Hort Innovation

## Final Report

# *Spongospora* Infection of Potato Roots – Ecology, Epidemiology and Control

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**Project code:**

PT14002

**Project:**

Spongospora Infection of Potato Roots – Ecology, Epidemiology and Control – PT14002



**Funding statement:**

This project has been funded by Hort Innovation, using the fresh potato and processed potato research and development levy, co-investment from Simplot Australia Pty Ltd – Tasmania and McCain Foods (Aust) Pty Ltd 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 4423 2

Published and distributed by: Hort Innovation

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Sydney NSW 2000

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www.horticulture.com.au

# Content

# Summary

Powdery scab disease of potato (caused by tuber infections with the protozoan pathogen *Spongospora subterranea*) has long been recognized as a common and economically important blemish disease. More recently potato root infection by this pathogen has been demonstrated to result in significant yield losses which greatly affect the economic competitiveness of the industry. This project focused on the root infection phase.

The broad aims of the project were to:

a) Provide new information on the fundamental understanding of the Spongospora root infection process essential for design and development of future management strategies.
b) Develop and optimize a new root pathogen detection tool which will be able to accurately quantify the efficacy of various management strategies in reducing or delaying root infection.
c) Develop a suite of novel disease control options that can reduce soil-borne inoculum and can interfere with the root infection processes through disruption of pathogen zoospore release and attraction.
d) Determine the pathogen's alternate host range (common weeds and alternate crops) and the relative resistance or tolerance of important processing potato varieties to root infection.
e) Develop a set of a recommended integrated disease control options.

We made significant breakthroughs in the fundamental understanding of the nature of pathogen resting spore dormancy, how spore germination is stimulated by root exudate chemicals released by host plants, and how released zoospores are chemotactically attracted to host roots via these same chemicals. Further studies noted influences of variety, plants stress, and environment on these processes. This led to development of highly novel approaches to soil inoculum management that may assist in reducing risk of disease on farm. From this foundational work, we utilized a series of laboratory and glasshouse trials to demonstrate 'proof of concept' for the use of these resting spore germination stimulants in absence of suitable host plants, to substantially deplete soil pathogen inoculum. This has provided a unique management opportunity between potato rotations to rehabilitate cropping soil through inoculum reduction strategies. We further showed that a combination of these approaches with fungicide treatments may provide additional benefits. Note: Fluazinam, FeEDTA and root exudate compounds are not currently registered for usage within Australia for controlling plant disease in potato crops and the use of these chemicals in commercial production is therefore not permitted.

We optimized a qPCR-based pathogen assay for quantifying infection in roots that allows quantification of the influence of management strategies and environment in reducing or delaying root infection. We demonstrated that any treatments that delay initial onset of root infection (reduced soil inoculum, chemical treatments, host resistance) will delay or slow the root infection cycle leading to reduced root infection, reduced impact on plant growth, and reduced subsequent tuber disease.

We demonstrated in pot and field trials that soil-applied fungicides such as fluazinam will provide partial disease control reducing zoosporangial infection and root galling. We showed why fungicides like fluazinam will only ever provide partial control by demonstrating while they provide efficient zoospore mortality they lack efficacy against dormant resting spores. We have postulated combinations of germination stimulants identified in this project with these fungicides should enhance the efficacy of fungicide treatments and is worthy of further

investigation. Again, we note that fluazinam is not currently registered for usage within Australia for controlling plant disease in potato crops and the use of these chemicals in commercial production is therefore not permitted.

In field and glasshouse trials we demonstrated the impact of elevated soil temperatures on reducing infection and disease impact and showed that soil Spongospora inoculum level correlated very well with tuber galling, and that root galling, in contrast to tuber disease, was a robust disease measurement that was not impacted, in these trials, by soil type, variety, plant date or crop duration.

We surveyed common weeds and select rotation crop plants for host status with *S. subterranea*. Several new or putative alternative hosts were identified, but none were as efficiently infected as potato (including volunteers). The role of volunteers in increasing *S. subterreanea* soil inoculum by up to 4-fold was subsequently demonstrated. This suggested potato volunteers are critical inoculum maintenance hosts and increased efforts at volunteer management are warranted. Blackberry nightshade (*Solanum nigrum*) was the most prevalent alternative weed host infection identified in cropping regions. Importantly both opium poppy and pyrethrum were identified as alternative hosts. Whilst these were both less susceptible than potato, given the density of these plants when cropped, their host status is significant, and soil testing following a commercial poppy crop showed greatly elevated levels of *S. subterranea* inoculum.

Glasshouse and field trials tested current and potential processing cultivars selected by the industry partners and identified root infection resistance levels ranging from highly susceptible through to moderate resistance. Assays including root zoosporangial scores, root galling and root pathogen levels were useful measures of resistance. A novel new zoospore root encystment assay, developed in the later stages of the project, appeared to be a cheaper, quicker and equally, if not more, robust measure of quantifying root resistance. This work identified root encystment efficiency as a key driver of host resistance and is worthy of further investigation.

Field trials that demonstrated early foliar applications of very low rates of the synthetic auxin, and herbicide PGR promoted potato root growth, mitigating against the damage caused by Spongospora root infection, and that the treatments provided small reductions in powdery scab disease. However, PGR treatments applied at moderate to high rates can result in foliar damage and can negatively impact yield, and there may be carry over residue issues if applied later in crop growth. Further investment in PGR for management of disease in potato crops is required to better inform whether the product could be considered for registration.

Key findings, novel detection and mitigation strategies on tools for pathogen detection, resting spore stimulation, root encystment and resting spore dormancy have been included in a series of refereed journal articles emanating from this project.

This project has achieved the goal of improving our understanding of this disease, has developed novel tools for subsequent study and evaluation of disease controls and host resistance, and has identified several opportunities for improved disease management. Many of the new disease management tools, require further development before commercial adoption including commercial field testing, refinement and generation of registration data. The project has furthermore identified key research areas for further investment that show strong promise for soil inoculum management and disease mitigation.

## Keywords

*Spongospora subterranea*; powdery scab; zoosporangia, root galls, zoospore encystment; resting spores; soil inoculum; alternative hosts; volunteers; qPCR; resistant cultivars

# Introduction

Potato is the fourth most important food crop in the world (after rice, wheat and maize), with annual production exceeding 320 million tonnes. It is the most valuable Australian vegetable crop with a farm gate value greater than $500M a year (Wilson, 2004). Australian production is expensive compared with world standards, a cost structure significantly worsened by disease. This has led to the supplanting of local potato products with imports from countries with lower labour and production costs. Disease impacts seed growers through: seed certification rejection and down-valuing; processing crop growers through yield loss and rejection by the factories; and the processing companies through requirements to over-contract seed and processing crops to allow for seasonal loss, as well as in-factory costs.

Powdery scab disease of potato (caused by tuber infections with the protozoan pathogen *Spongospora subterranea*) has long been recognized as a common and economically important blemish disease. More recently potato root infection by this pathogen has been demonstrated to result in significant yield losses which greatly affect the economic competitiveness of the industry. The Australian processing potato industry conservatively estimates losses of $13.4M p.a. (Wilson, 2016) with similar losses in the fresh market and indirect on-farm (labour, fertilizer, irrigation, lost opportunities, over-contracting crops, rejected crop loads) and off-farm (employment, rural community economy) costs greater still. Exacerbating these losses are the extremely high levels of the pathogen present in Australian (and especially Tasmanian) cropping soils (Sparrow & Wilson, 2012; Sparrow et al. 2015) and cultivars that are both susceptible and sensitive to root infection (Falloon et al. 2016). There are no effective management tools for *S. subterranea* root or tuber disease (Merz and Falloon, 2009; Falloon et al. 2016). Once infection occurs disease progresses rapidly.

*Spongospora subterranea* survives as a resting spore in the soil (and on seed tubers; Balendres et al 2016; 2017) from which motile zoospores actively swim toward and encyst on the root surface and penetrate the host root cell. Root infection progresses with the formation of a plasmodium which becomes a sporangium and produces secondary zoospores that are released and initiate new infections in the root and newly developing tubers in a polycyclic manner (Thangavel et al., 2015). At some point infections change from the zoosporangial to sporogenic with formation of root and tuber galls with production of resting spores. These are released into the soil providing new sources of inoculum. Very little is understood about the survival and activation of soil-borne inoculum of the pathogen and of the process of root infection which has greatly hampered our ability to generate effective control treatments and minimize losses due to root infection.

This project aimed to address these knowledge gaps and:

a) Provide new information on the fundamental understanding of the Spongospora root infection process essential for design and development of future management strategies.
b) Develop and optimize a new root pathogen detection tool which will be able to accurately quantify the efficacy of various management strategies in reducing or delaying root infection.
c) Develop a suite of novel disease control options that can reduce soil-borne inoculum and can interfere with the root infection processes through disruption of pathogen zoospore release and attraction.

d) Determine the pathogen's alternate host range (common weeds and alternate crops) and the relative resistance or tolerance of important processing potato varieties to root infection.
e) Develop a set of a recommended integrated disease control options.

# Methodology

There are four key programs of work with each summarized below – detailed Methodology for each section is available in the relevant manuscripts and sections in the appendices.

**Program 1: Epidemiology modelling and manipulation - delaying or slowing root infection**

We measured the dynamics of root infection and root disease development to determine the influence of a) host factors and b) external environmental factors (including various control strategies) on the rate of root infection, disease progression, root damage and resultant yield losses.

We developed and used DNA detection methods (qPCR) to precisely measure the quantity of pathogen present within individual plant root systems (see Appendix 1a, Thangavel et al 2015, for methodology). Three pot trials; two in potato (one winter planted, one summer planted) and one in tomato were conducted to quantify the impact of delayed infection on pathogen replication and disease development and to test this system (Appendix 1a).

Further trials assessing differing disease management approaches and environmental influences were conducted for which the root infection analysis tool was used. These included:

**Cultivar resistance testing** -Two glasshouse, two mini-plot trials and one field trial compared 15 varieties for resistance to root infection (Appendix 2). This is described under Program 3.

**Influence of environmental parameters on root disease** - A glasshouse trial was conducted to compare the impact of temperature on root pathogen build up and subsequent root disease. A field trial, in which 16 field sites were monitored through the growing season studied the impact of pathogen inoculum level, planting date, crop duration, cultivar and soil type on root infection and tuber disease (Appendix 3).

**Fungicide treatment** - Two pot and two field trials tested the impact of chemical seed and soil treatments on pathogen replication and root and tuber disease (Appendix 1a).

**Disease mitigation treatments** - Two field trials tested the application of foliar PGR for root stimulation, root infection, yield impact and powdery scab disease (Appendix 4).

**Program 2: Ecological study and manipulation of pathogen inoculum**

We examined resting spore dormancy and examined possible treatments for accelerating inoculum decay.

**Resting spore dormancy studies -** We demonstrated chemical stimulation of germination of resting spores (Appendix 1e), with activation stimulated by root exudate phytochemicals (Appendix 1c). This confirmed presence of stimuli-responsive dormancy. Furthermore in *in vitro* laboratory studies we confirmed presence of constitutive dormancy in a portion of resting spores by extended incubation with germination stimulants for up to 2.4 years (Appendix 1d).

**Host range studies** – We undertook four field surveys over two years of weed and volunteer crop plants in fields with known elevated soil inoculum levels. These identified several putative alternative host species including new records. A series of three glasshouse trials

tested confirmed host status of opium poppy and pyrethrum, both important rotation crops, and as such could be critical in inoculum maintenance. Field testing revealed elevated pathogen soil levels after a commercial poppy crop. Evidence for the importance of volunteer potatoes for inoculum maintenance was shown through surveys of soil inoculum levels (see Appendix 1g for complete methodology).

**Pathogen survival and decay** – we undertook *in vitro* studies with resting spores to examine the impact of wet:dry soil moisture cycles, temperature fluctuations and germination stimulants (Appendix 1e).

**Program 3: Assessment of potato germplasm for resistance and tolerance to Spongospora root infection**

**Cultivar resistance trials** - We quantified resistance to Spongospora root infection within 15 current and new processing cultivars identified by our project industry partners. These included numbered lines under assessment by the processing companies as possible new cultivars of importance to the Australian industry.

Plants were grown in a series of two *in vitro*, two glasshouse, two mini-plot and one field trial with amended or natural soil inoculum levels. Root infection (incl. qPCR quantitation of pathogen), root galling and tuber infection was assessed at multiple harvest dates and data compared across trials (Appendix 2a).

**Root encystment assay** - We further developed and tested a novel resistance assessment protocol using *in vitro* assessment of efficiency of zoospore root encystment of test cultivars. This provided a quick and robust assessment of resistance that was not influenced by environmental variables and avoided the confounding effect of polycyclic infections. This identified root encystment as a key resistance parameter (Appendix 2b).

**Program 4: Chemotaxis study – disruption of zoospore root interactions**

Here we studied the plant chemicals released by roots (root exudates) that have an effect on resting spore germination and zoospore attraction to roots.

In a large series of experiments (c. 20 trials) we confirmed that potato root exudates will chemically stimulate resting spore germination (Appendix 1c). We identified individual chemical constituents of potato root exudates and how production of these varied with cultivar, plant stress and environment (Appendix 1e). We tested purified individual chemicals at a standard concentration and identified which have the capacity to stimulate resting spore germination, and their relative efficiency at this activity. We also confirmed Hoagland's solution is also stimulatory and that FeEDTA is the stimulatory chemical within this solution.

We developed novel assays to test chemotaxis effect on released zoospores. Using *in vitro* assays with released zoospores in a water solution with microcapillary tubes containing specific root exudate chemicals we confirmed zoospores are chemotaxically attracted to the same chemicals that stimulate their release from resting spores.

We then tested the ability of stimulatory chemicals to accelerate resting spore germination within a soil environment, and testing this in absence of host plants, that stimulation can lead to soil inoculum depletion. This process was tested in a series of glasshouse trials which successfully confirmed proof of concept for a "germinate to exterminate" approach to inoculum management (Appendix 1f). Further testing at a small plot and field scale are now warranted.

# Outputs

## Key Results

## Program 1: Epidemiology modelling and manipulation - delaying or slowing root infection

**Impact of delayed *S. subterranea* infection on pathogen replication and disease (full results – Appendix 1a)**

Pathogen replication and root and tuber disease

Three pot trials (PT), two in potato (PT1 – winter planted, PT2 – summer planted) and one in tomato (PT3) showed that *S. subterranea* DNA within the root increased with time (Fig. 1). Where application of inoculum was delayed there was a delay in the onset of root infection, galling and tuber disease (see Appendix 1a) and a resultant decrease in pathogen DNA levels. This effect was consistent in multiple potato cultivars tested (Russet Burbank, Desiree) across multiple trials (3 in total) with PT2 presented in Fig 1.

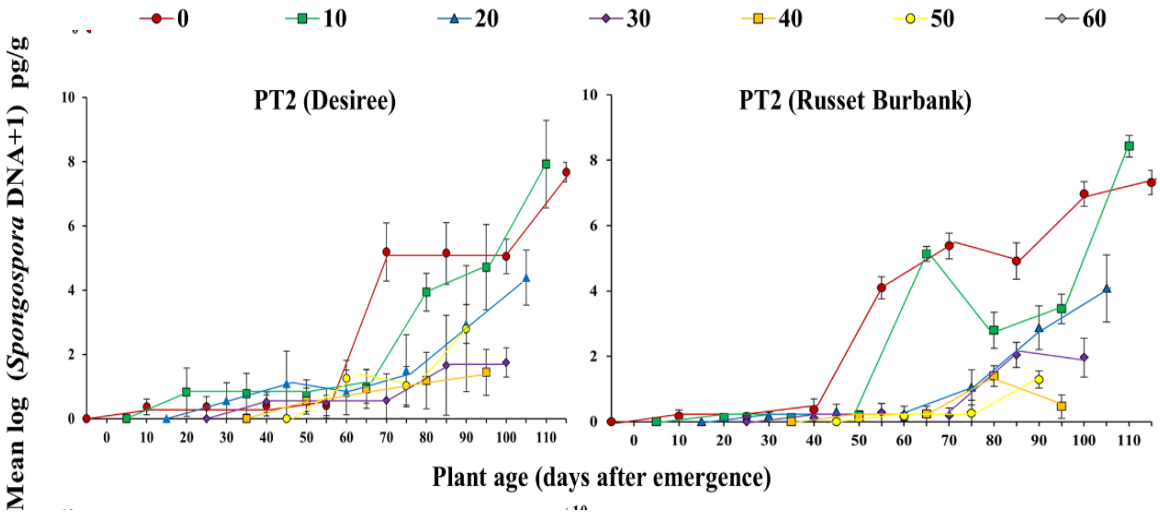


**Fig. 1.** The effect of delayed inoculation on initiation of root infection and replication of *S. subterranea*.

Results are for two potato cultivars in, PT2—summer planted crop. Markers represent the different inoculation treatment dates shown as days after emergence (DAE). Vertical bars are standard errors (n = 9).

**Impact of seed and soil chemical treatment on Spongospora infection and powdery scab (full results – Appendix 1a) NOTE THAT THE CHEMICALS TESTED IN THESE STUDIES, (E.G. FLUAZINAM) ARE NOT ALL CURRENTLY REGISTERED FOR USAGE WITHIN AUSTRALIA FOR CONTROLLING PLANT DISEASE IN POTATO CROPS AND THE USE OF THESE CHEMICALS IN**

**COMMERCIAL PRODUCTION IS THEREFORE NOT PERMITTED.**

Two pot trials (PT4, PT5) and two field trials (FT1, FT2) across multiple potato varieties (Russet Burbank, Innovator, Desiree) tested a range of seed and soil furrow fungicide treatments. While pathogen levels increased in all treatments with time the most successful treatments slowed the rate of pathogen increase. Essentially, as demonstrated in FT2 (Fig. 2) the untreated control and mancozeb soil furrow treatments were the least effective, as shown by an increased rate of pathogen replication in these two treatments. The other treatments (fluazinam seed dip, fluazinam soil furrow, formalin seed dip, mancozeb seed dip) had a lower rate of pathogen replication, suggesting these were the most effective field treatments.

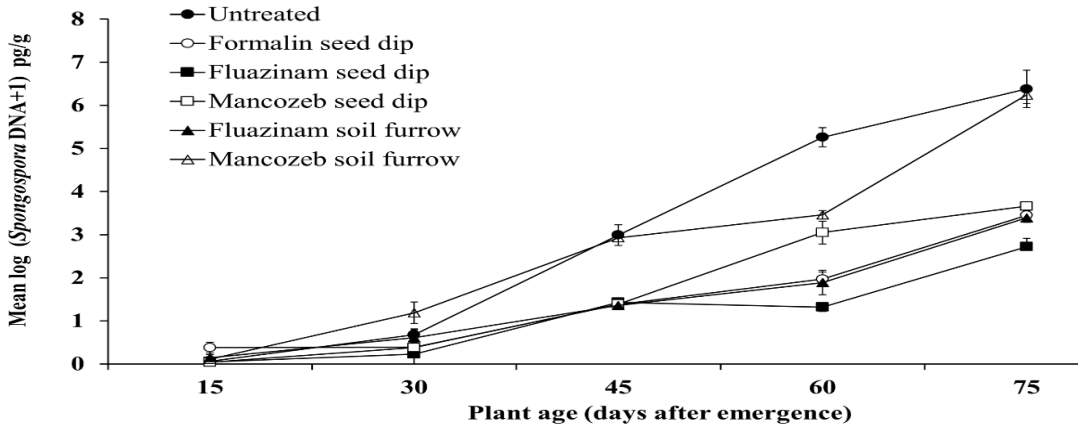


**Fig 2.** The effect of fungicide seed and soil treatments on the increase in pathogen content in FT2 (pg *S. subterranea* DNA/gm) in potato roots.

Measurements were made 15–75 days after emergence (DAE) in two field trials (A—FT1, B—FT2). Data was pooled across the cultivars Russet Burbank and Innovator; vertical bars are standard errors (n = 9).

**NOTE THAT THE CHEMICALS TESTED IN THESE STUDIES, (E.G. FLUAZINAM) ARE NOT ALL CURRENTLY REGISTERED FOR USAGE WITHIN AUSTRALIA FOR CONTROLLING PLANT DISEASE IN POTATO CROPS AND THE USE OF THESE CHEMICALS IN COMMERCIAL PRODUCTION IS THEREFORE NOT PERMITTED.**

Consistent with the pathogen multiplication data the soil treatment (fluazinam) and seed dips (formalin, fluazinam and mancozeb) consistently reduced both mean zoosporangial score ($p < 0.05$; 2 – 6 fold decrease, Fig. 3A) the root gall score ($p < 0.05$; 2 – 8 fold decrease, Fig. 3C), and mean tuber disease incidence and severity ($p < 0.01$, Appendix 1a) compared to the untreated control and the mancozeb soil treatment in both field trials.

Measurements included mean potato root zoosporangia infection score (0–4) in FT1 (A) and and root gall severity score (0–4) in FT1 (C). Data was pooled across the cultivars Russet Burbank and Innovator and two assessment dates, 45 and 60 days after emergence. Vertical bars are standard errors (n = 8).

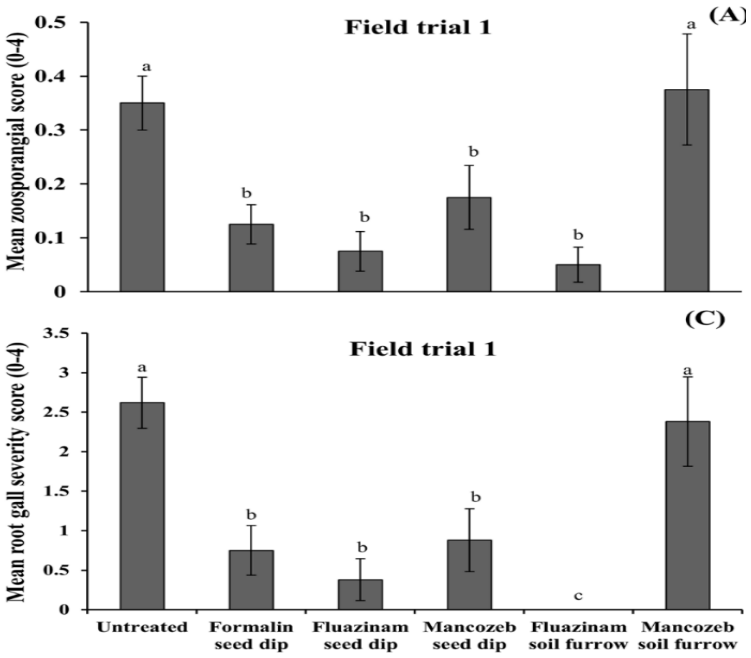


**Fig 3.** The effect of fungicide seed and soil treatments on root infection.

**Influence of environmental parameters on root disease** - We monitored 16 commercial fields through the 2017/18 growing season to broadly assess the impact of soilborne *Spongospora* on root galling and tuber disease (full results – see Appendix 3). We tested a number of parameters including cultivar type (program 1,3), soil type, planting date and length of time the crop was in the ground (program 3). We showed that soil *Spongospora* inoculum level correlated very high and moderately high with tuber galling and powdery scab disease respectively. We also showed that both soil type and crop duration had an impact on the expression of tuber disease, but not root galling. Essentially, lighter sandier soils or a shorter crop duration reduced tuber powdery scab compared to a heavier soil or a longer crop duration (Table 1).

**Table 1.** Interaction of factors impacting *Spongospora* root and tuber disease across 16 field sites

| Factor | Root galling | Tuber disease |
|---|---|---|
| | | |
| Soil type | ns | <0.05 |
| Cultivar | ns | ns |
| Plant Date | ns | ns |
| Crop duration | ns | <0.05 |
| Pathogen level | <0.01 | <0.05 |

**Disease mitigation treatments** - Two field trials over consecutive years tested the application of a foliar plant growth regulator (PGR) for root stimulation, root infection, yield impact and powdery scab disease (see Appendix 4 for full results). **NOTE THAT THE PGR IS NOT CURRENTLY REGISTERED FOR DISEASE CONTROL IN POTATOES IN AUSTRALIA AND ITS USE IN COMMERICAL PRODUCTION IS THEREFORE NOT PERMITTED.**

**Root growth stimulated:** There were significant increases in the fresh and dry weights of root material recorded from both PGR treatment rates at both assessment dates in 2016/17; shown is one assessment typical of the other results (Table 2).

**Table 2.** Effect of foliar PGR application on potato roots fresh and dry weights (grams) in late March 2016/17

| Treatment | Fresh Weight (g) | Dry Weight (g) |
|---|---|---|
| **Control (No PGR)** | 48.7 a | 7.3 a |
| **PGR (low rate)** | 70.2 b | 9.0 b |
| **PGR (very low rate)** | 61.2 b | 8.9 b |
| **LSD (0.05)** | 11.98 | 1.24 |
| **ANOVA** | 0.002 | 0.007 |

Means followed by the same letter are not significantly different at 0.05 level by Fisher's LSD test
Mean values ± SE of means (n =9)
Normality test and Skewness value indicate data are normally distributed

**Disease and yield impacts**

In 2016/17, powdery scab disease was decreased following PGR treatment; yield was increased slightly by the lower PGR treatment and decreased slightly by the higher PGR treatment (Table 3). There was no significant impact on root galling. Similar trends were seen in 2017/18 except that the PGR treatment increased the numbers of tubers (Appendix 4).

**Maximum Residue Limits (MRLs)** – A selection of tubers from both PGR treatments were sampled over both years and assessed for PGR residue. All samples recorded PGR levels at <0.01mg/kg (below detectable levels) which is at least 40 times below the current MRL for the PGR (0.4mg/kg).

**Table 3.** Effect of foliar PGR application on potato numbers, yield and powdery scab disease incidence and severity in 2016/17

| Treatment | Tuber Weight (kg) | Tuber Number | Disease incidence (%) | Disease severity (0-6) |
|---|---|---|---|---|
| **Control (No PGR)** | 15.91 | 80.6 b | 42.2 c | 0.549 c |
| **PGR (low rate)** | 15.50 | 69.2 a | 19.6 a | 0.255 a |
| **PGR (very low rate)** | 16.50 | 78.5 b | 30.8 b | 0.400 b |
| **LSD (0.05)** | ns | 8.83 | 9.92 | 0.129 |
| **ANOVA** | 0.45 | 0.042 | 0.004 | 0.004 |

Means followed by the same letter are not significantly different at 0.05 level by Fisher's LSD test

# Program 2: Ecological study and manipulation of pathogen inoculum

### Chemical activation of zoospore release

#### Effects of incubation of *S. subterranea* resting spores in HS and DDW on zoospore release

In eight separate experiments incubating resting spores in Hoaglands solution (HS) lead to a greater rate ($P = 0.028$ to $P < 0.01$) and greater number ($P = 0.023$ to $P < 0.001$) of zoospores being released compared to incubation in distilled water (DW) (Appendix 1f). This indicted that HS was encouraging zoospore release.

#### Effects of HS components and varying Fe-EDTA concentrations on zoospore release

Three experiments tested the individual constituents of HS and showed that zoospores were detected only in the Fe-EDTA solution (0.05 mM; all three experiments), compared to all other individual components (Appendix 1f). Fe-EDTA also increased the numbers ($P = 0.012$ to $P < 0.01$) and rate of zoospore production compared to DW (Appendix 1f).

**For impact of Fe-EDTA on soil pathogen levels see Program 4.**

#### Effects of HS on inoculum infectivity and root infection

To confirm that increased zoospore release (as seen with HS and Fe-EDTA) lead to greater infectivity we showed in three separate experiments with the key test species (tomato) that seedlings incubated in HS with *S. subterranea* resting spores had greater incidence and severity of zoosporangium root infection than tomatoes incubated in DDW with *S. subterranea* resting spores (Table 4).

**Table 4.** Mean *Spongospora subterranea* zoosporangium severity scores for roots of tomato bait plants grown in distilled water or Hoagland's solution in three experiments.

| Treatments | Experiment | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Distilled water | 2.0 ± 0.6 | 3.0 ± 0.6 | 1.8 ± 0.4 |
| Hoagland's solution | 5.0 ± 0.0 | 4.6 ± 0.4 | 5.0 ± 0.0 |
| *T-Test* | *P<0.001* | *P=0.065* | *P<0.001* |

Severity of zoosporangium were assessed using the scale: 0 (no infection), 1 (sporadic, zoosporangia covering approximately 1% of the roots), 2 (slight 2-10%), 3 (moderate, 11-25%), 4 (heavy, 26-50%) and 5 (very heavy, >50%).

Values are means ± standard errors (n = 3, Assay 1; n =5, Assays 2 and 3). Probability of difference between treatment means was tested using the independent t-test.

**Sporosori survival and longevity (see Appendix 1d)**

**Viability and infectivity of dry-stored sporosorus inoculum**

To investigate how long resting spores could survive in the soil and remain viable to cause infection we tested resting spores from 1-week old up to 5-year old from tuber and galls. We found that all resting spores were able to be germinated and cause infection on tomato bait plants confirming that the resting spore is able to survive for at least five years and that viable inoculum is produced regardless of the source (gall or tuber) and in all the ages tested in this study (1-week to 5-year old – see Appendix 1d).

**Viability and infectivity of sporosorus inoculum stored in a germination stimulatory environment**

To test whether inoculum could remain viable for long periods of time, even when incubated in a stimulatory nutrient solution, we incubated resting spores for up to 2.4 years. Dried inoculum incubated in nutrient solution remained infective for at least 2.4 years when subsamples were tested with the tomato bait plant assay (Appendix 1d). This indicates that not all zoospores are released from a resting spore when placed in a stimulatory environment; indicating adaption of the pathogen to both infect a plant but also retain a proportion of zoospores for maintaining persistence in the soil.

## Host range studies – see Appendix 1 g for complete results

Field surveys for natural infections in common weed and crop species

Four field surveys over two years of 16 weed and volunteer crop plants in fields with known elevated soil inoculum levels identified several putative alternative host species although most weed species were non-hosts. Only potato showed visual root galling compared to all other weed and volunteer host species tested.

Zoosporangia were found in root hairs from two blackberry nightshade (*Solanum nigrum*) plants sampled from one of the field sites. No other zoosporangia were observed in any other root samples from any species at the four sites. Quantitative PCR confirmed that the root samples from *S. nigrum* plants with visible zoosporangia contained high levels of *S. subterranea* DNA (500-fold greater than the non-template control). Similar high DNA levels were detected in *S. nigrum* (500-fold greater than NTC (non-template control)) and *Chenopodium murale* (150-fold greater than NTC) samples, both from another site, even though zoosporangia were not observed from root samples taken from these plants. Lower levels of *S. subterranea* DNA, well above that recorded in the NTC were detected in other weed species including *Sonchus arvensis*, *C. murale*, *Sisymbrium officinale* (all at 8-fold greater than NTC), and *Rumex conglomeratus* (at 4-fold greater than NTC). Elevated levels were also detected in *Papaver somniferum* (at 4-fold greater than NTC) at one of the sites.

Experimental challenge of opium poppy, pyrethrum and tomato plants with *S. subterranea*

Observations of plants from the first glasshouse trial indicated that the inoculation treatment affected early growth in opium poppy, pyrethrum and tomato plants compared to the un-inoculated controls. Tomato showed obvious reductions in shoot growth in the inoculated plants compared to the un-inoculated plants (Appendix 1g). Similarly, inoculated pyrethrum plants were slightly smaller than un-inoculated plants, and inoculated opium poppy plants

showed leaf yellowing, reduced vigour, smaller leaves and flower heads compared to the un-inoculated plants. Microscopic examination of root material from the three species showed presence of zoosporangia in roots of tomato (21/21) and opium poppy (5/21), but not pyrethrum (0/21) (Table 5). The differences between mean zoosporangium scores for the three species was significant ($P$<0.001), with tomato, opium poppy and pyrethrum giving, respectively, 3.39, 1.11 and 0.0 mean scores (Table 1). Un-inoculated plants of all species did not show any zoosporangium root infection. There were also significant differences in *S. subterranea* DNA levels between the three tested species ($P$<0.001) with tomato roots having the greatest amounts of pathogen DNA ($4.07*10^{10}$ pg/g; mean log 10.61), followed by opium poppy ($7.85*10^{8}$ pg/g; 8.89) and pyrethrum ($2.38*10^{7}$ pg/g; 6.37) (Table 5).

In the second challenge trial all three species were infected by *S. subterranea*. Incidence of zoosporangium infection varied across the three species, the greatest incidence was recorded in tomato (22/22), followed by opium poppy (7/22) and pyrethrum (4/22) (Table 5). In tomato, infection was frequently observed in root hair and epidermal cells, however in opium poppy and pyrethrum, infection was mostly restricted to the root hairs. Un-inoculated plants did not show any zoosporangium root infection. The severity of infection was again significantly different between the plant species with tomato, opium poppy and pyrethrum being, respectively, 1.85, 1.0 and 1.0 (Table 5). Despite no numerical difference between the mean scores for opium poppy and pyrethrum, infection in opium poppy root hairs was slightly more intense than in pyrethrum. Root infection in all three species was confirmed by qPCR. The quantity of *S. subterranea* DNA varied among the species with infected tomato, opium poppy and pyrethrum roots having averages, respectively, of $2.16*10^{11}$ (mean log 11.33), $6.65*10^{9}$ (9.82) and $6.94*10^{8}$ (8.84) pg *S. subterranea* DNA per gram of root (Table 5).

**Table 5.** Numbers of tomato, opium poppy and pyrethrum plants succumbing to zoosporangium root infections, mean zoosporangium score and mean amounts of pathogen DNA following *Spongospora subterranea* inoculation.

| | **Plants with zoosporangial infection / total plant inoculated** | **Zoosporangia score (0-4)** | ***S. subterranea* DNA pg/g (log)** |
|---|---|---|---|
| **Trial 1** | | | |
| Tomato | 21/21 | 3.39 | $4.07*10^{10}$ (10.61) |
| Opium poppy | 5/21 | 1.11 | $7.85*10^{8}$ (8.89) |
| Pyrethrum | 0/21 | 0.0 | $2.38*10^{7}$ (6.37) |
| *LSD (F probability)* | | *0.415 (<0.001)* | *0.426 (<0.001)* |
| **Trial 2** | | | |
| Tomato | 22/22 | 1.85 | $2.16*10^{11}$ (11.33) |
| Opium poppy | 7/22 | 1.00 | $6.65*10^{9}$ (9.82) |
| Pyrethrum | 4/22 | 1.00 | $6.94*10^{8}$ (8.84) |
| *LSD (F probability)* | | *0.167 (<0.001)* | *0.695 (<0.001)* |

Field observations of changes in *Spongospora subterranea* soil inoculum following rotation crops

The mean of log *S. subterranea* soil DNA levels measured at four marked plot locations prior to, during and after potato cropping and after a subsequent opium poppy crop varied significantly ($P<0.001$). At potato planting DNA levels were 6586 pg/g (mean log 3.81) on average at the sample site. This is considered a high disease risk level for subsequent potato cropping (Ophel Keller et al. 2009). Levels increased slightly, but not statistically significantly to 9311 pg/g (3.95) by mid-growth stage of the potato crop. At harvest the mean DNA levels were 16,964 pg/g (4.18) which was significantly greater than both the mid-growth and the initial inoculum levels. Following the subsequent opium poppy crop, S. *subterranea* soil inoculum levels had risen further to 37,893 pg/g (4.53), which was significantly greater than the values following potato harvest.

## Program 3: Assessment of potato germplasm for resistance and tolerance to Spongospora root infection

**Cultivar resistance testing** -Two glasshouse, two mini-plot trials and one field trial compared 15 varieties for resistance to root infection (for full results see Appendix 2a).

**Cultivar resistance trials** - Numbers of cultivars examined in each experiment varied based on availability of suitable quantities of tuber material. Root infection (incl. qPCR quantitation of pathogen), root galling and tuber infection from the glasshouse, mini-plot and field trial was assessed at multiple harvest dates and summary data provided (Table 6). Essentially, while there was a degree of variability in DNA levels quantified and root infection levels and galling in separate experiments (see typical results in Table 7, Fig 4, Appendix 2a) the same cultivars seemed to group with each other as to whether they showed any *Spongospora* resistance. Looking at Table 6 and the colour traffic signal guide it can be seen that Atlantic, Shepody, Desiree, AG and AE were often rated in the red category indicating poor levels of resistance. Russet Burbank, Ranger Russet, AI, Bondi, AF and AH were generally in the amber (orange category) showed intermediate resistance. AA, AB, AC and AD generally performed in the amber and green category (compared to other lines) so are showing a degree of resistance, although no cultivar demonstrated complete resistance.

**Table 6.** Summary of the relative resistance or tolerance of important processing potato varieties to root and tuber infection.

| Cultivar | Field trial | | | Glasshouse1 | | Glasshouse2 | | Mini Plot1 | | Mini Plot2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Root infection | | Tuber infection | Root infection | | Root infection | | root | tuber | Root infection | |
| | DNA | gall | | DNA | Galling | DNA | Galling | DNA | | DNA | galling |
| AA | | | | | | | | | | | |
| AB | | | | | | | | | | | |
| AC | | | | | | | | | | | |
| AD | | | | | | | | | | | |
| AE | | | | | | | | | | | |
| Bondi | | | | | | | | | | | |
| | | | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AF | | | | | | | | | | | |
| AG | | | | | | | | | | | |
| AH | | | | | | | | | | | |
| AI | | | | | | | | | | | |
| | | | | | | | | | | | |
| Russet Burbank | | | | | | | | | | | |
| Ranger Russet | | | | | | | | | | | |
| Desiree | | | | | | | | | | | |
| Shepody | | | | | | | | | | | |
| Atlantic | | | | | | | | | | | |
| | | | | | | | | | | | |

Nil or very low infection, Moderate infection, High infection

Below we present the results from the field trial:

**Field Trial.** The soilborne levels of *S. subterranea* were very high in this commercial paddock (>5000 pg S*. subterranea* DNA/g soil) resulting in both root and tuber disease in most cultivars tested (Table 7). Only one cultivar tested was shown to produce no galling, visible zoosporangia or powdery scab tuber infection (AA). Some industry standards included in the trial (Desiree, Shepody and Atlantic) and one of the commercial lines (AG) showed very significant to severe galling. Russet Burbank, Ranger Russet and AH produced moderate galling that was more severe, although not significantly so, than many of the newer lines tested. The zoosporangial scores recorded were generally very low and this may be related to the selection of suitable root tissue from the field and subsequent sub-sampling. Related DNA levels (Fig 4) show a general gradient of increasing DNA with time, however there was a few exceptions with Shepody, Ranger Russet and AD showing a decrease between sampling 2 and 3. This may be reflective of the sampling technique, perhaps with less viable roots and zoosporangia in the older roots. In general, those cultivars producing low root infection generally corresponded with lower DNA levels extracted.

Tuber infection was very high in some varieties with greater than 50% incidence for Shepody, Desiree, AE and Atlantic (Table 14). Bondi, AG, Russet Burbank and Ranger Russet also produce >10% incidence. Some varieties (AA, AD and AH) produce no tuber symptoms.

**Table 7.** Root infection response of fifteen cultivars grown in the field (FT) with high levels of S. subterranea. Shown are root galling assessed at 95 days after plant emergence, zoosporangial scores at 45, 75 and 95 days after emergence, and tuber disease severity at plant senescence.

| Cultivar | Number root galls[1] | Root gall severity[2] | Zoosporangia[3] | | | Tuber infection % | Average severity[4] |
|---|---|---|---|---|---|---|---|
| | | | DAE | | | | |
| | | | 45 | 75 | 95 | | |
| AA | 0 c | 0 h | 0 | 0 | 0 | 0 e | 0.00 d |
| AB | 5 c | 1.50 fg | 0 | 0 | 0 | 5.6 e | 0.04 d |
| AC | 2.5 c | 1.33 fg | 0 | 1.1 | 0 | 1.1 e | 0.01 d |
| AD | 4.3 c | 1.33 fg | 0 | 0 | 0 | 0 e | 0.00 d |
| AE | 5.7 c | 1.67 efg | 1.1 | 0 | 0 | 63.3 abc | 1.38 b |
| Bondi | 4.7 c | 1.33 fg | 0 | 0 | 0 | 24.6 cde | 0.22 d |
| AF | 3.0 c | 0.83 gh | 0 | 0 | 0 | 3.3 e | 0.04 d |
| AG | 44.0 a | 4.00 a | 0 | 0.9 | 0 | 22.2 cde | 0.27 cd |
| AH | 8.3 c | 2.33 cdef | 0 | 0 | 0 | 0 e | 0.00 d |
| AI | 4.7 c | 1.33 fg | 0 | 0 | 0 | 6.7 e | 0.11 d |
| Russet Burbank | 11.7 c | 2.67 bcde | 1.1 | 0 | 0 | 16.7 de | 0.21 cd |
| Ranger Russet | 9.0 c | 2.33 cdef | 1.0 | 0.9 | 0 | 12.2 e | 0.13 d |
| Desiree | 24.3 b | 3.17 abc | 0 | 0 | 0.5 | 67.8 ab | 0.84 bc |
| Shepody | 37.3 ab | 3.67 ab | 1.2 | 1.8 | 0 | 98.9 a | 2.60 a |
| Atlantic | 30.0 b | 2.83 bcd | 0 | 0.8 | 0 | 56.7 bcd | 0.99 b |
| LSD | 13.50 | 1.08 | | | | 41.1 | 0.66 |
| F prob (0.05) | <0.001 | <0.001 | | | | <0.001 | <0.001 |

[1] Number of root galls per plant. [2] Root gall severity score used a rating scale of 0-4 (Van De Graaf et al., 2007). [3]Zoosporangia score rating score 0-4. [4]Tuber severity score rating score 0-6. ns non-significant at P = 0.05. DAE = days after emergence. Means followed by the same letter within the same column are not significantly different at $P$ = 0.05 using Fisher's LSD test.

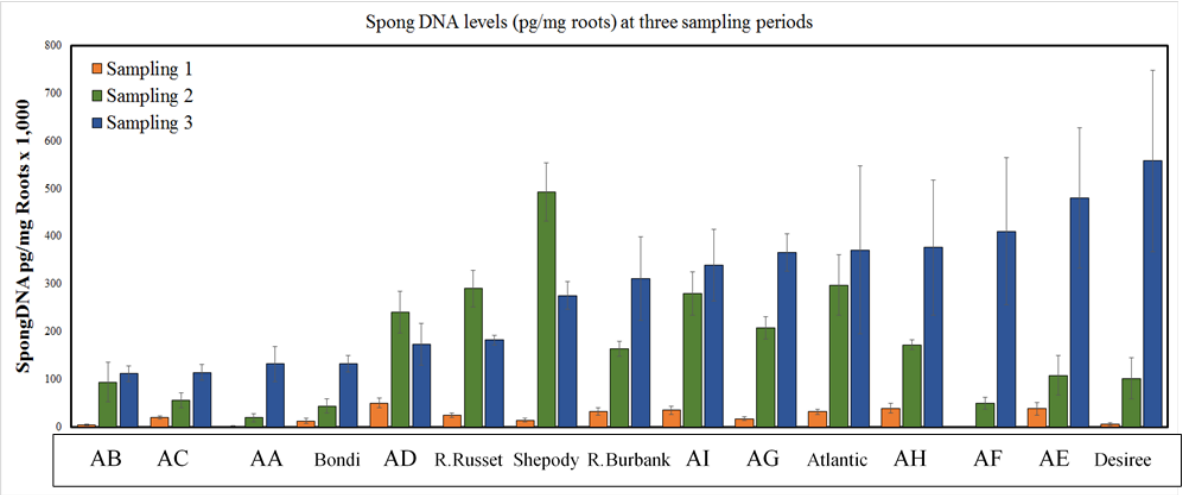


**Fig. 4.** Three consecutive samplings (45, 75 and 95 DAE) of 15 cultivars in a field trial for detection of *S. subterranea* from root tissue.

Root infection assessment methods including zoosporangial infection and pathogen DNA quantification provided a variable assessment of potential resistance. While the assays were useful, the impact of other factors (root age, sampling region) can lead to inherent variability.

**A better methodology for cultivar screening (see Appendix 2b)**

The glasshouse and field trials used typical inoculum challenge methodologies growing plants in soils either artificially or naturally infested with pathogen inoculum, and monitoring root infection rates by qPCR, microscopy and direct observation of galling (and tuber disease) incidence. Inherent variability in such glasshouse and field trials exists due to emergence and growth differences between cultivars, environmental factors between trials, distribution of pathogen inoculum within the soil, variation in onset of infection, and capacity to sample only a small segment of root tissues makes these traditional trials difficult, and potentially unreliable. Also, due to the polycyclic nature of root infection timing of the measurement of root infection is critical and direct comparisons within each experiment only are possible highlighting the need for known standards to be included in each trial.

These trials also are time and resource consuming requiring months of growth of potato plants with sufficient replication to allow for variable inoculum distribution.

We developed a new *in vitro* assay that focusses on the zoospores binding to and encysting on host roots (Fig 5) which is: a) much more rapid with results within 1 week, b) more robust with controlled inoculation conditions, c) requires much less resources (plants, land, time, labour, machinery), d) not time dependent and not influenced by polycyclic infection cycles.

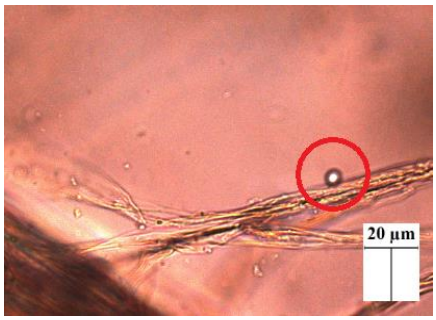


**Fig 5: Zoospore encysment on potato root**

## Program 4: Chemotaxis study – disruption of zoospore root interactions

Here we studied the plant chemicals released by roots (root exudates) that have an effect on resting spore germination and zoospore attraction to roots.

**Potato root exudates and resting spore germination (see Appendix 1c for full results)**

***Spongospora subterranea* Zoospore Identity.** Motile biflagellate (of unequal length) zoospores, of approximately 4-5 µm diameter, were observed in the majority of test potato root exudates and occasionally in control (sterile deionized water) solution. Appendix 1c shows that zoosporangia, indicative of *S. subterranea* infection, developed when test solutions with *S. subterranea* zoospores were exposed to tomato roots. *Similarly zoospores were observed attached to potato roots with a greater number of zoospores (P=0.008) attached to roots of the susceptible cv. 'Iwa' (16.67 ± 2.60) than the resistant cv. 'Gladiator' (3.67 ± 0.33; Appendix1c).*

**Stimulatory Effects of Potato Root Exudates to Resting Spore Germination**. In the first experiment, nine separate assays examined the impact of exposure to a range of potato root exudates on resting spore germination. Exposure to 25 of the 32 potato root exudates stimulated quicker (average first release – 4.4 days) and a greater number of zoospores released compared to the control water solution (average first release – 11.8 days) (Appendix 1c).

In the second experiment four cultivars with varying known resistance to *Spongospora* were tested. The interaction of incubation period and cultivar had a clear statistically significant effect (*P=0.033*), with differences between cultivar most evident in the 18 d incubation samples (Figure 6). This was best exemplified by the susceptible cultivar Iwa, whose exudates stimulated a significantly higher number of zoospores to release compared to a resistant variety such as Gladiator.

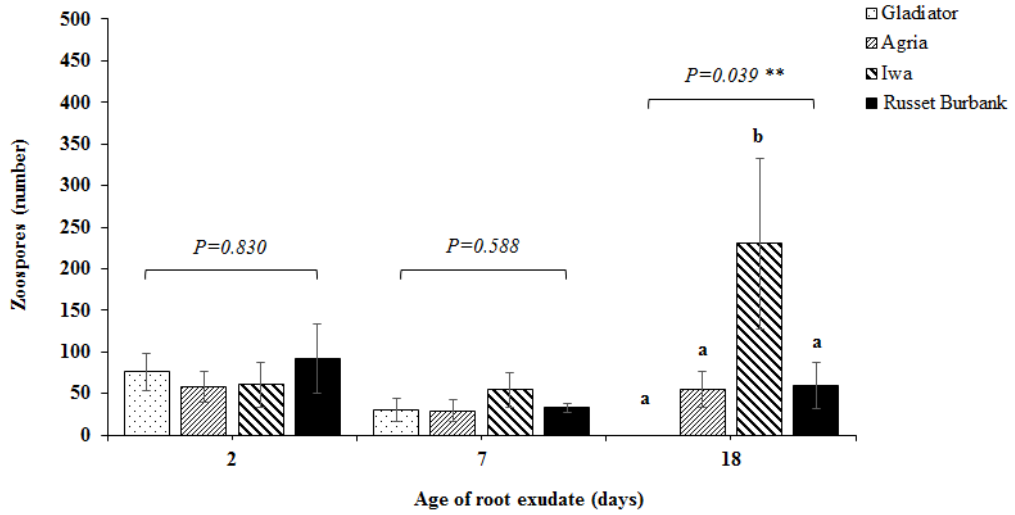


**Figure 6.** Resting spore germination (zoospore release) of *Spongospora subterranea* as influenced by the age (2, 7, and 18 d old) of potato root exudates. Vertical bars are standard error (*n*=6). Double asterisk indicates that treatment means, within a group (indicated by a horizontal bar), are statistically different by analysis of variance. Bars, within the 18 d old group, annotated with the same letter are not statistically different (*p<0.05*; Fisher's LSD test). *P* (incubation time) = 0.152; *P* (cultivar) = 0.050; *P* (incubation time x cultivar) = 0.033.

**Metabolite Constituents of Various Potato Root Exudates.** The HILIC UPLC-MS analysis detected a total of 24 LMWO compounds from potato root exudates (Appendix 1c) including eight amino acids, one sugar, three sugar alcohols, five organic acids and seven other organic compounds. Detection of these compounds varied among root exudates. Asparagine, glutamic acid, glutamine, proline, serine, pinitol, choline, trehalose and tyramine were detected in most of the potato root exudates. However, some compounds were uniquely observed in a particular potato cultivar root exudates or collection date. The hierarchical cluster analysis revealed that potato root exudates could be divided at 80% similarity into three groups based on the metabolite composition (Appendix 1c). Cluster 1 included all 'Iwa' root exudates, cluster 2 was composed of mostly 2 and 7 d incubation and all 'Russet Burbank' root exudates, and cluster 3 were mostly 18 d incubation and 'Gladiator' root exudates.

**Screening of Metabolites for Stimulants of Resting Spore Germination.** Testing individual compounds provided a more robust direct relationship of each compound to resting spore germination stimulation. Stimulation of germination of *S. subterranea* resting spores was chemical-specific (Appendix 1c). By comparing capacity and timing of zoospore release it was determined that five of the 18 compounds found in potato root exudates and seven of the 25 additional compounds tested were stimulant at 0.1 mg/ml, resulting in zoospore release at least 8 d earlier than the water control. L-Glutamine and tyramine had greater effects (*P=0.045* and *P=0.010*, respectively) on resting spore germination than the other compounds tested and the Hoagland's stimulant control. Mean accumulated zoospore numbers in other stimulant LMWO compounds, L-rhamnose, cellobiose, L-aspartic acid, *N*-acetyl cysteine, piperazine, glucoronic acid, L-serine, succinate, L-citrulline and citric acid, were not statistically different from the non-stimulant water control (*P*>0.05) but were deemed stimulant as zoospore release in the presence of these compounds was at least 8 d earlier than in the deionized water control (Figure 7).

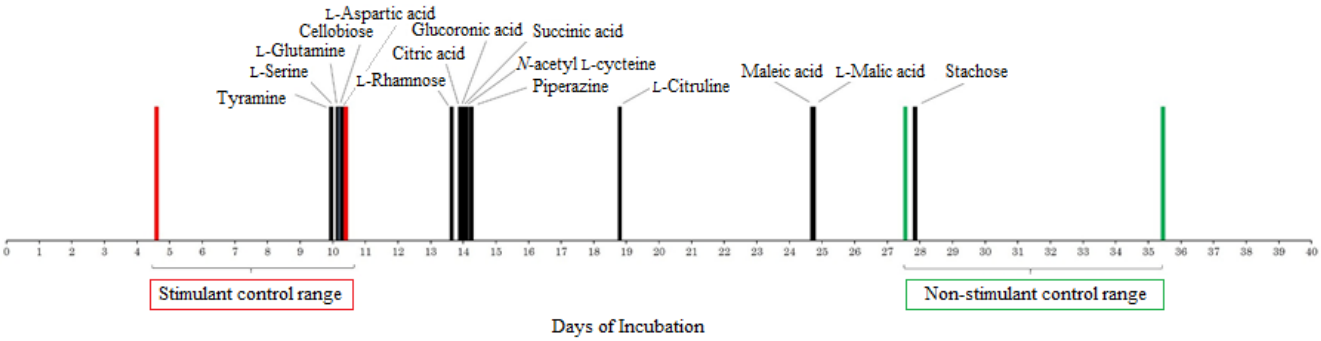


**Figure 7.** Days to initial *S. subterranea* resting spore germination (zoospore release) in low-molecular weight organic compound solutions (0.1 mg/ml), control (deionized water)

**Plant and environmental effects on potato root exudation and resting spore germination (see Appendix 1e for full results)**

Effect of Plant Physiological Conditions on Root Exudation

The potato plant's physiological conditions affected the number of compounds released by potato roots: a total of 24 compounds were detected which included amino acids (12), sugar alcohols (1), sugars (9), organic acids (3) and other LMWO compounds (12). Varying plant age produced no clear trend. Two month-old plants released more compounds than younger or older plants. Trimmed roots released more sugars than intact roots. Plants with stronger (larger) roots released more compounds in total than plants with weaker (smaller) root systems, however, the numbers of sugars released by the strong and weak plants did not differ. Cultivars also varied in their exudate metabolite profiles with Shepody releasing twice as many compounds as other cultivars (Appendix 1e).

Effect of the Environmental Conditions on Root Exudation

Environmental conditions during the plant's growth influenced the release of LMWO compounds in root exudates (Appendix 1e). The total number of compounds released in root exudates from plants grown in the dark were comparable to those receiving 16 hours of light. The release of LMWO compounds in plants which received additional nutrition (Hoagland's solution) was greatly suppressed. The plants with HS-supplementation produced seven times less LMWO compounds in their root exudates than the no nutrient supplemented plants, with substantially less amino acids, and no sugars, organic acids and other organic compounds detected. The number (density) of plants within the collection tube did not influence the number of LMWO compounds in the root exudates. Temperature showed no clear trend with the release of LMWO compounds (12 compounds) being greatest when plants were grown at 10, 24 and 30 °C, than at 4 and 15 °C (8).

Effect of Physiological and Environmental Factors on the Release of RSG-stimulants

Of the 24 LMWO compounds detected, 15 had been previously tested for *S. subterranea* RSG capacity (Balendres et al., 2016a), and of these only four (citruline, glutamine, rhamnose and tyramine) were identified as RSG stimulants (Appendix 1e). Various plant and environmental factors were found to affect release of these known RSG-stimulant compounds. Notably the stronger root systems were associated with three of the four RSG stimulants whilst weaker roots had none but otherwise there were no other obvious associations with any specific treatments (Appendix 1e).

**"Germinate to exterminate" approach to inoculum management** (Appendix 1f).

**Effects on pathogen inoculum of soil treatments with resting spore stimulants in absence of host plants.**

Two pot trials examined the impact of resting spore stimulants on pathogen DNA level.

Pre-treatment assays for pathogen DNA content revealed generally consistent amounts between each treatment within each trial. Trial 2 had a greater initial pathogen inoculum amount than trial 1 (Figure 8). HS and Fe-EDTA treatments (at 0.05 mM and 10 mM) applied to infested soil reduced *S. subterranea* DNA levels compared to the water only and dry control treatments in both glasshouse trials (Figure 12). In the first trial, the percentage reductions of *S. subterranea* DNA for each treatment were: Water control, 4%, Dry control, 59%, Fe-EDTA 10 mM, 83%, Fe-EDTA 0.05 mM, 88%, and HS, 89%. Confirmatory results from an independent laboratory (SARDI) supported these data estimating an 89% reduction in *S. subterranea* DNA levels with the Fe EDTA 10 mM treatment.

The extent of pathogen reduction in the second trial was less than the first trial, but the same treatment pattern of effects were observed (Figure 8). The percentage reduction of *S. subterranea* DNA for each treatment was: Water control, 17%, Dry control, 18%, HS, 44%, Fe-EDTA 10 mM, 54%, and Fe-EDTA 10 mM commercial formulation, 63%. The formulation (analytical grade or commercial) of Fe-EDTA did not significantly affect the reduction of amount of pathogen DNA (Figure 8).

Treatment "Dry" indicates the soils were not moistened. Initial (pre-treatment) and final (post-treatment) DNA concentration quantitation was carried out, respectively, the day before application of chemical treatments, and 36 d from first treatment. Verticals bars are standard errors of means.

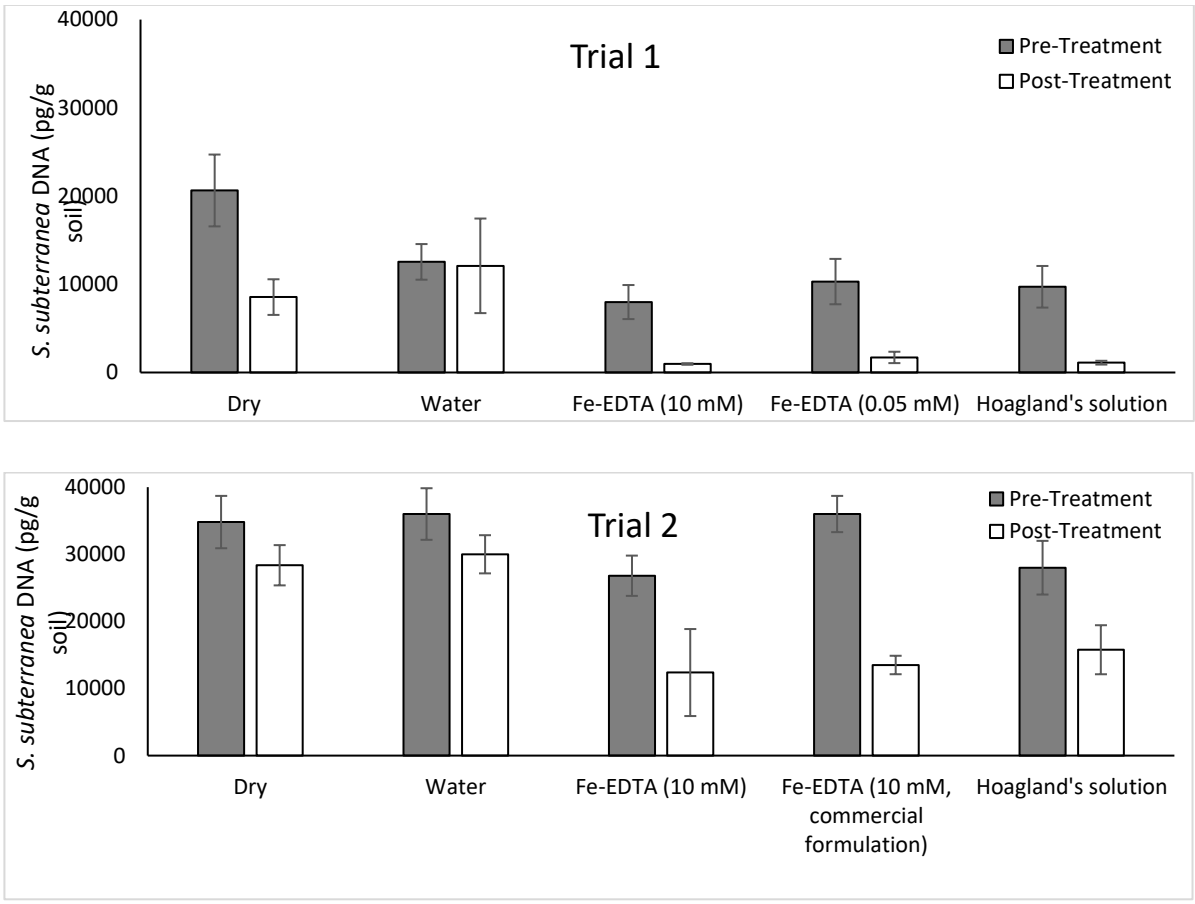


**Figure 8.** The impacts (in two trials) of soil treatment with resting spore germination stimulants on *Spongospora subterranea* DNA concentrations. Each treatment was applied to the soil six times within 34 d.

As well as this Project final report we have produced a number of industry and academic outputs including:

## Industry Communication

### Industry articles

We have provided the following technical articles to aid in the communication of the project outcomes to the industry:

- Potatoes Australia (Feb/March, 2018). Developing an understanding of soil inoculum persistence and management underway in Tassie. Page 8.

https://ausveg.com.au/app/uploads/publications/Potatoes-Australia_February-March-2018_Web.pdf

- Potatoes Australia (Aug/Sept 2018) Exploring effective management of Spongospora diseases. Page 28.

https://ausveg.com.au/app/uploads/publications/Potatoes-Australia_August-September-2018_Web.pdf

### Industry presentations

We presented on project progress and outcomes to industry partners by:

- Sharing of milestone reports and interim project reports with project partners
- Meetings with grower groups (NW region – Sisters Creek – region with v. high soil inoculum levels of Spongospora) which led to collaborative field trials testing cultivars, fungicides and hormonal sprays for controlling Spongospora root infection.
- Mid-project update to industry partners on 25th July, 2016 at Elizabeth Town, NW coast Tasmania.
- Presentation on Spongospora at the TIA potato industry presentation day at Forthside on Nov 14th 2017 https://www.youtube.com/watch?v=ryHPrvWcF8o&t=751s
- Presentations of final project outcomes at:
    - Potato Industry conference (Melbourne Arts Centre, Victoria, Aug 12-14)
    - Tasmanian (Serve-Ag) Potato Research day (Devonport, Tasmania, Aug 22)

The two presentations above featured similar presentations with the slides shown in Appendix 5.

## Scientific Outputs

We produced several scientific articles which are directly attributable to this project that are listed under the Refereed scientific publications section.

# Outcomes

1) An improved fundamental understanding of the Spongospora root infection process

**Measure:** Advances in our understanding of Spongospora disease will be measured through publication in peer reviewed scientific journals and conference papers by project completion.

**Outcome:** 6 refereed journal articles have been accepted in peer reviewed scientific journals, including a review article. This information has provided improved basic fundamental understanding in the key areas: novel detection and mitigation strategies on tools for pathogen detection, resting spore stimulation, root encystment and resting spore dormancy.

Key findings have been provided to industry at presentation days and in Potatoes Australia articles.

2) A root pathogen (quantitative DNA) detection tool for assessment of the efficacy of management strategies.

**Measure:** a quantitative DNA assessment research tool to accurately monitor pathogen content in roots developed by the end of year 1.

**Outcome:** a new qPCR technique was developed and optimized in the initial stages of the project which was able to track the progression of pathogen multiplication and therefore measure impact of potential controls including fungicides and new varieties.

3) Assessment of the efficacy and optimal application of traditional and novel control treatments to slow or reduce root infection, and the influence of environmental parameters.

**Measure:** The influence of a wide range of environmental parameters, and control strategies on root infection process determined. The efficacy of manipulating these parameters and controls to reduce disease determined by project completion.

**Outcome:** The qPCR assay (developed above) was able to quantify the total amount and rate of pathogen multiplication in order to identify successful treatment options (e.g. fluazinam) demonstrating the applicability of the assay. The assay was also used to quantify the relative resistance of a range of important processing cultivars belonging to the industry partners, although further assays (root encystment) have also been developed to complement the qPCR assay. Environmental conditions, particularly higher temperatures were shown to reduce disease progression. Evaluation of multiple field sites correlated levels of soilborne *Spongospora* with root infection and galling. We demonstrated that any treatments that delay initial onset of root infection (reduced soil inoculum, chemical treatments, host resistance) will delay or slow the root infection cycle leading to reduced root infection, reduced impact on plant growth, and reduced subsequent tuber disease.

4) A detailed description of the pathogen alternate host range (common weeds and alternate crops) and the relative resistance or tolerance of important processing potato varieties to root infection.

**Measure:** capacity of common crop weeds and alternate species to host the pathogen determined by DNA testing. Relative resistance of important processing potato cultivars to

root infection determined by project completion.

**Outcome:** We identified common weeds and select rotation crop plants that can host *S. subterranea*. None were as efficient as potato (including volunteers) at maintaining and increasing soil pathogen levels. This suggested potato volunteers are critical inoculum maintenance hosts and increased efforts at volunteer management are warranted. Blackberry nightshade (*Solanum nigrum*) was the most prevalent alternative weed host infection identified in cropping regions. Importantly, both opium poppy and pyrethrum were identified as alternative hosts. Whilst these were both less susceptible than potato, given the density of these plants when cropped, there host status is significant, and soil testing following a commercial poppy crop showed greatly elevated levels of *S. subterranea* inoculum.

Glasshouse and field trials tested current and potential processing cultivars selected by the industry partners and identified root infection resistance levels ranging from highly susceptible through to moderate resistance. Assays including root zoosporangial scores, root galling and root pathogen levels were useful measures of resistance. A novel new zoospore root encystment assay, developed in the later stages of the project, appeared to be a cheaper, quicker and equally, if not more robust measure of quantifying root resistance. This work identified root encystment efficiency as a key driver of host resistance.

5) Development of novel controls for manipulation of soil-borne inoculum and interfering with root infection processes through disruption of zoospore release and attraction.

**Measure:** Ability to disrupt chemotaxic responses important for zoospore release and root infection determined. Recommended treatments for effective disease control determined by project completion.

**Outcome:** We utilized a series of laboratory and glasshouse trials to demonstrate 'proof of concept' for the use of resting spore germination stimulants (e.g. Fe-EDTA) in absence of suitable host plants, to substantially deplete soil pathogen inoculum. This has provided a unique management opportunity between potato rotations to rehabilitate cropping soil through inoculum reduction strategies. We further showed that a combination of these approaches with fungicide treatments (e.g. fluazinam) may provide additional benefits.

6) Development of a recommended package of integrated disease control options optimised for efficacy.

**Measure:** Recommendations for optimal control treatments identified and distributed to industry by project completion:

Potatoes Australia articles:

https://ausveg.com.au/app/uploads/publications/Potatoes-Australia_August-September-2018_Web.pdf

and Youtube clip: https://www.youtube.com/watch?v=ryHPrvWcF8o&t=751s)

**Outcome:** We provided some disease control recommendations and future research direction priorities in the Recommendations section (see below). Presentations, meetings with industry partners have been regularly provided to industry partners. Key findings have also been made at presentation days and will be summarized in an August 2018 Potatoes Australia article.

# Monitoring and evaluation

This project was developed prior to the requirement to develop an M&E Plan. Nevertheless, this project has delivered against a number of key criteria typical of most research projects.

Overall, the project has delivered on the key criteria established during the planning stage of this project. A range of outputs and outcomes have been met as described above.

Key Evaluation questions (KEQ's) for the project are provided below with an indication of whether project expectations were met:

What has been the impact and/or outcome of the project?

- To what extent has the project contributed to increased knowledge
- To what extent has the project contributed to the goals of the stakeholders and Hort Innovation

Fundamental knowledge gain has been progressed substantially through peer-reviewed publication and regular meetings with project stakeholders. The fundamental knowledge gained has been utilized to develop novel and putative disease control options (e.g. resting spore stimulants (such as Fe-EDTA) to deplete soilborne pathogen levels), identify new hosts within the cropping rotation (e.g. poppy) and develop new (qPCR) and improved assays (root encystment) for quantifying management options (e.g. fungicide treatment, new cultivars). This improved understanding of the pathogen provides further insight into management options for this disease – a key goal of project stakeholders and Hort innovation.

The project has contributed significantly to the stakeholders and Hort Innovation by also providing the production of potential recommended projects or research areas of importance that will aid in combating *Spongospora* beyond this project.

To what extent has the project met participation objectives?

- How effectively has the project shared its findings with stakeholders and the broader agricultural community
- Did the project achieve its outcomes measured or otherwise

Communication with key stakeholders has been effective with frequent meetings and discussion of research ideas. Informal discussions and presentations at SIAP meetings have also enabled the sharing of findings with industry.

The broader industry has been provided with two summary articles in Potatoes Australia and a summary of key concepts/outcomes by oral presentation which is also available on-line https://www.youtube.com/watch?v=ryHPrvWcF8o&t=751s

As stated above, this project has meet key outcomes of increasing fundamental knowledge and unlocking putative control and management options.

How effectively was the project delivered?

- Timeliness, within budget
- What impacted on these?

The project has been undertaken on budget. The timeline for this project was extended by one-year with no impact on budget. The extension of the timeframe was due to the requirement to wait for and then receive and bulk up the key cultivar lines to be tested, provided by the two companies. This had negligible impact on project outcomes and actually allowed for publications to be completed prior to project end.

What resources have been used to run the project?

- How could the project have been run more efficiently
- What other resources, in-kind or otherwise were used to meet the project outcomes

Efficiency would have been improved with the supply of a larger number of the key industry potato lines earlier in the project – this may have enabled more pot and field trials to be conducted on cultivar resistance. Nevertheless, support from industry was very good and appreciated with the identification of grower sites for conducting fungicide, inoculum reduction testing with germination stimulants and cultivar assessment. Also industry (Frank Mulcahy, Simplot) supplied soilborne pathogen data from a poppy field trial which aided in testing hypotheses and subsequent publication. The stakeholders also provided their time frequently for informal discussions on research progress.

Other in-kind support has been provided by the University of Tasmania to provide and fund undergraduate and postgraduate students working on Spongospora. This included scholarship support and some financial assistance with research materials.

# Recommendations

Potato diseases induced by infection with *Spongospora subterranea* are complex, and their study complicated by the inability to culture the pathogen on artificial medium and its subterranean (underground) infection court.

It is clear the project has identified several significant opportunities for improved disease management, but there remains further work to refine and add to these tools to develop a robust and sustainable management system for all of Australian production. The project has provided data showing the promise of substantial disease control with novel approaches, which require further work for commercial adoption (registration data, refinement and field testing etc.). It has identified key research areas for further investment that show strong promise as inoculum management and disease mitigation tools

Some of the key findings for:

**Immediate use/adoption by industry:**

**Monitoring root infection in roots**. qPCR detection methodologies developed within this project are useful to identify early onset of infection and how delaying infection (through fungicide treatments and/or delayed host – pathogen contact) can alter the overall progression of the disease epidemic. This provides a useful research tool to test chemical efficacy and new cultivars and complements direct microscopic examination of root infection.

**Root encystment assay.** The new root encystment assay developed in this project offers much greater control and a more robust assessment methodology for identifying root infection response.

**Resistant varieties.** This project has identified varieties with enhanced (but not complete) resistance to root infection and galling. These varieties (owned by the co-investment partners) may have the potential to be used by growers in the near future and confirm cultivar choice as an important management tool.

**Alternate hosts (weeds and cropping species).** Weed control and volunteer potato control are essential for effective rotation with the weed species including blackberry nightshade, sow bane, sow thistle, hedge mustard and clustered dock noted to increase and/or sustain soilborne *Spongospora* levels. Additionally, the crop species, opium poppy and pyrethrum, were shown to partially support *S. subterranea*. Consideration of rotation scheduling should be given to avoid crops prior to potato exacerbating inoculum levels. Likewise, vigilant weed control, with a key focus on blackberry nightshade and volunteer potatoes is essential.

**Future research priorities:**

**Root encystment and understanding mechanisms of resistance**

While optimization of the root encystment assay may provide further enhancement of this assay also importantly it may enable the identification of key mechanistic areas of resistance to root encystment which may provide novel targets for future breeding, cultivar selection and disease mitigation tools that will be an important future research goal.

**Alternate hosts (weeds and cropping species)**

While preliminary studies identified the potential of poppy to host Spongospora further studies evaluating pathogen population dynamics following these crops is warranted to determine if (as one observation with a commercial poppy crop showed) these crops can maintain or even enhance pathogen soil inoculum levels. Additionally, the role of volunteers in increasing soil inoculum levels needs further study and specifically how vigilant volunteer control (whether through grazing, chemicals or tillage) can reduce soil inoculum levels. Scientific studies demonstrating this over the long term are warranted.

**Reducing soil borne inoculum.** The 'germinate to exterminate' approach is an area worth pursuing with further basic and applied studies. Of note, whilst increased germination and subsequent inoculum reduction will occur not all resting spores immediately respond to the stimulants. Studies to better understand resting spore dormancy have been and continue to be critical to this work. Further work is required to adapt this system to the field, but the promise of pathogen amelioration is very attractive.

We also believe these compounds may be able to improve the efficacy of fungicides which only partially control root and powdery scab disease due to inactivity against dormant resting spores.

**Enhanced root growth to mitigate against root disease.** Optimising plant growth and root function is essential when a pathogen attack by *Spongospora* is imminent. Sub-lethal levels of foliar applied PGR's were able to increase root mass in young plants, increase tuber yields and decrease powdery scab disease.

While these results show promise, other studies indicate that the PGR tested applied at higher rates can have detrimental effects. For example, it can result in foliar damage (Thorton et al. 2013, 2014), grade out from powdery scab (Waterer, 2010), and can reduce tuber size in stored potatoes (Thorton et al 2013). Thompson et al. (2014) emphasise that optimizing rate and timing of application is critical to avoid reduced yield, phytotoxic damage and residues. Further field testing is on-going, both within Tasmania and Internationally. Such treatments require full or off-label registration and commercial trials are required before formal recommendations could be made.

**Disease mitigation through a more robust root system**.

- Can root and stolon development be manipulated to compensate for root damage, and what effect would such treatments have on pathogen infectivity and soil inoculum accumulation?
- Can mycorrhizal and other microbial inoculants be beneficial for plant productivity and disease suppression?
- Improving the efficiency and reliability of disease control.
- What is the impact on soil health of commonly used prophylactic fungicides and can these be mitigated by strategic microbial inoculant use?
- Can we improve the efficacy of soil-applied fluazinam for S. subterranea management?
- Can the "germinate to exterminate" approach to S. subterranea soil inoculum management be optimised for commercial application?
- Can we disrupt S. subterranea zoospore root attraction and prevent infection?
- Host:pathogen interactions

- How does differing soil-borne pathogens interact to influence infection rate and host resistance? (e.g. root lesion nematode infections promoting Spongsopora root infections)
- What are the plant factors that encourage S. subterranea zoospores root encystment and can these be used to select for resistance or blocked?
- What plant factors promote gall formation and subsequent production of soil inoculum, and can this be inhibited?

# Refereed scientific publications

**Refereed journal articles published (also appended)**

Clark, T.J., Rockliff, L.A., Tegg, R.S., Balendres, M.A., Amponsah, J., Thangavel, T., Mulcahy, F., Wilson, A.J., Wilson, C.R. 2018. Susceptibility of opium poppy and pyrethrum to root infection by *Spongospora subterranea. Journal of Phytopathology* (in press). (Appendix 1g)

Balendres, M.A., Clark, T.J., Tegg, R.S., Wilson, C.R. 2018. Germinate to exterminate: chemical stimulation of Spongospora subterranea resting spore germination and its potential to diminish soil inoculum. *Plant Pathol*ogy **67**, 902-908. (Appendix 1f)

Balendres, M.A., Nichols, D.S., Tegg, R.S., Wilson, C.R. 2017. Potato root exudation and release of *Spongospora subterranea* resting spore germination stimulants are affected by plant and environmental conditions. *Journal of Phytopathology* **165**, 64-72. (Appendix 1e)

Balendres, M.A., Tegg, R.S., Wilson, C.R. 2017. Resting spore dormancy and infectivity characteristics of the potato powdery scab pathogen *Spongospora subterranea. Journal of Phytopathology* **165,** 323-330. (Appendix 1d)

Balendres, M.A., Nichols, D.S., Tegg, R.S., Wilson, C.R. 2016. Metabolomes of potato root exudates: compounds that stimulate resting spore germination of the soil-borne pathogen *Spongospora subterranea*. *Journal of Agriculture and Food Chemistry* **64,** 7466-7474. (Appendix 1c)

Balendres, M.A., Tegg, R.S. Wilson, C.R. 2016. Key events in pathogenesis of Spongospora diseases in potato: a review. *Australasian Plant Pathololog*y **45,** 229-240. (Appendix 1b)

Thangavel, T., Tegg, R., Wilson, C. 2015. Monitoring *Spongospora subterranea* development in potato roots reveals distinct infection patterns and enables efficient assessment of disease control methods. *Plos One* **10,** e0137647. doi:10.1371/ journal.p (Appendix 1a)

**Refereed conference papers**

Wilson, C.R., Balendres, M.A., Amponsah, J., Nichols, D.A., Tegg, R.S. 2018. Understanding the role of root exudation for pathogen germination and attraction, and their application for disease control. International Congress of Plant Pathology, Boston, USA.

Balendres, M.A., Nichols, D.S., Tegg, R.S., Wilson, C.R. 2014. Potato root exudates stimulate zoospore release of Spongospora subterranea. 8th Australasian Soilborne Diseases Symposium 10-13th Nov, Hobart Tasmania, p10.

Balendres, M.A., Nichols, D.S., Tegg, R.S., Wilson, C.R. 2014. Somaclonal selection for enhanced resistance to Spongospora root infection and studies on zoospore release. 2nd International Powdery Scab Workshop July29-Aug1, Pretoria, South Africa, 28pp.

**Research Training Outcomes**

***Postgraduate***

Balendres, M.A. 2017. Biology and Chemical ecology of *Spongospora subterranea.* PhD Thesis, Univ. Tasmania.

Waqas Md. 2016. Variety screening against root infection of potato caused by Spongospora subterranea. Masters by Coursework Thesis, Univ. Tasmania.

***Undergraduate***

Clark, T.J. 2016. *S. subterranea* – resistance, hosts, & management. Honours Thesis, Univ. Tasmania.

Rockliff, L.A. 2015. Optimising novel tuber treatments for common and powdery scab control and alternate host range for *Spongospora subterranea*. Honours Thesis, Univ. Tasmania.

***Training scholarship***

Tan, M.L. 2018. Method for screening Spongospora subterranea resistance in potato cultivars using zoospore encystment in roots. Deans Summer Research Scholarship award.

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Balendres, M. A., Tegg, R. S., & Wilson, C. R. 2017. Resting spore dormancy and infectivity characteristics of the potato powdery scab pathogen *Spongospora subterranea*. Journal of Phytopathology 165, 323-330.

Balendres, M.A., Tegg, R.S. Wilson, C.R. 2016. Key events in pathogenesis of Spongospora diseases in potato: a review. *Australasian Plant Pathology* **45,** 229-240.

Falloon, R., Merz, U., Butler, R., Curtin, D., Lister, R., & Thomas, S. 2016. Root infection of potato by *Spongospora subterranea*: knowledge review and evidence for decreased plant productivity. Plant Pathology 65, 422-434.

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Thangavel, T., Tegg, R. S., & Wilson, C. R. 2015. Monitoring *Spongospora subterranea* development in potato roots reveals distinct infection patterns and enables efficient assessment of disease control methods. PLoS ONE 10(9), e0137647. Doi:10.1371/journal.pone.0137647.

Thompson, H.K., Tegg, R.S., Corkrey, R. & Wilson, C.R. 2014. Optimal rates of PRG foliar application for control of common scab in potato, Annals of Applied Biology, 165, 293-302.

Thornton, M.K., Lee, J., John, R. et al. 2013. Influence of Growth Regulators on Plant Growth, Yield, and Skin Color of Specialty Potatoes. American Journal of Potato Research 90, 271-83.

Thorton, M.K., John, R. & Buhrig, W. 2014. The Influence of Plant Growth Regulators and Inflorescence Removal on Plant Growth, Yield, and Skin Color of Red LaSoda Tubers. Potato Research 57, 123-31.

Waterer, D. 2010. Influence of growth regulators on skin colour and scab diseases of red-skinned potatoes. Canadian Journal of Plant Science, 90, 745-753.

Wilson, C. R. 2004. "A summary of common scab disease of potato research from Australia", Proceedings of the International Potato Scab Symposium, 6-7 September 2004, Sapporo, Japan, 6-7 September.

Wilson, C. R. 2016. Plant pathogens - the great thieves of vegetable value. Acta Horticultuare 1123, 7-16.

# Intellectual property, commercialisation and confidentiality

This report has coded some varieties. A confidential version of this report that details the coded varieties will be shared with the relevant growers.

## Acknowledgements

We would like to acknowledge a range of people who have provided advice and assistance to this project. This assistance ranges from formal and informal discussions, providing detailed feedback and helping/undertaking field-based studies and providing cultivars for assessment.

We would like to acknowledge the key people who helped develop the project from Simplot (Frank Mulcahy) and McCains (Greg Bullock) and other company representatives. Also, for providing cultivars for testing (Angus Galloway, Daniel Grayling). We also thank Ed Blanchard for providing access to suitable field sites and in that regard acknowledge the two growers Leigh Elphinstone and Daryl Lohrey for providing their land, assistance with trials and technical knowledge.

Also, to technical TIA staff who have assisted with pot and field trials and accessing tuber inoculum from field stores, especially Phil Beveridge, Ann-Maree Donoghue, and Leonie White. We also acknowledge Dr David Nichols (CSL, University of Tasmania) for assistance with running chemical analytics. We also acknowledge Jemima Hamer (TIA) and Michelle De'Lisle (Ausveg) with publication assistance.

# Appendices

## Appendix 1 – List of peer reviewed journal publications

Thangavel, T., Tegg, R., Wilson, C. 2015. Monitoring *Spongospora subterranea* development in potato roots reveals distinct infection patterns and enables efficient assessment of disease control methods. *Plos One* **10,** e0137647. doi:10.1371/ journal.p **(Appendix 1a)**

Balendres, M.A., Tegg, R.S. Wilson, C.R. 2016. Key events in pathogenesis of Spongospora diseases in potato: a review. *Australasian Plant Pathololog*y **45,** 229-240. **(Appendix 1b)**

Balendres, M.A., Nichols, D.S., Tegg, R.S., Wilson, C.R. 2016. Metabolomes of potato root exudates: compounds that stimulate resting spore germination of the soil-borne pathogen *Spongospora subterranea*. *Journal of Agriculture and Food Chemistry* **64,** 7466-7474. **(Appendix 1c)**

Balendres, M.A., Tegg, R.S., Wilson, C.R. 2017. Resting spore dormancy and infectivity characteristics of the potato powdery scab pathogen *Spongospora subterranea. Journal of Phytopathology* **165,** 323-330. **(Appendix 1d)**

Balendres, M.A., Nichols, D.S., Tegg, R.S., Wilson, C.R. 2017. Potato root exudation and release of *Spongospora subterranea* resting spore germination stimulants are affected by plant and environmental conditions. *Journal of Phytopathology* **165**, 64-72. **(Appendix 1e)**

Balendres, M.A., Clark, T.J., Tegg, R.S., Wilson, C.R. 2018. Germinate to exterminate: chemical stimulation of Spongospora subterranea resting spore germination and its potential to diminish soil inoculum. *Plant Pathol*ogy **67**, 902-908. **(Appendix 1f)**

Clark, T.J., Rockliff, L.A., Tegg, R.S., Balendres, M.A., Amponsah, J., Thangavel, T., Mulcahy, F., Wilson, A.J., Wilson, C.R. 2018. Susceptibility of opium poppy and pyrethrum to root infection by *Spongospora subterranea. Journal of Phytopathology* (in press). **(Appendix 1g)**

Appendix 1a – Thangavel et al. 2015

PLOS ONE

RESEARCH ARTICLE

# Monitoring *Spongospora subterranea* Development in Potato Roots Reveals Distinct Infection Patterns and Enables Efficient Assessment of Disease Control Methods

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OPEN ACCESS

**Citation:** Thangavel T, Tegg RS, Wilson CR (2015) Monitoring *Spongospora subterranea* Development in Potato Roots Reveals Distinct Infection Patterns and Enables Efficient Assessment of Disease Control Methods. PLoS ONE 10(9): e0137647. doi:10.1371/journal.pone.0137647

**Editor:** Roberto Papa, Università Politecnica delle Marche, ITALY

**Received:** February 19, 2015

**Accepted:** August 20, 2015

**Published:** September 9, 2015



**Data Availability Statement:** All relevant data are within the paper.

**Funding:** This study was supported by Simplot Australia Pty. Ltd. and McCain Foods Australia Pty. Ltd., with matched funds from the Federal Government through Horticulture Innovation Australia Limited: project number PT14002 (http://www.horticulture.com.au/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

*Spongospora subterranea* is responsible for significant potato root and tuber disease globally. Study of this obligate (non-culturable) pathogen that infects below-ground plant parts is technically difficult. The capacity to measure the dynamics and patterns of root infections can greatly assist in determining the efficacy of control treatments on disease progression. This study used qPCR and histological analysis in time-course experiments to measure temporal patterns of pathogen multiplication and disease development in potato (and tomato) roots and tubers. Effects of delayed initiation of infection and fungicidal seed tuber and soil treatments were assessed. This study found roots at all plant developmental ages were susceptible to infection but that delaying infection significantly reduced pathogen content and resultant disease at final harvest. The pathogen was first detected in roots 15–20 days after inoculation (DAI) and the presence of zoosporangia noted 15–45 DAI. Following initial infection pathogen content in roots increased at a similar rate regardless of plant age at inoculation. All fungicide treatments (except soil-applied mancozeb which had a variable response) suppressed pathogen multiplication and root and tuber disease. In contrast to delayed inoculation, the fungicide treatments slowed disease progress (rate) rather than delaying onset of infection. Trials under suboptimal temperatures for disease expression provided valuable data on root infection rate, demonstrating the robustness of monitoring root infection. These results provide an early measure of the efficacy of control treatments and indicate two possible patterns of disease suppression by either delayed initiation of infection which then proceeds at a similar rate or diminished epidemic rate.

**Competing Interests:** Simplot Australia Pty. Ltd. and McCain Foods Australia Pty. Ltd. partly funded this study. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

## Introduction

*Spongospora subterranea* f.sp. *subterranea* is a soil-dwelling obligate cercozoan plant pathogen that invades potato roots, stolons and tubers, reducing root function [1,2] and causing the blemish disease powdery scab in tubers [3]. Losses to the Australian potato processing industries are estimated at A$13.4M per annum [4]. *S. subterranea* is also the vector of *Potato mop top virus*, a significant disease in many parts of the world [3], and may provide an entry point for other root and tuber-invading pathogens [3,5]. There are currently no sustainable and reliable controls for the pathogen [6].

The pathogen survives for prolonged periods in the soil and on seed tubers as conglomerates of resting spores (sporosori) from which motile primary zoospores are released. These actively swim toward host roots, encyst on the root surface and transfer the contents of the zoospore within root cells [1,6]. Root infection progresses with the formation of a plasmodium which becomes a sporangium and produces secondary zoospores that are released from the cell and initiate new infections within the root system and newly developing tubers in a polycyclic manner [1]. As the disease progresses there is a change from the zoosporangial to the sporogenic (resting spore) stage of the life cycle with formation of root and tuber galls where resting spores are produced within sporosori [1,7]. These are released into the soil providing new sources of inoculum.

As an obligate pathogen with infections occurring beneath the soil, the study of disease epidemics is technically difficult. Recent advances in pathogen detection and quantitation using qPCR have enabled researchers to measure the pathogen during root infection and assessments of cultivar susceptibility made [8]. Previous research has identified that the critical period for tuber infection is shortly after tuber initiation where the host cells are susceptible to pathogen penetration [9]. In controlled experiments where application of *S. subterranea* inoculum could be delayed, tuber symptoms were greatest when inoculum was applied early at tuber set, compared to later applications [10,11]. However, these experiments examined tuber disease only and did not assess root infection and root galling. Until this present study it was unknown whether a similar defined period of susceptibility existed in potato roots and what affect a delay in inoculum application would have on the dynamics of root infection.

In New Zealand and Europe a range of fungicides applied as to seed tubers or the soil at planting have successfully reduced the incidence of powdery scab increasing the yield of marketable tubers [12,13]. While, such fungicides may be an important part of an integrated management strategy for this disease [14], their impact on root infection and root disease has not been examined. Recent evidence suggests early associations between pathogen and the root are critical to subsequent disease expression [8]. Therefore, practices or strategies that can delay this interaction or slow subsequent disease progress may provide viable control options.

The aims of this study are to (1) develop a system for measuring temporal patterns of pathogen replication and disease within potato roots, and use these data to (2) identify whether potato roots, like tubers, have distinct periods of susceptibility to *S. subterranea* infection, (3) determine whether the epidemic rate within roots changes with respect to plant age at onset of infection, and (4) assess the effect of seed-tuber and soil applied fungicides on pathogen infection rate and epidemic patterns. We hypothesise that monitoring temporal root infection patterns can provide a reliable assessment of the ability of control strategies to mitigate root and tuber disease.

## Materials and Methods

### Ethics statement

No specific permissions were required for these pot and field trials. The studies did not involve endangered or protected species. Five pot trials were conducted in New Town (southern Tasmania, 147°17'57.21"E, 42°51'24.55"S), and two field trials in Wesley Vale (north-west Tasmania, 146° 24' 41"E, 41° 11' 38"S) over the period 2012–2014.

### Impact of delayed *S. subterranea* infection on pathogen replication and disease

Three pot trials (PT1, 2 and 3) examined the impact of delaying inoculation of *S. subterranea* on root infection and root and tuber disease in potato (PT1 and 2) and on root infection in tomato (PT3). Tomato was used to enable experimental effects to be tested across more than one species as it is a known susceptible host of the pathogen and provides a convenient model plant system. All trials occurred within a glasshouse environment with temperatures maintained at 16–22°C (PT1) or 16–35°C (PT2 and 3). Plastic pots (20 cm diameter, 4.5 L volume), were filled with pasteurized potting mix containing sand, peat, and composted pine bark (10: 10: 80; pH 6.0) and premixed with Osmocote 16–3.5–10 NPK resin coated fertiliser (Scotts Australia Pty Ltd.) at the rate of 6 kg/m3. In PT1 (11 August 2011—winter) and PT2 (8 January 2012—summer) pathogen-free mini-tubers of potato cultivars Russet Burbank and Desiree were planted at 10 cm depth (one tuber per pot) and in PT3 (22 January 2012—summer), 2-week-old healthy tomato seedlings of cv. Mortgage and Roma were transplanted into pots (one plant per pot).

*S. subterranea* inoculum was obtained from heavily diseased potato tubers that had been stored (for a maximum of three months) in ambient cool conditions (10°C) until use. Inoculum was prepared using a modification of previous methods [15,16]. Peel (to a depth of 1 cm) was removed from infected tubers, air-dried at 25°C, ground and sieved (35 μm); the resultant inoculum was stored at 4°C. Three days prior to application, inoculum powder was incubated in nutrient solution [17] for 3 days at 18–20°C in the dark. Ten mL of inoculum solution (adjusted to ~50 sporosori/10 μl using a haemocytometer i.e. 50,000 sporosori/pot) was applied to the soil surface of the pots [15]. In PT1 and PT2 pots were amended with *S. subterranea* inoculum at six different stages of plant development: emergence, 10 days after emergence (DAE), 20 DAE, 30 DAE, 40 DAE and 50 DAE. PT1 included an additional inoculum treatment at 60 DAE. Plants from individual replicate pots were destructively harvested at 15 days intervals for up to five harvest periods after initial inoculum treatment and root and tuber tissues sampled for further analysis. In PT3, pots were amended with *S. subterranea* inoculum at four different stages: transplanting, 20 days after transplanting (DAT), 40 DAT and 60 DAT. Plants were destructively harvested at 15 day intervals for up to seven harvest periods after initial inoculum treatment. For all PTs individual treatment combinations were replicated three times with pots arranged in a randomised complete block design and hand watered (when required) to maintain constant wet soil conditions. There were no pesticides or additional fertilizer applications. Harvested roots and tubers were assessed for disease in PT1 and PT2 while pathogen content in roots was quantified by qPCR in PT1-3.

### Impact of seed and soil applied fungicides on pathogen replication and disease

**Pot trials.** Two pot trials (PT4, PT5) examined the impact of mancozeb, applied as a soil treatment, on potato plants grown in potting soil inundated with *S. subterranea*. All trials were

conducted in an ambient, outdoor environment in New Town, Tasmania. PT4 was grown over summer (mean minimum of 12°C and mean maximum of 22°C) and PT5 over autumn/winter (mean minimum and maximum temperatures of 7 and 17°C). In PT4 disease-free mini-tubers of Russet Burbank were used and *S. subterranea*-infested field soil incorporated through the potting mix 1 week prior to planting, providing a high inoculum pressure (~ 40 ng *S. subterranea* DNA/g soil). Treatments included mancozeb (7.5 kg/ha i.e. 18L/ha in 1000 L of water) sprayed into a 10 cm-deep furrow in individual pots, just prior to planting and a water spray control. A control treatment without *S. subterranea* inoculum or fungicide was also included. Five tubers were planted at 10 cm depth (3 January 2012) in individual pots (20 cm diameter, 4.5 L volume) with six destructive harvests; 15, 30, 45, 60 and 75 DAE (root assessment), and after senescence (tuber assessment). There were three replicates for each harvest. In PT5 two-week old disease-free tissue cultured plantlets of Russet Burbank and Desiree were used with inoculum prepared as for PT1-3 trials and applied to potting soil just prior to planting. Treatments included three rates of mancozeb (equivalent to 3.25, 7.5, 15.0 kg/ha) sprayed into a 5cm-deep furrow just prior to planting and a water spray control. A control with no inoculum or fungicide was included. Seedlings were transplanted (4 April 2012) into individual pots (20 cm diameter, 4.5 L volume) with plants destructively harvested at 15 days intervals. In both trials, each treatment was replicated three times (one plant per pot) with pots arranged in a randomised complete block design and hand watered (when required) to maintain constant wet soil conditions. There were no additional pesticide or fertilizer applications. In PT4 pathogen content in roots by qPCR was quantified as well as root and tuber disease. In PT5 only root disease (zoosporangial score) was measured.

**Field trials.** Two field trials were established (12 October 2013) on commercial farms at Wesley Vale NW Tasmania (climate high rainfall season with mean minimum temperatures of 10°C and mean maximum temperatures of 20°C) on potato growing soils typical of the region; field trial 1 (FT1) on a red ferrosol soil and field trial 2 (FT2) on a brown dermosol soil. Powdery scab had been recorded in recent potato crops at both sites. Analysis of soil sampled just prior to planting by qPCR [18,19] gave very low *S. subterranea* inoculum levels of 415 and 33 pg DNA/g soil for F1 and F2 respectively. Planting material for all field trials was commercial seed of both 'Russet Burbank' and 'Innovator' with visually obvious powdery scab symptoms; each individual seed tuber having a surface cover score of 2 (surface cover percentage of *c.* 5–10% powdery scab) which corresponded to a qPCR in the tuber peel of ~100 ng *S. subterranea* DNA/g sample. Seed and soil chemical treatments were applied at planting. Treatments were seed-tubers dipped in (i) 1% formalin, (ii) fluazinam (7.5 g/10 kg seed) or (iii) mancozeb (32 g/10 kg seed), and soil in-furrow sprays of (iv) fluazinam (3.0 kg/ha i.e. 6L/ha in 1000 L of water) or (v) mancozeb (7.5 kg/ha i.e. 18L/ha in 1000 L of water) applied with a knap sack sprayer (Matabi 18L Elegance, Goizper Spraying, Portugal) and an untreated control.

Each treatment was replicated two times in each trial. Plots contained 15 seed tubers, spaced at 30 cm, with plots arranged in a randomized split-plot design. Fertiliser and irrigation scheduling followed standard commercial practice with no additional seed or soil pesticides applied. In both FTs the average emergence date was on 25$^{th}$ November 2013 with destructive sequential harvesting of one plant per plot made at 15, 30, 45, 60 and 75 DAE. Plants were gently uprooted with roots kept for infection, gall assessment and pathogen quantification by qPCR (stored at 4°C for up to two days). All other plants (10 per plot) were grown until senescence, tubers harvested and a random sample of 50 tubers per plot selected for disease assessment.

## Pathogen DNA quantification

DNA was extracted and quantified from soil material and tuber skin (peel) using established protocols of the commercial Root Testing Service of the South Australian Research Development Institute, Adelaide, South Australia, Australia [18,19,20]. This was to confirm the presence and quantity of pathogen in soil and tuber samples. DNA extraction from root tissue followed a modified technique with established quantification methodologies [8,21]. Essentially, whole root samples were washed thoroughly in running tap water to remove soil particles and representative sub-samples were stored at -80°C. Samples were dried at 30°C for 2–3 days and ground using mortar and pestle. Ground samples (50 mg) were mixed with 50μl of nuclease-free water prior to DNA extraction. DNA was extracted using Power plant ®pro DNA isolation Kit with RNAase treatment (MO BIO Laboratories, Inc, Canada), with DNA yield quantified using the Qubit® 2.0 Flurometer (Life technologies, Darmstadt, Germany). Primers for *S. subterranea* (SPO10, SPO11, probe) quantitation were from the ribosomal ITS region [8]. An internal control amplifying the conserved mitochondrial cytochrome oxidase (COX) gene from potato was run in all samples using previously reported primer (COX-F, COX-R) and probe (COX-P) sequences [21]. This was used as a positive internal control to confirm DNA quality, PCR amplification conditions and to normalise qPCR data for accurate quantification of the pathogen content.

Quantitative PCR was performed using 2 μl (10 ng/μl) of DNA template in 10 μl volume reactions using the Sensi FAST™ Probe No-ROX Kit (Bioline Pty.Ltd, Australia) [8]. All amplifications were carried out in a Rotor Gene 6000 instrument (Corbett Life Science, Sydney, Australia) with a thermocycle of 95°C for 15 mins, then 40 cycles of 94°C for 15 s and 60°C for 60s, with three replicates per sample. Amounts of *S. subterranea* DNA in samples were calculated by an absolute quantification method, using a standard curve determined from the amplification of six ten-fold dilutions (1.3 μg to 13 pg) of a plasmid DNA containing the *S. subterranea* ITS gene kindly supplied by the South Australian Research and Development Institute, as previously described [8]. The efficiency of the standard curve derived from the plasmid dilutions was determined as 99%.

## Disease assessment

**Root disease—root infection and galling.** Root hair infection was assessed by microscopic examination using a method modified from Merz [17]. From each plant, three samples of root (2–5 cm long) were cut at *c.* 30–50 mm from the crown region and thoroughly washed. Specimens were mounted on a glass slide, stained with aqueous 0.1% (w/v) aniline blue for 5 mins, rinsed with water, and a drop of 50% (v/v) aqueous glycerol added. Samples were examined microscopically at 200x magnification (Leica DMLB, Type LB 30T compound microscope; Leica Microsystems). Fifteen fields of view were examined per slide with presence of zoosporangia rated: 0 = no zoosporangia; 1 = only a few zoosporangia on root hairs; 2 = 3–5 root hairs with zoosporangia; 3 = 6–10 root hairs with zoosporangia, moderate infection; 4 = >10 root hairs with zoosporangia, heavy infection [17].

A root galling score was given per plant based on a visual rating scale modified from van de Graff *et al.* [9]: of 0 = no galls; 1 = 1–2 galls; 2 = 3–10 galls mostly <2mm in diameter, 3 = more than 10 galls some of which are >2mm in diameter; 4 = most major roots with galls some or all >4mm in diameter.

**Tuber disease—Powdery scab.** Harvested tubers were stored at 4°C for up to 4 weeks, prior to disease assessment. Tubers were washed and each tuber (> 4 g) was scored for powdery scab severity according to a visual tuber surface cover score ranging from 0 to 6 (0 = no visible disease on tuber surface, 0.5 = ≤ 1%; 1 = ≥ 1–5%; 2 = ≥ 5–10%; 3 = ≥ 10–30%; 4 = ≥ 30–50%; 5 = ≥ 50–70%; 6 = ≥ 70% tuber surface affected). The percentage of tuber surface

covered by lesions was then estimated by taking the mid values of these score ranges. The proportion of healthy tubers with no visible lesions was also recorded from which disease incidence was calculated [22].

### Data analysis

Data in all trials where time-course measurements were made (root DNA levels, root zoosporangia scores and root gall severity scores) were analysed for Area Under Disease Progress Curve (AUDPC) using the trapezoidal method—PROC ANOVA in SAS v9.3. All normalized DNA values were transformed to log (*Spongospora* DNA + 1) prior to AUDPC calculation. The mean AUDPC of each treatment were assessed by one-way analysis of variance (ANOVA) and using Tukey's method significant results were grouped at 5% difference level ($p = 0.05$). Additionally, where different inoculation dates were utilised (PT 1–3) a modified $AUDPC^i$ was also calculated measuring disease progress from the date of inoculum addition rather that the date of emergence. Data were truncated to ensure the same number of data points (three) was analysed for each curve. $AUDPC^i$ were assessed by one-way ANOVA. Where parameters were discrete, single measurements (e.g. tuber disease) data was analysed by one-way ANOVA using GENSTAT (version 14.2). Data was only used where the assumptions of the general linear model could be met and where graphical diagnostics showed a normal distribution.

## Results

### Impact of delayed *S. subterranea* infection on pathogen replication and disease

**Pathogen replication.** In PT1, 2 and 3 levels of *S. subterranea* DNA within the root increased with time (Fig 1). As expected, where application of inoculum was delayed there was a delay in the onset of infection and a resultant decrease in the AUDPC. In PT1 inoculation at 50 and 60 DAE resulted in a lower AUDPC than inoculation at emergence, 10, 20, 30 and 40 DAE in both Desiree ($p < 0.0001$, Fig 1A) and Russet Burbank ($p < 0.0001$, Fig 1B) cultivars (Table 1A). Likewise in PT2 similar trends were observed with the early application at emergence producing significantly greater pathogen levels in the root than the delayed applications at 40 and 50 DAE for Desiree ($p = 0.02$, Fig 1C) and at 20, 30, 40 and 50 DAE for Russet Burbank ($p = 0.001$, Fig 1D; Table 1B). In PT 3, the earliest inoculation treatment at transplant produced a greater AUDPC than the three later inoculation treatments, 20, 40 and 60 DAT, in cv. Mortgage ($p < 0.0001$, Fig 1E) and cv. Roma ($p < 0.0001$, Fig 1F; Table 1B).

Where disease progress was assessed from the date of inoculum addition and data truncated to three data points after inoculation ($AUDPC^i$, Table 1) there was no significant differences ($p > 0.05$) identified in any of the three PTs indicating that the rate of increase in pathogen over this period was consistent across all treatments.

**Root and tuber disease.** In PT1, zoosporangia were observed within 15–45 days after inoculation in all inoculation date treatments except the 60 DAE treatment where no zoosporangia were observed in either cultivar (Fig 2A and 2B). There was a significant decrease in the AUDPC for the mean zoosporangia score following delayed inoculation in cultivar Desiree ($p < 0.0001$, Table 1A) with the 50 and 60 DAE treatments having less infection than the 0 and 10 DAE treatments. Whilst similar trends were seen in Russet Burbank the effects were not significant ($p = 0.06$, Table 1A). Significant decreases in the AUDPC for mean root gall score was found in both cultivars, Desiree ($p = 0.003$, Fig 2C) and Russet Burbank ($p = 0.01$, Fig 2D), in response to delayed inoculation. In Desiree, pathogen inoculation at emergence, 10, 20 and 30 DAE led to a significantly greater root gall score than the inoculation at 60 DAE. In Russet

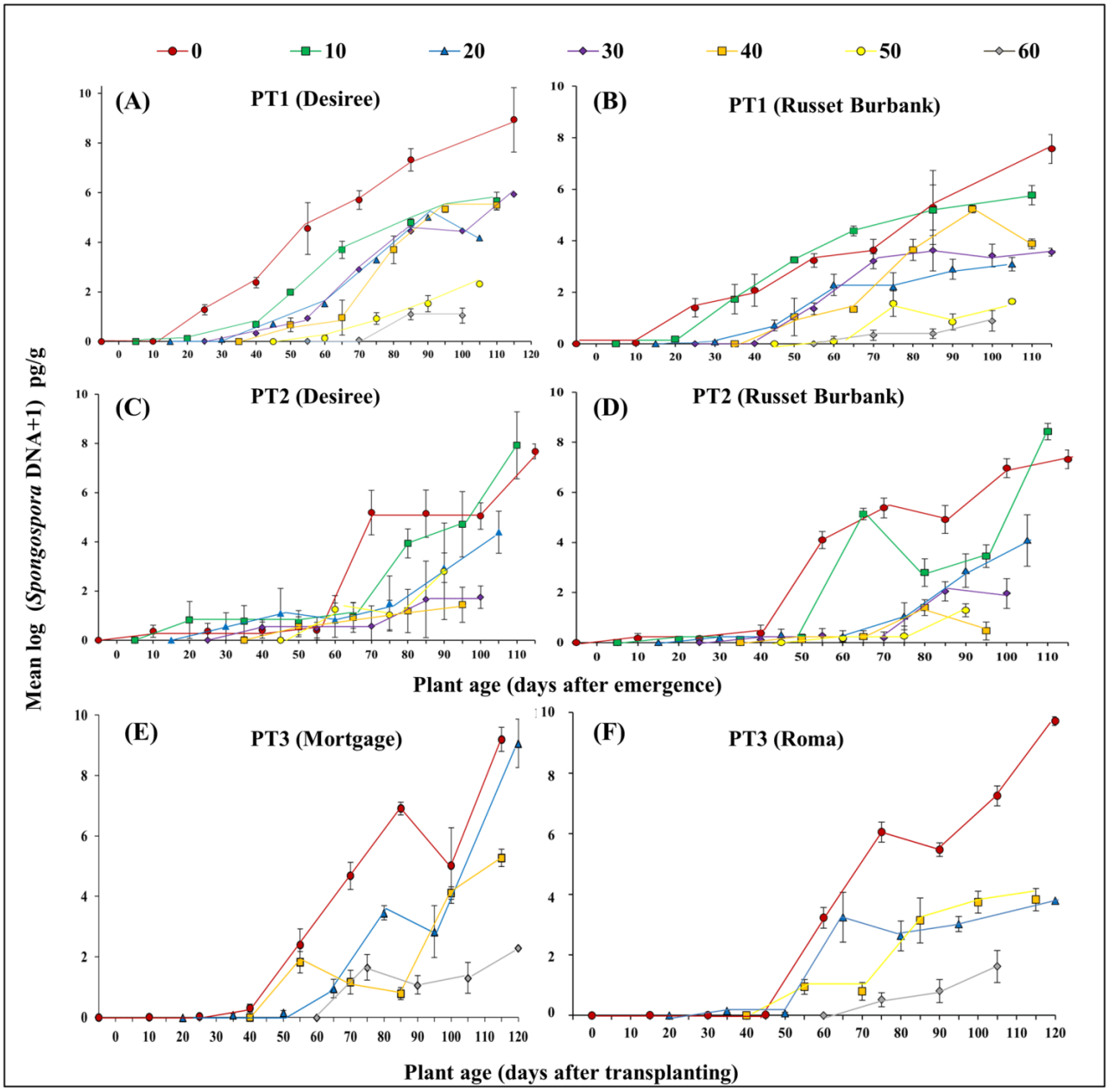


**Fig 1. The effect of delayed inoculation on initiation of root infection and replication of *S. subterranea*.** Results are for two potato cultivars in two pot trials (PT1—winter: A and B, PT2—summer: C and D) and in two tomato cultivars in one pot trial (PT3—summer: E and F). Markers represent the different inoculation treatment dates shown as days after emergence (DAE) or days after transplanting (DAT). Vertical bars are standard errors (n = 9).

doi:10.1371/journal.pone.0137647.g001

Burbank the inoculation at 10 DAE produced significantly greater gall score than the 60 DAE inoculation treatment (Table 1A). In PT1, where the analysis accounted for the altered application dates with data truncated to three data points after inoculation (AUDPC[i], Table 1A) there

**Table 1. Area Under Disease Progress Curve (AUDPC) for root pathogen and disease parameters following delayed inoculation (PT 1–3).**

| **A** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **PT1** | | | | | | | | | | | |
| **DAE** | **Pathogen DNA** | | | | **Zoosporangial score** | | | | **Root galling score** | | | |
| | **Desiree** | | **Russet Burbank** | | **Desiree** | | **Russet Burbank** | | **Desiree** | | **Russet Burbank** | |
| | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** |
| 0 | 1168.6 $^{a}$ | 85.3 | 893.1 $^{a}$ | 84.6 | 25.0 $^{a}$ | 0.5 | 10 | 0.5 | 50.0 $^{ab}$ | 2.5 | 27.5 $^{ab}$ | 0 |
| 10 | 665.7 $^{b}$ | 60 | 794.4 $^{a}$ | 119.1 | 15.5 $^{ab}$ | 1.5 | 12.5 | 1 | 50.0 $^{ab}$ | 12.5 | 50.0 $^{a}$ | 5 |
| 20 | 435.9 $^{b}$ | 51.8 | 334.7 $^{bc}$ | 65.5 | 6.0 $^{bc}$ | 1.5 | 6 | 2 | 47.5 $^{ab}$ | 7.5 | 30.0 $^{ab}$ | 15 |
| 30 | 547.3 $^{b}$ | 87.8 | 462.6 $^{b}$ | 102.5 | 6.0 $^{bc}$ | 1.5 | 23 | 2 | 62.5 $^{a}$ | 7.5 | 30.0 $^{ab}$ | 15 |
| 40 | 451.1 $^{b}$ | 108.5 | 438.1 $^{b}$ | 127.3 | 5.0 $^{bc}$ | 1.5 | 6 | 2 | 37.5 $^{abc}$ | 7.5 | 25.0 $^{ab}$ | 15 |
| 50 | 126.6 $^{c}$ | 60.2 | 113.4 $^{cd}$ | 70.3 | 1.0 $^{c}$ | 1 | 2 | 2 | 10.0 $^{bc}$ | 7.5 | 15.0 $^{ab}$ | 15 |
| 60 | 56.8 $^{c}$ | 56.9 | 34.5 $^{d}$ | 34.5 | 0.0 $^{c}$ | 0 | 0 | 0 | 0.0 $^{c}$ | 0 | 0.0 $^{b}$ | 0 |
| P | <0.0001 | 0.13 | <0.0001 | 0.1 | <0.0001 | 0.32 | 0.06 | 0.39 | 0.003 | 0.42 | 0.01 | 0.35 |
| F value | 69.32 | 2.48 | 46.43 | 2.89 | 13.1 | 1.17 | 2.75 | 1.13 | 5.91 | 1.08 | 4.16 | 1.22 |
| **B** | | | | | | | | | | | | |
| | **PT2** | | | | **PT3** | | | | | | | |
| | **Desiree** | | **Russet Burbank** | | **Mortgage** | | **Roma** | | | | | |
| | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** | **AUDPC** | **AUDPC$^{i}$** | | | | |
| 0 | 840.6 $^{a}$ | 92.6 | 789.6 $^{a}$ | 75.2 | 827.6 $^{a}$ | 53.2 | 929.1 $^{a}$ | 56.6 | | | | |
| 10 | 529.6 $^{ab}$ | 105.2 | 447.8 $^{ab}$ | 110.1 | - | - | - | - | | | | |
| 20 | 304.4 $^{ab}$ | 86.8 | 227.8 $^{b}$ | 82.2 | 557.8 $^{b}$ | 99.3 | 455.0 $^{b}$ | 162.2 | | | | |
| 30 | 132.5 $^{ab}$ | 85.5 | 94.8 $^{b}$ | 75.3 | - | - | - | | | | | |
| 40 | 107.3 $^{b}$ | 75.2 | 52.0 $^{b}$ | 45.2 | 474.8 $^{b}$ | 189.9 | 439.3 $^{b}$ | 216.9 | | | | |
| 50 | 105.1 $^{b}$ | 105.1 | 51.0 $^{b}$ | 51.0 | - | - | - | - | | | | |
| 60 | - | - | - | - | 116.0 $^{c}$ | 116.1 | 135.3 $^{c}$ | 135.3 | | | | |
| P | 0.02 | 0.29 | 0.001 | 0.27 | <0.0001 | 0.13 | <0.0001 | 0.33 | | | | |
| F value | 3.80 | 1.46 | 8.05 | 1.57 | 33.67 | 2.55 | 36.45 | 1.32 | | | | |

DAE is the number of days after plant emergence inoculum was applied to the potting soil.

AUDPC$^{i}$ is a modified measure such that all treatments were assessed for the same period after initial inoculation treatment. The data was truncated back to ensure the same number of data points (three) was analysed for each curve.

Different superscripts denote significant differences ($p < 0.05$) within same columns using Tukey's method.

doi:10.1371/journal.pone.0137647.t001

was no significant differences ($p > 0.05$) identified for either zoosporangial score or galling indicating that these parameters was consistent across all treatments. In PT1, there were also significant decreases in mean tuber disease incidence ($p < 0.001$, Fig 3A) and severity ($p < 0.001$, Fig 3B) following delaying inoculation in both cultivars. Inoculation at emergence, 10 and 20 DAE had significantly greater disease incidence and severity than at 30, 40, 50 and 60 DAE.

In PT2 and PT3 sporadic occurrence of zoosporangia were observed but not scored. No root galling was found in plants from either trial and no disease was present in tubers harvested from plants in PT2.

## Impact of seed and soil chemical treatment on *Spongospora* infection and powdery scab

**Pot trials.** In PT4 the amount of *S. subterranea* DNA present in Russet Burbank potato roots increased in both fungicide treated and control treatment but the rate of increase was

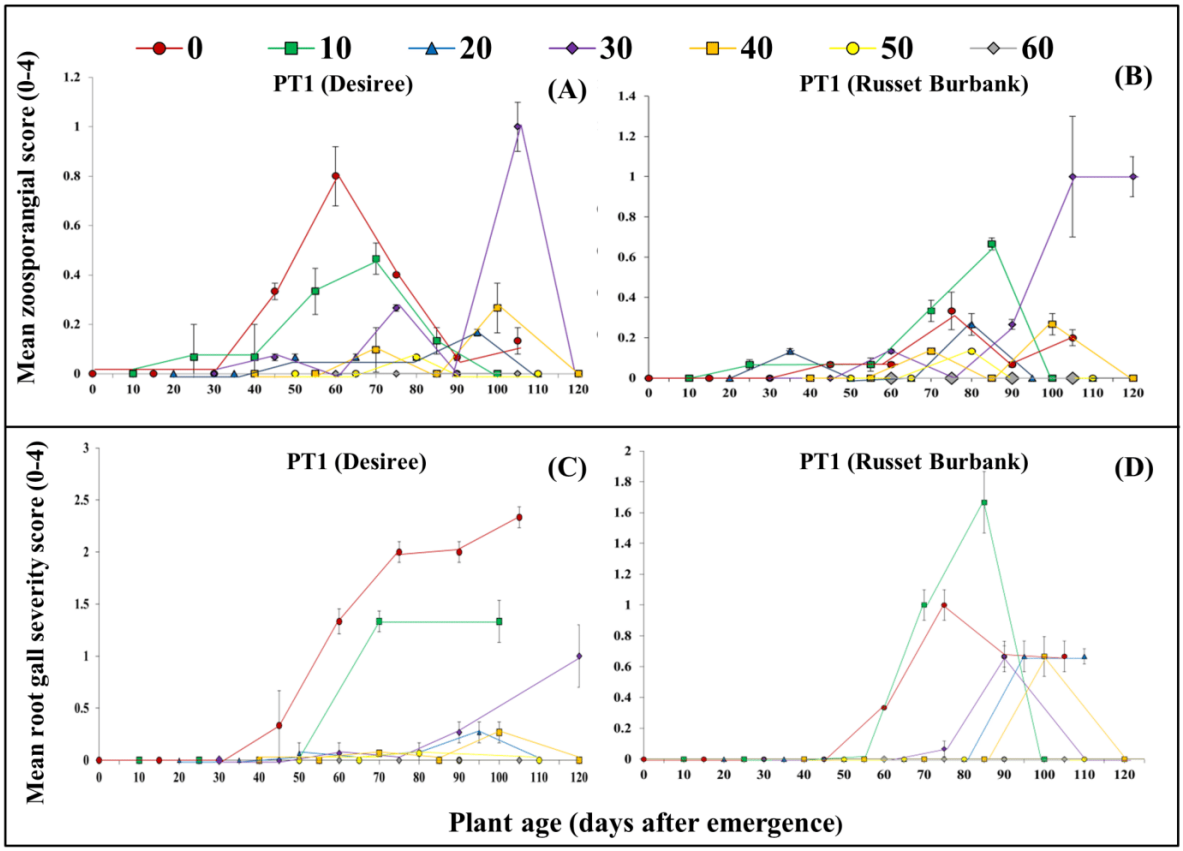


**Fig 2. The effect of delayed inoculation of *S. subterranea* on presence of zoosporangia and galls in roots.** Results are for two potato cultivars in pot trial 1 (winter) with A and B showing mean zoosporangial score (0–4) and C and D showing root gall severity score (0–4). Markers represent the different inoculation treatment dates shown as days after emergence (DAE). Vertical bars are standard errors (n = 6).

doi:10.1371/journal.pone.0137647.g002

significantly less, over time, in the soil furrow mancozeb treatment ($p < 0.001$, Fig 4A, Table 2). Zoosporangial score was also reduced by mancozeb treatment, with the effect greater in the earlier assessments contributing to a significant reduction in AUDPC compared to the untreated control ($p < 0.025$, Fig 4B, Table 2). Similarly, root galling (pooled at 45 and 60 days) was significantly reduced by mancozeb treatment ($p = 0.045$, Fig 4C) while tuber disease incidence (Fig 4D) and severity (Fig 4E) showed non-significant reductions by the fungicide soil treatment.

In PT5, where only the zoosporangial score was measured for a 25 day period, there were no significant differences seen between Desiree and Russet Burbank ($p > 0.05$) for each assessment and these data were pooled to provide a larger data set for subsequent analyses. All mancozeb treatments (3.25kg /ha,7.5kg/ha, 15.0 kg/ha) significantly reduced the zoosporangial score and slowed the rate of infection compared to the untreated control ($p < 0.001$, Fig 5, Table 2).

**Field trials.** Pre-plant soil inoculum levels were very low and seed inoculum levels very high in both field trials. Both trials produced high levels of *S. subterranea* root infection, root galling, and tuber disease in both cultivars. Exploratory analysis of the data indicated that there were no significant effects of cultivar on the disease outcomes assessed and thus data sets from Russet Burbank and Innovator were pooled to provide a larger data set.

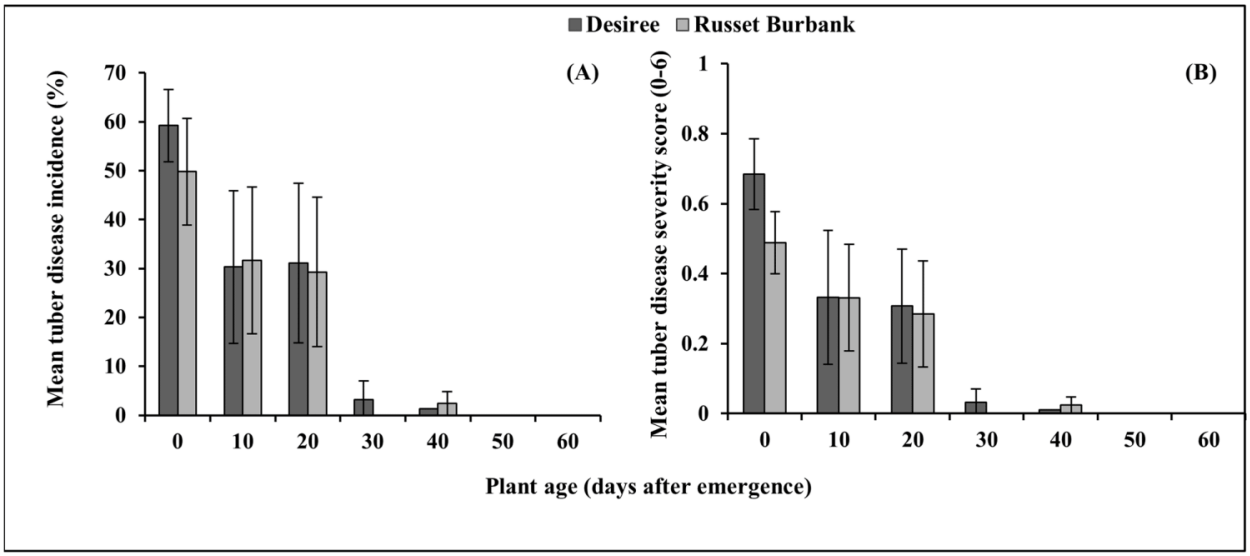


**Fig 3. Effect of delayed inoculation of *S. subterranea* on tuber disease incidence (A) and severity (B).** Results are for two potato cultivars in pot trial 1 (winter). Vertical bars are standard errors (n = 3).

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The amount of *S. subterranea* DNA detected within roots increased with time over the five sequential assessment dates. The AUDPC analysis identified significant treatment differences in FT1 ($p < 0.0001$, Fig 6A, Table 2) but not in FT2 ($p = 0.07$, Fig 6B, Table 2). Essentially, the untreated control and mancozeb soil furrow treatments had significantly higher AUDPC's than all other treatments in FT1, with analogous trends seen in FT2 indicating an increased rate of pathogen replication in these two treatments.

The soil treatment (fluazinam) and seed dips (formalin, fluazinam and mancozeb) consistently reduced ($p < 0.05$; 2–6 fold decrease, Fig 7A and 7B) mean zoosporangial score compared to the untreated control in both field trials. The mancozeb soil treatment produced a zoosporangial score equivalent ($p > 0.05$, Table 2) to the untreated control indicating that root infection developed more rapidly in these two treatments.

Root galls were first observed at 45 days after plant emergence with consistent root galling observed across all treatments at 60 and 75 days after emergence (galling data was pooled across these last two assessment dates for Fig 7C and 7D). Consistent with the root infection data, similar trends were observed with the soil treatment (fluazinam) and seed dips (formalin, fluazinam and mancozeb) reducing ($p < 0.05$; 2–8 fold decrease) root gall score and root gall production rate (AUDPC, Table 2) compared to the untreated control. Once again, the mancozeb soil treatment and the untreated control produced similar ($p > 0.05$) levels of moderately high galling (root gall scores of 2.3–2.6).

The soil treatment (fluazinam) and seed dips (formalin, fluazinam and mancozeb) also significantly reduced mean tuber disease incidence and severity in both trials (FT1 and FT2). In FT1, both disease incidence ($p = 0.01$, Fig 8A) and disease severity ($p = 0.003$, Fig 8C) were significantly lower in the soil furrow treatment (fluazinam) and seed dip treatments (fluazinam, formalin and mancozeb) than the untreated control and mancozeb soil furrow treatment. The same trend was found in FT2 for both disease incidence ($p = 0.001$, Fig 8B) and severity ($p = 0.003$, Fig 8D).

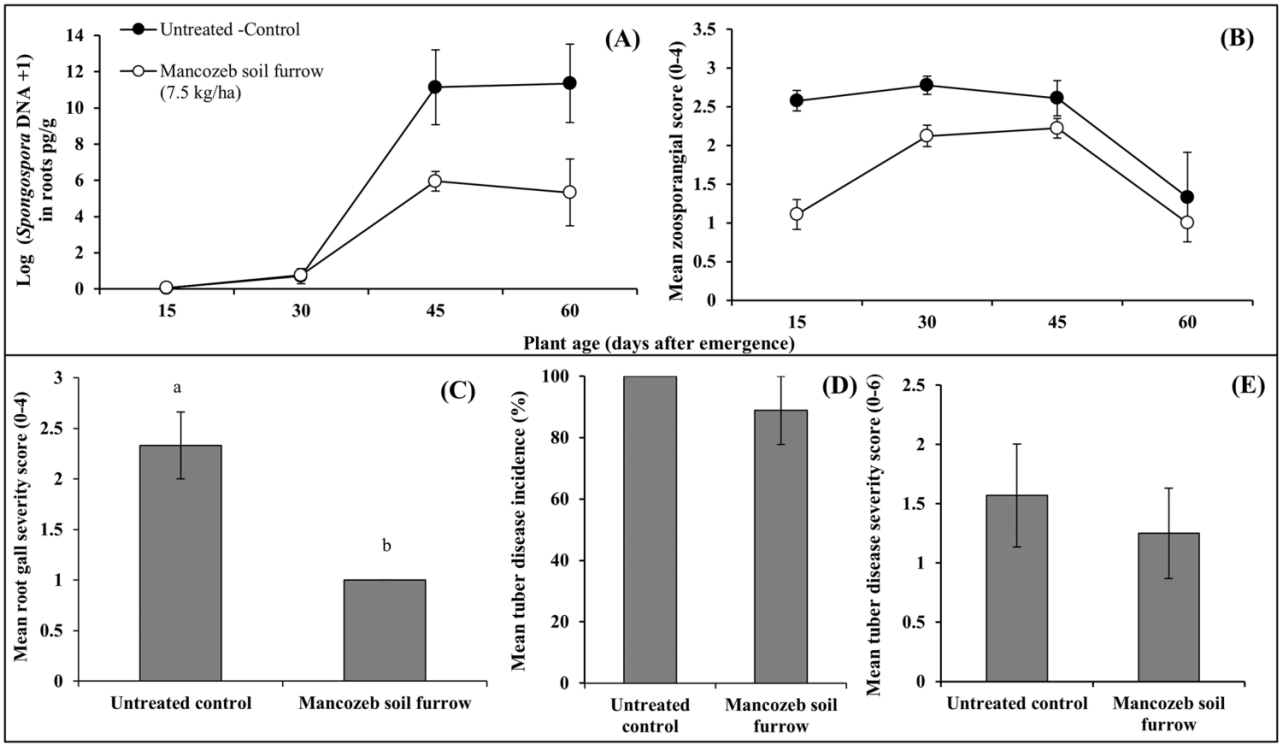


**Fig 4. The impact of mancozeb soil furrow treatment (7.5kg/ha) on root infection and disease in cultivar Russet Burbank in a pot trial (PT4—summer).** Multiple sequential assessments measured A- *S. subterranea* DNA concentration (pg/gm) in roots (n = 9); B- zoosporangia observation score (n = 3). Two assessments were made for C- root gall severity at 45 and 60 days (data pooled) after emergence (n = 3); and single assessments for D- mean tuber disease incidence and E- mean tuber disease severity at plant senescence (n = 3). Vertical bars are standard errors.

doi:10.1371/journal.pone.0137647.g004

## Discussion

Most previous studies with *S. subterranea* have focused on root galling and tuber lesions that occur late in the infection cycle. Yet the most critical interaction between pathogen and plant occurs much earlier, following initiation of root infection and subsequent polycyclic development of further root infections rapidly building pathogen populations which subsequently lead to expression of visual disease [6,7,9,10]. Recently, the use of qPCR to detect and quantify pathogen content within roots has been used to differentiate cultivars on the basis of resistance to root infection [8]. Other studies have emphasized the dynamics of root infection in informing subsequent disease progress [9]. Work presented here highlights the benefits of monitoring the temporal patterns of pathogen development during infection, demonstrating that two distinct pathways (delayed infection and a reduced epidemic rate) can substantially suppress disease using delayed inoculation and seed and soil treatments as test cases.

### Impact of delayed inoculum application

In this study, plants receiving inoculum earlier built pathogen levels faster and produced greater root and tuber disease. *S subterranea* DNA was detected 15–20 days after inoculation with zoosporangia and root galls observed 15–45 and 45–75 days after inoculation respectively. Tuber disease required inoculation by 40 DAE to occur. Within the average potato life cycle

**Table 2. Area Under Disease Progress Curve for root pathogen and disease parameters following the seed tuber and soil fungicide treatment (PT 4 and 5; FT 1 and 2).**

| | PT4 | | PT5 | FT1 | | | FT2 | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | Pathogen DNA | Zoosporangial score | Zoosporangial score | Pathogen DNA | Zoosporangial score | Root galling score | Pathogen DNA | Zoosporangial score | Root galling score |
| Untreated control | 344.7$^{a}$ | 64.2$^{a}$ | 36.3$^{a}$ | 393.3$^{a}$ | 6.7$^{a}$ | 48.8$^{a}$ | 238.8 | 10.5$^{a}$ | 54.4$^{a}$ |
| Formalin seed dip (1%) | | | | 205.6$^{b}$ | 0.7$^{c}$ | 13.1$^{b}$ | 139.7 | 1.8$^{b}$ | 9.4$^{b}$ |
| Fluazinam seed dip (7.5 g/10 kg seed) | | | | 180.4$^{bc}$ | 2.2$^{bc}$ | 11.3$^{b}$ | 94.5 | 2.3$^{b}$ | 16.9$^{b}$ |
| Mancozeb seed dip (32 g/10 kg seed) | | | | 115.3$^{bc}$ | 2.2$^{bc}$ | 15.0$^{b}$ | 174.3 | 1.5$^{b}$ | 18.8$^{b}$ |
| Fluazinam soil furrow (3.0 kg/ha) | | | | 257.8$^{b}$ | 3.0$^{b}$ | 0.0$^{c}$ | 162.2 | 1.5$^{b}$ | 18.8$^{b}$ |
| Mancozeb soil furrow (3.25 kg/ha) | | | 12.8$^{b}$ | | | | | | |
| Mancozeb soil furrow (7.5 kg/ha) | 133.1$^{b}$ | 32.2$^{b}$ | 10.5$^{b}$ | 368.9$^{a}$ | 3.8$^{b}$ | 56.3$^{a}$ | 173.1 | 5.3$^{a}$ | 43.8$^{a}$ |
| Mancozeb soil furrow (15 kg/ha) | | | 9.5$^{b}$ | | | | | | |
| P | <0.001 | <0.025 | 0.02 | <0.001 | 0.002 | 0.01 | 0.07 | 0.004 | 0.04 |
| F value | 9.24 | 3.71 | 3.91 | 12.60 | 5.39 | 3.66 | 2.35 | 7.65 | 3.21 |

Different superscripts denote significant differences ($p < 0.05$) within same columns using Tukey's method.

doi:10.1371/journal.pone.0137647.t002

(100–120 days), the expression of powdery scab tuber symptoms and root galls require 60 days [9,10]; this was evidenced in our study by altering the inoculation date. While both cultivars tested showed similar trends, the levels of infection, both in roots and tubers was greater in the moderately susceptible Desiree than the moderately resistant Russet Burbank [23]. From this study it is clear that any strategy that can delay the interaction between pathogen and host roots would be beneficial in reducing disease.

We also demonstrated that roots and tubers have different periods of susceptibility to pathogen infection. Roots are susceptible to *S. subterranea* throughout the whole plant growth cycle with infections initiating 15–20 days after inoculation and disease progressing in a rapid manner at a similar rate regardless of plant species (potato or tomato) or plant age at infection. Conversely, prior studies have shown tubers have a narrow window of susceptibility around early tuber initiation [9,10,11], with infections postulated to occur through immature lenticels [24,25,26]. Where infection can be restricted or delayed through this tuber development phase, tuber lesions can be reduced.

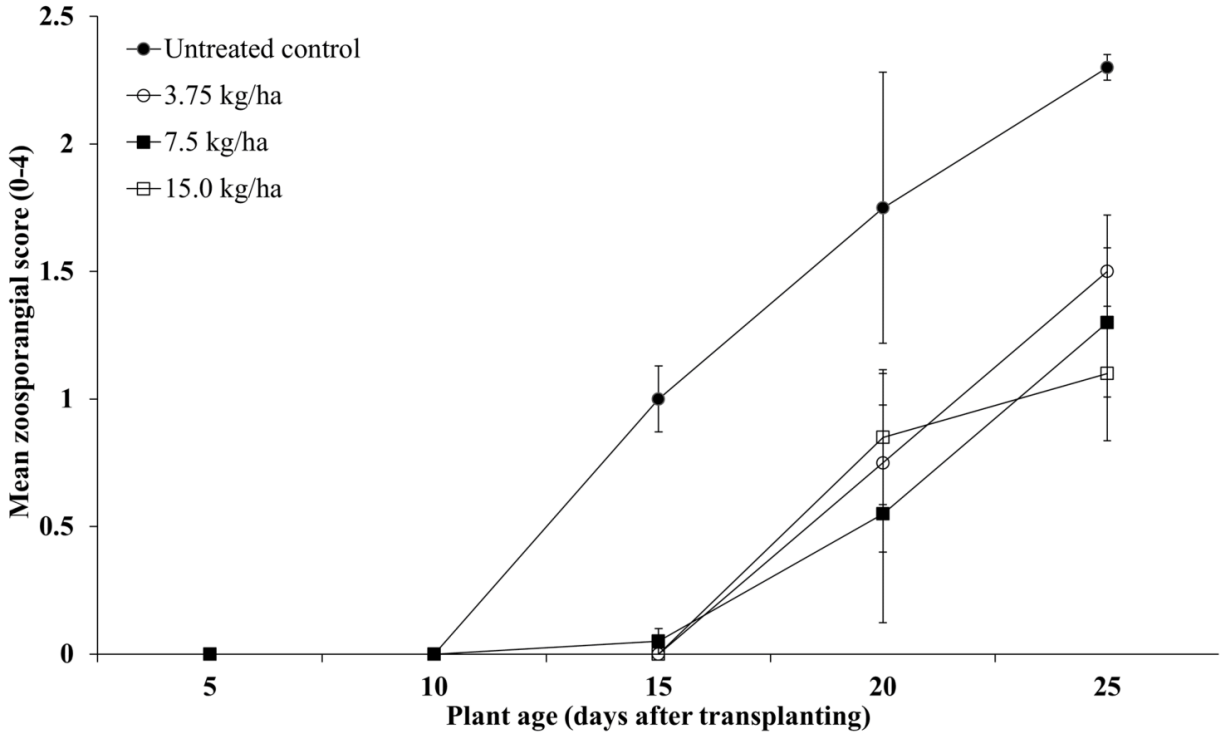


**Fig 5. The effect of different mancozeb soil furrow treatments (0, 3.25, 7.5 or 15.0 kg/ha) on mean zoosporangial score (0–4) in potato roots grown in a pot trial (PT5 –autumn/winter).** Data was pooled across the cvs. Desiree and Russet Burbank. Vertical bars are standard errors (n = 6).

doi:10.1371/journal.pone.0137647.g005

## Impact of seed and soil chemical treatments

Several fungicides have been tested for the control of powdery scab as soil or tuber treatments, with varying levels of efficacy [10,11,12]. High levels of elemental sulphur and Zinc-EDTA has been shown to reduce disease severity when applied to the soil but this has only limited efficacy [13]. Formalin has shown effective control but has been associated with poor plant emergence and growth [6,12]. Fluazinam and flusulfamide have shown promise as soil treatments, but the effects are not always consistent [3]. When testing a range of chemicals applied to the seed, foliage or furrow, Braithwaite and colleagues [12] identified alternatives including fluazinam, mancozeb, maneb, dichlorophen-Na for controlling powdery scab symptoms. These chemicals were able to significantly control powdery scab disease with reductions of 66%-95% recorded, although no treatment provided complete control. All of these aforementioned studies focused on tuber symptom development in field and pot trials with plants grown to full maturity. The sometimes erratic nature of the disease and uneven pathogen distribution within field sites can lead to inconsistent results when screening possible new chemical treatments [9,27]. Even in our studies, visual expression of root and tuber disease was not always found. However, tracking pathogen development within the root system proved to be highly reliable and provided an understanding of the efficacy and activity of these fungicide treatment on both root and tuber infection.

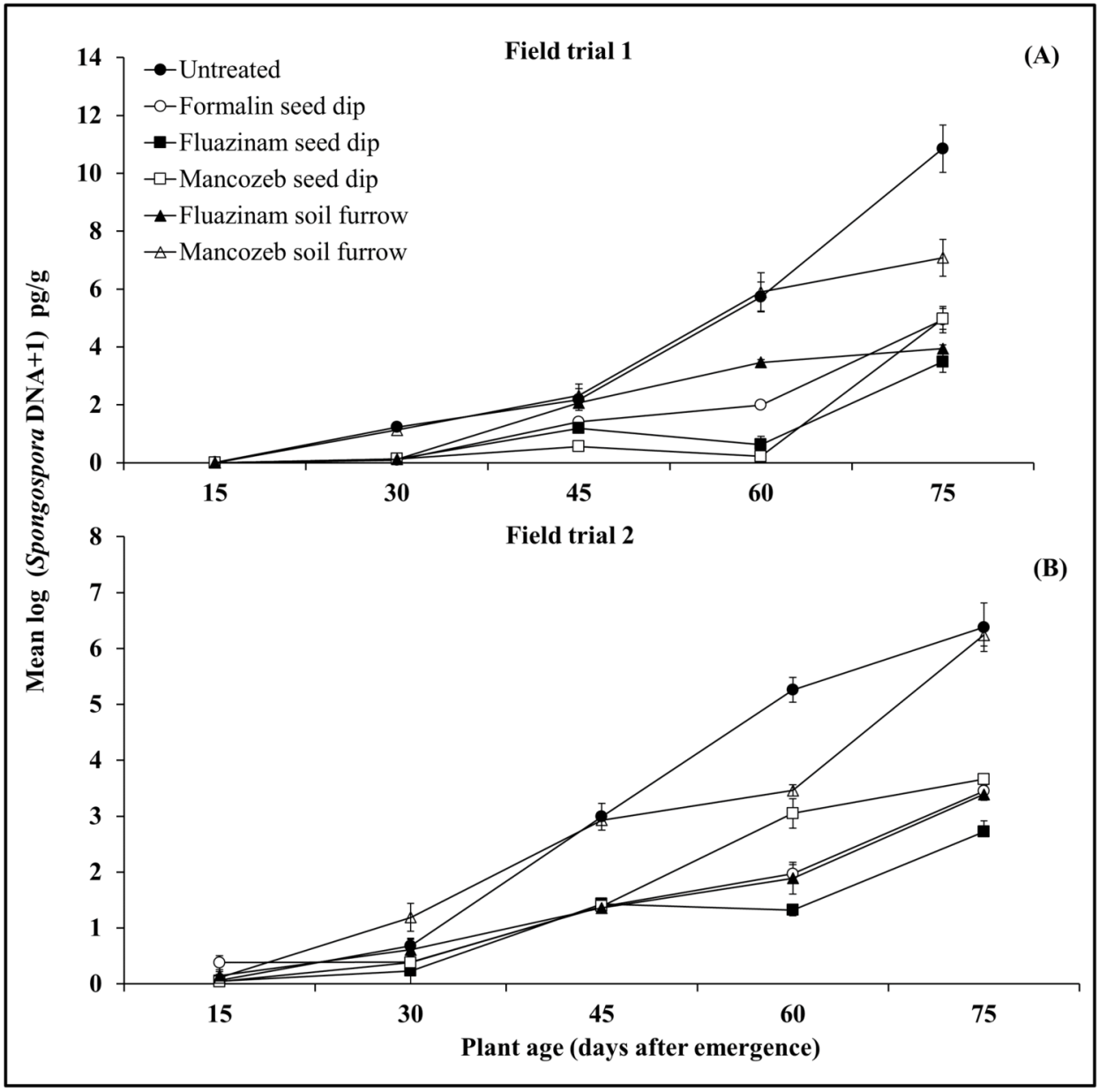


**Fig 6. The effect of fungicide seed and soil treatments on the increase in pathogen content (pg *S. subterranea* DNA/gm) in potato roots.** Measurements were made 15–75 days after emergence (DAE) in two field trials (A—FT1, B—FT2). Data was pooled across the cultivars Russet Burbank and Innovator, vertical bars are standard errors (n = 9).

doi:10.1371/journal.pone.0137647.g006

Consistent with previous work [6,12] fluazinam, mancozeb and formalin dips along with fluazinam soil furrow treatment was able to reduce root galling and tuber disease. Importantly we have identified the impact of these treatments on initiation of root infection and pathogen

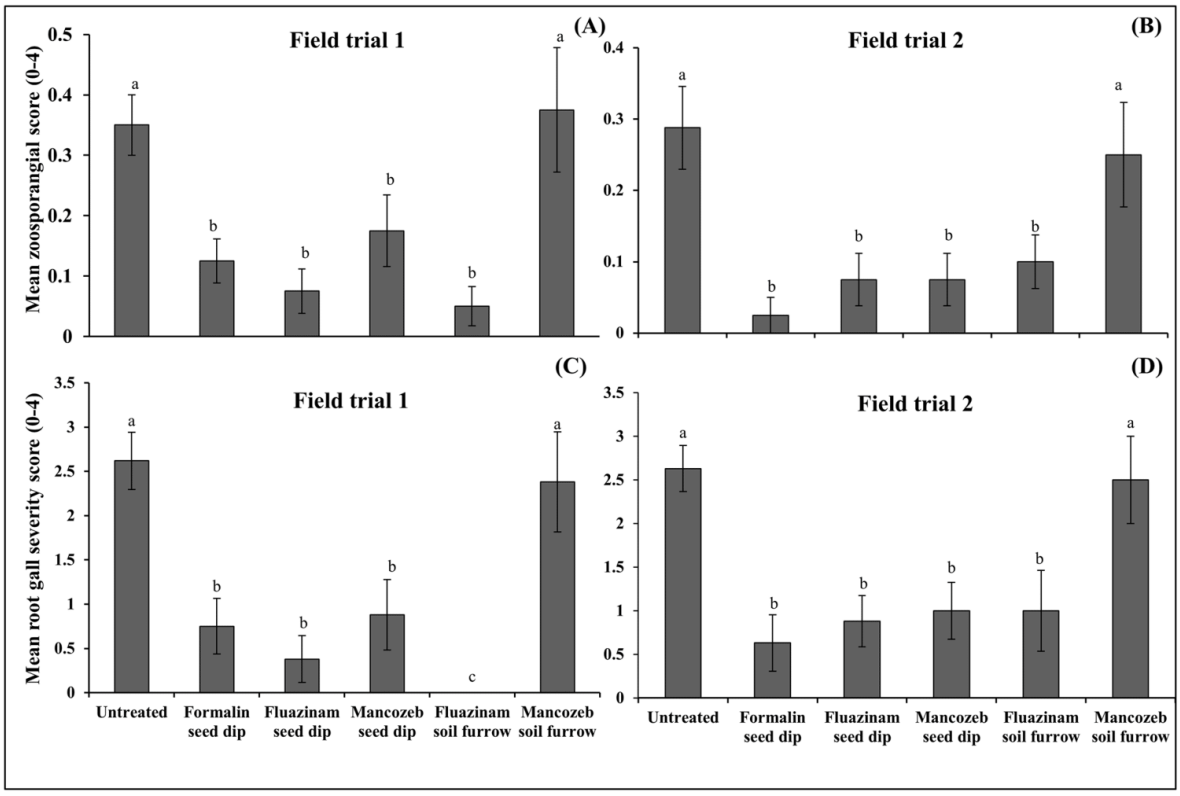


**Fig 7. The effect of fungicide seed and soil treatments on root infection.** Measurements included mean potato root zoosporangia infection score (0–4) in FT1 (A) and FT2 (B) and root gall severity score (0–4) in FT1 (C) and FT2 (D). Data was pooled across the cultivars Russet Burbank and Innovator and two assessment dates, 45 and 60 days after emergence. Vertical bars are standard errors (n = 8).

doi:10.1371/journal.pone.0137647.g007

development. In contrast to the delayed inoculation experiments, effective seed and soil-applied fungicide treatments (with the possible exception of some treatments in FT1) did not delay onset of infection but rather slowed pathogen development resulting in less disease. This early identification of treatment efficacy can provide a rapid means of screening and identifying fungicides effective against this disease.

The mancozeb soil treatment gave good control in pot trials but failed to suppress pathogen development in roots and tubers in the field trials. Similar erratic responses of this fungicide applied as a soil treatment have been seen (F. Mulcahy, Simplot Australia Pty Ltd, pers. comm.). The contrasting results in our trials may relate to the major inoculum sources used. In the pot trials, a high dose rate of soil inoculum was the sole source of pathogen. In the field trials despite presence of disease in previous potato crops on these sites, the soil tests prior to planting indicated low soil inoculum levels. The major inoculum source for these trials was rather from the infested seed tubers. With the unavailability of fluazinam as a registered fungicide for potato within Australia there is a need to assess other means of control which may form an integrated component of managing this disease [12,13].

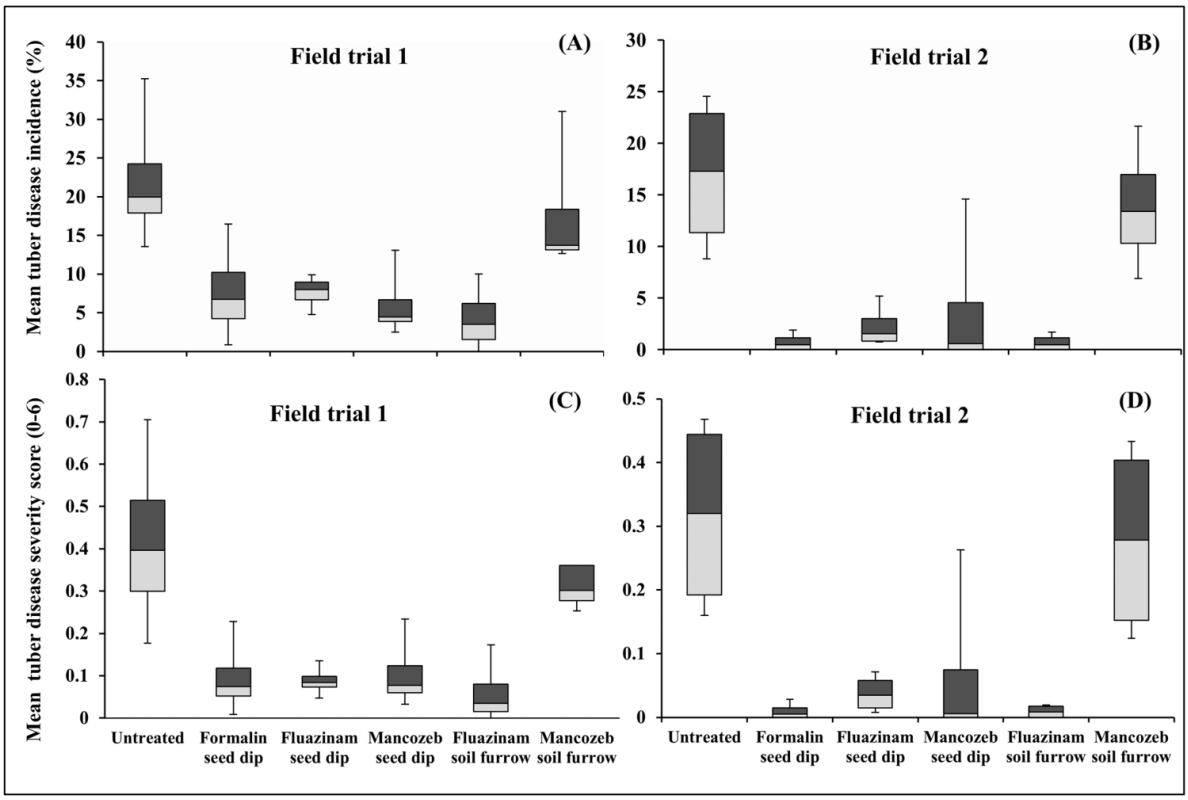


**Fig 8. The effect of fungicide seed and soil treatments on mean tuber disease.** Measurements included disease incidence in FT1 (A) and FT2 (B) and severity (0–6) in FT1 (C) and FT2 (D). Data was pooled across the cultivars Russet Burbank and Innovator. Vertical bars are the variation within the population (n = 8).

doi:10.1371/journal.pone.0137647.g008

Root galling and tuber disease is influenced by soil environmental conditions [6,23] at the critical period of tuber susceptibility [25,28]. Tuber disease is favoured by cool soil temperatures within the range of 9–17°C whilst root galling occurs within a warmer soil temperature range of 11–25°C) [9,29]. Plants in PT2 and PT3 failed to produce root galling and in PT2 tuber lesions. We can speculate that the high summer temperatures regularly experienced in the glasshouse over the trial period may have inhibited root galling and tuber disease expression. However, *S. subterranea* was successfully quantified within the roots of both trials and treatments were able to be compared for efficacy in reducing pathogen development highlighting the reliability benefits of this assay.

Tracking disease progress curves of *S. subterranea* within potato roots as we have done here has proven to be valuable in furthering our understanding of the epidemiology of this disease and in providing a reliable tool for rapid assessment of diverse disease mitigation strategies. The critical role of root infection in disease expression is clear, and we show both delaying infection and slowing epidemic rate provide substantial disease control.

## Acknowledgments

We thank Ross Corkrey for assistance with statistical analysis, Frank Mulcahy (Simplot Australia Pty Ltd) for chemical samples and useful discussion.

## Author Contributions

Conceived and designed the experiments: CRW RST TT. Performed the experiments: TT RST. Analyzed the data: TT RST CRW. Contributed reagents/materials/analysis tools: CRW. Wrote the paper: TT RST CRW.

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Appendix 1b – Balendres et al. 2016a

Australasian Plant Pathol. (2016) 45:229–240
DOI 10.1007/s13313-016-0398-3

REVIEW

# Key events in pathogenesis of *spongospora* diseases in potato: a review

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Received: 28 August 2015 / Accepted: 11 January 2016 / Published online: 5 February 2016


**Abstract** *Spongospora subterranea* (Wallr.) Lagerh. is the causative agent of powdery scab and root diseases of potato. Diseases induced by *S. subterranea* causes substantial economic losses to the global potato industry. The process of disease development in the *S. subterranea*-potato pathosystem have long been studied, but critical events prior to infection and disease development remain poorly understood because the subject have received relatively little attention. Nonetheless, new knowledge of host-pathogen-environment interactions have been gained in recent years. This paper provides the current knowledge of the key events which leads to the development of potato diseases caused by *S. subterranea* and highlights future research to address identified knowledge gaps. This will further our understanding of the interactions between *S. subterranea* and its potato host and contribute to improved disease control measures.

**Keywords** *Spongospora subterranea* · host-pathogen interaction · potato · root galling · plasmodiophorids

## Introduction

The plasmodiophorid pathogen *Spongospora subterranea* causes three distinct diseases in potato: powdery scab on tubers, zoosporangia root infection and root galling (Falloon et al. 2015). Powdery scab, the most widely recognised disease component, was first observed in 1841 in Germany by Wallroth (1842). The pathogen was initially described as *Erysibe subterranea*, but is now recognised as *Spongospora subterranea* (Wallr.) Lagerh. It was formerly assigned as a forma speciales to separate it from the crook root (of watercress) pathogen *S. nasturtii*, but Dick (2001) subsequently separated them into two distinct species. Recent genetic and phylogeny analysis based on the ITS rDNA and 18S rDNA confirms that the pathogen is distinct from the watercress pathogen (Qu and Christ 2004). In 1912, Pethybridge noted that galls are formed in *S. subterranea*-infected roots but made no clear indication of what preceded gall formation. It was Ledingham (1935) who reported that infection of *S. subterranea* in the roots results in the formation of zoosporangia – a structure common to plasmodiophorids.

That the pathogen originates from South America, the ancestral home of its primary Solanaceous hosts (Melhus 1913; Hawkes and Francisco-Ortega 1993), was first assumed by Lagerheim (1892)and Lyman and Rogers (1915). Further evidence for this assumption was provided by Gau et al. (2013, 2015) from their analysis of the DNA sequences of geographically diverse *S. subterranea* collections and the historical worldwide distribution of potato. To date, the global spread (Fig. 1) of the pathogen continues with recent new national records in Bulgaria (Bobev 2009), Iran (Norouzian et al. 2010), Latvia (Turka and Bimšteine 2011) and Sri Lanka (Babu and Merz 2011), and within country records in the US states of Colorado (Houser and Davidson 2010) and New Mexico (Mallik and Gudmestad 2014), and in the Greek island Crete (Vakalounakis et al. 2013). Most recently, the pathogen was detected in the island-nation of Cyprus (Kanetis et al. 2015).

The diseases induced by *S. subterranea* results in substantial economic impacts on potato production globally. Powdery

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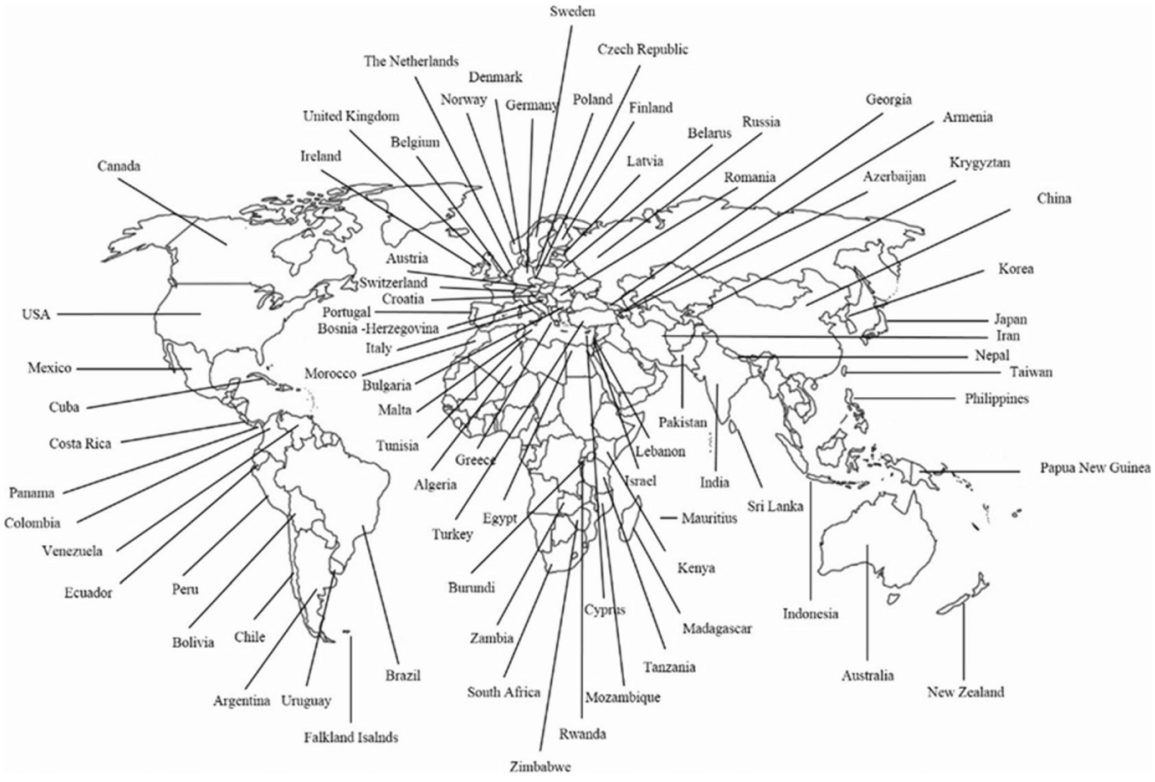


**Fig. 1** Global distribution of *S. subterranea* f. sp. *subterranea*. Pathogen was detected either on potato or tomato. Sources: National records compiled from CABI/EPPO (2012) and new reports

scab can result in significant losses for producers of seed potatoes as diseased crops may fail certification standards. For example in Australia seed tuber crops with >2 % of tubers with one or more powdery scab lesions will fail certification (Tegg et al. 2014; VICSPA 2007) resulting in seed crop devaluation and loss of company/grower reputation. Tuber lesions will also substantially downgrade the value of fresh market potatoes (Harrison et al. 1997). In the processing sector, grower's crops may be devalued or rejected if disease levels in harvested tubers are high and factories may require additional processing steps to remove tuber lesions; hence, increasing wastage and processing costs (Wilson 2015). Diseased tubers may also exacerbate water loss and tuber rots in storage with other pathogens gaining entry through *S. subterranea* tuber lesions. In the Australian potato processing sector which utilises more than half of Australia's domestic production (HAL 2011) losses due to powdery scab were estimated at A$ 13.4 million per annum, or approximately 4 % of the gross production value (Wilson 2015). In addition to such losses due to powdery scab, recent studies have indicated significant yield impacts following root infection where disease affects important plant physiological functions (e.g. water and nutrient uptake) and plant productivity (Falloon et al. 2015). To date, management of diseases caused by *S. subterranea* remains a major challenge with no single effective control strategy (Falloon 2008).

In the last decade, the development of new research tools and techniques has contributed to new knowledge of the interaction between *S. subterranea* and potato. Whilst this new information has improved our understanding of the *Spongospora*-potato pathosystem, studies relating to the events prior to pathogen infection, have received little attention. Hence, pathogen bionomics (interaction of *S. subterranea* to its environment) before it reaches the host is poorly understood and such a lack of clear understanding impedes the development of effective and durable management measures for diseases caused by *S. subterranea*. This paper reviews the current understanding of the critical pre- and post-infection events in the *S. subterranea*-potato pathosystem and examines the physical, biological, and chemical factors involved during these events. This paper highlights how these factors may influence disease development and how a better knowledge of these factors could be valuable in the development of control measures for potato diseases caused by *S. subterranea.*

## Pathogen morphology, genetics, and diversity

*Spongospora subterranea* is an obligate biotroph and as such requires a living host to complete its life cycle (Karling 1968, and Fig. 2). Motile (primary and secondary) zoospores are the means of initiating infection. Each spherical to ovoid zoospore measures on average 4.77± 0.15 μm in diameter, with short (4.35 ± 0.14 μm) and long (13.70 ± 0.20 μm) flagella attached at the zoospore's posterior (Ledingham 1934; Kole 1954). Zoospores have a swimming pattern faster than that of the bacteria commonly associated with inoculum extracts (Merz 1992). Resting spores provide prolonged survival of *S. subterranea* in soil and infected tubers. Each resting spore measures 4.0 μm in diameter (Osborn 1911; Lawrence and McKenzie 1981), although recently Falloon et al. (2011) observed resting spores collected from Switzerland that were larger (4.3 μm in diameter). Resting spores are aggregated into sporosori, and these structures measure 18–100 μm in diameter (Jones 1978). Sporosori can each contain approximately 150 to 1520 resting spores (Falloon et al. 2011). The chemical properties of the substance that binds individual resting spores is unknown. *Spongospora subterranea* shares some similar biological features to those of *S. nasturtii*, a pathogen of watercress. The longevity of viable resting spores in the soil without host plants has not been precisely determined but there are indications that *S. subterranea* resting spores can survive in natural soils for more than 4–5 years. De Boer (2000) detected viable spores buried for almost four years from an artificially inoculated field soil in Australia.

*Spongospora subterranea* is a phytomyxid species (Bulman et al. 2001) within the plasmodiophorid group, parasitising green plants (Neuhauser et al. 2014). Bulman and Marshall (1998) and Bell et al. (1999) initiated genetic characterisation of *S. subterranea* by sequencing the ribosomal transcribed spacer (ITS) DNA regions of the pathogen, which led to the classification of two *S. subterranea* groups (Type I and II). Qu and Christ (2004) found the same group types but in contrast to earlier characterisation (Bulman and Marshall 1998), they noted that North American *S. subterranea* DNA samples were Type II. In addition to the existing two types, Osorio et al. (2012) proposed a third group (Type III) based on the ITS rDNA sequences of 127 *S. subterranea* collected from four Colombian provinces. The diversity of South American isolates was further characterised in the work of Gau et al. (2013). They found that the more virulent collections (now spread worldwide) were nearly

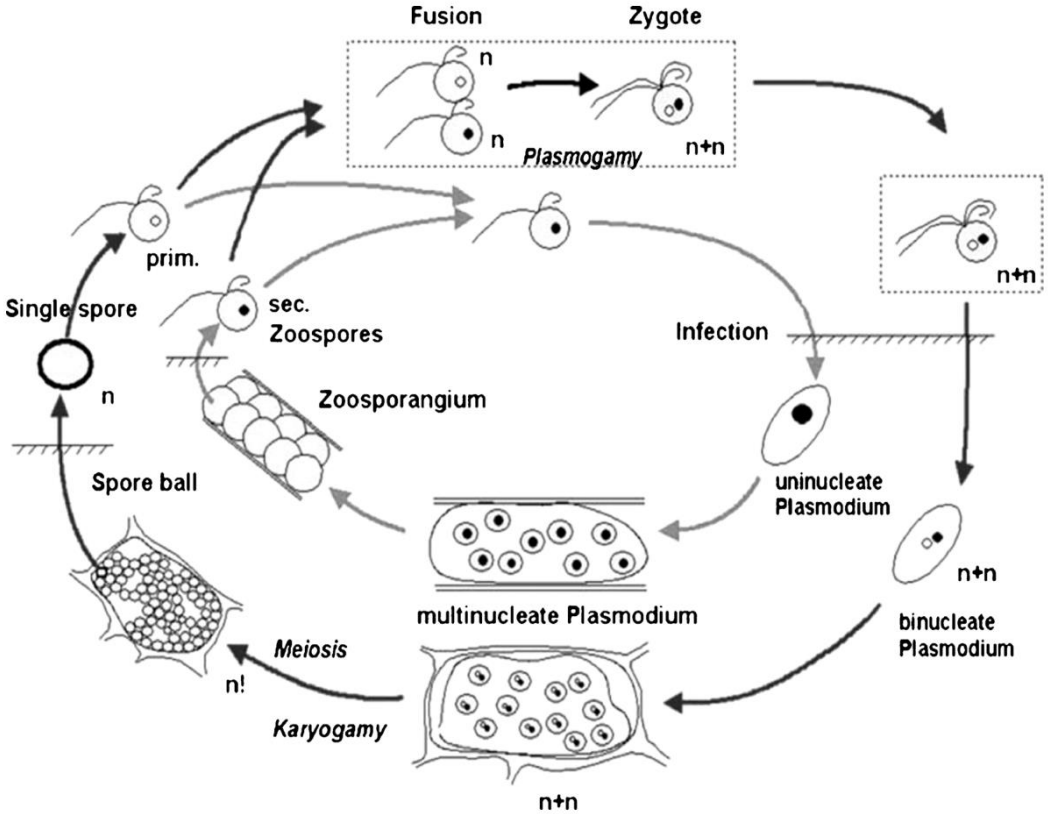


**Fig. 2** Tentative life cycle of *Spongospora* species with an asexual phase (inner circle) and a sexual phase (outer circle). Reproduced from Merz U. (2008). Powdery scab of potato - occurrence, life cycle, and epidemiology. American Journal of Potato Research 85:241–246 with permission of Springer

clonal, in contrast to the more diverse collections that are found in South America (Gau et al. 2015). Among the most significant developments on the genetics of *S. subterranea*, to date, is the complete sequencing of the pathogens mitochondrial (mtDNA) genome (Gutiérrez et al. 2014a). The mtDNA sequence of *S. subterranea* is the second complete mtDNA genome sequence of a cercozoan and the first of a plasmodiophorid. This sequence will be useful to further validate phylogenetic relationships of plasmodiophorids. Other new findings include the discovery of detailed non-long term repeat (non-LTR) retrotransposons (Bulman et al. 2011), and additional cDNA sequences (Burki et al. 2010) and RNA sequences of *S. subterranea* (Schwelm et al. 2015).

## Pre infection events

Resting spores each possess a triple wall (Lahert and Kavanagh 1985) functioning as a protective structure which enables the enclosed zoospores to tolerate unfavourable or extreme environmental condition (Harrison et al. 1997) for survival in the soil for many years. Resting spores of *S. subterranea* will tolerate passage through the digestive canal of farm animals (Pethybridge 1911; Morse 1914). Resting spores are however sensitive to the soil environment where physical (e.g. heat), biological (e.g. host root exudates), and chemical (e.g. chemical nutrients) factors may stimulate germination (release of short-lived primary zoospores) which if in absence of a suitable host will perish leading to reduced soil inoculum. Despite their importance on the epidemiology of root infection and powdery scab, knowledge of the specific factors stimulating resting spore germination and zoospore release is limited. Moreover, studies on the factors which influence the movement or attraction of zoospores towards the host (taxis) have not been conducted.

### Zoospore release

Germination of resting spores within the soil is the critical first step in pathogenesis (Melhus et al. 1916; Dorojkin 1936; Christ and Weidner 1988; Merz et al. 2012). Soil enviornmental factors are important for both resting spore germination and zoospore migration to host roots. The presence of adequate soil moisture, a favourable cool soil temperature, and an external chemical stimulus aid the release of primary zoospores from resting spores (Kole 1954; Merz 1989; Harrison et al. 1997; Merz 1997; Sparrow and Wilson 2012) although little is known of the nature of the chemical stimulus. Zoospores require free water to germinate and move through the soil solution (Kole 1954; Braselton 2001). Zoospore movement towards hosts root increases in presence of abundant water (Wale 1987) which is reflected in increased tuber disease incidence in soils with high water retention capacities (Prentice et al. 2007). Irrigated (Taylor and Flett 1981; Kirkham 1986; Adams et al. 1987; Jellis et al. 1987; Wale 1987) and water-logged conditions (Mol and Ormel 1946; Hughes 1980; Anonymous 1984; Parker 1984) are associated with high incidence of powdery scab on tubers. Cool temperatures of 9–17 °C favour the release of zoospores both in aqueous solutions (Fornier et al. 1996) and in the soil (Van De Graaf et al. 2005; Shah et al. 2012). Higher temperatures at a non-destructive range, however, can stimulate the germination of mature resting spores. For example, heating sporosori-infested soil for 8 days at 20 °C or for 2 days at 40 °C increased zoospore release and subsequent powdery scab severity (Kole 1954). It is unknown if heat can stimulate zoospore release of all sporosorus inoculum from different sources and of different maturities.

Host phytochemical compounds, released as root exudates probably play a role in the stimulus of *S. subterranea* resting spore germination (Harrison et al. 1997; Merz 2008; Merz and Falloon 2009) as they do for other soil-borne pathogens (Schroth and Hildebrand 1964; Nelson 1990; Suzuki et al. 1992). Merz (1989, 1992, 1997), conducted studies on the behaviour of *S. subterranea* zoospores in relation to host roots and suggested that root exudates stimulated zoospore release. He observed that the addition of bait-plants resulted in root infection and further suggest that resting spores do not exhibit dormancy with spores germinating in the presence of a host (Merz 1993). It is, however, unclear if all resting spores germinate when induced by presence of plant roots and perhaps dormancy may still occur in a proportion of the resting spores within a population (staggered dormancy). In the plasmodiophorid *P. brassicae*, both dormant and non-dormant resting spores exist and the mechanism of germination may differ. Non-dormant spores require only a favourable environment whilst dormant spores additionally require an external stimulus for germination (Hata et al. 2002; Ohi et al. 2003; Rashid et al. 2013). In practical terms, as pathogenesis follows a polycyclic pattern of infection, germination of only a proportion of resting spores will be sufficient to initiate root infection and subsequent disease while presence of spore dormancy will assist in pathogen inoculum longevity in the soil (Ogawa et al. 2001).

Fornier et al. (1996) have demonstrated in vitro that susceptible-host root exudates stimulate resting spore germination of *S. subterranea* by comparing the rate of zoospore release, using ELISA and microscopy (direct counts), following incubating of sporosori in distilled water and root exudate solutions. They found greater numbers of zoospores in root exudate solution than in distilled water after incubation at 15 °C for 5 days. Conversely, in three out of eight experiments, a suppressive effect of tomato root exudates (an alternative host of *S. subterranea*) caused a significant reduction in zoospore number compared to the distilled water control (Fornier et al. 1996). Both Merz (1993) and Fornier et al.

(1996) agreed that the root exudate factors stimulating resting spore germination (zoospore release) are non-host specific, similar to that of *P. brassicae* (Suzuki et al. 1992; Friberg et al. 2005) and *Pythium* sp. (Nelson 1990), because non-host plants may also stimulates zoospore release. It appears that the stimulatory or inhibitory effects of root exudates on *S. subterranea* resting spores depends on the phytochemical stimulant-constituent in the root exudates because this is the only factor that differentiates the root exudates which have been used. The biologically active chemical components of these root exudates is still unknown. In *P. brassicae*, resting spore germination stimulants are heat stable, fairly polar and low-molecular weight compounds (Suzuki et al. 1992; Kowalski 1996).

Of note, Merz (1997) showed that Hoagland's solution, commonly known as nutrient solution, can stimulate *S. subterranea* zoospore release. Merz (1997) prepared a pulse of zoospore inoculum by incubating sporosori in Hoagland's solution for ten days prior to using the inoculum in a tomato root-bait test. There was no direct observation or quantitation of zoospores in Hoagland's solution before tomato bait-plants were added, but the short time (5 h) required for observation of zoospore encystment on tomato roots indicated that zoospores may have been released in the solution prior to addition of the bait plants. Fornier et al. (1996); Harrison et al. (1997) and Balendres et al. (2014) reported that zoospores are initially released, in vitro, 4–5 days after the resting spores are exposed to water or to root exudates which stimulates zoospore release. A nutrient solution similarly stimulates germination of *P. brassicae* resting spores in the absence of its host (Asano et al. 2000; Friberg et al. 2005). Like root exudates, it is possible that only one or a few of the components of the Hoagland's solution has stimulatory effect and thus, requires assessment of individual Hoagland's solution components to identify the compound(s) responsible for stimulating *S. subterranea* zoospores.

### Zoospore taxis

Upon release, zoospores must travel through the soil water solution to host roots. Zoospores are short-lived (Harrison et al. 1997) and can travel only a short distance (Harrison et al. 1997) which means they must be in close proximity to their host and either possess an efficient means to locate their host or be produced in such abundance that random contact occurs frequently. There lacks a precise description on how *S. subterranea* zoospores find their way to their target host. Merz (1997) has shown encystment of zoospores in tomato bait plant roots, but it was unclear whether these zoospores were attracted to the roots by chemotactic response (movement stimulated by presence of a chemical gradient) or whether this occurred by chance through close proximity to host roots. Studies on zoosporic fungi (Chi and Sabo 1978; Zentmeyer 1961; Dukes and Apple 1961; Hickman and Ho 1966; Islam and Tahara 2001) and nematodes (Zuckerman and Jansson 1984; Rasmann et al. 2012; Hwang et al. 2015), strongly suggest that motile infective biological agents are attracted to roots via compounds in root exudates. For example, *Phytophthora* spp. zoospores are attracted to certain host isoflavones such as prunetin (Hosseini et al. 2014).

Recognition of the host phytochemicals by zoospores must involve activation of signalling pathways. The zoospores of the oomycete *P. sojae* are attracted to host roots by specific isoflavones (Hua et al. 2008; Hosseini et al. 2014). In this system, interaction of the attractant chemical with the pathogen zoospore results in a $Ca^{2+}$ influx activating signalling pathways involving heterotrimeric G proteins (Hua et al. 2008). Calcium and calmodium-regulated protein kinases are induced during zoospore release in *P. sojae*. Calcium channel blockers and protein kinase inhibitors suppressed zoospore release and root encystment (Judelson and Roberts 2002). Similarly, other studies with oomycetes have shown that swimming patterns may be influenced by $Ca^{2+}$, which regulates the modulation of flagella (or cilia) beat patterns in other eukaryotic cells (Bloodgood 1991; Wheeler et al. 2006). Addition of calcium channel blockers (e.g. $La^{3+}$), calcium chelators (e.g. EGTA), inhibitors of the calcium-binding protein calmodulin (e.g. trifluoperazine) and other compounds that interfere with intracellular calcium levels (e.g. caffeine) alters swimming patterns and removes responsiveness to chemotaxic attractants (Donaldson and Deacon 1993). $Ca^{2+}$ also plays a central role in autonomous encystment and adhesion of zoosporoic oomycetes (Kong et al. 2010). In *P. brassicae*, increased soil calcium (and increased soil pH) inhibits infection and disease development (Donaldson and Deacon 1993). High soil calcium levels may reduce the viability of resting spores, and probably interfere with zoospore motility (Donald and Porter 2009). This is not the case in *S. subterranea* as addition of soil calcium or increasing soil pH has not been found to affect disease (Harrison et al. 1997). Nonetheless, the association of ion influx and signalling has not been fully elucidated. Since they play an important role in taxis and encystment of zoospores, understanding the role of ion influx and signalling to *S. subterranea* and disease development could be advantageous. Upon release and after chemotactic responses, *S. subterranea* zoospores infect host roots within 5 h (Merz 1997) or 2 days (Qu and Christ 2006a) after exposure to host roots.

## Infection and disease development

Once zoospores have reached the plant (root or tuber) surface, the susceptibility of the host becomes a critical criterion for the establishment of infection. Successful zoospore infection of tubers leads to powdery scab whilst in roots lead to root

zoosporangial formation (internal symptom) and root galling. The disease cycle is illustrated in Fig. 3.

### Host recognition and encystment

Zoospores encyst to the outside of the host and penetrate the cell walls using a specialised 'Rohr' and 'Stachel' structure (Keskin and Fuchs 1969), common to most plasmodiophorid species, following which the contents of the zoospores enters the cells (Williams 1970; Kageyama and Asano 2009). It is understood that successful zoospore penetration requires some degree of host susceptibility which alongside soil environmental conditions (Merz et al. 2012) and crop agronomic treatments (Shah et al. 2014) dictates the rate of epidemic development (Brierley et al. 2013). In susceptible hosts, the greater the inoculum potential in the soil or on the seed tubers the higher the risk of the disease (Qu and Christ 2006a; Nakayama et al. 2007; Brierley et al. 2013; Tegg et al. 2015).

Binding to the host cell requires specific interaction between pathogen and the host cell wall. In the Oomycetes, host cell surface components are important in enabling induction of encystment to the host tissues. Various plant polymers have

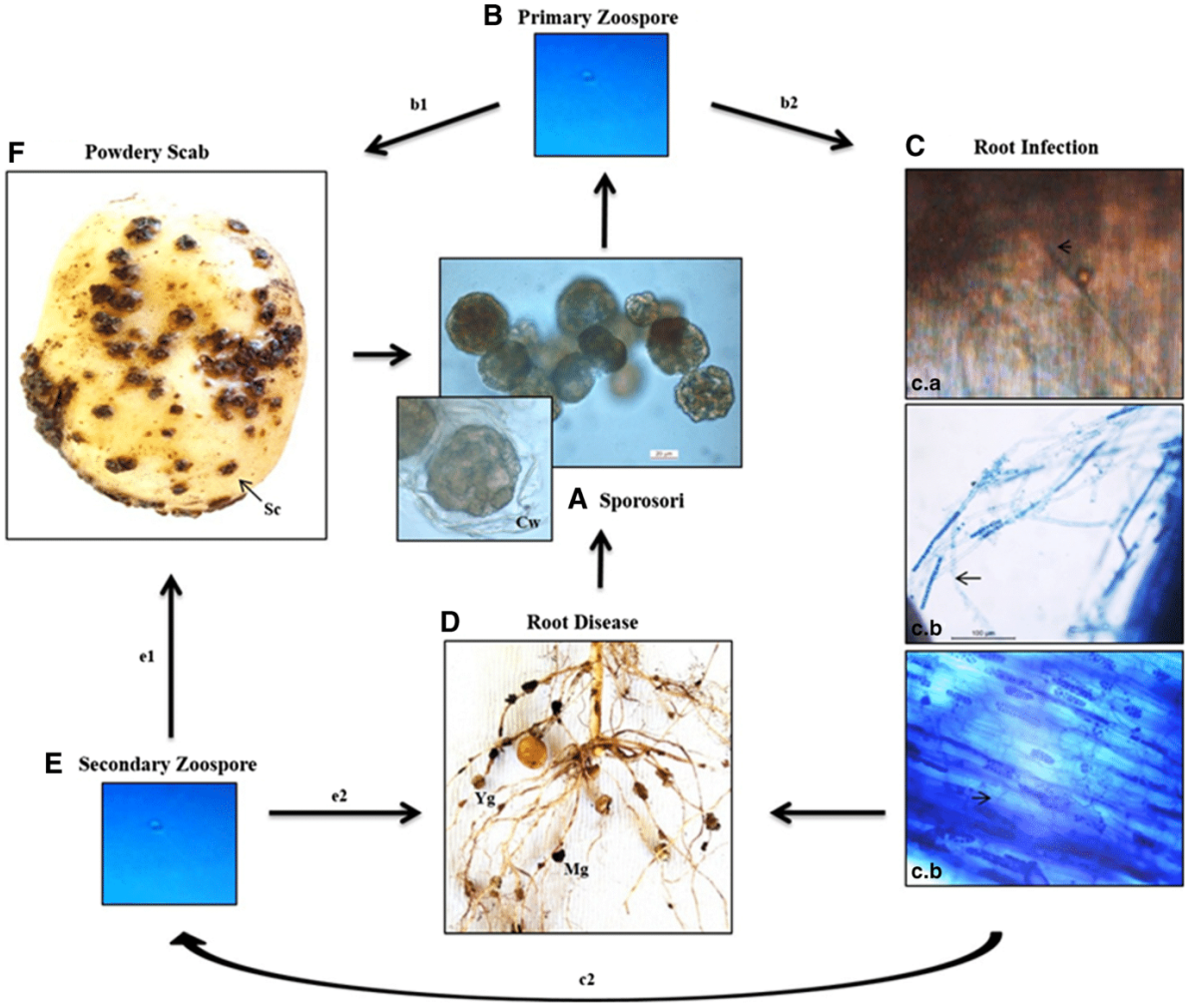


**Fig. 3** The *Spongospora subterranea* f. sp. *subterranea* disease cycle. The sporosori (**A**) release biflagellate heterocont primary zoospores (**B**) which then infect the tubers (**b1**) and/or the roots (**b2**). In the roots (**C**), zoospore attaches and encysts on roots (**C.a**), infection results to zoosporangia that are microscopically apparent in root hairs (**C.b**) and epidermal tissues (**C.c**). Symptoms of root disease (**D**) are browning of roots and formation of young creamy-white galls (**Yg**) – containing many sporosori. When galls mature (Mg) and rot, the sporosori (**A**) are released back into the soil. A cross section of a gall shows sporosori are wrapped in cell wall compartments (Cw). After discharge from zoosporangia (**c2**), secondary zoospores (**E**) infect the tubers (**e1**) leading to tuber "powdery scab" (**F**) and can re-infect the roots (**e2**) causing root diseases (**D**). Tuber scab lesions (**Sc**) contain sporosori (**A**) that are released into the soil when the tuber periderm ruptures

been found to induce encystment with some suggestion of specific recognition for certain pathogens (Hardham and Suzaki 1986). Falloon et al. (2015) suggested that host resistance to diseases caused by *S. subterranea* is likely to be influenced by different host genetic, biochemical or morphological factors such as the host cell walls. The cell wall components involved, if there is such, in *S. subterranea* zoospore recognition are not known nor is it known whether these have a role in cultivar resistance to disease expression.

### *Root "zoosporangia" infection*

Following zoospore encystment and root hair infection a multinucleate plasmodia forms within infected tissue (Braselton 1992). The plasmodium increases in size and forms into zoosporangia that each contain a nucleus (Kole 1954; Karling 1968; Hims and Preece 1975). Zoosporangia reaches maturity 4–5 days after infection (Merz 1989). Secondary zoospores are formed from the subsequent division of the nucleus (Lahert and Kavanagh 1985; Clay and Walsh 1990).

Zoosporangia is a common sign of infection in alternative hosts of *S. subterranea* (Jones and Harrison 1969; Jones and Harrison 1972; De Boer and Theodore 1997; Andersen et al. 2002; Arcila et al. 2013; Iftikhar and Ahmad 2005; Qu and Christ 2006b; Nitzan et al. 2009, Shah et al. 2010). Zoosporangia develops on root hairs and cortical cells (Lagerheim 1892). Infections are not restricted to dicotyledonous plants as Monocots are also susceptible. There are currently 28 plant families that have been reported susceptible to root "zoosporangia" infection. The Solanaceae (19 species), Poaceae (11 species) and Asteraceae (9 species) families have had the most number of susceptible plant species.

Secondary zoospores released into the soil infect stems (Link and Ramsey 1932), stolons (Salzmann 1950; Boyd AEW 1951) and tubers and can re-infect the roots. Resting spore formation does not occur during the zoosporangial stage in the life cycle but the increasing zoospore number following re-infection of secondary zoospores in the roots would likely increase the potential of two zoospores fusing into a binucleate zoospore. Root infection caused by binucleate zoospores may lead to sporosori development (Tommerup and Ingram 1971; Braselton 2001).

### Root gall formation

Galls (Fig. 3D) are produced after the pathogen completes the sporogenic stage. Tommerup and Ingram (1971) described how these resting spores are formed in the plasmodiophorid species *P. brassicae*. Accordingly, two uninucleate zoospores fused (now binucleate) and infect the host roots. Following infection, binucleate and multinucleate plasmodia (Braselton 1992; Braselton 2001) are formed. Karyogamy within the plasmodium occurs and meiosis follows. Finally, the plasmodial cytoplasm cleaves to give rise to haploid resting spores. To date, however, the final process of the fusing of two nuclei (karyogamy) has not been clearly documented (Braselton 1995). In *S. subterranea*, Kole (1954) has illustrated quadriflagellate zoospores as possible results of fusions between two biflagellate zoospores. Whilst galling (root hypertrophy) is a possible reaction to the rapid proliferation of sporosori within the infected host roots, such as occurs with *P. brassicae* (Kageyama and Asano 2009), the presence of galls do not indicate that resting spores are produced. In the United States (Qu and Christ 2006b), some alternative hosts (e.g. Eastern black nightshade and Penlate Orchard grass) of *S. subterranea* produces galls which do not contain resting spores. Conversely, galls may not indicate that *S. subterranean* has completed its life cycle. In Colombia Arcila et al. 2013 claims that some alternative hosts (e.g. Tamarillo, Kikuyu grass) do not produce galls but sporosori are still formed within infected root hairs.

Roots are susceptible to infection throughout the growth cycle of the host plants (Thangavel et al. 2015). As few as 1–10 sporosori g soil$^{-1}$ can initiate severe root infection (Shah et al. 2012). Root infection and subsequent root galling is favoured by soil temperatures of 12–17 °C (Van De Graaf et al. 2005) and 11–25 °C (Kole 1954; Van De Graaf et al. 2005; Van De Graaf et al. 2007), respectively. The galls, containing sporosori, are creamy-white to brown. Sporosori are released back into the soil when host roots and galls decay or when detached from the roots during early harvest.

### Tuber infection

Early symptoms of powdery scab are small, purple-brown, pimple-like swellings that later increase in size and when matured, rupture the tuber periderm (Osborn 1911; Lawrence and McKenzie 1981). Unlike roots, tubers are only susceptible to infection during a defined period early in their growth when they are actively elongating (0–6 weeks after tuber initiation (Hughes 1980; Van De Graaf et al. 2007)). Infection is favoured by soil temperatures of 9–17 °C but is most severe at 12 °C (Hughes 1980; Van De Graaf et al. 2005; Shah et al. 2012; Van De Graaf et al. 2007). The shape of the lesions vary, from round to irregular as they coalesce when abundant (Hughes 1980; Van De Graaf et al. 2007). Powdery scab symptoms can be confused with those of common scab (caused by *Streptomyces* spp.) but can be distinguished morphologically, by experienced disease inspectors. Detection of abundant sporosori by microscopic examination of lesions will confirm powdery scab (Obidiegwu et al. 2014; Bouchek-Mechiche and Wale 2014).

### Molecular host-pathogen interaction

Information on host-pathogen interactions at the molecular level is limited. The few available studies have provided insights of the genes involved during the interaction of *S. subterranea* and its hosts. Rodríguez et al. (2014) showed that methalothionein, phosphate 2C, and pectin methylesterase inhibitor genes were overexpressed in an infected susceptible *S. phureja* plant, and two different genes (one associated with α-Galactosidase) were transcribed 1000 times more than within a non-inoculated plant. The study was carried out using the diploid *S. phureja* and, therefore, warrants a separate analysis of these genes in the tetraploid *S. tuberosum.* Another study (Perla et al. 2014) on potato cultivars Mesa Russet, Centennial Russet and Russet Nugget which had shown resistance to powdery scab in repeated glasshouse experiments, found that the level of LOX (Lipoxygenase) gene expression was positively correlated with the russet skinned-genotypes and negatively correlated with tuber disease severity index. The LOX gene is also upregulated in host plants during the penetration of *P. brassicae* zoospores (Agarwal et al. 2011) and in root-knot nematode feeding sites (Gheysen and Fenoll 2002). Further studies on metabolic pathways (Bittara 2013) and gene expression (Gutiérrez et al. 2014b) have been reported.

## Future research

Characterisation of root exudates that stimulate resting spore germination and/or chemotactically attract zoospores to the host roots will provide important knowledge on the biology of the host-pathogen interaction. Characterised compounds may offer novel methods for control of *S. subterranea* infection by reducing soil inoculum level (stimulating zoospore release in the absence of the host) and reducing inoculum potential (inhibiting zoospore release). Moreover, if there are compounds that are responsible for chemotactic attraction there might also be compounds that cause repulsion. Compounds that repel zoospores offer a huge potential in the management of diseases caused by *S. subterranea* by disrupting zoospore movement towards the host. This approach has been well documented for nematode control (Chitwood 2002). Because the characterisation of these compounds are crucial, appropriate and effective analytical techniques will be needed to identify the compounds in the biologically-active root exudates. Previous research has characterised compounds (in biofluids) using a variety of techniques (e.g. inductively coupled plasma atomic emission spectroscopy, Friberg et al. 2005), but recent improvement in high-throughput analytical technologies provides more sensitive, rapid and selective detection. One such technology includes liquid chromatography coupled to mass spectrometry (LC-MS) and various improvements (such as ultra-high performance LC-MS) which can simultaneously detect more than a hundred low-molecular weight analytes (Gika et al. 2012). Specific compounds or phytochemicals will still need to be individually tested for their biological activity to *S. subterranea* resting spores (stimulation or inhibition) and zoospores (chemoattractant or repellent) and this will require appropriate bioassay methods.

Whilst the potato genome is large, with 844 megabases and as many as 39,031 protein-coding genes (The Potato Genome Consortium 2011), advances in molecular biology techniques coupled to powerful bioinformatics, statistics, data mining and network analysis software have provided tools which can assist in the study of the interaction of *S. subterranean* and potato at molecular level. Identification and characterisation of host genes associated and expressed during infection by *S. subterranea* would facilitate the elucidation of mechanisms of the compatible host-pathogen interaction, root gall and tuber lesion formation, and identification and characterisation of host defence processes and putative (*R*) genes. Gene sequences already available (Bulman et al. 2011; Rodríguez et al. 2014) can be further annotated for their structure and functions. Whilst it is expected that multiple (*R*) and susceptible (*r*) genes may be involved, quantitative trait loci (QTL) mapping and association analysis will be essential to characterise the gene(s) controlling resistance. Comparative proteomics have been successful in identifying 46 proteins that were differentially expressed between *P. brassicae* infected *Arabidopsis thaliana* and non-infected roots (Devos et al. 2006; Siemens et al. 2006; Siemens et al. 2009), and hence, a similar technique may be used in *S. subterranea.* Analysis of the roles of differentially expressed proteins will provide insight into pathogenesis processes.

## Concluding remarks

This review has focused on the empirical observations of the pre- and post-infection events of *S. subterranea* in potato. It discusses current understanding on how physical, biological, and chemical factors contributes to pre-infection processes and what changes occur, at the molecular level, during the post-infection stage. Pre-infection events are influenced by soil environmental factors and soil-chemical additives but available literature indicates that host factors involving root exudate phytochemicals have important roles which requires further elucidation. The review has highlighted the knowledge gaps in this pathosystem that requires further elucidation and research. There is much work that needs to be done to clarify the existence of dormancy within resting spores, to validate the longevity of resting spore survival in the soil in the absence of hosts and to identify the mechanisms of inoculum propagation in alternative host plants. Whilst work on pathogen genetics has progressed, signalling pathways and genetic expression of *S. subterranea* during zoospore release (resting

spore germination) and taxis are still unknown. Genes associated with metabolic pathways active during gall formation and resting spore production also needs to be identified to understand the genetic factors that triggers pathogen development in plants and the nature of spore production particularly in alternative host plants that do not form root galls. The lack of knowledge on the process of host recognition by zoospores is also evident and this impedes our understanding of the mechanisms of resistance by potato genotypes to infection. The identification of host receptor molecules that enable recognition of the pathogen and facilitate encystment would be valuable. Filling these knowledge gaps will significantly improve our understanding of the *Spongospora*/*Solanum* pathosystem which will contribute to new management options for the potato diseases caused by *S. subterranea.*

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Appendix 1c – Balendres et al. 2016b

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Article

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# Metabolomes of Potato Root Exudates: Compounds That Stimulate Resting Spore Germination of the Soil-Borne Pathogen *Spongospora subterranea*

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**ABSTRACT:** Root exudation has importance in soil chemical ecology influencing rhizosphere microbiota. Prior studies reported root exudates from host and nonhost plants stimulated resting spore germination of *Spongospora subterranea,* the powdery scab pathogen of potato, but the identities of stimulatory compounds were unknown. This study showed that potato root exudates stimulated *S. subterranea* resting spore germination, releasing more zoospores at an earlier time than the control. We detected 24 low molecular weight organic compounds within potato root exudates and identified specific amino acids, sugars, organic acids, and other compounds that were stimulatory to *S. subterranea* resting spore germination. Given that several stimulatory compounds are commonly found in exudates of diverse plant species, we support observations of nonhost-specific stimulation. We provide knowledge of *S. subterranea* resting spore biology and chemical ecology that may be useful in formulating new disease management strategies.

**KEYWORDS:** *powdery scab, root infection, Plasmodiophorid, HILIC UPLC-MS, zoospore release, metabolomics*

## INTRODUCTION

*Spongospora subterranea* (Wallr.) Lagerh. causes powdery scab and root disease in potato.[1] *Spongospora* diseases are major problems in potato production worldwide. The pathogen downgrades the tuber fresh market value, causes substantial economic losses in the potato processing industry,[2] and leads to failure of seed tuber certification and subsequent losses in product value for seed potato producers.[3] Primary zoospores initiate infection after release from germinating resting spores that are formed into aggregates known as sporosori.[4] Zoospores migrate to host roots, where they encyst and establish infections[5] which develop into plasmodia and may mature as zoosporangia or resting spores.[6] Zoosporangia contain secondary zoospores which are sources of secondary infection in tubers and roots after release into the soil environment. When resting spores are released back into the soil they become the new generation of primary inoculum. As such, the germination of *S. subterranea* resting spores is the first crucial step in disease development. Despite its importance, knowledge of the processes and the factors influencing resting spore germination is limited.[7] Root exudates play a critical role as stimulants of *S. subterranea* resting spore germination.[4,8,9] Prior studies report a lack of host specificity with exudates from both hosts and nonhosts capable of stimulating germination. However, no attempts have been made to identify any stimulatory compound(s) present in the root exudates.

An estimated 40−50% of the carbon fixed by plants is released as root exudates, mostly as low molecular weight organic (LMWO) compounds, making root exudation a significant carbon cost to the plant.[10,11] Root exudates play important roles in rhizosphere chemical ecology, influencing interactions between the plant and other soil biota. They may function as, but are not limited to, chemoattractants or inhibitors or stimulants of microbial growth[10] and may influence the colonization and activation of root-infecting pathogens.[12,13] The response of soil microbes may vary depending on the root exudate's organic composition. For instance, peanut root exudates differing in LMWO compound composition have varying influence on spore germination of soil-borne peanut pathogens.[14] The stimulatory effects of potato root exudates to *S. subterranea* resting spore germination might also be explained by their organic chemical composition.

Characterization of the root exudate metabolome can assist in gaining a better understanding of plant-microbial interactions. The choice of techniques to be used in metabolomic studies is important to achieve unbiased and successful characterization of a metabolome. Metabolite analysis of aqueous samples, including root exudates, generally uses gas or liquid chromatography which is usually coupled to mass spectrometry (MS). Gas chromatography is more suitable for volatile compounds, while liquid chromatography (LC) is commonly used for nonvolatiles. Recently, new improvements have been made to LC techniques, particularly for detecting polar, hydrophilic compounds. An improved hydrophilic interaction ultraperformance liquid chromatography mass spectrometry (HILIC UPLC-MS) approach has been used to simultaneously identify more than 100 analytes from grapefruit extracts in less than 30 min at detection levels as low as 5 ng/ml.[15]

Received: August 31, 2016
Revised: September 12, 2016
Accepted: September 18, 2016
Published: September 19, 2016





DOI: 10.1021/acs.jafc.6b03904
J. Agric. Food Chem. 2016, 64, 7466−7474

The present study examined metabolomes of potato root exudates to gain insight into their metabolite composition and to determine if specific chemical constituents stimulate *S. subterranea* resting spore germination. The bioassays were performed in a soil-less in vitro system[9] coupled with microscopy, and the targeted characterization of compounds in the root exudates was achieved using HILIC UPLC-MS methodology.[15] This work advances the understanding of the biology of *S. subterranea* resting spore germination and provides new insights into the pathogen's chemical ecology. Furthermore, the primary metabolic composition of potato root exudates is outlined, expanding known metabolite constituents. Potato genotypes used in this study have importance in potato breeding programs and in commercial production. Hence, the metabolite profile may compliment other "omics" approaches for potato improvement.

## MATERIALS AND METHODS

**Potato Root Exudate Collections.** Root exudates were collected from deionized water extracts of tissue-cultured potato (*Solanum tuberosum*). Four potato cultivars that varied in their known resistance to *S. subterranea* diseases were used. Cultivars 'Agria' and 'Iwa' are highly susceptible to both tuber and root disease, while cvs 'Russet Burbank' and 'Gladiator' have moderate to strong resistance to tuber infection and moderate resistance to root infection.[1,16] Preparation and collection of potato root exudates were performed aseptically. Potato tissue-cultured plantlets were grown in potato multiplication medium (MS salts and vitamins, 30 g/L of sucrose, 40 mg/L of ascorbic acid, 500 mg/L of casein hydrolysate, and 0.8% agar, pH 5.8) under a 16 h photoperiod using white fluorescent lamps (65 $\mu$mol/m$^2$/s) at 22 °C.[17] After 2 weeks, plants were gently uprooted from the media, and their roots were washed with sterile deionized water, blotted dry on sterile tissue paper, and then placed in a sterile polycarbonate bottle containing 20 mL of fresh sterile deionized water (instrument conductivity at 0.059 $\mu$S/cm). For exudate collection, plants were incubated in the water solution under the same light and temperature regime as before for varying incubation periods.

First, to confirm the capability of potato root exudates to stimulate resting spore germination and to assess possible influences of cultivar, a series of nine bioassays comparing 32 individual potato root exudates was conducted. Either 2 ('Gladiator' and 'Agria') or all 4 cultivars were incubated in the water solution for 7 d prior to collection and testing. Then, for analysis of root exudate composition and association with stimulation of resting spore germination, a further 12 exudate solutions were collected. Exudates from all four cultivars were collected following 2, 7, and 18 d incubation in the water solution. All exudate solutions were stored in sterile Falcon tubes at −20 °C in the dark until use. There were no signs of microbial contamination in the root exudate solutions prior to use. Unless otherwise stated, all chemicals used in this study were sourced from Sigma-Aldrich (St. Louis, MO).

**Inoculum Preparation.** The *S. subterranea* inoculum (resting spores) was prepared from powdery scab-affected tubers collected from Devonport, Tasmania, Australia (41.17 °S, 146.33 °E). Diseased tubers were washed with running tap water for 1−2 min, soaked in 2% sodium hypochlorite (White King, Pental Products Ltd. Pty, Melbourne, Australia) for 3 min, quickly rinsed, and air-dried. Lesions were excised using a scalpel, dried for 4 d at 40 °C, ground by mortar and pestle, and stored at 4 °C until use. The inoculum was approximately 1 y old when used. Inoculum contained approximately 6900 sporosori/mg, as determined by suspending 0.1 g of inoculum in 10 mL of water and quantification using light microscopy.

**Resting Spore Germination Assay.** Presence of motile zoospores in the test solutions indicated resting spore germination.[9] Briefly, 1 mg of inoculum (6900 sporosori) was suspended either in 1.5 mL of root exudate, individual compound solutions (0.1 mg/ml), or in deionized water (control) solutions, each in a 2 mL microcentrifuge tube covered with aluminum foil. Tubes were incubated for 30 d at 15−18 °C. At 3−7 d intervals, 35 $\mu$L from each treatment was subsampled. The number of motile zoospores in the subsample was determined with a DM 2500 LED microscope (Leica Microsystem, Wetzlar, Germany) at 200× magnification, scanning in an inverted "S" manner within a 22 × 22 mm area of each microscope slide. *S. subterranea* zoospores were identified by morphology[4] and motility behavior.[18] Assessments of each root exudate solution and 43 individual LMWO compounds were repeated, respectively, 7 and 2 times. Each assay consisted of three replications. In the individual chemical bioassay, Hoagland's solution and sterile deionized water were added, respectively, as a stimulant and nonstimulant controls.

Additional bioassays were performed to further validate *S. subterranea* zoospore identity. The first used a modified tomato-bait test.[19] A 100 $\mu$L subsample from each test and control solution was transferred into a McCartney bottle containing 5 mL of nonsterile deionized water and a healthy 2 week old tomato plant (cv. Grape). Tomato plants were left in the solution for 24−48 h at 15−18 °C to allow zoospores to encyst and were then removed. Roots were washed to remove any resting spores adhering to root surfaces. Each plant was transferred to a new McCartney bottle containing 20 mL of nonsterile deionized water and grown for an additional 2 weeks to allow the development of zoosporangia. This test was repeated using different zoospore solution sources. Sample roots were then cut, placed on microscope slides, and stained with 0.1% Trypan blue for at least 10 min, and zoosporangia[20] were examined under a light microscope. In the second test, the number of zoospores successfully attached to *S. subterranea* roots of cv. 'Gladiator' and cv. 'Iwa' host roots was determined. Single, 2 cm long roots were placed on microscope slides, which were flooded with 70 $\mu$L of test solutions and incubated at room temperature for 10 min. Each test was replicated three times. To ensure all roots received a similar exposure to zoospores, the test was performed one root/cultivar/replicate at a time. The number of zoospores attached on the roots was counted microscopically at 200× magnification by scanning the whole root.

**Phytochemical Analyses.** Detection of primary metabolites in potato root exudate solutions was done using HILIC UPLC-MS.[15] The UPLC assays were performed using a Acquity UPLC H-class system (Waters, Milford, MA), and MS analyses were performed using a Xevo triple quadrupole MS system (Waters). HILIC separation was done on a 2.1 × 150 mm Acquity 1.7 $\mu$m BEH amide VanGuard column maintained at 60 °C and eluted with a two-step gradient at 500 $\mu$L/min flow rate for 30 min. The mobile phases were composed of A (acetonitrile/water, 95/5 (v/v), 0.1% formic acid, and 0.075% $NH_4OH$) and B (acetonitrile/water, 2/98 (v/v), 0.2% formic acid, and 0.1% $NH_4OH$). LiChrosolv-grade acetonitrile was purchased from Merck (Darmstadt, Germany), while formic acid (>95%) and $NH_4OH$ were purchased from Sigma-Aldrich. The gradient started with a 4 min isocratic step at 100% mobile phase A, then rising to 28% mobile phase B over the next 21 min, and finally ending at 60% B over 5 min.[15] The column was then equilibrated for 12 min in the initial conditions. Two cycles of weak and strong solvent washing of the injection system were carried out between injections. The injection volume was 10 $\mu$L, and the column eluent was directed to the mass spectrometer. Metabolite detection was achieved using selected ion monitoring, and an electrospray ionization source was applied operating in both positive and negative ion mode. The parameters in the electrospray were set as follows: capillary voltage at −2.5 or 3 kV and cone and desolvation temperatures, respectively, at 150 and 400 °C with a desolvation gas flow of 950 L/h and cone flow of 100 L/h. The cone voltage was optimized for each individual analyte. The identity of metabolites was confirmed by detection of the expected $[M + H]^+$ or $[M - H]^-$ molecules at known retention indices[15] by the analysis of standard chemicals as both pure standards and spiked into root exudates samples. The identities of amino acid metabolites were also confirmed by tandem-MS multiple reaction monitoring experiments with the detection of known product molecules arising from selected precursor molecular species.[15]

**Data and Statistical Analyses.** Zoospore numbers were converted to counts per 100 $\mu$L solution prior to statistical analysis using SPSS statistical software version 22 (IBM, Armonk, NY). An



DOI: 10.1021/acs.jafc.6b03904
J. Agric. Food Chem. 2016, 64, 7466−7474

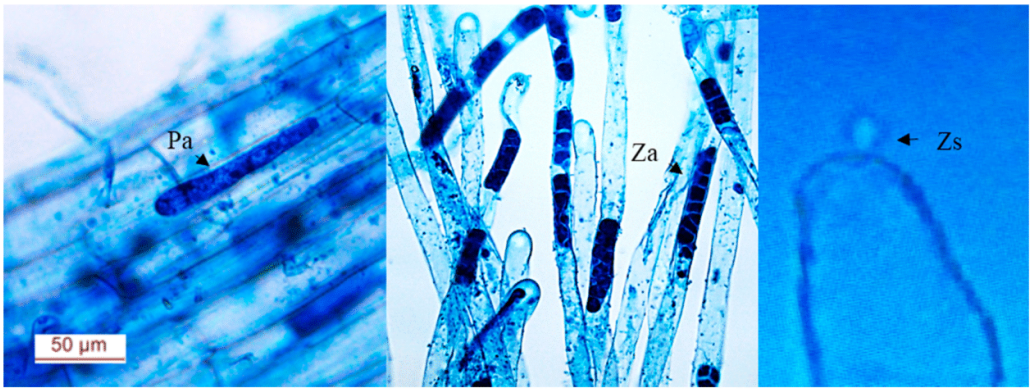


**Figure 1.** Photomicrographs of *S. subterranea* plasmodia (Pa) and zoosporangia (Za) in tomato roots and zoospores (Zs) attached on the potato root, which validates zoospore identity.

independent *t* test and one-way analysis of variance (ANOVA) were performed for data having, respectively, two and more than two treatments. Data containing two factors were analyzed by two-way ANOVA. Multiple comparison of means was carried out using the Fisher's least significant difference (LSD) test at a 0.05 level of probability. HILIC UPLC-MS analyses were done using MassLynx XS software.[15] The limit of detection and limit of quantitation of the analytes were determined at signal-to-noise ratios of 3 and 10, respectively. A hierarchical cluster dendrogram was constructed using the average linkage (between group) method with binary data measured using the squared Euclidean distance. A pattern (color) map was constructed to represent the distribution (numbers) of the metabolite/compound classes detected in various root exudates.

## RESULTS

***S. subterranea* Zoospore Identity.** Motile biflagellate (of unequal length) zoospores of approximately 4−5 μm diameters were observed in the majority of test potato root exudates and occasionally in the control (sterile deionized water) solution. The presence of the two flagella on each zoospore were best observed at 400× magnification, but observation of zoospore swimming patterns was best viewed at 200× magnification. Other uniflagellate and biflagellate organisms were present, but their numbers were minimal, and their movement patterns were distinct from those of the *S. subterranea* zoospores. Figure 1 shows that zoosporangia, indicative of *S. subterranea* infection, developed when test solutions with *S. subterranea* zoospores were exposed to tomato roots. Similarly, zoospores were observed attached to potato roots with the number of zoospores ($P$ = 0.008) attached to roots of the susceptible cv. 'Iwa' (16.67 ± 2.60) greater than those attached to the resistant cv. 'Gladiator' (3.67 ± 0.33; Figure 1).

**Stimulatory Effects of Potato Root Exudates to Resting Spore Germination.** In the first experiment across all nine assays, exposure of resting spores to 25 of the 32 potato root exudates stimulated release of *S. subterranea* zoospores (Figure 2). Zoospores were occasionally released in the control solution (four out of nine assays), but release was substantially later (average first release was at 11.8 d) than that in the potato root exudates (4.4 d). Within individual assays, statistically significant differences in zoospore numbers released were found between potato cultivars, but there was no consistent cultivar response between assays (Figure 2).

In the second experiment, the duration of incubation of potato roots prior to exudate collection (2, 7, and 18 d) had no statistically significant effect ($P$ = 0.152) on stimulation of resting spore germination, and while cultivar showed a statistically significant effect ($P$ = 0.050), there were again no obvious consistent trends (Figure 3). The interaction of incubation period and cultivar had a clear statistically significant effect ($P$ = 0.033) with differences between cultivar most evident in the 18 d incubation samples (Figure 3).

**Metabolite Constituents of Various Potato Root Exudates.** The HILIC UPLC-MS analysis detected a total of 24 LMWO compounds from potato root exudates (Table 1). These compounds ranged from 104 to 504 Da molecular weight and included eight amino acids, one sugar, three sugar alcohols, five organic acids, and seven other organic compounds. Detection of these compounds varied among root exudates. Asparagine, glutamic acid, glutamine, proline, serine, pinitol, choline, trehalose, and tyramine were detected in most of the potato root exudates. However, some compounds were uniquely observed in a particular potato cultivar root exudate or collection date. For instance, raffinose, dehydroascorbic and quinic acids, and adenosine were, respectively, present only in 'Iwa', 'Agria', and 'Gladiator' root exudates, and histamine was detected only in the exudates incubated for 18 d. The hierarchical cluster analysis revealed that potato root exudates could be divided at 80% similarity into three groups based on the metabolite composition (Figure 4). Cluster 1 included all 'Iwa' root exudates, cluster 2 was composed of mostly 2 and 7 d incubation and all 'Russet Burbank' root exudates, and cluster 3 was mostly 18 d incubation and 'Gladiator' root exudates.

**Screening of Metabolites for Stimulants of Resting Spore Germination.** Testing individual compounds provided a more robust direct relationship of each compound to resting spore germination stimulation (Figures 5 and 6). Stimulation of germination of *S. subterranea* resting spores was chemical-specific (Figure 5). By a comparison of capacity and timing of zoospore release, it was determined that 5 of the 18 compounds found in potato root exudates and 7 of the 25 additional compounds tested were stimulant at 0.1 mg/ml, resulting in zoospore release at least 8 d earlier than the water control (Figures 5 and 6). L-Glutamine and tyramine had effects ($P$ = 0.045 and $P$ = 0.010, respectively) on resting spore germination greater than those of the other compounds tested and the Hoagland's stimulant control. Mean accumulated zoospore numbers in other stimulant LMWO compounds (L-rhamnose,



DOI: 10.1021/acs.jafc.6b03904
J. Agric. Food Chem. 2016, 64, 7466−7474

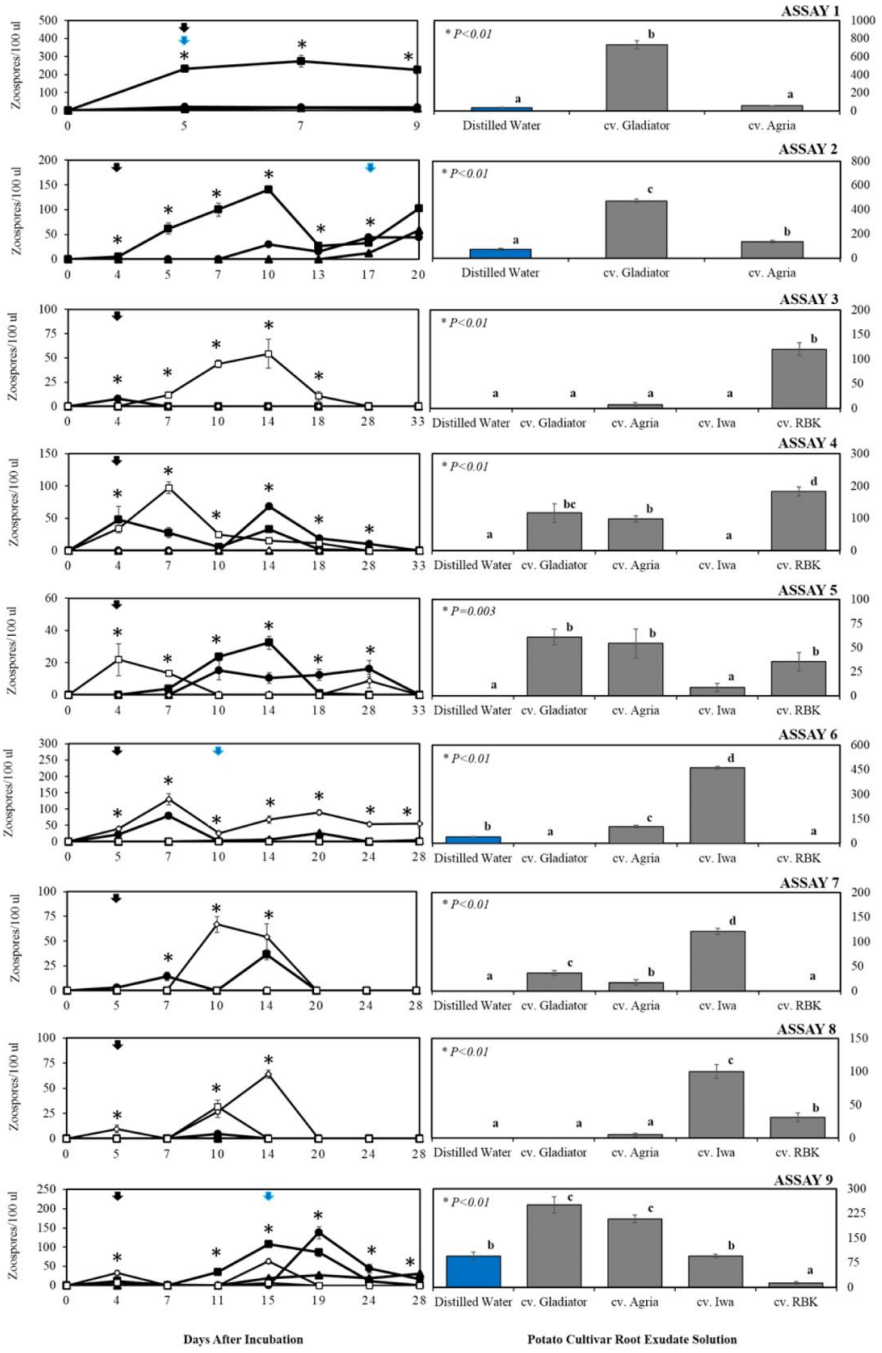


**Figure 2.** Resting spore germination (zoospore release) of *S. subterranea* as influenced by deionized water (▲) and root exudates of potato cultivars 'Gladiator' (■), 'Agria' (●), 'Iwa' (◊), and 'Russet Burbank' (□). Mean zoospore population at different time intervals (line graph, left) and the accumulated population at the end of incubation period (bar graph, right) are represented. Arrows in black and blue indicate initial zoospore release, respectively, in root exudates and deionized water (control). Vertical bars are standard errors ($n$ = 3). Asterisks indicate treatments means within the same day are statistically different ($p$ < 0.05) by analysis of variance. Bars with the same letter within an assay indicate means are not statistically different ($p$ < 0.05) by Fisher's LSD test.



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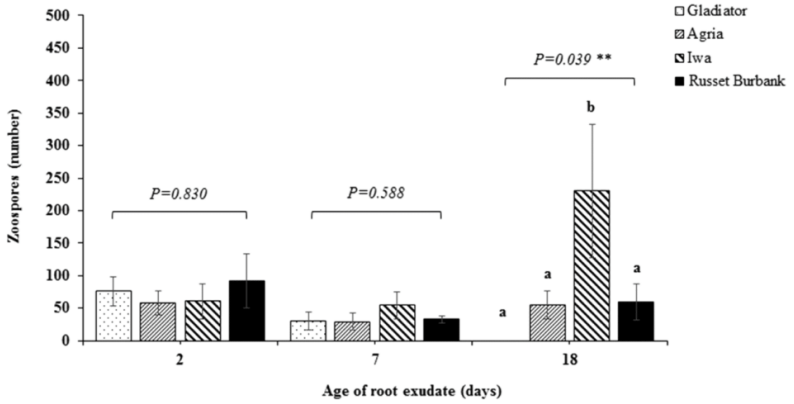


**Figure 3.** Resting spore germination (zoospore release) of *S. subterranea* as influenced by the age (2, 7, and 18 d old) of potato root exudates. Vertical bars are standard error ($n = 6$). Double asterisk indicates that treatment means within a group (indicated by a horizontal bar) are statistically different by analysis of variance. Bars within the 18 d old group annotated with the same letter are not statistically different ($p < 0.05$; Fisher's LSD test). $P$ (incubation time) = 0.152; $P$ (cultivar) = 0.050; $P$ (incubation time × cultivar) = 0.033.

**Table 1. Low Molecular Weight Organic Compounds in Potato Root Exudates from Different Cultivars Detected Using the HILIC UPLC-MS Technique**

| | | | | | 'Gladiator'[c] | | | 'Russet Burbank'[c] | | | 'Agria'[d] | | | 'Iwa'[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| no. | retention time (min) | compound | compound class | $m/z$[a] | 2[b] | 7 | 18 | 2 | 7 | 18 | 2 | 7 | 18 | 2 | 7 | 18 |
| 1 | 1.02 | nicotinamide | Oc | 123 | + | | + | | | | + | | + | + | | + |
| 2 | 1.27 | maleic acid[e] | Oa | 114 | | | | + | + | | | | | | | |
| 3 | 2.76 | dehydroascorbic acid[e] | Oa | 173 | | | | | | | | | + | | | |
| 4 | 2.78 | tyramine[ef] | Oc | 138 | + | | + | + | + | + | + | | + | | | |
| 5 | 2.86 | adenosine[e] | Oc | 268 | | + | | | | | | | | | | |
| 6 | 4.95 | choline | Oc | 104 | + | + | + | + | | + | + | + | + | | | + |
| 7 | 6.75 | pinitol | Sa | 192 | + | | + | + | + | + | + | + | + | | | |
| 8 | 6.99 | *N*-acetyl cysteine[ef] | Aa | 161 | + | | + | | | | | | + | + | | + |
| 9 | 7.45 | spermine[e] | Oc | 203 | + | + | + | | | | + | + | | | | |
| 10 | 10.39 | histamine | Oc | 111 | | | + | | | | | | + | | | |
| 11 | 11.48 | isoleucine[e] | Aa | 132 | | | + | | + | | | + | + | | | |
| 12 | 12.4 | proline[e] | Aa | 116 | + | + | + | | + | | + | + | + | + | | |
| 13 | 13.02 | quinic acid[e] | Oa | 190 | | | | | | | + | | | | | |
| 14 | 13.36 | malic acid[e] | Oa | 132 | | | | | | + | + | | + | | | |
| 15 | 13.90 | threonic acid | Oa | 134 | | | | | | + | + | | + | | | |
| 16 | 14.48 | lactose | Sg | 343 | | + | | + | | + | + | + | | + | | |
| 17 | 14.52 | 3-hydroxy proline[e] | Aa | 132 | | | + | | + | | | + | + | | | |
| 18 | 14.68 | trehalose[e] | Sg | 360 | + | + | + | + | + | + | + | + | + | + | + | + |
| 19 | 16.33 | glutamine[ef] | Aa | 147 | + | + | + | + | + | + | + | + | | + | | |
| 20 | 16.39 | serine[ef] | Aa | 106 | + | | + | + | + | + | + | + | + | | | |
| 21 | 16.59 | asparagine[e] | Aa | 133 | + | + | + | + | + | + | + | + | + | | | |
| 22 | 17.55 | citruline[ef] | Ac | 176 | | | | + | + | | | | | | | |
| 23 | 18.27 | glutamic acid[e] | Aa | 147 | + | + | | + | + | + | + | + | + | | | |
| 24 | 19.58 | raffinose[e] | Sg | 522 | | | | | | | | | | + | | |

[a]Mass/charge ratio of the analyte precursor molecule. [b]Age of the root exudate in days. [c]Cultivar is resistant to powdery scab in the field.[33] [d]Cultivar is susceptible to powdery scab in the field.[33] Aa, amino acid; Sa, sugar alcohol; Sg, sugar; Oa, organic acid; Oc, other organic compound. + indicates compound was detected (present); blank indicates the compound was absent. [e]Indicates compounds tested for *S. subterranea* RSG activity. [f]Indicates positive RSG stimulation activity.

cellobiose, L-aspartic acid, *N*-acetyl cysteine, piperazine, glucoronic acid, L-serine, succinate, L-citrulline, and citric acid) were not statistically different from the nonstimulant water control ($P > 0.05$) but were deemed stimulant as zoospore release in the presence of these compounds was at least 8 d earlier than in the deionized water control (Figure 6).

## DISCUSSION

This study aimed at elucidating, at a metabolite level, the stimulatory effects of potato root exudates on *S. subterranea* resting spore germination. LMWO compounds are the main constituents of plant root exudates and are known stimuli of soil-borne plant pathogens.[12,21] In an initial series of in vitro bioassays, we showed in most instances aqueous potato root



DOI: 10.1021/acs.jafc.6b03904
J. Agric. Food Chem. 2016, 64, 7466−7474

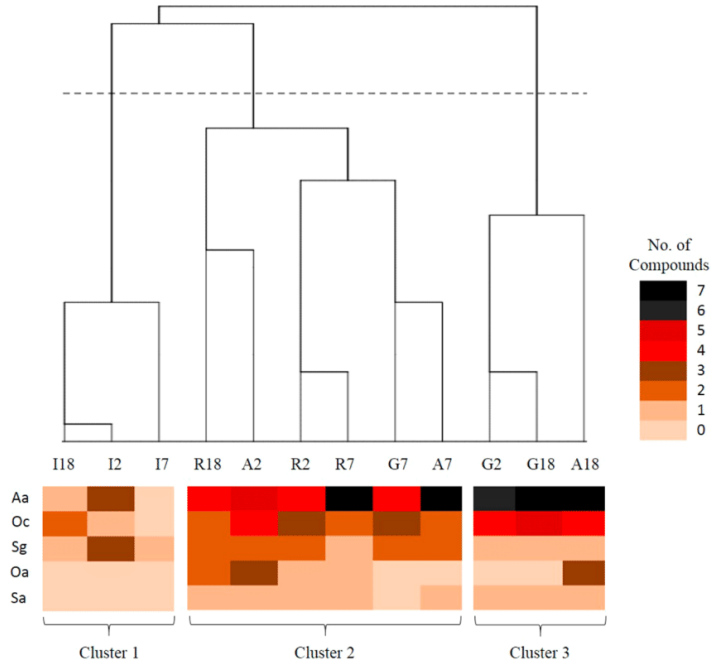


**Figure 4.** Hierarchical cluster analysis dendrogram (average linkage method) and pattern map of the compound class distribution for 12 potato root exudates using the metabolite/compound composition. Abbreviations: Aa, amino acid; Oc, other organic compounds; Sg, sugar; Oa, organic acid; Sa, sugar alcohol. Potato cultivars 'Iwa', 'Russet Burbank', 'Agria', and 'Gladiator' are denoted, respectively, by I, R, A, and G. The numbers 2, 7, and 18 are the incubation periods (days) of potato roots in water at the time of exudate collection. The dotted line indicates the 80% threshold point delineating three clusters.

exudates stimulated *S. subterranea* resting spore germination, releasing zoospores earlier and in greater numbers than the deionized water control. It was also clear that there was no consistent cultivar effect, with variability between assays likely due to variations in plant physiological condition. Using targeted HILIC UPLC-MS based metabolite profiling[15] covering major primary metabolites in a plant biological system, we identified 24 polar LMWO compounds within potato root exudates. The majority of these compounds were amino acids, which is in agreement with other plant species[22,23] and from prior metabolomic studies of potato tubers.[24,25] The LMWO compounds, however, were not uniformly detected across all potato root exudates with more compounds detected in the extracts from the cvs 'Agria', 'Gladiator', and 'Russet Burbank' than in the 'Iwa' extracts. Hierarchical cluster analysis of the potato root exudate components separated exudates into three clusters associated with cultivar and exudate incubation period. Similar variations have been observed with both plant and environmental factors influencing release of LMWO compounds.[26,27] Analysis of metabolites from tubers of different *Solanum* spp. indicated individual species could be identified by their metabolic composition.[24] Variability of root exudate composition with plant physiological and environmental factors[22] suggests these are less useful for biotyping potato cultivars, but with knowledge of their biosynthesis, it may be possible to investigate genetic and proteome changes in a plant grown under particular conditions. For example, the HILIC UPLC-MS-based metabolite profiling[15] used in this study could be extended to analyze metabolite change in *S. subterranea*-infected plants to gain further insights into genes expressed by host plants during root infection.

Pathogen germination or activation by host root exudates is a common phenomenon among many soil-borne pathosystems, and this is particularly the case for environmentally resistant spore producing pathogens.[12] The stimulation of resting spore germination by potato root exudates (Figure 2) corroborated the findings of earlier reports.[8,9] While there could be doubts that the zoospores identified, in this and other studies,[4,9,18] may be those of other flagellated contaminant organisms, additional testing showed that the detected zoospores produced zoosporangia after host infection[1] and attached to potato roots in a differential manner associated with known cultivar resistance,[28] as would be expected with *S. subterranea*. Furthermore, the initial release of zoospores (4−5 d) coincides with the other reports which have used in vitro approaches to examine *S. subterranea* resting spore germination.[7,21]

Root exudates from both host and nonhost plants and their chemical constituents have been suggested to play a part of the *S. subterranea* preinfection activation.[4,7,9,19] Our study supports these observations. In several other pathosystems, individual host-specific compounds found within root exudates activate and chemotaxically attract the pathogen spores to a susceptible host plant to facilitate infection.[10,12] Conversely, we have shown that a diverse range of LMWO compounds are stimulatory toward *S. subterranea* resting spore germination. While we show these are produced by the potato host, many of these compounds are commonly found in root exudates from other plant species, including nonhosts.[22,23] Thus, we conclude that resting spore germination is not host-specific. This is in



DOI: 10.1021/acs.jafc.6b03904
*J. Agric. Food Chem.* 2016, 64, 7466−7474

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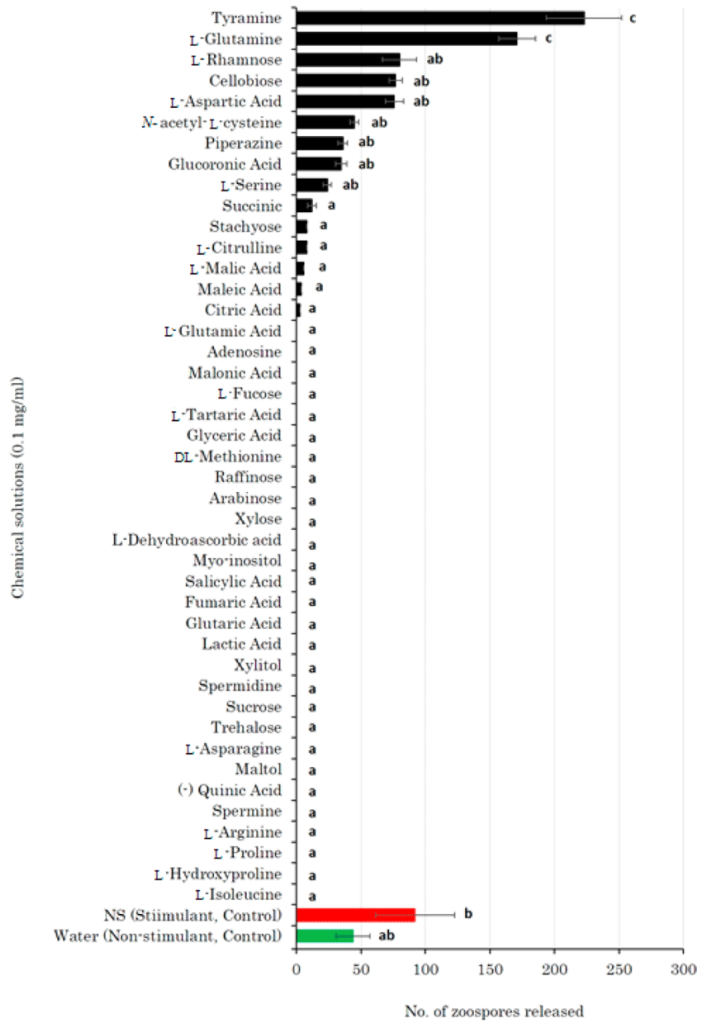


**Figure 5.** Mean accumulated resting spore germination (zoospore release) of *S. subterranea* as influenced by various organic compounds, the deionized water control, or stimulant (Hoagland's) solution. Vertical bars are standard errors ($n$ = 6). Treatments with the same letter are not statistically significant at $p > 0.05$ (Fisher's LSD test).

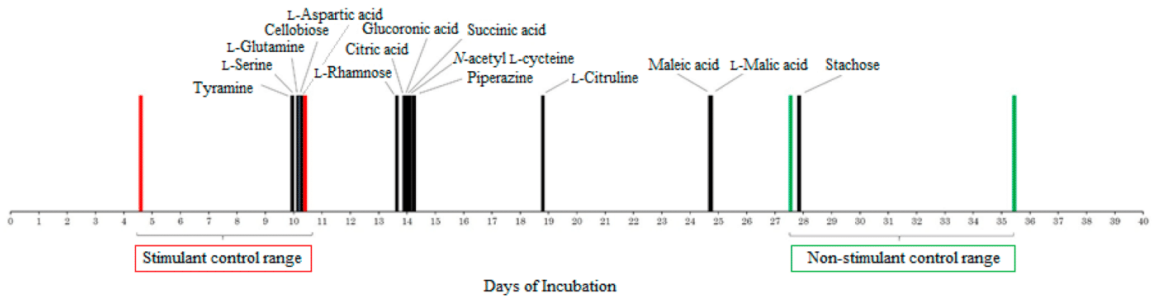


**Figure 6.** Days to initial *S. subterranea* resting spore germination (zoospore release) in low molecular weight organic compound solutions (0.1 mg/ml) and the control (deionized water)



DOI: 10.1021/acs.jafc.6b03904
*J. Agric. Food Chem.* 2016, 64, 7466−7474

agreement with studies of the closely related *Plasmodiophora brassicae*.[29−32] It is thus unsurprising that production of stimulatory compounds was not associated with known potato cultivar resistance with cultivar effects quite variable. Rather, it is likely that production of stimulatory LMWO compounds will be more strongly influenced by other factors such as the physiological condition of the plant.[27] It has been suggested that *S. subterranea* zoospores are likely attracted to root exudates[4] or specific chemical compounds within these exudates.[7] This has been well-documented in other pathosystems,[33,34] and variation in the susceptibility of plants can be associated with variation of chemoattraction by the zoospores.[35,36] We provide preliminary data indicating zoospore attachment to potato host roots is affected by resistance of the potato cultivar, but to date, no studies specifically examining chemotaxic attraction of *S. subterranea* zoospores have been conducted.

Control of potato diseases caused by *S. subterranea* has been partly successful.[37] Planting resistant cultivars has proven the most effective means for powdery scab control, but cultivars lack effective strong resistance to root infection. The use of fungicides provides partial efficiency, reducing disease impact[37,38] probably by reducing inoculum pressure or delaying initial zoospore-host interaction. However, there have been general restrictions on the use of synthetic chemicals for disease management due to potential health and environmental risks and on crop toxicity.[39] This has resulted in increased efforts to explore alternative chemical and biological control methods. Several investigations used a variety of plant extracts and their active chemical components to control major vegetable and grain crop diseases.[40] Others have used organic amendments[41] and biological control agents to eradicate or reduce inoculum levels and minimize disease outcomes,[42] while some continuously test other chemical substances.[43] Few of these types of investigations have been applied to *S. subterranea* diseases of potatoes.[37] The present study identified several root exudate compounds that stimulate *S. subterranea* resting spore germination, and application in the absence of a host plant could assist in reducing soil inoculum levels by removing viable resting spores.[7]

We acknowledge that the tests were done in vitro and that the root exudates were collected from plants grown in sterile aqueous solutions. Compounds detected in this study may differ from those exuded by plants grown under field conditions due to soil environmental and biological factors. Nevertheless, studies using root exudates from in vitro cultures provide clear indications of direct interactions of the pathogen with host root exudates due to the absence of potential compounding factors which could affect pathogen behavior.[44] Moreover, the main objective of this study was to determine whether compounds released by potato roots influence *S. subterranea* resting spore germination. Identifying physiological and environmental factors that may influence potato root exudation, particularly those influencing the release of chemical stimulants in potato root exudates, is worthy of further investigation.[7]

In conclusion, we demonstrated further support for and provide the first direct evidence of the previous findings that potato root exudates stimulate *S. subterranea* resting spore germination.[8,9] We also showed that these biologically active potato root exudates contain polar LMWO compounds, that specific compounds present within these exudates, and that related compounds stimulate resting spore germination. This study offers new knowledge of the LMWO chemical constituents in potato root exudates that directly influence resting spore germination and thus makes a contribution to knowledge of the pathogen's biology and chemical ecology. Active compounds are known from root exudates of other plants (including nonhosts), which supports the contention that resting spore stimulation is not host-specific. Our findings may also be of importance to other root-infecting pathogens of potatoes that may be influenced by root exudation. From an integrated pest management perspective, the use of nonpesticidal resting spore germination-stimulating chemical compounds to decrease the levels of infective agents (zoospores) during cropping breaks could provide a new method to manage *S. subterranea* soil inoculum and could potentially decrease subsequent disease outcomes.[7]

## ■ AUTHOR INFORMATION


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### Funding

M.B. is supported by a Tasmanian Graduate Research Scholarship provided by the University of Tasmania. This study was supported by Horticulture Innovation Australia Limited (Project PT14002, http://www.horticulture.com.au/) with funding from Simplot Australia Pty. Ltd., McCain Foods Australia Pty. Ltd., and matched funds from the Australian Federal Government. The University of Tasmania provided in-kind support.

### Notes

The authors declare no competing financial interest.


## ■ ACKNOWLEDGMENTS

We thank Annabel Wilson (University of Tasmania; UTAS, Australia) for providing tissue cultured potato plants, Sergey Shabala (UTAS) for certain chemicals, Ross Corkrey (UTAS) for assistance in statistical analysis, and James Braselton (Ohio University, Athens, OH) for technical advice and useful discussion.

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DOI: 10.1021/acs.jafc.6b03904
*J. Agric. Food Chem.* 2016, 64, 7466−7474

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Journal of Phytopathology

J Phytopathol

ORIGINAL ARTICLE

# Resting Spore Dormancy and Infectivity Characteristics of the Potato Powdery Scab Pathogen *Spongospora subterranea*

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**Keywords**
inoculum persistence, plasmodiophorid, potato pathogen, *Spongospora* root disease, tomato bait plants

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Received: October 9, 2016; accepted: February 7, 2017.

doi: 10.1111/jph.12565

## Abstract

The soil-borne potato pathogen *Spongospora subterranea* persists in soil as sporosori, which are aggregates of resting spores. Resting spores may germinate in the presence of plant or environmental stimuli, but direct evidence for resting spore dormancy is limited. A soilless tomato bait plant bioassay and microscopic examination were used to examine features of *S. subterranea* resting spore dormancy and infectivity. Dried sporosori inocula prepared from tuber lesions and root galls were infective after both short- and long-term storage (1 week to 5 years for tuber lesions and 1 week to 1 year for root galls) with both young and mature root galls inocula showing infectivity. This demonstrated that a proportion of all *S. subterranea* resting spores regardless of maturity exhibit characteristics of stimuli-responsive dormancy, germinating under the stimulatory conditions of the bait host plant bioassay. However, evidence for constitutive dormancy within the resting spore population was also provided as incubation of sporosorus inoculum in a germination-stimulating environment did not fully exhaust germination potential even after 2.4 years. We conclude that *S. subterranea* sporosori contain both exogenous (stimuli-responsive) and constitutively dormant resting spores, which enables successful host infection by germination in response to plant stimuli and long-term persistence in the soil.

## Introduction

The soil-borne pathogen *Spongospora subterranea* (Wallr.) Lagerh. causes the tuber disease powdery scab and root diseases of potato (Falloon et al. 2016). *Spongospora* diseases are one of the most economically significant problems in potato production worldwide (Balendres et al. 2016b; Wilson 2016). Presence of powdery scab lesions downgrades fresh market tuber value (Harrison et al. 1997) and can result in failure of seed tuber certification (Tegg et al. 2014). Root infection can impact plant growth and yield (Falloon et al. 2016). Current control practices for *Spongospora* diseases in potato are unsatisfactory. Some studies show success of pesticides in delaying disease development or reducing disease impact (Falloon 2008; Thangavel et al. 2015), but the mechanisms of disease suppression are not fully understood.

Dormancy is an important characteristic of many spore-forming plant pathogens that enables persistence in the soil in the absence of their hosts (Cochrane 1974; Deacon 2005; Feofilova et al. 2011). Dormant spores may be sensitive to exogenous factors (e.g. moisture, temperature or phytochemicals) which trigger spore activation or germination (stimuli-responsive, exogenous or environmental dormancy). The presence of stimuli probably indicates that an unfavorable condition has ceased and/or presence of a suitable host (Cochrane 1974; Deacon 2005; Feofilova et al. 2011). Exogenous dormancy may also require sources of energy for activation (Schroth and Hildebrand 1964; Cochrane 1974). Constitutive or endogenous spore dormancy also occurs, in which spores later respond to exogenous stimuli when such dormancy ceases. The germination of spores from this type of dormancy is delayed by the innate properties

of the dormant stage such as barriers to the penetration of nutrients, metabolic blocks or self-inhibitors (Sussman 1965; Cochrane 1974). Dormancy may also cease when the integrity of the spore walls is weakened (Sussman 1965; Cochrane 1974; Deacon 2005; Feofilova et al. 2011).

*Spongospora subterranea* is a plasmodiophorid (Braselton 1995). The plasmodiophorid plant pathogens (*S. subterranea*, *Plasmodiophora brassicae*, *Polymyxa* spp.) survive in the soil as resting spores and infect their hosts as motile zoospores. Resting spores have thick cell walls that protect the zoospores during unfavorable conditions (Lahert and Kavanagh 1985). *Spongospora subterranea* resting spores, and those of most other plasmodiophorids, are aggregated in sporosori, while *P. brassicae* has individual resting spores. The *S. subterranea* sporosorus is complex in which internal resting spores are protected by outer layers of the sporosorus (Falloon et al. 2011). Morphological assessment of *S. subterranea* sporosori indicated only a proportion of resting spores were released as zoospores over the period of assessment (Falloon et al. 2011). The authors suggested this allowed the pathogen to maintain inoculum potential over an extended period. Persistence of *S. subterranea* within the soil has been linked to resting spore dormancy (Harrison et al. 1997; Falloon 2008; Merz 2008; Balendres et al. 2016b), but dormancy has not been widely investigated, with few empirical observations reported to date. Different reports indicated that resting spores can survive for many years, although most were from observations on the re-occurrences of infection in the field (Harrison et al. 1997) with few reports based on direct resting spore examination (Kole 1954; Merz 1989; De Boer 2000). Persistence of resting spores may also vary with incubation conditions. Merz (1989) suggested infectivity of resting spores in moist soil decreased with increasing time of storage.

This study aimed to determine whether *S. subterranea* resting spores exhibit characteristics of exogenous and constitutive dormancy. Greater understanding of the survival and germination of *S. subterranea* could provide valuable knowledge for formulating and implementing *S. subterranea* disease management strategies.

## Materials and Methods

### Sources and preparation of sporosori inoculum

Seven *S. subterranea* sporosori samples were collected from infected potato plants from different fields in NW Tasmania. The samples included four sourced from powdery scab-affected tubers and three from mature potato root galls (dark brown to black colored) which were processed as described below and stored for periods of 1 week to 5 years (tuber inocula) or 1 week to 1 year (gall inocula; Table 1). Young, cauliflower-like, white- to cream-white-colored galls were also sampled and stored for 1 week after processing (Fig. 1; Table 1).

Sporosori were sampled and processed following the method of Merz (1989), with modifications. For each tuber sporosori sample, all powdery scab lesions (10–50 per tuber) were excised from *c.* 20 diseased tubers, collected from the same field, using a vegetable peeler. For the gall sporosori samples, all young or mature galls from *c.* 20 plants (50–60 galls per sample), collected from the same field, were cut from diseased potato roots and washed with running tap water. All sporosori samples were oven-dried for 4 days at 40°C, powdered using a mortar and pestle and stored at 4°C, in the dark, until testing. Tuber tissues free from visible lesions were similarly processed as controls.

### Tomato bait plant bioassay

The infectivity of each sporosori sample was assessed using a simplified soilless bioassay method (Merz 1989). Tomato was used as bait plants because they are very susceptible to *S. subterranea* root zoosporangium infection (Merz 1989), allowing rapid assessment of infection. Tomato (*Lycopersicon esculentum* cv. Grape) seeds were sown in nursery grade potting mix soil (1 : 1 : 8 mix of peat, course sand, and composted pine bark) in a glasshouse maintained at 20 ± 2°C. Two weeks after sowing, seedlings were each gently uprooted, roots washed with running tap water to remove adhering soil and placed in a McCartney bottle containing 20 ml of nutrient solution (Shah et al. 2012) for 24 h. Test plants were then individually placed in a McCartney bottle containing 20 ml of deionized distilled water (DW, 0.059 $\mu$S/cm conductivity) to which 1 mg of sporosorus inoculum was added. Plants were incubated at 15–18°C, 60% humidity with DW added as necessary to maintain a 20 ml of volume in each McCartney bottle. After 4 weeks, plants were removed from the solution, roots were excised and washed with running tap water. Separate root samples were selected from the top, mid-section and bottom of each root profile, mounted on microscopic slides and stained with 0.1% Trypan blue in lactophenol for 15–30 min. The intensity of zoosporangium infection was assessed using

 

**Table 1** Incidence and mean severity (0–5) of *Spongospora subterranea* zoosporangium root infection in tomato bait plant roots, from resting spores from different sources and different ages

| Resting spore source and age[a] | | Collection | Disease incidence[b] | | | Disease severity (0–5)[c] | | | Total no. of roots assessed | Mean disease severity[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Top | Middle | Bottom | Top | Middle | Bottom | | |
| Tuber lesions | | | | | | | | | | |
| 1-week-old | | This study | 8/8 | 8/8 | 8/8 | 3.9 ± 0.3 | 4.3 ± 0.4 | 4.6 ± 0.3 | 24 | 4.3 ± 0.2 [c]* |
| 1-year-old | | This study | 6/8 | 6/8 | 6/8 | 1.4 ± 0.4 | 1.3 ± 0.4 | 1.1 ± 0.3 | 24 | 1.3 ± 0.2[a] |
| 3-year-old | | TIA Collection | 8/8 | 8/8 | 8/8 | 4.0 ± 0.3 | 4.3 ± 0.3 | 4.5 ± 0.3 | 24 | 4.3 ± 0.1[c] |
| 5-year-old | | TIA Collection | 8/8 | 8/8 | 8/8 | 2.5 ± 0.5 | 2.6 ± 0.5 | 2.8 ± 0.4 | 24 | 2.6 ± 0.2[b] |
| L.S.D. | | | | | | | | | | 0.80 |
| P value | | | | | | | | | | <0.001 |
| Root galls | | | | | | | | | | |
| 1-week-old | Young | This study | 5/5 | 5/5 | 5/5 | 3.8 ± 0.4 | 3.6 ± 0.5 | 3.4 ± 0.4 | 15 | 3.6 ± 0.2[b] |
| 1-week-old | Mature | This study | 5/5 | 5/5 | 5/5 | 3.4 ± 0.2 | 3.4 ± 0.4 | 3.4 ± 0.5 | 15 | 3.4 ± 0.2[b] |
| 9-month-old | Mature | This study | 4/5 | 4/5 | 4/5 | 1.8 ± 0.6 | 1.6 ± 0.6 | 1.4 ± 0.4 | 15 | 1.6 ± 0.3[a] |
| 1-year-old | Mature | This study | 4/5 | 4/5 | 4/5 | 1.6 ± 0.5 | 1.6 ± 0.5 | 1.6 ± 0.4 | 15 | 1.6 ± 0.3[a] |
| L.S.D. | | | | | | | | | | 0.91 |
| P value | | | | | | | | | | <0.001 |
| Controls (no *S. subterranea*) | | | | | | | | | | |
| Dried and powdered healthy tuber skin | | | 0/3 | 0/3 | 0/3 | 0 | 0 | 0 | 9 | 0.0 |
| Water | | | 0/3 | 0/3 | 0/3 | 0 | 0 | 0 | 9 | 0.0 |

[a]Age of resting spores from the day of processing until testing. All inocula were dried at 40°C for 4 days and ground to a powder. Young root galls were white to cream-white and mature root galls were dark brown to black.
[b]Number of roots sample infected/total number of roots assessed.
[c]Root infection was evaluated using the scale 0 – no infection, 1 – sporadic, 2 – slight, 3 – moderate, 4 – heavy, 5 – very heavy (Merz et al. 2004).
*Means followed by different letters within each resting spore type were significantly different at P = 0.01, using Fisher's LSD test.

the scale 0 – no infection, 1 – sporadic, 2 – slight, 3 – moderate, 4 – heavy, 5 – very heavy (Merz et al. 2004). Root infection scoring and confirmation of zoosporangia were assessed under a light microscope (DM 2500 LED, Leica Microsystem, Germany) at 200× and 400× magnification, respectively (Hernandez Maldonado et al. 2012). Three to eight plants per treatment were tested with the three root samples per plant (top, middle and bottom) examined and recorded separately.

### Viability and infectivity of dry-stored sporosorus inoculum

To assess sporosori persistence in dry storage and capacity for host stimuli-induced germination, dried and powdered sporosorus inoculum from tuber lesions were stored for 1 week, 1, 3 or 5 years at 4°C in the dark before testing for infectivity using eight tomato bait plants per inoculum source using the method described above. Dried and powdered sporosorus inoculum from root galls incubated for 1 week (young and mature galls), 9 months or 1 year were similarly tested for infectivity using five tomato bait plants per inoculum source. Tomato plants (three per treatment) incubated in inoculum-free DW and in DW with powdered potato tuber skin free from powdery scab lesions were included as negative experimental controls. A total of 24 and 15 root samples were assessed for each tuber and root gall inoculum treatment, respectively.

### Viability and infectivity of sporosorus inoculum incubated in a germination stimulatory environment

To confirm the germination stimulatory effect of nutrient solution (modified Hoagland's solution; Falloon et al. 2003) on resting spore germination (Merz 1989, 1997; Balendres et al. 2016a), 1 mg of dried inoculum from tuber lesion material that had been stored for 1 year was added to 2 ml of nutrient solution and to 20 ml DW and incubated at 15–18°C in the dark. Three aliquots (35 $\mu$l each) were sampled daily and examined microscopically for presence of *S. subterranea* zoospores distinguished by morphology (size and biflagellate form; Kole 1954; Merz 1997) and characteristic swimming patterns (Merz 1992).


**Fig. 1** White-cream-colored, young (y) and dark brown to black mature (m) galls in potato roots.

To test longevity of infectivity under stimulatory conditions and presence of constitutive dormancy of resting spores, dried sporosorus inoculum from tuber lesion material stored for 6 months (20 mg) was added to 500 ml of nutrient solution and incubated at 18–20°C, in the dark for up to 29 months (2.4 years). Subsamples (1 ml) were taken at 2 weeks, 2 months and 2.4 years' incubation time and placed in McCartney bottles each containing 19 ml DW and the inocula was assessed for infectivity using the tomato bait plant assay described above.

### Data analyses

Data and statistical analyses were performed using SPSS® statistical software (Version 22, Armonk, NY, USA). Analysis of variance was performed to determine the variation between three or more treatments. The Fisher's least significant difference (LSD) test, at 0.05 level of probability, was used for multiple comparison of means. The means of zoosporangium severity scores from tuber and gall resting spores, within the same age, were compared using an independent *t*-test analysis. The association between zoosporangium severity and age of inoculum was determined using the Pearson's *r* correlation analysis.

## Results

### Viability and infectivity of dry-stored sporosorus inoculum

All dried sporosorus inocula, after the incubation period, were able to efficiently infect tomato seedlings once activated by suspending in DW with the host plant seedlings (Table 1; Fig. 2). For each assay, 18–24 root segments from the eight bait plants (tuber inocula) or 12–15 root segments of the five bait plants had zoosporangial infection. The control treatments (nine root segments from three plants treated with healthy powdery tuber tissue and DW) did not show any zoosporangia. While infection severity levels are indicative only, resultant mean disease severity statistically varied between tuber inocula ($P < 0.001$) and between gall inocula ($P < 0.001$) with dried tuber inocula samples stored for 1 week or 3 years showing greater disease severity than those stored for 1 year or 5 years, and root gall inocula stored for 9 months and 1 year having lower disease severity than those stored for 1 week. The age of tuber or root gall inocula did not correlate with severity of zoosporangium root infection ($r = -0.968$, $P = 0.929$: tuber; and $r = -0.071$, $P = 0.052$: root gall). There were no differences found in infection success (15 root segments from five plants each) nor disease severity between young (mean score = 3.6) and mature (3.4) gall inocula stored for 1 week.

### Viability and infectivity of sporosorus inoculum stored in a germination stimulatory environment

Adding dried sporosori inoculum to nutrient solution stimulated earlier and greater release of zoospores than the DW control (Table 2). Dried inoculum incubated in nutrient solution remained infective for at least 2.4 years when subsamples were tested with the tomato bait plant assay (Table 3). The length of incubation did not affect the number of infected root segments observed (all nine segments from three bait plants showing infection for each treatment) nor mean disease severity (3.3–3.7).

## Discussion

Resting spore dormancy is important for *S. subterranea* longevity in the absence of suitable host plants (Harrison et al. 1997), but there have been no empirical studies on resting spore dormancy. It is well known that *S. subterranea* resting spores in sporosori can persist in the soil for many years. Longevity of resting

 

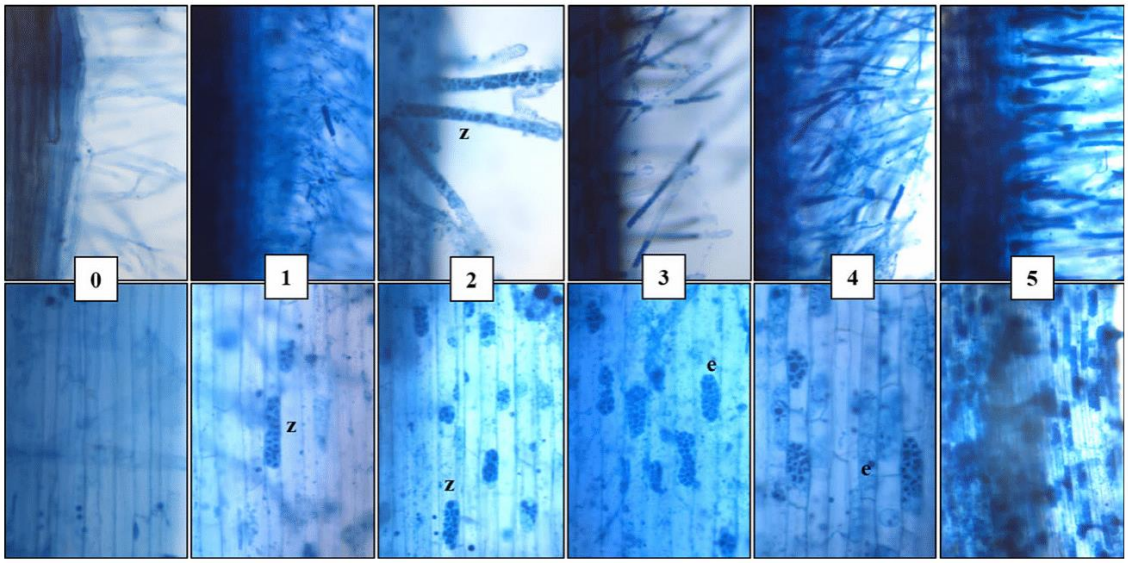


**Fig. 2** Representative photomicrographs of the intensity of zoosporangium infection by *Spongospora subterranea* in tomato (cv. Grape) root hairs (upper section; observed at 200× magnification) and root surface epidermal cells (lower section; observed at 400× magnification). Root infection was microscopically examined, and severity was evaluated using a 0–5 scale (Merz et al. 2004).

**Table 2** Mean numbers of *Spongospora subterranea* zoospores counted in 35-$\mu$l aliquots following incubation of 1 mg of sporosori inoculum in 2 ml of nutrient solution or distilled water

| | Days incubation | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 4 | 7 | 10 | 13 | 17 | 20 | Cumulative count |
| Nutrient solution | 0 | 9.7 ± 2.3 | 134.7 ± 6.8 | 65.3 ± 7.6 | 18 ± 2.4 | 18 ± 7.8 | 245.7 |
| Distilled water | 0 | 0 ± 0.0 | 0 | 0 | 12.3 ± 2.6 | 58.7 ± 2.0 | 71 |
| P value | – | 0.014* | <0.001* | 0.001* | 0.188 | 0.007* | |

Inoculum was from tuber lesions dried at 40°C for 4 days, ground to a powder and stored for 6 months prior to use. Inocula were incubated in treatment solution at 18–20°C.

*Means zoospore population between the solutions were significantly different at $P < 0.01$, using an independent *t*-test analysis. Values are means ± standard error.

spore viability and subsequent capacity to infect host plants has been demonstrated. De Boer (2000) found host plants introduced to 4-year-old resting spore-infested soils succumbed to infection. Resting spores persisting in dry soils for 6 years also remained capable of causing infection when host plants were introduced (Kole 1954). Furthermore, several authors have suggested that resting spores can survive for more than 10 years in the soil following observations from field sites with extended periods between potato crops (Falloon 2008; Merz 2008; Sparrow et al. 2015). However, it remains unclear whether resting spore dormancy was due to constitutive or exogenous mechanisms. Measurement of pathogen presence and abundance in soil and interpretation of these data can be problematic. Polymerase chain reaction and enzyme-linked immunosorbent assay methods can detect and enumerate pathogen levels in the soil (Brierley et al. 2009), but these methods do not necessarily indicate pathogen viability nor differentiate between dormant and infective inocula. Certain chemical stains can detect viability of spores (Riss et al. 2013), but while stains had been used to analyze *P. brassicae* resting spores (Takahashi 1994), the complex sporosorus structures of *S. subterranea* negates the usefulness of this approach. This has probably added to difficulties in obtaining direct empirical evidence linking dormancy to *S. subterranea* resting spore

**Table 3** Incidence and mean severity (0–5) of *Spongospora subterranea* zoosporangium root infection in tomato bait plant roots, from sporosori inoculum held in nutrient solution for varying periods

| Period of incubation[a] | Disease incidence[b] | | | Disease severity (0–5)[c] | | | Total no. of roots assessed | Mean disease severity[c] |
|---|---|---|---|---|---|---|---|---|
| | Top | Middle | Bottom | Top | Middle | Bottom | | |
| 2 weeks | 3/3 | 3/3 | 3/3 | 3.3 ± 0.35 | 3.3 ± 0.41 | 3.3 ± 0.26 | 9 | 3.3 ± 0.20 |
| 2 months | 3/3 | 3/3 | 3/3 | 3.7 ± 0.37 | 3.7 ± 0.41 | 3.7 ± 0.35 | 9 | 3.7 ± 0.21 |
| 2.4 years | 3/3 | 3/3 | 3/3 | 3.7 ± 0.26 | 3.3 ± 0.25 | 3.0 ± 0.27 | 9 | 3.3 ± 0.15 |
| P value | | | | | | | | n.s. (0.383) |

Inoculum was from tuber lesions dried at 40°C for 4 days, ground to a powder and stored for 6 months prior to use.
[a]Length of time resting spores were incubated in nutrient solution at 18–20°C.
[b]Number of plants infected over total number of plants assessed.
[c]Root infection was evaluated using the scale 0 – no infection, 1 – sporadic, 2 – slight, 3 – moderate, 4 – heavy, 5 – very heavy (Merz et al. 2004). n.s. = not significant at 0.05 level of significance by Fisher's LSD test. Values are means ± standard error.

persistence. The use of host bait plants allows the direct observation of infections and provides evidence of the presence of infective pathogen inoculum (Merz 1989). While only modest numbers of test plants were used in these trials, and thus the infection rates presented are approximate only, they do clearly indicate efficient infectivity occurred.

Our study clearly shows that sporosori inocula harvested from both tubers and root galls that were dried prior to storage were infective across a wide range of storage periods from 1 week to 1 year (root gall) or 5 years (tuber) and that both young (white-cream) and mature (brown-black) root galls stored for 1 week contain infective zoospores. This demonstrated that tomato bait plants were capable of stimulating zoospore release from both freshly produced and aged inocula providing evidence for stimuli-responsive dormancy of *S. subterranea* resting spores. Observations of the stimulation of *S. subterranea* resting spore germination have been previously suggested as providing evidence for exogenous dormancy processes. Merz (1993) first reported that host plant root exudates can stimulate or induce *S. subterranea* resting spore germination using root infection as an indicator. Fornier et al. (1996) demonstrated direct effects of root exudates to stimulate resting spore germination, quantifying zoospores in an exudate solution. We add to the current knowledge by demonstrating that both recently formed and aged *S. subterranea* resting spores may be stimulated to germinate by the presence of a stimulatory host and that there is no requirement for extended aging for at least some *S. subterranea* resting spores germinate in the presence of a host. We also confirm that nutrient solution provides a germination stimulus for *S. subterranea* resting spores (Merz 1989, 1997; Balendres et al. 2016a) resulting in more rapid and greater numbers of zoospores released than water alone. These data provide direct evidence for exogenous dormancy characteristics of *S. subterranea* resting spores.

We also show sporosori inocula maintained under stimulatory conditions (nutrient solution) for extended periods of time remain capable of releasing infective zoospores in the presence of tomato bait plants. This suggests a proportion of the resting spores within the sporosori inocula are not stimuli responsive; otherwise, we would expect germination to be fully exhausted within a relatively short period of exposure to such germination-conducive conditions. Instead, a proportion of the resting spores show characteristics of constitutive dormancy that become responsive to stimuli after constitutive dormancy factors have been removed over time. Indirect evidence for presence of constitutive dormancy within populations of *S. subterranea* resting spores was previously provided by Falloon et al. (2011). Through observation of sporosori over 5–8.5 h, they found the majority of resting spores within a sporosorus did not release zoospores, and were either non-viable or remained dormant.

Features of *S. subterranea* resting spore dormancy observed in this study were similar to those reported for *P. brassicae* (Ohi et al. 2003; Friberg et al. 2005). *Spongospora subterranea* and *P. brassicae* are both plasmodiophorids, and their resting spores have morphological similarities (Braselton 1995, 2001). *Plasmodiophora brassicae* persistence in the soil has also been linked to resting spore dormancy (Macfarlane 1970; Kageyama and Asano 2009) and their germination may be stimulated by nutrient solution (Friberg et al. 2005) and host root exudates (Ohi et al. 2003).

The findings from the present study strengthen understanding of the nature of survival and germination of *S. subterranea* resting spores. This knowledge could be valuable for disease management. In absence of the host, germination stimulants could be used to

 

prematurely trigger release of the relatively short-lived zoospores (Karling 1968), leading to a reduction of the soil inoculum potential (Donald and Porter 2014). An example of the use of this approach is shown by the addition of derivatives from garlic or onion containing diallyl disulfide to soil infested with *Sclerotium cepivorum*, the fungus which causes onion white rot. These compounds stimulated sclerotial germination in the absence of *Allium* hosts and reduced soil inoculum levels and resultant disease severity in subsequent onion crops (Davis et al. 2007). A similar approach using specific compounds from brassica root exudates to stimulate germination of *P. brassicae* resting spores in the absence of host plants has recently been proposed (Mattey and Dixon 2015).

Our study also highlights the limitations of such an approach. As a proportion of *S. subterranea* resting spores exhibit constitutive (non-stimuli-responsive) dormancy, inoculum reduction may only be partial. Similarly, certain constitutively dormant sclerotia of *S. cepivorum* failed to respond to diallyl disulfide stimuli (Coley-Smith et al. 1987). However, if *S. subterranea* soil inoculum could be reduced to a level that will reduce or delay infection (Thangavel et al. 2015), then the treatments may be highly beneficial. A detailed investigation of the form and concentration of stimulant and longevity of exposure of resting spores to germination stimulants is warranted.

In conclusion, this study confirms *S. subterranea* resting spores from tuber lesions and root galls may survive dried for an extended period of time without affecting resting spore infectivity. But evidence suggest that resting spores exhibit characteristics of a constitutive (innate) dormancy and thus, while it may be possible to utilize exogenous germination stimuli to encourage germination of resting spores in the absence of host plants to deplete soil inoculum and reduce disease (Davis et al. 2007; Falloon 2008; Balendres et al. 2016b), we would expect constitutively dormant resting spores to remain as infective sources of inoculum.

## Acknowledgements

We thank Dr Tamilarasan Thangavel (University of Tasmania, Australia) for assistance during the pilot setup of plant bait tests and for access to some of the sporosorus collections. The potato growers of Northern Tasmania provided for diseased potato material. We also thank Prof. Richard Falloon (Plant and Food Research, New Zealand), Dr Ueli Merz (ETH Zurich, Switzerland) and Prof. James Braselton (Ohio University, Athens, OH, USA) for feedback and some useful discussion of the photomicrographs. This project was partially funded by Horticulture Innovation Australia Limited (PT14002) with co-investment from Simplot Australia Pty. Ltd., McCain Foods Australia Pty. Ltd., and funds from the Australian Government. The first author was supported by a Tasmanian Graduate Research Scholarship provided by the University of Tasmania. The authors have no conflict of interest to declare.

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Appendix 1e – Balendres et al. 2016b

Journal of Phytopathology

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ORIGINAL ARTICLE

# Potato Root Exudation and Release of *Spongospora subterranea* Resting Spore Germination Stimulants are Affected by Plant and Environmental Conditions

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**Keywords**
HILIC UPLC-MS, plasmodiophorid, powdery scab, root exudates

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Received: September 22, 2016; accepted: October 26, 2016.

doi: 10.1111/jph.12537

## Abstract

Variation in plant and environmental conditions were studied to determine the effect thereof on the exudation of low-molecular-weight organic compounds by potato roots. The results of the phytochemical analyses showed that among the conditions investigated, root vigour, potato cultivar, nutrients in incubation solution and temperature influenced the number and the type of primary metabolites released. Moreover, these conditions influenced our detection of compounds known to stimulate germination of resting spores of the pathogen *Spongospora subterranea*, causal agent of powdery scab and root diseases of potato. We conclude that changes in plant and environmental conditions can affect the release of specific compounds that stimulate germination of *S. subterranea* resting spores. The impact of the factors affecting potato root exudation on subsequent disease development is discussed.

## Introduction

Plant roots release a substantial quantity of primary metabolites into the rhizosphere, equivalent to 40–50% of the total amount of carbon fixed by plants (Bais et al. 2006), the majority of which are low-molecular-weight organic (LMWO) compounds. These compounds play important roles in interactions between the plant, and other soil biota including soilborne plant pathogens (Schroth and Hildebrand 1964; Nelson 1990; Haichar et al. 2008; Micallef et al. 2009). Exudate compounds may benefit pathogens, promoting their germination, growth, survival, pathogenesis and reproduction, or may be detrimental inhibiting growth or disrupting motility. The composition of primary metabolites produced by plants including those found in root exudates will be influenced by both the plant's genetics and environmental growth condition (Bertin et al. 2003; Neumann and Romheld 2007). Various factors have been reported to influence the composition of root exudates for many plant species (Neumann and Romheld 2007). However, very little is known about the primary metabolites' released by potato (*Solanum tuberosum* L.) roots and how this is influenced by plant and environmental factors. This knowledge would assist the understanding of the dynamics of potato root phytochemical–soilborne pathogen interaction.

Recently, we have confirmed specific LMWO compounds detected in potato root exudates (L-Glutamine, tyramine, *N*-acetyl cysteine, L-serine and L-citrulline) and related compounds (L-rhamnose, cellobiose, L-aspartic acid, piperazine, glucoronic acid, succinate and citric acid) can stimulate the germination of *Spongospora subterranea* f.sp. *subterranea* (Wallr.) Lagerh. resting spores (Balendres et al. 2016a). *Spongospora subterranea* is a soilborne potato pathogen responsible for the tuber disease powdery scab (Wallroth 1842) and root diseases (Falloon et al. 2016). Powdery scab results in significant economic loss to producers of seed tubers, fresh market and processing potatoes (Wale 2000; Wilson 2016). Root infections can also negatively impact plant productivity and tuber yields (Falloon et al. 2016). There are no single effective management options for control of *S. subterranea* diseases (Falloon 2008). The use of

stimulant compounds to activate (germinate) pathogen resting spores in the absence of a host plant as an inoculum management strategy has been proposed (Balendres et al. 2016b). Primary metabolites in potato root exudates have been characterized, and some compounds known to stimulate the resting spore germination (RSG) process have been identified (Balendres et al. 2016a). However, the conditions or factors which influence the release of the specific germination-stimulant compounds are unknown. This knowledge may provide further insight into the epidemiology of *S. subterranea* diseases. For example, practices such as continuous (mono) cropping enhance the release of root exudates that activate soilborne peanut pathogens (Li et al. 2013).

In this study, the influence of plant age, root vigour, root injury, potato cultivar, light, nutrient availability, plant density and growth temperature on the composition of potato root exudates was investigated. We used a targeted metabolomics approach able to simultaneously screen a large number of polar LMWO compounds commonly found in plant extracts (Gika et al. 2012). Changes in root exudate metabolite profile that affected release of known *S. subterranea* germination-stimulating compounds were noted.

## Materials and Methods

### Plant Materials, growth conditions and treatments

Tissue-cultured potato (*S. tuberosum*) cultivars 'Desiree', 'Shepody', 'Gladiator', 'Iwa' and 'Russet Burbank' were used in this study. Cultivar 'Iwa' is highly susceptible to powdery scab, root infection by *S. subterranea* and root galling; 'Shepody' is moderately susceptible to powdery scab and highly susceptible to root galling; 'Russet Burbank' and 'Desiree' are moderately resistant to powdery scab, to root infection and to root galling; and 'Gladiator' is very resistant to powdery scab, root infection and root galling (Falloon et al. 2003, 2016; Nitzan et al. 2008). Plants were maintained in potato multiplication (PM) medium composed of MS salts and vitamins, 30 g/l sucrose, 40 mg/l ascorbic acid, 500 mg/l casein hydrolysate and 8 g/l agar, pH 5.8, and were grown under 16:8 h day:night cycle photoperiod in white fluorescent lamps (65 $\mu$mol $m^2$/s) at 24°C (Wilson et al. 2010).

Four plant physiological and four environmental factors were examined in the study. Unless otherwise stated, plants were grown for 2 weeks in PM medium prior to transfer to sterile deionized distilled water (SDW) for root exudate collection, were grown at 24°C and received 16 h of light in a 24-h cycle, and 'Russet Burbank' was used as the test cultivar. For the plant age factor, two-node potato cuttings were grown for 1, 2 and 3 months in PM medium prior to individual transfer to SDW for exudate collection. For the root vigour factor, each plant's root mass was measured after root exudate collection. Plants with a root mass greater than 90 g were considered to have strong (vigorous) root growth, whilst those less than 20 g were classed as a weak root system. For the root injury factor, roots were either trimmed by removing 2.5 cm from the root tip, using a sterile scalpel prior to transfer to SDW or left intact. For the cultivar factor, the six potato cultivars were assessed. For the light factor, plants were either incubated under the standard light cycle (16:8 h light–dark cycle) or were held in the dark, by encasing plants and their container in aluminium foil, after transfer to SDW. For the effect of nutrients, plants after 2 weeks in the PM media were transferred first into a Hoagland's solution (HS; Shah et al. 2012) for 7 days, then transferred to SDW for root exudate collection or were transferred to a SDW solution for 7 days, prior to transfer to fresh SDW for root exudate collection. Prior to transfer to SDW for exudate collection, roots were carefully washed in SDW several times and blotted dry to remove any remnant HS. For the plant density factor, either single plants or groups of five plants were placed in a single container with SDW for root exudate collection. For the effect of temperature, plants were incubated at 4, 10, 15, 24 or 30°C during root exudate collection. All treatments were replicated three times. Unless otherwise mentioned, each replicate used a single plant. Preparations were all performed in aseptic condition, to avoid microbial and exogenous chemical contamination.

### Root exudate collection

Root exudates were collected aseptically from the SDW solutions in which the tissue-cultured potato plants were incubated. Plants were lightly uprooted from the media, roots washed with SDW, blotted on sterile tissue paper and prepared based on their respective treatments. Individual or grouped plants were then placed in a polypropylene tissue culture bottle containing 20 ml of fresh SDW (0.059 $\mu$S/cm instrument conductivity). Root exudates were sampled 7 days from the date of plant's transfer and stored at −20°C, in the dark until use.

### Phytochemical analysis

Detection of LMWO primary metabolites or compounds in plant root exudate solution was achieved using a hydrophilic interaction ultra-high-performance liquid chromatography–mass spectrometry (HILIC UPLC-MS) procedure derived from Gika et al. (2012). Briefly, the UPLC and MS analysis were performed using the Acquity UPLC H-class and Xevo triple quadrupole MS system (Waters, Milford, MA, USA), respectively. The HILIC separation was performed on a 2.1 mm × 150 mm Acquity 1.7-$\mu$m BEH amide VanGuard column maintained at 60°C and eluted with a two-step gradient at 500 $\mu$l/min flow rate for 30 min. The gradient started with a 4-min isocratic step at 100% mobile phase A (acetonitrile–water, 95–5 (v/v), 0.1% formic acid and 0.075% $NH_4OH$), then rising to 28% mobile phase B (acetonitrile–water, 2–98 (v/v), 0.2% formic acid and 0.1% $NH_4OH$) over the next 21 min and finally to 60% B over 5 min (Gika et al. 2012). The column was then equilibrated for 12 min in the initial conditions. Two cycles of weak and strong solvent washing of the injection system were carried out between injections. The injection volume was 10 $\mu$l, and the column eluent was directed to the mass spectrometer. Metabolite detection was achieved using selected ion monitoring, and an electrospray ionization (ESI) source was applied operating in both positive and negative ion mode. The parameters in the electrospray were set as follows: capillary voltage, −2.5 kV or 3 kV; cone and desolvation temperatures, 150 and 400°C, respectively, with a desolvation gas flow of 950 l/h and cone flow of 100 l/h. The cone voltage was optimized for each individual analyte. The repeatability of the HILIC UPLC-MS method was confirmed by analysis of standard chemicals, root exudates and root exudates spiked with standard chemicals.

### Data and statistical analysis

Hydrophilic interaction ultra-high-performance liquid chromatography–mass spectrometry data were analysed using MASSLYNX XS software (Waters, Milford, MA, USA; Gika et al. 2012). The limit of detection and limit of quantitation of the analytes were determined at signal-to-noise ratios of 3 and 10, respectively. Compounds were identified based on their respective *m/z* ratio and typical retention time in the chromatogram (Gika et al. 2012). Only compounds detected in at least two of the three replicates were validated as 'present' in root exudate solutions. Compounds were designated '1' and '0' for 'present' and 'absent', respectively. Using the binary data, a hierarchical cluster analysis was performed using SPSS statistical software ver. 22, (IBM, Armonk, NY, USA). A dendrogram was constructed using the average linkage (between group) algorithm and the squared Euclidean distance method. A heat map was also constructed to present the distribution of the compound classes detected in various root exudates. The presence of known *S. subterranea* RSG-stimulant compounds (Balendres et al. 2016a) was ascertained using the binary data and plotted along with the heat map and in the dendrogram.

## Results

### Effect of plant physiological conditions on root exudation

The potato plant's physiological conditions affected the number of compounds released by potato roots (Table 1). A total of 24 compounds were detected which included amino acids (12), sugar alcohols (1), sugars (9), organic acids (3) and other LMWO compounds (12). Varying plant age produced no clear trend. Two-month-old plants released more compounds than younger or older plants. Trimmed roots released more sugars than intact roots. Plants with stronger (larger) roots released more compounds in total than plants with weaker (smaller) root systems; however, the numbers of sugars released by the strong and weak plants did not differ. Cultivars also varied in their exudate metabolite profiles with Shepody releasing twice as many compounds as other cultivars (Table 1).

### Effect of the environmental conditions on root exudation

Environmental conditions during the plant's growth influenced the release of LMWO compounds in root exudates (Table 2). The total number of compounds released in root exudates from plants grown in the dark was comparable to those receiving 16 h of light. The release of LMWO compounds in plants which received additional nutrition (Hoagland's solution) was greatly suppressed. The plants with HS supplementation produced seven times less LMWO compounds in their root exudates than the no nutrient-supplemented plants, with substantially less amino acids, and no sugars, organic acids and other organic compounds detected. The number (density) of plants within

**Table 1** The influence of plant factors on compounds detected in potato root exudates of 135 analytes screened by HILIC UPLC-MS

| | | | | | Age[d] | | | Cultivar[e] | | | | | Root injury[f] | | Root vigour[g] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Compounds[a] | ESI[c] | *m/z* | Rt | 1 Month | 2 Months | 3 Months | Iwa | Des | She | RBK | Glad | Intact | Trim | Strong | Weak |
| 1 | 3-hydroxyproline[a] | + | 132 | 13,33 | | | | + | | | | | | | | |
| 2 | Alanine | + | 90 | 14,42 | | | | | | | | | | | + | |
| 3 | Aminobutyric acid | + | 104 | 13,26 | + | | + | | + | + | | | + | + | + | + |
| 4 | Arginine[a] | + | 175 | 19,16 | + | + | + | | + | + | | | + | + | + | + |
| 5 | Asparagine[a] | + | 133 | 15,63 | | | + | | | | | | | | + | |
| 6 | Glutamic acid[a] | + | 147 | 17,35 | | + | + | | | + | | | + | + | + | + |
| 7 | Glutamine[a,b] | + | 147 | 15,17 | | | | | | | | | | | + | |
| 8 | Glycine | + | 76 | 15,51 | | | | | | | | | | + | | |
| 9 | Ornithine | + | 133 | 21,87 | + | | | | | + | + | | | | | + |
| 10 | Proline[a] | + | 116 | 11,48 | | + | | + | + | + | | + | + | + | + | + |
| 11 | Pyroglutamic acid | – | 128 | 11,27 | | | | | | | | | | | | |
| 12 | Tryptophan | + | 205 | 11,24 | | | | | | | | | | | | |
| Sugar alcohols | | | | | | | | | | | | | | | | |
| 13 | 5-carbon alcohol | – | 151 | 5,18 | | + | | | | | | | | | | |
| Sugars | | | | | | | | | | | | | | | | |
| 14 | C12 sugars [M-H] *m/z* 341 | – | 341 | 16.5 | | + | | | | | | | | | | |
| 15 | C6 sugars [M-H] *m/z* 179 | – | 179 | 8,17 | + | + | + | | | + | + | + | | + | | + |
| 16 | Lactose | + | 343 | 14,48 | | | | | | + | | | + | + | + | |
| 17 | Mannose | – | 179 | 9,14 | | | + | | | + | | + | | | | + |
| 18 | Rhamnose/fucose[a,b] | – | 163 | 2,35 | + | | | | | + | | | | + | + | |
| 19 | Ribose | – | 149 | 2,64 | | + | | | | | | | | | | |
| 20 | Sucrose[a] | – | 341 | 13,02 | | + | + | | + | + | | + | + | + | + | + |
| 21 | Trehalose[a] | + | 360 | 16,19 | + | + | | + | | + | | | | + | + | + |
| 22 | Xylose[a] | – | 149 | 3,97 | | | | | | | | | | | + | + |
| Organic acids | | | | | | | | | | | | | | | | |
| 23 | Glutaric acid[a] | – | 131 | 3,89 | | | | | | | | | | | + | |
| 24 | Carboxylic acid [M-H] *m/z* 87 | – | 87 | 4,69 | | + | | | | | | | | | | |
| 25 | Oxalic acid | – | 89 | 8,49 | | + | | | | | | | | | | |
| Other compounds | | | | | | | | | | | | | | | | |
| 26 | Choline | + | 176 | 4,95 | | | | | | + | | | + | + | + | + |
| 27 | Citrulline[a,b] | + | 223 | 16,30 | | | | + | | | | | | | + | + |
| 28 | Guanidine | + | 152 | 7,80 | | | | | | | | | | | + | |
| 29 | Histamine | + | 88 | 10,39 | | | | + | | | | | | | + | |
| 30 | Nicotinamide | + | 86 | 1,21 | | | | | | | | | | | | |
| 31 | Piperidine | + | 89 | 3,82 | | | | | | | | | | | | + |
| 32 | Putrescine | + | 168 | 19,13 | | | | | | | | | + | | + | |
| 33 | Pyridoxal | + | 169 | 1,38 | | + | | | | | | | | | | |
| 34 | Spermidine[a] | + | 203 | 24,30 | | | | + | | | | | + | + | | |
| 35 | Spermine[a] | + | 265 | 7,45 | + | + | + | | + | + | + | + | + | + | + | |
| 36 | Thiamine | – | 138 | 8,78 | | + | | | | | | | | | | |
| 37 | Tyramine[a,b] | + | 268 | 2,78 | | | | | | | | | + | | | |

[a]Compounds previously tested for *S. subterranea* RSG activity (Balendres et al. 2016a); [b]Positive RSG activity; [c]Electrospray ionization; [d]Roots incubated in distilled water for 1, 2 or 3 months prior to exudate sampling; [e]Des (Desiree), She (Shepody), RBK (Russet Burbank), Glad (Gladiator); [f]Roots were trimmed by 2.5 cm (trim) or left intact; [g]Roots were large (>90 g fresh weight) or weak (<20 g).

the collection tube did not influence the number of LMWO compounds in the root exudates. Temperature showed no clear trend with the release of LMWO compounds (12 compounds) being greatest when plants were grown at 10, 24 and 30°C, than at 4 and 15°C (8).

**Table 2** The influence of environmental factors on compounds detected in potato root exudates of 135 analytes screened by HILIC UPLC-MS

| | | | | | Light[d] | | Temperature[e] | | | | | Nutrients[f] | | Plant density[g] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Compounds[a] | ESI[c] | *m/z* | Rt | Dark | 16-h Light | 4°C | 10°C | 15°C | 24°C | 30°C | No HS | With HS | 5 Plants | 1 Plant |
| 1 | 3-hydroxyproline[a] | + | 132 | 13,33 | | | | | | | + | | | | |
| 2 | Alanine | + | 90 | 14,42 | | | | | | | | + | | | |
| 3 | Aminobutyric acid | + | 104 | 13,26 | + | + | + | + | + | + | + | + | | | + |
| 4 | Arginine[a] | + | 175 | 19,16 | + | + | | + | | + | | + | + | + | + |
| 5 | Asparagine[a] | + | 133 | 15,63 | | | | | | | | + | | | |
| 6 | Glutamic acid[a] | + | 147 | 17,35 | + | + | | | | + | + | + | | + | + |
| 7 | Glutamine[a,b] | + | 147 | 15,17 | | | | | | | | + | | | |
| 8 | Glycine | + | 76 | 15,51 | | | | | | | | | | | |
| 9 | Ornithine | + | 133 | 21,87 | | | | | | | | | + | + | + |
| 10 | Proline[a] | + | 116 | 11,48 | + | + | + | + | + | + | + | + | + | + | + |
| 11 | Pyroglutamic acid | – | 128 | 11,27 | | | | + | | | | + | | | |
| 12 | Tryptophan | + | 205 | 11,24 | | | | | | | | + | | | |
| Sugar alcohols | | | | | | | | | | | | | | | |
| 13 | 5-carbon alcohol | – | 151 | 5,18 | | | | | | | | | | | |
| Sugars | | | | | | | | | | | | | | | |
| 14 | C12 sugars [M-H] *m/z* 341 | – | 341 | 16.5 | | | | | | | | | | | |
| 15 | C6 sugars [M-H] *m/z* 179 | – | 179 | 8,17 | | + | + | + | + | | + | | | + | + |
| 16 | Lactose | + | 343 | 14,48 | + | + | | + | | + | + | + | | | |
| 17 | Mannose | – | 179 | 9,14 | | | + | + | + | | | | | | + |
| 18 | Rhamnose/fucose[a,b] | – | 163 | 2,35 | | + | | + | | | + | | | + | |
| 19 | Ribose | – | 149 | 2,64 | | | | | | | | | | | |
| 20 | Sucrose[a] | – | 341 | 13,02 | + | + | + | + | + | + | + | + | | + | + |
| 21 | Trehalose[a] | + | 360 | 16,19 | | | + | + | + | | | + | | | + |
| 22 | Xylose[a] | – | 149 | 3,97 | | | | | | | | + | | | + |
| Organic acids | | | | | | | | | | | | | | | |
| 23 | Glutaric acid[a] | – | 131 | 3,89 | | | | | | | | + | | | |
| 24 | Carboxylic acid [M-H] *m/z* 87 | – | 87 | 4,69 | | | | | | | | | | | |
| 25 | Oxalic acid | – | 89 | 8,49 | | | | | | | | | | + | |
| Other compounds | | | | | | | | | | | | | | | |
| 26 | Choline | + | 176 | 4,95 | + | + | | + | | + | + | + | | + | + |
| 27 | Citrulline[a,b] | + | 223 | 16,30 | | | | | | | | + | | + | + |
| 28 | Guanidine | + | 152 | 7,80 | | | | | | | | + | | | |
| 29 | Histamine | + | 88 | 10,39 | + | | | | | + | + | + | | | |
| 30 | Nicotinamide | + | 86 | 1,21 | | | + | | + | | | | | | |
| 31 | Piperidine | + | 89 | 3,82 | | | | | | | | | | | + |
| 32 | Putrescine | + | 168 | 19,13 | + | | | | | + | | + | | + | |
| 33 | Pyridoxal | + | 169 | 1,38 | | | | | | | | | | | |
| 34 | Spermidine[a] | + | 203 | 24,30 | + | | | | | + | + | | | + | |
| 35 | Spermine[a] | + | 265 | 7,45 | + | + | + | + | + | + | + | + | | + | |
| 36 | Thiamine | – | 138 | 8,78 | | | | | | | | | | | |
| 37 | Tyramine[a,b] | + | 268 | 2,78 | + | | | | | + | | | | | |

[a]Compounds previously tested for *S. subterranea* RSG activity (Balendres et al. 2016a); [b]Positive RSG activity; [c]electrospray ionisation; [d]Roots incubated in distilled water in full dark or 16-h light 8-h dark cycle prior to exudate sampling; [e]Roots incubated in distilled water at 4–30°C prior to sampling; [f]Roots were incubated in Hoagland's solution (with HS) or distilled water (no HS) for 7 days prior to exudate collection; [g]Incubation tubes held five plants or one plant prior to sampling.

### Effect of physiological and environmental factors on the release of RSG stimulants

Of the 24 LMWO compounds detected, 15 had been previously tested for *S. subterranea* RSG capacity (Balendres et al. 2016a), and of these, only four (citrulline, glutamine, rhamnose and tyramine) were identified as RSG stimulants (Tables 1 and 2). Various plant and environmental factors were found to affect release of these known RSG-stimulant compounds. Notably, the stronger root systems were associated with three of the four RSG stimulants whilst weaker

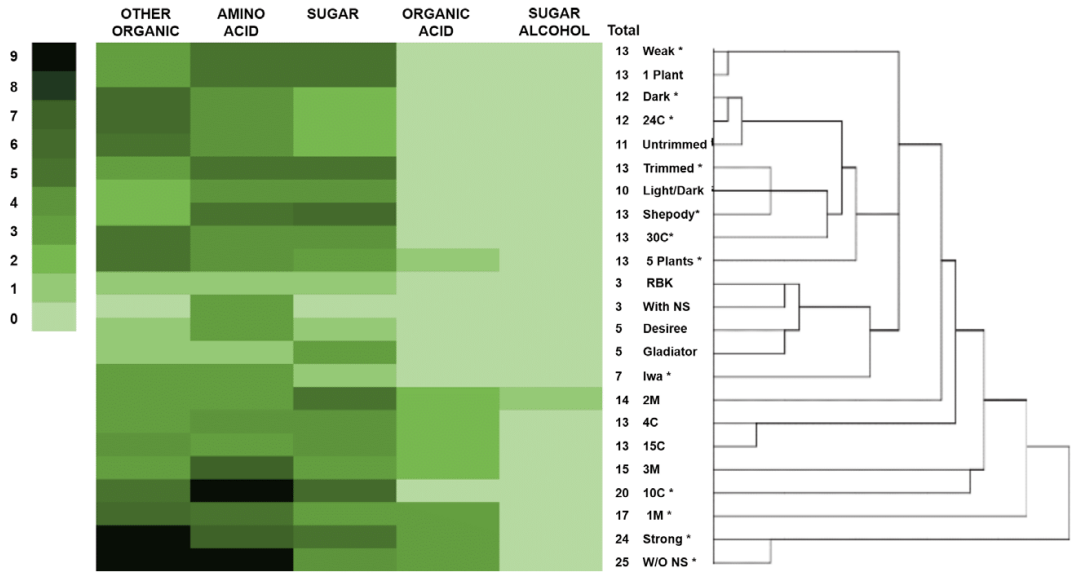


**Fig. 1** Hierarchical cluster analysis dendrogram (average linkage method) and heat map of the compound class distribution using the metabolite/compound composition in root exudates of potato grown in various physiological and environmental conditions. Treatments which led to release of *S. subterranea* resting spore germination-stimulant compounds are indicated with an asterix. [Colour figure can be viewed at wileyonlinelibrary.com].

roots had none but otherwise, there were no other obvious associations with any specific treatments (Fig. 1).

## Discussion

Potato root exudates contain primary LMWO metabolites some of which are stimulatory to RSG of the powdery scab pathogen *S. subterranea* (Balendres et al. 2016a). Factors influencing potato root exudation are poorly understood, and hence, the factors which affect the release of the known RSG-stimulant compounds are largely unknown. In this study, changes in some plant physiological and environmental conditions during exudate collection were found to affect the total number and distribution of primary metabolites in plant root exudates. In particular, plants of cv. 'Shepody', those with larger (vigorous) roots and those which did not receive nutrient supplementation released a greater number of LMWO compounds than their respective comparator treatments. These conditions also influenced the release of citrulline, rhamnose, tyramine and glutamine, known to be stimulants of RSG of *S. subterranea* (Balendres et al. 2016a).

Previous studies of primary metabolites of potato have primarily examined tubers, because of their economic value (Roessner et al. 2000; Dobson et al. 2010). Compounds released in potato root exudates have received less attention despite their importance in plant growth and productivity and their ability to manipulate the rhizosphere microbiota including pathogens (Schroth and Hildebrand 1964). Recently, the primary metabolite composition of potato root exudates from 2-, 7- and 18-day-old plants have been characterized (Balendres et al. 2016a), where it was shown that plant age and cultivar influenced the type of compounds released. The current study found that in addition to plant age and cultivar, other plant and environmental conditions can affect both the number and type of compounds detected in potato root exudates. The effect of these physiological factors and environmental conditions on the release of compounds in root exudates is similar to previous reports from other plant species (Rovira 1959, 1969). For instance, a greater number of sugars were released from trimmed than intact roots. This is observed when cell walls are damaged (Kumar et al. 2004). To our knowledge, this is the first study of factors influencing the release of compounds in potato root exudates

*in vitro*. Similar to studies on potato tuber metabolism where metabolites could be used as phenotype markers (Dobson et al. 2010), primary metabolite composition in root exudates presented in this study may be used to predict plant's response to different internal and external conditions. Moreover, these primary metabolite profiles may encourage studies on potato soilborne pathogen chemical ecology.

It has been shown that root exudates play a critical role as stimulants of *S. subterranea* RSG (Kole 1954; Merz 1993; Fornier et al. 1996). And recently, we have shown that potato root exudates contain certain LMWO compounds which are stimulant to RSG (Balendres et al. 2016a). In this study, we found that the release of some of these stimulant compounds could be influenced by the plant's growing conditions. This implies that these factors help predict the presence of these known stimulant compounds in plants grown *in vitro* and may affect disease development (Li et al. 2013). Potato roots at all development stages are susceptible to infection (Thangavel et al. 2015), and thus as roots develop, the RSG activation capacity and potential for infection will increase. However, whilst we demonstrate that many of these plant and environmental factors influence root exudation composition including the release of known *S. subterranea* RSG stimulants, it is important to acknowledge that many of these factors will have additional effects on disease expression through interaction with the pathogen or host plant. For example, temperature has been clearly demonstrated to influence both root and tuber infection (de Boer et al. 1985; van de Graaf et al. 2005, 2007).

One important factor identified in this study that affected the release of LMWO compounds was the addition of nutrients. The number of LMWO compounds was greater in plants which did not receive the nutrients (HS) and contained two known RSG stimulants. The higher exudation following incubation in distilled water rather than HS could possibly be attributed to the presence of phosphate substance in HS which can prevent leakiness of cell membranes and release of exudates (Rovira 1959, 1969) although the influence of other compounds cannot be discounted. However, the most important implication to emerge from this finding was the inhibition of the release of known RSG-stimulant compounds in nutrient-supplemented plants. It is thus interesting to speculate whether fertilisers may affect root exudation and indirectly reduce stimulation of *S. subterranea* RSG. Previous studies have shown that high rates of nitrogen applications to fields can increase powdery scab disease (Tuncer 2002; Shah et al. 2014) although the mechanism for increased disease is not known. Interestingly, detailed glasshouse studies of nutrient effects on potato root infection by *S. subterranea* suggested nitrogen in ammonium form could reduce root infections (Falloon et al. 2014).

There was speculation that cultivars with relative resistance to *S. subterranea* may produce less RSG stimulants than susceptible cultivars. Differences in exudate profiles were found between cultivars; however, we show that resistant cultivars also release the known germination-stimulant compounds, particularly if they have strong root system. This indicates that the initial interaction of the pathogen with its potato host is likely influenced by conditions that affect root exudation. However, this does not mean that the release of RSG-stimulant compounds from a resistant cultivar is necessarily followed by successful host infection or disease expression (Falloon et al. 2003).

One of the difficulties in the study of compounds affecting soilborne pathogen activities is distinguishing organic compounds or substances released by roots and those already present in the soil (Rovira 1969). This study used an *in vitro* soil-less culture system which allowed robust identification of plant root metabolites in the absence of soil-related confounding factors (Vranova et al. 2013), and thus more accurately determined the influence of specific plants and environmental factors on root exudation.

Our results support the hypothesis that, under an *in vitro* system, changes in potato root exudate composition and the release of *S. subterranea* RSG-stimulant compounds is influenced by both plant and environmental factors. Furthermore, we show that whilst cultivar affected root exudate composition, the release of *S. subterranea* germination-stimulant compounds was not associated with cultivar susceptibility to disease. Factors which affected the release of stimulant compounds may also influence disease outcomes; in particular, greater root mass may encourage greater release of RSG stimulants. Further studies examining the role of RSG stimulants in *Spongospora* disease epidemiology may inform development of new disease control tools (Balendres et al. 2016b).

## Acknowledgements

We thank Annabel Wilson (University of Tasmania; UTAS) for providing tissue-cultured potato plants and Sergey Shabala (UTAS) for certain chemicals. M. B. is supported by a Tasmanian Graduate Research Scholarship provided by the University of Tasmania. This project was funded by Horticulture Innovation

Australia Limited (PT14002) with co-investment from Simplot Australia Pty. Ltd., McCain Foods Australia Pty. Ltd. and funds from the Australian Government. TIA, UTAS provided in-kind support.

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*Plant Pathology* (2018) **67**, 902–908 Doi: 10.1111/ppa.12795

# Germinate to exterminate: chemical stimulation of *Spongospora subterranea* resting spore germination and its potential to diminish soil inoculum

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Hoagland's solution (HS), a defined nutrient supplement for plants, has been previously reported to stimulate zoospore release from resting spores of the potato pathogen *Spongospora subterranea* f. sp. *subterranea*. This study obtained direct empirical evidence for an increase in zoospore release with HS treatment, and identified Fe-EDTA as the stimulant component of HS. Stimulation of resting spores by HS and Fe-EDTA resulted in greater and earlier zoospore release compared to a distilled water control, and in the presence of a susceptible tomato host plant resulted in enhanced root infection. Given the labile nature of *S. subterranea* zoospores, it was postulated that stimulation of premature release of zoospores from the dormant resting spores in absence of susceptible hosts could reduce soil inoculum levels. In two glasshouse trials in the absence of host plants, both Fe-EDTA and HS soil treatments reduced *S. subterranea* soil inoculum levels, providing proof of concept for the 'germinate to exterminate' approach to inoculum management.

*Keywords:* chemical stimulants, Hoagland's solution, potato, powdery scab, soil inoculum management, *Spongospora* root infection

## Introduction

*Spongospora subterranea* f. sp. *subterranea* is a tuber- and soilborne plasmodiophorid pathogen that causes powdery scab on tubers (Wallroth, 1842) and root disease (Ledingham, 1935) in potato (*Solanum tuberosum*). These tuber and root diseases are responsible for substantial financial losses to the global potato industry (Balendres *et al.*, 2016b; Falloon *et al.*, 2016; Wilson, 2016). The pathogen persists in the soil for many years between potato crops (De Boer, 2000; Balendres *et al.*, 2017) as aggregates of resistant resting spores called sporosori (Kole, 1954; Falloon *et al.*, 2011). Each of the resting spores within a sporosorus contains a zoospore that, when released, is motile and is responsible for initiating infections in susceptible plant hosts. Unlike the resting spores, the zoospores, once released from their resting spore into the soil, are susceptible to environmental extremes and are short lived, needing to locate a susceptible host within approximately 5 h (Karling, 1968; Merz, 1997). Successful infection of the roots of a host plant leads to a polycyclic infection pattern with development of zoosporangia that produce and release secondary zoospores (Ledingham, 1935). The external conditions in which the resting spores persist before and after zoospore release are critical to the development of the disease (Harrison *et al.*, 1997; Merz, 2008). Knowledge and manipulation of these conditions may be valuable for inoculum management of *S. subterranea* (Balendres *et al.*, 2016b). The longevity of *S. subterranea* resting spores provides a significant challenge for soil inoculum management. Contributing to the problem is the ability of host plants to produce very large numbers of resting spores during infection cycles, which are released into the soil, particularly from decaying roots. Attempts have been made using a variety of strategies to reduce pathogen multiplication and inoculum propagation (Falloon, 2008), but none have proven to be fully effective.

White (1954) showed that disease development could be suppressed by growing jimsonweed (*Datura stramonium*), an alternative host of *S. subterranea*, in fields several weeks prior to planting potato. It was hypothesized that planting *D. stramonium* diminished *S. subterranea* soil inoculum by stimulating zoospore release from resting spores. Root infection in *D. stramonium* occurs, but no root galls form within which new resting spores develop. Thus, the pathogen is unable to complete its life cycle and produce a new generation of resting spores to replenish soil inoculum (Jones & Harrison, 1969; Qu & Christ, 2006). Similar observations were made for the plasmodiophorid pathogen *Plasmodiophora brassicae*, where planting daikon (*Raphanus sativus*) in infested fields reduced the disease potential in subsequent susceptible crops (Murakami *et al.*, 2000). An alternative strategy that has yet to be explored would be chemical

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*Published online 21 December 2017*





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stimulation of resting spore germination in the absence of any host plants, which should result in death of released zoospores and reduced soil inoculum potential. A similar approach has been demonstrated for inoculum management of *Sclerotium cepivorum*, which causes allium white root rot, where chemical stimulation of sclerotium germination in the absence of host plants led to enhanced reduction of soil inoculum potential and subsequent reduction in crop disease (Davis *et al.*, 2007). Previous studies (Ledingham, 1934; Kole, 1954; Merz, 1989, 1992, 1997) indicated that zoospore release could be stimulated by incubation of resting spores in Hoagland's solution (HS). The first direct measurement of stimulation of zoospore release by HS was recently reported, but the specific chemical(s) involved was not determined (Balendres *et al.*, 2016a).

This study aimed to identify the component(s) of HS that stimulates germination of *S. subterranea* resting spores and to demonstrate the potential of such chemical stimulation for *S. subterranea* inoculum management.

## Materials and methods

### Preparation of *S. subterranea* inoculum

Resting spore inoculum was obtained from powdery scab-infected potato tubers collected from Devonport, Tasmania, Australia (lat −41.17, long 146.33). Diseased tubers were washed with running tap water for 1–2 min, soaked in 2% sodium hypochlorite (Pental Products Pty Ltd) for 3 min, rinsed in sterile water, and air dried. Lesions were excised using a scalpel, dried for 4 days at 40 °C, ground by mortar and pestle, and stored at 4 °C until use. The inoculum was approximately 1 year old when used. Inoculum contained approximately 6900 sporosori $mg^{-1}$, as determined by suspending 0.1 g of inoculum in 10 mL of water and quantifying using a haemocytometer under light microscopy (Balendres *et al.*, 2016a).

### Hoagland's solution (HS) and chemical constituents

Hoagland's solution was prepared according to a standard recipe (Falloon *et al.*, 2003) in sterile deionized distilled water (DDW; Table 1). All chemicals used in this study were sourced from Sigma-Aldrich, except potassium phosphate (BDH Analytical Chemicals), ammonium nitrate (Prolab), calcium nitrate and potassium nitrate (Chem Supply). When tested individually, each chemical constituent of HS was prepared at the respective concentration that it occurs in HS. The seven chemicals that form the micronutrient group of HS were prepared as a single solution.

### Testing HS and its chemical constituents for stimulation of resting spore germination

A modified method of Fornier (1997) was used to test the stimulatory activity of HS and its chemical constituents on germination of *S. subterranea* resting spores and zoospore release. Aliquots (1 mg) of dried *S. subterranea* resting spore inoculum were added to 2 mL microcentrifuge tubes and suspended in 1.5 mL of HS, solutions containing one of its constituent chemicals or the micronutrient group of seven chemicals, or in DDW. The microcentrifuge tubes were covered with aluminium foil and incubated at 15–18 °C. In each experiment, seven subsamples (35 μL) were taken from each test solution tube at 2- to 6-day intervals for 28–29 days post-treatment. Subsamples were placed onto microscope slides, mounted under a 22 × 22 mm cover slip, and examined following an inverted S pattern with a light microscope (DM 2500 LED; Leica Microsystem) at ×200 magnification. The time to first observation of zoospores and the numbers of visible zoospores were recorded. To verify the identity of *S. subterranea* zoospores, observations on zoospore swimming pattern and zoospore morphology were made (Kole, 1954; Merz, 1992, 1997).

Table 1 Chemical composition of the Hoagland's solution used in this study (Falloon *et al.*, 2003).

| Chemical | Final concentration (mg $L^{-1}$) |
|---|---|
| Calcium nitrate | 253 |
| Magnesium sulphate | 722 |
| Potassium nitrate | 2.3 |
| Ammonium nitrate | 246 |
| Potassium phosphate | 40 |
| Iron-EDTA | 20 |
| Micronutrients group | |
| Boric acid | 0.14 |
| Potassium chloride | 0.4 |
| Manganese sulphate | 0.09 |
| Zinc sulphate | 0.115 |
| Copper sulphate | 0.05 |
| Molybdic acid | 0.016 |
| Sodium molybdate | 0.022 |

Eight independent experiments were performed comparing HS to DDW with treatments within each experiment replicated three times. A further three experiments tested HS, its individual chemical constituents at concentrations that they occur in HS, and DDW; treatments within each experiment were replicated three times. After identifying Fe-EDTA as the stimulant component within HS (present at 0.05 mM), the effect of six Fe-EDTA concentrations (0.05, 0.5, 1, 2, 5 and 10 mM) on resting spore germination was subsequently tested, using the method described above. Treatments within this experiment were replicated three times.

### Effects of HS treatment on infectivity of resting spore inoculum

The tomato bait test procedure of Balendres *et al.* (2017), modified from Merz (1989), was used to compare the effects of Hoagland's solution and distilled water on root infection. Three-week-old healthy tomato (cv. Grape) plants, grown in the glasshouse, were gently uprooted and their roots were washed to remove adhering soil, before placing the plants into individual vials containing 25 mL HS or DDW and 1 mg of dried sporosorus inoculum. Vials were placed on a tube rack and incubated in a growth chamber with 16 h daylight and 60% relative humidity at 15–18 °C. After 4 weeks, the roots were washed. The middle to bottom portions of the roots were cut, placed on microscope slides, stained with 0.1% trypan blue for 10 min and observed under a microscope at ×200 magnification. Presence and severity of zoosporangia were assessed using the following scale: 0 (no infection), 1 (sporadic, zoosporangia covering approximately 1% of the roots), 2 (slight, 2–10%), 3 (moderate, 11–25%), 4 (heavy, 26–50%) or 5 (very heavy, >50%; Merz *et al.*, 2004). The experiment was repeated three

times with three (experiment 1) and five (experiments 2 and 3) treatment replications in each.

### Effects on pathogen inoculum of soil treatment with resting spore stimulants in absence of host plants

The effects of HS and Fe-EDTA soil treatments on *S. subterranea* soil inoculum levels were evaluated in two glasshouse pot trials. In the first trial, plastic pots (6.5 L capacity) were filled with nursery grade potting mix (1:1:8 mix of peat, coarse sand and composted pine bark). With the exception of the non-pathogen amended controls, each pot received 500 mg of dried sporosorus inoculum that was thoroughly mixed through the soil. Treatments were three chemical treatments (aqueous solutions of Fe-EDTA at 0.05 mM, Fe-EDTA at 10 mM, or HS containing 0.05 mM Fe-EDTA), two inoculated soil control treatments (untreated dry soil and DDW-treated), and an untreated (dry) non-inoculated soil control (Fig. 1). Each treatment was replicated four times, with the pots arranged in randomized complete block designs. Soil samples (2 g) were taken from each pot 1 week after establishment and prior to the first treatment application for pathogen DNA quantification. Treatments were applied as 1 L applications of chemical or DDW solutions mixed manually throughout the soil. Treatment applications were repeated six times over 34 days to maintain soil wetness but avoid waterlogged condition. After 36 days from the first chemical treatment, a second soil sample (2 g) was taken from each pot for pathogen DNA quantification. The period of incubation (36 days) was based on the results obtained from the previous bioassays where no zoospores were observed after this period in solutions containing stimulants.

The second trial repeated the design of the first except that (i) 4.5 L capacity pots were used, (ii) a different pathogen inoculum source was used, which was produced from peel of 30 infected tubers blended in 2 L water (Tegg *et al.*, 2015) and was applied to the soil as a slurry (estimated to contain *c.* 20 000 sporosori $mL^{-1}$), with 30 mL slurry added to each pot, (iii) the three chemical treatments were HS, Fe-EDTA at 10 mM, and a solution prepared from a commercial iron chelate formulation (Rapisol Fe; AgSpec) that contained Fe-EDTA at 10 mM, and (iv) the volume of each treatment solution applied at each treatment time was 500 mL. The average glasshouse daily temperatures during both trials were 21–25 °C.

### Quantifying *S. subterranea* DNA in soil samples

*Spongospora subterranea* DNA was extracted and quantified from soil samples using methods adapted from the protocols of the commercial PreDicta Pt pathogen testing service of the South Australian Research Development Institute (SARDI) (Ophel-Keller *et al.*, 2008; Tegg *et al.*, 2014, 2015). Soil samples were oven dried at 30 °C for 2–3 days and ground using mortar and pestle. Ground samples (50 mg) were mixed with 50 µL of nuclease-free water prior to DNA extraction. As an internal control, 210 pg of purified potato DNA (cv. Russet Burbank) was added to each sample. Total DNA was extracted from 25 mg of each dried soil sample using a commercial DNA isolation kit (PowerLyzer PowerSoil DNA Isolation kit; Mo Bio Laboratories Inc.) following the manufacturer's protocol. DNA extracts were quantified using a Qubit 2.0 Fluorometer (Life Technologies) and a 10 ng $\mu L^{-1}$ DNA working stock was prepared.

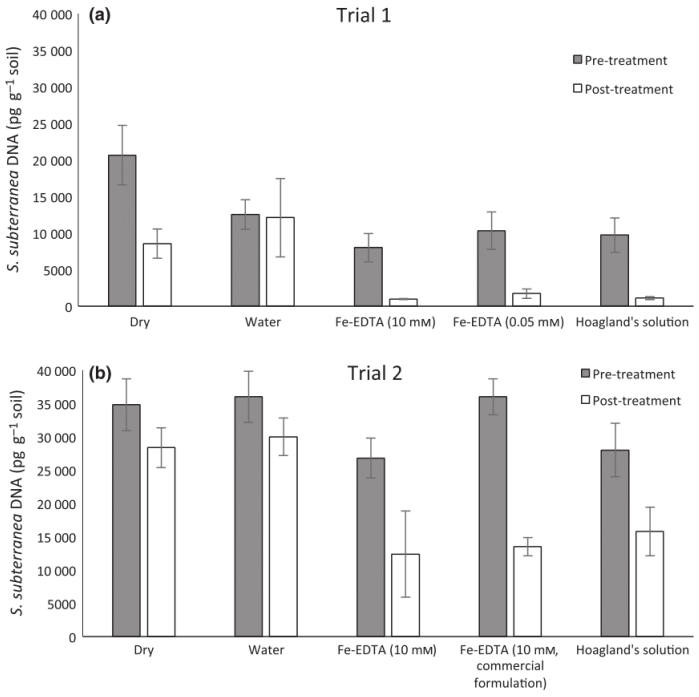


Figure 1 The impact (in two trials) of treatments of soil with stimulants of resting spore germination on mean DNA concentrations of *Spongospora subterranea* in the soil. Each treatment was applied to the soil six times within 34 days. Treatment 'Dry' indicates the soils were not moistened. Quantification of initial (before treatment) and final (after treatment) DNA concentration was carried out the day before application of chemical treatments and 36 days after the first treatment, respectively. Vertical bars are standard errors of means.

The concentration of *S. subterranea* DNA in each soil sample was determined by quantitative PCR (qPCR) following the protocol of Thangavel *et al.* (2015). Primers for quantification of *S. subterranea* (SPO10, SPO11, Probe) were from the ribosomal ITS region (Hernandez Maldonado *et al.*, 2012). An internal control, amplifying the conserved mitochondrial cytochrome oxidase (*COX*) gene from the spiked potato DNA, was run in all samples using previously reported primers (COX-F, COX-R) and probe (COX-P) sequences (Weller *et al.*, 2000). This was used as a positive internal control to confirm DNA quality and extraction efficiency, PCR amplification conditions and to normalize qPCR data for accurate quantification of the pathogen content. Quantitative PCR was performed using 2 μL (10 ng $\mu L^{-1}$) of the DNA template in a 10 μL reaction volume using the Sensi FAST Probe No-ROX kit (Bioline Pty. Ltd; Hernandez Maldonado *et al.*, 2012). All amplifications were carried out in a Rotor Gene 6000 instrument (Corbett Life Science) with a thermocycle of 95 °C for 15 min, then 40 cycles of 94 °C for 15 s and 60 °C for 60 s, with three biological replicates per soil sample and three technical replicates per biological replicate. The amount of *S. subterranea* DNA in each sample was calculated by an absolute quantification method, using a standard curve determined from the amplification of six tenfold dilutions (1.3 μg to 13 pg) of plasmid DNA containing the *S. subterranea* ITS sequence, supplied by the SARDI (Hernandez Maldonado *et al.*, 2012). The efficiency of the standard curve derived from the plasmid dilutions was determined as $R^2$ = 0.98–0.99.

Selected pre- and post-treatment soil samples from both trials were also sent to the SARDI for confirmatory testing of the soil *S. subterranea* DNA using the commercial PreDicta Pt test (http://www.pir.sa.gov.au/research/services/molecular_diagnostics/predicta_pt).

### Statistical analysis

For all experiments where numbers of zoospores were determined, they were converted to numbers per 100 μL prior to analysis. All data were tested for normality and homogeneity of variance before being subjected to further tests. An analysis of variance was performed using SPSS STATISTICS v. 22 (IBM) and Tukey's HSD test was used for multiple comparison of means at 0.05 probability level. For pairwise comparison of treatment means, the independent *t*-test ($\alpha$ = 0.05) was used.

## Results

### Effects of incubation of *S. subterranea* resting spores in HS and DDW on zoospore release

After 28 days' incubation, zoospores had been detected in all eight HS solutions, but in only four DDW controls from the eight experiments (Table 2). The mean total number of zoospores counted was greater ($P$ = 0.023 to $P$ < 0.001) in HS (35.0 to 246.3) than in the DDW control (0 to 71.7) in all experiments except experiment 8, where greater numbers of zoospores were found in the DDW control ($P$ = 0.023). In all experiments where zoospores were detected in both HS and DDW, including experiment 8, the time to the first observation of zoospores was significantly less ($P$ = 0.028 to $P$ < 0.01) in HS than DDW (Table 2).

### Effects of HS components and varying Fe-EDTA concentrations on zoospore release

Where the HS constituents were individually tested for their effect on stimulation of resting spore germination, zoospores were detected only in the Fe-EDTA solution (0.05 mM; all three experiments), the HS control (in both experiments in which it was tested) and the DDW control (in two of three experiments; Table 3). The numbers of zoospores detected varied between treatments within each experiment ($P$ = 0.012 to $P$ < 0.01), with the Fe-EDTA solution having similar numbers to that found in HS but substantially more than in DDW and all the other treatments. The time to first detection of zoospores was substantially earlier in HS (3–15 days) and Fe-EDTA (7–15 days) than in DDW (19–29 days; Table 3).

The testing of a range of Fe-EDTA concentrations (0.05–10 mM) revealed significant differences between treatments in both the number of zoospores released ($P$ < 0.01) and in the time to first zoospore observation ($P$ < 0.01) but no obvious trends could be seen in either the numbers or timing of zoospore release (Table 4).

Table 2 Mean numbers of zoospores and mean period (days) to zoospore release following treatment of *Spongospora subterranea* resting spores with distilled water or Hoagland's solution.

| | Experiment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Mean zoospore count[a] | | | | | | | | |
| Distilled water | 71.7 ± 7.2[b] | 0.0 ± 0.0 | 0.0 ± 0.0 | 13.7 ± 5.36 | 2.0 ± 5.4 | 0.0 ± 0.0 | 0.0 ± 0.0 | 39.7 ± 3.3 |
| Hoagland's solution | 246.3 ± 6.2 | 116.7 ± 22.3 | 35.0 ± 15.1 | 160.3 ± 69.3 | 126.0 ± 44.3 | 77.0 ± 13.6 | 35.0 ± 6.1 | 15.7 ± 4.1 |
| *P*-value | <0.001 | <0.001 | <0.01 | 0.023 | <0.01 | <0.001 | <0.001 | 0.023 |
| Days to initial zoospore release | | | | | | | | |
| Distilled water | 17.0 ± 0.0 | — | — | 7.0 ± 0.0 | 12.0 ± 0.0 | — | — | 11.3 ± 1.3 |
| Hoagland's solution | 7.0 ± 0.0 | 8.0 ± 0.0 | 8.0 ± 0.0 | 4.0 ± 0.0 | 4.0 ± 0.0 | 4.0 ± 0.0 | 7.0 ± 0.0 | 6.3 ± 0.7 |
| *P*-value | <0.01 | — | — | <0.01 | <0.01 | — | — | 0.028 |

[a]Zoospore numbers per 100 μL solution (cumulative count of seven subsamples taken at 2- to 6-day intervals for 28–29 days post-treatment).
[b]Values are means ± standard errors ($n$ = 3). Probability of difference between treatment means was tested using the independent *t*-test.

Table 3 Mean numbers of *Spongospora subterranea* zoospores released from resting spores treated with different Hoagland's solution components.

| | Mean zoospore number[a] | | | Days to first zoospore detection | | |
|---|---|---|---|---|---|---|
| Experiment | 1 | 2 | 3 | 1 | 2 | 3 |
| $CaNO_3$ | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | — | — | — |
| Micronutrients | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | — | — | — |
| $MgSO_4$ | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | — | — | — |
| $KNO_3$ | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | — | — | — |
| Fe-EDTA | 1046.3 ± 50.3 | 69.3 ± 30.9 | 87.3 ± 11.5 | 7 | 7 | 15 |
| $NH_4NO_3$ | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | — | — | — |
| $KH_2PO_4$ | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | — | — | — |
| Distilled water | 0.0 ± 0.0 | 16.3 ± 11.3 | 4.7 ± 1.2 | — | 19 | 29 |
| Hoagland's solution | nt | 157.7 ± 79.2 | 44 ± 14.1 | nt | 3 | 15 |
| *P*-value | <0.01 | 0.012 | 0.01 | | | |

nt, not tested in this assay.
[a]Zoospore numbers per 100 μL solution (cumulative count of seven subsamples taken at 2- to 6-day intervals for 28 days post-treatment). Values are means ± standard errors ($n = 3$). Probability of differences between treatment means was tested using Tukey's HSD.

Table 4 Mean numbers of *Spongospora subterranea* zoospores and periods to zoospore release from resting spores, for *in vitro* treatments with different Fe-EDTA concentrations.

| Fe-EDTA concentration (mM) | Mean zoospore count[a] | Days to initial zoospore release |
|---|---|---|
| 10 | 156.7 ± 7.2 | 7.0 ± 0.0 |
| 5 | 136.0 ± 1 | 5.0 ± 1.0 |
| 2 | 67.7 ± 6.7 | 10.0 ± 0.0 |
| 1 | 56.3 ± 5.2 | 4.0 ± 0.0 |
| 0.5 | 174.0 ± 9.1 | 10.0 ± 0.0 |
| 0.05 | 95.0 ± 12.9 | 14.0 ± 0.0 |
| *P*-value | <0.01 | <0.01 |

Values are means ± standard errors ($n = 3$). Probability of difference between treatment means was tested using Tukey's HSD.
[a]Zoospore numbers per 100 μL solution (cumulative count of seven subsamples taken at 2- to 6-day intervals for 28 days post-treatment).

Table 5 Mean infection severity scores by *Spongospora subterranea* zoosporangia on roots of tomato bait plants grown in distilled water or Hoagland's solution in three experiments.

| | Experiment | | |
|---|---|---|---|
| Treatment | 1 | 2 | 3 |
| Distilled water | 2.0 ± 0.6 | 3.0 ± 0.6 | 1.8 ± 0.4 |
| Hoagland's solution | 5.0 ± 0.0 | 4.6 ± 0.4 | 5.0 ± 0.0 |
| *P*-value | <0.001 | 0.065 | <0.001 |

Severity of zoosporangium infection was assessed using the scale: 0 (no infection), 1 (sporadic, zoosporangia covering approximately 1% of the roots), 2 (slight, 2–10%), 3 (moderate, 11–25%), 4 (heavy, 26–50%) and 5 (very heavy, >50%).
Values are means ± standard errors ($n = 3$, experiment 1; $n = 5$, experiments 2 and 3). Probability of difference between treatment means was tested using the independent *t*-test.

## Effects of HS on inoculum infectivity and root infection

Tomato seedlings incubated in HS with *S. subterranea* resting spores had greater incidence and severity of zoosporangium root infection than tomatoes incubated in DDW with *S. subterranea* resting spores (Table 5). The effects were highly significant in experiment 1 ($P < 0.001$) and experiment 3 ($P < 0.001$), while a similar but nonsignificant ($P = 0.065$) trend was recorded in experiment 2.

## Effects on pathogen inoculum of soil treatments with resting spore stimulants in absence of host plants

Pretreatment assays of soil for content of pathogen DNA revealed generally consistent amounts between each treatment within each trial. In Trial 1, the dry-treatment pots had a slightly elevated pretreatment content of pathogen DNA compared to the other treatments, which may reflect less even pathogen distribution within these pots during pretreatment sampling. Trial 2 had a greater initial pathogen inoculum applied than Trial 1, and this was reflected in the pathogen DNA contents detected (Fig. 1). HS and Fe-EDTA treatments (at 0.05 and 10 mM) applied to infested soil reduced *S. subterranea* DNA levels compared to the water only and dry control treatments in both glasshouse trials (Fig. 1). In the first trial, the percentage reductions of *S. subterranea* DNA for each treatment were: water control, 4%; dry control, 59%; Fe-EDTA 10 mM, 83%; Fe-EDTA 0.05 mM, 88%; and HS, 89%. Confirmatory results of treatment effects from an independent laboratory (SARDI) supported these data estimating an 89% reduction in *S. subterranea* DNA levels with the Fe-EDTA 10 mM treatment. The dry control showed a moderate but significant inoculum reduction that was not seen in the water control that may reflect the initial elevated pretreatment detection levels.

The extent of pathogen reduction in the second trial was less than the first trial, but the same pattern of treatment effects was observed (Fig. 1). The percentage reduction of *S. subterranea* DNA for each treatment was: water control, 17%; dry control, 18%; HS, 44%; Fe-EDTA 10 mM, 54%; and Fe-EDTA 10 mM commercial formulation, 63%. The formulation (analytical grade or

commercial) of Fe-EDTA did not significantly affect the reduction in amount of pathogen DNA (Fig. 1).

## Discussion

Previous studies have indicated that resting spores of *S. subterranea* suspended in HS increased severity of zoosporangium infection within host plant roots (Ledingham, 1934; Kole, 1954; Merz, 1989). Merz (1992, 1997) suggested this was due to enhanced stimulation of zoospore release. The present study corroborates these previous findings, confirming that HS treatment of resting spores in the presence of susceptible host plants exacerbates root zoosporangium infection. Furthermore, there was direct evidence that HS treatment of dried resting spore inoculum results in release of zoospores both earlier and in greater abundance than treatment with DDW. It is possible that earlier and greater zoospore release contributed to greater disease in the tomato bioassay. It has been previously demonstrated that early root infection results in greater root and tuber disease in potato hosts than if infection is delayed by several days (Thangavel *et al.*, 2015). However, while presence of more zoospores would probably increase the chance and rate of root infection, prior studies have shown that presence of large numbers of sporosori in soil does not always result in severe disease (Van De Graaf *et al.*, 2005).

It has been shown or suggested that HS stimulates zoospore release in other plasmodiophorids, including *P. brassicae* (Asano *et al.*, 2000; Friberg *et al.*, 2005) and *Polymyxa betae* (Ahm & Buchenauer, 1993). The similarity of response to HS by *S. subterranea* suggests that plasmodiophorids may share similar mechanisms for stimulating resting spore germination. However, these other plasmodiophorids may have varying responses to individual chemical components of HS, and the specific components of HS that affect resting spore germination of these pathogens are not known. For *S. subterranea*, although HS has been routinely used to create pulses of *S. subterranea* zoospore inoculum for biological and pathological studies (Merz, 1989, 1992, 1997; Falloon *et al.*, 2011), the stimulatory activity of the individual chemical components of HS has not been previously reported.

The present study identified Fe-EDTA as the only component that stimulated zoospore release. Adding chemical compounds that stimulate germination of resting spores or other structures of plant pathogens, in the absence of hosts, can be an effective approach for reducing pathogen inoculum levels and subsequently disease. For example, Davis *et al.* (2007) showed that application of garlic powder or diallyl disulphide to field soils infested with sclerotia of *S. cepivorum*, which causes allium white root rot, decreased the number of viable sclerotia by more than 90%. Similarly, Harman *et al.* (1980) identified organic compounds that stimulated germination of *Alternaria alternata* conidiospores, or conidiospores and chlamydospores of *Fusarium solani* f. sp. *pisi*; these authors suggested that chemical treatment in the absence of hosts may be expected to reduce inoculum potential. Recently, premature germination of *P. brassicae* resting spores was postulated as a possible tool for inoculum management by Mattey & Dixon (2015), following identification of root exudate compounds that stimulate resting spore germination. The present study demonstrated that resting spore germination stimulants could significantly reduce soil inoculum levels of *S. subterranea* as evidenced by a reduction in the content of pathogen DNA in the soil, providing proof of concept for this as a soil inoculum management tool. It is noted that quantitative PCR does not differentiate between viable and nonviable pathogen DNA; however, in a soil environment, rapid microbial degradation of unprotected DNA molecules is expected (Nielsen *et al.*, 2007). That Fe-EDTA provides stimulatory activity, rather than a more complex organic compound from root exudates that may be more expensive to obtain, may be beneficial in taking this proof of concept to practical disease management.

Soil inoculum was not eradicated by stimulant treatment over the trial periods considered in this study. It has recently been shown that not all resting spores of *S. subterranea* are responsive to germination stimulation, and that a level of constitutive dormancy exists (Balendres *et al.*, 2017). Thus, regardless of efficiency of distribution within the soil, it would be expected that soil treatment with stimulants might not provide complete disease control. Rather, soil treatments with germination stimulants to reduce preplant soil inoculum should be considered part of an integrated disease management system (Braithwaite *et al.*, 1994; Falloon *et al.*, 1996; Falloon, 2008; Merz & Falloon, 2009; Thangavel *et al.*, 2015; Wilson, 2016). Further optimization of the rate, frequency and timing of soil treatment, and consideration of the application technique is required when considering up-scaling treatments for field-testing. Effective reduction of soil inoculum may require repeated application and a substantial delay post-treatment prior to planting a susceptible host crop.

In conclusion, the findings from this study reveal that Fe-EDTA, a component of Hoagland's solution, stimulates resting spore germination and zoospore release in *S. subterranea*. This compound, if applied to infested soil in the absence of host plants, has the potential to reduce *S. subterranea* inoculum in the soil and subsequent disease development.

## Acknowledgments

The authors thank Tamilarasan Thangavel, Annabel Wilson and John Ireson (NTRL, UTAS-TIA, Australia) for technical support and advice. The first author is supported by a Tasmanian Graduate Research Scholarship from the University of Tasmania. This study was funded by Hort Innovation (PT14002), with coinvestment from Simplot Australia Pty. Ltd., McCain Foods Australia Pty. Ltd., and the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture The

University of Tasmania provided in-kind support. The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

# Field surveys for alternative weed hosts, and testing of the susceptibility of two important rotation crops, opium poppy and pyrethrum, to root infection by the causal agent of potato powdery scab, *Spongospora subterranea* in Australia

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**Keywords** *Solanum nigrum*, *Papaver somniferum, Tanacetum cinerariifolium*, tomato root infection, potato root galling, zoosporangia, qPCR analysis

## Abstract

*Spongospora subterranea*, the causal agent of powdery scab of potato, infects a diverse range of plant species. Crop rotation as a disease management tool will be compromised if pathogen hosts exist between potato crops. Field surveys of weed and volunteer crop species present within four commercial potato crops in North West Tasmania, Australia with a known history of powdery scab confirmed blackberry nightshade (*Solanum nigrum*) as a natural alternative host with zoosporangia present in roots with positive detection of *S. subterranea* by qPCR. Several additional species gave elevated qPCR results indicative of infection and host status but no zoosporangia were observed and thus remain putative natural hosts. One putative host, opium poppy (*Papaver somniferum*), is an important crop within intensive vegetable rotation production in NW Tasmania. Independent measurements of *S. subterranea* soil inoculum within a commercial field showed pathogen levels were substantially elevated following an opium poppy crop further suggestive of host status. In glasshouse testing we confirmed opium poppy and pyrethrum (*Tanacetum cinerariifolium*), another important crop within potato rotations, as hosts of *S. subterranea* with opium poppy the more susceptible of the two. Both species were less susceptible than tomato, a known host. Observations of early growth suggested inoculation impacts on all three plant species, although at 16 (tomato and opium poppy) or 26 (pyrethrum) weeks post inoculation only tomato had significantly reduced shoot and root development. We discuss the role of rotation crops and weeds in inoculum persistence and the possible role of *S. subterranea* as a minor pathogen of non-potato crops.

## Introduction

*Spongospora subterranea* f. sp. *subterranea* is the causal agent of powdery scab and root diseases of potato which are responsible for substantial economic losses to the global potato industries (Harrison et al. 1997; Merz and Falloon 2009; Falloon et al. 2016; Wilson, 2016). North West Tasmania, Australia is one of the most important potato production regions in Australia. Quantitative soil testing for abundance of *S. subterranea* in this region has revealed many fields with high soil inoculum levels (Sparrow and Wilson 2012; Sparrow et al. 2015). This is despite typical rotational practices in NW Tasmania of 5-8 years between potato crops, lengthy by national and global standards. Analyses of *S. subterreanea* soil inoculum levels in NW Tasmania throughout a typical cropping rotation found that following potato, inoculum levels in infested soils will be substantially raised, and will then gradually decline in the period between potato crops (Sparrow et al. 2015).

*Spongospora subterranea* is known to persist in the soil for many years between potato crops as aggregates of dormant resting spores called sporosori (Kole 1954; De Boer 2000; Falloon et al. 2011; Balendres et al. 2017). This may be influenced by presence of alternative host plants that can provide the opportunity to maintain and build soil inoculum during the intercropping period (Falloon 2008). Sporosori form within potato tuber lesions and in root galls (Kole 1954; Balendres et al., 2016). Whilst potato is recognised as the main economic host of this pathogen, surveys for field infections and experimental inoculum challenges have revealed a relatively broad and diverse range of host plants from over 20 plant families including both monocot and dicots, weeds and crop plants (Wurzer, 1964; Jones and Harrison, 1969; Jones and Harrison 1972; Foxe 1980; Andersen et al. 2002; Qu and Christ 2006; Aristizibal et al. 2013;). The non-potato hosts produce zoosporangia root infections of varying intensity, with only few also able to form root galls (Qu and Christ 2006).

In this study we firstly report the results of preliminary field surveys of Tasmanian potato fields known to be infested with *S. subterranea* inoculum for root infections in common weed and volunteer crop species found. We then describe an investigation of the susceptibility of two important industrial crop plants, opium poppy (*Papaver*

*somniferum*) and pyrethrum (*Tanacetum cinerariifolium*), that are a common part of potato rotations in Tasmania (Fist and Chung 2015), to *S. subterranea* root infection, and describe results from the testing of a commercial field site for *S. subterranea* inoculum levels following opium poppy cultivation. Furthermore, we make preliminary assessments of the possible impact of infection on early plant growth, and discuss the potential impact of crop plants as alternative hosts in maintenance of soil inoculum of *S. subterranea* in potato cropping systems.

## Materials and Methods

Field surveys for natural infections in common weed species

Samples of weeds, volunteer crop plants (opium poppy, oats, and barley) and potato plants growing within commercial potato crops were collected from four field sites in NW Tasmania in 2015 and 2016. The field sites were located near Riana (Riana 1, 41.22463 °S, 15.984103 °E; Riana 2, 41.223899 °S, 144.969408 °E), Sassafras (41.286780 °S, 146.499482 °E) and Red Hills (41.534722 °S 146.580639 °E). All potato fields sampled had a history of powdery scab and were rated as high disease risk based on pre-plant testing of DNA levels of *S. subterranea* inoculum in the soil using the Predicta Pt sampling and qPCR testing regime (http://www.pir.sa.gov.au/research/services/molecular_diagnostics/predicta_pt; Ophel Keller et al. 2009). At the time of collection of plant samples, potato crops were approximately 60-70 days after emergence. Weed, volunteer crop and potato plant sampling involved selecting plants of at least 2 months of age to ensure a greater opportunity for root infection. Two to three samples per plant species were collected by carefully uprooting intact plants and placing them in large containers and covering with soil to prevent root desiccation. All plant material was stored at 4 °C prior to assessment. Confirmation of weed species identity was done using appropriate taxonomic keys. Roots were observed for the presence of galling and then examined microscopically to identify incidence of zoosporangia infections.

Field observations of changes in *S. subterranea* soil inoculum following opium poppy cropping

At a commercial field site in Kapai Park, NW Tasmania, soil *S. subterranea* DNA levels were measured at four marked plot locations using the Predicta Pt sampling and qPCR testing regime (http://www.pir.sa.gov.au/research/services/molecular_diagnostics/predicta_pt; Ophel Keller et al. 2009). Samples were taken at potato planting (Oct 2015), mid growth of the potato crop (Jan 2016), at potato harvest (Apr 2016), and following harvest of the subsequent opium poppy crop (Mar 2017).

Experimental challenge of opium poppy, pyrethrum and tomato plants with *S. subterranea*

The susceptibility of opium poppy and pyrethrum to root infection with *S. subterranea* was tested and compared to tomato cv. Grape (*Lycopersicon esculentum*), a known host (Merz 1997, Balendres et al. 2018) in two glasshouse pathogen challenge trials.

*Spongospora subterranea* inoculum were prepared from infected potato tubers collected from a commercial crop in NW Tasmania. Tubers were washed in running water for 1-2 min and air-dried. Disease lesions were excised, oven-dried for 4 d at 40 °C, ground to a powder with a mortar and pestle and stored at 4 °C until use. The inoculum contained an estimated 6,900 sporosori/mg determined by enumerating with a haemocytometer.

In the first glasshouse trial, 42 plastic pots (20 cm diameter, 4.5 L capacity) were filled with nursery grade potting mix (1:1:8 mix of peat, course sand and composted pine bark; Horticultural Supplies, Hobart, Australia). Half of the pots were then amended with *S. subterranea* inoculum, prepared by the removal of peel from 15-20 powdery scab infected tubers and placing peel into a beaker with 2 L of water. The peel was then

homogenised in the solution using a stick blender. In the inoculum-amended pots, 30 mL of pathogen homogenate was added to the surface of each pot and the top 1/3 of the soil then thoroughly mixed. Seed of opium poppy, pyrethrum and tomato were sown into 14 pots each (7 inoculated and 7 un-inoculated) and on germination thinned to three plants per pot. Plants were grown under glasshouse conditions (22 °C +/- 2 °C) with water applied at regular intervals to maintain high soil moisture and favour disease (Falloon et al. 2016). A further addition of 30 mL of a new *S. subterranea* homogenate prepared in the same way as before was applied to each inoculated pot two weeks after thinning. Plants were regularly monitored for any visual effects of inoculation and plants harvested for root disease assessment at *c.* 9 weeks after emergence.

In the second trial, seeds of opium poppy, pyrethrum and tomato were sown in seedling trays and 132 plastic pots (20 cm diameter, 4.5 L capacity) were filled with potting mix. Three-week old seedlings of each plant species were individually transplanted into 44 pots (22 amended with *S. subterranea* inoculum and 22 unamended). After two weeks, 20 mL of pre-conditioned inoculum (2 g dried inoculum/1 L Hoagland's solution) was applied. Soil was excavated to a depth of 2-3 cm near the plants roots, the inoculum suspension applied and then the soil replaced. Inoculum pre-conditioning was done to stimulate zoospore release (Merz 1997). Plants were grown under glasshouse conditions with water applied to maintain high soil moisture as before. Plants were harvested at *c.* 16 weeks (tomato and opium poppy) or *c.* 26 weeks (pyrethrum) after emergence and stem height, shoot dry weight, root dry weight and root disease assessed.

Zoosporangia root infection assessment

Presence of *S. subterranea* zoosporangia indicating root infection was examined in three distinct zones (upper, mid and lower) of each whole root sample. All roots were triple-washed in running water before sub-samples were excised. Three to eight pieces of freshly cut roots, from each zone, each measuring *c.* 2cm, were placed on a glass slide, flooded with 0.1% trypan blue in lactophenol for 15 minutes, rinsed well with water and mounted with one drop of glycerol. Mounted specimens were examined for presence of

stained *S. subterranea* zoosporangia in the epidermal root cells and root hairs using a compound microscope at 200 × and 400 × magnifications for identification and confirmation of infection respectively. In the opium poppy, pyrethrum and tomato challenge experiments, infection intensity was scored using the rating scale of Merz et al. (2004): 0 (no infection), 1 (sporadic, zoosporangia covering approximately 1% of the roots), 2 (slight 2-10%), 3 (moderate, 11-25%), 4 (heavy, 26-50%) and 5 (very heavy, >50%). Additional sub-samples of root tissues were taken and stored at -20 °C for qPCR analysis to confirm *S. subterranea* identity.

Molecular confirmation of *Spongospora subterranea* infection

Testing for root infection by *S. subterranea* followed the method of Thangavel et al. (2015). Root samples from each plant (50 mg) were cut into small pieces and DNA extracted using DNeasy PowerPlant Pro Kit DNA isolation kit with RNAase treatment (Qiagen Group, Germany). DNA yields were quantified with Qubit 2.0 Flurometer (Life technologies, Darmstadt, Germany). DNA samples (*c.* 20 ng DNA per reaction) were tested for presence of *S. subterreanea* by qPCR in a 10 µL volume reaction using the Sensi FASTTM Probe No-ROX Kit (Bioline Pty.Ltd, Australia) with primers SPO10; 5'-CCGGCAGACCCAAAACC-3', SPO11; 5'-CGGGCGGTCACCCTTCA-3' and TaqMan probe 5'-/56-FAM/CAGACAATC/ZEN/GCACCCAGGTTCTCATG/3IABkFQ/-3'. All reactions were carried out in a Rotor Gene 6000 instrument (Corbett Life Science, Sydney, Australia), with a thermal cycling condition of 95°C for 5 mins, then 40 cycles of 95°C for 10 s, 60°C for 50s and 72°C for 20s, with three replicates per sample, together with a known positive and negative non-template control (NTC). The amount of *S. subterranea* DNA in each sample was calculated by an absolute quantification method, using a standard curve determined from the amplification of six ten-fold dilutions (1.3 µg to 13 pg) of plasmid DNA containing the *S. subterranea* ITS gene kindly supplied by the South Australian Agricultural Research and Development Institute (Hernandez Maldonado *et al.*, 2012). The efficiency of the standard curve derived from the plasmid dilutions was determined as $R^2$*=0.98-0.99*.

Data Analysis

Data were tested to ensure normality. Pathogen DNA data were log transformed prior to analysis. Data were assessed using one-way or two-way analysis of variance (ANOVA) to determine the statistical significance of variation between treatment means and their interactions. Where significant differences ($P<0.05$) were found the least significant difference (LSD) was determined using GENSTAT v. 14.2 (VSN International Ltd., Hemel Hempstead, UK).

## Results

Field surveys for natural infections in common weed species

Observation of harvested potato plants from all four survey sites showed presence of *S. subterranea* root galls and tuber lesions in all plants (Table 1). Microscopic examination confirmed the presence of zoosporangia clusters in potato root hairs. This was further supported by qPCR testing for *S. subterranea* in selected potato root samples. In contrast, none of the sampled weed and volunteer crop plants showed root galling. Zoosporangia were found in root hairs from two blackberry nightshade (*Solanum nigrum*) plants sampled from the Riana 2 site (Table 1). No other zoosporangia were observed in any other root samples from any species at the four sites (Table 1). Quantitative PCR confirmed that the root samples from *S. nigrum* plants with visible zoosporangia contained high levels of *S. subterranea* DNA (500-fold greater than the NTC). Similar high DNA levels were detected in *S. nigrum* (500-fold greater than NTC) and *Chenopodium murale* (150-fold greater than NTC) sample roots, both from the Sassafras site, even though zoosporangia were not observed from these plants. Elevated levels of *S. subterranea* DNA were detected from *S. nigrum* at Riana 1 (30-fold greater than NTC), *Sonchus arvensis*, *C. murale*, *Sisymbrium officinale* (all from Riana 1 at 8-fold greater than

NTC), *Rumex conglomeratus* at Sassafras, *S. arvensis* at Riana 2 and *Papaver somniferum* at Riana 2 (at 4-fold greater than NTC). Quantitative PCR testing was not conducted from samples taken from the Red Hills site.

Field observations of changes in *S. subterranea* soil inoculum following rotation crops

The mean of log *Spongospora subterranea* soil DNA levels measured at four marked plot locations prior to, during and post potato cropping and post a subsequent opium poppy crop varied significantly ($P<0.001$). At potato planting DNA levels were 6586 pg/g (mean log 3.81) on average at the sample site. This is considered a high disease risk level for subsequent potato cropping (Ophel Keller et al. 2009). Levels increased slightly, but not statistically significantly to 9311 pg/g (3.95) by mid-growth stage of the potato crop. At harvest the mean DNA levels were 16,964 pg/g (4.18) which was significantly greater than both mid-growth and initial inoculum level. Following the subsequent opium poppy crop S*. subterranea* soil inoculum levels had risen further to 37,893 pg/g (4.53), which was significantly greater than the values recorded following potato harvest.

Experimental challenge of opium poppy, pyrethrum and tomato plants with *S. subterranea*

Observations of plants from the first glasshouse trial suggested the inoculation treatment affected early growth in opium poppy, pyrethrum and tomato plants compared to the un-inoculated controls. Tomato showed an obvious visual reduction in shoot growth in the inoculated plants compared to the un-inoculated plants (Fig 1). Similarly inoculated pyrethrum plants appeared slightly smaller than un-inoculated plants, and inoculated opium poppy plants showed leaf yellowing, reduced vigour, smaller leaves and flower heads compared to the un-inoculated plants (Figure 1). Microscopic examination of root material from the three species showed presence of zoosporangia in roots of tomato (21/21) and opium poppy (5/21) but not pyrethrum (0/21) plants (Table 2). The

differences between mean zoosporangial score for the three species was significant ($P<0.001$) with tomato, opium poppy and pyrethrum giving 3.39, 1.11 and 0.0 mean scores respectively (Table 2). Un-inoculated plants of all species did not show any zoosporangial root infection. There was also a significant difference in *S. subterranea* DNA levels between the three tested species ($p<0.001$) with tomato roots having the highest pathogen DNA levels ($4.07*10^{10}$ pg/g; mean log 10.61) followed by opium poppy ($7.85*10^{8}$ pg/g; 8.89) and pyrethrum ($2.38*10^{7}$ pg/g; 6.37) (Table 2).

In the second challenge trial all three species succumbed to infection. Incidence of zoosporangial infection varied across the three species, the highest incidence was recorded in tomato (22/22), followed by opium poppy (7/22) and pyrethrum (4/22) (Table 2). In tomato infection was frequently observed in both root hair and epidermal cells, however in opium poppy and pyrethrum, infection was mostly restricted to the root hairs (Fig 1). Un-inoculated plants did not show any zoosporangial root infection. The severity of infection was again significantly different between species with tomato, opium poppy and pyrethrum scoring 1.85, 1.0 and 1.0 mean scores respectively (Table 2). Despite no numerical difference between opium poppy and pyrethrum using the rating scale infection in opium poppy root hairs appeared slightly more intense than in pyrethrum. Root infection in all three species was confirmed by qPCR. The quantity of *S. subterranea* DNA varied among the species with infected tomato, opium poppy and pyrethrum roots having an average of $2.16*10^{11}$ (mean log 11.33), $6.65*10^{9}$ (9.82) and $6.94*10^{8}$ (8.84) pg *S. subterranea* DNA per gram of root respectively (Table 2).

The roots of opium poppy plants 16 weeks following inoculation could not be recovered in full and thus were not assessed for root mass. *Spongospora subterranea* infection had no significant impact on pyrethrum or opium poppy shoot height or dry weight or pyrethrum root length or dry weight. Infected tomato plants had significantly reduced shoot and root dry weight, but not shoot height or root length compared to un-infected plants (Table 3).

## Discussion

Crop rotation is an important tool for the integrated management of potato powdery scab (Merz and Falloon 2009). The efficacy of rotation for disease suppression will be compromised if volunteer potato and weeds that can act as alternative hosts of *S. subterranea* persist between potato crops. Similarly, the resistance or immunity of crops used in rotation is very important to the success of controlling disease intensity and severity (Wale 1987).

Several studies have examined the possible host range of *S. subterranea*. It was first thought that host plants may be restricted to members of the Solanaceae (Würzer 1964). However subsequent studies demonstrated a broad host range for *S. subterranea* including species from at least 21 diverse plant families (Jones and Harrison 1972; Andersen et al. 2002; Qu and Christ 2006, Arcila Aristizabal et al. 2013). Infection has been generally confirmed by observation of zoosporangia within infected roots, however, few of these alternative hosts were found to form root galls within which form sporosori, a conglomerate of resting spores important for persistence of soil inoculum. Galling was seen on Eastern black nightshade (*Solanum plycanthum*), jimsonweed (*Datura stramonium*), oat (*Avena sativa*), penlate orchard grass (*Dactylis sanguinalis*), tomato and yellow mustard (*Brassica campestris*; Qu and Christ 2006) which were confirmed due to *S. subterranea* infection by PCR. As a cautionary note, microscopic evidence from alternative host studies suggest that root galls may not always contain sporosori (Qu and Christ 2006), and conversely in some alternative hosts sporosori have been found associated with infected root hairs in absence of galls (Arcila Aristizabal et al. 2013).

Blackberry nightshade, and related nightshade species, are common weeds in potato crops (Shah et al. 2010). The *S. subterranea* host status of blackberry nightshade has been thoroughly demonstrated (Jones and Harrison 1972; Andersen et al. 2002; Qu and Christ 2006, Shah et al. 2010; Arcila Aristizabal et al. 2013) and the results from our survey support this. Several other weed species were identified in our surveys as putative hosts determined by qPCR results but not confirmed by microscopic observation of zoosporangia in root samples. The discrepancy between molecular testing and microscopic observations may reflect the reduced amount of root tissue examined microscopically compared to the amount tested by qPCR where possible hosts had very limited root infection or alternatively the positive qPCR results could have been

influenced by possible contamination of samples from trace soil residues which was not successfully removed by rinsing. Controlled inoculation trials may be needed to make a definitive assessment of the host status of those putative hosts. Of the putative host species, *Sonchus arvensis* has previously been reported as a host (Andersen et al. 2002). There appears no previous record of infection of *Chenopodium murale, Sisymbrium officinale, Rumex conglomeratus* and *Papaver somniferum* by *S. subterranea,* although taxonomically close relatives have been identified for most of these in previous studies (Jones and Harrison 1972; Andersen et al. 2002; Qu and Christ 2006; Arcila Aristizabal et al. 2013).

In this study, opium poppy and pyrethrum, both important crops within the intensive vegetable production regions of NW Tasmania and commonly used in rotation with potato, were shown to be new hosts of *S. subterranea*. Previously the common agricultural weed and related species *Papaver rhoeas* (common or corn poppy), has been shown to be a host of *S. subterranea* (Jones and Harrison 1972), and thus susceptibility of opium poppy could have been suspected. The observation that soil inoculum levels of *S. subterranea* can substantially increase following an opium poppy crop grown immediately after a potato crop is of possible concern and suggests potential limitations of poppy as a rotation choice in potato production systems. In contrast, in a prior study that compared soil *S. subterranea* levels in field sites that had grown either opium poppy or pasture prior to potato production suggested pathogen levels were on average lower in field that had grown poppy, but that this did not result in less disease in the subsequent potato crop (Sparrow and Wilson 2012). Also, in a multi-year rotation trial conducted in NW Tasmania there was no significant effect of opium poppy crops on *S. subterranea* soil inoculum levels, but opium poppy crops did increase levels of *Rhizoctonia solani* AG2.1 (Sparrow et al. 2015). In the current study, inoculated opium poppy plants grown in the glasshouse did not show root gall development associated with zoosporangial infection. This suggests opium poppy infections may not necessarily contribute to sporosori populations in the soil, although there is the possibility of sporosori production in absence of root galls (Arcila Aristizabal et al. 2013), and field grown opium poppy plants have greater vigour than pot grown plants which could affect propensity of gall formation. If sporosori are not or are seldom produced, the increase in

field inoculum levels seen may be short-lived and perhaps associated with residual root material following harvest. Further examination of inoculum persistence following opium poppy production is warranted.

By comparison, tomato was more susceptible to *S. subterranea* root disease than opium poppy which was in turn more susceptible than pyrethrum. Inoculation appeared to reduce or slow early plant development in tomato, pyrethrum and opium poppy plants grown in pots under glasshouse conditions, although growth differences were not evident in a second trial measured at a later development stage. The impact of *S. subterranea* on glasshouse and field grown potato has been well documented (Lister et al. 2004; Falloon et al. 2004; Shah et al. 2012; Hernandez-Maldonodo et al. 2013; Falloon et al. 2016). Reduced growth in tomato grown in glasshouse or hydroponic culture has also been previously documented (Nielson and Larsen 2004; Balendres et al. 2018), although impact on growth in field grown plants and on quantity and quality of fruit remains to be tested. The extent of root infection in opium poppy and pyrethrum plants was less than that seen in tomato, and the impact on plant growth was not significant. However, it remains to be seen whether infection under field production may affect plant growth, persistence of the perennial pyrethrum plants, and/or pyrethrin and opiate yields for which these crops are primarily grown (Fist and Chung 2015). Given that the major cropping soils for opium poppy and pyrethrum production in Australia commonly contain high to very high levels of *S. subterreanea* inoculum, it may be worthwhile to further examine the possible impact of infection in the two species grown in commercial field conditions.

In this study we demonstrate new hosts of *S. subterranea* including two important crop species. This highlights the need for careful choice in designing rotations for soil inoculum management, and possible new disease impacts on productivity of these important crop species.

## Acknowledgements

This study was funded by Hort Innovation (PT14002), with co-investment from Simplot Australia Pty. Ltd., McCain Foods Australia Pty. Ltd., and the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture. The authors declare that there is no conflict of interest.

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## Figure legends

**Figure 1.** Effect of *Spongospora subterranea* inoculation on early plant development of (a) tomato (b) opium poppy and (c) pyrethrum plants in glasshouse culture and presence of S. subterranea zoosporangia in root tissues of (d) tomato, or root hairs of (e) opium poppy and (f) pyrethrum.

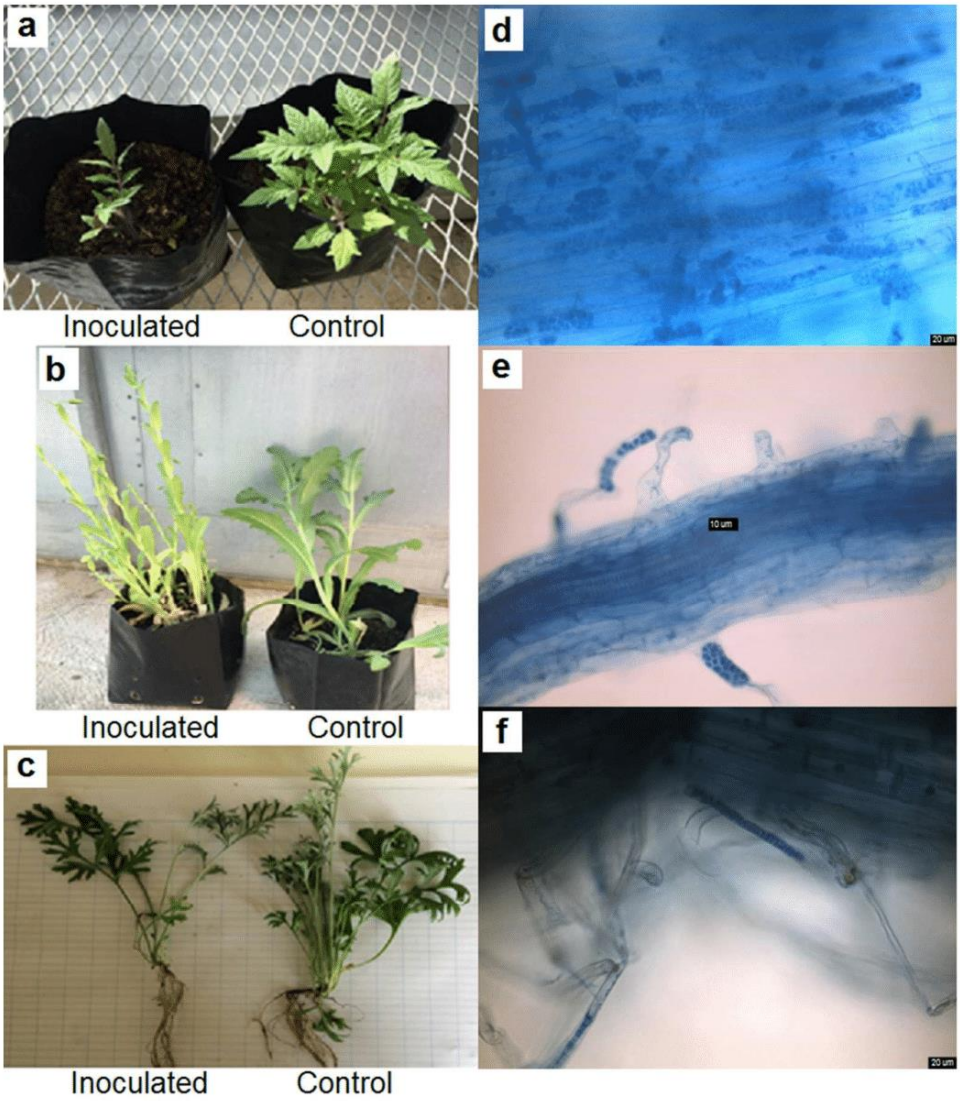


Figure 1. Effect of *Spongospora subterranea* inoculation on early plant development of (a) tomato (b) opium poppy and (c) pyrethrum plants in glasshouse culture and presence of *S. subterranea* zoosporangia in root tissues of (d) tomato, or root hairs of (e) opium poppy and (f) pyrethrum.

230x264mm (96 x 96 DPI)

**Table 1** Microscopic detection of *Spongospora subterranea* zoosporangia within root samples of weeds and volunteer crop plants collected from within potato fields in NW Tasmania.

| Common name | Scientific name | Riana 1 (2015) | Riana 2 (2015) | Sassafras (2015) | Red Hills (2016) |
|---|---|---|---|---|---|
| ***Solanaceae*** | | | | | |
| Potato | *Solanum tuberosum* | 3/3 | 3/3 | 3/3 | 3/3 |
| Blackberry nightshade | *Solanum nigrum* | 0/2* | 2/2* | 0/3* | - |
| ***Amaranthaceae*** | | | | | |
| Sow Bane | *Chenopodium murale* | 0/2* | 0/2* | 0/3 | 0/3 |
| ***Asteraceae*** | | | | | |
| Sow thistle | *Sonchus arvensis* | - | 0/2* | 0/2* | 0/3 |
| ***Brassicae*** | | | | | |
| Wild radish | *Raphanus raphanistrum* | - | - | - | 0/3 |
| Hedge mustard | *Sisymbrium officinale* | 0/2 | - | 0/2* | - |
| Shepherd's purse | *Capsella bursa-pastoris* | 0/2 | - | - | - |
| ***Caryophyllaceae*** | | | | | |
| Chickweed | *Stellaria media* | - | - | - | 0/3 |
| ***Fabaceae*** | | | | | |
| Sub Clover | *Trifolium subterraneum* | 0/2 | - | 0/3 | 0/3 |
| ***Papaveraceae*** | | | | | |
| Opium poppy | *Papaver somniferum* | - | 0/2* | - | - |
| Fumitory | *Fumaria officinalis* | - | - | 0/2 | - |
| ***Plantaginaceae*** | | | | | |
| Plantain | *Plantago lanceolata* | - | 0/2 | - | - |
| ***Poaceae*** | | | | | |
| Ryegrass | *Lolium multiflorum* | - | 0/2 | 0/3 | - |
| Oats | *Avena sativa* | - | - | - | 0/3 |
| Barley | *Hordeum vulgare* | - | - | - | 0/3 |
| ***Polygonaceae*** | | | | | |
| Clustered dock | *Rumex conglomeratus* | 0/2* | - | - | - |

* indicates putative detection of *S. subterranea* by qPCR from additional root segments tests.

**Table 2** Effect of *Spongospora subterranea* inoculation on zoosporangial root infection in Tomato, Opium poppy and Pyrethrum.

| | Plants with zoosporangial infection / total plant inoculated | Zoosporangia score (0-4) | *S. subterranea* DNA pg/g (log) |
|---|---|---|---|
| **Trial 1** | | | |
| Tomato | 21/21 | 3.39 | $4.07*10^{10}$ (10.61) |
| Opium poppy | 5/21 | 1.11 | $7.85*10^{8}$ (8.89) |
| Pyrethrum | 0/21 | 0.0 | $2.38*10^{7}$ (6.37) |
| *LSD (F probability)* | | *0.415 (<0.001)* | *0.426 (<0.001)* |
| **Trial 2** | | | |
| Tomato | 22/22 | 1.85 | $2.16*10^{11}$ (11.33) |
| Opium poppy | 7/22 | 1.00 | $6.65*10^{9}$ (9.82) |
| Pyrethrum | 4/22 | 1.00 | $6.94*10^{8}$ (8.84) |
| *LSD (F probability)* | | *0.167 (<0.001)* | *0.695 (<0.001)* |

**Table 3** Effect of *Spongospora subterranea* infection on shoot and root growth assessed 16 weeks (tomato, opium poppy) or 26 weeks (pyrethrum) after inoculation.

| | | Shoot height (cm) | Shoot dry weight (g) | Root length (cm) | Root dry weight (g) |
|---|---|---|---|---|---|
| Tomato | Uninoculated | 64.30 | 14.77 | 38.45 | 1.94 |
| | Inoculated | 59.60 | 9.55 | 37.82 | 1.50 |
| *LSD (F Probability)* | | *ns (0.11)* | *3.48 (0.005)* | *ns (0.40)* | *0.43 (0.046)* |
| Pyrethrum | Uninoculated | 23.48 | 17.05 | 35.38 | 13.37 |
| | Inoculated | 24.25 | 15.40 | 37.50 | 11.68 |
| *F Probability* | | *ns (0.43)* | *ns (0.21)* | *ns (0.52)* | *ns (0.89)* |
| Opium poppy | Uninoculated | 105.70 | 17.50 | - | - |
| | Inoculated | 114.40 | 19.53 | - | - |
| *LSD (F Probability)* | | *ns (0.19)* | *ns (0.47)* | | |

ns; not significant at P=0.05

## Appendix 2a – Cultivar resistance

**Determining the relative resistance or tolerance of important processing potato varieties to root infection.**

**Summary.** A series of two *in vitro*, two glasshouse, two mini-plot and one field trial have been conducted assessing resistance to root infection within selected processing cultivars including numbered lines under assessment by the processing companies as possible new cultivars of importance to the Australian industry.

Initially, within these trials we have measured and quantified root pathogen *S. subterranea* DNA levels, zoosporangial infection, root galling score and where present – powdery scab tuber disease. The trials produced disease outcomes ranging from low levels of disease incidence in one of the pot trials to high disease incidence in the field trial. The mini-plot trials produced intermediate disease levels.

The most detailed studies (GH1 and FT) were able to track increasing levels of pathogen DNA through the assessment dates (20, 40, 60 and 80 DAE – GH1; 30, 60, 90 DAE – FT) and this was generally associated with increasing levels of zoosporangial infection. GH1 had low incidence of root disease, but sufficient to infect 6 of the 15 cultivars tested; these cultivars included varieties with known susceptibilities to root infection and a couple of the industry varieties. DNA levels were low at 20DAE (<200 pg S. subterranea), increasing in the later assessment dates (60 DAE some varieties >5000 pg S. subterranea). Many of the varieties tested (putatively resistant) did not succumb to infection in this Glasshouse trial, but the identification of susceptible lines was useful.

The major FT tested 15 separate varieties and achieved greater incidence of root disease with all varieties showing some level of infection. Variability in PCR levels reduced the usefulness of the DNA data, but complemented by the zoosporangial data, provides further useful data on these important industry cultivars. We were able to confirm the susceptibilities of some of the putatively sensitive varieties and confirm high levels of resistance in 1-2 of the new industry lines. It is important to note that no line tested showed complete resistance.

The inherent variability that exists in glasshouse and field trials lead us to develop a faster, cheaper and possibly more useful assay for detecting resistance to Spongospora. We developed a new *in vitro* assay that focusses on the zoospores binding to and encysting on host roots which is: a) much more rapid with results within 1 week, b) more robust with controlled inoculation conditions, c) requires much less resources (plants, land, time, labour, machinery), d) not time dependant and not influenced by polycyclic infection cycles. An initial experiment showed that levels of encystment were consistent with resistance of key cultivars reported from the glasshouse and field experiments validating the efficacy and robustness of this new assay. Further development of this assay should be a priority for accurate assessment of potato cultivar root infection resistance.

Overall, these trials have identified lines ranging from highly susceptible through to those with a moderate degree of resistance. The results of these trials are confidential and will be provided to industry partners separately.

**Methods.**

**Experimental Set Up - Pot trials (PT1 and PT2)**

Two pot trials (PT1 and PT2) were conducted within a glasshouse with temperatures maintained between 16-22°C. PT1 was undertaken under ambient light conditions while PT2 was supplemented by artificial lighting (16 h light/8 h dark). Plastic pots (20cm diameter, 4.5L volume) were filled with a pasteurized potting mix containing sand, peat, and composted pine bark (10 : 10 : 80; pH 6.0) and premixed with Osmocote 16-3.5-10 NPK resin coated fertiliser (Scotts Australia Pty Ltd.) at the rate of 6 kg/m3. In both PTs there were control (non-treated pots) and pots amended with S. subterranea.

In both PTs the inoculated pots were amended by mixing *S. subterranea* infested soil with standard potting material in a 1:2 ratio. Additional *S. subterranea* inoculum was also added just after planting to the surface of all the treated pots to increase the chance of infection. The *S. subterranea* inoculum was prepared using a modification of previous methods (Bell, Roberts et.al. 1999, Tegg, Thangavel et.al 2015). Peel from ~30 infected tubers (to a depth of 1 cm) was removed and placed in a beaker that was made up to 2 L with tap water. The solution was mixed constantly with a stick blender and approximately 30 mL of inoculum solution was added to the surface of each pot. In addition to that, a previously created *S. subterranea* inoculum powder (Balendres, Mark A et al. 2016) was also added to the top of treated pots.

In PT1, 10 cultivars (mini-tubers) were planted at a depth of 15 cm on the 30$^{th}$ November 2015. Roots were assessed at 20 day intervals up to 80 DAE. The roots were kept for gall assessment, microscopy and pathogen quantification by qPCR. Three replicates of the 10 cultivars (AI, AD, AE, AA, AC, Bondi, Desiree, AF, AG & Ranger Russet) were assessed for tuber disease, yield and root infection. Both inoculated and controls were assessed from this completely randomized block experiment.

In PT2, 5 cultivars (mini-tubers) were planted at a depth of 15 cm on the 21$^{st}$ March 2016. The cultivars included Russet Burbank, Desiree, AD, RBK5 and Ranger Russet. Fifteen pots of each cultivar was planted; 9 pots amended with S. subterranea and 6 control pots. All were placed in a completely randomized design. The average emergence date was on the 26$^{th}$ April 2016 with the exception of Ranger Russet which was omitted from the study due to poor emergence.

Two replicates of each variety were destructively harvested ~30 days after emergence and then ~60 days after emergence. Tubers from each replicate were counted, collectively weighed and scored for tuber infection. The roots were kept for gall assessment, microscopy and pathogen quantification by qPCR. The remaining replicates were assessed for yield (number of tubers and combined weight per plant) on the 20$^{th}$ September just after plants had senesced.

**Mini-Plot Trials (MP1 and MP2)**

Two outside plot trials were conducted within a shadehouse subject to summer/autumn environmental growing conditions. The soil plots consisted of a sandy loam supplemented with a potting mix. Inoculated plots had *S. subterranea* infested potting mix incorporated into the plots as well as dried *S. subterranea* sporosori inoculum incorporated into the furrow at planting. The control plots had no *S. subterranea* added.

The experimental design was a split block design with three replicates. The control and inoculated treatments were split into separate blocks to avoid contamination. Within each block, 8 tubers each of the six cultivars Bondi, AD, Ranger, RBK5, Desiree and AH were planted in rows.

Mini-tubers of the cultivars were planted into a furrow (approx. ~20cm deep with 20 cm spacing) on 24th December 2015. The furrow was covered and hilled over manually similar to how planting occurs in a commercial field. An initial irrigation was applied by overhead sprinklers to thoroughly wet the soil. Tubers emerged approximately the 22nd of January 2016, after emergence overhead sprinkler irrigation occurred thrice weekly to ensure thorough wetting of the soil so as to encourage *S. subterranea* infection (Falloon REF). Throughout the duration of the trial no pesticides, fungicides of fertilisers were utilised.

Approximately 75 days after emergence the roots and tubers were harvested from all the plots, with the exception of Desiree and AH, where poor emergence and growth meant that some of these plots were not included in the harvest. From each inoculated and control block, 8 plants of each variety variety was carefully harvested and washed. Each plant had tubers collectively weighed and each was scored for tuber infection. Roots from each plant were assessed for the presence of galling. Two plants from the inoculated plots of each variety had their roots triple-washed to remove any adhering soil, these samples were assessed by microscopy for zoosporangia infection and a small sample was taken for drying prior to pathogen quantification by qPCR.

In MP2 the same methodology above was used except that 12 cultivars were assessed and the trial was planted a year later.

**Field Trial (FT)**

At the Leigh Elphinstone property (Field trial 1) we tested the germplasm (Program 3) supplied by the two companies (15 varieties in total: 5 varieties each from Simplot and McCain plus 5 control varieties) for root infection and galling. This included sequential assessments of root material on three separate dates (11th Jan 2017, 7th Feb 2017, 8th Mar 2017) and a tuber assessment at plant senescence (5th April 2017).

*Encystment trials*

The encystment work is prepared as a mini-paper in Appendix 2b, which provides complete methodology, results and discussion.

**Results.**

**Pot trial 1 (PT1)**

The first pot trial (PT 1) was destructively harvested at 80 days after plant emergence at which time the plants were showing signs of senescence. The root tissue obtained was scarce and in a slightly degraded state. At this assessment microscopic examination revealed no evidence for any zoosporangia on any of the root tissue samples. qPCR testing did however detect moderate to high levels of *S. subterranea* DNA (6619 – 484148 pg/g root tissues) associated with root samples, and light to moderate root galling numbers and severity was found on some cultivars (Table 1). There were no statistically significant differences found in pathogen DNA levels (p=0.513), average number of root galls (p=0.123) nor average gall severity score (p=0.235) between cultivars. The cultivars AD, AA and AF had no root galling although AA had the highest levels of pathogen *S. subterranea* DNA within the root tissue. AE and AG had the highest number of galls and gall severity score while AC had the lowest pathogen DNA levels.

There was no statistically significant interaction effect between individual cultivars and the inoculum treatments (p>0.05). However, a statistically significant difference was evident in mean tuber weight between cultivars (p=0.015) and between the inoculated and control plants (p=0.026). The average mean weight of tubers (across all ten cultivars) was significantly greater (15%) in the control treatments (163.9g) than the inoculated treatments (142.2g). Ranger Russet had the highest mean tuber weight in the inoculated soil (175.2g) whilst AC had the lowest (109.8g). AD and AC had the greatest difference between inoculated and control mean tuber weights with 43.5g and 43.3g, respectively. The inoculated AF had the smallest difference in tuber weights (3.8g heavier in the inoculated treatment) this was the only cultivar that showed a greater inoculated tuber weight than that of the controls.

**Table 1.** Root infection response of ten cultivars grown in pots (PT1) with S. subterranea. Shown are the mean pathogen levels of S. subterranea (pg/g root tissue) and root galling assessed at 80 days after plant emergence.

| Cultivar | DNA pg/g (log)[1] | Number of Root Galls[2] | Gall Severity[3] |
|---|---|---|---|
| AI | 701513 (4.54) | 0.7 | 0.33 |
| AD | 123775 (4.47) | 0 | 0 |
| AE | 94364 (4.17) | 15.3 | 2 |
| AA | 484148 (5.03) | 0 | 0 |
| AC | 6619 (3.82) | 1.3 | 0.67 |
| Bondi | 11219 (4.04) | 1.7 | 0.67 |
| Desiree | 144696 (4.38) | 2.3 | 1 |
| AF | 136128 (4.79) | 0 | 0 |
| AG | 162094 (4.77) | 19 | 1.67 |
| Ranger Russet | 42095 (4.31) | 2 | 0.67 |
| LSD | ns | ns | ns |
| P | 0.513 (0.860) | 0.123 | 0.235 |
| n | 3 | 3 | 3 |

[1] Shown are the actual (and log) levels of *S. subterranea* DNA (pg/g root tissue).

[2] Number of root galls per plant

[3] Root gall severity score used a rating scale of 0-4 (Van De Graaf et al., 2007).

ns non-significant at P = 0.05.

**Pot trial 2 (PT2)**

In the second pot trial (PT2) all four cultivars had zoosporangial root infections recorded following examination at the final harvest. Zoosporangia scores varied significantly between cultivars (p=0.005). The cultivar AD had the lowest mean zoosporangia score with 0.5 while Bondi and Desiree both had the highest levels with 2.67 (Table 2).

However, there were no statistically significant differences in the number of root galls or in gall severity score between the cultivars examined (Table 2). RBK5 had both the highest number of root galls and the highest severity score of 35 and 3.5 respectively. Bondi had the lowest number of galls.

There was no significant difference between levels of *S. subterranea* pathogen DNA across the cultivars examined at each assessment date. There was however a significant difference when comparing DNA levels at 30 days after emergence with 60 days after emergence (p= 0.037; LSD=0.494), in this case mean DNA levels were significantly higher at the later assessment date. Bondi had the lowest DNA levels at both 30 and 60 DAE. RBK5 had the highest DNA levels at 30 DAE while AD had the highest DNA levels at 60 DAE (Table 2).

***Table 1.*** *Root infection response of four cultivars grown in pots (PT2) with S. subterranea. Shown are the mean pathogen levels of S. subterranea (pg/g root tissue) at 30 and 60 days after emergence, root galling assessed at 60 days after plant emergence.*

| Cultivar | DNA 30 DAE[1] | DNA 60 DAE[1] | Number Root Galls[2] | Root Gall Severity[3] | Zoosporangia[4] |
|---|---|---|---|---|---|
| AD | 14537 (4.13) | 117579 (4.87) | 10 | 2.5 | 0.5[c] |
| Bondi | 11628 (4.02) | 30304 (4.39) | 3 | 1 | 2.67[a] |
| Desiree | 15100 (4.18) | 62392 (4.77) | 20 | 3 | 2.67[a] |
| RBK5 | 19487 (4.21) | 68224 (4.66) | 35 | 3.5 | 1.67[b] |
| LSD | ns | ns | ns | ns | 0.577 |
| p | 0.65 | 0.43 | 0.21 | 0.08 | 0.005 |
| n | 2 | 2 | 8 | 8 | 6 |

[1] Shown are the actual (and log) levels of *S. subterranea* DNA (pg/g root tissue).

[2] Number of root galls per plant.

[3] Root gall severity score used a rating scale of 0-4 (Van De Graaf et al., 2007).

ns non-significant at P = 0.05.

[4]Zoosporangia score rating score 0-4.

Means followed by the same letter within the same column are not significantly different at $P = 0.05$ using Fisher's LSD test.

**MP1.** There was no significant difference (p=0.577) in the zoosporangia score when comparing the cultivars grown in the outside plot trial (Table 3). AD had the lowest score (0.83) and Ranger Russet had the highest (1.67). There was no significant difference in the number of root galls but there was a significant difference in the mean gall severity score (p=0.006) with AD having a lower score than all other cultivars examined. Desiree cultivars grew poorly and only produced minimal root material so were harvested for tuber yield data only. There was a significant difference in the powdery scab incidence (p=<0.001). Desiree had the greatest incidence (42.77%) and RBK5 had the lowest incidence (3.21%). There was a significant difference in powdery scab severity scores (p=0.006). RBK5 had the lowest mean severity score (0.0321) and Desiree had the highest (0.6189) which was significantly greater than all other 4 cultivars examined.

***Table 3.*** *Root infection response of five cultivars grown in an outside plot (MP1) with S. subterranea. Shown are the mean pathogen levels of S. subterranea (pg/g root tissue), root galling assessment, zoosporangia and tuber disease assessment.*

| Cultivar | DNA pg/g (log) | Number of Root Galls AD | Root Gall score | Zoosporangia score | Powdery Scab Incidence (%) | Powdery Scab Severity |
|---|---|---|---|---|---|---|
| AD | 269621 (5.20) | 0.3 | 0.13[b] | 0.83[b] | 8.89[b] | 0.122[b] |
| Bondi | 98590 (4.19) | 1.5 | 0.53[a] | 1.17[a] | 20.00[b] | 0.300[b] |
| Ranger Russet | 96806 (4.74) | 1.7 | 0.87[a] | 1.67[a] | 9.44[b] | 0.105[b] |
| RBK5 | 46881 (4.62) | 2.8 | 1.07[a] | 1[b] | 3.21[b] | 0.032[b] |
| Desiree | n/a | n/a | n/a | n/a | 42.77[a] | 0.619[a] |
| LSD | ns | ns | 0.54 | ns | 17.54 | 0.309 |
| p | 0.09 | 0.053 | 0.006 | 0.577 | <0.001 | 0.006 |
| n | 6 | 15 | 15 | 6 | | |

[1] Shown are the actual (and log) levels of *S. subterranea* DNA (pg/g root tissue).

[2] Number of root galls per plant.

[3] Root gall severity score used a rating scale of 0-4 (Van De Graaf et al., 2007).

ns non-significant at P = 0.05.

[4]Zoosporangia score rating score 0-4.

Means followed by the same letter within the same column are not significantly different at $P = 0.05$ using Fisher's LSD test.

**MP2.** This mini-plot trial produced varying levels of pathogen DNA in the different cultivars with AC the best performed and Ranger Russet and AH recording the highest levels of pathogen DNA in their root tissue (Fig. 1). Galling was correspondingly the lowest in AC and also very low in AB. The varieties AE, AG and AH produced the highest levels of galling.

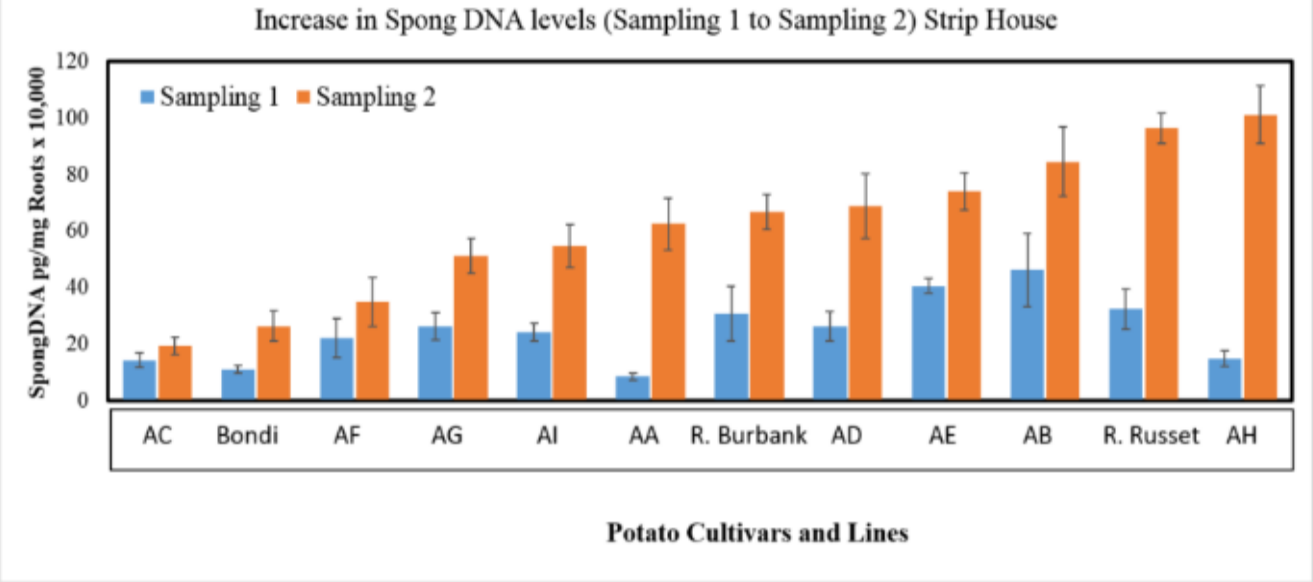


**Fig 1.** Two consecutive samplings of 12 cultivars in a mini plot trial for detection of *S. subterranea* from root tissue.

**Field Trial.** The soilborne levels of *S. subterranea* were very high in this commercial paddock (>5000 pg S. *subterranea* DNA/g soil) resulting in both root and tuber disease in most cultivars tested (Table 4). Only one cultivar tested was shown to produce no galling, visible zoosporangia or powdery scab tuber infection (AA). Some industry standards included in the trial (Desiree, Shepody and Atlantic) and one of the commercial lines (AG) showed very significant to severe galling. Russet Burbank, Ranger Russet and AH produced moderate galling that was more severe, although not significantly so, than many of the newer lines tested. The zoosporangial scores recorded were generally very low and this may be related to the selection of suitable root tissue from the field and subsequent sub-sampling. Related DNA levels (Fig 2) show a general gradient of increasing DNA with time, however there was a few exceptions with Shepody, Ranger Russet and AD showing a decrease between sampling 2 and 3. This may be reflective of the sampling technique, perhaps with less viable roots and zoosporangia in the older roots. In general, those cultivars producing low root infection generally corresponded with lower DNA levels extracted.

Tuber infection was very high in some varieties with greater than 50% incidence for Shepody, Desiree, AE and Atlantic (Table 4). Bondi, AG, Russet Burbank and Ranger Russet also produce >10% incidence. Some varieties (AA, AD and AH) produce no tuber symptoms.

**Table 4** Root infection response of fifteen cultivars grown in the field (FT) with high levels of S.

subterranea. Shown are root galling assessed at 95 days after plant emergence, zoosporangial scores at 45, 75 and 95 days after emergence, and tuber disease severity at plant senescence.

| Cultivar | Number root galls[1] | Root gall severity[2] | Zoosporangia[3] | | | Tuber infection % | Average severity[4] |
|---|---|---|---|---|---|---|---|
| | | | DAE | | | | |
| | | | 45 | 75 | 95 | | |
| AA | 0 c | 0 h | 0 | 0 | 0 | 0 e | 0.00 d |
| AB | 5 c | 1.50 fg | 0 | 0 | 0 | 5.6 e | 0.04 d |
| AC | 2.5 c | 1.33 fg | 0 | 1.1 | 0 | 1.1 e | 0.01 d |
| AD | 4.3 c | 1.33 fg | 0 | 0 | 0 | 0 e | 0.00 d |
| AE | 5.7 c | 1.67 efg | 1.1 | 0 | 0 | 63.3 abc | 1.38 b |
| Bondi | 4.7 c | 1.33 fg | 0 | 0 | 0 | 24.6 cde | 0.22 d |
| AF | 3.0 c | 0.83 gh | 0 | 0 | 0 | 3.3 e | 0.04 d |
| AG | 44.0 a | 4.00 a | 0 | 0.9 | 0 | 22.2 cde | 0.27 cd |
| AH | 8.3 c | 2.33 cdef | 0 | 0 | 0 | 0 e | 0.00 d |
| AI | 4.7 c | 1.33 fg | 0 | 0 | 0 | 6.7 e | 0.11 d |
| Russet Burbank | 11.7 c | 2.67 bcde | 1.1 | 0 | 0 | 16.7 de | 0.21 cd |
| Ranger Russet | 9.0 c | 2.33 cdef | 1.0 | 0.9 | 0 | 12.2 e | 0.13 d |
| Desiree | 24.3 b | 3.17 abc | 0 | 0 | 0.5 | 67.8 ab | 0.84 bc |
| Shepody | 37.3 ab | 3.67 ab | 1.2 | 1.8 | 0 | 98.9 a | 2.60 a |
| Atlantic | 30.0 b | 2.83 bcd | 0 | 0.8 | 0 | 56.7 bcd | 0.99 b |
| LSD | 13.50 | 1.08 | | | | 41.1 | 0.66 |
| F prob (0.05) | <0.001 | <0.001 | | | | <0.001 | <0.001 |

[1] Number of root galls per plant.
[2] Root gall severity score used a rating scale of 0-4 (Van De Graaf et al., 2007).
[3]Zoosporangia score rating score 0-4.
[4]Tuber severity score rating score 0-6.
ns non-significant at P = 0.05. DAE = days after emergence
Means followed by the same letter within the same column are not significantly different at *P* = 0.05 using Fisher's LSD test.

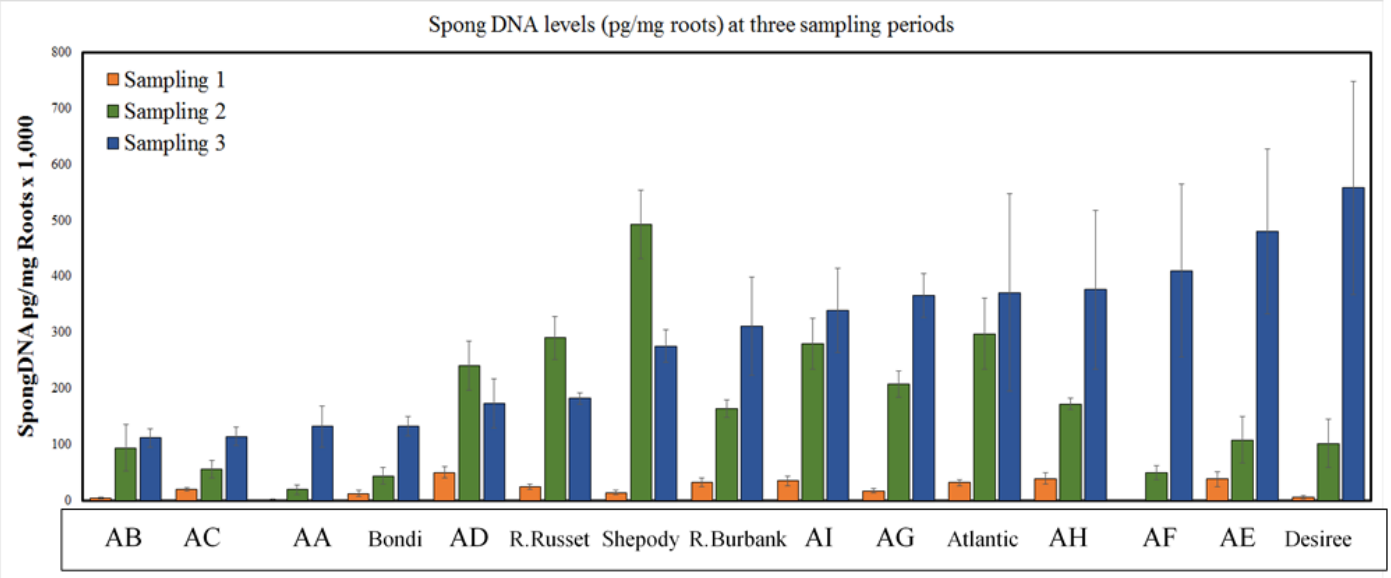


**Fig. 2.** Three consecutive samplings (45, 75 and 95 DAE) of 15 cultivars in a field trial for detection of *S. subterranea* from root tissue.

**Conclusions.**

Essentially, while there was a degree of variability in DNA levels quantified and root infection levels and galling in separate experiments the same cultivars seemed to group with each other as to whether they showed any Spongospora resistance (Table 5). Looking at Table 5 and the colour traffic signal guide it can be seen that Atlantic, Shepody, Desiree, AG and AE were often rated in the red category indicating poor levels of resistance. Russet Burbank, Ranger Russet, AI, Bondi, AF and AH were generally in the amber (orange category) showed intermediate resistance. AA, AB, AC and AD generally performed in the amber and green category (compared to other lines) so are showing a degree of resistance, although no cultivar demonstrated complete resistance.

**Table 5.** Summary of the relative resistance or tolerance of important processing potato varieties to root and tuber infection.

| Cultivar | Field trial | | | Glasshouse1 | | Glasshouse2 | | Mini Plot1 | | Mini Plot2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Root infection | | Tuber infection | Root infection | | Root infection | | root | tuber | Root infection | |
| | DNA | gall | | DNA | Galling | DNA | Galling | DNA | | DNA | galling |
| AA | | | | | | | | | | | |
| AB | | | | | | | | | | | |
| AC | | | | | | | | | | | |
| AD | | | | | | | | | | | |
| AE | | | | | | | | | | | |
| Bondi | | | | | | | | | | | |
| | | | | | | | | | | | |
| AF | | | | | | | | | | | |
| AG | | | | | | | | | | | |
| AH | | | | | | | | | | | |
| AI | | | | | | | | | | | |
| | | | | | | | | | | | |
| Russet Burbank | | | | | | | | | | | |
| Ranger Russet | | | | | | | | | | | |
| Desiree | | | | | | | | | | | |
| Shepody | | | | | | | | | | | |
| Atlantic | | | | | | | | | | | |
| | | | | | | | | | | | |

Nil or very low infection
Moderate infection
High infection

Root infection assessment methods including zoosporangial infection and pathogen DNA quantification provided a variable assessment of potential resistance. While the assays were useful, the impact of other factors (root age, sampling region) can lead to inherent variability.

**A better methodology for cultivar screening**

The glasshouse and field trials used typical inoculum challenge methodologies growing plants in soils either artificially or naturally infested with pathogen inoculum, and monitoring root infection rates by qPCR, microscopy and direct observation of galling (and tuber disease) incidence. Inherent variability in such glasshouse and field trials exists due to emergence and growth differences between cultivars, environmental factors between trials, distribution of pathogen inoculum within the soil, variation in onset of infection, and capacity to sample only a small segment of root tissues makes these traditional trials difficult, and potentially unreliable. Also, due to the polycyclic nature of root infection timing of the measurement of root infection is critical and direct comparisons within each experiment only are possible

highlighting the need for known standards to be included in each trial.

These trials also are time and resource consuming requiring months of growth of potato plants with sufficient replication to allow for variable inoculum distribution.

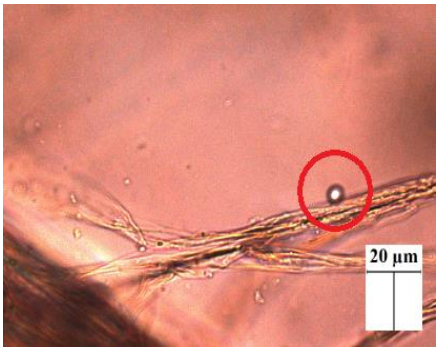


**Fig 3: Zoospore encysment on potato root**

We developed a new *in vitro* assay that focusses on the zoospores binding to and encysting on host roots (Fig 3) which is: a) much more rapid with results within 1 week, b) more robust with controlled inoculation conditions, c) requires much less resources (plants, land, time, labour, machinery), d) not time dependant and not influenced by polycyclic infection cycles.


**Fig 4.** Example of *in vitro* root assay results (nb cultivar names are commercial-in-confidence)

This assay gives a much clearer indication of resistance root infection (Fig 4). Importantly the assay only allows a single cycle of infection and as such the polycyclic infection process does not confound assessment. Further development of this assay should be a priority for accurate assessment of potato cultivar root infection resistance.

*The results support observations from glasshouse and field challenge experiments demonstrating robustness of the approach. Research in this new area should be continued to provide greater detail on levels of resistance in the industry cultivars.*

## Appendix 2b – Zoospore encystment

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Method for Screening *Spongospora Subterranea* Resistance in Potato Cultivars using Zoospore Encystment in Roots

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Abstract — Encystment rate of *S. subterranea* zoospores may be used to quickly determine resistant or susceptible potato cultivars within a short span of only 7 days by utilizing potato root samples. Results indicate that AC and AB are the most resistance varieties compared to known cultivars such as Russet Burbank.

## Introduction

*Spongospora subterranea f.sp. subterranea*, also known as powdery scab is a disease that affects potato crops globally by infection of potato tubers.[1] Fig. 1 below shows many *S. subterranea* lesions on an infected potato tuber. The appearance of the afflicted tuber is severely affected and the tubers are not marketable[1], so methods of detecting and screening cultivars' resistance to this disease is highly important to the Australian potato industry as lost production costs are approximately $13M p.a.[2] This disease is characterized by the occurrence of scab-like lesions on the surface of potato tubers, galls on infected roots, and zoosporangia.[3] Both tuber lesions and galls contain cytosori (fine brown powder) that are resting spores.[4] These spores may remain dormant in the soil for exceeding ten years.[3]

**Fig. 1 – *S. subterranea* lesions on an infected potato tuber**

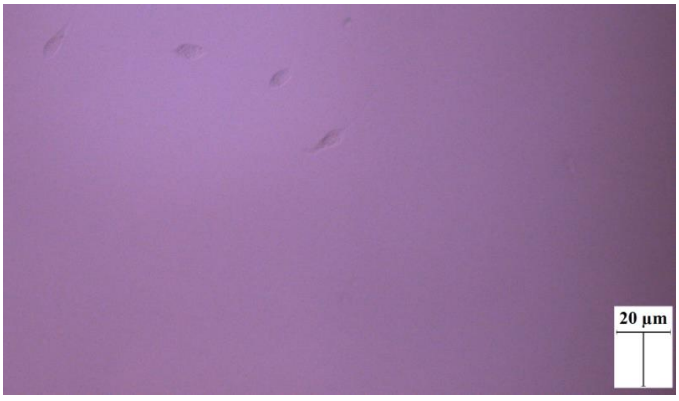
At present, there is no effective method of disease control.[1,3,4]. To minimize risk of disease, approaches such as long crop rotations or planting resistant cultivars may be implemented.[4] Another option is the use of in-furrow fungicides for soil treatment and to reduce infection levels. However, these treatments are less effective and costly.[1]

Two previous studies conducted at the University of Tasmania by researchers[8,9] report that potato root exudates can encourage resting spore germination of *S. subterranea*. The resulting zoospores are mobile but only have a short lifespan of a few hours. These zoospores will migrate towards and subsequently infect susceptible potato plant roots. Once a zoospore reaches a host root, it encysts on the root and transfers cell contents into the root cell, causing infection. In this experiment, the encystment of zoospores was used to quantify potato plant cultivar resistance.

Experimental

### Preparation of *S. subterranea* zoospore suspension and quantifying sporosori inoculum

Zoospore suspension of *S. subterranea* was prepared using Balendres et al's[9] method.



Dried resting spore inoculum was collected from diseased potato tubers, suspended in Hoagland's solution and incubated for 10 days at 18-22°C. The *S. subterranea* zoospore identity (Fig. 2) was verified by examination under a light microscope (DM 2500 LED, Leica Microsystem, Germany) at 40X magnification.

Fig. 2 – Zoospores as observed under a light microscope with 40x magnification

To quantify the average number of resting spores, 10mg of sporosori inoculum was suspended in 2mL of distilled water and vortexed. 10µL of this suspension was placed in a Haemocytometer chamber and covered with a glass cover. Sporosori (Fig. 3) counts in each grid was noted and multiplied by 700. An estimated quantity of 2,450,000 resting spores per mg of inoculum was obtained.

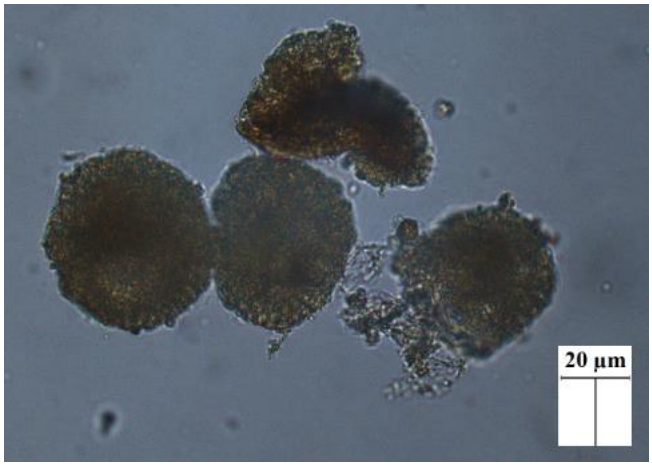



Fig. 3 – Sporosori as observed under a light microscope with 40x magnification

**Preparation of Potato Roots**

15 different cultivars of potato plants (Russet Burbank (RBK), AA, AB, AC, Ranger Russet (R. Russ), Bondi, AE, AI, AD, Atlantic, Desiree, AG, AF, Shepody and AH) used in this study was provided by the Tasmanian Institute of Agriculture. These cultivars demonstrated varying responses to powdery scab and root infection in the field[3,4] and are often used as references for screening studies.

**Zoospore-Root Encystment Assay**

2-cm long root segments were collected from each cultivar and placed in a petri dish. The petri dish was flooded with 50µL of zoospore suspension and 2mL of Hoagland's solution to prevent the roots from drying out. Each dish was covered and incubated in a dark room at 18-22°C and examined at Day 0, 1, 3 and 7. More Hoagland's solution was added to maintain moisture levels in root samples. A segment of Russet Burbank (RBK) was included in all dishes to serve as a reference point.

During examination, each root segment was placed on a microscope slide with 70µL of Hoagland's solution. The slide was covered with a cover slip and zoospore encystment (Fig. 4) recorded by scanning the whole sample with a light microscope at 40X

magnification. Three replicates per cultivar were conducted and each root sample was obtained from the same plant source.

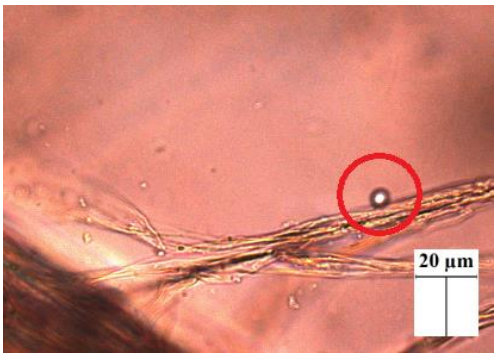
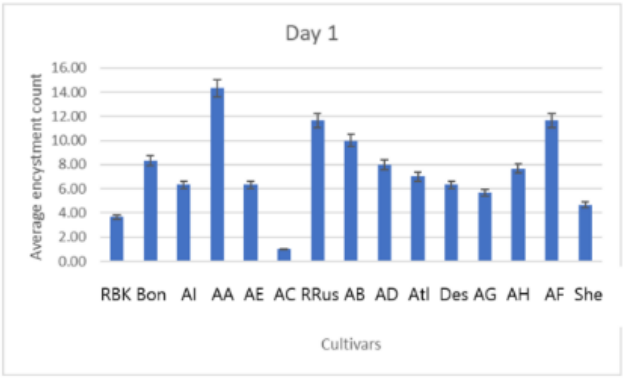
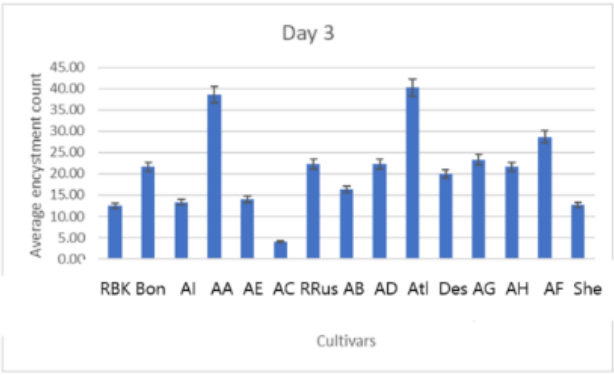



Fig. 4 – Encysted zoospore (circled in red) on potato root hair

**Results and Discussion**

*S. subterranea* zoospores encysted in all potato cultivars roots and all replicates. Day 1 shows the lowest encystment counts while Day 7 has the highest counts. Figures 5, 6 and 7 depict the average encystment counts for Day 1, 3 and 7 respectively. Cultivars such as AC appeared cleanest and free from encysted zoospores across all days. This suggests that this cultivar has high resistance to *S. Subterranea*.



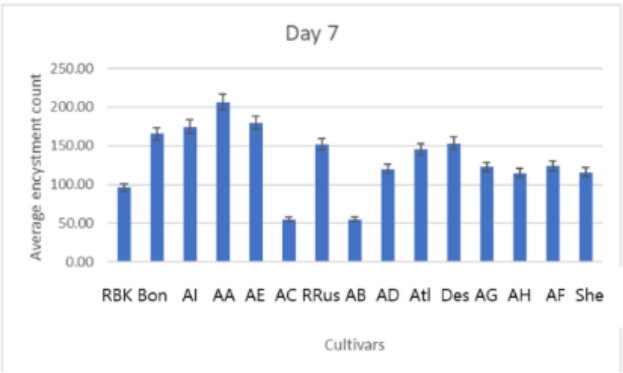
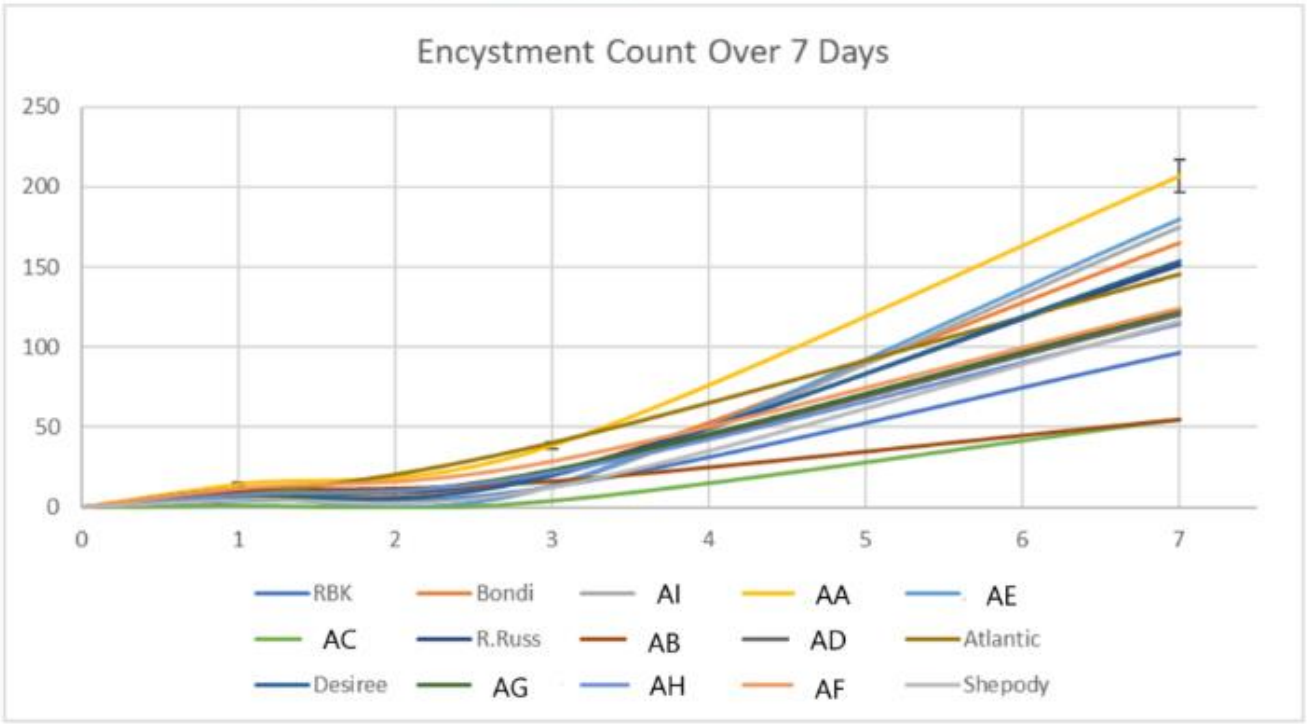



Fig 5 (top left), 6 (top right), 7 (left) – Average encystment count from 3 replicates of each cultivar on Day 1, 3 and 7.

Fig. 8 below shows the average encystment count in the form of a scatterplot across 7 days of incubation. From this graph, cultivars such as AB can be classed with high resistance to *S. subterranea*. Although its average encystment count was not as low as AC on Day 1, AB's ability to retain low encystment counts across 7 days of incubation results in this cultivar showing high resistance to the pathogen. From this study, AC and AB's roots were the most resistant to S. subterranea zoospore encystment. Russet Burbank demonstrates the highest resistance compared to all other named varieties. AA, AE, AI, Bondi, Ranger Russet, AH and Atlantic show lowest resistance to the pathogen. AD, Desiree, AG, AF and Shepody have moderate resistance to root encystment.

Fig. 8 – Zoospore encystment count over 7 days of incubation




Encystment occurs at a relatively slow pace. At Day 0 when sporosori was introduced to the root segments, no encystment was found after 1 hour of incubation. Thus, the samples were left to incubate and encystment counts collected on Days 1, 3 and 7. On Day 1, encystment was still occurring, so the results measured on this day show only a partial image of a cultivar's ability to resist this pathogen. On Day 3, encystment was still occurring but with a lower rate compared to Day 1. From Days 1 to 3, minor variation was observed between replicates. Although different cultivars were included

in the same petri dish as the RBK reference, similar encystment counts were noted for the same species. This suggests that the cultivar itself is the main factor in zoospore encystment in this study. On Day 7, higher variation of encystment counts were observed amongst replicates, hence, the standard error and subsequent error bars were also large. A few detached and dead zoospores were observed to be floating in solution (Fig. 9), however these loose zoospores were not counted as part of the encystment count for Day 7. This observation suggests that zoospores germinate, encyst on root surface and may detach a few days later. However, more studies should be conducted to determine if the zoospores naturally self-detach after a few days or if this observation is due to handling of the root segment when transferring onto the microscope slide.

Fig. 9 – Circled in red is a detached zoospore post-encystment. This was found in solution near but not attached to the root segment.


Future experiments should be conducted to further investigate the effects of the age of plant roots on encystment rates. This is because in this study, the numbered plants were younger compared to the named plants. The older plants have thicker roots, making observation and counting of encysted zoospores much more challenging. In addition to this, the segment where the root was obtained from may be studied to determine of the tip, middle or main root is more susceptible to attracting zoospores. Potato root exudate may be used in place of Hoagland's solution to encourage faster

zoospore germination and results could potentially be obtained in hours instead of this experiments' 7-day timeframe.

**Conclusions**

In summary, encystment rate of *S. subterranea* zoospores yield good indication of a cultivar's resistance. This method may be used to quickly distinguish resistant varieties within a short window of only 7 days using potato root segments. AC and AB demonstrate the most resistance to *S. subterranea* zoospores compared to other cultivars.

**Acknowledgments**

We thank Robert Tegg (University of Tasmania; UTAS, Australia) for providing potato plant roots and Annabel Wilson (UTAS) for chemicals used in this study.

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## Appendix 3 – Environmental and site influence

We monitored 16 commercial fields through the 2017/18 growing season to broadly assess the impact of soilborne *Spongospora* on root galling and tuber disease. We tested a number of parameters including cultivar type (program 1,3), soil type, planting date and length of time the crop was in the ground (program 3). We showed that soil *Spongospora* inoculum level correlated very high and moderately high with tuber galling and powdery scab disease respectively. We also showed that both soil type and crop duration had an impact on the expression of tuber disease, but not root galling. Essentially, lighter sandier soils or a shorter crop duration reduced tuber powdery scab compared to a heavier soil or a longer crop duration.

| Farmer ID | Location | Soil type (texture) | Cultivar | Plant date | Crop duration (d) | S. subterranea (pgDNA/g soil) | Root galling (0-4) | Tuber disease | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Incidence (%) | Severity (0-6) |
| 1 | Sassafras | Loam | Ranger | 1-Nov | 125 | 149 | 2 | 2 | 0.5 |
| 2 | Bakers Beach | Loamy sand | Ranger | 13-Nov | 125 | 1 | 0 | 0 | 0 |
| 3 | Moriarty | Clay loam ferrosol | Ranger | 3-Oct | 120 | 101 | 2 | 4 | 2 |
| 4 | Rocky Cape | Clay loam ferrosol | Innovator | 16-Nov | 135 | 5240 | 4 | 10 | 1 |
| 5 | Sisters Creek | Clay loam ferrosol | Ranger | 1-Nov | 130 | 971 | - | 14 | 1.84 |
| 6 | Sisters Creek | Clay loam ferrosol | Russet Burbank | 17-Nov | 140 | 3934 | 4 | 30 | 1.82 |
| 7 | Myalla | Clay loam ferrosol | Russet Burbank | 1-Nov | 140 | 4588 | 4 | 24 | 1.86 |
| 8 | Myalla | Clay loam ferrosol | Topcat | 18-Nov | 135 | 731 | 3.5 | 6 | 1 |
| 9 | Ulverstone | Clay loam ferrosol | Russet Burbank | 22-Nov | 135 | 341 | - | 4 | 1 |
| 10 | Scottsdale | Clay loam ferrosol | Russet Burbank | 23-Oct | 125 | 101 | 2.67 | 20 | 1.88 |
| 11 | Scottsdale | Clay loam ferrosol | Ranger | 3-Oct | 120 | 22 | 0 | 4 | 1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 12 | Branxholm | Clay loam ferrosol | Ranger | 4-Oct | 130 | 5938 | 3.75 | 90 | 3.1 |
| 13 | Bridport | Loamy sand | Ranger | 30-Oct | 130 | 518 | 2 | 0 | 0 |
| 14 | Bridport | Loamy sand | Russet Burbank | 28-Oct | 135 | 3972 | 2 | 2 | 1 |
| 15 | Myalla | Clay loam ferrosol | Ranger | 1-Nov | 130 | 6754 | - | 14 | 1.32 |
| 16 | Myalla | Clay loam ferrosol | Russet Burbank | 17-Nov | 140 | 20176 | 4 | 32 | 1.5 |

Interaction of factors impacting *Spongospora* root and tuber disease

| Factor | Root galling | Tuber disease |
|---|---|---|
| | | |
| Soil type | ns | <0.05 |
| Cultivar | ns | ns |
| Plant Date | ns | ns |
| Crop duration | ns | <0.05 |
| Pathogen level | <0.01 | <0.05 |
| | | |

This general matrix indicates the importance of various factors impacting the expression of disease. Root galling appears to be a very robust measurement and wasn't impacted, in these trials, by soil type, cultivar, plant date or crop duration. Basically galling was controlled by the levels of the pathogen present with levels of galling increasing as pathogen levels also increased.

Tuber disease occurs later in the crop cycle and was impacted by other factors. Like galling, it showed a significant relationship with pathogen levels (although the relationship was a little weaker). However, for tuber disease there was an impact of soil type, with the lighter sandier loams producing less tuber disease than the heavier clay loams. Also, there was an impact of crop duration with the crops that remained in the ground for longer more likely to express greater tuber disease.

## Appendix 4 – Foliar PGR for root stimulation and disease resistance

Testing the potential of foliar PGR for root stimulation and enhanced disease resistance

**NOTE THAT PGR IS NOT REGISTERED FOR DISEASE CONTROL IN POTATOES AND SO THEIR USE IN COMMERICAL PRODUCTION IS NOT PERMITTED.**

*Summary.* Over two consecutive years we tested the foliar application of low levels of PGR, applied just after plant emergence on root stimulation and disease performance in commercially grown 'Russet Burbank' crops. In the more substantial trial in Year 1, both PGR treatments (both low and very low rates) enhanced root growth significantly and decreased powdery scab incidence and severity with the higher application rate reducing disease more. There was a slight reduction in tuber yield from the low rate PGR treatment, although not significant, while the very low rate PGR treatment slightly enhanced tuber yield compared to the untreated control. In Year 2 which only tested the one level of PGR (very low rate) there was a slight but non-significant reduction in powdery scab incidence and severity while tuber yield was enhanced slightly. In both years, there was no significant impact of PGR treatment on the severity of galling. Maximum residue limit (MRL) tests in both years of all PGR treatments confirmed no detection at <0.01mg/kg. These trials have indicated that PGR can stimulate root growth and reduce powdery scab disease with negligible or sometimes an enhanced tuber yield impact. Given the above and the known benefits in reducing common scab the usage of PGR for enhancing tuber disease resistance and stimulating root growth warrants further investigation and uptake for commercial usage.

Methods.

Two field trials were conducted at Myalla, on the NW coast of Tasmania over two consecutive seasons (2016/17 and 2017/18) to test the efficacy of PGR foliar application on root growth and disease outcomes. Trials were conducted on a ferrosol with a known high level of *Spongospora subterranea* in the soil and 'Russet Burbank' was the cultivar tested.

In the first field trial we tested 2 levels of sub-lethal PGR (both low and very low rates) while in the second trial only the very low rate PGR was tested, compared to a water control. Plots were treated with a standard commercial tractor set-up and applications were made just after plant emergence, although in the second trial application was made 20 days after emergence.

In trial 1 we monitored pathogen and disease development in the roots on three separate dates (11$^{th}$ Jan 2017, 7$^{th}$ Feb 2017, 8$^{th}$ Mar 2017) carried out a root biomass assessment on the third sequential assessment date (8$^{th}$ Mar 2017) and a 4$^{th}$ root biomass assessment on 24$^{th}$ Mar 2017. We also took 4 replicates of 3m plot samples at plant senescence (28$^{th}$ April 2017) for tuber yield and disease assessment. In trial 2 we undertook a disease (gall) assessment of the roots on 27$^{th}$ March 2018 and we made a tuber yield and disease assessment (4 replicates each of 10 plants per plot) on the 18$^{th}$ May 2018.

Results and Discussion.

Effect of foliar PGR application on potato roots fresh and dry weights (grams) in 2016/17

## Early March (n=3-6)

| Treatment | Fresh Weight (g) | Dry Weight (g) |
|---|---|---|
| **Control (No PGR)** | 58.92 ± 4.70 a | 5.22 a |
| **PGR (low rate)** | 82.83 ± 5.96 b | 7.37 b |
| **PGR (very low rate)** | 82.07 ± 12.18 b | 7.30 b |
| **LSD (0.05)** | 22.9 | 2.01 |
| **ANOVA** | 0.05 | 0.04 |

## Late March (n = 9)

| Treatment | Fresh Weight (g) | Dry Weight (g) |
|---|---|---|
| **Control (No PGR)** | 48.7 a | 7.3 a |
| **PGR (low rate)** | 70.2 b | 9.0 b |
| **PGR (very low rate)** | 61.2 b | 8.9 b |
| **LSD (0.05)** | 11.98 | 1.24 |
| **ANOVA** | 0.002 | 0.007 |

Means followed by the same letter are not significantly different at 0.05 level by Fisher's LSD test

Mean values ± SE of means

Normality test and Skewness value indicate data are normally distributed

There were significant increases in the fresh and dry weights of root material recorded from both PGR treatment rates at both assessment dates.

Effect of foliar PGR application on potato numbers, yield and powdery scab disease incidence and severity

| Treatment | Tuber Weight (kg) | Tuber Number | Disease incidence (%) | Disease severity (0-6) |
|---|---|---|---|---|
| **Control (No PGR)** | 15.91 | 80.6 b | 42.2 c | 0.549 c |
| **PGR (low rate)** | 15.50 | 69.2 a | 19.6 a | 0.255 a |
| **PGR (very low rate)** | 16.50 | 78.5 b | 30.8 b | 0.400 b |
| **LSD (0.05)** | ns | 8.83 | 9.92 | 0.129 |
| **ANOVA** | 0.45 | 0.042 | 0.004 | 0.004 |

Overall, powdery scab disease was decreased with PGR treatment; yield was increased slightly by the very low rate PGR treatment and decreased slightly by the low rate PGR treatment.

Root galling assessment – *Spongospora* induced galling was visually assessed from a number of plants in February and March. There was no obvious difference between controls and PGR treatments with approximate galling scores of between 3 and 4. This represents a moderate-high gall count across all the plants assessed.

Maximum Residue Limits (MRLs) – A selection of tubers from both PGR treatments were sampled and assessed for PGR residue. Both samples recorded PGR levels at <0.01mg/kg (below detectable levels) which is at least 40 times below the current MRL for PGR (0.4mg/kg).

Spongospora DNA levels – Three soil samples were taken from the treatment plots with levels very high from all plots (control: 4587; PGR (25mg/l): 17493; PGR (6.25mg/l): 12396 pg *S. subterranea* DNA/g soil)

## 2017/18 trial

Effect of foliar PGR application on potato numbers, yield and powdery scab disease incidence and severity (harvest 180518)

| Treatment | Tuber Weight (kg) | Tuber Number | Average tuber weight | Disease incidence (%) | Disease severity (0-6) |
|---|---|---|---|---|---|
| **Control (No PGR)** | 17.42 | 75.2 | 235.1 | 90.4 | 1.78 |
| **PGR (very low rate)** | 17.57 | 89.0 | 196.9 | 73.0 | 1.38 |
| **LSD (0.05)** | ns | ns | ns | ns | ns |
| **ANOVA** | 0.937 | 0.270 | 0.104 | 0.267 | 0.259 |

Powdery scab incidence and severity was decreased with PGR treatment, although not significantly. Yield was increased slightly by the PGR treatment, although not significantly.

**Root galling assessment** – *Spongospora* induced galling was visually assessed from a number of plants. There was no obvious difference between controls and PGR treatments with approximate galling scores of between 3 and 4. This represents a moderate-high gall count across all the plants assessed.

**Maximum Residue Limits (MRLs)** – A selection of tubers from the PGR treatment was sampled and assessed for PGR residue. We are awaiting the results from Symbio Labs but last year the application of higher PGR levels failed to detect PGR at <0.01mg/kg (below detectable levels) which is at least 40 times below the current MRL for PGR (0.4mg/kg).

**Spongospora DNA levels** – Levels across the site were very high – 20,176 pg *S. subterranea* DNA/g soil.

### *Conclusions*

These trials have indicated that PGR can stimulate root growth and reduce powdery scab disease with negligible or sometimes an enhanced tuber yield impact. Given the above and the known benefits in reducing common scab the usage of PGR for enhancing tuber disease resistance and stimulating root growth warrants further investigation and uptake for commercial usage.

While these results show promise, other studies indicate that the PGR tested can have detrimental effects. For example, when applied at higher rates it can result in foliar damage (Thorton et al. 2013, 2014), increased grade out from powdery scab (Waterer, 2010), and can reduce tuber size in stored potatoes (Thorton et al 2013). Thompson et al. (2014) emphasise that optimizing rate and timing is critical to avoid reduced yield, phytotoxic damage and variety specific residues. Further field testing is on-going, both within Tasmania and Internationally. Such treatments require full or off-label registration and commercial trials are required before formal recommendations could be made.

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## Appendix 5– *Spongospora* slide presentation for industry

# Insights into powdery scab (*Spongospora*)

## PT14002 – *Spongospora* infection of potato roots – ecology, epidemiology & control

Calum Wilson
Robert Tegg
Tamil Thangavel
Annabel Wilson
Mark Balendres (PhD)
Mohammed Waqas (Masters)
Tamara Clarke (Honours)
Luke Rockliff (Honours)
Jonathan Amponsah (PhD)

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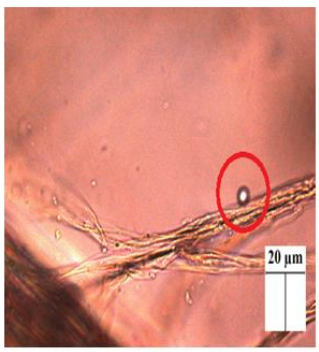
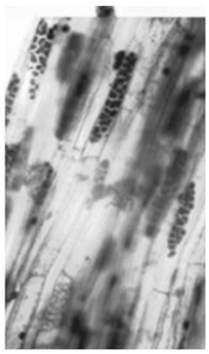
PT14002 – *Spongospora* infection of potato roots – ecology, epidemiology & control



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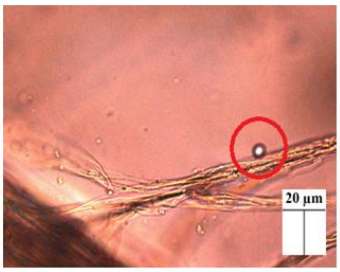
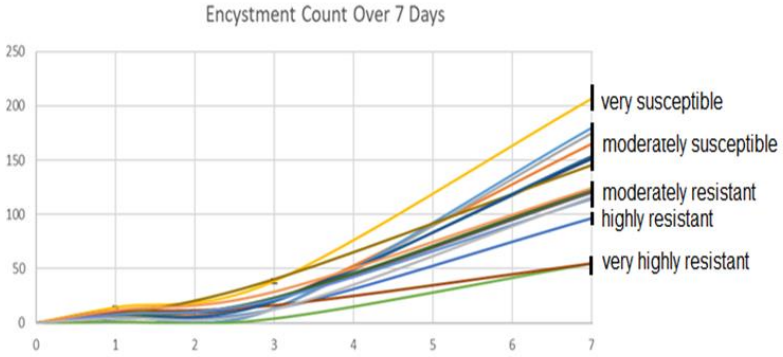
## Encystment – an alternative measure of plant resistance

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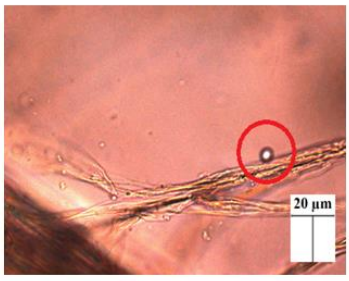
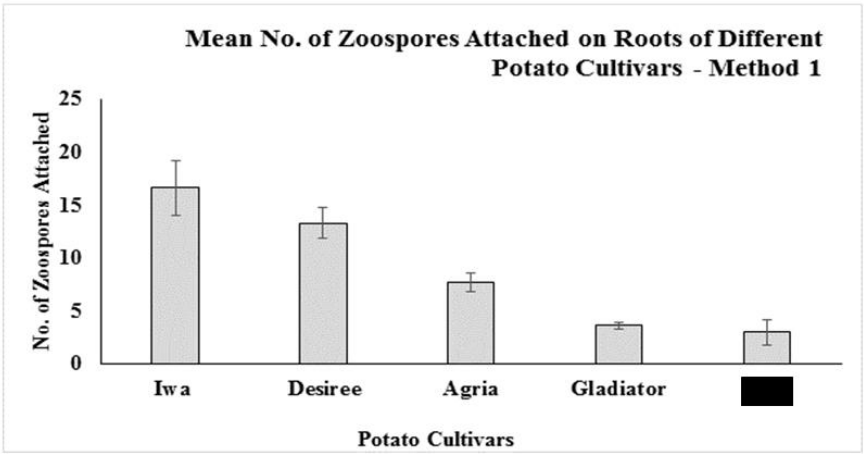
## Encystment – an alternative measure of plant resistance

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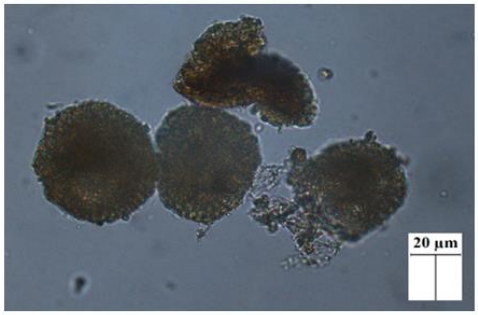
## The pathogen – susceptible and non-susceptible phases

- Long lived (resistant)

Short lived




Sporosori as observed under a light microscope with 40x magnification


Zoospores as observed under a light microscope with 40x magnification

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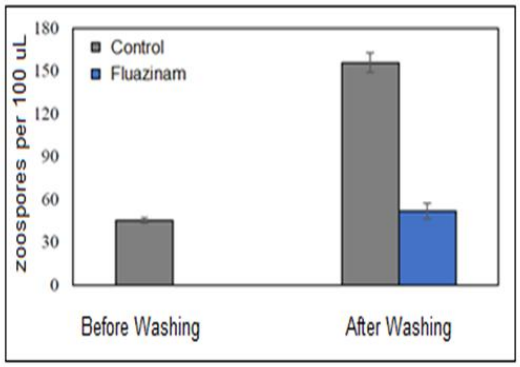
## Zoospores vulnerable but resting spores not




**Successful release of Spongospora zoospores from resting spores following treatment with Fluazinam demonstrates lack of fungicides ability to kill resting spores**

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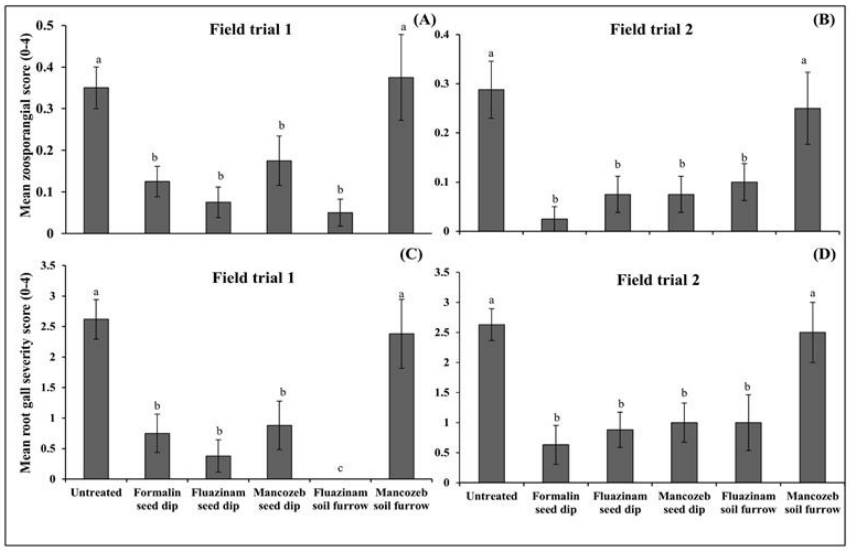
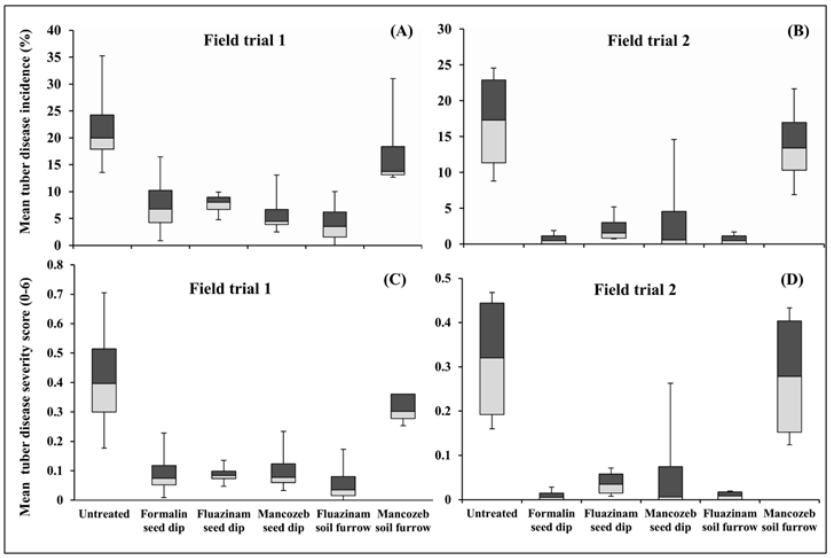
## Chemical impacts on root disease

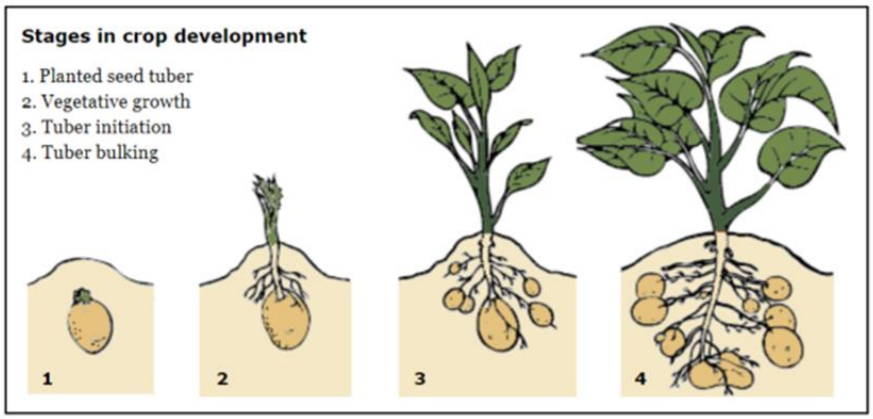



## Chemical impacts on tuber disease




- Chemical control tends to be partial – never complete – maybe 50-85% reduction.

## Is fluazinam effective through all stages of crop growth?




- Coverage and protection of root areas
- Fungicide longevity in soil

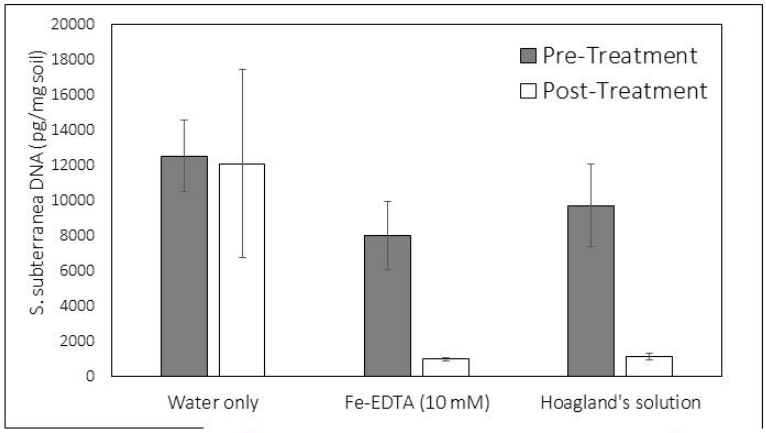
## Zoospore stimulants – Fe containing compounds

| | Experiment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Mean zoospore count[a] | | | | | | | | |
| Distilled water | 71.7 ±7[b] | 0 ±0.0 | 0 ±0.0 | 13.7 ±5 | 2.0 ±5 | 0.0 ±0.0 | 0.0 ±0.0 | 39.7 ±3 |
| Hoagland's solution | 246.3 ±6 | 116.7 ±22 | 35.0 ±15 | 160.3 ±69 | 126.0 ±44 | 77.0 ±13 | 35.0 ±6 | 15.7 ±4 |
| | *P<0.001* | *P<0.001* | *P<0.01* | *P=0.023* | *P<0.01* | *P<0.001* | *P<0.001* | *P=0.023* |
| Days to initial zoospore release | | | | | | | | |
| Distilled water | 17.0 ±0.0 | - | - | 7.0 ±0.0 | 12.0 ±0.0 | - | - | 11.3 ±1 |
| Hoagland's solution | 7.0 ±0.0 | 8.0 ±0.0 | 8.0 ±0.0 | 4.0 ±0.0 | 4.0 ±0.0 | 4.0 ±0.0 | 7.0 ±0.0 | 6.3 ±0.7 |
| | *P<0.01* | - | - | *P<0.01* | *P<0.01* | - | - | *P=0.028* |

# Zoospore stimulants – targeting the vulnerable phase

- To stimulate pathogen release when potato host is absent
- To deplete pathogen levels in soil.
- Carried out prior to cropping (winter period)
- Fe-EDTA and specific amino acids.

Surface spray and incorporate

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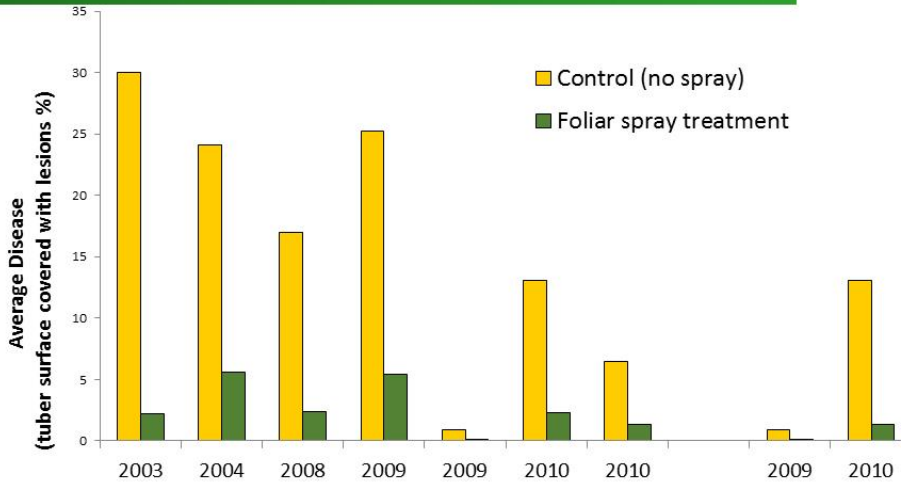
# Foliar PGR sprays for improved root production & disease resistance

Previous benefits

- Significant reduction in common scab
- Mechanism understood (targets thaxtomin)

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## Foliar PGR sprays for improved root production & disease resistance

| Treatment | Root Fresh Weight (g) | Root Dry Weight (g) | Tuber Weight (kg) | Tuber Number | Disease incidence (%) | Disease severity (0-6) |
|---|---|---|---|---|---|---|
| Control | 48.7 a | 7.3 a | 15.91 | 80.6 b | 42.2 c | 0.549 c |
| PGR (low rate) | 70.2 b | 9.0 b | 15.50 | 69.2 a | 19.6 a | 0.255 a |
| PGR (v. low rate) | 61.2 b | 8.9 b | 16.50 | 78.5 b | 30.8 b | 0.400 b |
| LSD (0.05) | 11.98 | 1.24 | ns | 8.83 | 9.92 | 0.129 |
| ANOVA | 0.002 | 0.007 | 0.45 | 0.042 | 0.004 | 0.004 |

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## Foliar sprays for improved root production & disease resistance

Further benefits

- Enhanced root growth
- Better ability to cope with other fungal root-attacking pathogens
- Increased resistance to powdery scab tuber disease

'Technology ready for industry usage'

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# Acknowledgments

**Thanks to the growers, industry representatives, government and research agencies that contributed to both these projects.**

**Supported by Hort Innovation (PT14002) with co-investment from Simplot Australia Pty. Ltd., McCain Foods Australia Pty. Ltd. and the Australian Government**



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