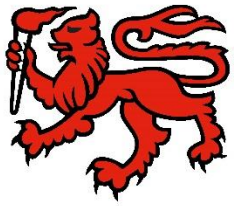

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Physiological and genetic basis of *Spongospora subterranea* zoospore attachment on potato roots

by

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Abstract

The plasmodiophorid, *Spongospora subterranea* f. sp. *subterranea* is an economically significant pathogen of potato (*Solanum tuberosum* L.) that is responsible for tuber powdery scab, root zoosporangium infection, and root galling diseases. Powdery scab has long been recognised as a major economic issue decreasing crop productivity while affected tubers have reduced market value. Options for managing *Spongospora* diseases are limited, and largely rely on using resistant cultivars, although no cultivar has been found to be completely resistant to the pathogen.

Traditionally, the identification of host resistance to *S. subterranea* has been based on the assessment of tuber powdery scab and/or root galling, in large, replicated field or glasshouse challenge trials, which are both time and resource intensive. Additionally, environmental factors and polycyclic infection issues can confound the accuracy of these evaluations. A potential alternative rapid in vitro assessment method for screening of cultivar resistance to potato tuber and root diseases is to observe relative propensity of initial zoospore root attachment at the very start of the infection process that would avoid issues of polycyclic infection.

The importance of zoospore root attachment as the initial stage of infection has been addressed in this study. The optimal conditions for zoospore release in Hoagland's solution and then the attachment of zoospores to plant hosts were first examined. Incubation of resting spores showed that the most sporosori germinated at 20°C. The extent of zoospore root attachment to host root hairs and other epidermis cells varied with cultivar, region of the root maturation zone, and temperature (with the most zoospore root attachment occurring at 15°C). Based on these preliminary experiments, a rapid and robust in vitro bioassay was developed to assess the resistance to zoospore root attachment for a total of 153 potato lines and cultivars. The presence of a continuum of varietal responses to zoospore root attachment provided evidence for the existence of polygenic regulation of resistance to this characteristic. Further comparisons revealed that the efficacy of zoospore root attachment was also substantially associated with known cultivar resistance to powdery scab, zoosporangium infection severity, and root galling, but there were notable exceptions to this generalisation.

Somatic cell selection was used in a further study to generate variants of potato with enhanced resistance to diseases. From the in vitro zoospore root attachment assessments, 31 (33%) of the

variants, from five potato cultivars and one clone, exhibited greater resistance to zoospore root attachment in comparison to their unselected parents. A glasshouse pathogenicity trial showed that most variants with enhanced resistance to zoospore root attachment also showed reduced powdery scab tuber disease than their parents, although these differences were not always statistically significant. This study demonstrated that somatic cell selection can be an effective approach for selecting disease-resistant variants to root infection, with the variants also showing reduced tuber disease.

To identify the potential basis for host resistance to *S. subterranea* at the molecular level, 12 potato cultivars differing in resistance to zoospore root attachment were used for comparative proteomic analysis. A total of 3723 proteins were quantified in root samples across the 12 cultivars using a data-independent acquisition mass spectrometry approach. Analysis identified 454 proteins that were more abundant, and 626 proteins were less abundant, in the resistant cultivars than in the susceptible cultivars. In the resistant cultivars, functional annotation of proteome data indicated that the gene ontology terms related to oxidative stress and metabolic processes were increased. KEGG pathway analysis confirmed the presence of phenylpropanoid biosynthesis pathway in the resistant cultivars, suggesting the potential role of lignin biosynthesis in host resistance to *S. subterranea*. Several enzymes involved in pectin biosynthesis and remodelling, such as pectinesterase and pectin acetylesterase, were more abundant in the resistant cultivars. Further investigation of the potential role of root cell wall pectin using the enzyme pectinase at a range of concentrations showed that pectinase reduced in zoospore root attachment both in the resistant and susceptible cultivars. These results suggest a unique role of cell wall pectin in regulating *S. subterranea* zoospore root attachment. This study provides the first insights into potato resistance to the zoospore root attachment at a proteomic level.

The enzyme study investigated the potential roles of surface root cell wall polysaccharides and proteins in zoospore root attachment by combining transcriptomic and proteomic datasets of a resistant and a susceptible potato cultivar. Effects of enzymatic removal of root cell wall proteins, *N*-linked glycans or polysaccharides on *S. subterranea* attachment to root tissue of a resistant and a susceptible potato cultivar. Subsequently, mass spectrometry analysis of peptides released by trypsin shaving (TS) of root segments identified a total of 1235 proteins of which 262 were differentially abundant between the resistant and susceptible cultivars. In particular, proteins associated with glutathione metabolism and lignin biosynthesis, were more abundant in the resistant cultivar. Comparison with whole-root proteomic analysis of the same

resistant and susceptible cultivars led to identification of 226 proteins that were unique to the TS dataset, of which 188 were significantly different between cultivars. Among these, the pathogen defence-related cell wall protein stem 28 kDa glycoprotein and two major latex proteins were identified as significantly less abundant in the resistant cultivar compared to the susceptible cultivar. A further major latex protein was detected at reduced levels in the resistant cultivar in both TS and whole-root proteomic datasets. In contrast, in the TS-specific dataset, three glutathione *S*-transferase (GST) proteins were more abundant in the resistant cultivar, while the protein glucan endo-1,3-beta-glucosidase was detected at increased levels in both the TS and whole-root datasets. These results imply particular roles of major latex proteins and glucan endo-1,3-beta-glucosidase in the regulation of host susceptibility to *S. subterranea*.

This study expands knowledge of the physiological and molecular basis of *S. subterranea* zoospore attachment to potato roots. *S. subterranea* zoospore root attachment and host resistance, which are important components of potato tuber and root disease management and potato breeding programmes, are now making significant progresses. Additional research is required, but the information provided by the present study will aid future investigations of potato *Spongospora* host/pathogen relationships.

Keywords: Zoospore root attachment, enhanced resistance, *Spongospora subterranea*, cultivar resistance, potato root proteomics, enzyme study

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Chapter 1. General introduction

1.1. Background

Global population growth, coupled with rising food consumption, is imposing unprecedented strain on agriculture and environment (Campos and Oritiz, 2020). In future decades, innovations to enhance food security will be necessary to provide sustainable, and nutrient-dense food for the world's expanding population. To feed this growing population, important changes are required that will help food producers make their livings and give consumers enough food to stay healthy, while protecting the environment.

The potato (*Solanum tuberosum* L.) is an annual cash crop, a member of the *Solanaceae* that evolved in the Andean highlands along the Bolivian and Peruvian border in South America 8000 years ago (Garzón, 2007). After arriving in Spain and the United Kingdom in the late 16th century, potato spread throughout Europe, followed by Asia, and then worldwide (Harris, 2012). Following maize, wheat, and rice, the potato ranks fourth in importance as a food crop, with 370 M tonnes of potato tubers produced annually around the world (FAOSTAT, 2021). When compared to grain crops, yield per hectare of potato crops can be two to four times greater. Potato crops are the only main crop that generates more food per unit of water than other crops, and their water use efficiency is up to seven times greater than that of cereals (Anonymous, 2007). Potatoes are the largest crop commodity grown in Australia by volume, with more than 1.3 million tons produced for fresh and processing potato industries in 2016 to 2017 (AUSVEG, 2017). Potatoes are also the most valuable crop commodity in Australia, with a value up to $717 million in 2016 to 2017 (AUSVEG, 2017). However, potato is vulnerable to over 40 pests and diseases, caused by nematodes, protists, viruses, bacteria, and fungi (Fiers et al., 2012).

Spongospora subterranea f. sp. *subterranea* is a plasmodiophorid soilborne pathogen that infects potato roots and tubers leading to root disease and tuber powdery scab (Falloon et al., 2016). The first record of tuber powdery scab of potato was that of Wallroth (1842), who observed the tuber disease was caused by *S. subterranea* in Germany. Originally identified as *Erysibe subterranea*, the pathogen is now known as *Spongospora subterranea* (Wallr.) Lagerh. Powdery scab of potatoes has long been recognised as a significant economic factor for potato industries, particularly in areas where potatoes are cultivated under intensive management practices (Harrison et al., 1997, Merz and Falloon, 2009). The disease costs the Australian

potato processing industry over $13.4 million annually in economic damage (Wilson, 2016), in addition to the fresh potato market and indirect on-farm and off-farm expenditures as a result of decreased quality and yields of infected potato tubers (Fiers et al., 2012).

When tuber scabs reach maturity, they form powdery masses of sporosori. Sporosori each contain a large number of resting spores, each of which has a triple-layer wall structure that protects the contained zoospores from exposure to environmental conditions for several decades (Lahert and Kavanagh, 1985a, Harrison et al., 1997). Sporosori are a main source of inoculum because they can stay in the soil for many years, making powdery scab a challenging disease to manage (de Boer, 2000).

In addition to tuber disease, *S. subterranea* also infects potato roots, causing zoosporangium infections and root galling, resulting in root dysfunction and reduced crop productivity (Falloon et al., 2016). The earliest report of *S. subterranea*-induced root disease was that of Pethybridge (1912), who stated that the *S. subterranea* infection of potato led to potato root and sprout galling and showed that root galls were a major source of sporosorus inoculum. *Spongospora subterranea* root galling was subsequently noted by Melhus et al. (1916) to occur widely across the Atlantic and Pacific coast production areas of the Americas. Similar to tuber powdery scab, Link and Ramsey (1932) described root infection in potato most likely occurred as a result of sufficient soil moisture and relatively low temperatures. A study in Scotland found that root and stolon galling on host plants, but not tuber powdery scab, provided the first indication that resistance mechanisms to root galling and tuber powdery scab may be distinct (Boyd, 1951).

There is now greater understanding of the biology of *S. subterranea*, which is the recognised as the causative agent of potato tuber and root diseases. However, management of *Spongospora* diseases still remains challenging, requiring a range of approaches including crop rotation, chemical applications, and the selection of disease or pathogen-free seed tubers and host resistance (Braithwaite et al., 1994b, Falloon et al., 1996, Tuncer, 2002, Larkin and Griffin, 2007, Shah et al., 2012). Further investigation of the molecular basis of this pathogen-host interaction, will help to understand how to potentially enhance host resistance, which is a key factor for potato growers and breeders to control the diseases and increase crop quality and yields.

1.1.1. Host pathogen interaction

Initial root infections by *S. subterranea* zoospores occur from swimming zoospores discharged from resting spores within the soil. The zoospores, whose heterokont flagella propel them through soil water or the aqueous environment, begin to migrate towards their hosts shortly after discharge (Harrison et al., 1997), with host root exudate production a key signalling attractant (Amponsah et al., 2021). Zoospores bind to host cell walls and each injects its contents through the cell wall via a characteristic 'Rohr' and 'Stachel' structure (Keskin and Fuchs, 1969, Williams, 1970). In addition to a suitable environment (Merz et al., 2012) and agronomic regime (Shah et al., 2014), the level of epidemic spread is determined by the level of host susceptibility (Brierley et al., 2013).

To date, no potato cultivar has been identified to have complete immunity to powdery scab (Falloon, 2008, Hernandez Maldonado et al., 2013), and host resistance varies among potato cultivars. Some cultivars show altered susceptibilities to the different stages of disease. For example, 'Russet Burbank' has high susceptibility to root infection and galling yet its tubers are moderately resistant to powdery scab. Generally, more resistant cultivars have fewer galls and zoosporangia in their roots, but this association is not perfect, and outliers do occur (Falloon et al., 2003).

Host resistance to *Spongospora* diseases can be regulated by genetic, biochemical, or morphological host variables, such as plant cell walls (Falloon et al., 2016). Specific interaction is required for zoospore attachment to host cells. Plant cell walls are a common entry point for pathogens that affect plants. At the most fundamental level, the cell walls act as physical barriers between pathogens and hosts (Vorwerk et al., 2004). As soon as zoospores reach the host surfaces, successful host recognition by the pathogen, and avoidance of host defence responses, are essential for subsequent infection. The attachment of zoospores on, and subsequent infection of roots results in zoosporangium infection and root galling, while zoospore root attachment to developing tubers can result in tuber powdery scab (Balendres et al., 2016b).

1.1.2. Cultivar susceptibility assessment to *Spongospora* tuber and root diseases

Resistance to *Spongospora* tuber and root diseases has been demonstrated (Genet et al., 1995, Falloon et al., 1996, Falloon et al., 2003, Merz et al., 2004, Nitzan et al., 2008, Nitzan et al., 2010, Merz et al., 2012, Falloon et al., 2016). However, despite the widespread availability of disease-resistant cultivars, genetic resistance to powdery scab remains a difficult factor to employ for disease control, because potato producers typically select cultivars for characteristics other than resistance to powdery scab (Harrison et al., 1997). Although host resistance has a substantial effect on disease establishment, the majority of cultivars sold commercially are susceptible to some extent (de Boer, 1991, Merz et al., 2012).

Plant disease assessment is important to the research of plant diseases, since it evaluates and quantifies plant disease (Jones, 1998). The major goal of plant disease assessment is to collect quantitative data on the incidence and development of plant diseases. This information can be used to assess the relative severity of various crop diseases by contrasting the frequency and severity with which they occur (James, 1971). Under particular conditions, disease assessment provides a crucial method for detecting treatment differences that cannot be distinguished by examining crop yields or produce quality. Therefore, disease assessment has been used in experiments to compare host resistance to disease (James, 1971).

Traditional field disease assessments of potato cultivars have relied on areas that have been artificially infected (Braithwaite et al., 1994a, Lees, 2000, Falloon et al., 2003) or that have been previously infested (Kirkham, 1986, Bus, 2000). Other disease evaluations have included pot trials (Gans and Vaughan, 2000) or glasshouse trials (de Boer, 1991).

Disease severity has been determined by examining the areas of scab coverage or counting the number of lesions on resultant tubers (Baldwin et al., 2008), or by scoring the zoosporangium infection via microscope. Several studies have been conducted to determine whether the degrees of root galling or the numbers of zoosporangia in the roots of plants are indicative of tuber resistance (Falloon et al., 2003, Merz et al., 2004). The levels of zoosporangium infection in the roots showed a stronger relationship with tuber powdery scab susceptibility than the severity of root galling (Falloon et al., 2003, Merz et al., 2004, Nitzan et al., 2008, Falloon et al., 2016). The results from the studies of Merz and Falloon (2009) and Thangavel et al. (2015) suggested that zoosporangium infection follows a polycyclic manner, which has proven to be an unreliable indicator of tuber resistance in breeding programs.

1.2. Thesis objectives

The present study investigates the physiological and biochemical basis of *S. subterranea* zoospore attachment to potato roots, the initial stage of host infection. In particular, the aims of the study are:

1 How can potato cultivars be assessed rapidly and reliably for resistance to root infection? (Chapter 3)

2 Is it possible to generate enhanced resistance to root and tuber disease within elite potato cultivars by using somaclonal variation techniques? (Chapter 4)

3 What are the constitutively expressed root proteins associated with resistance or susceptibility to zoospore root attachment? (Chapter 5)

4 What are the root surface compounds important for zoospore root attachment, and what are the root surface membrane associated proteins that may act as receptors to enable infection (susceptibility) or to induce host defence (resistance)? (Chapter 6)

These findings will be important for identifying host resistance to *S. subterranea* diseases and enhancing disease management. These findings will also motivate future research that will lead to increased understanding of the mechanisms of pathogen host attachment and host resistance to potato tuber and root diseases caused by *S. subterranea*. In addition to facilitating the development of effective and sustainable disease management approaches, the knowledge will also aid future potato breeding programme.

1.3. Thesis overview

This thesis includes peer-reviewed papers and articles awaiting peer review or revision. Consequently, there may be some repetition between chapters.

The thesis consists of seven chapters. Chapter 1 serves as an introduction to the study and its goals and scope, providing a brief explanation of the need for this research. Chapter 2 is a comprehensive literature review summarises the current management and assessment challenges for *Spongospora* tuber and root diseases. Chapters 3 to 6 have been published or are being prepared for publications in peer-reviewed international journals. Chapter 7 is a general discussion that concludes with recommendations for future research. The appendix includes additional investigations undertaken for this research.

Chapter 2. Literature review

2.1. Background

Potato (*Solanum tuberosum* L.) is an annual herbaceous crop belonging to the *Solanaceae* (Nasir and Toth, 2022). After maize, wheat and rice, potato is currently the fourth most important food crop in the world (FAOSTAT, 2021). It ranks first amongst root crops, followed by cassava, sweet potato, and yams (Park et al., 2009). There are now over 4500 potato cultivars worldwide since its spread from the Andean highlands 8000 years ago (Lutaladio and Castaldi, 2009, Campos and Oritiz, 2020).

Potato crops are grown in over 149 countries, with China and India accounting for around 40% of global potato production in 2019 (FAOSTAT, 2021). Potatoes can grow in temperate, subtropical, and tropical climates, exhibiting their resilience to a broad range of environment (FAO, 2008, Fiers et al., 2012, Campos and Oritiz, 2020, FAOSTAT, 2021). Potato is a valuable crop in developed and developing countries, produced for food, as seed tubers, and for animal feed (Lutaladio and Castaldi, 2009). An estimated 19 M hectares of potatoes are grown globally, with 378 million tonnes produced (Campos and Oritiz, 2020). Potatoes are primarily grown as cash crops and thus provide significant sources of income (Campos and Oritiz, 2020). However, potatoes are susceptible to more than 40 pests and pathogens, including insects, nematodes, protists, viruses, bacteria, and fungi (Fiers et al., 2012).

In the late 1960s, the transaction of infected seed potatoes led to the global spread of powdery scab (Karling, 1968). Powdery scab was regarded as a minor disease at the time because it had little impact on potato yield (Fornier, 1997) and the distribution of this disease was restricted to areas with cool and damp weather (Ramsey, 1918, Karling, 1968). In the 1970s and 1980s, powdery scab became a more prevalent and significant disease in potato industry in many countries (Fornier, 1997).

The obligate biotrophic protozoan pathogen, plasmodiophorid *Spongospora subterranea* f. sp. *subterranea* is responsible for powdery scab, an economically important disease complex of potato (Merz and Falloon, 2009). This pathogen was first reported by Lagerheim (1892) in South America (Melhus et al., 1916, Hawkes and Franciscoortega, 1993, Balendres et al., 2016b). Powdery scab, the most well-known component of the *Spongospora* disease complex, was first recognized in 1841 (Wallroth, 1842). Root galling was first observed forming in

Spongospora infected potato roots by Pethybridge (1912) and zoosporangium formation following the root infection by *S. subterranea* was noted by Ledingham (1935).

Powdery scab affects the majority of the world's temperate potato-growing regions, although it also occurs in hot and arid countries where irrigation is used (Wale, 2000). The pathogen causes tuber surface lesions each with a brown powder composed of sporosori, hence the common name "powdery scab" (Harrison et al., 1997). The potato root and tuber diseases caused by *S. subterranea* have significant economic impacts, reducing the quality and marketability of potatoes (Balendres et al., 2016b). In Australia, the presence of more than 2% in seed tuber harvests having one or more powdery scab lesions results in failure of the certification criteria, leading to depreciation of seed crops and deterioration of grower/breeder reputations (VICSPA, 2007, Tegg et al., 2014, Balendres et al., 2016b). Similarly, where potato crops are grown for fresh supermarket sale or processing, removal of surface layers are required for infected tuber lesions, which is more costly and wasteful (Wale, 2000). Moreover, there is increasing recognitions of the indirect impacts of *S. subterranea* on crop productivity, including reductions in water and nutrient uptake in infected roots (Merz and Falloon, 2009, Falloon et al., 2016).

New information and techniques have improved understanding of the interaction between *S. subterranea* and potato (Thangavel et al., 2015, Balendres et al., 2016b, Balendres et al., 2016a, Balotf et al., 2021b, Balotf et al., 2021a, Balotf et al., 2022b). However, the initial stage of *S. subterranea* infection has received little attention. Consequently, there is a lack of clarity on the biology of zoospore root attachment, which has hindered the development of an efficient and reliable disease assessment.

2.2. *Spongsopora subterranea* disease control and management

Effective management of *Spongospora* tuber and root diseases is challenging because resting spores can stay in the soil for several years while disease management chemicals are of low efficacy (Thangavel et al., 2015). As there is no one management strategy that provides complete control of powdery scab, the coordinated use of multiple methods is required for effective disease management, including crop rotation, soil selection, cultivar resistance, temperature, and effective crop management (Falloon et al., 2003, Falloon, 2008, Merz and Falloon, 2009). The current strategies for powdery scab disease control are outlined in Table 1.

Spongospora diseases still lack a single effective control method (Falloon, 2008). Existing *Spongospora* disease management tools have been investigated with variable and limited success. For example, treating the soil with pesticide chemicals to eliminate the pathogen is costly and may have adverse environmental effects (Falloon et al., 1996). Fluazinam treatment is currently available in New Zealand (Braithwaite et al., 1994b, Falloon et al., 1996, Tsror et al., 2020) but this is an unregistered fungicide for the commercial potato industry in Australia (Thangavel et al., 2015). There are cultivars with varying levels of resistance to the different phases of *Spongospora* tuber and root diseases, but no cultivar possesses complete immunity to the pathogen (Falloon et al., 2003, Merz et al., 2004, Falloon, 2008). It is anticipated that sustainable management of tuber and root diseases will need the development of potato cultivars and lines resistant to *S. subterranea*.

Table 1. Strategies for powdery scab disease management.

Treatments	Applications	Effects	References
Chemical control	Tuber dressing treatments: - Mancozeb - Formaldehyde - Fluazinam - Propineb - Dichlorophen-Na - Mixtures	Reduced the percentage of infected tubers to 29% compared with control (70%)	(Braithwaite et al., 1994b)
	Tuber dressing treatments: - Fluazinam - Mancozeb	Fluazinam (91% healthy tubers) and mancozeb (90%) compared with control (64% healthy tubers)	(Falloon et al., 1996)
	In-furrow treatments: - Fluazinam - Mancozeb	Fluazinam (95% healthy tubers) and mancozeb (86%) compared with control (64% healthy tubers)	(Falloon et al., 1996)
	Soil fumigation: - Metam sodium	98% reduction in powdery scab	(Tsror et al., 2020)
Crop rotation	*Brassica* crop rotation: - Perennial ryegrass - Mustard blend	31 - 55% reduction in powdery scab	(Larkin and Lynch, 2018)
	Green manure crop rotation: - Indian mustard - Rapeseed	15 - 40% reduction in powdery scab	(Larkin and Griffin, 2007)

	- Canola - Ryegrass		
	Crop rotation: - Pasture - Potato - Lucerne - Fallow - Broad bean - Canola - Tree plantation - Lupin	DNA concentration of *S. subterranea* increased from 3300 to 7300 pg DNA/g soil	(Sparrow et al., 2015)
Biocontrol	Antagonistic isolate *Aspergillus versicolor*	*A. versicolor* inhibited S*pongospora subterranea* with a protection value of 54 – 70% compared with 77 – 93% by fungicides fluazinam treatment	(Nakayama, 2017)
Agronomic management	Soil temperature	Powdery scab develops at 9 - 17℃ but most severe at 12℃	(Hughes, 1980, van de Graaf et al., 2005, van de Graaf et al., 2007, Shah et al., 2012)
	Irrigation	Lowest levels of powdery scab occurred at 673 mm irrigation	(Tuncer, 2002)
	Fertilizer	Lowest levels of powdery scab occurred at 150 kg N ha^{-1} nitrogen fertilizer	(Tuncer, 2002, Shah et al., 2014)
	Soil type	Infection level was higher in sandy soils than clay soils	(Van de Haar, 2000, Tuncer, 2002, van de Graaf et al., 2007, Brierley and Less, 2008)

2.3. Life cycle of *Spongospora subterranea* and disease development

Spongospora subterranea is a soilborne obligate parasite which relies on the living tissues of host plants to complete its life cycle (Karling, 1968, Campos and Oritiz, 2020). *S. subterranea* has been extensively studied, and its life cycle (Figure 1) has been documented (Harrison et al., 1997, Merz, 2008, Balendres et al., 2016b, O'Brien and Milroy, 2017). The important initial

stage in pathogenesis of *S. subterranea* is release of zoospores from resting spores into the soil (Melhus et al., 1916, Christ and Weidner, 1988, Merz et al., 2012, Balendres et al., 2016b). The soil environment is critical for motile zoospore recognition and attachment to the host. Zoospores attach to host roots or epidermis cells, including root hairs. Each zoospore penetrates the host cell wall using 'Rohr' and 'Stachel' structure (Keskin and Fuchs, 1969), to inject the zoospore contents into the host cell (Williams, 1970, Kageyama and Asano, 2009). A multinucleate plasmodium develops in each infected host cell (Braselton, 1992). Each plasmodium nucleus then further develops into a zoosporangium containing four nuclei, which progress into four secondary zoospores. This infection and secondary zoospore development process takes 4 to 5 days (Lahert and Kavanagh, 1985b, Merz, 1989, Clay and Walsh, 1990).

Zoosporangium is a typical indicator of infection by *S. subterranea* in host plants, in root hairs and other epidermis cells of roots and stolons (underground stems) (Lagerheim, 1892, de Boer, 1997, Nitzan et al., 2008, Balendres et al., 2016b). Secondary zoospores are released from zoosporangia into the soil to reinfect host tissues, but resting spores do not form within zoosporangia (Boyd, 1951, Braselton, 2001). Kole (1954) observed quadriflagellate zoospores of *S. subterranea*, possibly formed when two biflagellate zoospores fuse together, or possibly results from incomplete divisions in zoosporangia. Root galling may be a response to the sporosorus multiplication within infected host root tissues (Kageyama and Asano, 2009). In some alternative hosts of *S. subterranea*, root galling may not develop, but sporosori may still be produced after root hair infection occurs (Arcila et al., 2013, Balendres et al., 2016b).

There is potential for polycyclic infection when secondary zoospores reinfect host roots, so more zoospores are discharged into the soil and the amount of inoculum increases (Kole and Gielink, 1963, Harrison et al., 1997). Additionally, primary and secondary zoospores are capable of infecting tubers and normal cortical cells, so multiple host tissues are frequently infected concurrently (Diriwachter and Parbery, 1991). Tuber powdery scab is caused by zoospore infection of proliferating lenticels, but unlike roots, tubers are most susceptible to infection during the first 2 to 3 weeks following their initiation (Hughes, 1980, Diriwachter and Parbery, 1991, Harrison et al., 1997, van de Graaf et al., 2007, Balendres et al., 2016b).

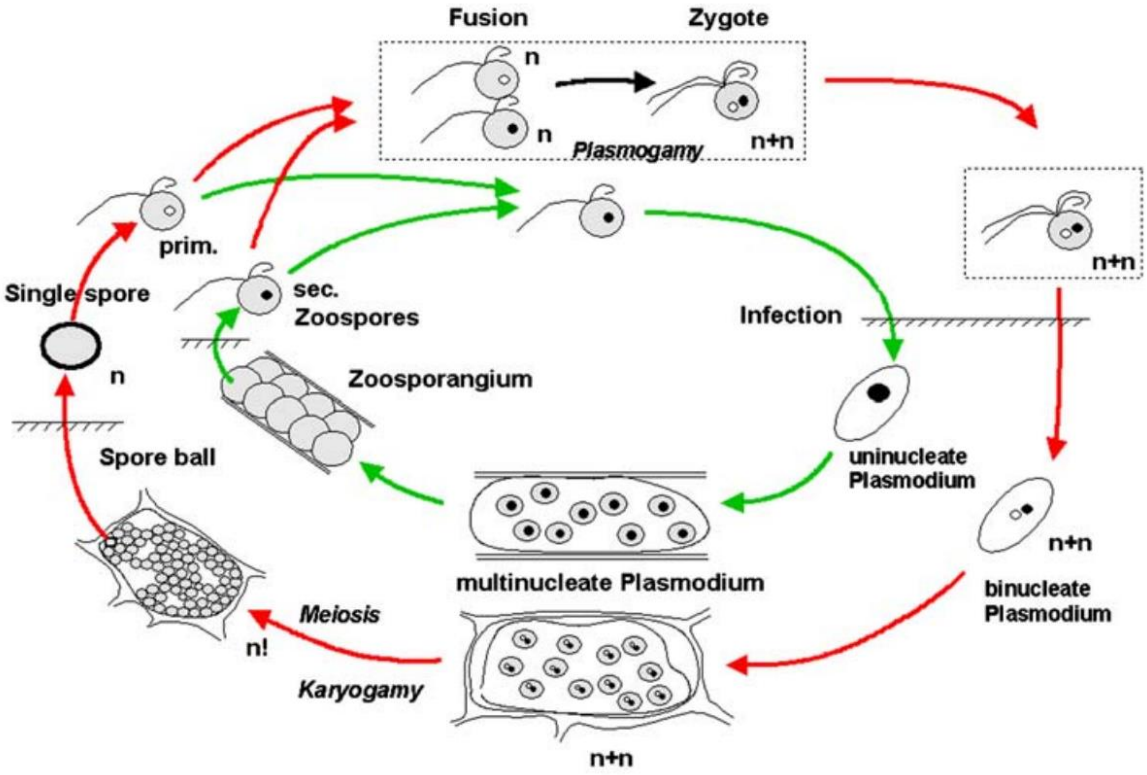


Figure 1. Lifecycle of *Spongospora* species with an asexual stage (inner circle) and a sexual stage (outer circle). Reproduced from Merz (2008).

2.4. Host range and infection

Previous studies have indicated that numerous members of several plant families are hosts for *S. subterranea* at the zoosporangium stage (Harrison et al., 1997). The known host range of *S. subterranea* includes plants in the following families: *Aizoaceae*, *Apiaceae*, *Asteraceae*, *Amarylidaceae, Brassicaceae*, *Cappardaceae, Caryophyllaceae*, *Chenopodiaceae*, *Coniferae, Convolvulaceae, Fabaceae*, *Lamiaceae*, *Oxalidaceae, Papaveraceae*, *Plantaginaceae*, *Poaceae*, *Polygonaceae*, *Ranunculaceae*, *Resedaceae*, *Solanaceae*, and *Urticaceae* (Würzer, 1964, Karling, 1968, Jones and Harrison, 1969, Jones and Harrison, 1972, Foxe, 1980, Mäkäräinen et al., 1994, Andersen et al., 2002, Qu and Christ, 2006, Simango et al., 2020), but sporosori (spore balls) only occur in *Solanaceae* host (Merz and Falloon, 2009).

As soon as zoospores reach the surface of host plant, infections can be established successfully (Balendres et al., 2016b). Successful zoospore infection in roots forms into zoosporangium and root galling, in which new sporosori are formed and discharged into the soil after the root decays (Balendres et al., 2018b). Pethybridge (1912) was the first to describe the root infection

process that led to root galling, speculating that root galling could have major effects on root function and plant health. In USA, field studies showed decreased potato yields (reduced by 5 to 12 metric tons/ha) of russet varieties resulting from *S. subterranea* root infection (Nitzan et al., 2008). Similarly, a pot trial conducted by Shah et al. (2012) and a greenhouse trial conducted by Falloon et al. (2016) found that root function (water and nutrient uptake), and plant growth and productivity were reduced, in the potato cultivar 'Iwa' infected by *S. subterranea*.

Tuber infection leads to powdery scab with sporosori forming within pustule lesions which will spread inoculum between soils and host plants (Tegg et al., 2015, Tegg et al., 2016, Balendres et al., 2016b). In contrast to roots, tubers are susceptible to infection only during a limited period at the time of tuber initiation, when they are actively elongating (Hughes, 1980, van de Graaf et al., 2007).

2.5. Zoospores and resting spores

Spongospora subterranea belongs to the plasmodiophorids, which is a group that release zoospores and form sporosori, and whose life cycles have primary (sporangium) and secondary (sporogenic) phases (Falloon, 2008, Merz, 2008, Schwelm et al., 2015, Simango et al., 2020). The zoospore stages are important in the pathogen life cycles because zoospores initiate infections in the field, spread viruses, and are the primary objectives of direct disease control methods (Merz, 1997). An individual *S. subterranea* zoospore has diameter of 5 µm with a spherical or oval shape (Kole, 1954). The zoospores are heterocont and biflagellate, each with a long flagellum (35 µm) and a short flagellum (14 µm) (Ledingham, 1935, Kole, 1954, Balendres et al., 2016b). Freshly released secondary zoospores were observed to have distinct movements, where each zoospore went from swimming in a straight line to circular motion in a very short time, then back to straight-line movement in a different direction (Merz, 1997). Primary zoospore movement was described as slow and steady (Diriwachter et al., 1979, Merz, 1992). Primary and secondary zoospores are identified by their size and distinguishable swimming pattern of circular movements (Merz, 1992).

Resting spores are produced in sporosori (Figure 2), which are aggregations of resting spores in most plasmodiophorids (Simango et al., 2020). The sporosori vary by plasmodiophorid genus, which is a key characteristic for distinguishing the *Plasmodiophoraceae* genera (Karling,

1968, Braselton, 2001). *Spongospora* has the most intricate sporosori of all known plasmodiophorids, with many resting spores in each sponge-like sporosorus (Figure 2), and sporosori have variable dimensions (Falloon et al., 2011). The thick walls of resting spores ensure long survival of the pathogen, allowing it to persist in soil for many years (Falloon et al., 2011). Primary zoospores are released from resting spores within sporosori, causing the pathogen spread to potato roots, stolons, and tubers (Merz, 1997). Sporosori on seed tubers are the most likely source of the pathogen's transmission over the majority of the world's potato growing regions, and the powdery scab is economically important in the majority of countries with intensive potato production (Harrison et al., 1997, Merz, 2008, Balendres et al., 2016b, Falloon et al., 2016).

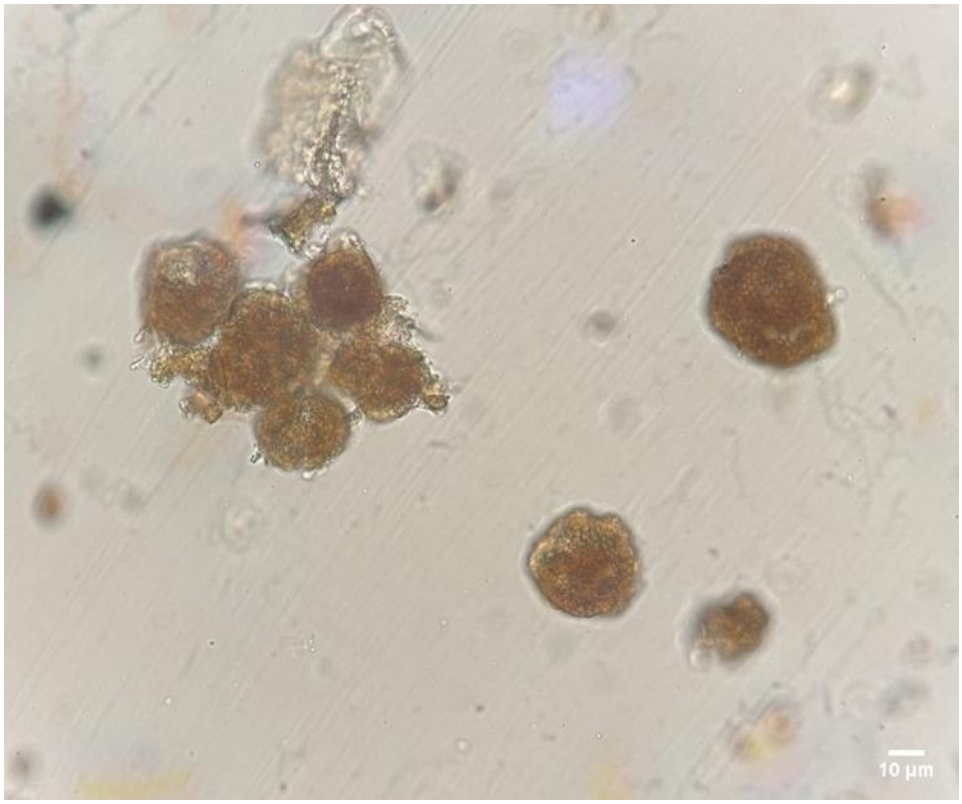


Figure 2. Sporosori of *Spongospora subterranea*.

2.6. Influence of temperature on zoospore release and disease development

Zoospore release from sporosori is the first step to developing disease in host plants (Melhus et al., 1916, Christ and Weidner, 1988, Merz et al., 2012, Balendres et al., 2016b, Balendres et

al., 2018a). Zoospore release and attachment to host plants are both influenced by soil environmental variables (Balendres et al., 2016b). Sufficient soil water, suitable soil temperature, and external host chemical stimuli all aid the germination of primary zoospores from resting spores (Kole, 1954, Merz, 1989, Harrison et al., 1997, Sparrow and Wilson, 2012, Balendres et al., 2016b). Low temperatures of 9 to 17°C promote zoospore germination in aqueous (Fornier, 1997) and soil conditions (van de Graaf et al., 2005, Shah et al., 2012). Zoospores are active at temperatures lower than 12°C (Harrison et al., 1997), and they are able to migrate at 0°C (unpublished paper), suggesting that zoospores may infect host plants at temperatures lower than 11°C. The observation of severe infection at 9°C corroborates this observation, and explains why tuber infection might be a problem in places with low average temperatures (van de Graaf et al., 2005).

At cooler temperatures, *S. subterranea* tuber powdery scab occurs more frequently. The optimal conditions for *S. subterranea* infection are believed to be saturated soils between 12 to 15°C (Melhus et al., 1916, Ramsey, 1918, Mol and Ormel, 1946, Hughes, 1980, Christ and Weidner, 1988, Carnegie et al., 2010). Severity of powdery scab has also been reported to increase with ascending altitude, mainly due to cool temperatures (Harrison et al., 1997), and plants developed more severe tuber powdery scab in cool and wet soils (Kirkham, 1986, Carnegie et al., 2010). van de Graaf et al. (2005) reported that tuber infection occurred at soil temperatures ranging from 9°C to 17°C, with the optimum temperature for disease development being 12°C.

After potato seedlings were planted with *Spongospora* inoculum for two weeks at 18°C, severe infection was observed in host root hairs (Ledingham, 1935). Similarly, zoosporangia were found by Würzer (1964) to develop in root hairs after nine days at 15°C. Kole (1954) reported that the most severe root hair infection occurred at 16 to 17°C while root hair infection can occur between 11 to 25°C.

These studies have shown that the temperature for zoospore release ranged from 9 to 25°C, but the optimum temperature for zoospore movement and tuber infection development are 12 to 13°C, whereas higher temperatures above 17°C encourage root infection (Kole, 1954, Würzer, 1964, Harrison et al., 1997, van de Graaf et al., 2005, Merz, 2008). The effects of temperature on zoospore release, tuber infection, and root infection by *S. subterranea* have been the subject of many studies, and the knowledge gained could be used to avoid cultivating potato plants

during times of the year when conditions are likely to encourage the development of powdery scab (Merz, 2008).

2.7. Pre-infection interactions

There are three major phases in the life cycle of plasmodiophorid species: survival as resting spores in soils, root hair infection, and cortical cell infection (Kageyama and Asano, 2009). Successful zoospore attachment to host cells requires some level of host susceptibility combined with favourable soil environmental conditions (Merz et al., 2012). Motile zoospores released from resting spores attached to host tissues to initiate pathogenesis. Once a zoospore attached to root epidermis cells, it releases the contents into the cells using 'Rohr' and 'Stachel' subcellular structures (Keskin and Fuchs, 1969, Williams, 1970, Kageyama and Asano, 2009, Balendres et al., 2016b). Primary plasmodia develop after successful zoospore penetration into the host plant. The plasmodia undergo nuclear divisions before cleaving into zoosporangia. Clusters of zoosporangia form in the infected cells. Subsequently, secondary zoospores develop within zoosporangia. Empty zoosporangia that have released zoospores remain in host root cells (Kageyama and Asano, 2009).

Plant pathogens can be removed from host surfaces by water, so adhesion (attachment) is critical to prevent dispersal from the infection courts (Epstein and Nicholson, 1997). In the past two decades, root infection by *S. subterranea* has received more attention (Falloon et al., 2003, Nitzan et al., 2008, Nitzan et al., 2010, Falloon et al., 2016), but less is known about the initial mechanisms involved with zoospore root attachment. Recognition and attachment to plant hosts requires particular interaction between pathogen and the host cell walls (Balendres et al., 2016b). A study of the root-infecting pathogen *Phytophthora cinnamomi* (Hardham, 1992) revealed that active zoospores attached to host roots was facilitated by chemical processes (Epstein and Nicholson, 1997). Similar findings for maize roots showed that zoospore root attachment was associated with root mucilage compounds (Hinch and Clarke, 1980).

In the past few decades, there have been many improvements in plant genetic and molecular technologies, which have provided increased understanding of plant/pathogen interactions. A variety of 'omics' tools can be used to monitor the different types of biological molecules in high-throughput ways, allowing comparisons between different biological states at genomic levels (Francisco et al., 2016). However, only a few studies have examined host-pathogen

interactions for *S. subterranea* at the molecular level (Table 2). For example, genes related to cell wall integrity, signal transduction, and stress, such as pectin methylesterase inhibitor, phosphatase 2C, and methalothionein were upregulated in a susceptible cultivar of *Solanum phureja* (Rodríguez Fuerte et al., 2014). In contrast, only two genes (a putative gene and galactosidase) were transcribed more than 1000 times in a resistant cultivar compared to the uninfected control, indicating a substantial constitutive defence response (Rodríguez Fuerte et al., 2014). In a proteomics investigation of pathogen-host interactions, Balotf et al. (2021a) examined the *S. subterranea* proteome during invasion of roots of a susceptible and a resistant potato host. Additionally, identification of differentially expressed genes (DEGs) involved in potato tuber defence against *S. subterranea* has been shown to be critical for elucidating molecular resistance mechanisms (Lekota et al., 2019).

Table 2. Omics studies on *Spongospora subterranea*.

Omics technique	Findings	References
Genomics *Spongospora subterranea* sporosori samples were used for DNA sequencing via 454 pyrosequencing technology	24 contigs associated with glycolysis, polysaccharide hydrolysis, chitin metabolism and sugar transport were detected	(Gutiérrez Sánchez et al., 2014)
Genomics A single tuber isolate (SSUBK13) of *S. subterranea* was used to produce DNA for Illumina sequencing	28.8 Mb draft genome with 2340 contigs and an L50 of 280 was generated	(Ciaghi et al., 2018)
Transcriptomics The transcriptome of *S. subterranea* was studied 4 weeks after root infection a susceptible and a resistant potato cultivar	Totally, 7560 transcripts were discovered in which 1377 transcripts were differentially expressed between two cultivars	(Balotf et al., 2021a)
Transcriptomics RNA-sequencing was used to differentiate the responses of two potato cultivars *S. subterranea* infection	Six defence-related genes, *StWRKY6, StTOSB, StSN2, StLOX, StUDP* and *StSN1*, were identified that may be used in the future molecular marker development	(Lekota et al., 2019)
Transcriptomics 454 transcriptomes were analysed for resistant and susceptible potato cultivars in response to *S. subterranea*	Methalothionein, phosphatase 2C and a pectin methylesterase inhibitor were most overexpressed in susceptible cultivars	(Rodríguez Fuerte et al., 2014)
Proteomics Shotgun proteomics was used to investigate the germination of *S.*	20 proteins were identified to be differentially abundant proteins during the germination	(Balotf et al., 2021b)

subterranea resting spores		
Proteomics *S. subterranea* proteome was examined during the invasion of roots of a susceptible and resistant potato host	117 proteins were identified, with 42 proteins showing significant differences between resistant and susceptible cultivars	(Balotf et al., 2021a)
Metabolomics Potato root exudates were analysed in the stimulation of *S. subterranea* resting spore germination	24 low molecular weight organic compounds and amino acid, sugar, organic acid, and other compounds were identified related to the stimulation of the resting spore germination	(Balendres et al., 2016a)
Metabolomics Potato roots and root exudates of resistant and susceptible potato cultivars inoculated with *S. subterranea* were used in identification of resistance-associated metabolites	In resistant cultivars, the number of amino acids, organic acids, fatty acids, phenolics, sugars, cell wall thickening chemicals, and phenolic-related root exudate increased	(Lekota et al., 2020)

2.8. Characteristics of zoospore-host attachment

Although attachment of zoospores to plant hosts (Figure 3) has been known for over a century, knowledge remains lacking of the adhesive compounds that regulate zoospore host attachment, and there is little data available illustrating the molecular basis of zoospore attachment to plant roots (Nicholson and Epstein, 1991, Epstein and Nicholson, 1997).

Figure 3. Attachment of *Spongospora subterranea* zoospores (orange circles) to potato root hairs.

Zoospore of some soilborne pathogens, such as those of *P. cinnamomi*, have the ability to travel long distances in running water (Zentmyer, 1980). When zoospores are in close proximity to a potential host, they can swim up to the plant roots by following gradients of root exudate components. The zoospores are frequently seen in the zones of elongation behind host root tips (Hickman, 1970). By reducing their moving speed and increasing the frequency of their turns, the zoospores are able to stay near the root surfaces, where they can proceed to attach (Carlile, 1983). Motility declines gradually during attachment, when flagella fall off or are withdrawn, and rapid exocytosis of the contents of host cortical vesicles covers the host cells with adhesive substances that firmly attach the zoospores to the root surfaces (Sing and Bartnickigarcia, 1975).

Extracellular substances have been observed in zoospore host attachment using light or fluorescence microscopy (Hamer et al., 1988). Other reports, however, indicate the presence of a very thin film of extracellular compounds detectable only via electron microscopy (Mims

and Richardson, 1989). Epstein and Nicholson (1997) suggest a link between the thickness of the adhesive layer and strength of the attachment.

Information on the chemistry of extracellular matrices of zoospores that trigger zoospore root attachment is limited. However, previous studies suggest that these matrices are biochemically complex and are detachable (Ramadoss et al., 1985, Moloshok et al., 1993). They are placed between the zoospore and the host and stimulate zoospore attachment. The majority of pathogen adhesive compounds are glycoproteins (McCourtie and Douglas, 1985, Bartnicki-Garcia and Sing, 1987, Chaubal et al., 1991, Tunlid et al., 1991, Kwon and Epstein, 1993, Xiao et al., 1994, Epstein and Nicholson, 1997). The presence of lectins implies that pathogen adhesives are frequently glycosylated (Hamer et al., 1988, Durso et al., 1993, Mercure et al., 1994). In attachment of the black yeast *Aureobasidium pullulans*, polysaccharides play a key role (Andrews et al., 1994).

Pathogen attachment can occur non-specifically to a wide range of matrices, or zoospores can be attracted to particular substances, such as receptors or lectin-hapten interactions. For example, lectin is required for mycorrhizal spore attachment to host roots (Bonfantefasolo and Spanu, 1992). Hinch and Clarke (1980) demonstrated that mucilage was detected on the surface of carbohydrate rich, hydrophilic roots, and that Oomycota attachment to fucosyl residues on root surface occurs via lectin-like interactions. Attachment of *Phialophora radiciola* and *Fusarium moniliforme* to maize root mucilage may be dependent on ionic interactions rather than lectin-hapten interactions (Northcote and Gould, 1988). Additionally, Estradagarcia et al. (1990) showed that mucilage polysaccharides on the surfaces of cress roots triggered zoospore root attachment. Similarly, mucilage polysaccharides on roots surface have been shown to have two functions in zoospore recognition, both of which promote pathogenesis: mucilage contains fucose-rich ligands that zoospores recognise during their attachment to root surfaces, and pectin-rich moieties possibly trigger zoospore root attachment (Callow et al., 1987).

Previous studies suggest that establishment of physical connections between pathogens and hosts is necessary for spore attachment. For fungi such as rusts and those causing anthracnose to successfully discharge their contents into host cells and cause disease, they must enter their hosts using specific structures such as appressoria (Epstein and Nicholson, 1997). Physical stimulation on the host surface can promote development towards a specific region and generate infection of cells (Hoch et al., 1987). If the pathogen propagules have been firmly

attached to the host surfaces, topographical stimuli on host surface will cause the pathogen to grow in a specific direction or change shape (Epstein et al., 1986).

Timing of propagule attachment is another important factor leading to successful infection. If attachment occurs too early or too late, the zoospores will not be positioned optimally at the host surfaces for further infection. Zoospores may detach from roots before penetration or before their flagella have reached host surfaces (Hardham and Suzaki, 1986). In some pathogen zoospores, adhesiveness is only present for short periods. For example, attachment of *P. cinnamomi* and *P. palmivora* is adhesion-competent within 4 min (Bartnicki-Garcia and Sing, 1987, Gubler and Hardham, 1988). In comparison, adhesion-competent spores of *Nectria haematococca* and *Colletotrichum musae* are able to bind at any time during the periods preceding germ tube emergence (Jones and Epstein, 1989, Selabuurlage et al., 1991).

2.9. Susceptibility of potato cultivars to *Spongsopora* tuber and root diseases

Resistance to powdery scab has been assessed on a broad scale (Genet et al., 1995). Although disease-resistant cultivars are now widely available, genetic resistance to powdery scab is still only a minor aspect, in disease control, because cultivars are typically selected for characteristics other than resistance to powdery scab (Harrison et al., 1997). Although host resistance has a significant impact on powdery scab establishment, the majority of commercially marketed cultivars are susceptible to some degrees (de Boer, 1991, Merz et al., 2012).

Potato resistance to *Spongospora* tuber and root infections has been reported in several studies (Table 3). The severity of *Spongospora* root infection (zoosporangia and root galling) varies in different potato cultivars. In a glasshouse trial with potato plants infected with *S. subterranea*, severity of zoosporangia and root galling were determined for 15 selected cultivars, which represented the range of field-assessed resistance to powdery scab (Falloon et al., 2003). Tuber powdery scab was related to root infection (zoosporangia and root galling). Cultivars that were resistant to tuber infection in the field had low numbers of root zoosporangia and root galls, whereas cultivars that were very susceptible to tuber infection in the field had high numbers of root infections (zoosporangia and root galling).

For some cultivars, there is evidence that host resistance to powdery scab is not directly correlated with root infection resistance (Falloon et al., 2016). For example, Nitzan et al. (2008) showed differences in resistance to root galling and tuber powdery scab for the cultivars 'Russet Burbank' and 'Ranger Russet'. Similarly, relative resistance varies between glasshouse and field trials (Houser and Davidson, 2010). For example, the cultivar 'Swift' in the study of Falloon et al. (2003) showed low levels of tuber infection in the field but high levels of root infection in glasshouse screening.

Table 3. Examples of field, glasshouse, and in vitro assessments of potato cultivar susceptibility to *Spongospora subterranea* diseases.

Cultivars	Assessment	Disease	References
10 commercial potato cultivars	Field trials Greenhouse trials	Powdery scab	(de Boer, 1991)
467 potato cultivars and clones	Field trials	Powdery scab	(Torres et al., 1995)
99 potato cultivars 13 breeding lines	Field trials	Powdery scab	(Falloon et al., 2003)
132 potato cultivars 18 germplasm lines	Field trials	Powdery scab	(Genet et al., 2007)
10 potato genotypes	Greenhouse trials	Powdery scab	(Perla et al., 2014)
1031 breeding lines	Field trials	Powdery scab	(Paget et al., 2014)
57 potato cultivars and clones	Field trials	Root galling	(Nitzan et al., 2008)
24 potato genotypes	Field trials	Root galling	(Nitzan et al., 2010)
8 potato cultivars	Greenhouse trials	Root galling	(Falloon et al., 2016)
11 potato cultivars	In vitro bioassay	Zoosporangia	(Merz et al., 2004)
15 potato cultivars	Greenhouse trials	Root galling Zoosporangia	(Falloon et al., 2003)
10 potato cultivars	Field trials	Powdery scab Root galling	(Merz et al., 2012)
30 potato cultivars 83 clones	Field trials	Powdery scab Root galling	(Bittara et al., 2016)
8 potato cultivars	Field trials	Root galling Zoosporangia	(Falloon et al., 2016)

2.10. Weaknesses of *Spongsopora* tuber and root disease assessments

Previous assessments of cultivar resistance to *S. subterranea* tuber and root infection have relied on field or greenhouse experiments. However, extraneous variables, including soil type, water content, temperature, and previous crop are often difficult to control in field trials and have been shown to affect disease ratings. Soil conditions, particularly water content and temperatures, are key factors for zoospore release from resting spores and migration to the hosts (Balendres et al., 2016b). Severity of powdery scab has been evaluated in a variety of soil types, with results demonstrating that infection levels are higher in sandy soils than in clay soils (Van de Haar, 2000, Tuncer, 2002, van de Graaf et al., 2007, Brierley and Less, 2008). Although resting spore germination and severe disease can occur over a range of soil pHs (5 to 8), (Merz, 1989, Falloon et al., 2005, Brierley and Less, 2008), other soil elements, such as sulphur and boron, have been found to lower powdery scab and root infection severity (Brierley and Less, 2008, Falloon, 2008). Wright et al. (2021) found that soil type, texture, pH, organic matter, and nutrient were related to tuber powdery scab severity, while the impacts of previous crop rotations varied according to the soil.

Resistance to powdery scab in potato cultivars is normally evaluated in field or glasshouse tests with soils infested with *S. subterranea* inoculum. However, these experiments are expensive, labour intensive, and time demanding, and have been significantly impacted by environmental circumstances (Merz et al., 2004). Merz et al. (2004) developed a laboratory bioassay that used the degree of zoosporangium infection as an indication of resistance for quick screening of potato cultivars during the early stages of potato variety selection, avoiding the disadvantages of traditional field and glasshouse trials. The screening period of the bioassay was 17 days, which normally takes a few months in field and glasshouse experiments. While several cultivars showed significant differences in resistance to zoosporangium infection, no correlation was found between the severity of root galling in the field trials and zoosporangium infection in this bioassay. The bioassay offers the ability to screen and select for resistant cultivars at the early stages of breeding, obviating the need for costly field trials (Merz et al., 2004). However, variation in the number of infection cycles over the incubation period can result in a major difference in observed root infection levels and lead to an inaccurate susceptibility ratings (Thangavel et al., 2015).

2.11. Conclusions and future directions

The soilborne biotrophic parasite *S. subterranea* requires a living host and cannot be cultured in vitro. This pathogen causes potato tuber and root infections by producing large numbers of sporosori that can survive in the soil for extended periods before resting spore germination and travelling to their plant hosts. So far, there is no fully effective approach to managing *Spongospora* diseases and no cultivar has been identified as completely immune to *S. subterranea*. There are varying degrees of resistance among potato cultivars to *S. subterranea*, but no potato cultivar has yet developed immunity (complete resistance) to this pathogen (Falloon et al., 2003, Merz, 2008, Balendres et al., 2016b). However, cultivar resistance is currently the most effective and sustainable management strategy for *Spongospora* disease control.

Spongospora subterranea disease-resistant cultivars can restrict plant growth disturbance, inhibit pathogen multiplication and dissemination, and increase the crop yield of healthy seed, fresh market, or processed potato industries (Falloon et al., 2016). Cultivar resistance to tuber and root infections have been carried out in glasshouse and field trials, which are time consuming and very expensive, and face environmental variables. Secondary zoospores released from zoosporangia re-infect potato root systems and freshly developing tubers in a polycyclic way. This renders resistance screening of the root infection unreliable, particularly for zoosporangium infection (Thangavel et al., 2015).

Zoospore root attachment is a critical stage of root infection which needs to be evaluated as an indicator for cultivar resistance to *S. subterranea* (Yu et al., Chapter 3). Additionally, the relationship between zoospore root attachment and *Spongospora* diseases needs to be validated if this is to provide an effective and rapid approach for cultivar resistance screening (Yu et al., Chapter 3, Yu et al., Chapter 4).

An understanding of the molecular and biochemical basis of zoospore root attachment is still lacking, but one of the objectives in the present study has been to elucidate effects of host root proteins on *S. subterranea* zoospores (Yu et al., Chapter 5, Yu et al., Chapter 6). Multiple investigations in different plant pathosystems have demonstrated that chemical components on host root surfaces that interact with pathogens can be employed as an alternative to pesticides for disease management. However, little is known about the chemicals on the surface of potato roots and those of other hosts during *S. subterranea* zoospore root attachment. Consequently, it has been necessary to initially characterise the root proteins (Yu et al., Chapter 5), and the

root surface chemical properties that are biologically active for *S. subterranea* zoospore root attachment (Yu et al., Chapter 6).

Effective *Spongospora* disease management requires comprehensive understanding of assessing resistance. As there is currently no rapid and effective screening system for cultivar resistance in the *S. subterranea* pathosystem, assessing cultivar resistance is challenging. Understanding zoospore root attachment offers a potential solution for rapid evaluation of potato cultivars and breeding lines for resistance to diseases caused by *S. subterranea*. When a repeatable and accurate method for resistance screening is developed, potato growers and breeders may find varietal assessment for root infection valuable for selecting plant material appropriate to particular disease risks.

Chapter 3. Development and validation of a novel rapid in vitro assay for determining resistance of potato cultivars to *Spongospora subterranea* zoospore root attachment

3.1. Abstract

Spongospora subterranea f. sp. *subterranea* is a major pathogen leading to losses in tuber quality and yield. Disease can be expressed as root infection, root galling and tuber lesions, the later known as powdery scab. Attachment of zoospores to potato root hairs is the first step before infection of roots and disease development. Root hair infection results in root disfunction leading to impaired plant productivity and yield. Varieties vary in their susceptibility to root and tuber disease; however, varietal screening is both time and resource intensive. Furthermore, traditional screens assess root galling or tuber disease and not root infection. In this study, optimal conditions for zoospore release and attachment of zoospores to plant roots were determined and, used this information to develop an in vitro bioassay to assess resistance to zoospore root attachment amongst 153 potato lines and cultivars. Optimal zoospore release occurred at 20℃ in Hoagland's solution in a rapid and synchronized manner over the first two days, followed by a steep decline. The extent of zoospore root attachment varied with cultivar (Iwa > Agria > RBK > Gladiator), region of the root maturation zone (lower > middle > upper), and temperature (greatest zoospore root attachment occurring at 15℃). Further comparisons suggested efficiency of zoospore root attachment was also generally associated with known variety resistance to powdery scab, zoosporangium infection, and root galling, with a few notable exceptions. The bioassay has been proved to be a rapid and robust method for screening cultivar resistance to zoospore root attachment.

Keywords: host resistance, in vitro assay, *Spongospora subterranea* f. sp. *subterranea*, zoospore root attachment, zoospore germination

3.2. Introduction

The soilborne pathogen *Spongospora subterranea* f. sp. *subterranea* infects potato roots and tubers, leading to root dysfunction and disease expression as zoosporangium root infection, root galling and powdery scab tuber lesions (Balendres et al., 2016b, Falloon et al., 2016). These commonly occurring root and tuber diseases are of great economic importance across most major potato production areas worldwide (Merz and Falloon, 2009). Root infections can result in diminished root function with subsequent yield losses (Falloon et al., 1996, Falloon et al., 2003, Nielsen and Larsen, 2004a, Nitzan et al., 2008, Falloon et al., 2016, Nielsen and Larsen, 2004b); in addition, infected tubers adversely affect seed and fresh tuber market quality and value, and can reduce the durability of cool stored potatoes prior to processing (Harrison et al., 1997, Merz and Falloon, 2009, Balendres et al., 2016b, Falloon et al., 2016). In Australia, for example, a conservative estimate of the economic loss to the Australian potato processing industry due to powdery scab is $13.4 million annually (Wilson, 2014a, Balendres et al., 2016b, Wilson, 2016).

The disease cycle of *S. subterranea* has been studied extensively. *Spongospora subterranea* persists in the soil as dormant resting spores in sporosori (Harrison et al., 1997) that can release motile zoospores under conducive environmental conditions stimulated by the presence of host plant root exudates (Balendres et al., 2016b). Zoospores are attracted by chemotaxis to host roots (Amponsah et al., 2021), to which they attach (encyst) and penetrate through the cell walls, and insert their cellular contents to facilitate infection (Merz, 1997, Harrison et al., 1997). A multinucleate plasmodium then forms which segments into uninucleate zoosporangia (Kole 1954). Secondary zoospores form within zoosporangia and are subsequently released into the soil where they can reinfect the roots or developing tubers in a polycyclic manner (Lahert and Kavanagh, 1985a, Clay and Walsh, 1990, Nitzan et al., 2008, Balendres et al., 2016b). Root infection generally leads to formation of root galls that are filled with sporosori; on root decay, these are released into the soil environment, adding to the soil inoculum load (Harrison et al., 1997, Nitzan et al., 2008, Balendres et al., 2016b). Tuber disease results from zoospore infection of young developing tubers (Hughes 1980; Van De Graaf et al. 2007).

Strategies to manage *S. subterranea*-induced diseases are very limited. Depending on market demands, growers may be able to select cultivars with resistance against root and tuber disease caused by *S. subterranea* infection (de Boer, 1991, Torres et al., 1995, Falloon et al., 2003, Hernandez Maldonado et al., 2013, Falloon et al., 2016); however, no cultivar has complete

immunity to infection, and significant disease may still result in moderately resistant cultivars (Merz et al., 2012). Reliable identification of host resistance amongst commercial cultivars is a critical step forward for management of disease caused by *S. subterranea* infection (Falloon et al., 2003, Nitzan et al., 2008, Falloon et al., 2016).

Traditionally, identification of host resistance has been based on assessment of tuber powdery scab and/or root galling in large, replicated field or glasshouse challenge trials (de Boer, 1991, Torres et al., 1995, Falloon et al., 2003, Nitzan et al., 2008, Falloon et al., 2016). These types of assessments are time (4 to 6 months) and resource intensive, coupled with the confounding impacts of variable environmental conditions and, in field trials, erratic distribution of soil inoculum (Falloon et al., 2003, Nitzan et al., 2008, Hernandez Maldonado et al., 2013). These glasshouse and field assays also fail to provide direct information on the relative host resistance to root hair infection, which now has been known to be critical for impact on plant productivity (Shah et al., 2012, Falloon et al., 2016). Previously, Merz et al. (2004) developed a laboratory bioassay that did examine resistance to root infection by observation of the relative abundance of zoosporangia within root hairs from tissue-cultured plantlets incubated with sporosori inoculum. While also much quicker than glasshouse and field assays, this method still required several weeks for observable infection to occur. Further, when assessing abundance of zoosporangia in roots, care must also be taken to ensure the roots of all test plants first come into contact with inoculum at the same time, as variation in the number of infection cycles over the incubation period can result in a difference in observed root infection levels and could lead to an inaccurate rating (Thangavel et al., 2015).

The need for an efficient in vitro assay for host resistance to root attachment/infection by zoospores is further emphasized by the variation in ratings of host resistance of some potato cultivars depending on the stage of disease being assessed. For example, cultivar ‘Swift’ produced low levels of tuber powdery scab in the field but high levels of root galling in the glasshouse, suggesting resistance to tuber and root diseases may not necessarily be related (Falloon et al., 2003). Similarly, cv. ‘Russet Burbank’ shows good resistance to tuber disease but has moderate susceptibility to root infection and galling (Boyd, 1951, van de Graaf et al., 2007, Falloon et al., 2016). It is also known that there are different temperature optima for expression of root infection (11 to 25°C) (Kole, 1954, van de Graaf et al., 2005, van de Graaf et al., 2007, Balendres et al., 2016b) and tuber disease (9 to 17°C) (Hughes, 1980, van de Graaf et al., 2005, van de Graaf et al., 2007, Shah et al., 2012, Balendres et al., 2016b). In addition, where soil temperatures may be warmer, substantial root infection can occur in the general

absence of tuber disease, further confounding assessments (Falloon et al., 2003, Falloon et al., 2016, Balendres et al., 2016b).

There is increasing concern on the impact of the early phases of root infection on plant productivity and yields for which demonstrated cultivar resistance data is largely lacking. In the past two decades, root infection has received more attention (Falloon et al., 2003, Nitzan et al., 2008, Nitzan et al., 2010, Falloon et al., 2016), but less is known about the initial mechanisms involved in zoospore root attachment. A potential alternative rapid in vitro assessment method for assessing cultivar resistance to root disease is to observe relative propensity of initial zoospore root attachment at the very start of the infection process, so avoiding issues of polycyclic infection. The present study postulates that this would provide a more robust method for screening for host resistance, with results obtained in significantly less time than previous assessments methods. In this study, in vitro conditions were first to optimize for zoospore release and zoospore attachment to potato roots. Then a zoospore root attachment assay was developed, which was used to screen 153 potato lines and cultivars for their relative resistance to zoospore root attachment against known standards. The results of zoospore root attachment within selected cultivars were compared with known resistance to root galling and tuber disease, and zoosporangium root infection.

3.3. Materials and methods

3.3.1. Preparation of sporosori inoculum

Spongospora subterranea sporosorus inoculum were obtained from powdery scab diseased potato cultivar ‘Kennebec’ tubers, harvested from a commercial crop grown in northwest Tasmania, Australia in 2019. Diseased potato tubers were washed with tap water and left to air-dry in a cool place for 24 to 48 h. Sporosori were scraped from tuber lesions with a scalpel and then sifted through a 600 µm sieve. The inoculum was then stored in a covered container at the ambient temperature in the dark until use.

3.3.2. Potato lines and cultivars

In total, 153 potato (*Solanum tuberosum* L.) lines and cultivars were assessed in this study and compared against four cultivar that differ in their resistance to powdery scab disease: ‘Gladiator’

(regarded as very resistant to powdery scab), 'Russet Burbank' (regarded as moderately resistant), 'Agria', and 'Iwa' (both are regarded as highly susceptible) (Falloon et al., 2003, Genet et al., 2007). Tissue-cultured plantlets of each variety or line were grown in potato multiplication (PM) medium (composed of Murashige and Skoog (MS) Salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; Phytagel, 2.2 g/L; at pH 5.8) under a 16 h photoperiod using white fluorescent lamps (65 µmol/m^2/s) at 22℃. For use in experiments, 1-month-old potato plantlets were transferred from PM medium to liquid potato multiplication (LPM) medium (composed of MS salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; at pH 5.8) and grown for 2 weeks under a 16 h photoperiod using white fluorescent lamps (65 µmol/m^2/s) at 22℃.

3.3.3. Optimum temperature for in vitro zoospore release

The optimum incubation temperature for zoospore release from sporosori was determined using a modified method of Balendres *et al.* (2018a). Aliquots of 3 mg of dried *S. subterranea* sporosorus inoculum (five replicates per temperature treatment) were each added to a 1.6 mL Eppendorf tubes and suspended in 1 mL of Hoagland's solution. The Hoagland's solution (Falloon et al., 2003) composition was KNO_3, 253 mg/L; $Ca(NO_3)_2 \cdot 4H_2O$, 722 mg/L; KH_2PO_4, 2.3 mg/L; $MgSO_4 \cdot 7H_2O$, 120 mg/L; NH_4NO_3, 40 mg/L; Fe-EDTA, 20 mg/L; H_3BO_3, 140 µg/L; KCl, 400 µg/L; $MnSO_4 \cdot H_2O$, 63 µg/L; $ZnSO_4 \cdot 7H_2O$, 115 µg/L; $CuSO_4 \cdot 5H_2O$, 50 µg/L; and $Na_2MoO_4 \cdot 2H_2O$, 22 µg/L, prepared in sterile deionized distilled water (DDW). The tubes were covered with aluminium foil and incubated in darkness at 10, 15, 20, 25 or 30℃ (plant growth chamber, Steridium Pty Ltd). This temperature range was selected based on successful zoospore release in field and laboratory studies (Fornier et al., 1996, Harrison et al., 1997, van de Graaf et al., 2007). Zoospore release was assessed by observation of subsamples taken after 2, 4, 7, 13, 20, 30 and 40 d after incubation. At each assessment, the tubes were briefly mixed to ensure a homogenous solution, then 1 µL sample was pipetted directly onto a glass microscope slide, covered with a coverslip, and the numbers of *S. subterranea* zoospores were determined by counting the total zoospore number at 200× magnification (with a DM 2500 LED light microscope; Leica Microsystems, Germany). Three 1 µL samples were taken and counted at each time from each tube, and five replicates were included in this experiment. Identification of zoospores used zoospore morphology (Kole, 1954) and motility behaviour (Merz, 1997).

3.3.4. Distribution of zoospore root attachment in four potato cultivars

The density of root hairs on roots of four potato cultivar standards, 'Iwa', 'Agria', 'Russet Burbank', and 'Gladiator', was determined by light microscopy. Roots from 2-week-old tissue-cultured potato plantlets grown in LPM were washed with deionized water. For each assessment, three primary roots (8 to 9 cm long) from each plant were divided into three segments representing the upper, middle, and lower parts of the root maturation region (Figure 4). Each segment was trimmed to a length of 10 mm, thus providing a total of nine root segments for each plant. Root segments were placed on a glass slide and covered with a coverslip. The number of root hairs was quantified by randomly scanning five fields of view for each root segment under light microscopy at 400× magnification. For each root zone, data from the three primary roots were averaged to provide a replicate value. Three plants of each cultivar were assessed, giving three replicates of each zone per cultivar.

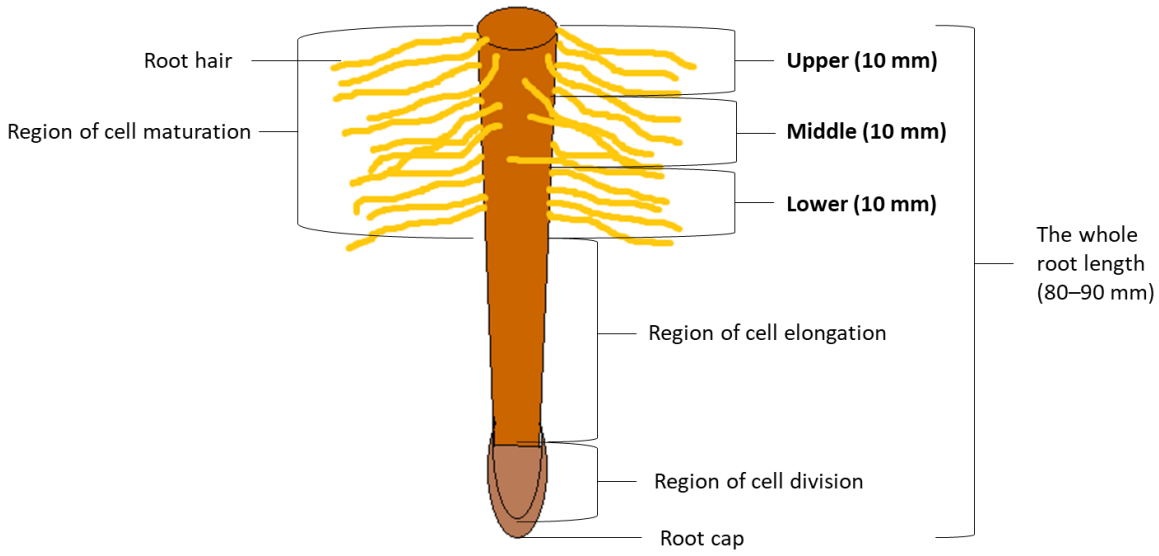


Figure 4. Schematic diagram of primary root of potato plantlets, showing the upper, middle and lower regions of the maturation zone from which root samples were taken.

A preliminary study evaluating zoospore attachment to entire primary roots revealed that zoospores attached exclusively to the root hairs within the maturation region of the root (Figure 4), and that 1000 zoospores/mL gave more reproducible data for attachment than 200 zoospores/mL. Subsequently, for each of the four cultivars, the preferred location within the root maturation region for attachment of *S. subterranea* zoospore was determined. Root

segments were cut from three regions of the maturation region (lower, middle, and upper), as described above, and placed in a plastic container. The three root segments of each individual root from each of the four cultivars, with three replicates per cultivar, were placed in a single container separated by a 100-micron mesh. Sixty milliliters of zoospore suspension (1000 zoospores/mL) were added to the treatment container, which was then incubated in the dark at 15℃ for 48 h. The number of zoospores attached to each root segment was quantified from five randomly selected fields of view under light microscopy at 400× magnification.

3.3.5. Optimum temperature and root tissue for attachment of zoospores to root hairs of four potato cultivars

Each assessment used three primary roots (8 to 9 cm long) that had numerous root hairs, excised from a single, 2-week-old in vitro propagated plantlet of cultivars ‘Iwa’, ‘Agria’, ‘Russet Burbank’, or ‘Gladiator’. Roots were washed, and a 10 mm segment was taken from the lower maturation region of each root, providing a total of three root segments from each individual plant. Washed root segments of each cultivar were equally distributed within the container, and a zoospore suspension (1000 zoospores/mL) added as described above. The containers were then incubated at 10, 15, 20, 25 or 30℃ in the dark for 48 h. After treatment, root segments were mounted on a glass slide with a coverslip and the number of attached zoospores was determined by light microscopy as described above. Data from the three roots of each plant were averaged to provide a replicate value and three plants of each cultivar were assessed, giving three replicates per cultivar.

3.3.6. In vitro screening of 153 potato lines and cultivars by zoospore root attachment assay

A total of 153 potato cultivars, breeding lines or clonal replicates were obtained from the potato germplasm collections of TIA, Agronico Pty Ltd, and Solan Pty Ltd. Three plants of each cultivar, line or clone were incubated for two weeks in LPM, as described above, and roots were harvested. A 10 mm section from the lower maturation region from each root was sampled and used for assessment of zoospore root attachment as previously described, with incubation at 15℃. The cultivars, lines and clones were tested in batches of eight with two reference cultivars (‘Iwa’ and ‘Gladiator’) included in each batch. This assessment of each cultivar or

line was performed with three independent biological replicates (three plants of each individual cultivar), each comprised of three technical replicates (three roots from each plant).

Score of zoospore root attachment for each cultivar/line in the screenings was standardized according to the reference cultivars, 'Gladiator' and 'Iwa', present in each batch. The mean scores for zoospore attachment to roots of 'Gladiator' and 'Iwa' in the first batch screened were 1.64 (G1) and 11.6 (I1), respectively, and this served as a reference score (G1 + I1) to adjust for batch differences in each subsequent batch. This was done by calculating a reference point correction coefficient (η_n) in each batch.

$$\eta_n = \frac{Gn + In}{G1 + I1}$$

Where Gn and In are the zoospore attachment scores for 'Gladiator' and 'Iwa' in batch n. This coefficient was used to linearly scale the attachment score for each cultivar/line.

3.3.7. Assessment of susceptibility of potato cultivar to zoosporangium infection

The relative susceptibility to development of zoosporangium root infection was determined in 12 cultivars ('Ida Rose', 'Nicola', 'Shepody', '10086', 'Krantz', 'Iwa', 'Tolaas', 'Toolangi delight', 'Granola', 'Gladiator', 'Russet Burbank', 'Russet Nugget') that varied in their response to the zoospore root attachment assay. In vitro propagated plantlets of the 12 cultivars (with three plantlets per cultivar) were grown for three weeks in LPM as described above. Each plantlet was suspended in an individual plastic container (30 × 130 mm) containing 10 mL Hoagland's solution with 25 mg of dried sporosorus inoculum prepared as described earlier A further three plantlets of each cultivar were suspended in a separate container filled with only 10 mL of Hoagland's solution as a uninoculated control. The plantlets were incubated in a plant growth chamber (Steridium Pty Ltd) at 15°C for 3 d in darkness. Following inoculation, each plantlet was transferred into a fresh container containing 10 mL of Hoagland's solution, arranged in a completely randomized pattern, and then grown in a plant growth chamber (Contherm Scientific Pty Ltd) under a 16 h photoperiod using white fluorescent lamps (65 µmol/m^2/s) at 20 ± 2°C. After one month, plantlets were each assessed for root zoosporangium infection as follows.

From each plant, about 0.1 g of fresh, intact washed roots were randomly selected, cut into 10 mm long sections and were stained with 0.1% trypan blue for about 15 min. Assessment of *S. subterranea* zoosporangium infection was conducted for three biological replicates under light microscopy at 200× magnification (Balendres et al., 2018b). The intensity of zoosporangium infection of each root segment was assessed using the rating scale of Merz, et al. (2004): 0 = no infection; 1 = < 10% of roots infected, sporadic; 2 = 2% - 10% of root infected, slight; 3 = 11% - 25% of root infected, moderate; 4 = 26% - 50% of root infected, heavy and 5 = > 50% of root infected, very heavy.

3.3.8. Statistical analyses

All data were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 27 following conformation of normality and homogeneity of variance. Data for zoospore release, root hair density, and zoospore root attachment to different root regions were assessed by two-way analysis of variance (ANOVA), with a Tukey's HSD test used to determine statistically significant differences between the means at the 5% level ($p = 0.05$). Zoospore root attachment and zoosporangium intensity scores were assessed by one-way ANOVA with a Tukey's HSD test used to determine statistically significant differences at the 5% level ($p = 0.05$). Multiple comparison of means was calculated with Fisher's least significant difference (LSD) analysis at a 0.05 level of probability.

3.4. Results

3.4.1. Optimum temperature for in vitro zoospore release

The mean number of zoospores released was influenced by assessment date ($p < 0.001$), incubation temperature ($p < 0.001$), and their interaction ($p < 0.001$; Figure 5). Maximum zoospore release occurred on day 2 at both 20 and 25°C, and thereafter decreased across all temperatures. No zoospores were observed after 2 d of incubation at 10 or 15°C, or at any time point at 30°C.

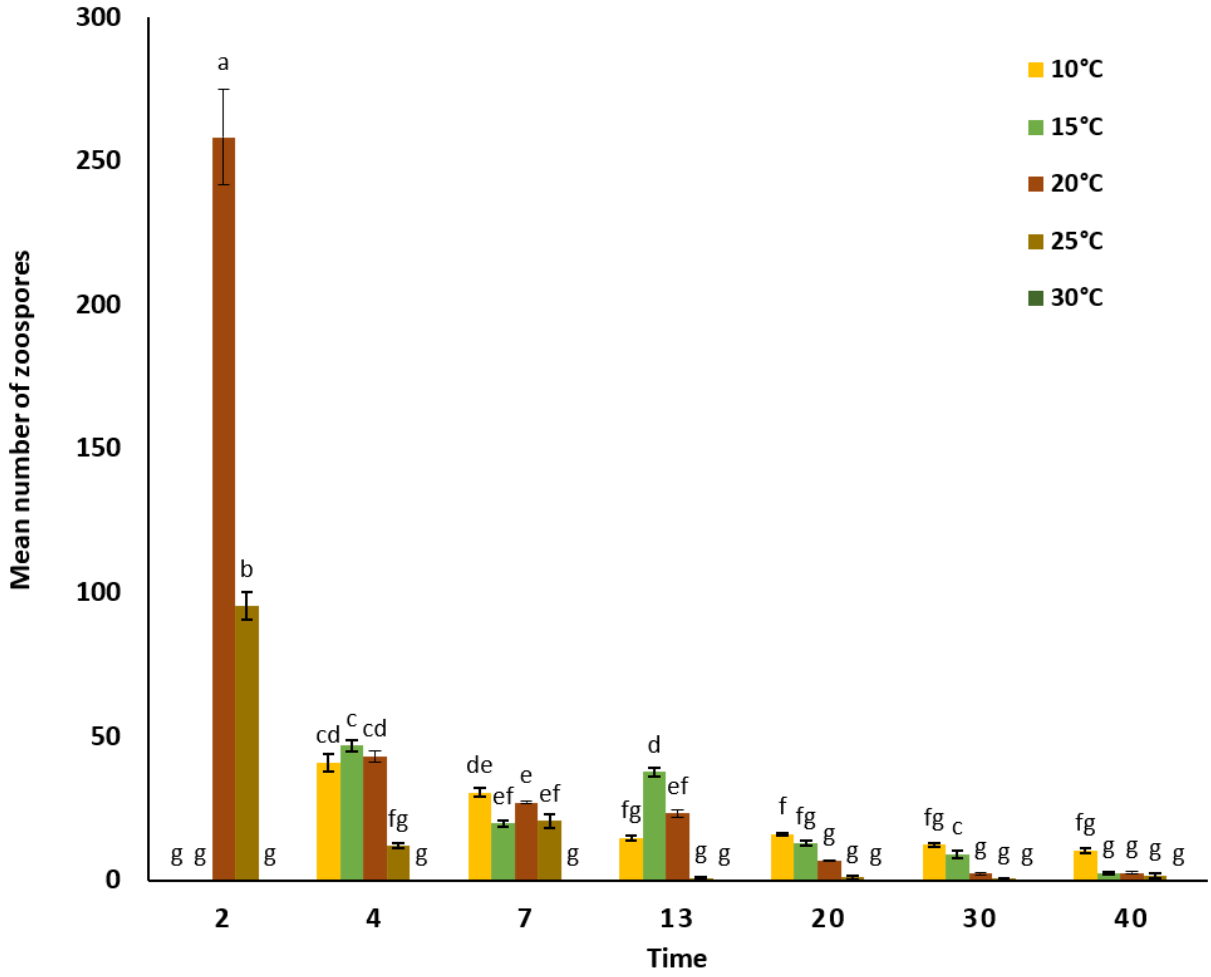


Figure 5. The impact of temperature treatment (10, 15, 20, 25 and 30°C) on release of *S. subterranea* zoospores in Hoagland's solution, assessed at 2, 4, 7, 13, 20, 30 and 40 d after incubation. Vertical bars represent standard errors ($n = 5$). p (temperature) < 0.001; p (time) < 0.001; p (time × temperature) < 0.001. Different letters above bars indicate significant temperatures × time interaction effect as determined by LSD (0.05) = 8.6.

3.4.2. Distribution of root hairs in the maturation zone of primary potato roots

Root hair density was significantly influenced by cultivar ($p < 0.05$; Table 4, Figure 6) and root region ($p < 0.05$). However, the interaction between cultivar and root region was not significant ($p = 0.751$). Mean root hair density was greater in the mid-section root region than in either the lower or upper regions. 'Russet Burbank' had the greatest mean numbers of root hairs followed by 'Iwa', 'Agria', and then 'Gladiator'.

Table 4. Mean numbers of root hairs in the upper, middle, and lower regions of the primary root maturation zones in the potato cultivars 'Iwa', 'Agria', 'Russet Burbank (RBK)', and 'Gladiator'. (400× magnification). Different letters indicate significant treatment effects ($p < 0.05$) of the cultivar and root region as determined by LSD (cultivar) = 1.26 and LSD (region) = 1.09.

	Mean number of root hairs per field of view		Mean number of root hairs per field of view
Cultivar		Region	
Iwa	50.9 b	Upper	48.2 b
Agria	49.5 c	Middle	50.5 a
RBK	52.5 a	Lower	49.5 a
Gladiator	44.6 d		
LSD ($p < 0.05$)	1.26		1.09

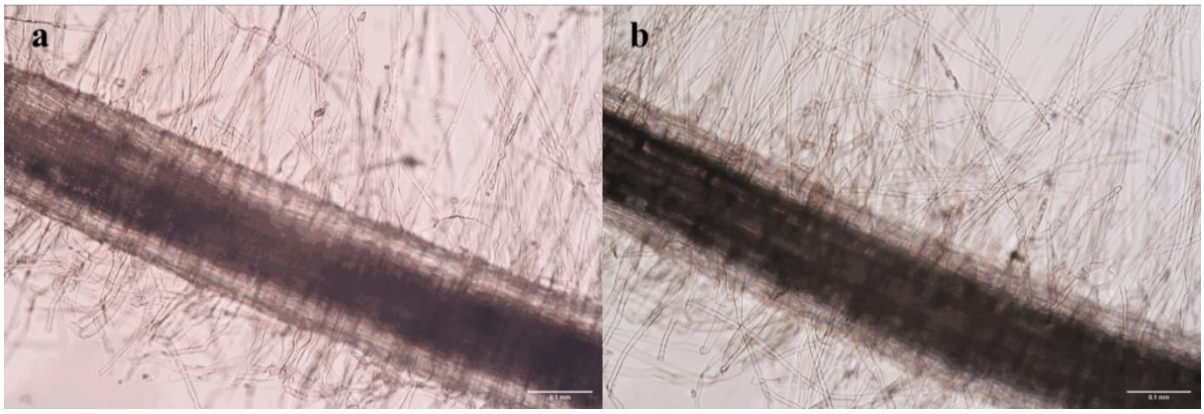


Figure 6. Representative images of root hairs from the maturation region of a susceptible 'Iwa' (a) and resistant cultivar 'Gladiator' (b). The root length of each field of view is 1.0 mm; scale bar = 0.1 mm

3.4.3. Optimum temperature for, and distribution of zoospore attachment to roots on four cultivars ('Iwa', 'Agria', 'Russet Burbank', and 'Gladiator')

Zoospore root attachment was influenced by potato cultivar ($p < 0.001$), and their interaction ($p < 0.001$; Figure 7). Mean number of zoospore attachment for 'Iwa' ($p < 0.001$) was greater (8.9 attached zoospores ± 0.3) than the other three cultivars (0.3 ± 0.1 to 4.9 ± 0.2) in the lower region of the primary root maturation zone. Zoospore root attachment was greater in the lower than in the upper and middle regions of root maturation zones of the cultivars 'Iwa', 'Agria' and 'RBK'.

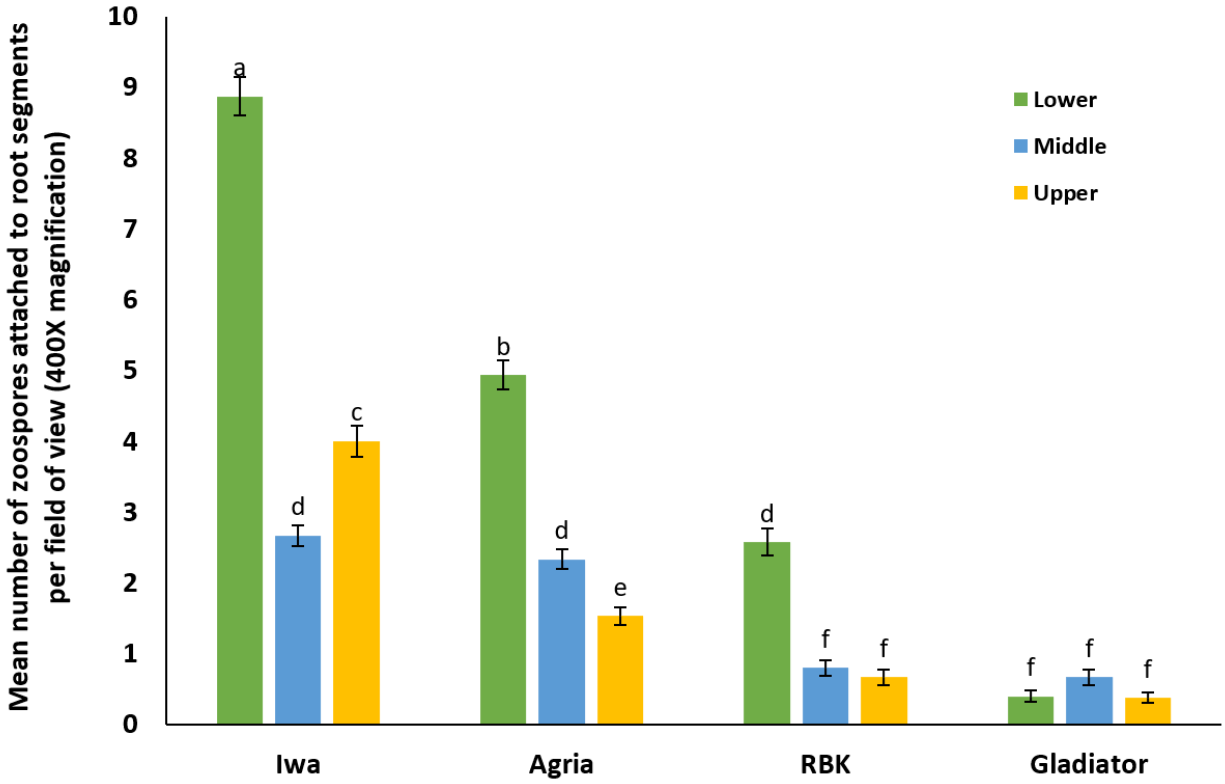


Figure 7. Scaled mean number of *Spongospora subterranea* zoospores observed per microscope field of view at 400× magnification attached to each of three root regions (lower, middle, upper) of the maturation zone of roots from potato cultivars 'Iwa', 'Agria', 'RBK', and 'Gladiator'. Vertical bars represent standard errors ($n = 3$). p (cultivars) < 0.001; p (region of root) < 0.001; p (cultivar × region of root) < 0.001. Different letters above bars indicate region × cultivar interaction effects as determined by LSD (0.05) = 0.44.

Zoospore root attachment was influenced by temperature ($p < 0.001$), cultivar ($p < 0.001$), and their interaction ($p < 0.001$; Figures 8 & 9). After 48 h' incubation, zoospore root attachment was greater in 'Iwa' and 'Agria' than in 'Gladiator' and 'Russet Burbank (RBK)' at all incubation temperatures except 30°C. In addition, for all cultivars, attachment was significantly higher at 15°C than at the other incubation temperatures, and very little zoospore root attachment was observed at 30°C.

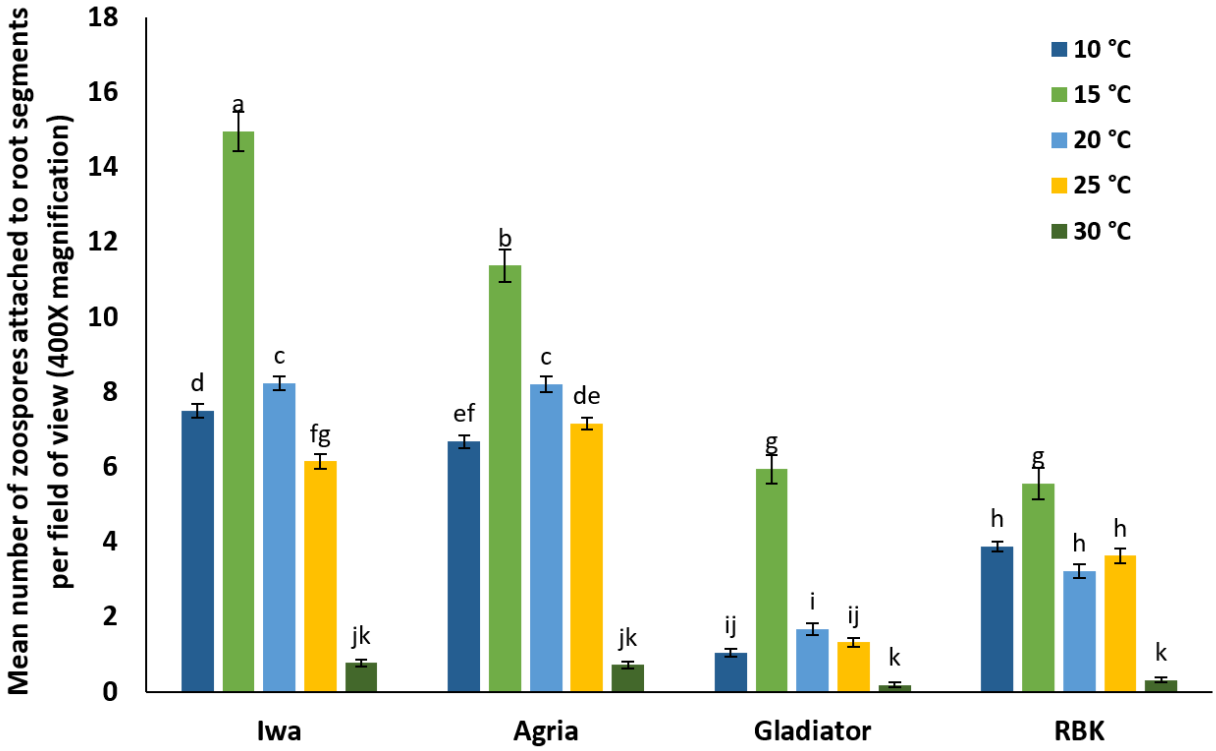


Figure 8. Scaled mean number of *Spongospora subterranea* zoospores observed per microscope field of view at 400× magnification attached to the lower maturation region of potato cultivars 'Iwa', 'Agria', 'Russet Burbank (RBK)', and 'Gladiator' at 10, 15, 20, 25 or 30°C. Vertical bars represent standard errors ($n = 3$). p (temperature) < 0.001; p (cultivar) < 0.001; p (temperature × cultivar) < 0.001. Different letters above bars indicate significant temperature × cultivar interaction effect (LSD (0.05) = 0.67).

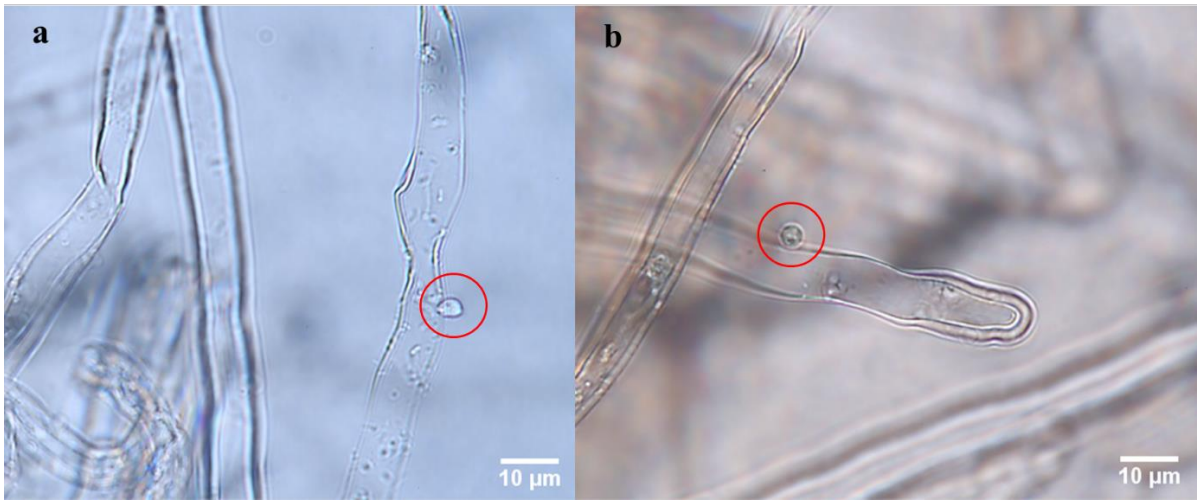


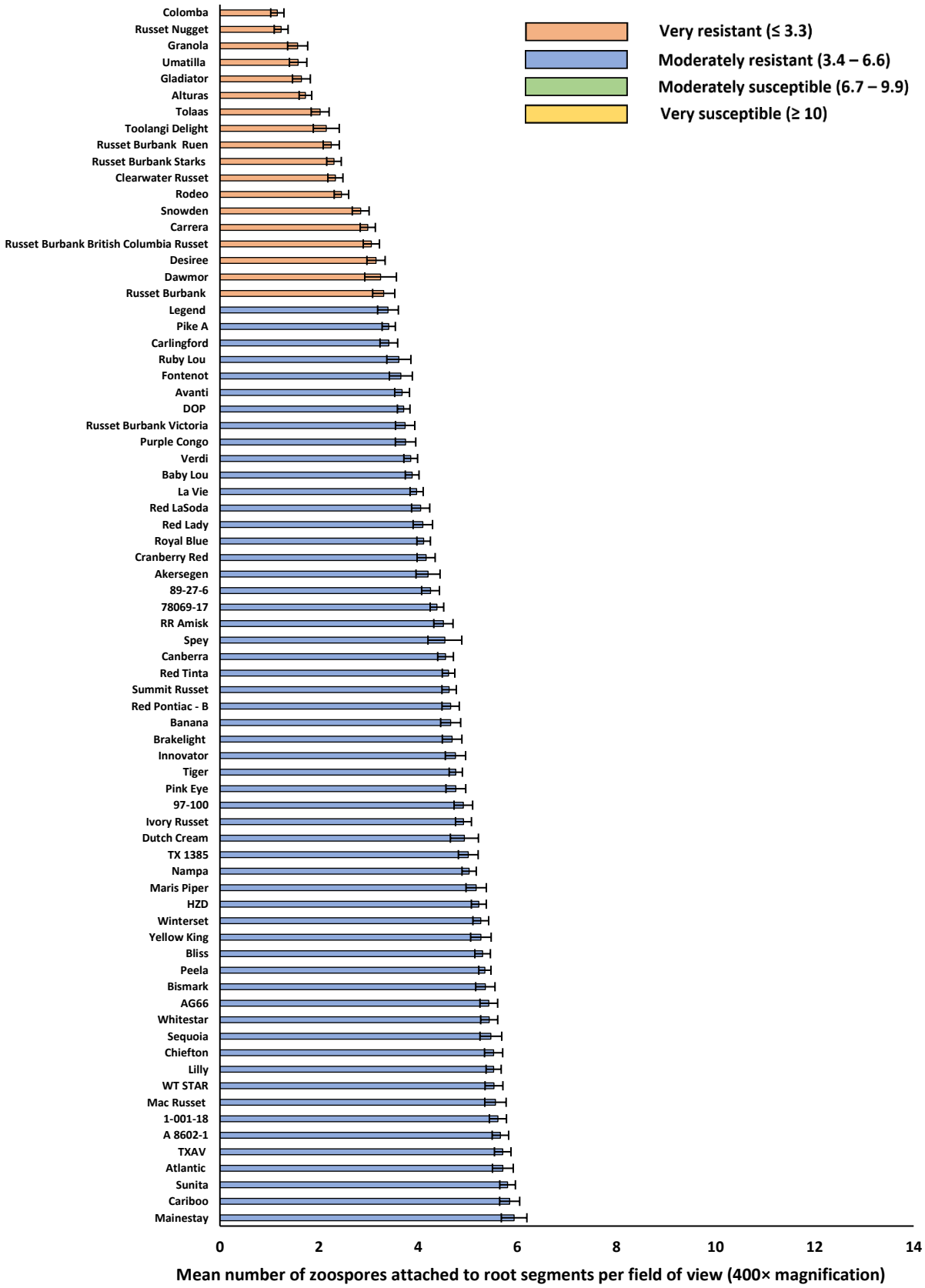
Figure 9. Attchment of *Spongospora subterranea* zoospores to potato root-hairs (lower zone of root maturation region) of potato cultivar 'Iwa'(a) and: cultivar Gladiator' (b), indicated by red circles.

3.4.4. Zoospore root attachment in potato cultivars with different resistance

The mean zoospore root attachment scores for the two standard cultivars 'Iwa' and 'Gladiator' differed across all the batches in which they were tested. The mean score for 'Gladiator' varied between 0.8 and 2.2 (*SD* = 0.5), whereas the mean score for 'Iwa' varied between 9.9 and 15.4 (*SD* = 1.9). All cultivar screenings for zoospore root attachment included these two standards as references, so linear scaling (see methods above) was required to account for batch-to-batch differences in zoospore root attachment, as observed for the two standards.

Figure 10 displays the scaled mean zoospore root attachment scores for the 153 potato cultivars/lines assessed using the in vitro bioassay. Zoospore attachment score (scaled mean severity score) exhibited a continuum of host susceptibility from very susceptible to very resistant. The host lines were arbitrarily classified as very resistant to zoospore root attachment (scaled mean severity score $\leq$ 3.3), moderately resistant (score 3.4 - 6.6), moderately susceptible (score 6.7 - 9.9) and very susceptible (score $\geq$ 10) (Figure 10). This categorisation classified 11.8% of the host lines as very resistant, 49.7% as moderately resistant, 28.8% as moderately susceptible, and 9.8% as very susceptible. Comparison of zoospore root attachment results of 13 cultivars measured in this study with root galling intensity scores published in previous studies (Falloon et al., 2003, Bittara et al., 2016, Falloon et al., 2016) showed that

cultivars ‘Gladiator’, ‘Kennebec’, ‘Yukon Gold’, ‘Russet Ranger’, and ‘Iwa’ were categorized into the same levels of resistance with both assessment methods (Table 5). In contrast, cultivars ‘Nicola’, ‘Agria’, and ‘Shepody’ were categorized as moderately susceptible, very susceptible, and very susceptible, respectively, based on root galling intensity scores, but were assessed as very susceptible, moderately susceptible, and moderately susceptible, respectively, in the zoospore root attachment bioassay. Similarly, cultivars ‘Umatilla Russet’, ‘Alturas’, and ‘Russet Burbank’ were considered as moderately susceptible by the root galling scores but were rated as very resistant, in the zoospore root attachment assay, while ‘Summit Russet’ was categorized as very resistant to root galling but moderately resistant to zoospore root attachment. Linear regression analysis of zoospore root attachment assay results with published field potato powdery scab (tuber disease) resistance scores revealed a negative linear relationship with a relatively weak R^2 value (0.42) (Figure 11). Notable outliers from the relationship were ‘Nicola’ (highly susceptible to zoospore root attachment but moderately resistant to tuber disease), ‘MacRusset’ (moderately resistant to zoospore root attachment but highly susceptible to tuber disease), and ‘Nooksac’ (moderately susceptible to zoospore root attachment but moderately resistant to tuber disease).


Very resistant (≤ 3.3)
Moderately resistant (3.4 – 6.6)
Moderately susceptible (6.7 – 9.9)
Very susceptible (≥ 10)
Colomba
Russet Nugget
Granola
Umatilla
Gladiator
Alturas
Tolaas
Toolangi Delight
Russet Burbank Ruen
Russet Burbank Starks
Clearwater Russet
Rodeo
Snowden
Carrera
Russet Burbank British Columbia Russet
Desiree
Dawmor
Russet Burbank
Legend
Pike A
Carlingford
Ruby Lou
Fontenot
Avanti
DOP
Russet Burbank Victoria
Purple Congo
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Baby Lou
La Vie
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89-27-6
78069-17
RR Amisk
Spey
Canberra
Red Tinta
Summit Russet
Red Pontiac - B
Banana
Brakelight
Innovator
Tiger
Pink Eye
97-100
Ivory Russet
Dutch Cream
TX 1385
Nampa
Maris Piper
HZD
Winterset
Yellow King
Bliss
Peela
Bismark
AG66
Whitestar
Sequoia
Chiefton
Lilly
WT STAR
Mac Russet
1-001-18
A 8602-1
TXAV
Atlantic
Sunita
Cariboo
Mainestay
0
2
4
6
8
10
12
14
Mean number of zoospores attached to root segments per field of view (400× magnification)

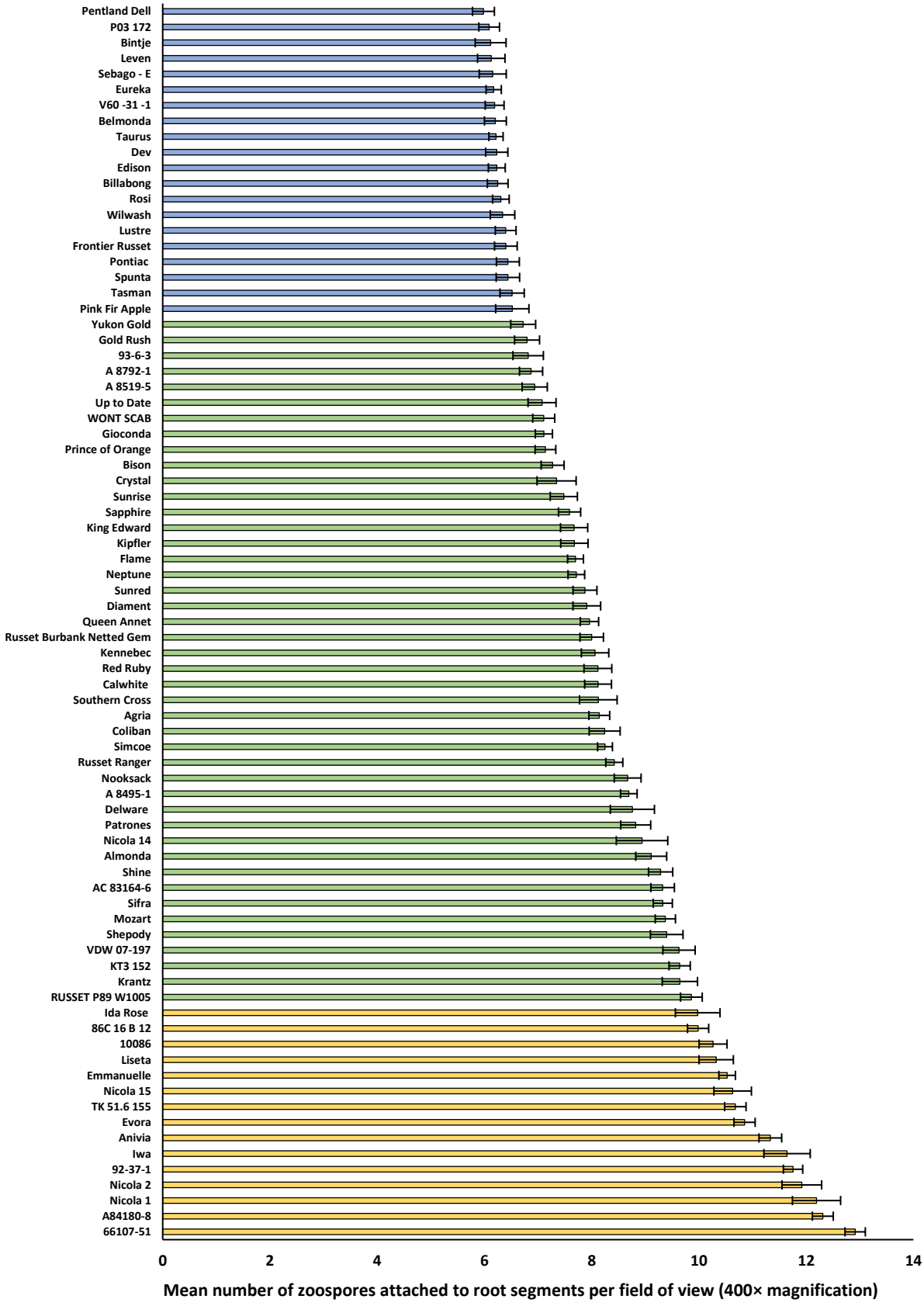


Figure 10. Scaled mean scores for *Spongospora subterranea* zoospore root attachment for 153 potato cultivars and lines assessed in the in vitro root attachment assays ($n = 3$) with $p < 0.001$.

The cultivars were arbitrarily classified as very resistant (score ≤ 3.3), moderately resistant (score 3.4 - 6.6), moderately susceptible (score 6.7 - 9.9) and very susceptible (score > 10). Horizontal bars represent standard errors (n = 3).

Table 5. Relationship between *Spongospora subterranea* zoospore root attachment severity rating of 13 potato cultivars and lines (very resistant to very susceptible) and published root galling severity rating (Falloon et al., 2003, Bittara et al., 2016, Falloon et al., 2016).

	Root galling (Published papers)			
	Very resistant	Moderately resistant	Moderately susceptible	Very susceptible
Root attachment (this study)				
Very resistant	Gladiator	Desiree	Umatilla Russet, Alturas, Russet Burbank	
Moderately resistant	Summit Russet			
Moderately susceptible			Kennebec, Yukon Gold, Russet Ranger	Agria, Shepody
Very susceptible			Nicola	Iwa

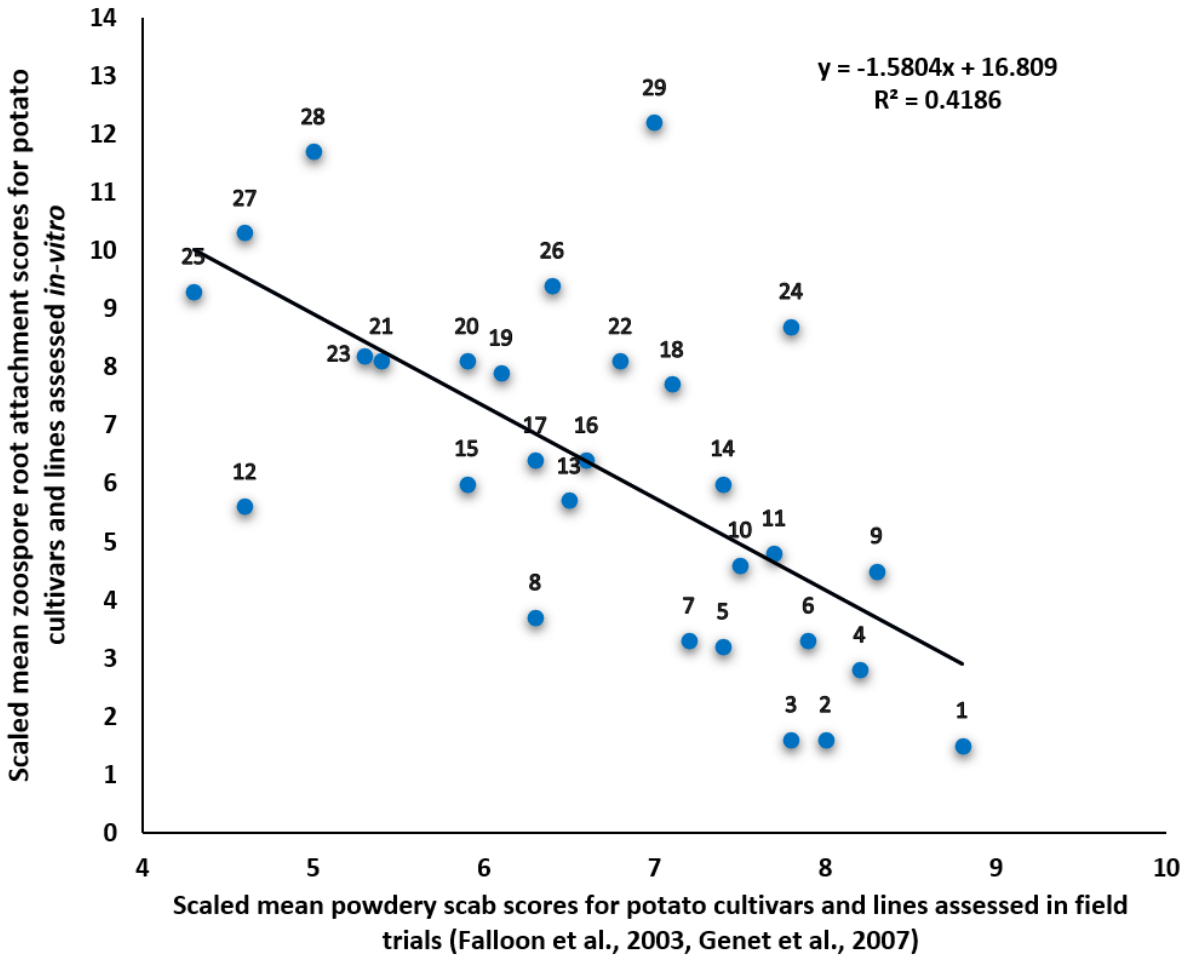


Figure 11. Relationship between scaled mean scores for *Spongospora subterranea* zoospore root attachment of 29 potato cultivars and the published powdery scab disease severity scores of these cultivars assessed in field trials (Falloon et al., 2003, Genet et al., 2007). For zoospore root attachment, the cultivars were arbitrarily classified as very resistant (score ≤ 3.3), moderately resistant (score 3.4 - 6.6), moderately susceptible (score 6.7 - 9.9) and very susceptible (score > 10). For powdery scab disease, the cultivars were classified as very resistant (score ≥ 8), moderately resistant (score 7.0 - 7.9), moderately susceptible (score 6.0 - 6.9) and very susceptible (score ≤ 5.9). 1 'Gladiator', 2 'Granola', 3 'Umatilla Russet', 4 'Snowden', 5 'Desiree', 6 'Russet Burbank', 7 'Sebago', 8 'Victoria', 9 'Spey', 10 'Summit Russet', 11 'Innovator', 12 'MacRusset', 13 'Atlantic', 14' Pentland Dell', 15 'Concorde', 16 'Spunta', 17 'Frontier Russet', 18 'Flame', 19 'Diamant', 20 'Kennebec', 21 'Agria', 22 'Red Ruby', 23 'Coliban', 24 'Nooksac', 25 'Shine', 26 'Shepody', 27 'Liseta', 28 'Iwa', and 29 'Nicola'.

3.4.5. Assessment of susceptibility to *Spongospora subterranea* zoosporangium infection for twelve potato cultivars

Zoosporangium infections were not observed in uninoculated control plants of any cultivar 30 d after inoculation. In contrast, all plants inoculated with *S. subterranea* showed zoosporangium infections, but the infection rates varied among the cultivars. Mean zoosporangium intensity scores were greater in cultivars assessed as susceptible to zoospore root attachment (i.e., cultivars 'Iwa', '10086', 'Krantz', 'Nicola', 'Shepody', and 'Ida Rose') than in cultivars that had greater resistance to zoospore root attachment (i.e., cultivars 'Russet Burbank', 'Russet Nugget', 'Gladiator', 'Granola', 'Tolaas', and 'Toolangi delight') (Figure 12).

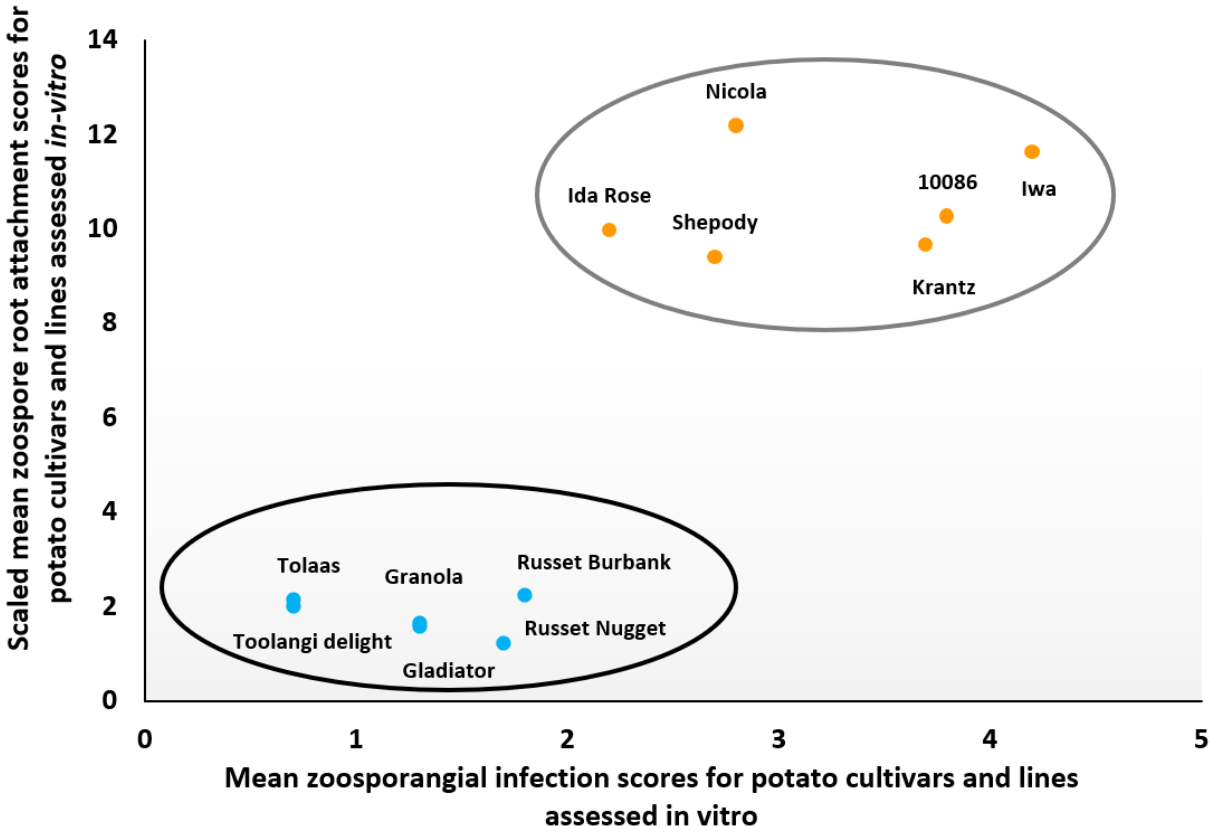


Figure 12. Relationship between scaled mean of *Spongospora subterranea* zoospore root attachment scores and zoosporangium infection severity score 0 = no infection; 1 < 10% sporadic; 2 = 2% - 10% slight; 3 = 11% - 25% moderate; 4 = 26% - 50% heavy, and 5 > 50% very heavy (Merz et al., 2004) of 12 selected potato cultivars.

3.5. Discussion

With an absence of effective control measures, host resistance is regarded as a critical tool for management of root and tuber diseases caused by *S. subterranea* infections (Bittara et al., 2016). However, the traditional methods used for cultivar screening for disease resistance have involved replicated glasshouse or field trials that are both time and resource intensive (Merz et al., 2004), and subject to variation of environmental conditions that can affect disease expression and assessment efficiency. These field and glasshouse trials assess resistance to root galling or tuber disease only, and the lack of information on resistance to root infection can be a limitation, as this phase of the disease is critical for impact on potato yield (Shah et al., 2012, Falloon et al., 2016). Prior in vitro assays that that assess plasmodia or zoosporangia within infected roots do provide this data (Merz et al., 2004), but can still require several weeks for completion. Results from in vitro assays that do not control timing of inoculation of roots may also be confounded by differing numbers of infection cycles between cultivars (Thangavel et al., 2015). This study has developed a novel in vitro assessment method that is rapid (results within 48 h), allows control over experimented environmental conditions, and assesses cultivar susceptibility to zoospore root attachment at the first point of pathogen interaction, thus avoiding issues associated with polycyclic infection.

This study provided data on the effect of temperature conditions for *S. subterranea* zoospore release and identified the optimal location on root tissue and temperature incubation conditions for zoospore root attachment. Maximum zoospore release occurred at 20℃ in Hoagland's solution, while the highest zoospore root attachment occurred at 15℃. Many studies have reported optimal temperatures for *S. subterranea* root infection or tuber disease development (Kole, 1954, Hughes, 1980, van de Graaf et al., 2005, van de Graaf et al., 2007, Shah et al., 2012), but this is the first to specifically determine the optimal temperature for zoospore root attachment. These results are consistent with the findings from previous studies. For example, germination of zoospores was shown to occur at temperatures between 9 to 17℃ in aqueous solution (Fornier et al., 1996) or soils (van de Graaf et al., 2005, Shah et al., 2012). Similarly, root galling and tuber infection were promoted by soil temperatures of 11 to 25℃ and 9 to 17℃, respectively, with more severe tuber infection at 12℃ (Hughes, 1980, van de Graaf et al., 2005, Shah et al., 2014, Balendres et al., 2016b).

This study showed that zoospore root attachment was generally higher in root hairs from the lower (younger) rather than the medium and upper maturation regions of the root. However, it

would appear the zoospore root attachment response is not associated with root hair density, given that root hair density was similar across the three root regions tested. Potentially, zoospores may have had greater attraction to younger root hairs. A similar observation noted that zoospore adhesion of *Pythium aphanidermatum* occurred largely in the younger root hairs (Jones et al., 1991).

The results of screening 153 potato lines and cultivars demonstrate that susceptibility to zoospore root attachment follows a continuum from very resistant to very susceptible, suggestive of control by polygenetic resistance factors similar to those observed for tuber disease (Falloon et al., 2003, Genet et al., 2007). There was a clear significant correlation between zoosporangium infection severity and zoospore root attachment for the 12 cultivars evaluated in this study; for example, 'Iwa', '10086' and 'Krantz' were very susceptible to both zoosporangium infection and root attachments, while 'Tolaas' and 'Toolangi delight' were very resistant in both assessments.

Although relationships between root infection (zoosporangium infection and root galling) and tuber infection (powdery scab) have been established with varying degrees of association, no previous studies have demonstrated a relationship between zoospore root attachment and cultivar resistance to both root and tuber infections. Here the resistance response of most potato cultivars has been shown that was generally consistent regardless of the method of assessment. This was best demonstrated by the response of cultivars 'Iwa' and 'Gladiator', whereby 'Iwa' had higher zoospore root attachment scores than cultivar 'Gladiator' reflecting the known higher susceptibility of 'Iwa' and the higher resistance of 'Gladiator' to zoosporangium infection, root galling, and tuber powdery scab (Falloon et al., 2003, Falloon et al., 2016). This is unsurprising as the present study expects that zoospore root attachment would impact the rate of root infection and thus the development of later disease expression such as root galls and tuber disease (Thangavel et al., 2015).

There are notable exceptions, however, that emphasize the difference between resistance expression to the root and tuber diseases. Among the 29 cultivars with known resistance to powdery scab, there are three outliers. 'MacRusset' was rated as moderately resistant to zoospore root attachment but moderately susceptible to tuber powdery scab. 'Nooksac' was rated as moderately susceptible to zoospore root attachment but moderately resistant to powdery scab. Additionally, 'Nicola' has been reported as possessing moderate resistance to

powdery scab but is highly susceptible to zoospore root attachment in our study with four independent clones tested ('Nicola 1', 'Nicola 2', 'Nicola 14', and 'Nicola 15').

Five of the 13 cultivars with documented resistance to root galling were in agreement with their resistance to zoospore root attachment. However, 'Umatilla Russet,' 'Alturas,' and 'Russet Burbank' were graded as resistant to zoospore root attachment but susceptible to root galling. Notably, 'Nicola' is rated as very resistant to zoospore root attachment and moderately susceptible to root galling. Thus, while growers often consider cultivar 'Nicola' as a cultivar with minimal impact from *S. subterranea* due to a lack of tuber disease, root disease may be prevalent, resulting in poor plant performance (Merz et al., 2012). These examples mentioned indicate the complexity of the diseases caused by *S. subterranea* and differences in optimal environmental conditions and genetic resistance for tuber and root infections (Harrison et al., 1997, Nitzan et al., 2008).

In conclusion, the in vitro screening technique developed in this study provides a novel and comprehensive assessment method of the relative susceptibility to zoospore root attachment by *S. subterranea*. Results from the screening of 153 cultivars and lines for zoospore root attachment bioassay generally agreed with the cultivar resistance scores obtained from published potato powdery scab severity scores field trial data, with a few notable varietal exceptions (Falloon et al., 2003, Falloon et al., 2016). This in vitro bioassay only requires root tissue samples. In the present study, tissue-cultured plantlets were used, but in unpublished studies root tissues harvested from glasshouse grown plants have also been successfully used for the in vitro screening. This increases the assays flexibility. For example, breeders could germinate true seed from select crosses and directly test roots from these seedlings without the need for first introducing these into tissue culture. This method avoids any confounding impact of polycyclic infections, which can result in subsequent variations in root infection scores, and the bioassay can be conducted under strictly controlled conditions in the laboratory, which minimizes variable environmental influences. This study proposes that breeders may find the use of this rapid screen highly valuable to rapidly screen for resistance to root attachment/infection, a trait of considerable interest to the commercial potato industries, decreasing generation time and improving screening efficiency, growers may find the varietal assessment cultivar rankings for root infection valuable in the selection of suitable planting material according to their disease risk.

Chapter 4. Enhanced resistance to powdery scab of potato through somatic cell selection and in vitro zoospore root attachment assays

4.1. Abstract

Potato root and tuber infection by the plasmodiophorid *Spongospora subterranea* f. sp. *subterranea* have impacts on potato production, and host plant resistance is an important component for management of diseases caused by this pathogen. Somatic cell selection was used to generate variants of commercial potato cultivars which were screened for altered susceptibility to zoospore root attachment. Thirty-one (33%) of the variants from five cultivars and one clone exhibited greater resistance to zoospore root attachment in comparison to their unselected parent cultivars or clones. A glasshouse pathogenicity trial showed that most variants with enhanced resistance to zoospore root attachment also had less tuber powdery scab than their parents, although these differences were not always statistically significant. This study demonstrated that somatic cell selection can be an effective approach for selecting variants resistant to root infection, with variants also showing reduced tuber powdery scab.

Keywords: Somatic cell selection, in vitro zoospore root attachment, regenerated variants, cultivar resistance, powdery scab disease

4.2. Introduction

The soilborne pathogen, *Spongospora subterranea* f. sp. *subterranea* (Wallr.) Lagerh. causes economically significant root and tuber diseases of potato (Harrison et al., 1997, Falloon et al., 2003, Merz et al., 2004). The lesions on potato tubers diminishes their suitability and value as fresh product, and, in processing, diseased tubers create challenges during skin removal (Falloon et al., 2003, Merz, 2008, Merz and Falloon, 2009, Wilson, 2014b, Falloon et al., 2016, Balendres et al., 2016b). Lesions on infected tubers may allow entry by other pathogens, exacerbating water loss and tuber rot during storage (Balendres et al., 2016b). As infected tubers carry inoculum, they also cannot be used as seed tubers. Root infection can reduce plant growth, through root disfunction and reductions in water and nutrient uptake, negatively impacting tuber yields (Falloon et al., 2003, Falloon et al., 2016).

Disease management remains difficult with no one effective control method to date (Falloon, 2008). Disease control strategies that have been employed include use of seed and soil chemical treatments (Braithwaite et al., 1994b, Falloon et al., 1996, Tsror et al., 2020), crop rotation (Larkin and Griffin, 2007, Sparrow et al., 2015, Larkin et al., 2010), biocontrol (Nakayama, 2017), other agronomic techniques (Hughes, 1980, Van de Haar, 2000, Tuncer, 2002, van de Graaf et al., 2005, van de Graaf et al., 2007, Shah et al., 2012, Shah et al., 2014, Balendres et al., 2018a), and host resistance (Falloon et al., 2003, Falloon, 2008, Merz and Falloon, 2009). The use of cultivars with resistance to *S. subterranea* is generally regarded as the most sustainable approach for management of this disease, and is a critical component of integrated disease management (Nitzan et al., 2008). Potato cultivars, breeding lines, and germplasm accessions exhibit different susceptibilities to *S. subterranea,* but none are immune to the pathogen (Karling, 1968, Hughes, 1980, Kirkham, 1986, Gans et al., 1987, Wastie et al., 1988, de Boer, 1991, Torres et al., 1995, Falloon et al., 2003). Furthermore, resistance breeding to date has focused on tuber disease expression which is not necessarily linked with root disease (Yu et al., Chapter 3).

Traditional potato breeding programs are time- and resource-consuming, with new cultivars taking up to 10 to 12 years before becoming commerically available (Bruines, 2018). Alternatively, it is possible to quickly produce new clones of exisiting cultivars, utilising plant tissue culture techniques that involve mutation and selection of somaclonal variants (Karp, 1991). As this does not involve sexual crosses, the approach avoids genetic recombination and preserves the desired agronomic traits and market acceptability of an original cultivar (Karp,

1991). Several important potato diseases have been effectively targeted for disease resistance selection, including common scab (caused by *Streptomyces scabies*), early blight (*Alternaria solani*), late blight (*Phytophthora infestans*) *Fusarium* dry rot (*Fusarium oxysporum*), and *Verticillium* wilt (*Verticillium dahliae*) using in vitro produced genetic diversity (Matern et al., 1978, Behnke, 1980, Sebastiani et al., 1994, Goyer et al., 1998, Kowalski and Cassells, 1999).

The present study aimed to determine whether somaclonal selection techniques could produce variants of commercial cultivars that showed reduced susceptibility to root infection by *S. subterranea*, using the in vitro zoospore root attachment assay (Yu et al; Chapter 3) for phenotyping potato clones. Promising variants were used to determine whether resistance to root infection is also expressed as reduced tuber disease, in glasshouse challenged plants.

4.3. Materials and methods

4.3.1. Sporosorus inoculum collection and zoospore release

Powdery scab infected potato tubers from a commercial potato production area in Devonport, Tasmania, Australia, were used to provide *S. subterranea* inoculum (sporosori). Diseased tubers were cleaned under running tap water for 1 min, immersed in 2% sodium hypochlorite (White King, Pental Products Ltd. Pty, Melbourne, Australia) for 3 min, then washed again and dried in a ventilated area. Tuber lesions were removed with a scalpel and dried for 4 d at 40°C, before grinding and storing at 4 °C in the dark until use.

Zoospore release was facilitated by incubation of 3 g of dried sporosorus inoculum in 10 mL Hoagland's solution in a sterile McCartney bottle. Hoagland's solution was prepared as described by (Falloon et al., 2003); KNO_3, 253 mg/L; $Ca(NO_3)_2 \cdot 4H_2O$, 722 mg/L; KH_2PO_4, 2.3 mg/L; $MgSO_4 \cdot 7H_2O$, 120 mg/L; NH_4NO_3, 40 mg/L; Fe-EDTA, 20 mg/L; H_3BO_3, 140 µg/L; KCl, 400 µg/L; $MnSO_4 \cdot H_2O$, 63 µg/L; $ZnSO_4 \cdot 7H_2O$, 115 µg/L; $CuSO_4 \cdot 5H_2O$, 50 µg/L; and $Na_2MoO_4 \cdot 2H_2O$, 22 µg/L, formulated in sterile deionized distilled water (DDW). The bottles containing sporosori were then incubated in the dark at 15°C for 3 d. Zoospore numbers were determined by taking a 1 µL subsample and counting the total number of zoospores present, using a light microscope (DM 2500 LED, Leica Microsystem, Germany) at 200× magnification.

4.3.2. Plant sample preparation

Parental potato cultivars ‘Desiree’, somaclone ‘Desiree-8’, ‘Sebago’, ‘Russet Burbank’, ‘Russet Ranger’, ‘Shepody’, their somaclonal variants, and the standard control cultivar ‘Agria’ were provided from the Tasmanian Institute of Agriculture in house collection. ‘Desiree-8’ is a somaclone of ‘Desiree’, that has shown enhanced resistance to common scab (unpublished data).

All variant clones had been created using the targeted somatic cell selection method described by Wilson et al. (2009) with minor modifications. Friable callus cells generated from each of the parent cultivars were suspended for 7 d in a callus inducing medium, containing thaxtomin A at concentrations from 2 to 4 mg/L (Murashige and Skoog (MS) Salts and vitamins, plus 5 g/L sucrose, 40 mg/L ascorbic acid, 500 mg/L casein hydrolysate, 2 mg/L Bone alkaline phosphatase (BAP), 0.2 mg/L *N*-Acetylaspartic acid (NAA), 5 mg/L Gibberellic acid (GA3) with pH adjusted to 5.8). Following treatment the cells were plated directly onto a sterile 7 cm diameter Whatman No. 1 filter paper placed on recovery media (MS salts and vitamins, plus sucrose, 10 g/L; mannitol, 40 g/L, glucose 10 g/L; BAP, 0.1 mg/L; NAA, 0.2 mg/L; and GA3, 0.2 mg/L, with pH adjusted to 5.8) and incubated under reduced light intensity (7 µmol/m^2/s) at 22℃ for 2 to 3 months. Surviving calli were transferred to regeneration medium (MS salts and vitamins plus myo-inositol, 100 mg/L; 2-(*N*-morpholino)ethanesulfonic acid (MES), 1 g/L; sucrose, 3 g/L; mannitol, 40 g/L; glucose, 10 g/L; casein hydrolysate, 1g/L; zeatin, 0.5 mg/L; kinetin, 0.5 mg/L; Iodoacetamide (IAA), 0.1 mg/L and GA3, 0.2 mg/L, with pH adjusted to 5.8). The variants were obtained from multiple selection experiments including eight for ‘Desiree’, two for ‘Desiree-8’, six for ‘Sebago’, three for ‘Russet Burbank’, nine for ‘Russet Ranger’ and two for ‘Shepody’, in– these experiments conducted between 2019 and 2021.

Regenerant clones were subsequently transferred to potato multiplication (PM) medium composed of MS salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; phytagel, 2.2g/L; at pH 5.8. Potato tissue-cultured plantlets were grown in PM under a 16 h photoperiod using white fluorescent lamps (65 µmol/m^2/s) at 22℃. Prior to use, 1-month-old plantlets were transferred from PM medium to liquid potato multiplication (LPM) medium composed of MS salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; at pH 5.8, and grown for 2 weeks under a 16 h photoperiod using white fluorescent lamps (65 µmol/m^2/s) at 22℃.

4.3.3. In vitro testing to evaluate susceptibility of potato variants to zoospore root attachment

All root segments were processed based on the in vitro zoospore root attachment bioassay described in Chapter 3 (Yu et al., Chapter 3). One primary root was harvested from each tissue-cultured plantlet, with six plants used for each parental cultivar or regenerated variant. A 10 mm segment from the lower maturation region from each root was sampled and used for assessment of zoospore root attachment. Washed root segments of each cultivar were equally distributed within container and a zoospore suspension (1000 zoospores/mL) added as previously described (Yu et al., Chapter 3). Root segments were incubated for 48 h in the dark at 15°C. Each container included root segments of 'Agria' as normalisation controls. Light microscopy at 400× magnification was used to count the zoospores attached to each root segments, randomly scanning five fields of view for each root segment.

Scores of zoospore root attachment data for each cultivar/variant in the screenings were standardized according to the reference cultivar 'Agria', present in each batch. The mean scores for zoospore root attachment to roots of 'Agria' in the first batch screened was 8.1 (A1), and this served as a reference score to adjust for batch differences in each subsequent batch. This was done by calculating a reference point correction coefficient (η_n) for each batch.

$$\eta_n = \frac{An}{A1}$$

where An is the zoospore root attachment scores for 'Agria' in batch n. This coefficient was used to linearly scale the attachment score for each cultivar/variant.

4.3.4. Glasshouse trial to evaluate susceptibility of potato variants to powdery scab

Selected variants and their parent cultivars were tested for resistance to tuber powdery scab in a glasshouse challenge trial. Plastic pots (4.5 L capacity) were filled with potting mix (1 part coarse sand: 1 part peat: 8 parts composted pine bark; at pH 6.0), with 500 mg of dried sporosorus inoculum (Balendres et al., 2018b) well mixed into the soil of each pot. Each pot was planted with a 3-week-old tissue-cultured plant, with five replicates (pots) per parent/variant tested. The plants were grown under glasshouse conditions (20 ± 2°C) with irrigation applied every 2nd d to maintain moist soil suitable for powdery scab development

(Falloon et al., 2003). No pesticides were applied throughout the trials. After 16 weeks, individual pots were harvested separately by uprooting plants and gently washing roots and tubers under running tap water.

4.3.5. Tuber infection assessment

From each pot individual tubers were counted and weighed, with each tuber > 2 g then assessed for powdery scab disease. Each tuber was assigned a score for diseased tuber surface coverage ranging from 0 to 6 (0 = no visible disease on tuber surface, 0.5 ≤ 1%, 1 ≥ 1 - 5%, 2 ≥ 5 - 10%, 3 ≥ 10 - 30%, 4 ≥ 30 - 50%, 5 ≥ 50 - 70%, 6 ≥ 70% tuber surface affected) (Tegg et al., 2015). An average disease cover score and percentage cover score was calculated per pot.

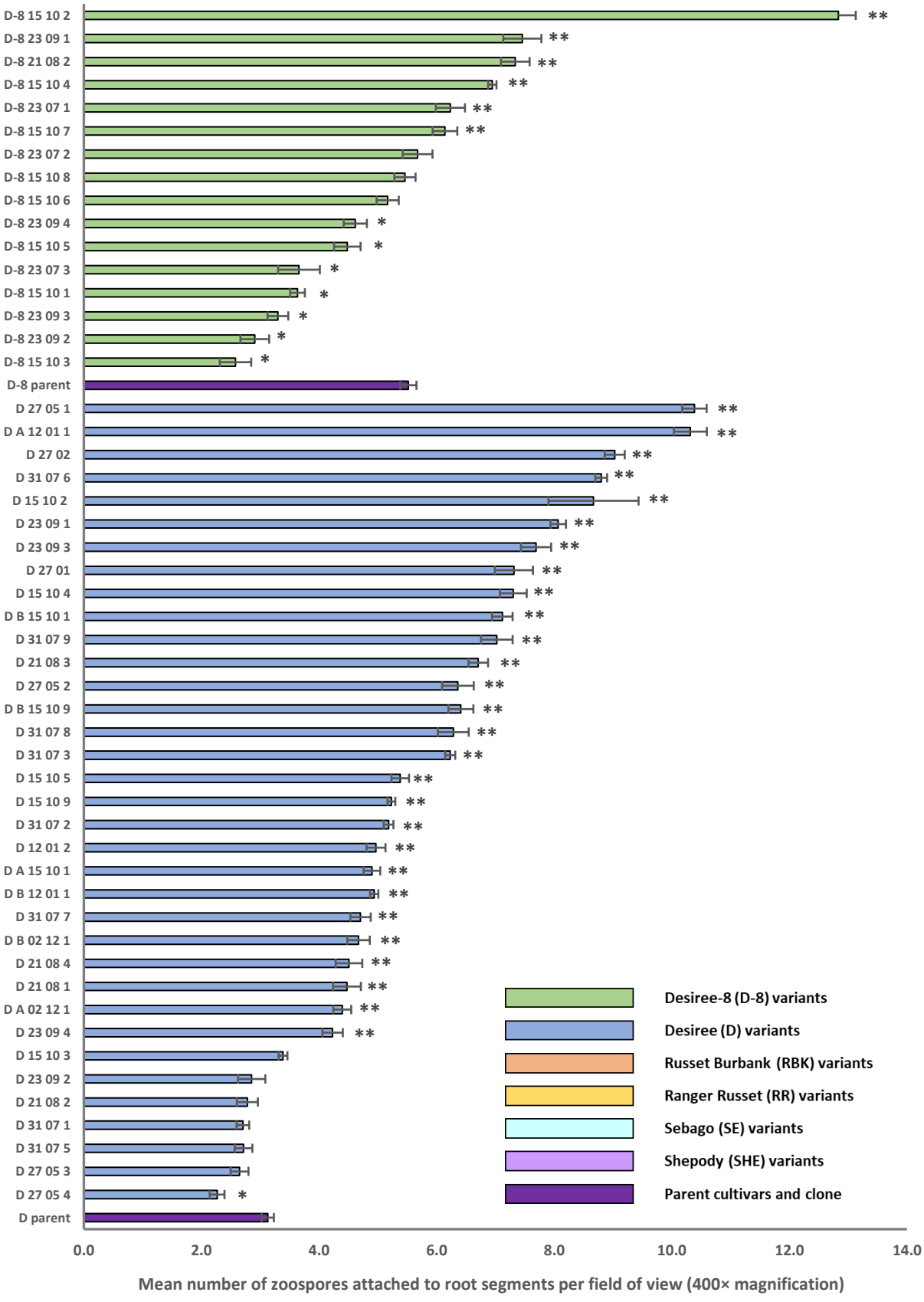
4.3.6. Statistical analyses

Analysis of variance (ANOVA) was performed using IBM SPSS Statistics 27 software after ensuring that all variances were normal and homogeneous. ANOVA with Protected Fisher's LSD was used to establish statistically significant means differences ($p < 0.05$) for zoospore root attachment and tuber infection severity scores.

4.4. Results

4.4.1. In vitro assessment of susceptibility of potato cultivars and variants to zoospore root attachment

Zoospore root attachment varied between potato cultivar variants ($p < 0.001$). Ninety-three variants and the selected parents were tested for zoospore root attachment using the in vitro zoospore root attachment bioassay. A total of 31 of the 93 regenerated variants (33%) had greater resistance to zoospore root attachment than the corresponding unselected parent cultivars, while 48 (52%) of variants exhibited significantly ($p < 0.05$) decreased resistance, and 14 variants showed no statistically significant difference from their parents (Figure 13). The zoospore root attachment severity score of the most resistant 'Desiree' variant, 'D 27 05 4', was 26% less than the 'Desiree' parent, while the score for the most resistant 'Desiree-8' variant, 'D-8 15 10 3' was 53% less than the parent. The two 'Shepody' variants with enhanced resistance, 'SHE 11 09 1' and 'SHE 23 07 1', exhibited, respectively, 45% and 32% less zoospore root attachment scores, and the Sebago variant with enhanced resistance, 'SE 04 01 2', had a 54% lower zoospore root attachment severity score than the parent. Two 'Russet Burbank' variants, 'RBK M12' and 'RBK HEAT 23 09 1', had increased resistance, with, respectively, 77% and 36% lower zoospore root attachment severity scores than their parent. All the 'Ranger Russet' variants had greater resistance to zoospore root attachment than their parent control with 14 to 92% lower severity scores than the parent.


D-8 15 10 2
D-8 23 09 1
D-8 21 08 2
D-8 15 10 4
D-8 23 07 1
D-8 15 10 7
D-8 23 07 2
D-8 15 10 8
D-8 15 10 6
D-8 23 09 4
D-8 15 10 5
D-8 23 07 3
D-8 15 10 1
D-8 23 09 3
D-8 23 09 2
D-8 15 10 3
D-8 parent
D 27 05 1
D A 12 01 1
D 27 02
D 31 07 6
D 15 10 2
D 23 09 1
D 23 09 3
D 27 01
D 15 10 4
D B 15 10 1
D 31 07 9
D 21 08 3
D 27 05 2
D B 15 10 9
D 31 07 8
D 31 07 3
D 15 10 5
D 15 10 9
D 31 07 2
D 12 01 2
D A 15 10 1
D B 12 01 1
D 31 07 7
D B 02 12 1
D 21 08 4
D 21 08 1
D A 02 12 1
D 23 09 4
D 15 10 3
D 23 09 2
D 21 08 2
D 31 07 1
D 31 07 5
D 27 05 3
D 27 05 4
D parent
Desiree-8 (D-8) variants
Desiree (D) variants
Russet Burbank (RBK) variants
Ranger Russet (RR) variants
Sebago (SE) variants
Shepody (SHE) variants
Parent cultivars and clone
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
Mean number of zoospores attached to root segments per field of view (400× magnification)

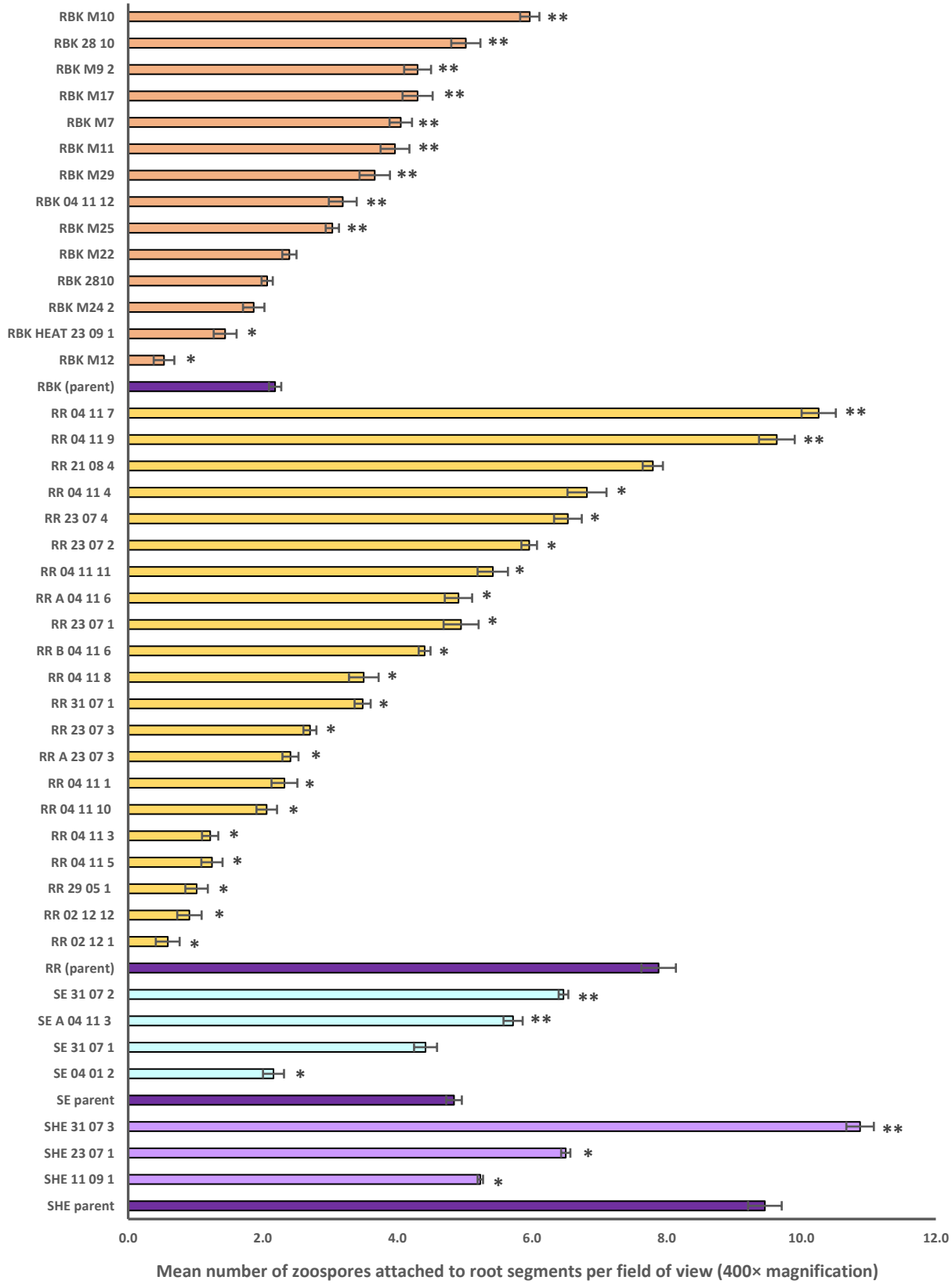


Figure 13. Scaled mean *Spongospora subterranea* zoospore root attachment scores for potato cultivar 'Desiree (D)', 'Shepody (SHE)', 'Sebago (SE)', 'Ranger Russet (RR)', 'Russet

Burbank (RBK)' and variant 'Desiree-8 (D-8)' with their variants assessed by the in vitro zoospore root attachment assay. p (cultivars) < 0.001. LSD (0.05) = 0.57. Horizontal bars represent standard errors (n = 6). Values marked * are significantly less, and ** significantly greater (p < 0.05) than the corresponding unselected parental cultivars.

4.4.2. Glasshouse trial to assess susceptibility of potato cultivars and variants to tuber infection

Initial in vitro assessments screened all 99 parent cultivars and variants for zoospore root attachment, so 31 regenerated variants showing enhanced resistance were rescreened in a subsequent glasshouse trial. For the cultivars 'Russet Burbank', 'Ranger Russet' and their variants, no tuber infection was observed in the glasshouse trial. Data for the pathogenicity testing of the other cultivars and variants are presented in Table 6. Several selected regenerated variants exhibited reduced tuber disease compared with their parent cultivars (p < 0.05). In comparison to the parent cultivar 'Desiree', variants 'D 23 09 2 (7)' and 'D 31 07 1 (5)' had a lower incidence with mean disease reduction of, respectively, 89.2 and 83.1% (mean cover score), and 86.1 and 44.4% (estimated percentage cover). For the variants of clone 'Desiree-8', 'D-8 23 09 2' and 'D-8 23 09 3' had the least disease incidence, with mean disease reductions of, respectively, 94.4 and 85.2% (mean cover score), and 91.4 and 86.2% (estimated percentage cover), in comparison to the parent cultivar.

'SHE 11 09 1' and 'SHE 23 07 1' showed decreases, respectively, of 44.9% and 17.0% in mean cover score and 41.0% and 2.6% in estimated percentage cover, compared to their parents. Compared to their parents, 'SE 04 01 2' and 'SE 31 07 1' had, respectively, 78.8% and 77.0% reduced mean cover scores, and 65.9% and 73.3% reduced estimated percentage cover.

Mean tuber number and tuber weights varied for some cultivars and variants (p < 0.001; Table 6). There was no a clear correlation between zoospore root attachment severity and mean tuber numbers or weights. Variants 'SE 04 01 2' and 'SE 31 07 1' produced greater (p < 0.05) numbers and weights of tubers than their parent. In contrast, all variants of 'Desiree-8 (D-8)' had reduced tuber numbers with two variants ('D-8 15 10 1' and 'D-8 15 10 5') also having reduced (p < 0.05) tuber weights than their parent. 'Desiree' variant (D 23 09 2) had increased tuber yield compared with the parent.

The zoospore root attachment scores and tuber infection intensity scores for ‘Sebago (SE)’, ‘Shepody (SHE)’, ‘Desiree (D)’, and ‘Desiree-8 (D-8)’ are summarized in Figure 14. Linear regression analysis of zoospore root attachment assay results with glasshouse tuber disease severity scores revealed a positive albeit weak linear relationship (R^2 = 0.39) (Figure 14). The parent cultivar ‘Sebago (20)’ was a notable outlier in that it was moderately resistant to zoospore root attachment but highly susceptible to tuber infection.

Table 6. Mean tuber powdery scab severity scores, tuber weights and number of tubers for 18 regenerated potato variants showing greater resistance to zoospore root attachment than unselected parental cultivars ‘Desiree (D)’, ‘Desiree-8 (D-8)’, ‘Shepody (SHE)’, and ‘Sebago (SE)’.

	Variants	Surface cover score (0 - 6)[a]	Surface cover (%)[b]	Mean tuber weight/plant (g)	Mean tuber number/plantt
1	D parent	0.65	48.00	18.8	7.4
2	D 27 05 4	0.20	20.00	6.6	2.4*
3	D 27 05 3	0.13	7.50*	16.6	4.4
4	D 31 07 5	0.37	11.33*	20.2	4.2*
5	D 31 07 1	0.11*	26.67	19.6	5.4
6	D 21 08 2	0.38	20.38	22.1	4.8
7	D 23 09 2	0.07*	6.67*	33.5**	4.0*
8	D-8 parent	0.54	38.67	29.1	13.0
9	D-8 15 10 3	0.47	33.17	34.0	8.0*
10	D-8 23 09 2	0.03	3.33*	18.6	3.0*
11	D-8 23 09 3	0.08	5.36*	27.7	4.2*
12	D-8 15 10 1	0.38	27.08	14.7*	8.2*
13	D-8 23 07 3	0.37	33.96	31.8	6.0*
14	D-8 15 10 5	0.20	11.67	14.4*	4.6*
15	D-8 23 09 4	0.44	29.00	20.5	3.4*
16	D-8 15 10 6	0.22	12.22	34.7	4.8*

17	SHE parent	1.47	71.90	19.8	3.8
18	SHE 11 09 1	0.81*	41.71	19.7	5.8
19	SHE 23 07 1	1.22	70.00	12.6	3.6
20	SE parent	2.17	100.00	1.6	3.3
21	SE 04 01 2	0.46*	34.09*	33.7**	8.2**
22	SE 31 07 1	0.50*	26.67*	17.4**	9.3**
	P	< 0.001	< 0.001	< 0.001	< 0.001
	LSD (0.05)	0.52	30.64	13.72	3.1

[a]Tuber disease surface cover score, 0 = no disease, 0.5 = 0 - 1% tuber surface affected, 1 = 1 - 5%, 2 = 5 - 10%, 3 = 10 - 30%, 4 = 30 - 50%, 5 = 50 - 70%, 6 ≥ 70% tuber surface affected.

[b]Estimated tuber surface coverage calculated from disease cover score using median percentile scores within the allocated range.

Values marked * are less, and ** greater (both at $p < 0.05$) than the corresponding unselected parental cultivars.

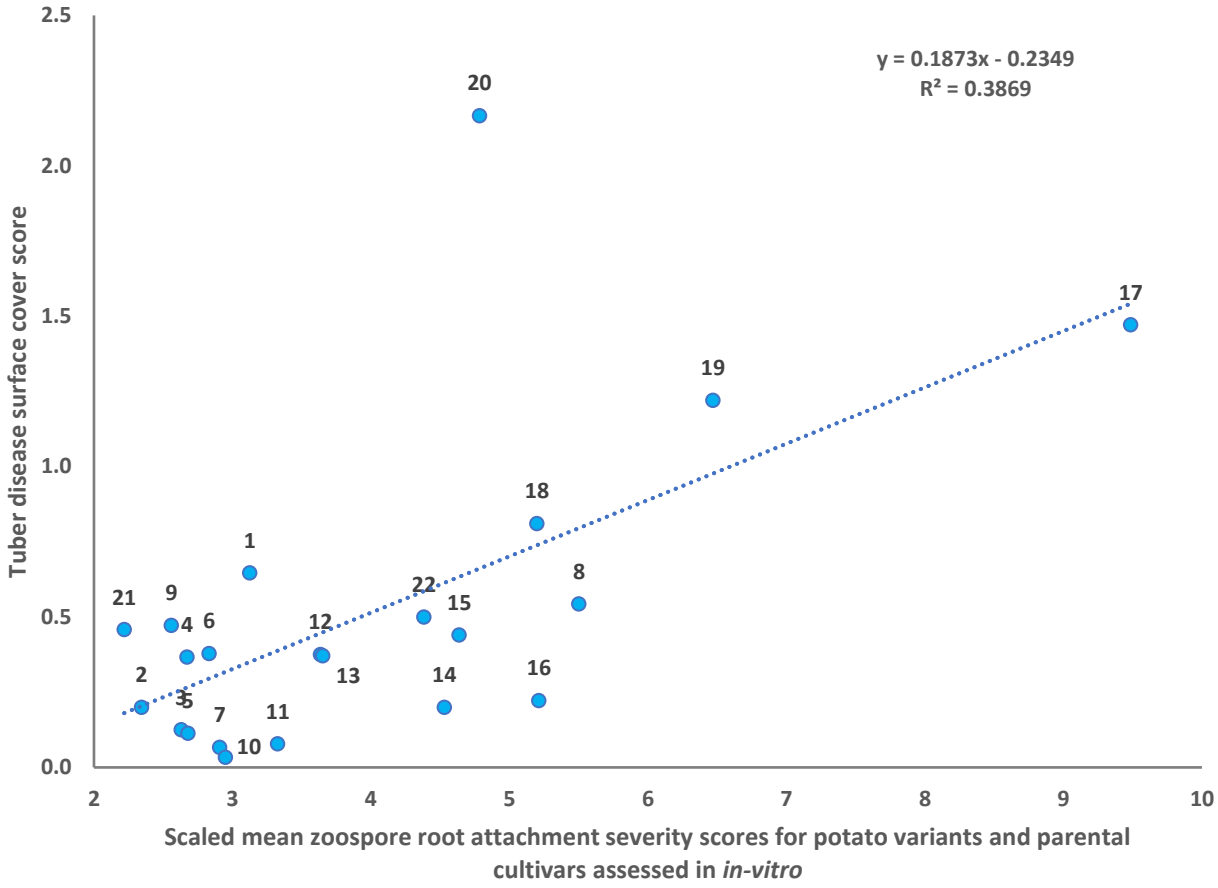


Figure 14. Relationship between mean scaled zoospore root attachment severity score of 22 potato cultivars and their variants. The glasshouse trial mean tuber disease (powdery scab) severity scores were 0 to 3 = no disease to 30% of tuber surface cover by lesions. Numbers 1 to 22 represent potato variants and their parents (see Table 6).

4.5. Discussion

This study has confirmed that a somatic cell selection strategy could be used to evaluate the potato cultivars 'Ranger Russet', 'Russet Burbank', 'Desiree', and a 'Desiree' variant, 'Shepody', and 'Sebago' could be used to select regenerated host variants for resistance to *S. subterranea* zoospore root attachment. The results showed that somaclonal selection techniques can be used to obtain variants of multiple potato cultivars that have reduced zoospore binding to roots and subsequent reduced root infection. This also led to reduced tuber powdery scab when the variant host plants were challenged under glasshouse trial conditions. The random selection of somaclonal variants for potato disease resistance has been shown to

be beneficial in previous studies, which have been conducted to obtain somaclonal variants with increased disease resistance. Application of somaclonal variation will depend on the frequency of specific, stable host variants and the efficiency of the procedures used to select these variants (Van den Bulk, 1991).

There are some advantages from using clonal selection for disease resistance traits, such as those achieved in this study, compared to conventional breeding methods. For example, there is minimal genetic change involved in the host plants, and derived variants are likely to retain the majority or all of the parent cultivar's desirable traits (Shepard et al., 1980, Karp, 1991, Wilson et al., 2009). Potato is a good candidate for somaclonal selection since it can easily be grown from single cells and callus (Shepard and Totten, 1977, De García and Martínez, 1995, Curry and Cassells, 1999).

Of the 93 somaclonal variants generated from six parents, one third showed significantly enhanced resistance to *S. subterranea* zoospore root attachment compared to their unselected parents. Previous studies found that 20 to 33% of regenerated variants showed greater resistance to common scab than the unselected parent cultivar in glasshouse assessment (Wilson et al., 2009, Wilson et al., 2010). A glasshouse trial confirmed the in vitro bioassay results. Selected variants and their parents with enhanced resistance to zoospore root attachment were tested in the glasshouse trial, and they generally maintained consistent resistance to tuber powdery scab (Figure 14).

These results indicate that the somatic cell selection strategy is effective for selecting resistant variants of an unselected parent cultivar. For resistant parent cultivars, somatic cell selection did not greatly increase host resistance. For instance, cultivar 'Desiree' is highly resistant to zoospore root attachment, with the only enhanced resistance variant 'D 27 05 4' exhibiting a 26% reduction in zoospore root attachment score relative to the parent cultivar. However, results were more encouraging for susceptible parent cultivars. For example, both 'Shepody' variants with enhanced resistance, 'SHE 11 09 1' and 'SHE 23 07 1', exhibited, respectively, 45% and 32% reductions in zoospore root attachment scores, when compared to their parent. In contrast, the 'Sebago' variant with enhanced resistance, 'SE 04 01 2', exhibited a 54% reduction in zoospore root attachment score compared with the parent cultivar. 'RR 02 12 1', the most resistant variant of 'Ranger Russet', recorded a 92% reduction in zoospore root attachment score compared to the parent cultivar.

Selection of somaclonal variants of potato cultivars through in vitro derived genetic variation has been successfully used to obtain enhanced resistance to several potato pathogens including *P. infestans* (Matern et al., 1978), *V. dahliae* (Sebastiani et al., 1994), *A. solani* (Shepard et al., 1980), *F. oxysporum* (Behnke, 1980) and *S. scabiei* (Wilson et al., 2009, Wilson et al., 2010).

The enhanced resistance observed in this study should be confirmed in further glasshouse and field trials. In this study, the mean tuber yields were low from 1.6 g to 34.7 g per pot. However, there is evidence that the enhanced resistance created by somatic clonal selection is robust and genetically stable. For example, Wilson et al. (2010) found pathogenicity and toxin tolerance to common scab remained consistent over a period of 6 years in glasshouse and field trials. In the present study, all the regenerated variants were selected according to responses to in vitro zoospore root attachment. Since zoospore root attachment can be detected at an early stage of host growth, compared to standard glasshouse and field challenge trials, this assay only requires root tissue samples from tissue-cultured propagated potato plantlets, saving time and money. The somatic cell selection approach described here is highly efficient for producing variants. The in vitro zoospore root attachment assay is a rapid way to screen host resistance to powdery scab, but standard cultivars with known resistance to the disease are required across all the screening as references for linear scaling of zoospore root attachment severity scores. This system is efficient for development of enhanced host resistance to potato powdery scab caused by *S. subterranea* in current commercial cultivars.

Chapter 5. Comparative proteomic analysis of potato roots from resistant and susceptible cultivars to *Spongospora subterranea* zoospore root attachment in vitro

5.1. Abstract

Potato (*Solanum tuberosum* L.) has broad cultivar variations in cultivar resistance to tuber and root infections by the soilborne, obligate biotrophic pathogen *Spongospora subterranea*. Host resistance has been recognised as an important approach in potato disease management, whereas zoospore root attachment has been identified as an effective indicator for the host resistance to *Spongospora* root infection. However, the mechanism of host resistance to zoospore root attachment is currently not well understood. To identify the potential basis for host resistance to *S. subterranea* at the molecular level, 12 potato cultivars differing in host resistance to zoospore root attachment were used for comparative proteomic analysis. In total, 3723 proteins were quantified from root samples across the twelve cultivars using a data-independent acquisition mass spectrometry approach. Statistical analysis identified 454 proteins that were significantly more abundant in the resistant cultivars; 626 proteins were more abundant in the susceptible cultivars. In resistant cultivars, functional annotation of the proteomic data indicated that Gene Ontology terms related to the oxidative stress and metabolic processes were significantly over-represented. KEGG pathway analysis identified that the phenylpropanoid biosynthesis pathway was associated with the resistant cultivars, suggesting the potential role of lignin biosynthesis in the host resistance to *S. subterranea*. Several enzymes involved in pectin biosynthesis and remodelling, such as pectinesterase and pectin acetylesterase, were more abundant in the resistant cultivars. Further investigation of the potential role of root cell wall pectin revealed that the pectinase treatment of roots resulted in a significant reduction in zoospore root attachment in both resistant and susceptible cultivars. This study provides a comprehensive proteome-level overview of resistance to *S. subterranea* zoospore root attachment across 12 potato cultivars and has identified a potential role for cell wall pectin in regulating zoospore root attachment.

Keywords: *Spongospora subterranea*, *Solanum tuberosum*, label-free proteomics, DIA, zoospore root attachment, host resistance

5.2. Introduction

The soilborne obligate biotrophic plant pathogen, *Spongospora subterranea* f. sp. *subterranea*, is responsible for root and tuber diseases, that cause quality reduction and yield losses in potato production (Harrison et al., 1997, Falloon et al., 2003, Merz, 2008, Merz and Falloon, 2009, Tegg et al., 2016, Wilson, 2016, Balendres et al., 2016b). Management of *Spongospora* diseases is difficult and requires a range of approaches, including crop rotation, chemical applications, and selection of disease- or pathogen-free seed tubers (Braithwaite et al., 1994b, Falloon et al., 1996, Tuncer, 2002, Larkin and Griffin, 2007, Shah et al., 2012). However, the most efficient strategy to control these diseases is likely to be the planting of resistant cultivars (Falloon, 2008). Despite recent research into understanding the biochemical processes underlying *Spongospora*-potato interactions (Balendres et al., 2016b, Balotf et al., 2021a), the mechanism of resistance to *S. subterranea* tuber and root infections have not yet been elucidated.

Proteomics are powerful tools for discovery of potential host resistance mechanisms and the protein biomarkers involved in host plant responses to pathogen infections (Balotf et al., 2022c). For example, quantitative proteomics was used to explore potato resistance to bacterial wilt caused by *Ralstonia solanacearum* (Park et al., 2016), leaf late blight caused by *Phytophthora infestans* (Xiao et al., 2019), and wart disease caused by *Synchytrium endobioticum* (Szajko et al., 2020). A recent study by Balotf et al. (2021a) also compared the in planta transcriptome and proteome of *S. subterranea* invading susceptible and resistant potato cultivars. Their results suggested that downregulation of enzyme activity and nucleic acid repair in a resistant cultivar could be related to resistance to *S. subterranea*.

Initial zoospore root attachment is a critical phase in disease development for this pathogen (Balendres et al., 2016b). In our previous study (Yu et al, Chapter 3), development was reported of an in vitro bioassay that efficiently assessed potato cultivar resistance to *S. subterranea* root disease, based on the efficiency of zoospore attachment. Reduced zoospore root attachment will likely manifest as less severe tuber and root infections (Yu et al, Chapter 3). During this critical stage of early infection, zoospores bind to the outside of host root cells and inject their contents through cell wall (Balendres et al., 2016b). Successful attachment of zoospores on potato roots either leads to the development of a plasmodium, in each cell, which subsequently develops zoosporangia, from which secondary zoospores are released (Ledingham, 1935), or to the formation of root galls and production of resting spores (Braselton, 1992). To date,

however, the basis for host resistance to *S. subterranea* zoospore root attachment is not well understood at the molecular level. To address this knowledge gap, label-free proteomic analysis was used to compare the root tissues of twelve potato cultivars with different resistance to zoospore root attachment, leading to the identification of a range of candidate pathways and proteins that may influence the host resistance to zoospore root attachment.

5.3. Materials and methods

5.3.1. Plant materials, and tissue cultures

Twelve potato cultivars with differential responses to zoospore attachment (Yu et al, Chapter 3) were selected for detailed analysis. These included six resistant (R) cultivars ('Gladiator', 'Granola', 'Toolangi Delight', 'Russet Burbank Ruen', 'Tolaas') and six susceptible (S) cultivars ('Iwa', 'Nicola', '10086', 'Shepody', 'Ida Rose', 'Kranz', 'Russet Nugget'). Plants were maintained in tissue cultures in liquid potato multiplication (LPM) medium, growing under a 16 h photoperiod, using white fluorescent lamps (65 µmol/m^2/s), at 22℃. The constituants of LPM medium included 4.43 g/L MS salts, 30 g/L sucrose, 0.5 g/L casein hydrolysate, 0.04 g/L ascorbic acid, at pH 5.8.

5.3.2. *Spongospora subterranea* inoculum preparation and zoospore germination

Sporosorus inoculum were obtained from powdery scab-infected potato tubers of cultivar 'Kennebec' harvested from a commercial crop grown on the northwest coast of Tasmania, Australia, in 2020. The tubers were washed with running tap water and air-dried in a cool place for 1 to 2 d. Powdery scab lesions were scraped as finely as possible using a scalpel and passed through a 600 µm mesh sieve. The resulting resting spore inoculum samples were kept at room temperature in the dark.

Zoospores were released by incubating 3 g sporosorus inoculum in 10 mL of Hoagland's solution in a McCartney bottle at 15℃ in the dark. Hoagland's solution was prepared following a standardised recipe (Falloon et al., 2003). The constituents of Hoagland's solution were dissolved in deionized water, including KNO_3, 253 mg/L; $Ca(NO_3)_2 \cdot 4H_2O$, 722 mg/L; KH_2PO_4, 2.3 mg/L; $MgSO_4 \cdot 7H_2O$, 120 mg/L; NH_4NO_3, 40 mg/L; Fe-EDTA, 20 mg/L; H_3BO_3, 140 µg/L;

KCl, 400 µg/L; $MnSO_4 \cdot H_2O$, 63 µg/L; $ZnSO_4 \cdot 7H_2O$, 115 µg/L; $CuSO_4 \cdot 5H_2O$, 50 µg/L; and $Na_2MoO_4 \cdot 2H_2O$, 22 µg/L. Zoospore numbers were determined by taking a 1 µL subsample and counting the numbers of zoospores present using light microscopy (DM 2500 LED, Leica Microsystem, Germany) at 200× magnification.

5.3.3. Zoospore root attachment assay

Confirmation of the relative resistance of each cultivar to zoospore root attachment was obtained using in vitro zoospore root attachment assays. Following a 2-week growth period in LPM, three primary roots (technical replicates) excised from each plant (biological replicates) of each cultivar or clone were washed in deionized water. A 10 mm section from the lower maturation region of each root was taken. The washed root segments were transferred into a treatment container (30 × 130 mm) and evenly immersed in 60 mL of deionized distilled water (DDW) containing 1000 zoospores/mL. This zoospore treatment was incubated for 48 h at 15°C in the dark, which has previously been shown to be optimal for zoospore root attachment (Yu et al, Chapter 3). The cultivars and variants were then examined in batches of eight, and each batch included two reference cultivars, 'Iwa' (very susceptible to powdery scab) and 'Gladiator' (very resistant). Five randomly chosen fields of view were used to count the number of zoospores attached to each root segment. Evaluation of each cultivar was carried out using three independent biological replicates (three plants), with each biological replicate consisting of three technical replicates (three roots from each plant).

Score of zoospore root attachment for each cultivar/clone in the screenings was standardized according to the reference cultivars, 'Gladiator' and 'Iwa', present in each batch, with the first batch screening serving as a reference point (G1 + I1) to adjust for batch differences in each subsequent batch. This was done by calculating a reference point correction coefficient (η_n) in each batch.

$$\eta_n = \frac{Gn + In}{G1 + I1}$$

Where Gn and In are the zoospore attachment scores for 'Gladiator' and 'Iwa' in batch n. This coefficient was used to linearly scale the attachment score for each cultivar/line.

Following checks of normality and homogeneity of variance, all data were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 27. Zoospore root attachment scores were

analysed using a one-way ANOVA, followed by Protected Fisher's LSD tests to determine mean differences at ($p \leq 0.05$).

5.3.4. Protein extraction and peptide sample preparation

Root proteins extracted from all 12 potato cultivars were then compared. Plants were grown in LPM medium for four weeks to provide sufficient root tissue, after which roots were excised for protein extraction. There were 4 independent biological replicates (plants) per cultivar. The total root tissue taken from each plant was washed with DDW and homogenised using a Fast Prep-24 bead beater (4000 rcf for 60 s) in PowerBead tubes containing ceramic 2.8 mm beads (Qiagen, Hilden, Germany) in 200 µL of protein extraction buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% glycerol, 10 mM Dithiothreitol (DTT)) and 20 µL protease inhibitor (one tablet of cOmplete Mini EDTA-free; Roche Diagnostics, North Ryde, NSW, Australia). The extracts were centrifuged at 12,000 rcf for 8 min at 4℃, the supernatant was collected and six volumes of cold acetone (-20℃) were added, and the sample was mixed by gently shaking the tubes five times. The precipitated protein sample was collected by centrifugation at 6800 rcf for 5 min at 4℃. The resulting pellets were each washed three times in chilled acetone before being dissolved in lysis buffer (6 M urea, 2 M thiourea).

The plant protein samples were quantified using the Qubit protein assay (Thermo Fisher Scientific, Waltham, MA, USA), and were diluted to 0.5 mg/mL in lysis buffer (6 M urea, 2 M thiourea). Aliquots of 30 µg protein were sequentially reduced using 10 mM DTT overnight at 4°C, were alkylated with 50 mM iodoacetamide for 2 h at ambient temperature, and then digested with 1.2 µg proteomics-grade trypsin/LysC (Promega, Madison, WI, USA) as per the SP3 protocol (Hughes et al., 2019). The digests were then acidified by addition of trifluoroacetic acid to 0.1%, and then centrifuged at 21,000 rcf for 20 min to collect peptides. Peptides were then desalted using ZipTips (Merck, Darmstadt, Germany), as per the manufacturer's instructions.

5.3.5. Proteomic analyses, and data processing and analyses

Peptide samples of approximately 1 µg were separated and analysed using an RSLCnano Ultimate 3000 and Q-Exactive HF mass spectrometer fitted with a nanospray flex ion source (Thermo Scientific, Waltham, MA, USA), essentially as described previously (Balotf et al.,

2021b). DIA-MS raw files were processed using Spectronaut software (v14.7, Biognosys AG, Schlieren, Switzerland) with the directDIA experimental analysis workflow. A spectral library was first generated by searching the DIA-MS data against the *Solanum tuberosum* L. UniProt reference proteome (UP000011115), comprising 53,106 entries, using the Pulsar search engine. This library, comprising 33,236 non-redundant peptide sequences and 4746 protein groups, was then used for targeted re-extraction of DIA-MS2 spectra and relative protein quantification between samples. With the exception of excluding single-hit proteins, default Spectronaut settings were used for protein quantification and normalization.

The Spectronaut protein group pivot report was imported into Perseus software for further processing. First, protein intensity values were log_2-transformed, and proteins identified in fewer than 50% of the samples were filtered out, with the remaining missing values then replaced using Perseus default settings. Differentially abundant proteins were identified based on t-test comparisons of all replicates (n = 4) of the six resistant and six susceptible cultivars, with a False discovery rate (FDR) < 0.05 and s0 value of 0.1 used as the criteria to define significant proteins. Gene Ontology classification and enrichment analysis of significant proteins were provided by the UniProt database (www.uniprot.org) and DAVID bioinformatics resources 6.8 (https://david.ncifcrf.gov; accessed in November 2020), and the KEGG database (www.genome.jp/kegg/) was used for pathway analysis. Perseus software was used to generate principal component analysis (PCA) and volcano plots.

5.3.6. Pectinase treatment

To provide further evidence of a possible role of root surface pectin, the potato cultivars ‘Iwa’ (S) and ‘Gladiator’ (R) were assessed for the impact of pectinase treatments of roots on the capacity and efficiency of zoospore root binding. Plantlets from tissue culture were further cultured in liquid potato multiplication (LPM) medium, under a 16 h photoperiod, using white fluorescent lamps (65 µmol/m^2/s) at 22°C. The constituents of LPM medium include Murashige and Skoog (MS) Salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; at pH 5.8. Pectinase solutions were made at four concentrations containing 0, 1, 2 or 3 mg pectinase (P4716; Sigma-Aldrich, Bayswater, Australia) each in 1 mL of 50 mM sodium acetate buffer at pH 5.0. The enzyme activity of pectinase at 37°C is 0.68 ± 0.020 µmol$min^{-1}mL^{-1}$ (Roy et al., 2018). Three primary roots (technical replicates) from each plant of each cultivar (biological replicates) were collected from propagated plantlets and

rinsed thoroughly with DDW. This experiment was carried out with three technical and three biological replicates. A segment trimmed to a length of 20 mm of the lower part of the root maturation region of each root was selected from each individual root (Yu et al, Chapter 3). The root segments comprising each biological replicate were added into one of three 1.5 mL capacity Eppendorf tubes, and 45 µL of 50 mM sodium acetate buffer and 5 µL pectinase solution were added into each of tubes. All three tubes were then incubated at 37℃ for 0.5 h (Roosdiana et al., 2013). This experiment was repeated at four selected pectinase concentrations (0, 1, 2, or 3 mg/mL). All treated root segments were then assessed for efficiency of zoospore root attachment by the in vitro zoospore root attachment assay.

5.4. Results

5.4.1. *Spongospora subterranea* zoospore attachment to potato roots of resistant and susceptible cultivars

Potato cultivars with a range of host resistance to *S. subterranea* zoospore root attachment, were selected and assessed using an in vitro assay; the results are summarized in Figure 15. Differences ($p < 0.001$) were detected amongst the twelve cultivars for zoospore root attachment. The mean scores of zoospore root attachment for cultivars designated R1 to R6 (Figure 15) ranged from 1 to 3, and S1 to S6 ranged from 9 to 13. This shows that R1 to R6 were much more resistant to the zoospore root attachment than S1 to S6, providing a basis for molecular analysis of the cultivars.

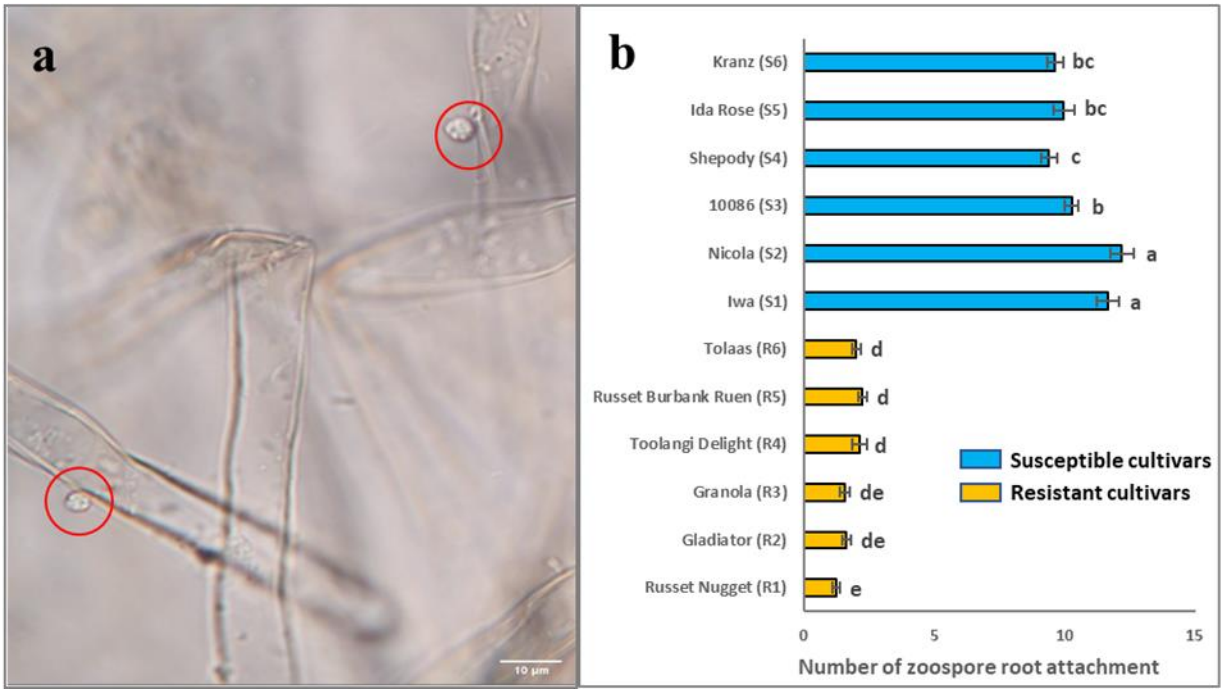
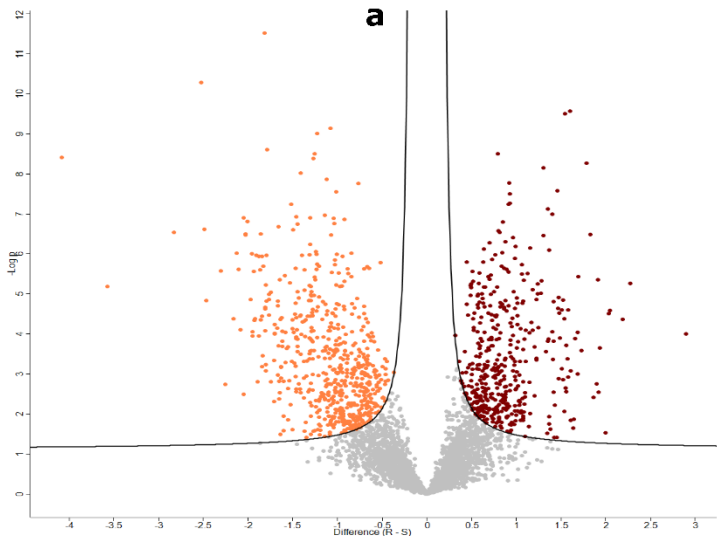
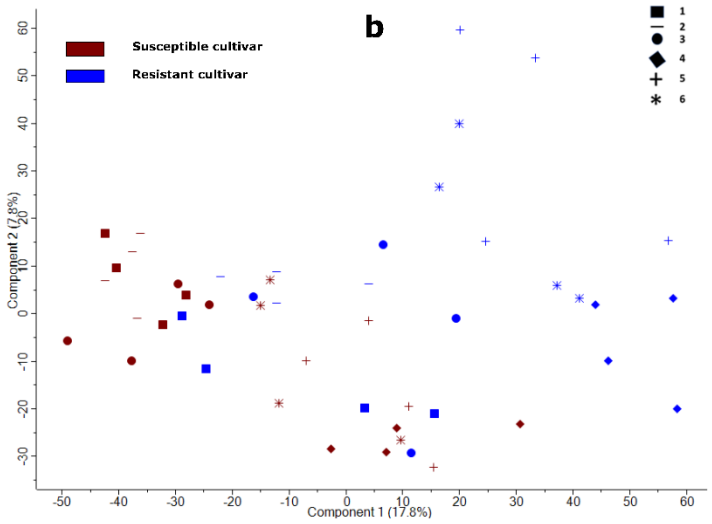


Figure 15. *Spongospora subterranea* zoospore root attachment to root hairs of potato cultivars. (a) Zoospore (red circles) attached to a potato root hair. (b) Mean numbers of zoospores attached to roots of 12 potato cultivars 48 h after incubation. Three independent biological replicates (from different plants) were assessed for each cultivar. Horizontal bars represent the standard errors ($n = 3$). $p < 0.001$. Different letters above bars indicate significant zoospore root attachment as determined by LSD (0.05) = 0.73. The blue bars represent *S. subterranea* susceptible cultivars (S), and yellow bars represent resistant cultivars (R) to zoospore root attachment.

5.4.2. Overview of the proteins in potato roots identified by label-free quantitative proteomics

Using a DIA-MS approach, 3723 proteins were quantified across the 48 samples comprising four replicates of each of the 12 cultivars (see Supplemental Excel file 1). Statistical analysis showed that 626 proteins were significantly less abundant in the resistant cultivars, whereas 454 proteins were significantly more abundant in these cultivars (Figure 16a and listed in full in Supplemental Excel file 2). Initially, PCA of the dataset comprising all proteins showed only partial separation of resistant and susceptible cultivar samples (Figure 16b). Although samples from the susceptible cultivars clustered tightly, those from the resistant cultivars were more dispersed and, in particular, root samples from R1, R2, and R3 overlapped with the samples from susceptible cultivars (Figure 16b). Subsequent PCA of the protein subset identified significant differences between resistant and susceptible cultivars showing greater separation of the two cultivar groups, but nonetheless indicated greater variation overall in the resistant cultivars (Figure 16c).


a
-Log
Difference (R - S)


b
Susceptible cultivar
Resistant cultivar
Component 2 (7.8%)
Component 1 (17.8%)

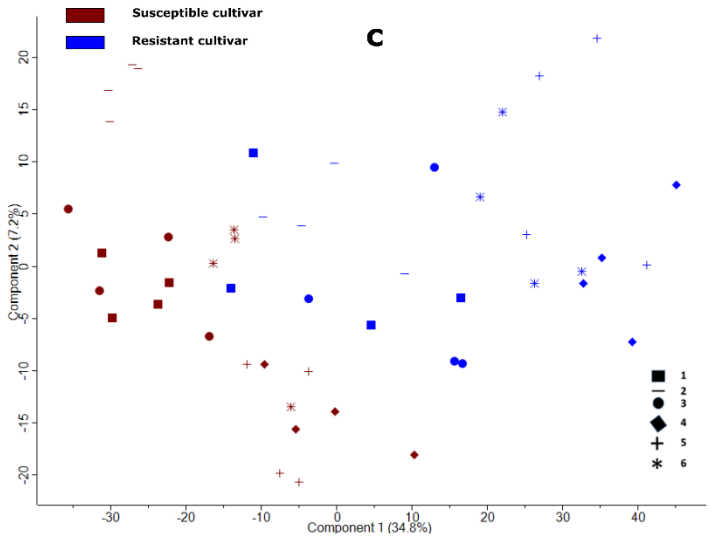

c
Susceptible cultivar
Resistant cultivar
Component 2 (7.2%)
Component 1 (34.8%)

Figure 16. (a) Volcano plot displaying the results of *t-test* comparisons of susceptible and resistant potato cultivars. The two lines show the threshold (FDR < 0.05; s0 = 0.1) separating the proteins increased (dark red data points) and decreased (orange data points) in resistant cultivars. (b) Principal component analysis (PCA) of the dataset comprising all proteins quantified across 12 potato cultivars. (c) PCA of the dataset restricted to the 1080 significant proteins between resistant and susceptible potato cultivars.

5.4.3. Overall functional classification of differentially abundant proteins

Gene ontology (GO) analysis was used to categorise the sets of differentially abundant proteins (DAPs) into groups according to molecular function (MF), cellular component (CC), and biological process (BP) GO terms (Figure 17a and b). In total, 19 functional categories were captured by the set of proteins that were significantly more abundant in resistant than susceptible cultivars, including several proteins related to oxidative stress (e.g., BP 'response to oxidative stress' and MF 'peroxidase activity') or metabolic processes (e.g., CC 'mitochondrion') (Figure 17a). In contrast, GO terms related to protein biosynthesis, such as CC 'cytosolic ribosome' and BP 'protein folding,' and chloroplast function (e.g., CC terms 'chloroplast stroma' and 'chloroplast envelope'), were associated with DAPs that were less abundant in the resistant cultivars than in the susceptible ones (Figure 17b).

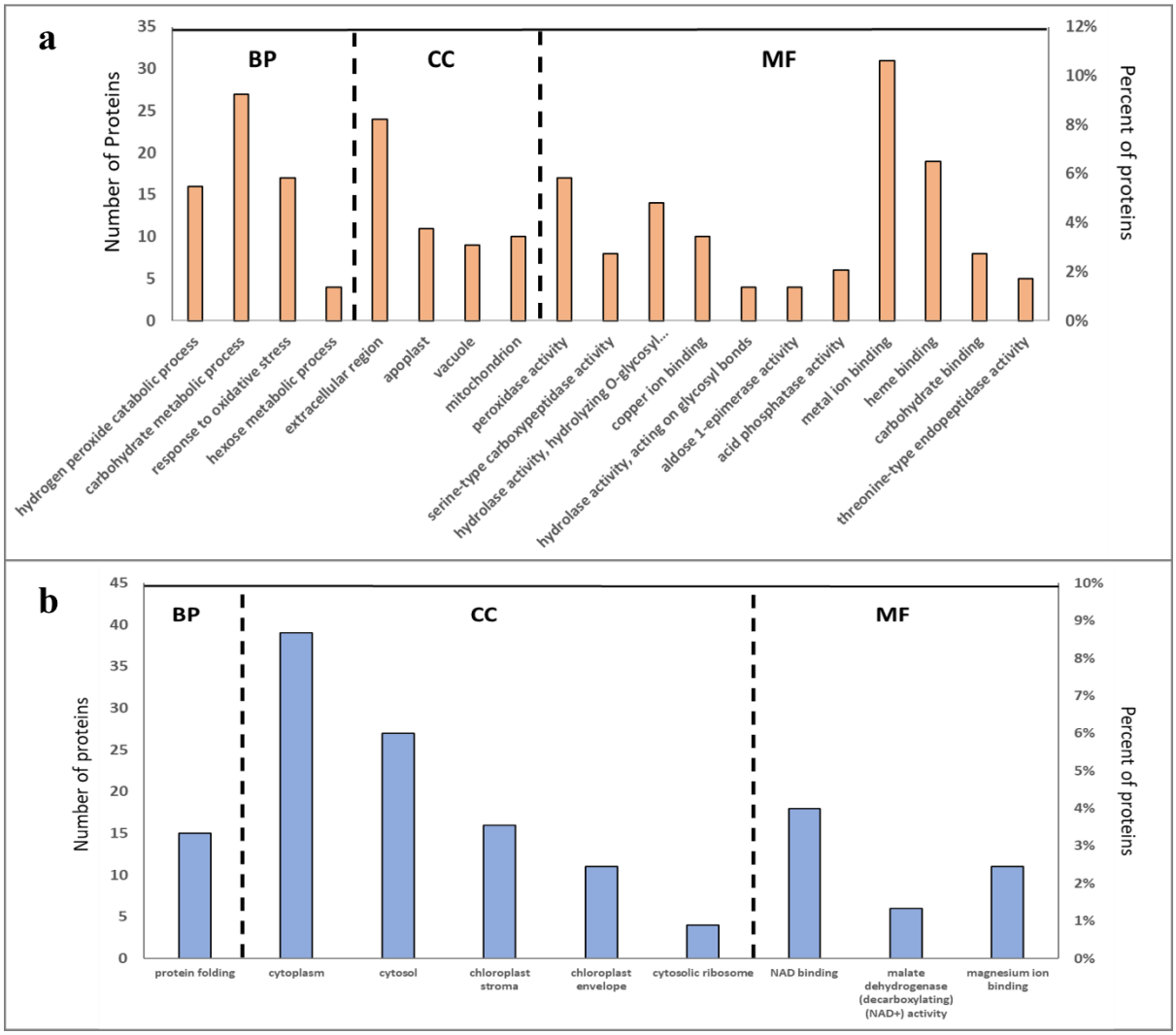


Figure 17. Classification of identified potato root proteins that were more abundant (a) or less abundant (b) in resistant cultivars from the proteome of potato (*Solanum tuberosum* L.). The proteins were classified into gene ontology (GO) categories, relating to their involvement in biological process (BP), cellular component (CC) or molecular function (MF).

5.4.4. Overall pathway analyses of differentially abundant proteins

To better understand how the metabolism of potato roots differed between resistant and susceptible cultivars, KEGG-based analysis was used to categorise the DAPs into their metabolic and genetic information pathways. The KEGG pathway enrichment analysis also showed common or specific pathways in the sets of DAPs either more or less abundant in the root tissues of resistant cultivars (Figure 18). Five pathways were identified as significant among the proteins abundant in resistant cultivars, while 17 pathways were identified as

significantly less abundant among the proteins in resistant cultivars. For proteins more abundant in resistant cultivars, most (*n* = 78) were related to metabolic pathways, including biosynthesis of secondary metabolites (*n* = 46) and phenylpropanoid biosynthesis (*n* = 22) (Figure 18). For proteins less abundant in resistant cultivars (Figure 18), two pathways were related to genetic information processing (Aminoacyl-tRNA biosynthesis) (*n* = 11) and the proteasome (*n* = 9)), while the remaining 15 significant pathways were also classified as metabolic pathways (*n* = 131). These included secondary metabolite biosynthesis (*n* = 90), antibiotic biosynthesis (*n* = 61), and carbon metabolism (*n* = 50).

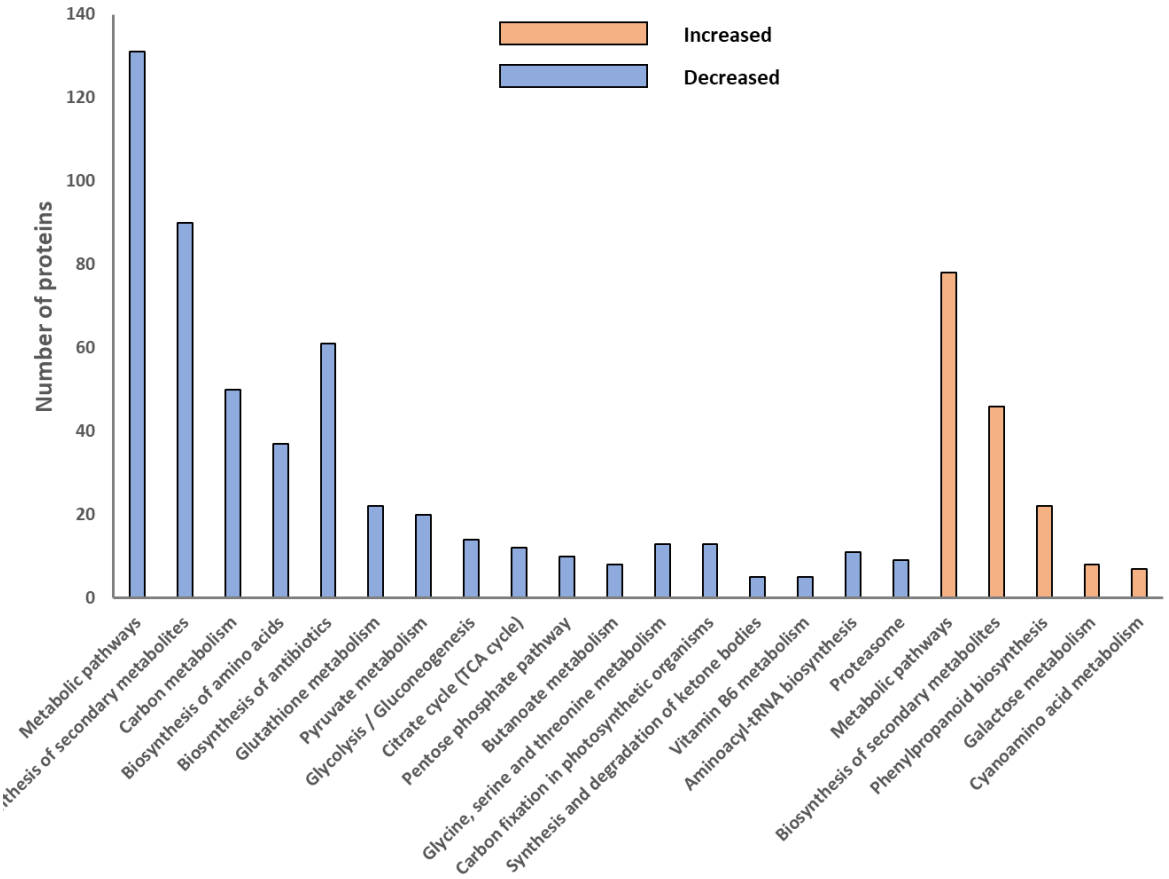


Figure 18. KEGG pathway classification and enrichment tests for proteins more or less abundant potato cultivars resistant to *Spongospora subterranea* zoospore attachment.

5.4.5. Differentially abundant proteins of root cell wall composition

In total, 39 DAPs involved in cell wall composition and modification were identified. Most (*n* = 37) were more abundant in the resistant cultivars (Table 7). Pathway analysis of the cell wall related proteins that were more abundant in resistant cultivars (Figure 19a) identified a number of significant pathways such as glycosaminoglycan degradation (*n* = 3), biosynthesis of secondary metabolite (*n* = 7), and phenylpropanoid biosynthesis (*n* = 7). Gene Ontology analysis of the cell wall DAPs that were more or less abundant in resistant cultivars according to their major biological functions are summarised in Figure 19b. In total, 30 functional categories were captured by the set of proteins that were significantly increased, including several GO terms related to oxidative stress (e.g., BP 'response to oxidative stress' and MF 'peroxidase activity'), and cell wall function (e.g., BP 'cell wall organization', 'cell wall biogenesis', 'cell wall modification' and CC 'plant-type cell wall', 'cell wall') (Figure 19b). In contrast, three functional categories (CC 'plasmodesma' and MF 'heme binding', 'metal ion binding') were associated with DAPs that were less abundant in resistant cultivars (Figure 19b). Notably, four categories involved in cell wall pectin biosynthesis and remodelling were associated with proteins that were more abundant in resistant cultivars, including MF 'pectin acetylesterase activity', 'pectinesterase inhibitor activity', 'pectinesterase activity' and BP 'pectin catabolic process'.

Table 7. Differentially abundant proteins in potato cell walls. The fold change is on a $\log_2$ scale. Positive fold changes indicate increased abundance in resistant cultivars; negative fold changes indicate reduced abundance in resistant cultivars.

Accession	Description	Fold change	Adjusted *p*-value
M1C976	Peroxidase	1.9	0.00
M1B051	Germin-like protein	1.7	0.00
M1BUZ0	Germin-like protein	1.5	0.00
M1A147	Beta-galactosidase	1.5	0.00
M1B041	Germin-like protein	1.1	0.00
M1BJ45	Pectinesterase	1.1	0.01
M1B6G3	Peroxidase	1.1	0.00

M1AQZ8	Xyloglucan	1.1	0.02
M1BFU7	Germin-like protein	1.1	0.00
M1D0Z2	Heparanase	1.0	0.00
M1BRR7	Pectin acetylesterase	1.0	0.00
M1AWV7	Polygalacturonase	1.0	0.00
M1C8D8	Pectin acetylesterase	1.0	0.00
M0ZQ51	Xyloglucan endotransglucosylase/hydrolase	0.9	0.03
M0ZJ69	Peroxidase	0.9	0.01
M1A385	Pectin acetylesterase	0.9	0.00
M1AZG9	Glycoside hydrolase family 28 protein	0.9	0.00
M1DTA0	Pectinesterase	0.9	0.02
M1BUZ2	Germin-like protein	0.9	0.00
M1AIV9	Pectinesterase	0.9	0.00
M1B6G2	Peroxidase	0.9	0.01
M1CV50	Expansin	0.9	0.01
M1CI69	Beta-galactosidase	0.8	0.00
M0ZGW4	Polygalacturonase	0.8	0.05
M1BTK5	Peroxidase	0.8	0.01
M1CE55	Peroxidase	0.8	0.01
M1A2Z2	Peroxidase	0.8	0.00
M1BYZ4	Peroxidase	0.8	0.01
M1AKA7	Periplasmic beta-glucosidase	0.8	0.01
M0ZJ70	Peroxidase	0.8	0.03
M1ARG0	Heparanase	0.7	0.02
M1BGD4	Xyloglucan	0.7	0.03
M0ZHI6	Beta-galactosidase	0.7	0.04
M1CAK9	Heparanase-2	0.6	0.00
M1CWU3	LEXYL2 protein	0.6	0.02
M1D155	Peroxidase	0.6	0.05

M1CCK1	Peroxidase	0.5	0.05
M1D1V1	Hemoglobin	-1.1	0.01
M1AY17	Peroxidase	-1.6	0.00

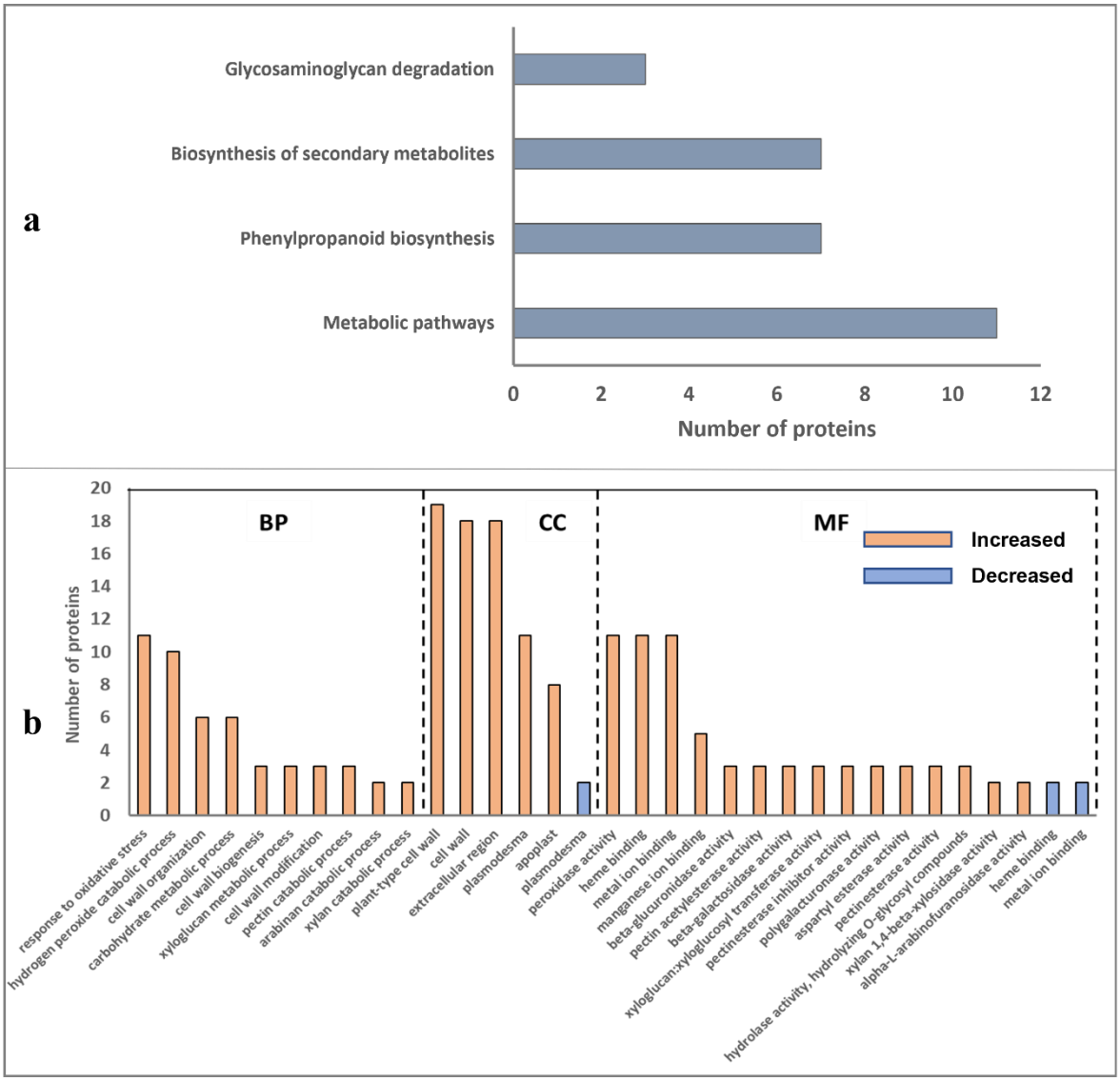


Figure 19. (a) Pathway analysis of cell walls related proteins that were increased in resistant potato (*Solanum tuberosum* L.) cultivars. (b) Classification of root cell wall related proteins that were increased (orange bars) or decreased (blue bars) in resistant cultivars by Gene Ontology (GO) categories for biological process (BP), cellular component (CC), or molecular function (MF).

5.4.6. Effects of pectinase treatment of potato roots on zoospore root attachment

The results from proteomic analysis indicated a potential role for cell wall pectin in the process of zoospore root attachment; therefore, the effect of pectinase treatment was assessed on zoospore attachment to on resistant ('Gladiator') and one susceptible ('Iwa') cultivar. Potato roots treated with pectinase exhibited a dose-dependent reduction in zoospore root attachment compared with the control in *S. subterranea* susceptible and resistant cultivars (Figure 20). Reductions in zoospore root attachment on resistant and susceptible cultivars were observed with pectinase concentrations of 1 and 2 mg/mL, with no zoospore root attachment observed following treatment with 3 mg/mL of pectinase solution.

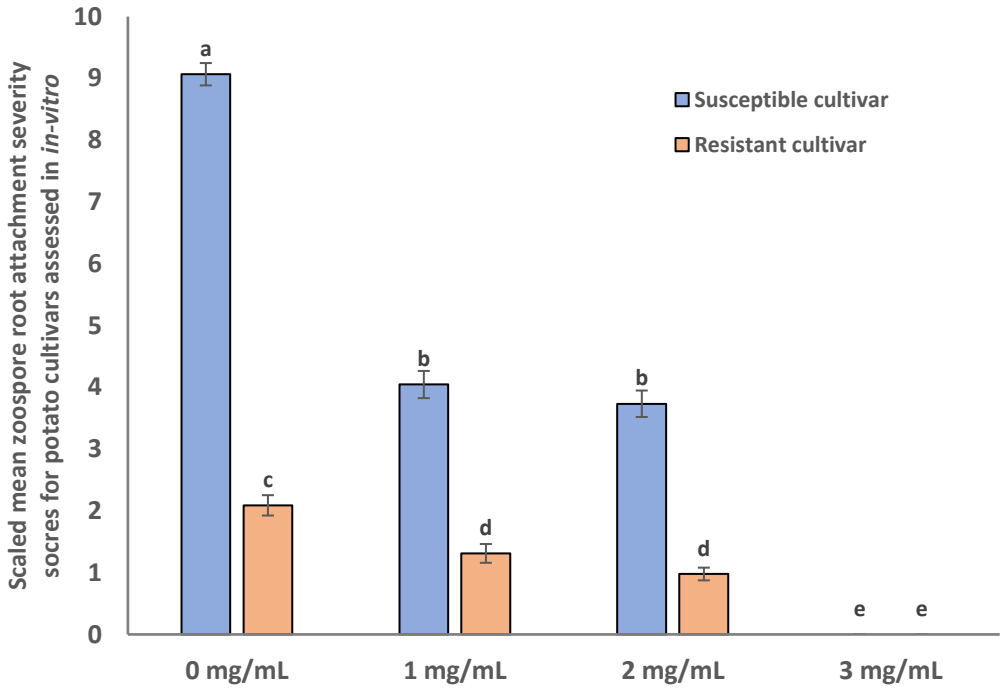


Figure 20. Mean severity of *Spongospora subterranea* zoospore infection in roots of resistant and susceptible potato cultivars, treated with different concentrations of pectinase. The vertical bars represent standard errors (n = 3). p (cultivars) < 0.001, p (concentration) < 0.001, p (cultivar × concentration) < 0.001. LSD (0.05) = 0.43. Bars that are labelled with different letters indicate values that are significantly different from each other.

5.5. Discussion

Root infection of potato by *Spongospora subterranea* is an under-explored area of research, despite the impact of infection on potato yield and subsequent tuber disease. Previously, an in vitro bioassay was developed for the rapid screening of potato resistance to zoospore root attachment (Yu et al, Chapter 3), the precursor to root infection. Using this assay in the current study, a very clear difference in zoospore root attachment was demonstrated between the six resistant and six susceptible cultivars selected. Subsequently, label-free proteomics was used to analyse root tissue from this set of twelve cultivars and identified proteins that were significantly different between the groups of resistant and susceptible potato cultivars. The zoospore root attachment assay revealed significant reductions in zoospore attachment in all resistant cultivars, but also some variation between cultivars, which may account for the greater dispersion in proteomic data for the resistant cultivars (Figure 16).

Analysis of the proteomic profile of potato roots revealed that most identified DAPs which were increased in resistant cultivars were for GO terms related to oxidative stress and metabolic processes, including ‘response to oxidative’, ‘peroxidase activity’ and ‘mitochondrion’. Peroxidases are well-known pathogenesis-related proteins that protect host tissues from pathogen attack, by producing physical barriers through mediating undefined cell wall components (Mehdy, 1994). They are reportedly involved in oxidative stress induced by pathogenic agents and the activation of defence-related activities in potato (Wu et al., 2009). Similarly, peroxidase activity has been found to play a key role in defending plants against bacterial and fungal pathogens (Wu et al., 1997). Peroxidases are also involved in phenol oxidation, IAA oxidation, lignification, plant defence, and plant cell elongation regulation (Schmid and Feucht, 1980, Boordman et al., 1981, Hammerschmidt et al., 1982, Goldberg et al., 1986, Beffa et al., 1990). Increases in peroxidase activity have been correlated with resistance in many plants including rice, tomato, and wheat. In these hosts, peroxidases are involved in the polymerisation of proteins and lignin or suberin precursors in plant cell walls, which could inhibit zoospore attachment and penetration (Young et al., 1995, Saikia et al., 2004). For proteins assigned to metabolic processes in resistant cultivars, they have important roles in the metabolism of carbohydrates, amino acids, nucleotides, and vitamins. These metabolic processes take place in organelles including the cytosol, chloroplast, mitochondria, and peroxisomes (Lim et al., 2012).

KEGG pathway analysis of the DAPs that were increased in resistant cultivars, identified metabolic pathways involved with the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, and cyanoamino acid, and galactose metabolism. Lignin biosynthesis, which is a part of phenylpropanoid metabolic process, contributes to resistance against pathogens in plants (Zhao and Dixon, 2014). Li et al. (2021) showed that phenylpropanoid pathway was associated with resistance to potato wart disease. The plasmodiophorid soilborne pathogen *Plasmodiophora brassicae*, which causes clubroot of brassicas, can involve changes in the cell wall composition of host roots (Lahlali et al., 2017, Ciaghi et al., 2019). Several genes involved in phenylpropanoid metabolic processes and cell wall synthesis were also upregulated in the transcriptome analysis of clubroot-infected *Brassica oleracea* (Ciaghi et al., 2019). Therefore, the establishment of mechanical barriers such as cell wall reinforcement of host root is likely to be a part of mechanism behind plant resistance/tolerance mechanisms against *P. brassicae* (Yadav et al., 2020, Rolfe et al., 2016). Balotf et al. (2022a) showed that the phenylpropanoid metabolic process plays a critical role in resistance of potato cultivars against root infection by *S. subterranea*. Their transcriptome analysis demonstrated upregulation of phenylpropanoid metabolic process and lignin genes in a resistant cultivar, but not in a susceptible cultivar. Our results from the proteomic analysis of twelve potato cultivars significantly expand on these previous findings and further suggest that lignin synthesis and cell wall thickening in the potato roots is likely to be an obstacle for *S. subterranea*. Therefore, constitutive and responsive gene/protein expression strategies are used by potato plants to increase resistance against *S. subterranea*.

The proteome study showed that several enzymes involved in pectin biosynthesis and remodelling were more abundant in resistant cultivars (Figure 19b). This included pectin acetylesterase, which, in *Nicotiana tabacum*, serves as a key structural regulator by changing the precise status of pectin acetylation to affect remodelling and physiochemical characteristics of cell wall polysaccharides (Gou et al., 2012). Activity of pectinesterase, a pectolytic enzyme that hydrolyses the ester linkages in pectin molecules; Maldonado and Strasser de Saad (1998), and inhibitor activity, were also abundant in resistant cultivars, as was the pectin catabolic process pathways, resulting in the degradation of pectin (Choi et al., 2020). Pectin on plant root cell walls has been demonstrated to induce rapid attachment of *Phytophthora cinnamomic* zoospores, implying that pectin-like materials on plant root surfaces may act as recognition signals, resulting in zoospore root attachment (Byrt et al., 1982b, Irving and Grant, 1984, Grant et al., 1985). The present in vitro study showed that potato roots pre-treated with pectinase had

reduced zoospore attachment, which also indicates an important role of potato root pectin in host resistance to zoospore root attachment. In this study, the effect of pectinase treatment on root morphology and plant growth was not analysed. However, it would be interesting to investigate the potential for the in vitro manipulation of cell wall pectin in modifying zoospore attachment and protection.

In summary, in the results of this study provide increased understanding of the constitutive basis of host resistance to zoospore root attachment among potato cultivars, which represent two ends of the spectrum of root resistance to zoospore attachment. Several candidate pathways and proteins were also identified that have the potential to influence cultivar resistance to zoospore root attachment. The biological importance of root pectin for zoospore root attachment has also been confirmed. An important unresolved issue in this study was how any of these proteins responded to in situ plant-pathogen interactions, which should be addressed in future research. However, this study is the first to examine the differences across a range of potato cultivars with different levels of resistance to *S. subterranea* on a proteomic level. This represents an important set of data from which to start exploring functional aspects of host resistance to *Spongospora* tuber and root infections.

Chapter 6. Enzymatic investigation of *Spongospora subterranea* zoospore attachment to roots of potato cultivars resistant or susceptible to powdery scab disease

6.1. Abstract

For potato (*Solanum tuberosum* L.) crops, host resistance is the most effective and sustainable strategy for management of potato root and tuber diseases caused by the plasmodiophorid, *Spongospora subterranea*. Zoospore root attachment is a critical phase in pathogen infection, but the mechanisms underlying zoospore root attachment remain unknown. This study investigated the potential roles of surface root cell wall polysaccharides and proteins in zoospore root attachment by combining transcriptomic and proteomic datasets of a resistant and a susceptible potato cultivar. Effects of enzymatic removal of root cell wall proteins, *N*-linked glycans or polysaccharides on *S. subterranea* attachment to root tissue of a resistant and a susceptible potato cultivar. Subsequently, mass spectrometry analysis of peptides released by trypsin shaving (TS) of root segments identified a total of 1235 proteins of which 262 were differentially abundant between the resistant and susceptible cultivars. In particular, proteins associated with glutathione metabolism and lignin biosynthesis, were more abundant in the resistant cultivar. Comparison with whole-root proteomic analysis of the same resistant and susceptible cultivars led to identification of 226 proteins that were unique to the TS dataset, of which 188 were significantly different between cultivars. Among these, the pathogen defence-related cell wall protein stem 28 kDa glycoprotein and two major latex proteins were identified as significantly less abundant in the resistant cultivar compared to the susceptible cultivar. A further major latex protein was detected at reduced levels in the resistant cultivar in both TS and whole-root proteomic datasets. In contrast, in the TS-specific dataset, three glutathione *S*-transferase (GST) proteins were more abundant in the resistant cultivar, while the protein glucan endo-1,3-beta-glucosidase was detected at increased levels in both the TS and whole-root datasets. These results imply particular roles of major latex proteins and glucan endo-1,3-beta-glucosidase in the regulation of host susceptibility to *S. subterranea*.

Keywords: *Spongospora subterranea*, *Solanum tuberosum* L., trypsin shaving, host resistance, cell wall modification, proteome

6.2. Introduction

The plasmodiophorid biotrophic pathogen, *Spongospora subterranea* f. sp. *subterranea*, is a threat to sustainable potato production wherever potato crops are grown (Balendres et al., 2016b). This soil-borne pathogen infects potato tubers, underground stolons, and roots, leading to tuber and root diseases (Burki et al., 2010, Balendres et al., 2016b, Falloon et al., 2016). These diseases can cause significant economic losses where potatoes are grown as intensively managed, highly productive crops (Harrison et al., 1997, Merz and Falloon, 2009, Falloon et al., 2016). Tubers infected by *S. subterranea* lead to powdery scab that affects tuber quality and storage longevity, while root infection affects root function (absorption of water and nutrients) and can reduce tuber yields (Falloon et al., 2016). Strategies to manage *S. subterranea* diseases are very limited. In some cases, farmers may be able to select cultivars that are relatively resistant to *S. subterranea* based on market demands, but no cultivar is completely immune to *S. subterranea* infection, and substantial disease can still result in varieties that are moderately resistant. Host resistance to *Spongospora* diseases has been assessed in glasshouse and field trials, and more recently, using a rapid and robust in vitro zoospore root attachment bioassay (Yu et. al; Chapter 3).

Infection of plant hosts by zoospores is preceded by a distinct sequence of initial zoospore recognition and attachment. Pathogen reactions to a host can be modelled on this pattern, making it a promising target for disease prevention (Deacon, 1988, Raftoyannis and Dick, 2006, Amponsah et al., 2021). Zoospore root attachment has long been identified as a critical step towards host infection. *Spongospora subterranea* zoospores discharge their contents through host plant cell walls using particular ‘Rohr’ and ‘Stachel’ subcellular structures (Keskin and Fuchs, 1969, Balendres et al., 2016b). Zoosporangia are formed four to five days after zoospore root attachment occurs (Merz, 1989, Merz, 2008). A previous study (Yu et. al; Chapter 3) showed that the efficiency of zoospore root attachment differed among potato cultivars, with more zoospore root attachment occurring in cultivars that are susceptible to disease than those that are resistant. However, the mechanisms underlying zoospore root attachment are unknown.

Previous studies in other pathosystems suggest that in pathogenesis and plant resistance management, molecular interactions between host plant cell wall surface components and the infective unit of the pathogens are crucial (Callow et al., 1987, Sarkar et al., 2022). The initiation of zoospore root attachment has been associated with the production of a range of high or low molecular weight root exudates (Estradagarcia et al., 1990, Amponsah et al., 2021),

including fucosyl residues (Hinch and Clarke, 1980, Longman and Callow, 1987), pectin (Irving and Grant, 1984, Grant et al., 1985), lectins (Hardham and Suzaki, 1986), particular nomoclonal antibodies (Hardham, 1989), amino acids (Dill and Fuller, 1971), and element ions (sodium, strontium, and calcium) (Grant et al., 1986). Zoospore attachment to host roots by *Pythium* spp. was affected by plant polysaccharides, whereas *Phytophthora* spp. zoospore root attachment varied depending on presence of pectin, polyuronates, and some inorganic cations (Byrt et al., 1982a, Byrt et al., 1982b, Irving and Grant, 1984, Grant et al., 1986, Shaw and Hoch, 2007).

Enzyme studies have been used to examine zoospore-host interactions (Hinch and Clarke, 1980, Irving and Grant, 1984, Callow et al., 1987, Longman and Callow, 1987, Jones and Epstein, 1989, Jones and Epstein, 1990, Jones et al., 1991, Donaldson and Deacon, 1993, Downer et al., 2001, Dunn and Smart, 2015). Longman and Callow (1987) investigated the role of protein and polysaccharide-based surface components involved in *P. aphanidermatum* zoospores binding to the root surface of cress (*Lepidium sativum*). They found that trypsin reduced the number of zoospore root attachments, and root surface mucus polysaccharides were modified by lectin and pectinase. Downer et al. (2001) showed treatment with cellulase reduced zoosporangium development by *P. cinnamomi* in avocado roots. However, no research has yet characterized the biochemical basis of the interactions between plant roots and attaching *S. subterranea* zoospores.

In this study, the roles were investigated of protein and polysaccharide-based root surface components in potato roots from a resistant and a susceptible cultivar, using three selected enzymes (trypsin, peptide:*N*-glycosidase F (PNGase F), and cellulase). Previous research (Yu et al; Chapter 5) identified the proteomic foundation of host resistance to zoospore root attachment by analysing the whole-root proteins of resistant and susceptible cultivars using quantitative proteomics. In the present study, quantitative proteomics combined with transcriptomics were used to identify putative protein biomarkers of resistance and susceptibility in potato roots, using trypsin shaving (TS) treatments on roots of resistant and susceptible cultivars. A comprehensive understanding of the protein profile of the TS treatment of potato roots may uncover targets for strategies to prevent zoospore attachment to host roots.

6.3. Materials and methods

6.3.1. *Spongospora subterranea* sporosori collection and germination

Spongospora subterranea sporosorus samples were collected in 2020, from powdery scab diseased tubers of potato cultivar 'Kennebec' from a commercial potato field in northwest Tasmania, Australia. Diseased tubers were washed with tap water and left to air-dry in a cool and dark place for one to two d. The lesions from diseased tubers were excised with a scalpel and then passed through a 600 µm mesh sieve. *Spongospora subterranea* inoculum was stored at room temperature in the dark until use.

Zoospores were released by incubation of sporosorus samples in Hoagland's solution (HS), which contained the following components: KNO_3, 253 mg/L; $Ca(NO_3)_2 \cdot 4H_2O$, 722 mg/L; KH_2PO_4, 2.3 mg/L; $MgSO_4 \cdot 7H_2O$, 120 mg/L; NH_4NO_3, 40 mg/L; Fe-EDTA, 20 mg/L; H_3BO_3, 140 µg/L; KCl, 400 µg/L; $MnSO_4 \cdot H_2O$, 63 µg/L; $ZnSO_4 \cdot 7H_2O$, 115 µg/L; $CuSO_4 \cdot 5H_2O$, 50 µg/L; and $Na_2MoO_4 \cdot 2H_2O$, 22 µg/L; in deionized distilled water (DDW) (Falloon et al., 2003). Aliquots of 3 mg of sporosorus inoculum were added to 1.6 mL Eppendorf tubes and suspended in 1.0 mL of HS. The tubes were then incubated at 15°C in darkness in a controlled environment chamber (Plant growth chamber, Steridium Pty Ltd, Brisbane, Australia). Zoospore release was examined after 3 d, by observation of subsamples (three 1 µL subsamples were examined with five replicates included) using light microscopy (DM 2500 LED, Leica Microsystem, Germany) at 200× magnification.

6.3.2. Plant material and growth conditions

Tissue cultured plantlets of potato cultivars 'Iwa', and 'Gladiator' were propagated in potato multiplication (PM) medium, containing the ingredients: 4.43 g/L of Murashige and Skoog (MS) salts, 30 g/L of sucrose, 0.5 g/L of casein hydrolysate, 0.04 g/L of ascorbic acid, 2.2g/L of phytagel, at pH 5.8. The plantlets were grown under a 16 h photoperiod using white fluorescent lamps (65 µmol/m^2/s) at 22°C. After one month, all plantlets were transferred into PM medium minus the phytagel and were grown for a further two weeks under a 16 h photoperiod using white fluorescent lamps (65 µmol/m^2/s) at 22°C.

6.3.3. Enzyme treatments

For trypsin, 20 µg of proteomics grade (T6567; Sigma-Aldrich) was dissolved in 100 µL of 50 mM ammonium bicarbonate buffer (pH 7.8) in each vial, to achieve a concentration of 0.2 mg/mL. For PNGase F, 50 units proteomics grade (P7367; Sigma-Aldrich) was dissolved in 100 µL of high purity water to provide a concentration of 500 units/mL, and the cellulase solutions were prepared at three concentrations, containing 1, 2 or 3 mg (Cellulase Onozuka™ RS, Yakult Pharmaceutical Industry Co., Ltd.), each in 1 mL of 50 mM sodium acetate buffer: pH 5.0.

Potato roots were collected from propagated plantlets and rinsed thoroughly with DDW. For each enzyme treatment, six primary roots from each plant of each cultivar were collected from propagated plantlets, and then rinsed thoroughly with DDW. An experiment was carried out with three technical and biological replicates. A segment of the lower part of the root maturation region trimmed to a length of 20 mm was selected from each root (Yu et al, Chapter 3). Three plantlets of each cultivar were used as biological replicates, thus providing a total of 18 root segments. These root segments were divided evenly into two groups evenly. In each group, the root segments comprising each biological replicate were added into one of three 1.5 mL capacity Eppendorf tubes. For TS treatment, 45 µL of 50 mM ammonium bicarbonate buffer and 5 µL of 0.2 mg/mL trypsin solution (final concentration of 20 µg/mL) were added into each tube in group 1, and the tubes were then incubated for 5 min at 37℃ (Sigma, NSW, Australia). Then, the TS experiment was repeated after 15-, 30-, or 60-min incubation at 37℃. For Peptide:*N*-glycosidase F (PNGase F) treatment, 45 µL of 50 mM ammonium bicarbonate buffer and 5 µL of PNGase F solution (final concentration 50 units/mL) were added into each tube in group 1. All three tubes were then incubated at 37℃ for 1 h (Sigma-Aldrich). For cellulase treatment, 45 µL of 50 mM sodium acetate buffer and 5 µL of 1 mg/mL cellulase solution were added into each tube in group 1. All the tubes were incubated at 37℃ for 0.5 h (Byrt et al., 2012). The cellulase experiment was repeated for 2 mg/mL and 3 mg/mL cellulase solutions. After enzyme treatment, all the processed root segments were assessed for in vitro zoospore root attachment. In group 2 (controls), all root segments were assessed for in vitro zoospore root attachment without any pre-treatment.

6.3.4. *Spongospora subterranea* zoospore root attachment assay

All root segments were assessed using to the in vitro zoospore root attachment assay described previously (Yu et al., Chapter 3). Root segments were placed in a plastic container (30 × 130 mm) with each replicate separated by a 100 µm mesh in the container, and then incubated in the dark at 15℃ for 48 h before further examination. The numbers of zoospores attached to each root segment was quantified from five randomly selected fields of view with a light microscope at 400× magnification. A preliminary study was conducted to test the effect of root segment incubation in enzyme buffers (ammonium bicarbonate and sodium acetate) and temperature (37℃) on zoospore root attachment and the results showed that both buffers and temperature did not affect zoospore root attachment. Therefore, the factors of buffers and temperature were excluded in this study.

Score of zoospore root attachment for each cultivar/clone in the screenings was standardized according to the reference cultivars, 'Gladiator' and 'Iwa', present in each batch, with the first batch screening serving as a reference point (G1 + I1) to adjust for batch differences in each subsequent batch. This was done by calculating a reference point correction coefficient (η_n) in each batch (Yu et al, Chapter 3).

$$\eta_n = \frac{Gn + In}{G1 + I1}$$

Where Gn and In are the zoospore attachment scores for 'Gladiator' and 'Iwa' in batch n. This coefficient was used to linearly scale the attachment score for each cultivar/line.

6.3.5. Time-course trypsin incubation sample proteomic analysis and data processing

C18 ZipTips (ZTC18S096; Merck) were used to prepare peptides released into solution following the TS treatment of potato roots for proteome analysis, according to the manufacturer's instructions. Samples were dehydrated through vacuum concentration and reconstituted in 12 mL HPLC loading buffer (2% acetonitrile and 0.05% trifluoroacetic acid in water). Thermo Scientific's Ultimate 3000 nano RSLC system and Q-Exactive HF mass spectrometer, both equipped with nanospray Flex ion sources, were used to analyse peptides with nanoflow HPLC-MS/MS and Xcalibur software (ver 4.3). Three mL aliquots of each sample were initially pre-concentrated on an analytical 20 mm × 75 mm PepMap 100 C18

trapping column, followed by separation over a 60 min segmented gradient on a 250 mm × 75 mm PepMap 100 C18 analytical column kept at 45°C, at a flow rate of 300 nL/min. The MS Tune software (version 2.9) parameters used for data acquisition were: 2.0 kV spray voltage, S-lens RF level of 60 and heated capillary set to 250°C. MS1 spectra (390 to 1500 m/z) were acquired at a scan resolution of 60,000 followed by MS2 scans using a Top15 DDA method, with 20 sec dynamic exclusion of fragmented peptides. MS2 spectra were acquired at a resolution of 15,000 using an AGC target of 2e5, maximum IT of 28 ms and normalized collision energy of 27.

Mass spectrometry raw files were processed using MaxQuant software (version 1.6.5.0) and the Andromeda search engine to search MS/MS spectra against the *Solanum tuberosum* L. UniProt reference proteome (UP000011115) comprising 53,106 entries. With the exception that match-between-runs function was activated, default parameters for mass error tolerances, missed trypsin cleavages, and fixed and variable modifications were used. The false discovery rate was set to 0.01 for both peptide-spectrum matches and protein identification. Protein intensity values were imported into Perseus software (version 1.6.15.0) for further analysis. Protein groups identified as potential contaminants and proteins only identified by site or by reverse database matching were removed, and LFQ intensity values were $\log_2$–transformed. The proteins were filtered to include only those detected in a minimum of eight samples and remaining missing values were replaced with random intensity values for low-abundance proteins based on a normal distribution of protein abundances using default Perseus parameters.

6.3.6. Bioinformatics and statistical analyses

Differentially abundant proteins were identified using a t-test comparison of all replicates (n = 12) for the resistant and susceptible cultivars, with a False Discovery Rate (FDR) of 0.05 and an s0 value of 0.1 used to define significant proteins. The differentially abundant proteins were classified using the UniProt database (www.uniprot.org), David bioinformatics resources 6.8 (https://david.ncifcrf.gov/), and the KEGG database (www.genome.jp/kegg/).

Following checks of normality and homogeneity variance, all data were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 27. Zoospore root attachment scores were analysed using a one-way ANOVA followed by a Protected Fisher's LSD test to determine statistically significant differences at ($p \leq 0.05$).

6.4. Results

6.4.1. Zoospore root attachment to potato roots from different enzyme treatments

Zoospore root attachment was significantly lower in root segments treated with trypsin and PNGase F showed less zoospore root attachment compared with the untreated controls in both susceptible ('Iwa') and resistant ('Gladiator') cultivars (Figure 21). In contrast, zoospore root attachment was unaffected by cellulase for both resistant and susceptible cultivars within the ranges of enzyme concentration tested. In 'Iwa' trypsin was the most effective treatment at reducing zoospore root attachment, while trypsin and PNGase F both reduced zoospore root attachment in 'Gladiator'. Zoospore root attachment to root segments occurred consistently during the 60 min incubation period (Supplemental Excel file 3), so 5 min was sufficient for potato root incubation.

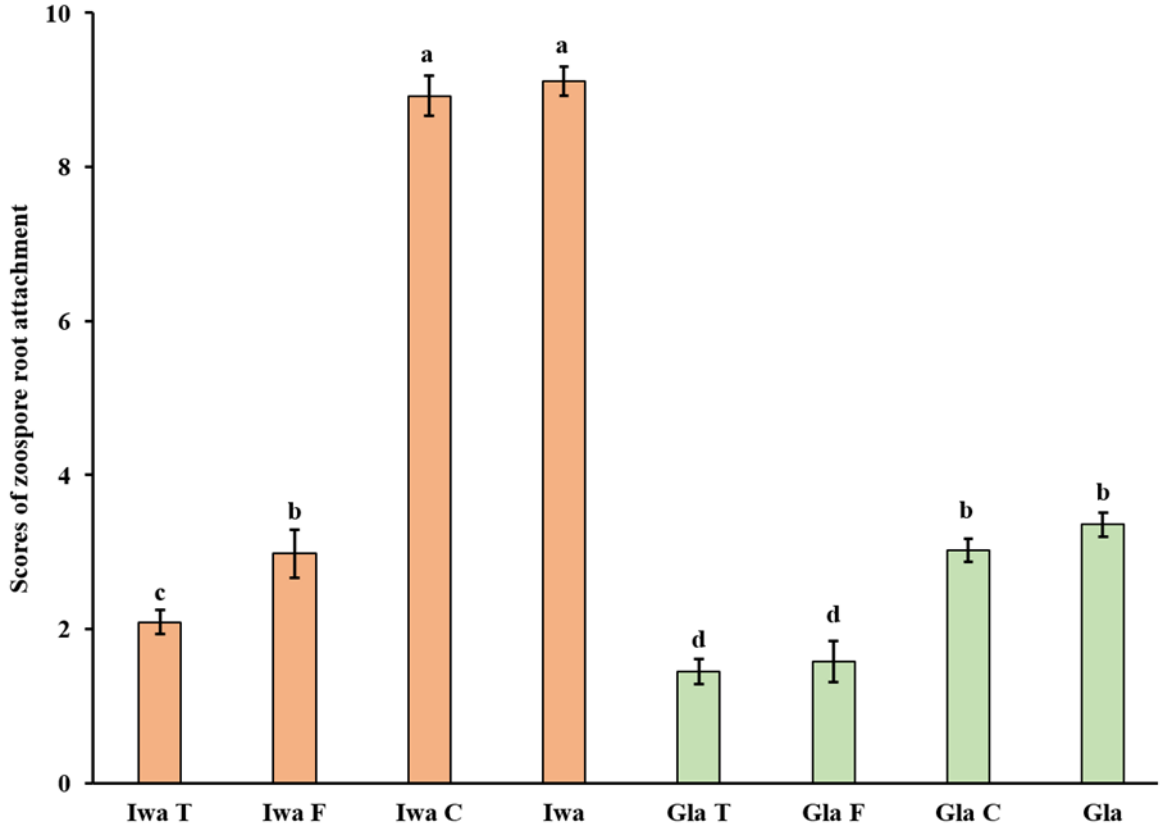


Figure 21. Mean *Spongospora subterranea* zoospore root attachment scores for roots pre-treated with different enzymes of potato cultivars 'Gladiator' (Gla, green bar); and cultivar 'Iwa' (orange bar). T: trypsin (20 µg/mL); F: PNGase F (50 units/mL); C: cellulase (1 mg/mL); Error bars represent standard deviations each based on three biological replicates. *p* (cultivars)

< 0.001, p (treatment) < 0.001, p (cultivar × treatment) < 0.001. Different letters above bars indicate significant zoospore root attachment as determined by LSD (p = 0.05) = 0.45.

6.4.2. Analysis of proteins released by trypsin shaving treatments of potato roots

The ability of PNGase F and trypsin to significantly reduce zoospore root attachment highlights a potential role for proteins, and in particular *N*-linked glycoproteins, in the process of plant-pathogen interactions. To gain a better understanding of potential mediators, a TS approach was used, in which peptides were collected from 'Iwa' and 'Gladiator' roots after incubation in trypsin for 5, 15, 30 or 60 min to allow for detection of proteins with different susceptibility to trypsin digestion under non-denaturing conditions. Following mass spectrometry analysis of the TS samples, a total of 1235 proteins were identified, of which 979 were quantified across the 24 samples after filtering the data to exclude proteins detected in fewer than eight samples (Supplemental Excel file 4). Principal component analysis of this dataset showed that 'Iwa' and 'Gladiator' samples were separated according to PC1, but the samples did not cluster according to time point (Figure 22a). On this basis, t-test analysis was used to identify differentially abundant proteins (DAPs) between the two cultivars. This identified 262 DAPs, of which 132 proteins were at significantly high abundance in 'Gladiator' and 130 proteins were low in 'Iwa' (Supplemental Excel file 5). Cluster analysis of the subset of DAPs (Figure 22b) also showed that samples collected at each time point did not cluster together, indicating that incubation time did not affect the profile of peptides released by TS treatment.

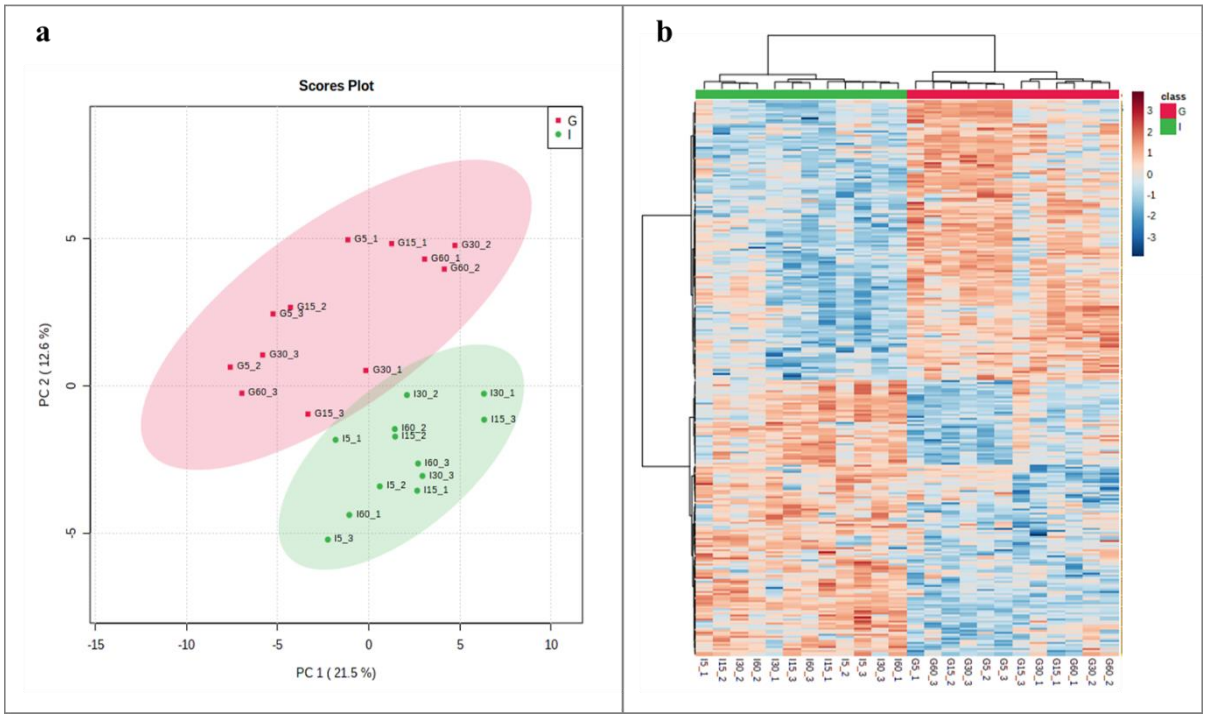


Figure 22. (a) Principal component analysis (PCA) of all identified proteins from a *Spongospora subterranea* resistant ('Gladiator', G) and susceptible ('Iwa', I) cultivar (n = 3) at four incubation times (5, 15, 30 or 60 min). (b) Heatmap of all significantly abundant proteins ('Gladiator' *vs* 'Iwa') at four incubation times (5, 15, 30 or 60 min).

6.4.3. Overall functional classification and pathway analyses of differentially abundant proteins

Functional enrichment analysis of the differentially abundant proteins (resistant *vs* susceptible cultivar) is shown in Figure 23a. For the proteins that were more abundant in the resistant cultivar 'Gladiator', the most highly enriched functional categories included glutathione transferase activity (GO_MF: 0004364), glutathione metabolic process (GO_BP: 0006749) and lignin biosynthetic process (GO_BP: 0009809). For the proteins that were reduced in resistant cultivar, significant functional categories included protein heterodimerization activity (GO_MF: 0046982), ATPase activity (GO_MF: 0016887) and nucleosome assembly (GO_BP: 0006334).

Pathway analysis revealed alterations in metabolic pathways in both subsets of DAPs (Figure 3b). Specific pathways associated with the proteins increased in the resistant cultivar included that oxidative phosphorylation (n = 8 proteins), biosynthesis of nucleotide sugars (n = 7

proteins) and amino sugar and nucleotide sugar metabolism (*n* = 7 proteins). In contrast, proteins that were less abundant in the resistant cultivar were related to the pathways carbon metabolism (*n* = 12 proteins), carbon fixation in photosynthetic organisms (*n* = 8 proteins), glyoxylate and dicarboxylate metabolism (*n* = 6 proteins) and pentose phosphate pathway (*n* = 5 proteins).

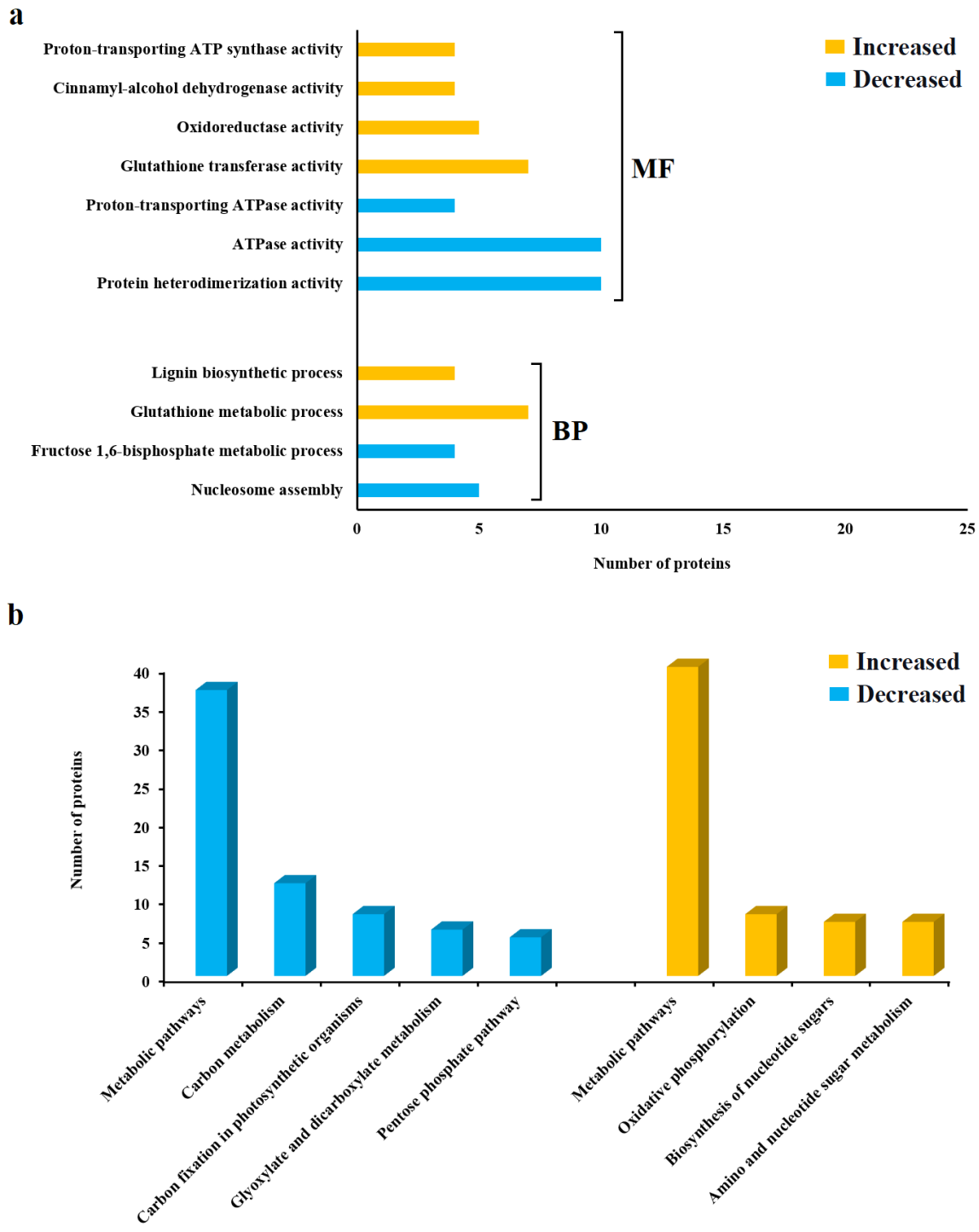


Figure 23. (a) Gene ontology (GO) categories of DAPs ('Gladiator' *vs* 'Iwa') from potato roots obtained from trypsin shaving (TS) treatments. BP, biological processes; MF, molecular function. (b) Pathway classification and enrichment analysis of DAPs ('Gladiator' *vs* 'Iwa') from potato roots from TS treatment.

6.4.4. Comparison of proteins identified in the TS experiment and the whole-root protein analyses

The bioinformatic analysis of the complete set of DAPs identified by TS experiment identified significant functional differences between the proteome of resistant and susceptible cultivars. However, this included a high proportion of cellular components that may not be directly involved in facilitating root attachment to the cell surface. Therefore, our previous proteomic dataset was used acquired from whole root tissue analysis to filter the TS dataset (Yu et al, Chapter 5), which enabled us to identify a subset of 226 proteins that were unique to the TS experiment (Figure 24a). Interestingly, a high proportion of these proteins (188) were significantly different in abundance between resistant and susceptible cultivars (Figure 24b).

Of the 188 significant proteins that were unique to the TS dataset, 92 were more abundant in the resistant cultivar while 96 were less abundant. Proteins that were detected at increased levels included globulin (M1C704), ER6 protein (M1AZC6) and B12D protein (M0ZLR3), while those that were reduced included wound-induced proteinase inhibitor 1 (P08454) and major latex proteins (M1CYU9 and M1BBE7) (Figure 24c). Of note, cell wall stem 28 kDa glycoprotein was significantly less abundant in the resistant cultivar while three glutathione *S*-transferase (GST) proteins (M0ZQ26, M1ARE1, and M0ZQ38) were significantly more abundant in resistant cultivar specific to TS experiment (Figure 24c). 59 proteins specific to the TS dataset were changed in abundance due to differential expression at the mRNA level, relative difference (log_2FC, resistant *vs* susceptible cultivars) was compared in respective their transcript and protein levels (Figure 24d). 39 proteins had changes in protein abundance that were in agreement between the two datasets, while 20 proteins had opposite changes in abundance between the RNA-seq and proteomics data. Globulin (M1C704) and two glutathione *S*-transferase (GST) proteins (M0ZQ26 and M0ZQ38) were among the proteins that were found at increased levels in both datasets.

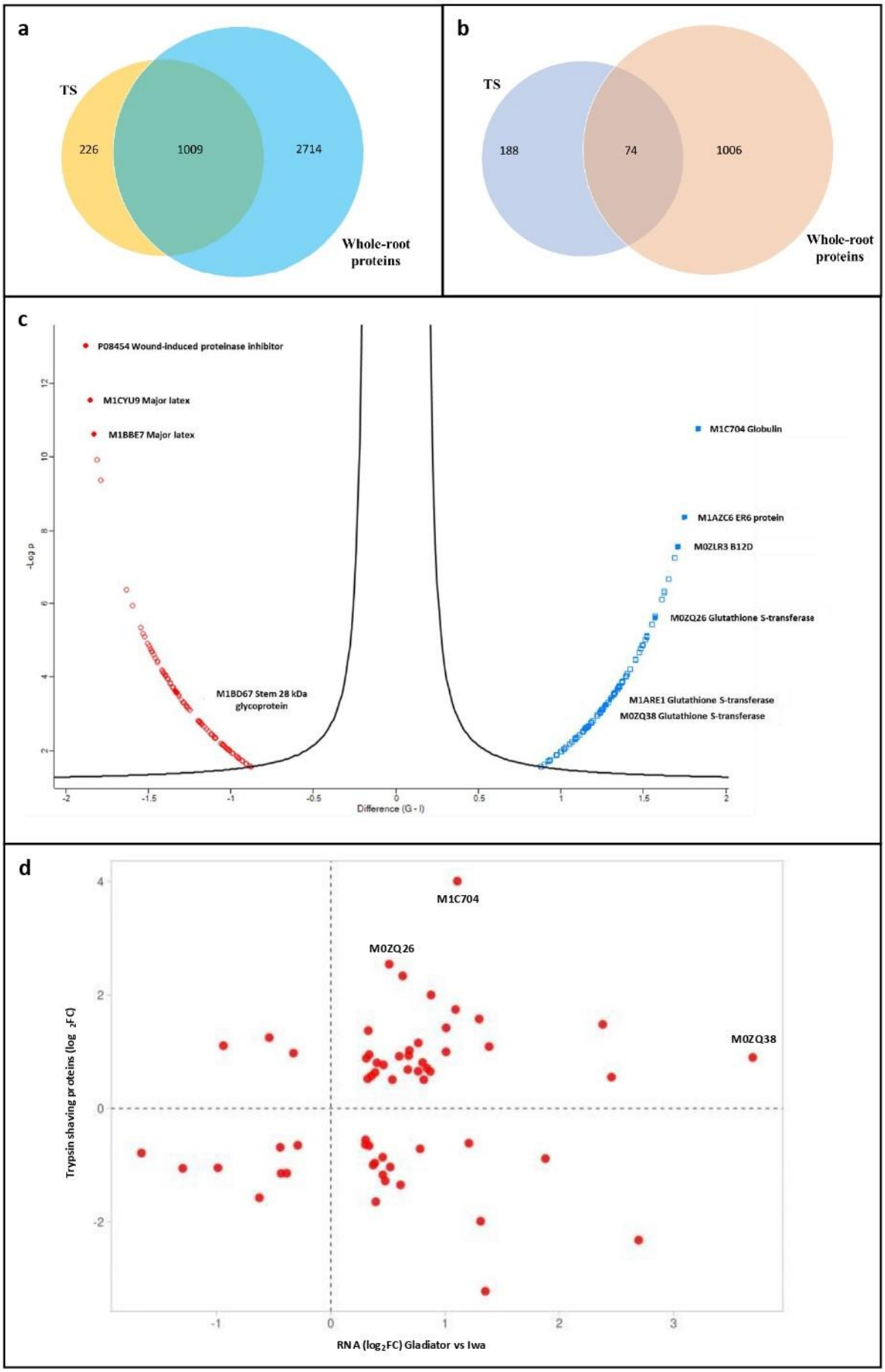

a
TS
226
1009
2714
Whole-root proteins
b
TS
188
74
1006
Whole-root proteins
c
P08454 Wound-induced proteinase inhibitor
M1CYU9 Major latex
M1BBE7 Major latex
M1C704 Globulin
M1AZC6 ER6 protein
M0ZLR3 B12D
M0ZQ26 Glutathione S-transferase
M1BD67 Stem 28 kDa glycoprotein
M1ARE1 Glutathione S-transferase
M0ZQ38 Glutathione S-transferase
-Log p
Difference (G - I)
d
M1C704
M0ZQ26
M0ZQ38
Trypsin shaving proteins (log 2FC)
RNA (log2FC) Gladiator vs Iwa

Figure 24. The Venn diagram (uses overlapping circles to illustrate the logical relationships between identified proteins from trypsin shaving (TS) treatment and the whole-root proteins) revealed (a) the groupings of the identified potato root proteins in the TS treatment and the whole-root proteins. (b) Groupings of the significant potato root proteins specifically identified in the TS treatment compared with the whole-root proteins. (c) Log_2 fold change of significant DAPs (resistant *vs* susceptible) (False discovery rate (FDR) < 0.05) specific to TS treatment (blue; significantly increased in the resistant cultivar and red; significantly decreased in the resistant cultivar compared to susceptible cultivar). (d) Distribution of proteins common between proteomics experiments specifically in TS treatment and transcriptomics (resistant *vs* susceptible) of roots.

Further comparison of the TS dataset with the whole-root proteome analysis enabled us to identify proteins with consistently high changes in abundance in both datasets. The 20 proteins were selected with the highest difference in abundance in the TS treatment (10 increased and 10 reduced in resistant *vs* susceptible cultivars), of which 17 were also identified in the whole-root proteomic dataset. The fold-changes (log_2) for these proteins are compared in Figure 25, where the values for the TS dataset are plotted against the respective values for the whole root proteomic dataset (Supplemental Excel file 6). The protein with the largest increase in the resistant cultivar (Glucan endo-1,3-beta-glucosidase: P52401) was highly modulated in both datasets (4.4-fold in the TS data and 6.0-fold in the whole-root proteomic data. Globulin (M1C704) was also significantly increased in the resistant cultivar in both datasets. Conversely, the Wound-induced proteinase inhibitor 1 (P08454) showed the largest decrease in abundance, with a 5.3-fold and 3.7-fold reduction in the TS treatment and whole-root samples, respectively. Major latex protein (M1AFT2) and an uncharacterized protein (M1AXR4) were also consistently and significantly decreased in the resistant cultivar. Notably, only one protein abscisic acid and environmental stress-inducible protein (M0ZVK4) showed opposite trends in the TS and whole root proteomic datasets, with a 3.3-fold decrease and 3.7-fold increase, respectively.

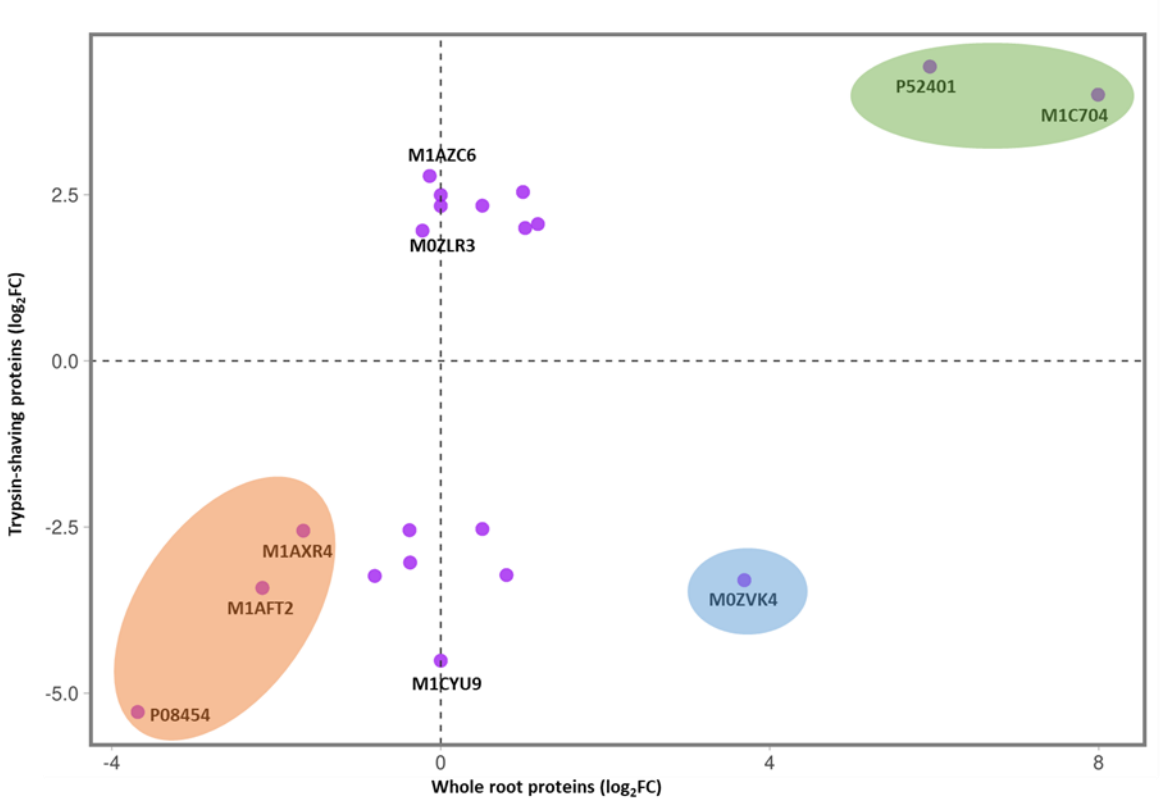


Figure 25. Comparison of the ten proteins with the greatest increased or decreased fold changes (log_2) in the *Spongospora* resistant cultivar ('Gla') from the trypsin shaving (TS) treatment and the whole-root protein analysis. Green ellipse: most increased in the resistant cultivar after TS treatment and the whole-root proteins, protein analysis. Orange ellipse: decreased in the resistant cultivar after TS treatment and the whole-root proteins, protein analysis. Blue ellipse: increased in the resistant cultivar in the whole-root protein analysis and decreased in the resistant cultivar after TS treatment.

6.5. Discussion

In this study, an in vitro zoospore root attachment assay and quantitative proteomics were used to investigate the *S. Subterranea*-potato interaction, based on the modification of plant root surface components with specific enzymes. Trypsin and PNGase F, assessed using an in vitro system, both reduced zoospore attachment to the potato roots. PNGase F catalyses the removal of *N*-linked oligosaccharide chains from glycoproteins in a full and efficient manner. This enzyme is commonly used for investigation of the structure-function relationships of glycoproteins (Sun et al., 2015). Plant cell wall polysaccharides and proteins may serve as dormant signal molecules during plant-pathogen interaction (Vorwerk et al., 2004, Castilleux

et al., 2018). Several studies have reported the biochemical basis of zoospore root attachments, and have demonstrated that root surface polysaccharides play important roles during zoospore root recognition and attachment (Byrt et al., 1982b, Hardham and Suzaki, 1986, Mitchell and Deacon, 1986, Longman and Callow, 1987, Gubler and Hardham, 1988, Hardham, 1989, Estradagarcia et al., 1990, Jones et al., 1991). The effects of plant cell wall proteins and polysaccharides on *Pythium* and *Phytophthora* zoospore host attachments have been demonstrated (Raftoyannis and Dick, 2006, Sarkar et al., 2022). Removal of polysaccharides of cress (*Lepidium sativum*) from root surfaces resulted in a reduction in *pythiaceous* zoospore attachment; and treatments that blocked or removed terminal fucosyl residues were particularly effective (Longman and Callow, 1987). Similarly, Estradagarcia et al. (1990) confirmed that cress root mucilage can encourage root attachment by zoospores. In the present study, while cellulase did not inhibit zoospore root attachment, trypsin and PNGase F significantly decreased attachment of *S. subterranea* zoospores to the roots of two potato cultivars (Figure 21). These results indicate that potato root proteins, especially *N*-glycoproteins, may affect zoospore root attachment processes.

The in vitro zoospore root attachment assay (Supplemental Excel file 3) indicated that a 5-min incubation was sufficient for the enzyme to take effect, while the time-course of TS found no significant differences between incubation times (Supplemental Excel file 5). Elsewhere, He and De Buck (2010) reported cell wall proteins of *Mycobacterium avium subsp. paratuberculosis* were digested with trypsin for 30 min at 37°C. Zahir et al. (2020) conducted a time-course investigation on the effects of trypsin digestion on soybean plants. They discovered that no intracellular protein was released during the initial 30 min of incubation, but it began to be detected after 60 min. In the present study, cytoplasmic proteins were found in the TS experiment at 5 min.

Among the 1235 proteins identified in the TS study, most of proteins that were significantly increased in resistant cultivar were associated with metabolic pathways such as oxidative phosphorylation, biosynthesis of nucleotide sugars, and the majority of amino acid biosynthesis pathways (Figure 23). These proteomics results were similar to those for proteome analyses of rice and sweet potato (Koller et al., 2002, Wang et al., 2013). According to the analysis of pathway and GO functional annotation, the present study showed that glutathione metabolism, including the glutathione metabolic process and glutathione transferase activity, were abundant in the resistant potato cultivar compared to the susceptible cultivar. Glutathione biosynthesis occurs in chloroplasts, cytosols, and mitochondria (Mahmood et al., 2010, Zechmann and

Müller, 2010). Glutathione-related enzymes have important roles in host resistance to different pathogen infections. For example, glutathione-related enzymes were abundant in a tomato cultivar resistant to *Oidium neolycopersici* (Pei et al., 2011), and in a rapeseed cultivar resistant to *Sclertinia sclerotiorum* (Zhao et al., 2009). The present study also detected three GST proteins that were abundant in the resistant cultivar specific to TS study, while two of them are also more abundant in resistant cultivar's RNA-seq data. Balotf et al. (2022a) reported that GST proteins were abundant in potato roots of a resistant potato cultivar after *S. subterranea* infection. In the potato genome, there are at least 90 GST proteins that are involved in plant immune systems (Islam et al., 2018). From study of the interaction between *S. subterranea* and potato, Balotf et al. (2022a) showed that more than 30 GST genes were induced after host infection.

The present study compared the proteomes of root cell surfaces of two potato cultivars in the absence of *S. subterranea* infection. Constitutive and responsive gene expression strategies were involved in the regulation of GST proteins, and these are used by potato to increase resistance to *S. subterranea*. Lignin biosynthesis processes were also identified in the functional analysis of DAPs in the *S. subterranea* resistant cultivar. Lignin is barrier against pest infestations and pathogen infections (Ithal et al., 2007). A previous study (Yu et al; Chapter 5), showed that the phenylpropanoid biosynthesis pathway was identified to be associated with *S. subterranea* zoospore root attachment in a resistant. Similar results were obtained by Balotf et al. (2022a) where the phenylpropanoid metabolic pathway, and especially lignin biosynthesis, played important roles in resistance of potato to *S. subterranea*.

Comparison of proteins from the TS proteome study with the whole-root proteins revealed 188 DAPs were significantly abundant after TS treatment (Figure 24). Major latex proteins (M1CYU9 and M1BBE7), which play roles in plant defence, had reduced abundance in the resistant cultivar. Additionally, the major latex protein M1AFT2 was consistently reduced in abundance in the resistant cultivar after TS treatment and whole-root protein analysis. Major latex proteins exist in different plant species, such as opium poppy (Nessler et al., 1985, Nessler et al., 1990), cucumber (Suyama et al., 1999), peach (Ruperti et al., 2002), melon (Inui et al., 2013), soybean (Zhu et al., 2014), and grape (Zhang et al., 2018). The number of major latex proteins varies among species. For instance, *Arabidopsis thaliana* contains 24 major latex proteins while grape has 14 (Lytle et al., 2009, Zhang et al., 2018). Major latex proteins respond to biotic and abiotic stressors and perform roles in plant growth and development, including disease resistance, stress tolerance, and development (Dai et al., 2002, Park et al., 2004, Lee

and Cho, 2012). He et al. (2020) showed that major latex proteins negatively regulate apple (*Malus domestica*) resistance to fungal infection through suppressing expression of genes and transcription factors associated with defence and stress. Similar to this result, the present study has shown that *S. subterranea* resistant potato cultivar 'Gladiator', had lower expression of major latex proteins than the susceptible cultivar 'Iwa'.

The cell wall stem 28 kDa glycoprotein (Figure 24c) was another protein identified less abundant in the resistant cultivar, which is consistent with the whole-root protein analysis (Yu et al; Chapter 5). Previous studies have reported that stem 28 kDa glycoprotein plays an important role in transformation of immature host elongation regions into mature, thickening tissues in the young tissues (Sergeant et al., 2019). Glycosylation and glycan processing are crucial posttranslational modifications that cell wall proteins undergo within cells, and these are regarded as essential for control of growth and defence mechanisms in plants (Nguema-Ona et al., 2014). In this study, treatment with PNGase F indicated that *N*-glycoproteins can inhibit the zoospore root attachment. Therefore, the cell wall stem 28kDa glycoprotein is promising for direct association with susceptibility of potato roots to zoospore root attachment.

Glycan endo-1,3-beta-glucosidase (P52401) was the protein identified as the most significantly abundant in the resistant cultivar after TS treatment. This result was consistent with the whole-root protein analysis (Figure 25). Glucan endo-1,3-beta-glucosidase is a hydrolytic enzyme that breaks down 1,3-β-D-glucosidic linkages in β-1,3-glucans, which widely exists in bacteria, fungi, and viruses (Doxey et al., 2007). Shinshi et al. (1988) reported that tobacco glucan endo-1,3-beta-glucosidase displayed complicated hormonal, developmental regulation, and was triggered by pathogen infection. In the line with these studies, the present results indicated glucan endo-1,3-beta-glucosidase contributed to potato resistance to *S. subterranea* infections. The abscisic acid and environmental stress-inducible protein (M0ZVK4) was differentially changed between TS and whole-root protein analysis. This protein decreased after TS treatment but increased in the whole-root proteins of the resistant cultivar. Abscisic acid is essential for numerous cellular processes, including seed development, germination, crop growth, and root architecture mediation (Xiong and Yang, 2003, Harris, 2015). According to Harris (2015), abscisic acid mediates responses to various environmental factors, including the presence of nitrate in soil, water stress, and salt, shaping root systems by regulating production of lateral roots and controlling root elongation by modulating cell division and elongation. Since only the lower part of mature potato roots was examined in the TS treatment, but entire roots were used in the whole-root protein analysis, the difference in fold changes of protein (M0ZVK4)

between the two experiments may be a consequence of different spatial distribution of abscisic acid across root zones.

6.6. Conclusions

This is the first study on the biochemical basis of *S. subterranea* zoospore attachment to potato roots. From the in vitro zoospore root attachment study, trypsin and PNGase F reduced zoospore root hair attachment, while cellulase did not affect zoospore root attachment. This detailed proteomic analysis showed broad-scale differences in root proteins between susceptible and resistant potato cultivars. Knowledge of these proteins within potato roots provides new insights into host resistance to zoospore root attachment. This study provides initial understanding of the biochemical and molecular basis of potato resistance to zoospore root attachment and is important for developing a novel approach for disease management.

This research contributes to knowledge of the biochemical and molecular basis of *S. subterranea* zoospore root attachment, but there are limitations. Firstly, high levels of intracellular proteins were detected in the TS protein analysis which may affect the identification of extracellular proteins. While different time points were assessed in this study, further refinement of the TS approach may help to minimize the background of intracellular proteins. Secondly, glycoproteomics can be used in the future study to discover the glycans and glycosylated proteins in cell walls to reveal how glycoproteins organize and function in cultivar resistance to zoospore root attachment.

Chapter 7. General discussion

7.1. Background

Spongospora subterranea infects potato (*Solanum tuberosum* L.) crops, leading to tuber and root infections (Harrison et al., 1997, Falloon et al., 2003, Falloon, 2008, Merz, 2008, Merz and Falloon, 2009, Balendres et al., 2016b, Falloon et al., 2016). Potato tuber and root diseases caused by *S. subterranea* cause major economic damage for potato growers (Wilson, 2014a) (Yu et al., Chapter 1). However, there are no efficient techniques to manage *Spongospora* tuber and root diseases, and no commercial cultivar has been found to be completely immune to this pathogen (Merz and Falloon, 2009). Growing potato cultivars resistant to the pathogen is the most sustainable and long-term strategy for disease management (Balendres et al., 2016b, Falloon et al., 2016) (Yu et al., Chapter 2).

Assessment of host resistance to tuber disease and to a lesser extent root galling has been well studied, primarily in glasshouse and field trials. Both these diseases are visually obvious and simple to score, hence their predominant use. However, invasion of the root system by the pathogen, irrespective of the extent of root galling, has increasingly been recognised to impair host root function and significantly reduce crop yields, and is probably the most critical phase of this host/pathogen relationship. In Chapter 3 a novel in vitro assay is described that directly screens for potato cultivar resistance to root infection, by measuring initial zoospore root attachment. The assay, while technically demanding, can be completed within 2 days rather than weeks or months, with less physical resources required than traditional field and glasshouse assays. This rapid and robust bioassay was used to screen 153 potato genotypes, and several commercially important cultivars and breeding lines showing strong resistance to *S. subterranea* root infection were identified.

In a complementary study, the in vitro zoospore root attachment assay was used to screen 93 somaclonal variants of important potato cultivars, which had been generated by somatic cell selection in an independent study. In vitro testing showed that several variants had enhanced resistance to *Spongospora* root disease. Subsequent glasshouse trials provided evidence that the identified resistance to zoospore root attachment was associated with enhanced resistance to potato tuber powdery scab (Yu et al., Chapter 4), suggesting this may be an approach for generating of disease resistance and improving resistance within elite varieties, without the need for conventional breeding approaches and genetic reassortment.

Chapter 5 provided data and insights on the molecular basis of constitutive resistance expression within potato roots with using a label-free quantitative proteomic approach with a wide set of six resistant and six susceptible cultivars. This gave us an important set of data from which to begin appraisal of functional aspects of host resistance to *S. subterranea*. Several candidate pathways and proteins were identified that have potential to influence potato cultivar resistance to the *S. subterranea* zoospore root attachment process (Yu et al., Chapter 5).

Increased understanding of the biochemical basis of potato root surfaces was shown to be important for developing a novel approach for the inhibition of zoospore root attachment and subsequent disease control. Therefore, this study investigated the effect of selected enzyme treatments on root cell wall polysaccharides and proteins including pectinase, cellulase, trypsin and PNGase F (Yu et al., Chapter 5, Yu et al., Chapter 6). Out of the four enzymes, PNGase F (50 units/mL), and trypsin (20 µg/mL) both reduced zoospore root attachment, suggesting potential roles for root surface proteins, especially *N*-linked glycoproteins (Yu et al., Chapter 6). However, the effect of pectinase on zoospore root attachment was concentration dependent, while cellulase had no effect on zoospore root attachment (Yu et al., Chapter 5, Yu et al., Chapter 6). Further detailed proteomic analysis showed broad scale differences in root proteins between susceptible and resistant potato cultivars which provides mechanistic insight into how the potato host resists zoospore root attachment (Yu et al., Chapter 5, Yu et al., Chapter 6).

7.2. Physiological basis for cultivar resistance to *Spongospora subterranea* zoospore attachment

Due to a lack of effective disease control strategies, it is important for potato breeders and growers to choose disease-resistant cultivars (Yu et al., Chapter 2). An in vitro zoospore root attachment assay for screening host resistance has been developed and optimised in this study, which greatly shortens the assessment period compared to the conventional glasshouse and field trials (Yu et al., Chapter 3).

Initial work to optimise the assay revealed new knowledge of the biology and pathology of *S. subterranea* in potato. Zoospore release from resting spores incubating in Hoagland's solution at 20°C occurred in a rapid and synchronised manner over 2 days, followed by a large decline (Yu et al., Chapter 3). Previous studies indicated the temperature requirements for the development of tuber and root diseases (Kole, 1954, Hughes, 1980, van de Graaf et al., 2005,

van de Graaf et al., 2007, Shah et al., 2012). This study also showed that the optimal temperature for zoospore root attachment was 15°C and the region of roots where most zoospore attachment occurred within root hairs present in the lower maturation zone (Yu et al., Chapter 3).

The assay directly measures the initial phase of invasion of root systems by the pathogen. While root infection is a necessary precursor for root galling and tuber powdery scab, expression of relative resistance to these different phases of disease varies (Yu et al., Chapter 3). It is therefore important that an efficient methodology is used for assessment of root infection that is independent of expression of root galling.

Rapid and efficient screening is very valuable. Previous studies showed that assessments of root galling and tuber powdery scab required 8 to 20 weeks from planting in glasshouse or field experiments (Falloon et al., 2003, Nitzan et al., 2008, Nitzan et al., 2010, Falloon et al., 2016). Previous in vitro assays measuring plasmodia or zoosporangia in infected roots still required 5 weeks to complete, and the results could be complicated by varying numbers of infection cycles among cultivars (Merz et al., 2004, Thangavel et al., 2015).

Another confounding factor in assessment of host resistance for powdery scab, is the polycyclic nature of disease development. Once established, *S. subterranea* can spread rapidly within host root systems. Initial onset of root infection requires resting spore germination, zoospore release, and migration to roots. In glasshouse or field trials, timing of this release and infection cannot be controlled. Delay of even a few days can greatly reduce the extent of infection and expression of disease (Thangavel et al., 2015), and thus alter cultivar resistance scores. The present study has developed a novel in vitro zoospore root attachment assessment tool that is rapid (results obtained within 48 h), enables strict control of experimental conditions, and assesses cultivar resistance to zoospore root attachment at the first point of pathogen/host interaction, thus avoiding issues associated with polycyclic infection (Yu et al., Chapter 3).

The assay was then used to screen somaclonal variants for relative resistance to root infection and then determine if these variants also expressed resistance to tuber disease. Somatic cell selection is a valuable tool for generating genetic and epigenetic variation within elite germplasm (Shepard et al., 1980, Karp, 1991). Potato breeding is confounded by requirements for many quality and productivity traits within new cultivars. Elite varieties that possess excellent quality, yield and processing characteristics, but lack strong disease resistance may be improved by selection of somaclonal variants. Selection of somaclonal variants of potato

cultivars through in vitro derived genetic variation has been successfully used to obtain enhanced resistance to several potato pathogens including *Phytophthora infestans* (Matern et al., 1978), *Verticillium dahliae* (Sebastiani et al., 1994), *Alternaria solani* (Shepard et al., 1980), *Fusarium oxysporum* (Behnke, 1980) and *Streptomyces scabiei* (Wilson et al., 2009, Wilson et al., 2010).

In this study, the use of somaclonal cell selection was shown to be a useful approach for generation of resistance to *S. subterranea* diseases. One third of the tested regenerated host variants exhibited significantly greater resistance to zoospore root attachment than the unselected parent cultivars (Yu et al., Chapter 4). Enhanced resistance to root attachment was then reflected in decreased tuber powdery scab in a subsequent glasshouse trial (Yu et al., Chapter 4).

7.3. Proteomic basis of cultivar resistance to *Spongospora subterranea* root infection

The present study results indicated that increase in the phenylpropanoid biosynthesis pathway in resistant cultivars was associated with cultivar resistance to zoospore root attachment. This is in agreement with the results of Balotf et al. (2022b) showing that the phenylpropanoid metabolic process plays an important role in the resistance of potato cultivars regarding root infection by *S. subterranea* (Yu et al., Chapter 5). Previous studies have demonstrated that oxidative stress is involved in the activation of defence-related activities in potatoes (Wu et al., 2009), and peroxidase activity is essential for preventing widespread resistance to bacterial or fungal infections (Wu et al., 1997). The majority of DAPs discovered in resistant cultivars were ascribed to oxidative stress and metabolic activities, such as oxidative stress response and peroxidase activity (Yu et al., Chapter 5). These results also showed that the majority of identified proteins in TS-treated plants were intracellular, classed as metabolic pathway proteins, such as those involve in oxidative phosphorylation, biosynthesis of nucleotide sugars, or amino acid biosynthesis pathways (Koller et al., 2002, Wang et al., 2013) (Yu et al., Chapter 6). Inspecting the types of proteins in a resistant cultivar indicated increased abundance of cytosol and cytoplasm-associated proteins. Numerous important biological processes occur in the plant cytosol (Yu et al., Chapter 6), the basic internal fluid that serves as a communication medium between organelles. This study identified glutathione metabolism, which is abundant in resistant cultivars (Yu et al., Chapter 6). Balotf et al. (2022b) reported that glutathione

metabolism was increased in a resistant cultivar following *S. subterranea* infection, which is also a key finding in the present study (Yu et al., Chapter 6). Lignin biosynthesis was also identified in the resistant cultivar, which is another significant result from GO functional annotation (Yu et al., Chapter 6).

Several enzymes involved in pectin biosynthesis and remodelling were abundant in resistant cultivars, suggesting that pectin-like substances on plant root surfaces may be recognition signals for zoospore root attachment (Yu et al., Chapter 5). This result was consistent with the treatment of potato roots with enzyme pectinase, which highlights the particular significance of pectin in the potato root cell wall in cultivar resistance to zoospore root attachment (Yu et al., Chapter 5).

Comparing the proteins identified from the TS proteome study (Yu et al., Chapter 6) with the whole-root proteins (Yu et al., Chapter 5), the major latex proteins (M1CYU9 and M1BBE7), which play essential roles in plant defence, were found to be less abundant in the resistant cultivar with the greatest negative fold change after TS treatment. In addition, the major latex protein M1AFT2 was consistently less abundant in resistant cultivars following TS treatment and whole-root protein analysis (Yu et al., Chapter 5, Yu et al., Chapter 6). Of note, cell wall stem 28 KDa glycoprotein was less abundant in the resistant cultivar specific to TS treatment, while three glutathione *S*-transferases (GST) (M0ZQ26, M1ARE1, and M0ZQ38) were more abundant in a resistant cultivar specific to TS study (Yu et al., Chapter 6). Unanswered is whether these or other membrane-bound proteins more commonly detected in susceptible than resistant cultivars' roots are connected directly with zoospore attachment.

Glucan endo-1,3-beta-glucosidase (P52401) was the protein with the largest difference identified as abundant in the resistant cultivar with TS treatment, and this was consistent with the whole-root protein analysis (Yu et al., Chapter 5, Yu et al., Chapter 6). The abscisic acid and environmental stress-inducible protein M0ZVK4 was 3.3-fold less abundant in the resistant cultivar when treated with TS but was 3.7-fold more abundant in the resistant cultivar in the whole-root protein analysis (Yu et al., Chapter 5, Yu et al., Chapter 6). The difference in fold change of the protein M0ZVK4 between the two studies may be because abscisic acid is distributed differently in different root zones.

7.4. Biochemical basis of cultivar resistance to *Spongospora subterranea* zoospore attachment

Results from this study have indicated that pectinase had a concentration-dependent influence on zoospore root attachment whereas cellulase had no effect on zoospore root attachment. This indicates a particular role of cell wall polysaccharides, especially pectin, in zoospore root attachment (Yu et al., Chapter 5, Yu et al., Chapter 6). Four categories involved in cell wall pectin biosynthesis and remodelling, including MF 'pectin acetylesterase activity', 'pectinesterase inhibitor activity', 'pectinesterase activity' and BP 'pectin catabolic process', were associated with proteins that were more abundant in resistant cultivars. This is consistent with the result of in vitro zoospore root attachment experiments with root modified with pectinase. Similarly, Estradagarcia et al. (1990) demonstrated that root mucilage can facilitate zoospore root attachment processes, which is consistent with the present study showing that cell wall pectin can inhibit zoospore root attachment.

PNGase F and trypsin inhibited zoospore root attachment, indicating a possible involvement of root surface proteins, particularly *N*-linked glycoproteins (Yu et al., Chapter 6). In this study, cell wall stem 28 kDa glycoprotein (M1BD67) was significantly less abundant in resistant cultivars after TS treatment (Yu et al., Chapter 6). This is consistent with the whole-root protein analysis (Yu et al., Chapter 5). Baumann and Doyle (1979) showed that 90% of glycoproteins were released from cell surface proteins treated with 20 µg/mL of trypsin within 10 min at 37°C. The present study indicates that 5 min of incubation at 37°C with 20 µg/mL trypsin was sufficient to initiate release of potato root surface proteins (Yu et al., Chapter 6).

7.5. Genetic basis of cultivar resistance to *Spongospora subterranea* zoospore attachment

An objective of this study was to conduct a genome-wide association analysis (GWAS) to identify genetic regions of interest underpinning potato resistance to the attachment of *S. subterranea* zoospores. The study had aimed to characterize a total of 250 potato lines sourced from the New Zealand Institute for Plant and Food Research Ltd, and from Australian germplasm. However, due to restrictions arising from COVID-19, it was only possible to phenotype Tasmania-sourced materials. Specifically, 170 potato cultivars and lines were assessed using an in vitro zoospore root attachment assay and extracted genomic DNA from

potato roots in New Town laboratory, Tasmania. All the DNA samples were sent to New Zealand for GWAS analysis using SNPchip data. The GWAS study found no resistance-related regions, most likely due to the small population size of the tetraploid *S. tuberosum* and the absence of shared resistance areas throughout the panel (Appendix). At the 5% threshold, the GWAS study failed to detect any meaningful marker relationships with zoospore root attachment scores. ST4.03ch02 36621867 on chromosomes 2 and PotVar0085803 on chromosomes 3, had the strongest relationship out of all the examined markers. These results show that some identified proteins were more or less abundant in resistant cultivars from TS treatment and the whole root protein analysis, and these proteins were located in chromosomes 2 and 3 (Yu et al., Chapter 5, Yu et al., Chapter 6). Abscisic acid and environmental stress-inducible protein (M0ZVK4), B12D (M0ZLR3), pectinesterase (M1DTA0), GTP-binding 2 (M1AYA4), and endoplasmic reticulum transmembrane protein (M1BZU4) were located in chromosome 2, while pectinesterase (M1BJ45), pectinesterase (M1AIV9), pectinacetylesterase (M1BRR7), glycoside hydrolase family 28 protein (M1AZG9), heparanase-2 (M1CAK9), and ATP synthase epsilon chain (M1AIX7) were identified in chromosome 3 (Yu et al., Chapter 5, Yu et al., Chapter 6). Further research is required to explore the relationship between these proteins and the two markers identified in the GWAS study.

7.6. Conclusions and recommendations

This research has developed a rapid and reliable approach for potato cultivar resistance screening at an early stage of *S. subterranea* root infection (zoospore root attachment), that bypasses the environmental factors involved in traditional glasshouse and field trials and reduces the cost and time required for screening (Yu et al., Chapter 3). Given the costs, resources and time necessary for cultivar improvement and breeding programs, whether through mutation breeding, targeted genetic engineering or traditional plant crossing techniques (Muthoni et al., 2012), a rapid technique that can aid the screening of host cultivars and germplasm lines is likely to be very worthwhile. Incorporation of this novel zoospore root attachment assay is an additional tool that can add value to evaluation of cultivar performance in current and newly developed varieties (Yu et al., Chapter 4). The present study showed that yield data (tuber numbers and weights) for most variants, collected from glasshouse assessments, was similar to that for the respective unselected parents. For the majority of variants, selection for disease resistance is, therefore, unlikely to be associated with detrimental

tuber yield characteristics. Further agronomic testing would be necessary to determine their commercial viability of these potato variants (Yu et al., Chapter 4).

In this study, new proteomic knowledge has been obtained about potato cultivars that exhibit different levels of resistance to *S. subterranea*. This gives a detailed set of data from which at functional aspects of host resistance to zoospore root attachment can be explored (Yu et al., Chapter 5, Yu et al., Chapter 6). An important issue unresolved in this study is how these candidate pathways and the proteins identified respond to plant-pathogen interactions, which should be addressed in future research. These induced responses would form an important component of understanding resistance as has been reported previously (Balotf et al., 2021a, Balotf et al., 2022b). Notwithstanding, the basal or constitutive host resistance mechanisms reported in this study represent a significant contribution to understanding of resistance mechanisms. A further avenue of study could assess whether these candidate pathways and proteins associated with resistance to *Spongospora* provide related resistance to other important soilborne pathogens. Given that multiple soilborne pathogens can occur in the field and the reported symbiotic relationships between pathogens e.g. *S. subterranea* can promote nematode (*Pratylenchus*) disease (Budahn et al., 2004), it would be of interest to determine cultivar resistances are pathogen specific or more general.

Increased understanding of the biochemical basis of potato root surfaces will be important for developing a novel approach for inhibition of zoospore root attachment and, potentially, subsequent disease control (Yu et al., Chapter 6). The present study contributes to knowledge of the biochemical and molecular basis of *S. subterranea* zoospore root attachment, but further research is required because of some limitations. For example, the high amounts of intracellular proteins detected in the TS protein analysis may affect the identification of extracellular proteins. In future studies, the TS approach can be improved to minimize the intracellular protein background. Secondly, glycoproteomes can be used to discover glycans and glycosylated proteins in cell walls to reveal how glycoproteins organize and function in cultivar resistance to zoospore root attachment. Some proteins, such as ER6 protein (M1AZC6) and B12D (M0ZLR3), which were detected in TS-treated cultivars and had significant impacts, have not been thoroughly explored. In addition, future research could use genetic manipulation to identify the functions of the putative proteins of interest, by silencing or over expressing them to see how this affects zoospore binding susceptibility/resistance. Future research could also assess specific blocking of some of these putative surface proteins (e.g., using specific

antibodies) to determine if this blocks/prevents zoospore binding and/or induction of host resistance.

Further potato genome searches for regions of interest for resistance- candidates, constructing appropriate markers for them, and testing them on additional host lines that have been phenotyped for zoospore root attachment, is likely to provide valuable new information. This should focus on host lines with known powdery scab severities, to determine the markers' predictive capabilities.

Chapter 8. References

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Chapter 9. Appendix

Mechanisms and manipulation of resistance to powdery scab in potato roots: QTL analysis

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Executive summary

The New Zealand Institute for Plant and Food Research Ltd (PFR) were sub-contracted to provide specialist services regarding undertaking research to identify genetic regions of interest underlying potato resistance to the initiation of root infection through root attachment to potato by *Spongospora subterranea* - a pathogen that causes root galls and the disease powdery scab on tubers. The preference was to undertake linkage-based mapping of QTL using a population of related individuals. However, because of COVID-19-related limitations and a lack of available mapping populations in Tasmania, it was decided in discussion with the client to access a panel of unrelated material and undertake a genome-wide association analysis (GWAS). Using genomic SNPchip data for 170 lines and their root attachment disease scores, a GWAS was unable to identify significant regions associated with resistance. This is likely because this population size was too limited for a tetraploid species, as well as lack of common regions of resistance across the panel. The analysis did, however,identify potential regions that while not significant were above background for multiple markers in the genomic regions. These would be key markers to explore further.

Introduction

Powdery scab disease of potato is a major disease impacting the quality and yield of tubers in commercial growing regions around the world. There are limited chemical control options and resistance remains a key target for breeding programmes.

The New Zealand Institute for Plant and Food Research Ltd (PFR) were approached by University of Tasmania (UTAS) to collaborate, given our expertise in molecular mapping and potato breeding, to assist in their project researching root attachment by the *Spongospora subterranea* pathogen.

Attachment is one of the first stages of the infection cycle.

The original plan was to undertake the root attachment assay in New Zealand on germplasm material held by PFR. However, COVID-19 and a block on international travel made this too challenging, and so the project became reliant on material that could be sourced in Tasmania.

Therefore, a genome-wide association study (GWAS) method that uses unrelated lines to identify marker:trait associations based on linkage disequilibrium was undertaken as opposed to QTL mapping that uses bi-parental populations.

Materials and methods

Genotype data generation

DNA was provided from UTAS. The QC values were checked and quantities normalised. Samples were then genotyped using the Geneseek GPP Potato Array V4 (Illumina).

Initial quality checks of resulting data were performed in GenomeStudio V2 and then further analysis undertaken using R statistical software. The raw X/Y intensity scores were exported from GenomeStudio into R. The fitPolyTools package was used to reformat and then package fitPoly (Voorrips et al., 2011) for genotype calling.

Genotype calling

Genotype calling was performed in fitPoly using the default settings, with the exception of a call threshold specified at 0.9, i.e. to pass, a marker must assign genotypes for greater than 90% of the samples, with the predicted genotype having a probability of $p>0.99$. Markers with >10% missing data and those with no positional information provided were discarded from further analysis.

COVID-19 interruptions meant the original plan of hosting a student at Lincoln was not possible. Instead PFR provided genotype data to the student based at UTAS and familiarised the student with PFR's programme of research, on the condition of confidentiality and recognition that the work programme contained PFR's Background Intellectual Property.

GWAS analysis

Discriminant analysis of principal components (DAPC) using the Adegenet package (Jombart, 2008) was used to explore the population structure within the samples based on the genotype data.

The R package GWASpoly (Rosyara et al., 2016) was used for GWAS. The genotype data from fitPoly were combined with the phenotype scores received from UTAS (Supplemental Table S1). Population structure was controlled using a random polygenic effect (Yu et al., 2006), using the 'Leave One Chromosome Out (LOCO)' method. Marker: trait associations were tested using two models: the additive model, which assumes an additive relationship between allele dosage and trait; and the '1-dom' model, which assumes complete dominance such that a single copy of a given allele is sufficient to control trait expression (two models are tested – dominance of the reference allele, and dominance of the alternate allele). The significance threshold for each marker was determined using the M.eff method at $p<0.05$. As this did not reveal any significant marker:trait associations, threshold setting was repeated with $p<0.1$ and $p<0.2$ to tentatively identify peaks of interest.

Identification of most-significant markers for each QTL was undertaken with the GWASpoly function get.QTL, using a window size of 5Mb; the fit.QTL function was used to estimate the partial R2 for each QTL identified.

Results

The phenotype of root attachment score was provided for 170 potato lines by UTAS. There was one line duplicated ('Anivia') and one line ('Norland wiscons super') with no screen result. The present study assumed 'Anivia' was a mistaken duplication of the same data, as the root attachment scores were the same for both entries.

As can be seen from Figure S1 there was a good distribution of root attachment scores from 1.16–12.92. (We were not provided with biological replicate scores to be able to understand how much variation there was between replicates or for replicates × genotypes) We assumed that a score of 1 is more resistant with less root attachment and a score of 12 is more susceptible. This fits with the powdery scab resistant standard 'Gladiator' and susceptible line 'Iwa'.

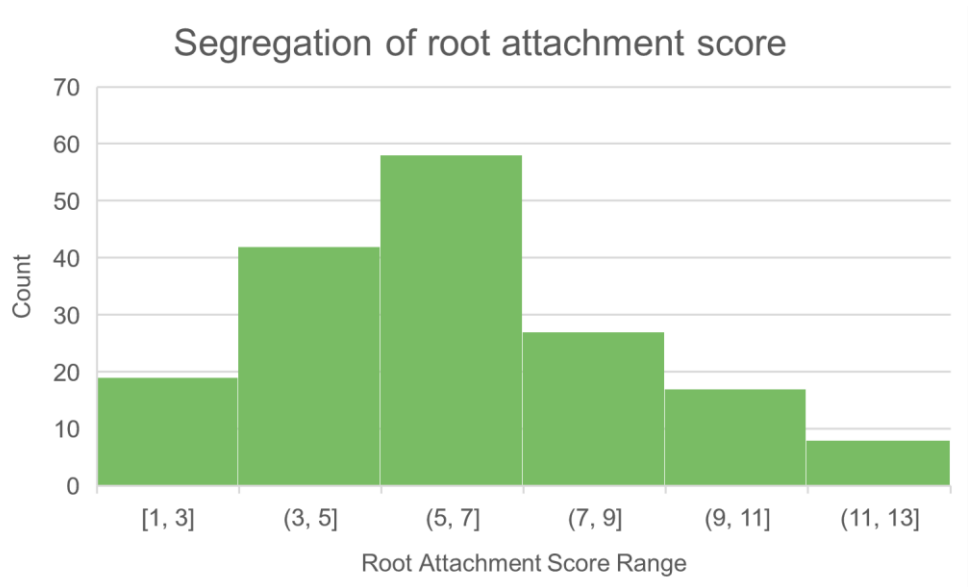

Figure S1. The segregation of the root attachment score (x-axis) by count (y-axis) in the panel of potato varieties used in the research.

DNA supplied for genotyping varied from 55 ng/µL to over 1000 ng/µL.

Genotype calling with fitPoly resulted in 17,953 markers (of a possible 30,991) for further analysis; of these 25 were discarded for lack of positional information against the reference genome. In total, 17,928 markers were used for subsequent GWAS. The Adegenet analysis suggested three sub- populations with most lines in the first two groups and an isolated subgroup that contained only 'Russet Burbank' somaclones.

Quantile-quantile plots indicated that the K model sufficiently accounts for population structure in thisgroup of accessions, with little inflation in observed versus expected $-\log_{10}(p)$ values for the association models tested (Figure S2). If required, the analysis could be repeated by including the population structure as calculated from Adegenet to determine if the model improves (a K+Q model).

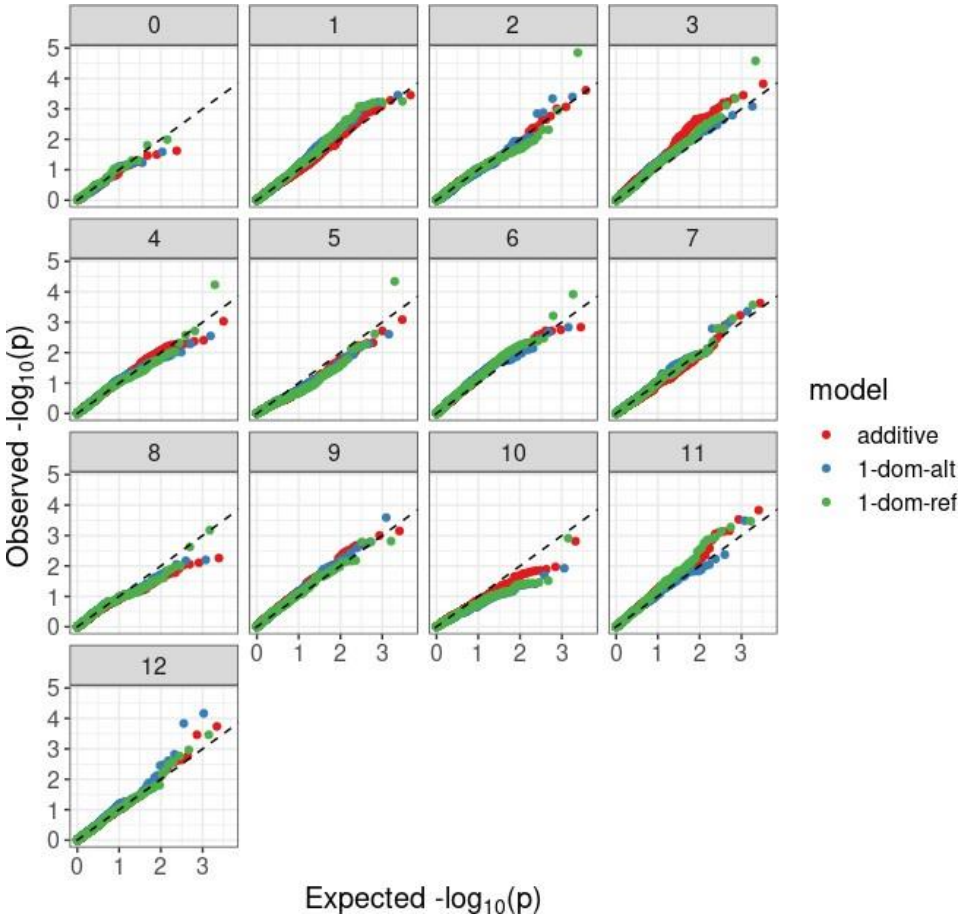

Figure S2. Quantile:quantile plots depicting deviation of observed from expected –log10(p) values from the null hypothesis (no association) for three models of association between the marker and trait: The additive model assumes an additive relationship between allele dosage and trait; the '1-dom' model assumes complete dominance such that a single copy of a given allele is sufficient to control trait expression (two models are tested – dominance of the reference allele ('1-dom-ref') and dominance of the alternate allele ('1-dom-alt) across the 12 chromosomes of potato.

The significance thresholds calculated at p<0.05, p<0.1 and p<0.2 for each method of associationanalysis are given in Table S1.

Table S1. -log10(p) significance thresholds calculated for each genetic model at three p values

Significance threshold	Model		
	additive	1-dom-alt	1-dom-ref
p<0.05	5.23	4.97	5.07
p<0.1	4.88	4.63	4.73

p<0.2	4.52	4.27	4.36

Initial assessment of marker:trait association at p<0.05 did not reveal any significantly associated loci (Figure S3 A) so different significance values were tested (Figure S3 B, C)

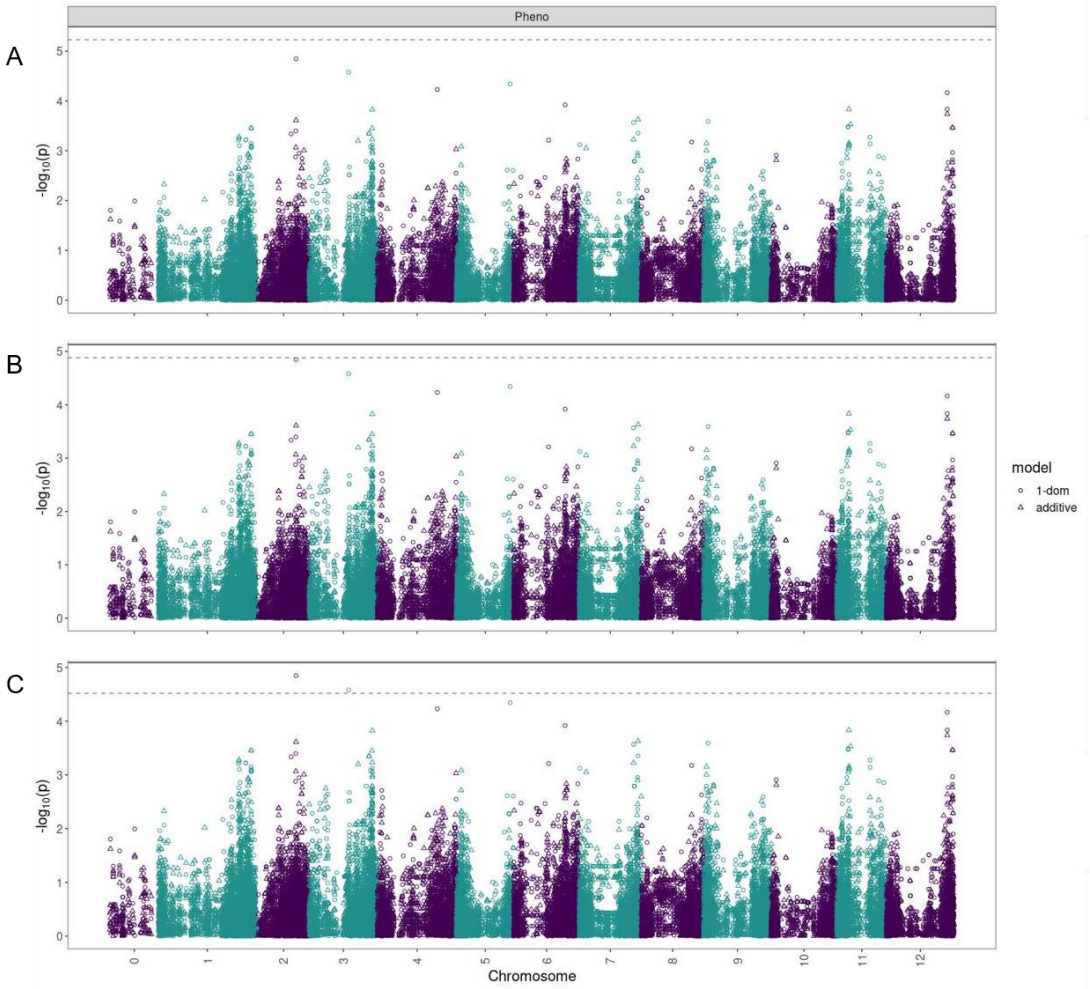

Figure S3. Manhattan plots showing marker: trait association across the potato genome. The dashed line in each plot represents the –log10(p) value above which markers are considered significantly associated with the trait, tested at M.eff $p < 0.05$ (A), $p < 0.1$ (B) and $p < 0.2$ (C). Note that each model (additive, 1-dom reference and1-dom alt) has a slightly different threshold; the additive model threshold is plotted here as the most stringent. Full details of this threshold for each p value and each association model are given in Table S1.

Relaxing the p-value threshold from p<0.05 to p<0.1 revealed a single QTL on chromosome 2, and at p<0.2 revealed a second QTL on chromosome 3; while the evidence for these is weak (Figure S4; Table S2), they may be real associations, but this study lacks power to detect them

at a significant score. Alternatively, they could be false positives.

The putative QTL on chromosomes 2 and 3 each explain approximately 10% of variation in the observed phenotype (Table S2). Both QTL are found under an association model that assumes a dominance effect of the reference allele on the trait. In Figures S5 and S6 the root attachment score for each of the different genotypes that were found in the panel for the markers ST4.03ch02_36621867 and PotVar0085803 are shown. In both cases the presence of the reference allele appears to suggest a potential increased resistance, as measured by a lower root attachment score. However, they are not significant at the $p<0.05$ in a population of this size.

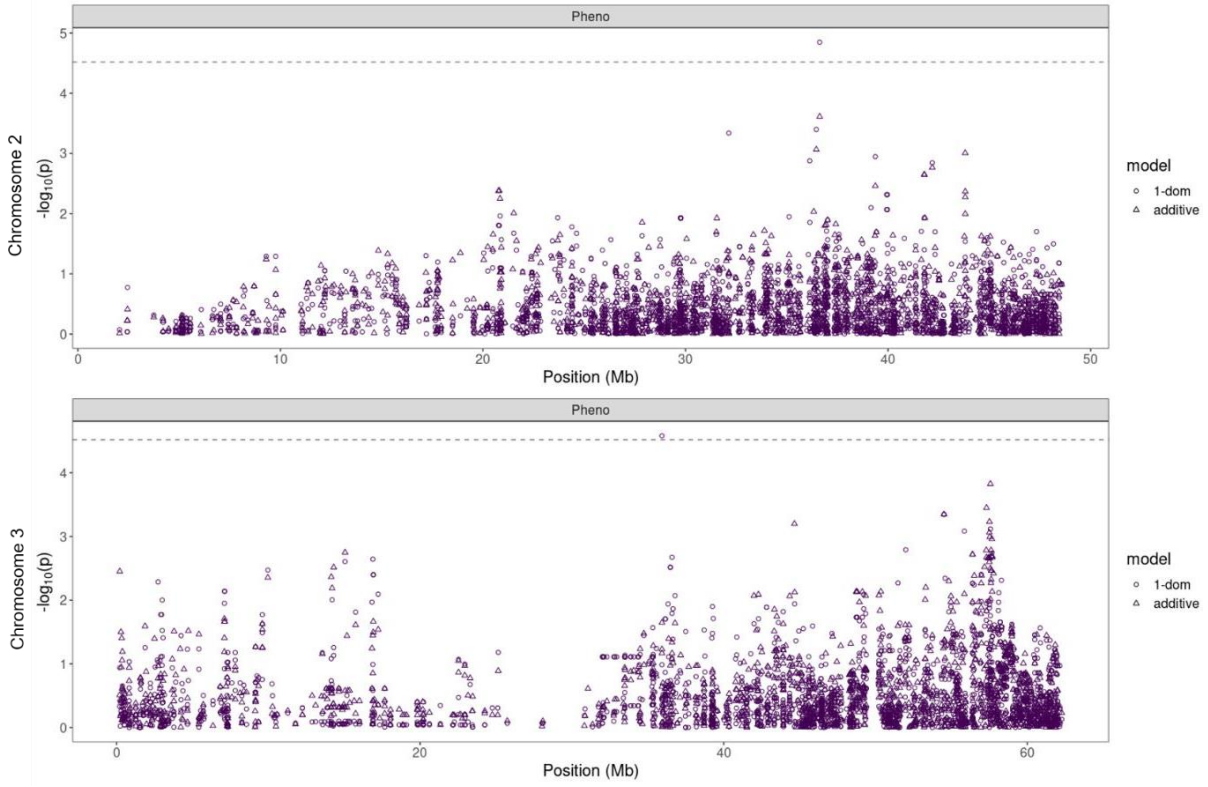

Figure S4. Manhattan plots showing marker:trait association at chromosomes 2 and 3. Markers above the threshold for association under the 1-dom-ref model at the $p<0.2$ level are identified at 36.6Mb on chromosome 2 and 35.9Mb on chromosome 3.

Table S2. Weak marker:trait associations identified. Two markers are found to be associated with the phenotype under a model where a single copy of the reference genome allele is present (1-dom-ref); each explains ~10% of variation in the trait (R^2) at either p<0.1 or p<0.2.

Model	p-value threshold	LOD Threshold	Marker	Chrom	Position	Score	R^2
1-dom-ref	P<0.1	4.73	ST4.03ch02_36621867	2	36621867	4.85	0.104
1-dom-ref	P<0.2	4.36	PotVar0085803	3	35930909	4.58	0.098

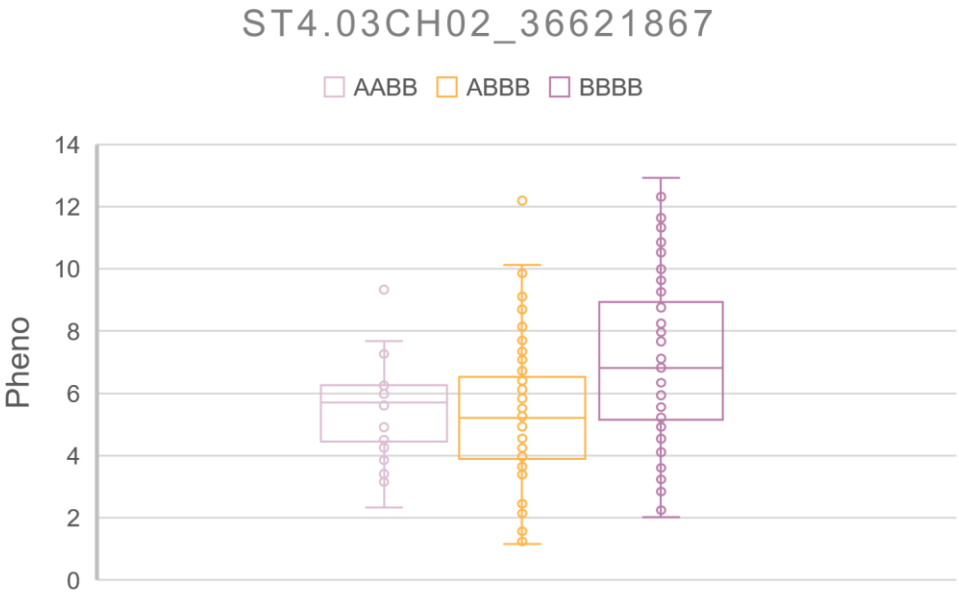

Figure S5. The phenotype (Pheno) as the average root attachment score for the three genotypes at marker ST4.03ch02_36621867. The different genotypes identified have a different dosage of the reference (A) SNP or alternate (B) SNP allele.

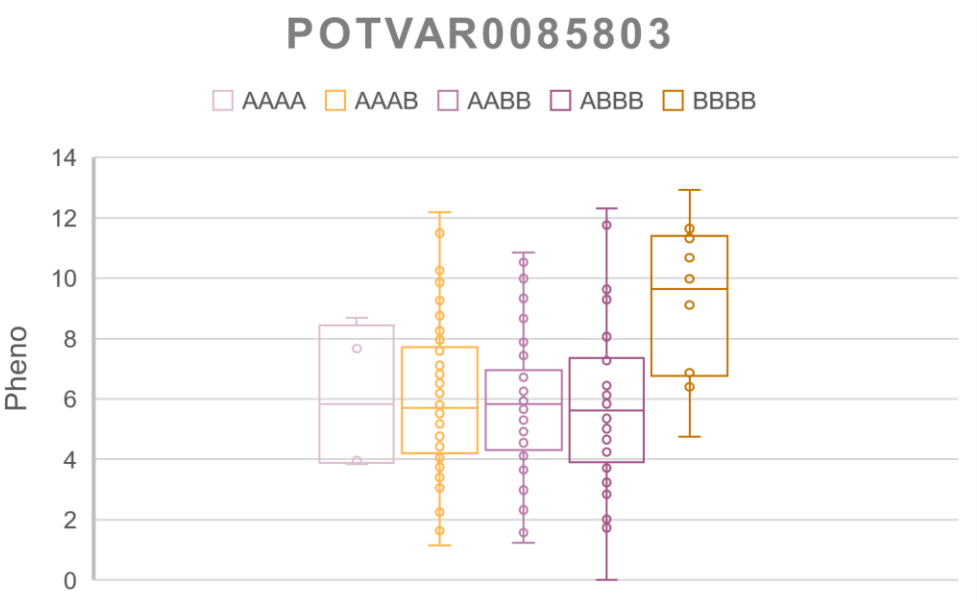

Figure S6. The phenotype (Pheno) as the average root attachment score for the five genotypes at marker POTVAR0085803. The different genotypes identified have a different dosage of the reference (A) SNP or alternate (B) SNP allele.

Discussion

The GWAS analysis was unable to identify any significant marker associations with root attachment score at the 5% threshold. Two markers had the most association out of all tested; ST4.03ch02_36621867 and PotVar0085803 on chromosomes 2 and 3, respectively.

Adding more phenotype data or more samples to the panel and repeating the analysis to improve the resolving power might identify significant associations, including for the two existing weak candidates. If there are multiple sources compared to a single source of resistance in the panel, then the power to detect these will be reduced due to a sampling and representation issue. The size and make-up of the tested population has likely put limitations on the power to detect associations with the phenotype, especially in a tetraploid where there are extra possible genetic combinations compared to a diploid. The presence of many small effect QTL would also require an even larger population to generate the necessary power in the analyses.

The project team could examine the sequence of the regions identified to determine if there are known resistance genes or QTL in the region based on the literature.

Future collaborative work

It is possible to look further in the regions of interest to see if there are any resistance-like candidates,design markers to these and test them on more lines that have been phenotyped for root attachment or on lines that have a known powdery scab score to see if there is any predictive ability of the markers. We suggest, given the lack of significance, that further sources of either biological (known overlapping candidates) or statistical (further lines tested) evidence be sought before assuming thesemarker regions are associated with root attachment.

PFR have powdery scab resistance scores for a potato mapping population with the potential for further work or collaboration comparing the root scores to the tuber disease. PFR also have existingbreeding data on lines for powdery scab scores as well as QTL data for powdery scab resistance, which could be potentially followed up with the team with further collaborative funding.