virulent on Hyuuga, but avirulent on UG5.

The South African isolate SA-14-01 was the most virulent of the African isolates, while the Malawian and six of the 12 Tanzanian isolates were the least virulent. Of all the isolates tested, only six from Tanzania were virulent on PI 567102B (*Rpp6*), while none of the African isolates were virulent on cultivars Hyuuga and UG5. There were no virulence differences observed between samples collected from the same field.

Pathotype evaluation and analysis.

The 25 isolates were distributed into 10 distinct *P. pachyrhizi* pathotypes (Table 3). Isolates from Kenya and South Africa each had distinct pathotypes, 3212 and 5312, respectively. Tanzanian isolates were grouped into two distinct pathotypes, 1212 and 1232, each comprising 50% of the isolates. One of these pathotypes, 1212, was also associated with all three isolates from Malawi and the single isolate from Taiwan (TW). This was the only pathotype that was common in three different countries while the other pathotypes were distinct to the country of origin. Argentinian isolates ARG-14-02 and ARG-14-04 were grouped into one pathotype (1353), while ARG-14-01, ARG-14-03, FL-07-01 (U.S.A.), TX-13-

01 (U.S.A.), and AUS-14-01 (Australia) had distinct pathotypes. The number of virulences on the 11 differentials (virulence complexity) varied from four to eight. Pathotype 1753 from Argentina had the highest number of virulences (73%) including cultivars Hyuuga and UG5 (Table 2). The least virulent pathotype (1212) from Malawi, Tanzania, and Taiwan was virulent on *Rpp1*, *Rpp4*, and Rpp5 genes and on the susceptible check.

The isolates were clustered into four major groups (Fig. 1). Group I comprised a single isolate (FL-07-01) from the United States, while all the four isolates from Argentina were grouped into cluster II. The South African isolate was grouped in cluster III. Cluster IV includes the Taiwanese and Australian isolates, one isolate from Texas, and all the isolates from Malawi and Tanzania.

Table 2. Infection types induced by *Phakopsora pachyrhizi* isolates on the 11 soybean host differentials.

	Differential [a]											
Isolates [b]	*Rpp1*	*Rpp1b*	*Rpp 2*	*Rpp3*	*Rpp 4*	*Rpp5a*	*Rpp5b*	*Rpp6*	Hyuuga	UG5	Williams 82	TAN (%)[c]
KE-12-01	TAN	TAN	RB	RB	TAN	RB	TAN	RB	RB	IM	TAN	45
MAL-14-01	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	IM	TAN	36
MAL-14-02	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	IM	TAN	36
MAL-14-03	TAN	IM	RB	RB	TAN	RB	TAN	IM	RB	IM	TAN	36
SA-14-01	TAN	IM	TAN	TAN	TAN	RB	TAN	RB	RB	IM	TAN	54
TZ-14-01	TAN	RB	RB	RB	TAN	RB	TAN	TAN	RB	IM	TAN	45
TZ-14-02	TAN	RB	RB	RB	TAN	RB	TAN	TAN	RB	IM	TAN	45
TZ-14-03	TAN	RB	RB	RB	TAN	RB	TAN	TAN	RB	IM	TAN	45
TZ-14-04	TAN	RB	RB	RB	TAN	RB	TAN	TAN	RB	RB	TAN	45
TZ-14-05	TAN	RB	RB	RB	TAN	RB	TAN	TAN	RB	IM	TAN	45
TZ-14-06	TAN	RB	RB	RB	TAN	RB	TAN	TAN	RB	RB	TAN	45
TZ-14-07	TAN	IM	RB	IM	TAN	RB	TAN	IM	RB	RB	TAN	36
TZ-14-08	TAN	IM	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	36
TZ-14-09	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	36
TZ-14-10	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	36
TZ-14-11	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	36
TZ-14-12	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	36
AUS-14-01	TAN	IM	RB	RB	TAN	RB	TAN	IM	TAN	IM	TAN	45
ARG-14-01	TAN	RB	RB	TAN	TAN	TAN	TAN	RB	TAN	TAN	TAN	73
ARG-14-02	TAN	RB	RB	TAN	TAN	RB	TAN	RB	TAN	TAN	TAN	64
ARG-14-03	TAN	RB	RB	TAN	TAN	RB	TAN	RB	RB	TAN	TAN	54
ARG-14-04	TAN	RB	RB	TAN	TAN	RB	TAN	RB	TAN	TAN	TAN	63
FL-07-01	IM	TAN	RB	RB	TAN	TAN	TAN	TAN	TAN	IM	TAN	64
TW-07-01	TAN	RB	IM	RB	TAN	RB	TAN	RB	RB	IM	TAN	36
TX-13-01	TAN	IM	IM	RB	TAN	RB	TAN	IM	RB	IM	TAN	36
RB/IM (%)[c]	4	92	96	80	0	92	0	72	80	84	0	

[a]*Rpp1* = LD09-16057, *Rpp1b* = LD10-30052, *Rpp2* = PI 417125, *Rpp3* = PI 462312, *Rpp4* = PI 459025B, *Rpp5a* = LD10-14274, *Rpp5b* = PI 200526, *Rpp6* = PI 567102B. LD10-14205 (Hyuuga) = *Rpp3* and *5,* UG5 = *Rpp1* and *3.* TAN = Tan colored infection type with abundantly sporulating uredinia, RB = reddish brown infection type (with little or no sporulation) and IM indicates the presence of an immune response with no visible symptoms.

[b]Isolate name consisting of three parts: (i) country or state of origin; AUS = Australia, ARG = Argentina, FL = Florida, KE = Kenya, MAL = Malawi, SA = South Africa, TW = Taiwan, TX = Texas, TZ = Tanzania; (ii) year of collection 07 (2007), 12 (2012), 13 (2013),14 (2014); and (iii) isolate number in each location.

[c]Percentage of TAN or RB/IM of the isolates and the differentials, respectively.

Table 3. *Phakopsora pachyrhizi* isolates from samples collected in eight countries, the year of collection and pathotype designation.

Isolate name[a]	Country of origin	Year of collection	Pathotype designation[d]
TZ-14-01	Tanzania	2014	1232
TZ-14-02	Tanzania	2014	1232
TZ-14-03	Tanzania	2014	1232
TZ-14-04	Tanzania	2014	1232
TZ-14-05	Tanzania	2014	1232
TZ-14-06	Tanzania	2014	1232
TZ-14-07	Tanzania	2014	1212
TZ-14-08	Tanzania	2014	1212
TZ-14-09	Tanzania	2014	1212
TZ-14-10	Tanzania	2014	1212
TZ-14-11	Tanzania	2014	1212
TZ-14-12	Tanzania	2014	1212
TW-07-01	Taiwan	2007	1212
MAL-14-01	Malawi	2014	1212
MAL-14-02	Malawi	2014	1212
MAL-14-03	Malawi	2014	1212
AUS-14-01[c]	Australia	2014	1252
ARG-14-02[c]	Argentina	2014	1353
ARG-14-01[c]	Argentina	2014	1753
ARG-14-03[c]	Argentina	2014	1313
ARG-14-04[c]	Argentina	2014	1353
TX-13-01[b]	USA	2013	1712
FL-07-01[b]	USA	2007	2652
KE-12-01	Kenya	2012	3212
SA-14-01[c]	South Africa	2014	5312

[a]The isolate name consists of three parts: (i) country or state of origin; AUS = Australia, ARG = Argentina, FL = Florida, KE = Kenya, MAL = Malawi, SA = South Africa, TW = Taiwan, TX = Texas, TZ = Tanzania; (ii) year of collection 07 (2007), 12 (2012), 13 (2013), 14 (2014); and (iii) isolate number in each location.

[b]Isolates maintained at the University of Illinois-Urbana, Soybean Pathology laboratory provided by Yi-Shou Huang in 2007.

[c]Leaf samples provided by Dr. Natalie Moore (NWS, Australia). Dr. Antonio Ivancovich (INRA Argentina) and Dr. Jarvie (Pannar Seed, South Africa).

[d]Pathotype designation was based on octal nomenclature. Octal digits were assigned as follows: 000 = 0, 100 = 1, 010 = 2, 110 = 3, 001 = 4, 101 = 5, 011 = 6, and 111 = 7. The octal digits in this system are sorted according to the number of plants susceptible to the particular isolate per triplet (Herman *et al*. 1999).

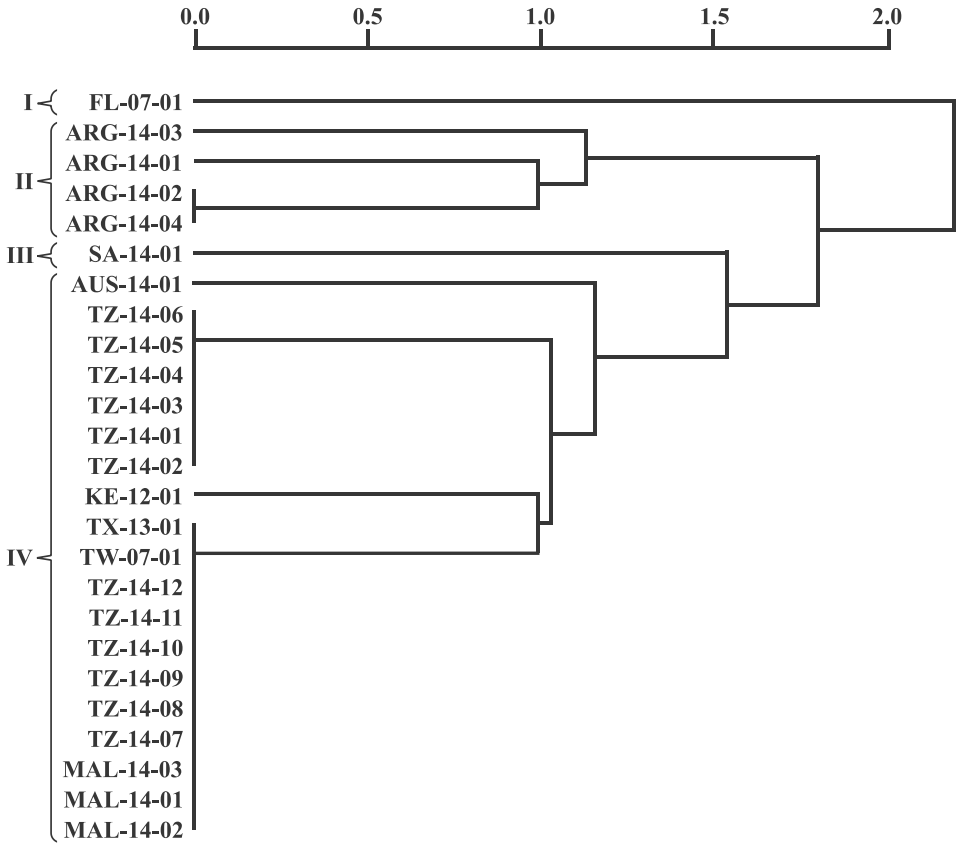

Figure 1. Cluster dendrogram of the 25 *Phakopsora pachyrhizi* isolates from eight different countries based on their virulence on the differential set of soybean. Cluster analysis was performed using R software version 3.2.0 (R Development Core Team 2008). The distance matrix was entered into a hierarchical clustering function of the software and the dendrogram was constructed using the minimum variance (Ward's method). Group I consist of an isolate from the United States (FL07-1), group II consists of isolates from Argentina (ARG-14-1-4). A South African isolate (SA-14-01) was grouped in III. The remaining isolates were grouped in IV (AUS = Australia, KE = Kenya, MAL = Malawi, TX = Texas, TW = Taiwan, and TZ = Tanzania).

DISCUSSION

The recent global spread of *P. pachyrhizi* requires a rapid diagnosis and consistent monitoring for the changes in virulence of different pathotypes in order to guide the development and deployment of soybean germplasm with durable host resistance. We discovered 10 distinct pathotypes with specific virulence patterns from the 25 isolates of *P. pachyrhizi* from eight countries when tested against a set of 11 differential hosts with known resistance genes. Four pathotypes were identified among the 17 isolates collected from Africa, with Kenyan and South African isolates classified into two distinct pathotypes (Table 3). The three isolates from Malawi and 50% of the Tanzanian isolates were grouped into one common pathotype, suggesting the presence of an identical *P. pachyrhizi* population in those countries. Isolates obtained from a single field did not differ in virulence, which suggested that they belonged to the same *P. pachyrhizi* population. We report for the first-time classification of *P. pachyrhizi* isolates into pathotypes from multiple East African countries. In Nigeria, located in West Africa, seven pathotypes among 116 isolates were reported (Twizeyimana *et al.,* 2009), while three pathotypes among 45 isolates were reported in Uganda (Tukamuhabwa and Maphosa 2012).

In our study, the South African isolate was the most virulent of the African isolates with virulence on six of the host differentials, including differentials with the resistance genes *Rpp2* and *Rpp3* that were effective against the pathotype from Kenya, Malawi, and Tanzania. An isolate from South Africa, collected in 2001, was shown to be avirulent on PI 462312 (*Rpp3*) (Bonde *et al.,* 2006), suggesting that a more virulent pathotype in South Africa may have appeared since the initial introduction of *P. pachyrhizi*. Pathotype 1212 was represented by six Tanzanian, the three Malawian, and the one Taiwanese isolate, which was the least virulent isolate in our study. Soybean cultivation in Malawi and Tanzania occurs only once per year, which implies that the pathogen population size may be reduced due to periods without a host. This may limit gene diversity in the pathogen since only a small subset of genetic diversity from the original population may be present in the new population. The isolate from Taiwan represents one of the older cultures within the collection in this study. It is possible that the culture has lost some of its original virulence due to prolonged storage.

Despite the limited number of isolates tested, four from Argentina were classified into three distinct pathotypes, while those from Australia, Taiwan, and the United States were each grouped into distinct pathotypes, suggesting a broader variation among *P.*

pachyrhizi isolates from the Americas used in our study as compared with those from Africa. Pathotype 1732 from Argentina was the most virulent, with virulence on eight differentials including the two cultivars Hyuuga and UG5 with multiple resistance genes. Greater virulence has previously been observed among isolates from Argentina compared with those from Brazil, Japan, and Paraguay (Akamatsu *et al.,* 2013). Twizeyimana and Hartman (2012) reported three distinct pathotypes among 72 *P. pachyrhizi* isolates collected from three different regions in the United States, while Yamaoka *et al.,* (2014) identified six races out of the 26 isolates collected within a single district in Japan. This suggests that *P. pachyrhizi* is highly dynamic across the globe, and underscores the need to screen and deploy effective and novel sources of resistance.

We did not find any isolates that differentiated *Rpp1*, *Rpp4*, and *Rpp5b* genes as all isolates produced TAN lesion types except for FL-07-01 from the United States on *Rpp1*. Previous studies on *Rpp1* reported TAN type infections among South American isolates (Akamatsu *et al.,* 2013; Miles *et al.,* 2008; Silva *et al.,* 2008), Thailand (Miles *et al.,* 2011), and Vietnam (Pham *et al.,* 2010). In contrast, *Rpp1* was effective against some isolates from the United States (Paul *et al.,* 2015; Twizeyimana and Hartman 2012) and Japan (Yamanaka *et al.,* 2010; Yamaoka *et al.,* 2014).

Our study showed the ineffectiveness of *Rpp4*, which was reported to confer resistance against rust populations from Brazil (Laperuta *et al.,* 2008; Pham *et al.,* 2009; Silva *et al.,* 2008; Yamanaka *et al.,* 2010), Japan (Yamanaka *et al.,* 2010; Yamaoka *et al.,* 2014), Vietnam (Pham *et al.,* 2010), Nigeria (Twizeyimana *et al.,* 2009), Uganda (Maphosa *et al.,* 2013), and Zimbabwe (Pham *et al.,* 2009). Others have similarly reported the ineffectiveness of *Rpp4* to isolates from Argentina and Paraguay (Akamatsu *et al.,* 2013). Our study showed the ineffectiveness of *Rpp5b*, which was reported to be effective against rust from South America and Japan (Akamatsu *et al.,* 2013; Lemos *et al.,* 2011; Yamaoka *et al.,* 2014), and from the United States and Vietnam (Kendrick *et al.,* 2011; Pham *et al.,* 2010; Twizeyimana and Hartman 2012). In Africa, TAN infection types previously have been reported on *Rpp5b* induced by isolates from South Africa and Zimbabwe (Kendrick *et al.,* 2011).

The *Rpp2* gene was highly effective against most isolates, but ineffective to one South African isolate that was similar to reports from South America (Akamatsu *et al.,* 2013; Laperuta *et al.,* 2008; Miles *et al.,* 2008; Yamanaka *et al.,* 2010) and Japan (Akamatsu *et al.,*

2013). There are no previous reports on testing of this genotype carrying *Rpp2* with rust isolates from Africa.

The *Rpp3* gene was mostly effective with only five isolates producing a TAN reaction; four from Argentina and the one South African isolate. In other studies, this gene was effective against rust populations in Japan (Yamanaka *et al.,* 2010; Yamaoka *et al.,* 2014), the United States (Paul *et al.,* 2015; Twizeyimana and Hartman 2012), Nigeria (Twizeyimana *et al.,* 2009), Uganda (Maphosa *et al.,* 2013), and Zimbabwe (Pham *et al.,* 2009). In contrast, *Rpp3* was ineffective against rust isolates from Argentina (Akamatsu *et al.,* 2013), Brazil (Laperuta *et al.,* 2008; Silva *et al.,* 2008), and Vietnam (Pham *et al.,* 2010). Gene *Rpp1b* was reported ineffective against rust isolates from Vietnam (Pham *et al.,* 2010) and the United States (Paul *et al.,* 2015), but was effective against rust in Nigeria (Twizeyimana *et al.,* 2009), where it conferred complete resistance. This gene was effective against most of the isolates in our study. *Rpp5a* gene was effective against rust from Colombia, Hawaii, South Africa, Vietnam, Zimbabwe (Kendrick *et al.,* 2011), and the United States (Paul *et al.,* 2015). This gene also was effective against 92% of the rust isolates in our study. The *Rpp6* gene discovered in PI 567102B (Li *et al.,* 2012) produced a resistance response to *P. pachyrhizi* populations from Paraguay (Miles *et al.,* 2008) and the United States (Paul *et al.,* 2015). Despite most of the isolates from our study inducing RB infection types on genotypes containing this gene, half of the isolates from Tanzania induced a TAN infection type. This suggests that its future as a source of resistance may be limited.

Our study, as well as other similar studies suggest an evolving virulence of the *P. pachyrhizi* population and that the *Rpp* genes can easily lose their durability for resistance and therefore may not be uniformly useful for resistance breeding. The breakdown of resistance could be attributed to evolution of local pathogen populations through mutation, recombination, immigration, or direct selection on the cultivars deployed (Burdon and Silk 1997; McDonald and Linde 2002).

Gene pyramiding stacks multiple resistance genes in a single cultivar has been suggested as a method to develop more durable resistance against *P. pachyrhizi* (Goellner *et al.,* 2010; Lemos *et al.,* 2011; Maphosa *et al.,* 2012; Yamanaka *et al.,* 2015; Yamaoka *et al.,* 2014). In the current study, cultivars UG5 and Hyuuga each carrying two genes had a resistance response to over 80% of the isolates providing evidence that gene stacking may be effective in certain cases. Cultivar UG5 was developed in Uganda and carries *Rpp1* and

Rpp3 (Paul *et al.,* 2015). The cultivar has been reported to consistently confer resistance against soybean rust in Nigeria (Twizeyimana *et al.,* 2009), the United States (Paul *et al.,* 2015; Twizeyimana and Hartman 2012), and Uganda (Maphosa *et al.,* 2013; Oloka *et al.,* 2008). Cultivar UG5 has been deployed in breeding programs in Nigeria and Uganda (Paul *et al.,* 2010; Tukamuhabwa and Maphosa 2012) and appears to be effective as was observed among the African isolates in our study. Despite its effectiveness, isolates from Argentina induced susceptible reactions on this cultivar, suggesting the presence of a more virulent population in South America. Cultivar Hyuuga, containing both *Rpp3* and *Rpp5*, also consistently exhibited resistant reactions against isolates from Africa. Kendrick *et al.,* (2011) reported the effectiveness of this cultivar among seven of the eight isolates from different countries as well as rust from Brazil (Yamanaka *et al.,* 2013). This cultivar could potentially be deployed in African soybean breeding programs to provide sustainable resistance to soybean rust. Virulence of AUS-14-01, FL 07-01, and three of the Argentinian isolates on cultivar Hyuuga and all the Argentinian isolates on cultivar UG5 suggests that stacking of only two resistance genes may not be sufficient to produce durable resistance. Yamanaka *et al.,* (2013) reported the effectiveness of a line carrying three *Rpp* genes (*Rpp1*, *Rpp4*, and Rpp5), as compared with lines carrying either one (*Rpp2*) or two (*Rpp2* and *Rpp4*) genes against single-lesion rust isolates from Brazil, demonstrating the need to stack multiple genes. Gene stacking has been reported to be effective in providing more durable resistance against for example bacterial blight of rice (Huang *et al.,* 1997; Singh *et al.,* 2001), rice leaf blast (Fukuoka *et al.,* 2015; Yasuda *et al.,* 2015), and leaf rust and stripe rust of wheat (Charpe *et al.,* 2012).

Although the number of isolates in our study was limited, it is clear that comparable virulence diversity exists among *P. pachyrhizi* populations from Africa, as well as from other continents. Therefore, to obtain a more detailed insight into the extent of this diversity, additional studies involving more isolates are required. The knowledge on *P. pachyrhizi* virulence and the available effective resistance genes from this study is especially important for Africa, where soybean rust has spread rapidly. Our study demonstrates that soybean lines carrying *Rpp2*, *Rpp3*, and *Rpp5a* genes and the cultivars Hyuuga and UG5 are effective against rust in eastern Africa. The information gained from our study will help breeders to determine what resistance genes should be used in their breeding programs. The current virulence diversity of *P. pachyrhizi* populations and its anticipated increase necessitate the continuous

monitoring of virulence changes and screening for more sources of novel resistance to guide targeted breeding to safeguard soybean production.

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CHAPTER 4

Diversity and distribution of pathotypes of the soybean rust fungus Phakopsora pachyrhizi in East Africa

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ABSTRACT

Phakopsora pachyrhizi is a biotrophic fungus that causes rust on soybean leading to devastating yield losses. Breeding for resistance is the preferred method for disease control. The development of resistant cultivars for deployment in different geographic regions requires a comprehensive understanding of the prevalent *P. pachyrhizi* pathotypes. To determine the pathotypes existing in four East African countries, 65 isolates were tested on 11 soybean host differentials. In addition, the virulence of isolates collected from the same region over multiple years and from different countries was compared. Out of the 12 pathotypes identified, pathotype 1000 that was found in all four countries accounted for the majority of isolates. Isolates from Kenya and Malawi were virulent on four differentials. All pathotypes were virulent on soybean genotypes carrying *Rpp1* and avirulent on cultivars carrying *Rpp1b*, *Rpp2* or *Rpp3* genes, as well as on cultivar No6-12-1 that carries *Rpp2*, *Rpp4* and *Rpp5*. Two of the pathotypes were virulent on cultivar UG 5 that carries *Rpp1* and *Rpp3* and on Hyuuga that carries *Rpp3* and *Rpp5*. The isolates collected from different countries differed in virulence across the years. This is the first study involving *P. pachyrhizi* isolates collected from four different countries in East Africa and tested on a common set of host differentials. The resistance genes identified in this study can be deployed for soybean breeding aimed at durable rust resistance.

INTRODUCTION

Soybean is a legume crop of significant and increasing importance for Africa. It contains up to 40% of high quality proteins and 20% of oil, making it suitable for human consumption and livestock feeding. In sub-Saharan Africa, about 6.8 million households grow soybean and the production is predicted to increase at a rate of 2-3% per annum (Abate *et al*., 2012). Soybean cultivation improves soil fertility due to the ability of the plant to fix nitrogen from the atmosphere via symbiosis with nitrogen fixing bacteria. Overall, soybean has the potential to significantly improve the nutrition of people in sub-Saharan Africa, and it contributes to increased economic opportunities and higher living standards for farmers. However, soybean yields in Africa are low (1.5 t/ha), compared to the yields obtained in the most productive countries like the USA (3.5t/ha), Brazil (2.9 t/ha) and Argentina (3 t/ha) (FAOSTAT, 2018). This is partly due to the numerous diseases and insect pests that limit soybean production.

Soybean rust caused by a biotrophic fungus, *Phakopsora pachyrhizi,* is the major disease of soybean. It has been recognized as one of the most important fungal pathogens worldwide (Dean *et al*., 2012)*,* because of its rapid spread across soybean growing regions and the potential to devastate the crop. The fungus produces numerous spores that are easily dispersed over short (Wen *et al.*, 2017) and long distances (Isard *et al*., 2007) by wind currents, leading to new infections. Soybean rust was first observed in Africa in 1996 (Kawuki *et al*., 2003) and has spread to more than 10 countries in the continent since then (Murithi *et al*, 2015). The yield losses from soybean rust infection range from 10 to 80% worldwide (Levy, 2005), raising concerns about its impact on the rapidly growing soybean sector in Africa. The yield losses are the result of lesions caused by the fungus on the soybean leaves that can result in complete defoliation, reducing the photosynthetic capacity leading to fewer pods and smaller seeds (Kumudini *et al*., 2008). Huge losses have been reported in countries like Brazil where, in the 2011/2012 growing season, grain losses caused by rust were estimated at over US$192 million while the costs of controlling it were approximately US$1.54 billion (Godoy *et al.*, 2016).

P. pachyrhizi pathotypes are groups of isolates that are classified based on their infection type (IT) on a set of host differentials carrying specific (combinations of) resistance genes. A tan-coloured (TAN) lesion with abundant sporulation indicates susceptibility, while reddish brown (RB) lesions with little to no sporulation and immune (IM) reactions signify resistance. *P. pachyrhizi* pathotypes were described for the first time in Taiwan based on the IT caused by nine different isolates on six soybean genotypes and five other legumes. Each pathotype induced identical IT on plants carrying specific resistance loci (Lin, 1966). Since then, the soybean host differentials are used for characterization of pathogenic variability among *P. pachyrhizi* isolates across the globe. The host differentials used in different studies may vary, but the most commonly used hosts are six lines carrying well characterized *P. pachyrhizi* resistance (*Rpp*) loci, namely *Rpp1* (Mc Lean & Byth, 1980), *Rpp2* (Bromfield & Hartwig, 1980), *Rpp3* (Bromfield & Melching, 1982), *Rpp4* (Hartwig, 1986), *Rpp5* (Garcia *et al*., 2008) and *Rpp6* (Li *et al*., 2012). In addition, soybean cultivars carrying multiple (two or three) resistance loci are also used (Paul *et al*, 2015, Yamanaka *et al*., 2013). The lack of a universal set of soybean host differentials limits the comparison of *P. pachyrhizi* pathotypes across the globe (Hartman *et al*., 2011). The emergence of novel rust pathotypes that overcome the major resistance genes (Yorinori *et al*., 2008, Akamatsu *et al*., 2017) limits the use of these cultivars in breeding,

since they only confer resistance to a limited number of isolates (Hartman *et al*., 2005; Miles *et al*., 2008). Similar pathogenic diversity has been observed for other pathogens like in *Puccinia graminis* f.sp. *tritici*, against which resistance broke down within a short period following deployment of particular resistance genes in breeding programs (Singh *et al*., 2015).

Development of durably resistant cultivars in different geographic regions requires a comprehensive understanding of the prevalent *P. pachyrhizi* pathotypes that are occurring in a particular region, due to the race-specificity of the available resistance genes. Variable numbers of *P. pachyrhizi* pathotypes have been reported in South America (Akamatsu *et al*., 2013), Japan (Yamaoka *et al*., 2014) and the USA (Twizeyimana and Hartman, 2012). A recent study involving 83 isolates from Argentina, Brazil and Paraguay, collected between 2010 and 2015 and tested on 16 host differentials, did not result in any identical virulence profile (Akamatsu *et al*., 2017). Thus, the pathotypes identified in all these studies demonstrate high variability among global *P. pachyrhizi* isolates in a population. In Africa, few studies have been conducted to determine the variability of *P. pachyrhizi*. To date, seven pathotypes have been reported in Nigeria and three in Uganda (Tukamuhabwa and Maphosa 2012; Twizeyimana *et al*., 2009). In Nigeria, three of the pathotypes were identified in all the three regions that were surveyed, two of which accounted for the majority (85%) of the isolates, and only one pathotype was unique for one of the regions. Four pathotypes were recently identified among a total of 17 isolates including those from Kenya, Malawi, South Africa and Tanzania. The most virulent pathotype was from South Africa, while the least virulent pathotype was found in both Malawi and Tanzania (Murithi *et al*., 2017). Due to the dynamic nature of *P. pachyrhizi*, similar studies are required to provide more insight into pathogen diversity at a regional level to guide deployment of disease management strategies.

The objectives of this study were to evaluate: (i) the virulence diversity of *P. pachyrhizi* populations in East Africa, (ii) the prevalence and distribution of particular *P. pachyrhizi* pathotypes and (iii) the effectiveness of the *Rpp* genes against the *P. pachyrhizi* population currently existing in East Africa.

MATERIALS AND METHODS

Field surveys, sample collection and isolation of *P. pachyrhizi* strains.

Samples of *P. pachyrhizi*-infected leaves were collected across the major soybean growing regions in Kenya (western region), Malawi (central and southern region), Tanzania (southern

highlands and eastern region) and Uganda (central, eastern, western and northern regions) in the growing seasons of 2015, 2016 and 2017. Disease severity (the percentage of affected leaf area) was evaluated on five randomly selected plants, based on a modified nine-point scale developed for soybean rust disease severity evaluation (Walker *et al*., 2011). The median severity percentage for the scale is as follows: 1 = 0%, 2 = 1%, 3 = 4%, 4 = 8%, 5 = 13%, 6 = 20%, 7 = 30%, 8 = 51% and 9 ≥ 68%. The soybean growth stages ranged from R4 (pod forming) to R6 (seed filling). In all locations, three individual leaflets at the bottom, middle and top canopy of the five randomly selected plants in each plot were rated individually. Disease severity of the entire plant was based on the mean disease severity at the three canopy levels. Five to ten leaf samples bearing sporulating uredinia from each field and from different cultivars were air-dried, wrapped in paper towels and transported to the lab in a cooling box and subsequently stored at 4˚C. Individual site details, including latitude, longitude and elevation, were recorded using a hand-held GPS (Garmin-eTrex 10; Garmin International Inc., Olathe, KS).

Establishment of *P. pachyrhizi* single pustule isolates

The recovered isolates were revived by hydrating overnight on a weigh boat placed in a petri dish containing a few drops of water. The spores were used to inoculate the abaxial surface of healthy detached leaves of the susceptible soybean cultivar Soya 1. The inoculated leaves were placed on moist filter papers in a 30 x 23 x 5 cm transparent plastic container. The filter papers were kept moist by adding distilled water at 3-day intervals or when needed. To promote infection, the containers were first incubated in a tissue chamber (Percival Scientific, Inc. Boone, IA) in the dark for 24 h at 23˚C, followed by 12-hour light/dark cycles at a temperature between 20 and 23˚C, and a humidity of 70% for 14 days. To purify the isolates, spores were collected from an isolated uredinium and were re-inoculated and multiplied on fresh leaves of Soya 1 under the same conditions as described above.

***P. pachyrhizi* isolate characterization on soybean differentials.**

Virulence of purified *P. pachyrhizi* isolates was assessed using 11 soybean host differentials with known rust resistance genes. The differential cultivars were obtained from the USDA Soybean Germplasm Collection and JIRCAS (Table 1). Soya 1 was used as universally susceptible control. Four seeds of each of the 11 differential lines were sown in plastic pots

filled with Kekilla Professional Peatmoss (Balton, Tanzania), mixed with 10 mg 18:46:00: N:P:K: DAP fertilizer (Yara, Tanzania). The plastic pots were placed in a growth chamber under alternating dark and light periods of 12 h each, at temperatures between 22˚C and 25˚C and humidity between 65% and 75%, under rust-free conditions. Four-week-old leaflets from the 2nd and/or 3rd trifoliate leaves were used for inoculation. Plastic containers were used with each containing one leaflet from each of the 11 soybean differentials and a single *P. pachyrhizi* isolate. The experiment was arranged in a randomized complete block design with three replications for each isolate. For inoculation, the abaxial side of the leaflets was moistened with distilled water. Leaves bearing fresh sporulating uredinia from each isolate were lightly tapped to dislodge the spores onto the leaflets of the differentials. The inoculated leaflets were then incubated in a tissue culture chamber at similar conditions as described above. To determine the ITs for each of the isolates, the leaflets were observed under a stereomicroscope (Olympus, Japan) at ×80 magnification at 14 days after inoculation. The *P. pachyrhizi* ITs were classified as immune (IM), reddish brown (RB) (avirulent) and TAN (virulent).

Table 1. The set of soybean host differentials employed for determining the virulence spectrum of the collected *Phakopsora pachyrhizi* isolates, their resistance genes, origin and the frequency of virulence of the different isolates used in this study.

Line/cultivar	Resistance gene(s)	Origin	Proportion of virulent isolates (%)	Reference
PI 200492	*Rpp1*	Japan	100	Mc Lean and Byth, 1982
PI 587855	*Rpp1b*	China	0	Yamanaka *et al*., 2016
PI 230970	*Rpp2*	Japan	0	Hartwig and Bromfield, 1983
PI 462312	*Rpp3*	India	0	Bromfield and Melching, 1982
PI 459025B	*Rpp4*	China	18.5	Hartwig, 1986
PI 200526	*Rpp5*	Japan	7.6	Garcia *et al*., 2008
PI 567102B	*Rpp6*	Paraguay	34	Lin, 2012
No6-12-1	*Rpp2,4* & *5*	Japan	0	Lemos *et al*., 2011
Hyuuga	*Rpp3* & *5*	Japan	4.6	Kendrick *et al*. 2011
UG 5	*Rpp1* & *3*	Uganda	3.1	Paul *et al*., 2015
Soya 1	None	Tanzania	100	This study

Comparison of ITs among isolates collected from 2014 to 2016.

To evaluate the possible variation in virulence between isolates collected from the 2014 to 2016 cropping seasons, we selected *P. pachyrhizi* isolates collected from same regions in the respective years. The isolates were collected from sites that were about 5-10 km apart in each region. The data on the ITs that were caused by isolates collected in 2014 were obtained from Murithi *et al*. (2017). To ensure uniformity of the data, we compared the ITs on seven identical host differentials for both studies and these included PI 200492 (*Rpp1*), PI 462312 (*Rpp3*), PI 459025B (*Rpp4*), PI 200526 (*Rpp5*), PI 567102B (*Rpp6*) and the soybean cultivars Hyuuga (*Rpp3,5*) and UG 5 (*Rpp1,3*). Evaluation of the ITs was conducted as described above.

Analysis of *P. pachyrhizi* pathotype and virulence spectrum.

The three IT classifications (TAN, RB and IM) were used to assign a pathotype to each *P. pachyrhizi* isolate that was obtained in our surveys. The octal nomenclature system (Goodwin *et al*. 1990) was used for pathotype classification, by coding "1" for a TAN IT (compatible) while both RB and IM ITs were coded "0" (incompatible) (Twizeyimana *et al*. 2009). The Habgood-Gilmour Spreadsheet (HaGiS) program V.3.1 (Hermann *et al*. 1999) was used to summarize the virulence data of each isolate by converting the data into an octal. In this format, the 11 host differentials were arranged in groups of three and assigned one octal digit as follows. The first octal digit contains PI 200492 (*Rpp1*), PI 587855 (*Rpp1b*), and PI 230970 (*Rpp2*); the second octal digit includes PI 462312 (*Rpp3*), PI 459025B (*Rpp4*), and PI 200526 (*Rpp5*); the third octal digit consists of PI 567102B (*Rpp6*), cultivar Hyuuga (*Rpp3* and *Rpp5*) and UG 5 (*Rpp1* and *Rpp3*), and the fourth octal digit is represented by cultivar No6-12-1 (*Rpp2*, *Rpp4*, and *Rpp5*). Octal digits are assigned as follows: 000 = 0, 100 = 1, 010 = 2, 001 = 4, 110 = 3, 101 = 5, 011 = 6, and 111 = 7, sorted according to the number of virulence per triplet. The virulence frequencies and mean virulence complexity were calculated for each isolate.

Statistical Analysis

Analysis of variance for disease severity was conducted using PROC GLIMMIX in SAS version 9.3 (SAS Institute, Cary, NC). Disease severity of the entire plant was based on the mean severity of the three canopy levels. The disease severity of five randomly selected plants in each plot was used for data analysis.

RESULTS

Field surveys, sample collection and virulence of the various P. pachyrhizi isolates on soybean host differentials.

In the field surveys conducted in the 2015, 2016 and 2017 soybean growing seasons, a total of 150 isolates were collected from Kenya (37), Malawi (15), Tanzania (51) and Uganda (47). In all countries, the disease severity ranged between 5 and 90%, the highest of which (20-90%) were recorded in Uganda. A total of 65 isolates were eventually recovered from infected leaf samples that were collected from the four countries (Table 2). The highest number of isolates recovered was from Kenya (25), followed by Uganda (18), Tanzania (15) and Malawi (7) (Table 3). The variable number of the isolates recovered from each country may reflect differences in sample handling during transportation from the field to the laboratory.

When tested on the 10 host differentials, all the 65 isolates (Table 2) were found to be virulent on PI 200492 (*Rpp1*) and on the susceptible Soya 1 cultivar that lacks known resistance genes, indicating that the *Rpp1* locus is not effective against isolates from East Africa (Table 2). Furthermore, Table 2 shows that 12(18%) of the isolates were virulent on PI 459025B (*Rpp4*) and 23 (35%) on PI 567102B (*Rpp6*), while less than 8% of the isolates were virulent on PI 200526 (*Rpp5*). Four (57%) of the isolates from Malawi, three (20%) from Tanzania and five (28%) from Uganda were virulent on PI 459025B (*Rpp4*). Three (42%) of the isolates from Malawi and two (13%) of the isolates from Tanzania were virulent on PI 200526 (*Rpp5*). Ten (40%) of the isolates from Kenya, five (71%) from Malawi, four (27%) from Tanzania and three (17%) from Uganda were virulent on PI 567102B (*Rpp6*). None of the isolates from Kenya were virulent on lines with the *Rpp4* and *Rpp5* loci. 48% of the Kenyan isolates induced immune IT on cultivar No6-12-1, while all other isolates induced RB ITs suggesting that this cultivar is highly resistant to the rust populations in Kenya. All the isolates were avirulent on PI 587855 (*Rpp1b*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*) and soybean cultivar No6-12-1, indicating that the corresponding resistance genes are effective against all rust pathotypes identified in this study and could therefore be used for developing durable resistance.

Table 2. Infection types (ITs), virulence complexity and pathotype of *Phakopsora pachyrhizi* isolates sampled in East Africa on 11 soybean differentials, including the universally susceptible Soya 1 cultivar.

	P. pachyrhizi resistance genes												
Isolates[a]	*Rpp1*	*Rpp1b*	*Rpp2*	*Rpp3*	*Rpp4*	*Rpp5*	*Rpp6*	*Rpp1,3*	*Rpp3,5*	*Rpp2 Rpp4, Rpp5*	Soya 1[b]	Complexity[c]	Pathotype
KE-17-01	TAN[d]	RB[e]	RB	RB	RB	RB	TAN	TAN	TAN	IM[f]	TAN	4	1070
KE-17-02	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-03	TAN	RB	RB	RB	RB	RB	TAN	RB	TAN	IM	TAN	3	1050
KE-17-04	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	IM	TAN	2	1010
KE-17-05	TAN	RB	RB	RB	RB	RB	RB	RB	RB	IM	TAN	1	1000
KE-17-06	TAN	RB	RB	RB	RB	RB	TAN	TAN	RB	IM	TAN	3	1030
KE-17-08	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-09	TAN	RB	RB	RB	RB	RB	RB	RB	TAN	IM	TAN	2	1040
KE-17-13	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
KE-17-14	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-15	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
KE-17-16	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	IM	TAN	2	1010
KE-17-17	TAN	RB	RB	RB	RB	RB	RB	RB	RB	IM	TAN	1	1000
KE-17-20	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-21	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-22	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-23	TAN	RB	RB	RB	RB	RB	RB	RB	RB	IM	TAN	1	1000
KE-17-27	TAN	RB	RB	RB	RB	RB	RB	RB	RB	IM	TAN	1	1000
KE-17-28	TAN	RB	RB	RB	RB	RB	RB	RB	RB	IM	TAN	1	1000
KE-17-30	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-31	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000

KE-17-32	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
KE-17-33	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
KE-17-36	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
KE-17-37	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	IM	TAN	2	1010
Mal 15-01	TAN	RB	RB	RB	TAN	TAN	RB	RB	RB	RB	TAN	3	1600
Mal 15-02	TAN	RB	RB	RB	TAN	TAN	TAN	RB	RB	RB	TAN	4	1610
Mal 15-03	TAN	RB	RB	RB	TAN	TAN	TAN	RB	RB	RB	TAN	4	1610
Mal-16-01	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	3	1210
Mal-16-02	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Mal-16-11	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
Mal 16-20	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
Tz-15-51	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	3	1210
Tz-15-64	TAN	RB	RB	RB	RB	TAN	TAN	RB	RB	RB	TAN	3	1410
Tz-15-88	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Tz-15-48m	TAN	RB	RB	RB	TAN	RB	RB	RB	RB	RB	TAN	2	1200
Tz-16-08	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
Tz-16-09	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Tz-16-10	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Tz-16-11	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Tz-16-12	TAN	RB	RB	RB	TAN	RB	RB	RB	RB	RB	TAN	2	1200
Tz-15-22	TAN	RB	RB	RB	RB	TAN	RB	RB	RB	RB	TAN	2	1400
Tz-15-29	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Tz-15-36	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Tz-17-01	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010

TZ-17-02	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
Tz-17-03	TAN	RB	RB	RB	RB	RB	TAN	RB	RB	RB	TAN	2	1010
UG-16-01	TAN	RB	RB	RB	TAN	RB	RB	RB	RB	RB	TAN	2	1200
UG-16-03	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	3	1210
UG-16-08	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	3	1210
UG-16-15	TAN	RB	RB	RB	TAN	RB	TAN	RB	RB	RB	TAN	3	1210
UG-16-30	TAN	RB	RB	RB	TAN	RB	RB	RB	RB	RB	TAN	2	1200
UG-17-02	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-04	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-05	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-09	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-10	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-11	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-12	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-14	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-16	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-17	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-18	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-19	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000
UG-17-20	TAN	RB	RB	RB	RB	RB	RB	RB	RB	RB	TAN	1	1000

[a] KE-Kenya, Mal-Malawi, Tz-Tanzania, UG-Uganda.
[b] Universally susceptible control.
[c] Average number of soybean differentials with which a given isolate has a compatible interaction.
[d] TAN: tan infection type.
[e] RB: reddish-brown infection type.
[f] IM: immune infection type.

Table 3. List of recovered *Phakopsora pachyrhizi* isolates that were used to analyse the virulence spectrum with the country of origin and the year of collection.

	Number of recovered isolates			
Country of origin	2015	2016	2017	**Total**
Kenya	0	0	25	25
Malawi	3	4	0	7
Tanzania	7	5	3	15
Uganda	0	5	13	18
Total	**10**	**14**	**41**	**65**

Notably, two Kenyan isolates, KE-17-01 and KE-17-03, were virulent on cultivars UG 5 and Hyuuga, while isolates KE-17-6 and KE-17-09 were virulent on Hyuuga but not on UG 5 (Table 2). Interestingly, isolate KE-01-17 was avirulent on PI 462312 (*Rpp3*) and PI 200526 (*Rpp5*) but it was virulent on cultivar Hyuuga that contains both resistance genes (Table 2). This may suggest that the alleles for resistance in cultivar Hyuuga are different from the alleles in *Rpp3* and *Rpp5.* 48% of the Kenyan isolates induced immune ITs on cultivar No6-12-1, while all other isolates induced RB ITs suggesting that the cultivar is highly resistant to the rust populations in Kenya (Table 2).

Comparison of ITs caused by the various *P. pachyrhizi* isolates collected in East Africa from 2014 to 2016.

When the infection types of the isolates collected in different years were compared, all the 34 isolates collected from Malawi and Tanzania caused TAN IT on PI 200492 (*Rpp1*), while RB ITs were produced on PI 462312 (*Rpp3*) and cultivar Hyuuga (Table 4). This again confirms that the *Rpp1* locus is ineffective and that the *Rpp3* locus and cultivar Hyuuga and are highly effective against rust populations in Malawi and Tanzania. All the isolates collected from Malawi over the years triggered TAN ITs on PI 459025B (*Rpp4*), except for one isolate. Likewise, all the 12 isolates from Tanzania collected in 2014 had TAN ITs, while from the seven isolates collected in 2015; two of them induced TAN ITs on the *Rpp4*-containing differential (Table 4). Interestingly, all five isolates collected in 2016 from Tanzania caused RB IT. All six isolates collected in Malawi in 2014 and 2015 triggered TAN ITs on PI 200526 (*Rpp5*), while all the 4 isolates collected in 2016 caused RB IT (Table 4). Similar observations were made in Tanzania, were all the 12 isolates collected in 2014 caused TAN ITs, whereas of the seven

isolates collected in 2015 six caused RB IT and only one still caused a TAN IT on the *Rpp5*-containing soybean differential (Table 4). Only one isolate caused a TAN IT in 2016, while the other four caused RB ITs on this differential (Table 4). These results demonstrate that isolates collected from various regions in different years differed in their virulence spectrum.

In 2014, two of the *P. pachyrhizi* isolates collected in Malawi induced RB ITs on PI 567102B (*Rpp6*), while only one induced an IM IT. Of the three isolates collected in 2015, one resulted in an RB IT, while the other two produced a TAN IT on this differential. All but one isolate collected in 2016 in Malawi induced TAN IT (Table 4). For Tanzania, 50% (6) of the isolates collected in 2014 induced an RB IT, while the other six isolates resulted in a TAN IT on the *Rpp6* differential. In contrast, only one isolate of the 12 isolates collected in 2015 and 2016 had a TAN infection type, while the remainder of the isolates showed an RB IT (Table 4). The isolates from Malawi collected in 2014 caused an IM IT on UG 5, while those collected in 2015 (3) and 2016 (4) produced RB ITs. Either RB or IM ITs were observed on UG 5 when inoculated with the Tanzanian isolates collected in 2014, while those collected in 2015 and 2016 resulted in RB IT only (Table 4). This indicates that UG 5 is highly effective against rust populations in Malawi and Tanzania.

Table 4. Comparison of infection types (ITs) among *Phakopsora pachyrhizi* isolates sampled during the 2014 to 2016 growing seasons in Malawi (Mal) and Tanzania (Tz).

Isolates	Year of collection	Differential resistance genes[a]						
		Rpp1	***Rpp3***	***Rpp4***	***Rpp5***	***Rpp6***	***Rpp3* and *Rpp5***	***Rpp1* and *Rpp3***
Mal-14-01	2014[b]	TAN	RB	TAN	TAN	RB	RB	IM
Mal-14-02		TAN	RB	TAN	TAN	RB	RB	IM
Mal-14-03		TAN	RB	TAN	TAN	IM	RB	IM
Mal 15-01	2015	TAN	RB	TAN	TAN	RB	RB	RB
Mal 15-02		TAN	RB	TAN	TAN	TAN	RB	RB
Mal 15-03		TAN	RB	TAN	TAN	TAN	RB	RB
Mal 16-01	2016	TAN	RB	TAN	RB	TAN	RB	RB
Mal-16-11		TAN	RB	TAN	RB	TAN	RB	RB
Mal-16-20		TAN	RB	TAN	RB	TAN	RB	RB
Mal-16-21		TAN	RB	RB	RB	RB	RB	RB
Tz-14-01	2014[b]	TAN	RB	TAN	TAN	TAN	RB	IM
Tz-14-02		TAN	RB	TAN	TAN	TAN	RB	IM
Tz-14-03		TAN	RB	TAN	TAN	TAN	RB	IM
Tz-14-04		TAN	RB	TAN	TAN	TAN	RB	RB
Tz-14-05		TAN	RB	TAN	TAN	TAN	RB	IM
Tz-14-06		TAN	RB	TAN	TAN	TAN	RB	RB
Tz-14-07		TAN	RB	TAN	TAN	RB	RB	RB
Tz-14-08		TAN	RB	TAN	TAN	RB	RB	RB
Tz-14-09		TAN	RB	TAN	TAN	RB	RB	RB
Tz-14-10		TAN	RB	TAN	TAN	RB	RB	RB
Tz-14-11		TAN	RB	TAN	TAN	RB	RB	RB
Tz-14-12		TAN	RB	TAN	TAN	RB	RB	RB
Tz-15-51	2015	TAN	RB	TAN	RB	RB	RB	RB
Tz-15-64		TAN	RB	RB	RB	RB	RB	RB
Tz-15-88		TAN	RB	RB	RB	RB	RB	RB
Tz-15-48M		TAN	RB	TAN	RB	RB	RB	RB
Tz-15-22		TAN	RB	RB	RB	RB	RB	RB
Tz-15-29		TAN	RB	RB	TAN	RB	RB	RB
Tz-15-36		TAN	RB	RB	RB	RB	RB	RB
Tz-16-01	2016	TAN	RB	RB	RB	TAN	RB	RB
Tz-16-02		TAN	RB	RB	RB	RB	RB	RB
Tz-16-08		TAN	RB	RB	RB	RB	RB	RB
Tz-16-10		TAN	RB	RB	RB	RB	RB	RB
Tz-16-11		TAN	RB	RB	RB	RB	RB	RB

[a] *Rpp1* (PI 200492), *Rpp3* (PI 462312), *Rpp4* (PI 459025B), *Rpp5* (PI 200526), *Rpp6* (PI 567102B) and cultivars Hyuuga (*Rpp3*,*5*) and UG 5 (*Rpp1*,*3*).

[b] ITs data for differentials originally reported in Murithi *et al*., 2017.

***P. pachyrhizi* pathotype evaluation, analysis and distribution**.

In total, 12 pathotypes were identified among the 65 *P. pachyrhizi* isolates tested in this study. The highest number of pathotypes (6) was found among the isolates collected in Kenya and Tanzania, while five pathotypes were identified in Malawi and three in Uganda (Table 5). Pathotype 1000 was the most frequent and comprised 35 isolates, representing 54% of all the collected isolates. This pathotype was present in all four countries. Another frequently isolated pathotype was 1010, which included 12 (18%) isolates and was found in three countries, while each of the remaining pathotypes occurred infrequently and represented less than 8% of all the isolates (Table 5).

About 56% (14) of all isolates from Kenya and 72% (13) of all isolates from Uganda belonged to pathotype 1000. Of the six pathotypes identified in Kenya, four (1030, 1040, 1050, 1070) were unique, as they were not found in any of the other countries and each was represented by one isolate. In the same way, pathotypes 1600 and 1610 were unique for Malawi and were represented by one and two isolates, respectively. Pathotype 1200 was unique for Uganda and Tanzania, while pathotypes 1400 and 1410 were only found in Tanzania. Pathotype 1210 was found in all countries, except in Kenya (Table 5). This means that the virulence changes that occur among isolates in each country are independent. The virulence complexity of the different *P. pachyrhizi* pathotypes, defined as the average number of soybean differentials with which an isolate has a compatible interaction, varied between one and four, with the majority of the isolates (54%) having a complexity of 1 (Table 5). A virulence complexity of 2 to 3 was observed for about 42% of the isolates tested, while only 4.6% of the *P. pachyrhizi* isolates had a virulence complexity of 4, indicating that only a few isolates were compatible with four of the 11 differentials.

Table 5. Pathotypes for *Phakopsora pachyrhizi* isolates sampled in East Africa, their virulence complexity and distribution in different countries.

Pathotype[a]	Virulence complexity[b]	No of isolates				
		Kenya	Malawi	Tanzania	Uganda	Total
1000	1	14	1	7	13	35
1010	2	7	2	3		12
1030	3	1				1
1040	2	1				1
1050	3	1				1
1070	4	1				1
1200	2			2	2	4
1210	3		1	1	3	5
1400	2			1		1
1410	3			1		1
1600	3		1			1
1610	4		2			2
No. of isolates		**25**	**7**	**15**	**18**	**65**
No. of different pathotypes		**6**	**5**	**6**	**3**	**12**

[a] Pathotypes were determined using the octal nomenclature system (Goodwin *et al*. 1990) classification by coding "1" for TAN reactions (susceptible) while IM and RB reactions were coded "0" (resistant).
[b] Average number of soybean differentials with which a given isolate has a compatible interaction.

Pathotypes 1070 and 1610, found in Kenya and Malawi respectively, were virulent on four of the ten host differentials, being *Rpp1*, *Rpp4, Rpp5* and *Rpp6*, while pathotype 1000 was virulent on only one of the differentials (*Rpp1*) (Table 6). Pathotype 1070 was virulent on *Rpp6* and on both cultivars UG 5 and Hyuuga, while pathotype 1030 was virulent on *Rpp6* and on cultivar UG 5. Additionally, more than 50% of the isolates were virulent on *Rpp6* (Table 6), indicating that this differential may be used to distinguish the majority of isolates from this region. The most striking observation from this study was for pathotypes 1040 and 1070, both of which were virulent on cultivar Hyuuga (carrying both *Rpp3* and *Rpp5*), but were avirulent on the soybean differentials carrying only *Rpp3* or *Rpp5* (Table 6). This suggests that the *Rpp3* and *Rpp5* resistance loci in Hyuuga convey a different recognition specificity, and are likely allelic variants.

Table 6. Virulence profile and frequency of pathotypes of *Phakopsora pachyrhizi* isolates collected in East Africa and tested on ten *Rpp* gene differentials and the universally susceptible cultivar Soya 1.

	***P. pachyrhizi* resistance genes**[a]											
Pathotype	*Rpp1*	*Rpp1b*	*Rpp2*	*Rpp3*	*Rpp4*	*Rpp5*	*Rpp6*	*Rpp1,3*	*Rpp3,5*	*Rpp2,4,5*	Soya 1[b]	Frequency (%)[c]
1000	+	-	-	-	-	-	-	-	-	-	+	53.8
1010	+	-	-	-	-	-	+	-	-	-	+	18.5
1030	+	-	-	-	-	-	+	+	-	-	+	1.5
1040	+	-	-	-	-	-	-	-	+	-	+	1.5
1050	+	-	-	-	-	-	+	-	+	-	+	1.5
1070	+	-	-	-	-	-	+	+	+	-	+	1.5
1200	+	-	-	-	+	-	-	-	-	-	+	6.2
1210	+	-	-	-	+	-	+	-	-	-	+	7.7
1400	+	-	-	-	-	+	-	-	-	-	+	7.7
1410	+	-	-	-	-	+	+	-	-	-	+	1.5
1600	+	-	-	-	+	+	-	-	-	-	+	1.5
1610	+	-	-	-	+	+	+	-	-	-	+	3.1

[a] *Rpp1* (PI 200492), *Rpp1b* (PI 587855), *Rpp2* (PI 230970), *Rpp3* (PI 462312), *Rpp4* (PI 459025B), *Rpp5* (PI 200526), *Rpp6* (PI 567102B), UG 5 (*Rpp1,3*), Hyuuga (*Rpp3,5*), No-6-12 (*Rpp2,4,5*).
[b] Susceptible check.
[c] Pathotype frequency; Symbols: +, compatible interaction; -, incompatible interaction.

DISCUSSION

To deploy durable resistance, knowledge of the virulence spectrum that exists in the local *P. pachyrhizi* population is required. We investigated the virulence diversity of *P. pachyrhizi* isolates using a set of 11 soybean differentials and identified 12 different pathotypes among the 65 isolates collected from East Africa. Virulence of *P. pachyrhizi* isolates on the soybean differentials (Table 6) supports the observation that more virulent races have evolved that overcome the resistance conferred by the majority of the resistance loci worldwide (Yorinori, 2008). For instance, resistance conferred by *Rpp1* was overcome within three years of its discovery in Australia (Hartman *et al*., 2011). This is consistent with previous reports where all *P. pachyrhizi* isolates from Africa and some isolates from South America were virulent on plants that carry *Rpp1* (Bonde *et al*., 2006, Twizeyimana, *et al*., 2009, Akamatsu *et al*., 2013, Murithi *et al*., 2017). Although *Rpp1* was ineffective against most African isolates, it may still confer resistance to rust isolates in Japan, Mexico and the USA (Twizeyimana and Hartman 2012, Yamaoka *et al*., 2014).

For the first time, isolates that are virulent on cultivars UG 5 (*Rpp1* and *Rpp3*) and Hyuuga (*Rpp3* and *Rpp5*), both carrying multiple resistance loci (Paul *et al*., 2015, Kendrick *et al*., 2011) were identified in Kenya (Tables 5 and 6). Previously, *P. pachyrhizi* isolates from Nigeria, Uganda and the US induced immune infection types on cultivar UG 5 (Twizeyimana *et al*., 2009 and 2012). Virulence on UG 5 and Hyuuga could be due to the emergence of more virulent pathotypes that may have developed as a result of mutation, recombination, immigration, or direct selection (McDonald and Linde, 2002).

The high frequency of pathotype 1000 in the region may be attributed to a limited genetic variation among *P. pachyrhizi*, caused by the utilization of a single resistance gene over a long time. This may have selected for this particular pathotype and shaped *P. pachyrhizi* to be present at a low diversity in the region. Deployment of similar resistance genes over large areas was shown to contribute to the uniform virulence spectra among a global collection of wheat stripe rust (*Puccinia striiformis* f.sp *tritici*) isolates (Sharma-Poudyal *et al*., 2013).

Pathotypes 1040 and 1070 were both virulent on cultivar Hyuuga, which has a natural stack of the *Rpp3* and *Rpp5* locus (Kendrick *et al*., 2011). It is surprising that both pathotypes were avirulent on soybean differentials carrying either *Rpp3* or *Rpp5* (Table 6). Pathotype 1070 was also virulent on UG 5 that contains *Rpp1* and *Rpp3.* Although pathotype 1070 is virulent on *Rpp1*, it is avirulent on *Rpp3*. This suggests that the *Rpp3* and *Rpp5* alleles in UG 5 and Hyuuga are different from those in PI 462312 and PI 200526, respectively. Multiple alleles of *Rpp5* have previously been reported and the *Rpp5* locus and recognition spectrum in PI 200526 was found to be different from the one present in Hyuuga (Garcia *et al*., 2008). Likewise, although the *Rpp3* in PI 462312 maps to the same locus as Hyuuga they possess different recognition specificities (Kendrick *et al.*, 2011), and are therefore likely not identical. Interestingly, Hyuuga is a cross between Akasaya and Ako musume (Hossain *et al*., 2014). Akasaya (PI 416764) is a Japanese landrace with a resistance locus similar to *Rpp3* that clusters with PI 462312 (*Rpp3*) (Hossain *et al*., 2014). Although these two accessions are closely related, some Argentinian *P. pachyrhiz*i isolates caused different infection types on these accessions (Akamatsu *et al*., 2013). Likewise, a Brazilian isolate produced TAN infection types on PI 462312, while it caused an RB infection type on PI 506764 (Silva *et al*., 2008). Our results corroborate these earlier observations and therefore PI 462312 and PI 506764 likely carry different alleles of the *Rpp3* locus.

In this study, a cultivar with a stack of three resistance loci was tested for the first time against *P. pachyrhizi* isolates from Africa, and was found to be immune. This finding is consistent with reports on the resistance of the No6-12-1 cultivar against isolates from Brazil and Japan (Yamanaka *et al*., 2015). Cultivar No6-12-1 originates from Japan and it contains three resistance loci: *Rpp2*, *Rpp4*, and *Rpp5* (Lemos *et al*., 2011). Arguably, the stacking of multiple major resistance genes helps to cope with the rapid evolution of pathogen virulence, as the pathogen will have to mutate multiple avirulence genes simultaneously to gain virulence on plants with the stacked *R* genes. This becomes less likely with the deployment of several novel resistance genes. Therefore, stacking of resistance genes is likely to enhance the durability of resistance of soybean against *P. pachyrhizi* (Mundt *et al*., 2014, McDonald and Linde, 2002). Nevertheless, some of the gene combinations, like *Rpp1* + *Rpp2* + *Rpp4*, were found to be ineffective against a highly virulent Brazilian isolate (Yamanaka *et al*., 2015). Thus, careful selection of the resistance gene combinations is important to develop durable resistance. Furthermore, as most genes are deployed individually already, the pathogen may overcome each of these resistance genes in a sequential fashion.

The present study identified *Rpp1b, Rpp2*, *Rpp3* and the cultivar No6-12-1 with three resistance genes (*Rpp2, Rpp4* and *Rpp5*), as the most suitable genes for deployment in the African soybean breeding program (Table 6). *Rpp1b, Rpp2* and *Rpp3* are effective, for instance, in Japan (Yamanaka *et al*., 2010, Yamaoka, 2014), the United States (Paul *et al*., 2015), Nigeria (Twizeyimana *et al*., 2009), Tanzania, Malawi (Murithi *et al*., 2017) and Uganda (Maphosa *et al*., 2012). Although *Rpp2* and *Rpp3* are ineffective in South America (Yorinori, 2008, Akamatsu *et al*., 2013), our study confirms that they provide resistance to *P. pachyrhizi* isolates in East Africa, and therefore can be deployed in the soybean breeding programs. However, care needs to be taken to prevent the introduction of South American *P. pachyrhizi* isolates into Africa.

Cultivar UG 5 has been widely used in breeding programs in Nigeria and Uganda. However, the susceptibility of this cultivar to some of the Kenyan isolates implies that its use might be short-lived, as pathotypes that are also virulent on UG 5 have emerged. Similarly, although cultivar Hyuuga was previously identified as a suitable candidate for breeding programs in Africa (Murithi *et al*., 2017), the susceptibility of this cultivar to some of the isolates tested in this study (Table 6), implies that the *Rpp3* and *Rpp5* combination may no longer be effective against isolates from Kenya.

The results of our study reveal the highly dynamic virulence diversity among *P. pachyrhizi* isolates. This calls for a consistent surveillance of the virulence profile of the occurring strains in order to understand the changes that continuously occur in the field and to inform breeders on the most suitable resistance genes to deploy. Pyramiding of the most effective resistance genes should be pursued for developing the most durable, and most broad-spectrum resistance against *P. pachyrhizi.*

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CHAPTER 5

Evaluation of soybean accessions for resistance against soybean rust in East Africa

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ABSTRACT

Soybean rust caused by the biotrophic fungus *Phakopsora pachyrhizi* is the most important foliar disease of soybean (*Glycine max*) worldwide. Deployment of resistant soybean cultivars is the preferred method of managing the disease. Genes conferring resistance to *P. pachyrhizi* have been identified, but rust pathotypes that overcome these resistance genes have been found as well. In order to identify novel resistance genes, 77 soybean accessions from both local and international sources were screened at multiple locations in Tanzania and Uganda in the years 2016 and 2017. The results from this screening revealed that infection types, disease severities and sporulation levels varied among the accessions and locations. The majority of the accessions displayed tan-coloured (TAN) lesions and developed moderate sporulation, implying susceptibility, while a handful of accessions showed a low disease severity and displayed reddish brown (RB) lesions, signifying resistance. We identified seven accessions that were the most resistant to rust in most locations over the two years. These accessions are useful for further study and, ultimately, rust management.

INTRODUCTION

Soybean (*Glycine max* (L.) Merill) is an important legume crop as it is a major source of protein and oil in Africa (Hartman *et al*., 2011). Soybean is used as a component in livestock feed, but also for human consumption in the form of soymilk, tofu, soybean oil and also as a vegetable (Ali *et al*., 2010). Besides its importance as food/feed source soybean cultivation is important for the improvement of soil quality, as soybean cultivation leads to the fixation of nitrogen into the soil, leading to improved soil fertility in turn. Hence, it is a preferred crop for intercropping and rotation with non-leguminous crops (Sanginga *et al*., 2003). More than 2.1 million tonnes of soybeans were produced in Africa in 2016, representing a 67% increase since 2007 (FAOSTAT, 2018). Soybean production has intensified in eastern and southern Africa; a trend that is expected to continue. For instance, in Malawi, soybean production has more than tripled since 2005, while the production area increased by about 50% within the same period (FAOSTAT, 2018). Soybean production in Tanzania is concentrated in the southern highlands and the production area and quantity has doubled over the past ten years (FAOSTAT, 2018). Similar trends have been observed in Kenya, Rwanda and Uganda (Murithi *et al*. 2016). Average yields range between 0.8 and 1.2 tonnes/ha while the yield potential is predicted to range between 2.5 and 4 tonnes/ha (FAOSTAT, 2018). The relatively low

productivity of soybean is largely due to abiotic factors (soil fertility, drought and poor nodulation) and biotic ones (diseases and insect pests) (Wrather *et al.*, 1997).

Rust, caused by the biotrophic fungus *Phakopsora pachyrhizi*, is one of the most damaging foliar diseases of soybean. The disease is native to Asia but has spread to Australia, India (Goellner *et al*., 2010), and Africa where it was first reported in Uganda in 1996 (Levy, 2005). It subsequently spread to Brazil in 2002 (Yorinori *et al*., 2005) and to the United States in 2004 (Schneider *et al*., 2005). Its introduction into Africa probably occurred through urediniospores blowing from western India to the African east coastal areas by moist northeast monsoon winds (Levy, 2005). The fungus spread rapidly and was reported after its introduction into Uganda on soybean in South Africa in 2001 (Pretorius *et al.*, 2001), in western Cameroon in 2003 (Levy, 2005), and in Ghana and the Democratic Republic of Congo in 2007 (Bandyopadhyay *et al.*, 2007; Ojiambo *et al*., 2007). The disease was also confirmed in Ethiopia, Malawi and Tanzania (Tesfaye *et al*., 2017; Murithi *et al*., 2015; Murithi *et al*., 2014). A second species causing rust on soybean, *Phakopsora meibomiae*, has not been reported in Africa or elsewhere outside the Americas (Hartman *et al*., 2011).

During infection, *P. pachyrhizi* differentiates within 7-9 days to form uredinia, fruiting bodies that erupt through the epidermis and release numerous urediniospores (Goellner *et al.*, 2010) that appear as loosely woven to compact masses of mycelium in the palisade or spongy mesophyll of the soybean leaves (Marchetti *et al*., 1979). Temperature and moisture play a vital role in soybean rust establishment and epidemics. The optimum temperature for spore germination ranges from 17˚C to 29˚C (Bonde *et al*., 2012), with a relative humidity greater than 85% and moisture on the leaf surface for a period of 6 to 12 hours (Melching *et al*., 1989). The tropical climate in Africa favours the infection of *P. pachyrhizi* throughout the year (Pivonia and Yang, 2004). The leaf tissue around the first uredinia appears in light brown/tan colour to reddish brown (Hartman *et al*., 1999; Goellner *et al*., 2010). Severe infection results in premature plant defoliation (Kumudini *et al*., 2008), leading to yield losses normally ranging between 18-55%, but losses can be as high as 80%, as has been reported in Uganda and Zimbabwe (Levy, 2005; Oloka *et al*., 2008).

The use of fungicides currently is the most widely employed method for management of soybean rust disease, although fungicides are not easily accessible to many smallholder farmers in developing countries. However, their use significantly increases production costs, poses environmental risks and can result in fungicide resistance in the pathogen. Such

resistance has been reported in South America and efforts are now directed towards developing novel, broad spectrum activity fungicides (Godoy *et al*., 2016). Nevertheless, it is increasingly recognized that deployment of resistant soybean cultivars is the preferred disease control method because it is economical, safe, environmentally friendly and complements other control methods. Several sources of rust resistance have been identified and seven resistance loci, designated *Rpp* (for resistance to *P. pachyrhizi*) have been characterized. These resistance loci comprise *Rpp1* (McLean & Byth, 1980), *Rpp2* (Bromfield & Hartwig, 1980), *Rpp3* (Bromfield & Melching, 1982), *Rpp4* (Hartwig, 1986), *Rpp5* (Garcia *et al*., 2008), *Rpp6* (Li *et al*., 2012) and *Rpp7* (Childs *et al*., 2018). Nevertheless, none of these resistance genes is effective against all known soybean rust pathotypes (Childs *et al*., 2018).

Several studies have been conducted to identify additional resistance sources. For instance, over 16,000 soybean accessions were screened in 2006 in the US, using a mixture of four different rust isolates, and about 50% of the accessions were identified as resistant (Miles *et al*., 2006). In 2008, 25% of 530 accessions screened in Paraguay under field conditions were found to be resistant (Miles *et al*., 2008). In the USA, 64 resistant accessions were identified among 576 accessions evaluated at seven locations (Walker *et al*., 2011). Pham *et al*. (2009) identified about ten resistant accessions out of the 63 that were tested in Vietnam. In Africa, screening of soybean accessions has been conducted only in a few countries. Out of the 178 accessions developed at the International Institute of Tropical Agriculture (IITA) and tested at three different locations in Nigeria, three breeding lines that display low rust severities across the three locations were identified (Twizeyimana *et al*., 2008). In the same study, only three resistant accessions were identified out of the 101 accessions sourced from the United States Department of Agriculture (USDA) that were tested at a single location in Nigeria (Twizeyimana *et al*., 2008). In the 2005 and 2006 soybean growing seasons, 25 soybean accessions sourced from the World Vegetable Centre (AVRDC) were tested in Uganda. Out of these, ten resistant accessions were identified and among them was accession PI 230970 (carrying *Rpp2*) that was found to be highly effective when compared with other accessions carrying the *Rpp1*,*3* or *Rpp4* genes (Oloka *et al*., 2008). In South Africa, all 26 soybean cultivars tested from 2003 to 2005 were susceptible to rust (Mc Laren, 2008). Due to the high variability among *P. pachyrhizi* isolates, which includes shifts in virulence, no resistant soybean varieties are commercially available yet. Continuous screening

of germplasm for resistance to soybean rust is important, as it could aid in the identification of durable resistance for use in breeding programs.

The objective of this study was to identify soybean accessions that are resistant to *P. pachyrhizi* at multiple locations in Tanzania and Uganda, and evaluate their potential to manage soybean rust. To this end, 77 soybean accessions were screened at five different locations in the 2016 and 2017 cropping seasons. The infection type, disease severity and sporulation levels were scored for all accessions.

MATERIALS AND METHODS

Establishment of soybean accessions and experimental design

A total of 77 soybean accessions (Tables 1 and 2) were evaluated during the 2016 growing season, and a subset of the accessions that showed some level of resistance were further evaluated in the 2017 growing season. Germplasm was obtained from the International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria), AVRDC (currently the World Vegetable Centre, Arusha, Tanzania) and the United States Division of Agriculture (USDA Urbana, USA). Furthermore, local cultivars were included (Table 1). Field experiments were established at Mufindi (S 08.11828, E 35.41100, 1737 m) in Iringa, Mikumi (S 36.89931, E 7.47968, 725 m) in Morogoro and at Suluti (S 10.54414, E 36.07763, 894 m) in Ruvuma, Tanzania. In Uganda, the experiments were established at Ngetta (N 2.29741° E 32.91204°, 1073 m) in Lira and at Mubuku (N 0.22343, E 30.13135, 1005 m) in Kasese.

The soybean accessions were evaluated using a randomized complete block design (RCBD) with two replications. In 2016, plots consisted of three rows of 1 m in length for each accession with 50 cm spacing between the rows and 5 cm within the rows. In 2017, plots consisted of four rows of 5 m in length with the same spacing as in 2016. A row of the highly susceptible variety Soya 2 was planted around the blocks in Tanzania, while Wonder soya was used in Uganda to increase the amount of rust inoculum. At all locations common cultural practices, including weeding, were applied, but fungicides were not used.

Table 1. Resistance gene(s) present in the soybean accessions evaluated for resistance to *Phakopsora pachyrhizi* in the Mikumi region of Tanzania in the 2016 growing season.

	Accession[a]	Donor[b]	Resistance gene[c]		Accession[a]	Donor[b]	Resistance gene[c]
1	AGS 129	AVRDC		40	TGx 1989 11F	IITA	*Rpp1, Rpp3*
2	AGS 292	AVRDC		41	TGx 1989 4F	IITA	*Rpp1, Rpp3*
3	AGS 329	AVRDC		42	TGx 1989 53FN	IITA	*Rpp1, Rpp3*
4	AGS 338	AVRDC		43	TGx 1989 5F	IITA	*Rpp1, Rpp3*
5	AGS 339	AVRDC		44	TGx 1989 19F	IITA	*Rpp1, Rpp3*
6	AGS 406	AVRDC		45	TGx 1989 21F	IITA	*Rpp1, Rpp3*
7	AGS 423	AVRDC		46	TGx 1989 3F	IITA	*Rpp1, Rpp3*
8	AGS 432	AVRDC		47	TGx 1989 41F	IITA	*Rpp1, Rpp3*
9	AGS 447	AVRDC		48	TGx 1989 42F	IITA	*Rpp1, Rpp3*
10	AGS 458	AVRDC		49	TGx 1989 48FN	IITA	*Rpp1, Rpp3*
11	AGS 459	AVRDC		50	TGx 1989 49FN	IITA	*Rpp1, Rpp3*
12	AGS 461	AVRDC		51	TGx 1989 4F	IITA	*Rpp1, Rpp3*
13	GC 4051321	AVRDC		52	TGx 1989 53FN	IITA	*Rpp1, Rpp3*
14	TZA 3826	AVRDC		53	TGx 1989 5F	IITA	*Rpp1, Rpp3*
15	TZA 3829	AVRDC		54	TGx 1989 62F	IITA	*Rpp1, Rpp3*
16	TZA 448	AVRDC		55	TGx 1989 68F	IITA	*Rpp1, Rpp3*
17	PI 200492	USDA	*Rpp1*	56	TGx 1989 75FN	IITA	*Rpp1, Rpp3*
18	PI 594538A	USDA	*Rpp1b*	57	TGx 1990 114FN	IITA	*Rpp1, Rpp3*
19	PI 230970	USDA	*Rpp2*	58	TGx 1990 55F	IITA	*Rpp1, Rpp3*
20	PI 462312	USDA	*Rpp3*	59	TGx 1990 110FN	IITA	*Rpp1, Rpp3*
21	PI 459025B	USDA	*Rpp4*	60	TGx 1990 11F	IITA	*Rpp1, Rpp3*
22	PI 200526	USDA	*Rpp5*	61	TGx 1990 15F	IITA	*Rpp1, Rpp3*
23	PI 567102B	USDA	*Rpp6*	62	TGx 1990 2F	IITA	*Rpp1, Rpp3*
24	SC Safari	SeedCo		63	TGx 1990 3F	IITA	*Rpp1, Rpp3*
25	SC Samba	SeedCo		64	TGx 1990 40F	IITA	*Rpp1, Rpp3*
26	SC Saga	SeedCo		65	TGx 1990 46F	IITA	*Rpp1, Rpp3*
27	SC Sequel	SeedCo		66	TGx 1990 4F	IITA	*Rpp1, Rpp3*
28	SC Squire	SeedCo		67	TGx 1990 52F	IITA	*Rpp1, Rpp3*
29	Soya 2	ARI-		68	TGx 1990 57F	IITA	*Rpp1, Rpp3*
30	TGx 1835 10E	IITA		69	TGx 1990 5F	IITA	*Rpp1, Rpp3*
31	TGx 1987 10F	IITA	*Rpp1, Rpp3*	70	TGx 1990 67F	IITA	*Rpp1, Rpp3*
32	TGx 1987 31F	IITA	*Rpp1, Rpp3*	71	TGx 1990 78F	IITA	*Rpp1, Rpp3*
33	TGx 1987 14F	IITA	*Rpp1, Rpp3*	72	TGx 1990 80F	IITA	*Rpp1, Rpp3*
34	TGx 1987 32F	IITA	*Rpp1, Rpp3*	73	TGx 1990 95F	IITA	*Rpp1, Rpp3*
35	TGx 1987 34F	IITA	*Rpp1, Rpp3*	74	TGx 1990 97F	IITA	*Rpp1, Rpp3*
36	TGx 1987 62F	IITA	*Rpp1, Rpp3*	75	TGx 1991 10F	IITA	*Rpp1, Rpp3*
37	TGx 1987 64F	IITA	*Rpp1, Rpp3*	76	TGx 1993 4FN	IITA	*Rpp1b*
38	TGx 1987 8F	IITA	*Rpp1, Rpp3*	77	TGx 1995 5FN	IITA	*Rpp1b*
39	TGx 1988 3F	IITA	*Rpp1, Rpp3*				

[a]TGx: Tropical *Glycine max* crosses

[b]AVRDC: Asian Vegetable Research Development Centre (currently known as the World Vegetable Centre), Taiwan; USDA: United States Department of Agriculture, USA; SeedCo: Seed Company from Zimbabwe; ARI: Agricultural Research Institute, Tanzania; IITA: International Institute of Tropical Agriculture, Nigeria.

[c]*Rpp*: Resistance to *P. pachyrhizi*.

Table 2. Resistance gene(s) present in the soybean accessions evaluated for resistance to *Phakopsora pachyrhizi* in Ngetta and Mubuku regions of Uganda in the 2016 growing season.

	Accession[a]	Donor[b]	Resistance gene[c]		Accession[a]	Donor[b]	Resistance gene[c]
1	AGS 292	AVRDC		29	TGx 1989 42F	IITA	*Rpp1, Rpp3*
2	AGS 329	AVRDC		30	TGx 1989 48FN	IITA	*Rpp1, Rpp3*
3	AGS 339	AVRDC		31	TGx 1989 49FN	IITA	*Rpp1, Rpp3*
4	AGS 3829	AVRDC		32	TGx 1989 53FN	IITA	*Rpp1, Rpp3*
5	Hyuuga	USDA	*Rpp3, Rpp5*	33	TGx 1989 68FN	IITA	*Rpp1, Rpp3*
6	PI 200492	USDA	*Rpp1*	34	TGx 1989 75FN	IITA	*Rpp1, Rpp3*
7	PI 594538A	USDA	*Rpp1b*	35	TGx 1990 110FN	IITA	*Rpp1, Rpp3*
8	PI 200526	USDA	*Rpp5*	36	TGx 1990 114 FN	IITA	*Rpp1, Rpp3*
9	Samba	SeedCo		37	TGx 1990 15F	IITA	*Rpp1, Rpp3*
10	SC Saga	SeedCo		38	TGx 1990 21F	IITA	*Rpp1, Rpp3*
11	SC Sequel	SeedCo		39	TGx 1990 2F	IITA	*Rpp1, Rpp3*
12	SC Squire	SeedCo		40	TGx 1990 3F	IITA	*Rpp1, Rpp3*
13	TGx 1835 10E	IITA		41	TGx 1990 40F	IITA	*Rpp1, Rpp3*
14	TGx 1987 10F	IITA	*Rpp1, Rpp3*	42	TGx 1990 52F	IITA	*Rpp1, Rpp3*
15	TGx 1987 14F	IITA	*Rpp1, Rpp3*	43	TGx 1990 55F	IITA	*Rpp1, Rpp3*
16	TGx 1987 31F	IITA	*Rpp1, Rpp3*	44	TGx 1990 57F	IITA	*Rpp1, Rpp3*
17	TGx 1987 32F	IITA	*Rpp1, Rpp3*	45	TGx 1990 5F	IITA	*Rpp1, Rpp3*
18	TGx 1987 34F	IITA	*Rpp1, Rpp3*	46	TGx 1990 67F	IITA	*Rpp1, Rpp3*
19	TGx 1987 62F	IITA	*Rpp1, Rpp3*	47	TGx 1990 78F	IITA	*Rpp1, Rpp3*
20	TGx 1987 64F	IITA	*Rpp1, Rpp3*	48	TGx 1990 80F	IITA	*Rpp1, Rpp3*
21	TGx 1987 8F	IITA	*Rpp1, Rpp3*	49	TGx 1990 95F	IITA	*Rpp1, Rpp3*
22	TGx 1988 3F	IITA	*Rpp1, Rpp3*	50	TGx 1990 97F	IITA	*Rpp1, Rpp3*
23	TGx 1988 5F	IITA	*Rpp1, Rpp3*	51	TGx 1991 10F	IITA	*Rpp1, Rpp3*
24	TGx 1989 11F	IITA	*Rpp1, Rpp3*	52	TGx 1993 4FN	IITA	*Rpp1b*
25	TGx 1989 19F	IITA	*Rpp1, Rpp3*	53	TGx 1995 5FN	IITA	*Rpp1b*
26	TGx 1989 21F	IITA	*Rpp1, Rpp3*	54	Maksoy	Makerere	
27	TGx 1989 40F	IITA	*Rpp1, Rpp3*				
28	TGx 1989 41F	IITA	*Rpp1, Rpp3*				

[a]TGx: Tropical *Glycine max* crosses.
[b]AVRDC: Asian vegetable Research Development Centre (currently known as World Vegetable Centre); USDA: United States Department of Agriculture, USA; SeedCo: Seed Company from Zimbabwe; IITA: International Institute of Tropical Agriculture, Nigeria.
[c]*Rpp*: Resistance to *P. pachyrhizi*.

Evaluation of disease severity and reactions of the various accessions to *P. pachyrhizi*

Disease severity (the percentage of leaf area affected by soybean rust) was evaluated based on a modified nine-point disease severity scale (Walker *et al*., 2011) (Table 3). Evaluations were conducted between the R4 (pod forming) and R6 (seed filling) soybean growth stages.

Table 3. Disease severity assessment scale used to evaluate soybean accessions for resistance to *Phakopsora pachyrhizi* in field trials.

Soybean rust rating	% of leaflet surface covered by lesions	
	Range	Mid point[a]
1	0	0
2	0 to 2.5	1.25
3	2.5 to 5	3.75
4	5 to 10	7.5
5	10 to 15	12.5
6	15 to 25	20
7	25 to 35	30
8	35 to 67.5	51.25
9	67.5 to 100	83.75

[a]The midpoint value is used for all statistical analyses.

At all locations, three leaflets from the bottom, middle and top canopy of five randomly selected plants in each plot were rated separately per replication. Disease severity of the entire plant was based on the mean severity of the three leaflets per plant.

Three infection types were used for distinguishing compatible and incompatible reactions among soybean accessions infected by *P. pachyrhizi*. Both RB and IM infection types signify incompatibility between the accession and the rust fungus. Sporulation levels were recorded based on a 0 to 3 scale, in which 0 equals no sporulation, 1 stands for 1-10 lesions with spores (little), 2 stands for 10-15 lesions with spores (moderate) and 3 represents >15 lesions with spores (abundant). The sporulation level of each accession was based on the average of three ratings.

Data analysis

Analysis of variance (ANOVA) for soybean rust severity was conducted using PROC GLIMMIX in SAS version 9.3 (SAS Institute, Cary, NC, USA). A Bartlett test of homogeneity of variances across locations was performed to assess whether the variances were equal for all locations as significant differences ($P < 0.001$) were observed between the locations, the analysis was conducted for individual locations. Mean separations were performed using Tukey-Kramer

Grouping of least significant difference at P=0.05. Accessions with disease severities of less than 10%, a sporulation level of 0 or 1 and RB infection type, relative to the susceptible checks were categorized as resistant to *P. pachyrhizi*.

RESULTS

We screened a collection of 77 soybean accessions at five different locations in Tanzania and Uganda in the 2016 and 2017 cropping seasons. In both years, poor seed germination occurred due to low seed viability. Furthermore, soybean rust did not appear at some of the locations due to drought conditions. Nevertheless, data were collected for the observed infection types, disease severity and sporulation levels.

Upon *P. pachyrhizi* infection, susceptible soybean accessions typically exhibit a TAN infection type that is characterized by tan-coloured lesions with abundant sporulation, signifying compatibility between the accession and *P. pachyrhizi*. In contrast, resistant accessions typically develop reddish-brown (RB) lesions, with little or no sporulation. Some accessions do not show any observable symptoms and are therefore classified as immune (IM). RB or IM infection types that are combined with low sporulation levels and lower means for disease severities are considered to be resistant.

Overall, infection types and sporulation levels did not differ significantly between locations for the majority of the accessions that displayed TAN infection types and moderate to abundant sporulation (Tables 4 and 5). For instance, accessions such as SC Saga, SC squire, and SC Sequel that are known to possess partial resistance only (Tichagwa, 2004), displayed TAN infection types at Mubuku, Ngetta and Iringa (Tables 4 and 5). This finding demonstrates that soybean rust resistance is scarce and that resistance-breaking isolates of the fungus are common and widespread. Some of the accessions containing single resistance genes, including PI 459025B (*Rpp4*), PI 200526 (*Rpp5*) and PI 567102B (*Rpp6*) displayed RB infection types with little sporulation at the Mikumi site (Table 4). Although this could be interpreted as the absence of resistance-breaking isolates at that region, we noted that Mikumi was the only site where the majority of the accessions did not display TAN infection types and the RB infection type was the most common.

Collectively, these findings suggest a generally lower disease pressure of the pathogen at that site. However, accession PI 594538A that carries *Rpp1b* displayed RB infections at all the three locations in 2016 (Table 4) and at Mikumi and Iringa in 2017 (Table 5). Accession Hyuuga

that carries *Rpp3* and *Rpp5* also displayed RB infections in all the three locations in 2016 (Table 4). This finding suggests that *P. pachyrhizi* isolates that are able to overcome *Rpp1b*, or *Rpp3* in combination with *Rpp5* are not generally present in the *P. pachyrhizi* population, in contrast to isolates that have broken the other resistance genes.

Table 4. Soybean rust disease severity ratings, infection types and sporulation levels for selected genotypes in Uganda (Mubuku and Ngetta) and Tanzania (Mikumi) in the 2016 growing season.

Accession	Mubuku			Ngeeta			Mikumi		
	Severity[a] Mean ± SE	IT[b]	SL[c]	Severity[a] Mean ± SE	IT[b]	SL[c]	Severity[a] Mean ± SE	IT[b]	SL[c]
AGS 3829	29 ± 0.9	TAN	1	56.1 ± 2.6	TAN	3	4.6 ±0.6	RB	1
AGS 339	22.5 ± 2.6	TAN	3	33.2 ± 1.2	TAN	2	1.9 ± 0.5	RB	1
Hyuuga	2.4 ± 2	RB	1	3.2 ± 1	RB	1	8.6 ± .8	RB	1
PI 594538A	4.6 ± 2.2	RB	1	2.3 ± 2.1	RB	1	8.7 ± 1.1	RB	1
SC Saga	46.8 ± 3.2	TAN	3	23.4 ± 2.3	TAN	2	3.5 ± 0.7	RB	1
SC Sequel	15 ± 0.7	TAN	2	20.3 ± 1.5	TAN	1	4.2 ± 0.7	RB	1
SC Squire	16.2 ± 0.6	RB	1	34 ± 0.9	TAN	3	4.8 ± 3.3	RB	1
TGx 1989 42F	29.6 ± 2.5	TAN	2	6.6 ± 1.2	TAN	1	11.3 ± 1.4	TAN	2
TGx 1989 19F	43.2 ± 3.8	TAN	3	24.6 ± 2.3	TAN	3	3.9 ± 0.8	RB	2
TGx 1987 14F	47.8 ± 3.2	TAN	3	6.8 ± 0.8	TAN	2	5.6 ± 0.7	RB	1
TGx 1987 34F	16.8 ± 1.3	RB	1	6.4 ± 0.7	RB	1	2.2 ± 0.5	RB	1
TGx 1990 110FN	37 ± 2.6	TAN	2	24.6 ± 2.3	TAN	2	6 ± 0.7	RB	1
TGx 1993 4FN	3.2 ± 0.7	IM	0	5.6 ± 0.7	RB	2	2.2 ± 1.1	RB	1
TGx 1990 114FN	11.6 ± 1.7	RB	2	4.3 ± 0.9	RB	1	6.5 ± 1	RB	1
TGx 1990 55F	33.9 ± 2.6	TAN	3	4.2 ± 0.7	RB	1	4.5 ± 0.7	RB	1
TGx 1990 2F	25.7 ± 4.1	TAN	3	15.3 ± 1.4	TAN	2	1.9 ± 0.5	RB	1
TGx 1987 62F	14.6 ± 1.5	TAN	3	6.8 ± 0.6	TAN	1	5.5 ± 1.5	RB	1
TGx 1990 21F	11.1 ± 1	RB	2	6.4 ± 0.7	RB	1	3.4 ± 0.5	RB	1
TGx 1990 5F	10.8 ± 0.8	RB	1	18 ± 2.4	TAN	2	2.2 ± 0.5	RB	1
TGx 1995 5FN	1.0	IM	0	2.1 ± 1.2	RB	1	4.6 ± 0.8	RB	1
TGx 1989 45F	22.9 ± 2.5	TAN	1	6.4 ± 0.7	RB	2	nd		
TGx 1990 48FN	18.6 ± 2.2	TAN	2	6.9 ± 0.9	TAN	2	nd		
TGx 1990 57F	5.2 ± 0.3	RB	1	13.4 ± 1.3	TAN	2	nd		
TGx 1990 78F	18.5 ± 2.6	TAN	2	3.5 ± 0.7	RB	1	nd		
TGx 1990 80F	15.6 ± 1.4	TAN	2	3.6 ± 0.9	RB	1	nd		
TGx 1990 95F	40.5 ± 2.9	TAN	3	13.4 ± 1.3	RB	2	nd		

TGx 1990 15F	15.3 ± 2	TAN	2	nd			nd		
TGx 1990 97F	18.8 ± 1.6	TAN	2	nd			nd		
TGx 1990 3F	11.2 ± 0.7	TAN	1	nd			nd		
TGx 1987 10F	14.6 ± 0.7	RB	1	nd			nd		
TGx 1989 41F	nd			14.1 ± 1.4	TAN	2	2.6 ± 0.7	RB	1
TGx 1989 68FN	nd			15.1 ± 1.1	TAN	3	5.2 ± 0.6	RB	1
TGx 1989 11F	nd			20.9 ± 2.2	TAN	3	nd		
TGx 1989 40F	nd			7.8 ± 1	TAN	2	nd		
TGx 1989 49FN	nd			6.8 ± 0.6	TAN	2	nd		
TGx 1989 53FN	nd			6.8 ± 0.3	RB	1	nd		
TGx 1990 40F	nd			8.2 ± 1	TAN	2	5.2 ± 0.6	RB	1
TGx 1998 5F	nd			9.5 ± 0.8	RB	2	nd		
TGx 1990 52F	nd			25.3 ± 2	TAN	3	nd		
TGx 1990 67F	nd			19.2 ± 2.1	TAN	2	nd		
AGS 423	nd			nd			10 ± 0.8	RB	2
AGS 459	nd			nd			12.4 ± 1.5	RB	1
PI 200492	nd			nd			37.4 ± 3.8	TAN	3
PI 200526	nd			nd			11.9 ± 1.1	RB	1
PI 459025B	nd			nd			9.4 ± 1.3	RB	1
PI 567102B	nd			nd			10.7 ± 1	RB	1
TGx 1987 31F	nd			nd			4.5 ± 0.7	RB	1
TGx 1987 32F	nd			nd			2.9 ± .7	RB	1
TGx 1987 8F	nd			nd			9.5 ± .8	RB	1
TGx 1988 5F	nd			nd			6.1 ± 1	RB	1
TGx 1989 21F	nd			nd			4.6 ± 0.8	RB	1
TGx 1990 110FN	nd			nd			12 ± 1.6	RB	3
TGx 1990 46F	nd			nd			2.5 ± 0.5	RB	1
TGx 1990 52F	nd			nd			4.5 ± 0.7	RB	1
TZA 448	nd			nd			10.2 ± 1.3	RB	1
TGx 1987 64F	nd			nd			9.1 ± 0.9	RB	1
Line 8	nd			nd			29.5 ± 1.5	TAN	2
Maksoy 3N	47.2 ± 1.1	TAN	3	53.6 ± 4.7	TAN	3	nd		

[a]Severity (mean ± standard error) was rated on a scale of 1-9 (Table 2); SE: Standard error.
[b]IT: Infection type; TAN: tan coloured; RB: reddish brown; IM: immune.
[c]SL: sporulation level with 0 = no sporulation, 1 = little sporulation, 2 = moderate sporulation and 3 = abundant sporulation.
nd: not determined.

Table 5. Soybean rust (*Phakopsora pachyrhizi*) disease severity ratings, infection types and sporulation levels for selected soybean accessions in Uganda (Mubuku and Ngetta) and Tanzania (Mikumi, Iringa and Suluti) in the 2017 growing season.

Accession	Uganda						Tanzania								
	Mubuku			Ngeeta			Mikumi			Iringa			Suluti		
	Severity[a] Mean ± SE	IT[b]	SL[c]	Severity[a] Mean ± SE	IT[b]	SL	Severity[a] Mean ± SE	IT[b]	SL[c]	Severity[a] Mean ± SE	IT[b]	SL[c]	Severity[a] Mean ± SE	IT[b]	SL[c]
AGS 339	37.4 ± 3.8	TAN	3	23.1 ± 1.6	TAN	2	6 ± 1.4	TAN	1	34.3 ± 5.4	TAN	1	24.7 ± 5.2	TAN	2
TGx 1990 114FN	11.3 ± 3.1	TAN	1	12.5 ± 1.6	TAN	1	4.6 ± 0.8	TAN	1	16.4 ± 3.3	TAN	1	11.5 ± 3.8	TAN	1
TGx 1993 4FN	1.0 ± 0.2	IM	0	2.2 ± 0.5	RB	1	1.6 ± 0.4	RB	1	0.9 ± 0.1	RB	0	9.2 ± 2.6	RB	1
TGx 1995 5FN	1.0 ± 0.1	IM	0	4.5 ± 1.3	RB	1	1.3 ± 0.5	RB	1	5.7 ± 0.8	RB	0	4 ± 1	RB	1
TGx 1987 34F	11.4 ± 2.6	TAN	2	19 ± 6.9	TAN	2	2.6 ± 0.7	TAN	1	16.3 ± 3.2	TAN	1	19.3 ± 2	TAN	2
TGx 1987 62F	16.1 ± 2.5	TAN	3	13.3 ± 2.6	TAN	2	2.1 ± 0.6	TAN	1	10.7 ± 3.2	TAN	1	7.3 ± 2	TAN	1
TGx 1987 14F	14.3 ± 2	RB	1	18.2 ± 1.3	TAN	2	3.1 ± 0.5	TAN	1	12.2 ± 1.8	TAN	1	12.7 ± 1.8	TAN	2
TGx 1989 45F	27.3 ± 3.3	TAN	3	24.3 ± 2	TAN	3	7.1 ± 1.3	TAN	1	41.9 ± 6.9	TAN	3	19 ± 2.3	TAN	1
TGx 1990 15F	24.3 ± 2	TAN	2	15.2 ± 2.1	TAN	2	4.1 ± 2.4	TAN	1	43.9 ± 6.3	TAN	3	30.9 ± 6.1	TAN	2
TGx 1990 21F	12.7 ± 1	TAN	2	41.6 ± 5.5	TAN	3	4.3 ± 1	RB	1	16.4 ± 3.3	TAN	2	16.2 ± 3	TAN	2
TGx 1990 3F	10.4 ± 1.6	TAN	2	14.2 ± 2.3	TAN	1	5.1 ± 0.4	RB	1	28.8 ± 4.3	TAN	2	14.1 ± 2.4	TAN	1
TGx 1987 10F	16.3 ± 2	TAN	2	19.4 ± 4	TAN	1	3.1 ± 0.5	TAN	1	28.3 ± 1.2	TAN	1	21.2 ± 2.6	TAN	1
TGx 1990 48FN	17.2 ± 1.1	TAN	2	22.6 ± 6.6	TAN	2	4.3 ± 0.3	RB	1	44.3 ± 4.2	TAN	3	14.9 ± 2.4	TAN	2
TGx 1990 57F	32 ± 5.4	TAN	3	12 ± 1.6	TAN	1	8.5 ± 1.3	TAN	1	24.6 ± 3.5	TAN	2	14.4 ± 1.8	TAN	1
TGx 1990 5F	12 ± 1.6	TAN	2	23 ± 2.4	TAN	2	5 ± 1.5	TAN	1	34.8 ± 6.6	TAN	2	19.9 ± 4.2	TAN	2
TGx 1990 78F	28.1 ± 6.7	TAN	2	21.1 ± 4.1	TAN	2	nd			33.3 ± 4.1	TAN	2	nd		
TGx 1990 80F	23.3 ± 1.9	TAN	1	16.1 ± 2.3	TAN	2	nd			48.4 ± 6.1	TAN	2	25.8 ± 4.5	TAN	2

TGx 1990 97F	28.4 ± 3.2	TAN	1	nd			nd			42.9 ± 5.4	TAN	2	17.8 ± 2.7	TAN	2
MAK 2N	16 ± 1.4	TAN	3	14.8 ± 6.1	TAN	2	nd			nd			nd		
MAK 3N	33.1 ± 1.4	TAN	3	50.2 ± 4	TAN	3	nd			nd			nd		
MAK 4N	21.2 ± 1.3	TAN	2	14.6 ± 2.2	TAN	2	nd			nd			nd		
AGS 3829	nd			20.2 ± 2.3	TAN	2	nd			nd			nd		
AGS 461	nd			nd			nd			33.3 ± 4.1	TAN	2	18.4 ± 4.2	TAN	2
GC 4051321	nd			nd			nd			36.8 ± 6.3	TAN	2	27.1 ± 4.6	TAN	3
PI 594538A	nd			nd			3.2 ± 1.9	RB	1	4.8 ± 1.2	RB	1	nd		
PI 230970	nd			nd			6.7 ± 1.3	RB	1	49.8 ± 4.9	TAN	3	nd		
SC Saga	nd			nd			3.6 ± 0.9	TAN	1	19.4 ± 2.2	TAN	2	nd		
SC Sequel	nd			nd			3.5 ± 0.7	TAN	1	50.2 ± 4	TAN	3	nd		
SC Squire	nd			nd			5.6 ± 1.2	TAN	1	32.2 ± 1.3	TAN	3	nd		
TGx 1987 32F	nd			nd			7.4 ± 6.5	TAN	3				nd		
TGx 1987 64F	nd			nd			10.3 ± 2	TAN	2	49.4 ± 5.7	TAN	3	nd		
TGx 1987 8F	nd			nd			3.2 ± 0.7	TAN	2	9.1 ± 2.2	RB	1	nd		
TGx 1988 5F	nd			nd			7.1 ± 1.2	TAN	1	29.5 ± 4	TAN	2	16.6 ± 2.8	TAN	2
TGx 1989 19F	nd			nd			9.9 ± 2.1	TAN	1	33 ± 9.6	TAN	2	28.4 ± 3.2	TAN	3
TGx 1989 42F	nd			nd			6.4 ± 1.4	TAN	2	36.4 ± 5.6	TAN	3	nd		
TGx 1990 2F	nd			nd			3.9 ± 0.8	TAN	1	46.4 ± 3.3	TAN	2	34 ± 6.5	TAN	3
TGx 1990 95F	nd			nd			4.6 ± 0.8	TAN	1	41.2 ± 4.8	TAN	3	7.1 ± 1.8	TAN	1
TGx 1991 10F	nd			nd			5.3 ± 0.8	TAN	1	21.6 ± 2	TAN	2	10.6 ± 1.8	TAN	1
TGx 1987 129F	nd			nd			nd			29.2 ± 6.6	TAN	3	6.8 ± 1.4	TAN	1
TGx 1987 31F	nd			nd			nd			36.1 ± 6.7	TAN	2	nd		
TGx 1987 65F	nd			nd			nd			41.9 ± 5.8	TAN	2	12.7 ± 1.8		

TGx 1987 88F	nd			nd			nd			7.8 ± 1.7	RB	2	28.3 ± 5.8	TAN	3
TGx 1988 3F	nd			nd			nd			48.8 ± 6.1	TAN	2	nd		
TGx 1989 11F	nd			nd			nd			35.4 ± 4.4	TAN	2	20.9 ± 2.2	TAN	2
TGx 1989 20F	nd			nd			nd			41.2 ± 6.1	TAN	3	34 ± 6.3	TAN	3
TGx1989 48FN	nd			nd			nd			30.5 ± 3.9	TAN	2	14 ± 2.7	TAN	1
TGx 1989 53FN	nd			nd			nd			28.1 ± 3	TAN	2	15.2 ± 2.6	TAN	2
TGx 1989 68FN	nd			nd			nd			24.5 ± 5.4	TAN	2	nd		
TGx 1989 75FN	nd			nd			nd			34.3 ± 6.6	TAN	2	20.1 ± 3.9	TAN	2
TGx 1990 40F	nd			nd			nd			40.7 ± 7.4	TAN	3	12.5 ± 1.6	TAN	2
TGx 1990 52F	nd			nd			nd			20.6 ± 1.8	TAN	2	10.5 ± 1.7	TAN	1
TGx 1990 55F	nd			nd			nd			27.8 ± 4.4	TAN	2	32.1 ± 6	TAN	2
TGx 1990 67F	nd			nd			nd			24.9 ± 4.9	TAN	2	25.1 ± 4.6	TAN	3
MAK 1N	33.1 ± 1.1	TAN	2	38.8 ± 1.9	TAN	2	nd			nd			nd		
Soya 2	nd			nd			18.9 ± 5.1	TAN	2	59.1 ± 4.1	TAN	3	37 ± 4.6	TAN	3

[a]Severity (mean ± standard error) was rated on a scale of 1-9 (Table 2); SE: Standard error.
[b]IT: Infection type; TAN: tan coloured; IM: immune, RB: reddish brown.
[c]SL: sporulation level with 0 = no sporulation, 1 = little sporulation, 2 = moderate sporulation and 3 = abundant sporulation.
nd: not determined.

Although the majority of the accessions displayed similar infection types, a few accessions displayed different infection types between locations. For instance, whereas accession SC Squire displayed an RB infection type at Mubuku and at Mikumi, the same accession displayed a TAN infection type at Ngeeta (Table 4). Similarly, accession TGx 1990 55F displayed a TAN infection type at Mubuku, while the same accession displayed an RB infection type at both Ngeeta and Mikumi (Table 4). IM infection types were only displayed on two accessions, namely TGx 1934 4FN and TGx 1995 5FN, at the Mubuku site, while the same accessions displayed RB infection types in the other locations (Tables 4 and 5). These findings demonstrate that the rust populations differ between the various locations within the same cropping season. Moreover, infection types for some of the accessions also differed between the two years. For example, whereas accession TGx 1990 114FN and TGx 1987 34F displayed RB infection types in 2016 (Table 4), both accessions displayed TAN infection type in 2017 (Table 5). Although this may similarly be attributed to differences in the rust populations between the two years, this may also be attributed to environmental changes between the two years, such as changes in weather conditions that affected the disease development.

To assess disease development with a higher resolution, disease severities (the percentage of leaf area affected by soybean rust) were assessed based on a modified nine-point disease severity scale (Walker *et al*., 2011) (Table 3). This is particularly relevant for infections that were classified as TAN, as considerable differences in disease severity were observed once disease occurred. Intriguingly, we observed significant differences in disease severities for the majority of the accessions between locations (Tables 4 and 5). For instance, accession TGx 1987 14F which displayed the highest severity (47.8%) at Mubuku, showed a significantly lower disease severity at both Ngeeta (6.8%) and Mikumi (5.6%) in 2016 (Table 4). Similarly, accession AGS 3829 which displayed a high disease severity (56%) at Ngeeta showed a lower disease severity at Mubuku (29%) and even much lower at Mikumi (4.6%) (Table 4). These data provide further support for the notion that significant variation in *P. pachyrhizi* populations exists between the various locations.

Besides differences in disease severities for the same accessions between locations, disease severities for some of the accessions also differed between the two years. For example, accession TGx 1990 57F showed a low disease severity (5.2%) at Mubuku in 2016 (Table 4), while the same accession showed high disease severity (32%) at that location in 2017 (Table 5). Similarly, accession AGS 3829 showed a high disease severity (56.1%) at Ngeeta in 2016 and a significantly lower disease severity at that same location (20.2%) in 2017. These data suggest that not only significant variation exists in the *P. pachyrhizi* populations between the various locations, but also between the two years at the same location. However, at some of the locations disease severities on the same accessions did not differ significantly between the two years. For instance, accession TGx 1990 48FN at Mubuku displayed disease severities of 18.6% and 17.2% in 2016 and 2017 (Table 5), respectively. Other accessions that displayed similar diseases severities at the same location between the years include TGx 1990 21F and TGx 1990 114FN at Mubuku and Mikumi (Tables 4 and 5). These findings may suggest that same isolate is present at these locations in both years, although this may also be the consequence of similar susceptibilities of these accessions to different isolates. Overall, our data point towards significant variation in local *P. pachyrhizi* populations between sites and between years, and shows that resistance-breaking isolates within those populations are common. Thus, most soybean genotypes are susceptible and provide little basis for promising soybean disease resistance management. Nevertheless, accessions Hyuuga, PI 594538A, TGx 1987 34F, TGx 1990 21F, TGx 1990 114FN, TGx 1993 4FN and TGx 1995 5FN displayed an RB infection type with little sporulation and relatively low disease severities across all the three locations in 2016 (Table 4). Of these seven accessions, TGx 1934 4FN and TGx 1995 5FN also displayed an RB infection type with little to no sporulation and very low disease severities across the five tested locations in 2017 (Table 5). Thus, these two accessions appear to be resistant against the various rust populations that occurred in the different locations in the two years, and may provide a basis for improved soybean rust resistance management in the future.

DISCUSSION

Deployment of host resistance is the preferred approach to manage soybean rust caused by *P. pachyrhizi* (Hartman *et al.,* 2005). High virulence diversity exists among *P. pachyrhizi* isolates and populations with differential virulence spectra occur across soybean growing regions worldwide (Akamatsu *et al*., 2017; Murithi *et al*., 2016; Godoy *et al*., 2016). Furthermore, several pathotypes have been identified among field isolates of *P. pachyrhizi* in Africa (Murithi *et al*., 2017), Japan (Yamaoka *et al*., 2014), South America (Akamatsu *et al*., 2013) and the USA (Twizeyimana and Hartman, 2012). Considering the geographical variability of the pathogen, it is important to identify sources of resistance that can be deployed to effectively control *P. pachyrhizi* populations in different locations. Therefore, continuous screening of soybean accessions at different locations is important and may aid in the identification of novel resistance sources that can be introduced into local breeding programmes. However, field screening for resistance of soybean to rust is challenging due to variable weather conditions during the cropping seasons, which may affect seed germination as well as the occurrence of soybean rust infections. Sometimes, the growth stages from flowering to seed filling for the early maturing soybean varieties may not coincide with favourable conditions for rust infection (Twizeyimana *et al*., 2011). Such variable environmental factors could have contributed to the low disease pressure that led to the low disease severities observed at the Mikumi site in both years (Table 4 and 5). Previous studies have shown that soybean rust establishment is negatively affected by temperatures above 28˚C and low rainfall (Bonde *et al*., 2012; Narvaez *et al*., 2010; Del Ponte *et al*., 2006).

In our current study, the majority of the accessions that were tested were susceptible to soybean rust, although disease severities differed between locations and years. This finding confirms the existence of rust populations at various locations that have overcome most of the known resistance genes. However, of the 77 soybean accessions tested in different locations and years in this study, two accessions were found to be able to control the rust populations that occurred in all locations, namely TGx 1934 4FN and TGx 1995 5FN. The source of rust resistance in these lines is thought to be the USDA accession PI 594538A

that carries *Rpp1b* (Bandyopadhyay R, *personal communication*, July 2017). This accession was also tested in our study and also showed a low disease severity and RB infection type at the three locations that were assessed in 2016, namely Mubuku, Ngeeta and Mikumi (Table 4), as well as at the Mikumi and Iringa sites in 2017 (Table 5). No data were obtained for this accession at the remaining three locations in 2017 due to poor seed germination (Table 5). Our findings are consistent with previous studies in which PI 594538A displayed RB or IM infection types upon challenge with rust isolates from African countries (Murithi *et al*., 2017; Twizeyimana *et al*., 2009) and also with isolates from South America and the USA (Paul *et al*., 2015; Akamatsu *et al*., 2013; Twizeyimana *et al.*, 2011; Twizeyimana *et al*., 2009).

In addition to USDA accession PI 594538A, three other USDA accessions that carry single previously characterized resistance genes were found to be resistant to soybean rust, namely PI 459025B (*Rpp4*), PI 200526 (*Rpp5*) and PI 567102B (*Rpp6*) (Table 4), although it needs to be noted that these were tested in only one location. Moreover, also USDA accession Hyuuga (*Rpp3,5*) was found to be resistant to soybean rust in the three locations that were tested in 2016 (Table 4). Unfortunately, accession PI 462312 (*Rpp3*) did not germinate at any of the locations tested in this study. Remarkably, USDA accessions such as PI 200492 (*Rpp1*), PI 594538A (*Rpp1b*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), PI 459025B (*Rpp4*), PI 200526 (*Rpp5*) and PI 567102B (*Rpp6*) have been reported to be effective against rust infections across the globe (Twizeyimana *et al*., 2008; Oloka *et al*., 2008; Pham *et al.*, 2009; Walker *et al*., 2014) and have been successfully deployed in soybean breeding programs (Childs *et al*., 2018). However, our study revealed high disease severities and TAN infection types on accessions PI 200492 (*Rpp1*) and PI 230970 (*Rpp2*) at the Mikumi and Iringa locations in Tanzania. High disease severity and sporulation levels on PI 230970 (*Rpp2*) were also reported in Nigeria in 2005 (Twizeyimana *et al*., 2008). The high disease severity on PI 200492 (*Rpp1*) is not surprising, as it has previously been shown that the *Rpp1* gene is ineffective against rust isolates from East Africa (Murithi *et al.*, 2017). Therefore, our findings imply the occurrence of novel *P. pachyrhizi* pathotypes that overcome resistance conferred by the *Rpp1* and *Rpp2* genes. In contrast, the *Rpp4*, *Rpp5*

and *Rpp6* genes were still able to prevent rust infection in the single location where they were tested. Although accession PI 462312 (*Rpp3*) did not germinate at any of the locations, recent studies found this accession to be resistant to rust isolates collected in Uganda and Tanzania (Murithi *et al*., 2017; Chapter 4, this thesis).

Besides PI 594538A (*Rpp1b*), the other known source of rust resistance for the majority of IITA breeding lines that were tested in this study is soybean cultivar UG 5 (Hartman *et al.*, 2011) that contains two resistance genes; *Rpp1* and *Rpp3* (Paul *et al*., 2015). Cultivar UG 5 has been reported as highly resistant against *P. pachyrhizi* isolates in Nigeria, Uganda and the US (Paul *et al*., 2015, Twizeyimana *et al*., 2008, Oloka *et al*., 2008). This cultivar was not included in our current study. However, considering that we found that the majority of the IITA soybean accessions are susceptible to soybean rust in this study (Tables 4 and 5), we anticipate that more virulent pathotypes have evolved that have overcome the resistance conferred by *Rpp1* and *Rpp3* in East Africa.

The rapid evolution of *P. pachyrhizi* continues to threaten the available resistance genes, as soybean rust populations are able to quickly overcome resistance once it is deployed. Thus, efforts should be directed towards enhancing the durability of the resistance genes that are still effective (Johnson, 2000). Durability can be achieved through the use of resistant cultivars that carry different resistance genes at regular intervals over time and space (McDonald, 2014). Alternatively, resistance gene pyramiding that involves combining (stacking) of multiple resistance genes in a single cultivar can contribute to the durability of resistance, provided that these genes recognize different effector proteins (Mundt, 2018). In this manner, the different resistance genes present in a stack confer recognition of multiple effectors simultaneously, which makes it difficult for the pathogen to overcome, as it requires mutations in multiple effector genes to occur simultaneously (McDonald and Linde, 2002). In our study, accessions TGx 1993 4FN (presumably *Rpp1b*), TGx 1995 5FN (presumably *Rpp1b*), PI 594538A (*Rpp1b*) and cultivar Hyuuga (*Rpp3* and *Rpp5*) were resistant against soybean rust at different locations in both years. Potentially, also accessions PI 459025B (*Rpp4*), PI 200526 (*Rpp5*) and PI 567102B (*Rpp6*) can be used, but these should first be tested in other locations to confirm their effectiveness in these

regions as well. Thus, by making combinations of these *R* genes, as well as those identified in our previous studies (Murithi *et al*., 2017; Chapter 4) may aid in the development of durable resistant soybean cultivars for use in Tanzania and Uganda. Finally, more efforts should be put into screening for novel sources of resistance, especially among the wild relatives of soybean and non-host legumes, to have novel resistance genes to combine in *R* gene stacks to prevent the erosion of stacks that are based on *R* genes that have been deployed as singular genes.

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CHAPTER 6

Comparative transcriptome analysis of Phakopsora pachyrhizi isolates to predict candidate avirulence effectors

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ABSTRACT

Soybean rust caused by the biotrophic fungus *Phakopsora pachyrhizi* is the most important foliar disease of soybean. Similar to other pathogenic fungi, *P. pachyrhizi* secretes effector proteins that facilitate host colonization, often through the suppression of host immunity. However, some effectors activate host immunity when they are recognised by specific immune receptors in a resistant host plant. Understanding the processes that control virulence or avirulence of pathogens can inform the development of durable plant disease resistance. In this study, we used RNA-Seq to monitor differences between the transcriptomes of ten *P. pachyrhizi* isolates that are differentially virulent on soybean cultivars that carry particular resistance genes. For each isolate, about 27 million 250 base pair, paired-end reads were generated from infected tissue of universally susceptible soybean plants. Between 5% and 37% of the reads were found to map to *P. pachyrhizi*, and we could assemble these into ~23,500 to ~45,000 contigs per sample. Collectively, a total of 7,061 secreted protein sequences were predicted for the ten isolates, putatively containing effector proteins. Effectors that could be the product of potential avirulence genes corresponding to particular soybean rust resistance genes were identified. If their avirulence activity can be confirmed, such effectors can be used for assessing the recognition specificity of the various *Rpp* genes, and to identify effective combinations for *Rpp* gene pyramiding in soybean.

INTRODUCTION

Soybean rust caused by the obligate biotrophic fungal pathogen *Phakopsora pachyrhizi* is a devastating disease of soybean. The pathogen is found in almost all soybean-growing areas and can cause up to 80% yield loss if not controlled (Levy, 2005). Upon landing on the leaf surface of a host plant, urediniospores of the fungus germinate and produce a single germ tube with an appressorium that mediates direct penetration into the interior of the leaf (Goellner *et al*., 2010). The penetration hypha grows through the epidermal cells into the intracellular space and forms a haustorium mother cell from which a haustorium develops inside a mesophyll cell (Goellner *et al*., 2010). Haustoria of fungal and oomycete

pathogens are important sites for exchange of information between the pathogen and the host (Panstruga and Dodds, 2009; Petre and Kamoun, 2014). They are used to absorb nutrients from the plant and to deliver effector proteins. In a susceptible host plant, these effector proteins manipulate the host, for instance by suppressing host immunity, to facilitate infection and support colonization. However, in a resistant host plant effector proteins activate immunity when they are recognised by specific immune receptors (Petre and Kamoun, 2014).

Plants perceive pathogens and respond to pathogen invasion through their innate immune system (Jones and Dangl, 2006). This immune system detects pathogen invasion through sensing of pathogen-produced or -induced ligands that are termed invasion patterns (IPs), via receptors that are referred to as invasion pattern receptors (IPRs) (Cook *et al*., 2015). Recognition of IPs triggers local and systemic responses in the host plant to stop pathogen invasion. Such responses include ion influxes, accumulation of reactive oxygen species (ROS) and a hypersensitive response (HR) that limits spread of the pathogen from the infection site (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Pathogens, in turn, respond by employing various virulence strategies to counter plant immunity. These include the secretion of effectors that prevent host recognition, inhibit host defence responses or hijack host metabolism (Rodriguez-Moreno *et al*., 2018). Effectors are secreted, and often small and cysteine-rich proteins (Petre *et al*., 2015). Effectors from plant-pathogenic bacteria are the most well-studied, especially those that are delivered into host plant cells through the type III secretion system, and that target various cellular processes (Deng *et al*., 2017). Likewise, the majority of effectors from plant-pathogenic fungi and oomycetes are thought to target host cellular processes (Stergiopoulos and de Wit, 2009). Considering their importance for the infection process of the pathogen, it is not surprising that evolution has led to the selection of plant lines that have evolved recognition specificities for important effectors in order to activate an immune response (Jones and Dangl, 2006; Cook *et al.*, 2015). Consequently, within pathogen populations effector genes are typically highly variable, as pathogens have devised different ways of overcoming host resistance, including deletion or sequence diversification of the

recognized effector (Petre *et al*., 2015; Dodds *et al*., 2004). Furthermore, effector genes are frequently located at genomic sites that promote accelerated evolution through mutation or recombination (Raffaele and Kamoun, 2012).

Effectors can play a functional role in both pathogen attack and host immunity, and understanding the processes that control virulence and pathogenicity on the one hand, and plant immunity on the other, could aid in the development of durable plant disease resistance (Petre and Kamoun, 2014). Practically, effectors can be used as tools to accelerate the identification of matching recognition specificities encoded by potential resistance genes. In addition, they can serve as markers for evaluating field resistance that would be difficult to evaluate under laboratory conditions (Du and Vleeshouwers 2014). Bioinformatics tools are currently available to predict the presence of effector genes in pathogen genomes (Lowe *et al*., 2017; Saunders *et al*., 2012). Currently, RNA sequencing (RNA-Seq) is one of the most robust methods for the analysis of various aspects of fungal transcriptomes, including gene identification, secretome analysis and gene annotation (Wang *et al*., 2009). This is especially relevant for genomes that are bloated with transposable elements and other types of repeats, in such a way that sometimes the major part of the genome sequence is comprised of repetitive elements and assembly of a genome sequence becomes cumbersome (Seidl and Thomma, 2017). Such is for example the case for powdery mildews and rust fungi (Spanu *et al*., 2010, Duplessis *et al.*, 2011). For rust fungi, RNA-Seq has previously been used to gain insight into the mechanisms behind host interactions (Petre *et al*., 2015; Nemri *et al*., 2014; Cantu *et al*., 2013; Link *et al*., 2013; Saunders *et al*., 2012). Understanding the function of effector proteins secreted by rust fungi can help to unravel mechanisms underlying pathogen virulence and host resistance, thereby supporting the development of durable disease resistance.

Among the rust fungi, flax rust (*Melampsora lini*) has been extensively studied for decades already and seven avirulence (*Avr*) genes of this fungus have been identified and characterized (Ellis *et al*., 2007, Anderson *et al*., 2016). For *P. pachyrhizi*, transcriptome studies on isolated haustoria by Link *et al*. (2014), revealed 156 effector candidate genes (PpECs), while Kunjeti *et al*. (2016) identified 35 candidate secreted effector proteins

(CSEPs). In addition, recent prediction of the *P. pachyrhizi* secretome based on RNA-Seq identified 851 proteins, some of which were classified as effector candidates (de Carvalho *et al*., 2017). Some of the identified PpECs were shown to suppress immunity *in planta* and to activate immunity in non-host plants, while others were shown to suppress immunity in general (Qi *et al*., 2018). Furthermore, it was shown that PpEC23 interacts with the soybean transcription factor GmSPL2I that functions as a negative regulator of defence to suppress immunity (Qi *et al.*, 2016).

In this study, we have investigated the occurrence of effector genes among various *P. pachyrhizi* isolates that were selected based on their virulence on a set of soybean varieties with different (combinations of) known *Rpp* (for resistance to *P. pachyrhizi*) genes. To date, seven *Rpp* resistance loci have been mapped to the soybean genome, although none of the actual resistance genes has been cloned (Childs *et al*., 2018). *Rpp* genes are pathotype-specific and virulent pathotypes of *P. pachyrhizi* that overcome *Rpp* resistance have evolved. Compatible interactions between *P. pachyrhizi* and soybean are characterized by TAN-coloured sporulating lesions on the leaf surface, while incompatible interactions are represented by reddish-brown (RB) lesions with little or no sporulation. In some cases, no observable response of the plant is present, a phenomenon that is termed immune (IM) (Goellner *et al*., 2010). More than ten *P. pachyrhizi* pathotypes have been identified in Africa (Murithi *et al*., 2017; Twizeyimana *et al*., 2009), and with the intensification of soybean production more virulent pathotypes are likely to appear, as has similarly occurred in South America (Akamatsu *et al*., 2017). We have used RNA-Seq to gain insight into the transcriptome differences between different isolates of *P. pachyrhizi*. The selected isolates display differential virulence on some of the soybean lines carrying particular *Rpp* genes; while some are virulent, others are avirulent on these lines, suggesting the presence of particular recognised avirulence factors in the avirulent isolates. Thus, the objectives of this study were to: (i) evaluate the virulence of *P. pachyrhizi* isolates on the host differentials and (ii) to identify potential effectors responsible for the different virulence patterns observed among the various *P. pachyrhizi* isolates.

MATERIALS AND METHODS

Isolate recovery, multiplication and virulence evaluation

Virulence of single-spored *P. pachyrhizi* isolates was assessed using eight differential soybean varieties with known *Rpp* genes, sourced from the USDA soybean germplasm collection (Table 1). Four seeds of each of the differential lines were sown in plastic pots filled with Kekilla Professional Peatmoss (Balton, Tanzania), mixed with 10 mg 18:46:00: N:P:K: DAP fertilizer (Yara, Tanzania). The plastic pots were placed in a growth chamber under alternating dark and light periods of 12 h each, at temperatures between 22˚C and 25˚C and humidity between 65% and 75%, under rust-free conditions.

Table 1. The set of soybean host differentials used for determining the virulence spectrum of the collected *Phakopsora pachyrhizi* isolates, their resistance genes, and their origin.

Soybean line[a]	Resistance gene(s)[b]	Country of Origin	Reference
PI 200492	*Rpp1*	Japan	McLean and Byth, 1980
PI 230970	*Rpp2*	Japan	Hartwig and Bromfield, 1983
PI 462312	*Rpp3*	India	Bromfield and Melching, 1982
PI 459025B	*Rpp4*	China	Hartwig, 1986
PI 200526	*Rpp5*	Japan	Garcia *et al*., 2008
PI 567102B	*Rpp6*	Paraguay	Li *et al*., 2012
Hyuuga	*Rpp3,5*	Japan	Kendrick *et al*. 2011
UG 5	*Rpp1,3*	Uganda	Paul *et al*., 2015

[a] PI, plant introduction.
[b]*Rpp*, resistance to *Phakopsora pachyrhizi*.

Four-week-old leaflets from the 2nd and/or 3rd trifoliate leaves were used for inoculation in a detached leaf assay. For this, plastic containers were used, containing one leaflet from each of the eight soybean differentials that were inoculated with a single *P. pachyrhizi* isolate. The experiment was arranged in a randomized complete block design, with three replications for each isolate. For the inoculation, the abaxial side of the leaflets was moistened with distilled water, after which leaves bearing fresh sporulating uredinia from each isolate were lightly tapped to dislodge the spores onto the leaflets of the differential soybean varieties. The inoculated leaflets were subsequently incubated in a tissue culture

chamber under similar conditions as described above. The infection types (TAN, RB, IM) of each of the isolates were determined by observing the inoculated leaflets under a stereomicroscope (Olympus, Tokyo, Japan) at 80 times magnification, at 14 days after inoculation.

Isolate multiplication and leaf harvest

Eight *P. pachyrhizi* isolates from Kenya and Tanzania that displayed differential virulence patterns on the soybean differential varieties were multiplied on the susceptible soybean variety Soya 1 in a growth chamber, at similar conditions as described above (Table 2). For each of the isolates, infected leaf sections showing abundant sporulation were harvested at 14 days post inoculation (dpi) and preserved in RNA later solution (Kruse *et al*., 2016). The isolates were shipped to The Sainsbury Laboratory (TSL, Norwich, UK). In addition, two Japanese isolates that were collected from the wild host plant kudzu (*Pueraria montana* var *lobata*) and on a local soybean variety were multiplied on the susceptible soybean accession Williams 82 at TSL in a similar fashion (Yamaoka *et al.*, 2014).

Table 2. Infection types of the *Phakopsora pachyrhizi* isolates tested on eight differential soybean lines with different (combinations of) *Rpp* genes.

	***Rpp* genes present in soybean lines**[a]								
Isolate[b]	***Rpp1***	***Rpp2***	***Rpp3***	***Rpp4***	***Rpp5***	***Rpp6***	***Rpp1,3***	***Rpp3,5***	**Soya 1/W82**
T1	TAN	RB	RB	RB	RB	TAN	TAN	TAN	TAN
T2	TAN	RB	RB	RB	RB	TAN	RB	TAN	TAN
T3	TAN	RB	RB	RB	RB	TAN	TAN	TAN	TAN
T4	TAN	RB	RB	RB	RB	TAN	TAN	TAN	TAN
T5	TAN	RB	RB	RB	TAN	RB	RB	RB	TAN
T6	TAN	TAN	RB	RB	RB	RB	RB	RB	TAN
T7	TAN	TAN	RB	RB	RB	RB	RB	RB	TAN
T8	TAN	RB	RB	RB	RB	RB	RB	TAN	TAN
T9	RB	TAN	RB	TAN	RB	NT	NT	NT	TAN
T10	RB	RB	RB	RB	RB	TAN	NT	RB	TAN

[a]For the identity of the soybean lines, see Table 1; *Rpp*: resistance to *Phakopsora pachyrhizi*; TAN: tan coloured lesion; RB: reddish-brown lesion; NT: not tested.
[b] Isolates were collected in Kenya (T1, T2, T3, T4 and T8), Tanzania (T5, T6 and T7) and Japan (T9 and T10).

RNA isolation and sequencing

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), followed by treatment using DNase 1 (Promega, Madison, WI, USA) to remove possible DNA contamination. RNA purification was subsequently performed using the RNeasy MiniElute Clean up Kit (Qiagen, Hilden, Germany). The quality and quantity of the isolated RNA was determined using a Nanodrop 8000 (Thermo Scientific Inc, Wilmington, DE, USA). RNA-Seq library preparation and sequencing was conducted by Novogene (Hong Kong, China). Libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Beverly, MA, USA) and were sequenced using a Illumina HiSeq 2500 sequencing system (Illumina, San Diego, CA, USA), generating 250 base pair paired-end reads.

Read quality and alignment

A total of 30 million raw paired-end reads were generated per sample. Before assembly, the quality of the reads was assessed using FASTQC and the sequences were trimmed to remove adaptor sequences. Low-quality reads were discarded using Trimmomatic (Bolger *et al*. 2014). The reads were aligned against the *P. pachyrhizi* draft genome, generated using the PacBio long read sequencing platform (Gupta *et al*., unpublished), using Bestus Bioinformaticus map (BBmap) aligner version 37.38 (Bushnell, 2014). The mapped reads were *de novo* assembled using the Trinity version 2.5.1 (Grabherr *et al*., 2011). Genome completeness was assessed using the benchmarking universal single copy orthologs (BUSCO v3) based on the *Basidiomycota* odb9 ortholog dataset (Simao *et al*., 2015). Open reading frames (ORFs) were identified using TransDecoder 5.0, and the start and end points of putative signal peptides were identified using SignalP, version 4.0 (Petersen *et al*., 2011). The TMHMM (version 2.0) software package (Krogh *et al*., 2001) was used to predict and exclude sequences with putative transmembrane helices. The sequences without such transmembrane helices were then used for putative effector identification.

Putative effector sequences from all ten isolates were combined and redundant sequences were filtered using the CD-Hit algorithm (Fu *et al*., 2012; Li and Godzik, 2006)

with a sequence identity set at 98%. A database of all identified secreted proteins was generated by combining all putative effector sequences from the ten isolates and used as a BlastP query against putative effectors from the individual isolates to identify homologous sequences using an expectation value of 1e-10. The output from the BlastP query comprised of an identifier protein from the database of all secreted proteins (subject) and an identifier protein from each of the ten isolates (query). The output also included identical sequence matches for the proteins, alignment lengths and protein mismatches. Partial assemblies were identified by matching the length of the query proteins and the subject proteins. These partial assemblies were further filtered into proteins that were identical and that had a sequence length of >50 amino acids. Using the Microsoft Excel VLOOKUP function, protein sequences from each isolate were compared against the secreted protein database to identify the proteins present in each of the isolates and to check whether these were identical, with mismatches or a partial assembly. Selection of *P. pachyrhizi* proteins that potentially correspond to soybean *Rpp* genes as avirulence factors was conducted by comparison of the individual putative effector sets from each isolate, while taking into account whether it is virulent or avirulent on the particular soybean accessions. The BlastP function of the Blast2Go software (Gotz *et al*., 2008) tool was then used for functional annotation of the proteins, compared to the non-redundant (nr) protein database of NCBI. The maximum expectation value was set at 1.0e-10, the maximum alignment at 20 amino acids and the highest scoring pair length at 33 amino acids. To identify avirulence proteins possibly matching *Rpp* proteins, we filtered for proteins that were absent in an isolate showing virulence on a soybean line carrying a particular *Rpp* gene and present in a *P. pachyrhizi* strain avirulent on that specific line. The gene ontology (GO) terms analysis and annotation of the top BLAST hits for these putative avirulence proteins were determined using default parameters.

RESULTS

Virulence of *P. pachyrhizi* isolates

In this study, the virulence of ten *P. pachyrhizi* isolates was assessed, five of which (T1, T2, T3, T4 and T8) were collected from different locations in Kenya, three isolates (T5, T6 and T7) were collected in Tanzania, and two isolates (T9 and T10) were collected in Japan (Table 2) (Yamaoka *et al*., 2014). The isolates were assessed for their virulence on eight differential soybean varieties with different (combinations of) the known *Rpp* genes, *Rpp1* to *Rpp6* (Table 1). The seventh known *Rpp* gene, *Rpp7* (Child *et al*., 2018), was not included in our analysis. No IM infection types were recorded and only TAN-coloured sporulating lesions of compatible interactions and RB lesions of incompatible interactions were found. Each of the isolates was virulent on at least one of the soybean lines, but most isolates were virulent on multiple soybean lines (Table 2). Except for the two Japanese isolates, all were virulent on the soybean line carrying the *Rpp1* gene, while none of the isolates was virulent on soybean carrying the *Rpp3* gene. Isolate T10 was avirulent on all soybean lines, except for the line carrying *Rpp6.* Three Kenyan isolates (T1, T3 and T4) were found to display the widest virulence spectrum, with compatible interactions on four of the eight soybean accessions.

Transcriptome sequencing of *P. pachyrhizi* isolates

To determine possible differences between the transcriptomes of the various *P. pachyrhizi* isolates, each of them was inoculated onto the susceptible soybean variety Soya 1 or Williams 82 that both lack any of the known *Rpp* genes, and RNA from infected leaf sections was extracted at 14 dpi. RNA-Seq was performed using an Illumina HiSeq2500 platform and about 30 million raw 250 base pair, paired-end reads were generated for each of the ten isolates, out of which about 27 million clean reads were obtained (Table 3). Between 5% and 37% of the clean reads from each interaction sample mapped to a *P. pachyrhizi* draft genome (Gupta *et al*., unpublished), which is likely due to the variable leaf colonisation rates as a consequence of differences in the aggressiveness of the various isolates. The number of assembled contigs for *P. pachyrhizi* varied between ~23,500 for T4 to ~45,000

for T1 (Table 3). With BUSCO analyses, the completeness of the assembled gene space was assessed. BUSCO examines the presence of single copy orthologs that are conserved between closely related species in the Ortholog DB v9 database (Simao *et al*., 2015).

Table 3. Summary of the amounts of raw reads, clean reads, reads mapping to the genome and the assembled contigs for the ten *Phakopsora pachyrhizi* isolates.

Isolate	Raw reads	Clean reads	% of reads mapped to *P. pachyrhizi*	Assembled contigs
T1	31,054,558	27,578,848	37.24	45,092
T2	31,332,082	28,264,845	9.17	26,916
T3	29,304,105	26,409,318	15.48	32,868
T4	30,394,138	26, 783,762	5.08	23,598
T5	30,643,546	26,692,104	28.25	40,560
T6	31,755,034	28,107,312	21.93	40,874
T7	31,336,128	27,437,733	12.02	33,547
T8	30,836,190	27,435,451	19.19	36,944
T9	30,690,398	28,308,189	23.86	37,310
T10	30,743,897	27,856,359	18.55	31,707

From a total of 1,335 *Basidiomycota* orthologs in the database, the number of complete sequences detected among the different isolates ranged from 83% to 90%, suggesting that the assemblies covered most of the genes. For less than 11% of the orthologs only a partial assembly was obtained, while less than 5% was missing (Table 4). We also found duplicated BUSCO orthologs in our transcriptome assemblies, indicative of the relatively high degree of variation between the two haplotypes in the dikaryotic fungus that is known for its high degree of heterozygosity (Loehrer *et al.,* 2014). A total of 7,061 putatively secreted protein sequences, characterized by the presence of a predicted signal peptide for extracellular targeting and the absence of transmembrane domains, were generated for the ten isolates collectively. Out of these putatively secreted protein sequences, 353 sequences were found in a previously predicted *P. pachyrhizi* secretome (de Carvhalo *et al.,* 2017), and 93 sequences were previously identified in the *P. pachyrhizi* haustorial transcriptome (Link *et al.,* 2014).

Table 4. Benchmarking Universal Single Copy Orthologs (BUSCO)[a] assessment for the ten *Phakopsora pachyrhizi* isolates.

Isolate	% Complete[b]	% Complete single-copy[c]	% Complete duplicated[d]	% Fragmented[e]	% Missing[f]
T1	87	39	48	9	4
T2	86	50	36	10	4
T3	86	47	39	10	4
T4	85	54	30	11	4
T5	89	40	49	8	4
T6	86	40	46	9	4
T7	83	45	38	11	7
T8	85	41	44	10	5
T9	90	43	47	7	3
T10	90	45	45	7	3

[a]Based on a reference database containing 1,335 *Basidiomycota* genes.
[b]Orthologs for which a full-length match is found.
[c]Orthologs for which a single full-length match is found in the transcriptome assembly.
[d]Orthologs for which two or more full-length matches are found in the transcriptome assembly.
[e]Orthologs for which only a partial match is found in the transcriptome assembly.
[f]Orthologs for which no match is found in the transcriptome assembly.

The majority of the gene ontology (GO) terms that could be associated with the secreted proteins were of the category “Cellular Components” and “Molecular Functions”, and a few belonged to “Biological processes” (Table 5). The “Cellular Component” proteins were related to “Integral Component of Membrane”. Some of the proteins that were related to “Molecular Functions” are presumably chitinase-binding, calcium ion-binding, zinc ion-binding, and proteins with catalytic activity, peptidase activity and hydrolase activity. The proteins related to “Biological Processes” were associated with cellular, metabolic and oxidation-reduction processes.

Table 5. Annotation of secreted effector protein candidates of the *Phakopsora pachyrhizi* isolates.

Protein ID	Putative function	Length[a]	GO IDs[b]
PP4597	No match identified	525	P:GO:0005975; F:GO:0016798
PP6875	Glycoside hydrolase family 7 protein	306	
PP12957	Non-catalytic module family EXPN	228	
PP4529	CSEP-09, partial	182	
PP4709	Family 61 glycoside hydrolase	270	C:GO:0016020; C:GO:0016021; F:GO:0016787
PP5023	CSEP-12, partial	192	
PP4153	Endoplasmic reticulum mannosyl-oligosaccharide-alpha-mannosidase protein	485	C:GO:0005737; F:GO:0016462
PP2569	Inositol phosphoryl ceramide synthase	527	
PP10181	Hypothetical protein PCANC_09428	430	C:GO:0016020; C:GO:0016021
PP3724	Uncharacterized protein VP01_831g8	380	F:GO:0005179; C:GO:0005576; P:GO:0007275; P:GO:0010469; C:GO:0016020; C:GO:0016021
PP4293	Carbonic anhydrase	260	F:GO:0004089; F:GO:0008270; P:GO:0015976; F:GO:0016829
PP4432	Peptidyl-prolyl cis-trans isomerase B (cyclophilin B)	220	P:GO:0000413; F:GO:0003755; P:GO:0006457
PP809	Hypothetical protein PGTG_17065	134	C:GO:0016020; C:GO:0016021
PP6611	Family 5 glycoside hydrolase	498	F:GO:0016985; P:GO:0046355
PP6875	Glycoside hydrolase family 7 protein	456	P:GO:0000272; F:GO:0016798
PP5327	Hypothetical protein PSTG_03700	357	C:GO:0016020; C:GO:0016021; F:GO:0016765
PP3512	Secreted protein (*Melampsora larici-populina*)	300	
PP5640	Hypothetical protein PCANC_20687	296	F:GO:0005507; F:GO:0009055; C:GO:0016020; C:GO:0016021; P:GO:0022900
PP1738	Prohibitin phb1	276	P:GO:0000001; P:GO:0001302; C:GO:0005743; P:GO:0006457; P:GO:0007007; P:GO:0045861; P:GO:0070584; C:GO:1990429
PP4781	Hypothetical protein PSTG_05339	266	
PP936	Hypothetical protein PGTG_00898	251	
PP6661	Hypothetical PGTG_14956	256	

PP3752	Hypothetical protein PSTG_06809	247		
PP3901	Hypothetical protein PTTG_01125	249		
PP2145	Hypothetical protein PCANC_22744	238		
PP5499	Hypothetical protein PSTG_10345	231		
PP5630	Hypothetical protein PCANC_20687	185		
PP3162	Uncharacterized protein VP01_444g8	179		
PP6324	AN1-type zinc finger protein	163	F:GO:0008270	
PP2237	FK506-binding protein 2	161	P:GO:0000413; F:GO:0005528; P:GO:0061077	F:GO:0003755; C:GO:0005737;
PP2132	Hypothetical protein PTTG_27691	137		
PP3127	CSEP-13, partial	142		
PP9297	CSEP-21, partial	131		
PP195	60s acidic ribosomal protein p2	113	F:GO:0003735; P:GO:0006414	C:GO:0005840;
PP5624	UPF0357 protein, putative	129	C:GO:0016021	
PP3803	Uncharacterised protein	124		
PP5239	Uncharacterised protein	96		
PP6239	Hypothetical protein H113_06952	87		
PP5059	DUF1748-domain-containing protein	89		
PP4033	Hypothetical protein PGTG_04510	86		
PP65	Uncharacterised protein	78		
PP3047	Hypothetical protein PCASD_04835	80		
PP4016	Hypothetical protein ALC57_13858	82		
PP4788	Cell division control protein 48	75		
PP3036	hypothetical protein PHLGIDRAFT_209497	69		

[a]Protein size (amino acids).
[b]GO IDs: Gene ontology identifiers, P: biological processes, C: cellular component, F: molecular function.

Selection of *P. pachyrhizi* secreted proteins putatively matching *Rpp* resistance proteins

Next, we tried to correlate the presence or absence of particular secreted proteins in the different *P. pachyrhizi* isolates with incompatibility or compatibility of those isolates with soybean lines that possess or lack particular *Rpp* genes (Table 6). Ten proteins that were identical in the isolates T9 and T10, both of which are avirulent on soybean carrying the *Rpp1* gene, and that are absent in isolates T1 to T8 that are virulent on soybean carrying the *Rpp1* gene, were found (Table 6). These ten proteins can therefore be regarded as the product of a potential *AvrRpp1* avirulence gene candidate. Out of these 10 proteins, only for one protein (PP6875) a functional annotation was found, as it shows homology to glycoside hydrolase family 7 proteins, which belong to the cell wall-degrading enzymes (Table 5).

Eleven proteins are absent from isolate T9 that is virulent on soybean carrying the *Rpp4* gene, while they are present in all other isolates that are avirulent on *Rpp4*-containing soybean. Thus, one of these eleven proteins potentially is the *AvrRpp4* gene product. Of these candidates, protein PP4529 was previously identified as candidate secreted effector protein (CSEP) CSEP-09 (Kunjeti *et al*., 2016).

Seventeen proteins are absent from isolate T5 that is virulent on soybean that carries the *Rpp5* gene, while they are present in all other isolates that are avirulent on this particular soybean line, suggesting that one of these proteins may be AvrRpp5. Of these candidates, protein PP4709 shows homology to family 61 glycoside hydrolase proteins.

Four proteins are absent from T1, T2, T3 and T4, while they are all present in all other isolates, corresponding with virulence and avirulence of the isolates on soybean with the *Rpp6* gene, respectively. None of these proteins has homology to previously described proteins.

Our analysis based on differential virulence on the soybean lines carrying *Rpp2* or *Rpp3* did not yield any avirulence gene candidates.

We did not only base our analysis on isolates that are virulent on soybean lines that carry single *Rpp* genes, but we also used isolates that are virulent on soybean cultivars that carry multiple *Rpp* genes. Thirty-three proteins are absent in *P. pachyrhizi* isolates T1, T3

and T4 that are virulent on soybean cultivar UG 5 (not shown), which combines alleles of the *Rpp1* and *Rpp3* genes (Paul *et al*., 2015), while they are present in the other isolates that are avirulent on this cultivar. Three of the proteins have homology to particular enzymes: an inositol phosphoryl ceramide synthase (PP2569), a carbonic anhydrase (PP4293) and a peptidylprolyl isomerase B (Cyclophilin B) (PP4432) (Table 5). Among the 30 remaining proteins, protein PP809 has homology to a hypothetical protein from wheat stem rust (*Puccinia graminis* f.sp. *tritici*), PP10181 has homology to a hypothetical protein from crown rust of oats (*Puccinia coronata* f.sp *avenae*), while PP3724 has homology to an uncharacterized protein from common rust of maize (*Puccinia sorghi*). Importantly, no proteins were found in common with the ten AvrRpp1 candidates that were identified based on soybean carrying the *Rpp1* gene only.

Seven proteins are absent in isolates T1, T2, T3, T4 and T8 that are virulent on cultivar Hyuuga (not shown) that combines *Rpp3* with *Rpp5* (Kendrick *et al*., 2011), while they are present in all other isolates that are avirulent on this cultivar. Importantly, no proteins were found in common with the analysis based on avirulence on soybean carrying the *Rpp5* gene only, which yielded 17 AvrRpp5 candidates (Table 6).

Table 6. Selection of putative avirulence (Avr) proteins, of which the presence and absence is correlated with avirulence or virulence on cultivars that carry matching *Phakopsora pachyrhizi* resistance genes.

		***P. pachyrhizi* isolate**									
	Effector[a]	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
AvrRpp1	PP4597	-	-	-	-	-	-	-	-	+	+
	PP6875	-	-	-	-	-	-	-	-	+	+
	PP6909	-	-	-	-	-	-	-	-	+	+
	PP7020	-	-	-	-	-	-	-	-	+	+
	PP2684	-	-	-	-	-	-	-	-	+	+
	PP9172	-	-	-	-	-	-	-	-	+	+
	PP2289	-	-	-	-	-	-	-	-	+	+
	PP6278	-	-	-	-	-	-	-	-	+	+

	PP6895	-	-	-	-	-	-	-	-	+	+
	PP6063	-	-	-	-	-	-	-	-	+	+
AvrRpp4	PP10104	+	+	+	+	+	+	+	+	-	+
	PP4529	+	+	+	+	+	+	+	+	-	+
	PP2780	+	+	+	+	+	+	+	+	-	+
	PP1805	+	+	+	+	+	+	+	+	-	+
	PP6235	+	+	+	+	+	+	+	+	-	+
	PP5220	+	+	+	+	+	+	+	+	-	+
	PP4679	+	+	+	+	+	+	+	+	-	+
	PP5006	+	+	+	+	+	+	+	+	-	+
	PP3643	+	+	+	+	+	+	+	+	-	+
	PP6481	+	+	+	+	+	+	+	+	-	+
	PP3353	+	+	+	+	+	+	+	+	-	+
AvrRpp5	PP4709	+	+	+	+	-	+	+	+	+	+
	PP5023	+	+	+	+	-	+	+	+	+	+
	PP5685	+	+	+	+	-	+	+	+	+	+
	PP4810	+	+	+	+	-	+	+	+	+	+
	PP5639	+	+	+	+	-	+	+	+	+	+
	PP6223	+	+	+	+	-	+	+	+	+	+
	PP6947	+	+	+	+	-	+	+	+	+	+
	PP6189	+	+	+	+	-	+	+	+	+	+
	PP5378	+	+	+	+	-	+	+	+	+	+
	PP6701	+	+	+	+	-	+	+	+	+	+
	PP6186	+	+	+	+	-	+	+	+	+	+
	PP6511	+	+	+	+	-	+	+	+	+	+
	PP6723	+	+	+	+	-	+	+	+	+	+
	PP5070	+	+	+	+	-	+	+	+	+	+
	PP7065	+	+	+	+	-	+	+	+	+	+

	PP6392	+	+	+	+	-	+	+	+	+	+
	PP5508	+	+	+	+	-	+	+	+	+	+
AvrRpp6	PP2291	-	-	-	-	+	+	+	+	NT	+
	PP6880	-	-	-	-	+	+	+	+	NT	+
	PP7608	-	-	-	-	+	+	+	+	NT	+
	PP14566	-	-	-	-	+	+	+	+	NT	+

[a]NT, Not tested; +, present; -, absent.

DISCUSSION

Rust fungi comprise one of the largest group of plant fungal pathogens that are responsible for substantial yield losses of crops globally (Kolmer *et al*. 2009). They use haustoria to secrete effectors into the cytoplasm of host cells to counter host defence (Panstruga and Dodds, 2009). Effector genes are highly expressed during host colonization (Dodd and Rathjen, 2010) and thus expression *in planta* can be reliably used as a criterion to predict candidate effectors (Petre *et al*., 2015). Following the gene-for-gene theory, R proteins from the host detect matching Avr proteins from the pathogen, resulting in an incompatible interaction, whereas the absence of either the R protein or the Avr protein leads to a lack of detection, thereby resulting in a compatible interaction (Jones and Dangl 2006). A pathogen can evade detection by mutating *Avr* genes, losing them entirely or evolving novel effectors that suppress Avr recognition (Rodriguez-Moreno *et al*., 2018; Lo Presti *et al*., 2015). Thus, the variation in virulence among the ten isolates that were used in this study can likely be attributed to the presence of particular effector alleles in isolates that are avirulent on soybean genotypes carrying particular *Rpp* genes, and that act as Avr factors.

This study focussed on presence/absence polymorphisms to identify potential Avr gene candidates in *P. pachyrhizi*. However, it needs to be noted that mutations as subtle as a single point mutation can be sufficient to evade recognition. For example, a single base pair change in the *Avr4* gene of the tomato leaf mould fungus *Cladosporium fulvum* is sufficient for the isolate to become virulent on tomato genotypes carrying the *Cf-4* resistance gene

(Joosten *et al*., 1994). However, assessing allelic sequence variation in our analysis would not be straightforward because of the sequencing quality and also the possibility that different mutations can occur among isolates within the same *Avr* gene. Thus, in our study we have limited the analysis to potential presence/absence polymorphisms. For several resistance genes, it has been shown that *Avr* gene deletion is the preferred means for the pathogen to overcome recognition. For instance, strains of the vascular wilt fungus *Verticillium dahliae* that evolved to escape recognition by the tomato Ve1 immune receptor, all have lost the corresponding *Ave1* gene (de Jonge *et al*., 2012). Also for the *C. fulvum* effectors Avr4E, Avr5 and Avr9, gene deletion frequently occurs (Stergiopoulos *et al*., 2007; Iida *et al*., 2015). Clearly, the approach to focus on presence/absence polymorphisms still yielded lists with candidate genes for most of the *AvrRpps*. However, we did not identify candidates for *AvrRpp2* and *AvrRpp3* in our analysis based on presence/absence polymorphisms. Moreover, we also did not observe overlap among candidates identified based on differential pathogenicity on the soybean line carrying *Rpp1* and the cultivar carrying a combination of *Rpp1* and *Rpp3*. Similarly, we did not find overlap among candidates identified based on differential pathogenicity on the soybean line carrying *Rpp5* and the cultivar carrying a combination of *Rpp3* and *Rpp5*. This may be explained by the presence of different alleles of the same *R* gene with somewhat different recognition specificities, as we have observed in our own studies for *Rpp3* (Chapter 4). However, this may also indicate that the true matching Avr is not absent in virulent isolates, but rather present as an allelic, mutated variant.

Recent studies on *P. pachyrhizi* transcriptomes have focused on comparing the secretome of virulent versus avirulent genotypes, with the aim to gain insight in differential gene expression during infection (de Carvalho *et al*., 2017). Previously, Link *et al.* (2014) also identified haustorially secreted proteins in leaves of susceptible soybean plants infected with *P. pachyrhizi*. Some of these secreted proteins were also reported in the study by de Carvalho *et al*. (2017). In this study, we performed a transcriptome analysis of *P. pachyrhizi* isolates that displayed differential virulence patterns on a set of soybean accessions that carry different *Rpp* genes to identify potential avirulence genes that match

particular *Rpp* genes. To our knowledge, no studies have been conducted to identify Avr effectors from *P. pachyrhizi* matching with soybean *Rpp* genes.

Among the rust fungi, 11 effectors have been identified so far, which are rust-transferred protein 1 (*RTP1*) from *Uromyces fabae* (Kemen *et al*., 2005), *AvrM*, *AvrL567*, *AvrP123*, *AvrP, AvrP4, AvrL2* and *AvrM14,* from *M. lini* (Dodds *et al*., 2004; Ellis *et al*., 2005, Anderson *et al*., 2016) and PGTAUSPE-10-1, *AvrSr35* and *AvrSr50* from *P. graminis* f. sp. *tritici* (Salcedo *et al*., 2017; Chen *et al*., 2017; Upadhyaya *et al*., 2014). As can be deduced from their names, most of these act as Avr factors on flax and wheat plants.

Transcriptome annotations might provide insight into the function and biological processes the identified effector proteins are involved in. Most predicted proteins from our study did not have any significant matches in the database, as is commonly observed for fungal effectors (Saunders *et al*., 2012). However, most of the annotated proteins in our study corresponded to hypothetical proteins and uncharacterized proteins with unknown functions (Table 5). The GO terms point to a possible enrichment of glycoside hydrolase (GH) family 61 and 7 proteins. These two families consist of cell wall-degrading enzymes that can aid in the penetration of host cells by fungal pathogens (Van Vu *et al*., 2012). These two types of proteins are for example significantly upregulated during infection of populus leaves by *M. larici-populina* and of wheat by *P. graminis* f. sp. *tritici* (Duplessis *et al*., 2011). Cellulase, a member of the GH7 family, has also been shown to contribute to the penetration of the host epidermis by *Magnaporthe oryzae* (Van Vu, *et al*., 2012). Other pathogens, like *Phytophthora infestans*, also secrete glycoside hydrolase enzymes that are associated with carbohydrate metabolism (Raffaele *et al*., 2010). The expression of these proteins by some of the isolates in this study suggests that they play an important role in the colonization of soybean plants by *P. pachyrhizi*.

Future studies will focus on cloning and characterization of the *Avr* gene candidates using a soybean mosaic virus (SMV) assay (Seo *et al*., 2016). To this end, the candidate effectors will be cloned in an SMV expression vector and inoculated onto soybean leaves. The virus will systemically spread in the plant, express the inserted effector gene and will induce a cell death response, once recognition by the corresponding *Rpp* gene product

occurs. Transient expression of the Avrs could also be evaluated by delivering effector candidates into soybean leaves using microparticle-bombardment (Dou *et al*., 2008). Furthermore, we will characterize the expression of the genes encoding some putative effectors, followed by elucidation of the mechanism by which they promote fungal virulence through functional analyses.

Overall, by using RNA-Seq and *de novo* assembly of the *P. pachyrhizi* transcriptome, we have identified avirulence gene candidates of *P. pachyrhizi*. Understanding the molecular mechanisms of *P. pachyrhizi* infection will guide the development of better disease management strategies. Once confirmed, Avrs can be used for assessing the novel recognition specificity of resistance genes. This can be achieved through transient expression of effector genes in germplasm by *Agrobacterium*-mediated expression or by SMV assays (Mangano *et al*., 2014; Seo *et al*., 2016). Polymerase chain reaction (PCR)-based assays can also be used to determine the presence or absence of the specific Avrs among *P. pachyrhizi* isolates.

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CHAPTER 7

General discussion

INTRODUCTION

Soybean (*Glycine max*) is a globally important legume that is cultivated on more than 120 million hectares, producing more than 330 million tonnes of soybeans annually (FAOSTAT, 2018). The multiple utilization of soybean for human food and animal feed is linked to its relatively high protein and oil content, when compared with other commonly cultivated legumes. Soybean production in Africa has been relatively moderate in the past years, but the trend is rapidly changing as the production has gradually intensified driven by increased demand for soybean and soybean products. Soybean production in Africa is expected to increase by an average of 2.3% per year (Abate *et al*., 2012). However, the rapid expansion of the area under soybean production in Africa provides a vast niche for threats, such as soybean pathogens that affect crop production. A better understanding of interactions of the soybean plant with various pathogens is needed in order to guide the development of appropriate disease control methods.

Worldwide, soybean production is threatened by *Phakopsora pachyrhizi*, an obligate fungus that causes rust on soybean plants. Rust is one of the most devastating foliar diseases of soybean that causes yield losses of up to 80% when left uncontrolled. Generally, infection starts at the flowering stage and continues through to maturity. Consequently, the disease affects flowering, pod and seed formation, resulting in fewer, smaller and lighter soybean seeds. Severe infections lead to complete premature defoliation of the plant (Kumudini, 2008). The spread of fungal spores via wind currents has led to infections in almost all soybean-producing regions globally (Isard *et al*., 2007), a situation that is expected to continue as soybean production intensifies across the globe. *P. pachyrhizi* is already causing substantial yield losses in Africa and the majority of the cultivated soybean cultivars are susceptible (Murithi *et al*., 2016).

P. pachyrhizi is a highly diverse fungus, encompassing many races that show different virulence spectra on various soybean genotypes, making the pathogen difficult to control.

So far, there are no resistant soybean varieties commercially available (Hartman *et al.,* 2005), and several studies have confirmed the high diversity of pathogenic *P. pachyrhizi* isolates (Yamaoka *et al.*, 2014; Murithi *et al*., 2016; Akamatsu *et al.*, 2017).

For various pathogens it has been shown that new pathotypes continuously arise through migration, mutation and somatic recombination (McDonald and Linde, 2002). Therefore, the greatest challenge facing effective plant disease control is reducing the rate at which pathogens evolve to overcome resistance (Burdon *et al*., 2014). Understanding the virulence diversity of a pathogen helps in the identification of the suitable genes that can be employed in resistance breeding, improves the knowledge about the major forces that drive pathogen evolution and aids in developing systems for predicting pathogen evolution. Furthermore, breeders can use this knowledge to select resistant cultivars, in addition to guiding the use of host resistance in different locations and over various crop seasons. Thus, understanding the virulence of *P. pachyrhizi* populations in Africa will aid in the identification and deployment of durable resistance, leading to increased soybean productivity in turn.

The main aim of this thesis research was to gain insight into the distribution, virulence and genetic diversity of *P. pachyrhizi* isolates from East Africa, in order to generate knowledge that can guide appropriate management of the fungus. This final chapter discusses the key findings of the thesis in perspective with published literature, thereby drawing conclusions and making recommendations for future studies.

Significant virulence diversity exists among *P. pachyrhizi* isolates causing disease in Africa

Knowledge of the virulence dynamics among different *P. pachyrhizi* isolates is important for guiding the deployment of resistant soybean cultivars. The virulence spectrum of *P. pachyrhizi* isolates in Africa has been previously investigated in only a limited number of countries (Twizeyimana *et al*. 2009; Tukamuhabwa and Maphosa, 2012). These studies differed in the number of isolates that were tested and the differential set of soybean genotypes that was used. Moreover, only a limited number of *P. pachyrhizi* isolates from East Africa was evaluated for their virulence spectrum (Murithi *et al*., 2017). In addition,

little is known about the actual effectiveness of the so-called resistance to *Phakopsora pachyrhizi* (*Rpp*) genes against the rust population in the region. To this end, we characterized the virulence spectrum of a set of *P. pachyrhizi* isolates collected in East Africa using a set of eleven well-defined soybean host differentials with particular *Rpp* genes, and compared these isolates with isolates from other countries outside Africa (Murithi *et al*, 2017, Chapter 3). Previously, four distinct pathotypes were identified among the 17 isolates collected from different countries in Africa (Murithi *et al*, 2017). There were no identical virulence patterns observed between isolates from Africa and those from other continents (Murithi *et al.,* 2017). To gain more insight into the *P. pachyrhizi* virulence diversity in East Africa, further virulence spectrum evaluations on 65 single pustule *P. pachyrhizi* isolates obtained from Kenya, Malawi, Tanzania and Uganda were conducted (Chapter 4). This resulted in the identification of 12 additional pathotypes. Evaluation of the virulence spectrum of the *P. pachyrhizi* isolates enabled us to determine the distribution of these pathotypes over the different countries, and their virulence frequencies on soybean differentials carrying different *Rpp* genes.

The virulence of Kenyan isolates on the soybean cultivars UG 5 and Hyuuga, carrying *Rpp1* and *Rpp3*, and *Rpp3* and *Rpp5*, respectively (Chapter 4) demonstrated the emergence of new isolates with a broader virulence spectrum in the region. Both cultivars were reported to be currently resistant to the majority of the isolates present in Nigeria and the USA (Twizeyimana *et al*., 2009; Paul *et al*., 2015). Notably, the same Kenyan isolates were avirulent on soybean differentials carrying either *Rpp3* (PI 462312) or *Rpp5* (PI 200526) (Chapter 5), which is a remarkable observation. However, although the *Rpp3* gene in PI 462312 maps to the same locus as in Hyuuga, these loci may possess different recognition specificities (Kendrick *et al.*, 2011). Previous studies revealed that the pedigrees of Hyuuga (PI 506764) are the soybean varieties Akasaya and Ako musume (Hossain *et al*., 2014). Akasaya (PI 416764) is a Japanese landrace with a resistance locus similar to *Rpp3* and it also clusters with PI 462312 (*Rpp3*) (Hossain *et al*., 2014). Although these two accessions, PI 416764 and PI 462312, are closely related, they showed different infection types when inoculated with particular Argentinian *P. pachyrhiz*i isolates (Akamatsu *et al*., 2013).

Similarly, an isolate from Brazil was virulent on PI 462312 but avirulent on Hyuuga (PI 506764) (Silva *et al*., 2008). Thus, the results from our studies further support the hypothesis that the *Rpp3* loci in PI 462312 and PI 506764 carry different *Rpp* alleles.

When compared with South American isolates, the virulence spectrum of *P. pachyrhizi* isolates from Africa is narrower (Murithi *et al.*, 2017; Chapter 4). Recent evidence showed variable virulence patterns among 83 isolates collected from three different countries in South America (Akamatsu *et al*., 2017). Our study showed that Argentinian isolates have the broadest virulence spectrum among 25 isolates from eight different countries of four continents (Murithi *et al.*, 2017; Chapter 4). This may be the consequence of the intensive monoculture of soybean in South America that may promote the emergence of virulent pathotypes. In comparison, soybean production in Africa occurs on a much smaller scale and involves the cultivation of multiple soybean varieties. Nevertheless, the 16 pathotypes found among the African isolates that were tested in this study (Chapters 4 and 5), confirm that considerable diversity exists among the African *P. pachyrhizi* population, which may reflect the simultaneous cultivation of multiple soybean genotypes. With the intensification of soybean cultivation in Africa, *P. pachyrhizi* pathotypes with broader virulence spectra are likely to evolve, especially when large monocultures of soybean genotypes carrying only one particular *Rpp* gene will be employed. Therefore, continuous monitoring of changes in the virulence spectrum of the *P. pachyrhizi* population is necessary in order to guide the exploitation of soybean resistance sources in the region. The number of samples used in the studies described in Chapters 4 and 5 were relatively low, due to limitations like low rust spore recovery from field samples and the absence of rust infections in some growing seasons. Therefore, improved methods for spore recovery and better tools for rapid screening of the virulence patterns are needed for better monitoring of virulence changes among *P. pachyrhizi* populations over time in Africa.

Only a few *Rpp* genes are effective against local rust populations in Africa

At least seven major resistance loci against *P. pachyrhizi*, *Rpp1* to *Rpp7*, have been identified (Childs *et al*., 2018). Resistance conferred by these genes has been broken in a

number of African countries shortly after their deployment. For instance, the effectiveness of the *Rpp1* (PI 200492) resistance gene was overcome about nine years after its discovery (Bromfield, 1984). Among the earlier reports of ineffectiveness of *Rpp1* was in Taiwan in 1983 (Bromfield 1984). Similarly, resistance conferred by *Rpp2* and *Rpp3* was broken within seven and ten years, respectively, after its introduction (Bromfield 1984). Therefore, it is important to test the currently available *Rpp* genes against the locally occurring soybean rust population to evaluate their effectiveness before their deployment in breeding programs. As new germplasm screens are conducted and new resistance genes are being discovered, it is also necessary to challenge soybean genotypes carrying these newly discovered genes with *P. pachyrhizi* isolates having the broadest virulence spectrum, in order to evaluate the effectiveness of the novel *Rpp* genes. This could help in recommending the local utilization of the most effective *Rpp* genes. One limitation in identifying and comparing *P. pachyrhizi* virulence across the globe is the lack of an internationally accepted standard set of soybean host differentials that can be challenged with *P. pachyrhizi* isolates (Hartman *et al*., 2011). Currently, there is no common standardized method of evaluating the virulence of *P. pachyrhizi* isolates, which makes it difficult to compare the different pathotypes described in different studies. Thus, there is an urgent need to agree on a defined set of differentials to enhance the global comparison of pathotypes and to guide the deployment of resistance genes.

The use of cultivar UG 5 (*Rpp1,3*) in Africa may no longer be sustainable. Similarly, cultivar Hyuuga (*Rpp3*,*5*) may not be suitable for deployment in particular African countries (Chapter 4), because of the occurrence of resistance-breaking pathotypes. Breeders must therefore identify new sources of resistance, which can also include exploring the potential of stacking particular *Rpp* genes. Resistance gene pyramiding can be an effective method to enable the development of durable resistance (Koller *et al*., 2018, Mundt 2014), as the resistance genes present in the stack may enable the plant to recognize multiple effectors secreted by the same isolate simultaneously which may be difficult for the pathogen to overcome (McDonald and Linde, 2002). However, a careful choice of resistance gene combinations is necessary, as some of the *Rpp* gene combinations have proven to be

ineffective (Chapter 4; Yamanaka *et al*., 2015). This is because the level of resistance that is provided differs based on the genetic background of the breeding material (Yamanaka *et al*., 2013). Stacking of at least three *Rpp* genes (as is the case for the line No6-12-1 that combines *Rpp2*, *Rpp4* and *Rpp5*), was effective against all the isolates tested in this study (Chapter 4), as well as against isolates from Brazil and Japan (Yamanaka *et al*., 2015). With careful selection of *Rpp* genes that are effective against local populations, gene pyramiding remains a valuable option for developing cultivars with durable resistance against *P. pachyrhizi*. This study identified soybean genotypes carrying the *Rpp1b, Rpp2* and *Rpp3* resistance genes and line No6-12-1 that carries *Rpp2*, *Rpp4* and *Rpp5*, as the most resistant against soybean rust in Africa (Murithi *et al*., 2017; Maphosa *et al*., 2013; Twizeyimana *et al*., 2009). Therefore these *Rpp* genes may be used as components of resistance gene pyramiding in the soybean-breeding programs in Africa.

Transcriptome analysis reveals candidate avirulence genes matching *Rpp* resistance genes

P. pachyrhizi resistance genes are race-specific, and therefore they confer resistance only to particular isolates of the soybean rust fungus (Hartman *et al*., 2011). Following the gene-for-gene theory, race-specific effectors induce resistance responses only in host plant varieties carrying the matching resistance gene (Flor 1942). Fungal and oomycete pathogens secrete effectors during host colonization to suppress defence responses conferred by so-called pattern recognition receptors (PRRs) present at the surface of the host cells (Jones and Dangl 2006; Dodds and Rathjen, 2010). Identifying effector candidates may be challenging, but the advent of robust and affordable high-throughput sequencing tools has significantly accelerated various types of effector discovery strategies. These include whole genome analyses, but also transcriptome analyses through RNA-Seq. Despite these developments, up till now only 11 effectors have been identified for rust fungi, including *AvrM*, *AvrL567*, *AvrP123*, *AvrP*, *AvrL2*, *AvrM14* and *AvrP4* from *Melampsora lini*, *RTP1* from *Uromyces fabae* and PGTAUSPE-10-1, *AvrSr35* and *AvrSr50* from *Puccinia*

graminis f.sp. *tritici* (Dodds *et al*., 2004; Ellis *et al*., 2007; Kemen *et al*., 2005; Anderson *et al*., 2016, Salcedo *et al*., 2017; Chen *et al*., 2017; Upadhyaya *et al*., 2014).

Efforts have been put into the identification and characterization of *P. pachyrhizi* candidate effectors (Link *et al*., 2014; de Carvalho *et al*., 2017), but no studies have been conducted to identify the effectors that match the various *Rpp* genes. Through transcriptome analyses, effector proteins associated with some of the *Puccinia triticina* races have been identified (Bruce *et al*., 2014). We applied a similar approach to compare the transcriptomes of ten selected *P. pachyrhizi* isolates that were either virulent or avirulent on soybean carrying a particular *Rpp* gene. A total of 7,061 putative effector proteins were identified for the ten isolates collectively (Chapter 6). Some of the proteins were found previously as well (de Carvalho *et al*., 2017; Link *et al*., 2014). Combining the results from infection types on the respective soybean differentials with the RNA-Seq data, candidate avirulence genes matching the various *Rpp* genes were identified (Chapter 6). The majority of the annotated proteins corresponded to hypothetical proteins, secreted proteins or uncharacterized proteins with unknown function (Chapter 6). This is a typical feature commonly associated with effectors of fungal pathogens (Saunders *et al*., 2012). Functional characterization of some of the identified putative effectors revealed the presence of glycoside hydrolase (GH) family proteins that comprise cell wall-degrading enzymes that might aid in the penetration of host cells (van Vu *et al*., 2012). Proteins from this family were demonstrated to facilitate host infection by *Melampsora larici-populina* and *Puccinia graminis* f.sp. *tritici* (Duplessis *et al*., 2011), *Magnaporthe oryzae* (van Vu, *et al*., 2012) and *Phytophthora infestans* (Raffaele *et al.,* 2010). Further studies will be conducted to determine which of the identified avirulence effector candidates genuinely match the various *Rpp* genes. To this end, the candidate effectors will be cloned in a soybean mosaic virus (SMV) expression vector and inoculated onto soybean leaves. The virus will systemically spread in the plant, express the inserted effector gene and will induce a cell death response once recognition by the corresponding *Rpp* gene product occurs. Furthermore, we will characterize the expression of the genes encoding some putative

effectors, followed by elucidation of the mechanism by which they promote fungal virulence, through functional analyses.

Knowledge on the effectors responsible for virulence of the *P. pachyrhizi* isolates will lead to a better understanding of the molecular basis of the host-pathogen interaction and should facilitate the screening of germplasm for sources of resistance by focussing on effectors that are important for virulence. In addition, our work will aid the identification of resistance mechanisms and should guide the efficient deployment of soybean resistance genes. Compared to lengthy field or greenhouse screening of soybean genotypes, effectors can be used to mine for the presence of new resistance genes within a period of weeks (Du and Vleeshouwers 2014). To this end, effector genes are expressed in a panel of germplasm that can be used for breeding. If expression of the effector triggers a hypersensitive response in a particular genotype, recognition specificity is present that may be introduced into a cultivar to mediate *P. pachyrhizi* resistance (Vleeshouwers, *et al*., 2008).

Screening of soybean accessions by inoculation with a defined set of *P. pachyrhizi* strains identifies novel resistance sources

Although fungicides are commonly used for controlling rust in the major soybean-producing countries, such as Brazil and the USA (Miles *et al*., 2007; Godoy *et al.*, 2016), their use increases production costs and causes environmental and health risks. Furthermore, resistance of *P. pachyrhizi* to several fungicides has already been reported among isolates collected in Brazil, a phenomenon probably resulting from selection pressure placed on the rust pathogen by repeated fungicide applications (Reis *et al*., 2015; Godoy *et al.*, 2016). Therefore, deployment of resistant cultivars remains the most sustainable method for managing *P. pachyrhizi* (Hartman *et al*., 2005). Previous studies have identified resistance sources against African *P. pachyrhizi* populations (Twizeyimana *et al*., 2008; Oloka *et al*., 2008), but the majority of these resistance sources are no longer effective (Maphosa *et al*., 2013). Screening of soybean accessions is key to identifying novel resistance sources that can be deployed in breeding programs. Seven accessions that appeared most resistant to rust in most locations over the two years were identified and

these may provide useful material for further study and, ultimately, rust management in Tanzania and Uganda (Chapter 5). The source of resistance of two (TGx 1993 4FN and TGx 1995 5FN) of the seven accessions is *Rpp1b*, as is found in PI 594538A. *Rpp1b* also confers resistance against isolates from East Africa, Nigeria, South America and the USA (Chapter 4; Twizeyimana *et al*.; 2009; Akamatsu *et al*., 2013; Paul *et al*., 2015; Murithi *et al*., 2017). This was the first study to identify soybean cultivars and accessions that are resistant to rust across Tanzania and Uganda. Further evaluations to assess the yield potential are recommended before these two accessions are released for cultivation in the respective countries. Preferably, before release also other *Rpp* genes are introduced into the cultivar to prevent rapid defeat of the *Rpp1b* gene. Some of the USDA accessions that carry *Rpp1b*, a combination of *Rpp3* and *Rpp5*, or *Rpp6* were also resistant across various locations and these should also be deployed in breeding programs as a source of resistance. Screening of accessions in other countries was unsuccessful due the limited number of seeds of particular soybean genotypes that were available and because, in some cases, drought conditions affected plant establishment. Nevertheless, the resistant accessions identified in Tanzania and Uganda are likely to be effective across the region. Also PI 594538A that carries *Rpp1b* can be deployed in breeding programs to develop resistant varieties.

The resistant parent used for breeding the accessions sourced from IITA is cultivar UG 5, which contains two resistance genes (*Rpp1*, *3*). Although this cultivar has been effective in other countries (Twizeyimana *et al*., 2009; Paul *et al*., 2015), our findings show that most of the IITA lines have now succumbed to soybean rust in Tanzania and Uganda. This is further confirmed by the identification of Kenyan *P. pachyrhizi* isolates that overcome the resistance provided by cultivar UG 5 (Chapter 4). Susceptibility of the majority of accessions points to the presence of common *P. pachyrhizi* pathotypes across the two countries. At least three common pathotypes were reported to exist in Tanzania and Uganda (Chapter 4). Future studies can also focus on exploring non-host resistance to identify novel resistance genes among the Fabaceae family. For example, recently genes from pigeon pea (*Cajanus cajan*) were reported to be effective against over 80 *P. pachyrhizi* isolates (Kawashima *et al*., 2016).

Conclusion and future perspectives

The rapid expansion of soybean cultivation in Africa, coupled with changes in climate conditions in the areas of cultivation, will contribute to the establishment of new *P. pachyrhizi* pathotypes with a broader virulence spectrum. The emergence of pathotypes with a higher complexity, meaning that these isolates have broken the resistance provided by several resistance genes at the same time, presents a challenge to breeders to identify novel resistance genes. The knowledge gathered from the studies described in this thesis can help soybean breeders to select and target the genes that are suitable for specific locations. The availability of information about the genes that are effective in East Africa will help with the selection of the best resistance genes for gene pyramiding. The methods developed for virulence analysis in our studies will be essential for plant pathologists to consistently test the rust populations with the aim to identify changes in virulence among rust isolates.

Future research should also focus on the identification of a common set of soybean differentials and the development of universally accepted methods to measure virulence changes. Moreover, the deployment of novel effective resistance genes across the globe should be guided. Developing cheaper virulence evaluation methods is necessary, as this will enhance frequent phenotyping, with the aim to generate accurate virulence information that should guide rust management. Our studies suggest that pyramiding of at least three *Rpp* genes has potential for taming the rapid virulence gain among *P. pachyrhizi* isolates. Therefore, future research should focus on developing soybean cultivars containing multiple *Rpp* genes. Breeding programs can for example start by deploying cultivar No6-12-1, containing resistance genes *Rpp2*, *Rpp4* and *Rpp5*, that has been shown to be effective against *P. pachyrhizi* isolates from Africa, Brazil and Japan. This should go hand in hand with testing of other multiple combinations of genes to diversify the breeding target. The use of effectors for screening for resistance is a growing field and elucidating the role of these secreted proteins in host-pathogen interactions will enhance our understanding of the underlying mechanisms of infection. The application of effectors in

identifying *P. pachyrhizi* resistance genes may accelerate the breeding process and should ultimately lead to a timely and effective management of *P. pachyrhizi.*

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SUMMARY

Soybean is an important legume crop that is cultivated worldwide on more than 120 million hectares, producing more than 330 million tonnes of soybeans annually. Soybeans contain 40% of protein and 20% of oil and the crop is therefore used both for human and livestock consumption. Generally, soybean has the potential to significantly improve the nutrition of the inhabitants of sub-Saharan Africa. Soybean production has intensified in Africa over the past 15 years, owing to the raising demand driven by a population increase and a desire for diversification of diets. In addition, soybean cultivation contributes to increased economic opportunities and higher living standards for farmers. However, soybean yields in Africa on average range between 0.8 to 1.2 tonnes/ha, while the potential of the crop can be as high as 4 tonnes/ha. The relatively poor yields can largely be attributed to abiotic factors (soil fertility, drought, poor nodulation) as well to as biotic ones (diseases and insect pests).

Rust caused by the biotrophic fungus *Phakopsora pachyrhizi* is a major threat to soybean cultivation, as it can cause up to 80% yield loss. The fungus is widely spread and found in most soybean-producing regions worldwide. Unfortunately, no rust-resistant soybean cultivars are commercially available yet. However, eventually the use of resistant cultivars is the preferred option for disease control, as the use of fungicides is expensive and it complements other disease management strategies. The development of resistant cultivars requires a comprehensive understanding of the prevalent *P. pachyrhizi* pathotypes in different geographic regions. In this thesis research, the aim was to gain more insight into *P. pachyrhizi* diversity and distribution in East Africa, in order to guide the design and deployment of proper disease management strategies.

Chapter 1 is an introduction to the work described in this thesis and puts forward the importance of soybean as a crop and provides a biological background of the biotrophic soybean rust fungus *P. pachyrhizi*. The chapter presents mechanisms that operate in soybean-pathogen interactions, highlighting the major *Rpp* (for resistance to *P. pachyrhizi*) genes that have been characterized and our knowledge on virulence diversity among African *P. pachyrhizi* isolates. The chapter also presents an outline of the thesis.

Chapter 2 is a review on the economic importance of soybean as a crop, highlighting soybean rust as a major constraint for global soybean production. The epidemiology of the soybean rust fungus is described and the global status of the pathogen virulence spectrum is discussed. Furthermore, the review highlights some of the on-going research efforts in eastern Africa to identify effective soybean rust disease control measures.

In **Chapter 3**, the diversity of the various *P. pachyrhizi* isolates collected in East Africa and from other continents is compared. Our study identified four distinct pathotypes among the isolates from East Africa. The isolates that were virulent on most resistance genes originated from Argentina and South Africa. Among the *Rpp* genes, four (*Rpp1b*, *Rpp2*, *Rpp3* and *Rpp5*) were identified as resistant to most of the isolates tested in this study, and therefore these are candidates for deployment in breeding programs in Africa.

Chapter 4 investigates the diversity and distribution of *P. pachyrhizi* pathotypes in East Africa. A set of 11 host differentials carrying specific (combinations of) resistance genes was used to determine the pathotypes present among 65 *P. pachyrhizi* isolates and their distribution over four countries in East Africa. Twelve pathotypes were identified that were virulent on soybean genotypes carrying *Rpp1* and avirulent on cultivars carrying *Rpp1b*, *Rpp2* or *Rpp3*, as well as on a cultivar that contains *Rpp2*, *Rpp4* and *Rpp5*.

In **Chapter 5**, soybean accessions were evaluated for resistance to *P. pachyrhizi*. To this end, 77 soybean accessions from both local and international sources were evaluated at multiple locations in Tanzania and Uganda in 2016 and 2017. Infection types, disease severities and sporulation levels varied among the accessions and locations. The majority of accessions displayed tan-coloured (TAN) lesions and developed moderate sporulation, implying susceptibility, while a handful of accessions showed a low disease severity and displayed reddish brown (RB) lesions, signifying resistance. We identified seven accessions that appeared most resistant to rust in most locations over the two years, and these may provide useful material for further study and, ultimately, rust management.

In **Chapter 6**, a comparative transcriptome analysis was conducted for *P. pachyrhizi* isolates that have different virulence patterns on a set of soybean cultivars carrying specific (combinations of) resistance genes. The transcriptomes of ten different isolates were

compared using RNA-Seq, and candidate avirulence proteins matching the different soybean *Rpp* genes were identified. Collectively, a total of 7,061 secreted protein sequences were predicted for the ten isolates, putatively containing effector proteins. Once confirmed, Avrs can be used for assessing the recognition specificities of resistance genes and other effectors can be used to screen for novel recognition specificities.

Chapter 7 discusses the major findings of this thesis research and relates these to findings from previous studies on *P. pachyrhizi*. Furthermore, research gaps that require further investigations are identified.

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IITA and with whom the idea of this PhD started. Thanks for believing in me and supporting my research ideas.

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Special thanks to my paranymphs; Jinbin Wu and Laurens Deurhof; I am grateful for your support. I always enjoyed your company in the Laboratory of Phytopathology.

IITA Dar es Salaam remains a family to me. This is where my research on soybean pathology commenced. I have spent seven years with you and words are not enough to express my gratitude. I thank the East Africa Hub director, Dr Victor Manyong for his support and guidance throughout my stay at IITA. Thanks for the financial support through the Lukas Brader Fellowship Award that enabled me start my PhD at Wageningen University. Thanks also to my external supervisor Dr. Bandyopadhyay Ranajit for the inspiring discussions and for challenging me to take up the soybean rust research.

I am sincerely grateful to all my colleagues whom I worked with in the field and in the lab at IITA. Special thanks to Ms Mary Njala for her vast support in the lab and during field work. My PhD could not have been completed without your support. Dr Fredrick Baijukya, working with you and your soybean team was fulfilling, thanks for the massive support especially with the field work. I am also grateful to my many other colleagues in Uganda, Kenya, Malawi, Zambia and Nigeria for their support during my field work.

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During my PhD research, I had the opportunity to visit and work with Dr. Glen Hartman at the soybean pathology lab at the University of Illinois. Thanks for teaching me the various aspects of the soybean rust fungus, including spore recovery, soybean inoculation and evaluating the virulence spectra of isolates. It is with you that I published my first article and this marked a beginning of many others that came later. Thanks also for providing me with the soybean germplasm that I have used in my research. I am greatly inspired by your research on soybean pathology.

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I do not have enough words to thank my dear wife. Thanks for braving all the moments when I was away and you had to take care of our family all alone. I admire your patience, courage, strength and encouragement. My two sons: Jayden and Adriel, thanks for holding on, waiting for daddy to come back from field trips; both locally and abroad and from the extended periods that I spent in the lab. I hope when you grow up you will understand the reason for such moments.

Finally, I thank my brother, my sisters, my relatives and friends for their spiritual and moral support that they offered me during my PhD studies. It takes a whole village to raise a child (African proverb). I am forever grateful to all.

ABOUT THE AUTHOR

Harun Muthuri Murithi was born on September 19th, 1981 in Meru, Kenya. After finishing his secondary education at Kibirichia Boys Secondary School (2001), he joined Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya, where he pursued his BSc in Horticulture. After completion of his BSc in 2007, he worked for both public and private institutions in Kenya, before securing a scholarship to study his MSc in Plant protection at the University of Pannonia, Hungary in 2009. His MSc thesis on the characterization of heirloom tomato varieties as sources of resistance to tomato viruses was supervised by Dr. Takacs Andras. In 2011, he joined the International Institute of Tropical Agriculture (IITA), Dar es Salaam, Tanzania, as an associate professional officer to work on the soybean rust disease. In 2014 he started his "sandwich" PhD at Wageningen University in the *Solanaceae* (SOL) group at the Laboratory of Phytopathology, under the supervision of Dr Matthieu Joosten and at IITA under the supervision of Dr. George Mahuku. The results from his research are presented in this thesis.

LIST OF PUBLICATIONS

Murithi, H.M., Soares, R.M., Mahuku, G., van Esse, H.P and Joosten, M.H.A.J. Diversity and distribution of pathotypes of the soybean rust fungus *Phakopsora pachyrhizi* in East Africa (in preparation).

Murithi H.M., Namara, M., Tamba M., Tukamuhabwa P., Mahuku, G., van Esse, H.P., Thomma, B.P.H.J and Joosten, M.H.A.J. Evaluation of soybean accessions for resistance against soybean rust in East Africa (in preparation).

Murithi, H.M., Haudenshield, J., Beed, F., Mahuku, G., Joosten, M.H.A.J. and Hartman, G.L. 2017. Virulence diversity of *Phakopsora pachyrhizi* isolates from East Africa compared to a geographically diverse collection. *Plant Disease* 101:1194-1200.

Murithi, H.M., Beed, F., Tukamuhabwa, P., Thomma, B. P. H. J. and Joosten, M. H. A. J., 2016. Soybean production in eastern and southern Africa and threat of yield loss due to soybean rust caused by *Phakopsora pachyrhizi*. *Plant Pathology* 65: 176-188.

Murithi, H. M., Owati, A., Madata, C.S., Joosten, M.H.A.J., Beed, F. and Kumar, P.L., 2015. First report of 16SrII-C subgroup phytoplasma causing phyllody and witches'-broom disease in Soybean in Tanzania. *Plant Disease*, 99:886-886.

Murithi H.M., Beed, F., Soko, M., Haudenshield, J., and Hartman, G.L. 2015. First report of *Phakopsora pachyrhizi* causing rust on soybean in Malawi. *Plant Disease* 99:420.

Murithi, H.M., Beed F, Madata, C., Haudenshield, J., and Hartman G.L. 2014. First report of *Phakopsora pachyrhizi* on soybean causing rust in Tanzania. *Plant Disease* 98:1586.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Harun Muthuri Murithi
Date: 25 February 2019
Group: Laboratory of Phytopathology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
Risk assessment of soybean rust based on its distribution, variance and influence of environmental parameters in eastern and southern Africa	16 Oct 2013	1,5
► **Writing or rewriting a project proposal**		
Genetic characterization of soybean rust in eastern and southern Africa to optimize strategies for disease management	Oct 2013	6,0
► **Writing a review or book chapter**		
Murithi et al., Soybean production in eastern and southern Africa and threat of yield loss due to soybean rust caused by *Phakopsora pachyrhizi.* Plant Pathology (2016) 65:176-188, DOI: 10.1111/ppa.12457	Jan 2016	6,0
► **MSc courses**		
Molecular Aspects of Bio-Interactions (PHP 30806)	Oct-Nov 2013	6,0
Subtotal Start-Up Phase		19,5

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
EPS PhD student days 'Get2Gether', Soest, NL	28-29 Jan 2016	0,6
EPS PhD student day, 'Get2Gether', Soest NL	09-10 Feb 2017	0,6
► **EPS theme symposia**		
EPS theme 2 - Interactions between Plants and Biotic Agents, Leiden University, The Netherlands	22 Jan 2016	0,3
EPS theme 1 - Developmental Biology of Plants Leiden University, The Netherlands	28 Feb 2017	0,3
► **National meetings (e.g. Lunteren days) and other National Platforms**		
IITA annual Research for Development (R4D) week, Ibadan, Nigeria	25-29 Nov 2013	1,5
IITA-Tanzania Science Day 'Increasing our impact through shared learning', Dar es Salaam, Tanzania	29-30 Nov 2016	0,5
► **Seminars (series), workshops and symposia**		
IITA Monthly Seminar: Functional analysis of sequences that enhance Gemini virus symptoms - Dr. Joseph Ndunguru	14 May 2014	0,1
IITA Monthly Seminar: Studying the genotype by environment interactions for resistance to CBSD and CMD in eastern and southern Africa - Dr. Silver Tumwegamire	15 Aug 2014	0,1
IITA Monthly Seminar: Fast-tracking access to popular and improved varieties of root crops by small holder farmers - Dr. Collins Abuga Marita	25 Mar 2015	0,1
IITA Monthly Seminar: Engaging in IITA gender research aiming at enhancing equity and social inclusion in African agriculture and rural development - Dr Amare Tegbaru	18 Jun 2015	0,1
Flying Seminar: Prof.dr. Jane Parker, 'Plant intracellular immunity: evolutionary and molecular underpinnings'	21 Jan 2016	0,1
IITA Monthly Seminar: Odor-guided behavior of insects with particular interest to cassava whitefly (Bemisia tabaci) - Ms Latifa Mrisho	05 May 2016	0,1
IITA Monthly Seminar: Potential for RNA interference in management of *Bemisia tabaci* - Dr Wosula Everlyne	25 Aug 2016	0,1
Symposium publish for impact -Wageningen Campus	07 Feb 2017	0,2
Mini-Symposium Applied Phytopathology-From the lab to the field-Wageningen Campus	01 Mar 2017	0,2
JIC seminar: Professor Jijie Chai, Recognition of plant peptide hormones by their receptors	15 Sep 2017	0,1
JIC seminar: Professor L. Mahadevan, Some physical aspects of plant morphogenesis	22 Sep 2017	0,1
TSL Seminar: Dr. Piingtao Ding, Capture sequencing enpowers the study of signal transduction in plant innate immunity	03 Oct 2017	0,1
JIC seminar: Professor KJ Patel,"Toasting" our genomes: how alcohol and aldehydes cause damage to us	06 Oct 2017	0,1
TSL Seminar: Dr. Kasia Lyabak, Searching for general pathogenesis determinants	24 Oct 2017	0,1
TSL Seminar: Dr. Dae Sung Kim, Investigating effectors from downy mildew of Arabidopsis	31 Oct 2017	0,1
JIC seminar: Professor Miltos Tsiantis, Development and diversity of leaf shape: towards trait reconstruction	03 Nov 2017	0,1
TSL Seminar: Dr. Marta Bjorson, TranscriPTIon: transcriptional regulation in PAMP-triggered immunity	14 Nov 2017	0,1
TSL PhD Colloquium with Rebecca Nelson	21 Nov 2017	0,3
JIC seminar: Professor Hopi Hoekstra, From the field to the lab: the genetic basis of evolutionary change	24 Nov 2017	0,1
JIC seminar: Professor Christina Smolke, Synthetic biology platforms for plant natural product biosynthesis and discovery	01 Dec 2017	0,1
TSL Seminar: Dr. Marco Trujillo, Ubiquitination – at the crossroads between vesicle trafficking and immune signaling	14 Dec 2017	0,1
► **Seminar plus**		
► **International symposia and congresses**		
International Plant Pathology Congress, Berlin, Germany	24-27 Aug 2015	1,2
Tropentag Conference, Bonn, Germany	20-22 Sep 2017	0,9

CONTINUED ON NEXT PAGE

►	**Presentations**		
	Poster: Soybean rust (*Phakopsora pachyrhizi*) and Witches broom (16SrII *Candidatus phytoplasma*); the two emerging and devastating diseases of soybean in Tanzania, IPPC Conference, Berlin, Germany.	24-27 Aug 2015	1,0
	Poster: Virulence diversity of soybean rust isolates from east Africa compared to a geographicaly diverse collection, IITA R4D Week, Ibadan, Nigeria.	21-25 Nov 2016	1,0
	Oral presentation: Tackling the threat of soybean rust in eastern Africa, IITA-Tanzania Science Day, Dar es Salaam, Tanzania.	29-30 Nov 2016	1,0
	Oral presentation: Virulence diversity of soybean rust isolates from Africa,Tropentag Conference, Bonn, Germany.	20-22 Sep 2017	1,0
►	**IAB interview**		
►	**Excursions**		
	Subtotal Scientific Exposure		12,4

3) In-Depth Studies		*date*	*cp*
►	**EPS courses or other PhD courses**		
	TSL Biological safety training	14 Sep 2017	0,3
	TSL Chemical safety for scientists	04 Oct 2017	0,3
►	**Journal club**		
	2Blades/TSL literature discussion club	Sep 2017- Dec 2017	0,3
►	**Individual research training**		
	Molecular characterization of fungal pathogens, University of Illinois-Crop Science Urbana, Illinois	15 May - 30 Jun 30 2014	1,0
	Training on molecular diagnostics of soybean disease, EMBRAPA, Londriana, Brazil	21 Sep - 02 Oct 02 2015	1,0
	The Sainsbury Lab PhD student research visit	07 Sep - 21 Dec 2017	1,0
	Subtotal In-Depth Studies		3,9

4) Personal Development		*date*	*cp*
►	**Skill training courses**		
	Scientific paper writing-ILRI- Addis Abeba, Ethiopia	26-30 Oct 2015	1,0
	WGS Course: Data management planning	06 Feb 2017	0,4
	WGS Course: Essentials of scientific writing and publishing	10-21 Feb 2017	1,2
	WGS Course: Interpersonal communication for PhD candidates	2-3 Mar 2017	0,6
	2Blades retreat on subject personal development	24 Oct - 26 Oct 2017	0,6
	TSL Research Integrity Workshop	06 Nov 2017	0,3
►	**Organisation of PhD students day, course or conference**		
	Trainer for international outreach course 'Diagnosis of pests and diseases of root, tuber and banana crops', IITA, Dar es Salaam, Tanzania	30 Nov - 4 Dec 2015	1,5
►	**Membership of Board, Committee or PhD council**		
	Subtotal Personal Development		5,6

TOTAL NUMBER OF CREDIT POINTS*	**41,4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

This research was conducted at the International Institute of Tropical Agriculture (IITA), Dar es Salaam, Tanzania, The Sainsbury Laboratory, Norwich, UK and the Laboratory of Phytopathology, Wageningen University & Research, The Netherlands. The work was financially supported by a Lukas Brader Scholarship Award, The 2Blades Foundation and the International Institute of Tropical Agriculture (IITA).

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