

Progress Report: ARC Linkage Project LP160100758

Project Title: Development of a risk management system for systemic downy mildew of poppy

Research Team: Dr Jason Scott, Prof Calum Wilson, Dr Tamil Thangavel, Mrs Krithika Krishnamoorthy and Ms Dharushana Thanabalasingam

Date: 23/4/2020

Summary:

This project is investigating the factors that promote outbreaks of systemic downy mildew (SDM) in Tasmanian poppy crops. Work has been ongoing since March 2017 and is due for completion in March 2021. Key observations from work and/or analyses undertaken since the previous progress report (April 2019) are presented below.

PhD candidate Mrs Krithika Krishnamoorthy submitted her thesis for examination in April 2020. Mrs Krishnamoorthy's studies involved comparisons between the two downy mildew pathogens *P. somniferi* (SDM) and *P. meconopsidis* (localised downy mildew; LDM). She compared and contrasted the infection processes of the two pathogens using both air- and soil-borne inoculum sources. She also tested the host range of the two species to examine the potential for weed and ornamental poppy species to act as reservoirs for the disease outside of the cropping season. Some of the key findings of her studies were:

- Soil borne infections:
 - Both downy mildew species can infect poppy roots and can move intercellularly into the leaf tissue of the poppy plant at approximately the same rate.
 - Once they reach the host cells in the leaves the typical symptoms of localised or systemic infection are then triggered and expressed.
- Air borne infections:
 - Infection rates by *P. somniferi* were slightly quicker, with symptoms present from four days after inoculation compared to six days for *P. meconopsidis*.
 - Hydrogen peroxide, which is produced by the plant as a defence response to invasion, was detected at much higher rates when plants were infected with *P. meconopsidis* than *P. somniferi*. This suggests that poppy plants can detect and respond to infection

by *P. meconopsidis* more easily, and hence its impact on plant growth (LDM) is less than for *P. somniferi* (SDM).

- Host range:
 - Both species are able to infect multiple weed and ornamental poppy species, including rough poppy (*Papaver dubium*) which is a major weed of poppy cultivation and Flanders poppy (*Papaver rhoeas*) which is a common ornamental.
 - This work has been submitted the Journal of Phytopathology for publication; it is currently in its second round of review.

During the 2019/2020 growing season soil samples were collected from 20 commercial fields around the state with the help of field staff from both SunPharma and Tasmanian Alkaloids. Five samples were collected from each field. To measure SDM inoculum loads within the samples, DNA is currently being extracted from all samples and will be used for analysis using a new soil-specific qPCR test that has been developed in this project. To date, approximately 80% of samples collected have been processed. To help calibrate the laboratory-based measurements, estimates of SDM within the sampled fields at approx. the 10-leaf stage were also recorded. Additionally, clean poppy seed was sown into seed raising trays containing soil collected from the sampled fields and the incidence of seedlings infected with SDM calculated. This will provide an estimate of disease transmission rate independent of regional and commercial production factors that can differ between commercial fields.

Air-borne spore trapping continued over the 2019/2020 growing season at six sites around the state. Spores were trapped using rotor-rod impaction traps and a real-time PCR assay was used to quantify the amount of *P. somniferi* (SDM) DNA present in the samples. Data from these sites will be combined with data from the previous two seasons prior to analysis.

Two field trials were conducted at the TIA Vegetable Research Facility at Forthside in the 2019/20 growing season. The first trial, conducted by PhD candidate Ms Dharushana Thanabalasingam, examined the role of different inoculum sources (soil vs air borne) on the timing and severity of SDM. The second trial examined the influence timing of infection has on SDM severity. In both trials, SDM incidence was monitored over the growing season and poppy capsules were manually harvested to estimate yield. Capsule material was then passed to SunPharma for alkaloid content measurements. Disease data from these trials are currently being collated for analysis. Final yield estimates are currently awaiting data from SunPharma.

In February 2020, Dr David Gent (United States Department of Agriculture) visited TIA at the Cradle Coast campus for 10 days. During this visit Drs Gent and Scott analysed the spatial and temporal data

collected from commercial poppy fields from 2014 to 2019. The results of these analyses indicated that SDM outbreaks across these years were driven primarily via early season infections. This suggests that soil or seed inoculum, possibly in combination with infected regrowth poppies, as having the largest impact on the disease spread. The results from these analyses are currently being written up for publication.

Further work in this project is planned to:

1. Determine environmental conditions that favour soil and/or seed to seedling transmission; include soil temperature and soil type
2. Assess potential strain variation within the SDM pathogen
3. Assess the spatial variation of SDM in poppy fields to enable the development of sampling protocols for soil and plant testing for inoculum/disease levels
4. Continue the development of a PCR-based soil testing protocol for the estimation of SDM risk
5. Undertaken initial validation of the SDM risk model during the 2020/21 growing season

Project outputs:

Journal papers

Thangavel, T., Scott, J., Jones, S., Gugalothu, R., and Wilson, C. 2020. Effect of physio-chemical seed treatments on opium poppy downy mildews caused by *Peronospora meconopsidis* and *P. somniferi*. PLoS ONE 15:e0230801.

Conference papers

Thangavel, T., Scott, J., Krishnamoorthy, K., Singaloda, C., Jones, S., Ramakrishnan, H. and Wilson, C. 2019. Disease risk assessment of poppy downy mildew using qPCR analysis of two *Peronospora* spp. in naturally infested soil. In Proceedings of the Australasian Plant Pathology Society Conference 2019, Melbourne, Australia.

Thanabalasingam, D., Thangavel, T., Krishnamoorthy, K., Jones, S., Wilson, C., and Scott, J. 2019. Determining *Peronospora somniferi* genotype diversity and its potential impact on management of



systemic downy mildew of opium poppy. In Proceedings of the Australasian Plant Pathology Society Conference 2019, Melbourne, Australia.

Newspaper stories

Options for poppy plight, Tasmanian Country, April 10, 2020



TIA is a joint venture between the University of Tasmania and the Tasmanian Government



Appendix 1: PLoS ONE journal article

Journal papers

Thangavel, T., Scott, J., Jones, S., Gugalothu, R., and Wilson, C. 2020. Effect of physio-chemical seed treatments on opium poppy downy mildews caused by *Peronospora meconopsidis* and *P. somniferi*. PLoS ONE 15:e0230801.



TIA is a joint venture between the University of Tasmania
and the Tasmanian Government

RESEARCH ARTICLE

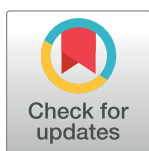
Effect of physio-chemical seed treatments on opium poppy downy mildews caused by *Peronospora meconopsidis* and *P. somniferi*

Tamilarasan Thangavel^{1*}, Jason Scott², Suzanne Jones², Ramya Gugalothu^{1a}, Calum Wilson^{1b}

1 Research Laboratories, Tasmanian Institute of Agriculture (TIA), University of Tasmania (UTAS), New Town Australia, **2** TIA, UTAS, Burnie, Tasmania, Australia

✉ Current address: Eden Farms, Bundaberg, Queensland, Australia

* tamilarasan.thangavel@utas.edu.au



OPEN ACCESS

Citation: Thangavel T, Scott J, Jones S, Gugalothu R, Wilson C (2020) Effect of physio-chemical seed treatments on opium poppy downy mildews caused by *Peronospora meconopsidis* and *P. somniferi*. PLoS ONE 15(4): e0230801. <https://doi.org/10.1371/journal.pone.0230801>

Editor: Sabrina Sarrocco, Universita degli Studi di Pisa, ITALY

Received: July 19, 2019

Accepted: March 9, 2020

Published: April 10, 2020

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0230801>

Copyright: © 2020 Thangavel et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript. Raw data are available from DOI: [10.5061/dryad.m0cfxpp0r](https://doi.org/10.5061/dryad.m0cfxpp0r).

Abstract

Downy mildew of opium poppy is the single biggest disease constraint afflicting the Australian poppy industry. Within the pathosystem, the transmission of infections via infested seed is of major concern. Both downy mildew pathogens of poppy; *Peronospora meconopsidis* and *P. somniferi*, are known contaminants of commercial seed stocks. Using seed naturally infested with these pathogens, the effect of physio-chemical seed treatments on seedling health and disease transmission were evaluated. Individual seed treatments were tested to determine optimal treatment parameters for each; including incubation time, temperature and treatment concentration. Optimised physiochemical treatments were then compared. The most effective treatment methods were seed washes in acidified electrolytic water (400 ppm hypochlorous acid for 5 min) and hypochlorite solution (2% NaOCl for 5 min). In seed to seedling transmission assays, these two treatments reduced transmission of *P. somniferi* by 88.8% and 74.61%, and *P. meconopsidis* by 93.3% and 100%, respectively. These methods are recommended for seed treatment of commercial opium poppy seed to assist in the control of the downy mildew diseases.

Introduction

Opium poppy (*Papaver somniferum*) is a highly valuable annual crop grown primarily for its opiate alkaloids. Around 40 alkaloids, including morphine, thebaine, codeine and noscapine, can be isolated from poppy. These alkaloids are used primarily by pharmaceutical industries in the synthesis of opiate-based medicinal products [1, 2]. Australia is the world's largest producer of licit opiate alkaloids, contributing over half the world's production [3, 4]. Within Australia, the majority of poppy production occurs within the island state of Tasmania [2].

Downy mildew is one of the most destructive diseases of poppy worldwide and causes substantial economic losses to the Australian poppy industry [5, 6]. Two pathogen species, *Peronospora meconopsidis* and *P. somniferi*, can cause downy mildew, with each associated with a distinct set of symptoms. Infections by *P. meconopsidis* are characterized by localised angular

Funding: The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. This work was financially supported by a grant from the Department of Primary Industry, Parks, Wildlife and Environment, Tasmania, Australia with contributions from Tasmanian Alkaloids Pty Ltd, Sun Pharma Pty Ltd and TPI Enterprise Ltd, Poppy Growers of Tasmania Pty Ltd and an ARC linkage grant (LP 160100758).

Competing interests: The authors have declared that no competing interests exist.

lesions on the adaxial surface of leaves and sparse sporulation on the abaxial surface [7, 8]. Prior to 2014, this was the only form of the disease known in Australia. However since 2014, systemic plant infections resulting in stunted plant growth, leaf deformation and heavy sporulation on the abaxial leaf surfaces have been observed. These symptoms are associated with infections by *P. somniferi* [6].

Understanding the drivers for pathogen spread is a large component of developing management for plant diseases. In poppy, transmission of downy mildew pathogens from seed-borne inocula to seedlings has been demonstrated [5, 9]. While transmission rates are generally low, seed transmission is an important mechanism for initiating epidemics and creating in field foci of inocula for subsequent secondary spread within the crop [8, 10]. As such, minimising seed transmission is seen by the poppy industry as an important component of managing the disease.

A large number of seed treatments, that are either physical, chemical, or biological in nature have been considered for management of seed-borne pathogens, including downy mildews [11]. Chemical fungicides are already a major component of the disease management strategy of the poppy industry, particularly for the control of secondary spread of downy mildew. Thus, alternative seed treatments that do not further increase reliance on fungicides are perceived as beneficial by the industry. Many alternatives to chemical fungicides can be used for such treatments. These include treating seed with oxidised chlorine compounds such as hypochlorite [12–15] or electrolyzed water solutions [16–20] and incubating seed at elevated treatments in hot water [21–24] or with aerated steam [25–28]. However, such treatments require evaluation and optimisation for individual pathosystems, as treatment protocols involve balancing the effects on pathogen inoculum control with maintaining physio-chemical seed health and vigour [26, 28–32].

This study aimed to evaluate the potential of select non-fungicide seed treatments for reducing transmission of both *Peronospora* spp. from seed to developing poppy seedlings. That such treatments, ensured optimal seed viability and performance was also a key consideration.

Materials and methods

Poppy seed

Poppy commercial seed lines SL21 and SL22, previously shown to contain high levels of both *P. somniferi* and *P. meconopsidis* inocula [5], were supplied by the Australia poppy industry for this study. Seed was stored at ambient room temperature (18–22°C) in the dark before use. In all experiments 10 g of seeds (equating to ~10,000 seeds) were used per seed treatment.

Seed treatments

Four seed treatment methods were evaluated in this study. These can be broadly grouped as either chlorine- (hypochlorite or electrolyzed water) or heat- (hot water or aerated steam) based. To optimise the protocols, each treatment was first evaluated individually. This was followed by two experiments comparing the efficiency of select individual seed treatments (Table 1). In all experiments, untreated controls were included; for studies involving electrolyzed water, hot water and hypochlorite, control seeds were soaked at ambient room temperature (18–22°C) in sterile water for an equivalent time-period. The controls for steam treatment studies were seed exposed to ambient room air (18–22°C). Following treatment, all replicates from each treatment were assessed for seed germination, microbial contamination, and seedling emergence. In all experiments, seed lot SL 21 was used unless otherwise mentioned. All treatments in all experiments were replicated in triplicate.

Table 1. Outline of seed treatment experiments and treatment parameters conducted in this study.

Experiment code	Treatment type	Incubation time (min)	Chlorine concentration ^a	Incubation Temperature (°C)
BT1	Hypochlorite solution	0, 5, 10, 15 & 20	0, 1, 2, 3 & 4	RT ^b
BT2		5	0, 1, 2, 3 & 4	RT and 5
EW1	Hypochlorous acid	0, 5, 10, 15, 30 & 60	0, 50, 100, 200 & 400	RT and 0, 5, 10, 15, 30 & 60
EW2		0, 5, 10, 15 & 20	0, 200 & 400	RT and 0, 5, 10, 15 & 20
HW1	Hot water	5, 15, 20 & 30	-	RT, 30, 40, 50 & 60
HW2		15	-	RT, 30, 40, 50 & 60
HW3		15	-	RT, 40, 50, 60 & 70
ST1	Aerated steam	15, 30 & 60	-	RT, 40, 50 & 60
ST2		10, 20, 30 & 40	-	RT, 40, 50 & 60
ST3		15	-	RT, 40, 50, 55, 60, 65 & 70
Protocol comparison 1	Untreated	0	-	RT
	Hypochlorite solution	5	2	RT
	Hypochlorous acid	15	200	RT
		15	400	RT
	Hot water	15	-	40
		15	-	50
		15	-	60
		15	-	70
	Aerated steam	15	-	50
		15	-	60
	Hypochlorous acid + Hot water	15 (HOCl), 15 (Hot water)	200	RT, 40 (Hot water)
		15 (HOCl), 15 (Hot water)	200	RT, 60 (Hot water)
	Hypochlorous acid + Aerated steam	15 (HOCl), 15 (Aerated steam)	200	RT, 60 (Aerated steam)
	Protocol comparison 2	Untreated	0	-
Hypochlorite solution		5	1	RT
		5	2	RT
		5	4	RT
Hypochlorous acid		15	100	RT
		15	200	RT
		15	400	RT
Hot water		15	-	30
		15	-	40
		15	-	50
		15	-	60
Aerated steam		15	-	40
		15	-	50
		15	-	60
		15	-	70
Hypochlorite solution + Hypochlorous acid		5 (NaOCl), 15 (HOCl)	2 & 400	RT, RT
Hypochlorite solution + Hot water		5 (NaOCl), 15 (HW)	2 & 400	RT, 40 (Hot water)
Hypochlorite solution + Aerated steam		5 (NaOCl), 15 (Aerated steam)	2	RT, 60 (Aerated steam)
Hypochlorous acid + Aerated steam		5 (NaOCl), 15 (Aerated steam)	200	RT, 60 (Aerated steam)
Hot water + Hypochlorous acid		5 (NaOCl), 15 (HOCl)	200	40 (Hot water), RT
Hot water + Aerated steam	15 (Hot water), 15 (Aerated steam)	-	40 (Hot water), 60 (Aerated steam)	

^a Concentration of active chlorine in solution measured as a percentage (%; hypochlorite treatments) or parts per million (ppm; hypochlorous acid treatments).

^b RT, ambient room temperature (18–22°C).

<https://doi.org/10.1371/journal.pone.0230801.t001>

Hypochlorite treatment

The optimal condition for hypochlorite treatment of seed was evaluated in two rounds of testing (BT1 and BT2; Table 1). A commercial bleach solution with sodium hypochlorite (42 g/L), 4% available chlorine (White King Bleach Standard, Victoria, Australia) was used in both experiments. In BT1, 10 g of seed were placed in a 100 ml conical flask and 50 ml of NaOCl solution added. Concentrations of 0 (control), 1, 2, 3 and 4% available chlorine were used, with incubation times of 0, 5, 10, 15 and 20 min. Treated seed was then rinsed three times in sterile deionised water, drained on sterile paper towel, and air-dried at ambient room temperature (18–22°C) in a laminar flow chamber for 24 h. In BT2, the same NaOCl concentrations were repeated, but for only a single incubation time (5 min). Treated seeds were rinsed, drained and air dried as previously described.

Electrolysis water treatment

Electrolysis water solution was obtained as electrolyzed saline water (generated using the Envirolite[®] EL-400, Envirolite Industries International OÜ, Tallinn, Estonia) from Prof Roger Stanley (Centre for Food Safety, Newnham campus, University of Tasmania). The chlorine concentration of the electrolysis water was determined by colorimetric analysis using a commercial kit (Compact ClO₂+ meter, Palintest, Australia). In both experiments, 10 g of seed was placed in a 100 ml conical flask and then 50 ml of electrolysis water solution was added. In EW1, seeds were treated in 0 (control), 50, 100, 200 and 400 ppm ClO₂ (pH = 6.7) for 0, 10, 15, 30 and 60 min. In EW2, seeds were treated at 0, 200 and 400 ppm (pH = 6.7) for 0, 5, 10, 15 and 20 min. Following treatment, seeds were drained onto a sterile paper towel and air dried at ambient room temperature (18–22°C), as previously described.

Hot water treatment

In three experiments (HW1, HW2 and HW3), 10 g of seed was placed into a 100 ml conical flask that contained 50 ml of sterile deionised water at ambient room temperature (18–22°C). The flasks were then incubated in a hot water bath (Ratek Instruments Pty Ltd, Australia) set at the various test temperatures. A thermometer (76mm N₂ filled immersion Zeal, England) was placed inside the flask to monitor temperature. The duration of individual treatments commenced from the time the water attained the desired temperature. In HW1, seeds were incubated at 30°C, 40°C, 50°C and 60°C for durations of 0, 5, 15, 20 and 30 min. In HW2, seeds were incubated at 40°C, 50°C, 60°C and 70°C for 15 min only. In HW3, seeds incubated at 30°C, 40°C, 50°C and 60°C for 0, 5, 15 and 30 min. Following treatment, seeds were drained and air dried at ambient room temperature (18–22°C), as previously described.

Aerated steam treatment

In three experiments (ST1, ST2 and ST3), 10 g of seeds were spread evenly across the base of open Petri plates (60 × 15 mm) and placed in a steamer oven (Royston steamer DS745, Hospitality World Direct, Australia) at ambient room temperature (18–22°C). Target temperatures were set and monitored using a thermometer within the chamber. The duration of individual treatments commenced from the time the internal chamber attained the desired temperature. In ST1, seeds were treated at 40°C, 50°C and 60°C for 0, 15, 30 and 60 min. In ST2, seeds were treated at 40°C, 50°C and 60°C for 0, 10, 20, 30 and 40 min. In ST3, seeds were treated at 40°C, 50°C, 55°C, 60°C, 65°C and 70°C for 0 and 15 min. Treated seeds were then air dried at room ambient room temperature (18–22°C), as previously described.

Treatment protocol comparison

Selected optimised treatments of the four seed disinfestation methods and selected combinations of treatments were directly compared in two protocol comparisons experiments (PC1 and PC2). Each experiment used 10 g of seed; PC1 used seed lot SL21 and PC2 used seed lot SL22. Both experiments tested a set of individual treatment types as well as compound treatments of varying conditions (Table 1).

A completely randomized block design was adopted for the seed emergence and seed-disease transmission experiments. In all experiments, three biological replicates were conducted for each treatment.

Seed health assessment

Seed germination and contamination rate. Germination capacity and microbial contamination of treated seed was tested in all experiments. Twenty-five seeds were sampled following each seed treatment and were aseptically placed onto four 2% water agar plates ($n = 100$) and incubated at 20°C with a 16 h photoperiod under cool white fluorescent lamps ($65 \mu\text{mol}/\text{m}^2\text{s}^{-1}$). After 10 days, seeds were assessed for germination and presence of any fungal or bacterial contaminants on the medium. For germination, only seeds that produced true leaves were counted. For contamination, both non-germinated and germinated seeds with fungal or bacterial colonies present were considered contaminated.

Seedling emergence. From each experiment, 100 seeds ($n = 100$) were sampled and sown in plastic trays (54 cm x 28 cm) filled with pasteurised potting mix containing sand, peat, and composted pine bark (10:10:80, pH 6.0) premixed with Osmocote 16–3.5–10 NPK resin coated fertilizer (Scotts Australia Pty Ltd.) to a depth of 0.5 cm. Glasshouse temperatures were maintained at 15–18°C by day with relative humidity at 55–60% and 10–14°C at night with 70–75% relative humidity. Trays were hand watered daily to maintain soil moisture. Trays were arranged in a randomized complete block design. Seedling emergence was determined three weeks after sowing.

Seed-disease transmission and disease incidence. To test the efficacy of individual treatments on seedborne-pathogen transmission to seedlings, and on subsequent visual disease incidence, glasshouse trials were conducted using the seeds treated in experiments BT1, EW2, HW1, ST1, PC1 and PC2. For each treatment, 3 g of seeds were sown on seed raising trays (60 × 40 × 12 cm) that were filled with a potting mix. In each tray, 0.5 g seeds were sown and covered with 0.5 cm layer of potting mix. In total six replicate trays were sown for each treatment. Experiments were conducted within a glasshouse environment with temperatures maintained as above. Hand watering occurred twice daily avoiding watering directly onto leaves.

Above ground foliage from three-week-old seedlings from each tray were harvested and tested for the presence of *P. somniferi* and *P. meconopsidis* using a pathogen species-specific PCR assay [5]. For each treatment, 15 groups of 100 seedlings were sampled from each replicate. Grouped seedlings were ground using a mortar pestle and 200 μl of homogenised plant tissue used for DNA extraction according to manufacturer's protocol using a Power Plant Pro DNA isolation kit (Mo BIO laboratories, Australia). Samples were then tested for each pathogen using the species-specific PCR assay [5]. Pathogen incidence was subsequently estimated using the group testing methodology of Gibbs and Gower [33].

After PCR testing, plants were visually assessed for disease. Individual trays were sealed with a plastic bag to enhance sporulation, raising the relative humidity to 100% for 24 hrs. After three days, 100 plants ($n = 100$) were assessed for disease based on the following symptoms: for *P. meconopsidis* infection symptoms were localized polyangular lesions surrounded

by chlorosis and symptoms; for *P. somniferi* infections symptoms were localized or systemic infection, stunted plants or leaves and strongly distorted stems.

Data analysis

Estimates of seed germination, contamination, emergence, and the incidence of *Peronospora* spp. in seedlings were analysed individually for treatment differences in the all experiments. Observations were fit using logistic regression modelling in the R statistical language framework v. 3.4 [34]. For *in vitro* seed testing, the counts of germinated or contaminated seeds were regressed with the total number of seedlings per replicate as the negative case. For seedling germination, the count of germinated seedlings at three weeks post-sowing was regressed with total number of seeds sown per replicate. Incidence of *P. somniferi* and *P. meconopsidis* in germinated seedlings was assessed by analysing the number of positive and negative qPCR reactions ($n = 15$) obtained per replicate. Post hoc comparisons were undertaken using least-squares means estimates incorporating the Tukey correction for multiple pairwise comparisons using the lsmeans package in R [35].

Results

Hypochlorite treatment

Following hypochlorite treatment, seed germination rates were typically improved relative to untreated seed (Table 2). Across both experiments, the highest rate of germination achieved was for seed treated with 2% hypochlorite solutions for 5 min (95 and 94% for experiments BT1 and BT2, respectively). Increasing either treatment duration or hypochlorite concentrate resulted in decreased seed germination rates. The highest observed reduction in seed contamination occurred when treatment duration was increased to 15 or 20 min (Table 2). Contamination rate was significantly reduced relative to untreated seed when concentrations were greater than 1%, irrespective of treatment duration, and when treated with a 1% solution for 15 min or more in BT1 (Table 2). However, in BT1 differences between individual treatment combinations were not significant at concentrations greater than 1%. In BT2, contamination rate was lowest when treated with a 4% solution, which was significantly less than all other concentrations. Seedling emergence was highest in seed treated with 2% hypochlorite for 5 min in BT1, but significantly decreased with increasing hypochlorite concentration in BT2 (Table 2).

No significant difference was found in the mean visual disease incidence of localised or systemic downy mildew symptoms with incidence low ($\leq 2\%$) even in untreated seed (Table 2). However, it is worth noting that localised lesions were not observed in any seedling treated with hypochlorite prior to planting and systemic symptoms were only observed sporadically. Similarly, no significant differences were found in the incidence of *P. meconopsidis* in germinated seedlings between treatments, however incidence was only estimated to be 1% in untreated seedlings. Estimated incidence of *P. somniferi* in untreated seedlings was 5.4% and was reduced to 2% or less by treatment with 2% hypochlorite or higher. Treatment with 4% hypochlorite reduced *P. somniferi* incidence below detection limits regardless of treatment duration.

Electrolysis water treatment

Seed treatment with 400 ppm electrolysis water significantly increased seed germination rate compared to the untreated controls regardless of treatment duration (Table 3). At lower concentrations, germination response varied with treatment duration. However, in EW1 optimal

Table 2. The effect of hypochlorite solution treated at various concentration and time on the opium poppy seeds. The mean proportion of germination, emergence, contamination and disease incidences were estimated using logistic regression model. Significant differences were grouped using HSD mean separation.

Hypochlorite solution (%)	Time (min)	Germination (%) ^a	Contamination (%) ^b	Emergence (%) ^b	Systemic downy mildew incidence ^c	Localised downy mildew incidence ^c	<i>P. somniferi</i> transmission/incidence in seedlings ^d	<i>P. meconopsidis</i> transmission/incidence in seedlings ^d
0	0	52.7 a	17.7 e	47.7 ab	2.0	0.3	5.3 c (2.9)	1.0 (0.47)
1	5	62.4 ab	10.7 cde	61.4 bcd	0.0	0.0	3.3 bc (1.73)	0.3 (0.16)
1	10	80.0 cdef	6.7 abcd	56.0 abcd	0.3	0.0	2.3 abc (1.14)	0.3 (0.16)
1	15	80.0 cdef	7.7 bcd	52.0 abc	0.0	0.0	2.0 abc (0.97)	0.0 (0.00)
1	20	86.7 efgh	3.0 ab	60.0 bcd	0.3	0.0	0.3 ab (0.16)	0.3 (0.16)
2	5	96.0 i	6.7 abcd	74.7 e	0.0	0.0	0.3 ab (0.16)	0.3 (0.16)
2	10	84.0 defg	2.4 ab	63.4 cde	0.0	0.0	0.3 ab (0.16)	0.0 (0.00)
2	15	94.7 hi	13.7 de	58.7 abcd	0.0	0.0	0.3 ab (0.16)	0.0 (0.00)
2	20	91.4 ghi	5.7 abcd	64.0 cde	0.0	0.0	0.3 ab (0.16)	0.0 (0.00)
3	5	88.0 fgh	4.7 abc	52.0 abc	0.0	0.0	0.3 ab (0.16)	0.0 (0.00)
3	10	72.0 bc	3.4 ab	65.4 cde	0.0	0.0	0.0 a (0.00)	0.0 (0.00)
3	15	86.7 efgh	1.4 a	66.7 de	0.0	0.0	0.3 ab (0.16)	0.0 (0.00)
3	20	74.7 bcd	2.7 ab	54.7 abcd	0.0	0.0	0.3 ab (0.16)	0.0 (0.00)
4	5	81.4 cdef	6.1 abcd	49.4 ab	0.0	0.0	0.0 a (0.00)	0.0 (0.00)
4	10	73.4 bcd	4.0 abc	54.0 abcd	0.0	0.0	0.0 a (0.00)	0.0 (0.00)
4	15	77.4 cde	2.0 ab	45.7 a	0.0	0.0	0.0 a (0.00)	0.0 (0.00)
4	20	84.0 defg	5.4 abc	48.0 ab	0.0	0.0	0.0 a (0.00)	0.0 (0.00)
Probability ^d		<0.001	<0.001	<0.001	0.49	1.00	<0.001	0.60
0	5	71.9 a	0.2 d	69.5 c	-	-	-	-
1	5	82.0 a	0.1 c	65.0 bc	-	-	-	-
2	5	93.7 c	0.0 bc	0.8 d	-	-	-	-
3	5	85.0 a	0.0 b	57.4 ab	-	-	-	-
4	5	63.4 a	0.0 a	53.7 a	-	-	-	-
Probability ^d		<0.001	<0.001	<0.001				

^a Values were obtained from 100 seeds plated onto water agar.

^b Values were obtained from 100 plants in three-week-old seedlings for both emergence and visual disease symptoms.

^c Results were obtained from 15 subsamples of 10 three-week-old seedlings from individual seed treatment by PCR. In PCR, weak positive amplifications were also considered as a positive detection [5]. Values in parentheses indicate the estimated pathogen transmission incidence (%) of based on the testing procedure of Gibbs and Gower [33]

^d Treatment differences were analysed via logistic regression. Germination, contamination, emergence and symptom incidences were analysed using number of positive seeds/seedlings out of the total number assessed. Incidence of *P. somniferi* and *P. meconopsidis* were analysed using the number of positive PCR reactions out of the total number of tests conducted. Post hoc comparisons were undertaken using least square means estimates with a Tukey correction for multiple pairwise comparisons. Treatment estimates sharing a lowercase letter within an experiment are not significantly different from each other ($P \geq 0.05$).

<https://doi.org/10.1371/journal.pone.0230801.t002>

germination was achieved with 15 min treatment for 50, 100 and 200 ppm concentrations and was significantly greater than nontreated seed. In EW2, treatment with electrolysis water significantly increased germination for all concentrations and durations, with maximal germination achieved with treatment for 15 min with 400 ppm. Similarly, microbial contamination was significantly reduced by treatments at 400 ppm electrolysis water at all tested durations across both experiments. Seedling emergence was significantly greater than nontreated when treated with either 200 or 400 ppm for 15 min or longer in EW1 and 5 min or longer in EW2.

The estimated visual disease incidence of symptoms of systemic downy mildew in seedlings grown from seed treated with all hypochlorous acid rates and durations, except 200 ppm for

Table 3. The effect of electrolysis water treated at various concentration and time on the opium poppy seed health and controlling poppy downy mildew.

Experiment	Hypochlorous acid (ppm)	Time (min)	Germination (%) ^a	Contamination (%) ^a	Emergence (%) ^b	Systemic symptom incidence (%) ^c	Localised symptom incidence (%) ^c	<i>P. somniferi</i> incidence in seedlings ^d	<i>P. meconopsidis</i> incidence in seedlings ^d
EW1	0	0	55.3 ab	25.3 c	46.7 a	-	-	-	-
	50	5	72.3 cde	20.7 bc	55.7 abc	-	-	-	-
	50	10	59.3 abc	16.3 b	45.3 ab	-	-	-	-
	50	15	69.7 cd	20.3 bc	49.7 abc	-	-	-	-
	50	30	62.0 bc	17.3 bc	50.3 abc	-	-	-	-
	50	60	64.0 bc	16.7 b	44.7 ab	-	-	-	-
	100	5	65.3 bc	16.0b	46.7 abc	-	-	-	-
	100	10	84.0 efg	17.7 bc	45.7 ab	-	-	-	-
	100	15	92.3 gh	18.0 bc	45.7 ab	-	-	-	-
	100	30	90.0 fgh	15.0 b	57.7 abcd	-	-	-	-
	100	60	81.7 def	19.7 bc	48.7 abc	-	-	-	-
	200	5	59.7 abc	16.3 b	48.0 abc	-	-	-	-
	200	10	81.3 def	15.7 b	49.7 abc	-	-	-	-
	200	15	93.0 gh	13.0 b	60.7 cd	-	-	-	-
	200	30	87.3 fg	13.7 b	69.7 d	-	-	-	-
	200	60	47.0 a	17.7 bc	58.0 bcd	-	-	-	-
	400	5	85.0 fg	11.3 b	56.7 abcd	-	-	-	-
	400	10	96.3 h	10.3 b	51.3 abc	-	-	-	-
	400	15	92.7 gh	1.7 a	59.0 bcd	-	-	-	-
	400	30	90.7 fgh	1.7 a	58.3 bcd	-	-	-	-
400	60	72.7 cde	0.3 a	59.0 bcd	-	-	-	-	
Probability ^d			<0.001	<0.001	<0.001				
EW2	0	0	45.7 a	17.4 c	41.0 a	6.4 b	2.4	3.6 c (6.3)	2.0 b (4.0)
	200	5	75.0 bc	15.4 bc	65.0 bc	0.0 a	0.0	1.3 bc (2.6)	0.0 a (0.0)
	200	10	84.0 cd	12.7 bc	75.7 cd	0.0 a	0.0	1.6 bc (3.3)	0.7 ab (1.6)
	200	15	71.0 b	11.7 bc	69.7 bc	0.0 a	0.0	1.6 bc (3.3)	0.0 a (0.0)
	200	20	74.7 bc	8.7 b	63.4 b	2.4 ab	0.0	1.3 bc (2.6)	0.1 a (0.3)
	400	5	71.7 b	2.0 a	68.0 bc	0.0 a	0.0	0.4 ab (1.0)	0.1 a (0.3)
	400	10	85.4 d	1.7 a	73.0 bc	0.0 a	0.0	0.0 a (0.0)	0.1 a (0.3)
	400	15	93.4 e	0.0a	85.0 d	0.0 a	0.0	0.0 a (0.0)	0.0 a (0.0)
	400	20	74.0 bc	0.0 a	64.7 bc	0.0 a	0.0	0.0 a (0.00)	0.0 a (0.0)
Probability ^d			<0.001	<0.001	<0.001	<0.001	0.16	<0.001	<0.001

^a Values were obtained from 100 seeds plated onto water agar.

^b Values were obtained from 100 plants in three-week-old seedlings for both emergence and visual disease symptoms.

^c Results were obtained from 15 subsamples of 10 four-week-old seedlings from individual seed treatment by PCR. In PCR, weak positive amplifications were also considered as a positive detection [5]. Values in parentheses indicate the estimated pathogen transmission incidence (%) of based on the testing procedure of Gibbs and Gower [33]

^d Treatment differences were analysed via logistic regression. Germination, contamination, emergence and symptom incidences were analysed using number of positive seeds/seedlings out of the total number assessed. Incidence of *P. somniferi* and *P. meconopsidis* were analysed using the number of positive PCR reactions out of the total number of tests conducted. Post hoc comparisons were undertaken using least square means estimates with a Tukey correction for multiple pairwise comparisons. Treatment estimates sharing a lowercase letter within an experiment are not significantly different from each other ($P \geq 0.05$).

<https://doi.org/10.1371/journal.pone.0230801.t003>

20 min, was below detection limits and significantly less than the nontreated seed (Table 3). Localised downy mildew symptoms were only detected in nontreated seedlings. Incidence of *P. somniferi* and *P. meconopsidis* within seedlings was significantly reduced by treatment with

400 ppm electrolysis water at all durations. Additionally, *P. meconosidis* incidence in seedlings treated with 200 ppm for 15 and 20 min was also significantly reduced compared to non-treated seedlings (Table 3).

Hot water treatment

Treatment of seed with hot water at 40°C, regardless of duration, or 50°C for 20 min or less was associated with significant increases in germination rate, relative to the control, across all three experiments (Table 4). However, contamination rate was not reduced unless treated at 40°C for 30 min, or at higher temperatures. Both seed germination and contamination were significantly reduced by treatment at 60°C or higher. Seedling emergence was not significantly different from the control at 30°C and was significantly reduced by treatment at 60°C or higher. Incidence of downy mildew was $\leq 1.2\%$ for both systemic and localised symptoms with no significant treatment effects detected (Table 4). However, incidence of both symptom types was below detection limits when treated with 50 or 60°C regardless of treatment duration. Similarly, incidence of *P. meconosidis* infection was low and no significant treatment effects were observed. Conversely, incidence of *P. somniferi* infection was significantly reduced by treatment at 50°C for 15 or 30 min and at 60°C for 15 min or longer.

Aerated steam treatment

Seed germination was improved by aerated steam treatment for 30 min or less at temperatures of 50°C or less (Table 5). Treatment up to 60°C for 15 min or less also either significantly improved or did not reduce germination rates relative to the control across all experiments. However, treatment for 30 min or more at 60°C or higher was typically detrimental to germination rate. When treated with 55°C or higher for 15 min or longer seed contamination consistently demonstrated significant reductions, relative to the control. At lower temperatures effects on contamination were less consistent across experiments. No positive effects of aerated steam treatment were detected in ST1 and ST3. However, in ST2, 60°C for 10 and 15 min, 50°C for 30 min or less and 40°C for all durations significantly improved germination (Table 5). Incidence of both *P. somniferi* and *P. meconosidis* in germinated seedlings was significantly reduced by treatment at 60°C for all durations, while *P. somniferi* was also reduced at 50°C for 60 min and *P. meconosidis* at 40°C for either 30 or 60 min. No significant differences in symptom incidence were observed.

Treatment protocol comparison

Across both treatment comparison experiments, the effect of single treatments on seed germination was consistent (Table 1). All rates of hypochlorite and electrolysis water significantly improved germination relative to the controls of each experiment. Furthermore, hot water at 50°C and aerated steam at 50 or 60°C significantly improved germination in both experiments. In PC1, highest germination rates were observed when seed was treated with 400 ppm electrolysis water (91%; Table 6). In PC2, the highest germination rates were achieved with 2% hypochlorite. Except for hot water (40°C) followed by aerated steam (60°C) in PC2, no significant improvements in germination rate relative to the first treatment in the combination were observed. In the case of hot water and aerated steam, this treatment was not significantly better than aerated steam alone.

Hypochlorite and electrolysis water also significantly reduced seed contamination rates in both experiments at all concentrations. The effects of aerated steam and hot water was less consistent across experiments. However, contamination was lowest when treated with hot water at 60°C in both experiments. Combining electrolysis water, hypochlorite or aerated steam with other treatments did not reduce contamination below that of those treatments alone.

Table 4. The effect of hot water treatment at various temperature and time on the opium poppy seed health and controlling poppy downy mildew.

Experiment code	Temp (°C)	Time (min)	Germination (%) ^a	Contamination (%) ^a	Emergence (%) ^b	Systemic symptom incidence (%) ^b	Localised symptom incidence (%) ^b	<i>P. somniferi</i> incidence in seedlings ^c	<i>P. meconopsidis</i> incidence in seedlings ^c
HW1	RT ^e	0	48.0 b	16.1 d	44.7 cd	0.3	0.3	2.3 def (4.3)	0.2 (0.5)
	30	5	64.0 d	15.5 d	46.2 cd	1.2	0.2	3.0 f (5.5)	0.3 (0.6)
	30	15	64.0 d	14.5 d	46.0 cd	1.2	0.2	2.0 def (3.8)	0.5 (1.1)
	30	20	50.0 bc	15.1 d	40.2 c	1.2	0.2	1.6 cdef (3.1)	0.2 (0.5)
	30	30	50.0 bc	10.0 cd	40.0 c	1.2	0.2	2.0 def (4.0)	0.4 (0.8)
	40	5	75.0 e	13.7 d	58.0 ef	0.2	0.2	2.6 ef (4.8)	0.5 (1.1)
	40	15	75.0 e	14.0 d	57.9 ef	0.2	0.2	2.9 f (5.3)	0.1 (0.1)
	40	20	61.0 d	15.1 d	52.0 de	0.2	0.2	2.7 f (5.0)	0.3 (0.7)
	40	30	61.0 d	4.1 b	63.2 fg	0.2	0.2	1.8 cdef (3.5)	0.1 (0.3)
	50	5	78.0 e	5.5 bc	66.4 fg	0.0	0.0	2.1 def (4.1)	0.1 (0.3)
	50	15	82.0 e	2.3 b	69.2 g	0.0	0.0	0.4 abc (1.0)	0.0 (0.1)
	50	20	58.0 cd	3.1 b	52.0 de	0.0	0.0	1.3 bcdef (2.6)	0.2 (0.5)
	50	30	43.0 b	3.1 b	14.9 b	0.0	0.0	0.8 abc (1.6)	0.2 (0.5)
	60	5	0.6 a	0.0 a	0.4 a	0.0	0.0	1.0 abcde (2.0)	0.2 (0.5)
	60	15	0.6 a	0.0 a	0.2 a	0.0	0.0	0.1 a (0.3)	0.2 (0.5)
	60	20	0.6 a	0.0 a	0.4 a	0.0	0.0	0.2 ab (0.5)	0.2 (0.5)
60	30	0.6 a	0.0 a	0.4 a	0.0	0.0	0.4 abc (1.0)	0.1 (0.3)	
Probability ^d			<0.001	<0.001	<0.001	0.17	0.68	<0.001	0.55
HW2	RT	15	43.4 b	21.7 c	38.0 b	-	-	-	-
	40	15	59.4 c	14.0 c	63.0 c	-	-	-	-
	50	15	80.7 d	6.7 b	66.7 c	-	-	-	-
	60	15	0.0 a	0.0 a	0.0 a	-	-	-	-
	70	15	0.0 a	0.0 a	0.0 a	-	-	-	-
Probability ^d			<0.001	<0.001	<0.001				
HW3	RT	0	44.6 cd	17.0 d	56.6 bc	-	-	-	-
	30	5	35.6 c	16.4 d	57.0 c	-	-	-	-
	30	15	51.3 de	16.7 d	44.6 bc	-	-	-	-
	30	30	62.0 ef	14.4 cd	57.0 c	-	-	-	-
	40	5	58.3 ef	15.4 cd	54.3 bc	-	-	-	-
	40	15	67.6 f	11.7 cd	44.0 bc	-	-	-	-
	40	30	70.6 f	13.7cd	52.0 bc	-	-	-	-
	50	5	68.6 f	9.7 bcd	48.0 bc	-	-	-	-
	50	15	68.3 f	7.0 bc	53.6 bc	-	-	-	-
	50	30	50.6 de	4.0 ab	43.3 b	-	-	-	-
	60	5	4.3 b	0.4 a	1.0 a	-	-	-	-
	60	15	0.0 a	0.4 a	0.0 a	-	-	-	-
60	30	0.0 a	0.4 a	0.0 a	-	-	-	-	
Probability ^d			<0.001	<0.001	<0.001				

^a Values were obtained from 100 seeds plated onto water agar.

^b Values were obtained from 100 plants in three-week-old seedlings for both emergence and visual disease symptoms.

^c Results were obtained from 15 subsamples of 10 four-week-old seedlings from individual seed treatment by PCR. In PCR, weak positive amplifications were also considered as a positive detection [5]. Values in parentheses indicate the estimated pathogen transmission incidence (%) of based on the testing procedure of Gibbs and Gower [33].

^d Treatment differences were analysed via logistic regression. Germination, contamination, emergence and symptom incidences were analysed using number of positive seeds/seedlings out of the total number assessed. Incidence of *P. somniferi* and *P. meconopsidis* were analysed using the number of positive PCR reactions out of the total number of tests conducted. Post hoc comparisons were undertaken using least square means estimates with a Tukey correction for multiple pairwise comparisons. Treatment estimates sharing a lowercase letter within an experiment are not significantly different from each other ($P \geq 0.05$).

^e RT, ambient room temperature (18–22°C)

<https://doi.org/10.1371/journal.pone.0230801.t004>

Table 5. The effect of aerated steam treatment at various temperature and time on the opium poppy seed health and controlling poppy downy mildew.

Experiment	Temperature (°C)	Time (min)	Germination (%) ^a	Contamination (%) ^a	Emergence (%) ^b	Systemic symptom incidence (%) ^b	Localised symptom incidence (%) ^b	<i>P. somniferi</i> incidence in seedlings ^c	<i>P. meconopsidis</i> incidence in seedlings ^c
ST1	RT ^c	0	49.5 cd	18.8 f	46.0 c	0.6	0.0	6.8 c (4.1)	0.7 b (1.3)
	40	15	61.0 d	17.6 ef	55.6 c	0.3	0.0	6.3 bc (3.6)	0.1 ab (0.3)
	40	30	63.6 d	13.6 def	53.3 c	0.0	0.0	5.0 bc (2.6)	0.0 a (0.0)
	40	60	41.3 c	10.3 cde	31.3 b	0.3	0.0	3.3 abc (1.7)	0.0 a (0.0)
	50	15	62.6 d	12.6 def	51.0 c	0.3	0.0	7.3 bc (4.4)	0.1 ab (0.4)
	50	30	40.3 bc	7.0 bcd	34.3 b	0.0	0.0	3.3 abc (1.7)	0.1 ab (0.4)
	50	60	28.6 b	7.3 bcd	26.0 b	0.0	0.0	1.0 a (0.4)	0.1 ab (0.4)
	60	15	62.6 d	4.0 bc	53.6 c	0.0	0.0	3.0 ab (1.4)	0.0 a (0.0)
	60	30	28.6 b	2.6 ab	28.0 b	0.0	0.0	1.0 a (0.4)	0.0 a (0.0)
	60	60	0.6 a	0.0 a	0.3 a	0.0	0.0	0.3 a (0.1)	0.0 a (0.0)
<i>Probability</i> ^d			<0.001	<0.001	<0.001	0.49	1.00	<0.001	<0.001
ST2	RT	0	56.0 bc	13.6 b	43.5 c	-	-	-	-
	40	10	72.3 d	10.0 b	68.3 e	-	-	-	-
	40	15	67.3 d	13.0 b	64.6 de	-	-	-	-
	40	30	68.8 d	10.0 b	66.3 de	-	-	-	-
	40	40	63.8 cd	12.0 b	62.0 de	-	-	-	-
	50	10	69.7 d	10.0 b	63.0 de	-	-	-	-
	50	15	67.0 d	9.3 b	62.3 de	-	-	-	-
	50	30	67.3 d	8.3 b	58.6 de	-	-	-	-
	50	40	46.6 b	9.0 b	26.3 b	-	-	-	-
	60	10	69.6 d	7.6 b	62.0 de	-	-	-	-
	60	15	64.6 cd	1.0 a	54.6 d	-	-	-	-
	60	30	24.0 a	0.0 a	14.6 a	-	-	-	-
	60	40	25.0 a	0.0 a	11.0 a	-	-	-	-
	<i>Probability</i> ^d			<0.001	<0.001	<0.001			
ST3	RT	0	62.0 b	0.2 c	48.7 c	-	-	-	-
	40	15	70.4 bc	0.2 c	55.0 c	-	-	-	-
	45	15	77.4 c	0.1 bc	52.4 c	-	-	-	-
	50	15	59.7 b	0.1 b	49.4 c	-	-	-	-
	55	15	63.0 b	0.1 b	55.4 c	-	-	-	-
	60	15	60.7 b	0.0 a	55.7 c	-	-	-	-
	65	15	59.4 b	0.0 a	13.7 b	-	-	-	-
	70	15	17.4 a	0.0 a	0.00 a	-	-	-	-
<i>Probability</i> ^d			<0.001	<0.001	<0.001				

^a Values were obtained from 100 seeds plated onto water agar.

^b Values were obtained from 100 plants in three-week-old seedlings for both emergence and visual disease symptoms.

^c Results were obtained from 15 subsamples of 10 four-week-old seedlings from individual seed treatment by PCR. In PCR, weak positive amplifications were also considered as a positive detection [5]. Values in parentheses indicate the estimated pathogen transmission incidence (%) of based on the testing procedure of Gibbs and Gower [33]

^d Treatment differences were analysed via logistic regression. Germination, contamination, emergence and symptom incidences were analysed using number of positive seeds/seedlings out of the total number assessed. Incidence of *P. somniferi* and *P. meconopsidis* were analysed using the number of positive PCR reactions out of the total number of tests conducted. Post hoc comparisons were undertaken using least square means estimates with a Tukey correction for multiple pairwise comparisons. Treatment estimates sharing a lowercase letter within an experiment are not significantly different from each other ($P \geq 0.05$).

^cRT, ambient room temperature (18–22°C)

<https://doi.org/10.1371/journal.pone.0230801.t005>

In PC1, the mean germination rate was significantly greater than the untreated control in all treatments except the combined 200 ppm HOCl for 15 min & hot water at 60°C for 15 min, hot water at 40°C, 60°C and 70°C for 15 min.

Seedling emergence was only significantly greater than the control when treated with aerated steam at 50 or 60°C for 15 min, and when electrolysis water was used with aerated steam at 60°C in PC1 (Table 6). In the latter case, aerated steam in combination with electrolysis water was not significantly different from aerated steam at 60°C alone. In PC2 all hypochlorite concentrations, 200 and 400 ppm electrolysis water, hot water at 50°C and aerated steam at 40, 50 and 60°C significantly increased emergence. No combined treatment resulted in a significant increase in emergence above that of its constituent treatments.

In PC1, incidence of *P. somniferi* in control seedlings was 8.0% (Table 6). Treatment with hypochlorite (2%), electrolysis water (400 ppm) and hot water (60 and 70°C) all significantly reduced incidence of *P. somniferi* below the control. Electrolysis water (200 ppm) in combination with aerated steam (60°C) also significantly reduced *P. somniferi* incidence relative to both the control and each of the individual constituent treatments. In PC2, hypochlorite (1, 2, and 4%), electrolysis water (100, 200 and 400 ppm), hot water (50 and 60°C) and aerated steam (60 and 70°C) all significantly reduced *P. somniferi* incidence relative to the 3.0% incidence observed in the control. All combination treatments also significantly reduced *P. somniferi* incidence, but none were significant reductions relative to either one or both of their individual constituents. No *P. somniferi* infected seedlings were detected when seed was treated with hypochlorite at 1 or 4%, or 2% in combination with another treatment, nor when treated with electrolysis water in combination with hot water at 40°C. Incidence of *P. meconopsidis* in seedlings was low ($\leq 1.7\%$) for all treatments across both experiments and no significant treatment effects were detected.

Discussion

This study was undertaken to examine the potential of physiochemical seed treatments to manage seed-borne transmission of downy mildew in opium poppy. Many *Peronospora* spp. are known to be seed-borne, including those infecting poppy [5, 9, 36, 37]. Seed-borne transmission of disease can initiate inocula foci from which secondary spread occurs [5, 8, 10]. Furthermore, seed-borne infestations can be transported across great distances by anthropogenic mean allowing the introduction of pathogens to regions with no previous history of the disease.

Hypochlorite treatments have been commonly used as sanitisers and as seed disinfectants [13, 38]. In the range of hypochlorite treatments tested, 2% hypochlorite for 5 min gave the greatest seed germination and emergence, whilst minimising microbial contamination and reducing *P. somniferi* transmission to seedlings. This suggests hypochlorite at this rate could be an effective seed treatment to both reduce disease risks and improve seed health. Similar outcomes have been also reported in barley and lettuce to eradicate *Alternaria* sp., and *Xanthomonas* sp. from seed lots respectively [38–40]. However, hypochlorite treatment requires careful removal of the chemical by multiple water rinses, otherwise the residues on the seed coat increase the risk of damage to seed lines during emergence [13]. Also handling of hypochlorite solutions poses potential risk to users as it is highly caustic and produces dangerous volatiles. Thus, for commercial use, hypochlorite-based seed treatments need careful attention to handling procedure and seed processing to minimise risks.

Electrolysis water is widely used in food industries, healthcare environments and in agriculture as a sanitiser [41, 42]. Recent studies have shown that electrolysis water application can reduce the spore germination of species in the genera *Botrytis*, *Fusarium*, *Penicillium*,

Table 6. Comparison and efficacy of four seed treatment on enhancing opium poppy seed health and controlling poppy downy mildew.

Experiment	Treatment	Incubation Temperature (°C)	Hypochlorite (%)	Hypochlorous acid (ppm)	Time (min)	Germination (%) ^a	Contamination (%) ^a	Emergence (%) ^b	<i>P. somniferi</i> incidence in seedlings ^c	<i>P. meconosoidis</i> incidence in seedlings ^c	
Protocol comparison (PCI)	Untreated	RT ^e	-	-	-	49.4 bc	17.3 d	60.5 cde	5.3 de (8.00)	0.9 (1.7)	
	Hypochlorite solution	5	2	-	5	86.7 efg	2.3 ab	68.0 def	0.6 abc (1.34)	0.0 (0.0)	
	Electrolysis water	RT	-	-	200	15	80.0 cdef	8.3 bc	65.3 cdef	3.3 de (5.67)	0.0 (0.0)
		RT	-	-	400	15	91.4 g	2.3 ab	70.6 efg	0.4 ab (1.00)	0.0 (0.0)
		40	-	-	-	15	37.7 b	12.6 cd	55.0 cd	6.3 e (9.34)	0.0 (0.0)
	Hot water	50	-	-	-	15	73.4 d	7.6 bc	54.6 c	4.6 de (2.60)	0.3 (0.7)
		60	-	-	-	15	0.0 a	0.6 a	0.0 a	0.0 a (0.00)	0.0 (0.0)
		70	-	-	-	15	0.0 a	0.6 a	0.0 a	0.0 a (0.00)	0.0 (0.0)
	Aerated steam	50	-	-	-	15	85.4 defg	12.3 cd	76.67 fg	4.3 de (7.00)	0.0 (0.0)
		60	-	-	-	-	76.7 cde	10.0 cd	73.3 fg	2.9 cde (5.00)	0.2 (0.3)
	Electrolysis water + Hot water	RT+ 40	-	-	200	15 (HOCl), 15 (Hot water)	76.0 cd	9.3 cd	12.0 b	2.2 bcd (4.00)	0.0 (0.0)
		RT + 60	-	-	200	15 + 15	0.0 a	2.3 ab	0.0 a	0.0 a (0.00)	0.0 (0.0)
	Electrolysis water + Aerated steam	RT + 60	-	-	200	15 + 15	87.7 fg	8.0 bc	81.6 g	0.5 ab (1.00)	0.0 (0.0)
		Probability ^d					<0.001	<0.001	<0.001	<0.001	0.70

(Continued)

Table 6. (Continued)

Experiment	Treatment	Incubation Temperature (°C)	Hypochlorite (%)	Hypochlorous acid (ppm)	Time (min)	Germination (%) ^a	Contamination (%) ^a	Emergence (%) ^b	<i>P. somniferi</i> incidence in seedlings ^c	<i>P. meconopsidis</i> incidence in seedlings ^c
Protocol comparison (PC2)	Untreated	RT	-	-	-	71.2 c	24.5 g	40.4 cd	3.0 c (2.18)	0.2 (0.3)
	Hypochlorite solution	RT	1	-	5	87.8 de	4.4 cd	82.9 gh	0.0 a (0.00)	0.0 (0.0)
		RT	2	-	5	96.7 g	1.9 abc	90.9 i	0.2 a (0.08)	0.0 (0.0)
		RT	4	-	5	89.0 de	4.4 cd	86.7 hi	0.00 a (0.00)	0.0 (0.0)
		RT	-	100	15	92.4 defg	11.7 ef	48.0 de	0.7 abc (0.3)	0.0 (0.0)
	Electrolysis water	RT	-	200	15	96.0 fg	5.5 cd	97.2 j	0.3 a (0.16)	0.0 (0.0)
		RT	-	400	15	95.4 fg	0.4 ab	96.0 j	0.2 a (0.08)	0.0 (0.0)
		30	-	-	15	60.7 b	26.2 g	45.0 cde	2.7 bc (1.51)	0.0 (0.0)
	Hot water	40	-	-	15	74.0 c	18.4 fg	36.0 c	1.7 abc (0.98)	0.0 (0.0)
		50	-	-	15	86.9 d	9.0de	71.2 f	0.5 ab (0.24)	0.0 (0.0)
		60	-	-	15	0.6 a	0.0 a	0.0 a	0.0 a (0.00)	0.0 (0.0)
		40	-	-	15	79.0 c	15.9 f	72.0 f	1.5 abc (0.8)	0.0 (0.0)
	Aerated steam	50	-	-	15	87.8 de	8.2 de	74.7 fg	0.8 abc (0.41)	0.0 (0.0)
		60	-	-	15	92.5 defg	5.4 cd	89.2 hi	0.5 ab (0.24)	0.0 (0.0)
		70	-	-	15	9.0 a	0.0 a	0.0 a	0.0 a (0.00)	0.0 (0.0)
Hypochlorite solution + Electrolysis water	RT + RT	2	200	5 + 15	93.5 efg	0.9 ab	75.5 fg	0.0 a (0.00)	0.0 (0.0)	
	RT + 40	2	-	5 + 15	96.5 g	2.0 abc	90.2 i	0.0 a (0.00)	0.0 (0.0)	
	RT + 60	2	-	5 + 15	91.0 def	2.9 bc	43.9 cd	0.0 a (0.00)	0.0 (0.0)	
Electrolysis water + Aerated steam	RT + 60	-	200	15 + 15	70.8 c	0.90 ab	26.2 b	0.0 a (0.00)	0.0 (0.0)	
	RT + 40	-	200	15 + 15	89.7 de	5.4 cd	54.9 e	0.5 ab (0.25)	0.0 (0.0)	
	40 + 60	-	-	15 + 15	88.9 de	4.4 cd	23.5 b	0.2 a (0.08)	0.0 (0.0)	
Hot water + Aerated steam	40 + 60	-	-	15 + 15	88.9 de	4.4 cd	23.5 b	0.2 a (0.08)	0.0 (0.0)	
Probability ^d					<0.001	<0.001	<0.001	<0.001	<0.001	0.90

^a Values were obtained from 100 seeds plated onto water agar.

^b Values were obtained from 100 plants in three-week-old seedlings for both emergence and visual disease symptoms.

^c Results were obtained from 15 subsamples of 10 four-week-old seedlings from individual seed treatment by PCR. In PCR, weak positive amplifications were also considered as a positive detection [5].

^d Values in parentheses indicate the estimated pathogen transmission incidence (%) of based on the testing procedure of Gibbs and Gower [33]. Treatment differences were analysed via logistic regression. Germination, contamination, emergence and symptom incidences were analysed using number of positive seeds/seedlings out of the total number assessed. Incidence of *P. somniferi* and *P. meconopsidis* were analysed using the number of positive PCR reactions out of the total number of tests conducted. Post hoc comparisons were undertaken using least square means estimates with a Tukey correction for multiple pairwise comparisons. Treatment estimates sharing a lowercase letter within an experiment are not significantly different from each other ($P \geq 0.05$).

^e RT, ambient room temperature (18–22°C)

Cladosporium, *Helminthosporium* and *Phytophthora* [43, 44]. In this study, poppy seeds treated with 400 ppm of electrolysis water for 15 min had improved germination, decreasing microbial contamination and a significant reduction in the transmission of *P. somniferi*. In contrast to hypochlorite treatments, electrolysis water does not require rinsing as the breakdown products are sodium chloride and water, and it poses less risk to the operator [41, 45].

Conventional heating methods, such as hot water and aerated steam, have been reported as a valuable for controlling many seed-borne pathogens. These include black leg and black rot in cabbage caused by *Phoma lingam* and *Xanthomonas campestris* pv. *campestris*, respectively [46, 47]. However, these were less efficacious than chlorine-based treatments in this study. The most suitable temperature and duration of hot water was 50°C for 15 min with higher temperatures (>50°C) and longer durations (>20 min) having a significant detrimental effect on germination and emergence suggesting loss of seed viability. In addition, seed treated with hot water often results in a reduction in viability if not stored appropriately, as the seed coat can form cracks during treatment [48]. Long term viability of poppy seeds post treatment was not assessed in this study. However, this should be evaluated prior to commercial adoption of any treatment protocol by the poppy industry. Aerated steam treatment at 60°C for 15 min was found sufficient to enhance the seed health and minimise transmission of *Peronospora* spp. in seedlings. However, as with hot water treatment, increased temperatures and durations had a negative effect on seed performance.

An additional benefit of seed treatments observed in this study was the reduction in fungal contaminants other than *Peronospora* spp.. While, not the focus of this study, poppy fire, caused by *Alternaria penicillata* and *A. papavericola* [49], is another seed-borne disease of opium poppy [50–52]. Poppy fire is known to be present in Australian crops [53], however the significance of the disease for Australian production is currently unknown. Whether or not the co-occurrence of reduced seed fungal contamination and increased seedling emergence following physiochemical treatment in this study is due to poppy fire contamination of seed bears further study.

Physical seed treatment methods can also enhance seed viability. Physical treatments can break the dormancy of the seed and that enhance the uniformity in germination and emergence [54–56]. Seed scarification methods, including chlorine and temperature-based treatments, can soften the seed coat, increasing the seed gas exchange rate and moisture content [54–57]. Our standardized treatments from these both methods provide satisfactory germination, emergence and effective microbial suppression. Treating poppy seeds either with 2% hypochlorite solution for 5 min or 400 ppm electrolysis water results in higher germination rates and adequate pathogen elimination compared to temperature-based treatments. This could be because chlorine-based treatments enhance the water regulation within the seed [55] and therefore, provide more synchronized germination and emergence.

A major driver for the evaluation of non-chemical fungicide seed treatments in this work is the perceived risk of fungicide resistance development. In season fungicide applications provide the principle mechanism for in season disease management for the Australian poppy industry [58]. However, such reliance carries with it the risk of resistance development. While fungicide resistance has not been detected in the poppy pathogens, it has occurred in other downy mildew species with resistance to metalaxyl [59, 60] and mefenoxam [61, 62] recorded. Thus, to mitigate the risk of this occurring, seed treatment options based on alternatives to chemical fungicides as seen as beneficial for poppy production.

In conclusion, in this study we have demonstrated the benefits of four physiochemical seed treatments for enhancing the poppy seed health and performance, and minimising transmission of *P. somniferi* and *P. meconopsidis*. Of the four physio-chemical treatments, the chlorine-based hypochlorite and electrolysis water treatments appeared superior to be temperature-

based treatments in controlling pathogen transmission whilst retaining seed performance. Combining multiple treatments in series was not observed to give an advantage over individual treatments. We recommend consideration of hypochlorite or electrolysis water seed treatments for treatment of commercial poppy seed lots to minimise downy mildew transmission and improve seed performance.

Author Contributions

Conceptualization: Tamilarasan Thangavel, Jason Scott, Suzanne Jones, Calum Wilson.

Data curation: Tamilarasan Thangavel, Jason Scott, Ramya Gugalothu.

Formal analysis: Tamilarasan Thangavel, Jason Scott, Calum Wilson.

Investigation: Tamilarasan Thangavel, Jason Scott, Ramya Gugalothu, Calum Wilson.

Methodology: Tamilarasan Thangavel, Ramya Gugalothu.

Supervision: Jason Scott, Calum Wilson.

Validation: Tamilarasan Thangavel, Jason Scott, Suzanne Jones, Calum Wilson.

Writing – original draft: Tamilarasan Thangavel, Calum Wilson.

Writing – review & editing: Tamilarasan Thangavel, Jason Scott, Suzanne Jones, Calum Wilson.

References

1. Guo L, Winzer T, Yang X, Li Y, Ning Z, He Z, et al. The opium poppy genome and morphinan production. *Science*. 2018; 362(6412):343–7. <https://doi.org/10.1126/science.aat4096> PMID: [30166436](https://pubmed.ncbi.nlm.nih.gov/30166436/)
2. Frappell B, editor Fifty years of poppies in Tasmania: The first ten years, 1960 to 1970. Tasmanian Historical Research Association; 2010.
3. Miltenburg J. Supply chains for illicit products: case study of the global opiate production networks. *Cogent Business and Management*. 2018; 5(1).
4. INCB. Narcotic drugs: Estimated World Requirements for 2019- Statistics for 2017. Available from: <https://www.incb.org/incb/en/publications/incb-publications.html> Cited 1 May 2019. New York: United Nations: 2019.
5. Thangavel T, Jones S, Scott J, Livermore M, Wilson CR. Detection of two *Peronospora* spp., responsible for downy mildew, in opium poppy seed. *Plant Dis*. 2018; 102(11):2277–84. <https://doi.org/10.1094/PDIS-03-18-0503-RE> PMID: [30211657](https://pubmed.ncbi.nlm.nih.gov/30211657/)
6. Thangavel T, Wilson C, Jones S, Scott J, Voglmayr H. First report of systemic downy mildew of opium poppy caused by *Peronospora somniferi* in Australia. *Plant Dis*. 2016; 101(2):392.
7. Cotterill P, Pascoe I. Downy mildew of *Papaver somniferum* in Tasmania. *Australas Plant Pathol*. 1998; 27(4):263–4.
8. Scott J, Hay F, Wilson C. Adaptation of the forecasting model DOWNCAST for determination of downy mildew epidemics of oilseed poppy in Tasmania, Australia. *J Phytopathol*. 2008; 156(4):205–13.
9. Montes-Borrego M, Munoz-Ledesma FJ, Jimenez-Diaz RM, Landa BB. Real-time PCR quantification of *Peronospora arborescens*, the opium poppy downy mildew pathogen, in seed stocks and symptomless infected plants. *Plant Disease*. 2011; 95(2):143–52. <https://doi.org/10.1094/PDIS-07-10-0499> PMID: [30743414](https://pubmed.ncbi.nlm.nih.gov/30743414/)
10. Scott J, Hay F, Wilson C, Cotterill P, Fist A. Spatiotemporal analysis of epiphytotics of downy mildew of oilseed poppy in Tasmania, Australia. *Phytopathology*. 2003; 93(6):752–7. <https://doi.org/10.1094/PHYTO.2003.93.6.752> PMID: [18943064](https://pubmed.ncbi.nlm.nih.gov/18943064/)
11. Thakur R, Shetty H, Khairwal I. Pearl millet downy mildew research in India: progress and perspectives. *International Sorghum and Millets Newsletter*. 2006; 47:125–30.
12. Ali S, Paterson J, Crosby J. A standard technique for detecting seed-borne pathogens in peas, chemical control, and testing commercial seed in South Australia. *Aust J Exp Agric*. 1982; 22(117):348–52.

13. Chun S-C, Schneider R, Cohn M. Sodium hypochlorite: effect of solution pH on rice seed disinfestation and its direct effect on seedling growth. *Plant Dis.* 1997; 81(7):821–4. <https://doi.org/10.1094/PDIS.1997.81.7.821> PMID: 30861901
14. Dumroese RK, James RL, Wenny DL, Gilligan CJ. Douglas-fir seed treatments: effects on seed germination and seedborne organisms. In: Landis TD, editor. *Proc. Combined Meeting of the Western Forest Nursery Associations; Colorado, USDA: General Technical Report; 1988.* p. 155–60.
15. Wenny DL, Dumroese RK. Germination of conifer seeds surface sterilized with bleach. *Tree Planters' Notes.* 1987; 38(3):18–21.
16. Al-Haq MI, Seo Y, Oshita S, Kawagoe Y. Disinfection effects of electrolyzed oxidizing water on suppressing fruit rot of pear caused by *Botryosphaeria berengeriana*. *Food Res Int.* 2002; 35(7):657–64.
17. Bonde M, Nester S. Acidic electrolyzed water (AEW) for surface sterilization of teliospores. *Phytopathology.* 2002; 92(6):S142.
18. Lazarovits G, Abbasi P, Earl R. Use of acidic electrolyzed water for reducing populations by *Xanthomonas campestris* pv. *vesicatoria* on tomato seed and foliage. *Canadian J Plant Pathol.* 2003; 25:429.
19. Mi A-H, Seo Y, Oshita S, Kawagoe Y. Acidic electrolyzed water suppresses fungal growth. *J Jpn Soc Agric Mach.* 2000; 62:479–80.
20. Yoshida K, Min J-S, Park J-B, Isobe S, Suzuki T. Disinfecting rice seeds using acidic electrolyzed water. *J Soc Agric Struc.* 2003; 33(4):247–53.
21. Hayasaka T, Ishiguro K, Shibutani K, Namai T. Seed disinfection using hot water immersion to control several seed-borne diseases of rice plants. *Jpn J Phytopathol.* 2001; 67(1):26–32.
22. Hermansen A, Brodal G, Balvoll G. Hot water treatments of carrot seeds: effects on seed-borne fungi, germination, emergence and yield. *Seed Sci Technol.* 1999; 27(2):599–613.
23. Masum M, Islam S, Fakir M. Effect of seed treatment practices in controlling of seed-borne fungi in sorghum. *Sci Res Essays.* 2009; 4(1):22–7.
24. Rahman M, Ali M, Ali M, Rahman M, Islam M. Hot water thermal treatment for controlling seed-borne mycoflora of maize. *Int J Sustain Crop Prod.* 2008; 3(5):5–9.
25. Forsberg G, Johnsson L, Lagerholm J. Effects of aerated steam seed treatment on cereal seed-borne diseases and crop yield. *J Plant Dis Protect.* 2005; 112(3):247–56.
26. Mancini V, Romanazzi G. Seed treatments to control seedborne fungal pathogens of vegetable crops. *Pest Manag Sci.* 2014; 70(6):860–8. <https://doi.org/10.1002/ps.3693> PMID: 24293285
27. Navaratnam S, Shuttleworth D, Wallace D. The effect of aerated steam on six seed-borne pathogens. *Aust J Exp Agric.* 1980; 20(102):97–101.
28. Schmitt A, Koch E, Stephan D, Kromphardt C, Jahn M, Krauthausen H-J, et al. Evaluation of non-chemical seed treatment methods for the control of *Phoma valerianellae* on lamb's lettuce seeds. *J Plant Dis Protect.* 2009; 116(5):200–7.
29. Groot S, Birnbaum Y, Kromphardt C, Forsberg G, Rop N, Werner S. Effect of the activation of germination processes on the sensitivity of seeds towards physical sanitation treatments. *Seed Sci Technol.* 2008; 36(3):609–20.
30. Groot S, Birnbaum Y, Rop N, Jalink H, Forsberg G, Kromphardt C, et al. Effect of seed maturity on sensitivity of seeds towards physical sanitation treatments. *Seed Sci Technol.* 2006; 34(2):403–13.
31. Koch E, Schmitt A, Stephan D, Kromphardt C, Jahn M, Krauthausen H-J, et al. Evaluation of non-chemical seed treatment methods for the control of *Alternaria dauci* and *A. radicina* on carrot seeds. *Eur J Plant Pathol.* 2010; 127(1):99–112.
32. Schärer H, Schnueriger M, Hofer V, Herforth-Rahmé J, Koller M, editors. Effect of different seed treatments against seed borne diseases on corn salad. III International Symposium on Organic Greenhouse Horticulture 1164; 2016.
33. Gibbs A, Gower J. The use of a multiple-transfer method in plant virus transmission studies—some statistical points arising in the analysis of results. *Ann Appl Biol.* 1960; 48(1):75–83.
34. RCoreTeam. R: A language and environment for statistical computing V 3.1.0 Vienna, Austria: R Foundation for statistical computing; 2015. Available from: <http://www.R-project.org/>.
35. Lenth RV. Least-squares means: The R package lsmeans. *J Stat Softw* 2016; 69(1):1–33.
36. Garibaldi A, Minuto G, Bertetti D, Gullino ML. Seed transmission of *Peronospora* sp. of Basil. *J Plant Dis Protect.* 2004:465–9.
37. Pathak V, Mathur S, Neergaard P. Detection of *Peronospora manshurica* (Naum.) Syd. in seeds of soybean, *Glycine max.* *Eppo Bull.* 1978; 8(1):21–8.
38. Sauer D, Burroughs R. Disinfection of seed surfaces with sodium hypochlorite. *Phytopathology.* 1986; 76(7):745–9.

39. Mweetwa A, Welbaum G, Tay D. Effects of development, temperature, and calcium hypochlorite treatment on in vitro germinability of Phalaenopsis seeds. *Sci Hort.* 2008; 117(3):257–62.
40. Ramakrishna N, Lacey J, Smith J. Effect of surface sterilization, fumigation and gamma irradiation on the microflora and germination of barley seeds. *Int J Food Microbiol.* 1991; 13(1):47–54. [https://doi.org/10.1016/0168-1605\(91\)90135-c](https://doi.org/10.1016/0168-1605(91)90135-c) PMID: 1863528
41. Rood L, Koutoulis A, Bowman JP, Evans DE, Stanley RA, Kaur M. Control of microbes on barley grains using peroxyacetic acid and electrolysed water as antimicrobial agents. *Food Microbiol.* 2018; 76:103–9. <https://doi.org/10.1016/j.fm.2018.05.002> PMID: 30166129
42. Tango CN, Hussain MS, Oh D-H. Application of electrolyzed water on environment sterilization. In: Ding T, Oh D-H, Liu D, editors. *Electrolyzed Water in Food: Fundamentals and Applications.* Singapore: Springer; 2019. p. 177–204.
43. Fujiwara K, Fujii T, Park J-S. Comparison of foliar spray efficacy of electrolytically ozonated water and acidic electrolyzed oxidizing water for controlling powdery mildew infection on cucumber leaves. *Ozone-Sci Eng.* 2009; 31(1):10–4.
44. Buck J, Van Iersel M, Oetting R, Hung Y-C. In vitro fungicidal activity of acidic electrolyzed oxidizing water. *Plant Dis.* 2002; 86(3):278–81. <https://doi.org/10.1094/PDIS.2002.86.3.278> PMID: 30818607
45. Dewi FR, Stanley R, Powell SM, Burke CM. Application of electrolysed oxidising water as a sanitiser to extend the shelf-life of seafood products: a review. *J Food Sci Technol.* 2017; 54(5):1321–32. <https://doi.org/10.1007/s13197-017-2577-9> PMID: 28416883
46. Sharma K, Singh U, Sharma P, Kumar A, Sharma L. Seed treatments for sustainable agriculture-A review. *J Nat Appl Sci.* 2015; 7(1):521–39.
47. Agrios G. *Plant Pathology.* Fifth ed. San Diego CA: Elsevier Academic Press; 2005.
48. Mohamed-Yasseen Y, Barringer S, Splittstoesser WE, Costanza S. The role of seed coats in seed viability. *Botanical Review.* 1994; 60(4):426–39.
49. Woudenberg J, Groenewald J, Binder M, Crous P. *Alternaria* redefined. *Stud Mycol.* 2013; 75:171–212. <https://doi.org/10.3114/sim0015> PMID: 24014900
50. Bailey BA, Apel-Birkhold PC, O'Neill NR, Plaskowitz J, Alavi S, Jennings JC, et al. Evaluation of infection processes and resulting disease caused by *Dendryphion penicillatum* and *Pleospora papaveracea* on *Papaver somniferum*. *Phytopathology.* 2000; 90(7):699–709. <https://doi.org/10.1094/PHTO.2000.90.7.699> PMID: 18944488
51. O'Neill N, Jennings J, editors. *Dendryphion penicillatum*, a destructive seed-borne pathogen of *Papaver somniferum*. (Abstr.). *Phytopathology*; 1997.
52. Spitzer T, Spitzerová D, Matušinský P, Kazda J. Possibility of using seed treatment to suppress seed-borne diseases in poppy. *Plant Protect Sci.* 2014; 50(2):78–83.
53. D M. *Dendryphion penicillatum* (Corda) Fr. on opium poppy. *APPS Newsletter.* 1978; 7(1):8.
54. Khare D, Bhale MS. *Seed Technology.* Second ed. India: Scientific Publishers; 2014.
55. Taylor A, Harman G. Concepts and technologies of selected seed treatments. *Annu Rev Phytopathol.* 1990; 28(1):321–39.
56. Bennett MA, Fritz VA, Callan NW. Impact of seed treatments on crop stand establishment. *Hort Tech.* 1992; 2(3):345–9.
57. Khasa P. Scarification of limba seeds with hot water, bleach, and acid. *Tree planters' Notes.* 1992; 43(4):150–2.
58. Fist AJ, Chung B. Pyrethrum and poppy: two Tasmanian industries of world significance. In: Chung B, editor. *Acta Horticulturae.* Leuven, Belgium: International Society for Horticultural Science (ISHS); 2015. p. 85–90.
59. Bruck RI, Gooding GV Jr, Main CE. Evidence for resistance to metalaxyl in isolates of *Peronospora hyoscyami*. *Plant Disease.* 1982; 66(1):44–5. 46.
60. Crute I, Norwood J, Gordon P, editors. Resistance to phenylamide fungicides in lettuce and brassica downy mildew. *Proc. of the Mixture Centenary Meeting, Bordeaux, BCPC Monograph No 31*; 1985.
61. Pintore I, Gilardi G, Gullino ML, Garibaldi A. Detection of mefenoxam-resistant strains of *Peronospora belbahrii*, the causal agent of basil downy mildew, transmitted through infected seeds. *Phytoparasitica.* 2016; 44(4):563–9.
62. Cohen Y, Vaknin M, Ben-Naim Y, Rubin AE, Galperin M, Silverman D, et al. First Report of the occurrence and resistance to mefenoxam of *Peronospora belbahrii*, causal agent of downy mildew of basil (*Ocimum basilicum*) in Israel. *Plant Disease.* 2013; 97(5):692



Appendix 2: Tasmanian Country article

Newspaper stories

Options for poppy plight, Tasmanian Country, April 10, 2020



**TIA is a joint venture between the University of Tasmania
and the Tasmanian Government**



Options for poppy plight

CLOSE LOOK: Dharushana Thanabalasingam's research project is aiming to help the poppy industry combat systemic downy mildew.

A PhD student's research will help support the prosperity of Tasmania's poppy industry which has been battling a relatively new disease for six years.

Dharushana Thanabalasingam moved from Sri Lanka in 2018 to join the Tasmanian Institute of Agriculture and pursue a PhD to understand a disease of opiate poppy known as systemic downy mildew.

TIA is leading the scientific response to the disease since its discovery in Tasmania in 2014 threatened the viability of the industry and shook grower confidence. TIA is also working with industry to implement recommendations for best-practice disease management.

CUPPA TIA

Miss Thanabalasingam's PhD research aims to gain a greater understanding of the relative importance of inoculum sources that spread systemic downy mildew.

"My main aim is to identify the roles played by the critical inoculum sources to spread systemic downy mildew disease in opiate poppies.

"The inoculum sources include seed, soil and air and my aim is to find out which factor plays the biggest role in spreading the disease and how best to target it," she said.

"The findings of the research will inform effective management strategies. This could include advice around the most effective times to apply fungicide, further insight about crop rotations, or possible biocontrol options."

Poppy Growers Tasmania chief executive Keith Rice said a strength of TIA's research was that it was conducted in collaboration with local farmers and poppy processors.

"The Tasmanian poppy industry has its foundations anchored in the application of sophisticated science and technology to a natural plant.

"It has faced and conquered numerous challenges since its

inception 55 years ago, and this research is essential to the ongoing success of this globally important industry," he said.

In February, Miss Thanabalasingam was at TIA's Forthside research farm to harvest a trial crop of poppies. The trial included two different treatments – one section was subjected to possible infection from soil, seed and air, and another which was sprayed regularly with fungicide to reduce the possibility of infection from airborne sources.

Healthy plants were grown in greenhouse conditions using potting mix with pathogen-free seeds. She brought the healthy plants to the field

trial before each sample collection, leaving them in the field for a week to assess the impact of airborne spores without the additional impact of infected seed or soil. Disease progression in the field trial was also monitored and recorded.

"I am so excited to find out the results from this trial as it is a major part of my PhD project. I am harvesting the poppies to check for differences between the two treatments over time, including variations in yield and alkaloid content.

"This trial will help to identify whether it is seed, soil or air that plays the major role in spreading the disease."

The project has included

other experiments including greenhouse trials looking at the role of environmental conditions such as soil type, soil moisture and soil temperature in how the disease is spread via seed and soil and the potential plant-growth stage for airborne disease spread.

Her project is supervised by TIA senior research fellow Dr Jason Scott as part of an Australian Research Council funded project also supported by processors Tasmanian Alkaloids and Sun Pharmaceuticals, Poppy Growers Tasmania and the State Government.

The project is expected to run until early 2021.

WRITE-OFF
\$150,000

Terrano 3.5FX
TerraGrip tines, Rigid levelling discs, Cage roller 64cm
Carbide Mulchmix point HM Pjus
Carbide Mulchmix coultter wing



LONGFORD 24-26 Wellington St, Longford 7301 P 03 6391 2922
MONTROSE 108 Duncan Street, Montrose 7010 P 03 6273 6080
LATROBE 6 Speedway Drive, Latrobe 7307 P 03 6426 1666



CULTIVATION WITHOUT COMPROMISE

Joker 6RT Classic
FarmFlex Packer
52cm discs, Disc thickness 6mm
Maintenance free bearings
SoftRide, Two front support wheels
HP - 180 to 240hp
Adjustable drawbar hitch
5 to 8 m working width



08 TASMANIAN COUNTRY, Friday, April 10, 2020

TSCE01201MA - V1

Appendix 3: 2019 APPS Conference paper abstract

Thangavel, T., Scott, J., Krishnamoorthy, K., Singaloda, C., Jones, S., Ramakrishnan, H. and Wilson, C. 2019. Disease risk assessment of poppy downy mildew using qPCR analysis of two *Peronospora* spp. in naturally infested soil. In Proceedings of the Australasian Plant Pathology Society Conference 2019, Melbourne, Australia.

Concurrent Session 2 - Monitoring and Diagnostics 1
Tuesday 26 November 2019, 1:15 PM - 2:45 PM

Disease risk assessment of poppy downy mildew using qPCR analysis of two *Peronospora* spp. in naturally-infested soil

Dr Tamilarasan Thangavel¹, Dr Jason Scott², Mrs Krithika Krishnamoorthy¹, Miss Chiranthika Singaloda¹, Miss Suzie Jones², Miss Harshitha Sri Ramakrishnan¹, Dr Calum Wilson¹

¹Tasmanian Institute of Agriculture, 13, St. John's Avenue, New Town, Australia, ²Tasmanian Institute of Agriculture, Cradle Coast Campus, 16-20 Mooreville Road, Burnie, Australia

Poppy downy mildew, caused by *Peronospora meconopsidis* and *P. somniferi*, is one of the most devastating diseases of opium poppy and continues to be a major constraint to commercial production in Australia. A real-time polymerase chain reaction assay was developed for the quantification of the two *Peronospora* spp. in leaves, soil and in spore traps. Based on the nucleotide differences within the cytochrome oxidase regions, species-specific Taqman and locked nucleotide acid probes were designed to perform a quantitative assay for the specific detection and quantification of *P. meconopsidis* and *P. somniferi*, respectively. To normalise between samples, an exogenous internal positive control, the marine bacterium *Pseudoalteromonas prydzensis* was included with samples prior to DNA extraction, and a primer and probe set targeting the control was also developed. Specificity of the assay to detect *P. meconopsidis* and *P. somniferi* was confirmed through testing against nine other closely related *Peronospora* spp. The assay was able to reliably detect a minimum of 30 pg/ μ l and 2 ng/ μ l of DNA for *P. meconopsidis* and *P. somniferi*, respectively. The capacity of the *P. somniferi* assay to quantify pathogen levels in soil was validated by testing 95 naturally-infested field soils collected between 2015 and 2017. Pathogen detection levels were 0.00 - 5,568 ng DNA/g of soil for *P. somniferi*, with only low levels recorded for *P. meconopsidis* (<1.0 ng DNA/g of soil). Successful disease transmission was shown in seven of the field soils, through measurement of infection in seedlings grown in tested soils under controlled conditions. A threshold of 20 ng DNA/g of soil was observed for reliable transmission of *P. somniferi*. No transmission of *P. meconopsidis* was detected from these samples. Utilization of this assay will enable risk prediction of field sites prior to planting. This assay can also be deployed for monitoring of pathogen dissemination via seed and airborne conidia, through spore trapping.

Appendix 4: 2019 APPS Conference paper abstract

Thanabalasingam, D., Thangavel, T., Krishnamoorthy, K., Jones, S., Wilson, C., and Scott, J. 2019. Determining *Peronospora somniferi* genotype diversity and its potential impact on management of systemic downy mildew of opium poppy. In Proceedings of the Australasian Plant Pathology Society Conference 2019, Melbourne, Australia.

Poster Session 3 - Evolution & Diversity
Thursday 28 November 2019, 12:00 PM - 12:30 PM

Poster Board 74

Determining *Peronospora somniferi* genotype diversity and its potential impact on management of systemic downy mildew of opium poppy

Miss Dharushana Thanabalasingam¹, Dr Tamil Thangavel², Mrs Krithika Krishnamoorthy², Dr Calum Wilson², Dr Suzie Jones¹, Dr Jason Scott¹

¹Tasmanian Institute of Agriculture, University of Tasmania, Burnie, Australia, ²Tasmanian Institute of Agriculture, University of Tasmania, New Town, Australia

Opium poppy (*Papaver somniferum*) is grown for its pharmaceutically important opiates (1). Australian production comprises over 50% of the world's licit production and is primarily based in Tasmania with limited production in mainland Australia. Outbreaks of a new systemic form of downy mildew (SDM) in 2014 raised significant industry concern (1). SDM infections were characterised by stunted and deformed plant growth, often leading to early plant death. *Peronospora somniferi* was identified as the causal organism of SDM (1). The level of diversity within Australian populations of *P. somniferi* is unknown. However, recently an isolate of *P. somniferi* that caused localized lesions rather than a typical systemic infection was noted. This suggests the presence of races in the *P. somniferi* population of Australia. To address this knowledge gap, simple sequence repeat (SSR) markers were developed following construction of a whole genome assembly of a *P. somniferi* individual using Illumina paired end reads. Repeats of tri- and quad- base pair motifs were targeted for further marker development. Primers were developed for screened SSR marker regions and sanger sequencing was used to confirm target region and marker polymorphism. Polymorphic SSR markers were tested against different field *P. somniferi* individuals to identify the level of diversity present, with marker fragments sized by capillary electrophoresis. The outcomes of this study will be presented at the meeting. Studies to examine potential links between pathogen genotype and host symptom response are planned to follow.

1. Thangavel, T. et al. 2017. Plant Disease 101: 392.