THESIS

INVESTIGATING ECOLOGICAL FACTORS INVOLVED IN THE INCIDENCE AND SEVERITY OF *Puccinia punctiformis* INFECTION IN *Cirsium arvense*

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ABSTRACT

INVESTIGATING ECOLOGICAL FACTORS INVOLVED IN THE INCIDENCE AND SEVERITY OF *Puccinia punctiformis* INFECTION IN *Cirsium arvense*

The rust fungus *Puccinia punctiformis* has been proposed as a biocontrol agent against the widespread and noxious weed *Cirsium arvense* due to its specificity and ability to systemically infect and eventually kill *C. arvense*. However, the incidence of *P. punctiformis* is low in naturally infected systems. The environmental conditions of temperature and humidity to which *P. punctiformis* teliospores are exposed after production and until germination could affect their viability, and consequently, compromise the occurrence of infections. The first chapter of this thesis investigated teliospore viability for germination after exposure to different relative humidity (5 %, 22 %, 62 %, 90 % RH) and temperature levels (-20 °C, 6 °C, 23 °C) over the course of a year. Results showed that teliospore germinability decreases significantly faster when exposed to 23 °C and 62 % RH, or 90 % RH, followed by -20 °C and 5 % RH, compared to all other conditions.

Teliospore priming is a stimulation process prior to germination that has been associated with increased germination in some rust fungi (Morin et al., 1992; Bruckart & Eskandari, 2002; Fisher et al., 2009). The second chapter examined how priming *P. punctiformis* teliospores, either in water or in a 250 µl/L solution of the germination stimulator dodecyl-NCS dissolved in water, influences their germinability, as well as the incidence and severity of systemic infection in *C. arvense* plants inoculated with the primed teliospores. Results showed that priming teliospores in water, without a germination stimulator, results in significantly higher germination rates compared to priming them in the 250 µl/L dodecyl-NCS solution. Furthermore, both priming treatments

enable significantly greater germination proportions compared to not priming the teliospores. Statistically, the incidence and severity of the systemic infections caused by water-primed teliospores were not significantly different from those caused by teliospores primed in the 250 µl/L dodecyl-NCS solution.

Ultimately, our findings suggest that environmental factors such as warm-humid and cold-dry conditions, reduce teliospore germinability. Consequently, these conditions can also limit *P. punctiformis* infection in *C. arvense*. For biocontrol purposes, we recommend to collect and store teliospores avoiding these adverse conditions to maximize their viability. Furthermore, we propose using water-primed teliospores for *P. punctiformis* inoculations on *C. arvense*, as they exhibited increased germination rates and may facilitate systemic infections in both axillar and new shoots.

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CHAPTER 1:

INFLUENCE OF ENVIRONMENTAL TEMPERATURE AND HUMIDITY ON TELIOSPORE VIABILITY

1.1 Introduction

Cirsium arvense is a Eurasian Asteraceae species accidentally introduced to North America in 1600s (Guggisberg et al., 2012; Tiley, 2010). It is the third most detrimental weed in Europe (Schroeder et al., 1993), and the most widespread noxious weed in the United States and Canada (Skinner et al., 2000, USDA Plant Database, 2024). *Cirsium arvense* causes large economic losses in agricultural systems (Moyer et al., 1991; Mamolos & Kalburtji, 2001; Grekul & Bork, 2004; Bourdôt et al., 2016), and ecosystem alterations in natural forest landscapes (Pritekel et al., 2006; Humber & Hermanutz, 2011).

Wherever *C. arvense* has spread to, its host specific pathogen *Puccinia punctiformis* has also been found (Wilson, 1940; Buller, 1950; Menzies, 1953; Aliabadi & Abbasi, 2013; Savoskina et al., 2023; Bradshaw et al., 2023; Liang et al., 2024). *Puccinia punctiformis* causes a systemic infection that compromises entire *C. arvense* plant shoots, penetrates the host vascular system, and colonizes the root and new shoots (Buller, 1950; Menzies, 1953). *Puccinia punctiformis* consumes host energy and resources, alters host hormonal machinery for development (Bailis & Wilson, 1967); affects host clonal reproduction and seed formation, limits patch expansion (Berner, Smallwood, Cavin, et al., 2015; Bean et al., 2024); and leads to plant withering and eventual host death (Czechinsky, 1929).

This systemic infection is initiated by basidiospores produced from teliospore germination (Van Den Ende et al., 1987). Basidiospore infection leads to the spermogonial phase where sexual

reproduction results in uredinoid aecia. Uredinoid aeciospores spread to adjacent leaf tissue and originate uredia. As a brachy-form rust fungi, *P. punctiformis* produces teliospores in both uredinoid aecia and uredia (also referred as primary and secondary uredia, respectively, by Baka & Lösel in 1992a and 1992b). Teliospore production occurs during late spring and summer, and their amount become massive by the end of the season to start the cycle again (Jackson, 1931; Petersen, 1974). Sometimes, the uredinoid aecial phase will occur directly if the basidiospore was heterokaryotic and had both mating types, and the cycle will continue as described (Jackson, 1931; Petersen, 1974). The spermogonia-uredinoid aecia phase of *P. punctiformis* corresponds to the systemic infection observed in *C. arvense*. It is characterized by an intercellular mycelium that possesses intracellular filamentous unspecialized haustoria invading the host vascular system (Baka & Lösel, 1992a; Baka & Lösel, 1992b) and causes all the morphological and physiological alterations previously described.

The occurrence of systemic infections depends on the availability of basidiospores, whose production occurs after teliospore germination. After being produced during late summer and fall, teliospores remain in plant debris and/or become soilborne until optimal condition for germination occur (Turner et al., 1986). Teliospore germination and basidiospore formation thrive in darkness, high humidity, and temperatures between 15 °C and 21 °C, and require the presence of a germination stimulator produced by *C. arvense* (Turner et al., 1986). This germination stimulator is produced mainly by roots and, to a lesser amount, by stems (Turner et al., 1986), and is also released into the host rhizosphere (French & Lightfield, 1990). It is a volatile compound, presumably a C_{13} polyacetylene hydrocarbon, present in germinating seeds, and increases throughout seedlings growth (French et al., 1994). Therefore, the occurrence of systemic infections

is subject to the contact of teliospores with the host and environmental conditions that enable their germination (Frantzen, 1994a).

Teliospore production progressively increases until the end of the season (Buller, 1950; Menzies, 1953; Petersen, 1974). Although *P. punctiformis* teliospores disperse over several months (Buller, 1950; Petersen, 1974), the incidence of systemic infections is low (Frantzen, 1994a). In the Netherlands, *C. arvense* patches naturally infected with *P. punctiformis* had no more than 4 % of individual shoots systemically infected within the same season, increasing up to 14 % the following year (Frantzen, 1994a). In North America, *P. punctiformis* is less commonly seen in arid regions (Savile, 1970) and rarely reaches epidemic levels (Buller, 1950). Instead, systemically infected shoots are observed at low rates (for instance: 13/141 in 2022 and 2/99 in 2023, personal observation in a Fort Collins, Colorado location (40°34'39.6" N 105°06'13.1" W)).

The environmental conditions to which teliospores are exposed prior to germination may influence their viability. According to Turner et al. (1986), *P. punctiformis* teliospores stored at 4 °C for 6 months showed higher germination percentages (25.65 % ± 10.95) than those stored for 3 months (2.0 % ± 0.66); while teliospores collected later on the sporulating season (September - October) germinated in a greater percentage (36 %) compared to those collected earlier (July – August, 0 %), suggesting that longer exposure to outdoor conditions may reduce teliospore germinability. In some microcyclic (*Puccinia mesnieriana*, *P. lagenophorae*) and macrocyclic rusts (*P. hordei*, *P. graminis*, *P. avenae*, *P. recondita*, *Uromyces viennon-bourginii*, *U. scillarum*), teliospores lose germinability after having been exposed to outdoor conditions for a year, but when stored at 5 °C in dry conditions, retained viability for several years (Anikster, 1986). In *P. graminis* and *Austropuccinia psidii*, exposure to high temperatures (22 °C – 35 °C) and dry conditions (< 30 %) reduced both their ability to germinate (Roelfs, 1985) and survival time (Lana et al., 2012).

For the microcyclic chrysanthemum white rust, *P. horiana*, teliospores lose viability faster in moist soil under warm temperatures (22 °C – 26 °C) compared to cold temperatures (5 °C, -20 °C) (Bonde et al., 2013). These findings suggest that exposure to diverse temperature and humidity conditions impacts teliospore germinability.

Based on these reports, we hypothesize that the environmental conditions of temperature and humidity to which *P. punctiformis* teliospores are exposed after production and until germination influences their germinability, and consequently, affects the occurrence of infections as well. This study investigated teliospore germinability after exposure to different humidity and temperature levels during the course of one year. Understanding the effects of these conditions on teliospore germination could help explain the low occurrence of systemic infection by *P. punctiformis* in natural systems of *C. arvense*.

1.2 Material and Methods

1.2.1 Plant material

Cirsium arvense seeds were collected in 2021 from multiple individuals at one location (40°33’37.3” N 105°01’57.1” W) in Fort Collins, Colorado, and stored at 4 °C for 5 months. Before germination, the seeds were sanitized in a 2.5 % sodium hypochlorite solution with 0.1 % Tween-20 for 15 minutes and rinsed in deionized water. Sanitized seeds were then incubated in 0.001 M gibberellic acid (GA) solution (French et al., 1994) for 24 hours at 23 °C to stimulate germination.

1.2.2 Spore sample production and collection

To obtain fresh spore samples of *P. punctiformis*, ten sanitized and GA-incubated seeds of *C. arvense* were inoculated with 2 mg of spores resuspended in 1 ml of isopentane (Merck, USA), which evaporated, leaving the spores attached to the seeds (French et al., 1994). Inoculated spores

were collected from two sources: 1) Fort Collins, CO, collected in 2018 by Clark et al. (2020) and stored at 4 °C for 4 years, and 2) Grand Junction, CO, collected and shared by Karen Rosen (Biological Control Specialist, CDA) in 2021 and stored at 4 °C for 5 months. The inoculated seeds were placed in moistened general-purpose soil (PRO-MIX, Premier Tech Horticulture, USA) in 30-ml square pots of 1.0-inch depth, incubated at 18 °C in darkness for two weeks (French et al., 1994), transferred to the greenhouse (21-30 °C, ~30% relative humidity, and 12-hour photoperiod), and finally re-potted in 1-gallon pots.

In both cases, two plants per inoculation became infected at different days post-inoculation (*dpi*). Spermogonium-bearing shoots were observed at 45 and 62 *dpi* in plants inoculated with teliospores from Fort Collins, and at 47 and 81 *dpi* in those inoculated with teliospores from Grand Junction. Only the plants that developed spermogonia at 62 and 87 *dpi* started sporulating uredinoid aeciospores and/or uredospores at 90 *dpi*, and subsequently produced mostly teliospores. As at 141 *dpi*, teliospore clumps from the Fort Collins progeny were collected by shaking the spore-bearing tissue into a paper funnel, while the Grand Junction progeny required to be scraped out of the infected tissue with a scalpel. Both samples were isolated from systemically infected shoots only, and stored in sealed plates at 4 °C for three months prior to the start of the experiment. Fort Collins progeny consisted of 88 % teliospores and 12 % uredinoid aeciospores and/or uredospores, while Grand Junction progeny, had 56 % teliospores and 44 % uredinoid aeciospores and/or uredospores.

1.2.3 Spore treatments and experimental design

To determine how exposure of teliospores to different conditions of moisture and temperature prior to their germination affects their viability; teliospores were stored under 5, 22, 62, and 90 % relative humidity (RH), and at 23, 6, and -20 °C, constituting 12 different storage

treatment combinations (4 humidities x 3 temperatures). Both spore samples produced in section 1.2.2. were used as biological replicates, resulting in a total of N = 24 experimental units (2 biological replicates x 12 treatments). Each sample was homogenized by sieving it twice to break up teliospore clumps, then 1 mg of the homogenized sample was dispensed into 1.5 ml-capacity microtubes with perforated-lid, which were placed inside 760 ml-capacity sealed containers with a 50-ml jar holding either glycerol-water solutions to create relatives humidities (RH) of 22, 62, and 90 % RH, or 46 g of orange-indicator silica gel (DRY&DRY, USA) for 5% RH. Glycerol-water solutions were prepared according to Forney & Brandl's (1992) protocols. Finally, the containers were stored at 23, 6, and -20 °C. Temperature and humidity were monitored throughout the experiment using HOBO Mx1101 data loggers (ONSET, USA), and glycerol-water solutions as well as silica gel were changed as required to maintain desired humidity.

1.2.4 Teliospore germination

Teliospore germination rate was assessed prior to the exposure to any conditions (time = 0), and then after 1, 2, 3, 4, 5, 6, 9, and 13 months of exposure to the 12 treatments. In all cases, each 1-mg-exposed sample was resuspended in 1 ml cold isopentane (Sigma-Aldrich Solutions, USA), evenly pipetted in one plate of 0.8 % water agar mixed with 250 µl/L dodecyl isothiocyanate (dodecyl-NCS, from Thermo Fisher Scientific, 90 % purity, 227.41 g/mol), and incubated at 18 °C in darkness (French, 1990). A single plate was used for each 1-mg-exposed sample per biological replicate, per treatment. At 21 days post-incubation (dpi), germination data were collected from five 0.7 cm-diameter agar sections from each plate. Each agar section was stained with 20 µl of 10 % crystal violet, and visualized at 40X microscope magnification. Teliospores were considered germinated when a germ-tube was observed from either or both cells of the spore, and was longer than the teliospore (Van Den Ende et al., 1987).

Statistical analysis was performed using R software, version 4.3.2 (R Core Team, 2023). The response variable was the proportion of teliospores that germinated, with each agar section examined used as the statistical unit for analysis. The 'glmmTMB' package (Brooks et al., 2017) was used to build a logit model using a beta-binomial distribution of teliospore germination as a function of months after exposure (continuous variable), temperature, and relative humidity (categorical variables) and all possible interactions. Source of original spores (Fort Collins, Grand junction) was included as additional categorical factor. Plates, counts nested within plate, and assay date (months as categorical variable) were included as random factors. Residuals diagnostics of model were performed using the 'DHARMa' package (Hartig, 2022). Factors significance was determined through the analysis of deviance using Type III Wald chi-square test executed in the 'car' package (Fox & Weisberg, 2019) with p-values less than 0.05 considered significant. Differences between humidity slopes within temperatures were tested using the 'emmeans' package (Lenth, 2024).

1.3 Results

The teliospore germination model (Appendix 1) exhibited a distribution of observed versus simulated residuals that confirmed normality and good model fit (Appendix 2, A and B), with no significant overdispersion of residuals (Appendix 2, C and D). A significant three-way interaction between humidity, temperature, and time (p value < 0.001, Table 1) suggests that teliospore germination was influenced by the interaction of these factors.

The highest teliospore germination rate occurred at time zero and was around 0.65 for all treatments. With the exception of the -20 °C, 90 % RH treatment, germination decreased through time in all treatments (Figure 1, Appendix 3). At -20 °C, there was no significant difference among

the three highest humidity treatments (22 %, 62 %, 90 %). At 6 °C, no significant differences were detected among RH treatments, and averaged germination dropped to 0.35 – 0.50 by the 13th month. At 23 °C and 90 % and 62 % RH, teliospore germination declined earlier and faster than in all the other treatments, dropping below 0.1 by the 5th and 6th month of exposure, respectively, with no germination thereafter. At 22 % RH and 5 % RH, germination decreased to 0.16 and 0.41 by the 13th month, respectively (Figure 1).

Table 1: Analysis of deviance using Type III Wald chi-square test for teliospore germination model, where the three-way interaction between temperature, humidity, and time was significant (***).

Analysis of Deviance Table (Type III Wald chi-square test)				
Response: Teliospore_germination_proportion				
	Chisq	Df	Pr(>Chisq)	Significance
(Intercept)	7.3849	1	0.0065775	**
Temperature	10.0472	2	0.0065807	**
Humidity	6.7167	3	0.0814960	.
Month	26.6363	1	2.456e-07	***
Spore_source	0.4773	1	0.4896487	
Temperature:Humidity	3.0319	6	0.8048267	
Temperature:Month	3.5539	2	0.1691491	
Humidity:Month	16.3551	3	0.0009589	***
Temperature:Humidity:Month	165.3260	6	< 2.2e-16	***

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1				

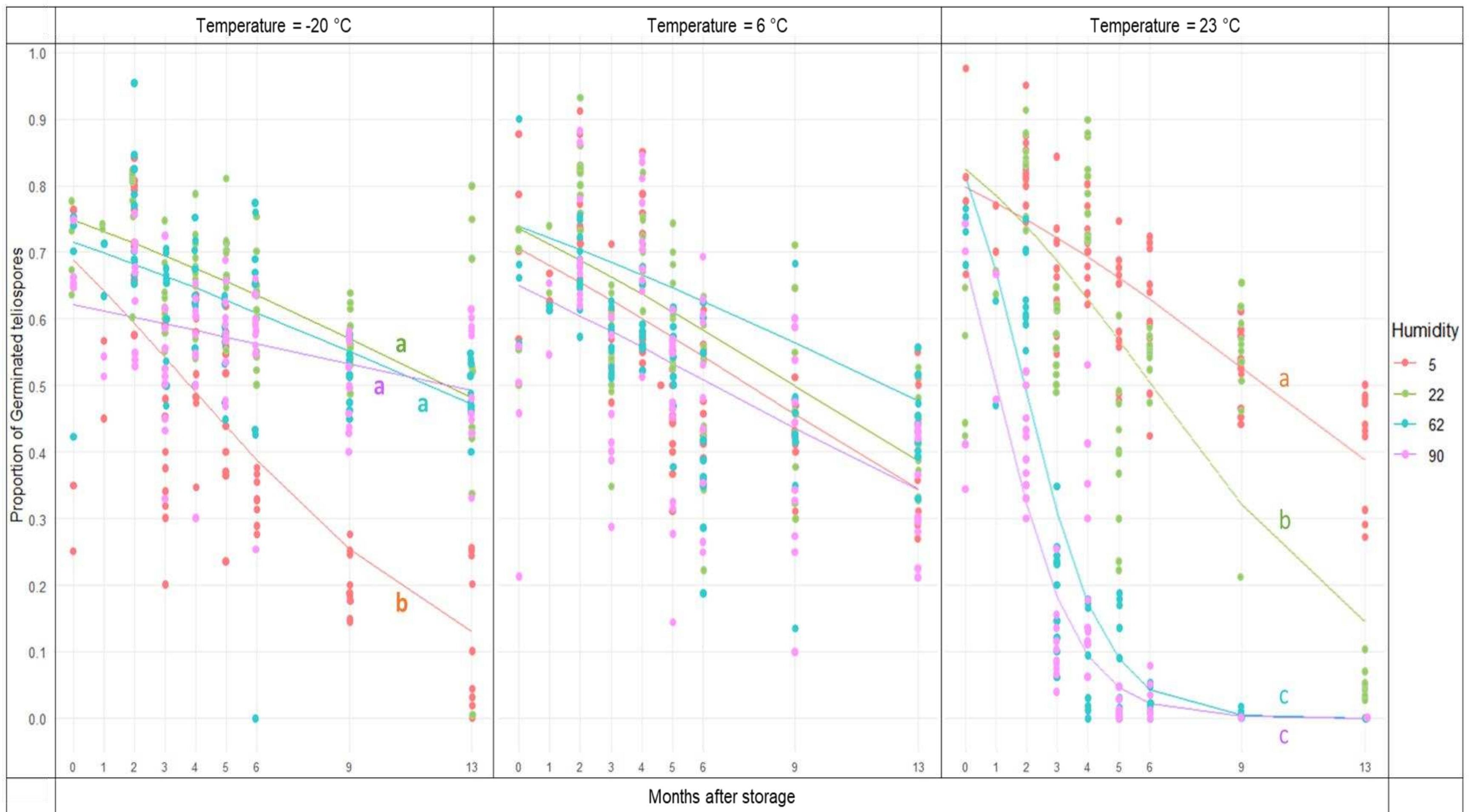

Figure 1: Proportion of teliospore germination as affected by length of exposure to different humidities and temperatures, where germination reduced to zero after 13 months at 23 °C and 62 % and 90 % humidity. Slopes represent modeled germination while dots represent the raw data. Letters (a, b, c) show the significant differences within slopes of the same temperature with p-values less than 0.05 considered significant.

1.4 Discussion

Our results support the hypothesis that the temperature and humidity to which *P. punctiformis* teliospores are exposed until their germination affect their viability. At -20 °C, teliospore viability decreased significantly more under low humidity (5 %) compared to higher humidity levels (22 %, 62 %, 90 %), indicating a positive influence of humidity on teliospore survival at low temperatures. Bonde et al. (2013) reported similar effects for the microcyclic rust fungus *P. horiana* at -20 °C, where teliospores remained viable for longer periods under moist soil (112 days) compared to dry soil (< 60 days). They also described that at around 60 days, teliospores in moist soil germinated at a greater rate (50 %) compared to those in dry soil surfaces (20 %).

At 6 °C, there was no significant difference in the decrease of viability across different humidity conditions. Teliospores stored at 6 °C for 6 months did not show higher germination rates than those stored for 3 months as previously observed by Turner et al. (1986) at 4 °C. These results suggest that *P. punctiformis* teliospores can retain viability at 5 °C, which is also the case for several other microcyclic (*P. mesnieriana*, *P. lagenophorae*) and macrocyclic (*P. hordei*, *P. graminis*, *P. avenae*, *P. recondita*, *Uromyces viennon-bourginii*, *U. scillarum*) species (Anikster, 1986). However, the dry conditions that those species required to retain higher viability do not seem necessary for *P. punctiformis* since there was no significant difference in viability across relative humidity levels at 6°C. Similarly, Bonde et al. (2013) observed that *P. horiana* teliospores at 5 °C survived longer (77 days) in damp soil compared to dry soil (< 60 days).

At room temperature (23 °C), germinability was greater earlier in the experiment, similar to the observations of Turner et al. (1986), where teliospores exposed for shorter periods to outdoor conditions germinated at a greater percentage (36 %) compared to those exposed for longer (0 %).

However, we observed that the loss of teliospore viability occurred earlier and faster under the higher relative humidity levels. This observation suggests a negative influence of humidity on teliospore survival at 23 °C, which concurs with Menzies (1953) who reported a 1 % germination rate after storing teliospores at 5 °C and then keeping them moistened at room temperature for 6 weeks. Similar effects of humidity have been reported for *A. psidii*, which exhibited longer survival time (maximum of 90 days) at lower temperatures (15 °C) and relative humidity levels (35 %, 55 %) compared to higher temperatures (25 °C – 35 °C) and relative humidity levels (70 %, 85 %), where viability declined to zero earlier (20 – 40 days) (Lana et al., 2012). Conversely, Bonde et al. (2013) reported that *P. horiana* teliospores remained viable for longer periods in moist soil (28 days) compared to dry soil (7 days) between 22 °C and 26 °C. Additionally, the greater survival rate of *P. punctiformis* under low humidity levels at 23 °C contrasts with the reduced viability reported for *P. graminis* under dry conditions (Roelfs, 1985). Overall, these observations indicate that humidity plays a variable role in the survival of teliospores.

Higher viability was observed at -20 °C and 6 °C, which is consistent with the assumption that teliospores are overwintering structures, as noted by Buller (1950), Menzies (1953), and Turner et al. (1986). This suggests that the overwintering process enables the survival of *P. punctiformis* teliospores. However, since teliospore germination rate prior to exposure to any treatments was 0.65, and significantly declined over time on eleven of the twelve treatments; overwintering is not necessary for *P. punctiformis* to achieve germination as referred by Buller (1950) and Menzies (1953). Furthermore, cold storage may not increase teliospore germination in our experiments, as referred Turner et al. (1986).

1.5 Conclusion

We found that relative humidity plays a key role in the survival of *P. punctiformis* teliospores. Our results also suggest that *P. punctiformis* teliospores are capable of overwintering but this process may not increase teliospore germination.

For biocontrol purposes, we recommend the collection of teliospores from systemically infected shoots before rainy conditions as Turner et al. (1986) also suggested. In the case of teliospores from localized infections, their collection at the end of the fall or beginning of winter will ensure higher viability by avoiding low humidity levels.

According to our results, storage of teliospores at room temperatures around 23 °C or above are not recommended for *P. punctiformis* particularly in humid environments. Storing at 6 °C could be useful for short-term periods, and keeping at -20 °C with some source of humidity could work for longer periods but no longer than a year since *P. punctiformis* teliospores lose viability over time, regardless of the temperature and humidity conditions.

CHAPTER 2:

TELIOSPORE PRIMING EFFECTS IN GERMINATION AND PLANT INFECTION

2.1 Introduction

The noxious Eurasian weed *Cirsium arvense* has spread widely across the United States since its accidental introduction to North America in the 1600s (Guggisberg et al., 2012; Tiley, 2010). *Cirsium arvense* successfully spreads by producing 1,500 to 5,000 airborne seeds per plant for long distance dispersion (Amor & Harris, 1974), while its extensive perennial roots (up to 6 - 15 ft deep and wide) generate additional stems and maintain dense colonies (Thomsen et al., 2013). *Cirsium arvense* colonies compete for water, nutrients, and light, and displace crops and native species by allelopathy (Stachon & Zimdahl, 1980).

Managing *C. arvense* poses significant challenges in both organic and conventional systems. In organic systems, cultural practices such as cover crops, tillage, and mowing are not effective (Lukashyk et al., 2008; Bicksler & Masiunas, 2009; Gruber & Claupein, 2009; Wedryk & Cardina, 2012). Instead, these practices may promote vegetative extension of *C. arvense* colonies due to the regenerative nature of the roots (Thomsen et al., 2013). In conventional systems, populations of *C. arvense* have also developed herbicide resistance (Solymosi et al., 1987 cited by Zargar et al., 2019; Hodgson, 1970; Schroeder et al., 1993; Solymosi & Nagy, 1998 cited by Szigeti & Lehoczki, 2003; Fogelfors & Lundkvist, 2008; Zargar et al., 2019). Although the aboveground portion can be reduced by herbivore biological agents (Bourdôt et al., 2024; Katovich et al., 2023; Cripps et al., 2019; Moore, 1975), this damage is not sufficient to control the thistle population since the root system persists until next season to sprout new healthy shoots and forms abundant patches again.

Utilizing microorganisms known to be pathogenic against *C. arvense* represents an additional strategy for the weed management (TeBeest, 1991). Several pathogenic fungi such as *Albugo tragoponis, Erysiphe cichoraceum, Fusarium, Ophiobolus porphyrogonus, Phyllosticta cirsii, Puccinia cirsii, Puccinia punctiformis, Pyrenochaeta erisiphoides, Sclerotonia sclerotorum, S. rolfsii*, and *Septoria cirsii* affect *C. arvense* in North America (Conners, 1967; USDA, 1960). However, with the exception of *P. punctiformis*, the wide host range of these pathogens presents risks to native plants as well as crops (Müller & Nentwig, 2011).

The biocontrol potential of *P. punctiformis* against *C. arvense* relies on its autoecious, brachy-type, and biotrophic nature (Jackson, 1931; Buller, 1950; Menzies, 1953; Petersen, 1974). Being autoecious and host specific, all life stages of *P. punctiformis* occur in *C. arvense* exclusively, posing no threat to crops and native plants (Buller, 1950). As a brachy-type basidiomycete (Jackson, 1931; Petersen, 1974), teliospores are increasingly produced in both systemic uredinoid aecia and localized uredia stages soon after sporulation begins (also called primary and secondary uredia, respectively, by Baka & Lösel in 1992a and 1992b). Consequently, systemic uredinoid aecia and localized uredia serve as primary and secondary sources of inoculum for systemic infection over several months and until the end of the growing season (Frantzen, 1994a). As a biotroph, the spermogonial – uredinoid aecial stage is characterized by systemic intercellular mycelium with intracellular haustoria extending through the parenchymal and vascular tissue (Bailiss & Wilson, 1967; Baka & Lösel, 1992a), draining the host of energy and resources (Bailiss & Wilson, 1967) and leading to a generalized biomass reduction (Clark et al., 2020; Chichinsky et al., 2023). Systemically infected shoots become etiolated, chlorotic, floppy, and brittle, with deformed apical shoots and reduced or absent floral buds that dry out before seed formation (Plowright, 1889, cited by Buller, 1950; Halsted 1894, cited by Wilson, 1969;

Czechinsky, 1929), thus limiting the host long-distance spread. Infected roots produce new infected shoots that wilt and eventually die, thereby limiting *C. arvense* colonies expansion (Czechinsky, 1929; Berner, Smallwood, Cavin, et al., 2015; Bean et al., 2024).

The dynamics of teliospore production, dispersion, and longevity influence the incidence of systemic infection caused by *P. punctiformis* in natural *C. arvense* colonies (Frantzen, 1994a), as teliospores are necessary for the production of basidiospores that initiate systemic infections (Van Den Ende et al., 1987). The gradual increase in the production of teliospores in both systemic uredinoid aecia and localized uredia (Jackson, 1931; Petersen, 1974) suggests that teliospores disperse over several months, increasing the likelihood of contact with susceptible host root buds (Frantzen, 1994a). However, before the interaction with the root buds, teliospores may become soilborne (Turner et al., 1986) or cling to the leaves (Petersen, 1974). Thereby, teliospores are subjected to hot and humid summers (23° C or above, and 60 % humidity or higher) and dry winters (freezing temperatures and less than 20 % humidity) that gradually reduce teliospore viability over time, as described in the previous chapter. This exposition may reduce the probability of interaction between viable teliospores and susceptible root buds, as suggested by Frantzen (1994a), consequently leading to a natural low occurrence of systemic infections in *C. arvense* colonies.

When employing *P. punctiformis* as a biocontrol agent against *C. arvense*, researchers strive to overcome the previously described challenges by collecting naturally produced teliospores and artificially spreading them under favorable environmental conditions to initiate infection during the fall, and infected shoots appear the following spring. First, teliospores are collected in naturally infected patches, either from systemic (Buller, 1950; Menzies, 1953; Turner et al., 1986; French & Lightfield, 1990; Baka & Lösel, 1992; Clark et al., 2020) or localized

infections (Berner et al., 2013; Berner, Smallwood, Vanrenterghem, et al., 2015; Bean et al., 2024). Then, these teliospore-bearing leaves are air-dried at room temperature and are placed in cold-storage or frozen until inoculation. Finally, the crown of young rosettes is repeatedly inoculated 2, 4, 6, or even 8 times every 3 - 4 days, using teliospore-water suspensions (0.5 ml of 1 or 2 x 10^6 teliospores/ml) or ground telia-bearing leaves (0.5 – 1 g per rosette) (Berner et al., 2013; Berner, Smallwood, Vanrenterghem, et al., 2015; Bean et al., 2024).

Despite these efforts, the resulting incidence of systemic infections in the following spring remains below one-third of the inoculated plants (Berner et al., 2013; Berner, Smallwood, Cavin, et al., 2015), or field sites (Bean et al., 2024). These low levels of systemic infection suggest that *P. punctiformis* demands further manipulation to increase the infection of the host. Given that temperature and humidity can impact the germinability of *P. punctiformis* teliospores (Chapter 1), other factors may stimulate their germination, and their manipulation could enhance systemic infections and resulting biocontrol efforts.

Teliospore priming is a process to stimulate teliospores prior to germination, and water incubation has been the predominant priming method used across different rust fungi. Priming conditions and effects have been primarily studied in teliospores of major macrocyclic and microcyclic rust crop pathogens. For instance, teliospore submersion in water increased the germination of the safflower rust *Puccinia carthami* (Klisiewicz, 1977). Similarly, water pre-soaking and water floating have been reported to ensure teliospore germination of *P. hordei*, *P. recondita*, *P. graminis* f. sp. *avenae*, *P. lagenophorea*, *Uromyces scillarum*, and *U. s. viennon-bourginii* (Anikster, 1986). With potential biocontrol agents, teliospore priming studies are limited. Incubation in high relative humidity and darkness enhanced both teliospore germination and basidiospore formation of the Noogoora burr rust, *P. xanthii* (Morin et al., 1992), and water

soaking under cold temperatures increased the germination rates of the yellow star thistle rust *P. jaceae* var. *solstitialis* (Bruckart & Eskandari, 2002; Fisher et al., 2009).

For *P. punctiformis*, anecdotal evidence suggests that floating teliospores in water (Buller, 1950) or keeping them moist (Menzies, 1953) could enable germination and seedling infection. However, further research indicates that exposing teliospores to stimulatory compounds produced by *C. arvense* is also necessary for germination and infections. According to Turner et al. (1986) and Van Den Ende et al. (1987), teliospores flooded in aqueous root extracts had greater germination rates compared to those flooded in aqueous stem extracts, while no germination was observed in leaf extracts or water alone. Similarly, root bud inoculations with teliospores previously stimulated by the vapor phase of the *C. arvense* root extract resulted in a greater incidence of systemic infections compared to non-stimulated teliospores (French & Lightfield, 1990). These host compounds are primarily found in the root (Turner et al., 1986; Van Den Ende et al., 1987) and secondarily in the stem (Turner et al., 1986), consisting of C_{13} polyacetylene hydrocarbons produced since the seed germination (Binder & French, 1994; French, Nester, & Binder, 1994) and even released to the rhizosphere (French & Lightfield, 1990). These reports suggest that the synergy of high humidity conditions and host compounds in the field facilitates teliospore germination and host infection. Ultimately, the synthetic volatile compound dodecyl-isothiocyanate (dodecyl-NCS, or C_{12}-NCS) has been reported to emulate the germination stimulating activity of *C. arvense* root extract (French, 1990).

Based on the studies described above, we hypothesize that priming *P. punctiformis* teliospores by incubating them either in water or in water containing the germination stimulator dodecyl-NCS will increase germination rates and infection occurrence. Our objectives were to determine: 1) which priming solution, water or water with dodecyl-NCS, promotes greater

germination rates compared to non-primed teliospores; 2) if the presence of the germination stimulator is required after teliospore priming to achieve germination; 3) whether primed teliospores remain viable to germinate and infect after freezing; and 4) which priming treatment enables greater incidence and severity of plant infection. Understanding the effects of teliospore priming on germination and infection occurrence will contribute to optimizing the employment of *P. punctiformis* as a biocontrol agent against *C. arvense*.

2.2 Material and Methods

2.2.1 Plant material

C. arvense plants were obtained from seeds collected from multiple locations in Fort Collins, Colorado, during 2020, 2021, and 2022 and stored at 4 °C. Prior to germination, seeds were sanitized in a 2.5% sodium hypochlorite solution with 0.1% tween-20 for 15 minutes and rinsed in deionized water. Sanitized seeds were incubated in 0.001 M gibberellic acid (GA) solution for 24 hours (French et al., 1994) at 23 °C, and placed on a moistened paper towel in a sealed plate at 23 °C and 16-hour photoperiod to germinate (adapted from Van Den Ende et al., 1987). Emerging seedlings with fully expanded cotyledons were transferred to wetted general-purpose soil (PRO-MIX, Premier Tech Horticulture, USA) mixed with ¾ tbsp of Osmocote Plus 15-9-12 slow-release fertilizer (ICL Specialty Fertilizers, USA) in 2.5-inch-deep square pots, and grown in the greenhouse at 21 °C – 30 °C, ~ 30 % relative humidity, and 12-hour photoperiod until they became two-month-old rosettes.

2.2.2 Spore sample production and collection

To obtain a spore sample of *P. punctiformis*, 2 mg of spores collected in Grand Junction, Colorado in September 2021, and stored at 4 °C for 5 months were resuspended in 1 ml of

isopentane (Merck, USA). This isopentane-spore suspension was applied to ten sanitized and GA-incubated seeds of *C. arvense*, and the spores remained attached to the seeds after isopentane evaporated completely (French et al., 1994). The inoculated seeds were placed in moistened general-purpose soil (PRO-MIX, Premier Tech Horticulture, USA) in 1.0-inch-deep square pots, incubated at 18 °C in darkness for two weeks (French et al., 1994), and transferred to the greenhouse between 21 °C and 30 °C, around 30 % relative humidity, and 12-hour photoperiod. This resulted in two infected plants exhibiting spermogonium-bearing shoots at 47- and 87-days post-inoculation (dpi), and only the shoots of the 87-dpi-infected plant bore uredinoid aeciospores/uredospores around 90 dpi and progressively produced mostly teliospores. The teliospore-bearing tissue was collected at 141 dpi and stored at 4°C for three months. To separate the teliospores, the tissue was vigorously washed in 0.01 % tween-20 solution, and the resulting spore suspension was filtered through gauze followed by grade 1 filter paper (Whatman, Cytiva, Fisher Scientific) where teliospores deposited, and dried overnight at room temperature. Finally, the collected teliospores were stored on the folded filter paper inside of a sealed petri dish, at 4 °C until the beginning of the priming treatment. The spore sample was 81 % teliospores and 19 % uredinoid aeciospores/uredospores, and it was used in both trials as specified in section 2.2.3.

2.2.3 Teliospore priming treatments and experimental design

To ascertain if priming teliospores increases germination rates compared to non-primed teliospores, 1 mg of the spore sample was incubated for two weeks at 18 °C under darkness in a 2-ml solution of either sterile deionized water (water priming treatment), or 250ul/L dodecyl-NCS (Thermo Fisher Scientific, 90 % purity, 227.41 g/mol) dissolved in deionized water (dodecyl priming treatment).

To determine if primed teliospores retain their ability to germinate and infect after freezing at -20 °C, glycerol was added as a cryoprotectant at a 10 % final concentration (v/v) to half of both 14-day incubated samples of both water or dodecyl priming treatments. Prior to placing them in the freezer, teliospores were cooled at 4 °C for 24 hours and then placed at -20 °C for 3 days. Frozen primed teliospores were then thawed at 4 °C for 24 hours. Each of the four resulting treatments (water priming treatment, water priming treatment and freezing, dodecyl priming treatment, and dodecyl priming treatment and freezing) had five 2-ml repetitions, and the experiment was replicated in two trials. The first trial was started on March 15th, 2023, while the second began on May 24th, 2023.

To allow for comparisons with non-primed teliospores in both trials, 1 mg of teliospores was not subjected to any of the priming treatments described above. Instead, these teliospores were taken directly from the sample stored on folded filter paper inside a sealed petri dish at 4 °C, as specified in section 2.2.2, and immediately used in the germination assessment.

2.2.4 Teliospore germination assessment

To determine the effects of the teliospore priming treatments, germination was measured for non-primed, primed, and primed-and-frozen teliospores. First, the germination of non-primed teliospores was measured by resuspending 1 mg of spore sample previously collected on filter paper (section 2.2.2) in 1 ml cold isopentane to plate it on 0.8 % water agar mixed with 250 µl/L dodecyl-NCS (dodecyl agar) as germination media (French, 1990). Two plates were used as repetitions. Second, teliospore germination of the four resulting treatments (water priming, water priming with freezing, dodecyl priming, and dodecyl priming with freezing) was measured by pipetting one 10-µl drop of each of the five 2-ml treatment repetitions in a plate of 0.8 % water agar mixed with 250 µl/L dodecyl-NCS using 5 plates as repetitions. In both cases, teliospores

were also plated onto 0.8 % water agar without the germination stimulator (water agar) to measure germination rates in the absence of the germination stimulator and assess whether primed teliospores still require the germination stimulator to germinate.

All plates were incubated at 18 °C in darkness (French, 1990). At 0,1,7, and 14 days after incubation (*dai*), germination data was collected from five 0.7 cm-diameter sections per plate of germination media of non-primed teliospores, and from the one 0.7 cm-diameter section of germination media with the 10-µl drop per plate of each of the five replicates of primed teliospores. Each agar section was stained with 20 µl of 10 % crystal violet and visualized at 40X microscope magnification. Teliospores were considered germinated when at least the germ-tube was observed from either or both cells (Van Den Ende et al., 1987). For statistical comparison across treatments, only teliospore germination data from 14 *dai* were analyzed, as at this time point all non-primed, primed, and primed-and-frozen treatments yielded their greatest percentages.

Statistical analysis was performed using R software, version 4.3.2 (R Core Team, 2023). The 'glmmTMB' package (Brooks et al., 2017) was used to build a logit model with a beta-binomial distribution where teliospore germination proportion per agar section was a function of three categorical variables: treatment (not priming, water priming, water priming with freezing, dodecyl priming, and dodecyl priming with freezing), germination media, experiment trial, and their interactions. Residuals diagnostics of the model were performed using the 'DHARMa' package (Hartig, 2022). Factor significance was determined through the analysis of deviance using Type III Wald chi-square test executed in the 'car' package (Fox & Weisberg, 2019) with p-values less than 0.05 considered significant. Differences between the teliospore germination means were tested using the 'emmeans' package (Lenth, 2024).

2.2.5 Infection occurrence assay

To determine which priming treatment enables greater incidence and severity of plant infection, each 2-ml priming replicate was inoculated with a spore suspension of 3000 teliospores per ml (50 ml total volume). Five 2-month-old rosettes were inoculated per treatment per rep, and 25 plants were inoculated per treatment for each trial. Each rosette was immersed into 10 ml of inoculum until wet, and the remaining inoculum was poured onto the plant soil evenly. Additionally, a set of 25 rosettes were inoculated with sterile deionized water as a negative control. All inoculated rosettes were incubated (MIR-554-PE Cooled Incubator, from SANYO Electric) at 18 °C and 100 % relative humidity in darkness for 14 days. To ensure 100 % humidity, all plants were bagged (Morin et al., 1992; Van Den Ende et al., 1987) in their trays before been placed inside the incubator. Then, plants were moved to the greenhouse (21-30 °C, 30 % relative humidity, 12-hour photoperiod), plastic bags were removed, and plants were randomly interspersed in different trays.

Plant infection data was collected for each plant at 2, 3, 4, 5, 6, 8, 12 and 16 weeks after inoculation (*wai*). Plant infections were divided in two main categories: 1) Localized infections that manifest as foliar yellowing of ~ 5 mm-diameter (Buller, 1950), with spermogonial growth in one or both sides of the leaf or midrib, that may or not sporulate later (French & Lightfield, 1990) (Figure 2, A – K); and 2) Systemic infections: Spermogonial or direct uredinoid-aecial phase committing the leaves, stem, and eventual axillar ramets of a shoot. This shoot could be a new secondary shoot different from the initially inoculated shoot but grown from the same root system (Buller, 1950; Menzies, 1953; French & Lightfield, 1990; Van Den Ende et al., 1987) (Figure 2, L – N); or an axillar shoot grown in the initially inoculated shoot (French & Lightfield, 1990; Van Den Ende et al., 1987) (Figure 2, M).

The incidence and severity of both localized and systemic infections for each priming treatment were analyzed at the time point of its highest occurrence: 6 *wai* for localized infections and 16 *wai* for systemic infections. The occurrence (1) or no occurrence (0) of the infection in each plant was the response variable, and plant was the statistical unit of replication to determine the incidence of localized or foliar infections. The proportion of shoots (new and axillar) infected per plant was the response variable to determine the severity of systemic infections, while the proportion of leaves presenting local infections was the statistical unit to determine severity of systemic infections.

Statistical analysis of the incidence and severity of systemic and localized infections were performed using R software, version 4.3.2 (R Core Team, 2023). The 'glmmTMB' package (Brooks et al., 2017) was used to build a logit model for incidence or severity for both localized and systemic infections separately, using a beta-binomial distribution of each as a function of the priming treatment, experiment trial (categorical variables), and their interactions. In each of the four models, the priming treatment repetition (5 tubes) was included as random factor. Residuals diagnostics of models were performed using the 'DHARMa' package (Hartig, 2022). Factor significance was determined through the analysis of deviance using Type III Wald chi-square test executed in the 'car' package (Fox & Weisberg, 2019) with p-values less than 0.05 considered significant. Differences among incidence and severity means for localized and systemic infections were tested using the 'emmeans' package (Lenth, 2024).

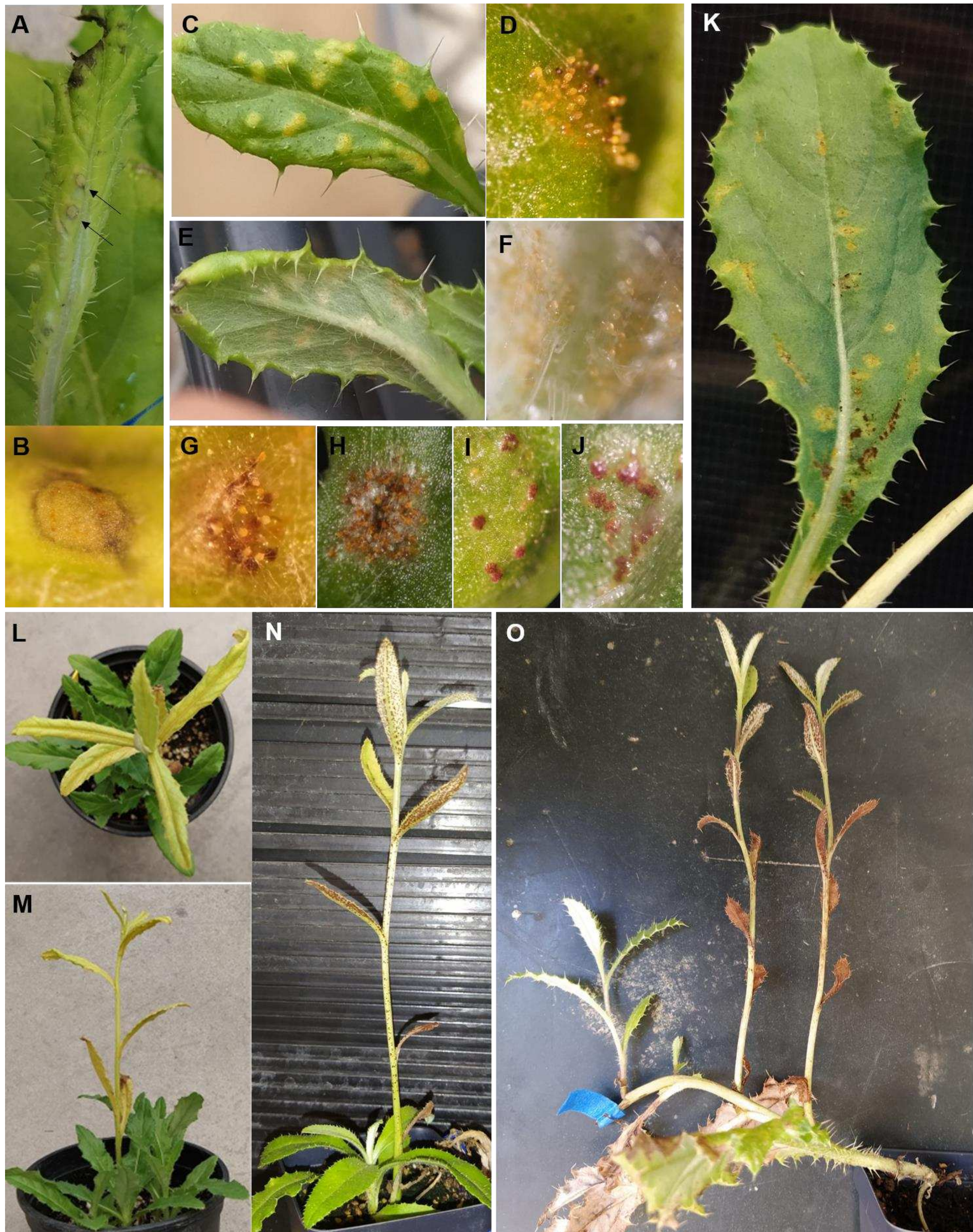

Figure 2: Types of infections caused by basidiospores in C. arvense: Localized infections at 2 wai (A, B) with spermogonia development on the adaxial (C, D) and abaxial leaf surfaces (E, F) at 3 wai. Localized lesions dry out (G, H) or sporulate (I, J, K). Systemic infection of new shoots with spermogonia (L, M), or uredinoid aecia (N), also in axillar shoots (O).

2.3 Results

2.3.1 Teliospore germination assessment

The teliospore germination model for non-primed, primed, and primed-and-frozen teliospores (Appendix 4), exhibited a distribution of observed versus simulated residuals that confirmed normality and good model fit (Appendix 5). A significant two-way interaction was detected between priming treatment and germination media (p value < 0.001, table 2).

At 14 *dai*, the germination proportion of non-primed, water-primed, dodecyl-primed, water-primed-and-frozen, and dodecyl-primed-and-frozen teliospores on dodecyl agar was significantly greater than on water agar (Figure 3). Non-primed teliospores exclusively germinated on dodecyl agar (0.15). Similarly, the germination rates of water-primed (0.01) and water-primed-and-frozen teliospores (0.01) on water agar were significantly lower compared to their germination on dodecyl agar (0.88 for water priming, and 0.82 for water priming and freezing treatment). The germination rates of dodecyl-primed teliospores (0.23) as well as dodecyl-primed-and-frozen teliospores (0.19) on water agar were greater compared to non-primed, water-primed, and water-primed-and-frozen teliospores. However, their germination rates on dodecyl agar were greater than on water agar: 0.63 for dodecyl priming, and 0.58 for dodecyl priming and freezing treatment.

Water-primed and dodecyl-primed teliospores yielded significantly greater germination rates compared to non-primed teliospores when germination occurred on dodecyl agar, with water priming treatment exhibiting the greatest germination rates. On water agar, only the germination rates of dodecyl-primed teliospores were significantly greater than not primed teliospores. The same pattern was observed for both water-priming-and-freezing and dodecyl-priming-and-freezing treatments (Figure 3).

Finally, except for the water-priming treatment yielding significantly greater germination rates than water-priming-and-freezing on dodecyl agar, the germination rates of the water-priming-and-freezing and dodecyl-priming-and-freezing treatments were not statistically different compared to water-priming and dodecyl-priming treatments, respectively, on both dodecyl agar and water agar. Although the germination rate of water-priming-and-freezing treatment was significantly lower compared to water-priming on dodecyl agar, it was still statistically greater than the germination in both dodecyl-priming and dodecyl-priming-and-freezing treatments (Figure 3).

Table 2: Analysis of deviance using Type III Wald chi-square test for the model of germination of non-primed, primed, and primed-and-frozen teliospores at 14 *dai*.

Analysis of Deviance Table (Type III Wald chi-square test)				
Response: bin_y_all				
	Chisq	Df	Pr(>Chisq)	Significance
(Intercept)	32.2267	1	1.372e-08	***
Priming treatment	57.4286	4	1.006e-11	***
Germination media	27.4255	1	1.633e-07	***
Experimental trial	0.1188	1	0.7304	
Priming treatment:Germination media	81.7004	4	< 2.2e-16	***
Priming treatment:Experiment trial	4.1138	4	0.3908	
Germination media:Experiment trial	2.4152	1	0.1202	
Priming treatment:Germination media: Experiment trial	0.1325	4	0.9979	

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1				

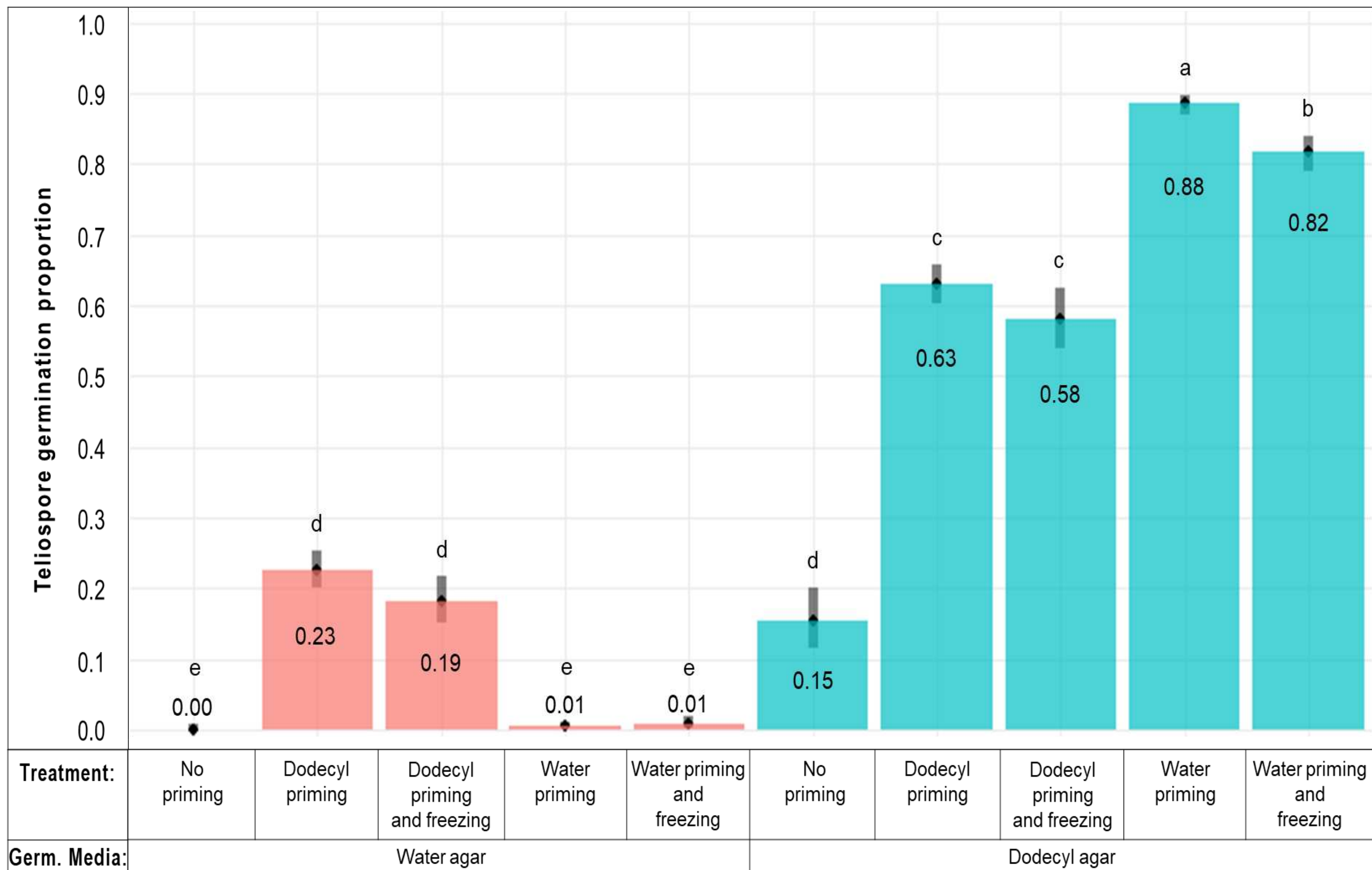

Figure 3: Germination of non-primed, primed, and primed-and-frozen teliospores as affected by germination media (Germ. media) at 14 dai. Confidence intervals are display as gray bars. Letters represent the significant differences within means, at a 0.95 confidence level.

2.3.2 Infection occurrence assay

Water inoculation used as negative control treatment showed no localized or systemic infections, indicating no evidence of cross-contamination between treatments during the experiment. Localized infections were observed at 3 weeks after inoculation (*wai*) in all treatment of both trials, and their highest incidence was at 6 *wai*; declining through time as older leaves abscised and new foliage developed (Figure 4A). Systemic infections first appeared at 3 *wai* in plants inoculated with water-primed teliospores of both trials, and in the dodecyl priming treatment of the second trial. At 5 *wai*, all treatments presented systemic infections that increased as axillar and new shoots developed. The highest incidence of systemic infection was at 16 *wai* (Figure 4B).

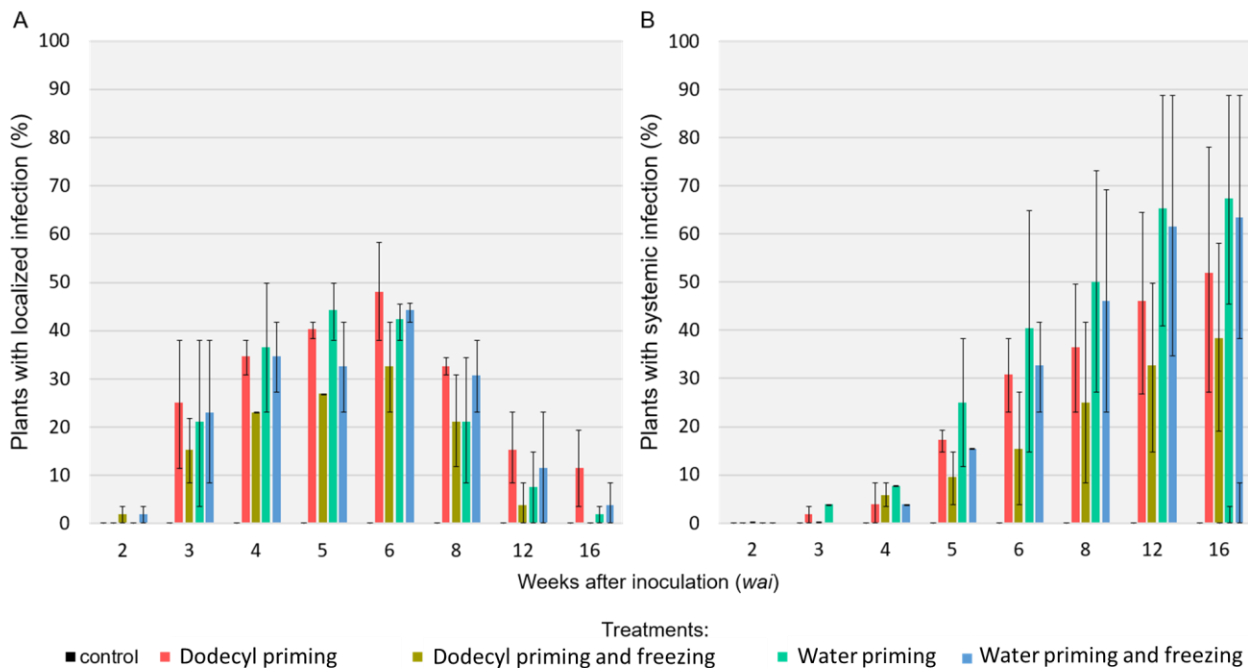

Figure 4: Incidence of localized (A) and systemic infections (B) from 2 to 16 weeks after inoculation (wai) with primed and primed-and-frozen teliospores, with incidence averaged across trials (Incidence per trials in Appendices 6 and 7). Error bars at the 95 % confidence interval.

The distribution of observed versus simulated residuals confirmed normality and good models fit for both incidence (Appendix 9) and severity (Appendix 11) of localized infection

models at 6 *wai* (Appendices 8 and 10, respectively), as well as for incidence (Appendix 13) and severity (Appendix 15) of systemic infections at 16 *wai* (Appendices 12 and 14, respectively). There was no significant interaction between priming treatment and experimental trial in any of these models (Tables 3, 4, 5 and 6).

Since trial was not a significant factor in the incidence and severity models of localized infections ($p > 0.1$, Tables 3 and 4), the infection rates were plotted in function on the priming treatments. No significant differences across priming treatments were detected but all were statistically different from the negative control as expected (Figures 5 and 6).

Table 3: Analysis of deviance using Type III Wald chi-square test for incidence of localized infections at 6 *wai*.

Analysis of Deviance Table (Type III Wald chi-square test)				
Response: bin_y_local				
	Chisq	Df	Pr(>Chisq)	Significance
(Intercept)	0.5508	1	0.45800	
treatment	10.4795	4	0.03308	*
trial	1.7143	1	0.19043	
treatment:trial	2.2104	4	0.69712	

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1				

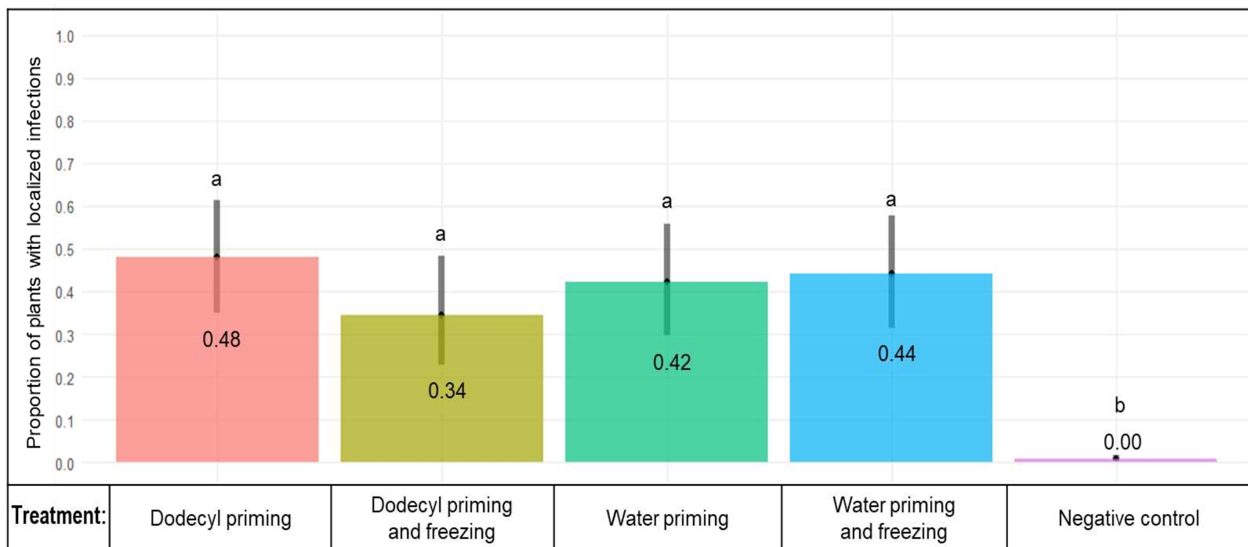

Figure 5: Incidence of the localized infections at 6 weeks after incubation (wai). Confidence intervals are displayed as gray bars for each priming treatment inoculated. Means significant differences are represented by letter (a, b) with p-values less than 0.05 considered significant.

Table 4: Analysis of deviance using Type III Wald chi-square test for severity of localized infections at 6 *wai*.

Analysis of Deviance Table (Type III Wald chi-square test)				
Response: bin_y_local_SEV				
	Chisq	Df	Pr(>Chisq)	Significance
(Intercept)	46.3478	1	9.902e-12	***
treatment	7.6564	4	0.1050	
trial	0.8514	1	0.3561	
treatment:trial	3.3295	4	0.5043	
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

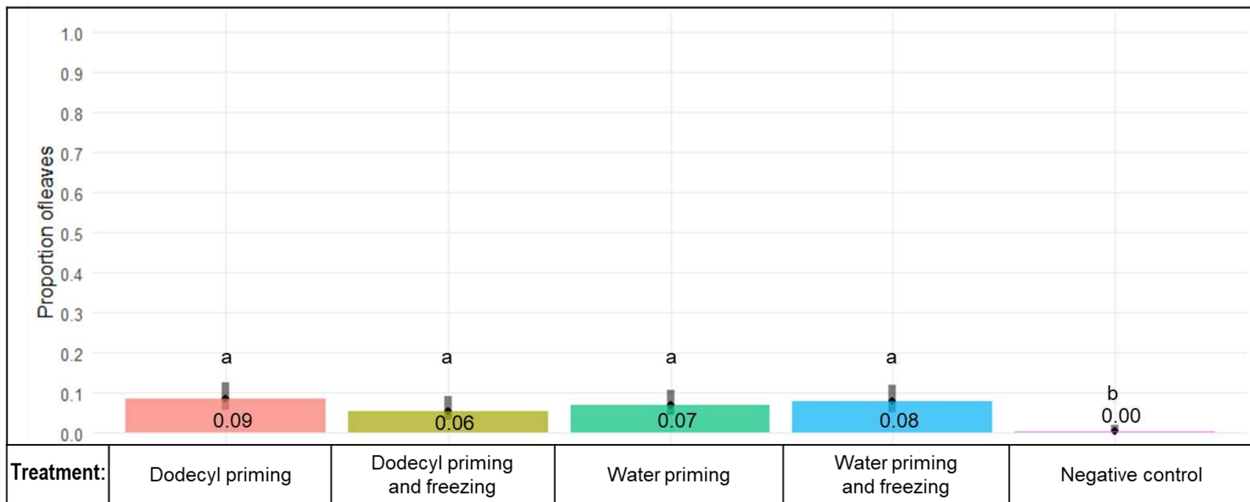

Figure 6: Severity of the localized infections at 6 weeks after incubation (wai). Confidence intervals are display as gray bars for each priming treatment inoculated. Means significant differences are represented by letter (a, b) with p-values less than 0.05 considered significant.

In both incidence and severity models of systemic infection at 16 *wai*, trial and priming treatment were found independently significant factors (p value < 0.01, Tables 5 and 6). However, there were no significant differences between priming treatments withing trials (Figures 7 and 8).

Table 5: Analysis of deviance using Type III Wald chi-square test for incidence of systemic infections at 16 *wai*.

Analysis of Deviance Table (Type III Wald chi-square test)				
Response: bin_y_sys2				
	Chisq	Df	Pr(>Chisq)	Significance
(Intercept)	5.8233	1	0.015816	*
treatment	24.3796	4	6.703e-05	***
trial	10.0027	1	0.001563	**
treatment:trial	5.5614	4	0.234382	
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1				

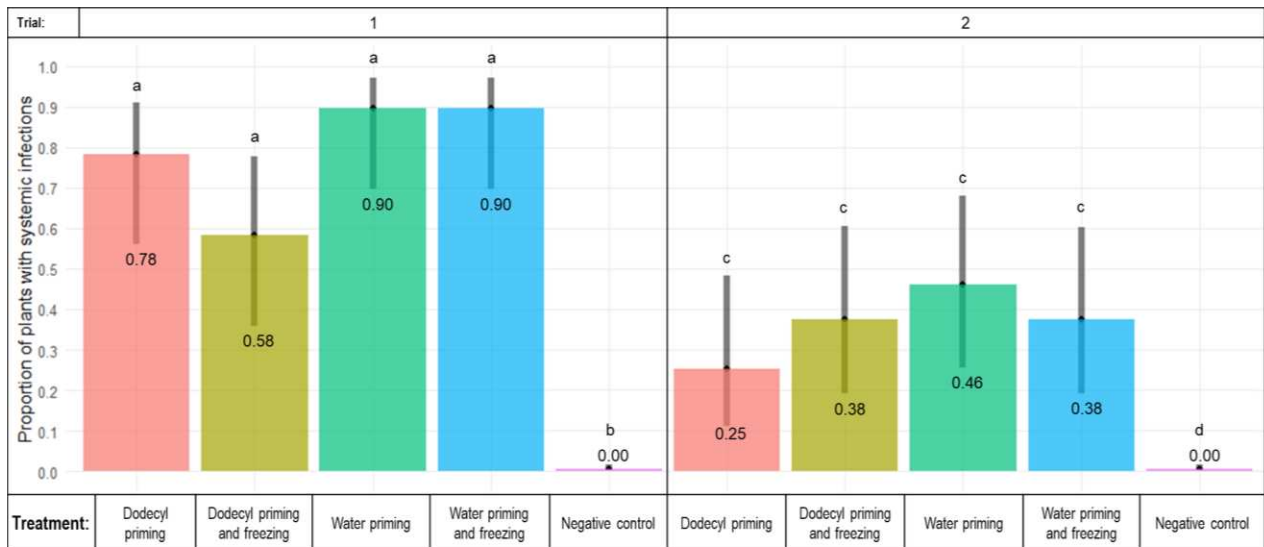

Figure 7: Incidence of the systemic infections at 16 weeks after incubation (wai). Confidence intervals are display as gray bars for each priming treatment inoculated. Means significant differences are represented by letter with p-values less than 0.05 considered significant.

Table 6: Analysis of deviance using Type III Wald chi-square test for severity of systemic infections at 16 *wai*.

Analysis of Deviance Table (Type III Wald chi-square test)				
Response: bin_y_sys2				
	Chisq	Df	Pr(>Chisq)	Significance
(Intercept)	10.9255	1	0.0009485	***
Priming_treatment	27.8454	4	1.341e-05	***
trial	8.7522	1	0.0030923	**
Priming_treatment:trial	2.7682	4	0.5973322	
Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1				

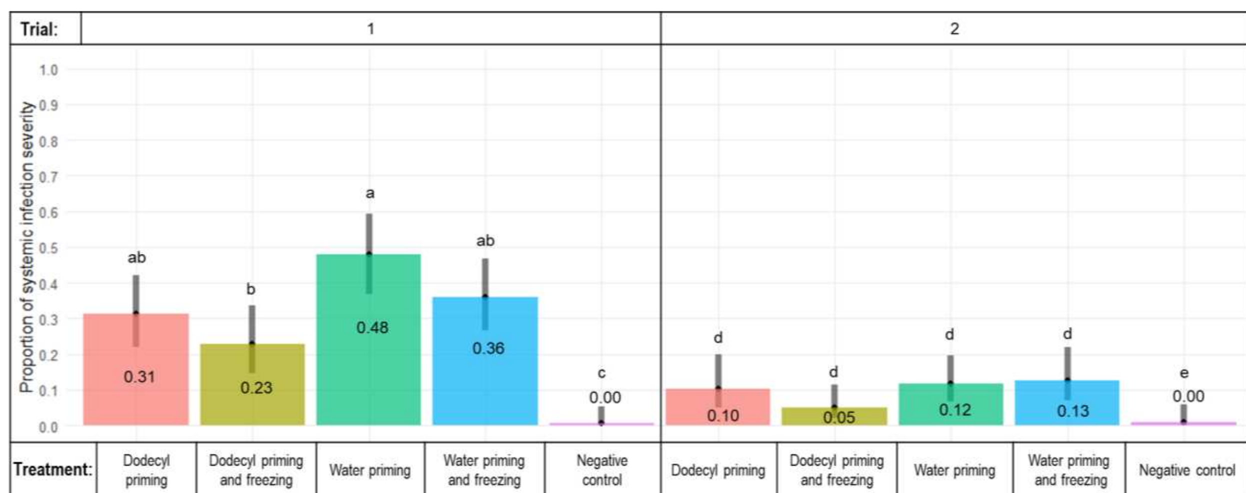

Figure 8: Severity of the systemic infections at 16 weeks after incubation (wai). Confidence intervals are display as gray bars for each priming treatment inoculated. Means significant differences are represented by letter with p-values less than 0.05 considered significant.

2.4 Discussion

Our results demonstrate that *P. punctiformis* teliospores can benefit from priming treatments to achieve greater germination proportions. The significantly greater germination rates achieved by priming teliospores in water without the germination stimulator align with previous reports of increased teliospore germination in rust fungi employing only water for teliospore priming by flooding in *P. carthami* (Klisiewicz, 1977), pre-soaking and floating in *P. hordei*, *P. recondita*, *P. graminis avenae*, *P. lagenophorea*, *U. scillarum*, and *U. viennon-bourginii* (Anikster, 1986), wetting and drying cycles in *Phakopsora pachyrhizi* (Saksirirat & Hoppe, 1991), and soaking in *P. jaceae* var *solstitialis* (Bruckart & Eskandari, 2002; Fisher et al., 2009). These reports, along with our findings, suggest that a germination stimulator is not necessary for teliospore priming, at least in *P. punctiformis* and the aforementioned species. Our observations indicate that using a germination stimulator in the priming process would not be more effective than using water alone to achieve the greatest germination rates. In fact, the use of the germination stimulator during priming resulted in significantly lower germination rates compared to using only water. These findings suggest that water priming could be the most effective method to achieve the greatest teliospore germination rates in *P. punctiformis*.

Although the germination stimulator seems not necessary during teliospore priming, the germination results of primed and primed-and-frozen teliospores indicate that the presence of the stimulator compound during germination (as amended in the media) is crucial; since low or no germination (≤ 0.01) was observed for the water-primed teliospores when incubated in water agar. These observations concur with previous reports of minimal or zero germination in the absence of the stimulator during germination (Turner et al., 1986; Van Den Ende et al., 1987; French, 1990;

French & Lightfield, 1990; French, 1990) and indicate that priming teliospores does not exempt them from the need for the germination stimulator to achieve germination.

The need for a host compound to germinate is known for other rust fungi with potential biocontrol activity like *P. carthami* (Klisiewicz, 1972, 1973; Klisiewicz, 1977; Binder et al., 1977) and *Puccinia jaceae var. solstitialis* (Bruckart & Eskandari, 2002). In contrast, no need for a germination stimulator has been reported for pathogenic rusts like *A. psidii* (Lana et al., 2012), *P. horiana* (Bonde et al., 2013), *P. hordei*, *P. recondite*, *P. graminis avenae*, *P. lagenophorea*, *U. scillarum*, and *U. viennon-bourginii* (Anikster, 1986). These outcomes suggest that *P. punctiformis* priming requirements differ from its germination requirements regarding the need for the germination stimulator.

We observed that the germination rates of non-primed teliospore in dodecyl agar differed across trials (trial 1 = 0.40, trial 2 = 0.04) but were closely similar after priming in water (trial 1 = 0.86, trial 2 = 0.90), dodecyl (trial 1 = 0.64, trial 2 = 0.62), as well as after priming and freezing them in water (trial 1 = 0.83, trial 2 = 0.80), and dodecyl (trial 1 = 0.61, trial 2 = 0.55). This suggests that the percentage of viable teliospores might have remained constant despite the additional two months of storage experienced by teliospores in the second trial since each started at different dates (first trial, March 15th, 2023; second trial, May 24th, 2023). These observations suggest that even when teliospores did not germinate at the same rates prior to priming, they were still viable; therefore, germinating at similar rates after being primed and primed and then frozen. Lana et al. (2012) reported a similar behavior in *A. psidii*, suggesting that live but dormant spores may not germinate readily. These observations open the possibility that *P. punctiformis* teliospores, instead of unviable as assumed in the previous chapter, may instead become dormant. Dormant teliospores require an activation process to break the dormancy (Mendgen, 1983), and

our observations suggest that water priming could serve as a dormancy-breaker. Further research is required to determine if *P. punctiformis* teliospores are indeed dormant and to reveal whether the priming process in fact acts as a dormancy breaker.

The incidence and severity rates of systemic infection caused by primed-and-frozen teliospores were not statistically different from those originated by primed teliospores (Figures 7 and 8). These observations concur with the germination results and suggest that teliospores retain priming effects after being frozen for 3 days at -20 °C, which could facilitate inoculum delivery and storage. However, further research is required to determine if primed teliospores can survive after longer freezing periods while still maintaining their viability to germinate and cause infections.

The incidence rates of systemic infection observed when plants were inoculated with water-primed (0.90 in both trials) and water-primed-and-frozen teliospores (0.38 in trial 1, 0.46 in trial 2) (Figure 7) are greater than the one-third proportions or less reported in field experiments in which teliospores were spread without any prior stimulation (Berner et al., 2013b; Berner, Smallwood, Cavin, et al., 2015; Bean et al., 2024). Similarly, our results showed greater infection rates compared to greenhouse experiments where inoculations were followed by water spray applications (17 % - 48 %) (Clark et al., 2020) or directly performed in root buds (26 %) (French & Lightfield, 1990). The rates of systemic infection incidence obtained in our experiments with water-primed teliospores are similar to experiments where teliospores were either stimulated with the vapor phase of a concentrated *C. arvense* root extract before direct root bud inoculation (52 %) (French & Lightfield, 1990), pre-germinated in a liquid solution of *C. arvense* seedlings before inoculation and followed by spray applications of jasmonic acid (58 % - 66 %) (Clark et al., 2020), or applied directly in root segments (34/47 = 72 %) (Van Den Ende et al., 1987) that exudate the

germination stimulator (Turner et al., 1986; French & Lightfield, 1990; French et al., 1994). These rates are also greater than those observed in our previous experiments where *C. arvense* rosettes inoculated with teliospores without any prior stimulation or plant incubation period resulted in 4/100, 1/35, and 3/60 systemic infection incidence observable only after 4 – 8 months, even after trimming the plants down (Astete-Farfan and Norton, personal communication). These differences suggest that teliospore priming in water may increase the incidence of systemic infections as effectively as treatments where teliospores are exposed to a germination stimulator, or even more.

The statistically higher germination rates of water-primed teliospores compared to dodecyl-primed teliospores did not translate in significantly greater proportions of incidence and severity of systemic infection. The incidence (0.90 in trial 1, and 0.46 in trial 2) and severity (0.48 in trial 1, and 0.12 in trial 2) observed for water-priming treatment were not statistically different from those in the dodecyl-priming treatment (incidence = 0.78 in trial 1, and 0.25 in trial 2; severity = 0.31 in trial 1, and 0.10 in trial 2). This discrepancy suggests that other factors might affect in-plant teliospore germination, basidiospore production, and host penetration, possibly leading to non-significant differences across treatments. Additionally, this discrepancy may indicate the need for a control group using non-primed teliospores to improve contrast in the statistical analysis, and additional replicates to enhance statistical power and reduce variation.

The infection results provided insights into *P. punctiformis* disease progression, suggesting that teliospore priming might also facilitate the earlier occurrence of systemic infections. The earliest systemic infections occurred at 3 *wai* and resulted from water-primed and dodecyl-primed teliospores, whereas all other treatments exhibited systemic infections at 4 *wai* (Appendix 16). Overall, the systemic infections from inoculations performed with primed teliospores appeared earlier compared to what has been reported for field inoculations with non-primed teliospores in

the fall that typically show infections the following spring (Berner et al., 2013b; Berner, Smallwood, Cavin, et al., 2015; Bean et al., 2024), and inoculation with pre-germinated, two-year-old teliospores accompanied with jasmonic acid applications, that exhibit systemic infections 4 months later (Clark et al., 2020). Instead, our primed teliospore inoculations results appeared around the same time as reported for inoculations with pre-germinated, six-month-old teliospores accompanied of jasmonic acid applications that exhibited systemic infections (5 *wai*) (Clark et al., 2020), and direct inoculations of root buds that developed systemically infected shoots after 27 – 40 days (French & Lightfield, 1990). These findings reinforce the importance of priming *P. punctiformis* teliospores for use as a biocontrol agent against *C. arvense*.

In general, priming and priming-and-freezing treatments resulted in lower systemic infection incidence and severity rates in the second trial compared to the first one (Figures 7 and 8), despite using the same spore sample stored at 4 °C for both trials. Similar incidence variation rates between trials have also been reported by Van Den Ende et al. (1987), suggesting teliospore age or plant age as possible causes. Since teliospore germination rates after both priming and priming-and-freezing processes were similar in both trials in our experiment (Figures 6 and 8), we believe that teliospore age was not the reason for the different systemic infections rates in our experiment. Instead, other factors involved during the infection process (greenhouse conditions, seasonal factors, host susceptibility) could be the cause, and further research is necessary to identify them.

The development of systemically infected axillar shoots, whether or not originating from the node of localized infected leaves, could represent a benefit of priming teliospores. In our study, the first systemically infected axillar shoot emerged from the node at the base of a leaf with localized infections (Figure 9) in a plant inoculated with water-primed teliospores at 3 *wai*.

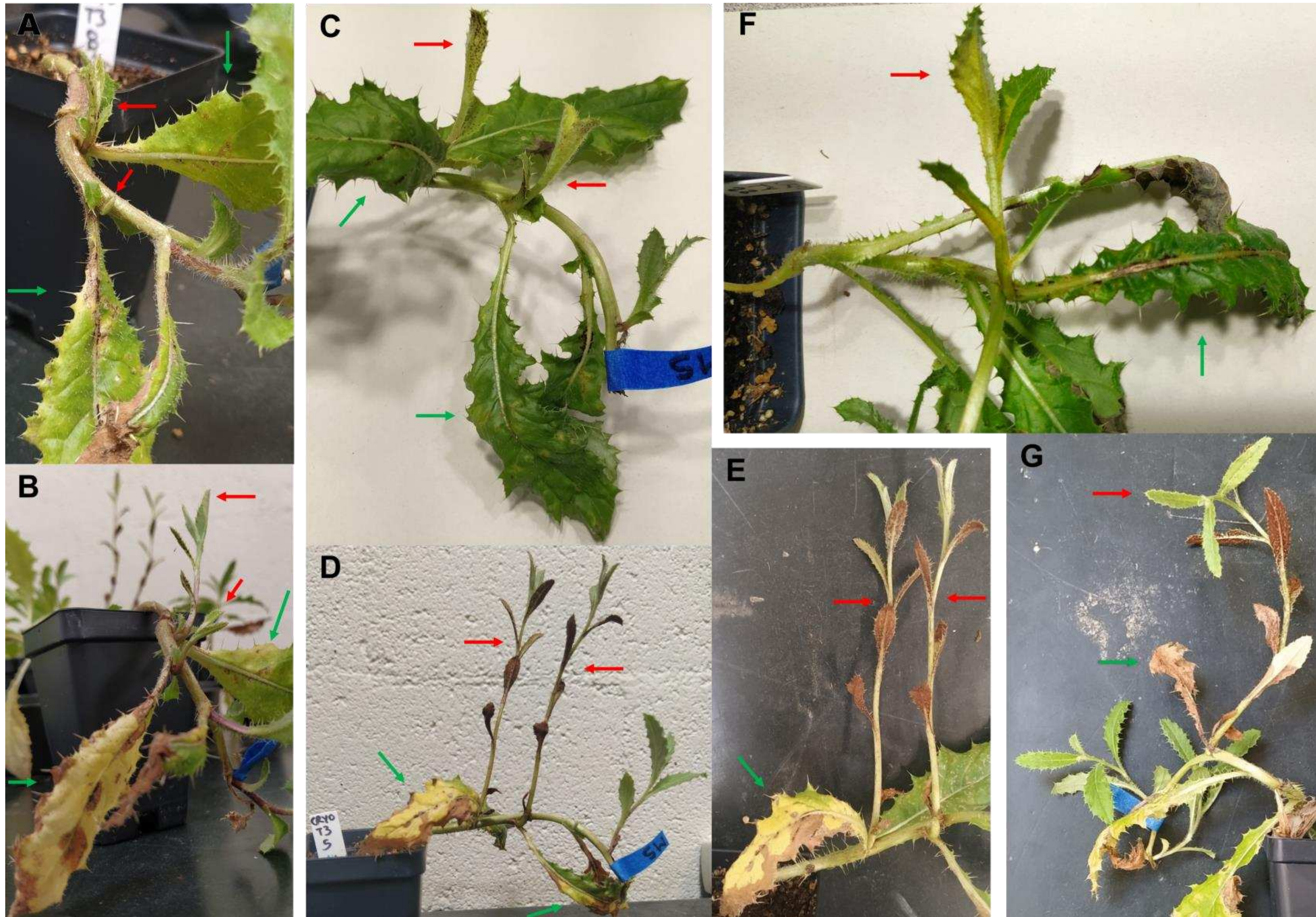

Figure 9: Plants developing systemically infected axillar shoots (red arrows) from the node at the base of leaves with localized infections (green arrows) observed on July 13th, 2023 (A, C, F): Growth of the systemically infected axillar shoots in plant A after one week (B), in plant C after two weeks (D, E), and in plant F after three weeks (G).

This type of infection, which we define as ‘Localized infection, then systemic infection in axillar shoots’ (Appendix 16), was observed in at least one plant from each of the other priming treatments by 4 *wai*, and its incidence gradually increased until the end of the experiment. Some of these plants also developed new infected shoots, which we defined as ‘Localized infection, then systemic infection in axillar and new shoots’ (Appendix 16). Similarly, the incidence of systemically infected shoots not associated with localized leaf infections also incremented across all treatments, either as ‘Systemic infection in axillar shoots’ or ‘Systemic infection in axillar and new shoots’ (Appendix 16). At 16 *wai*, 64 %, 54 %, 47 %, and 40 % of the plants inoculated with water-primed, water-primed-and-frozen, dodecyl-primed, and dodecyl-primed-and-frozen teliospores, respectively, developed systemically infected axillar shoots and more than half of them associated with localized infections (33 %, 33 %, 35 %, and 25 % of the plants inoculated with water-primed, water-primed-and-frozen, dodecyl-primed, and dodecyl-primed-and-frozen teliospores, respectively) (Appendix 16). These findings reinforce the idea that primed teliospores may cause infection of axillar shoots in the initially inoculated shoot of *C. arvense*. Therefore, we strongly encourage implementing water-primed teliospores in field inoculations.

This close association between localized foliar infections and systemically infected axillar shoots has previously been observed by French & Lightfield (1990) in very few plants. Cytological studies indicate that the spermogonial stage of *P. punctiformis* is capable of expanding systemically through the host vascular system (Baka & Lösel, 1992a, 1992b). However, Van Den Ende et al. (1987) suggested that the systemic infections in axillar and new shoots may originate from the colonization of *C. arvense* axillar and root primordia, respectively. Further research is required to determine if axillar shoots can indeed derive from foliar infections of *P. punctiformis*.

Not all localized infections were associated with systemic infections. The infected foliage of some plants abscised as the localized spermogonia simply dried out or sporulated, and no further symptoms of plant infection were observed after abscission. Therefore, we categorized this observation as 'No symptoms after localized infection die' (Appendix 16).

The most appropriate tissue (roots or rosettes) to inoculate and achieve systemic infection should be further studied. Van Den Ende et al. (1987) suggested that the penetration places for *P. punctiformis* are the primordia, and since their number is higher in the root than in the shoot system, root inoculations will be more convenient to achieve systemic infections. However, Frantzen (1994b) reported that *C. arvense* uses the dynamic clonal growth of the root system as a scape from the disease since shoots produced vegetatively show less probability to be infected by *P. punctiformis*. In our experiments, the incidence of systemic infections in axillar shoots was higher compared to the incidence observed in new shoots, suggesting that primed teliospores could enter axillar primordia in the stem.

It would also be important to investigate other factors that could be manipulated as part of the priming treatment to enhance the efficacy of *P. punctiformis* as a weed biocontrol agent as well. Based on the incubation conditions of our infection occurrence assay, other important factors could include dew point or humidity since we maintained 100 % humidity with plastic coverage (adapted from Van Den Ende et al., 1987; Morin et al., 1992), and also using other temperatures other than 18 °C, and photoperiod influence during the priming prior to germination since we used darkness only. It also should be noted that our experiments were performed with less concentrated spore suspensions (3000 teliospores per ml, delivering a total of 10 ml = 30,000 teliospores per plant) compared to the 0.5 ml of 2 x 10^6 teliospores/ml used for greenhouse (Clark et al., 2020) and field experiments (Berner et al., 2013; Berner, Smallwood, Vanrenterghem, et al., 2015; Bean

et al., 2024); as well as with a single time inoculation instead of multiple inoculation (Berner et al., 2013; Berner, Smallwood, Vanrenterghem, et al., 2015; Bean et al., 2024), and still exhibited higher efficiency suggesting that teliospore priming could also decrease the amount or required inoculum, however, it requires more investigation.

2.5 Conclusion

We found higher rates of *P. punctiformis* teliospore germination following priming treatments, with priming in water being the most efficient method to increase germination rates. Our results also indicate that primed teliospores can remain viable for germination and plant infection after being frozen for 3 days at -20 °C without losing priming effects.

For biocontrol purposes, we recommend inoculating with water-primed teliospores because they ensure greater germination rates than both dodecyl-primed and non-primed teliospores, and the germination stimulator is not necessary during the priming process. Moreover, water-primed teliospores could cause similar or greater incidence and severity rates of systemic infection compared to dodecyl-primed teliospores, and potentially lead to earlier and larger rates of systemic infection in both axillar shoots of the initially inoculated shoots and new secondary shoots, even simultaneously.

Furthermore, we encourage inoculating primed teliospores in stems and roots to facilitate their contact with stimulator compounds required in the germination process and known to be produced in these organs. Additionally, we suggest performing inoculations during spring, when the host is in its earliest growth stages and actively developing primordia, which have been referred as potential penetration sites to start systemic infections. Leaf inoculations in young stages may also lead to systemic infections preceded by spermogonial localized infections in the foliage.

Finally, for assessing germination, we suggest always incorporating dodecyl-NCS in the germination media since teliospores achieve their greatest germination rates under its influence, regardless of whether they are primed or not. However, we recommend priming teliospores in water before their germination in order to assess their maximum germinability, as teliospores have shown increased germination percentages after priming.

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APPENDICES

Appendix 1: Summary of the statistical model of teliospore germination showing significant three-way interactions involving temperature, humidity and time (p value < 0.1).

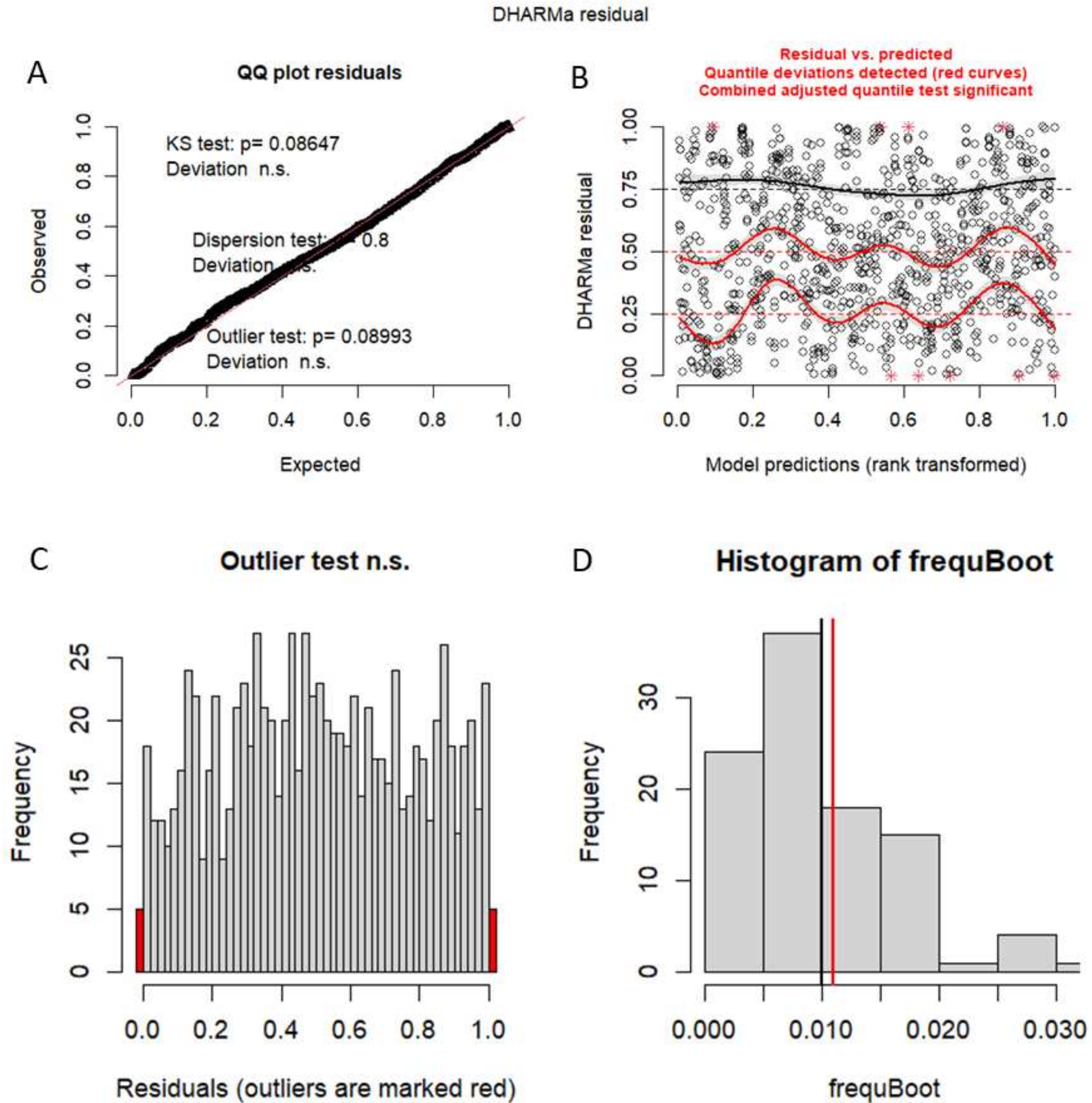
Family: betabinomial (logit)				
Formula: Teliospore_germination_proportion ~ Temperature * Humidity * Month + Spore_source + (1 | Plate * Counts) + (1 | assay_date)				
Data: spore_longevity				
AIC: 6176.8	BIC: 6321.3	logLik: -3058.4	Deviance: 6116.8	Df.resid: 884

Random effects:			
Conditional model:			
Groups	Name	Variance	Std.Dev.
Plate	(Intercept)	1.635e-01	4.043e-01
Counts	(Intercept)	2.583e-09	5.083e-05
Plate:Counts	(Intercept)	2.583e-09	5.083e-05
assay_date	(Intercept)	7.605e-02	2.758e-01
Number of observations: 914, groups: Plate, 215; Counts, 914; Plate:Counts, 914; assay_date, 9			
Dispersion parameter for betabinomial family (): 97.9			

Conditional model:					
	Estimate	Std. Error	z value	Pr(>|z|)	Significance
(Intercept)	0.61824	0.22750	2.718	0.006577	**
Temperature6	0.30400	0.24162	1.258	0.208320	
Temperature23	0.77854	0.24714	3.150	0.001631	**
Humidity22	0.44341	0.24038	1.845	0.065094	.
Humidity62	0.32774	0.24096	1.360	0.173782	
Humidity90	-0.09803	0.24438	-0.401	0.688329	
Month	-0.19042	0.03690	-5.161	2.46e-07	***
SourceK	-0.03993	0.05780	-0.691	0.489649	
Temperature6:Humidity22	-0.40236	0.33949	-1.185	0.235945	
Temperature23:Humidity22	-0.48939	0.34924	-1.401	0.161125	
Temperature6:Humidity62	-0.14863	0.34141	-0.435	0.663316	
Temperature23:Humidity62	-0.40375	0.37383	-1.080	0.280124	
Temperature6:Humidity90	-0.31074	0.34286	-0.906	0.364766	
Temperature23:Humidity90	-0.45127	0.37941	-1.189	0.234282	
Temperature6:Month	0.07218	0.03896	1.852	0.063956	.
Temperature23:Month	0.04979	0.03943	1.263	0.206651	
Humidity22:Month	0.11198	0.03992	2.805	0.005028	**
Humidity62:Month	0.11263	0.03910	2.881	0.003966	**
Humidity90:Month	0.15155	0.03932	3.854	0.000116	***
Temperature6:Humidity22:Month	-0.09697	0.05502	-1.763	0.077956	.
Temperature23:Humidity22:Month	-0.21827	0.05679	-3.844	0.000121	***
Temperature6:Humidity62:Month	-0.08320	0.05464	-1.523	0.127806	
Temperature23:Humidity62:Month	-0.74705	0.07740	-9.652	< 2e-16	***
Temperature6:Humidity90:Month	-0.11843	0.05474	-2.164	0.030502	*
Temperature23:Humidity90:Month	-0.83963	0.08316	-10.096	< 2e-16	***

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Appendix 2: Residuals diagnostics of the teliospore germination model described in Appendix 1: QQ plot of expected versus observed residual showed normality (A), observed versus predicted residuals showed deviations (B) which were not significant (C) and their frequency was close to the expected outlier frequency (0.01) (D) according to the outlier tests.



Appendix 3: Slopes for teliospore germination variation through time for each Temperature-humidity combination, from model in Appendix 1. Boldface indicates slopes with confidence intervals that spanned 0. Results were averaged between spore sources, and a 0.95 confidence level was used for the analysis.

Temperature = -20 °C			
RH	Slope	asymp.LCL	asymp.UCL
5	-0.1904	-0.263	-0.11811
22	-0.0784	-0.151	-0.00630
62	-0.0778	-0.148	-0.00737
90	**-0.0389**	**-0.110**	**0.03203**
Temperature = 6 °C			
RH	Slope	asymp.LCL	asymp.UCL
5	-0.1182	-0.188	-0.04810
22	-0.1032	-0.173	-0.03320
62	-0.0888	-0.159	-0.01813
90	-0.0851	-0.156	-0.01464
Temperature = 23 °C			
RH	Slope	asymp.LCL	asymp.UCL
5	-0.1406	-0.212	-0.06948
22	-0.2469	-0.321	-0.17267
62	-0.7750	-0.902	-0.64768
90	-0.8287	-0.969	-0.68833

Appendix 4: Summary of the model for germination of non-primed, primed, and primed-and-frozen teliospores at 14 *dai*; showing significant two-way interactions between priming treatment and germination media (p value < 0.001).

Family: betabinomial (logit)

Formula: bin_y_all ~ Priming treatment * Germination media * Experiment trial + (1 | Rep)

Data: priming_all

AIC: 786.1	BIC: 848.8	logLik: -371.0	Deviance: 742.1	Df. resid: 106

Random effects:

Conditional Model:

Groups	Name	Variance	Std. Dev.	
Rep	(Intercept)	1.161e-10	1.078e-05	

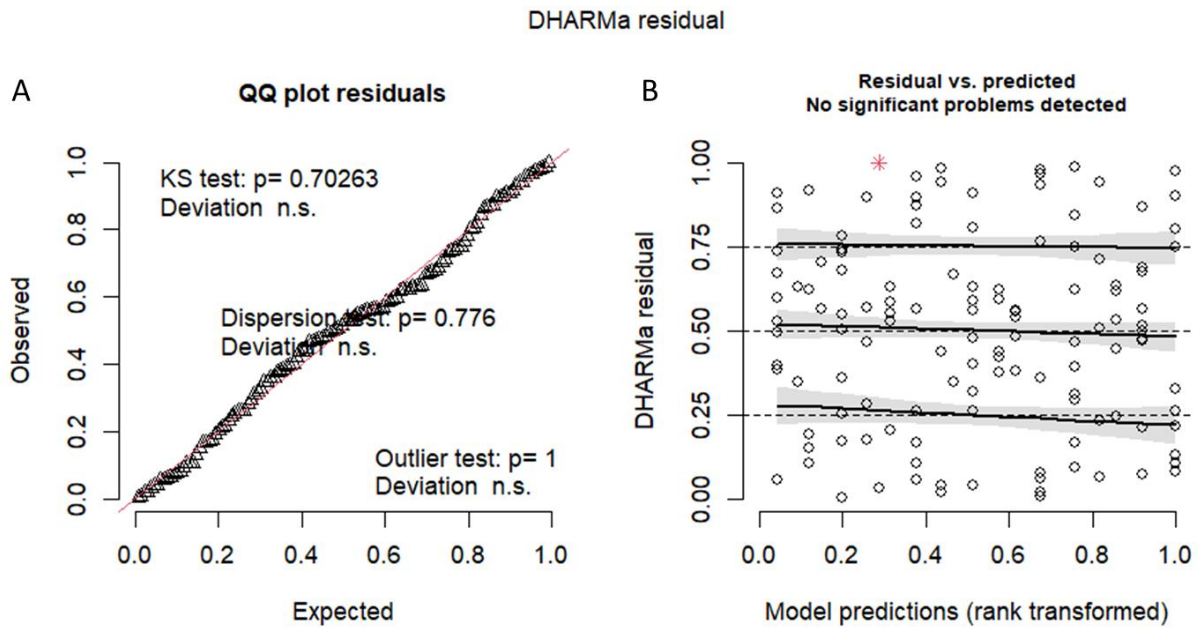
Number of Obs:128, groups: Rep, 5

Dispersion parameter for betabinomial family (): 172

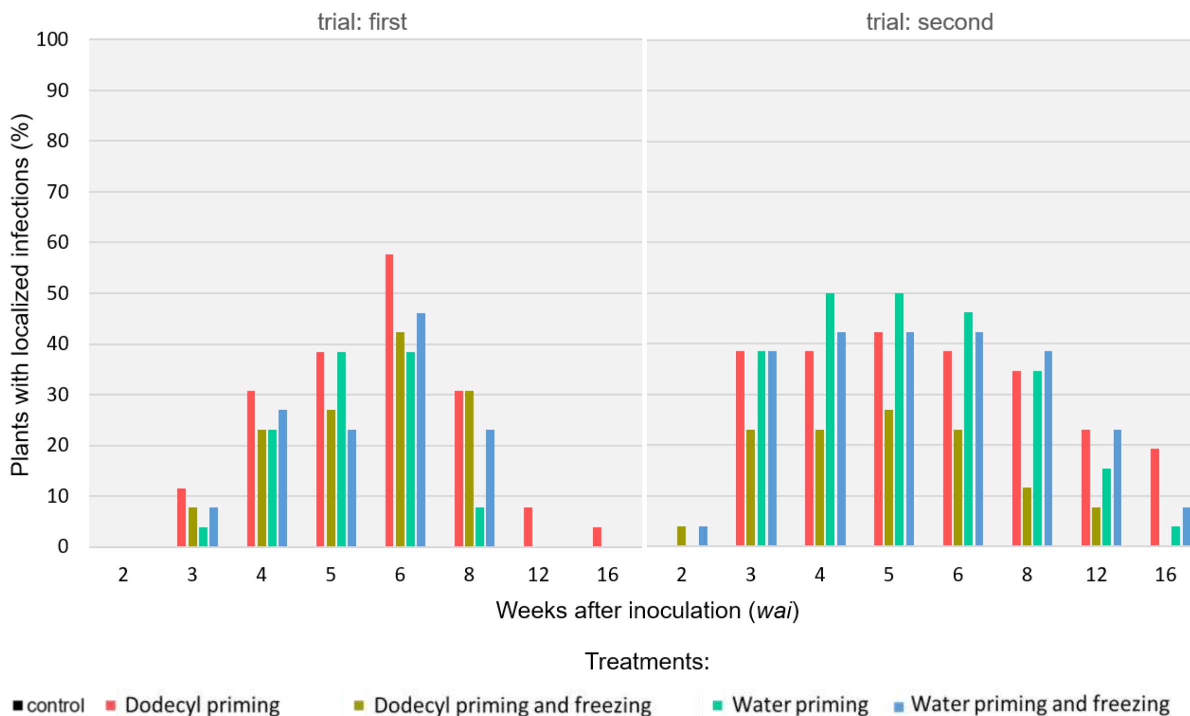
Conditional model:					
	Estimate	Std. Error	z value	Pr(>|z|)	Significance
(Intercept)	-5.71371	1.00649	-5.677	1.37e-08	***
Priming treatment: Dodecyl	3.53999	1.01143	3.500	0.000465	***
Priming treatment: Dodecyl and freezing	3.37610	1.01903	3.313	0.000923	***
Priming treatment: Water	-0.68863	1.22704	-0.561	0.574653	
Priming treatment: Water and freezing	-0.07194	1.41777	-0.051	0.959529	
Germination media: Dodecyl agar	5.32521	1.01686	5.237	1.63e-07	***
Experiment trial: 2	-0.48851	1.41739	-0.345	0.730353	
Priming treatment: Dodecyl Germination media: Dodecyl agar	-2.59269	1.02458	-2.530	0.011390	*
Priming treatment: Dodecyl and freezing Germination media: Dodecyl agar	-2.52284	1.03553	-2.436	0.014840	*
Priming treatment: Water Germination media:Dodecyl agar	2.92013	1.23913	2.357	0.018443	*
Priming treatment:Water and freezing Germination media:Dodecyl agar	2.05648	1.43002	1.438	0.150412	
Priming treatment:Dodecyl Experiment trial:2	2.40720	1.42512	1.689	0.091197	.
Priming treatment:Dodecyl and freezing Experiment trial:2	2.20369	1.43466	1.536	0.124529	
Priming treatment:Water Experiment trial:2	2.99685	1.60209	1.871	0.061403	.
Priming treatmentd:Water and freezing Experiment trial:2	2.49215	1.75874	1.417	0.156480	
Germination media: Dodecyl agar Experiment trial:2	-2.28979	1.47338	-1.554	0.120161	
Priming treatment: Dodecyl Germination media: Dodecyl agar Experiment trial:2	0.30185	1.48495	0.203	0.838920	
Priming treatment:Dodecyl and freezing Germination media: Dodecyl agar Experiment trial:2	0.30119	1.49855	0.201	0.840709	
Priming treatment:Water Germination media: Dodecyl agar Experiment trial:2	0.14383	1.65802	0.087	0.930873	
Priming treatment:Water and freezing Germination media: Dodecyl agar Experiment trial:2	0.05511	1.81157	0.030	0.975731	

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

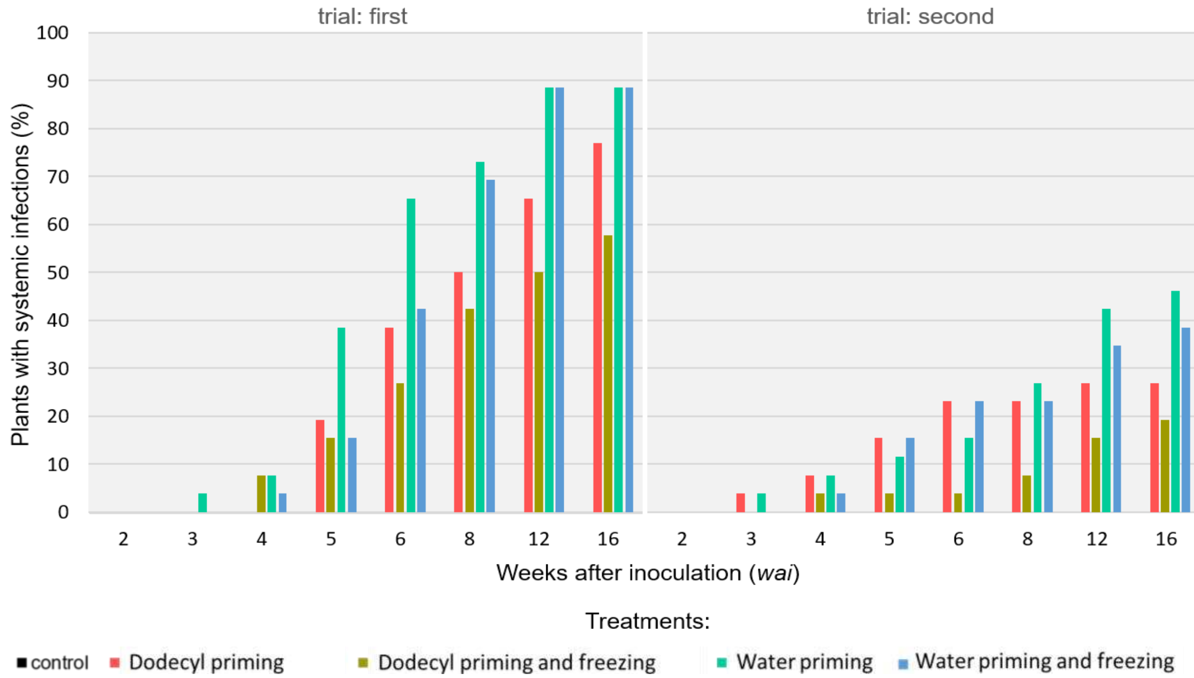
Appendix 5: Residuals diagnostics of germination model described in appendix 4: QQ plot of expected versus observed residual showed normality (A), and observed versus predicted residuals showed no significant deviations (B).



Appendix 6: Incidence of localized infections from 2 to 16 weeks after inoculation (wai) with primed and primed-and-frozen teliospores by trial.



Appendix 7: Incidence of systemic infections from 2 to 16 weeks after inoculation (wai) with primed and primed-and-frozen teliospores by trial.

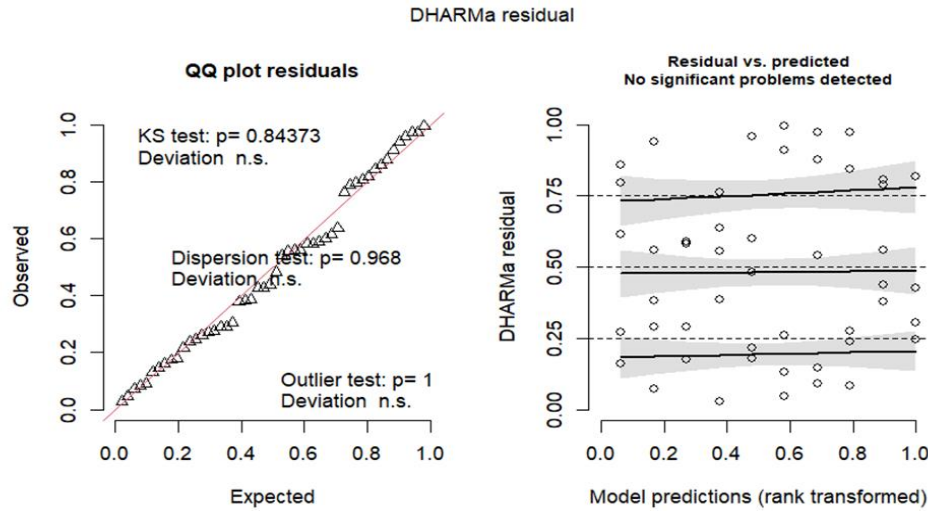


Appendix 8: Summary of the statistical models of the incidence of localized infections at 6 weeks after inoculation (*wai*): No significant interaction between priming treatment and experiment trial; however, priming treatment was significant independently (p value < 0.01).

Family: betabinomial (logit)					
Formula: bin_y_local ~ treatment * trial + (1 | tube_rep)					
Data: local					
AIC: 318.2	BIC: 360.9	logLik: -147.1	Deviance: 294.2	Df.resid: 248	
Random effects:					
Conditional model:					
Groups	Name	Variance	Std.Dev.		
tube_rep	(Intercept)	0.1217	0.3488		
Number of obs: 260, groups: tube_rep, 50					
Dispersion parameter for betabinomial family (): 0.00262					
Conditional model:					
	Estimate	Std. Error	z value	Pr(>|z|)	Significance
(Intercept)	0.32068	0.43210	0.742	0.45800	
Treatment Dodecyl solution and freezing	-0.63586	0.61248	-1.038	0.29919	
Treatment Water solution	-0.79948	0.61587	-1.298	0.19424	
Treatment Water solution and freezing	-0.48860	0.61007	-0.801	0.42320	
Treatment Negative control	-3.59339	1.12659	-3.190	0.00142	**
trial2	-0.80736	0.61663	-1.309	0.19043	
Treatment Dodecyl solution and freezing:trial2	0.09939	0.88924	0.112	0.91100	
Treatment Water solution:trial2	1.13082	0.87007	1.300	0.19371	
Treatment Water solution and freezing:trial2	0.66175	0.86770	0.763	0.44567	
Treatment Negative control:trial2	0.80734	1.58734	0.509	0.61103	

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

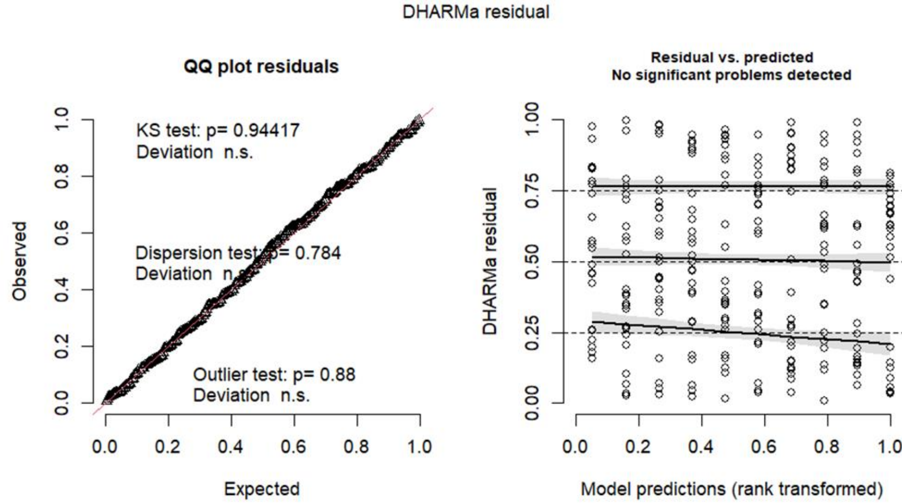
Appendix 9: Residuals diagnostics of the incidence model of localized infections at 6 wai described in Appendix 8: QQ plot of expected versus observed residual showed normality (left), and observed versus predicted residuals showed good model fit (right).



Appendix 10: Summary of the statistical model of the severity of localized infections at 6 *wai*: No significant interaction between priming treatment and experiment trial; however, priming treatment was significant independently (p value < 0.01).

<table>
<tr><td colspan="6">Family: betabinomial (logit)</td></tr>
<tr><td colspan="6">Formula: bin_y_local_SEV ~ treatment * trial + (1 | tube_rep)</td></tr>
<tr><td colspan="6">Data: local_severity</td></tr>
<tr><td>AIC: 482.2</td><td>BIC: 525.0</td><td colspan="2">logLik: -229.1</td><td>Deviance: 458.2</td><td>Df.resid: 248</td></tr>
<tr><td colspan="6">Random effects:</td></tr>
<tr><td colspan="6">Conditional model:</td></tr>
<tr><td>Groups</td><td>Name</td><td colspan="2">Variance</td><td colspan="2">Std.Dev.</td></tr>
<tr><td>tube_rep</td><td>(Intercept)</td><td colspan="2">0.1117</td><td colspan="2">0.3342</td></tr>
<tr><td colspan="6">Number of obs: 260, groups: tube_rep, 50</td></tr>
<tr><td colspan="6">Dispersion parameter for betabinomial family (): 22.6</td></tr>
<tr><td>Conditional model:</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td>Estimate</td><td>Std. Error</td><td>z value</td><td>Pr(>|z|)</td><td>Significance</td></tr>
<tr><td>(Intercept)</td><td>-2.17424</td><td>0.31937</td><td>-6.808</td><td>9.9e-12</td><td>***</td></tr>
<tr><td>Treatment Dodecyl solution and freezing</td><td>-0.04062</td><td>0.45411</td><td>-0.089</td><td>0.92873</td><td></td></tr>
<tr><td>Treatment Water solution</td><td>-0.37655</td><td>0.47626</td><td>-0.791</td><td>0.42915</td><td></td></tr>
<tr><td>Treatment Water solution and freezing</td><td>-0.19902</td><td>0.46050</td><td>-0.432</td><td>0.66562</td><td></td></tr>
<tr><td>Treatment Negative control</td><td>-2.84427</td><td>1.06364</td><td>-2.674</td><td>0.00749</td><td>**</td></tr>
<tr><td>trial2</td><td>-0.41142</td><td>0.44587</td><td>-0.923</td><td>0.35615</td><td></td></tr>
<tr><td>Treatment Dodecyl solution and freezing:trial2</td><td>-0.84384</td><td>0.69556</td><td>-1.213</td><td>0.22506</td><td></td></tr>
<tr><td>Treatment Water solution:trial2</td><td>0.33564</td><td>0.64918</td><td>0.517</td><td>0.60514</td><td></td></tr>
<tr><td>Treatment Water solution and freezing:trial2</td><td>0.25809</td><td>0.63457</td><td>0.407</td><td>0.68422</td><td></td></tr>
<tr><td>Treatment Negative control:trial2</td><td>-0.11229</td><td>1.50464</td><td>-0.075</td><td>0.94051</td><td></td></tr>
<tr><td colspan="6"></td></tr>
<tr><td colspan="6">Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1</td></tr>
</table>

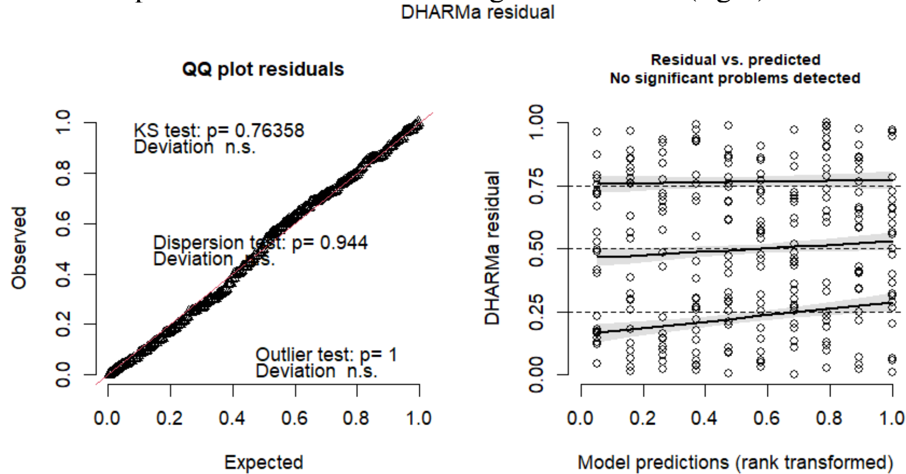
Appendix 11: Residuals diagnostics of the severity model of localized infections at 6 weeks after inoculation described in Appendix 10: QQ plot of expected versus observed residual showed normality (left), and observed versus predicted residuals showed good model fit (right).



Appendix 12: Summary of the statistical models of the incidence of systemic infections at 16 weeks after inoculation (*wai*): No significant interaction between priming treatment and experiment trial; however, both factors were significant independently (p value < 0.01).

Family: betabinomial (logit)					
Formula: bin_y_sys2 ~ treatment * trial + (1 | tube_rep)					
Data: sys2					
AIC: 304.3	BIC: 347.1	logLik: -140.2	Deviance: 280.3	Df.resid: 248	
Random effects:					
Conditional model:					
Groups	Name	Variance	Std.Dev.		
tube_rep	(Intercept)	0.2514	0.5014		
Number of obs: 260, groups: tube_rep, 50					
Dispersion parameter for betabinomial family (): 0.00262					
Conditional model:					
	Estimate	Std. Error	z value	Pr(>|z|)	Significance
(Intercept)	1.28363	0.53193	2.413	0.01582	*
Treatment Dodecyl solution and freezing	-0.94822	0.70519	-1.345	0.17875	
Treatment Water solution	0.87927	0.84902	1.036	0.30038	
Treatment Water solution and freezing	0.86344	0.84755	1.019	0.30832	
Treatment Negative control	-4.68250	1.19444	-3.920	8.85e-05	***
trial2	-2.35930	0.74597	-3.163	0.00156	**
Treatment Dodecyl-NCS solution and freezing:trial2	1.51884	0.99187	1.531	0.12570	
Treatment Water solution:trial2	0.03724	1.09021	0.034	0.97275	
Treatment Water solution and freezing:trial2	-0.29861	1.09385	-0.273	0.78486	
Treatment Negative control:trial2	2.35932	1.66115	1.420	0.15552	
Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

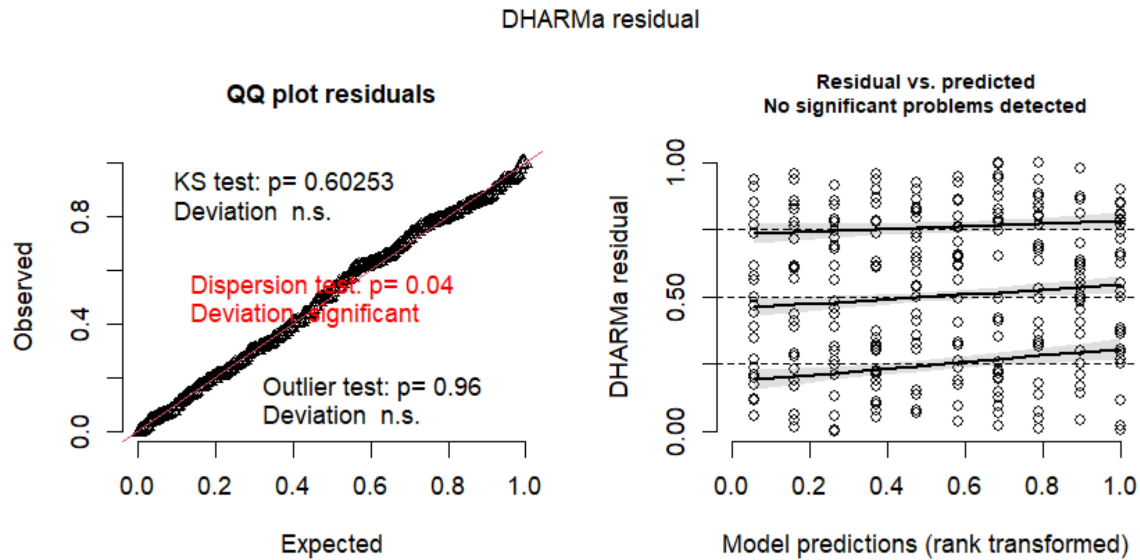
Appendix 13: Residuals diagnostics of the incidence model of systemic infections at 16 wai described in Appendix 12: QQ plot of expected versus observed residual showed normality (left), and observed versus predicted residuals showed good model fit (right).



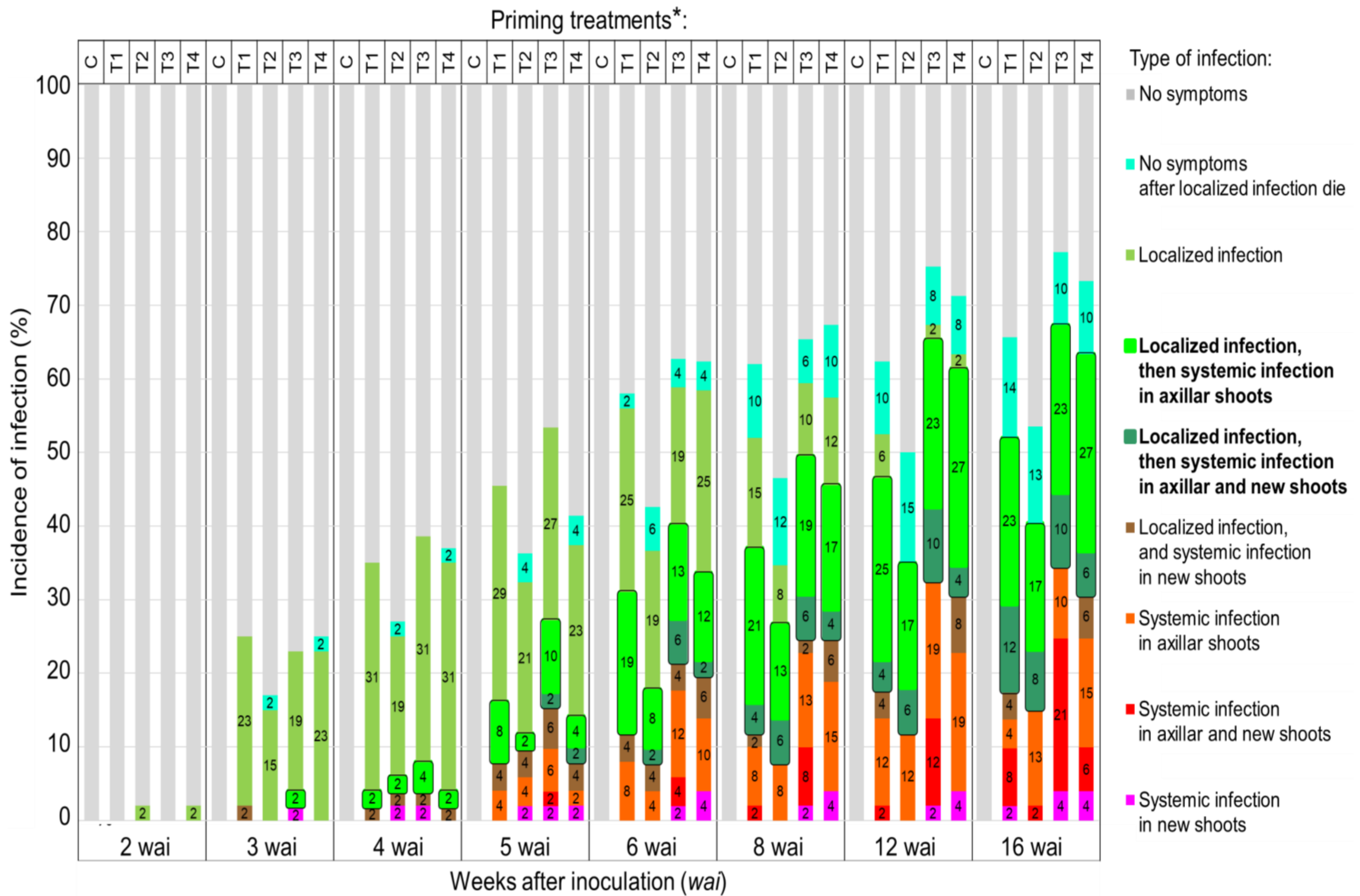
Appendix 14: Summary of the statistical model of the severity of systemic infections at 16 *wai*: No significant interaction between priming treatment and experiment trial; however, both factors were significant independently (p value < 0.01).

Family: betabinomial (logit)					
Formula: bin_y_sys_SEV ~ treatment * trial + (1 | tube_rep)					
Data: sys_SEVERITY					
AIC: 754.4	BIC: 797.1	logLik: -365.2	Deviance: 730.4	Df.resid: 248	
Random effects:					
Conditional model:					
Groups	Name	Variance	Std.Dev.		
tube_rep	(Intercept)	4.603e-08	0.0002145		
Number of obs: 260, groups: tube_rep, 50					
Dispersion parameter for betabinomial family (): 2.71					
Conditional model:					
	Estimate	Std. Error	z value	Pr(>|z|)	Significance
(Intercept)	-0.79312	0.23995	-3.305	0.000948	***
Treatment Dodecyl-NCS and freezing	-0.43175	0.36624	-1.179	0.238455	
Treatment Water	0.70846	0.33447	2.118	0.034161	*
Treatment Water and freezing	0.22228	0.32849	0.677	0.498607	
Treatment Negative control	-4.06159	1.03477	-3.925	8.67e-05	***
trial2	-1.37336	0.46422	-2.958	0.003092	**
Treatment Dodecyl-NCS and freezing:trial2	-0.35155	0.71086	-0.495	0.620929	
Treatment Water:trial2	-0.55415	0.60280	-0.919	0.357944	
Treatment Water and freezing:trial2	0.01231	0.61372	0.020	0.983994	
Treatment Negative control:trial2	1.47081	1.49646	0.983	0.325677	
Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

Appendix 15: Residuals diagnostics of the severity model of systemic infections at 16 *wai* described in Appendix 14: QQ plot of expected versus observed residual showed normality (left), and observed versus predicted residuals showed good model fit (right).



Appendix 16: Disease progress of localized and systemic infections from 2 to 16 weeks after incubation (*wai*): 1) No symptoms (plants that never show infection symptoms); 2) No symptoms after localized infection die (plants initially exhibited localized infections on leaves that later abscised, and no further symptoms were observed); 3) Localized infections (plants presented only localized infections on leaves); 4) Localized infection, then systemic infection in axillar shoots (plants with localized leaf infections from which node later developed a systemically infected axillar shoot); 5) Localized infection, then systemic infection in axillar and new shoots (plants with localized leaf infections from which node later developed a systemic infected axillar shoot. Later, plants also developed new shoots systemically infected); 6) Localized infection, and systemic infection in new shoots (plants with localized leaf infections. Later plants also developed new shoots systemically infected); 7) Systemic infection in axillar shoots (plants developed systemic infected axillar shoots exclusively); 8) Systemic infection in axillar and new shoots (plants developed axillar and new shoots systemically infected); 9) Systemic infection in new shoots (plants developed systemically infected new shoots exclusively).



*Priming treatments: **C**: negative control**, T1**: dodecyl priming, **T2**: dodecyl priming with freezing, **T3**: water priming, **T4**: water priming and freezing.