Hort Innovation

Final Report

Mechanisms and manipulation of resistance to powdery scab in potato roots

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Project code:

PT17003

Project:

Mechanisms and manipulation of resistance to powdery scab in potato roots (PT17003)

Disclaimer:

Funding statement:

This project has been funded by Hort Innovation, using the Fresh Potato and Processing Potato research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Publishing details:

ISBN 978-0-7341-4883-4

Published and distributed by: Hort Innovation

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www.horticulture.com.au

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Public summary

Spongospora subterranea is an important pathogen responsible for powdery scab of tubers that affects appearance and value of tubers, and for root disease that disrupts root function leading to reduced growth and yields. Managing these diseases is extremely difficult, and largely relies on resistant cultivars. Traditionally, the identification of disease resistance has been based on the visual assessment of disease in large field or glasshouse trials, which are both time and resource intensive. This also focusses on assessment of resistance to tuber disease which we now know is not always linked to resistance to root disease.

In this project we developed a rapid cost-effective screen for cultivar resistance to root infection. It focused on the very first phase of interaction between the pathogen and plant where pathogen spores bind to potato roots initiating infection. Using this assay, we screened 153 potato cultivars and lines for their relative resistance to root infection showing a continuum of resistance expression.

We then attempted to generate better disease resistance within commercial cultivars. Using a cell selection technique, we generated variants of important fresh market and processing cultivars which were screened using the new assay. Lines of each cultivar appeared to have enhanced resistance to root infection. We then tested these in repeated glasshouse trials which were able to confirm enhanced disease resistance. These lines require further agronomic testing to determine commercial potential but show great promise for reducing root disease and demonstrate the potential of cell selection techniques for generating disease resistance across a range of potato cultivars.

The project then focused on obtaining a better understanding of the nature of pathogen root attachment. Firstly, we examined the whole root of resistant and susceptible varieties for expression of proteins which may indicate genes responsible for resistance (or susceptibility). More abundant proteins in the resistant lines include those associated with the phenylpropanoid biosynthesis pathway, suggesting the potential role of lignin biosynthesis in host resistance, glutathione metabolism and enzymes involved in pectin biosynthesis and remodelling.

We then used sophisticated proteomic and enzyme digestion techniques to focus on proteins found on the root surface. We found evidence for the importance of glycoproteins on the root surface that enable pathogen binding. For example a 28 kDa glycoprotein was less abundant in resistant varieties which suggests it could be a pathogen recognition factor. Glutathione metabolism and lignin biosynthesis pathway were again found to be more abundant in resistant cultivars. These findings provide possible targets that could be useful in breeding for resistance (or removal of susceptibility).

Lastly, we undertook a genetic screen of a large range of potato cultivars to look for markers specific to the root attachment trait. Covid-restrictions during the project meant we were unable to source as many varieties as we wished, and the power of the analysis was subsequently limited, however linking the gene marker data with the proteomic data should clarify useful markers.

In summary, the project successfully created a new rapid and cost-effective screen for resistance to root infection, and using this generated a listing of relative resistance rankings across 153 varieties. It then confirmed that cell selection techniques can be used to generate potato variants with enhanced disease resistance selecting several lines worthy of further testing. Lastly, fundamental studies improved our understanding of how the pathogen binds to potato roots and identified possible targets for breeding of resistant potatoes in the future.

Keywords

Spongospora subterranea, powdery scab, root disease, disease resistance, zoospore root binding, proteomics

Introduction

Powdery scab is a globally important potato disease. The Australian processing potato industry conservatively estimates losses of $13.4M p.a. with similar losses in the fresh market and indirect on-farm and off-farm costs greater still. Historically most emphasis has been placed on the blemish tuber disease "powdery scab" which reduced tuber value. However, in recent years the importance of root disease caused by this pathogen on diminishing yield has been recognised. Root infection directly affects potato yields through disruption of root function, and necessitates increased use of irrigation, fertiliser and fungicides to compensate for poor root development.

Whilst caused by the same pathogen, the root and tuber infection processes are separate and different plant resistance processes operate against each. Varieties that show resistance to tuber disease do not necessarily show resistance to root infection. Diminishing root infection however will slow disease epidemics and will reduce tuber disease. During root infection, the powdery scab pathogen releases motile zoospores that swim toward and encyst (bind) on the root surface leading to infection and disease. In preliminary studies we have shown that reduced root binding by zoospores is correlated with reduced disease and yield loss and that potato cultivars vary in their efficiency of encystment with resistant cultivars showing reduced binding and subsequent infection. Virtually nothing is known about the nature of the root binding sites and the basis of this resistance.

This project had multiple aims addressing the lack of knowledge around pathogen binding, assessment of resistance and using this information to strategically support resistance selection using traditional or novel breeding approaches.

Specifically, the project had the following major objectives:

1) Develop a rapid, robust and cost-effective means for assessment of cultivar resistance to the root infection (attachment) phase of *S. subterranea* interaction with the potato host.
2) Use this assay to select variants of commercial cultivars developed through the use of somaclonal cell selection techniques that show enhanced resistance to pathogen root binding and subsequent disease whilst retaining important agronomic traits.
3) Develop a better understanding of the nature of pathogen root attachment, attempting to identify possible pathogen receptors on roots that could be novel breeding targets for resistance and/or targets for blockers of pathogen attachment.
4) Assess the genetic basis of root attachment to develop gene markers to assist breeding for this trait.

This project was envisaged to provide benefits for the fresh and processing Australian potato industries by increased potato industry productivity and profitability through:

a) Reduced losses due to root disease caused by infections with *S. subterranea* achieved by deployment of cultivars with greater resistance and/or blockers to reduce pathogen root binding.
b) Reduced requirements for agronomic inputs (fertiliser, water) needed to compensate for reduced yields and root function in disease plants and greater quality produce with better storage capability.
c) Development and adoption of more efficient resistant screening technologies to test new varieties of interest to industry for resistance to root disease, which will enable more rapid adoption of new varieties.

This project will be of direct relevance to the fresh and processing strategic investment plans providing input into the Hort Innovation investment priorities "Support industry efficiency and sustainability" and Processing potato SIP outcome: Outcome 3: Losses from pest and disease are reduced, resulting in improved quality and increased marketable yield and Fresh potato SIP outcome: Outcome 3: Average yields have significantly improved resulting in reduced cost of production.

Methodology

This project included numerous and extensive laboratory and glasshouse experimentation that would be cumbersome to include here within the main text of this report. Rather, full details of all experiments are presented within the attached appendices.

Results and discussion

The full results and detailed discussions of these results are again overly substantial for inclusion within the main text of the report but are once again available within the attached appendices.

Rather, here I provide an extended summary of the major outcomes from the various studies.

Development of a rapid, cost-effective assay to screen potato varieties for resistance to root infection.

In this part of the project, we aimed to develop a lab-based screen for resistance to root disease that is more rapid, more cost-effective, and more reliable than traditional field and glasshouse trials. The assay relies on a good understanding of the biology and pathogenicity of the pathogen. *S. subterranea* persists in the soil as dormant resting spores that release motile zoospores which are attracted by chemotaxis to host roots to which they attach (encyst) and penetrate through the cell wall, inserting their cellular contents to facilitate infection. Infection eventually leads to formation of zoosporangia from which further zoospores are released allowing further root infections to occur. Root galling and tuber disease follows as the infection progresses. Infections proceed in a polycyclic manner (many cycles in the one season) which can be influenced by environmental conditions. A greater or lesser infection rate can influence the expression of disease and ability of traditional assays to provide an accurate resistance rating.

Critically, the very first interaction occurs when the zoospore binds to the potato root, which occurs before the polycyclic infections take place. By examining the relative capacity of roots to allow zoospore binding we can quickly assess their relative resistance to root infection independent of tuber disease.

In our work we developed a lab assay that uses excised root segments from potato cultivars placed into an incubation jar which contains a suitable buffer and a population of pathogen zoospores. The numbers of zoospores attached to root segments after 48 hours incubation were counted using light microscopy and compared to reference resistant and susceptible cultivars. Firstly, we undertook a study to understand optimal conditions for zoospore germination and root attachment. Optimal zoospore release occurred at 20°C in Hoagland's solution in a rapid and synchronized manner over the first 2 days, followed by a steep decline. The extent of zoospore root attachment varied with cultivar (Iwa > Agria > Russet Burbank > Gladiator), region of the root maturation zone (lower > middle > upper) and temperature (greatest zoospore root attachment occurring at 15°C). Following this, we developed the screening assay using optimal temperature and incubation parameters. We screened 153 cultivars and lines that were available to us and for which we gained permissions from variety owners. The results showed a continuum of disease resistance expression indicative of multigenic control of resistance.

Resistance to zoospore root attachment generally agreed with the cultivar resistance rankings obtained from published potato powdery scab severity scores field trial data (where available), with (as we expected) a few notable varietal exceptions. This laboratory bioassay only requires potato root tissue samples. In the present study, we used tissue-cultured plantlets, but, we have also successfully used root tissues harvested from glasshouse-grown plants for the in vitro screen. This increases the bioassay's 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. We propose 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.

Generation of enhanced resistance to root infection in select potato varieties through somaclonal cell selection techniques.

This part of the project looked to use techniques we successfully developed and deployed for generation of enhanced resistance to common scab disease against Spongospora root and tuber diseases.

In short, we can take elite potato cultivars with superior agronomic traits, and through tissue culture techniques generate cell cultures of these varieties. We then treat these cell cultures with stresses to induce somaclonal mutations. For these experiments we used both a crude extract derived from Spongospora induced potato root galls and thaxtomin A, the toxin produced by the common scab pathogen which was known to induce broad spectrum disease resistance from previous work.

In consultation with industry, we selected a range of fresh market and processing varieties that were publicly owned. Of those selected we successfully obtained a total of 97 variants from Russet Burbank., Ranger Russet, Shepody, Sebago, Desiree and Nicola. These variants were screened using our laboratory assay for resistance to root attachment and those clones that showed potential for enhanced resistance selected from further screening. Of the 97 somaclonal variants, eight showed significantly enhanced resistance to *S. subterranea* zoospore root attachment compared to their unselected parents with a further 20-30% showing enhanced resistance (but not significantly so). Previous studies found that 20 to 33% of regenerated variants showed greater resistance to common scab than the unselected parent cultivar in glasshouse assessment.

These were then tested in repeated glasshouse trials with tuber production and tuber disease parameters measured. The glasshouse trials generally confirmed the laboratory bioassay results with enhanced resistance to tuber powdery scab observed. Whilst we note that varieties vary in their ease of cell selection, the trials successfully demonstrated the capacity to select for enhanced disease resistance across all varieties we tested.

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 laboratory zoospore root attachment assay is a rapid way to screen host resistance to powdery scab. This system is efficient for development of enhanced host resistance to potato powdery scab caused by *S. subterranea* in current commercial cultivars.

We now have several lines which show greater disease resistance than their parent variety. These require further agronomic testing to determine their potential commercial application but provide an exciting alternate approach to generating disease resistance without genetic reassortment associated with traditional breeding approaches.

Understanding the physiological and genetic basis of zoospore binding to potato roots

This part of the project aimed to determine the nature of pathogen attachment to roots that leads to infection, to identify putative receptor genes and gene products that could be targeted for enhancing disease resistance, and to test disruption of these putative receptors.

This was done in two major steps. Firstly, a whole of root analysis comparing root proteins isolated from a population of resistant and susceptible cultivars for their relative abundance. This allows identification of key proteins and pathways consistently associated with disease resistance or susceptibility. 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 also 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.

The second approach used enzyme digestion treatments and analysis of root surface proteins to (a) identify the likely type of molecule responsible for pathogen binding reception and (b) develop a database of putative target protein and polysaccharide molecules. We first compared the effects of enzymatic removal of root cell-wall proteins, N-linked glycans and polysaccharides on *S. subterranea* attachment. Subsequent analysis of peptides released by trypsin shaving (TS) of root segments identified 262 proteins that were differentially abundant between cultivars. These were enriched in root-

surface-derived peptides but also included intracellular proteins, e.g., proteins associated with glutathione metabolism and lignin biosynthesis, which were more abundant in the resistant cultivar. Comparison with whole-root proteomic analysis of the same cultivars identified 226 proteins specific to the TS dataset, of which 188 were significantly different. Among these, the pathogen-defence-related cell-wall protein stem 28 kDa glycoprotein and two major latex proteins were significantly less abundant in the resistant cultivar. A further major latex protein was reduced in the resistant cultivar in both the TS and whole-root datasets. In contrast, three glutathione S-transferase proteins were more abundant in the resistant cultivar (TS-specific), while the protein glucan endo-1,3-beta-glucosidase was increased in both datasets. These results imply a particular role for major latex proteins and glucan endo-1,3-beta-glucosidase in regulating zoospore binding to potato roots and susceptibility to *S. subterranea*.

These studies have both dramatically improved our understanding of the nature of pathogen attachment and infection, and identified putative target proteins and glycoproteins associated with resistance or susceptibility that can be further tested to determine their specific role in disease. Manipulation of the expression of these genes through breeding, gene editing or cell selection may provide novel options for highly effective and robust disease resistance.

Analysis of potential gene markers associated with root attachment resistance phenotype.

In this part of the project, we worked in collaboration with potato geneticists at New Zealand Plant and Food Research. The original plan was to travel to NZ to access a large genotype collection and conduct the genetic analysis. Covid-restrictions on travel made this impossible during the project period. Rather we diverted resources into accessing as many genotypes from Australian sources as possible. We successfully obtained 153 cultivars and lines which was less than the anticipated 200-250 suggested might be necessary for statistical power in the GWAS analysis. The attached report notes that whilst some potential gene marker peaks were identified, these were at low confidence/significance and this not ready at this stage for adoption within breeding programs. However, we believe overlaying proteomics data using new software recently developed by NZP&F may assist in clarifying target genes (and reinforcing data generated from the proteomics experiments). We will continue not examine this option with NZP&F post project completion.

Outputs

The outcomes of the project were extensively reported to industry and scientific audiences. The table below provides reference to the published industry and journal articles, and the industry and scientific conference presentations.

Published industry reports: 2 articles (PotatoLink Magazine) – see appendices.

Published scientific papers: 3 refereed journal articles with 1 further article planned.

Industry presentations and conference: 4 presentations at potato R&D meetings and 7 ad hoc presentations to industry groups in Australia and overseas.

Scientific conferences: 3 national and international conference presentations

Table 1. Output summary

Output	Description	Detail
Project plans, milestones and report	Project initial planning, milestone reports and final report.	All project reporting has been completed with acceptance of this final report. Milestone reports and final report are available via the Hort Innovation web site.
Stakeholder communication activities.	Industry publication, industry conferences and industry meetings	Articles published in PotatoLink (May – June 2021, and Summer 2022). Presentation at all annual potato R&D meetings that were able to be held (incl. in Melbourne, 2019, Ballarat, 2021, and zoom meetings in 2020 and 2021).

		Presentations on project outcomes at ad hoc industry meetings Forthside open day, 2019; Simplot Agronomists, 2020; Simplot Growers, 2021, ServeAg Agronomy team, 2021, CSS Farms, USA (online) 2021, Potato NZ (online) 2021; and Potato-Link industry session, Forthside, 2021.
A rapid and robust disease screening system to assist in variety choice.	A novel system for rapidly screening potato varieties for resistance to pathogen root attachment (and subsequent infection) was developed.	Details of the assay are provided in journal article and summarised in industry articles listed in the appendix of this report.
A listing of potato varieties for relative resistance to root disease.	A listing of available varieties for their relative resistance to pathogen root attachment was developed and shared.	Details of the list are provided in industry and journal articles listed in the appendix of this report.
Variant clones of industry selected varieties with reduced susceptibility to root disease.	Somaclonal variants of select potato varieties have been generated and screened for disease resistance.	Details of the assay are provided in journal article listed in the appendix of this report.
Scientific peer review publications and conference presentations.	Project results have been published or presented in journal articles and/or conferences.	Three refereed journal articles have been published (and one further one flagged). See appendix. Conference presentations were given at the European Association of Potato Research meeting, Switzerland, 2019; Australasian Plant Pathology Society meeting (on line) 2021; and the INIA Semana de la Sanidad Vegetal Y Medio Ambiente, in Chile (on line) 2021.
Analysis of the surface proteins of resistant and susceptible potato roots	Studies undertook analysis of root surface proteins to determine putative pathogen receptor molecules	Two scientific articles published.
New knowledge of physiological and genetic basis of resistance to zoospore attachment to roots.	Proteomic and genetic analyses have revealed new knowledge about the nature of zoospore binding to potato roots	Three scientific articles published.

Outcomes

The key outcomes of this project are listed in the table below.

This project supported the fresh and processing strategic investment plans providing input into the Hort Innovation investment priorities "Support industry efficiency and sustainability" and Processing potato SIP outcome: Outcome 3: Losses from pest and disease are reduced, resulting in improved quality and increased marketable yield and Fresh potato SIP outcome: Outcome 3: Average yields have significantly improved resulting in reduced cost of production.

We expect project outcomes will have direct benefits for potato breeders, and those within industry interested in understanding the relative merits of the cultivars they grow in terms of disease impact. Pending further work on testing agronomic performance and commercial viability, this project may also benefit growers through provision of lines of commercial cultivars with enhanced disease resistance. Lastly, it will benefit applied scientists in identification of new research targets to facilitate improved disease resistance.

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Adoption of more efficient and targeted screening technologies	*Outcome:* The Australian potato industry has increased profitability, efficiency and sustainability through innovative research and development (R&D) and sustainable best management practices (BMPs). *Strategy*: Identify and support opportunities to improve productivity and sustainability through effective IPDM, weed control, soil health and cover crops. *KPI:* Pest and disease management strategies are developed that mitigate crop loss in collaboration with growers.	A new system for screening varieties for resistance to root attachment by the target pathogen has been developed that provides a much more efficient method than traditional assays.	The new screening system has been published in both an industry magazine (PotatoLink) and in a scientific journal (Plant Pathology).
A listing of potato varieties selected by industry stakeholders for their relative resistance	As above	A large collection of potato varieties we were sourced from repositories around Australia. These were all screened using the new root attachment system and relative resistance determined. The full listing for which we have permission from variety owners has been published.	A listing of potato varieties has been published in both an industry magazine (PotatoLink) and in a scientific journal (Plant Pathology). We have received feedback and had discussions from both industry and scientists on our listing which resulted in some further testing.
Disease-resistant variant	As above	A selection of fresh market	We have selected and

clones of industry selected potato varieties		and processing varieties were selected through industry consultation. Somaclonal variants from these were generated and screened using both the new root screening technique and more traditional glasshouse pathology testing.	screened 97 somaclonal variants from six fresh and processing varieties selected by industry (some varieties proved difficult to efficiently obtain variants). These clones are available for further agronomic testing prior to evaluation of potential use.
New knowledge of physiological and genetic basis of resistance to zoospore attachment to roots.	As above	Greater understanding of pathogen:host interaction relating to resistance.	Peer reviewed journal publications
Increased productivity and profitability of the potato industries.	As above	Adoption of more resistant varieties identified by the project should decrease disease and improve productivity and profitability. More resistant potato cultivar variants will require further work prior to adoption.	We expect adoption to occur post project completion

Monitoring and evaluation

Table 3. Key Evaluation Questions

Key Evaluation Question	**Project performance**	**Continuous improvement opportunities**
To what extent has the project achieved its expected outcomes?	The project achieved it major outcomes as listed in table 2. These have been communicated widely to both industry and science communities. The project has developed novel outcomes in new disease assessment assays, listings of cultivar resistance and somaclonal variants with enhanced resistance, alongside important advances in knowledge of this disease system which will provide targets for future research.	Recruitment of an associated PhD student enabled a greater volume and depth of research to be undertaken. The project had quite a basic research focus, but has clearly identified areas for further strategic investment to acehve goals of limiting disease impact.
How relevant was the project to the needs of intended beneficiaries?	We believe the project achieved its major goals. We provided a novel cultivar assessment assay that can	Further testing of somaclonal variants over multi-year trials will be required to fully engage industry and

	easily be adopted by beneficiaries. Contact from potato breeding programs have indicated interest in such screening. Feedback from industry groups through extension activities was universally positive, with excitement generated around the potential of novel controls in the near future.	provide substantial benefits from this aspect of the study.
How well have intended beneficiaries been engaged in the project?	The major outcome readily adoptable was published in PotatoLink magazine. Project CI attended all industry R&D for a when held (limited due to covid19 travel restrictions)	Restrictions on extension opportunities may have limited project outreach, but these were supplemented by numerous ad hoc engagement activities which were universally well received and provided valuable feedback to the project.
To what extent were engagement processes appropriate to the target audience/s of the project?	The major external engagement activities from this project were with variety owners and breeders (to obtain lines and gain permissions for testing), project collaborators (on gene markers, and various industry groups and science audiences updating project outcomes.	The communication activities were very well received. Covid-travel restriction meant there were limited opportunities for face-to-face extension activities outside of Tasmania which was unfortunate, however on-line activities did achieve significant reach both nationally and internationally.
What efforts did the project make to improve efficiency?	Access to potato lines was restricted by our ability to travel to our partners facilities in New Zealand. We managed this through accessing materials from various sources within Australia, rediverting project resources to obtaining these materials. We also recruited a UTAS funded PhD student to work alongside the project and project greater in depth studies that would have been possible within the project budget alone. This also provided industry specific training for a new scientist in potato research.	

Recommendations

Practical application of the project findings

The new screening tool can now be used to rapidly and efficiently evaluate cultivar resistance to root infection by *S. subterranea*. This may be of immediate value to potato breeders, and also of value to major companies and growers that look to establish new varieties within their business and wish to understand the likelihood of impact of root infection on these varieties before significant investment in plantings have been made.

The listing of cultivars provided gives growers, companies and breeders critical information on the relative resistance or susceptibility of a wide range of potato genotypes. This allows growers and companies to make strategic selection of varieties with the knowledge of the likely impact of root infection.

Enzymatic inhibition/blocking root treatments, whilst effective at preventing infection, are unlikely to be commercially valuable for industry. They do however assist in identifying the nature of pathogen attachment and infection.

Possibilities of future RD&E that directly flow from the work undertaken and its results

The somaclonal study revealed several lines of the target cultivars that show enhanced resistance to root infection in laboratory and glasshouse screening. Further evaluation of these lines under field assessment to test both robustness of disease resistance and agronomic performance is required. This will identify those with potential for commercial deployment.

Furthermore, the project has demonstrated proof of concept for using cell selection techniques to generate enhanced resistance to root infection and powdery scab. This could now be further strategically employed to develop enhanced resistance in other cultivars of immediate or strategic interest.

NEW PROJECT – Somaclonal selection for enhancing disease resistance in commercial cultivars. We recommend that further multi-year evaluation of key somaclonal variants developed in this project be undertaken to test their agronomic and disease resistance performance under commercial production practices. We further recommend that additional variants of other strategically important potato cultivars be developed. Importantly, these variants clones also provide a unique resource to evaluate mechanisms of enhanced resistance within these resistant variants which should be undertaken.

The root-pathogen interaction studies identified several potato genes and gene products that may be receptors or linked to receptors of pathogen binding to potato roots. Elimination of these genes/gene products may therefore result in comprehensive resistance to infection and subsequent disease with the possibility of immunity. Further studies to empirically test these specific genes for their role in disease resistance/susceptibility are certainly warranted.

Furthermore, by overlaying proteomic and gene marker data using newly developed software tools, identification of further important genes associated with resistance may be possible.

NEW PROJECT – Selection of disease resistance through targeting pathogen root receptors. We recommend that identified genes and gene products be empirically tested for their role in pathogen root attachment to roots. Once confirmed, we recommend that options for using this information to select or develop resistant varieties be examined. As these are susceptibility factors, non-GMO CRISPR approaches could be employed to specially remove these genes from existing elite varieties strategically enhancing disease resistance. Alternately, presence or absence of the identified genes could be used as a specific gene marker in selection of new varieties in traditional breeding approaches or indeed in approaches such as somaclonal selection for greater efficiency of selection. We further recommend that new software tools be used to overlay resistance data and mine datasets for new gene targets.

Development and adoption activities that would ensure full value from the project's findings for industry.

The efficiency of the root attachment assay could perhaps be further improved by assessing the possibility of replacing microscopic examination with sensitive detection tools such as qPCR. This developmental work may make adoption of the assay easier for those with less direct experience in observation of pathogen spores.

Further expansion of the testing of potato cultivars and lines could be done where new lines of strategic importance are identified by industry.

One could also consider further field assessment of somaclones as a development activity to ensure industry confidence in selection lines, and provide bulked materials for commercial testing.

Refereed scientific publications

Journal articles

1 Yu, X., Tegg, R.S., Eyles, A., Wilson, A.J. and Wilson, C.R., 2022. Development and validation of a novel rapid in vitro assay for determining resistance of potato cultivars to root attachment by *Spongospora subterranea* zoospores. Plant Pathology, 72 (2) 392-405. https://doi.org/10.1111/ppa.13659.

2 Yu, X., Wilson, R., Balotf, S., Tegg, R.S., Eyles, A. and Wilson, C.R., 2022. Comparative Proteomic Analysis of Potato Roots from Resistant and Susceptible Cultivars to *Spongospora subterranea* Zoospore Root Attachment In Vitro. Molecules, 27(18), 6024; https://doi.org/10.3390/molecules27186024.

3 Yu, X., Wilson, R., Eyles, A., Balotf, S., Tegg, R.S. and Wilson, C.R., 2023. Enzymatic Investigation of *Spongospora subterranea* Zoospore Attachment to Roots of Potato Cultivars Resistant or Susceptible to Powdery Scab Disease. Proteomes, 11(1), 7; https://doi.org/10.3390/proteomes11010007

References

Citation lists accompany each output listed in the appendices.

Intellectual property

No project IP or commercialisation to report.

Acknowledgements

We acknowledge the financial support for UTAS in providing a PhD stipend for the student aligned to the project activities.

Appendices

Potato Link articles (May-June 2021 & Summer 2022).

Development and validation of a novel rapid in vitro assay for determining resistance of potato cultivars to root attachment by Spongospora subterranea zoospores

- Experimental data providing new knowledge on *S. subterranea* zoospore germination and root binding parameters, development of a novel resistance screening assay and provision of a comprehensive listing of variety resistance to zoospore root attachment.

Selection of disease resistant variants of elite fresh market and processing potato varieties through somatic cell

selection

- Experimental data developing and assessing somaclonal variants of elite fresh market and processing potato cultivars for enhanced resistance to S. subterranea zoospore root attachment and resultant powdery scab disease.

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

- Whole root proteomic analysis comparing populations of resistant and susceptible potato cultivars for likely gene factors associated with resistance or susceptibility to disease.

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

- Direct investigation of root surface proteins and polysaccharides to determine the nature and identity of potato pathogen receptor molecules.

Mechanisms and manipulation of resistance to powdery scab: QTL analysis

- Data and results from our NZ partners looking at putative gene markers for resistance to root Infection trait. Layering of results with proteomic data should assist in revealing genetic linkages.

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MAY • JUNE 2021

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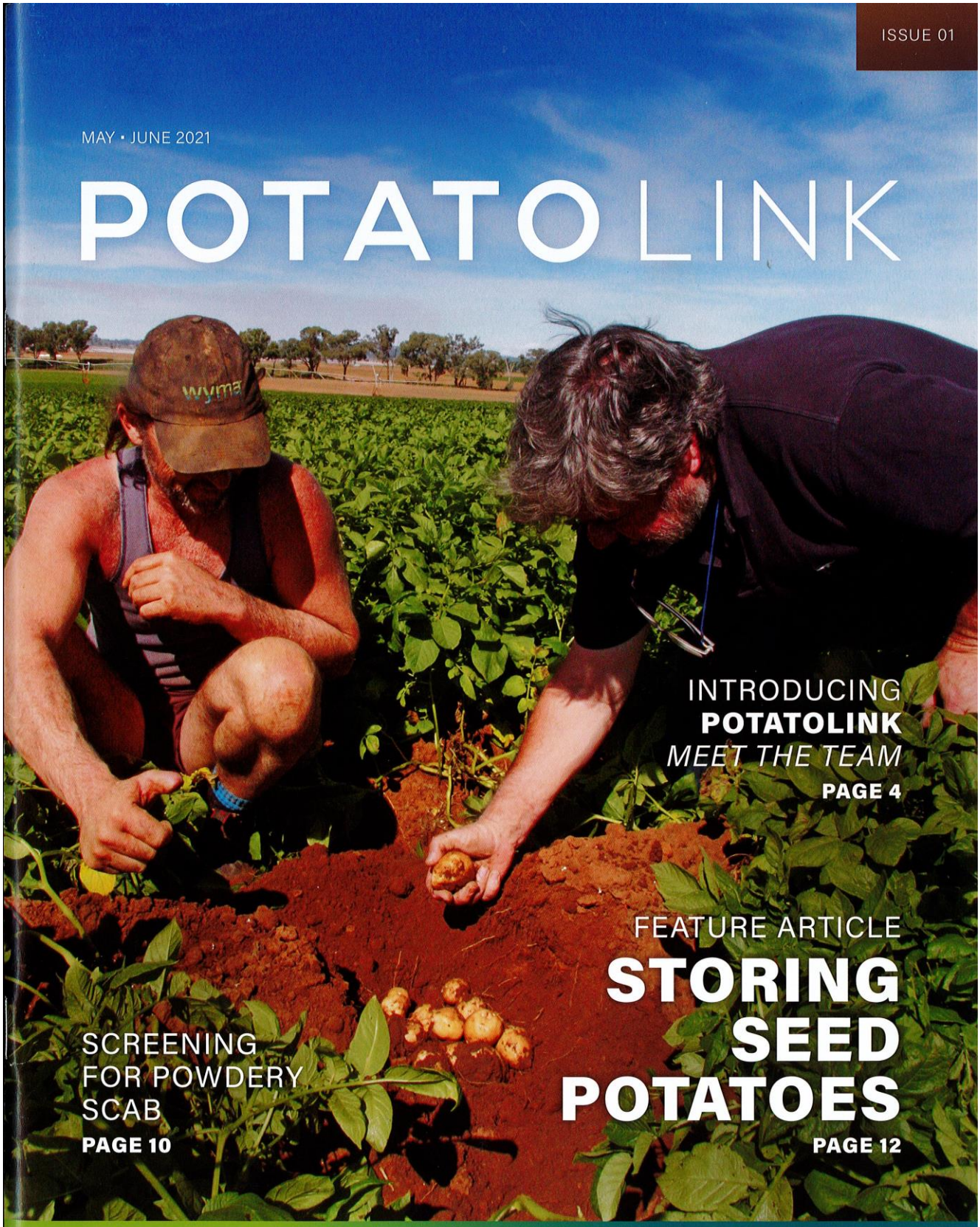

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AUSTRALIAN POTATO INDUSTRY EXTENSION PROJECT

A NEW, RAPID TOOL TO SCREEN FOR POWDERY SCAB RESISTANCE

Half-way through a three-year project to improve understanding of *Spongospora* (powdery scab), the Tasmanian Institute of Agriculture has successfully developed a method that rapidly identifies varietal differences in susceptibility to this disease. *PotatoLink* spoke to Professor Calum Wilson about the novel root attachment screening component of the project.

Powdery scab can be a devastating disease. Not only does it reduce quality and marketability, it also disrupts root function, and can greatly reduce yield.

However, there are clear differences in disease susceptibility between varieties. Traditionally, varietal screening requires either glasshouse or field trials. Potatoes are grown in pathogen-infested soil in large, replicated experiments.

Such trials take many months to complete as the potato plant needs to grow to full maturity. This makes them expensive in both resources and skilled labor. The results, especially in field trials, can also be variable as soil-borne sources of pathogen may be patchy across the trial site.

The research team, headed by Professor Callum Wilson at the Tasmanian Institute of Agriculture (TIA) at the University of Tasmania, has developed a rapid and robust laboratory test to screen potato varieties for resistance to the root infection phase of powdery scab disease. Professor Wilson says, "The new test we have developed allows varieties to be tested within a period of days without even having to leave the laboratory. This greatly reduces the time required for screening, as well as costs associated with resource inputs."

Root infection is the first and most important phase of this disease. Successful root infection allows the pathogen to multiply exponentially within the root zone, leading to extensive root damage and eventually to tuber disease. Varieties that have increased resistance to root infection will be impacted less by the pathogen in terms of both tuber yields and quality.

"Targeting early stages of root infection was the key to this screening technique. This is when the powdery scab pathogen releases motile spores. The spores swim through the water in the soil and bind to potato roots, leading to infection and disease. The efficiency of potato root attachment by the pathogen spore has been demonstrated to impact subsequent root and tuber disease development" explains Professor Wilson.

PhD student Xian Yu has been working on developing this assay and has used it successfully to screen a large number of potato varieties for resistance to root infection.

The varietal resistance rankings determined by the new laboratory assay for a selection of lines are shown in Table 1.

Highly susceptible		Moderately susceptible		Moderately resistant		Good resistance	
Nicola	12.19	Gold Rush	6.8	Dutch Cream	4.93	Toolangi Delight	2.5
Liseta	10.32	Yukon Gold	6.72	Pink Eye	4.76	Tolaas	2.02
Kranz	9.65	Tasman	6.52	Banana	4.66	Granola	1.57
Shepody	9.4	Pink Fir Apple	6.51	Red Pontiac	4.66	Russet Nugget	1.24
Shine	9.29	Spunta	6.44	Spey	4.54		
Patrones	8.82	Frontier Russet	6.4	Nampa	4.26		
Delaware	8.76	Lustre	6.4	Cranberry Red	4.16		
Nooksack	8.67	Wilwash	6.34	Purple Congo	3.75		
Coliban	8.24	Leven	6.13	Fontenot	3.65		
Red Ruby	8.12	Bintje	6.12	Ruby Lou	3.61		
Southern Cross	8.12	Pentland Dell	5.98	Brake light	3.36		
Kennebec	8.06	Mainstay	5.94	Sebago	3.3		
Diament	7.91	Cariboo	5.85	Russet Burbank	3.29		
King Edwarwd	7.67	Atlantic	5.71	Dawmore	3.24		
Sapphire	7.59	Pontiac	5.55	Desiree	3.15		
Sunrise	7.48	Chieftain	5.52				
Crystal	7.35	Sequoia	5.47				
Bison	7.27	Bismark	5.36				
Kipfler	7.25	Yellow King	5.27				
Up-to-Date	7.08	Maris Piper	5.17				

Table 1. Selected variety resistance rankings based on the novel root attachment assay.

Figure 1. Powdery scab pathogen spores (arrowed) attached to potato root hairs (left)

Figure 2. Professor Calum Wilson and PhD student Xian Yu inspecting tissue culture potatoes (right)

The team at TIA found that higher numbers of zoospores attached to the roots of varieties known to be susceptible to powdery scab root infection by traditional assays. Similarly, those varieties with known resistance to root infection had low numbers of zoospores attached to the root. These results validate the effectiveness of this robust, quick assay.

This may now enable the screening of hundreds of potato cultivars for resistance to powdery scab within weeks, instead of the traditional glasshouse or field methods that take many months and are more expensive.

"The importance of this early phase of root infection cannot be underestimated. Interventions at this stage can provide major benefits in disease outcomes later in the season. The project is also looking closely at how the spores bind to roots and how resistant varieties reduce root infection. These may lead to novel controls focused on preventing root infection and tools to assist breeding for disease resistance," Professor Wilson says.

Hort Innovation
Strategic levy investment

■ **For more information from this project, please contact Dr Calum Wilson at** calum.wilson@utas.edu.au

This project, *Mechanisms and manipulation of resistance to powdery scab in potato roots* (PT17003) is being funded by Hort Innovation, using the processing and fresh potato research and development levy, in-kind contributions from the Tasmanian Institute of Agriculture and contributions from the Australian Government.

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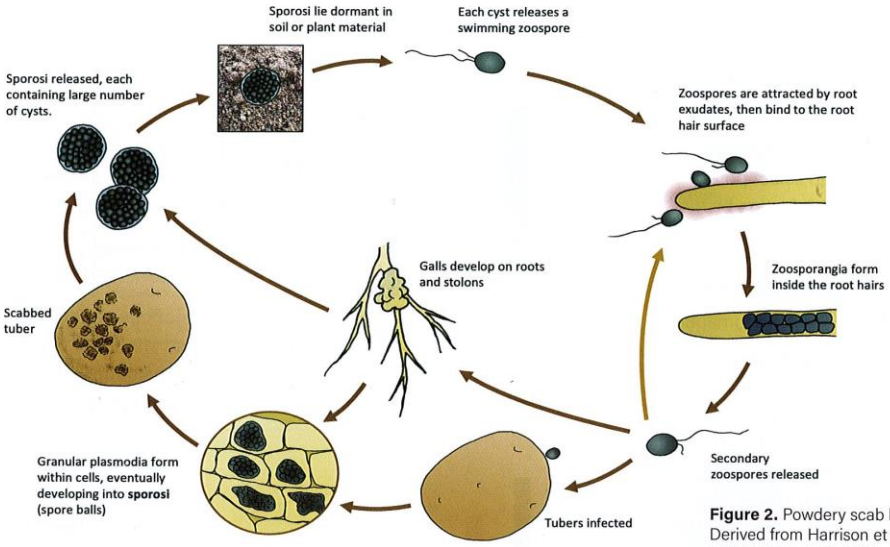

Figure 2. Powdery scab lifecycle. Derived from Harrison et al., 1997 and Falloon et. al., 2015.

- Improve quality and storage life of tubers
- Develop new disease management and resistance screening tools

To achieve this, the project has progressed in a series of activities.

The first was to develop a new way to search for resistant varieties. Traditional screening relies on growing different potato varieties in a glasshouse over many months, then examining root galls and lesions formed on tubers. This is not only time and resource intensive, but may not provide an accurate indication of infection.

Professor Wilson's new method examines the numbers of zoospores able to attach to the potato root hairs. This technique can provide reliable results in as little as 48 hours, potentially allowing hundreds of cultivars to be screened for resistance.

"We wanted to measure how much infection occurs in those roots," said Professor Wilson, "you get varieties where there are almost no symptoms

Table 1. Variety resistance to powdery scab, based on the root attachment assay.

Highly susceptible	Moderately susceptible	Moderately resistant	Good resistance
Nicola	Gold Rush	Russet Burbank	Russet Nugget
Liseta	Yukon Gold	Desiree	Granola
Kranz	Tasman	Dawmore	Tolaas
Shepody	Spunta	Sebago	Tolangi Delight
Shine	Pontiac	Brake Light	
Red Ruby	Frontier Russet	Ruby Lou	
Patrones	Lustre	Fontenot	
Delaware	Wilwash	Purple Congo	
Nooksack	Leven	Cranberry Red	
Coliban	Bintje	Nampa	
Southern Cross	Pentland Dell	Spey	
Kennebec	Mainstay	Banana	
Diment	Cariboo	Pink Eye	
Kipfler	Atlantic	Dutch Cream	
Up-to-Date	Chiefton		
	Sequoia		
	Bismark		
	Yellow King		

POWDERY SCAB PROJECT UPDATE

POTATO R&D FORUM 2022

If you grow potatoes in Tasmania, you are probably familiar with powdery scab. While sometimes regarded as a southern disease, this devastating pathogen has been detected from Queensland to South Australia, and even parts of Western Australia. Annual losses are estimated to top $13.4 million annually. However, incidence varies hugely by cultivar and area, raising the question – **WHY?**

Jenny Ekman reports.

Professor Calum Wilson from the Tasmanian Institute of Agriculture (TIA) is a world leader when it comes to managing *Spongospora subterranea*, the cause of powdery scab. He is currently conducting a three-year Hort Innovation project "Mechanisms and manipulation of resistance to powdery scab in potato roots".

Professor Wilson provided an update on the project at the recent R&D Forum in Ballarat.

While the most obvious symptoms of powdery scab are lesions on tubers, the pathogen also causes formation of galls on roots, shoots and stolons (Figure 1).

Figure 1. Galls can form on roots, stolons or shoots. Source: C. Wilson.

However, it is the effects on the roots which have the greatest influence on yield. Symptoms of root infection are not always visible, with the result powdery scab can be an underestimated disease. However, recognition of the importance of this pathogen has been increasing worldwide.

It is also possible that intensification of production, use of susceptible varieties and more frequent irrigation have increased incidence of disease[1].

HOW DOES INFECTION OCCUR?

Infection mainly occurs by zoosporangia binding to and then infecting the root hairs (Figure 2). This early, critical stage can reduce root function by 20% or more, even though no symptoms are outwardly visible. Root infection is often unrelated to the physical symptoms of galls and lesions on tubers and roots. However, it is the most important factor reducing plant growth (Figure 3, Figure 4).

Professor Wilson has therefore focussed primarily on the root hair infection phase, rather than the symptoms on tubers. The aims of the project are to:

- Reduce losses
- Reduce input requirements, including water and fertiliser

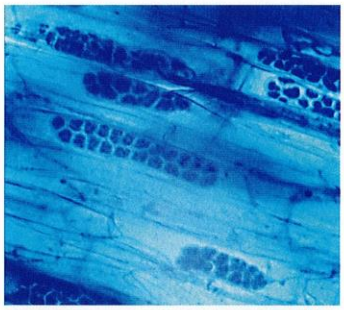

Figure 3. Zoosporangia forming in root cells. Source: C. Wilson.

Figure 4. Healthy potato seedlings (top), compared to seedlings infected with powdery scab (bottom). Although no scab or root galls have formed, there are clear effects on both root and shoot growth of the infected plants. Source: C. Wilson.

on the tubers, but very high levels of infection in the roots – and vice versa (Nicola is an example of this). Our screen directly examines that initial stage of root infection."

The results from the root assays can then be used to select potato varieties that resist infection by the *Spongospora* zoospores.

INDUCING RESISTANCE TO ROOT INFECTION

Professor Wilson's team also looked at a novel tecnique that allows selection of resistant variants of existing cultivars. 'Somaclonal variants' are produced by exposing shoot tips to a stress, then growing the young plantlet in tissue culture. The plantlets often look and behave almost identically to the parent plant, but can have tiny genetic changes.

"Using our new assay we can screen for those variants that have altered susceptibility to root infection" explained Professor Wilson.

The TIA laboratory produced a large number of somaclonal variants, exposed them to the *Spongospora* pathogen, and examined infection of the root hairs by zoospores. As shown in Figure 5, while most are similar to the parental line, some variants proved highly resistant to zoospore binding.

The next stage was to conduct greenhouse trials with lines that appeared to have resistance. Plants were grown to maturity and tubers examined for signs of disease.

While there was considerable variability, several of the variants that strongly resisted binding by zoospores also had less physical evidence of powdery scab (Figure 6). Of course, these variants still need to be field tested to examine yield and other agronomic characteristics, but such results are highly promising.

PREVENTING INFECTION

The third phase of the project is finding ways to prevent infection in the first place. "We are interested in just how the pathogen attaches to the roots and how we can basically stop it happening," Professor Wilson commented, "one possibility is to find a receptor, something that the pathogen recognises and allows it to bind."

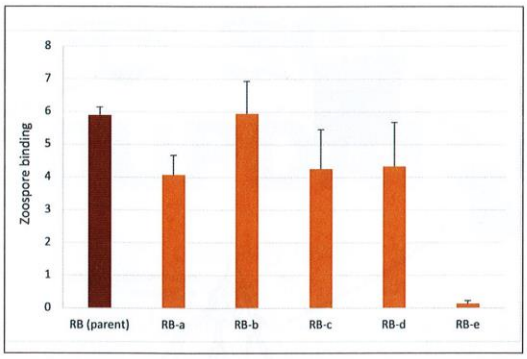

Figure 5. Somaclonal variants, here labelled a to e, were produced from parental line RB. While zoospore binding to most variants was similar to their parent, RB-e was highly resistant.

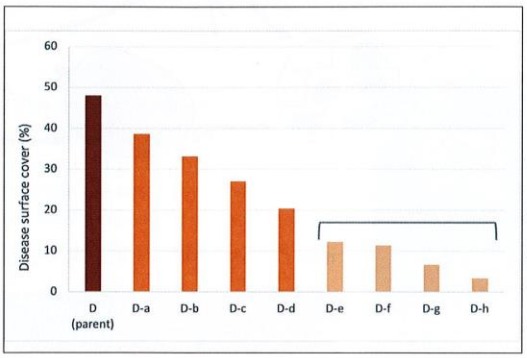

Figure 6. Somaclonal variants (here labelled a to h) that resisted zoospore infection were grown in the glasshouse, then tubers examined for scab. Variants D-e to D-h were significantly different to parental line D.

The team compared resistant and sensitive varieties. Sure enough, they came up with a candidate – a 'glycosolated protein receptor.' When they used enzymes to remove glycosylated proteins from a root hair, zoospores no longer attacked it. "We now have a candidate for where the pathogen binds – if we can knock this out, we could have an immune variety," explained Professor Wilson.

Another avenue is to look at the natural root exudates potato plants produce. These can either attract or repel the swimming zoospores.

"What we found was that the resistant varieties had less of the attractants, and more of the inhibitors, so this

Powdery scab symptoms on a tuber

again could be a really useful screen for resistance."

An Australian Research Council (ARC) grant has enabled the TIA team to examine whether a bacterial inoculant could interfere with this process. The bacteria consume the attractant root exudates, reducing potential infection.

A large number of bacteria have been screened for this ability.

"We got one!" announced Professor Wilson, "and not only did the bacteria digest away the key attractant exudates, it also grew bigger potatoes. It both boosted productivity **and** reduced disease." While the bacteria was not a cure for powdery scab, it meant that susceptible varieties produced a better crop in the presence of the pathogen (Figure 7). If the disease was not present, adding inoculant still produced a bigger crop.

NEXT STEPS

Professor Wilson feels the best option is to target the root receptors. "Potentially, we can use genetic approaches to knock those out, and get an immune variety."

Other good options involve improving root health using beneficial bacteria as a seed dressing, helping the plant tolerate infection. It will also be important to examine interactions between interventions for powdery scab and incidence of other root diseases.

One thing is clear. Powdery scab is both a fascinating and challenging organism. However, with these new management tools, plus a better understanding of how it infects the potato plant, we are now developing a range of innovative options to manage this devastating disease.

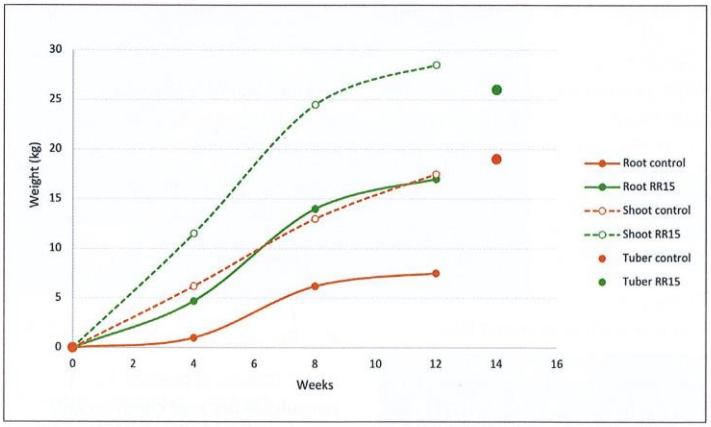

Figure 7. Effect of a bacterial inoculant (RR15) on root growth, shoot growth and tuber weight of potatoes exposed to the powdery scab pathogen. Images at right show the difference between the control (left) and inoculated (right) plants after 12 weeks.

Some of the TIA team

PT17003 - Mechanisms and manipulation of resistance to powdery scab in potato roots has been funded by Hort Innovation, using the potato – fresh and potato – processing research and development levies and contributions from the Australian Government. Hort Innovation is the grower owned, not-for-profit research and development corporation for Australian horticulture

This research was also partially funded by the Australian Government through the Australian Research Council grant DP180103337

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Development and validation of a novel rapid in vitro assay for determining resistance of potato cultivars to root attachment by *Spongospora subterranea* zoospores

This has been published as: Yu, X., Tegg, R.S., Eyles, A., Wilson, A.J. and Wilson, C.R., 2022. Development and validation of a novel rapid in vitro assay for determining resistance of potato cultivars to root attachment by Spongospora subterranea zoospores. Plant Pathology, 72 (2) 392-405. https://doi.org/10.1111/ppa.13659

Spongospora subterranea f. sp. *subterranea* is a major pathogen of potatoes leading to losses in tuber quality and yield. Disease can be expressed as root infection, root galling and tuber lesions, the latter 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 dysfunction 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, we determined optimal conditions for zoospore release and attachment of zoospores to plant roots and used this information to develop an in vitro bioassay to assess resistance to zoospore root attachment among 153 potato lines and cultivars. Optimal zoospore release occurred at 20°C in Hoagland's solution in a rapid and synchronized manner over the first 2 days, followed by a steep decline. The extent of zoospore root attachment varied with cultivar (Iwa > Agria > Russet Burbank > Gladiator), region of the root maturation zone (lower > middle > upper) and temperature (greatest zoospore root attachment occurring at 15°C). Further comparisons suggested efficiency of zoospore root attachment was also generally associated with known variety resistance to powdery scab, zoosporangial infection and root galling, with a few notable exceptions. The bioassay 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

Introduction

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

The disease cycle of *S. subterranea* has been studied extensively. *S. subterranea* persists in the soil as dormant resting spores (Harrison et al., 1997) that can release motile zoospores under conducive environmental conditions, stimulated by the presence of host plant root exudates (Balendres, Nichols, et al., 2016). Zoospores are attracted by chemotaxis to host roots (Amponsah et al., 2021), to which they attach (encyst) and penetrate through the cell wall, inserting their cellular contents to facilitate infection (Merz, 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 (Balendres, Tegg, et al., 2016; Clay & Walsh, 1990: Lahert & Kavanagh, 1985; Nitzan et al., 2008). 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 (Balendres, Tegg, et al., 2016; Harrison et al., 1997; Nitzan et al., 2008). Tuber disease results from zoospore infection of young developing tubers (van de Graaf et al., 2007; Hughes, 1980).

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; Falloon et al., 2003, 2016; Hernandez Maldonado et al., 2013; Torres et al., 1995); however, no cultivar has complete immunity to infection, and significant disease may still result in moderately resistant varieties (Merz et al., 2012). Reliable

identification of host resistance among commercial cultivars is a critical step forward for management of disease caused by *S. subterranea* infection (Falloon et al., 2003, 2016; Nitzan et al., 2008).

Traditionally, identification of host resistance has been based on the assessment of tuber powdery scab and/or root galling in large, replicated field or glasshouse challenge trials (de Boer, 1991; Falloon et al., 2003, 2016; Nitzan et al., 2008; Torres et al., 1995). These types of assessments are both time (4–6 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; Hernandez Maldonado et al., 2013; Nitzan et al., 2008). These glasshouse and field assays also fail to provide direct information on the relative host resistance to root hair infection, which we now know to be critical for impact on plant productivity (Falloon et al., 2016; Shah et al., 2012). 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. Whilst 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; Falloon et al., 2016; van de Graaf et al., 2007). It is also known that there are different temperature optima for expression of root infection (11–25°C; van de Graaf et al., 2005, 2007; Kole, 1954) and tuber disease (9–17°C; van de Graaf et al., 2005, 2007; Hughes, 1980; Shah et al., 2012). Thus, 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, 2016).

There is increasing concern about 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, 2016; Nitzan et al., 2008, 2010), 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 the relative propensity of initial zoospore root attachment at the very start of the infection process, so avoiding issues of polycyclic infection. We postulate that this would provide a more robust method of screening for host resistance to root infection, with results obtained in significantly less time than previous assessment methods. In this study, we first optimized in vitro conditions for zoospore release and zoospore attachment to potato roots. We then developed a zoospore root attachment assay, which was used to screen 153 potato lines and cultivars for their relative resistance to zoospore root attachment against known standards. We compared the results of zoospore root attachment within selected cultivars against known resistance to root galling and tuber disease, and zoosporangial root infection.

Materials and methods

Preparation of sporosori inoculum

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

Potato lines and cultivars

In total, 153 potato (Solanum tuberosum) lines and cultivars were assessed in this study and compared to four cultivars that differ in their resistance to powdery scab: Gladiator (regarded as very resistant to powdery scab), Russet Burbank (regarded as moderately resistant), and Agria and Iwa (both regarded as highly susceptible) (Falloon et al., 2003; Genet et al., 2007). Tissue-cultured plantlets of each potato 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°C. 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°C.

Optimum temperature for *in vitro* zoospore release

The optimum incubation temperature for zoospore release from sporosori was determined using a modified method of Balendres, Clark, et al. (2018). Aliquots of 3 mg of dried S. subterranea sporosori inoculum (five replicates per temperature treatment) were added to 1.6 ml Eppendorf tubes and suspended in 1 ml 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. The tubes were covered with aluminium foil and incubated in darkness at 10, 15, 20, 25 or 30°C (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; van de Graaf et al., 2007; Harrison et al., 1997). Zoospore release was assessed by observation of subsamples taken at 2, 4, 7, 13, 20, 30 and 40 days after incubation. At each assessment, the tubes were briefly mixed to ensure a homogenous solution, then 1 µl was pipetted directly onto a glass slide with a coverslip and the numbers of S. subterranea zoospores were determined by counting the total zoospore number at 200× magnification (DM 2500 LED; Leica). Three 1 µl samples were taken and counted each time from each tube, and five replicates were included in this experiment. Identification of zoospores used both zoospore morphology (Kole, 1954) and motility behaviour (Merz, 1997).

Distribution of zoospore root attachment in four potato cultivars

The density of root hairs on four potato cultivar standards, Iwa, Agria, Russet Burbank and Gladiator, was determined by light microscopy at 400× magnification. Roots from 2-week-old tissue-cultured potato plantlets grown in LPM were washed with deionized water. For each assessment, three primary roots (8–9 cm long) from the same plant were divided into three segments representing the upper, middle and lower parts of the root maturation region (Figure 1). 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, the 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 root zone per cultivar.

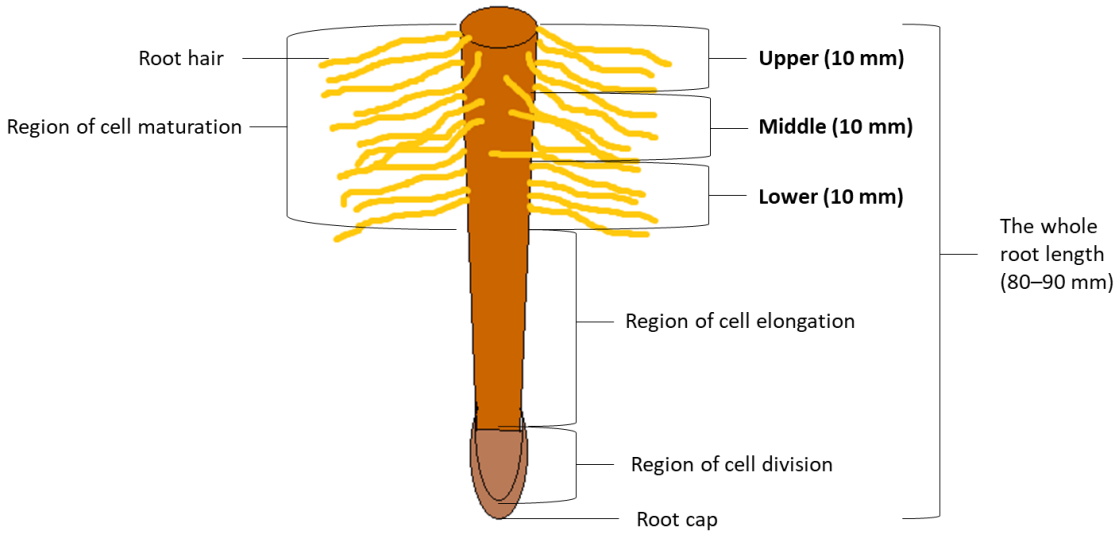


Figure 1. Schematic diagram of primary root of potato plantlets, showing the upper, middle and lower regions of the maturation zone from which root samples were taken.

A preliminary study evaluating zoospore attachment to the entire potato primary root revealed that zoospores attached exclusively on the root hairs within the maturation region of the root (Figure 1), 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 zoospores 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-µm mesh. Sixty millilitres of zoospore suspension (1000 zoospores/ml) was added to the treatment container, which was then incubated in the dark at 15°C 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

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

Each assessment used three primary roots (8–9 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) was added as previously described. The containers were then incubated at 10, 15, 20, 25 or 30°C 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 counted 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.

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

A total of 153 individual 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 2 weeks in LPM, as previously described, 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°C. 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 individual cultivar or line was performed with three independent biological replicates (three plants of each individual cultivar) and each comprised three technical replicates (three roots from each plant).

Scores of zoospore root attachment for each cultivar/line in the screenings were standardized according to the two 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 (ηn) for 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.

Assessment of susceptibility of potato cultivar to zoosporangium infection

The relative susceptibility to development of zoosporangial 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 zoospore root attachment assay. In vitro propagated plantlets of the 12 cultivars (with three plantlets per cultivar) were grown for 3 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 sporosori inoculum prepared as described earlier. A further three plantlets of each cultivar were suspended in a separate container filled with only 10 ml Hoagland's solution as a noninoculated control. The plantlets were incubated in a plant growth chamber (Steridium Pty Ltd) at 15°C for 3 days in darkness. Following inoculation, each plantlet was transferred into a fresh container containing 10 ml of Hoagland's solution only, arranged in a completely randomized pattern and grown in a plant growth chamber (Contherm Scientific Pty Ltd) under a 16-h photoperiod using white fluorescent lamps (65 µmol m−2 s−1) at 20 ± 2°C. After 1 month, plantlets were each assessed for root zoosporangial 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 stained with 0.1% trypan blue for about 15 min. Assessment of S. subterranea zoosporangial infection was conducted for three biological replicates under light microscopy at 200× magnification (Balendres, Tegg, et al., 2018). The intensity of zoosporangial infection of each root segment was assessed according to 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.

Statistical analysis

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 ANOVA, with a Tukey's honestly significant difference (HSD) test used to determine statistically significant differences between the means at the 5% level ($p = 0.05$). Zoospore root attachment and zoosporangia 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 analysis at a 0.05 level of probability.

Results

Optimum temperature for *in vitro* zoospore release

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

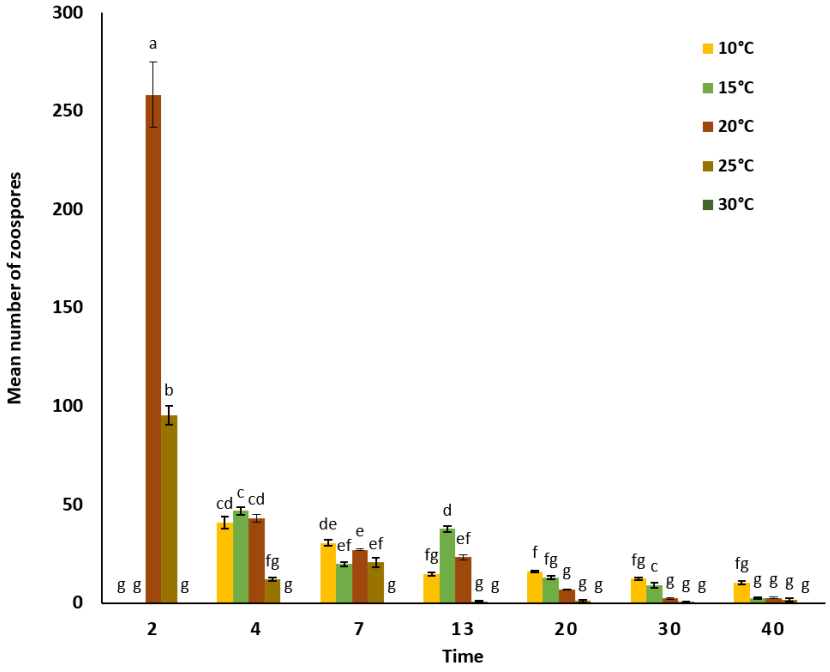


Figure 2. The impact of temperature treatment (10, 15, 20, 25 and 30°C) on release of *Spongospora subterranea* zoospores in Hoagland's solution, assessed at 2, 4, 7, 13, 20, 30 and 40 days after incubation. Vertical bars represent standard errors ($n = 5$). p (temperature) < 0.001; p (time) < 0.001;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

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

Root hair density was significantly influenced by cultivar ($p < 0.05$; Table 1, Figure 3) 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 higher in the

mid-section than in either the lower or upper regions of the maturation zone. Russet Burbank had the highest mean root hair number followed by Iwa, Agria and then Gladiator.

Table 1. Root hair density in upper, middle and lower regions of the maturation zone of primary roots in potato cultivars Iwa, Agria, Russet Burbank and Gladiator (n = 3)

	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

Note: Field of view was at 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.

Abbreviation: LSD, least significant difference

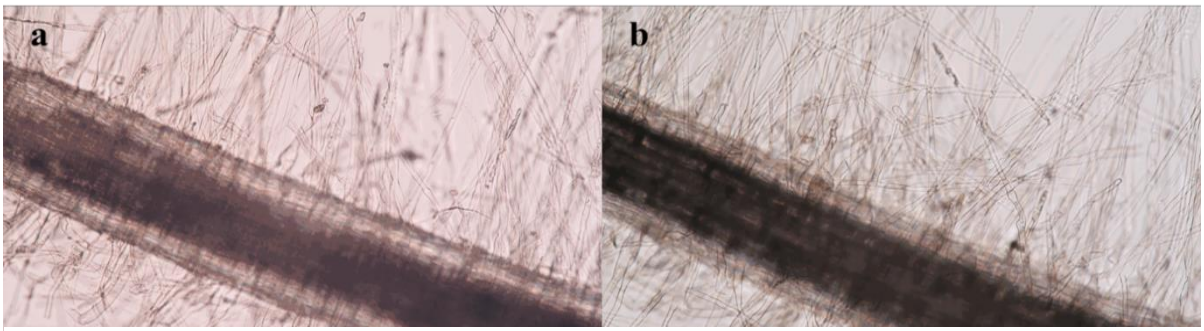


Figure 3. Representative images of root hairs from the maturation region of a susceptible 'Iwa' (a) and resistant cultivar 'Gladiator' (b). The root length of each field of view is 1.0 mm; scale bar = 0.1 mm

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

Zoospore root attachment was significantly influenced by potato cultivar ($p < 0.001$), root region tested ($p < 0.001$) and their interaction ($p < 0.001$; Figure 4). Zoospore root attachment for Iwa ($p < 0.001$) was significantly 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 significantly higher in the lower region than in the upper and middle regions of root maturation zones of the cultivars Iwa, Agria and Russet Burbank.

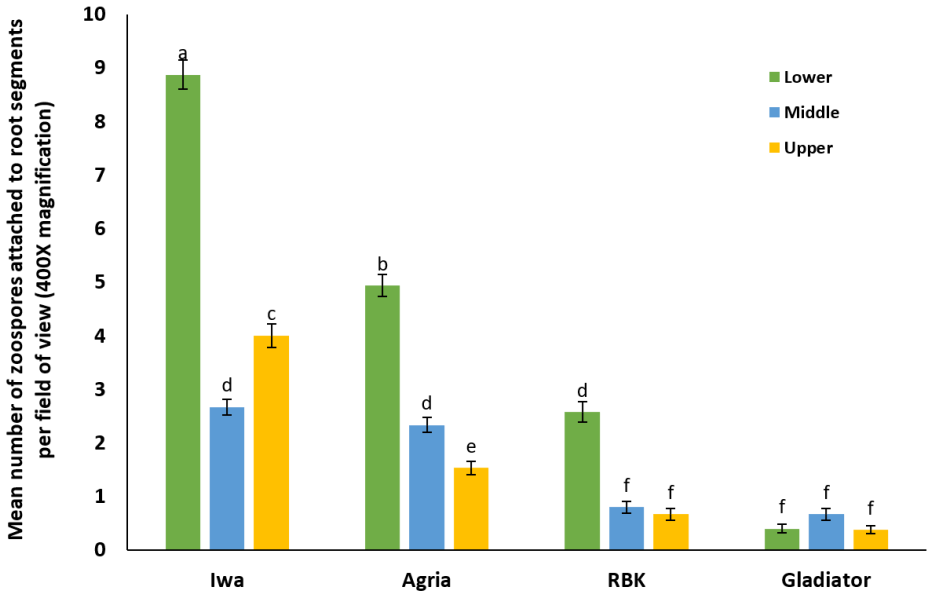


Figure 4. Scaled mean number of *Spongospora subterranea* zoospores observed per microscope field of view at 400× magnification attached to each of three root regions (lower, middle, upper) of the maturation zone of roots from potato cultivars 'Iwa', 'Agria', 'RBK', and 'Gladiator'. Vertical bars represent 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 significantly influenced by temperature ($p < 0.001$), cultivar ($p < 0.001$) and their interaction ($p < 0.001$; Figures 5 and 6). After 48 h incubation, zoospore root attachment was significantly higher in Iwa and Agria than Gladiator and Russet Burbank at all incubation temperatures except 30°C. In addition, for all cultivars, attachment was significantly higher at 15°C than at other incubation temperatures and very little zoospore root attachment was observed at 30°C.

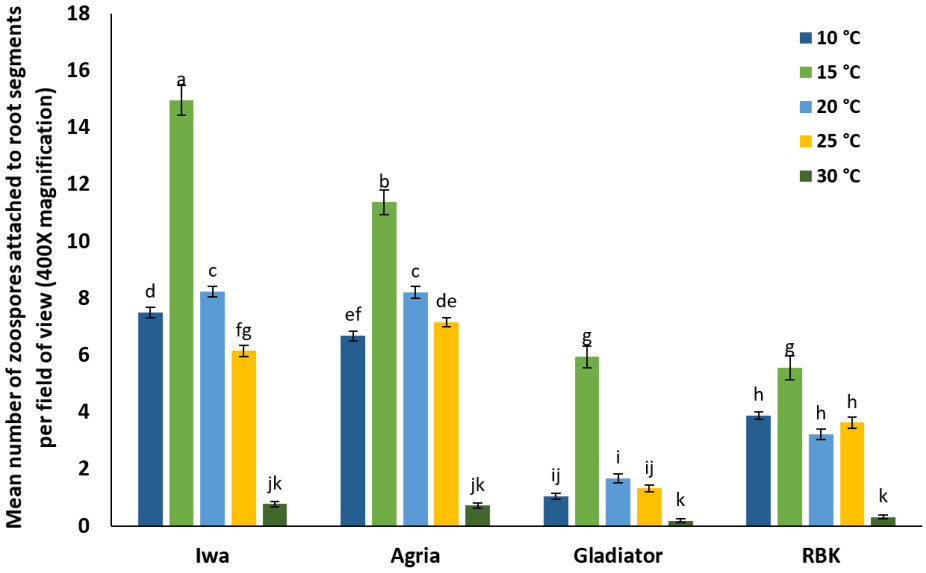


Figure 5. Scaled mean number of *Spongospora subterranea* zoospores observed per microscope field of view at 400× magnification attached to the lower maturation region of potato cultivars 'Iwa', 'Agria', 'Russet Burbank (RBK)', and 'Gladiator' at 10, 15, 20, 25 or 30°C. Vertical bars represent standard 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).

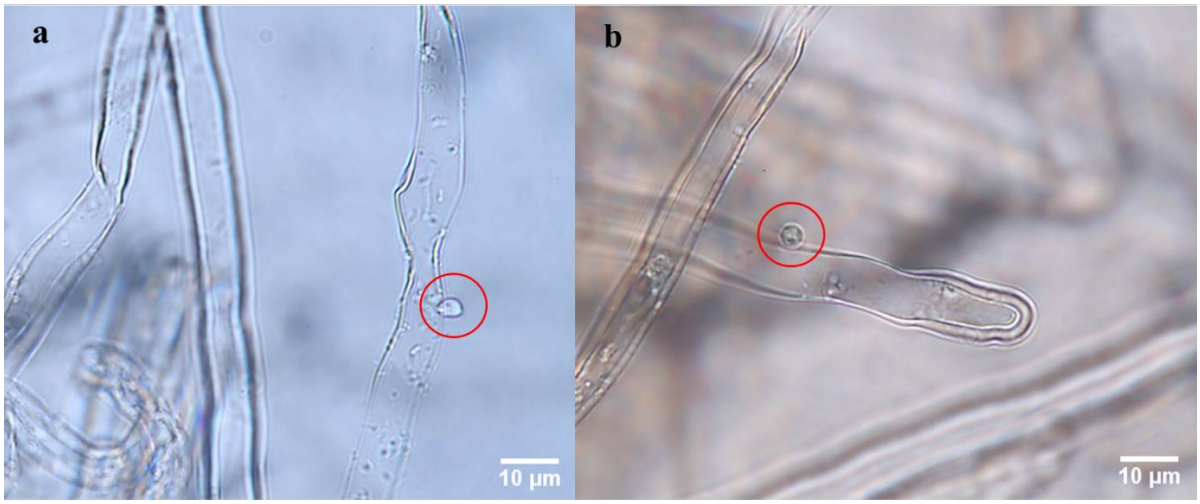
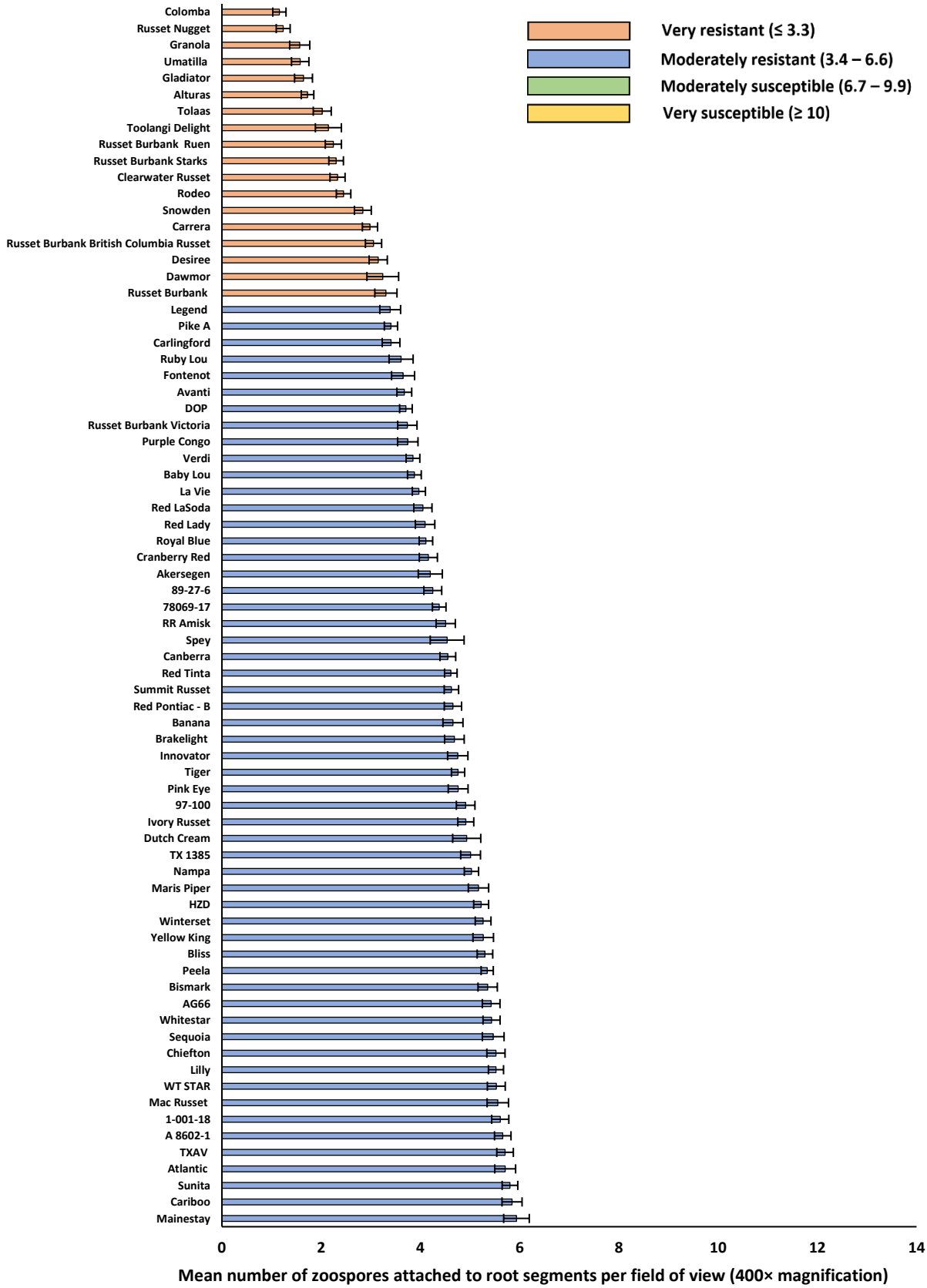


Figure 6. Attachment of *Spongospora subterranea* zoospores to potato root-hairs (lower zone of root maturation region) of potato cultivar 'Iwa'(a) and: cultivar Gladiator' (b), indicated by red circles.

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 (standard deviation [SD] = 0.5), whereas the mean score for Iwa varied between 9.9 and 15.4 (SD = 1.9). All cultivar screening for zoospore root attachment included these two standards as a reference point; hence, 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 7 displays the scaled mean zoospore root attachment scores for the 153 cultivars/lines of potato assessed using an in vitro bioassay. The zoospore root attachment score (scaled mean severity score) of all cultivars/lines exhibited a continuum of susceptibility from very susceptible to very resistant. The cultivars were arbitrarily classified as very resistant to zoospore root attachment (scaled mean severity score ≤3.3), moderately resistant (score 3.4–6.6), moderately susceptible (score 6.7–9.9) and very susceptible (score ≥10) (Figure 7). This categorization classified 11.8% of the cultivars 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 (Bittara et al., 2016; Falloon et al., 2003, 2016) showed that cultivars Gladiator, Gold Kennebec, Yukon, Russet Ranger and Iwa were categorized into the same levels of resistance with both assessment methods (Table 2). 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 moderately susceptible by the root galling scores but were rated as very resistant in the zoospore root attachment assay, whilst 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 R2 value (0.42) (Figure 8). 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
Verdi
Baby Lou
La Vie
Red LaSoda
Red Lady
Royal Blue
Cranberry Red
Akersegen
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)

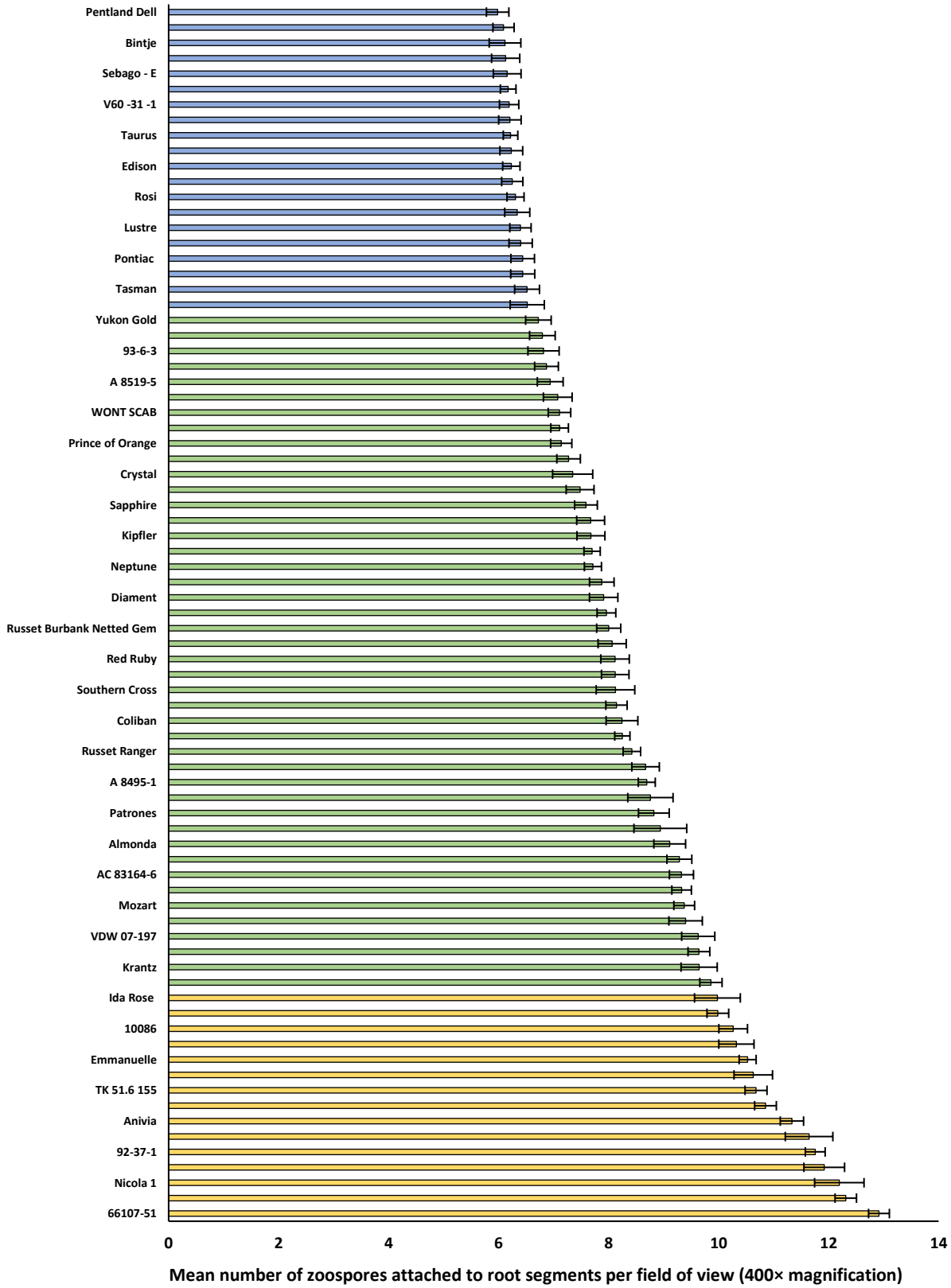


Figure 7. Scaled mean scores for *Spongospora subterranea* zoospore root attachment for 153 potato cultivars and lines assessed in the in vitro root attachment assays (n = 3) with $p < 0.001$. The cultivars were arbitrarily classified as very resistant (score ≤ 3.3), moderately resistant (score 3.4 - 6.6), moderately susceptible (score 6.7 - 9.9) and very susceptible (score > 10). Horizontal bars represent standard errors (n = 3).

Table 2. Relationship between categorization of 13 potato cultivars and lines as very resistant to very susceptible by Spongospora subterranea zoospore root attachment assay and similar published root gall resistance categories.

Root Attachment (this study)	**Root galling (Bittara et al., 2016; Falloon et al., 2003, 2016)**			
	Very resistant	**Moderately resistant**	**Moderately susceptible**	**Very susceptible**
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

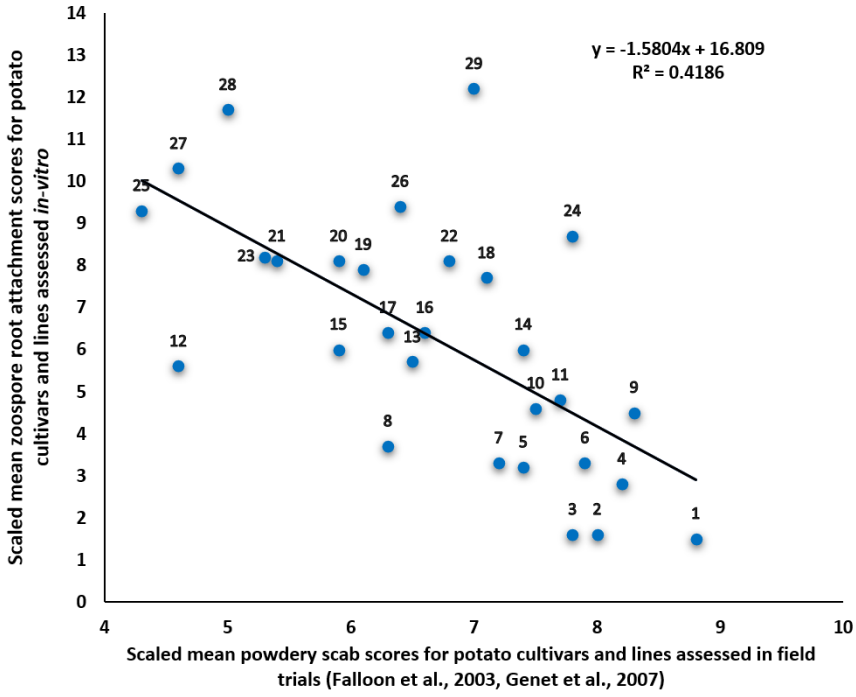


Figure 8. Relationship between scaled mean scores for *Spongospora subterranea* zoospore root attachment of 29 potato cultivars and the published powdery scab disease severity scores of these cultivars assessed in field trials (Falloon et al., 2003; Genet et al., 2007). For zoospore root attachment, 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 diseases, 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 Desirée, 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, 29 Nicola.

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

Zoosporangial infections were not observed in uninoculated control plants of any cultivar 30 days after inoculation. In contrast, all plants inoculated with S. subterranea showed zoosporangial root infections, but the infection rate varied

among cultivars. Mean zoosporangial intensity scores were significantly greater in cultivars assessed as susceptible to zoospore root attachment (i.e., cultivars Iwa, 10086, Krantz, Nicola, Shepody, Ida Rose) than in cultivars that had greater resistance to zoospore root attachment (i.e., cultivars Russet Burbank, Russet Nugget, Gladiator, Granola, Tolaas, Toolangi Delight) (Figure 9).

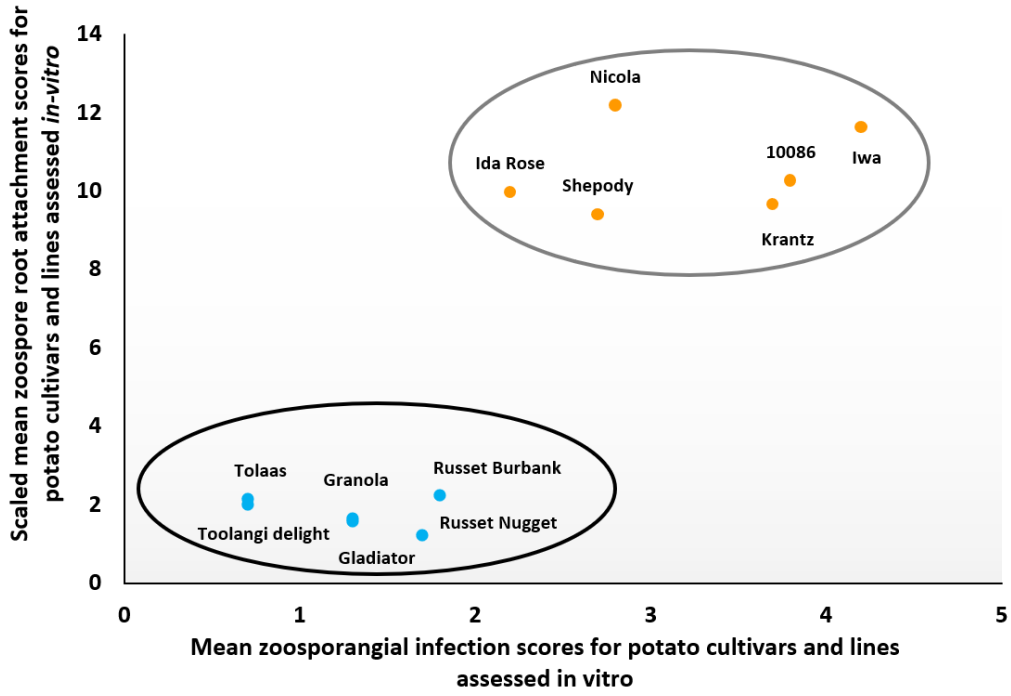


Figure 9. Relationship between scaled mean of *Spongospora subterranea* zoospore root attachment scores and zoosporangium infection severity score 0 = no infection; 1 < 10% sporadic,; 2 = 2% - 10% slight; 3 = 11% - 25% moderate; 4 = 26% - 50% heavy, and 5 > 50% very heavy (Merz et al., 2004) of 12 selected potato cultivars.

Discussion

With an absence of effective control measures, host resistance is regarded as a critical tool in the 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 involve 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 (Falloon et al., 2016; Shah et al., 2012). Prior in vitro assays 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 very rapid (results within 48 h), allows control over experimental 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°C in Hoagland's solution, while the highest zoospore root attachment occurred at 15°C. Many studies have reported optimal temperatures for S. subterranea root infection or tuber disease development (van de Graaf et al., 2005, 2007; Hughes, 1980; Kole, 1954; 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 and 17°C 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 11–25°C and 9–17°C, respectively, with more severe tuber infection at 12°C (van de Graaf et al.,

2005; Hughes, 1980; Shah et al., 2014).

This study showed that the zoospore root attachment was generally higher in root hairs from the lower (younger) rather than the medium and the upper maturation regions of the root. However, it would appear that 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 zoosporangial 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 zoosporangial infection and root attachments, while Tolaas and Toolangi Delight were very resistant in both assessments.

Although relationships between root infection (zoosporangial 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 we showed that the resistance response of most potato cultivars 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 zoosporangial infection, root galling and tuber powdery scab (Falloon et al., 2003, 2016). This is unsurprising as we would expect 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 susceptible 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 exceptions 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 of potato cultivars to zoospore root attachment by S. subterranea. Results from the screening of 153 cultivars and lines by the 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, 2016). This in vitro bioassay only requires potato root tissue samples. In the present study, we used tissue-cultured plantlets, but in unpublished studies we have also successfully used root tissues harvested from glasshouse-grown plants for the in vitro screen. This increases the bioassay's 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. We propose 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.

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Selection of disease resistant variants of elite fresh market and processing potato varieties through somatic cell selection

At the time of report preparation, this work is yet to be formally published

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

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.

Materials and methods

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℃ 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.

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℃.

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. 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., 2022). Root segments were incubated for 48 h in the dark at 15°C. Each container included root segments of the relevant parent as comparative 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 relative to the score of the parent variety.

Glasshouse trials to evaluate susceptibility of potato variants to powdery scab

Selected variants and their parent cultivars were tested for resistance to tuber powdery scab in two glasshouse challenge trials.

For one trial, the planting material was mini-tubers (approximately 2 g) of each clone, for the other trial, the ploanitng material was 3-week-old tissue-cultured plantlets of each clone.

For both trials, 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 1g of dried sporosorus inoculum (Balendres et al., 2018b) well mixed into the soil of each pot. Each pot was then planted with either a mini-tuber or a tissue culture plantlet, with five replicates (pots) per parent/variant tested. The plants were grown under glasshouse conditions (20 ± 2°C) with irrigation applied daily to maintain moist soil suitable for powdery scab development (Falloon et al., 2003). No pesticides were applied throughout the trials. After full senecsence, individual pots were harvested separately by uprooting plants and gently washing roots and tubers under running tap water.

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, 1 ≤ 1%, 2 ≥ 1 - 5%, 3 ≥ 5 - 10%, 4 ≥ 10 - 30%, 5 ≥ 30 - 50%, 6 ≥ 50% tuber surface affected) (Tegg et al., 2015). An average disease cover score and percentage cover score was calculated per pot.

Statistical analyses

Analysis of variance (ANOVA) was performed using GENSTAT 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 glasshouse trial datasets.

Results

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

Zoospore root attachment varied between potato cultivar variants. The unselected parents and 97 variants lines were tested for zoospore root attachment using the in vitro zoospore root attachment bioassay. A total of 38 of the variants had greater than or equal resistance to zoospore root attachment than the corresponding unselected parent cultivars. Eight lines showed significantly less zoospore binding rate comparent to the parent and eight lines showed significantly greater binding than the control (Table 1).

These scores were used to select supervior lines for subsequent glasshouse screening.

Table 1. Mean relative zoospore binding efficiency of variant clones compared to their respecitiv unselected parent

Zoospore binding relative to parent variety			
Russet Burbank	**Mean**	**Desiree**	**Mean**
M10	10.17**	A1201-1	3.46**
HEAT 2309-1	7.49**	2702-	3.35**
M9.2	7.34**	2705-1	3.12**
M17	7.24**	B1510-1	2.66
M7	6.9**	2309-1	2.55
0411-12	0.86	2108-3	2.36
M22	0.79	3107-2	2.34
M11	0.74	2701-	2.31
M24.2	0.74	3107-3	2.18
A2810	0.66	A0212-1	1.76
M29	0.49	B1201-1	1.74
M25	0.43	3107-8	1.73
M12	0.22*	2108-4	1.72
		3107-6	1.66
Ranger Russet		A3107-1	1.57
2108-4	3.29	1510-4	1.55
0411-7	1.76	D8 1510-2	1.52
2307-3	1.71	1510-3	1.41
0411-11	1.66	1510-2	1.39
0411-4	1.65	2705-2	1.37
3107-4	1.64	D8 2309-3	1.37
0411-9	1.56	A1510-1	1.37
2307-2	1.41	B1510-9	1.37
2307-1	1.33	3107-7	1.33
2307-4	1.22	3107-9	1.32
0411-8	1.1	1510-5	1.3
B0411-6	1.09	2705-4	1.27
A0411-6	0.89	B0212-1	1.19
0411-1	0.83	D8 2307-1	1.19
3107-1	0.79	2309-2	1.15
0411-10	0.79	2108-1	1.14
0411-3	0.75	3107-5	1.09
2307-3A	0.66	D8 2309-1	1.08
0411-5	0.46*	2309-4	1.06
2905-1	0.32*	D8 2108-2	1.06
0212-1	0.19*	D8 1510-3	1.05
0212-2	0.18*	D8 2309-4	1.04
		D8 2307-3	1
Shepody		D8 2309-2	0.99
3107-3	1.13	1510-9	0.93
1109-1	0.57*	2705-3	0.87
2307-1	0.55*	D8 2307-2	0.83
		D8 1510-1	0.83
Nicola		D8 1510-6	0.76
0309-2	0.87	2108-2	0.72
		1201-2	0.69
Sebago		D8 1510-8	0.67
3107-1	1.37	D8 1510-5	0.66
3107-2	1.07	2309-3	0.53
A0411-3	0.86	D8 1510-7	0.51
0401-2	0.36*	D8 1510-4	0.35
		1510-8	0.67
		2309-2	0.60
		1510-7	0.51

Red colours denote lines with lowermean zoospore binding, bold indicates binding less than half of parent value. * or ** indicates value significantly less than or greater than unselected parent.

Glasshouse trials to assess susceptibility of potato cultivars and variants to tuber infection

Following selection of best performing clones through *in vitro* screening, 44 available varients were subsequently tested across two glasshouse trials were lines were available.

The trial using mini-tubers as planting stock tested 34 clones across five varieties. Powdery scab disease levels were low to moderate across the trial. Within each variety tested there were variant clones identified that possesed less than half the disease score of the unselected parent. No significant differences were noted for disease score in this trial. For Russet Burbank, all ten variants had on average less disease than the parent with eight having scores less than half, and three completely disease free. For Ranger Russet, two varients showed disease levels less than half of the unselected parent, with the remaining seven varients showing on average greater disease levels than the parent. Some of the lines with enhanced disease lacked tuber skin russeting indicating a delitarious mutation. For Desiree, four of the 11 varients had disease scores less than half of the parent with all others except one showing on average less disease than the parent. With Shepody and Sebago, all tested variants (two each) had disease levels less than half of the unselected parent (Table 2).

In the trial using tissue cultured plantlets as planting stock no disease was recorded for the parent or variant clones of Russet Burbank or Ranger Russet. Disease was observed for Desiree, Shepody and Sebago parents and clones with many lines again showing disease scores less than half of the unselected parent. Disease scores were significantly different for Desiree and Shepody in this trial with 11 and two lines showing significantly less disease than their parents for these cultivars respectively. Where tested in both trials, variants lines generally perfomed similarly (Table 2).

Mean tuber numbers and tuber weights significantly varied between clones. A few variants had significantly reduced tuber yields suggestive of additional delitarious mutations, while the majority performed equivalent to or exceeded (soemtimes significantly so) the tuber yields of the parent cultivars. We note however, that glasshouse growth of potato plants provides a poor estmate of tuber yield potential of any line. Tuber numbers set were occasionally greater than the parent cultivar.

Table 2. Powdery scab disease scores and tuber yields of variants tested under pathogen challenge in glasshouse trials

Variety / Clone	Mini-tuber trial			Tissue culture trial		
	Disease Score	Tuber Number	Tuber weight	Disease Score	Tuber Number	Tuber weight
Russet Burbank	0.27	3.8	431.4	no disease	*	*
M11	0.17	11.8	370.4	no disease	*	*
M25	0.15	6.8	301.3*	no disease	*	*
M22	0.084	8.8**	480.1	no disease	*	*
A2810	0.084	8.2**	418.7	no disease	*	*
M9.2	0.05	5	307.4*	no disease	*	*
A380	0.044	6.2	342.4*	*	*	*
M24.2	0.018	8.6**	315.7*	no disease	*	*
M6	0.0	7.6	336.5*	no disease	*	*
M12	0.0	8	390.7	no disease	*	*
M29	0.0	11.2**	360.2	no disease	*	*
	P=0.39	*P=0.02*	*P=0.001*			
Ranger Russet	0.08	8	361.7	no disease	*	*
0411-10	0.55	4.75	102*	no disease	*	*

B0411-6	0.348	15.4**	414.5**	no disease	*	*
0212-1	0.296	22.4**	436.7**	no disease	*	*
0212-2	0.294	12.2	441.3**	no disease	*	*
A0411-6	0.234	12	431.2**	no disease	*	*
0411-1	0.142	8	359.3	no disease	*	*
0411-3	0.13	23.2**	493.2**	no disease	*	*
0411-5	0.028	10.2	280.8*	no disease	*	*
0411-9	0.026	7.8	373.4	no disease	*	*
	P=0.20	*P<0.001*	*P<0.001*			
Shepody	0.414	8.4	360.2	1.47	3.8	19.76
SH-11-09-1	0.176	9.6	408.4	0.81	5.8	19.74
SH-23-07-1	0.15	7	348.5	1.22	3.6	12.57
SH-15-10-2				0.58	5	19.63
	P=0.61	*P=0.54*	*P=0.32*	*P=0.11*	*P=0.29*	*P=0.22*
Desiree	0.44	8.2	514	0.65	7.4	18.77
2705-3	0.51	6.8	412.9*	0.13*	4.4	16.6
2108-2	0.392	10.8	519.7	0.38	5	22.1
2309-2	0.344	10	418.7*	0.07*	4.8	33.54**
3107-1	0.286	8.4	450	0.37	5.4	19.55
D8-1510-3	0.28	7	224.9	0.0*	4.4	34.05
D8-2309-2	0.274	9.2	435.1	0.03*	3*	18.59
3107-5	0.26	11.6	425.3	0.11*	4.2	20.24
D8-2309-3	0.124	5.2	143*	0.08*	4.2	27.73
D8	0.11	9	408.5*	0.54	7.4	29.08
D8-1510-5	0.078	13.2	484.6	0.47	8	14.35
D8-1510-6	0.0	9.6	434.2	0.2*	4.6	34.72
D8-1510-1				0.54	13	14.66
D8-2309-4				0.44	3.4	20.51
D8-1510-2				0.38	8.2	33.27**
1510-9				0.38	4.4	20.51
D8-2307-3				0.3	5.8	27.26
2705-4				0.2*	2.4*	6.64
2705-2				0.17*	3.8*	17.05
1201-2				0.15*	4.8	19.16
3107-8				0.0*	6	29.87
	P=0.70	*P=0.11*	*P<0.001*	*P=0.04*	*P<0.001*	*P=0.002*
Sebago	3.79	4	28.75	2.17	3.33	1.57
SE-31-07-1	1.28	9.6	93.3	0.5*	9.33**	17.35**
SE-04-01-2	1.5	12.4**	294.4**	0.46*	8.2**	33.71**
	P=0.12	*P=0.04*	*P=0.001*	*P=0.04*	*P=<0.001*	*P=0.005*

red colours denotes disease score less than half of parent control. * or ** indicates value significantly less than or greater than unselected parent

Linear regression analysis of zoospore root attachment assay results of variants of cv.s Desiree, Shepody and Sebago from

the glasshouse treial using tissue cultured plantlets with glasshouse tuber disease severity scores revealed a positive albeit weak linear relationship (R^2 = 0.39) (Figure 1). 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.

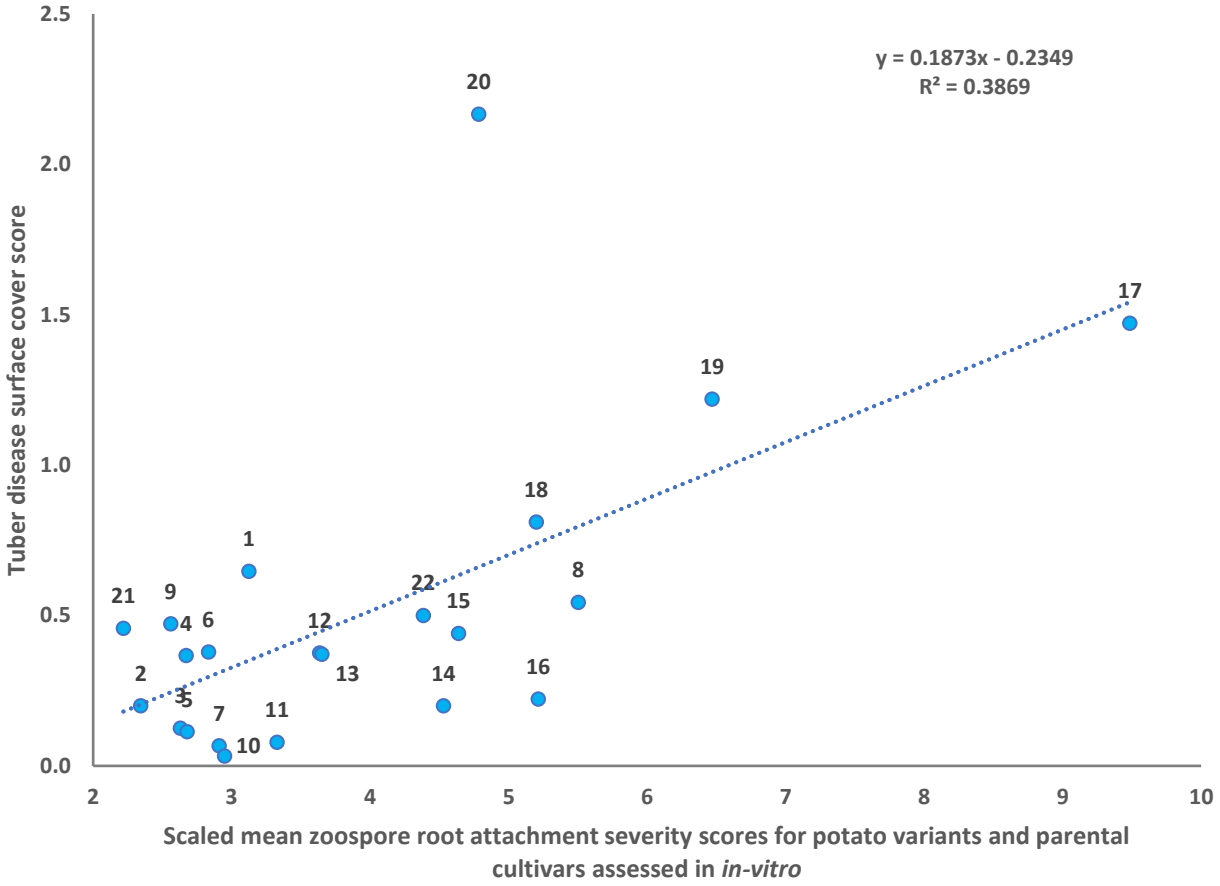

Figure 1. Relationship between mean scaled zoospore root attachment severity score of 22 potato cultivars and their variants. The glasshouse trial mean tuber disease (powdery scab) severity scores were 0 to 3 = no disease to 30% of tuber surface cover by lesions. Numbers 1 to 22 represent potato variants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

Desiree (1)	SE 3107-1 (22)
2705-4 (2)	SE 0401-2 (21)
2705-3 (3)	Sebago (20)
3107-5 (4)	SHE 2307-1 (19)
3107-1 (5)	SHE 1109-1 (18)
2108-2 (6)	Shepody (17)
2309-2 (7)	1510-6 (16)
D8 (8)	2309-4 (15)
D8 1510-3 (9)	1510-5 (14)
2309-2 (10)	2307-3 (13)
2309-3 (11)	1510-1 (12)

Discussion

This study has confirmed that a somatic cell selection strategy can be used to generate variants of the potato cultivars 'Ranger Russet', 'Russet Burbank', 'Desiree', 'Shepody', 'Sebago' and 'Nicola' and that within the population of variants exists lines with enhanced resistance to *S. subterranea* zoospore root attachment and subsequent powdery scab tuber disease. 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 97 somaclonal variants generated from six parents, 8% showed significantly improved resistance to *S. subterranea* zoospore root attachment compared to their unselected parents with a further 20-30% of lines showing improved (but not significant) resistance. 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). Selected variants and their parents with enhanced resistance to zoospore root attachment tested in the glasshouse trials generally maintained consistent resistance to tuber powdery scab. These results indicate that the somatic cell selection strategy is effective for selecting resistant variants of an unselected parent cultivar and that the in vitro test can enhance the efficiency of screening process.

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 from the plants grown from tissue culture were low from 1.6 g to 34.7 g per pot. Whilst better tuber development was observed when grown from mini-tubers tuber yields are only approximate in glasshouse grown plants. The indicative yields suggest that some of the lines with enhanced disease resistance produce similar yields to the unselected parents which is encouraging for potential development of resistant cultivars.

There is prior 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.

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

This has been published as: Yu, X., Wilson, R., Balotf, S., Tegg, R.S., Eyles, A. and Wilson, C.R., 2022. Comparative Proteomic Analysis of Potato Roots from Resistant and Susceptible Cultivars to Spongospora subterranea Zoospore Root Attachment In Vitro. Molecules, 27(18), 6024; https://doi.org/10.3390/molecules27186024.

Potato (*Solanum tuberosum* L.) exhibits broad 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, twelve 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 twelve 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

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 [1,2,3,4,5,6,7]. *S. subterranea* disease management is difficult and requires a range of approaches, including crop rotation, chemical application, and the selection of disease- or pathogen-free seed tubers [8,9,10,11,12]. However, the most efficient strategy to control *S. subterranea* diseases is arguably the planting of resistant cultivars [13]. Despite recent research into understanding the biochemical processes underlying Spongospora–potato interactions [7,14], the mechanism of resistance to *S. subterranea* tuber and root infections has not yet been elucidated.

Proteomics has been shown to be a powerful tool for the discovery of potential resistance mechanisms and protein biomarkers involved in the response of host plants to pathogen infection [15]. For example, quantitative proteomics was used to explore potato resistance to bacterial wilt caused by *Ralstonia solanacearum* [16], leaf late blight disease caused by *Phytophthora infestans* [17], and wart disease caused by Synchytrium endobioticum [18]. In addition, a recent study by Balotf, Wilson, Tegg, Nichols, and Wilson [14] compared the in planta transcriptome and proteome of *S. subterranea* invading susceptible and resistant potato cultivars. Their results suggested that the downregulation of enzyme activity and nucleic acid repair in the resistant cultivar could be related to resistance to *S. subterranea*.

Initial zoospore root attachment is one of the most critical phases of disease development in *S. subterranea* [7]. In our previous study [19], we reported the development of a novel in vitro bioassay that efficiently assessed potato cultivar resistance to *S. subterranea* root disease based on the efficiency of zoospore root attachment. We showed that reduced zoospore root attachment will likely manifest as less severe tuber and root infections [19]. During this critical stage of early infection, zoospores bind to the outside of the host roots and inject their contents into the root's cell wall [7]. Successful attachment of zoospores on potato roots either leads to the development of a plasmodium, which subsequently forms a zoosporangium and can subsequently release further secondary zoospores [20], or to the formation of root galls and production of resting spores [21]. 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, we used label-free proteomic analysis to compare the root tissues of twelve potato cultivars with various 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.

Materials and methods

Plant materials

Twelve potato cultivars with differential response to zoospore root attachment [19] were selected for detailed analysis: six resistant (R) cultivars ('Gladiator', 'Granola', 'Toolangi Delight', 'Russet Burbank Ruen', and 'Tolaas') and six susceptible (S) cultivars ('Iwa', 'Nicola', '10086', 'Shepody', 'Ida Rose', 'Kranz', and 'Russet Nugget'). Plants were maintained in tissue culture in liquid potato multiplication (LPM) medium, growing under a 16 h photoperiod, using white fluorescent lamps (65 µmol/m2/s) at 22 °C. The constitutes of LPM medium include MS salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; pH 5.8.

Spongospora subterranea inoculum preparation and zoospore germination

Sporosori inocula 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. Tubers were washed with running tap water and air-dried in a cool place for one to two days. Powdery scab lesions were scrapped as fine as possible using a scalpel and sifted through a 600 µm sieve. The resting spore inoculum samples were kept at room temperature in the dark.

Zoospores were released by incubating 3 g sporosori inoculum in 10 mL of Hoagland's solution in a McCartney bottle at 15 °C in the dark. Hoagland's solution was prepared following Falloon's standardised recipe [2]. 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; KH2PO4, 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 total number of zoospores present by light microscopy at 200× magnification (DM 2500 LED, Leica Microsystem, Wetzlar, Germany).

Zoospore root attachment assay

Confirmation of the relative resistance to zoospore root attachment of each cultivar was obtained by undertaking an in vitro zoospore root attachment assay. Following a two-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 (70 mm diameter) 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 [19]. The cultivars and variants were examined in batches of eight, and each batch contained two reference cultivars ('Iwa' and 'Gladiator'). Five randomly chosen fields of view were used to count the number of zoospores attached to each root segment under 400× magnification. This evaluation of each specific cultivar was carried out using three independent biological replicates (three plants of each specific cultivar or clone), with each biological replicate consisting of three technical replicates (three roots from each plant).

Zoospore root attachment scores for each cultivar/clone in the screenings were normalised against the reference cultivars, 'Gladiator' and 'Iwa', with the first batch screening serving as a reference point (G1 + I1) to adjust for across-batch differences. Cultivar/clone scores were further linearly scaled according to the reference point correction coefficient (ηn) in each batch [19].

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

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 a protected Fisher's LSD test to determine statistically significant differences at the 5% level (p = 0.05).

Protein extraction and peptide sample preparation

Root proteins extracted from all twelve 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 four independent biological replicates (plants) per cultivar. The total root tissue taken from each individual plant was washed with DDW and homogenised using a Fast Prep-24 bead beater (4000 rcf for 60 s) in PowerBead tubes with 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 DTT) and 20 µL protease inhibitor (one tablet of cOmplete Mini EDTA-free; Roche Diagnostics, North Ryde, NSW, Australia). The extract was centrifuged at 12,000 rcf for 8 min at 4 °C, the supernatant was collected and 6 volumes of cold acetone (−20 °C) was added, and the sample was mixed by shaking the tubes gently five times. The precipitated protein sample was collected by centrifugation at 6800 rcf for 5 min at 4 °C. The pellet was 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 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, 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 [45]. The digests were acidified by the 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.

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 [46]. DIA-MS raw files were processed using Spectronaut software v14.7) (Biognosys AG, Schlieren, Switzerland) using the directDIA experimental analysis workflow. A spectral library was first generated by searching the DIA-MS data against the *Solanum tuberosum* 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 the targeted reextraction of DIA-MS2 spectra and relative protein quantitation between samples. With the exception of excluding single-hit proteins, default Spectronaut settings were used for protein quantitation and normalisation.

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 cultivars and six susceptible cultivars, with an 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 was 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.

Pectinase treatment

To provide further evidence of a possible role of root surface pectin, cultivars 'Iwa' (S) and 'Gladiator' (R) were assessed for the impact of pectinase treatment of roots on the capacity and efficiency of zoospore root binding. Tissue-cultured plants were cultured in liquid potato multiplication (LPM) medium, growing under a 16 h photoperiod, using white fluorescent lamps (65 µmol/m2/s) at 22 °C. The constitutes of LPM medium include MS Salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; pH 5.8. Pectinase solutions were made at four concentrations containing 0, 1, 2, and 3 mg pectinase (P4716; Sigma-Aldrich, Bayswater, Australia) in 1 mL of 50 mM sodium acetate buffer with pH 5.0, respectively. The enzyme activity of pectinase at 37 °C is 0.68 ± 0.020 µmolmin−1mL−1 [47]. Three primary roots (technical replicates) from each individual plants (biological replicates) of each cultivar were collected from propagated plantlets and rinsed thoroughly with DDW. This experiment was performed 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 individual root [19]. The root segments comprising each biological replicate were added into one of three 1.5 mL Eppendorf tubes. Subsequently, 45 µL 50 mM sodium acetate buffer and 5 µL pectinase solution were added into all the tubes. Then, all three tubes were incubated at 37 °C for 0.5 h [48]. This experiment was repeated at four selected concentration levels (0, 1, 2, and 3 mg/mL) of pectinase solution. All treated root segments were then assessed

for efficiency of zoospore root attachment by the in vitro zoospore root attachment assay

Results

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

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

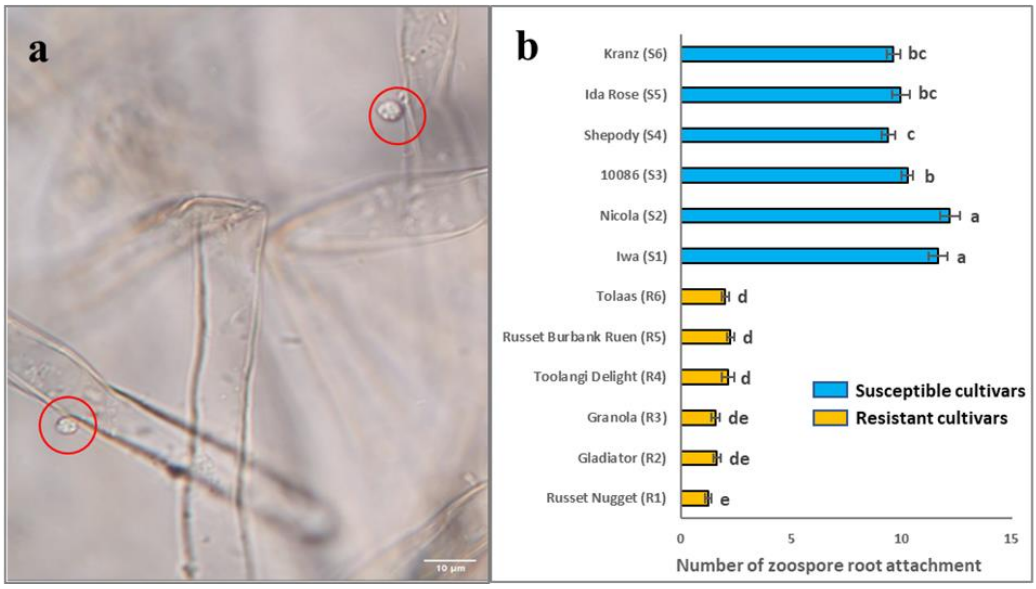

Figure 1. Zoospore root attachment of twelve potato cultivars. (a) Zoospore (in red circle) attached to potato root; (b) statistical analysis of zoospore root attachment severity scores of twelve potato cultivars 'Iwa (S1)', 'Nicola (S2)', '10086 (S3)', 'Shepody (S4)', 'Ida Rose (S5)', 'Kranz (S6)', 'Russet Nugget (R1)', 'Gladiator (R2)', 'Granola (R3)', 'Toolangi Delight (R4)', 'Russet Burbank Ruen (R5)', and 'Tolaas (R6)' at 48 h after inoculation. Three independent biological replicates (from different plants) were assessed for each cultivar. Horizontal bars represent the standard error (n = 3). $p < 0.001$. LSD (0.05) = 0.73. The blue bars represent all the susceptible cultivars (S), and the yellow bars represent all the resistant cultivars (R) to zoospore root attachment. Bars that are labelled with different letters indicate values that are significantly different from each other.

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 twelve cultivars (provided in Table S1). According to the statistical analysis results, 626 proteins were significantly less abundant in resistant cultivars, whereas 454 proteins were significantly more abundant in resistant cultivars (Figure 2a, and listed in full in Table S2). Initially, PCA of the dataset comprising all proteins showed only partial separation of resistant and susceptible cultivar samples (Figure 2b). Although samples from the susceptible cultivars clustered quite tightly, those from the resistant cultivars were more dispersed and, in particular, root samples R1, R2, and R3 overlapped with the samples from susceptible cultivars (Figure 2b). Subsequent PCA of the protein subset identified significant differences between resistant and susceptible cultivars, showing stronger separation of the two groups, but nonetheless indicated greater variation overall in the resistant cultivars (Figure 2c).

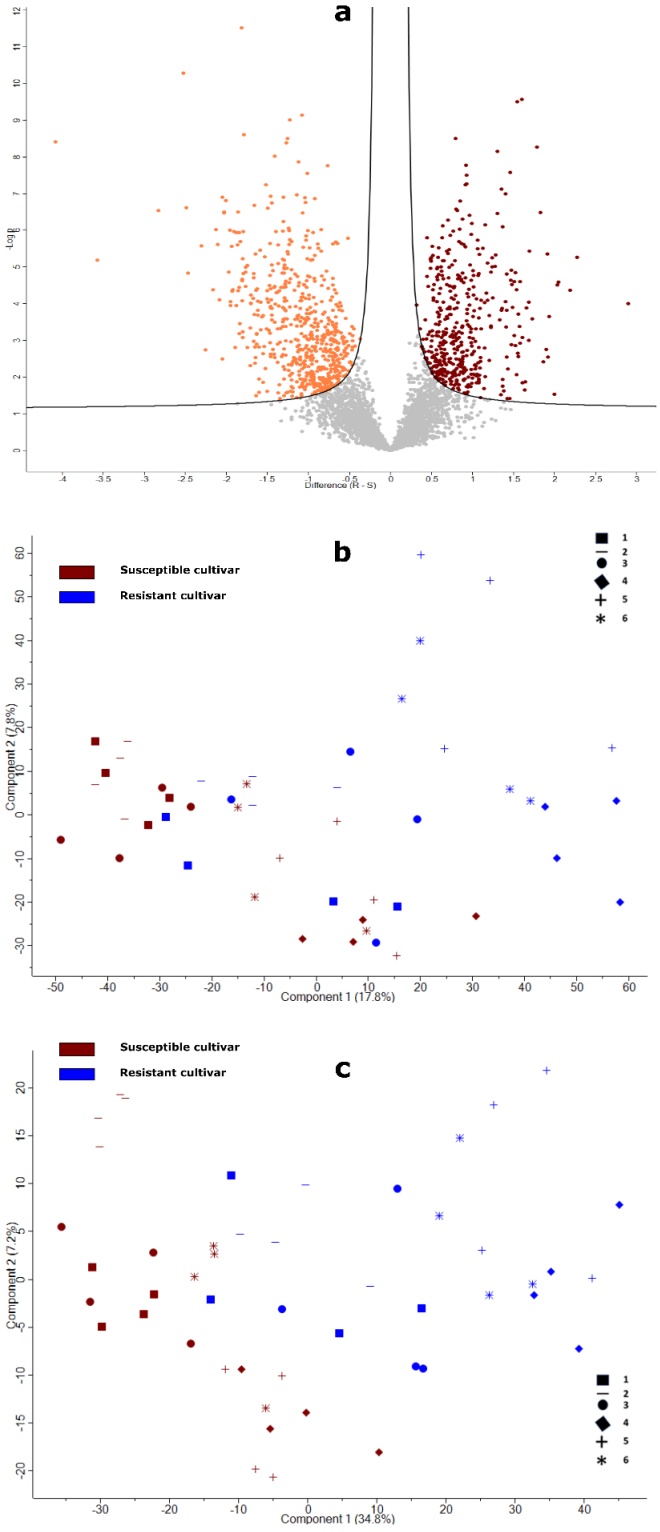

Figure 1. (a) Volcano plot displaying the results of t-test comparisons of susceptible and resistant potato cultivars. The two lines show the threshold (FDR < 0.05; s0 = 0.1) separating the proteins increased (dark red data points) and decreased in resistant cultivars (orange data points); (b) principal component analysis (PCA) of the dataset comprising all proteins quantified across the 12 potato cultivars; (c) PCA of the dataset restricted to the 1080 significant proteins between resistant and susceptible potato cultivars.

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 3a,b). In total, 19 functional categories were captured by the set of proteins that were significantly more abundant in resistant cultivars, including several related to oxidative stress (e.g., BP "response to oxidative stress" and MF "peroxidase activity") and metabolic processes (e.g., CC "mitochondrion") (Figure 3a). In contrast, GO terms related to protein biosynthesis such as CC "cytosolic ribosome" and BP "protein folding," and chloroplast functions (e.g., CC terms "chloroplast stroma" and "chloroplast envelope") were associated with DAPs that were less abundant in the resistant cultivars (Figure 3b).

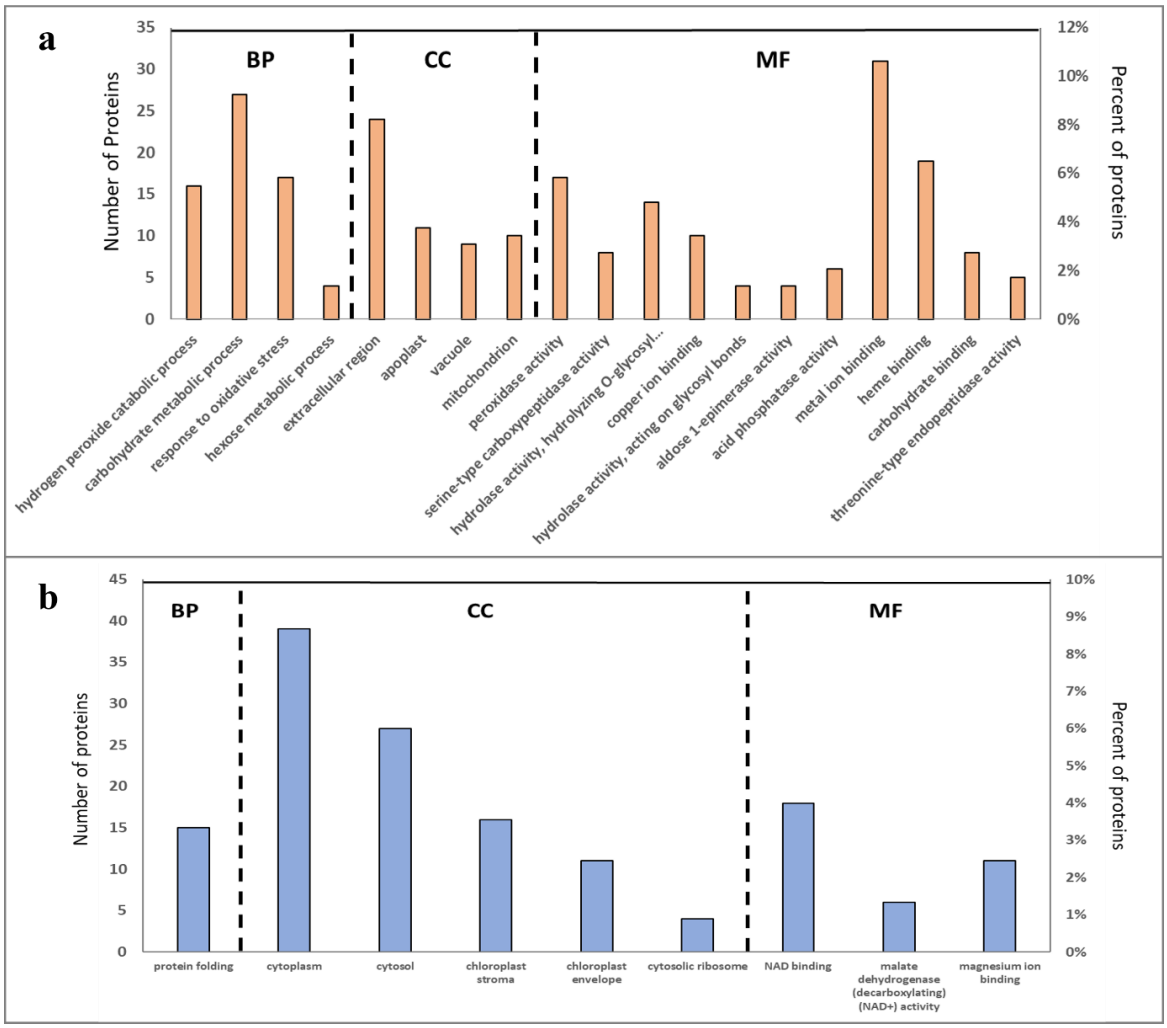

Figure 3. Classification of identified proteins of potato roots as (a) more abundant and (b) less abundant in resistant cultivars from the proteome of potato (Solanum tuberosum) into Gene Ontology (GO) categories, in terms of their involvement in biological process (BP, orange bar), cellular component (CC, green bar), and molecular function (MF, blue bar).

Overall pathway analyses of differentially abundant proteins

To better understand how the metabolism of potato roots differed between resistant and susceptible cultivars in this study, KEGG-based analysis was used to categorise the DAPs into metabolic and genetic information pathways. The KEGG pathway enrichment analysis further revealed common or specific pathways in the sets of DAPs either more or less abundant in the root tissues of resistant cultivars (Figure 4). In total, 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. Accordingly, for proteins more abundant in resistant cultivars, most proteins were related to metabolic pathways (n = 78) including biosynthesis of secondary metabolites (n = 46) and phenylpropanoid

biosynthesis (n = 22) (Figure 4). For the proteins less abundant in resistant cultivars (Figure 4), 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) including secondary metabolite biosynthesis (n = 90), antibiotic biosynthesis (n = 61), and carbon metabolism (n = 50).

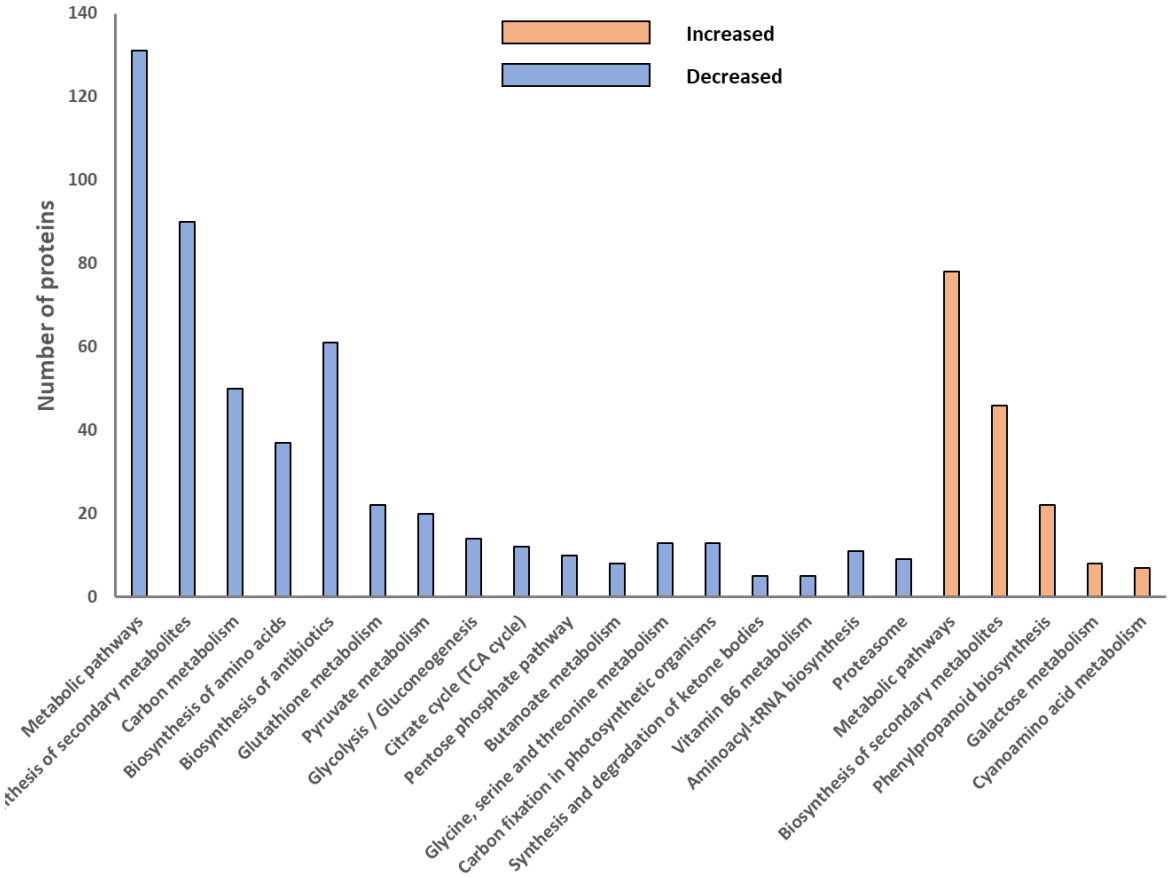

Figure 4. KEGG pathway classification and enrichment tests for proteins more or less abundant in resistant cultivars.

Differentially abundant proteins of root cell wall composition

In total, 39 DAPs involved in cell wall composition and modification were identified. Notably, the vast majority of them (n = 37) were more abundant in resistant cultivars (Table 1). Pathway analysis of the cell wall related proteins that were more abundant in resistant cultivars (Figure 5a) identified a number of significant pathways such as glycosaminoglycan degradation (n = 3 proteins), 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 5b. 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 functions (e.g., BP “cell wall organization”, “cell wall biogenesis”, “cell wall modification”, and CC “plant-type cell wall”, “cell wall”) (Figure 5b). 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 5b). 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 1. 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

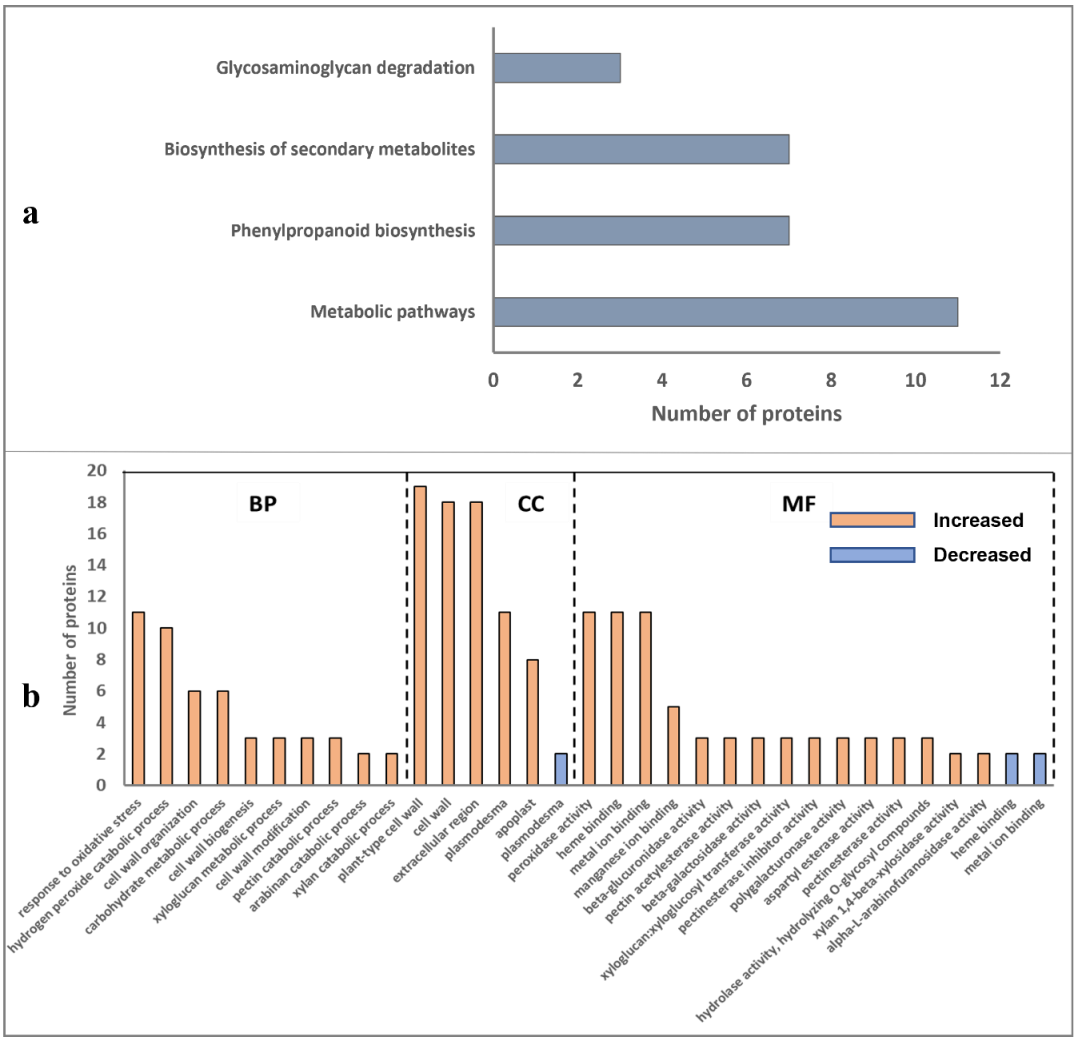

Figure 5. a) Pathway analysis of cell wall related proteins that were increased in resistant potato (Solanum tuberosum) cultivars; (b) classification of root cell wall related proteins that were increased (orange bars) or decreased (blue bars) in resistant cultivars by Gene Ontology (GO) categories for biological process (BP), cellular component (CC), and molecular function (MF).

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, we assessed the effect of pectinase treatment on zoospore attachment to one 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 both susceptible and resistant cultivars (Figure 6). Significant reductions in zoospore root attachment on both resistant and susceptible potato cultivars were observed with pectinase concentrations of 1 and 2 mg/mL, with no zoospore root attachment observed following 3 mg/mL of pectinase solution.

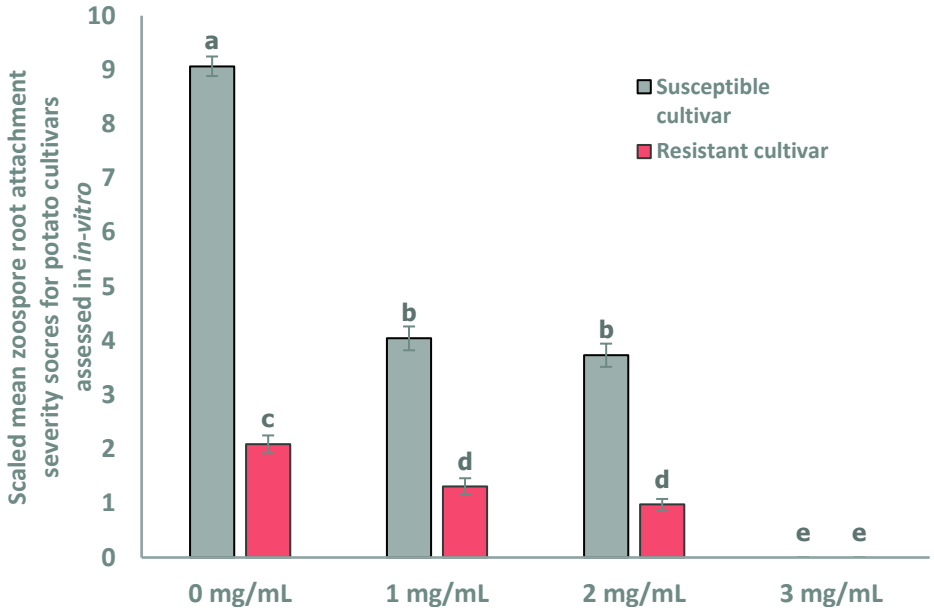

Figure 6. Mean severity of *Spongospora subterranea* zoospore infection in roots of resistant and susceptible potato cultivars, treated with different concentrations of pectinase. The vertical bars represent standard errors (n = 3). p (cultivars) < 0.001, p (concentration) < 0.001, p (cultivar × concentration) < 0.001. LSD (0.05) = 0.43. Bars that are labelled with different letters indicate values that are significantly different from each other.

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, we developed an in vitro bioassay for the rapid screening of potato resistance to zoospore root attachment [19], the precursor to root infection. Using this assay in the current study, we demonstrated a very clear difference in zoospore root attachment between the six resistant and six susceptible cultivars selected. Subsequently, we used label-free proteomics 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 2).

Analysis of the proteomic profile of potato roots revealed that most of DAPs which were increased in resistant cultivars were assigned to GO terms related to oxidative stress and metabolic processes, including “response to oxidative stress”, “peroxidase activity” and “mitochondrion”. Peroxidases are well-known pathogenesis-related proteins that protect host tissue from pathogen attack by producing physical barriers through mediating undefined cell wall components [22]. They are reportedly involved in oxidative stress induced by pathogenic agents and the activation of defence-related activities in potatoes [23]. Similarly, peroxidase activity has been found to play a key role in defending plants against bacterial and fungal pathogens [24]. Peroxidases are also involved in phenol oxidation, IAA oxidation, lignification, plant defence, and plant cell elongation regulation [25,26,27,28,29]. Increases in peroxidase activity have been correlated with resistance in many species including rice, tomato, and wheat. In these plant hosts, peroxidases are involved in the polymerisation of proteins and lignin or suberin precursors into plant cell walls, which could inhibit zoospore attachment and penetration [30,31]. 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 [32].

KEGG pathway analysis of the DAPs that were increased in resistant cultivars identified metabolic pathways such as the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, cyanoamino acid metabolism, and galactose metabolism. It has been shown that lignin biosynthesis, which is part of the phenylpropanoid metabolic process, contributes to resistance against pathogens in plants [33]. Li et al. [34] showed that the phenylpropanoid pathway was associated with resistance to potato wart disease. The plasmodiophorid soilborne pathogen Plasmodiophora brassicae, which causes clubroot disease, can involve drastic changes in the cell wall composition of host roots [35,36]. Several

genes involved in phenylpropanoid metabolic process and cell wall synthesis were also upregulated in the transcriptome analysis of clubroot-infected Brassica oleracea [36]. Therefore, the establishment of mechanical barriers such as cell wall reinforcement of the host root seems to be a part of the mechanism behind plants' resistance/tolerance mechanisms against P. brassicae [37,38]. In a recent study, Balotf et al. [39] showed that the phenylpropanoid metabolic process plays a critical role in the resistance of potato cultivars against root infection by S. subterranea. Their transcriptome analysis revealed upregulation of the phenylpropanoid metabolic process and lignin genes in the resistant cultivar, but not in the susceptible cultivar [39]. 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 a considerable obstacle for *S. subterranea*. We concluded that both constitutive and responsive gene/protein expression strategies are used by potato plants to increase resistance against *S. subterranea*.

Our proteome study showed that several enzymes involved in pectin biosynthesis and remodelling were identified as more abundant in resistant cultivars (Figure 5b). This included pectin acetylesterase which, in tobacco (Nicotiana tabacum), serves as a key structural regulator by changing the precise status of pectin acetylation to impact the remodelling and physiochemical characteristics of the cell wall's polysaccharides [40]. Pectinesterase (a pectolytic enzyme that hydrolyses the ester linkages in pectin molecules; Maldonado and Strasser de Saad [41]) activity 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 the rapid attachment of Phytophthora cinnamomic zoospores, implying that pectin-like materials on plant root surfaces may act as a recognition signal, resulting in zoospore root attachment [42,43,44]. Our current in vitro study revealed that potato roots pre-treated with pectinase exhibited significant reductions in zoospore root attachment, which further suggests 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 vivo manipulation of cell wall pectin in modifying zoospore attachment and protection.

In summary, our findings in this study provide a better understanding of the constitutive basis of host resistance to zoospore root attachment among potato cultivars, representing two ends of the spectrum of root resistance to zoospore attachment. We have further identified several candidate pathways and proteins that have the potential to influence the cultivar resistance to zoospore root attachment process. Moreover, we have confirmed the biological importance of root pectin for zoospore root attachment. An important issue unresolved in this study is how any of these proteins respond 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.

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

This has been published as: Yu, X., Wilson, R., Eyles, A., Balotf, S., Tegg, R.S. and Wilson, C.R., 2023. Enzymatic Investigation of Spongospora subterranea Zoospore Attachment to Roots of Potato Cultivars Resistant or Susceptible to Powdery Scab Disease. Proteomes, 11(1), 7; https://doi.org/10.3390/proteomes11010007

For potato crops, host resistance is currently the most effective and sustainable tool to manage diseases caused by the plasmodiophorid *Spongospora subterranea*. Arguably, zoospore root attachment is the most critical phase of infection; however, the underlying mechanisms remain unknown. This study investigated the potential role of root-surface cell-wall polysaccharides and proteins in cultivars resistant/susceptible to zoospore attachment. We first compared the effects of enzymatic removal of root cell-wall proteins, N-linked glycans and polysaccharides on *S. subterranea* attachment. Subsequent analysis of peptides released by trypsin shaving (TS) of root segments identified 262 proteins that were differentially abundant between cultivars. These were enriched in root-surface-derived peptides but also included intracellular proteins, e.g., proteins associated with glutathione metabolism and lignin biosynthesis, which were more abundant in the resistant cultivar. Comparison with whole-root proteomic analysis of the same cultivars identified 226 proteins specific to the TS dataset, of which 188 were significantly different. Among these, the pathogen-defence-related cell-wall protein stem 28 kDa glycoprotein and two major latex proteins were significantly less abundant in the resistant cultivar. A further major latex protein was reduced in the resistant cultivar in both the TS and whole-root datasets. In contrast, three glutathione S-transferase proteins were more abundant in the resistant cultivar (TS-specific), while the protein glucan endo-1,3-beta-glucosidase was increased in both datasets. These results imply a particular role for major latex proteins and glucan endo-1,3-beta-glucosidase in regulating zoospore binding to potato roots and susceptibility to *S. subterranea*.

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

Introduction

The plasmodiophorid biotrophic pathogen *Spongospora subterranea* f. sp. *subterranea* is a significant threat to sustainable potato production wherever potato crops are grown [1]. This soil-borne pathogen infects potato tubers, underground stolons and roots, leading to tuber and root diseases [1-3]. Tubers infected by S. subterranea develop powdery scabs that affect tuber quality and storage longevity, whilst root infection affects root function (absorption of water and nutrients) and can reduce tuber yields [3]. 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; nevertheless, no cultivar is immune to the infection, and substantial disease can still result in varieties that are only moderately resistant. To date, host resistance to Spongospora diseases has been assessed in traditional glasshouse and field trials and, more recently, using a rapid and robust in vitro zoospore root-attachment bioassay [4].

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 [5-7]. Following attachment to a host root, S. subterranea zoospores discharge their contents into the plant cell walls via a particular 'Rohr' and 'Stachel' structure [1,8]. Zoosporangia form 4 to 5 d after zoospore root attachment occurs [9,10]. Our previous study showed that the efficiency of zoospore root attachment differs among potato cultivars [4]. However, the mechanisms underlying the differences in the efficiency of zoospore root attachment remain unknown.

Previous studies on other pathosystems suggest that the molecular interactions between host-plant cell-wall surface components and the infective units of pathogens are critical in the management of pathogenesis and plant resistance [11,12]. The initiation of zoospore root attachment has been associated with the production of a range of high- or low-molecular-weight root exudates [7,13] including fucosyl residues [14,15], pectin [16,17], lectins [18], certain monoclonal antibodies [19], amino acids [20] and ions (sodium, strontium and calcium ions) [21]. Zoospore attachment to host roots by Pythium spp. was found to be affected by different plant polysaccharides, whereas Phytophthora spp. zoospore root attachment varied with the presence of pectin, polyuronates and some inorganic cations [16,21-24] [16,21,22,24,25].

Enzymatic studies have been extensively used to examine zoospore–host interactions [11,14-16,25-30]. Longman and

Callow [15] investigated the role of protein- and polysaccharide-based surface components involved in the zoospores of *P. aphanidermatum* which bind to the root surfaces of cress (Lepidium sativum). They found that trypsin was effective in reducing the number of zoospore root attachments, as was root-surface mucus–polysaccharide modification with lectin and pectinase. Downer, Menge and Pond [29] showed that treatment with cellulase significantly reduced zoo-sporangia development by *P. cinnamomi* in avocado roots. However, no study has yet characterized the biochemical basis of the interaction between plant roots and *S. subterranea* zoospore attachment.

Our previous research investigated the basis of host resistance to zoospore root attachment by analysing the whole-root proteins of resistant and susceptible cultivars using label-free proteomics [31] and differential mRNA expression analysis [32]. In this study, we sought to investigate the role of protein- and polysaccharide-based root-surface components through the modification of potato roots from resistant and susceptible cultivars, using three selected enzymes (trypsin, PNGase F and cellulase). In addition, we compared the proteins identified by TS with our published whole-root proteomic analysis [31] and transcriptomic dataset for the same two cultivars [32]. A comprehensive understanding of protein profiles following TS treatment of potato roots may uncover novel targets for zoospore root-attachment control strategies.

Materials and methods

Spongospora subterranea sporosori collection and germination

S. subterranea sporosori samples were collected from powdery-scab-infected tubers of the potato cultivar 'Kennebec' from a commercial potato field in North-West Tas-mania, Australia, 2020. Infected tubers were washed with tap water and left to air-dry in a cool and dark place for 1 to 2 d. The lesions from infected tubers were excised with a scalpel and then sifted through a 600 µm sieve. S. subterranea inoculum was stored at room temperature in the dark until use.

Zoospores were released by incubation of sporosori samples in Hoagland's solution, 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) [33]. Aliquots of 3 mg of sporosori inoculum were divided into 1.6 mL Eppendorf tubes and suspended in 1.0 mL of Hoagland's solution. All tubes were incubated at 15 °C in darkness in a test chamber (Plant growth chamber, Steridium Pty Ltd., Brisbane, QLD, Australia). Zoospore release was examined by observation of subsamples (three 1 µL of subsample were examined each time, with five replicates included) by light microscopy at 200× magnification (DM 2500 LED, Leica Microsystem, Wetzlar, Germany) after 3 d of incubation [4].

Plant material and growth conditions

Tissue-cultured plantlets of the cultivars 'Iwa' and 'Gladiator' were further propagated in tissue culture in potato multiplication medium containing the following 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, 2.2 g/L of phytagel (pH 5.8) under a 16 h photo-period using white fluorescent lamps (65 µmol/m2/s) at 22 °C. After one month, all plantlets were transferred into potato multiplication medium minus the phytagel grown for a further two weeks under a 16 h photoperiod using white fluorescent lamps (65 µmol/m2/s) at 22 °C.

Enzyme treatments

Potato roots were collected from propagated plantlets and rinsed thoroughly with DDW. For each enzymatic treatment, six primary roots from each individual plant of each cultivar were collected from propagated plantlets and rinsed thoroughly with DDW. This experiment was performed with three technical and three biological repli-cates. A segment of the lower part of the root-maturation region trimmed to a length of 20 mm was selected from each individual root [4]. Three plantlets of each cultivar were used as biological replicates, thus providing a total of 18 root segments. The eighteen root segments were divided into two groups evenly (i.e., groups 1 and 2). In each group, the root segments comprising each biological replicate were added to one of three 1.5 mL Eppendorf tubes.

A vial of 20 µg proteomic-grade trypsin (T6567; Sigma-Aldrich, Macquarie Park, NSW, Australia) was dissolved in 100 µL of 50 mM ammonium bicarbonate buffer (pH 7.8) to achieve a concentration of 0.2 mg/mL. A vial of 50 units of proteomic-grade PNGase F (P7367; Sigma-Aldrich) was dissolved in 100 µL of high-purity water to pro-vide a concentration of 500 units/mL. A quantity of 1 mg of the cellulase solution was prepared (Cellulase Onozuka™ RS, Yakult Pharmaceutical Industry Co., Ltd. Tokyo, Japan) in 1 mL of 50 mM sodium acetate buffer (pH 5.0).

For the PNGase F treatment, 45 µL of 50 mM ammonium bicarbonate buffer and 5 µL PNGase F solution (final concentration

of 50 units/mL) were added to each tube in group 1. Further, all three tubes were incubated at 37 °C for 1 h [34].

For the cellulase treatment, 45 µL of 50 mM sodium acetate buffer and 5 µL of 1 mg/mL cellulase solution were added to each tube in group 1. Then, all tubes were incubated at 37 °C for 0.5 h [35].

For the TS treatment, 45 µL of 50 mM ammonium bicarbonate buffer and 5 µL 0.2 mg/mL trypsin solution (final concentrations of 20 µg/mL) were added to each tube in group 1, with 5 min of incubation at 37 °C (Sigma-Aldrich Pty Ltd Macquarie Park, NSW, Australia). Then, the TS experiment was repeated with 15, 30, and 60 min incubations at 37 °C.

After enzymatic treatment, all the processed root segments were assessed for in vitro zoospore root attachment using the method described below. Similarly, all root segments in group 2 (control) were assessed via in vitro zoospore root-attachment as-says, directly without any pre-treatment.

***Spongospora subterranea* zoospore root attachment assay**

All root segments were assessed according to the in vitro zoospore root-attachment assay, as previously described [4]. Root segments were placed in a plastic container (70 mm in diameter), with each replicate separated by a 100 µ mesh in the container, and then incubated in the dark at 15 °C for 48 h before further examination. The number of zoospores attached to each root segment was quantified from five randomly selected fields of view via light microscopy at 400× magnification. A preliminary study tested the effects of root-segment incubation in enzyme buffers (ammonium bicarbonate and sodium acetate) and temperature (37 °C) on zoospore root attachment, and the results showed that neither buffer nor temperature affected zoospore root attachment (data not presented).

The zoospore root-attachment score for each cultivar/line in the screenings was normalized against the reference cultivars, 'Gladiator' and 'Iwa', with the first batch screening serving as a reference point (G1 + I1) to adjust for across-batch differences. The cultivar/line scores were further linearly scaled according to the reference-point correction coefficient (ηn) for each batch [4].

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

Proteomic analysis and data processing

Following TS treatment, root samples for all incubation times (i.e., 5, 15, 30 and 60 min) were prepared for proteomic analysis using C18 ZipTips (ZTC18S096; Merck Pty, Ltd, Bayswater, VIC, Australia), according to the manufacturer's instructions. The samples were dehydrated through vacuum concentration and reconstituted in 12 µL 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 analyze peptides with nanoflow HPLC-MS/MS and Xcalibur software (ver 4.3). Three ml aliquots of each sample were initially pre-concentrated in an analytical 20 mm × 75 µm PepMap 100 C18 trapping column, followed by separation over a 60 m segmented gradient in a 250 mm × 75 µm PepMap 100 C18 analytical column kept at 45 °C, at a flow rate of 300 nL/m. 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 (390–1500 m/z) were acquired at a scan resolution of 60,000, followed by MS2 scans using a Top15 DDA method, with 20 s dynamic exclusion of fragmented peptides. MS2 spectra were acquired at a resolution of 15,000 using an AGC target of 2e5, a maximum IT of 28 ms and a normalized collision energy of 27.

Mass spectrometry raw files were processed using MaxQuant software (version 1.6.5.0), using the Andromeda search engine to search MS/MS spectra against the *Solanum tuberosum* UniProt reference proteome (UP000011115) comprising 53,106 entries. With the exception of the activation of the match-between-runs function, 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 identifications. 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 log2-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.

Bioinformatics and statistical analyses

Differentially abundant proteins were identified using a t-test comparison of all replicates (n = 12) of both 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) (accessed on June 6th 2020), DAVID bioinformatics resources 6.8 (https://david.ncifcrf.gov/) (accessed on March 2021) and the KEGG database (www.genome.jp/kegg/) (accessed on March 2021).

Following normality and homogeneity of variance checks, all data were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 27. Zoospore root-attachment scores were analyzed using one-way ANOVA followed by protected Fisher's LSD test to determine statistically significant differences at the 5% level ($p = 0.05$). The TS time-course incubation study revealed that zoospore root attachment was comparable for all TS incubation times (Supplementary Table S1). Therefore, only data for the 5 min incubation times are presented in the results.

Results

In this study, we first assessed the effect of three different enzymatic treatments (trypsin, PNGase F and cellulase) on zoospore attachment to potato roots, followed by a detailed proteomic analysis of the products of the trypsin shaving treatment (Supplementary Figure S1).

Effects of enzyme treatments on zoospore root root attachment

Zoospore root attachment was significantly reduced in root segments treated with trypsin and PNGase F when compared with the untreated control in both susceptible ('Iwa') and resistant ('Gladiator') cultivars (Figure 1). In contrast, zoospore root attachment was unaffected by cellulase for both resistant and susceptible cultivars within the enzyme concentration ranges tested. In 'Iwa', trypsin was the most effective treatment with respect to reducing zoospore root attachment, whilst trypsin and PNGase F both significantly reduced zoospore root attachment in 'Gladiator'.

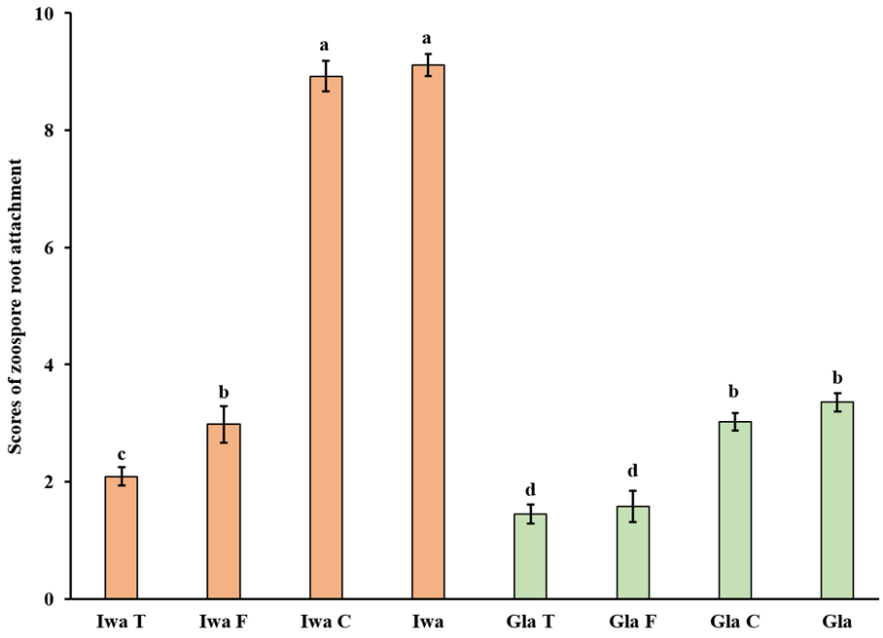

Figure 2. Effects of pre-enzyme treatment on zoospore root attachment for the 'Gladiator' (Gla, green bars) and 'Iwa' cultivars (orange bars). T: trypsin (20 µg/mL); F: PNGase F (50 units/mL); C: cellulase (1 mg/mL). Error bars represent standard deviations based on three biological replicates. Lower case letters denote values that are significantly different from each other p (cultivars) < 0.001, p (treatment) < 0.001, p (cultivar x treatment) < 0.001. LSD (0.05) = 0.4.

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—in particular, N-linked glycoproteins—in plant–pathogen interactions. To gain a better understanding of potential mediators, we used a TS approach, in which peptides were collected from 'Iwa' and 'Gladiator' roots after incubation in trypsin for 5, 15, 30 and 60 min to allow for the detection of proteins with different susceptibilities 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 8 samples (Supplementary Excel File S2). Principal component analysis of this dataset showed that 'Iwa' and 'Gladiator' samples were separated according to PC1; however, the samples did not cluster according to time points (Figure 2a). On this basis, t-test analysis was used to identify differentially abundant proteins (DAPs) between the two cultivars. This analysis identified 262 DAPs, of which 132 and 130 proteins were found to be significantly higher or lower in abundance in 'Gladiator' compared to 'Iwa', respectively (Supplementary Excel File S3). Cluster analysis of the subset of DAPs (Figure 2b) also showed that samples collected at each time point did not cluster together, indicating that incubation time did not affect the profiles of peptides released in the TS experiment.

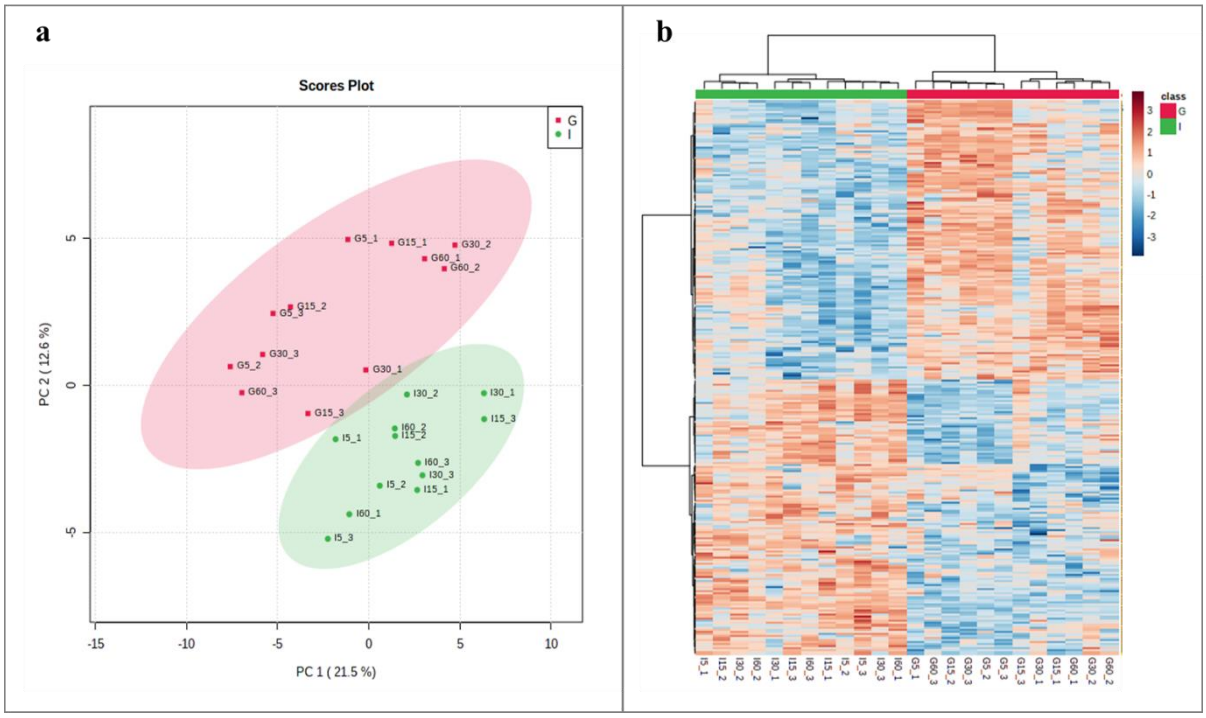

Figure 3. (a) Principal component analysis (PCA) of all identified proteins from a *Spongospora subterranea* resistant ('Gladiator', G) and susceptible ('Iwa', I) cultivar (*n* = 3) at four incubation times (5, 15, 30 or 60 min). (b) Heatmap of all significantly abundant proteins ('Gladiator' *vs* 'Iwa')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

Overall functional classification and pathway analyses of differentially abundant proteins

The functional enrichment analysis of the differentially abundant proteins (resistant vs. susceptible) is shown in Figure 3a. 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), the glutathione metabolic process (GO_BP: 0006749) and the lignin biosynthetic process (GO_BP: 0009809). For the proteins that were reduced in the 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 that were increased in the resistant cultivar included 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 carbon metabolism (n = 12 proteins), carbon fixation in photosynthetic organisms (n = 8 proteins), glyoxylate and dicarboxylate metabolism (n = 6 proteins), and the pentose phosphate pathway (n = 5 proteins).

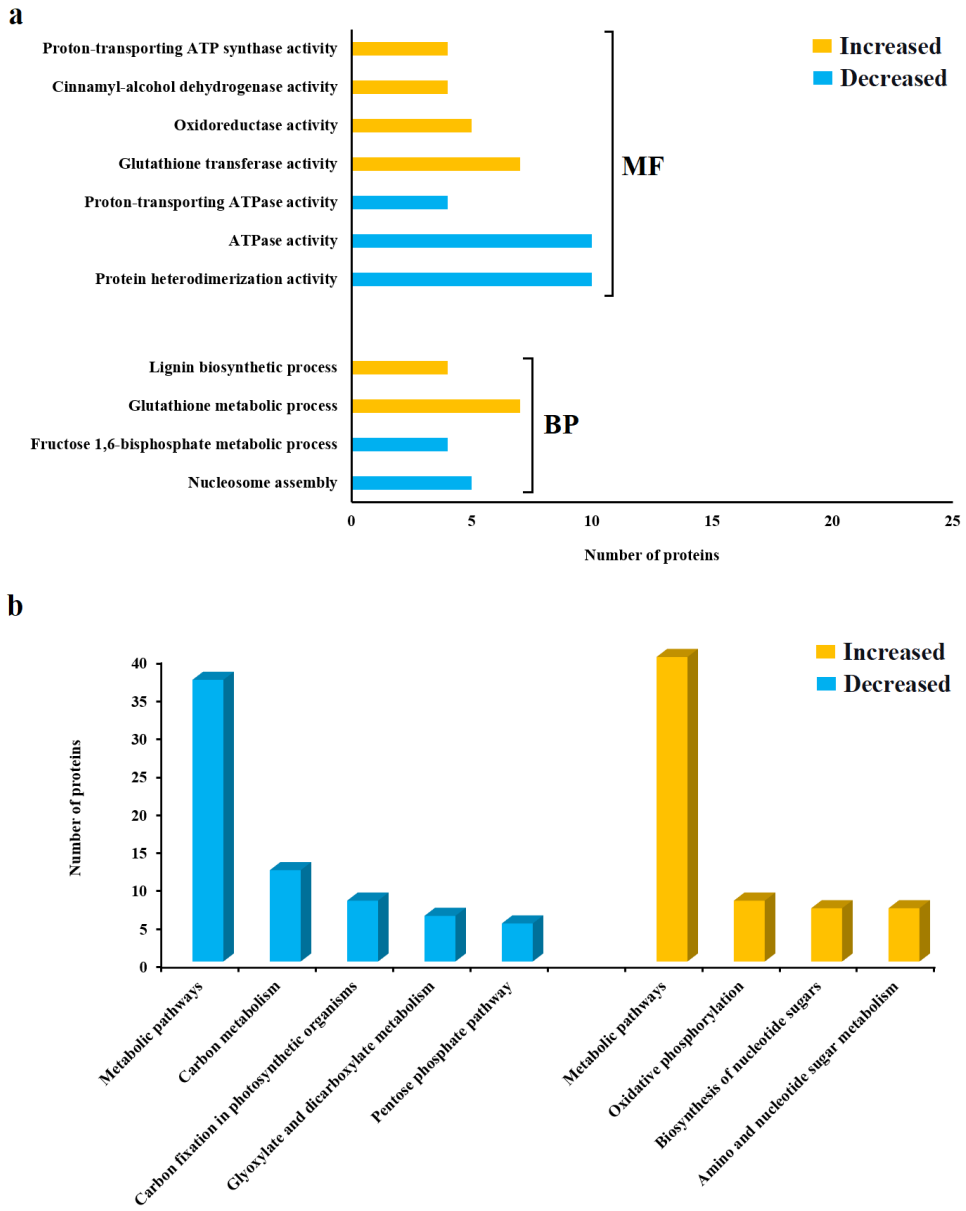

Figure 4. a) Gene ontology (GO) categories of DAPs ('Gladiator' vs. 'Iwa') from potato roots as determined with the trypsin shaving (TS) treatment. BP: biological process; MF: molecular function. (b) Pathway classification and enrichment analysis of DAPs ('Gladiator' vs. 'Iwa') from potato roots via TS treatment.

Comparison of proteins identified by TS with whole-root proteomics and transciptomics

The bioinformatic analysis of the complete set of DAPs identified by the TS experiment identified significant functional differences between the proteomes of resistant and susceptible cultivars. However, this included a high proportion of cellular components that may not be directly involved in facilitating attachment to root surfaces. Therefore, we used our previous proteomic dataset acquired from whole-root tissue analysis to filter the TS dataset [31], which enabled us to identify a subset of 226 proteins that were unique to the TS experiment (Figure 4a). Interestingly, a high proportion of these proteins (188) were significantly different in terms of abundance between resistant and susceptible cultivars (Figure 4b).

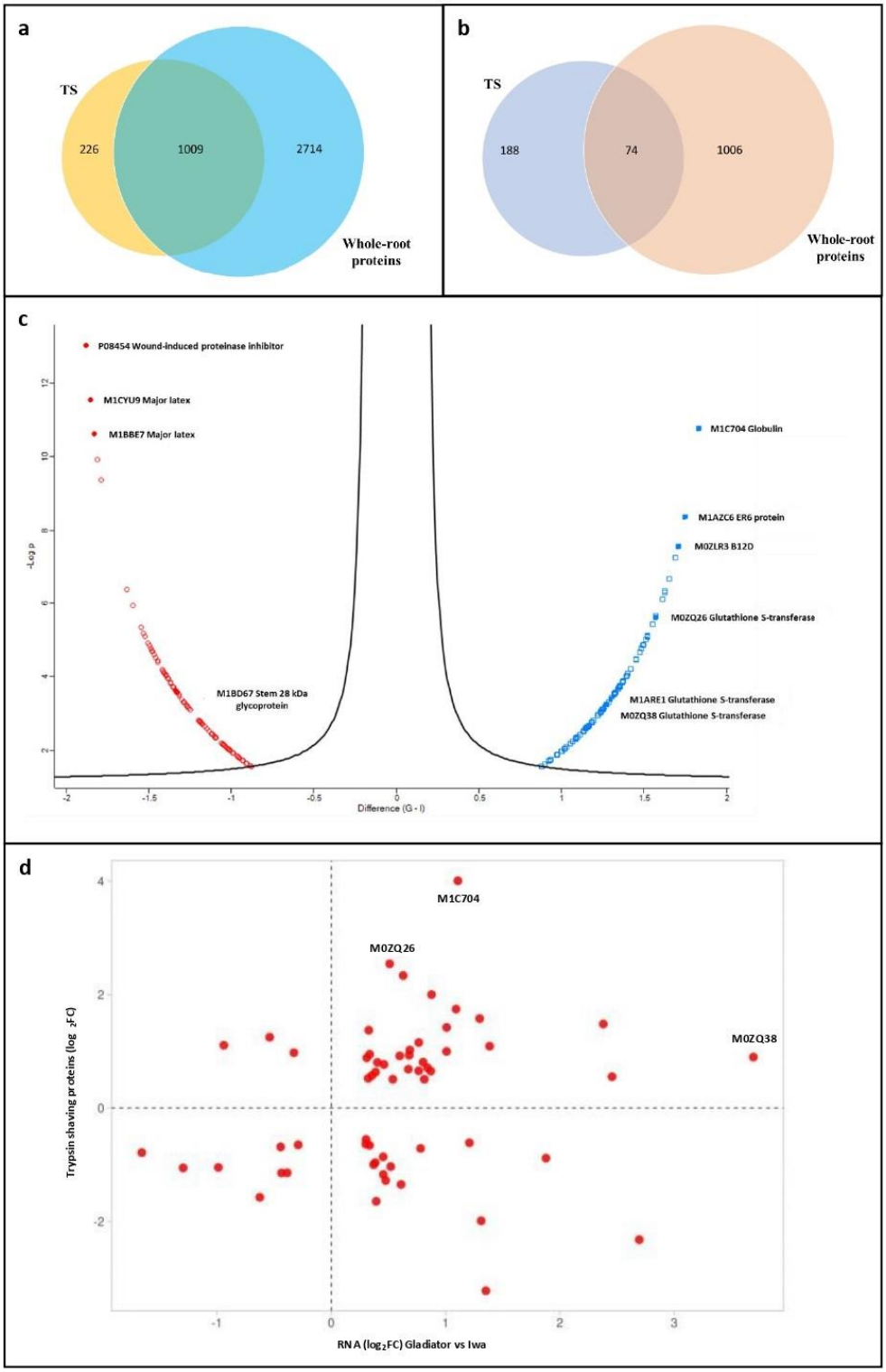

Figure 4. *a) Venn diagram representing the total number of potato root proteins identified specifically in the trypsin shaving (TS) treatment or the whole-root proteome analysis or in both (overlap). (b) Venn diagram representing the subsets of significant potato root proteins (resistant vs. susceptible DAPs) identified specifically in the TS treatment or whole-root proteome analysis or both (overlap). (c) Volcano plot displaying the 188 significant DAPs (resistant vs. susceptible) specific to the TS treatment plotted according to their log2 fold changes (t-test differences) on the x-axis and –log10 p-values on the y-axis. Data points in blue represent the proteins significantly increased in the resistant cultivar and those in red the proteins significantly increased in the susceptible cultivar. (d) Scatter plot representing the subset of 59 proteins that were significantly altered at both the mRNA and protein levels. Data points are displayed as the log2 fold changes (resistant vs. susceptible) at the mRNA level on the x-axis vs. the log2 fold changes (resistant vs. susceptible) at the protein level on the y-axis. The three datapoints labelled with their accession numbers are Globulin (M1C704) and two glutathione S-transferase proteins (M0ZQ26 and M0ZQ38)*

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 de-tected 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 4c). Of note, the cell-wall stem 28 kDa glycoprotein was significantly less abundant in the re-sistant cultivar, whilst three glutathione S-transferases (GSTs) (M0ZQ26, M1ARE1 and M0ZQ38) were significantly more abundant in the resistant cultivar specific to the TS experiment (Figure 4c). Fifty-nine proteins specific to the TS dataset were also altered in abundance due to differential expression at the mRNA level, based on a comparison with our previously published transcriptomic analysis of the cultivars Iwa and Gladiator [32]. The relative differences (log2FC, resistant vs. susceptible cultivars) in their tran-script and protein levels were compared (Figure 4d). Thirty-nine proteins underwent changes in abundance that were in agreement between the two datasets, while 20 pro-teins underwent opposite changes in abundance between the RNA-seq and proteomic data. Globulin (M1C704) and two glutathione S-transferases (GSTs) (M0ZQ26 and M0ZQ38) were among the proteins that were found at increased levels in both datasets.

Further comparison of the TS dataset with the whole-root proteome analysis ena-bled us to identify proteins with consistently large changes in abundance in both da-tasets. We selected the 20 proteins with the greatest differences in abundance in the TS treatment (ten increased and ten reduced in resistant vs. susceptible cultivars), of which 17 were also identified in the whole-root proteomic dataset. The fold changes (log2) for these proteins are compared in Figure 5, where the values for the TS dataset are plotted against the respective values for the whole-root proteomic dataset (Supplementary Excel Files S4 and S5 show all proteins and significant DAPs, respectively). 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 in-hibitor 1 (P08454) showed the largest decrease in abundance, with 5.3-fold and 3.7-fold reductions in the TS treatment and whole-root samples, respectively. Major latex pro-tein (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 a 3.7-fold increase, respectively.

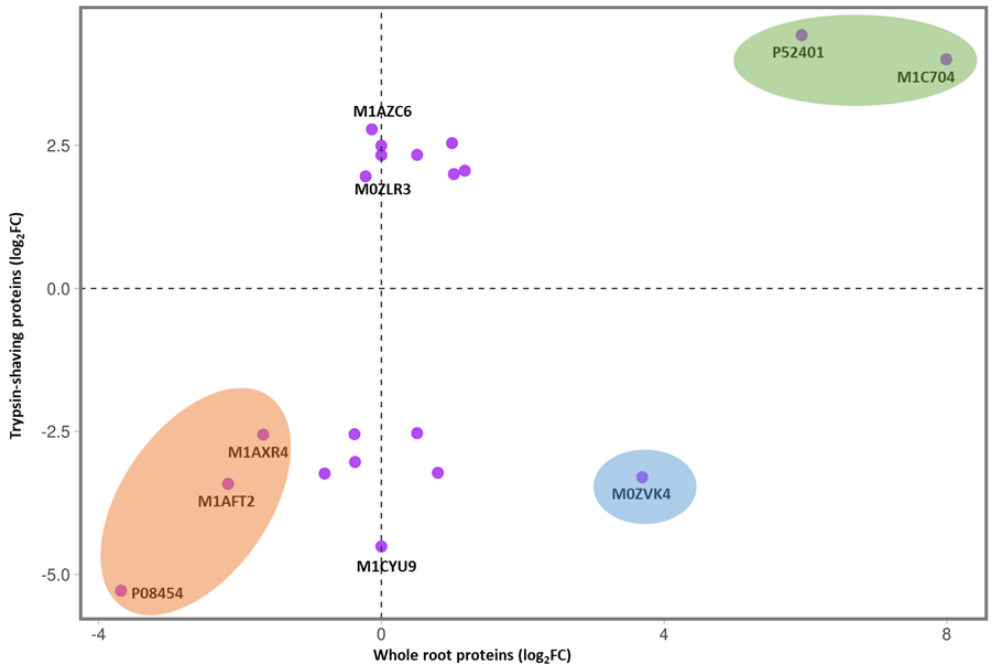

Figure 5. Comparison of the ten proteins with the largest increased or decreased fold changes (log2) in the resistant cultivar ('Gla') from the trypsin shaving (TS) treatment and the whole-root protein analysis. Proteins in green ellipse: most significantly increased in resistant cultivar in TS treatment and whole-root proteins; proteins in orange ellipse: significantly decreased in resistant cultivar in TS treatment and whole-root proteins; proteins in blue ellipse: significantly increased in resistant cultivars in whole-root proteins and significantly decreased in resistant cultivars in TS treatment.

Discussion

In this study, a combination of the in vitro zoospore root-attachment assay and label-free proteomic analysis was used to investigate pathogen–host interactions based on the modification of plant root-surface components with specific enzymes. We showed that trypsin and PNGase F, assessed in an in vitro model in this study, both reduced *S. subterranea* zoospore attachment to potato roots. PNGase F is an enzyme that catalyzes the removal of N-linked oligosaccharide chains from glycoproteins in a full and efficient manner. This enzyme is commonly used to investigate structure–function relationships of glycoproteins [36]. Plant cell-wall polysaccharides and proteins may serve as inactive signal molecules during plant–pathogen interactions [37,38]. Several studies have reported the biochemical basis of zoospore root attachments and demonstrated that root-surface polysaccharides play a critical role in zoospore root recognition and attachment [13,15,18,19,23,27,39,40]. The effects of plant cell-wall proteins and polysaccharides on Pythium and Phytophthora zoospore host attachment have been demonstrated previously [6,12]. The removal of polysaccharides of cress (*Lepidium sativum*) from root surfaces resulted in a reduction in Pythiaceous zoospore attachment; treatments that block or remove terminal fucosyl residues were particularly effective [15]. Similarly, Estradagarcia, Ray, Green, Callow and Kennedy [13] confirmed that cress-root mucilage can encourage the process of zoospore root attachment. In the present study, while cellulase had no effects on inhibiting zoospore root attachment, both trypsin and PNGase F significantly decreased the attachment of *S. subterranea* zoospores to the roots of two potato cultivars (Figure 1). These results suggests that potato root proteins, especially N-glycoproteins, may impact the zoospore root-attachment process.

Following the preliminary assessment of the effect of enzymatic treatment on root attachment, which indicated the potential involvement of cell-surface proteins, we used the trypsin shaving approach as the most practical first step towards the identification of cultivar-specific glycoproteins. Among the 1235 proteins identified in the TS study, most of the proteins that were significantly increased in the resistant cultivar were as-sociated with metabolic pathways, such as oxidative phosphorylation, biosynthesis of nucleotide sugars and the majority of amino acid biosynthesis pathways (Figure 3). These proteomic results were in line with the findings of similar proteome analyses of rice and sweet potato [41,42]. According to the analysis of pathways and GO functional annotation, we observed that glutathione metabolism, including the glutathione metabolic process and glutathione transferase activity, occurred at a high rate in the resistant cultivar compared to the susceptible cultivar. Glutathione biosynthesis occurs in chloroplasts, cytosol and mitochondria [43,44]. A few studies revealed the critical role of glutathione-related enzymes in host resistance to different pathogen infections. For example, glutathione-related enzymes were abundant in a tomato cultivar resistant to *Oidium neolycopersici* [45] and a rapeseed cultivar resistant to *Sclerotinia sclerotiorum* [46]. Three GST proteins were found to be highly abundant in the resistant cultivar specific to the TS study, while two of them were also more abundant according to the resistant cultivar's RNA-seq data. Balotf et al. [32] reported that GST proteins were significantly abundant in the 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 the plant immune system [47]. In a study of the interaction between S. subterranea and potato, it was shown that more than 30 GST genes were induced after infection [32].

In our present study, we compared the proteomes of root cell surfaces of two potato cultivars in the absence of S. subterranea infection and concluded that both constitutive and responsive gene expression strategies are involved in the regulation of GST proteins and used by potato hosts to increase resistance to S. subterranea. Lignin biosynthesis processes were also identified in our functional analysis of DAPs in the resistant cultivar. Lignin serves as a crucial barrier against pest and pathogen infection [48]. In our previous study [4], the phenylpropanoid biosynthesis pathway was identified in resistant cultivars associated with S. subterranea zoospore root attachment. Similar results were obtained by Balotf et al. [32], in whose study the phenylpropanoid metabolic pathway and especially lignin biosynthesis were shown to play important roles in the constitutive resistance of potato to *S. subterranea.*

The in vitro zoospore root-attachment assay (Supplementary Table S1) indicated that a 5 min incubation was sufficient for the enzyme to take effect, while, with respect to the time course for TS, no significant differences were found between incubation times (Supplementary Excel File S3). Elsewhere, He and De Buck [49] reported that di-gestion of cell-wall proteins of Mycobacterium avium subsp. paratuberculosis with trypsin required 30 min and a temperature of 37 °C. In contrast, Zahir, et al. [50] found that intracellular proteins were detected only after increasing the trypsin incubation period from 30 min to 60 min. In our investigation, cytoplasmic proteins were detected after trypsin shaving at all time points tested, suggesting that further optimization is re-quired to increase the specificity for cell-surface proteins. However, we were able to use our whole-root proteomic analysis to filter the TS dataset and target potential cell-surface peptides of interest.

Comparison of proteins from the TS proteome study with the whole-root proteins revealed 188 DAPs that were significantly abundant in the TS treatment (Figure 4b). Major latex proteins (M1CYU9 and M1BBE7), which play crucial roles in plant defence, were significantly reduced in abundance in the resistant cultivar. The major latex protein (M1AFT2)

was also found to be consistently reduced in abundance in the resistant cultivar via both TS treatment and whole-root protein analysis. Major latex proteins exist in different plant species, such as opium poppy [51,52], cucumber [53], peach [54], melon [55], soybean [56] and grape [57]. The number of major latex proteins varies among species; for instance, Arabidopsis thaliana contains 24 major latex proteins, while grape has just 14 [57,58]. Major latex proteins respond to biotic and abiotic stressors and perform crucial roles in plant growth and development, such as disease resistance, stress tolerance and development [59,60]. He, et al. [61] revealed that major latex proteins negatively regulate resistance to fungal infection in apple (Malus domestica) by suppressing the expression of genes and transcription factors associated with defence and stress. Similar to this result, we showed that the resistant potato cultivar, 'Gladiator', had lower expression of major latex proteins than the susceptible cultivar 'Iwa'.

The cell-wall stem 28 kDa glycoprotein (Figure 4c) was another protein that was found to be less abundant in the resistant cultivar, which was consistent with the whole-root protein analysis [31]. Previous studies have reported that stem 28 kDa glycoprotein plays a critical role in the transformation of immature elongation regions into mature, thickening tissues in the youngest regions [62]. Glycosylation and glycan processing are crucial post-translational modifications that cell-wall proteins undergo within the cell and are regarded as essential for the control of growth and defence mechanisms in plants [63]. PNGase F treatment demonstrated that N-glycoproteins can suppress the zoospore root attachment considered in this study. Together with these results, the cell-wall stem 28 kDa glycoprotein is an interesting candidate for direct association with the susceptibility of potato roots to zoospore root attachment.

Glycan endo-1,3-beta-glucosidase (P52401) was the protein with the largest fold change, being identified as significantly more abundant in the resistant cultivar with the TS treatment, and this finding is consistent with the whole-root protein analysis (Figure 5). Glucan endo-1,3-beta-glucosidase is a type of hydrolytic enzyme that breaks down 1,3-β-D-glucosidic linkages in β-1,3-glucans, which exist widely in bacteria, fungi and viruses [64]. Shinshi, et al. [65] reported that tobacco glucan endo-1,3-beta-glucosidase displays complicated hormonal and developmental regulation and is triggered by pathogen infection. In line with these studies, our results indicated that glucan en-do-1,3-beta-glucosidase contributes to potato resistance against S. subterranea infection. The abscisic acid- and environmental-stress-inducible protein (M0ZVK4) was one of the proteins that was differentially changed between the TS and whole-root protein analysis. This protein decreased in the TS treatment but increased in the whole-root protein analysis for the resistant cultivar. Abscisic acid is essential for numerous cellular processes, including seed development, germination, crop growth and root architecture mediation [66,67]. According to Harris [67], abscisic acid mediates responses to various environmental factors, including the presence of nitrate in the soil, water stress and salt, shaping the root system by regulating the production of lateral roots and controlling root elongation by modulating cell division and elongation. Since only the lower part of the mature potato root was examined in the TS treatment, while the entire root was used in the whole-root protein analysis, the difference in the fold changes of the protein (M0ZVK4) between the two studies may be a consequence of different spatial distributions of abscisic acid across different root areas.

Conclusions

This is the first report of an investigation of the biochemical basis of potato root-surface components in relation to S. subterranea zoospore attachment. From the in vitro zoospore root-attachment study, the enzymes trypsin and PNGase F were found to significantly reduce zoospore root attachment, whilst cellulase had no effect on zoo-spore root attachment. Our detailed proteomic analysis revealed broad-scale differences of root proteins between susceptible and resistant potato cultivars. These proteins within potato roots provide new insights into host resistance to zoospore root attachment at a proteomic level. Overall, this study provides an initial understanding of the biochemical and molecular bases of potato resistance to zoospore root attachment and is important for developing novel approaches in future disease management.

This work contributes to knowledge of the biochemical and molecular bases of *S. subterranea* zoospore root attachment, but there are some limitations that ought to be mentioned. Firstly, the TS peptide analysis identified a large number of intracellular proteins, which may have hindered the identification of lower-abundance cell-surface 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, trypsin shaving is inherently a peptide-centric approach that cannot easily distinguish between different proteoforms and therefore may underestimate proteome complexity. However, future studies using glycoproteomics may lead to a better understanding of the role of protein glycosylation in cultivar resistance to zoospore root attachment.

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The following report was prepared by our project collaborators at Plant and food Research relating to work examining gene markers associated with resistance phenotypes.

Plant & Food™ Research
Rangahau Ahumāra Kai

PFR SPTS No. 22468

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

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May 2022

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 whilst not significant were above background for multiple markers in the genomic regions. These would be key markers to explore further.

1 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.

2 Materials and methods

2.1.1 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.

2.1.2 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.

2.1.3 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 (the K model, as per 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 R^2 for each QTL identified.

3 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. We 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 1 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 x 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'.

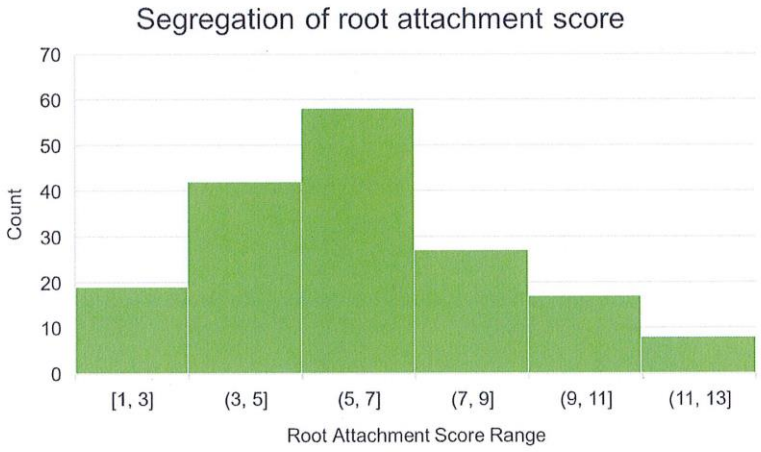

Figure 1. The segregation of the root attachment score (x-axis) by count (y-axis) in the panel of potato varieties used in the research.

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 this group of accessions, with little inflation in observed versus expected $-\log_{10}(p)$ values for the association models tested (Figure 2). 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).

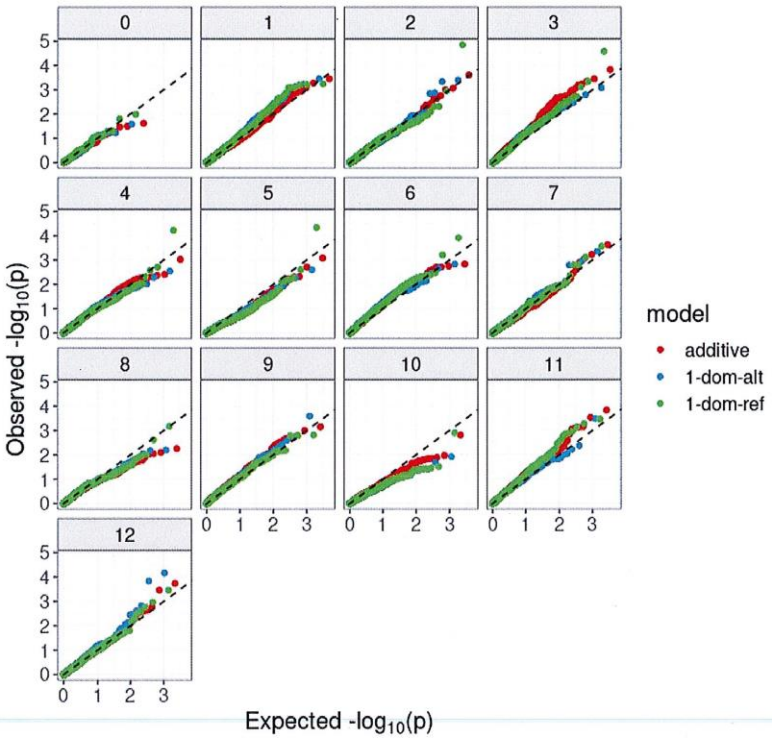

Figure 2: Quantile:quantile plots depicting deviation of observed from expected –log10(p) values from the null hypothesis (no association) for three models of association between the marker and trait: The additive model assumes an additive relationship between allele dosage and trait; the "1-dom" model 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 association analysis are given in Table 1.

Table 1. $-\log_{10}(p)$ 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 3 A) so different significance values were tested (Figure 3 B, C)

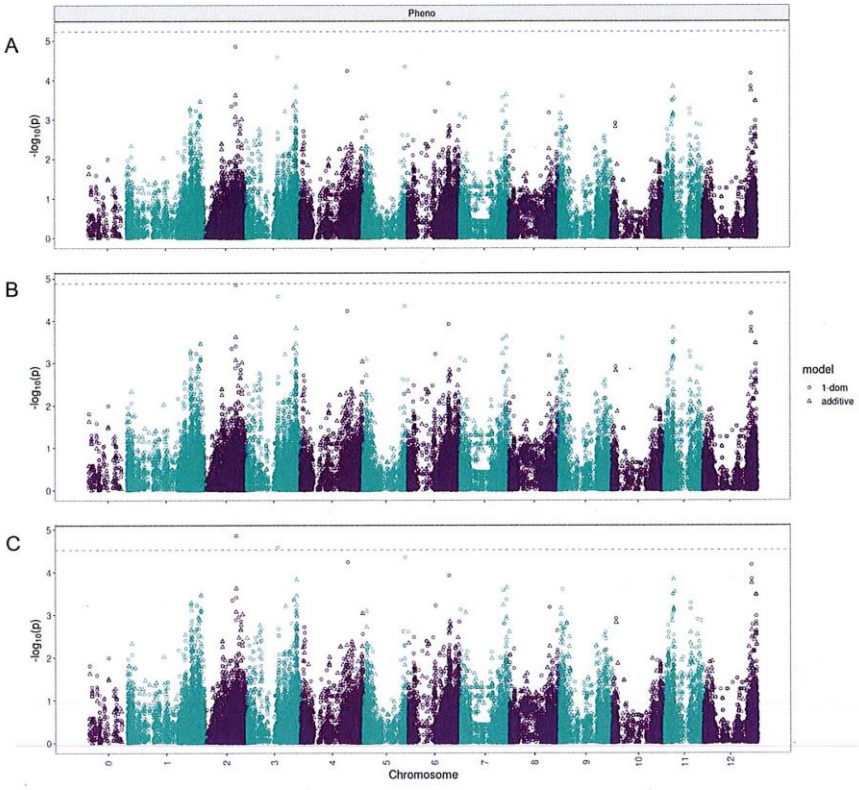

Figure 3. Manhattan plots showing marker:trait association across the potato genome. The dashed line in each plot represents the –log10(p) value above which markers are considered significantly associated with the trait, tested at M.eff p<0.05 (A), p<0.1 (B) and p<0.2 (C). Note that each model (additive, 1-dom reference and 1-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 1.

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 4; Table 2), 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 2). Both QTL are found under an association model that assumes a dominance effect of the reference allele on the trait. In Figures 5 and 6 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.

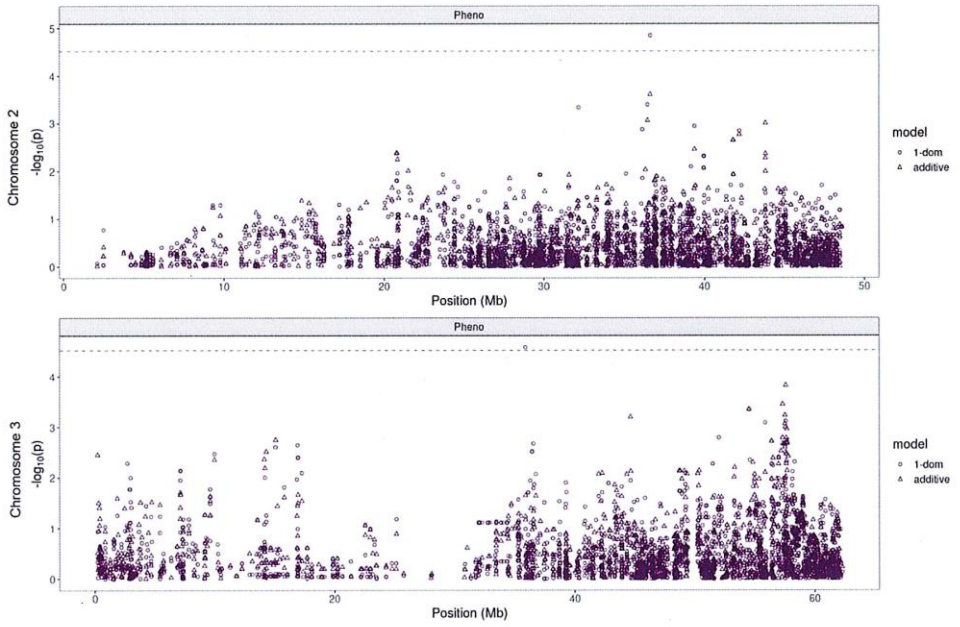

Figure 4. Manhattan plots showing marker:trait association at chromosomes 2 and 3. Markers above the threshold for association under the 1-dom-ref model at the p<0.2 level are identified at 36.6Mb on chromosome 2 and 35.9Mb on chromosome 3.

Table 2: 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

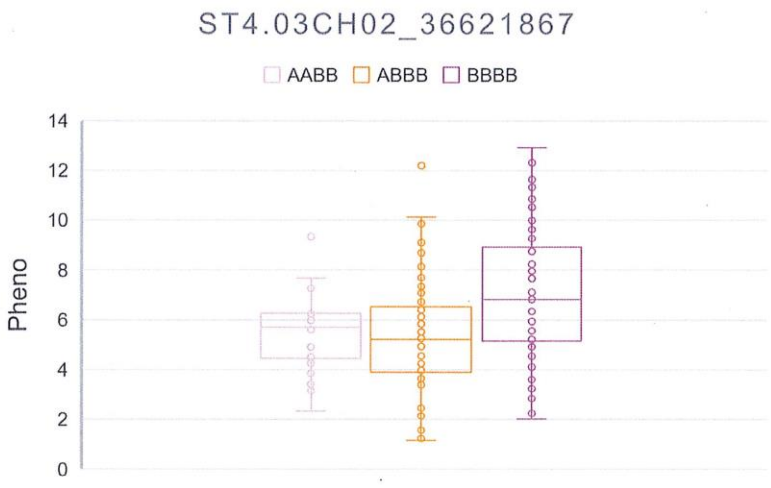

Figure 5. The phenotype (Pheno) as the average root attachment score for the three genotypes at marker ST4.03ch02_36621867. The different genotypes identified have a different dosage of the reference (A) SNP or alternate (B) SNP allele.

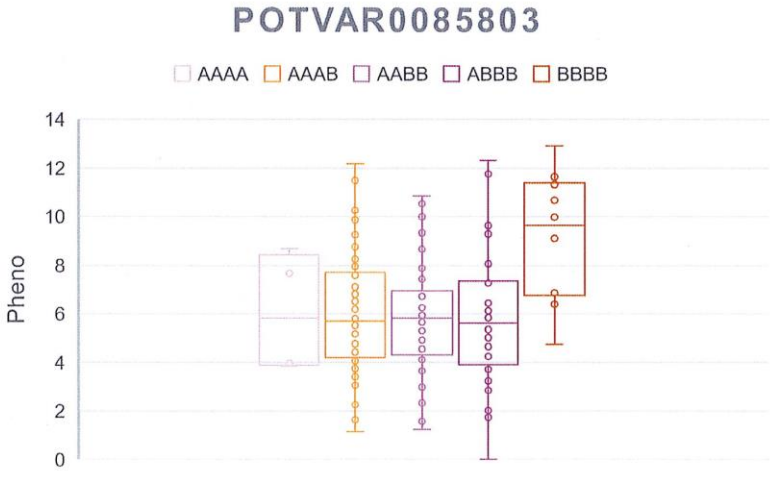

Figure 6. The phenotype (Pheno) as the average root attachment score for the five genotypes at marker POTVAR0085803. The different genotypes identified have a different dosage of the reference (A) SNP or alternate (B) SNP allele.

4 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.

4.1.1 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 these marker 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 existing breeding 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.

5 References

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Confidential report for:

University of Tasmania
Client ref: W0026170

PUBLICATION DATA

Baldwin S, Bloomer R, Monaghan K. May 2022. Mechanisms and manipulation of resistance to powdery scab in potato roots: QTL analysis. A Plant & Food Research report prepared for: University of Tasmania. Milestone No. 92558. Contract No. 37752. Job code: P/190502/01. PFR SPTS No. 22468.

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